Transcriptional regulation of histone H2A variants in liver cancer

By

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Tata Memorial Centre

Mumbai

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DECLARATION

I, hereby declare that the investigation presented in the thesis has been carried out by me. This work is original and has not been submitted earlier as a whole or in part for a degree / diploma at this or any other Institution / University.

Navi Mumbai,
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Monica Tyagi

List of Publications arising from the thesis

1. Monica Tyagi, Bharat Khade, Shafqat A Khan, Arvind Ingle, Sanjay Gupta. Expression of histone variant, H2A.1 is associated with the undifferentiated state of hepatocyte. (*Exp Biol Med (Maywood)1535370214531869, first published on April 24, 2014*

2. Database submission: Monica Tyagi, Shafqat A Khan, Sanjay Gupta. Sequence submission to GenBank-NCBI, USA. Accession Numbers ***JX661508*** and ***JX661509*** are assigned to submission of complete gene sequence of H2A.1 and H2A.2 to GenBank data. The data are simultaneously made available to *EMBL, Europe and the DNA Data Bank, Japan.2012*

3. Molecular cloning, characterization and promoter analysis of rat H2A.1 and H2A.2 gene. (*manuscript ready for communication)*

Other Publications:

1. Ketan Patel, **Monica Tyagi**, Jasmin Monpara, Lalit Vora, Sanjay Gupta, Pradeep Vavia. Arginoplexes: an arginine-anchored nanoliposomal carrier for gene delivery. *J Nanopart Res (2014) 16:2345*

2. Shafqat A Khan, **Monica Tyagi**, Ajit K Sharma, Savio G Barreto, Bhawna Sirohi, Mukta Ramadwar, Shailesh V Shrikhande, Sanjay Gupta. Cell-type specificity of β-actin expression and its clinicopathological correlation in gastric adenocarcinoma. *World Journal of Gastroenterology, in press*

3. Monica Tyagi, Shafqat A Khan, Saikat Bhattacharya, Divya Reddy, Ajit K Sharma, Bharat Khade, Sanjay Gupta. Techniques to Access Histone Modifications and Variants in Cancer. 'Cancer Epigenetics: Risk Assessment, Diagnosis, Treatment, and Prognosis', *Methods in Molecular Biology Series* (Humana Press/Springer) 2014, *in press.*

4. Lalit Vora, **Monica Tyagi**, Ketan Patel, Sanjay Gupta and Pradeep Vavia, Self assembled nanocomplexes of anionic pullulan and polyallylamine for DNA and pH sensitive intracellular drug delivery, *Journal of Nanoparticle Research (Accepted for publication, Nov'2014)*

5. **NewsLetter: Monica Tyagi**, Shafqat A Khan and Sanjay Gupta. Histone Post-translational Modifications as biomarks in cancer: Dream or Reality? *PSI News Letter Vol. 2 (No.1) April 2014*, Proteomics Society, India (PSI)

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1. Attended and presented poster entitled "Differential expression of histone variants and their transcriptional regulation during hepatocarcinogenesis" in **Chromatinasia conference '2010 December** held in JNCSAR Bangalore, India.
2. Attended and presented poster entitled "Transcriptional regulation of differentially expressing variants of histone H2A during hepatocarcinogenesis" in **Spetses Summer School on Chromatin and Systems Biology, Spetses, Greece 17-23 September 2011**.
3. Attended and presented poster entitled "Transcriptional regulation of homomorphous variants of histone H2A." in **31st Annual Convention of IACR**, Advanced Centre for Treatment, Education and Research in Cancer, Tata Memorial Centre, Kharghar, Navi Mumbai,. **26-29 January, 2012**.
4. Attended and oral presentation entitled "Transcriptional regulation of differentially expressed H2A variants during liver development and carcinogenesis" **Epigenetics Mechanism in Development and Diseases, 4th Meeting of the Asian Forum of Chromosome and Chromatin Biology**, CSIR-CCMB, Hyderabad. **22-24 November, 2012**
5. Attended and presented poster entitled "Insight into histone alterations during liver development and carcinogenesis." **33rd Annual Convention of IACR**,RGCB, Kerala,**13-15 Feb, 2014**

Monica Tyagi

Dedicated to
Mommy and Papa

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"It is good to have an end to journey toward, but it is the journey that matters in the end."

– Ursula K. Le Guin

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Monica Tyagi

CONTENTS

Synopsis

Homi Bhabha National Institute

Ph. D. PROGRAMME

1. Name of the Student:	Monica Tyagi
2. Name of the Constituent Institution:	Tata Memorial Centre**,** Advanced Centre for Treatment, Research and Education in Cancer.
3. Enrolment No. :	LIFE09200804003
4. Title of the Thesis:	Transcriptional regulation of histone H2A variants in liver cancer
5. Board of Studies:	Life Sciences

SYNOPSIS

Synopsis

1. INTRODUCTION:

Genomic DNA is packaged by histones into a basic unit i.e. nucleosome and the array of nucleosomes assemble to form a compact chromatin structure inside the eukaryotic nucleus (1). The packaging of the genome into the nucleosomes, their accessibility for regulatory proteins and RNA polymerase requires a fine balance for eukaryotic gene transcription. Therefore, distinct mechanisms have evolved to regulate the dynamic alterations in the chromatin structure to allow well orchestrated transcription regulation of genes (2). The dynamic mode of chromatin architecture is mediated majorly by post-translational modifications of histones, replacement by histone variants and ATP-dependent chromatin remodeling resulting in reorganization, repositioning or eviction of nucleosomes.

Multiple variants of H1, H2A, H2B, H3 and H4 histones have been identified (3, 4). Histone variants are the non allelic subtypes of canonical histones. On the basis of amino acid sequence similarity, variants are grouped as homomorphous and heteromorphous; also, on the basis of their expression during different stages of cell cycle, as replication-dependent and replication-independent (5). The incorporation of histone variants into the chromatin may alters their post-translational modification (PTM) profiles due to amino acid sequence differences from their conventional histone counter parts and thereby changes the structure, stability and dynamics of the nucleosome (6-8).

H2A variants have been studied during development stages, cell cycle and cancer; however their regulation needs to be elucidated further. H2A.Z has been reported to be recruited in transcriptionaly active regions, whereas, macro H2A and H2ABbd is reported to be involved in gene inactivation (9-11). Studies on histone variants, H2AZ and macroH2A are emerging in embryonic development and cancer

suggesting their role in epigenetic reprogramming. H2A.Z has been shown to over-express in cancers and has also associated with development and differentiation [1,2,3]. However, macroH2A has been reported to suppress tumor progression and its loss positively correlates with malignant phenotype of cancer [4]. Further, the redundancy of macroH2A has been shown by developmental defects in zebra fish embryos ([5] and cell fate decisions in human male pluripotent cells [6]. Previous report from lab has also shown over-expression of H2A.1 with decrease in expression of H2A.2 with the sequential progression in NDEA induced rat hepatocellular carcinoma (HCC) [7]. Recently, replication dependent histone coded by human HIST1H2AC gene locus, sequence similar to rat H2A.1, has been associated with cell proliferation and tumorigenicty of bladder cancer cells [8]. Therefore, *in vivo* replacement of H2A.2 by H2A.1 may modulate the highly dynamic nature of the nucleosomes and reorganizes the chromatin landscape of *cis*-regulatory and coding regions for specific transcription required for different biological programs in different physiological states (19).

The homomorphous histone variants, H2A.1 and H2A.2 encode for a protein of 130 amino acids, each. H2A.1 and H2A.2 differ at three amino positions viz. 16 (Serine to Threonine), 51 (Leucine to Methionine) and 99 (Arginine to Lysine). Leucine to Methionine alteration at 51st amino acid position is highly conserved across species. The genes encoding for these variants are mapped on chromosome 17p for H2A.1 and H2A.2 on 2q, thereby, suggesting their specific gene promoters and transcription regulation. Also, the differential expression profile of H2A.1 and H2A.2 mRNA during sequential HCC has shown increased level of H2A.1 in HCC with decrease of H2A.2 (20). This suggests that the substantial alteration in their expression in various patho-physiological states like cancer might be contributed by differential

transcriptional regulatory mechanisms. To understand the transcriptional regulation of these genes, the prime requisite is to have the experimentally identified complete genomic organization including transcriptional start site, promoter and 3'untranslated region. Therefore, to dissect the regulatory mechanism of H2A.1 and H2A.2 gene expressions following objectives were proposed.

2 OBJECTIVES:

2.1 Cloning and characterization of H2A variants transcriptional start site and promoter.

2.2 Identification of protein binding sites regulating expression of H2A variant promoter.

2.3 DNA-protein interaction studies to characterize the transcription factors.

3. WORK PLAN:

Objective 1: Cloning and characterization of variants transcriptional start site and promoter.

3.1.1 Mapping of 5'UTR of H2A.1 and H2A.2 gene by 5'RNA Ligase Mediated-Rapid Amplification of cDNA (5'RLM RACE)

3.1.2 Mapping of 3'UTR for H2A.1 and H2A.2 mRNA by RNA circularization coupled with PCR Amplification (3'RCPA)

3.1.3 Cloning of H2A.1 and H2A.2 gene promoter in pGL3 basic Luciferase vector

Objective 2: Identification of protein binding sites regulating expression of H2A variant promoter.

3.2.1 *In silico* identification of H2A.1 and H2A.2 promoters and transcription factor binding sites by Motif finder and Bio-base software analysis.

3.2.2 Identification of *cis-acting* regulatory elements on H2A.1 and H2A.2 gene promoter using Dual Luciferase assay.

3.2.3 Profiling of H2A.1 and H2A.2 during development of liver in embryogenesis and post-partial hepatectomy by Acetic Acid-Urea-Triton PAGE (AUT-PAGE) and semi-quantitative PCR analysis.

Objective 3: DNA-protein interaction studies to characterize the transcription factors

3.3.1 *In vitro* identification of DNA binding transcription factors for H2A.1 and H2A.2 gene promoter by Electrophoretic mobility shift assay (EMSA)

3.3.2 *In vivo* identification of transcription factors binding to H2A.1 and H2A.2 gene promoter by Chromatin Immuno precipitation coupled with promoter specific PCR amplification. (ChIP-PCR)

4. RESULTS:

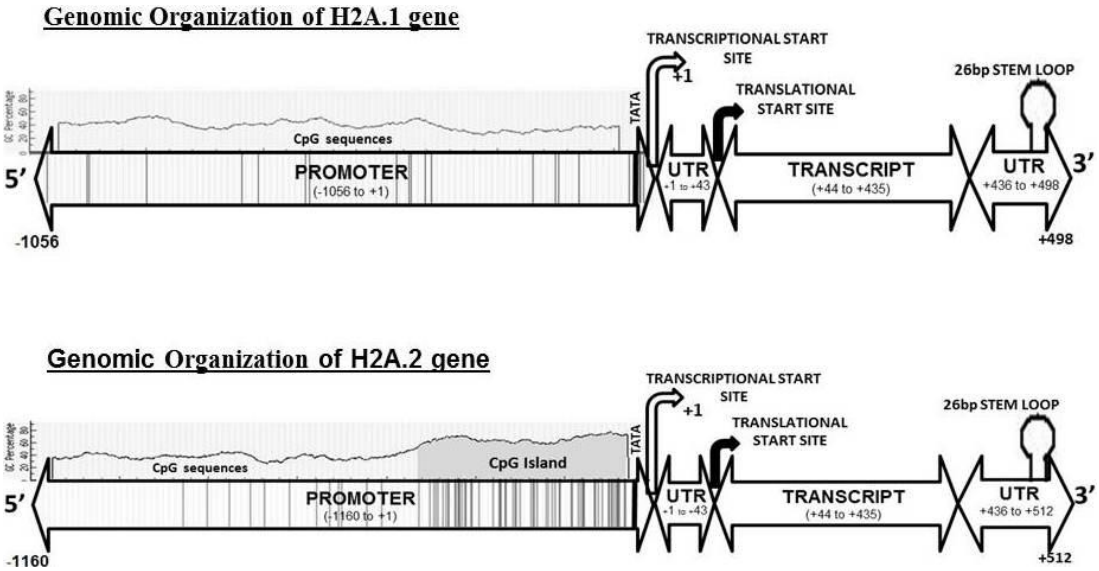
4.1 Cloning and characterization of variants transcriptional starts site and promoter

4.1.1 *Mapping of 5'UTR of H2A.1 and H2A.2 gene*: Histone variants H2A.1 and H2A.2 fall under the category of homomorphous variants sharing 89% homology between their predicted cDNA sequences. Therefore, the cDNA for both the variants were cloned and sequenced for designing of gene specific primers for further experiments. The NCBI database (XM-577573 for H2A.1) and (XM-345255 for H2A.2) suggested the predicted transcriptional start site (TSS) for H2A.1 is positioned 10bp upstream the translational start site (ATG), and for H2A.2, 88bp upstream of the first ATG site. The experimentally identified TSS is positioned 31bp upstream the predicted TSS in the NCBI data base and/or 43bp upstream the Translational Start site. Further TSS of H2A.2 gene is 46bp downstream of the predicted start site in the NCBI data base and /or 43bp upstream the ATG site as identified using 5'RLM-RACE technique.

4.1.2 *Mapping of 3'UTR for H2A.1 and H2A.2 mRNA*: The expression of the histone is majorly dependent on the 3' end of mRNA i.e. poly-A tail or the conserved stem loop. The experimentally identified 3'UTR of H2A.1 and H2A.2 using mRNA circularization approach followed by cloning and sequencing confirmed the presence of 26bp conserved stem loop in both the variants. However, the 3'UTR is 62bp long in H2A.1, whereas, 88bp long in H2A.2 mRNA. This

further suggests that H2A.1 and H2A.2 variants might transcribe in replication-dependent manner.

4.1.3 *Cloning of H2A.1 and H2A.2 gene promoter*: Rat genome database (http://rgd.mcw.edu/) was used to locate the upstream sequence from experimentally identified TSS for H2A.1 and H2A.2 gene from chromosome 17 arm q11 (GeneID:502125, region 48507120 to 48508520) and chromosome 2 arm q34 (GeneID:365877, region 191050439 to 191052653), respectively. The predicted H2A.1 and H2A.2 gene promoters ranging from -1056 to +144, and -1160 to +219 downstream of the +1 site were PCR amplified and sequenced using gene specific primers, respectively. The amplified product of 1200bp (-1056 to +144) and 1379bp (-1160 to +219) encompassing H2A.1 and H2A.2 gene promoters were cloned in pGL3 basic luciferase vector to study transcriptional activity of these variant promoters.



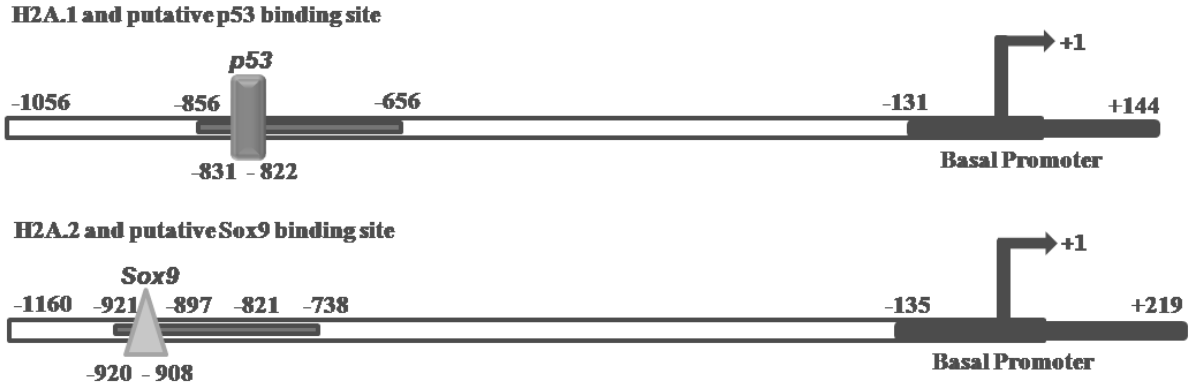
4.2 Identification of protein binding sites regulating expression of H2A variant promoter

In silico studies suggested presence of multiple putative transcription factor binding sites in region encompassing -1056 to +144 for H2A.1, and -1160 to +219 for H2A.2

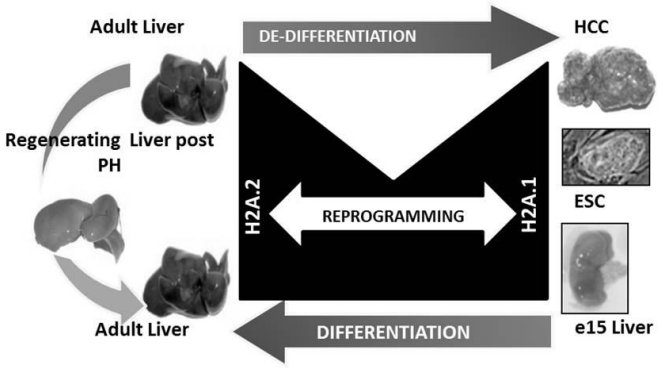
gene. Further, the data suggested that both the genes were TATA driven with location of TATA box (TATAAA) at 25-30bp upstream and CCAAT box at 63-67bp upstream from the transcription initiation site. Further, H2A.1 gene promoter contains various putative *cis-acting* binding sites, like p53, Oct-1, cEBPα and cdxA, whereas, H2A.2 contains putative binding sites for AP4, E2F, cEBPβ and family of Sox-binding sites for transcription factors. Additionally, differential mapping of the CpG islands or –CCGG- sequences were observed in the promoter regions of these histone variants. The H2A.2 gene promoter (-1160 to +219) contains CpG Island, having greater than 90% G+C composition in the 5' untranslated segment spanning from -290 to +219, while, H2A.1 (-1056 to +144) has no CpG islands but contains multiple –CCGG- sites within its promoter.

4.2.1 Dual Luciferase assay with cloned H2A.1 (-1056 to +144) and H2A.2 (-1160 to +219) promoters showed transcriptional activity. Further, luciferase assays using a series of constructs containing unidirectional deleted fragments revealed that -656 to -456 region was required for the maximal, and -131 to +144 region required for basal promoter activity of H2A.1 gene. The basal promoter activity of H2A.2 promoter was observed with -135 to +219, whereas -921 to -738 regions have shown minimal promoter activity. Moreover, the unidirectional deletion constructs of -831 to +144, deleting out the putative p53 binding region showed increase in transcriptional activity. Thus, signifying repressor binding element site, -856 to -832, on H2A.1 gene promoter. Similarly, the multiple unidirectional deletion constructs of H2A.2 gene promoter, -921 to -738, showed repression in transcriptional activity. Subsequently, to delineate the putative Sox-factor binding sites, following deletion constructs were prepared, -920 to +219, -897 to +219 and -821 to +219 and

transcriptional activity measured. The luciferase assay suggested cumulative repressive effect of *cis-acting* elements having putative Sox-family transcription factors in regulation of H2A.2 gene promoter.



4.3 Expression of histone variant, H2A.1 is associated with the undifferentiated state of hepatocyte: The alterations in expression of H2A.1 and H2A.2 variants during rat liver embryogenesis and regeneration was delineated. Each patho-physiological stage of liver was characterized by well established specific genetic marker (PCNA, AFP and Sox9) and epigenetic markers (H3K27me3, H3K9Ac).The expression of H2A.1 and H2A.2, at protein and mRNA level, does not alter in normal cellular proliferation associated with regeneration of liver post PH. In contrast, gradual decrease of H2A.1 with increase of H2A.2 was observed during differentiation of embryonic to adult liver. Further, the accumulation of H2A.1 was higher in embryonic stem cells compared to normal adult liver. Collectively, the data support a strong correlation of H2A.1 expression and overall epigenetic reprogramming in dedifferentiation and maturation of undifferentiated cells, rather than with normal cellular proliferation.



4.4 DNA-protein interaction studies to characterize the transcription factors

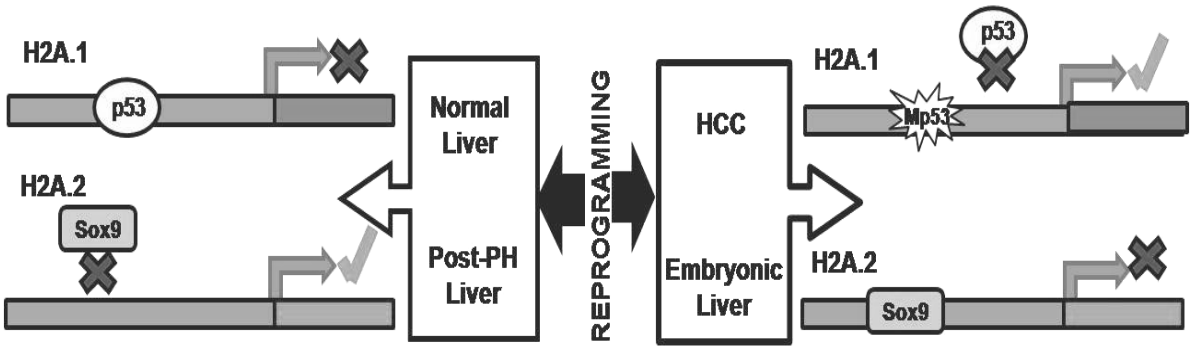
4.4.1 In vitro binding of putative p53 binding on *cis-acting* regulatory regions, -831 to -822, of H2A.1 and Sox9 binding region, -920 to -908, of H2A.2 gene promoters by EMSA showed enhanced binding with nuclear extract of HCC compared to normal liver. Further, the specificity of binding in the above mentioned specific regions of p53 (H2A.1) and Sox9 (H2A.2) was confirmed by competitive titration experiment with respective, specific, non-specific and mutant oligonucleotides. Additionally, super-shift assay with p53 and Sox9 specific antibody confirmed their binding with H2A.1 and H2A.2 promoters, respectively.

4.4.2 In vivo binding of p53 and Sox9 on H2A.1 and H2A.2 promoter regions was confirmed by mono-nucleosomal ChIP coupled with gene specific (H2A.1: -856 to -756 and H2A.2: -921 to -913) PCR amplification. The 90bp amplified product with gene specific primers from immuno-precipitated DNA confirmed the *in vivo* binding of p53 and Sox 9 within the promoter region of H2A.1 (-831 to -822) and H2A.2 (-920 to -908).

5. SUMMARY AND CONCLUSION:

The present study demonstrates a strong correlation of H2A.1 expression with undifferentiated hepatocytes, overlapping signatures of histone modifications and

chromatin architecture. H2A.1 and H2A.2 mRNA has different 5'UTR and conserved stem-loop with 62bp and 88bp long 3'UTR, suggesting their replication-dependent expression. Further, both H2A.1 and H2A.2 are TATA-driven with conserved CAAT sequence with different *cis-acting* elements in their promoters signifying their different regulatory mechanisms. The binding of transcription factors, p53 and Sox-9 have been identified, *in vitro* and *in vivo,* as negative transcriptional regulators of H2A.1 and H2A.2 gene promoters, respectively. The over-expression of Sox9 and mutation of p53 in HCC corroborates with our findings and therefore, p53 and Sox9 are one of the key regulators for transcriptional repression of H2A.1 and H2A.2 genes in HCC compared to normal liver, respectively. Finally, H2A.1 and H2A.2 variants might play a potential role in defining highly specialized chromatin organization during different biological processes, differentiation and/or de-differentiation suggesting their role in epigenetic reprogramming of genome.



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7. Publications in Refereed Journal:

a. **Published:**

1. Monica Tyagi, Bharat Khade, Shafqat A Khan, Arvind Ingle, Sanjay Gupta. Expression of histone variant, H2A.1 is associated with the undifferentiated state of hepatocyte. (*Exp Biol Med (Maywood)1535370214531869, first published on April 24, 2014*

2. Monica Tyagi, Shafqat A Khan, Sanjay Gupta. Sequence submission to GenBank-NCBI, USA. Accession Numbers ***JX661508*** and ***JX661509*** are assigned to submission of complete gene sequence of H2A.1 and H2A.2 to GenBank data. The data are simultaneously made available to *EMBL, Europe and the DNA Data Bank, Japan.2012*

b. **To be communicated**:

1. Monica Tyagi, Bharat Khade, Shafqat A Khan, Sanjay Gupta. Molecular cloning, characterization and promoter analysis of rat H2A.1 and H2A.2 gene. (*manuscript ready for communication)*

2. Monica Tyagi, Bharat Khade, Shafqat A Khan, Arvind Ingle, Sanjay Gupta Alteration in chromatin organization is associated with specific pattern of histone modification during differentiation, de- differentiation and regeneration of liver. (*manuscript to be prepared*)

c. **Other Publications:**

1. Monica Tyagi, Shafqat A Khan and Sanjay Gupta. Histone Post-translational Modifications as biomarks in cancer: Dream or Reality? *PSI News Letter Vol. 2 (No.1) April 2014*, Proteomics Society, India (PSI)

2. Ketan Patel, **Monica Tyagi**, Jasmin Monpara, Lalit Vora, Sanjay Gupta, Pradeep Vavia. Arginoplexes: an arginine-anchored nanoliposomal carrier for gene delivery. *J Nanopart Res (2014) 16:2345*

3. Shafqat A Khan, **Monica Tyagi**, Ajit K Sharma, Savio G Barreto, Bhawna Sirohi, Mukta Ramadwar, Shailesh V Shrikhande, Sanjay Gupta. Cell-type specificity of β-actin expression and its clinicopathological correlation in gastric adenocarcinoma. *World Journal of Gastroenterology, in press*

4. Monica Tyagi, Shafqat A Khan, Saikat Bhattacharya, Divya Reddy, Ajit K Sharma, Bharat Khade, Sanjay Gupta. Techniques to Access Histone Modifications and Variants in Cancer. 'Cancer Epigenetics: Risk Assessment, Diagnosis, Treatment, and Prognosis', *Methods in Molecular Biology Series* (Humana Press/Springer) 2014, *in press.*

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List of abbreviations

ATP	Adenosine Tri phosphate
ATM	Ataxia-Telangiectasia
AUT	Acetic acid Urea Triton
AFP	Alpha Feto Protein
BSA	Bovine Serum Albumin
CHZ1	Nuclear Chaperon for H2A.Z
CENP-A	Centromeric Protein A
CAF-1	Chromatin Assembly Factor-1
DTT	DL-Dithio Threitol
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal Bovine Serum
HCC	Hepato Cellular Carcinoma
HRPO	Horseradish peroxidase
HFD	Histone Fold Domain
HAT	Histone Acetyl-Transferases
HDAC	Histone Deacetylase
HIRA	Histone Regulation A
HMT	Histone methyltransferases
H3Lys (9,14) Ac	Histone H3Lysine (9,14) Acetylation
H3K9me3	Histone H3 Lysine 9 methylation-tri
MALDI	Matrix-assisted laser desorption/ionization
MOPS	3- (N-morpholino)propanesulfonic acid
MS	Mass spectrometry
PCR	Polymerase chain reaction
PH	Partial Hepatectomy

List of abbreviations

PTM	Post-Translational Modification
PBS	Phosphate Buffer Saline
PVDF	Poly Vinyliene Di-Fluoride
RT-PCR	Reverse transcription PCR
RLM-RACE	RNA Ligase mediated Rapid Amplification of cDNA
RPMI	Roswell Park Memorial Institute medium
SAM	S-Adenosyl Methionine
SSB	Single Strand Breaks
SDS-PAGE	Sodium-Dodecyl-Sulphate –Poly-Acrylamide Gel Electrophoresis
SEM :	Standard error of the mean
TSS	Transcriptional Start Site

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Chapter 1
Introduction

1. INTRODUCTION:

Multilayered network of regulatory mechanisms tightly control the organization and function of cell, tissue and organ system resulting in highly complex animal. The identity of individual cell type is achieved by the combination of mechanisms involved in regulating chromatin structure and transcription factor (TF) distribution within the nucleus on generalized and specific regulatory regions [1]. Transcription initiation is determined by the interplay of several tightly controlled processes: (i) the recruitment, assembly and structure of RNA polymerase II (RNA pol II) and the initiating complex that actively transcribes DNA into RNA, (ii) the TFs binding to proximal and distal regulatory regions, (iii) the maintenance of chromatin architecture of loci to be transcribed via combinatorial outcome of nucleosome positioning, histone variants and modifications [2], histone remodelling complexes [3], DNA methylation [4], chromatin structural proteins and enhancer-promoter interactions and (iv) the non-coding RNAs (ncRNAs) [5]. Together these factors result in highly specific regulatory landscapes, with discrete characteristics for individual cell type during differentiation, dedifferentiation and organismal development.

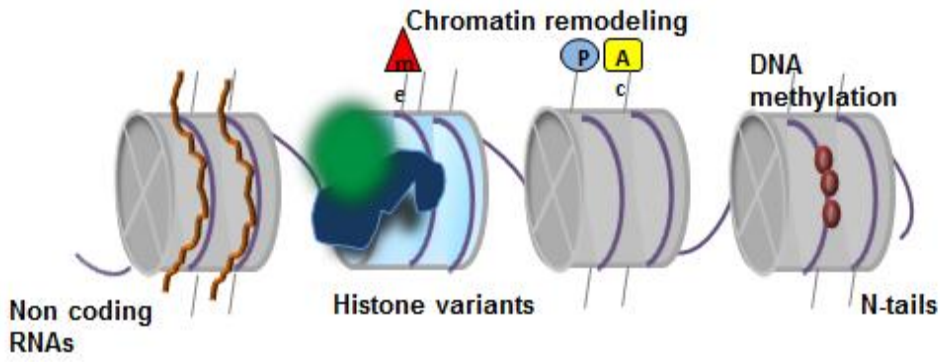

Figure 1.1: Diagrammatic representation of different regulatory mechanisms involved in altering chromatin architecture. Chromatin remodeling is achieved broadly by post translation modifications at the N-terminal tail of histones, exchange of canonical histone with variants, chromatin remodelers and non-coding RNAs.

Introduction

1.1. Epigenetics:

Historically, the word "epigenetics" was used to describe events that could not be explained by genetic principles. Conrad Waddington (1905–1975), coined the term epigenetics that is defined as "the branch of biology which studies the causal interactions between genes and their products, which bring the phenotype into being" [6]. Epigenetics in broad sense is a bridge between a genotype and a phenotype- a phenomenon that change the final outcome of a locus or a chromosome without changing the underlying DNA sequence. There are number of actors involved in this play namely; DNA methylation, chromatin variations: covalent, non-covalent modifications and exchange of histone variants [7].

In interphase nuclei the genome is distributed in a non-random fashion reflecting the major nuclear functions. The genomic DNA forms complexes with proteins which represent not only important structural components of chromatin but exert prominent regulatory control functions on the genome such as transcriptional activation or repression, which is reflected in the intranuclear extent and distribution pattern of the respective chromatin compartments. Histones are associated with the DNA of eukaryotes to form chromatin. The basic unit of chromatin is the nucleosome core particle, which contains 145–147 base pairs of DNA. The DNA wraps around the surface of the histone octamer, composed of two copies of the four core histones, H2A, H2B, H3, and H4. The H3 and H4 histones form a tetramer $(H3–H4)_2$; this tetramer organizes the central 70 bp of the DNA for the further addition of the two flanking H2A–H2B dimers [8]. In each cell cycle, a sufficient amount of core histones must be synthesized to provide 20 million new nucleosomes for packaging the newly replicated daughter strands. Not surprisingly, the synthesis of the canonical core histones is tightly coupled to the cell cycle [9,10].

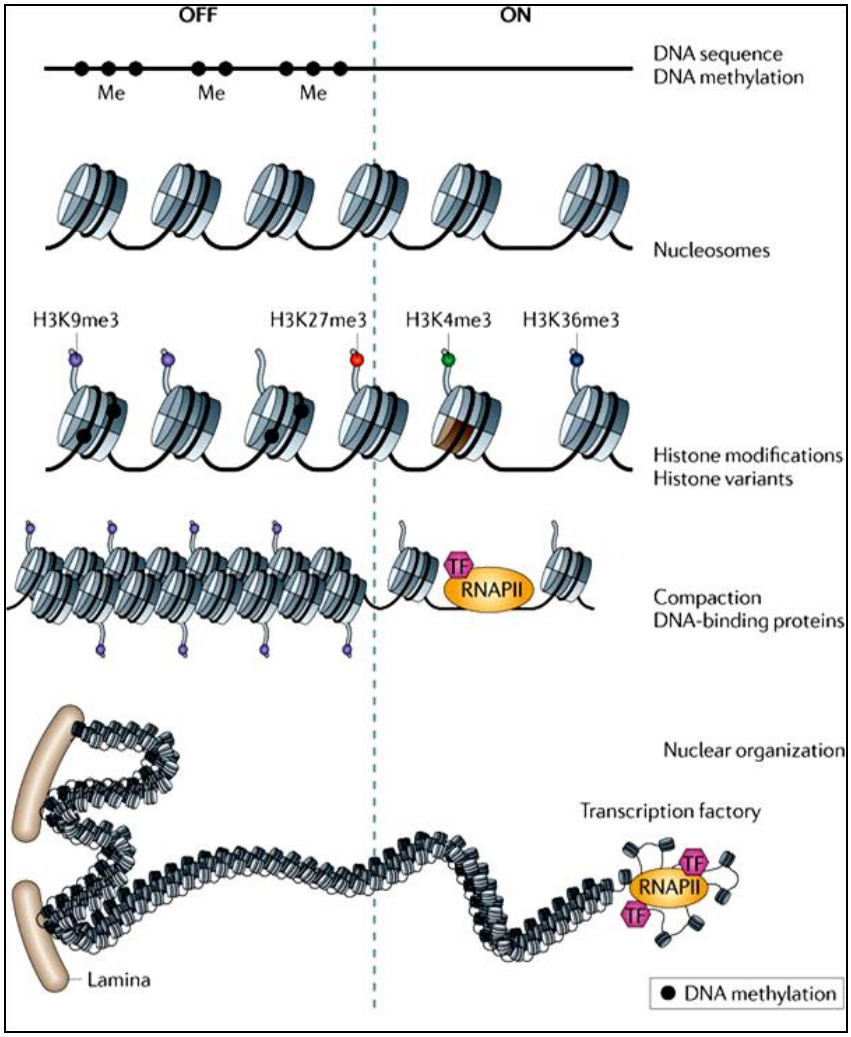

Figure 1.2: Broadly, features at different levels of chromatin organization are generally are associated with inactive (off) or active (on) transcription. From the top, genomic DNA is methylated (Me) on cytosine bases in specific contexts and is packaged into nucleosomes, which vary in histone composition and histone modifications; these features constitute the primary layer of chromatin structure. Here, different histone modifications are indicated by colored dots and histone variants such as H2A.Z are brown. DNA in chromatin may remain accessible to DNA-binding proteins such as transcription factors (TFs) and RNA polymerase II (RNAP II) or may further be compacted. Chromatin can also organize into higher order structure such as nuclear lamina-associated domains and transcription factories. Each layer of organization reflects aspects of gene and genome organization [11].

Both transcriptional and posttranscriptional regulatory controls exist to ensure sufficient histone levels and prevent the accumulation of excess histones. Interestingly, histone mRNAs lack a poly (A) tail and instead have a stem-loop structure at the 3`end, which is important for stability and translation in mammals [12]. Failure to regulate histone levels can have profound consequences on cell-cycle progression and genome stability. DNA methylation and PTMs of histones are

widely viewed to be conveyors of epigenetic information that is independent from the underlying genomic sequence. PTMs of histones are crucial for defining and maintaining the epigenetic state of chromatin [13,14]. The abundance of lysine residues within the non conserved histone tails and their ability to be post translationally modified provides for a massive combinatorial repertoire, denoted a "histone code," and has the potential to regulate many chromatin-templated functions [15]. These regulatory functions potentially include transcription, replication, repair, recombination, and chromatin condensation and segregation. In addition, the histone tails not only contact the DNA wrapped around the histone octamer, but also bind to linker DNA and the acidic patches of the neighboring nucleosomes. These interactions between histone tails and DNA may play critical roles in the formation of higher-order chromatin structure [16]. Finally, several of these PTMs and histone variants can contribute to the specific marking of chromatin states, which in some cases can be stably propagated through multiple cell divisions; as such, they are believed to be purveyors of epigenetic information.

Further, multiple histone variants further contribute to the diversity of chromatin structure and function and adding another layer of complexity to the regulation of gene expression [17,18,19].These mechanisms have to be plastic and able to dynamically respond to tissue-specific and developmental demands.

1.2. Histone modifications:

Histones are subjected to wide variety of histone modifications at their N-terminal tails that serves as regulatory registers on to which epigenetic signals can be imprinted. The PTMs of histones includes, but is not limited to, methylation, acetylation, SUMOylation, ubiquitination, and ribosylation of lysine residues and phosphorylation on serine and threonine residues. Notably, these modifications are

reversible. Acetylation of histones has long been implicated in the control of transcription, whereas loss of acetylation on histones is correlated with silencing [20]. Histone acetylases purified from *Tetrahymena thermophila* is homologous to the yeast transcription factor GCN5, whereas a mammalian histone deacetylases is related to Rpd3p [21], a negative regulator of transcription in yeast [22], both suggest a close relationship between acetylation and transcriptional regulation. Histone modifications pattern and regulation of gene expression have converged into the 'histone code' hypothesis, initially proposed by Strahl and Allis and Turner [23]. According to the hypothesis, different combinations of histone covalent posttranslational modifications influence chromatin organization that leads to diverse transcriptional outputs and regulate cellular functions.

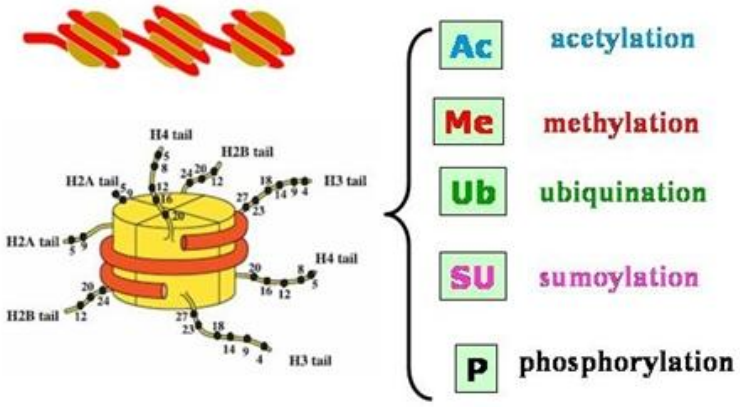

Figure 1.3: The figure illustrates nucleosome models and major post-translational modifications that play essential roles in gene expression regulation and disease processes (https://lithium.gsu.edu/Zheng.php).

The most studied histone PTMs are acetylation and methylation of lysine residues on H3 and H4 histones [24]. Addition and removal of these histone marks occurs by specific enzymes; histone acetyl transferases (HATs) and histone methyltransferases (HMTs) introduce acetyl and methyl groups respectively whereas removal of these marks take place by the activity of histone deacetylases (HDACs) and histone demethylases (HDMs) [25,26,27]. The active or repressed state of gene expression

associates with the specific amino acid residue and type of modification it undergo. Usually, transcriptionaly active state is marked by the histone acetylation whereas histone methylation associates either with an active or a repressed state, depending upon the modification of specific amino acid and its precise location [28]. Moreover, histone modifications functions in a well orchestrated complex network of multiple "cross talk" mechanisms [2,29] besides their interconnection with DNA methylation machinery [30,31]. This accounts for their possible role in several cellular processes such as chromatin organization, DNA repair, replication and gene transcription, leading to cancer development.

1.3. Histone variants:

Histone variants are non-allelic isoforms of canonical histones that differ in both, their primary sequence as well as their expression timing. As mentioned earlier histone variants differ in their genomic location and usually present outside the clusters. They can be grouped as replication dependent (RD) and independent (RI) on the basis of their phase of expression during cell cycle [32]. Having said that, variants differ from their canonical counterpart in the amino acid sequences; the extent of divergence becomes the base for categorizing them as homomorphous (few amino acids) and heteromorphous variants. These slight differences in the histone variants may result in distinctive PTMs marks that in turn act as target site for the recruitment of specific chromatin modifier altering the chromatin architecture. Histone variants can be expressed irrespective of tissue type or they may be highly tissue specific (H2ABbd, testis H1) [33,34] and their incorporation into the nucleosome need assistance of supplementary factors, termed as histone chaperones.

Histone variants have been associated with number of cellular functions such as DNA repair, transcription regulation, and replication thus, presenting their emerging

importance in various disease conditions as well, markedly cancer. Variants for H1, H2A, H2B and H3 with their functional implication on the cellular processes have been reported so far [35]. However, H4 variants being the slowest evolving eukaryotic proteins are only described in Tetrahymena [36] trypanosomes [37]and the urochordate, *Oikopleura dioica* [38].

