"Comparative Study of SATB Family Proteins"

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by

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Under the guidance of

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Dedicatedtomyfamily...

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CERTIFICATE

Certified that the work incorporated in the thesis entitled "Comparative Study of SATB Family Proteins" Submitted by Sunita Singh was carried out by the candidate, under my supervision. The work presented here or any part of it has not been included in any other thesis submitted previously for the award of any degree or diploma from any other University or institution.

Prof. Sanjeev Galande

(Supervisor)

Date:

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Abbreviations

ACF	ATP-Utilizing Chromatin Assembly And Remodeling Factor Complex
ACF1	ATP-Dependent Chromatin Assembly Factor Large Subunit-1
ATF4	Activating Transcription Factor 4
ATP	Adenosine Triphosphate
BALB/C	Bagg Albino
Bcl2	B-Cell Leukemia/Lymphoma 2
BIO	6-Bromoindirubin-3′-Oxime
β–ME	β–mercaptoethanol
BMP	Bone Morphogenetic Protein
BMSCs	Bone Marrow Stromal Cells
bp	base pair
Bsp	Bone Sialoprotein
BURs	Base Unpairing Regions
CBP	CREB-Binding Protein
CD	Cut repeat containing Domain
Cdk	Cyclin-Dependent Kinase
CEAS	Cis-regulatory Element Annotation System
CHD	Chromodomain Helicase DNA binding protein 1
ChIP	Chromatin Immunoprecipitation
ChIP-seq	Chip-Sequencing
CHRAC	Chromatin-Accessibility Complex
CNS	Central Nervous System
CoIP	Co-Immunoprecipitation
CP	Cortical Plate
Cnot3	CCR4-NOT transcription complex, subunit 3
CREB	cAMP Response Element Binding Protein
CSBS	Consensus SATB1 Binding Sequence
Ct	Threshold Cycle
CtBP1	C-terminal Binding Protein 1
Ctip	Chicken ovalbumin upstream promoter transcription factor interacting protein
DAPI	4',6-Diamidino-2-Phenylindole
Dax1	Dosage sensitive sex reversal (DSS), Adrenal hypoplasia congenita (AHC) critical region on the X chromosome, gene 1
DFCs	Dental Follicle Cells
Dkk1	Dickkopf Homolog 1
DL	Deep Layers
DMEM	Dulbecco'S Modified Eagle'S Medium
DNMT	DNA (cytosine-5)-Methyl Transferase
dpc	days postcoitum
DTT	Dithiothreitol
E	Embryonic Day
EDTA	Ethylene Di-amine Tetra-Acetic acid
EGTA	Ethylene Glycol Tetra-Acetic acid
EMSA	Electrophoretic Mobility Shift Assay
ERK	Extracellular Receptor Kinases
ERas	Embryonic stem cell expressed Ras
Esrrb	Estrogen related receptor, beta
FBS	Fetal Bovine Serum
FCS	Fetal Calf Serum

FGF	Fibroblast Growth Factor
FGFR	Fibroblast Growth Factor Receptor
FL	Full Length
FOXP3	Forkhead box P3
GATA	Glutamyl-tRNA Amido Transferase subunit-A
GDF	Growth Differentiation Factor
GO	Gene Ontology
gp130	glycoprotein-130
GSK-3	Glycogen Synthase Kinase-3
GST	Glutathione S-Transferase
h	hour
H3K14ac	Histone H3 Lysine 14 Acetylation
H3K27me3	Histone H3 Lysine 27 Trimethylation
H3K4me3	Histone H3 Lysine 4 Trimethylation
H3K9ac	Histone H3 Lysine 9 Acetylation
H3K9me	Histone H3 Lysine 9 Trimethylation
HAT	Histone Acetyltransferases
HD	Homeodomain
HDAC	Histone Deacetylase
HEPES	(4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid)
hESCs	human Embryonic Stem Cells
HIV	Human Immunodeficiency Virus
HMG-1	High Mobility Group Protein-1
Hoxa2	Homeobox Protein Hox-A2
HRP	Horseradish Peroxidase
HS	Hypersensitive Site
IB	Immunoblot
ICM	Inner Cell Mass
Id	Inhibitor Of Differentiation
IEGs	Immediate Early Genes
IFNγ	Interferon γ
IgH	Immunoglobin Heavy Chain
IL	Interleukin
IP	Immunoprecipitation
iPSCs	induced Pluripotent Stem Cells
IPTG	Isopropyl β- D -thiogalactoside
ISWI	Chromatin Remodeling By Imitation Switch
JAK	Janus-Associated Tyrosine Kinase
kb	kilobase
kDa	kilo Dalton
Klf	Kruppel-like factor
LB	Luria Broth
LCR	Locus Control Region
LIF	Leukemia Inhibitory Factor
LIFR	LIF Receptor
M	Molar
M2H	Mammalian Two-Hybrid
MACS	Model-based Analysis of Chip-Seq
MAPK	Mitogen Activated Protein Kinase
MARs/SARs	MatrixAttachment Regions /Scaffold Attachment Regions
MBP	MAR-Binding Protein
MEF	Mouse Embryonic Feeder