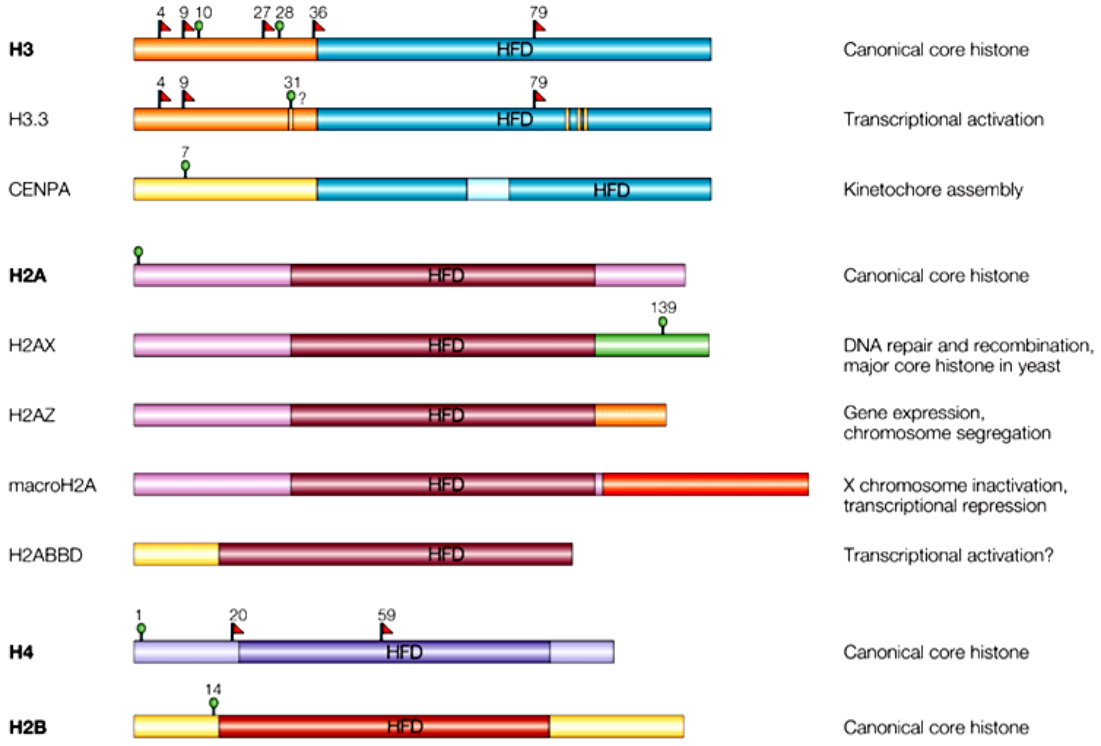

Figure 1.4: Histone variants: Histones contain a conserved histone fold domain (HFD) and N- and C-terminal tails that harbor post-translational modifications. (Red flag: K-me, green circles: S-ph). H3.3 variant residues that differ from canonical variant H3.1 are highlighted in yellow. CENP-A has a unique N-terminus, which does not resemble other core histones. The C-terminus of H2A.X harbors a conserved serine residue (S139), the phosphorylation of which is an early event in response of DNA double strand breaks. MacroH2A has a C-terminal macrodomain, exact function of which is unknown. H2A.Bbd is smallest of H2A variants and lacks all of the conserved modification sites. C-terminus is truncated [35].

In the H3 family of histone variants the homomorphous variants H3.1 and H3.2 show strictly replication dependent behavior whereas H3.3 express in replication independent manner. H3.1 and H3.2 differ only at single amino acid position i.e., 96 amino acid Cys-to-Ser substitution. H3.3 is almost identical to H3.1 and differs at

only four positions; one in the N-terminal tail (A31) and three in the histone fold domain (S87, V89, M90).

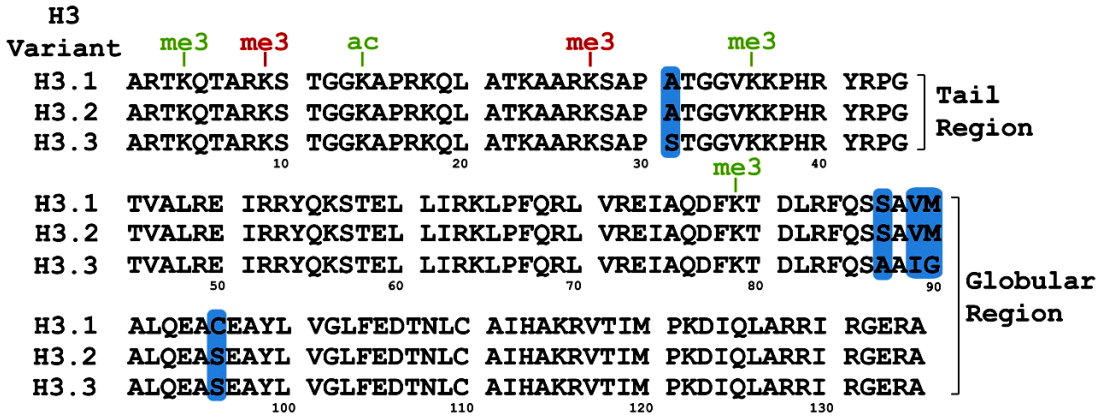

Figure 1.5 Sequence alignment of human canonical H3 variants. Changes in the amino acid composition are highlighted in blue. Modifications generally correlating with active transcription are shown in green, and those correlating with silenced chromatin are shown in red [39].

Inspite being expressed in replication independent manner [40], H3.3 is located within the histone gene cluster. These changes inspite being few in numbers surprisingly results alterations in their function and deposition pathways. H3.3 can be incorporated into the functional chromatin regions by distinct factors, such as HIRA (HIR histone cell cycle regulation defective homolog A), DAXX (death-domain associated protein), ATRX (X-linked a-thalassaemia retardation syndrome protein), CHD1 (chromodomain helicase DNA binding protein 1), CHD2 and DEK protein [41]. HIRA has been assigned to deposit H3.3 throughout the cell cycle across the genome. However, recently it has been reported that ATRX, and its co-factor DAXX, regulate H3.3 deposition at non-coding genomic loci, including telomeres and pericentric heterochromatin. Other well-illustrated H3 variants are the centromeric H3 variant (CenH3; CENP-A in humans, Cse4 in yeast and CID in fruit fly), rapidly evolving and essential for kinetochore assembly and is crucial for mitosis [42]. In particular, histone variants restrain an inimitable capability to control

key cellular and developmental processes, and, when deregulated, might contribute to cancer initiation and progression.

Certainly, emerging reports indicate towards the association histone variants to cancer biology [43,44]. Large number of H2A variants has been meticulously studied over the past decade; with respect to the expression level of particular variants altering with tumor progression in a number of different tumor types. Remarkably, the first histone variant mutations have recently been revealed in cancer (H3.3 in GBM) [45] and, thus, pointing towards better understanding of histone variants biology in various pathophysiological conditions and switching them from bench to bed side as prognostic indicators in cancer.

1.4. Epigenetics and cancer:

The epigenetic regulations of gene expression in combination with genetic alterations augment cancer development.

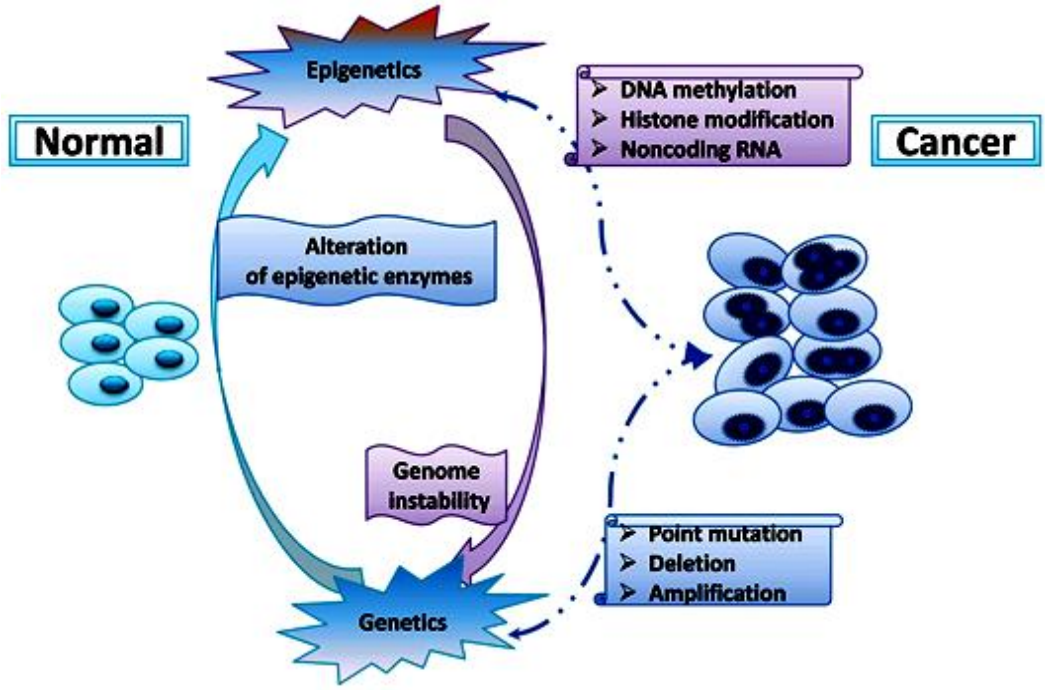

Figure 1.6 Epigenetic alteration in cancer (adapted from [46]) During cancer formation, a large number of epigenetic modifiers are mutated or abnormally activated. At the same time, epigenetic changes such as DNA methylation, histone modifications and microRNAs lead to abnormal gene expression which evoke genome instability.

This is apparent from all facets of tumor biology including cell growth and differentiation, cell cycle control, DNA repair, angiogenesis, migration, and circumvention of host immuno surveillance. On the other hand, such mechanisms are also essential in maintaining various normal physiological processes including cell differentiation, embryogenesis, genomic imprinting, inactivation of chromosome X and anti-viral properties [47,48].

1.4.1. Liver cancer:

Alterations in the epigenetic machinery have been correlated with the pathophysiology of many diseases [49,50] including Hepatocellular carcinoma (HCC) [51,52,53]. Hepatocellular carcinoma (HCC) is the most prevalent primary malignancy of the liver, ranked as 6th most occurring type of cancer and the 3rd in causing extensive rates of lethality worldwide, that is ~700,000 deaths occurring each year [54].The tumors are unexpectedly resistant to conventional therapies such as chemotherapy and radiotherapy. Liver transplantation or tumor ablation is the only effective therapy for HCC treatment with restriction based on tumor size. However, recurrence or metastasis is quite common in patients who have had a resection and survival rate is 30% to 40% at 5 years postoperatively.

Hepatocellular carcinoma is grouped into 4 different categories; well, moderately, poorly differentiated and undifferentiated tumors, respectively. HCC arises as a well differentiated cancer and continues with a stepwise dedifferentiation process. HCC can originate from mature hepatocytes or stem cells and thereby, categorized as an epithelial tumor [55]. HCC development is a deliberate process, and generally requires 10-30 years from the initiation step to the fully malignant phenotype. This process is mediated by the damage and death of hepatocytes occurring due to

structurally aberrant chromosomes and genes repeatedly as a result of telomerase reactivation with marked genomic instability. The heterogeneous malignant phenotype is the consequence of irreversible alteration of genes and chromosomes. The pathological changes observed are chronic inflammation, massive cell death and necrosis, fibrosis and cirrhosis, with the cycles of regeneration, and finally dysplasia and followed by HCC. Recent studies indicate that cirrhosis is associated with senescence arrest of hepatocytes, from which HCC cells must emerge through the bypass of senescence control. A number of risk factors have been associated with HCC and these may alter according to the geographical region, adding complexity to the extrapolation of data obtained from one region and applying it to others. For example, chronic hepatitis B virus (HBV) infection is prevalent in many Asian countries and Africa, whereas hepatitis C virus (HCV) is dominant in Japan and the United States [56].Other factors include exposure to hepatitis viruses [57,58], vinyl chloride [59], tobacco [60], foodstuffs contaminated with aflatoxin B1 (AFB1) [61], heavy alcohol intake [62], nonalcoholic fatty liver disease[63], diabetes[64], obesity [63], diet, coffee [65], oral contraceptives [66], and hemochromatosis [67].

The study in the animal models of carcinogenesis compensate for the unavailability of biopsies from the patients along with the additive benefit of reproducibility of particular stage of cancer and corresponding normal tissue for comparative analysis. HCC can be induced by various methods that includes; exposure of chemicals such as N-nitrosodiethylamine (NDEA), transgenic expression of protooncogenes, chronic liver inflammation by genetic manipulation using surface antigen of hepatitis B virus. Within the hepatic lineage, there are four levels and each may respond differently on the exposure to carcinogenic treatment.

1.4.2. Rat model for hepatocellular carcinoma:

Hepatocarcinogenesis is a multifarious process associated with accumulation of genetic and epigenetic changes occurring during the initiation, promotion, and progression of the disease. The increasing incidence of HCC has generated intense research to understand the physiological, cellular, and molecular mechanisms of the disease with the hope of developing new treatment strategies. In NDEA induced HCC, the mature hepatocytes respond giving rise to sequential cellular changes from clear and acidic cell foci to mixed and basohillic cell foci, along with the abundance in ribosomes and loss of glycogen content in the neoplastic nodules of HCC [69]. NDEA is DNA reactive post bio activation facilitated by p450 isozymes in liver, resulting in formation of an electrophile ethyl diazonium ion intermediate that reacts with the neutrophils including nitrogenous bases to form DNA adduct.

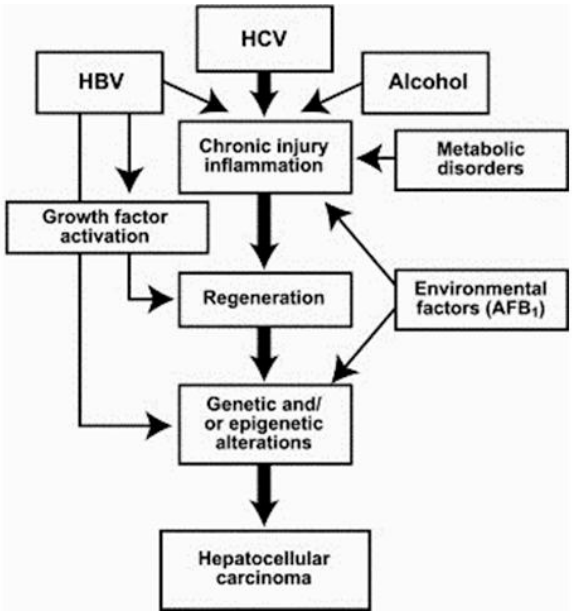

Figure 1.7: Pathogenesis of hepatocellular carcinoma adapted from [68] Hepatocellular carcinoma (HCC) associates with chronic hepatitis or chirrhosis. Chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV) and chronic alcohol abuse results in sustained inflammation and regenerative hyperplasia. Aneuploidy and multiple genetic alterations are majorly present in HCC.

1.4.3. DNA methylation and cancer:

Perhaps the finest categorized epigenetic modification is DNA methylation. The cytosine is methylated in the C-5 position by a family of DNA (cytosine-5) methyltransferases (DNMTs) mediated by the universal methyl donor Sadenosyl- L-methionine (SAM). CpG dinucleotides are underrepresented in the human genome due to spontaneous deamination of 5-methylcytosine to thymine during evolution. A significant fraction of the CpG dinucleotides left in the genome are located in CpG islands, which are GC-rich regions that possess high relative densities of CpG. They are primarily positioned at the 5` ends of majority of genes and are usually unmethylated regardless of the expression status of the associated gene. This covalent addition of a methyl group to cytosine residues is inherited through mitotic divisions and, at some loci, transcends meiosis as well [70]. Cancer cells display global hypomethylation and a hypermethylation of CpG islands. Loss of DNA methylation is associated with activation of proto-oncogenes like c-jun, c-myc as well as H-Ras and also responsible for genomic instability [71].

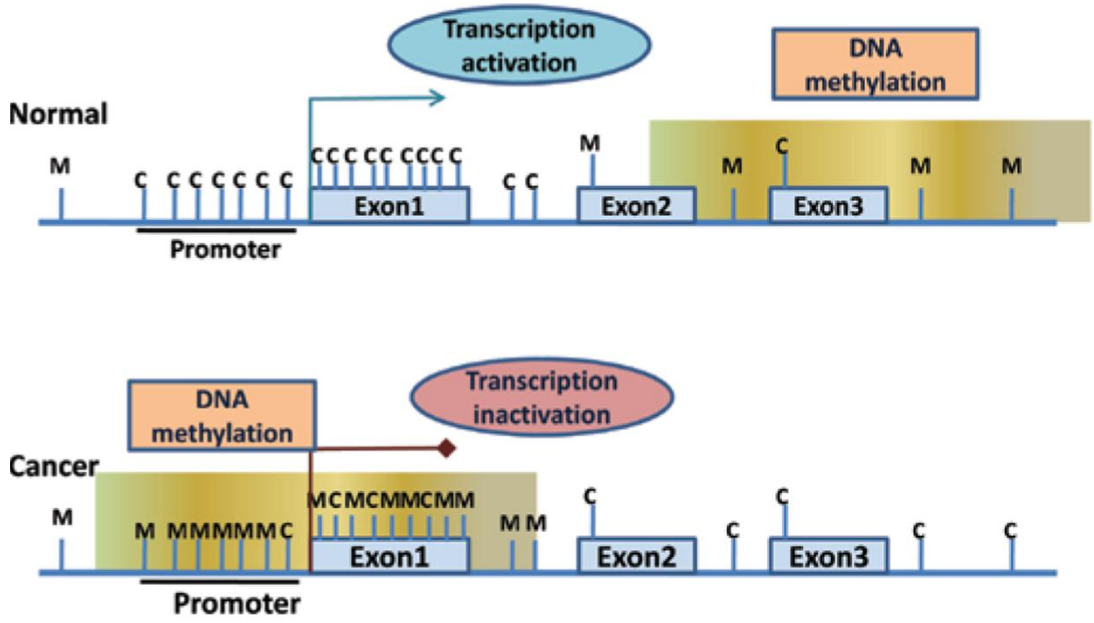

Figure 1.8: DNA methylation adapted from [46]: The diagram represents the differential DNA methylation at the gene promoter during normal and cancer conditions. The hypermethylation of the promoter region inactivates the downstream gene transcription.

On the other hand, methylation of CpG dinucleotides is associated with silencing of tumor suppressor genes. APC, E-cadherin, p16Ink4a, SOCS-1, GSTP1, RASSF1A, Cox-2, CFTR, CCND2, and RIZ1 genes are found to be frequently methylated and thus silenced in HCC [72] hTERT expression is also regulated by DNA methylation [73]. Some micro RNAs including miR-127, that is strongly silenced and/or down-regulated in cancer cells that was mediated by hypermethylation of the putative miRNA promoter region and could be reversed only by the combination of a DNA demethylating agent (5-aza- 2'-deoxycytidine) and a histone deacetylase inhibitor (4-phenylbutyric acid) [74]. Also, miRNA-1 has shown to be silenced by DNA methylation and associated with liver cancer [75]. Abnormal hypermethylated or hypomethylated genes in cancer can serve as biomarkers for clinical use in early detection, tumor classification, and response to treatments such as target therapy, epigenetic agents, and traditional chemotherapy drugs. A number of studies have verified that methylated tumor-related genes in the peripheral blood or tissue of cancer patients are potential markers for disease, metastatic potential prediction, and therapeutic response. The aberrant methylation of the Vimentin [76] and WIF-1 gene has been reported as common event in hepatocarcinogenesis [77]. The methylated SPINT2, SRD5A2, AFP, and PIVKA-II were the best predictors for detection and RELN gene hypermethylation coincides with the occurrence of tumor [78]. Moreover, an analysis of the clinical data revealed an inverse correlation between RELN expression and HCC recurrence. The methylation status of RASSF1A, CCND2, and SPINT2 showed high sensitivity, specificity, and accuracy in discriminating between HCC and non-HCC tissues and correctly diagnosed all early cases of HCC [72]. The reports suggests that the epigenetic changes in the RASSF1A, p16, and p15 tumor suppressor gene loci in serum DNA may be valuable

biomarkers for early detection in populations at high risk of HCC [79]. DNA methylation changes in HCC are not isolated events; they occur in an environment of large-scale disruptions of the cellular epigenome that also include alterations in histone modification patterns.

1.5. Histone Modifications and cancer:

Altered profile of histone modification have been reported in cancer, and discuss how these epigenetic changes, caused by alterations in histone modifying enzymes, can contribute to the development of a variety of human cancers, referred as histone onco-modifications and these subsequently changes critical cellular processes, like aberrant gene expression, DNA repair, genomic instability etc [80].

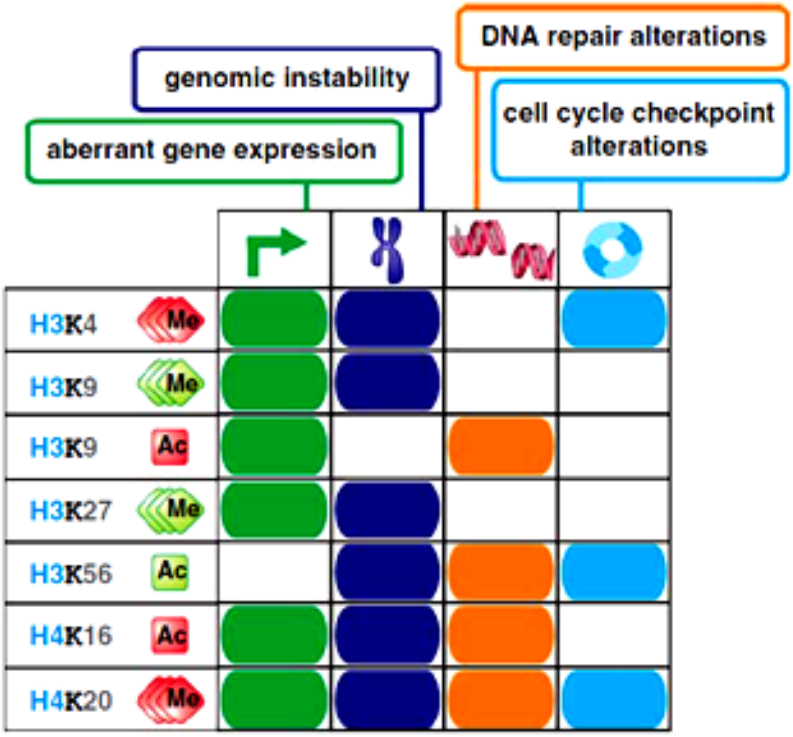

Figure 1.9.Functional consequences of histone onco-modifications Specific histone modifications, which have been shown to occur in cancer cells, are displayed and their implication in cancer associated processes, such as aberrant gene expression (in green), genomic instability (in purple), DNA repair (in orange) and cell cycle checkpoint alterations (in blue). ac, acetylated; H, histone; K, lysine; me, methylated[80].

Multiple PTMs have been reported to alter in case of Hepato Cellular Carcinoma (HCC) [52]. For instance, hypoacetylation of lysine residues at histone H3 and H4 associates with a number of transcriptionally repressed genes in human HCC. Predominantly HCC associates with chronic viral infections of hepatitis B and C [81,82] several studies have reported aberrant alterations in histone modifying enzymes in viral-induced hepato-carcinogenesis. Direct interaction of oncogenic X-protein (HBx) of hepatitis B virus (transcriptional regulator acting on both viral and cellular promoters) has been shown with histone acetyl-transferase complex CBP/p300 thereby altering gene expression leading to tumorigenesis [83]. Similarly, an increased level of repressive methylation marks at histone H3 lysine 9 and histone H3 lysine 27 on the promoter of RIZ1, p16INK4A and RASSF1A tumor-suppressor genes are the characteristic feature of human HCC. Histone methyltransferases EZH2, RIZ1, and SMYD3 specifically methylates lysine residues 4, 9 and 27 at histone, respectively. EZH2 (KMT6) encodes the catalytic domain of the Polycomb Repressive Complex 2 (PRC2) responsible for transcriptionaly silencing H2K27Me3 histone marks resulting in transcription repression. EZH2 over expression has been associated with the silencing of growth-suppressive Wnt antagonists, which in turn activates constitutive Wnt/b-catenin signaling and extensive proliferation of HCC cells [84]. In contrast, SMYD3 over expression promotes HCC proliferation by creating transcription active H3K4Me marks thus resulting in subsequent activation of downstream genes, NKX2-8 gene, reported to be upregulated in HCC. Lastly, another HMT RIZ1 responsible for trimethylation of H3K9 has been frequently found to be down regulated in HCC. Along with the gene specific aberration of histone modifications, HCC presents genome wide alterations in the histone

modification pattern, in particular, increase of H3K27Me3, H3 phosphorylation and loss of H4K20Me3 [47].

1.6. H2A variants and cancer:

The H2A family is the largest family of variants among the core histones studied so far (19 in humans). These are coded by 26 genes with majority of them present in cluster 1 and 2 and a single gene in cluster 3. Out of these, 9 genes are not part of any cluster and code for atypical histone H2A such as macro-H2A, H2A-Bbd, H2A.X etc. which exhibit significant variability in amino acid sequence between each other [85].

H2A.X

In 1980 H2A.X was first identified in human cells and from then on extensive work has been done in understanding the functional aspect of this variant especially in response to DNA damage and nominated as "histone guardian" of the genome [86]. H2A.X is highly conserved and constitutes 2-25% of total H2A depending upon cell and tissue type. The probable reason for such difference in the relative amounts of H2AX remains to be unknown, although they might occur due to the unique regulation of H2AX synthesis.

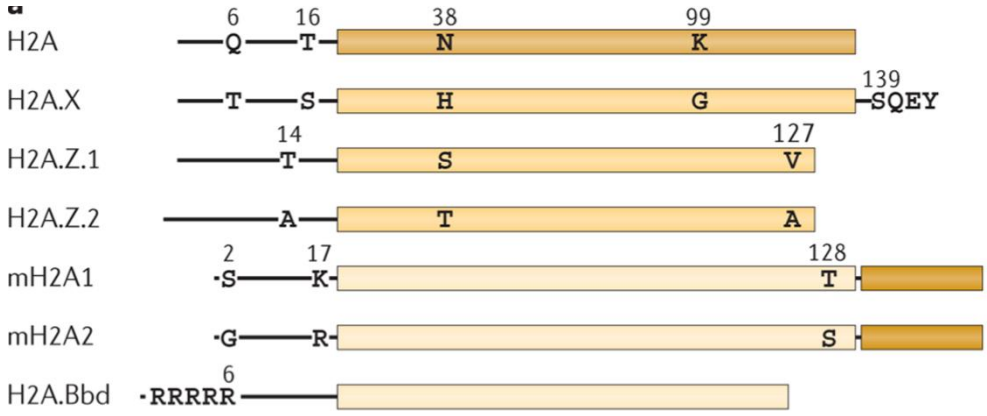

Figure 1.10: Human H2A core histone and variants. Variants of the core histones H2A are shown. Unstructured amino-terminal tails are shown as black lines. Specific amino acid residues are depicted at key differences among variants of a common histone protein family (for example, H2A.X). Different shades of colour are used to indicate protein sequences that are highly divergent between canonical histones (which is listed first) and its variants [44].

H2AFX gene coding for H2A.X has been mapped to chromosome 11 positioned 11q23 in humans. The H2AFX gene coding for H2A.X contains landscape of both replication dependent and replication-independent histone species. The small intronless gene code for H2A.X transcript having a stem-loop structure at its 3`end, characteristic of replication dependent histones, moreover, it also encodes transcript with additional poly (A) site read approx 1kb downstream of the stem loop, peculiar to replication independent histone species. The plausible role of this dual mechanism for translational regulation might be to ensure the presence of sufficient H2A.X during genome replication for efficient DSB detection and in G1 and G0 phases of cell cycle [87].

H2A.X is highly similar to canonical H2A in primary structure however, its unique feature is a longer C-terminal extension having a serine–glutamine (SQ) motif followed by an acidic and hydrophobic residue [SQ (E/D)φ] [88]. This motif is conserved throughout species with respect to its sequence and position relative to the C-terminus [89]. The immediate response to DNA damage in eukaryotic cells is the phosphorylation of serine 139 in the C-terminal tail of H2A.X, this yields a particular modified form known as γH2AX, and act as target site for the recruitment of DNA repair machinery factors to the DSBs with the simultaneous increase of γH2AX proportional to the severity of DNA damage [90].

H2AFX gene coding for H2A.X has been mapped to chromosome 11 positioned 11q23 in humans, reported to be frequently mutated or deleted in extensive number of human cancers with majority of haematopoietic malignancies such as acute lymphoid leukemia and acute myeloid leukaemia. Recently, a study demonstrated a strong association of a single nucleotide polymorphism upstream H2AFX and

follicular lymphoma, a subtype of non-Hodgkin lymphoma, and mantle cell lymphoma [91,92]. The tumor suppressing ability of H2AX has been studied in gastrointestinal stromal tumour (GIST) cell lines and on treatment of cells with Imatinib mesylate the clinically permitted protein kinase inhibitor H2A.X expression gets upregulated and triggers apoptosis. Additionally, promoter hypermethylation of H2AFX is responsible for reduction of H2A.X in lung squamous cancer and decrease in the H2AFX gene copy number has been demonstrated in MCF7 breast cancer cell line, corroborating with the latest study on tumours from patients with breast cancer that showed 37% alteration in H2AFX copy numbers and. The amplification of 11q13 and loss of distal 11q regions of chromosome 11 containing H2AFX gene is characteristic of Head and neck squamous cell carcinoma [92]. The potential role of H2A.X in DNA repair and maintaining genomic stability, it has been proposed to utilize H2A.X/ γH2AX as markers for initial cancer detection, prognosis and therapeutics. Therefore, the number of enriched γH2AX foci at DSBs, may serve as a sensitive technique to monitor either cancer progression or response to treatment. Collectively, these findings indicate toward the contribution of H2A.X in tumour development, progression and resistance to therapy in certain cancer subtype.

MacroH2A

Among all other variants of H2A histone, macroH2A is the most divergent from its canonical counterpart. The tripartite structure of macroH2A consists of a N-terminal histone-fold that 64% identical to canonical H2A, an intrinsically unstructured linker domain and a C-terminal macro domain of 30kDa, resulting in protein three times larger than canonical H2A [85,93]. Localization of macroH2A has been reported near transcription start sites (TSSs) and CTCF-binding sites of the transcriptionaly

silenced senescence-associated heterochromatic foci [94,95]. The possible reason for this selective genome wide localization on silenced regions has been associated with the hindrance due to incorporation of macroH2A in transcription factor binding if the binding site is in proximity to the nucleosome dyad axis and nucleosome remodeling by SWI/SNF complexes [96]. *In vitro* macroH2A inhibits p300-dependent histone acetylation and it also interacts with histone deacetylases resulting in co-precipitation with hypoacetylated chromatin [97].

In mammals, two macroH2A genes (*macroH2A1/H2AFY* and *macroH2A2 /H2AFY2*) are present that codes for proteins macroH2A1 and macroH2A2 that share the similar overall domain structure with 68% identity in their amino acid sequences.

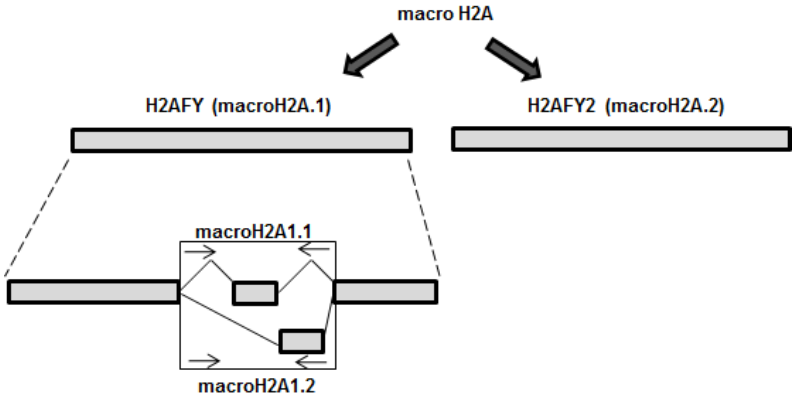

Figure 1.11 Schematic representation of macroH2A isoforms:
macroH2A1/H2AFY and macroH2A2 /H2AFY2 genes code for macro H2A.1 and macro H2A.2. Alternative splicing of macro H2A.1 gene results in two isoforms; macro H2A1.1 and macro H2A1.2

The macroH2A.1/*H2AFY* gene contains two mutually exclusive exons that undergo alternative splicing to generate isoforms, macroH2A1.1 and macroH2A1.2 [98,99]. The difference within the two splice variants is at a short amino acid stretch affecting the size and hydrophobicity of the binding pocket of the macro domain that in turn contributes to their differential ability in interacting with NAD metabolites. MacroH2A1.1 ability to bind NAD metabolites suggests its unique role in chromatin

remodeling in response to poly-ADP-ribose polymerase (PARP) activity that gets stimulated by metabolic stress and DNA damage [100].

The first insight into the functional aspect of macroH2A came from its discovery as a part of the heterochromatin of inactive X chromosome (Xi) and its roles in mediating X chromosome silencing and transcription repression [101]. A growing body of evidence suggests potential role of macroH2A regulating cellular proliferation. The redistribution of macroH2A into a diffuse pattern occurs upon cells re-entering the cell cycle. ATP dependent SWI/SNF helicases ATRX (Alpha Thalassemia/ MR, X-linked) has been identified to interact with all three isoforms of macroH2A [102].ATRX loss has been associated with the increased incorporation of macroH2A at α-globin gene cluster, with suppression of α-globin gene expression usually observed in patients with ATRX syndrome [103]. However, regardless of these findings, the mechanisms of macroH2A incorporation at distinct genomic loci still remain ambiguous. Interestingly, it has been reported recently; in differentiated cell the pluripotency genes are enriched with macroH2A.1 and macroH2A.2, these isoforms act as an 'epigenetic barrier' for the set of genes required during reprogramming [104]. Thus, suggesting the role of macroH2A in inhibiting cellular reprogramming and plasticity, and therefore might be potential candidate acting as barrier to tumor development.

The first report in context of macroH2A and cancer was published by Ladurner group, where they have shown expression of macroH2A1.1 and macroH2A.2 inversely correlates with cellular proliferation studied on human breast and lung tumor biopsies samples [105]. In continuation to the above finding other groups have shown similar results with significant decrease in levels of macroH2A1.1 in tumor compared to normal counterpart in wide range of tumor types such as testicular,

colon [106], lung, melanoma [107], bladder, cervical, breast, ovarian and endometrial cancer [108]. This decrease in the level of macroH2A.1.1 has been attributed to the splicing of macroH2A.1 transcript that is in particular governed by QKI (QuaKIng), a factor reported to be down regulated in certain cancer types [109]. Thus, results in down regulation of macroH2A1.1 and subsequent increase in expression of macroH2A1.2 transcript as a result of enhanced splicing. Additionally, DDX5 and DDX17 RNA helicases have been identified in regulation of alternative splicing of macroH2A.1 in breast cancer [110].

In melanoma, predominantly strong inverse correlation of macroH2A levels with metastatic potential and tumor grade has been reported. Comparative studies conducted on metastatic cell lines and lesions from metastatic patients with primary and benign tumors have shown significant correlation of melanoma malignancy with loss of macroH2A [107].The down regulation of macroH2A isoforms occurs due to the loss of transcriptional regulation of macroH2A.1 and macroH2A.2 and in particular, the silencing of macroH2A.2 is attributed to promoter DNA hypermethylation [111].

Moreover, macroH2A loss exerts melanoma progression through deregulation of CDK8 (Cyclin-Dependent-Kinase 8), identified as oncogene in colorectal carcinoma that promotes extensive cellular proliferation due to enhanced malignant transformation by β-catenin [112]. Collectively, these findings implicate macroH2A as a leader in cancer mostly due to its effect on cell proliferation and tumor invasiveness [107].

H2A.Z

H2A.Z is highly evolutionarily conserved H2A variants right from malaria-causing protozoan of *Plasmodium falciparum* to *Homo sapiens*, and share 60% sequence

identity with canonical H2A. H2A.Z is constitutively expressed throughout the cell cycle and is incorporated into chromatin in a replication independent manner [113]. Once incorporated into the nucleosome H2A.Z governs plethora of cellular processes such as maintenance of heterochromatic boundaries, epigenetic memory, genome stability, chromosome segregation, transcription regulation and many more such events. The best studied role of H2A.Z is in the context of transcriptional regulation where it has been demonstrated that H2A.Z marks the promoter regions of gene to poise the chromatin for rapid transcriptional activation upon stimulation [114]. Interestingly, it has been found that H2A.Z demonstrates both, activating as well as repressive effect on transcription. This contrasting impact on transcription can be explained by the accumulating evidences that suggest the effect of H2A.Z on nucleosome mobility and positioning. As a result of such alterations, the incorporation of H2A.Z could differentially affect the binding of both activating and repressive regulatory factors on their respective target sequences. In addition to gene promoters, H2A.Z is associated with other regulatory regions like enhancers and insulators. H2A.Z incorporation also prevents the spread of heterochromatin into euchromatin on associating with the heterochromatin protein HP1α at a range of constitutive heterochromatic domains in various mammalian cell types. The incorporation of H2A.Z into the chromatin is governed by ATP-dependent chromatin remodeling complexes, including the Swr1 complex in yeast, or by its orthologs p400 and SRCAP (Snf2-Related CBP activator protein) in mammals [115]. INO80 complex has recently been found to play crucial role in H2A.Z eviction from the nucleosome in yeast [116], thus, opening up a new challenge to figure out the existence and implication of this process in higher eukaryotes. Overall, it is the combinatorial effect of H2A.Z, TFs and other targeting factors working in

cooperation with the subsequent PTMs on nearby histone thereby influencing gene expression.

The first incidence of H2A.Z over-expression in cancer was reported in sporadic colorectal tumors using genome wide gene expression profiling [117]. The over-expression of H2A.Z has also been reported in genitor-urinary cancers, such as prostate and bladder cancer [118]. In bladder cancer, the alteration in the expression and localization of H2A.Z has been correlated with activation of cell growth regulatory genes. The genome wide study in bladder cancer cells have reported enrichment of H2A.Z near TSS of majority of proliferative and growth regulatory genes, thus establishing H2A.Z as potential candidate facilitating cancer progression. H2A.Z has been extensively studied in hormone dependent breast cancer progression. H2AFZ and SCRAP up regulation was first time reported in invasive and metastatic breast cancer compared to normal mammary epithelium [119]. Further, immunostaining and tissue microarray based screening of over 700 breast cancer patients tumor samples revealed over-expression of H2AZ.1[120,121]. Enhanced metastasis to lymph nodes and poor patient survival has been observed with over expression of H2A.Z.1. The mechanism of H2A.Z.1 induction in breast cancer cells has been recently studied using genome ChIP-chip method for binding sites of estrogen receptor α (ERα) and Myc along with estrogen-stimulated gene expression arrays. In response to estrogen, stimulated ERα activates c-myc in turn positively influence H2AFZ promoter for transcription. In addition, out of the two isoforms of H2A.Z, i.e. H2A.Z.1 and H2A.Z.2, it is H2A.Z.1 found to be specifically unregulated in response to androgen treatment expression, as a result of increased binding of Myc on the *H2AFZ* promoter, as observed for estrogen-stimulated *H2AFZ* expression in breast cancer cells [122].

H2A.1 and H2A.2

H2A.1 and H2A.2 are homomorphous variants of H2A and are coded by different histone coding gene clusters localized on chromosome 1st and 6th in humans and 2nd and 17th in rats, respectively. In 1977 on the basis of electrophoretic mobility H2A.1 and H2A.2 were identified as the isoforms of canonical H2A that differ at 51st amino acid[123]. However, so far, no functional specialization of these canonical H2A isoforms has been demonstrated. Earlier studies from that lab have reported, that the over-expression of H2A.1 and decrease in the level of H2A.2 variant associates with the sequential progression of hepato cellular carcinoma (HCC) [124].

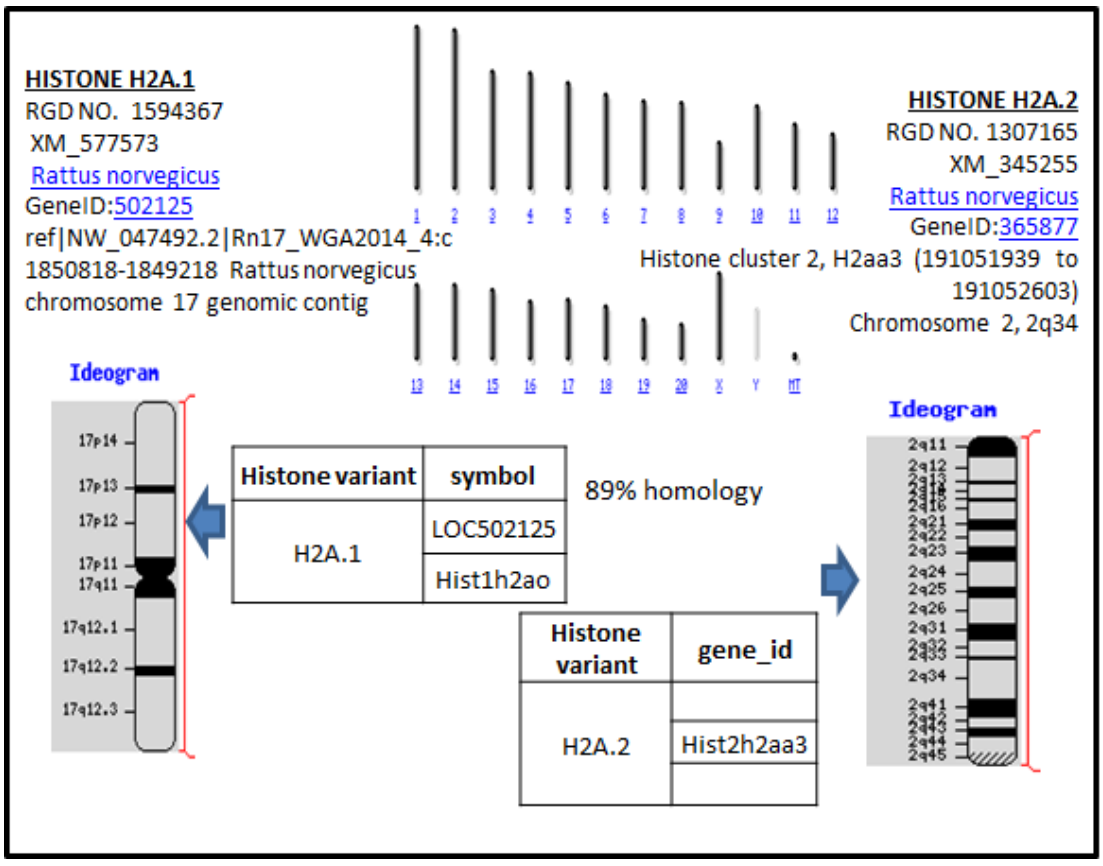

Figure 1.12: Chromosomal distribution of rat histone H2A.1 and H2A.2 variants: Rat genome comprises of 20 autosomes and 2 sex chromosomes out of which chromosomes 17, 2 and 10, harbor large clusters of histone coding genes. The major variants are often coded by multiple, intronless genes located in these clusters whereas the minor variants on the other hand are coded by isolated, single genes. The H2A.1coding gene has been mapped on chr.17p11 and H2A.2 at chr.2q34 of the rat genome.

On similar lines another report got published recently where the authors have shown the abundance of specific replication- dependent isoforms of histone H2A (HIST1H2AC) locus that is altered in patients with chronic lymphocytic leukemia (CLL) Comparable changes in the abundance of these H2A isoforms also associates with the cellular proliferation and tumorigenicity of bladder cancer cells [125]. Collectively these limited findings, suggests the involvement of these histone variants having the potential to significantly facilitating the construction of distinct epigenetic landscape performing specialized functions in various pathological conditions.

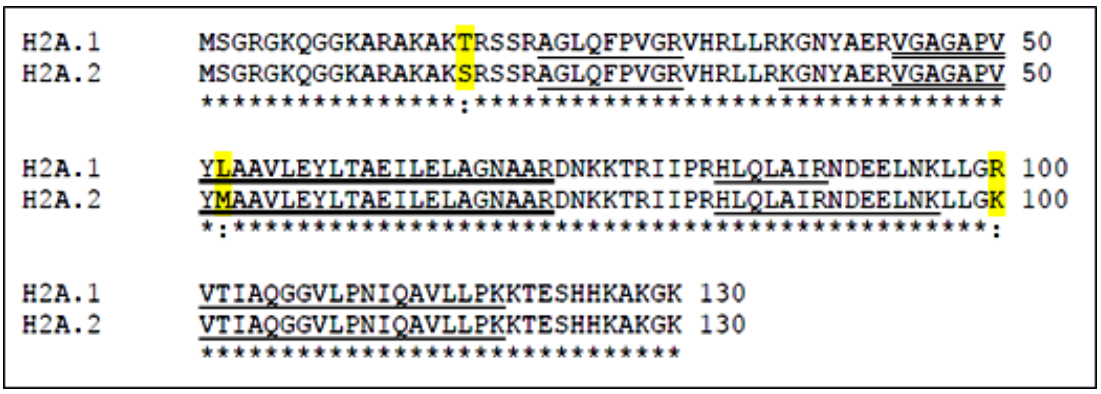

Figure 1.13: Amino acid Sequence alignment of H2A.1 and H2A.2: The amino acid sequence differs at three amino acid position i.e. 16th, 51st and 99th adapted from [124].

However, these core histone isoforms are yet to be studied in detail, to understand the mechanism and potential targets implementing known small but significant changes to the cellular physiology. This can probably be explained in light of transcription or post transcriptional regulation of these histone gene expressions.

1.7. Histone gene organization:

Each of the five histone types are encoded by a family of genes consisting of 20 to 40 copies of the coding sequences. Simple tandem repeat organization of histone genes have been demonstrated previously in sea urchin, *Drosophila* and other lower eukaryotes. However, in the higher eukaryotes including human, mouse and chicken

the histone genes are organized as random clusters containing combination of the HI and core histone genes. In mouse, the histone gene clusters are localized on chromosome 3 and 13 and in rat the cluster is present on chromosome 17, 2 and 10 whereas, in humans these clusters have been mapped at least three different chromosomes 1, 6, and 12 [126,127]. This organization possibly has to role to play in regulating their expression by bringing them together in a domain enriched with required factors, for example, the Cajal bodies in mammals. The evolution of multi-gene family of histones might be associated with the presence of interspersed repetitive elements within the clusters, giving rise to gene duplication [128].

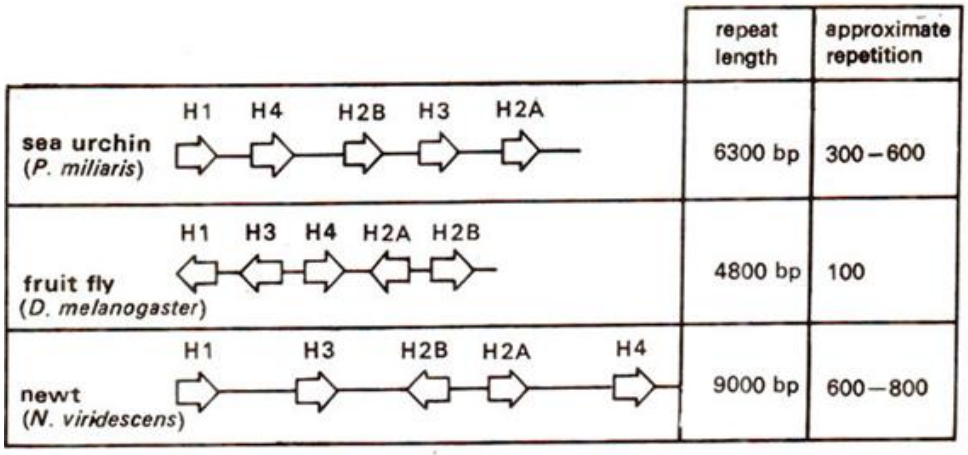

Figure 1.14: Organization of highly repeated histone gene clusters in several organisms; genes are separated by non-transcribed spacer (NTS) regions; the direction of transcription is shown by arrows; the clusters are tandemly repeated in sea urchin and fruit fly, but not in the newt (http://www.eplantscience.com/index/genetics/multigene_families_in_eukaryotes/multigene_families_with_identical_genes.php).

Histone genes have been grouped into three classes; cell cycle regulated genes, cell cycle independent genes, and pseudo-genes. Majority of the histone genes are cell cycle regulated and possess an atypical yet simple gene structure. They usually lack intron region and possess very short 5` untranslated region and a short 3` untranslated sequence which terminates in a unique 26 bp conserved stem-loop structure instead of a typical polyadenylated tail [129]. These unique characteristics

of cell cycle regulated histone gene are ideally compatible to sustain exhaustive demand of histone required during DNA synthesis for packaging into nucleosome. Whereas, the cell cycle independent histone genes codes for the minority of histone classified as histone variants [130]. They may possess a more typical structure with both introns and a polyadenylation site. Histone variants have been mentioned further in detail. At last, the pseudo-genes that have been implicated as are non-functional copies having frame-shifts in the coding region or deletions of the promoter sequences.

1.8. Histone gene promoters and transcription regulation:

Inspite of importance laid down on histone and their variants in context to various biological processes, only limited promoter sequence information for these genes is available. The well studied histone gene promoter is of H4 gene. The histone H4 proximal promoter element, designated Site II, mediates cell cycle transcriptional control. The transcription factors interacting with Site II include cdc2, cyclin A, an RB-related protein and interferon regulatory factors (IRFs) The mutational analysis indicated the critical role of distal part of Site II during cell cycle regulation. Equally important for the developmental transcriptional control, histone gene expression is repressed when differentiation is initiated. In the adipocytes, the deletion analysis indicates that the proximal Site II mediates the differentiation response of H4 gene transcription [131]. The most specific information concerning the regulation of histone gene expression during S phase has been reported for histone H2b gene expression. The cell-cycle regulation of H2B transcription has been reported to be mediated by the presence of highly conserved subtype-specific consensus element, containing the core octanucleotide ATTTGCAT that is positioned immediately upstream from the TATA element [132]. Reports on the regulation of the human H3

variant genes; H3.3A have shown the promoter region lacking a TATA box and any CCAAT boxes but a GC rich sequence upstream the transcription start had been identified. On the contrary, H3.3B demonstrated a common promoter organization with one TATA and six CCAAT boxes in addition to an Oct and a CRE/TRE motif [133,134]

1.8.1. Transcriptional Regulation:

Several crucial steps are involved that lead to productive transcription that are determined by the arrangement of a functional pre-initiation complex (PIC), followed by the initiation of transcription. Transcription initiation is the first major determining step on the intricate path from DNA to a completely functional protein tightly regulated through multiple mechanisms.

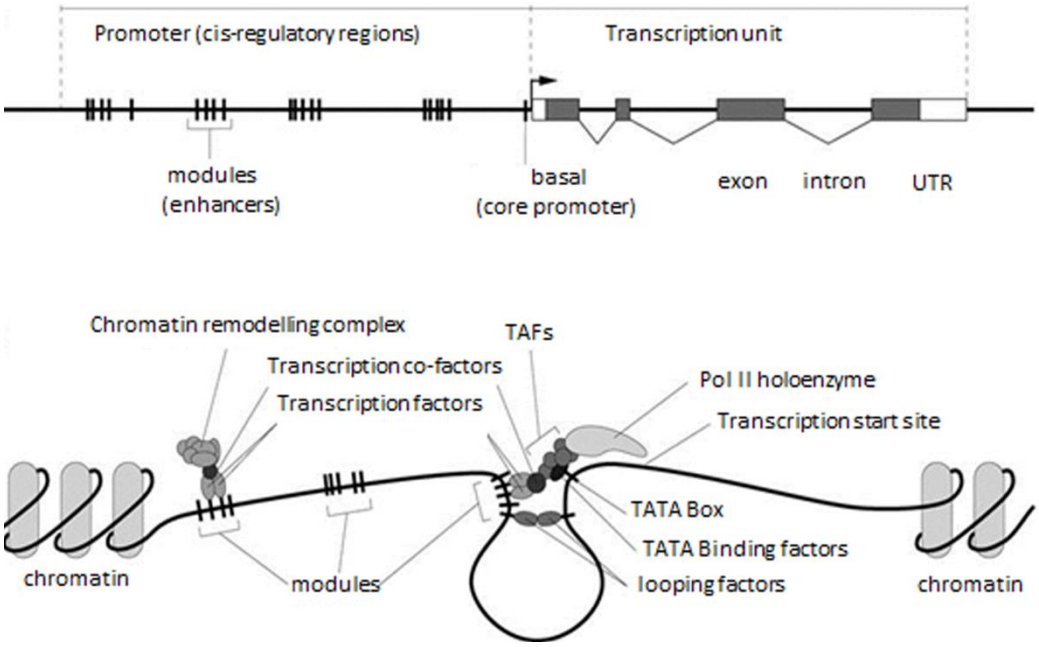

Figure 1.15: Promoter structure and function. Organization of a generalized eukaryotic gene, showing the relative position of the transcription unit, basal promoter region (black box with bent arrow), and transcription factor binding sites(vertical bars). The position of transcription factor binding sites differs enormously between loci; although they often reside within a few kb 59 of the start site of transcription (as shown here), many other configurations are possible. Idealized promoter in operation. Initiating transcription requires several dozen different proteins which interact with each other in specific ways. These include the RNA polymerase II holoenzyme complex (15 proteins); TATA-binding protein (TBP; 1 protein); TAFs (TBP-associated factors, also known as general transcription factors; ;8 proteins); transcription factors (precise composition and number bound differs among loci and varies in space and time and according to environmental conditions, but several to many any time transcription is active); transcription cofactors (again,

precise composition and number will vary); and chromatin remodeling complexes (which can contain a dozen or more proteins)[135].

Genomic locations essential in transcription initiation are (1) the core promoter, where the pre-initiation complex assembles and general TFs bind; (2) the proximal promoter, bound by tissue-specific TFs and (3) distal regulatory regions, known as enhancers or repressors, on the basis of their influence of targeted gene activity.

The core promoter elements (CPEs) are the distinctive DNA sequences on the promoter region, which discretely categorize the gene. TATA box is one of the CPEs, on the basis of its presence and absence the promoters can be broadly categorized as TATA driven or TATA-less promoters. The TATA Binding Protein (TBP) recognizes the TATAA sequence and directly binds to the DNA in association with several TBP associated factors (TAFs) as the TFIID complex. PIC formation is strictly regulated by the recruitment of TBP facilitating both positive and negative gene regulation depending on the binding of subsequent TFs on the promoter region [136]. Downstream elongation and mRNA processing is achieved by the recruitment of factors on the phosphorylated CTD of Pol II [137].

Nucleosome positioning and chromatin structure of the genomic region adds extra layers of complexity by varying the accessibility of the RNA pol II complex and TFs to the DNA. Termination of transcription is brought about by the 3` end processing machinery, that tightly regulates and couple the cleavage and polyadenylation of the nascent transcript at specific sequence elements in the pre-mRNA. This coordination helps manage gene expression and ensures insulation of nearby genes (pause play repeat). Termination is essential for the release of RNA Pol II from bound template DNA [138] and aberrant gene expression is prevented by termination of transcription by avoiding formation of antisense RNAs that can interfere with normal pre-RNA

production. However, the mechanisms underlying transcription termination remain least understood so far.

1.8.2. H2A gene promoters:

H2A families of histones in humans have altogether 15 genes. Fourteen of these are organized as H2A/H2B gene pairs, while one H2A gene is a solitary gene. Two H2A genes are reported as pseudogenes. Thirteen H2A genes code for at least 6 different H2A proteins with different amino acid sequences, referred as H2A isoforms [139]. Each H2A/H2B gene pair is controlled by a divergent promoter spanning 300 to 330 nucleotides between the coding regions of the two genes. The highly conserved divergent H2A/H2B promoters can be classified in two groups based on the patterns of consensus sequence elements. Group I promoters contain a TATA box for each gene, two Oct-1 factor binding sites, and three CCAAT boxes. Group II promoters contain the same elements as group I promoters and an additional CCAAT box, a binding motif for E2F and adjacent a highly conserved octanucleotide (CACAGCTT) that has not been described so far. Five of the 6 gene pairs and 4 solitary genes with group I promoters are localized in the large histone gene cluster at 6p21.3–6p22, and one gene pair is located at 1q21. All group II promoter associated genes are contained within the histone gene subcluster which is located at a distance of about 2 Mb from the major subcluster at 6p21.3–6p22 containing histone genes with group I promoters. Almost all group II H2A genes encode identical amino acid sequences, whereas group I H2A gene products vary at several positions [140]. The mouse H2A.2 promoter has been mapped on chromosome no.3, the gene possesses TATA and CAAT box and Sp1 transcription factor binding site [141].

Other studied members of H2A family in context of gene regulation are H2A.Z and H2A.X. Histone H2A.Z is a distinct and evolutionarily conserved member of the histone H2A family whose synthesis, in contrast to that of most other histone species, is not dependent on DNA replication. The H2A.Z gene proximal promoter contains three CCAAT and two GGGCGG elements as well as a consensus TATA element. The central CCAAT element was found to be the most important determinant of promoter activity; the two GGGCGG elements both were found to bind transcription factor Sp1, but the distal element bound Sp1 with higher affinity. Thus, suggesting the critical role of the central and proximal CCAAT elements and the distal GGGCGG element in determining transcriptional activity of the H2A.Z gene [142].

H2A.X gene encodes for two mRNA species; one is replication dependent having stem loop at 3`end and another is replication independent and polyadenylated 0.8kb downstream of the processing site. The elaborate study on the H2A.X gene of mouse indicated the region of 280bp upstream the TSS as important regulatory region with maximal promoter activity. The presence of two activating sequence within the promoter regions; one element is E2F binding site and another is CCAAT box, however these elements are not same in mouse and human inspite having conserved promoter sequences [143,144].

Therefore, from the previous finding from our lab where we have reported differential expression of H2A.1 and H2A.2 variants during sequential development of HCC. We went a step ahead to study the expression profile of these variants during embryonic liver development compared to regenerating liver. Simultaneously, we emphasized on understanding the transcriptional regulation of these gene promoters that arise one of the possible reason for differential expression of H2A

variants during HCC. We have identified the important regions of gene promoter associated with the transcription regulatory mechanism of H2A.1 and H2A.2 variants genes. This information will be significant in providing essential understanding of the regulatory factors responsible for differential expression of these variants during differentiation and de-differentiation.

Chapter 2
Aims and Objectives

Aims and objectives

Hypothesis:

Earlier report from lab has shown the differential expression of the homomorphous histone variants of H2A; H2A.1 and H2A.2 during sequential hepatocarcinogenesis. H2A.1 and H2A.2 differ at three amino positions viz. 16 (Serine to Threonine), 51 (Leucine to Methionine) and 99 (Arginine to Lysine). Leucine to Methionine alteration at 51st amino acid position is highly conserved across species. The genes encoding for these variants are mapped on chromosome 17p for H2A.1 and H2A.2 on 2q, thereby, suggesting their specific gene promoters and transcription regulation. This suggests that the substantial alteration in their expression might be contributed by differential transcriptional regulatory mechanisms. To understand the transcriptional regulation of these genes, the prime requisite is to have the experimentally identified complete genomic organization including transcriptional start site, promoter and 3'untranslated region. Therefore, to dissect the regulatory mechanism of H2A.1 and H2A.2 gene expressions following objectives were proposed.

Objectives:

1. Cloning and characterization of H2A variants transcriptional start site and promoter.
2. Identification of protein binding sites regulating expression of H2A variant promoter.
3. DNA-protein interaction studies to characterize the transcription factors.

Aims and objectives

Objective 1: Cloning and characterization of variants transcriptional start site and promoter.

3.1.1 Mapping of 5'UTR of H2A.1 and H2A.2 gene by 5'RNA Ligase Mediated-Rapid Amplification of cDNA (5'RLM RACE)

3.1.2 Mapping of 3'UTR for H2A.1 and H2A.2 mRNA by RNA circularization coupled with PCR Amplification (3'RCPA)

3.1.3 Cloning of H2A.1 and H2A.2 gene promoter in pGL3 basic Luciferase vector

Objective 2: Identification of protein binding sites regulating expression of H2A variant promoter.

3.2.1 *In silico* identification of H2A.1 and H2A.2 promoters and transcription factor binding sites by Motif finder and Bio-base software analysis.

3.2.2 Identification of *cis-acting* regulatory elements on H2A.1 and H2A.2 gene promoter using Dual Luciferase assay.

3.2.3 Profiling of H2A.1 and H2A.2 during development of liver in embryogenesis and post-partial hepatectomy by Acetic Acid-Urea-Triton PAGE (AUT-PAGE) and semi-quantitative PCR analysis.

Objective 3: DNA-protein interaction studies to characterize the transcription factors

3.3.1 *In vitro* identification of DNA binding transcription factors for H2A.1 and H2A.2 gene promoter by Electrophoretic mobility shift assay (EMSA)

3.3.2 *In vivo* identification of transcription factors binding to H2A.1 and H2A.2 gene promoter by Chromatin Immuno precipitation coupled with promoter specific PCR amplification. (ChIP-PCR)

Chapter 3
Material and Methods

MATERIALS AND METHODS:

3.1. Animal Experiments:

All the experiments were performed on Sprague-Dawley rats (spp. *Rattus norvegicus*). Experiments were performed after approval of Institute Animal Ethics Committee (IAEC), ACTREC vide project no's.09/2010 and 29/2011. Procedures for maintenance and conducting experiments were as per Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), India, standards.

3.1.1 N-Nitrosodiethylamine treatment

Hepatocellular-carcinoma (HCC) was induced in Sprague-Dawley rats as per the protocol described [69]. Sprague-Dawley rats were exposed to chemical carcinogen N-Nitrosodiethylamine (NDEA) through drinking water for a period of 45 and 120 days. Male Sprague-Dawley rats (n=30), 6-8 weeks old, were obtained from Animal House, ACTREC and were used in all experiments. Rats were randomized by weight and divided into three groups (n=6). First group which was given normal drinking water for 120 days, acted as control. Group 2 was given NDEA (Sigma, cat. N0756) at a concentration of 1ppm (v/v) per gram of animal body weight in drinking water for 45days followed by normal drinking water and group 3 was given NDEA at a concentration of 1ppm (v/v) per gram of animal body weight in drinking water for 120 days followed by normal drinking water. NDEA containing water was provided during weekdays and normal water on weekends. One week after completion of treatment all the animals were euthanized in carbon dioxide chamber. Liver, lung, kidney and brain tissues were excised, washed with ice cold PBS. A section of tissues were fixed in formalin for hematoxylin and eosin (H&E) staining for histological examination and remaining tissue was snap-frozen in liquid nitrogen for further storage at -80°C until required for histone or RNA isolation.

3.1.2. Liver Regeneration after partial hepatectomy:

Male Sprague-Dawley rats (n=6) were obtained and maintained as described earlier. On age normalization (22-24 weeks of age) animals were subjected to partial hepatectomy. All surgeries were performed under aseptic conditions under general anesthesia by ketamine (Ketmin-50, Themis medicare) at a dose of 10mg/ml per kg of body weight intramuscularly. Each animal's abdomen was shaved and disinfected. A horizontal incision was made, approximately 2-3cm long, just below the Xiphoid cartilage. Left lateral and median lobes of liver (50-70% liver) were resected and muscle and skin layers were closed. Animals were provided with an external source of heat during recovery and were maintained post operatively under optimal conditions. Animals were sacrificed at different time points (16hr, 24hr, 48hr, 36hr, and 72hrs) post partial hepatectomy. Excised liver tissues were stored at -80^0C for further experiments and also processed for haematoxylin and eosin staining.

3.1.3. Embryonic liver development:

Pregnant Sprague-Dawley rats confirmed by the vaginal plug on embryonic day 0 (E0) were obtained. On e15, the liver buds were excised, followed by e18 and e20. The day of birth is recorded as D0, the liver tissue were obtained from D0, 1wk and 3wk and stored at -80^0C for further experiments. Also, the tissue sections were fixed in formalin for haematoxylin and eosin staining.

3.1.4. Histological examination:

Formalin-fixed and paraffin-embedded liver tissues were processed for H&E staining. Liver, lung, brain and kidney histology was evaluated on the basis of cellular degeneration, fibrosis, steatosis and inflammation.

3.2. Total RNA preparation

All solutions were prepared in di-ethylpyrocarbonate (DEPC) treated water for isolation of RNA. Glassware ware was baked at 240°C for 4hr and compatible plastic ware was rinsed with chloroform. Nitrile gloves were used to prevent RNase contamination.

Total RNA was isolated from liver tissue by guanidine isothiocynate method. Frozen liver tissue (0.1gm) was powdered with liquid nitrogen using mortar/ pestle. The powder was suspended in 1ml denaturing solution (4M guanidine thiocyanate, 25mM sodium citrate, 0.5% sarkosyl and 0.1M 2-βME) in microcentrifuge tube. To this suspension, 0.1ml of 2M sodium acetate pH 4.0, 1ml of water saturated phenol and 0.2ml of chloroform/isoamyl alcohol (49:1) were added. The suspension was thoroughly mixed and incubated at 4°C for 15min. The suspension was then centrifuged for 20min at 10000g at 4°C. The upper aqueous phase was transferred to fresh microcentrifuge tube. RNA was precipitated with 0.3ml of 100% isopropanol for 30min at -20°C and centrifuged for 10min at 10000g at 4°C. RNA pellet was resuspended in 75% ethanol, vortexed, incubated for 10-15min at RT and re-centrifuged for 5min at 10000g at 4°C. RNA pellet was dried for 5min at RT and dissolved in DEPC treated water. RNA was stored at -70°C until required. RNA was quantitated by diluting 5µl in 1ml alkaline water (1mM Na_2HPO_4) and absorbance at A_{260}. Quality of RNA was confirmed by A_{260}/A_{280} (1.9-2.0), A_{260}/A_{230} (2.0-2.2) and agarose formaldehyde gel electrophoresis.

3.3. Agarose formaldehyde gel electrophoresis

Agarose (0.5gm) was dissolved in 36ml water and cooled to ~60°C. After cooling, 5ml of 10X MOPS running buffer (0.2M MOPS pH 7.0, 0.5M sodium acetate pH 7.0 and 0.01M EDTA pH 8.0) and 9ml of 12.3M formaldehyde were added. The gel

(1%) was poured and placed in gel tank filled with 1X MOPS running buffer. For electrophoresis RNA sample (5µg) was prepared as follow: RNA volume was increase to 11µl by water and 5µl of 10X MOPS buffer, 9µl of 12.3M formaldehyde and 25µl of formamide were added and sample was incubated for 15min at 55°C. To this mixture 10µl formaldehyde loading buffer (1mM EDTA pH8.0, 0.25% w/v BPB, 0.25% w/v xylene cyanol, 50% v/v glycerol) was added and loaded onto the gel. The gel was run at 5V/cm until dye migrated two-third length of the gel. The gel was removed, transferred to RNase free glass dish with water and soaked twice for 20min each. After sufficient removal of formaldehyde, gel was soaked in 0.5µg/ml ethidium bromide (EtBr) and allowed to stain for 40min. The gel was destained in water for 1hr and examined on a UV transilluminator to visualize RNA.

3.4. cDNA synthesis

Total RNA was subjected to cDNA synthesis using Revert Aid H-Minus first strand cDNA synthesis kit (Fermentas, cat#K1632) according to manufacturer. In brief, 5µg total RNA was used for 20µl total reaction volume using random hexamer primers and synthesized cDNA were subjected to PCR analysis.

3.5. Preparation of genomic DNA from liver

Genomic DNA was prepared from liver tissues as described [145]. Frozen tissue (0.1gm) was powdered with liquid nitrogen using mortar/ pestle. The tissue powder was suspended in 1.2ml digestion buffer (100mM NaCl, 10mM Tris-Cl pH8.0, 25mM EDTA pH8.0, 0.5% SDS, 0.1mg/ml proteinaseK). Samples were incubated with shaking at 50°C for overnight. Samples were extracted with equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and centrifuged for 10min at 1700 g. Aqueous layer was transferred to fresh tube to which half volume of 7.5M ammonium acetate and 2 volumes of 100% ethanol were added. Precipitated DNA

was centrifuged at 1700g for 2min. The pellet was washed with 70% ethanol. DNA pellet was resuspended in TE buffer (10mM Tris-Cl pH8.0, 1mM EDTA pH8.0) until dissolved. The residual RNA was removed by adding 0.1% SDS and 1μg/ml DNase free RNase and incubated for 1hr at 37°C. DNA was re-extracted by phenol/chloroform/isoamyl alcohol method and ethanol precipitated as described earlier. DNA pellet was again resuspended at ~1mg/ml concentration in TE buffer until dissolved. Quality of DNA was confirmed by A_{260}/A_{280} (~1.8) and A_{260}/A_{230} (2.0-2.2). The DNA was stored at 4°C until required for agarose gel electrophoresis or PCR analysis.

3.6 Agarose gel electrophoresis

Agarose gel electrophoresis was carried out in 1X TAE buffer to analyze levels of PCR and RT-PCR products of different samples. DNA samples were prepared in appropriate amount of 6X loading buffer (12% Ficoll-400, 60mM EDTA pH 8.0, 0.6%w/v SDS, 0.15%w/v BPB and 0.15%w/v xylene cyanol) and resolved with appropriate DNA marker on 1% or 1.5% agarose gel with 0.5mg/ml EtBr. The gel was electrophoresed at 5V/cm of gel and the progress of separation was monitored by migration of dyes in the loading buffer. After separation of tracking dyes, the gel was examined on UV transilluminator to visualize DNA.

3.7. RNA ligase mediated rapid amplification of cDNA (RLM RACE) for mapping of the Transcription Start Site of the H2A variant Gene

Rapid amplification of cDNA ends (RACE) is a polymerase chain reaction-based technique which facilitates the cloning of full-length cDNA sequences. (RLM-RACE) represents a major improvement to the classic RACE technique [146].RLM-RACE is designed to amplify cDNA only from full-length, capped mRNA, usually producing a single band after PCR.

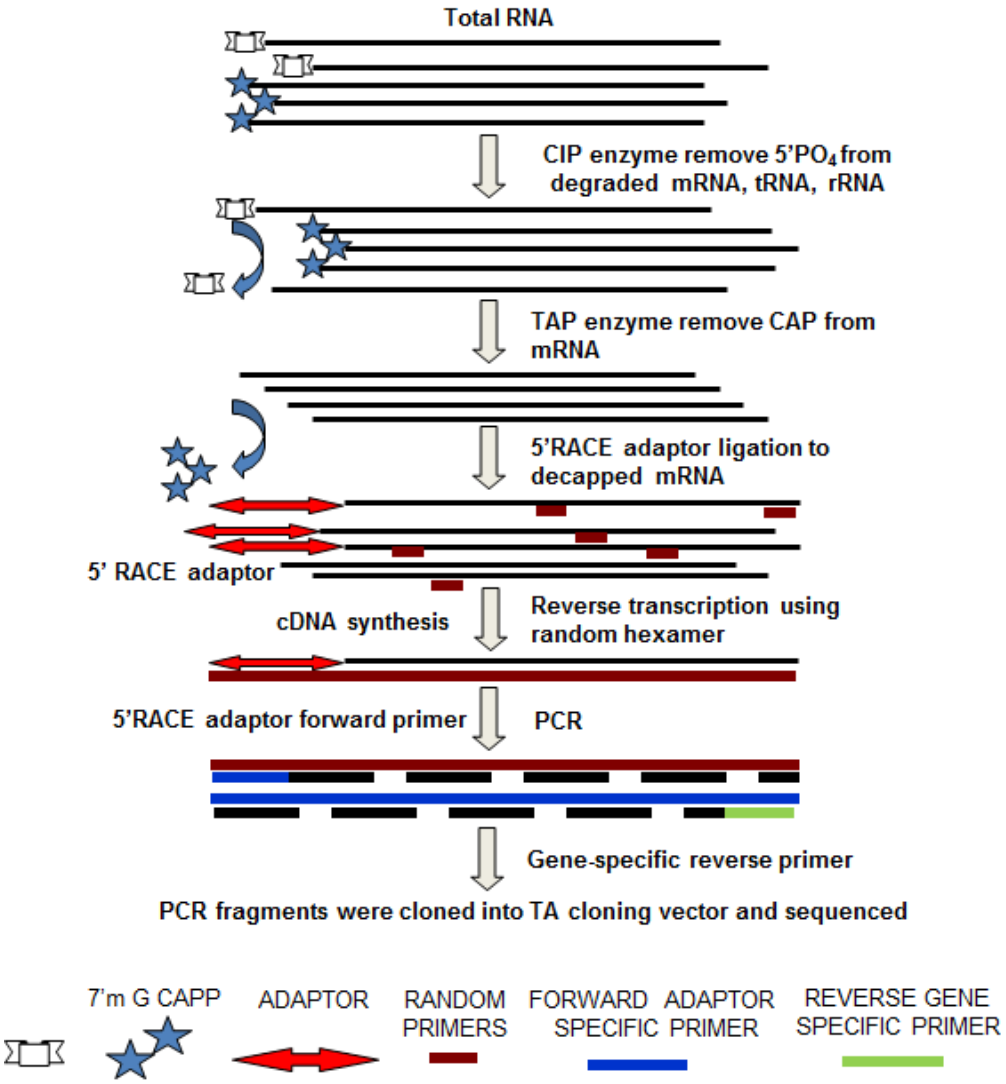

Figure 3.1: Schematic representation of experimental strategy for 5'RLM RACE. The technique involves the use of RNA as template which has its +1 site protected with intact 5'cap structure. The PCR amplification using gene specific primer and primer for adaptor sequence ligated to the 5'end of the mRNA results in amplification of TSS and the 5'UTR region as identified by sequencing.

Experimentally, the transcriptional start site of H2A variants has not been characterized. Therefore, to characterize the transcriptional start site for H2A.1 and H2A.2 genes RLM-RACE using Ambion kit (Cat# AM1700) was carried out according to manufactures instructions. Briefly, total RNA was treated step-wise with Calf Intestinal Phosphatase (CIP) and Tobacco Pyrophosphatase (TAP) enzymes to specifically select 7'mCAP protected mRNA. Random decamers were used for cDNA synthesis. The primary PCR was done using antisense gene (H2A.1

and H2A.2) specific primer and outer adapter primer (Table I) provided with the kit. The PCR conditions were as follows: 94ºC for 3mins; 35cycles of 94ºC for 45 sec; 56.4ºC for 30sec; 72ºC for 30 sec and 72ºC for 25min. The PCR product was resolved on 1.5% agarose gel and the DNA band was excised, cloned in pTZ57R/T vector and sequenced.

Table I: Primers designed for 5`UTR identification by RLM-RACE

5'UTR	Primers
H2A.1	5'-GTCGAATTCCTATTATTTGCCCTTGGCCTTGTG-3'
H2A.2	5'-GTCGAATTCTTATCACTTGCCCTTCGCCTTATG-3'
Adaptor	5'-GCTGATGGCGATGAATGAACACTG-'3

3.8. RNA circularization strategy for characterization of 3' untranslated region

Histone 3' UTR stem-loop is an RNA element involved in nucleo-cytoplasmic transport of the histone mRNAs, and in the regulation of stability and of translation efficiency in the cytoplasm.

Total RNA (10ug) was treated sequentially with CIP (Calf Intestine Phosphate) followed by treatment with TAP (Tobacco Pyrophosphatase). These enzymes treatment exposed the 5'phospahate of full length CAP protected mRNA for ligation of the 3' hydroxyl group of mRNA end. The intra mRNA circularization was carried out by RNA ligase at 18^{o}C for 16hrs.

This circularized RNA was used as a template for cDNA synthesis using single gene specific primers (H2A.1R or H2A.2R), followed by PCR amplification using specific primers for H2A.1 and H2A.2 genes (Table II).

The PCR amplified product was resolved on 1.5% agarose gel, DNA fragments were gel eluted and cloned in pTZ57R/T vector. Multiple clones were screened using Restriction Enzyme (RE) EcoRI/ HindIII for H2A.1 and EcoRI/ BamHI for H2A.2

digestion for fragment of interest. The positive clones obtained after RE digestions were confirmed by sequencing.

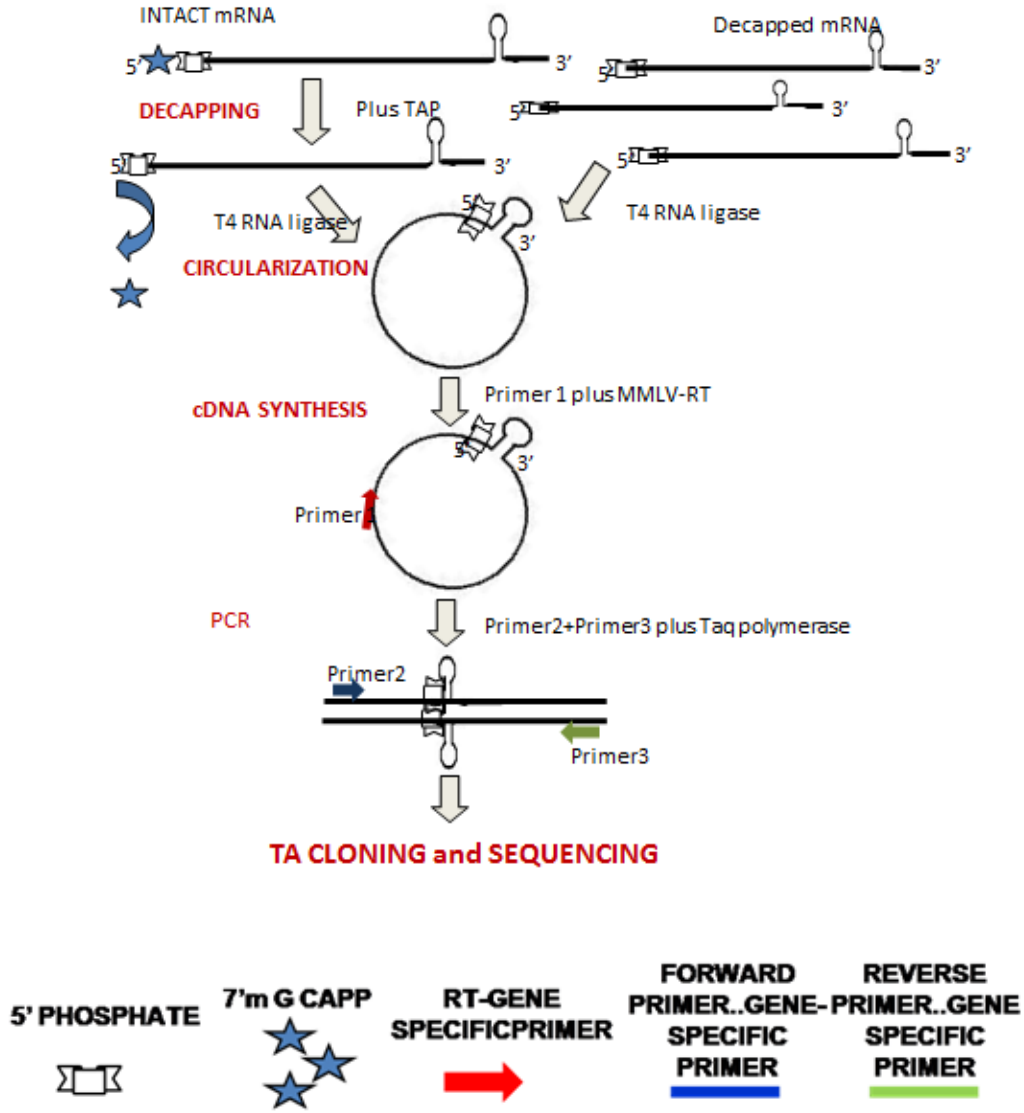

Figure 3.2: Schematic representation of 3'UTR identification using RNA circularization strategy. The mRNA circularization involves circulation of single RNA followed by ligation of 5'end to the 3'end of the transcript. The use of gene specific primers results in PCR amplified product from the cDNA used as template. The 3'end is identified upon sequencing.

Table II: Primers designed for 3`UTR identification by RNA circularization

3'UTR	Primers
H2A.1 F	5'-CGTAGTTGCCTTTGCGAAGCAAACGGTGCACA-3'
H2A.1R	5'-ATGAGGAACTCAACAAGCTGCTGGGCCGTGTGA-3'
H2A.2F	5'-TTGCCCGCCAGCTCCAGGATCTGGGCGGTCAG-3'
H2A.2R	5'-ACCCACAGAATCAGATAAAGAGTTGTGTCA-3'

3.9. TA cloning of PCR amplified DNA

TA cloning was carried out as per manufacturer's instructions (MBI, Ferments Cat#K1213). The vector and the insert with the molar ratio of the ends 1:3 were incubated in the presence of ligation buffer and T4 DNA ligase for 16hr at 21^0C

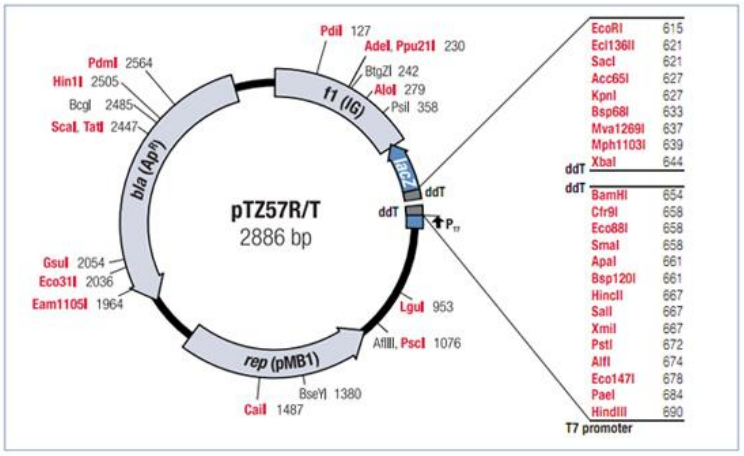

Figure 3.3 pTZ57R vector map for TA cloning (Fermentas): pTZ57R/T has lacZ promoter, with multiple cloning sites and have ampicillin selection marker gene. The ligation occurs at the T-over hangs compatible to the A-overhang generated post PCR using Taq polymerase.

3.10. Ultra- competent cell preparation

E.coli DH5α-MCR bacterial strain was made ultra-competent to transform plasmid DNA. A single colony was inoculated in chilled 100ml SOB media (2% bactoptryptone, 0.5% yeast extract,10mM NaCl,10mM $MgCl_2$, 10mM $MgSO_4$) and incubated at 18^0C at 100rpm till the OD_{600} reached to 0.4. Culture was chilled on ice for 10min and centrifuged at 2500g at 4^0C for 10min. The cell pellet was resuspended in 80ml of chilled transformation buffer (10 mM PIPES pH 6.7, 5N KOH, 55mM $MnCl_2$, 15mM $CaCl_2$, and 250mM KCl) and incubated on ice for 10min. The cell suspension was centrifuged at 2500g for 10min at 4^0C to pellet down the cells. The cell pellet was further resuspended in 20ml of transformation buffer with 7% DMSO, followed by 10min incubation on ice. Aliquots of 100µl were prepared; subsequently snap frozen and stored at -80^0C. The 100µl aliquot was used for transformation, the cells were quickly thawed followed by addition of 2-5µl

of ligation reaction mix and incubated on ice for 30min. The reaction mix was transferred to 42^0C water bath for 55sec and immediately transferred back on ice for 2-3min incubation. LB media (900 µl) was added to the cells and incubated at 37^0C for 1hr at 200rpm. The cells were pelleted down and suspended in 50µl of LB media and spread on X-Gal (40µl from 20mg/ml)/IPTG (40µl from 0.1M/ampicllin (100µg/ml) LB agar plate. The plates were incubated at 37^0C for overnight. The white colonies were picked, cultured and screened further.