mESCs	mouse Embryonic Stem Cells
mg	milligram
MHC	Major Histocompatibility Complex
min	minutes
miRNAs	microRNAs
ml	millilitre
mM	milli Molar
MTA2	Metastasis Associated 1 Family, Member 2
Na3VO4	Sodium Orthovanadate
Ni-NTA	Nickel-Nitriloacetic Acid
NLS	Nuclear Localization Sequence
NMTS	Nuclear Matrix Targeting Sequence
NT2D1	NTERA-2 clone D1
NuRD	Nucleosome Remodeling and Deacetylase Complex
Ocn	Osteocalcin
OCT4	Octamer-binding transcription factor 4
P	Postnatal Day
PARP-1	Poly (ADP-Ribose) Polymerase-1
PBS	Phosphate Buffered Saline
PCAF	P300/CBP Associated Factor
PcG	Polycomb Group
PCR	Polymerase Chain Reaction
PDGF	Platelet-Derived Growth Factor
PDZ	Post Synaptic Density Protein (Psd95), Drosophila Disc Large Tumor Suppressor (Dlg1), And Zonula Occludens-1 Protein (Zo-1)
PI3K	Phosphoinositide 3-Kinase
PKC	Protein Kinase C
PMA	Phorbol 12-Myristate 13-Acetate
PML	Promyelocytic Leukemia
PMSF	Phenyl Methyl Sulfonyl Fluoride
poly dI-dC	poly(deoxyinosinic-deoxycytidylic) acid
PRC	Polycomb Repressive Complex
PRDM	PR Domain Containing 14
p-TEFb	positive Transcription Elongation Factor b
PVDF	Polyvinylidene Difluoride
RA	Retinoic Acid
RNAi	RNA interference
RPMI-1640	Roswell Park Memorial Institute-1640
RTFs	Runt-related transcription factor 2
Runx2	Runt-Related Transcription Factor 2
SAF-A	Scaffold Attachment Factor-A
Sall4	Sal-like protein 4
SATB1	Special AT-rich sequence Binding protein 1
SATB2	Special AT-rich sequence Binding protein 2
SBS	Satb Binding Sequence
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis
SeqChIP	Sequential ChIP
SetDB1	SET domain bifurcated 1
Sin3a	SIN3 transcription regulator homolog A
SINE	Short Interspersed Nuclear Element
SMAD	Similar to Mothers Against Decapentaplegic homologue

SOLiD	Sequencing By Ligation
SOX2	SRY-box containing gene 2
SRY	Sex determining region Y
STAT	Signal Transducer and Activation of Transcription
Tat	Transactivator of transcription
Tbx3	T-box transcription factor
TCF3	T-Cell Factor-3
TCF-4	Transcription Factor-4
TF	Transcription Factor
TGF-β	Transforming Growth Factor-β
Th1	T-helper 1 Cells
Th2	T-helper 2 Cells
Treg	T-regulatory Cells
Trim28	Tripartite motif-containing 28
TrxG	Trithorax Group
TSS	Transcription Start Site
UL	Upper Layer
UTR	Untranslated Region
VEGF	Vascular Endothelial Growth Factor
Wnt	Wingless type protein
Xist	X-inactive specific transcript
Zfx	zinc finger protein, X-linked

Abstract

Eukaryotic chromosomes are organized inside the nucleus in a three-dimensional assembly involving topological attachments of chromatin loops to a nuclear matrix or scaffold. The bases of loops which mediate attachment to the nuclear matrix for the formation of chromatin loops are known as matrix attachment regions (MARs). Formation of these matrix-bound chromatin loops are facilitated by matrix-binding proteins which tether chromatin loops to the nuclear matrix. Matrix/Scaffold attachment regions (MARs/SARs) have been identified as AT-rich sequences which exhibit high affinity to nuclear matrix. These AT-rich regions are represented by a specialized DNA context that contains a cluster of sequences where one strand consists exclusively of well-mixed As, Ts, and Cs, excluding Gs (ATC sequences). Within MARs there are 100-150 bp sequences which have an intrinsic property of unwinding under negative super helical strain and are called as base unpairing regions (BURs). Since MARs often colocalize or exist in close proximity to the regulatory sequences including enhancers, the MAR-binding proteins are very important for regulating cell type specific gene expression. Proteins which preferentially bind to MARs include SATB1 (Special AT-rich Sequence Binding Protein 1), PARP-1 (Poly (ADP-Ribose) Polymerase-1), SAF-A (Scaffold Attachment Factor-A), Ku 70/86 (Auto antigen Ku), HMG-I(Y) (High Mobility Group Proteins) and nucleolin and a novel cell type-specific MAR-binding protein, SATB2 (Special AT-rich Sequence Binding Protein 2).

Special AT-rich Sequence Binding (SATB) family of MAR-binding proteins includes SATB1 and SATB2. SATB1 (Special AT-rich Sequence Binding Protein 1) is a nuclear protein that was originally cloned by virtue of its ability to bind to a core unwinding element (BUR) within the matrix attachment regions (MARs) located 3' of the immunoglobin heavy chain (IgH) gene enhancer. SATB1 selectively binds to double stranded DNA sequences possessing high unwinding capability believed to consist of specialized AT-rich sequence context where one strand consists of mixed A's, T's, and C's, excluding G's (ATC sequences) along the minor groove with very little contact with the bases. SATB1 knockout mice revealed drastic effect on the lymphoid organs, particularly thymus and spleen which are reduced in size and the lymph nodes contain reduced number of lymphocytes. At the molecular level, derepression of multiple genes including cytokine receptor genes, chemokine genes, and apoptosis related genes was observed in thymocytes along with impaired T-cell development and function. Gene expression

profiling of cells overexpressing wild type, acetylation and phosphorylation defective mutants of SATB1 indicated that approximately 10% of total genes are regulated by SATB1, suggesting that SATB1 may act as a global gene regulator. SATB1 binds to the IL-2R locus in thymocytes and regulates its expression by acting as a landing platform for several chromatin remodeling complexes such as the histone deacetylase (HDAC1) of NURD chromatin remodeling complex, ACF1 and ISWI, subunits of the CHRAC and ACF nucleosome mobilizing complexes. In mouse thymocyte nuclei SATB1 forms a three dimensional 'cage-like' network structure presumably by tethering specific MAR sequences to the nuclear matrix, thereby giving rise to tissue-specific chromatin architecture. In case of HIV infection one of the early synthesized transactivator of transcription protein (Tat) displaces HDAC1 from the IL-2 locus and its receptor bound SATB1-HDAC1 complex to derepress transcription of these genes. SATB1 mediated loop organization leads to coordinated expression of the TH2 locus- and MHC class I locus-linked genes. A close relative of SATB1 was later discovered which expresses predominantly in pre B cells, brain and osteoblast; with significant similarity to SATB1 at amino acid level and referred to as SATB2. SATB2 plays an important role in mouse palate development and osteoblast formation. Similar to SATB1, SATB2 possesses all the three important functional domains viz. PDZ, CD, and HD. It was shown to be involved in regulation of the immunoglobulin μ heavy chain gene expression in pre-B cells. The transcriptional activity of SATB2 is dependent on its sumoylation status. Very recently SATB2 was shown to activate γ-globin genes in erythroid cells by binding to MARs in their promoters and recruiting histone acetylase PCAF. SATB2 has been alsodemonstrated playing active role in neuronal differentiationin brain. Therefore it is very evident that SATB family of proteins not only organize chromatin into a distinct loop structure but also play a very important role in transcriptionalregulationof genes at the global level to execute important developmental processes. Both act as active gene regulators directly (acting at the level of target genes' promoters) as well as indirectly by interacting with other co-repressors or co-activators.