3.11. Plasmid Isolation:

3.11.1. Mini preparation of plasmid:

Overnight grown 1.5ml bacterial culture was pellet down followed by addition of 150µl of TELT buffer (50mM Tris-Cl pH 7.5, 62.5mM EDTA pH 8.0, 0.4%Triton X-100, 2.5M LiCl). After resuspension of bacterial cells, 5µl of lysozyme (1µg/µl) treatment was done to rupture the bacterial cell wall. Followed by pulse centrifugation, supernatant containing plasmid DNA was collected in fresh tube, and allowed to precipitate by two volumes of ethanol. Plasmid quality was checked on 1% TAE agarose gel.

3.11.2. Maxi preparation of plasmid:

Overnight grown 500ml of bacterial culture was pelleted down at speed of 2500g for 10min at 4˚C. The pellet was resuspended in 18ml of sol-1 (Glucose 0.9g, 25mM Tris-Cl pH 8.0, 10mM EDTA pH8.0 in 100ml). To this suspension freshly prepared lysozyme solution (10mg/ml) was added and left at RT for 10min, further 40ml of sol 2 (2M NaOH, 10% SDS) was added by gently invert mixing and was allowed to be at RT for 10 min. To this suspension, 20ml of ice cold sol 3 (3M potassium acetate, pH 5.5) was added followed by invert mixing and incubated on ice for 10min. The suspension was centrifuged at 8,000g for 15min at 4˚C. The supernatant

was filtered through 4 layers of cheese cloth into fresh tube. Plasmid precipitation was carried out by addition of 0.6 volume of isopropanol at RT for 10min, followed by centrifugation at 8000g at RT for 10 min. Finally, pellet was dissolved in 8.5 ml TE buffer pH 8.0.

3.11.3. CsCl/Ethidium Bromide equilibrium centrifugation:

To the above dissolved plasmid 8.5gm of CsCl (1gm/ml) and 0.4 ml EtBr (10mg/ml) was added, this mixture was centrifuged at 5000g at RT for 2min to remove the air bubbles. The suspension was sealed in polycarbonate ultratubes and centrifuged at 48,000g for 22hrs at 20˚C. After the run, tubes were taken out and the lower linear plasmid band was removed with the help of 16 gauge needle. Further EtBr was removed from the plasmid by water saturated butanol treatment. Precipitation of DNA was carried out by addition of 2 volumes of distilled water and 6 volumes of 100% ethanol at 4˚C for overnight. Plasmid DNA containing mixture centrifuged at 11000g for 20min at 4˚C. The pellet was washed with 70% ethanol and spun at 74,000g for 10 min at 4˚C. Finally pellet was air dried and dissolved in water and the concentration was adjusted to 1µg/µl and stored at -20˚C. The quality of plasmid was checked on 1% agarose gel and the ratio of A_{260}/A_{280} was measured.

3.12. *In silico* promoter analysis and cloning

In silico analysis showed presence of TATA box in both H2A.1 and H2A.2 promoters. The 1.2 kb and 1.3kb genomic fragment was amplified from genomic DNA using gene specific primers (Table III). The amplified PCR fragment was gel eluted cloned into pGL3 basic vector at KpnI /XhoI site. Further positive clones were confirmed by sequencing. The promoter activity of the cloned putative promoters was confirmed by dual luciferase assay as described later.

Table III: Primers for H2A.1 and H2A.2 promoters

Promoter	Primers
-1056FH2A.1	5'-GGTACCAGCAATGTGAATGTGTAAGCG-3'
+144RH2A.1	5'-CTCGAGAAACGGTGCACACGGCCCACG-3'
-1160FH2A.2	5'-GGTACCCTGAACCCTTGATCCATCTCTC- 3'
+219RH2A.2	5'-CTCGAGATCTCGGCGGTCAGGTACT-3'

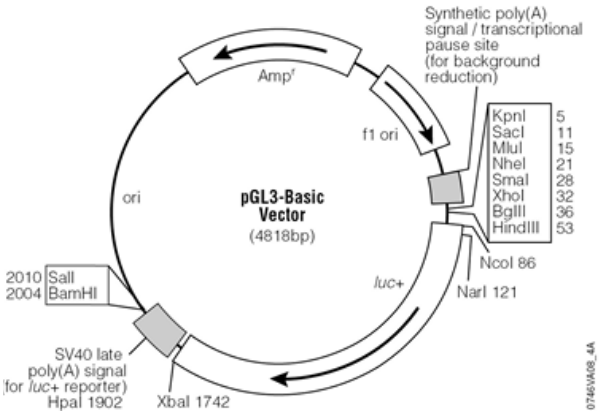

Figure 3.4 pGL3 basic vector map (Promega): The vector contains luciferase reporter gene located beneath the MCS. The vector is used for measuring the transcriptional activity of given gene sequence that corresponds to the luciferase activity.

3.12.1. Preparation of 5'promoter deletion constructs:

The promoter deletion constructs were prepared using full length promoter plasmid as template for PCR amplification. Primers for both the gene promoter deletion constructs were designed as mentioned in the Table IV.

Table IV: Primers for H2A.1 and H2A.2 5`promoter deletion constructs

5'Promoter deletions	Primers
-856FH2A. 1	5'-GGTACCATTGCAGGCTTTCCTGGGTCA-3'
-656FH2A. 1	5'-GGTACCAAGACAGTGTATAGCCAATT-3'
-456FH2A. 1	5' -GGTACCAATTAAACCTTAGCAGGACGAC-3'
-256FH2A. 1	5'-GGTACCGATCTCAACTATTAATCTACCCTT-3'
-131FH2A. 1	5'-GGTACCAAGAGTACGCCTTATGCAAATGA-3'
+144RH2A.1	5'-CTCGAGAAACGGTGCACACGGCCCACG-3'
-921FH2A.2	5'-GGTACCCAAGAACAATGTGACCATGC -3'
-738FH2A.2	5'-GGTACCTACTGCCAGTCTACACCCAAA -3
-560FH2A.2	5'-GGTACCTACTGCCAGTCTACACCCAAA -3
-315FH2A.2	5'-GGTACCGGTTCTTAACTCAGGCCTTCTTG -3'
-135FH2A.2	5'-GGTACCGGTTCTTAACTCAGGCCTTCTTG- 3'
+219RH2A.2	5'-CTCGAGATCTCGGCGGTCAGGTACT-3'

All PCR amplified products were gel extracted and cloned into pGL3 basic vector at Kpn I /XhoI site. Further all positive clones were confirmed by sequencing.

3.13. Basic tissue culture techniques:

Cell lines used for the study:

Table V: Cell lines

Cell line	Media	Property	Origin (rat)	
CL38	MEM	Neoplastic	NDEA induced HCC derived	[147]
CL44	MEM	Pre-neoplastic	NDEA induced preneoplastic	[148]

3.13.1. Thawing of Cell lines

CL38 cells were maintained in Earle's minimum essential medium supplemented with 6%v/v fetal bovine serum (MEM-6) in humidified incubator maintained at 37˚C, 5%CO_2. Complete media was prepared by adding 10% FBS and 1% antibiotic. The frozen vials of cells stored in liquid nitrogen were allowed to thaw at 37˚C water-bath. The thawed cell suspension was transferred to sterile centrifuge tube containing 2ml MEM-6 media and centrifuged for 10min at 1400g at RT. The cell pellet obtained was resuspended in 1ml MEM-6 and transferred to sterile culture plates with sufficient amount of media (2ml media for 35mm dish). Cells condition was tracked after 24hr under microscope and media was changed if it turned from red to yellow.

3.13.2. Trypsinization and Sub-culturing

The culture plates were sub cultured by trypsinization. These cells were then counted and transferred into new sterile plates with sufficient media. The numbers of viable cells were determined by adding 20µl of trypan blue into 180µl of cell suspension.

The drop from this mixture was loaded on haemocytometer and viable cells were counted.

Cells/ml = average number of cells per WBC chamber x dilution factor (10) $x10^4$

3.13.3. Transfection

Rat CL38 hepatoma cells grown on 24 well plate to approximately 40% confluence was transfected with either 1.5µg pGL3-Basic plasmid or an equivalent molar amount of test plasmid was co-transfected along with 2.5ng of pRL-TK plasmid using cation based transfecting reagent, Turbofect (Fermentas, cat# R0531), according to the manufacturer's instructions. The pRL-TK vector containing the *Renilla* luciferase gene under control of the herpes simplex virus thymidine kinase promoter (Promega) was used as an internal control for differences in transfection efficiency and cell number. After pre-incubation, the transfectants were incubated for 48h in the same medium supplemented. At the end of the culture period, the transfectants were lysed, and the luciferase activity in the cell lysate was measured by dual-luciferase reporter assay system (Promega, cat#E1910).

3.14. Protein Estimation by Lowry's Method

Histone and total protein concentrations in various samples were determined by Lowry method [149] with Bovine Serum Albumin (BSA, Sigma) 2-16µg as standard. The freshly prepared Copper Tartrate Carbonate (CTC- 0.1% $CuSO_4$, 0.2% Sodium potassium tartrate, 10% Na_2CO_3; CTC mixture: CTC, 0.8N NaOH, 10% SDS and D/W in 1:1:1:1 ratio) mixture was added and vortexed. After incubation for 10min at RT, 500µl of Folin Ciocalteau reagent (1:6 dilutions with D/W, 0.33N) was added, tubes were vortexed and incubated in dark for 30 min at RT. Absorbance at 750 nm was measured and standard curve of BSA was plotted to determine unknown protein concentration.

3.15. Histone Isolation from rat liver tissue

Histones were isolated as described [150] from rat liver tissue of e15, -18, -20, post birth D0, 1wk and 3wk adult. The tissue (400mg) was homogenized in chilled buffer A (0.34M Sucrose, 50mM Tris-Cl pH 7.5, 25mM KCl, 5mM $MgCl_2$, 1mM DTT, 0.1% TritonX100, 0.1mM EDTA pH 8.0, 0.15mM EGTA, 0.1mm PMSF, 0.5mM spermidine). The homogenate was centrifuged at 3000g for 15min at 4°C. The pellet was suspended in chilled buffer A followed by centrifugation at 2,000g, 15min at 4°C. The pellet obtained was further used for histone extraction by addition of 0.2N H_2SO_4. Intermittent vortexing was done for 2-3hr to extract the chromatin bound histone followed by high speed centrifugation at 14,000g for 20min at 4°C. The supernatant containing histone protein was precipitated with four volumes of chilled acetone and left overnight at -20°C. The histone pellet obtained was washed twice with acidified acetone (50mM HCl) followed by two washes of chilled acetone. The histone pellet was dried and dissolved in 0.1% βME. Histones were stored at -20°C.

3.16. SDS PAGE

Histones were resolved on 18% SDS-PAGE using modification of traditional Laemmli buffer system [151]. Increased concentrations of buffers provide better separation between the stacked histones and SDS micelles. In brief, glass plate sandwich was assembled using 0.1cm thick spacers. Separating gel solution (17.5% w/v acrylamide, 0.5% bisacrylamide, 0.75M Tris pH8.8, 0.1% w/v SDS, 0.033%w/v APS, 0.66%v/v TEMED) was prepared and poured into the glass plate sandwich and allowed to polymerize. Stacking gel solution (3.8%w/v acrylamide, 0.1%w/v bisacrylamide, 0.125M Tris-Cl pH 6.8, 0.1%w/v SDS, 0.05%w/v APS, 0.1%v/v TEMED) was then prepared and poured between the glass plate with Teflon combs. Histone samples to be analyzed were diluted 1:1 (v/v) with 2X SDS sample buffer

(0.05M Tris-Cl pH6.8, 20%v/v glycerol, 4%w/v SDS, 2%v/v 2-βME, 0.01%w/v bromophenol blue, BPB) and incubated for 5min in boiling water bath. Samples were loaded on the gel attached to the electrophoresis chamber with 2X SDS electrophoresis buffer (0.05M Tris-Cl, 0.384M glycine, 0.2%w/v SDS, pH8.3-8.6). The gel was run at 20mA of constant current until the BPB tracking dye entered the separating gel and then at 30mA until the BPB dye reached the bottom of the gel. The power supply was then disconnected and gel was subjected to Coomassie or silver staining for protein visualization.

3.17. Coomassie staining

After the electrophoresis gel was transferred to tray containing Coomassie Brilliant Blue R-250 (CBBR) staining solution (0.1%w/v CBBR, 50% methanol and 10% acetic acid in water). The gel was stained for ~3 hrs on shaker. Gel was then transferred to destaining solution (50% methanol and 10% acetic acid in water). The gel was destained on shaker with several changes of destaining solution.

3.18. SDS-silver staining:

To achieve more sensitive detection of histone on the gel, the gel was further subjected to ammoniacal silver staining [152]. The gel was soaked in ten volumes of freshly prepared ammoniacal silver nitrate solution (0.38% NaOH solution, 2.8ml of liquid ammonia, 20% $AgNO_3$) for 30min. The gel was washed thrice with water each time for 2min. The gel was then developed by using developing solution (0.01%w/v citric acid monohydrate and 0.1%w/v formaldehyde). The development of gel was stopped when protein spots appeared on clear gel background using stopping solution (40% methanol and 10% acetic acid)

3.19. Resolution of histones on AUT PAGE

Histones (20µg) were resolved on 15% AUT-PAGE gel as described [153]. Separating gel solution (15%w/v acrylamide, 0.1%w/v bisacrylamide, 1%v/v acetic acid, 0.05M ammonium hydroxide, 8M urea, 0.56%w/v Triton X-100, 0.5% TEMED, 0.0004%w/v flavin mononucleotide) was prepared and poured into glass plate sandwich and was allowed to polymerize in light. Stacking gel solution (4% w/v acrylamide, 0.16%w/v bisacrylamide, 1%v/v acetic acid, 0.05M ammonium hydroxide, 0.5%v/v TEMED and 0.0004%w/v flavin mononucleotide) was then prepared, poured and polymerized into the glass plate sandwich. The sandwich was attached to electrophoresis chamber with electrophoresis buffer (1M acetic acid and 0.1M glycine). The gel was pre electrophoresed for 2hrs at constant voltage of 200V before loading the samples. The gel was stained by Coomassie or amido black-silver or SDS-silver staining method [154].

3.20. Electro blotting from SDS-PAGE

Histones (5-10µg) were electroblotted from SDS-PAGE gels to PVDF membranes for western blot analysis [110]. The transfer tank of electroblotting apparatus (Trans-Blot Cell, Bio-Rad) was filled with 1X transfer buffer (10mM Tris-Cl, 0.1M glycine and 10%v/v methanol). PVDF membrane was activated with 100% methanol for 5sec. The activated membrane and SDS-PAGE gel were equilibrated in 1X transfer buffer. The gel membrane transfer sandwich was prepared and inserted into the transfer tank with gel on cathode side and membrane on anode side. Transfer was conducted at a constant current of 300mA for 2hr. Proteins transferred onto the membrane were detected by staining with Fast green (0.5%w/v Fast green in destainer; 50% methanol, 10% acetic acid) and destaining with several changes of

water. Histones transferred onto PVDF membrane were probed for global and site specific histone H3 acetylation and methylation antibodies according to manufacturer's instructions.

Immunoblots detection:

In general, membrane with transferred proteins was incubated in "blocking buffer" i.e. 5% BSA in Tween20/Tris-buffered saline (TTBS, 100mM Tris-Cl pH7.5, 0.9% w/v NaCl and 0.1%v/v Tween20) for 1hr at RT on orbital shaker. Blocking buffer was then replaced by recommended dilutions of primary antibodies mentioned in table VI in TTBS and incubated for 1hr at RT in orbital shaker. The membrane was vigorously washed four times with TTBS for 15min each at RT. Further, the membrane was incubated with recommended concentrations of HRPO labeled secondary antibodies in TTBS for 1hr at RT on orbital shaker. The membrane was again washed vigorously four times with TTBS at RT and developed using Immobilon Western reagent (Miilipore). The membrane was exposed to X-ray film in dark room and developed using Optimax X-ray film processor. Band intensities were analyzed using ImageJ and GraphPad prism software was used for plotting graphs.

Table VI: List of antibodies used for immunoblotting assay

Antibody	**Amount of Histone (µg)**	**Working dilution of primary Ab**	**supplier**
H3 PanAc	2	1:1000	Abcam
H3Lys9Ac	2	1:3000	Abcam
H3Lys14Ac	2	1:3000	Abcam
H3Lys9Me3	2	1:2000	Millipore
H3Lys27Me3	2	1:2000	Millipore

3.21. Electrophoretic-mobility Shift Assay (EMSA)

The principle of EMSA is that the protein specifically binding to an end-labelled DNA fragment retards the mobility of DNA-protein complex during electrophoresis and resulting in discrete bands corresponding to the individual protein-DNA complexes.

3.21.1. Nuclear extract preparation for EMSA

Preparation of nuclear extracts was done as described by Gorski et al with slight modifications as per the requirement. Liver (10%) homogentate/cell lysate was prepared in 0.34M sucrose concentration in buffer A (10mM HEPES pH 7.6, 0.15mM Spermine, 0.5mM, Spermidine, 1mM EDTA, 10mM KCl, 0.1mM EGTA, 1mM DTT, 0.5mM PMSF) was filtered and centrifuge at 1500g for 15min, 4°C. The pellet was suspended in buffer B (Buffer A with 2M sucrose) and was layered on the cushion of buffer B. Ultra centrifugation was carried out at 23,000g, 90 min at 4°C. The nuclear pellet was re-suspended in 1ml of nuclear lysis buffer (10mM HEPES pH 7.6, 100mM KCl, 0.1mM EDTA, 3mM $MgCl_2$, 10% glycerol, 1mM DTT, 0.1mM PMSF). Absorbance at 260nm was measured and nuclei suspension was diluted to $10A_{260}$ units/ml. Nuclei were lysed by adding 0.1volume of 4M ammonium sulphate pH 7.9 (pH adjusted by NaOH) and lysate was incubated on ice for 30min with gentle mixing. Chromatin was removed by centrifugation at 90,000g for 60min at 4°C. The supernatant was collected and solid ammonium sulphate (0.33g/ml) was added, dissolved by gentle shaking on ice for 60min. The precipitated proteins were centrifuged at 90,000g, 30min at 4°C. The protein pellet was re-suspended in nuclear dialysis buffer (20% glycerol, 20mM HEPES pH 7.6, 0.1 M KCl, 0.2mM EDTA, 2mM DTT, 0.1mM PMSF and 1mM $NaMoO_4$) and dialysed for 2hrs at 4°C against 100 volume of dialysis buffer. The nuclear extracts

were stored at -80^{o}C until further use. Protein estimation was carried out by Bradford reagent as per manufacturer's instructions.

3.21.2. Oligonucleotide Annealing

The equimolar concentration of individual oligonucleotides were taken and boiled at 95^{o}C in annealing buffer (10mM Tris-Cl pH7.9, 2mM $MgCl_{2,}$ 50mM NaCl and 1mM EDTA pH 8.0). The reaction mixture was kept at RT for overnight annealing of the oligonucleotides. The annealed oligo were checked on 12% 1XTBE-PAGE, followed by EtBr staining.

Table VII: List of oligonucleotides for EMSA

Oligonucleotides	
p53 (1)	5'TTGTCATGGGACATGCTCTCCTTCTC 3'
P53 (2)	5'TTGAGAAGGAGAGCATGTCCCATGAC3'
Mutant p53 (1)	5'TTGTCATGGGCAGCACTCTCCTTCTC 3'
Mutant p53 (2)	5'TTGAGAAGGAGAGTGCTGCCCATGAC3'
Sox9 (1)	5'TTAAAATACAAGAACAATGTGACCATGC3'
Sox9 (2)	5'TTGCATGGTCACATTGTTCTTGTATTTT3'
Mutant Sox9 (1)	5'TTAAAATACAAGAAAGGCGTGACCATGC3'
Mutant Sox9 (2)	5'TTGCATGGTCACGCCTTTCTTGTATTTT3'

3.21.3. Radioactive labelling of annealed oligonuclotides

The annealed oligos 5' overhangs were filled in using radiolabelled ^{32}Pα-dATP by Klenow enzyme. The probes were purified using Sepharose G-25 Columns as described (Current protocols, chapter 12). The labeling efficiency was checked by measuring the beta-count (cpm) on β-counter.

3.21.4. EMSA reaction

Nuclear extract was incubated with oligonucleotide (0.01 pmole, ~10,000 cpm) in the presence of 1X EMSA buffer (1mM Tris-Cl pH 7.5, 5mM NaCl, 0.5mM $MgCl_{2,}$ 1mM EDTA pH 8.0, 1mM DTT, 0.1% Triton X-100, 5% glycerol, 1mM PMSF) and poly dI-dC for 10 min on ice. The competitive binding assay was done using cold specific, non-specific and mutant oligonucleotides as mentioned in table VII.

Further, the specificity of binding was confirmed by super-shift assay using specific antibodies (p53-Cell signaling, Sox9-Abcam, PCNA-Santacruz). Loading dye was added and samples were resolved on pre-run 5% 1X TBE PAGE at 4^0C till the BPB dye migrated 2/3rd of the gel length. The gel was fixed (10% acetic acid, 50% methanol), dried and exposed for autoradiography.

3.22. Chromatin Immunoprecipitation (ChIP):

The ChIP assay has become one of the most practical and useful techniques to study the mechanisms of gene expression, histone modification, and transcription regulation. This assay determines whether a certain protein-DNA interaction is present at a given location, condition, and time point.

3.22.1. Preparation of mononucleosomes by MNase digestion:

Confluent plates of CL38 cells ($1x10^7$- $5x10^7$ cells per plate) were used for the experiment. Protein and DNA were cross-linked by drop wise addition of formaldehyde directly into the media for a final concentration of 0.75% and allowed to gently rotate for 10min at RT. The reaction was stopped by the addition of glycine to a final concentration of 125mM to the media and incubated with shaking for 5min at RT. The cells were washed thrice and scrapped with chilled 1XPBS. The cells were pellet down and resuspended in solution I (10mM Tris-Cl pH 7.4, 75mM NaCl, 24mM EDTA pH8.0, 0.2mM PMSF) followed by centrifugation at 2500g for 15min at 4^0C. The nuclear pellet obtained was resuspended in Solution III {Buffer A (15mM Tris-pH 7.4, 15mM NaCl, 60mM KCl), sucrose (0.34M), protease inhibitors (15mM β-mercaptoethanol, 0.5mM spermidine, 0.15mM spermine and 0.2 mM PMSF). The DNA quantitation was done and the nuclei were resuspended in required amount of solution III to attain concentration of 1mg/ml. The obtained nuclei was subjected to MNase digestion at a concentration of 50U MNase/mg DNA

for 2-3 hrs, and intermittently checked for mononucleosome on 1.8% agarose gel. The protein concentration of the mononucleosome was quantified by Bradford's method as per manufacturer's information.

3.22.2. Immunoprecipitation:

Mononucleosomes equivalent to 25µg protein concentration was used for immuno – precipitation (IP) using specific (p53 and Sox 9) and IgG antibodies (as per Abcam ChIP protocol). 20µl of protein A/G beads (pre-absorbed with sonicated single stranded salmon sperm DNA at 1.5µg / 20µl beads) was added to all samples and IP overnight with rotation at 4°C. The A/G beads were centrifuged for 1min at 1500g and the supernatant was removed. The beads were washed thrice with 1ml of wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA pH8.0, 150mM NaCl, 20mM Tris-Cl pH8.0) followed by single wash with final wash buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA pH8.0, 500mM NaCl, 20mM Tris-Cl pH8.0). This was followed by elution of DNA by addition of 120µl of Elution Buffer to the protein A/G beads with continuous rotation for 15 min at 30°C.

Table VIII: Primers for ChIP-PCR

Promoter region (ChIP)	**Primers**
F-p53 H2A.1	5'-GGTACCATTGCAGGCTTTCCTGGGTCA-3'
R-p53 H2A.1	5'-TTATGGCTGAATTATCCTTAAACTGC-3'
F-SOX9 H2A.2	5'-GGTACCCAAGAACAATGTGACCATGC-3'
R-SOX9 H2A.2	5'- TGGCATTAAGAATCACAGATTAGA-3'

The beads were pellet down by short spin and supernatant was transferred into fresh tube. The INPUTs were included at this stage to which elution buffer along with the 2µl RNase A (0.5mg/ml) and 5 µl of proteinase K (20 mg/ml) for purifying DNA. The eluates (INPUTs and IP material) were heated at 65°C for 4-5hrs (or overnight) for re-crosslinking of protein and DNA. DNA was then purified and ethanol

precipitated in presence of 10µl glycogen and dissolve in H_20. The purified DNA samples were stored at -20°C and later preceded for PCR. The PCR amplified product using gene specific primers (Table VIII) was resolved on 2% agarose gel and analyzed for specific loci occupancy of ChIPed DNA with specific transcription factor antibody

Chapter 4
Results

Results

4.1. NDEA induced Hepatocellular carcinoma:

Carcinogenesis is a general term used to denote the development of neoplasia. Hepatocellular carcinoma (HCC) is multistage processes with the sequential appearance of hyperplastic foci, nodular adenomas and finally heptomas. Altered foci and neoplastic nodules are two types of hepatocellular lesions. Altered foci are small lesions of hepatocyte that do not disturb the parenchymal architecture. They are equivalent to hyperplastic foci. Neoplastic nodules correspond to lesions described as adenomas are longer lesions that disrupt and compress the surrounding parenchyma.

Male Sprague-Dawley rats were exposed to liver specific chemical carcinogen NDEA through drinking water for period of 45 days and 120 days to induce pre-and neoplastic stages of liver cancer. Animals survived until their scheduled euthanization. Liver, brain, lung and kidney were excised and processed for histopathological analysis. Liver is the target organ, site of NDEA metabolism and develops cancer whereas brain, lung and kidney are considered as non-targeted organs.

Histopathological analysis of H&E stained slides of normal liver showed hepatic architecture comprising of radiating cords of polygonal cells, each with central hepatic vein (Figure 4.1 upper left panel). The normal liver parenchymal cells reveal granulated cytoplasm. The trabeculae were interspersed with sinusoids confirming no alteration in normal liver architecture. Whereas sections from the nodules after 45 days NDEA treatment showed focal lesion formation, serosis, and vacuolar degeneration but no dysplastic changes suggesting towards pre-neoplasia, however, animals treated for 120 days showed gross alteration of hepatocellular architecture. Phenotypically altered hepatocyte populations of 120 days treatment appear in the

form of altered cell foci and nodules (Figure 4.1. upper left panel). These were much enlarged, assumed oval shape majorly include dysplasia, necrosis, inflammation and fibropathy of tissue in between liver cells accompanied by eosinophilic and plasma infiltration of tissue. Nuclei showed marked degenerative changes and were largely vesciculated suggesting neoplastic changes. Thus, the histopathology of liver confirms HCC, further, to validate the preneoplastic and neoplastic changes, the tissue sections were transplanted in SCID mice and development of tumor was seen within period of 4-5 week from the 120 days tissue transplant and no tumor development was observed with 45 days treated liver tissue transplant confirming them as neoplastic and preneoplastic stages of liver tumor development respectively (Appendix IV).

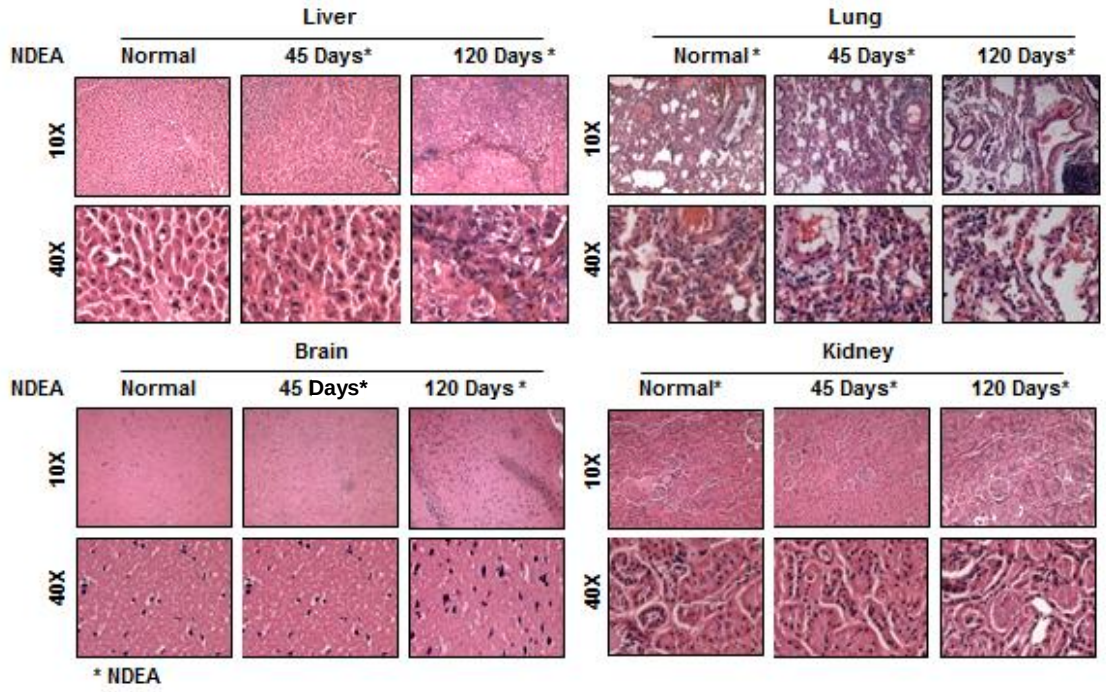

Figure 4.1: Histopathological analysis of various tissues pre- and post- N-Nitrosodiethyamine treatment:
Tissue sections (5µm) of adult rat liver, lung, brain and kidney pre- and post-NDEA treatment for period of 45 days and 120days were stained with haematoxylin and eosin stain. The untreated group (Normal) of animals showed normal architecture of liver, lung, brain and kidney, whereas NDEA treatment resulted in preneoplastic and neoplastic changes only in liver. No significant alterations were observed in; lung, brain and kidney. Magnification: 10X and 40X

Further, the histopathological analysis of lung tissue at 45 and 120 days compared to control confirmed no significant change in the tissue architecture of lung which showed normal appearance of fine lace because most of the lung is composed of thin-walled alveoli (Figure 4.1 upper right panel). The alveoli are composed of a single layer of squamous epithelium. Between the alveoli thin layer of connective tissue and numerous capillaries are also lined with simple squamous epithelium. The brain tissue section does not show any alteration in the architecture both pre- and post-NDEA treatment (Figure 4.1 lower left panel) whereas inflammatory changes were observed in kidney of 120days NDEA treated animals, though the normal architecture was maintained as the glomeruli were normo-cellular, the tubules were closely spaced and the interstitium and blood vessels were not prominent (Figure 4.1 lower right panel). The above result confirmed the specificity of carcinogen for liver and therefore, the target and non-target organ tissues were further used for profiling the alteration in histone variants associated with phenomenon of carcinogen and carcinogenesis.

4.1.1. H2A.1 and H2A.2 accumulation in chromatin is tissue-specific and their differential expression associates with HCC

The histone variant profile of HCC liver tissue (120 days NDEA treatment) showed increase in H2A.1 expression compared to normal liver. The other organs (kidney, lung and brain) tissue of NDEA treated animal showed no alteration in the histone variant profile of H2A.1 and H2A.2 compared to their normal counterpart (Figure 4.2 left panel). This suggests that increase in H2A.1 expression is associated with the process of carcinogenesis and shows no correlation with exposure to carcinogen or inflammatory changes observed in kidney.

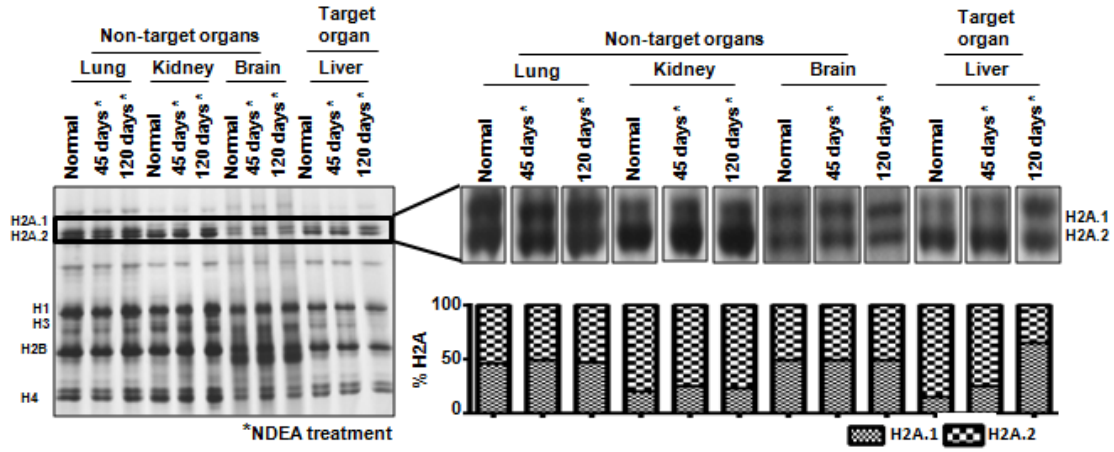

Figure 4.2: Separation of histones on Acetic acid-Urea- Triton (AUT) PAGE: (A) Acid extracted total histones (20 µg) isolated from lung, kidney, brain and liver tissues with or without treatment of NDEA were resolved on 15% AUT-PAGE and detected by ammoniacal silver nitrate staining.(B) The higher magnification of H2A region is depicted and the ratio of H2A.1/H2A.2 was analyzed. (C) H2A.1 and H2A.2 showing differential accumulation specific to neoplastic changes were observed in liver tissue post NDEA treatment compared to other organs. The analysis of band intensity is represented graphically as an average of three independent experiments

Further analysis suggests that the ratio of H2A.1 and H2A.2 variants was tissue specific as plotted after densitometric analysis (Figure 4.2 right panel). H2A.2 was predominantly observed in adult liver and kidney, whereas, H2A.1 and H2A.2 were accumulated equally in the chromatin of lung and brain (Figure 4.2 right panel). Thus, the differential expression of histone variants in various tissues suggests further investigation to understand their role in chromatin remodeling.

4.2. Cloning and characterization of H2A.1 and H2A.2:

Histone H2A.1 and H2A.2 are homomorphous variants having difference of only three amino acids positioned at 16^{th}, 51^{st} and 99^{th} and they are intron less genes having predicted 5`UTR and 3`UTR. Histone mRNA contains either poly A tail, stem loop or both. The 26bp conserved stem loop structure is present on the 3`end depending on their cell cycle phase of expression.

4.2.2. H2A.1 and H2A.2 cDNA clones in pTZ57R/T vector:

The cDNA sequences of H2A.1 (XM_577573) and H2A.2 (XM_345255) were obtained from NCBI database. Multiple sequence alignment of H2A.1 and H2A.2 using CLUSTAL W shows 89% homology between their sequences (Figure 4.3).

RNA isolated from the rat liver tissue was resolved on denaturing MOPS gel and the data suggested that the lane 3 RNA sample was of good quality with integrity of 2:1

```
CLUSTAL 2.1 multiple sequence alignment


gb|JX661508.1|_1100-1492      ATGTCTGGACGCGGCAAACAAGGCGGTAAGGCTCGCGCCAAGGCCAAGAC 50
gb|JX661509.1|_1204-1596      ATGTCCGGCCGTGGCAAGCAAGGAGGCAAGGCCCGCGCCAAGGCCAAGTC 50
                              ***** ** ** ***** ***** ** ***** *************** *

gb|JX661508.1|_1100-1492      CCGCTCCTCCCGGGCCGGCCTGCAGTTCCCCGTGGGCCGTGTGCACCGTT 100
gb|JX661509.1|_1204-1596      GCGGTCGTCCCGCGCCGGGCTGCAGTTCCCAGTGGGTCGCGTGCACCGGC 100
                               ** ** ***** ***** *********** ***** ** ********

gb|JX661508.1|_1100-1492      TGCTTCGCAAAGGCAACTACGCGGAGAGGGTGGGCGCCGGCGCCCCGGTG 150
gb|JX661509.1|_1204-1596      TGCTGCGCAAGGGCAACTACGCGGAGCGCGTGGGCGCCGGCGCGCCGGTA 150
                              **** ***** *************** * ************** *****

gb|JX661508.1|_1100-1492      TACCTGGCGGCTGTGCTGGAGTACCTGACGGCCGAGATCCTGGAGCTGGC 200
gb|JX661509.1|_1204-1596      TACATGGCGGCGGTGCTGGAGTACCTGACCGCCGAGATCCTGGAGCTGGC 200
                              *** ******* ***************** ********************

gb|JX661508.1|_1100-1492      GGGCAACGCGGCGAGGGACAACAAGAAGACACGCATCATCCCGCGCCACC 250
gb|JX661509.1|_1204-1596      GGGCAACGCGGCCCGCGACAACAAGAAGACCCGCATCATCCCGCGCCACC 250
                              ************  * *************** ********************

gb|JX661508.1|_1100-1492      TACAGCTGGCCATTCGGAACGATGAGGAACTCAACAAGCTGCTGGGCCGT 300
gb|JX661509.1|_1204-1596      TGCAGCTGGCCATCCGCAACGACGAAGAACTCAACAAGCTGCTGGGCAAA 300
                              * ************ ** ***** ** **********************

gb|JX661508.1|_1100-1492      GTGACCATCGCCCAGGGCGGCGTTCTGCCCAACATCCAAGCGGTGCTGCT 350
gb|JX661509.1|_1204-1596      GTGACGATCGCGCAGGGCGGCGTCCTGCCCAACATCCAGGCCGTGCTGCT 350
                              ***** ***** ************ *************** ** ********

gb|JX661508.1|_1100-1492      GCCCAAGAAGACTGAGAGCCACCACAAGGCCAAGGGCAAATAA 393
gb|JX661509.1|_1204-1596      GCCCAAGAAGACGGAGAGCCACCATAAGGCGAAGGGCAAGTGA 393
                              ************ ************ ***** ******** * *
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Figure 4.3: Sequence alignment of H2A.1 and H2A.2 cDNA: The coding sequences cDNA obtained from NCBI database of rat H2A.1 (XM_577573) and H2A.2 (XM_345255) gene are of 393bp each and CLUSTALW multiple alignment data analysis showed 89% homology between their sequences.

(Figure 4.4A) and had a ration of A_{260}/A_{280}: 2.0, therefore, used as template for cDNA synthesis. Gene specific primers (Appendix I) for both H2A.1 and H2A.2 were designed and PCR was performed with cDNA as template at the annealing temperature of 56.8^{o}C for H2A.1 and 61.4^{o}C for H2A.2 as standardized after gradient PCR.

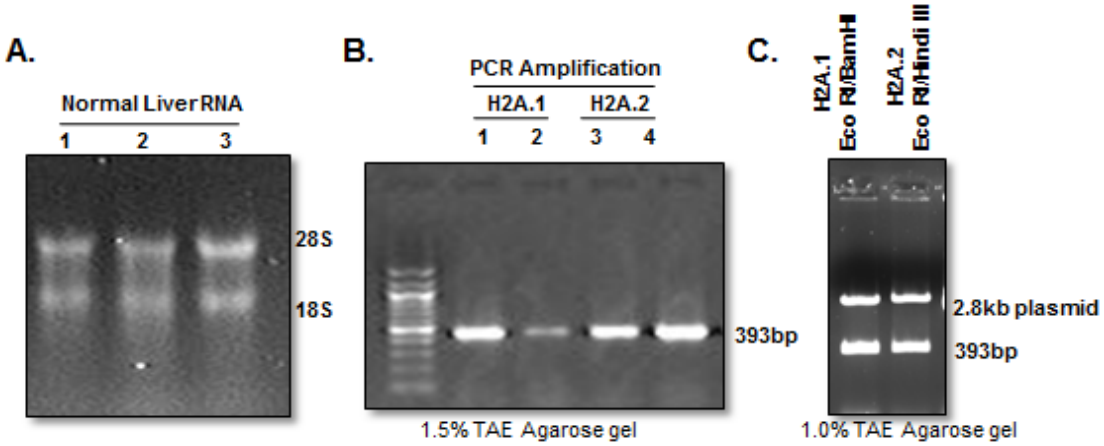


Figure 4.4: cDNA cloning of H2A.1 and H2A.2 (A) Total RNA extracted from adult rat liver tissue was resolved on denaturing MOPS gel. The sample in lane 1 and 3 showed the ratio of 28S: 18S as 2:1 confirming the integrity of RNA (B) The cDNA was synthesized from the RNA sample 3 and was PCR amplified using H2A.1 (lane 1,2) and H2A.2 (lane 3,4) cDNA specific primers. The amplified products were resolved on 1.5% TAE gel and correspond to the product size of 393bp for both the cDNA (C)The cDNA was cloned into pTZRT vector and clones containing H2A.1 and H2A.2 were screened using restriction enzyme digestion (EcoRI/ BamHI and EcoRI /HindIII) and resolved on 1% TAE agarose gel. The positive clones were further confirmed by sequencing.

The PCR amplified product of 393bp (Figure 4.4B) was cloned into pTZ57R/T vector; positive clones for H2A.1 were selected after EcoRI/BamHI digestions and for H2A.2 with EcoRI/HindIII digestions (Figure 4.4C). The plasmids having the fragment release of 393bp were further confirmed by sequencing.

4.2.3. Identification of transcriptional start site and 5`UTR of H2A.1 and H2A.2 genes

In order to identify the promoter of a gene to study its role in transcription regulation the transcription start site is mandatory. *In silico* data suggested that the predicted start site for H2A.1 was 10 nucleotide of upstream of the translational start site (ATG) and for H2A.2, it was 88bp upstream of the first ATG site. In order to experimentally identify the start site, 5`RLM RACE was carried out.

The experiment was performed using total RNA extracted from normal liver tissue. cDNA was synthesized using gene specific primers for H2A.1 and H2A.2 (Table I, Material & methods, 3.7) followed by PCR amplification using primers specific for

gene sequence and the ligated adaptor sequence (Table I, Material & method 3.7.). PCR amplification of cDNA synthesized using total RNA treated with CIP (+) and TAP (+) gave single band with estimated size of 458 bp for H2A.1 (Figure 4.5A.I) and H2A.2 (Figure 4.5B.I) genes. Whereas, the CIP (+) and TAP (-) treated samples showed no PCR amplification and therefore, served as negative controls.

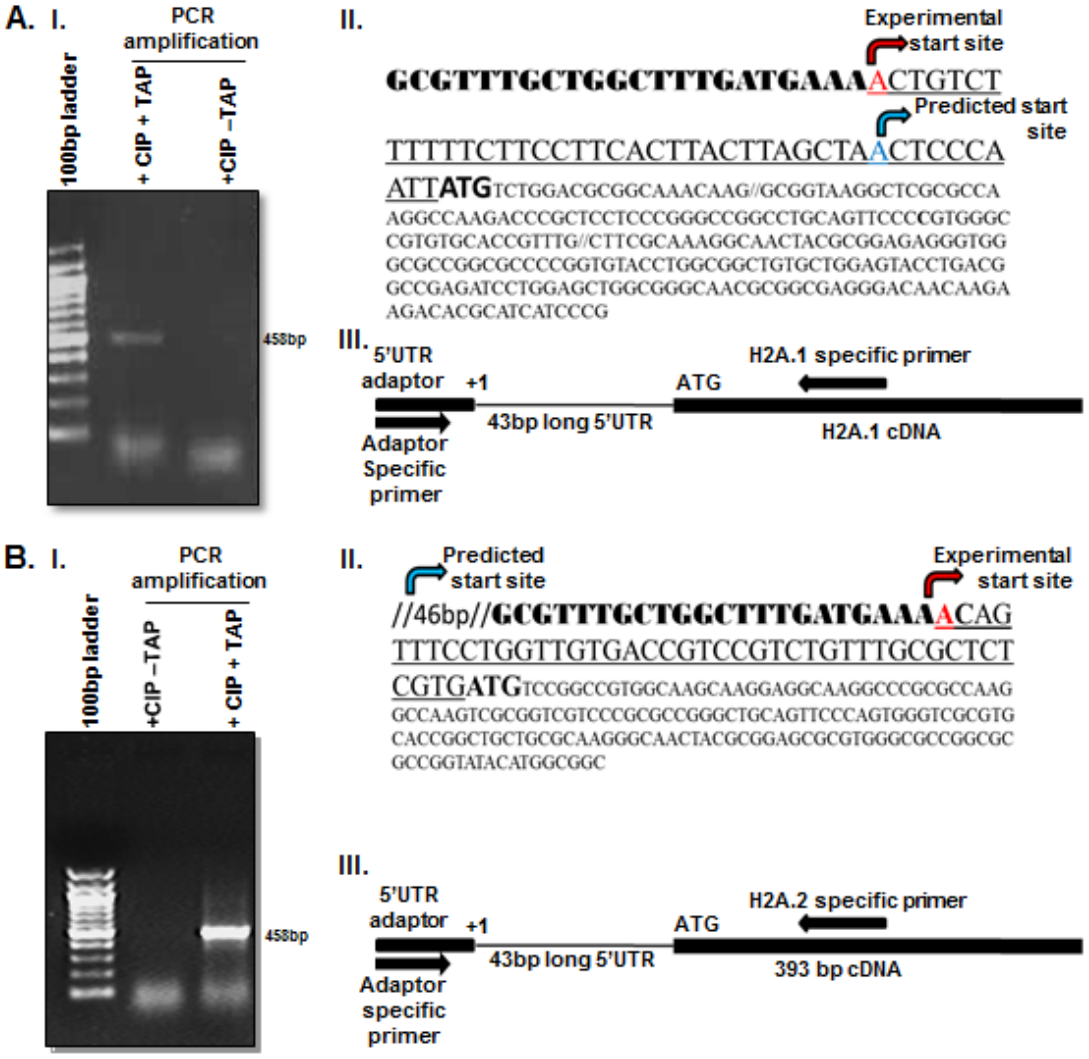


Figure 4.5: Identification of 5`Untranslated Region and Transcription Start Site of H2A.1 and H2A.2 mRNA.** 5`RLM-Rapid Amplification of cDNA was carried out using total RNA from rat liver tissue as template. The CIP/TAP treated samples on PCR amplification showed single band amplification that indicated single transcriptional start site for H2A.1 (A.I) and H2A.2 (B.I). **GCGTT...GAAA sequence indicates the ligated adaptor sequence to the 5`end of the mRNA, the red arrow (experimental start site) demarcates the first nucleotide just after the adaptor sequence corresponding to +1, the blue arrow shows predicted start site in NCBI data base (H2A.1 :XM_577573 and H2A.2 : XM_ 345255), in between sequence ACTGTC....AATT (A.II) and ACTGTTT....CGTG (B.II) are the 5`Untranslated region. Schematic diagram represents the +1 that indicates the transcriptional start site for H2A.1 (A.III) and H2A.2 (B.III) mRNA positioned 43bp upstream of translational start site (ATG) with the primer complimentary to the adaptor sequence and H2A.1 and H2A.2 cDNA.

This also confirmed the ligation of adaptor sequence to the authentic 5`end protected by mRNA cap. Cloning in pTZR57/T vector and sequencing data of the PCR products identified the experimental TSS which precedes sequence corresponding to the RNA oligonucleotide adapter and thus representing authentic cap sites (Figure 4.5AII bold sequence and Figure 4.5.BII bold sequence). The experimentally identified TSS of H2A.1 is positioned 31bp upstream of the predicted TSS (Figure 4.5 AII underlined sequence)and/or 43bp upstream the Translational Start site.

Whereas in H2A.2 gene the experimentally identified TSS is 46bp downstream of the predicted start site and /or 43bp upstream the ATG site (4.5B.II underlined sequence). The identified 5`UTR and TSS are represented as schematic diagram (Figure 4.5 AIII, BIII).

4.2.4. Mapping of 3`UTR for H2A.1 and H2A.2 mRNA

Stabilization and localization of the histone mRNA is majorly mediated by the 3` end .Therefore, we mapped the 3`UTR of H2A.1 and H2A.2 gene. using RNA ligase.

The isolated RNA from rat normal liver tissue treated with CIP (calf intestine phosphatase enzyme) and TAP (tobacco pyrophosphatase enzyme) was circularized cDNA was synthesized from circularized mRNA using forward gene specific primer (Table II, Material & methods, 3.8) and used as template for gene specific amplification. The PCR amplified product of 406bp (H2A.1) (Figure 4.6A.I) and 206bp (H2A.2) (Figure 4.6B.I) was cloned in pTZ57R/T vector, screened using EcoRI/BamHI digestion and the positive clones were further confirmed by sequencing. The analysis of sequencing data confirmed the presence of 26bp conserved stem loop in H2A.1 and H2A.2 mRNA. However, the 3`UTR is 62bp long

in H2A.1 (Figure 4.6A.II), whereas, 88bp long in H2A.2 (Figure 4.6B.II) mRNA including the conserved stem loop structure.

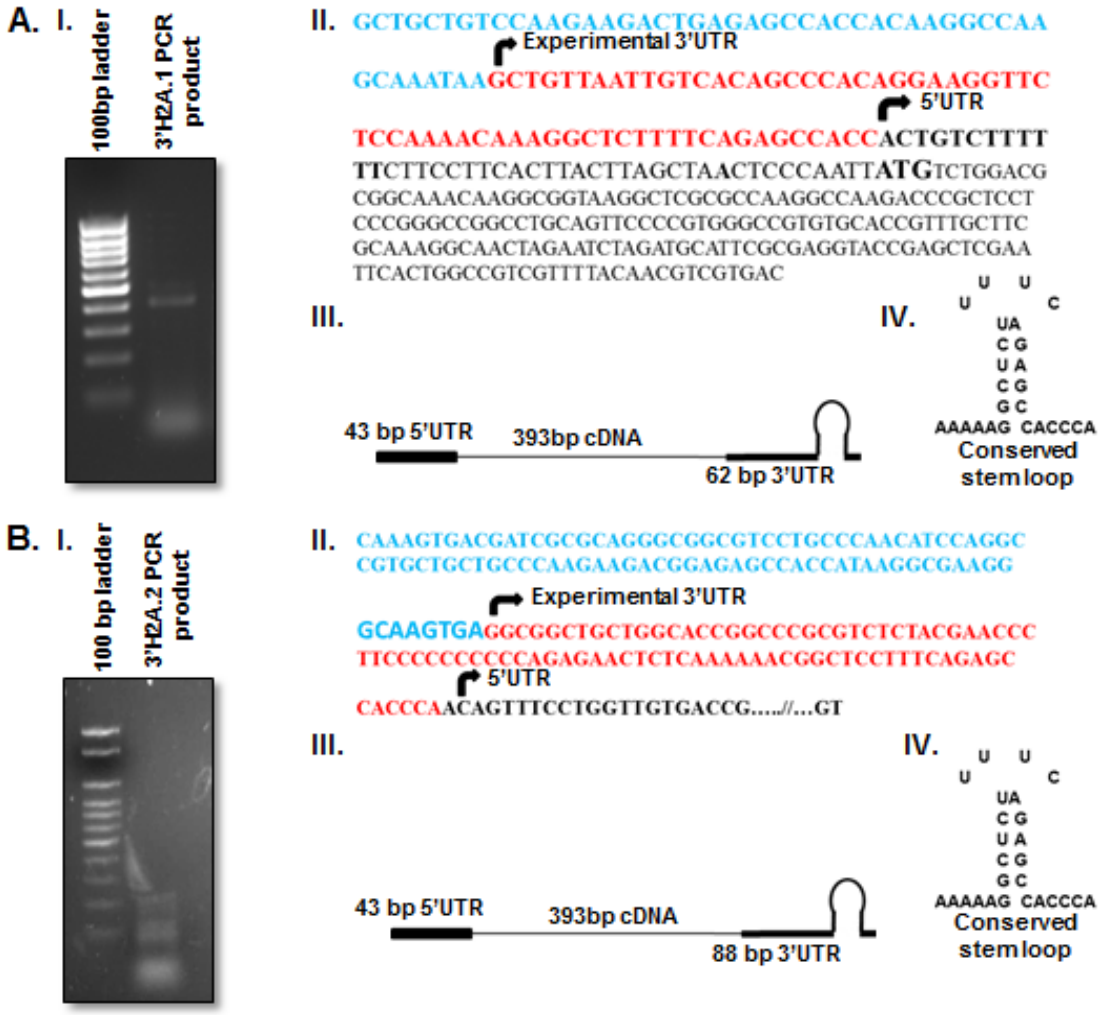


Figure 4.6: Identification of 3` untranslated region of histone variants, H2A.1 and H2A.2 mRNA: The PCR amplified product obtained from cDNA template of circularized RNA was resolved on 1.5% TAE agarose gel and Etbr stained (A.I, B.I).The amplified product of H2A.1 (406bp) and H2A.2 (206bp) was cloned in pTZ57R/T vector and the sequencing data confirmed the 3`untranslated region (highlighted in red colour) for H2A.1 (A.II) and H2A.2 (B.II) lying just ahead of the 5`UTR and downstream the predicted end of 3`UTR (B.II) as reported in NCBI data base (H2A.1 : XM_577573 and H2A.2 : XM_345255). The line diagram showed the entire transcript of H2A.1 and H2A.2 gene with 43 bp long 5`UTR, 393bp of cDNA and 62 bp H2A.1 in 3`UTR (A.III) and 88bp 3`UTR in H2A.2 (B.III) of 3`UTR that include 26bp of conserved stemloop.

4.2.5. Cloning of H2A.1 and H2A.2 gene promoters

Rat genome database (http://rgd.mcw.edu/) was used to locate the upstream sequence from experimentally identified TSS for H2A.1 and H2A.2 gene on chromosome 17 arm q11 and chromosome 2arm q34, respectively.

Promoter prediction and identification of protein binding sites regulating expression of H2A variant promoter

In silico analysis was done using motif finder (http://www.genome.jp/tools/motif/) and bio-base (http://www.biobase- international.com/product/transcription-factor-binding-sites) software for identification of putative transcription factor binding sites in the predicted promoter sequence (NCBI database) upstream of experimentally confirmed TSS for H2A.1 and H2A.2 genes.

Figure 4.7: Nucleotide sequence of Rat H2A.1 gene promoter: The H2A.1 gene sequence from -1056 to+144 was obtained from Rat Genome Database. The +1 indicates denotes the transcriptional start site as confirmed by 5`RLM RACE experiments. The putative promoter from -1056 to +1 and 5`UTR from +1 to +43 was used for transfec analysis to identify putative transcription factor binding sites using motif-finder and Biobase software. Position -1056 to ATG is the non-coding region and from ATG to +144 is the coding region. The H2A.1 putative promoter sequence showed the presence of TATA Box at position from -30 to -25 and CAAT box. The list of detailed transcription factor binding sites have been tabulated as Annexure V

Results

The region encompassing -1056bp to +144bp for H2A.1 and -1160 to +219 for H2A.2 gene suggest presence of multiple putative transcription factor binding sites. In putative H2A.1 gene promoter, a TATA box (TATAAA) was identified at 25-30 bp upstream from the transcription initiation site; a CCAAT box was located at 63-67bp upstream from the initiation site and 37 bp upstream from the TATA box along with multiple putative binding sites such as Oct-1, cEBP-α and cdxA transcription factors (Figure 4.7).

In H2A.2 gene promoter, a TATA box (TATAAA) was identified at 25-30 bp upstream from the transcription initiation site; a CCAAT box was located at 63-67bp upstream from the initiation site and 36bp upstream from the TATA box along with various putative binding sites of are AP4, E2F, cEBP-β, Sox-family (Figure 4.8).

These *in silico* results suggest the possible importance of *cis-acting* elements in transcription regulation of the H2A variants gene differential expression during HCC development. Additionally, differential mapping of the CpG islands or –CCGG- sequences were observed in the promoter regions of these histone variants. The H2A.2 gene promoter (-1160 to +219) contains CpG island, having greater than 90% G+C composition in the 5` untranslated segment spanning from -290 to +219. However, H2A.1 (-1056 to +144) has no CpG islands, but contains multiple –CCGG- sites within its promoter. Thus, the 5` region of these genes has CpG islands or CpG dinucleotides which may be correlated directly with transcriptional activity of H2A.1 and H2A.2 gene in cancer. Thus, to perform functional characterization of the regulatory structural elements identified in the 5` flanking regions of histone variants gene, DNA constructs containing full length promoter.

Figure 4.8: Nucleotide sequence of Rat H2A.2 gene promoter: The H2A.2 gene sequence from -1160 to +219 was obtained from Rat Genome Database. The +1 indicates denotes the transcriptional start site as confirmed by 5`RLM RACE experiments. The putative promoter from -1160 to +1 and 5`UTR from +1 to +43 was used for transfec analysis to identify putative transcription factor binding sites using motif-finder and Biobase software. Position -1160 to ATG is the non-coding region and from ATG to +219 is the coding region. The H2A.2 putative promoter sequence showed the presence of TATA Box at position from -30 to -25 and CAAT box. The list of detailed transcription factor binding sites have been tabulated as Annexure V

The predicted promoter ranging from -1056bp upstream of the +1 site and +144bp downstream TSS was PCR amplified using genomic DNA isolated form rat liver (Figure 4.9A) for H2A.1 using gene specific primers (Table III, Material & methods, 3.12). The amplified PCR product of 1200 bp (-1056 to +144) (Figure 4.9B) was

cloned in pTZ57R/T vector and positive clones were screened by EcoRI/BamHI digestion and further confirmed by sequencing (Figure 4.9C).

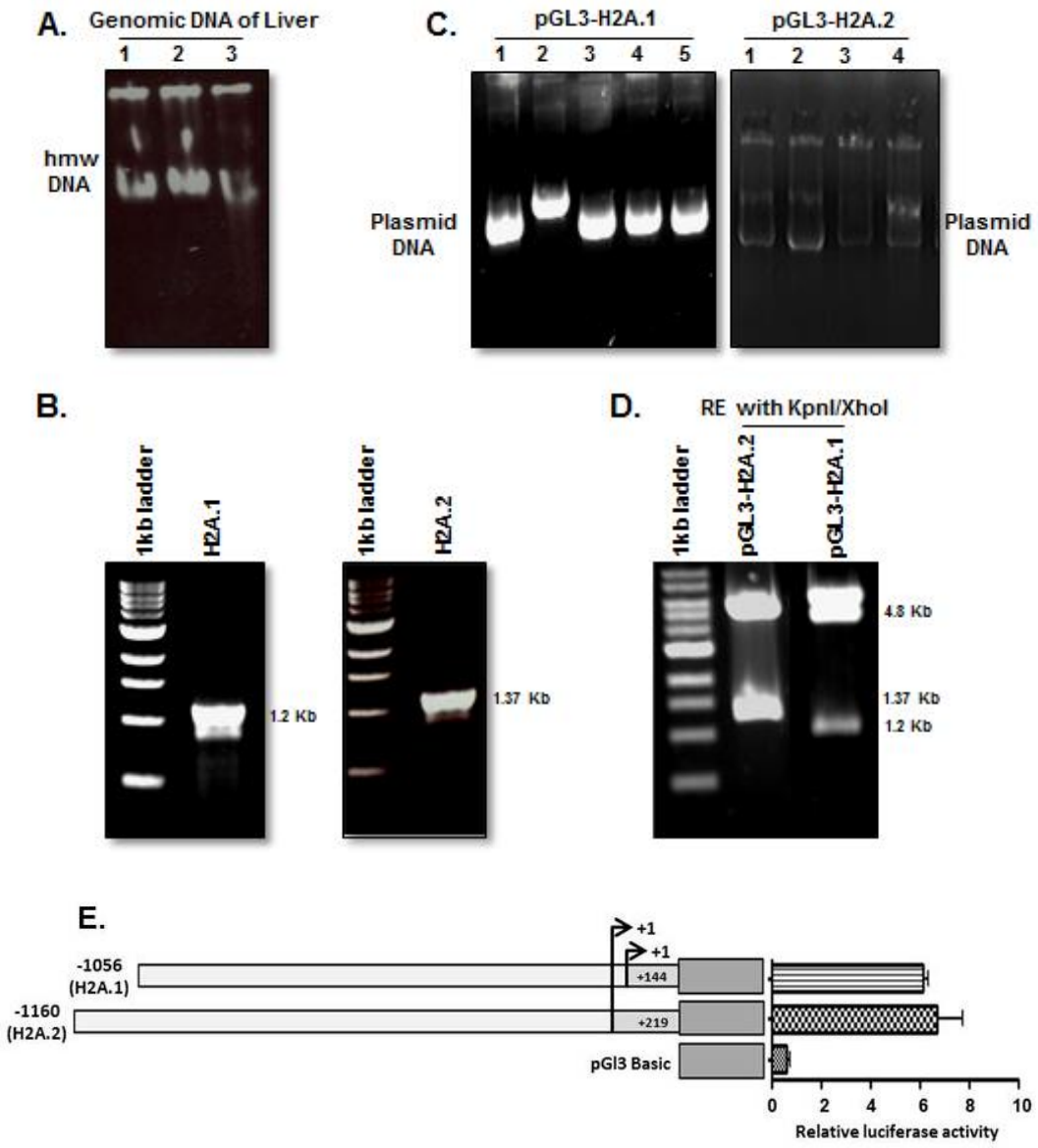


Figure 4.9: Cloning and characterization of H2A.1and H2A.2 promoter: (A) Genomic DNA was isolated from three adult rat liver and resolved on 0.4% TAE-agarose gel lane 1,2,3 (B) PCR amplification was carried out using genomic DNA as template and the amplified product corresponding to 1.2kb of H2A.1 (-1056 to +144) and 1.37kb of H2A.2 (-1160 to +219) was resolved on 1% TAE agarose gel (C) The gel purified fragments of H2A.1 (1.2kb) and H2A.2 (1.37kb) putative promoters were cloned in pGL3 Basic vector at KpnI/XhoI restriction sites. The cloned and purified plasmids containing H2A.1 and H2A.2 promoter insert were resolved on 1% TAE gel. (D) The clones were confirmed by restriction enzyme (KpnI/XhoI) digestion followed by sequencing. (E) The promoter activity of the cloned putative H2A.1 and H2A.2 gene promoters was confirmed by luciferase assay. The empty pGL3Basic vector served as negative control. The data presented are the means ± SD from triplicate plates and are representative of multiple independent experiments

The H2A.1 gene has been mapped Chromosome 17 from region encompassing within 48507120 to 48508520 (GeneID:502125). The 1.2kb insert was further

cloned at KpnI/XhoI site in pGL3 basic vector encoding the luciferase gene and lacking eukaryotic promoter and enhancer sequences to study transcriptional activity of H2A.1 gene (Figure 4.9D).

The H2A.2 is localized on (GeneID:365877) Chromosome 2, positioned from 191050439 to 191052653 region. The predicted promoter ranging from 1160bp upstream of the +1 site to +219bp downstream TSS for H2A.2 was PCR amplified using gene specific primers (Table III, Material & methods, 3.12) with genomic DNA isolated from liver tissue (Figure 4.9A). The amplified product of 1379 bp was cloned in pTZ57R/T vector and positive clones were screened by EcoRI/HindIII digestion, further confirmed by sequencing (Figure 4.9B, C). The 1.37kb insert was later cloned at KpnI/XhoI in pGL3 basic vector encoding the luciferase gene and lacking eukaryotic promoter and enhancer sequences to study transcriptional activity of H2A.2 gene (Figure 4.9D).Further, the promoter activity of cloned 1.2Kb H2A.1 and 1.37kb H2A.2 putative promoter sequences was confirmed by luciferase assay (Figure 4.9E)

4.3. Identification of *cis-acting* regulatory elements involved in transcription regulation

4.3.1. Preparation 5`deletion constructs of H2A.1 and H2A.2

On the basis of i*n silico* transcription factor binding site prediction the multiple 5` deletion constructs for H2A.1 and H2A.2 gene promoters were prepared as mentioned in Figure 4.10 using promoter specific primers (Table IV, Material & methods, 3.12.1). The PCR amplified product specific to each construct were cloned at KpnI/XhoI site of pGL3 basic vector and confirmed by restriction enzyme digestion and sequencing.

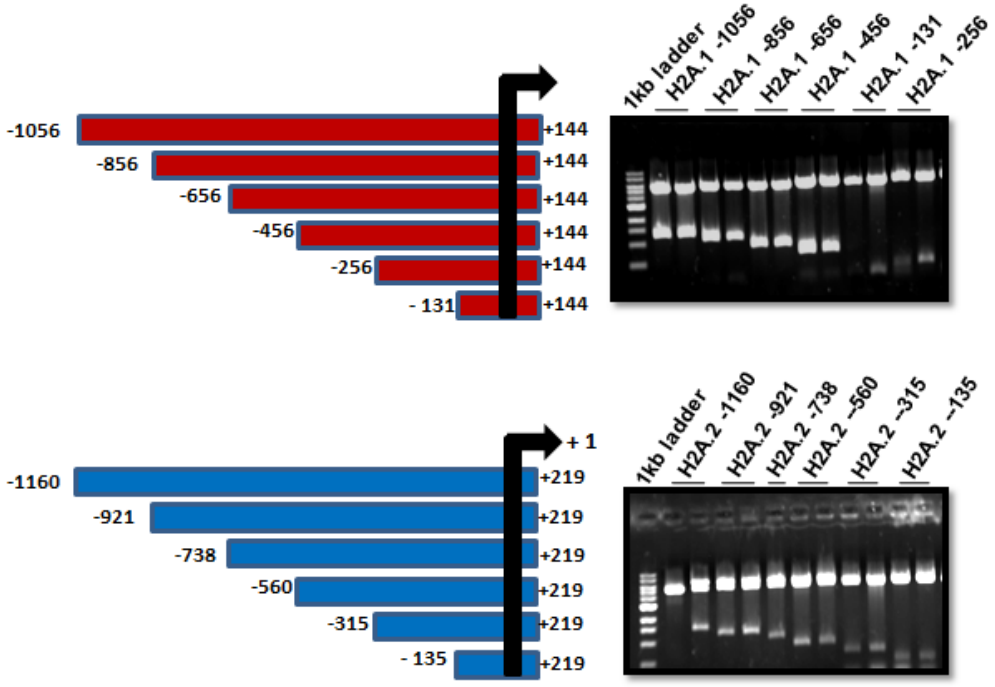


Figure 4.10: H2A.1 and H2A.2 promoter deletion constructs. The line diagrams at the left panel represents the 5`promoter deletion constructs prepared on using specific primers represented as "➡" arrows for H2A.1(-1056 to +144, -856 to +144, -656 to +144, -456 to +144, -256 to +144 & -131 to +144) and H2A.2 genes (-1160 to +219, -921 to +219, -738 to +219, -560 to +219, -315 to +219 & -135 to +219). The right panel represents the positive clones in pGL3 basic vector obtained after restriction enzyme digestion using KpnI/XhoI with the release of subsequent expected size of promoter insert for H2A.1 and H2A.2 gene promoters as resolved on 1.2%TAE agarose gel.

4.3.2. Dual luciferase assay of H2A.1 and H2A.2 gene promoter

The multiple deletion constructs of H2A.1 (-1056 to +144) promoters showed differential transcriptional activity on basis of dual luciferase reporter assay

The 200bp region from -656 to -456 bp showed maximum promoter activity thus suggesting presence of activator binding sites. The region encompassing from -131 to +144 region includes the TATA and CAAT box along with the few putative transcription factor binding sites such as Oct-1/2, Smad 3, ELF-1 and showed minimal promoter activity ,thus suggesting the region is required for basal promoter activity of H2A.1 gene (Figure 4.11A).The promoter region from -856 to -656 showed decrease in the transcription activity thus, suggesting the presence of putative repressor binding sites that were predicted to be p53 and ElF-1 (Figure 4.11B). Further, to delineate the significant decrease in the transcription activity in

region from -856 to -656, the unidirectional deletion constructs of -831 to +144 were prepared.

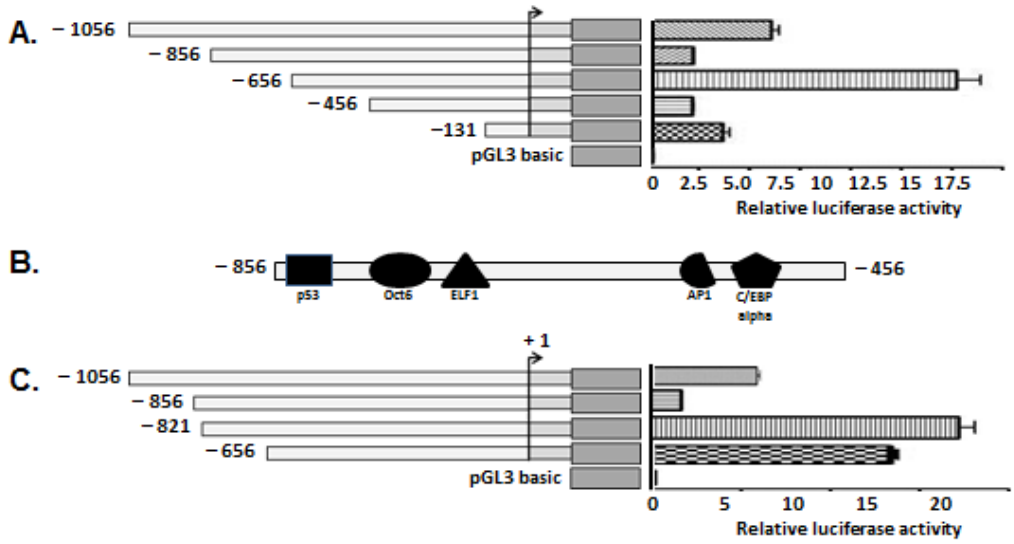


Figure 4.11: Transcriptional activity of H2A.1 promoter deletions. The size and orientation of each construct is indicated by the numbers, which are relative to the start of transcription site, designated +1. The rat histone H2A.1 gene promoter fragments were cloned upstream of the luciferase gene in the pGL3 basic vector. The promoter activity was the measure of luciferase activity normalized by co-transfecting renilla vector (A) Multiple deletion constructs spanning from -1056 to +144 are represented with their corresponding promoter activity. (B) Schematic representation of predicted transcription factor binding sites on region encompassing from -856bp to -456bp that shows presence of potential *cis*-acting regulatory elements. (C) Deletion constructs of the potential repressor binding site within -821 to -856 region and their promoter activity. The luciferase activity obtained from transfection of the empty pGL3 Basic vector used as the negative control. The data presented are the means ± SD from triplicate plates and are representative of multiple independent experiments

Deleting out the putative p53 binding region from -856 to+144 showed increase in transcriptional activity (Figure 4.11C). Thus, the results confirm presence of repressor binding element within -856 to -832, on H2A.1 gene promoter.

The multiple deletion constructs of 1.37kb region of H2A.2 promoter region encompassing from -1160 to +219bp were prepared and the transcription activity was measured on the basis of luciferase reporter assay (Figure 4.12A). The 200bp region from -921 to -738bp showed minimal promoter activity thus, suggesting the possibility of repressor binding elements in this region. The i*n silico* data analysis

identified putative transcription factor binding sites that include Sox-5,-9,-10 and Oct (Figure 4.12 B).

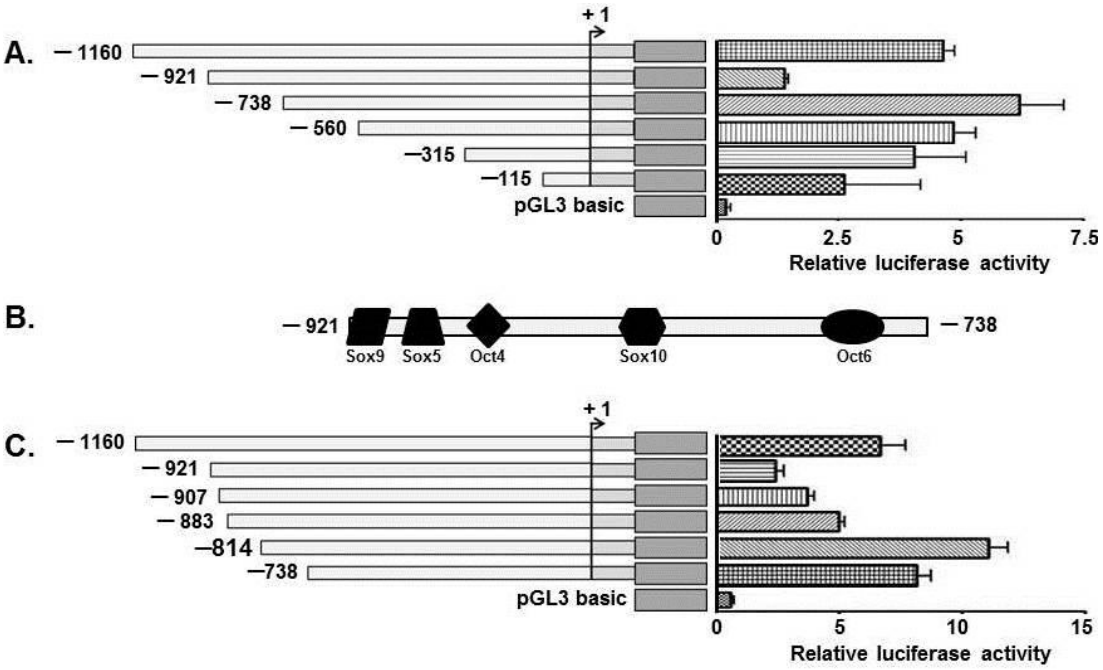


Figure 4.12: Transcriptional activity of H2A.2 promoter deletions. The size and orientation of each construct is indicated by the numbers, which are relative to the start of transcription site, designated +1. The rat histone H2A.2 gene promoter fragments were cloned upstream of the luciferase gene in the pGL3 basic vector. The promoter activity was the measure of luciferase activity normalized by co-transfecting renilla vector (A) Multiple deletion constructs spanning from -1160 to +219 are represented with their corresponding promoter activity. (B) Schematic representation of predicted transcription factor binding sites on region encompassing from -921bp to -738bp that shows presence of potential *cis*-acting regulatory elements. (C) Deletion constructs of the potential repressor binding site within -921 to -738 region and their promoter activity. The luciferase activity obtained from transfection of the empty pGL3 Basic vector used as the negative control. The data presented are the means ± SD from triplicate plates and are representative of multiple independent experiments

The basal promoter activity was observed in region from -135 to +219bp, that includes TATA and CAAT box along with other putative transcription factors like, E2F-1, Nf-y and Oct-1. Further, to delineate the possible *cis acting* regulatory region within 200bp from -921 to -738, subsequent deletion constructs were prepared on the basis of putative repressor Sox-family binding proteins. The stepwise deletion (920 to +219, -897 to +219 and -821 to +219) of each Sox-factor binding sites resulted in enhanced promoter activity as measured by luciferase assay (Figure 4.12C).

4.4. Expression of histone variant H2A.1 and H2A.2 in embryonic and regenerating liver post partial hepatectomy

In order to gain insight in relevant epigenetic overlapping mechanism involved in embryonic liver, regeneration and cancer development, a comprehensive analysis of differentially expressing histone variants, H2A.1 and H2A.2 was performed.

4.4.1. Histopathology analysis of embryonic and regenerating liver

The histopathological analysis of regenerating liver post PH at different time points (16hr, 24hr, 36hr, 48hr and 72hr) showed normal liver architecture having maintained hexagonal structure with sinusoidal spaces though the nuclei looks enlarged at 16hr and 24hr time points, indicating toward high level of DNA replication coupled with cellular proliferation (Figure 4.13B).

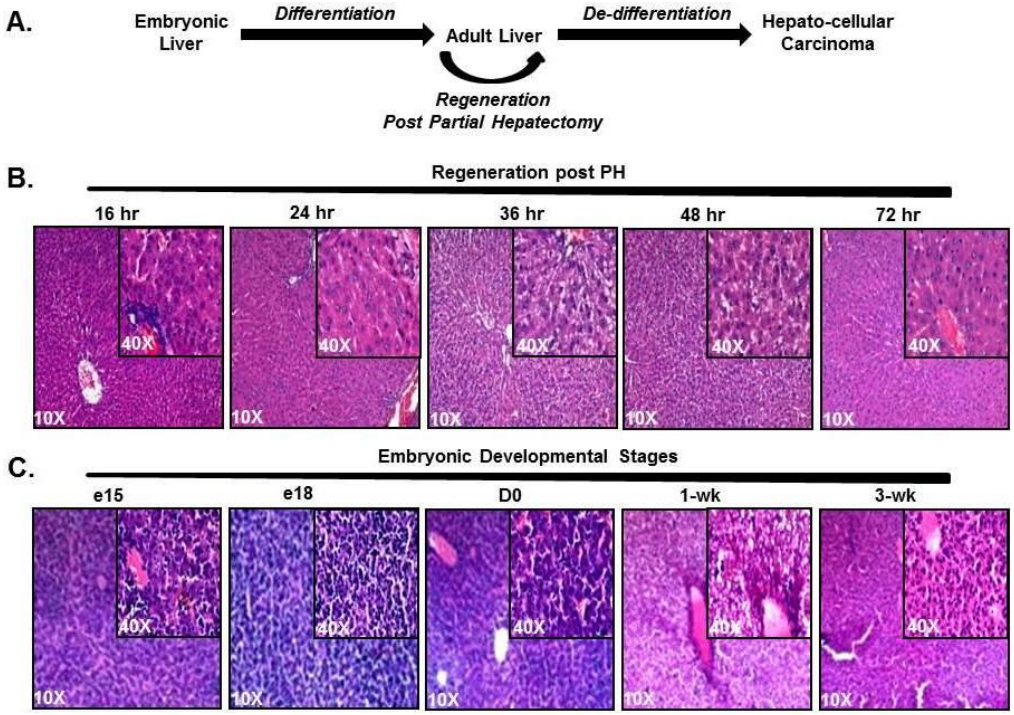


Figure 4.13: Histopathological analysis of embryonic liver development and regenerating liver post Partial Hepatectomy (PH) (A) Schematic representation of various pathophysiological liver stages associated with differentiation (-e15), dedifferentiation (HCC) and normal cellular proliferation (PH) (B) Tissue sections (5µm) of rat stained tissue of regenerating liver post partial hepatectomy at different time points (16hr, 24hr, 36hr, 48hr and 72hr) stained with haematoxylin and eosin. (C) Tissue section (5µm) of progressive stage of embryonic rat liver development (-e15,-e18, D0, 1-wk and 3wk) stained with haematoxylin and eosin. The liver sections showed normal liver architecture and higher nuclear to cytoplasmic ratio at earlier stages of liver regeneration and embryonic development. Magnification: 10X and the inset 40X

The e15 liver showed hyperplasia, hyperchromatic, dissimilar sized hepatocytes with polyploidy and a spongy appearance due compactness of nuclei.

The -e18 stage had multinucleate giant cells with hexagonal hepatocyte with increased cytoplasm to nuclear ratio at D0. The histo-pathological analysis along with the development of new blood vessels confirmed the maturation of hepatoblast to hepatocyte during liver development from –e15 to 3wk (Figure 4.13C).

4.4.2. Histone profiling of different patho-physiological stages of liver

H2A variant expression during different stages of embryonic development and time chase regeneration post PH were studied. Histones were purified from different stages of liver development, e15, e18, D0, 1wk, and 3wk and subjected to AUT-PAGE analysis (Figure 4.14A). The expression profiling of H2A.1 and H2A.2 showed highest accumulation of H2A.1 variant at e15 stage with sequential decrease as liver progress towards maturation. On contrary, the level of H2A.2 variant was highest in differentiated liver at 3wk stage. Also, densitometric analysis shows that H2A.1 and H2A.2 variants are equimolar at D0, whereas, H2A.2 becomes predominant in 3wk compared to maximally expressed H2A.1 in e15 (Figure 4.14A). Further, the histone profile of H2A.1 and H2A.2 variants of mouse Embryonic stem Cell (ESC) was similar to that of –e15 and HCC, whereas, the adult mouse liver showed low level of H2A.1 compared to H2A.2 as observed in normal liver. Thus, suggesting the association of H2A.1 variant with un/-dedifferentiates stage of liver. The H2A.1 and H2A.2 protein bands for embryonic liver were confirmed by MALDI-MS-MS (Figure 4.15 and Appendix III). Liver has the capacity to regenerate with high demand of cellular proliferation with peak of DNA and histone synthesis in 5-7days even after 70% of liver lobe resection. Therefore, the different time points of (16hr, 24hr, 36hr, 48hr and 72hr) regenerating liver post

PH were considered for the study. The higher amount of H2A.2 compared to H2A.1 suggests no alteration in the histone profile compared to normal liver (Figure 4.14B).

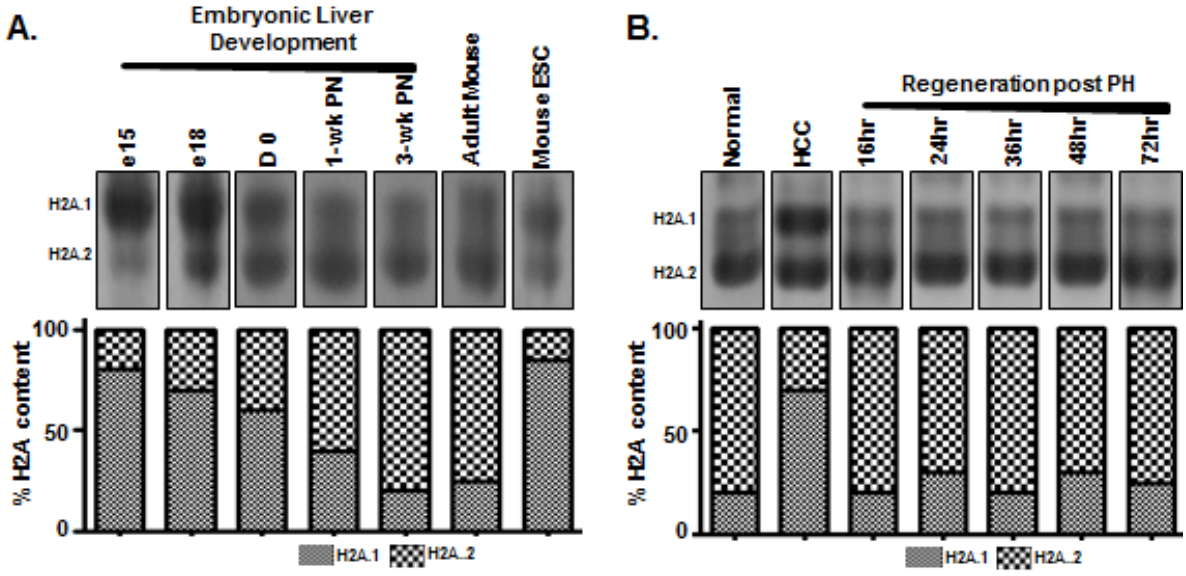


Figure 4.14: Profiling of H2A.1 and H2A.2 during liver regeneration and embryonic liver development: Acid extracted total histones (25µg) from various pathophysiological stages of liver tissue were resolved on 15% AUT-PAGE and stained with ammonical silver method. H2A region was magnified and represented in (A) and (B) panel. (A) Representative gel image of histones extracted from e15 to 3-wk adult rat, mouse ESC and adult mouse liver, (B) The panel shows the resolved histones as extracted from normal adult liver, HCC, 16-, 24-, 36-, 48-, and 72hrs regenerating liver post PH. The higher ratio of H2A.1/H2A.2 was observed in e15, HCC and mESC compared to normal adult and regenerating liver tissue. The densometric analysis of band intensity is represented graphically as an average of three independent experiments

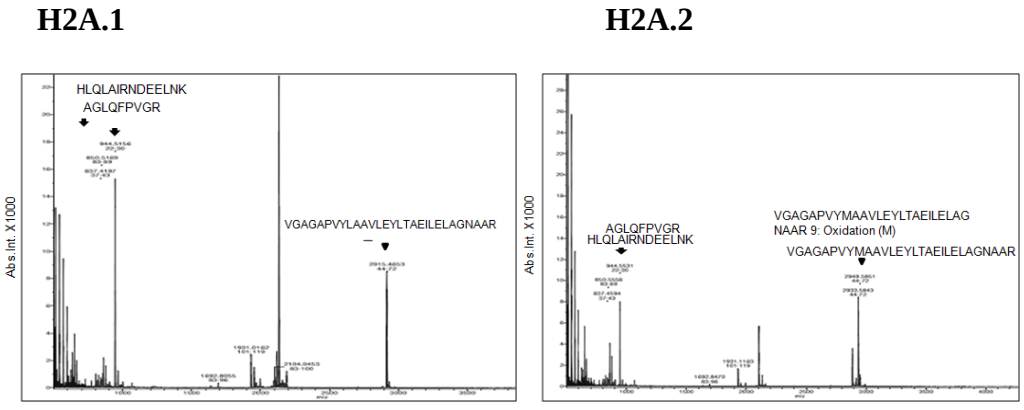


Figure 4.15: Mass Spectra of H2A.1 and H2A.2 in embryonic liver (e15)
Mass of identified peak and range of identified peptide sequence of the respective protein is indicated at the top of each peak.

Thus, suggesting relatively higher expression of H2A.1 than H2A.2 variant is associated only with cellular differentiation and dedifferentiation and does not couple with normal cellular proliferation.

4.5. Expression profiling of H2A.1 and H2A.2 during liver regeneration, differentiation and de-differentiation

The comparative analysis of mRNA expression was carried out within normal adult, embryonic (e15), HCC and 24hr-post PH regenerating liver (24hr-PH) using gene specific primer for each pathophysiological liver stage (Table IX). The level of H2A.1 was significantly high in e15 and HCC compared to normal and regenerating liver (Figure 4.16).

Table IX: Primers for RT-PCR

Gene	Primer
H2A.1(F)	5`-CTGTGCTGGAGTACCTGACG-3`
H2A.1(R)	5`-TGTGGTGGCTCTCAGTCTTC-3`
H2A.2(F)	5`- GAAGACGGAGAGCCACCATA-3`
H2A.2(R)	5`- GGAAGAGTAGGGCACACGAC-3`
PCNA(F)	5`- TCACAAAAGCCACTCCACTG-3`
PCNA(R)	5`- CATCTCAGAAGCGATCGTCA-3`
Sox9 (F)	5`- AGTACCCGCATCTGCACAAC-3`
Sox9 (R)	5`- ACGAAGGGTCTCTTCTCGCT-3`
AFP (F)	5`- ACCTGACAGGGAAGATGGTG-3`
AFP(R)	5`- GCAGTGGTTGATACCGGAGT-3`
18S(F)	5`-CGCGGTTCTATTTTGTTGGT-3`
18S(R)	5`- AGTCGGCATCGTTTATGGTC-3`

However, H2A.2 was highly expressed in normal and regenerating liver. Further, α-fetoprotein (AFP) showed increased expression in e15 compared to adult and regenerating liver. Sox9, a proto-onco gene, showed over-expression in HCC. However, its expression remains unaltered in normal, embryonic and regenerating liver. The cell proliferation marker, PCNA was high in e15, whereas, the increase was similar in HCC and regenerating liver compared to normal. Collectively, the differential expression of markers, PCNA, SOX9 and AFP confirmed the distinct stages of liver development, cancer and regeneration.

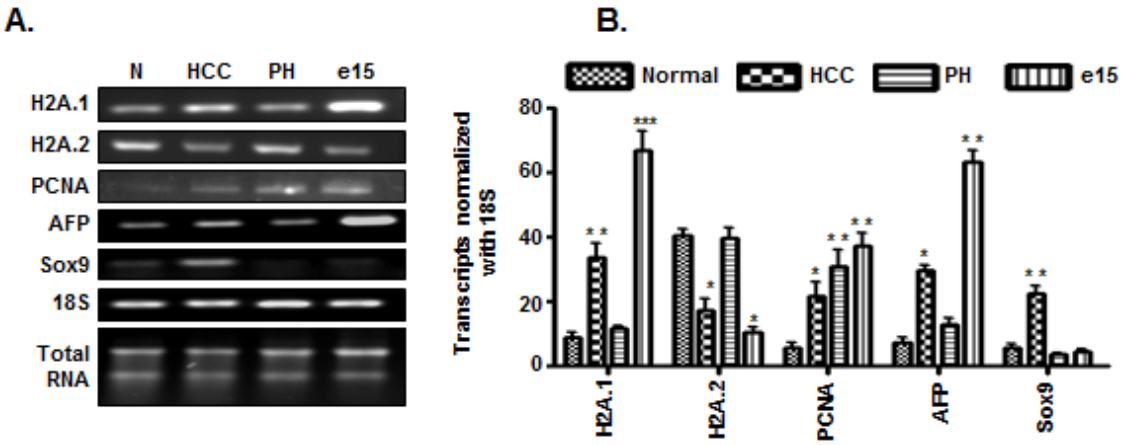


Figure 4.16: Semi-quantitative Reverse Transcription (RT)-PCR analysis of liver stage specific markers: Total RNA was isolated from different pathophysiological stages of liver normal (N), Hepato-cellular carcinoma (HCC), regenerating liver post partial hepatectomy (PH) and embryonic liver (-e15); and subjected for RT-PCR analysis. The expression level of proliferation marker, Proliferating cell nuclear anitgen (PCNA), embryonic liver marker α-feto protein (AFP) and SOX9 at different liver stage was studied. (A) The amplified PCR product was resolved on 1.5% TAE gel and Etbr stained. (B) The densitometry analysis of H2A.1, H2A.2 and other markers was done on normalizing with 18S rRNA as loading control. Each histogram represents mean density ± SEM (*p≤0.05, **p≤0.01)

4.6. Post-translational modifications of histone during hepatocytes proliferation, differentiation and de-differentiation

Histone PTMs play an important role in chromatin structure and function. Active and inactive chromatin marks alter as per change in cellular environment. Histone H3 in regenerating liver shows increase in global acetylation compared to e15 and HCC.

The site-specific modification H3Lys9Ac increases in HCC and decreases in regenerating liver. H3Lys9me3 decreases in HCC and e15, whereas, increases in regenerating liver compared to normal liver. A higher H3Lys14Ac is observed in regenerating and e15 liver compared to normal and HCC. The increased tri-methylation of H3Lys27 is associated with e15 liver and HCC, whereas decreases in regenerating liver compared to normal liver (Figure 4.17).

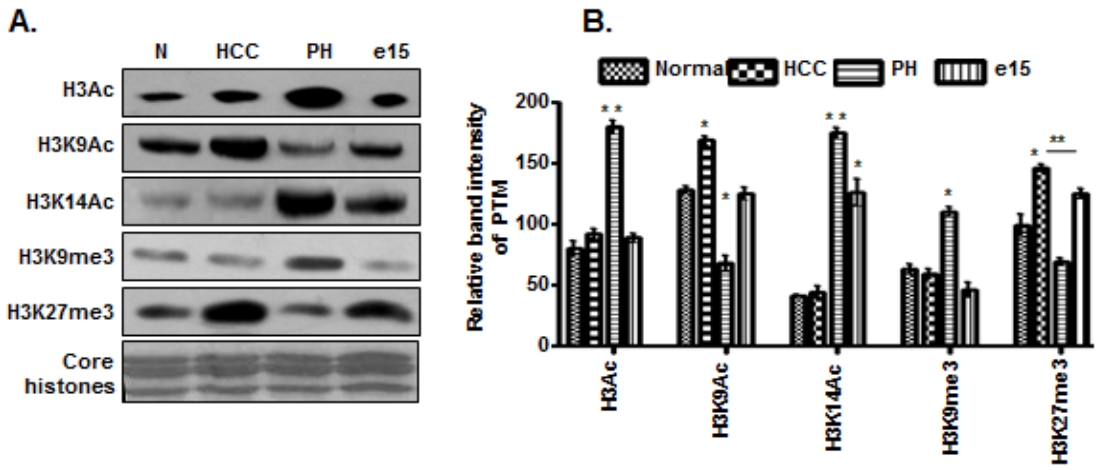


Figure 4.17: Immuno blotting for histone modification marks during different pathophysiological stages of liver: Immuno blot analysis of acid extracted histones from various pathophysiological stages (N-normal, HCC-Hepato Cellular carcinoma, PH-regenerating liver post partial hepatectomy and e15-embryonic liver) of liver was performed. (A) The total histones were resolved on 18% SDS-PAGE and transferred on nitrocellulose membrane for profiling the active and inactive chromatin markers such as; H3Ac, H3Lys9Ac, H3Lys14Ac, H3Lys9me3 and H3Lys27me3. (B) The densitometry analysis was done by normalizing with core histone on fast green stained membrane as loading control. Each histogram represents mean density ± SEM (*p≤0.05, **p≤0.01)

4.7. DNA-protein interaction studies to characterize the transcription factors

Dual luciferase assay performed using 5`promoter deletion construct identified *cis-acting* regulatory region on the promoter of H2A.1 and H2A.2 genes. p53 and Sox 9 emerged as the possible transcription factors for H2A.1 and H2A.2 gene, that gained attention for further investigation.

4.7.1. *In vitro* binding assay was carried out by Electrophoretic mobility shift assay (EMSA) for the binding of putative p53 and Sox9, respectively on H2A.1 and H2A.2 promoter regions.

H2A.1

The p53 binding on H2A.1 promoter region (-831 to -822) as identified to be important *cis-acting* regulatory element from luciferase assay was confirmed by titrating nuclear lysate prepared of Cl38 and CL44 cell line. The increasing nuclear extract concentration (2.5 and 5.0µg) shows a direct correlation with the binding observed as the shift in the labeled DNA. The high level of binding was observed in

the nuclear extract prepared from the neoplastic CL38 cell line compared to pre-neoplastic CL44 cell line (Figure 4.18A). The binding specificity of p53 specific oligonucleotides was confirmed by titration with specific p53 oligos, non specific (ELF1) oligos and mutant p53 oligos (Table VII, Material & methods, 3.21.2.). The loss in binding was observed on titration with 100X cold specific oligo to the reaction mixture whereas the binding was not hampered on addition of 100X of cold non specific or mutant p53 oligo. Thus, confirming the binding specificity of p53 oligos with nuclear extract (Figure 4.18B).

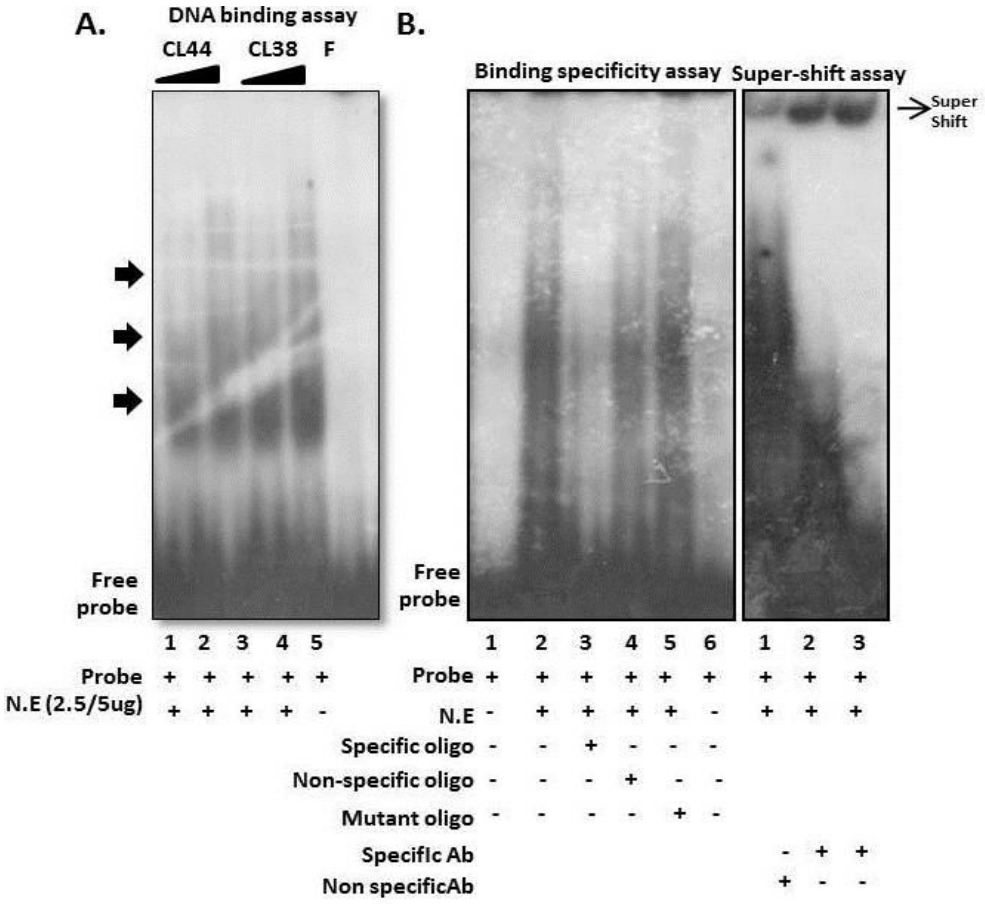


Figure 4.18: In vitro binding assay: (A) The Nuclear extracts prepared from CL38 (neoplastic) and CL44 (Pre-neoplastic) cells were incubated with increasing concentration (2.5µg and 5µg) in EMSA buffer with 32p labelled p53 oligonucleotide and the samples were resolved on 5% TBE gel. (B) Binding specificity assay: nuclear extract of CL38 was incubated with either specific or non-specific or mutated oligonucleotide in presence of 32p labelled p53 oligonucleotide. Super-shift assay: The nuclear extract of CL38 was incubated with either specific (p53) or non-specific (PCNA) antibody in the presence of 32p p53 oligonucleotide.

The p53 binding was confirmed by super shift assay using p53 specific antibody (concentration of 1.5 and 3µg). The data showed that on addition of non-specific antibody (PCNA) in similar concentration does not result in super shift of the protein-DNA complex as observed with p53 specific antibody (Figure 4.18B).

H2A.2

In H2A.2 promoter region the presence of important *cis-acting* repressive element (-921 to-738) were identified by luciferase assay. The 200bp region contains multiple putative Sox family of transcription factor binding sites.

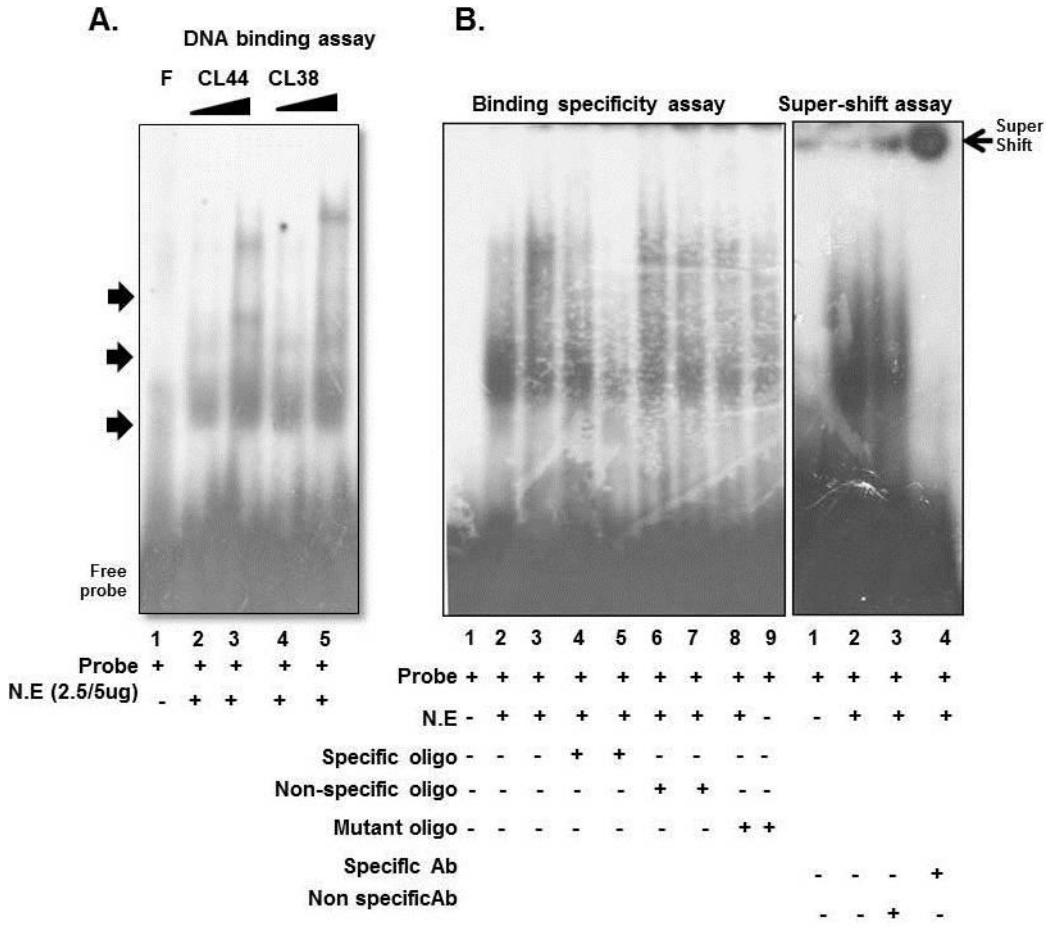


Figure 4.19: In vitro binding assay: (A) The Nuclear extracts prepared from CL38 (neoplastic) and CL44 (Pre-neoplastic) cells were incubated with increasing concentration (2.5µg and 5µg) in EMSA buffer with 32p labelled Sox9 oligonucleotide and the samples were resolved on 5% TBE gel. (B) Binding specificity assay: nuclear extract of CL38 was incubated with either specific or non-specific or mutated oligonucleotide in presence of 32p labelled p53 oligonucleotide. Super-shift assay: The nuclear extract of CL38 was incubated with either specific (Sox9) or non-specific (PCNA) antibody in the presence of 32p labelled p53 oligonucleotide.

The Sox9 binding on H2A.2 gene promoter was observed on titration with nuclear extract (CL38 and CL44 cell lines) at 2.5 and 5 µg concentration. The sox9 binding was comparatively less in CL44 lystae (Figure 4.19A). The binding of Sox9 oligos was confirmed by titration with sox9 specific oligo, non specific ELF1 oligo and mutant Sox9 oligo (Table VII, Material & methods, 3.21.2) on the promoter region (-920 to -908) of H2A.2 gene. The loss in binding was observed on addition of specific 100X Sox9 oligo whereas addition of mutant Sox9 (10X and 100X) and non specific oligo (10X and 100X) does not hinder Sox9 binding, thus confirming the binding specificity of Sox9 oligos (Figure 4.19B). The binding specificity of Sox9 was confirmed by super shift assay using specific (Sox9) antibody and non specific (PCNA) antibody (Figure 4.19B). The specificity of Sox9 was confirmed by super shift assay against Sox9 specific antibody, resulting shift in mobility of protein DNA complex whereas addition of nonspecific antibody does not alter the mobility pattern (Figure 4.19B).

4.7.2 ***In vivo*** **binding assay of p53 and Sox9 at H2A.1 and H2A.2 promoter:**

Mononucleosomal ChIP was performed to confirm *in vivo* binding of p53 and Sox9 on H2A.1 and H2A.2 promoter regions. The cross-linked nuclei were digested with MNase to convert chromatin into mononucleosomes. The completion of reaction was confirmed by resolving digested chromatin on 1.8% TAE agarose gel. The repeat length of DNA is 147bp around the octamer in mammals, so on completed digestion the migration of DNA corresponds to ~147bp on agarose gel (Figure 4.20A).

H2A.1 promoter occupancy:

The mononucleosomes (Figure 4.20A) were immunoprecipitated using p53 specific and IgG antibody. Immunoblots on probing confirmed the specificity of p53 binding,

as signal was seen in INPUT and p53 ChIPed chromatin whereas no signal was obtained from the immuno-precipitates of IgG (Figure 4.20C).

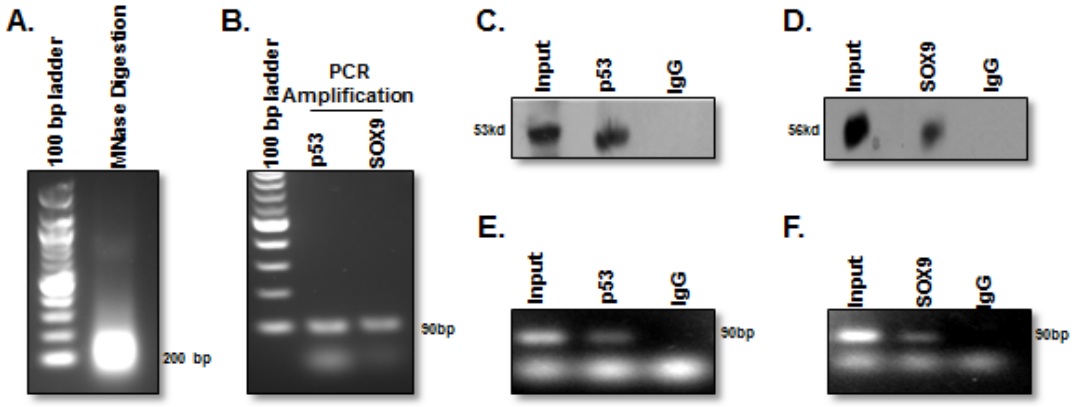


Figure 4.20: I*n vivo* binding ChIP assay identifying p53 and Sox9 occupancy on H2A.1 and H2A.2 gene promoter: (A) Purified nuclei from formaldehyde cross linked CL38 cell line were digested with Micrococcal nuclease (MNase) digestion. The purity of mononucleosomes was checked on 1.8% 1xTAE agarose gel (B) The primer specificity for p53 binding region of H2A.1 and sox9 binding region of H2A.2 was confirmed by PCR amplification (90bp) using mononucleosomal DNA as template. (C and D) The chromatin immunoprecipitated protein lysate was resolved on 10% SDS-PAGE, transferred and immunoblotted with either p53 (H2A.1) or Sox9 (H2A.2) antibody. (E and F) DNA was extracted from the chromatin immune precipitated mononucleosome and subjected for PCR amplification with either p53 (H2A.1) or Sox 9 (H2A.2) primer. The IgG served as the negative control whereas Input served as positive control.

The promoter occupancy on the H2A.1 region encompassing from (-831 to -822) was confirmed by PCR amplification of the reverse cross-linked ChIPed chromatin using H2A.1 gene promoter specific primers (Table VII, Material & methods 3.22.2). The presence of 90bp amplified DNA obtained from the INPUT and p53 ChIPed chromatin and no amplification from negative control (IgG) using H2A.1 p53 binding promoter region specific primers confirmed *in vivo* occupancy of p53 transcription factor (Figure 4.20E).

H2A.2 promoter occupancy:

The *in vivo* binding specificity of Sox9 transcription factor on H2A.2 promoter from -921 to -913 was confirmed by performing ChIP assay. The mononucleosomes immuno-precipitated using Sox9 and IgG (negative control) when immuno-blotted

confirmed the specificity of Sox9; signal was obtained in INPUT and Sox9 precipitates and no signal seen in negative control (IgG) (Figure 4.20D). The 90bp amplified PCR product obtained from the ChIPed DNA against Sox9 antibody using primers specific for H2A.2 gene promoter region (Table VII, Material & methods 3.22.2) confirmed the in vivo occupancy of Sox9 transcription factor (Figure 4.20F).

Chapter 5

Discussion

Discussion

Epigenetic regulation supervises the underlined genetic map and there by becomes one of the prime players associated with various pathophysiological conditions. Various epigenetic mechanisms have been reported to play crucial role in regulating gene expression such as DNA methylation, post-translational modifications of histone and exchange of canonical histone with variants. Lately, emphasis on histone variants has been given on their specific cellular functions apart from their association with modulation in chromatin architecture.

Histone H2A and its variants have been reported to control higher degree of genome reprogramming, chromatin folding and organization of the genome into functionally distinct chromatin territories. These play a crucial role in differentiation and dedifferentiation, ageing and various diseases [155,156]. Our earlier lab reports have shown differential expression of H2A.1 and H2A.2 variants during the NDEA induced sequential development of hepato-cellular carcinoma. There is increase in the expression of H2A.1 variant and decrease in the expression of H2A.2 variant. Further, other reports have shown changes of the H2A.1 and H2A.2, histone variants during aging. The similar alterations are also reported during the post-natal development of chicken and mouse tissues [157] and in early differentiating neurons [158]. In addition, there is recent evidence that the relative abundance of the H2A.1 gene expression increases during cellular differentiation. The level of H2AHISTIC, human H2A.1, is also reported to increase in tumor bladder cells compared to normal [125]. These provide a clue that specific histone variants might share a common mechanism with other different differentiating systems studied so far.

In our earlier study, NDEA has been used as a liver carcinogen to induce hepatocarcinogenesis and also NDEA is known to be metabolized in liver by microsomal membrane bound enzyme, cytochrome p-450 IIEI. Reactive oxygen

species (ROS) generated by the P450-dependent enzymatic system might induce oxidative stress by the formation of hydrogen peroxide and superoxide anions. These ROS circulate in blood stream and might be affecting the genetic and epigenetic profiles in non-target organs. Therefore, we were interested to profile histone variants if there was any effect on non-target organs over a period of carcinogen treatment. Interestingly, the studied showed no histopathological neoplastic changes in the non-target organs (lung, kidney, brain), except few inflammatory changes were seen in kidney. The inflammatory changes in the kidney may be due to excretion of by-products of NDEA metabolism through kidney. Earlier studies have shown that ROS generated deteriorates cell membrane and cause cellular injury and impair the function. The ROS is known to cause DNA, protein and lipid damage; therefore, oxidative stress can play an important role in hepato-carcinogenesis.

The organs and tissues are comprised by a wide range of cell types, possessing same genome. Remarkably, the level of expression of gene vary among different tissues, relatively small numbers of genes are expressed in tissue-specific fashion to contribute to the morphological and biochemical characteristics that distinguish the phenotypes of these cell types. The tissue-specific profile of H2A.1 and H2A.2 in different organs showed predominance of H2A.2 in liver and kidney, whereas, H2A.1 and H2A.2 are equal in brain and lung. Thus, contradicting earlier report of higher level of H2A.2 in adult tissues. These are in agreement with similar developmental change in other histone variants, macroH2A.1 composition in rat liver and kidney [159]. The H2A.Z isoforms H2A.Z.2.2 is putatively primate-specific and present at much lower levels in most tissues in contrast to the highly conserved major isoforms H2A.Z.2.1. In brain tissues, however, Z.2.2 is significantly enriched with abundances similar to Z.2.1 [160]. Further, H2A.Bbd is highly tissue specific

and strongly expressed in testis and to a much lesser extent in brain [161]. In rat, testis specific histones have been characterized for each histone type except H4. Also, H3.2 is reported to be most abundant H3 variant in all tissues examined except for testes. The abundance of H3.3 was comparable to H3.2 in brain, testes and heart, but H3.2 was 2-3 times more abundant than H3.3 in most cases. Recently, H3.3 variant has also been reported to be preferentially associated with transcriptionaly active gene loci [162] and undergo PTM associated with gene activation [163]. Thus, the association of different histone variants with the chromatin in various differentiated tissues may contribute to different level of nucleosomal stability, chromatin structure and dynamics to carry out tissue or stage specific functions. However, the functional relevance of tissue- or stage-specific distribution of these histone variants is still to be delineated.

To delineate whether these histone variants were reactivated in previously differentiated cells or whether represent in early developmental stage during stem/ progenitor cell differentiation further studies were carried out on embryonic and regenerating liver post-PH. Thus, studying embryonic liver development may not only be important from an embryologic perspective but also to contribute to a better understanding of the pathogenesis of liver cancer. The expression profiling of H2A.1 and H2A.2 variants in different pathophysiological conditions demonstrated that the increase in level of H2A.1 expression associates with the un-/de-differentiated states of hepatocytes as observed during embryonic liver development and HCC. The liver development and de-differentiation of mature hepatocyte to HCC are in coherence with the expression profile of cell proliferation marker, PCNA and liver differentiation marker, AFP. The expression level of AFP is high in the liver of the developing fetus and silenced after birth. Adult expression of AFP is monitored as a

tumor marker, aberrant reactivation occurs in up to 85% of all hepatocellular carcinoma cases [164,165] whereas Sox9, remains minimal during differentiation [166] and increases in HCC [167], which is in accordance with earlier studies. The observed higher level of H2A.1 expression in mouse embryonic stem cells (ESC) along with e15 and HCC strengthens that H2A.1 is associated with undifferentiated cells. H2A.Z and macroH2A incorporation has been well reported to alter chromatin architecture. Therefore, we looked for the known active and inactive histone marks that also had been reported to as significant markers of HCC. Our data showed increase in H3Ac suggesting open chromatin organization in different patho-physiological states. The alteration in active and inactive marks on H3 suggests similar pattern of modification(s) during early differentiation and dedifferentiation but further studies are required to understand their significance. The report on differentiation and cancer development have shown increase of macroH2A in maturation of new born to young adult liver, though macroH2A decrease in cancers. The incorporation of different homomorphous histone variants of H2A and increase in global acetylation may have potential implication on chromatin architecture, downstream gene expression and thereby maintaining high plasticity for genomic reprogramming during differentiation and dedifferentiation.

Hepatocarcinogenesis and liver regeneration share a common important feature i.e. cellular proliferation though in one case it is well regulated and in other scenario its uncontrolled dysregulated process. We investigated on this query with respect to the differential expression of H2A.1/H2A.2 being specific to the process or de-/un-differentiation or it is associated only with normal cellular proliferation. Liver regeneration post partial hepatectomy provides excellent model to study normal cellular proliferation. The DNA replication in liver regeneration is coupled with

histone synthesis, modification (s) imprinting and increase in PCNA for assembly of chromatin. Studies of Zweidler *et al* [157] have reported that non-dividing adult tissues, i.e. after cell growth and cessation of DNA replication, contain accumulation of variants, H2A.2, H2A.3, H2B.1 and H3.3, whereas embryonic tissue and dividing cells of adult tissue have higher amount of H2A.1, H2B.2, H3.1 and H3.2 variants. On contrary, our studies on regenerating liver showed no alteration in the accumulation and expression of H2A.1 and H2A.2 variants. Also, the chromatin marks are different compared to e15 and HCC liver. The observed significant increase of H3Lys14Ac after PH is in accordance with previous reports. Thus, increase in normal cell division for tissue maintenance or compensatory growth is not associated with change in expression of histone variant but only associated with different post-translational modification(s) to regulate change in gene expression. The dual regulatory mechanism comprising of exchange of canonical histone with variants and the site-specific PTM has been reported in case of other histone such as cellular localization experiments highlight that ubiquitination of H2AZ is found at transcriptionaly silent facultative heterochromatin on the inactive X chromosome [168]. Remarkably, H2AZub is portrayed as a silencing mark, while H2AZac is associated with transcriptional activity, suggesting that bivalent domains are marked by antagonistic modifications on both H3 (H3K4me3 versus H3K27me3) and H2AZ (H2AZac versus H2AZub1) [169]. In *Sacchromyces cerevisiae*, sumoylation of HtZ1 (H2AZ) on lysine 26 and 133 play important role in DNA double strand break by relocating the unrepaired chromosomal break to the nuclear prephiry [170]. Interestingly, comparison of the H3.1 and H3.2 PTMs in mammals shows that H3.2 is enriched in modifications associated with transcriptional repression, such as lysine27 is di-methylated (K27me2) and tri-methylated (K27me3), whereas H3.1

carries both active and repressive marks [171]. Earlier studies have shown broadly overlapping genetic mechanism leading to the biological changes during embryonic liver development and HCC. The majority of the biological mechanisms such as cell cycle control, proliferation and growth essential for both embryonic development and cancer de-differentiation. A pioneer study analysed a comprehensive microarray data set of mouse liver development during multiple stages. Li et al. reported that genes enhanced in early stages of liver development are also enriched in HCC development [172,173]. These reports in corroboration with our findings suggest that H2A.1 and H2A.2 have potentially different roles in organisation of chromatin structure for differential regulation of their gene expression in different pathophysiological conditions.

The differential expression in various pathophysiological liver stages might be due to their mode of transcription regulation. With the limitation in availability of literature related to regulatory mechanisms for the expression of H2A.1 and H2A.2 variants it becomes immensely important to delineate this mechanism. The control of histone gene expression at the level of transcription is complex and only few histone and its variants transcription regulation have been studied so far with exception of human H4 genes that is best studied and is reviewed in [174]. The majority of histone genes are organized into clusters, persists throughout the course of evolution from yeast to human and add to a layer of complexity in their transcription regulation [175]. Histone gene clusters in mammals are heterogeneously organized and contain one or more copies of the five histone subtypes, i.e., core (H2A, H2B, H3, H4 and variants) and linker (H1) histone genes [176,177]. The functionally characterized vast majority of the 74 known human histone genes were mapped within the major and minor clusters located on chromosomes 6p21 and 1q21, respectively [177]. In rat,

Discussion

H2A.1 and H2A.2 gene are localized on Chr. no 17 and Chr. no. 2, respectively in the histone clusters. Both the genes are intron-less, the cloned 393bp cDNA was sequenced and confirmed by aligning with the predicted sequence deposited in NCBI database (XM-577573 for H2A.1 and (XM-345255 for H2A.2). Thus, the location of these variants on the genome suggests that they acquire independent basal transcription regulatory unit. The cDNA sequence for both the variants share 89% homology and differs at 3 amino acids position in their protein sequence. The coding region of several histone genes share high level of sequence homology but differs significantly in the untranslated regions. Primarily, the untranslated sequences in the 5' region, presumably affects the transcription rate, as well as the 3' region is involved in mRNA processing, contributing to the differences in mRNA accumulation [178]. The identified and characterized the 5’ and 3’ un-translated region of rat showed the positioning of transcriptional start site (+1) for H2A.1 and H2A.2 gene at 43bp upstream the ATG contradicting *in silico* identified TSS. Our sequence and mapping analysis also confirm the presence of single transcript for H2A.1 and H2A.2 gene as both are having only one TSS. The 5’UTR of H2A.1 and H2A.2 genes has been identified to be 43bp long and as other RNA polymerase II transcribed genes the functional significance of these untranslated regions may be the regulation of post-transcriptional gene expression and the control of mRNA nucleo-cytoplasmic transport, translation efficiency, sub cellular localization and stability [179]. Another critical regulatory step in gene expression occurs via regulation of the half-life of mRNA. It provides the best mechanism for inactivating the translation of an mRNA and hence halting synthesis of a particular protein until more mRNA is synthesized. The degradation of histone mRNA plays a critical role in the regulation of histone protein biosynthesis, helping to maintain the balance

between DNA and histone biosynthesis [180]. Histone synthesis is coupled with DNA replication and upon entry into the S-phase, histone gene transcription is induced 5-fold approximately [181]. This induction is cell cycle-regulated and involves *cis*- and *trans*-acting elements including subtype-specific consensus elements in histone gene promoters and the transcription regulator NPAT (nuclear protein, ataxia–telangiectasia locus) [182]. NPAT have been shown to interacts with the pro-apoptotic protein FLASH [FADD (Fas-associated death domain)-like IL-1β (interleukin 1β)-converting enzyme associated huge protein], which has interestingly been implicated in both histone gene transcription and histone RNA processing [183,184]. This indicates that histone RNA synthesis and processing may be coupled, as is the case for other genes transcribed by RNA polymerase II.

Like polyadenylated mRNAs, the 3'end of histone mRNA plays a critical role in translation [185]. The regulation of the half life of histone mRNA is mediated by the unique 3'end of histone mRNA [186,187]. The metazoan replication-dependent histone mRNAs are the only eukaryotic mRNAs that are not polyadenylated. The mRNA circularization experiments done in our studies have shown that the histone variant H2A.1 has 62bp long 3'untranslated region including 26bp long conserved stem loop structure, typical of the histone family. Similar set of experiment carried for identification of 3'UTR in H2A.2 mRNA shows presence of 88bp long 3'UTR with a conserved stem loop structure. The stem-loop sequence at the 3' end of histone mRNAs contains a six-base stem and a four-base loop. The five nucleotides 5' to the stem are a conserved AC-rich sequence with the adenosines at positions two and three being the most highly conserved and essential for function. There is also an AC-rich sequence after the stem-loop, with the consensus ACCA in *Drosophila*, sea urchins and *Caenorhabditis elegans*, and ACCCA in vertebrates. This 25–26

nucleotides sequence is the binding site for the stem-loop binding protein (SLBP), which is involved in multiple steps of histone mRNA metabolism. The processing of stem loop containing mRNA occur by SLBP binding to stem loop followed by nucleo-cytoplasmic transport and later on proceeding for translation. Earlier studies have reported formation of the 3' end requires two *cis-acting* sequences, the stem-loop and a 3' purine-rich sequence termed the histone downstream element (HDE) [188,189,190]. The decision to degrade an mRNA (in particular when the half-life of the mRNA is changing as the result of a signaling event) generally results in degradation of an mRNA that is being translated. In the case of histone mRNA, mRNA translation is obligatory for mRNA degradation; histone mRNA is stable when its translation is blocked [187]. Collectively, their comparable UTR structures suggest that H2A.1 and H2A.2 expression might be coupled with replication phase of the cell cycle and their stage specific expression in various pathophysiological conditions might be contributed by transcriptional regulation but not due to post-transcription regulation. (*The identified H2A.1 and H2A.2 mRNA are 498bp and 525bp long containing 5'UTR, 3'UTR and stem loop that are submitted to NCBI database with accession number JX_661508 and JX_661509, respectively*)

The histone gene promoters are very similar to the promoters of other mRNAs, and they bind many of the same factors involved in synthesis of polyadenylated mRNAs. The H2A.1 as well as in H2A.2 gene promoter analysis confirmed presence of TATA box (TATAAA) and CCAAT box that are well positioned as earlier reported in replication dependent gene promoters and play an important role in determining basal promoter activity of the histone. The functional characterization of 5' upstream region, by using reporter plasmids consisting of the 5'-deletion fragment linked to the luciferase reporter gene demonstrated that the minimum sequence necessary for

basal promoter activity resides within 131bp upstream of transcription start site in H2A.1 gene and 135bp upstream of transcription start site in H2A.2 gene.

The deletion constructs were prepared on the basis of *in silico* identified putative transcription factor binding sites within the promoter region of H2A.1 and H2A.2 genes. The H2A.1 promoter region analysis suggested binding of important TFs such as p53 [191], ELF1 [192], BRCA-1 [193], C/EBPα [194], NFAT-1 [195] that independently or in combination have been associated with activation and repression of downstream gene expression. The luciferase based reporter assay identified the 400bp region from -856 to -456bp with significant change in promoter activity suggesting presence of *cis*-acting regulatory elements. The significant decrease in the transcriptional activity from -856 to -656 suggest presence of repressor binding site, whereas presence of activator binding site cannot be ruled out in the 200bp region (-656 to-456) in H2A.1 gene promoter. Further, delineation of the 5'-flanking promoter region of H2A.1 (-856 bp to -656 bp) showing transcriptional repression showed presence of putative half p53 binding site. p53 is frequently portrayed as an emergency response molecule, activated only under conditions of high stress or DNA damage [196]. The p53 protein is known to either activates or represses the transcription of a gene. The first step in p53-mediated transcription is the binding of the protein to its recognition site in DNA. p53 binds in a sequence-specific manner to DNA binding sites consisting of two decameric motifs or half-sites. After various types of genotoxic insults, p53 is known to be stabilized, translocates to the nucleus, and binds as a dimer of dimers to its response element (RE) [197]. In general, the functional consequence of p53 DNA binding is transcription activation of target genes with role in cellular stress response, in developmental pathways [198]. However, recently examples of p53-mediated transcription repression through

sequence-specific DNA binding have been reported. The luciferase data suggests the presence of transcriptional activator binding site in 200bp region from -1056 to -856, the identified putative factors include, Sox10 and NFAT-1. NFAT-1 is highly expressed in HCC and mediate p53 inactivation, apoptosis and degradation via MDM-2 dependent pathways. On the contrary, the non-significant cell death observed post transfection of H2A.1 promoter in CL38 cell line and the high level of binding to H2A.1 promoter region in EMSA suggest the importance of stabilization or increase in transcription of p53 and rules out the possibility of NFAT-1 mediated p53 degradation and transcription regulation of H2A.1 gene promoter. The possible explanation for this could due to very short half life of p53 in normal liver condition as reported, whereas in case of liver cancer p53 gets mutated which stabilizes the protein but inactivates the repressive ability of p53 domain [199]. Thus, it corroborates with our earlier finding where we observe increase in H2A.1 during liver cancer. The binding of p53 on H2A.1 gene promoter region (-856 to -656) was confirmed by *in vitro* assay, where in the maximum binding of p53 was observed in the neoplastic cell line compared to pre-neoplastic cell line. α-fetoprotein (AFP), a known marker for liver embryogenesis and carcinogenesis, gene has also been reported to be transcriptionaly regulated by p53. The genes repression is mediated by the method of p53 steric interference. The binding of p53 physically excludes a tissue-specific activator of AFP transcription and may tether a complex of tissue-specific co-repressors which actively interfere with gene expression. The aberrant expression of AFP during tumorigenesis (HCC) may require functional inactivation of the p53 protein [196]. Thus, the binding assay results corroborates with our earlier finding where we observe increase in H2A.1 and AFP during HCC in coherence of AFP gene regulation report mentioned above. Further, the *in vivo* binding assay

confirmed the p53 occupancy on H2A.1 promoter region and suggests the potential functional role of p53 as transcription factor that in combination with other transcription factors regulate the intricate balance of H2A.1 gene expression during various pathophysiological conditions.

In silico analysis of H2A.2 gene promoter identified several putative transcription factors such as Oct, Nanog [200], Sox9, -5, -10, p300 [200]. The *cis-acting* regulatory elements of H2A.2 promoter region were identified as 200bp long (-921 to -738) with presence of repressor binding sites, that are predicted to be of Sox-family 5, 9 and 10. Sox5 has been reported to act as a strong transcriptional repressor. As mentioned earlier Sox9 is a member of the large class of SOX (SRY-type HMG BOX) transcription factors related to the testis determining factor, SRY, through their HMG domains that bind and bend DNA in a sequence-specific manner. Sox9 binds the consensus DNA sequence, (A/T)(A/T)CAA(A/T)G, and belongs with its closest homologues, Sox8 and Sox10, to the group E of SOX genes. A dimerization domain has been identified in Sox10 in a 40 amino acid region immediately N-terminal to the HMG domain, that allows cooperative binding in the presence of target gene promoters such as the promoter of myelin protein zero (P0), present in Schwann cells. This region is highly conserved amongst all other class E members (Sox8 and Sox9) [201]. *In vitro* and *in vivo* binding assay confirmed the occupancy of Sox9 on the H2A.2 gene promoter region. Thus, Sox9 is a transcription factor that might in combination with other factors facilitate the regulation of H2A.2 gene expression. We have also reported the decrease in expression of H2A.2 in liver cancer and embryonic liver with increase of Sox9 expression, this further corroborates with the current study suggesting repressive ability of Sox9 transcription factor on H2A.2 gene promoter. Sox9 plays a pivotal

role in a number of developmental processes and its levels need to be strictly controlled for normal embryogenesis. Recently, SOX transcription factors have been found to be associated with human cancers. Sox9 is often over-expressed in cancers of the skin, prostate, lung, brain and liver [167, 202, 203, 204, 205].

Collectively, the findings signify that homomorphous histone variants, H2A.1 and H2A.2 with conserved stem loop plays a potential role in defining highly specialized biological mechanisms, differentiation and de-differentiation opposed to normal cellular proliferation during liver regeneration and suggests overlapping functional relevance in epigenetic reprogramming of genome. The in-depth study on the transcription regulation of H2A.1 and H2A.2 gene confirms the presence of the independent promoter region having binding sites for oncogenic protein p53 and Sox9, respectively. It would be interesting to further investigate functional relevance of these transcription factors and their interaction with other transcription factors on regulation H2A.1 and H2A.2 gene expression in various pathophysiological conditions.

Chapter 6
Summary and Conclusion

Summary and conclusion

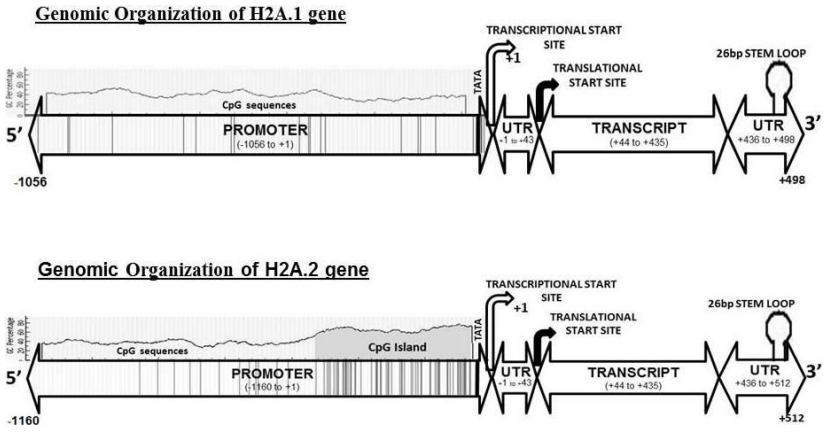
Chromatin is highly organized structure that acts as regulatory switch for genetic information and the possible mechanisms so far demonstrated involves PTMS, DNA methylation and histone variants. Recent studies report number of histone variants that account for specialized cellular function under various pathophysiological conditions. On the basis of previous lab report on differential expression of H2A.1/H2A.2 variant during sequential development of HCC, in the present study we demonstrate a strong correlation of H2A.1 expression with undifferentiated hepatocytes, overlapping signatures of histone modifications and chromatin architecture.

The plausible reason hypothesized for such observation is the transcriptional regulatory mechanism of H2A.1 and H2A.2 variant gene. Therefore, in the current study we have delineated mechanism of H2A.1 and H2A.2 transcription regulation by step wise identification of TSS, 5'UTR and conserved stem-loop with 62bp and 88bp long 3'UTR, suggesting their replication-dependent expression. Further, both H2A.1 and H2A.2 are TATA-driven with conserved CAAT sequence with different *cis-acting* elements in their promoters signifying their different regulatory mechanisms. The binding of transcription factors, p53 and Sox-9 have been identified, *in vitro* and *in vivo,* as negative transcriptional regulators of H2A.1 and H2A.2 gene promoters, respectively. The reports on over-expression of Sox9 and mutation of p53 in HCC corroborates with our findings and therefore, p53 and Sox9 are one of the key regulators for transcriptional repression of H2A.1 and H2A.2 genes in HCC compared to normal liver, respectively. Finally, H2A.1 and H2A.2 variants might play a potential role in defining highly specialized chromatin organization during different biological processes, differentiation and/or de-differentiation suggesting their role in epigenetic reprogramming of genome.

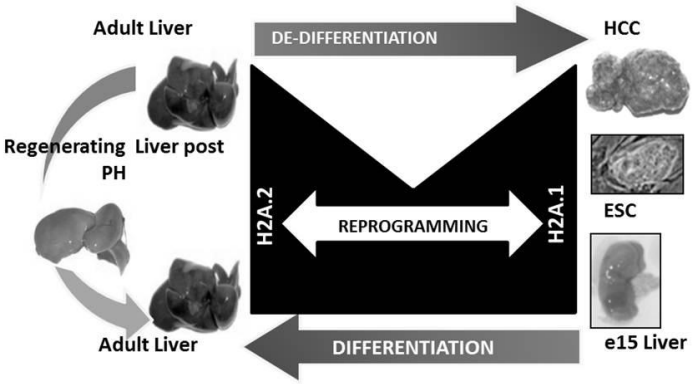
Summary and conclusion

The salient findings of the work done are:

- The H2A.1 and H2A.2 cDNA of 393 bp showed 88% homology in their sequences.
- The TSS of H2A.1 and H2A.2 gene is positioned 43bp upstream the Translation start site (ATG) and they do not share any sequence similarity.
- The region encompassing from -1056 to +144 of H2A.1 and -1160 to +219 for H2A.2 showed promoter activity.
- *In silico* studies suggests presence of CpG Island spanning from -290 to +219 in the promoter region of H2A.2 gene whereas CCGG repeats are identified in H2A.2 gene promoter region.



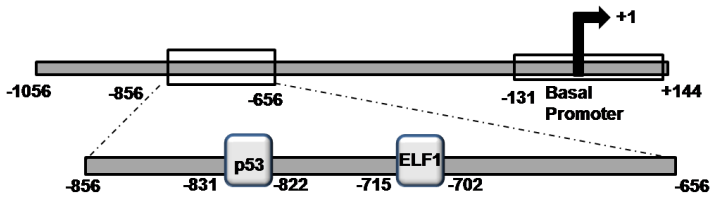
- Over expression of H2A.1 associates with un/de-differentiated hepatocytes, whereas, H2A.2 correlates with differentiated hepatocyte



Summary and conclusion

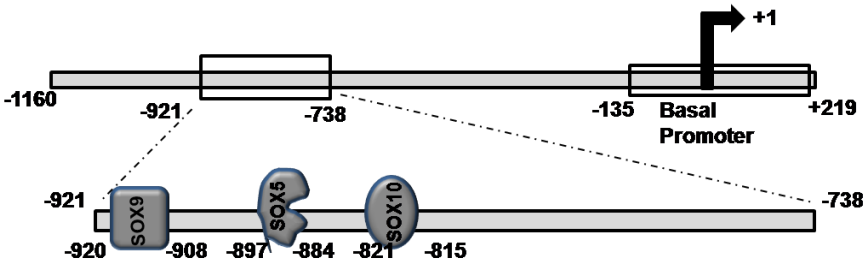
- The important cis regulatory regions identified in H2A.1 promoter region -856 to -656 bp possessing activator as well as repressor activity identified by dual luciferase assay.
- Within the 200bp the region encompassing from -856 to -456bp posses putative p53 binding sites (-831 to -822) that might be responsible for transcriptional repression.

H2A.1



- In H2A.2 gene promoter the 200bp region encompassing from -921 to -738bp posses repressor activity having putative Sox-family of transcription factors binding sites.
- Sox9 is identified as the transcription factor binding to the promoter region of H2A.2 gene from (-921 to -908bp) and possibly acting as key repressive factor in H2A.2 gene expression

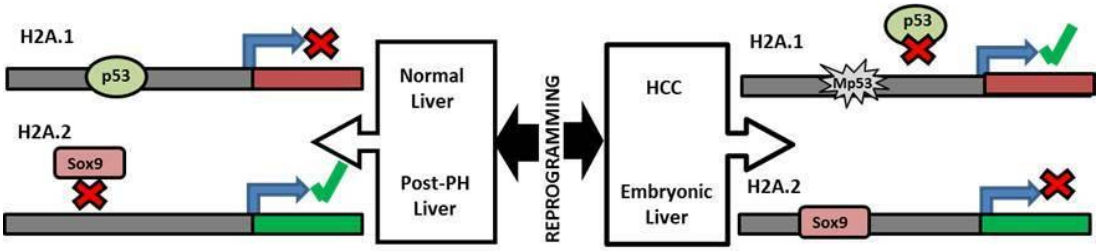
H2A.2



Conclusion:

In the present study we decipher the transcription regulatory mechanism possibly involved in H2A.1 and H2A.2 histone variants gene expression. The significance of

the work is beneficial in the better understanding of the epigenetic influences in pathophysiological conditions. In continuation with our previous lab reported on differential expression of H2A.1/H2A.2 variants during HCC, here we have studies their expression in embryonic and regenerating liver. This finding suggests H2A.1 association with the un-/de-differentiated hepatocytes. Collectively, we propose p53 and Sox9 as the possible transcription factors that might be regulating the expression of H2A.1 and H2A.2 genes and thereby altering the chromatin architecture



Future directions:

- Functional significance of p53 and Sox9 in combinatorial effect of other transcription factors in regulating H2A.1 and H2A.2 gene expression.
- Promoter methylation studies to understand regulation of H2A.1 and H2A.2 gene expression in different pathophysiological conditions.

Chapter 7
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Chapter 8
Appendix

Appendix

Appendix I

List of Chemicals

1	Acrylamide	Sigma
2	Agarose	USB
3	Ammonium persulfate	USB
4	Acetic Acid	Qualigens
5	Aprotinin	Sigma
6	Amido Black	Sigma
7	Ammonical Silver nitrate	Qualigens, SD fine
8	Ammonium hydoxide	Qualigens
9	Bovine Serum Albumin fraction V	Roche-Sigma
10	Bradford reagent	Biorad
11	BME	Sigma
12	Commassie brilliant blue R-250	USB
13	Calcium chloride	SD fine
14	Cysteamine hydrochloride	Sigma
15	Disodium Hydrogen Phosphate	Qualigens
16	Dihydrogen Sodium phosphate	SD fine
17	Dimethyl Sulphoxide	Qualigens
18	EDTA	Sigma
19	EtBr	Sigma
20	ECL detection reagent	Millipore
21	Formaldehyde	Qualigenes
22	Femto	Pierce
23	Glycerol	Merck
24	Glycine	Sigma
25	Hydrochloric Acid	Qualigens
26	Leupeptin	Sigma
27	Magnesium Chloride	Qualigens
28	Methanol	Merck
29	Mnase	USB
30	N-N Methylene Bis-Acrylamide	Sigma, Amresco
31	Nonidet P-40	Sigma
32	Potassium Acetate	SD fine
33	PMSF	Sigma
34	Potassium Chloride	Glaxo
35	Pepstatin A	Sigma
36	Protamine Sulfate	Sigma
37	Potassium Acetate	SD fine
38	PVDF membrane	Millipore
39	Paraformaldehyde	Sigma
40	Riboflavin 5 phosphate	Sigma
41	RNASE A	Amresco
42	Sodium Chloride	Qualigens
43	Sodium Orthovandate	Sigma
44	Sodium Lauryl sarcosine	Sigma
45	Sodium Citrate	Merck
46	Sodium Deoxycholate	BDH
47	Sodium Bicarbonate	SD fine
48	Sodium Hydroxide	SD fine
49	Sulphuric Acid	Qualigens
50	Spermine	USB

51	Spermindine	USB
52	SDS	Sigma
53	Sucrose	Sigma
54	Tween-20	Sigma
55	Triton X-100	Sigma
56	Tris	Sigma
57	Trypsin	Sigma
58	TEMED	Sigma
59	Urea	Sigma

Appendix

Appendix II

List of Primers

GENE PROMOTER	
-1056 FH2A. 1	5'GGTACCAGCAATGTGAATGTGTAAGCG3'
+144 RH2A. 1	5'CTCGAGAAACGGTGCACACGGCCCACG3'
-1060 FH2A.2	5'GGTACCCTGAACCCTTGATCCATCTCTC 3'
+229 RH2A.2	5'CTCGAGATCTCGGCGGTCAGGTACT3'
5'Deletion constructs primers	**H2A. 1**
-656FH2A. 1	5' GGTACCAAGACAGTGTATAGCCAATT3'
-456FH2A. 1	5' GGTACCAATTAAACCTTAGCAGGACGAC3'
-256FH2A. 1	5'GGTACCGATCTCAACTATTAATCTACCCTT3'
-131FH2A. 1	5'GGTACCAAGAGTACGCCTTATGCAAATGA3'
-821FH2A.1	5'GTCGGTACCCTCCTTCTCCTCAGGC3'
5'Deletion constructs primers	**H2A.2**
-738FH2A.2	5'GGTACCTACTGCCAGTCTACACCCAAA 3'
-560FH2A.2	5'GGTACCTACTGCCAGTCTACACCCAAA 3'
-315FH2A.2	5'GGTACCGGTTCTTAACTCAGGCCTTCTTG 3'
-135FH2A.2	5'GGTACCGGTTCTTAACTCAGGCCTTCTTG 3'
-907FH2A.2	5'GTCGGTACCCCATGCTACTGTGTAA3'
-883FH2A.2	5' GTCGGTACCCCTGTTTCTAGTGCTA3'
-814FH2A.2	5' GTCGGTACCGCCTATCACACATTTC3'
+219RH2A.2	5'CTCGAGATCTCGGCGGTCAGGTACT3'
CHIP PCR PRIMERS	
F-SOX9 H2A.2	5'GGTACCCAAGAACAATGTGACCATGC 3'
R-SOX9 H2A.2	5'TGGCATTAAGAATCACAGATTAGA3'
F-p53 H2A.1	5'GGTACCATTGCAGGCTTTCCTGGGTCA'3
R-p53 H2A.1	5'TTATGGCTGAATTATCCTTAAACTGC3'

Appendix

Appendix III

MALDI-TOF Mass Spectrometry analysis of H2A.1 and H2A.2.

H2A.1 **(GeneID: 502125)**

Meas. M/z Calc.	MH+ Int.	Dev.(Da)	Dev.(ppm)	Range	Sequence
850.5169	495.3839	-0.0088	-10.3800 7	83 – 89	HLQLAIR
944.5156	12784.9104	-0.0157	-16.5910	22-30	AGLQFPVGR
1931.0162	1607.2530	-0.1526	-79.0478	110-119	VTIAQGGVLPNIQAVLLPK
2915.4653	4363.4239	-0.1224	-41.9831	44-72	VGAGAPVYLAAVLEYLTAEILELAGNAAR

H2A.2 (**GeneID: 365877)**

Meas. M/z Calc.	MH+ Int.	Dev.(Da)	Dev.(ppm)	Range	Sequence
850.5558	495.1984	0.0301	35.4624	83 – 89	HLQLAIR
944.5531	6926.5611	0.0219	23.2077 6	22-30	،GLQFPVGR
1931.1163	1463.5483	-0.0525	-27.1931	101-119	VTIAQGGVLPNIQAVLLPK
2933.5843	4694.9618	0.0402	13.7171	44-72	VGAGAPVYMAAVLEYLTAEILELAGNAAR
2949.5851	614.5437	0.0461	15.6461	44-72	VGAGAPVYMAAVLEYLTAEILELAGNAAR 9: Oxidation (M)
1692.8470	142.8310	-0.0557	-32.9270	83-96	HLQLAIRNDEELNK

Appendix

Appendix IV

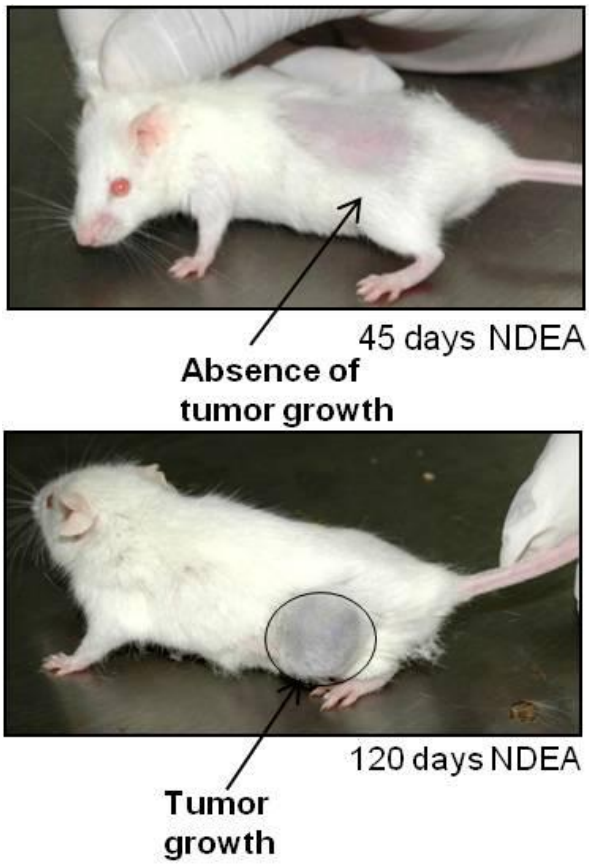

Figure represents the tumor growth observed after implantation of neoplastic liver tissue (120days of NDEA treatment) in SCID mice, however no tumor development was seen on implanting pre-neoplastic liver tissue from 45 days NDEA treated animal.

Appendix

Appendix V

List of putative transcription factors and their respective binding sites on H2A.1 gene-promoter identified using Biobase and Moti finder softwares.

Predicted transcription factors binding on H2A.1 gene promoter (-1056/+144)	DNA binding region
NFAT	-1036/-1028
SOX 10	-993/-986
P53	-831/-822
ELF-1	-714/-709,-625/-619
BRCA-1	-549/-542
C/EBP alpha	-499/-487
CDX	-378/364
GATA3	-252/-241
Oct-1/2	-121/-105
CAAT-Box	-69/-60
SMAD3	-54/-46
v-Myb	-35/-30
TATA-Box	-29/-23
E2f-1	+78/+83

Appendix

List of putative transcription factors and their respective binding sites on H2A.2 gene-promoter identified using Biobase and Moti finder softwares.

Predicted transcription factors binding on H2A.2 gene promoter (-1160/+219)	DNA binding region
Nanog	-1077/-1057
BRCA-1	-1007/-999, -513/-496
Sox9	-921/-907
Sox5	-895/-884
Oct-4	-880/-865
Sox10	-816/-809
P300	-553/-539
C/EBP beta	-532/-515
NFAT-1	-410/-403
ELF-1	-380/-374
C-ETS-2	-270/-263
E2F-1	-173/-166, +77/+83,+104/+110
Oct-1	-130/-119
TATA-box	-29/-23
C-myc	+168/+177

Chapter 9

Publications

Publications

Published:

✓ **Monica Tyagi**, Bharat Khade, Shafqat A Khan, Arvind Ingle, Sanjay Gupta. Expression of histone variant, H2A.1 is associated with the undifferentiated state of hepatocyte. (*Exp Biol Med (Maywood)1535370214531869, first published on April 24, 2014*

✓ **Database submission: Monica Tyagi**, Shafqat A Khan, Sanjay Gupta. Sequence submission to GenBank-NCBI, USA. Accession Numbers ***JX661508*** and ***JX661509*** are assigned to submission of complete gene sequence of H2A.1 and H2A.2 to GenBank data. The data are simultaneously made available to *EMBL, Europe and the DNA Data Bank, Japan.2012*

To be communicated:

✓ Molecular cloning, characterization and promoter analysis of rat H2A.1 and H2A.2 gene. (*manuscript ready for communication)*

Other Publications:

✓ Ketan Patel, **Monica Tyagi**, Jasmin Monpara, Lalit Vora, Sanjay Gupta, Pradeep Vavia. Arginoplexes: an arginine-anchored nanoliposomal carrier for gene delivery. *J Nanopart Res (2014) 16:2345*

✓ Shafqat A Khan, **Monica Tyagi**, Ajit K Sharma, Savio G Barreto, Bhawna Sirohi, Mukta Ramadwar, Shailesh V Shrikhande, Sanjay Gupta. Cell-type specificity of β-actin expression and its clinicopathological correlation in gastric adenocarcinoma. *World Journal of Gastroenterology, in press*

✓ **Monica Tyagi**, Shafqat A Khan, Saikat Bhattacharya, Divya Reddy, Ajit K Sharma, Bharat Khade, Sanjay Gupta. Techniques to Access Histone Modifications and Variants in Cancer. 'Cancer Epigenetics: Risk Assessment, Diagnosis, Treatment, and Prognosis', *Methods in Molecular Biology Series* (Humana Press/Springer) 2014, *in press.*

✓ **Newsletter : Monica Tyagi**, Shafqat A Khan and Sanjay Gupta. Histone Post-translational Modifications as biomarks in cancer: Dream or Reality? *PSI News Letter Vol. 2 (No.1) April 2014*, Proteomics Society, India (PSI)

Publications

List of conference presentations:

Attended and presented poster entitled "Differential expression of histone variants and their transcriptional regulation during hepatocarcinogenesis" in **Chromatinasia conference '2010 December** held in JNCSAR Bangalore, India.

Attended and presented poster entitled "Transcriptional regulation of differentially expressing variants of histone H2A during hepatocarcinogenesis" in **Spetses Summer School on Chromatin and Systems Biology, Spetses, Greece 17-23 September 2011**.

Attended and presented poster entitled "Transcriptional regulation of homomorphous variants of histone H2A." in **31st Annual Convention of IACR**, Advanced Centre for Treatment, Education and Research in Cancer, Tata Memorial Centre, Kharghar, Navi Mumbai,. **26-29 January, 2012**.

Attended and oral presentation entitled "Transcriptional regulation of differentially expressed H2A variants during liver development and carcinogenesis" **Epigenetics Mechanism in Development and Diseases, 4th Meeting of the Asian Forum of Chromosome and Chromatin Biology**, CSIR-CCMB, Hyderabad. **22-24 November, 2012**

Attended and presented poster entitled "Insight into histone alterations during liver development and carcinogenesis." **33rd Annual Convention of IACR**,RGCB, Kerala,**13-15 Feb, 2014**

Attended and presented poster entitled "Transcription regulation of histone H2A.1 and H2A.2 variants"**, Carcinogenesis 2015,** ACTREC, Navi-Mumbai, **Feb, 2015**

Expression of histone variant, H2A.1 is associated with the undifferentiated state of hepatocyte

Monica Tyagi, Bharat Khade, Shafqat A Khan, Arvind Ingle and Sanjay Gupta

Exp Biol Med (Maywood) published online 24 April 2014
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Exp Biol Med (Maywood) OnlineFirst, published on April 24, 2014 as doi:10.1177/1535370214531869

Original Research
Brief Communication

Expression of histone variant, H2A.1 is associated with the undifferentiated state of hepatocyte

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Abstract

Recent studies suggest the incorporation of histone variants into the chromatin regulate cellular proliferation, differentiation, and de-differentiation. We have earlier reported the increase of H2A.1 variant during sequential de-differentiation of hepatocyte to hepatocellular carcinoma. Here, we decipher the alterations in expression of H2A.1 and H2A.2 variants during rat liver embryogenesis and regeneration. The expression of H2A.1 and H2A.2, at protein and mRNA level, does not alter in normal cellular proliferation associated with regeneration of liver post PH. In contrast, gradual decrease of H2A.1 with increase of H2A.2 is observed during differentiation of embryonic to adult liver. Furthermore, the accumulation of H2A.1 is higher in embryonic stem cells compared to normal adult liver. Collectively, these data support a strong correlation of H2A.1 expression with undifferentiated cells and overall epigenetic reprogramming in dedifferentiation and maturation of undifferentiated cells, rather than with normal cellular proliferation.

Keywords: H2A.1, H2A.2, differentiation, de-differentiation, normal cellular proliferation

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Introduction

Chromatin, a dynamic structure, is composed of nucleosome having octameric histone core of H2A, H2B, H3, and H4 wrapped by 146 bp of DNA. Histones have variants along with the canonical forms and can be categorized on the basis of sequence similarity and replication behaviour.[1–3] Studies on histone variants, H2AZ, and macroH2A are emerging in embryonic development and cancer, suggesting their role in epigenetic reprogramming. Furthermore, macroH2A association with the repression of gene expression correlates with the occupancy of H3K27me3 during maintenance of pluripotency.[4] H2A.Z has been shown to over-express in cancers and has also associated with development and differentiation.[5–7] However, macroH2A has been reported to suppress tumor progression, and its loss positively correlates with malignant phenotype of cancer.[8] Furthermore, the redundancy of macroH2A has been shown by developmental defects in zebra fish embryos[9] and cell-fate decisions in human male pluripotent cells.[10] Previous report from laboratory has also shown over-expression of H2A.1 with decrease in expression of H2A.2 during de-differentiation of mature hepatocytes to HCC.[11] Recently, replication-dependent histone coded by human HIST1H2AC gene locus, sequence similar to rat H2A.1, has been associated with cell proliferation and tumorigenicty of bladder cancer cells.[12]

In order to define alteration in H2A.1 and H2A.2 variants in the same organ system during development, de-differentiation, and normal cellular proliferation, their comparative profiling at RNA and protein level was studied during liver regeneration post PH and differentiating embryonic liver. Liver has remarkable capacity to regenerate and, therefore, serves as an excellent model for normal cellular proliferation, whereas HCC development occurs due to uncontrolled proliferation of oval cell or de-differentiation of hepatocytes and, during embryonic liver development from hepatoblasts to hepatocytes, can be used to delineate the process of differentiation.

Our studies have shown no alteration in H2A.1/H2A.2 ratio, at protein and transcript level, during normal cellular proliferation. However, studies on differentiating embryonic liver from e15 to post-natal liver show decreased expression of H2A.1 with increased level of H2A.2 variant in adult liver. Collectively, the profile of H2A.1 and H2A.2 suggests increased level of H2A.1 with undifferentiated hepatocytes to probably impart epigenetic reprogramming for performing overlapping chromatin function for differentiation in different physio-pathological states.

Materials and methods

All the experiments were carried out on male Sprauge–Dawley rats (*Rattus norvegicus*). The proposal to use the

animals was duly approved by Institute Animal Ethics Committee, ACTREC, and the Committee, which is endorsed by the committee for the Purpose of Control and Supervision on Animals, Government of India. All excised tissues were washed with normal saline, snap frozen in liquid nitrogen, and stored at −80°C for further experiments unless otherwise mentioned. Tissues were fixed in formalin for hematoxylin and eosin staining for histopathological analysis (Supplementary Figure 1).

Animal experiments

The normal and *N*-nitrosodiethylamine-treated animals (n = 5, for each set of experiments) were euthanized after 4 months of treatment.[11,13] The animals (22–24 weeks) for liver regeneration studies were subjected to two-third PH. The surgeries were performed under sterile conditions as per protocol.[14] A horizontal incision was made, approximately 2–3 cm long, just below the Xiphoid cartilage. Left lateral and median lobes of liver (50–70% liver) were resected, and muscle and skin layers were closed. Animals were provided with an external source of heat during recovery and were maintained post operatively under optimal conditions. Animals were sacrificed, and regenerating liver tissues were collected at different time points (n = 5, for different time points and each set of experiment) (16, 24, 36, 48 and 72 h) post PH in synchrony with DNA synthesis and were processed as mentioned earlier. Furthermore, for embryonic liver development studies, pregnant rats (n = 6, for each set of experiments) confirmed by the vaginal plug were obtained. The developing liver (n = 6, for each time point) was excised on e15, e18, D0 (day of birth), 1 week, and 3 weeks and were processed as mentioned earlier.

Resolution of histones on AUT PAGE

Histones were extracted by acid-extraction method from nuclei purified by sucrose density-gradient centrifugation.[15] Histone (25 μg) and their variants were separated on AUT PAGE as described.[16] H2A.1 and H2A.2 variants from complete gel were cropped, enlarged, and quantified by Image-J software (v1.42q, NIH) as shown in Figure 1. Individual spot intensities were normalized to total, H2A.1 and H2A.2 intensities, and alterations in band intensities as percentage difference were plotted using GraphPad Prism software.

Immunoblots detection

Histones (2 μg) were resolved on SDS–PAGE, transferred, and probed with global and site-specific H3 antibodies (pan acetyl, H3Lys9Ac, H3Lys14Ac, H3Lys9Me3, and H3Lys27Me3) according to the manufacturer's instructions (Abcam). The dilutions of primary and HRPO labeled secondary antibodies in 1% BSA were as follows: H3K9me3 (1:8000), H3K27me3 (1:5000), H3K9Ac (1:3000), H3K14Ac (1:3000), and anti-rabbit (1:8000). Band intensity was normalized with H3 intensity on transferred membrane and

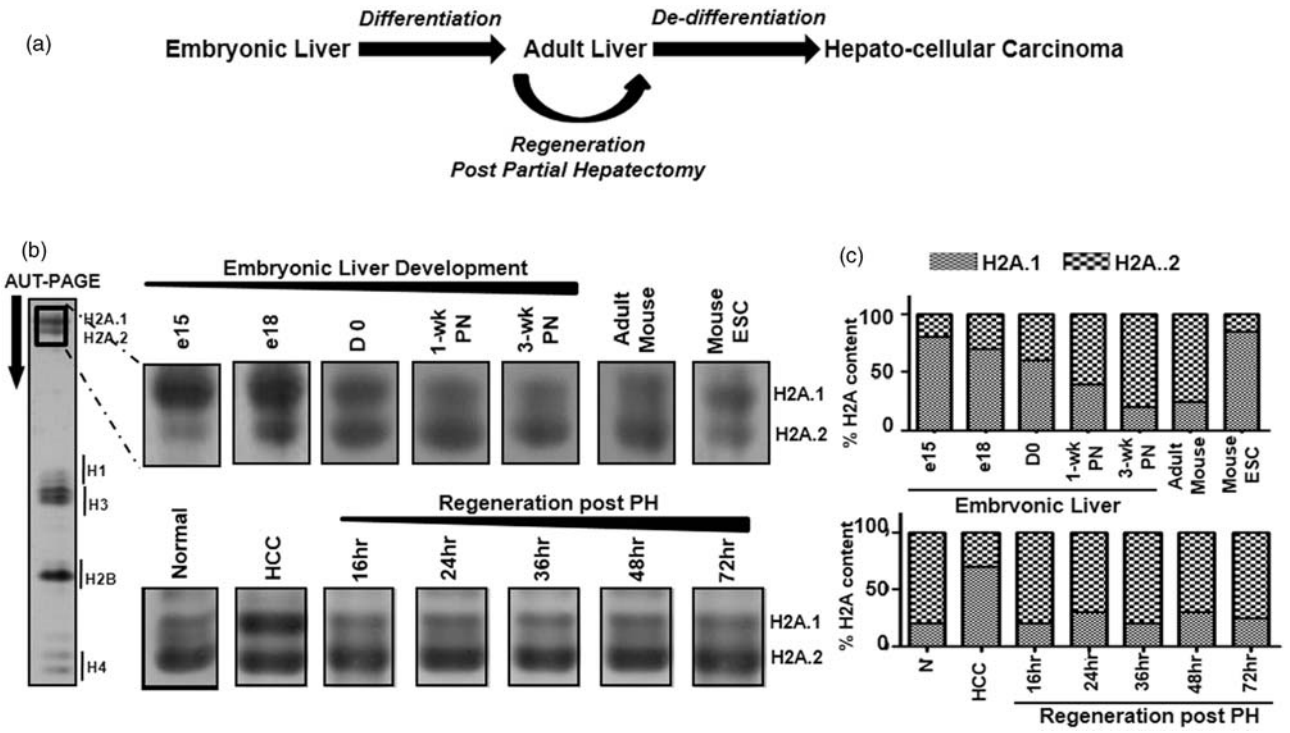

Figure 1 Profiling of H2A.1 and H2A.2 during step-wise liver regeneration and sequential embryonic liver development. (a) Schematic representation of different patho-physiological stages of liver and (b) histones (25 μg) that are resolved on AUT PAGE, silver-stained, and H2A.1/H2A.2 is represented in a square. The enlarged images of H2A.1 and H2A.2 are shown for e15 to 3-week-old adult rat, mouse ESC, mouse liver (*upper panel*), and normal adult liver, HCC, 16, 24, 36, 48, and 72 h regenerating liver post PH (*lower panel*). (c) Densitometric analysis of individual spot intensities was normalized to total, H2A.1 and H2A.2 intensities, and alterations in band intensities as percentage difference was plotted using GraphPad Prism software in different patho-physiological states of liver

analyzed by performing unpaired t-test within the respective groups, HCC, PH, and e15 with normal using Graph Pad prism software (version 5.0 for windows), and $p < 0.05$ is considered as significant values.

Semi-quantitative reverse transcriptase-polymerase chain reaction analysis

Total RNA (5 μg) prepared using TRIzol (Invitrogen, Cat#15596-026) was subjected to cDNA synthesis using random primers (Fermentas, Cat#K1632) as per the manufacturer's instructions. cDNA was subjected to PCR (NEB, Cat#M0271L) for the analysis of H2A.1 (216 bp, F:CTGTGCTGGAGTACCTGACG, R:TGTGGTGGCTCTC AGTCTTC), H2A.2 (220 bp, F:GAAGACGGAGAGCCAC CATA, R:GGAAGAGTAGGGCACACGAC), proliferating cell nuclear antigen (PCNA) (193 bp F: TCACAAAAG CCACTCCACTG, R: CATCTCAGAAGCGATCGTCA), SOX9 (88 bp F: AGTACCCGCATCTGCACAAC, R: ACG AAGGGTCTCTTCTCGCT), α-feto protein (AFP) (155 bp F: ACCTGACAGGGAAGATGGTG, R: GCAGTGGTTGAT ACCGGAGT), and 18SrRNA (219 bp F:CGCGGTTCTATT TTGTTGGT, R:AGTCGGCATCGTTTATGGTC). Band intensities of PCR products were normalized to 18 S rRNA and analyzed by performing unpaired t-test within the respective group, HCC, PH, and e15 with normal using Graph Pad prism software (version 5.0 for windows), and $p < 0.05$ is considered as significant values.

Results

In order to gain insight in relevant epigenetic overlapping mechanism involved in embryonic liver, regeneration, and cancer development (Figure 1(a)), we performed a comprehensive analysis of differentially expressing histone variants, H2A.1 and H2A.2.

H2A.1 loss is associated with liver differentiation and remains unaltered during liver regeneration

H2A variant expression during time -chase regeneration post PH and different stages of embryonic development was studied (Figure 1). The regenerating liver with higher rate of cellular proliferation and DNA replication at 16, 24, 36, 48, and 72-h post PH showed no alteration in the profile of H2A.1 and H2A.2 compared to normal liver (Figure 1(b) and (c)). The histopathological analysis of regenerating liver post PH showed normal liver architecture having maintained hexagonal structure with sinusoidal spaces though the nuclei look enlarged indicating toward high level of DNA replication coupled with cellular proliferation (Supplementary Figure 1(a)). The profiling of H2A variants during different stages of liver development, e15, e18, D0, 1 week, and 3 weeks, showed highest accumulation of upper band at e15 (Figure 1(b)). Differentially expressing bands (upper and lower) of H2A variants were identified as H2A.1 (upper band) and H2A.2 (lower band) by mass spectroscopy and further confirmed by MS–MS of identified peptides (Supplementary Figure 2 and Supplementary Table). The sequential decrease of H2A.1 was observed with progression of hepatoblast toward maturation; on the contrary, the level of H2A.2 increases with differentiation of embryonic liver to 3-week stage (Figure 1(b) and (c)). The e15 liver showed hyperplasia, hyperchromatic, dissimilar-sized hepatocytes with polyploidy, and a spongy appearance due to compactness of nuclei. The e20 stage had multinucleate giant cells with hexagonal hepatocyte having increased cytoplasm to nuclear ratio at D0. The histo-pathological analysis along with the development of new blood vessels confirmed the maturation of hepatoblast to hepatocyte during liver development (Supplementary Figure 1(b)). Thus, the higher expression of H2A.1 than H2A.2 variant is associated with cellular differentiation and not coupled with the regeneration of liver.

Expression profiling of H2A.1 and H2A.2 and their association with chromatin marks during liver regeneration, differentiation, and de-differentiation

The comparative analysis of mRNA expression was carried out within normal adult, embryonic (e15), HCC, and 24 h post-PH regenerating liver (PH) (Figure 2(a)). The expression of H2A.1 was significantly high in e15 and HCC compared to normal and PH. However, H2A.2 was higher in normal and PH. Along with histo-pathological analysis of different patho-physiological state of liver, the liver specific, and proliferation markers, AFP, a proto-onco gene, SOX9, and PCNA were also studied. AFP shows increased expression in e15 and HCC compared to adult and regenerating liver. SOX9 in consent with other report[17] showed overexpression only in HCC but not during normal cellular proliferation, neither in development nor post-PH. PCNA was significantly higher in e15 compared to the increase in HCC and PH. Collectively, the differential expression of markers, PCNA, SOX9, and AFP in coherence with stages confirms H2A.1 is associated with the process of cellular proliferation during differentiation or dedifferentiation but not with cellular proliferation during regeneration.

To understand whether change in H2A.1 and H2A.2 variant is associated with specific chromatin architecture, the active and inactive acetylation and methylation marks of histone H3 are studied by western blotting (Figure 2(b)). The pan-acetylation on histone H3 increases in all the patho-physiological states of liver compared to control but the increase was significant in regenerating liver. The active chromatin marks, H3Lys9Ac and H3Lys14Ac, showed an inverse correlation with different patho-physiological states of liver. H3Lys9Ac increases in HCC and decreases after PH, whereas H3Lys14Ac increases in PH and e15 liver compared to normal and HCC. The inactive marks, H3Lys9Me3 increases in PH, whereas in e15 liver and HCC, no significant change was observed compared to normal. The increased tri-methylation of H3Lys27 is associated with e15 liver and HCC, whereas the level decreases after PH is compared to normal liver.

Discussion

Earlier studies have shown broadly overlapping genetic mechanism leading to the biological changes during embryonic liver development and HCC.[18] Interestingly, our studies on H2A variants, H2A.1 and H2A.2, have shown the

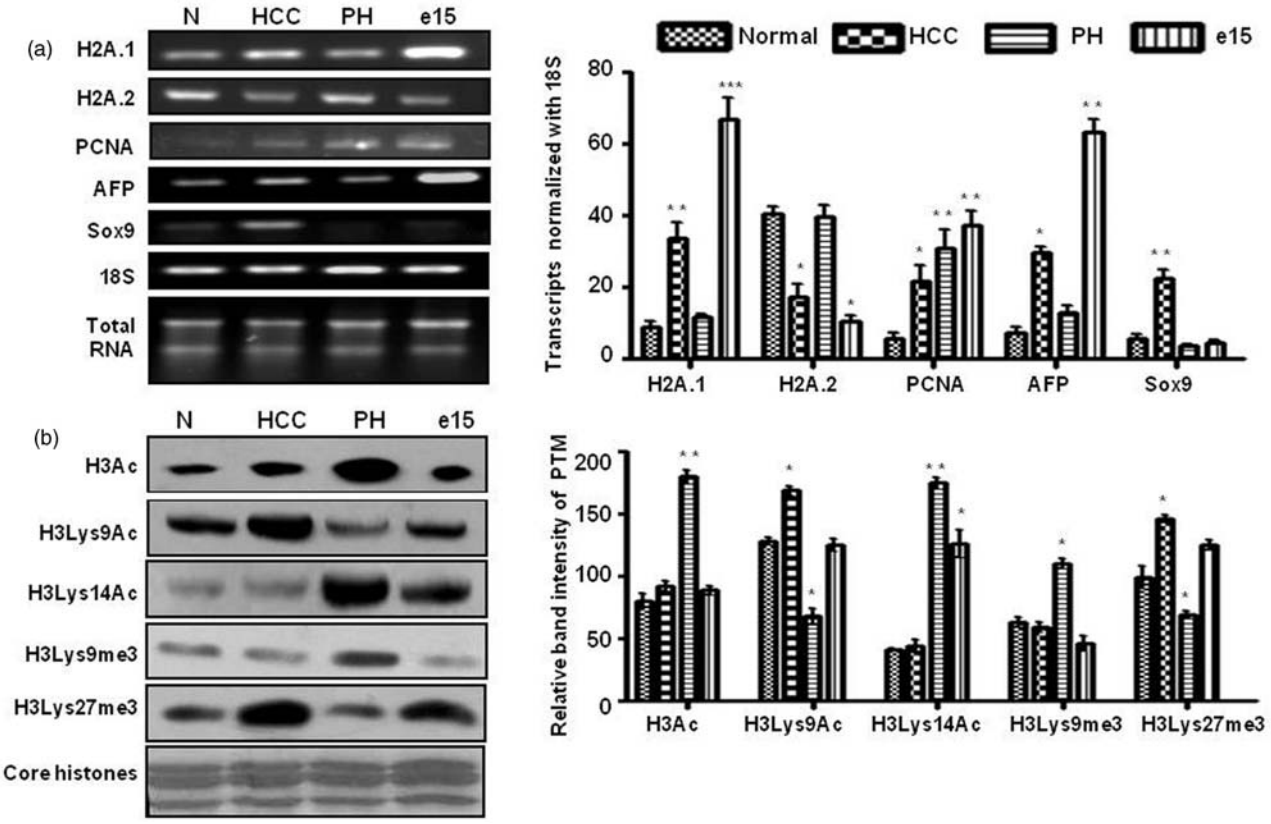

Figure 2 Stage-specific expression profiling of H2A.1 and H2A.2 and chromatin marks. Total RNA and histones purified from liver of normal adult, embryonic (e15), HCC, and 24 h post-PH regenerating liver (PH). (a) Semi quantitative RT-PCR of H2A.1, H2A.2, PCNA, AFP, and SOX9. 18 S is used as loading control and (b) immunoblots with antibodies against pan-Ac, H3Lys9Ac, H3Lys14Ac, H3Lys27me3, and H3Lys9me3. Densometric analysis of band intensity is represented graphically as an average of three independent experiments. Each histogram represents mean densitometry ± SEM of normal compared to HCC, PH, and e15. $p < 0.05$ was considered as significant

decrease in the expression of H2A.1 with simultaneous increase of H2A.2 during liver development and also confirmed our report of H2A.1 increase in HCC.[11] The level of H2AHISTIC, human H2A.1, is also reported to increase in tumor bladder cells compared to normal.[12] The liver development and de-differentiation of mature hepatocyte to HCC are in coherence with the expression profile of cell-proliferation marker, PCNA, and liver differentiation marker, AFP, whereas Sox9 remains minimal during differentiation and increases in HCC, which is in accordance with the earlier studies.[17,19] The observed higher level of H2A.1 expression in mouse embryonic stem cells (ESC) along with e15 and HCC strengthens that H2A.1 is associated with undifferentiated cells (Figure 1(b)). Furthermore, report on differentiation and cancer development has shown increase of macroH2A in maturation of new born to young adult liver,[20] though macroH2A decreases in cancers. The data have shown that altered transcription is contributing to change in histone variants, H2A.1 and H2A.2, that share 97.6% homology in their protein sequence but differ significantly in their promoter sequences (H2A.1:NCBI-*JX-661508* and H2A.2:NCBI-*JX-661509*). The transcriptional regulation in different patho-physiological states needs further investigation. Furthermore, our data have also shown increase in H3Ac suggesting open chromatin organization in different patho-physiological states. The alteration in active and inactive marks on H3 suggests similar pattern of modification(s) during early differentiation and dedifferentiation but further studies are required to understand their significance. The incorporation of different homomorphous histone variants of H2A and increase in global acetylation may have potential implication on chromatin architecture, downstream gene expression, and thereby maintaining high plasticity for genomic reprogramming during differentiation and dedifferentiation.

The DNA replication in liver regeneration is coupled with histone synthesis, modification(s) imprinting, and increase in PCNA for assembly of chromatin. Our studies on regenerating liver showed no alteration in the accumulation and expression of H2A.1 and H2A.2 variants. Also, the chromatin marks are different compared to e15 and HCC liver. The observed significant increase of H3Lys14Ac after PH is in accordance with the previous reports. Thus, increase in normal cell division for tissue maintenance or compensatory growth is not associated with change in expression of histone variant but only associated with different post-translational modification(s) to regulate change in gene expression.

The three amino acid differences at 16th, 51st, and 99th position make it difficult to generate highly specific

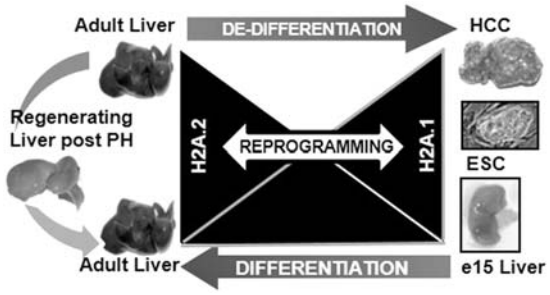

Figure 3 Proposed model for association of histone variants, H2A.1 and H2A.2 with regeneration, de-differentiation, and differentiation. Hepatocytes of adult and regenerating liver are H2A.2 enriched, whereas undifferentiated cells, e15 hepatoblasts, ESC, and de-differentiated hepatocytes of HCC are enriched with H2A.1. ESC: embryonic stem cell

antibodies against H2A.1 and H2A.2. Therefore, the limitation of the study is to understand and directly correlate the in vivo observation of change in histone variant profiles with the group of genes, expressing differentially or overlapping, during differentiation and de-differentiation. Based on our study, a model has been proposed (Figure 3) signifying H2A.1 and H2A.2. H2A.2 variants play a potential role in defining highly specialized biological mechanism, differentiation, and de-differentiation opposed to normal cellular proliferation during liver regeneration and suggest the overlapping functional relevance in epigenetic reprogramming of genome.

Authors' contributions

SG and MT conceived and designed the experiments. MT, BK, and SAK performed the experiments. MT, SAK, and SG analyzed the data and wrote the manuscript. AI performed histopathological analysis.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY FIGURE LEGENDS:

Supplementary Figure 1: Histopathological analysis of embryonic liver development, HCC, and regenerating liver post PH. H & E stained tissue section of (A) adult liver, HCC and regenerating liver post partial hepatectomy at different time points of regeneration (16hr, 24hr, 36hr, 48hr and 72hr), (B rat liver development from progressive stages of liver development, e15 to 3wk post natal.

Supplementary Figure 2: Mass Spectra of differentially expressing protein bands during progressive stages of liver development

Mass of identified peak and range of identified peptide sequence of the respective protein is indicated at the top of each peak. The proteins are identified as H2A.1 (upper band) and H2A.2 (lower band) as identified earlier in normal and HCC. The differentially expressing bands are henceforth labelled as H2A.1 and H2A.2 during liver development in the manuscript.

SUPPLEMENTARY TABLE:

Table: MS-MS details of peptides identified differentially expressing proteins bands, as H2A.1 and H2A.2.

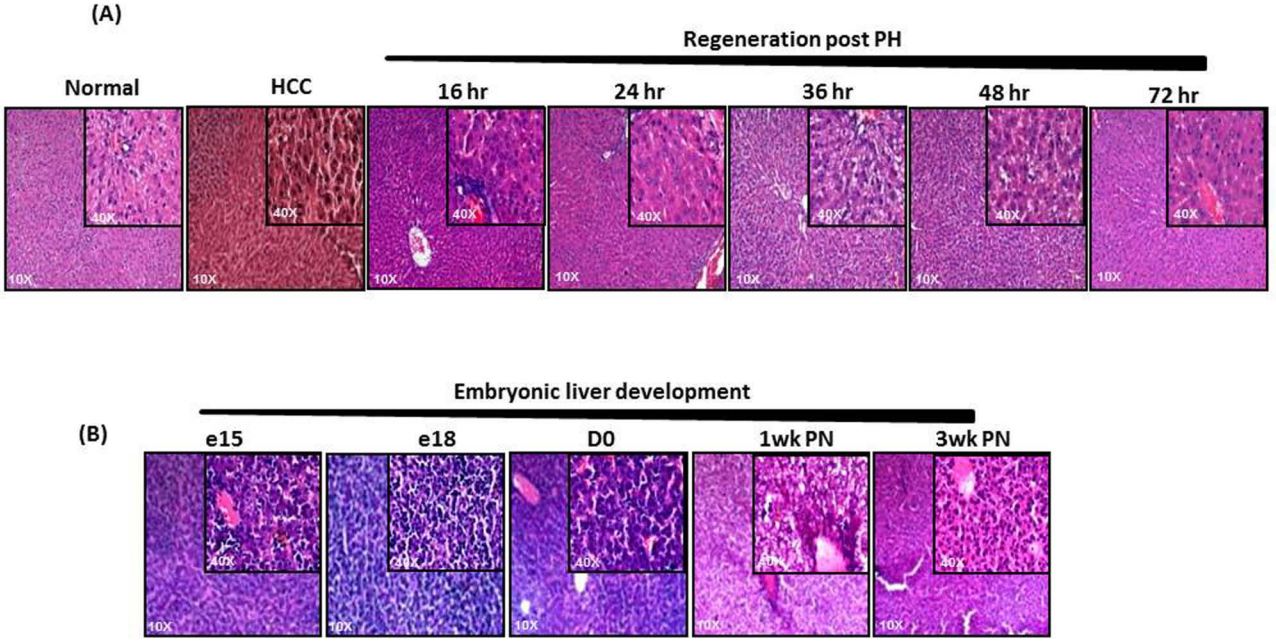
H2A.1 (GeneID: 502125)

Meas. M/z Calc.	MH+ Int.	Dev.(Da)	Dev.(ppm)	Range	Sequence
850.5169	495.3839	-0.0088	-10.3800 7	83 – 89	HLQLAIR
944.5156	12784.9104	-0.0157	-16.5910	22-30	AGLQFPVGR
1931.0162	1607.2530	-0.1526	-79.0478	110-119	VTIAQGGVLPNIQAVLLPK
2915.4653	4363.4239	-0.1224	-41.9831	44-72	VGAGAPVYLAAVLEYLTAEILELAGNAAR

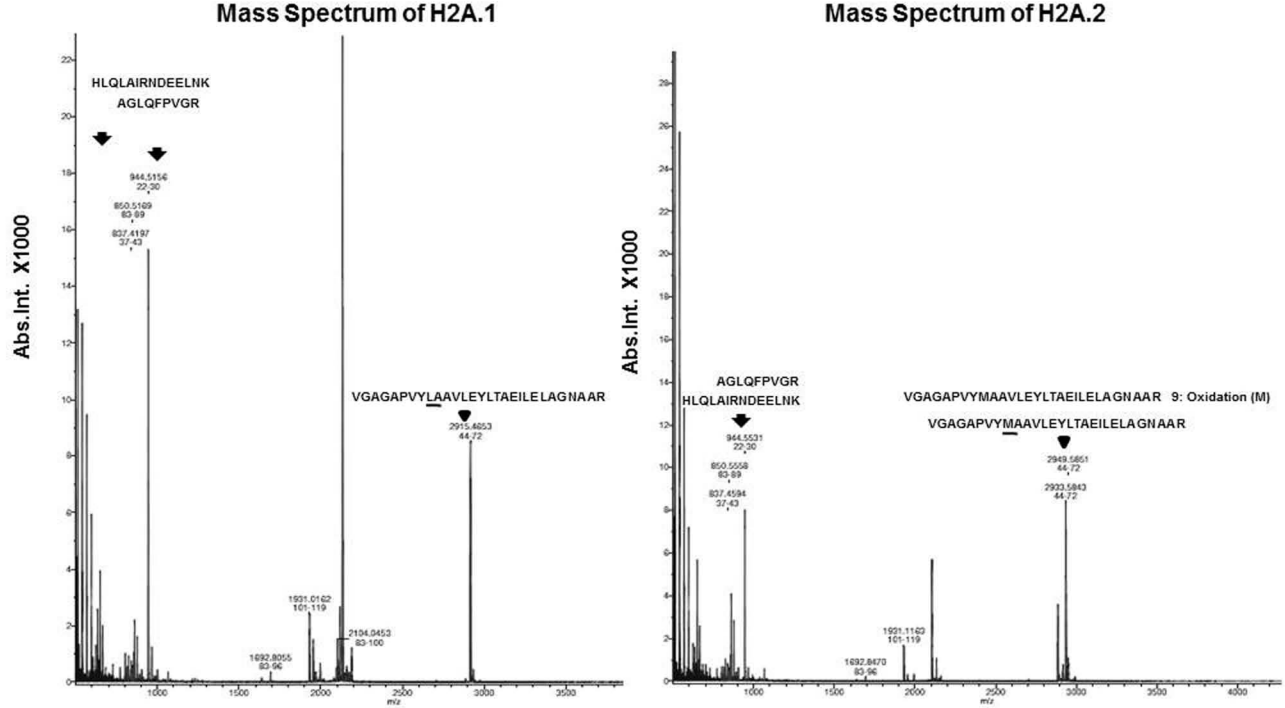
H2A.2 (GeneID: 365877)

Meas. M/z Calc.	MH+ Int.	Dev.(Da)	Dev.(ppm)	Range	Sequence
850.5558	495.1984	0.0301	35.4624	83 – 89	HLQLAIR
944.5531	6926.5611	0.0219	23.2077 6	22-30	\GLQFPVGR
1931.1163	1463.5483	-0.0525	-27.1931	101-119	VTIAQGGVLPNIQAVLLPK
2933.5843	4694.9618	0.0402	13.7171	44-72	VGAGAPVYMAAVLEYLTAEILELAGNAAR
2949.5851	614.5437	0.0461	15.6461	44-72	VGAGAPVYMAAVLEYLTAEILELAGNAAR 9: Oxidation (M)
1692.8470	142.8310	-0.0557	-32.9270	83-96	HLQLAIRNDEELNK

Supplementary Figure 1



Supplementary Figure 2



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Original Research
Brief Communication

Expression of histone variant, H2A.1 is associated with the undifferentiated state of hepatocyte

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Abstract

Recent studies suggest the incorporation of histone variants into the chromatin regulate cellular proliferation, differentiation, and de-differentiation. We have earlier reported the increase of H2A.1 variant during sequential de-differentiation of hepatocyte to hepatocellular carcinoma. Here, we decipher the alterations in expression of H2A.1 and H2A.2 variants during rat liver embryogenesis and regeneration. The expression of H2A.1 and H2A.2, at protein and mRNA level, does not alter in normal cellular proliferation associated with regeneration of liver post PH. In contrast, gradual decrease of H2A.1 with increase of H2A.2 is observed during differentiation of embryonic to adult liver. Furthermore, the accumulation of H2A.1 is higher in embryonic stem cells compared to normal adult liver. Collectively, these data support a strong correlation of H2A.1 expression with undifferentiated cells and overall epigenetic reprogramming in dedifferentiation and maturation of undifferentiated cells, rather than with normal cellular proliferation.

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Materials and methods

All the experiments were carried out on male Sprauge–Dawley rats (*Rattus norvegicus*). The proposal to use the

animals was duly approved by Institute Animal Ethics Committee, ACTREC, and the Committee, which is endorsed by the committee for the Purpose of Control and Supervision on Animals, Government of India. All excised tissues were washed with normal saline, snap frozen in liquid nitrogen, and stored at −80°C for further experiments unless otherwise mentioned. Tissues were fixed in formalin for hematoxylin and eosin staining for histopathological analysis (Supplementary Figure 1).

Animal experiments

The normal and *N*-nitrosodiethylamine-treated animals (n = 5, for each set of experiments) were euthanized after 4 months of treatment.[11,13] The animals (22–24 weeks) for liver regeneration studies were subjected to two-third PH. The surgeries were performed under sterile conditions as per protocol.[14] A horizontal incision was made, approximately 2–3 cm long, just below the Xiphoid cartilage. Left lateral and median lobes of liver (50–70% liver) were resected, and muscle and skin layers were closed. Animals were provided with an external source of heat during recovery and were maintained post operatively under optimal conditions. Animals were sacrificed, and regenerating liver tissues were collected at different time points (n = 5, for different time points and each set of experiment) (16, 24, 36, 48 and 72 h) post PH in synchrony with DNA synthesis and were processed as mentioned earlier. Furthermore, for embryonic liver development studies, pregnant rats (n = 6, for each set of experiments) confirmed by the vaginal plug were obtained. The developing liver (n = 6, for each time point) was excised on e15, e18, D0 (day of birth), 1 week, and 3 weeks and were processed as mentioned earlier.

Resolution of histones on AUT PAGE

Histones were extracted by acid-extraction method from nuclei purified by sucrose density-gradient centrifugation.[15] Histone (25 μg) and their variants were separated on AUT PAGE as described.[16] H2A.1 and H2A.2 variants from complete gel were cropped, enlarged, and quantified by Image-J software (v1.42q, NIH) as shown in Figure 1. Individual spot intensities were normalized to total, H2A.1 and H2A.2 intensities, and alterations in band intensities as percentage difference were plotted using GraphPad Prism software.

Immunoblots detection

Histones (2 μg) were resolved on SDS–PAGE, transferred, and probed with global and site-specific H3 antibodies (pan acetyl, H3Lys9Ac, H3Lys14Ac, H3Lys9Me3, and H3Lys27Me3) according to the manufacturer's instructions (Abcam). The dilutions of primary and HRPO labeled secondary antibodies in 1% BSA were as follows: H3K9me3 (1:8000), H3K27me3 (1:5000), H3K9Ac (1:3000), H3K14Ac (1:3000), and anti-rabbit (1:8000). Band intensity was normalized with H3 intensity on transferred membrane and

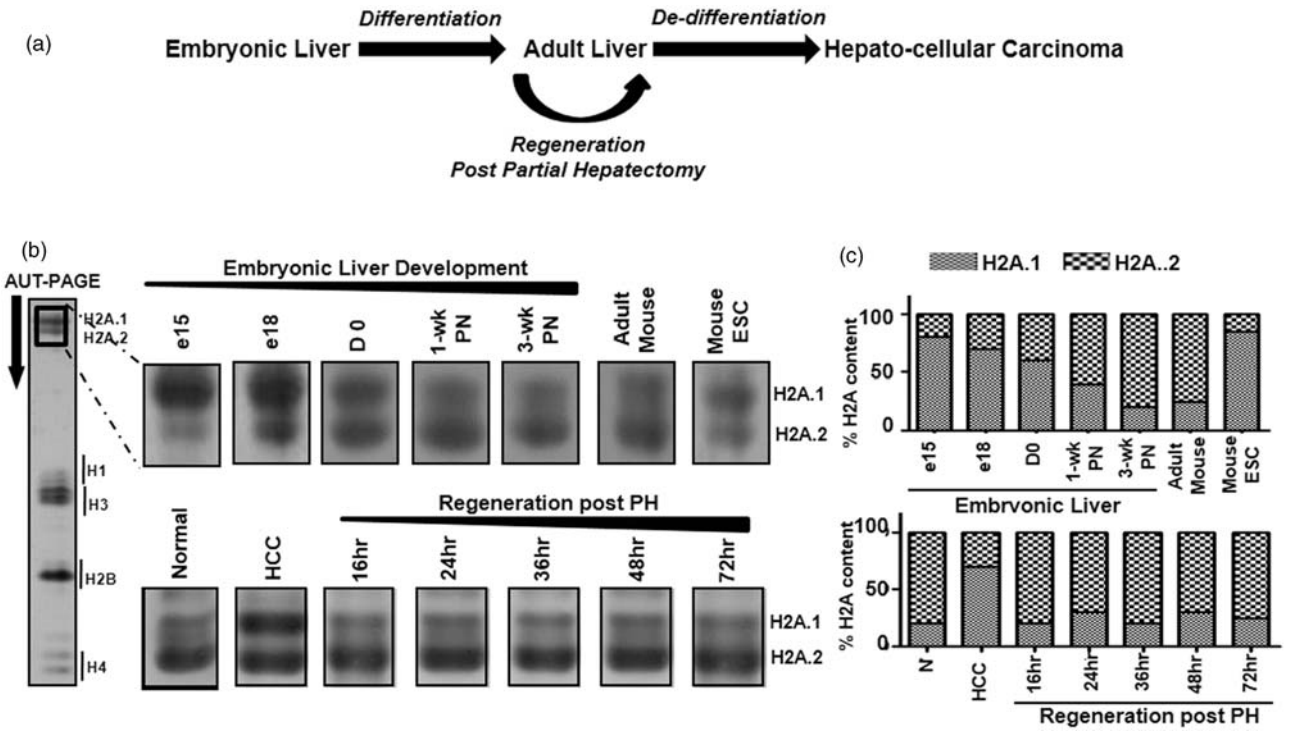

Figure 1 Profiling of H2A.1 and H2A.2 during step-wise liver regeneration and sequential embryonic liver development. (a) Schematic representation of different patho-physiological stages of liver and (b) histones (25 μg) that are resolved on AUT PAGE, silver-stained, and H2A.1/H2A.2 is represented in a square. The enlarged images of H2A.1 and H2A.2 are shown for e15 to 3-week-old adult rat, mouse ESC, mouse liver (*upper panel*), and normal adult liver, HCC, 16, 24, 36, 48, and 72 h regenerating liver post PH (*lower panel*). (c) Densitometric analysis of individual spot intensities was normalized to total, H2A.1 and H2A.2 intensities, and alterations in band intensities as percentage difference was plotted using GraphPad Prism software in different patho-physiological states of liver

analyzed by performing unpaired t-test within the respective groups, HCC, PH, and e15 with normal using Graph Pad prism software (version 5.0 for windows), and $p < 0.05$ is considered as significant values.

Semi-quantitative reverse transcriptase-polymerase chain reaction analysis

Total RNA (5 μg) prepared using TRIzol (Invitrogen, Cat#15596-026) was subjected to cDNA synthesis using random primers (Fermentas, Cat#K1632) as per the manufacturer's instructions. cDNA was subjected to PCR (NEB, Cat#M0271L) for the analysis of H2A.1 (216 bp, F:CTGTGCTGGAGTACCTGACG, R:TGTGGTGGCTCTC AGTCTTC), H2A.2 (220 bp, F:GAAGACGGAGAGCCAC CATA, R:GGAAGAGTAGGGCACACGAC), proliferating cell nuclear antigen (PCNA) (193 bp F: TCACAAAAG CCACTCCACTG, R: CATCTCAGAAGCGATCGTCA), SOX9 (88 bp F: AGTACCCGCATCTGCACAAC, R: ACG AAGGGTCTCTTCTCGCT), α-feto protein (AFP) (155 bp F: ACCTGACAGGGAAGATGGTG, R: GCAGTGGTTGAT ACCGGAGT), and 18SrRNA (219 bp F:CGCGGTTCTATT TTGTTGGT, R:AGTCGGCATCGTTTATGGTC). Band intensities of PCR products were normalized to 18 S rRNA and analyzed by performing unpaired t-test within the respective group, HCC, PH, and e15 with normal using Graph Pad prism software (version 5.0 for windows), and $p < 0.05$ is considered as significant values.

Results

In order to gain insight in relevant epigenetic overlapping mechanism involved in embryonic liver, regeneration, and cancer development (Figure 1(a)), we performed a comprehensive analysis of differentially expressing histone variants, H2A.1 and H2A.2.

H2A.1 loss is associated with liver differentiation and remains unaltered during liver regeneration

H2A variant expression during time -chase regeneration post PH and different stages of embryonic development was studied (Figure 1). The regenerating liver with higher rate of cellular proliferation and DNA replication at 16, 24, 36, 48, and 72-h post PH showed no alteration in the profile of H2A.1 and H2A.2 compared to normal liver (Figure 1(b) and (c)). The histopathological analysis of regenerating liver post PH showed normal liver architecture having maintained hexagonal structure with sinusoidal spaces though the nuclei look enlarged indicating toward high level of DNA replication coupled with cellular proliferation (Supplementary Figure 1(a)). The profiling of H2A variants during different stages of liver development, e15, e18, D0, 1 week, and 3 weeks, showed highest accumulation of upper band at e15 (Figure 1(b)). Differentially expressing bands (upper and lower) of H2A variants were identified as H2A.1 (upper band) and H2A.2 (lower band) by mass spectroscopy and further confirmed by MS–MS of identified peptides (Supplementary Figure 2 and Supplementary Table). The sequential decrease of H2A.1 was observed with progression of hepatoblast toward maturation; on the contrary, the level of H2A.2 increases with differentiation of embryonic liver to 3-week stage (Figure 1(b) and (c)). The e15 liver showed hyperplasia, hyperchromatic, dissimilar-sized hepatocytes with polyploidy, and a spongy appearance due to compactness of nuclei. The e20 stage had multinucleate giant cells with hexagonal hepatocyte having increased cytoplasm to nuclear ratio at D0. The histo-pathological analysis along with the development of new blood vessels confirmed the maturation of hepatoblast to hepatocyte during liver development (Supplementary Figure 1(b)). Thus, the higher expression of H2A.1 than H2A.2 variant is associated with cellular differentiation and not coupled with the regeneration of liver.

Expression profiling of H2A.1 and H2A.2 and their association with chromatin marks during liver regeneration, differentiation, and de-differentiation

The comparative analysis of mRNA expression was carried out within normal adult, embryonic (e15), HCC, and 24 h post-PH regenerating liver (PH) (Figure 2(a)). The expression of H2A.1 was significantly high in e15 and HCC compared to normal and PH. However, H2A.2 was higher in normal and PH. Along with histo-pathological analysis of different patho-physiological state of liver, the liver specific, and proliferation markers, AFP, a proto-onco gene, SOX9, and PCNA were also studied. AFP shows increased expression in e15 and HCC compared to adult and regenerating liver. SOX9 in consent with other report[17] showed overexpression only in HCC but not during normal cellular proliferation, neither in development nor post-PH. PCNA was significantly higher in e15 compared to the increase in HCC and PH. Collectively, the differential expression of markers, PCNA, SOX9, and AFP in coherence with stages confirms H2A.1 is associated with the process of cellular proliferation during differentiation or dedifferentiation but not with cellular proliferation during regeneration.

To understand whether change in H2A.1 and H2A.2 variant is associated with specific chromatin architecture, the active and inactive acetylation and methylation marks of histone H3 are studied by western blotting (Figure 2(b)). The pan-acetylation on histone H3 increases in all the patho-physiological states of liver compared to control but the increase was significant in regenerating liver. The active chromatin marks, H3Lys9Ac and H3Lys14Ac, showed an inverse correlation with different patho-physiological states of liver. H3Lys9Ac increases in HCC and decreases after PH, whereas H3Lys14Ac increases in PH and e15 liver compared to normal and HCC. The inactive marks, H3Lys9Me3 increases in PH, whereas in e15 liver and HCC, no significant change was observed compared to normal. The increased tri-methylation of H3Lys27 is associated with e15 liver and HCC, whereas the level decreases after PH is compared to normal liver.

Discussion

Earlier studies have shown broadly overlapping genetic mechanism leading to the biological changes during embryonic liver development and HCC.[18] Interestingly, our studies on H2A variants, H2A.1 and H2A.2, have shown the

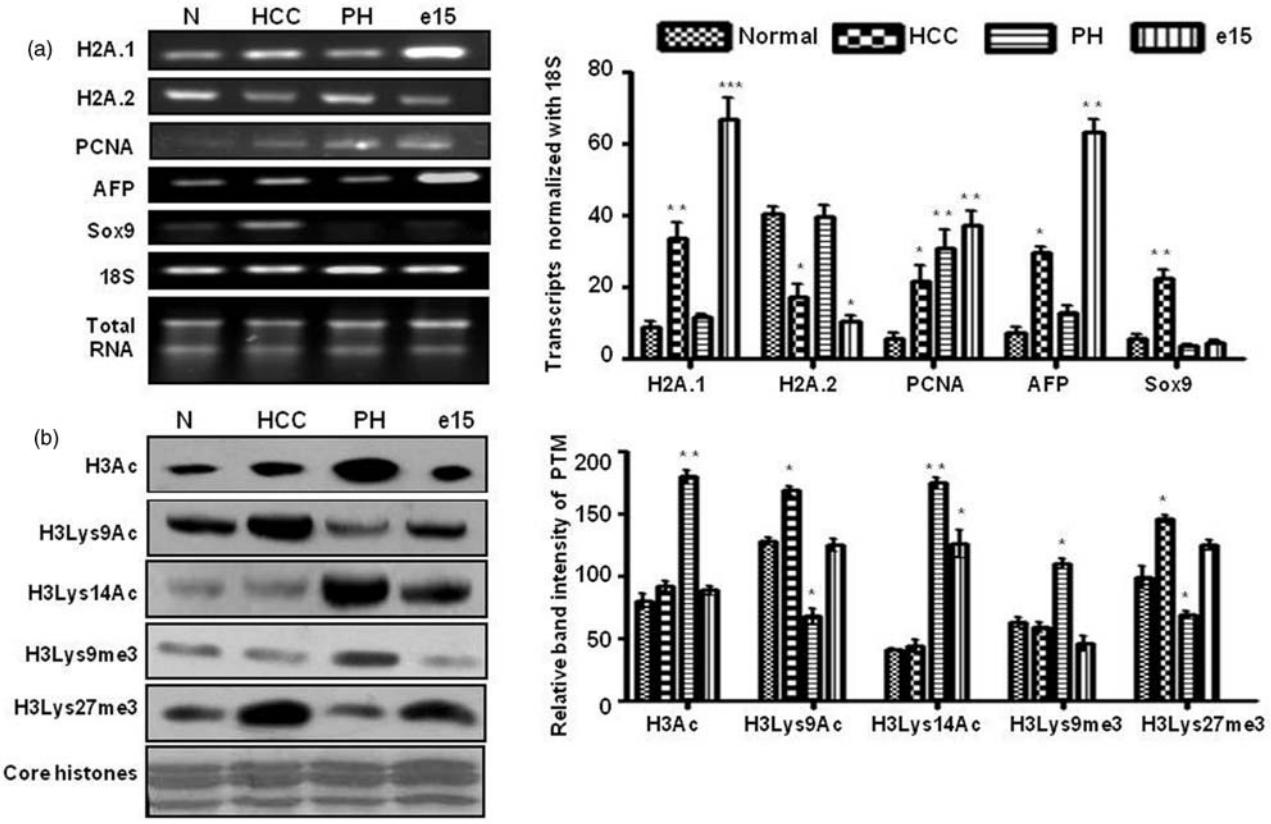

Figure 2 Stage-specific expression profiling of H2A.1 and H2A.2 and chromatin marks. Total RNA and histones purified from liver of normal adult, embryonic (e15), HCC, and 24 h post-PH regenerating liver (PH). (a) Semi quantitative RT-PCR of H2A.1, H2A.2, PCNA, AFP, and SOX9. 18 S is used as loading control and (b) immunoblots with antibodies against pan-Ac, H3Lys9Ac, H3Lys14Ac, H3Lys27me3, and H3Lys9me3. Densometric analysis of band intensity is represented graphically as an average of three independent experiments. Each histogram represents mean densitometry ± SEM of normal compared to HCC, PH, and e15. $p < 0.05$ was considered as significant

decrease in the expression of H2A.1 with simultaneous increase of H2A.2 during liver development and also confirmed our report of H2A.1 increase in HCC.[11] The level of H2AHISTIC, human H2A.1, is also reported to increase in tumor bladder cells compared to normal.[12] The liver development and de-differentiation of mature hepatocyte to HCC are in coherence with the expression profile of cell-proliferation marker, PCNA, and liver differentiation marker, AFP, whereas Sox9 remains minimal during differentiation and increases in HCC, which is in accordance with the earlier studies.[17,19] The observed higher level of H2A.1 expression in mouse embryonic stem cells (ESC) along with e15 and HCC strengthens that H2A.1 is associated with undifferentiated cells (Figure 1(b)). Furthermore, report on differentiation and cancer development has shown increase of macroH2A in maturation of new born to young adult liver,[20] though macroH2A decreases in cancers. The data have shown that altered transcription is contributing to change in histone variants, H2A.1 and H2A.2, that share 97.6% homology in their protein sequence but differ significantly in their promoter sequences (H2A.1:NCBI-*JX-661508* and H2A.2:NCBI-*JX-661509*). The transcriptional regulation in different patho-physiological states needs further investigation. Furthermore, our data have also shown increase in H3Ac suggesting open chromatin organization in different patho-physiological states. The alteration in active and inactive marks on H3 suggests similar pattern of modification(s) during early differentiation and dedifferentiation but further studies are required to understand their significance. The incorporation of different homomorphous histone variants of H2A and increase in global acetylation may have potential implication on chromatin architecture, downstream gene expression, and thereby maintaining high plasticity for genomic reprogramming during differentiation and dedifferentiation.

The DNA replication in liver regeneration is coupled with histone synthesis, modification(s) imprinting, and increase in PCNA for assembly of chromatin. Our studies on regenerating liver showed no alteration in the accumulation and expression of H2A.1 and H2A.2 variants. Also, the chromatin marks are different compared to e15 and HCC liver. The observed significant increase of H3Lys14Ac after PH is in accordance with the previous reports. Thus, increase in normal cell division for tissue maintenance or compensatory growth is not associated with change in expression of histone variant but only associated with different post-translational modification(s) to regulate change in gene expression.

The three amino acid differences at 16th, 51st, and 99th position make it difficult to generate highly specific

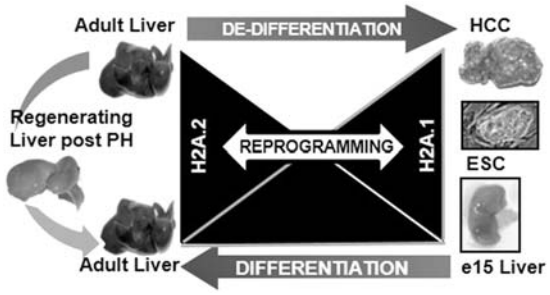

Figure 3 Proposed model for association of histone variants, H2A.1 and H2A.2 with regeneration, de-differentiation, and differentiation. Hepatocytes of adult and regenerating liver are H2A.2 enriched, whereas undifferentiated cells, e15 hepatoblasts, ESC, and de-differentiated hepatocytes of HCC are enriched with H2A.1. ESC: embryonic stem cell

antibodies against H2A.1 and H2A.2. Therefore, the limitation of the study is to understand and directly correlate the in vivo observation of change in histone variant profiles with the group of genes, expressing differentially or overlapping, during differentiation and de-differentiation. Based on our study, a model has been proposed (Figure 3) signifying H2A.1 and H2A.2. H2A.2 variants play a potential role in defining highly specialized biological mechanism, differentiation, and de-differentiation opposed to normal cellular proliferation during liver regeneration and suggest the overlapping functional relevance in epigenetic reprogramming of genome.

Authors' contributions

SG and MT conceived and designed the experiments. MT, BK, and SAK performed the experiments. MT, SAK, and SG analyzed the data and wrote the manuscript. AI performed histopathological analysis.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY FIGURE LEGENDS:

Supplementary Figure 1: Histopathological analysis of embryonic liver development, HCC, and regenerating liver post PH. H & E stained tissue section of (A) adult liver, HCC and regenerating liver post partial hepatectomy at different time points of regeneration (16hr, 24hr, 36hr, 48hr and 72hr), (B rat liver development from progressive stages of liver development, e15 to 3wk post natal.

Supplementary Figure 2: Mass Spectra of differentially expressing protein bands during progressive stages of liver development

Mass of identified peak and range of identified peptide sequence of the respective protein is indicated at the top of each peak. The proteins are identified as H2A.1 (upper band) and H2A.2 (lower band) as identified earlier in normal and HCC. The differentially expressing bands are henceforth labelled as H2A.1 and H2A.2 during liver development in the manuscript.

SUPPLEMENTARY TABLE:

Table: MS-MS details of peptides identified differentially expressing proteins bands, as H2A.1 and H2A.2.

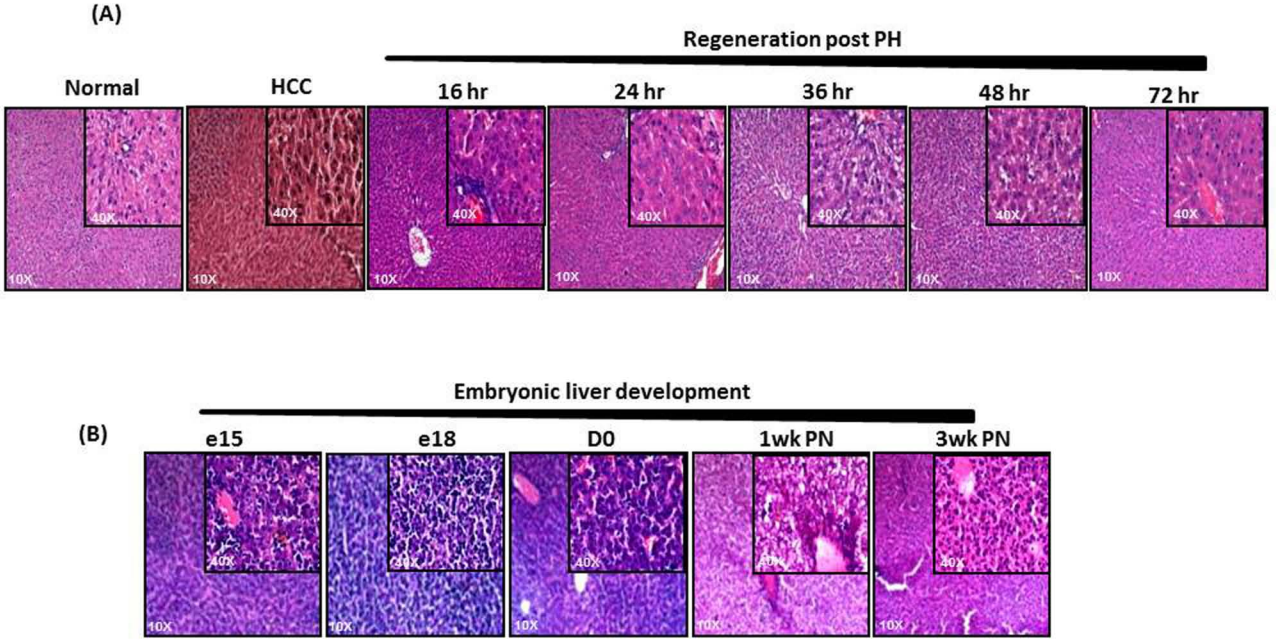
H2A.1 (GeneID: 502125)

Meas. M/z Calc.	MH+ Int.	Dev.(Da)	Dev.(ppm)	Range	Sequence
850.5169	495.3839	-0.0088	-10.3800 7	83 – 89	HLQLAIR
944.5156	12784.9104	-0.0157	-16.5910	22-30	AGLQFPVGR
1931.0162	1607.2530	-0.1526	-79.0478	110-119	VTIAQGGVLPNIQAVLLPK
2915.4653	4363.4239	-0.1224	-41.9831	44-72	VGAGAPVYLAAVLEYLTAEILELAGNAAR

H2A.2 (GeneID: 365877)

Meas. M/z Calc.	MH+ Int.	Dev.(Da)	Dev.(ppm)	Range	Sequence
850.5558	495.1984	0.0301	35.4624	83 – 89	HLQLAIR
944.5531	6926.5611	0.0219	23.2077 6	22-30	\GLQFPVGR
1931.1163	1463.5483	-0.0525	-27.1931	101-119	VTIAQGGVLPNIQAVLLPK
2933.5843	4694.9618	0.0402	13.7171	44-72	VGAGAPVYMAAVLEYLTAEILELAGNAAR
2949.5851	614.5437	0.0461	15.6461	44-72	VGAGAPVYMAAVLEYLTAEILELAGNAAR 9: Oxidation (M)
1692.8470	142.8310	-0.0557	-32.9270	83-96	HLQLAIRNDEELNK

Supplementary Figure 1



Supplementary Figure 2