At the protein level, SATB1 and SATB2 share 61% sequence homology. Both proteins share common conserved domains which include: N-terminal PDZ-like domain, middle CUT repeat containing domain (CD) and C-terminal homeo domain (HD). PDZ-like domain is mediates the dimerization of SATB1 and may therefore have a similar function in case of SATB2. SATB1 differs from SATB2 in possessing a caspase-6 cleavage site, which is

responsible for cleavage of SATB1 during T-cell apoptosis. Additionally, SATB1 contains a glutamine rich region, which is absent in SATB2. In addition, both proteins also differ in having different kinds of post-translational modifications. In case of SATB1 acetylation and phosphorylation are the major post-translational modifications whereas sumoylation is the only reported post-translational modification of SATB2, even though the possibility of other kinds of modifications in either protein cannot be ignored. SATB1 has also been shown to be sumoylated at lysine 744 residue. Sumo conjugation to SATB1 is important for targeting it to the PML bodies where it undergoes caspase-mediated cleavage during apoptotic induction.

Since both these proteins performcommon role of gene regulation, we were interested in studyingthe comparative functions of SATB1 and SATB2 and their role in gene regulation. Thus, this study was undertaken with the following objectives:

1. To study the various functional and biochemical features of SATB1 and SATB2.

SATB1 and SATB2 are members of Special AT-rich binding proteins characterized by their ability to bind AT-rich sequence *in vivo*. Both possess a C-terminal DNA-binding region consisting of a cut domain and homeo domain which provides sequence-specific binding and an N-terminal dimerization domain consisting of PDZ-like domain which provides dimerization and interaction interface to SATB1 and SATB2. A number of post-translational modifications are known for SATB1 and SATB2 which regulate their transcription regulatory activity. Under this objective, we set out to study the comparative roles of SATB1 and SATB2 *in vivo* and the effect of various co-repressors and co-activators on the transcription regulatory activity of SATB1 and SATB2. The functional and biochemical features of SATB1 have been extensively studied. SATB1 has been shown to undergo variety of post-translational modifications which in turn regulate its transcription regulatory activity. SATB1 has been shown to interact with several co-repressors and co-activators depending upon its modification status. SATB1 is also known to form higher- order structures via its PDZ domain mediated dimerization. We compared these features to that of SATB2 and asked if SATB2 functions in similar manner. We show here that SATB2 also regulates MAR-linked reporter activity in a mannersimilar to that of SATB1. Both proteins show the effect of various co-repressors such as HDAC1 and co-activators such as P300 and PCAF. However, we found that unlike SATB1, SATB2 does not interact with CtBP1.

We have shown earlier that SATB1 forms homodimer via its PDZ-like domain and also interacts with other PDZ containing proteins. Since SATB2 also harbors a PDZ-like domain, we asked whether SATB1 and SATB2 interact *in vivo*. We show by co-immunoprecipitation experiments that SATB1 and SATB2 interact *in vivo*.

SATB1 is known to play an important role during tumor progression and development. It has been also shown that level of SATB1 increases while that of SATB2 decreaseswith progression of metastasis. Using a colorectal cancer cell line SW480 we have shown that the knockdown of SATB1 results in upregulation of SATB2 and *vice versa*. Next, we asked if SATB1 and SATB2 regulated each other's expression *in vivo*. Using chromatin immunoprecipitation assay we show that SATB1 and SATB2 occupy each other's promoter thereby regulate respective expression.

2. To study the roles of SATB1 and SATB2 in cellular context.

SATB1 and SATB2 are cell type specific MAR-binding proteins. Until now, multiple groups have studied roles of SATB1 and SATB2 usingdiverse types of cells and tissues. SATB1 has been mainly in studied in T-cells where it has been shown to regulate the development and differentiation of various T-cell subpopulations. Oncontrary, SATB2 has been mainly implicated in the development of neurons in the mouse brain cortex and in osteogenesis. Recently, SATB family proteins have been shown to play important roles in the progression of metastatic tumors. They also have been implicated in the differentiation of mouse embryonic stem cells. Under this objective we have performed a tissue wide expression profiling and show that various tissues show differential expression of SATB1 and SATB2. Next, we performed immunoblot analysis to detect the expression of these proteins in cell lines of different lineages. We found that while SATB1 expression was higher in T-cells, SATB2 expressed predominantly in B-cells. However, various neuronal cell lines were found to express comparable levels of SATB1 and SATB2.

To address the cell type-specific roles of SATB1 and SATB2, we used a human embryonic carcinoma cell line; NT2D1 which is widely used as a model for human embryonic stem cells. We show that levels of SATB1 and SATB2 increase upon retinoic acid (RA) mediated differentiation of NT2D1 cells while level of various pluripotency markers go down.

siRNA mediated knockdown of SATB1 and SATB2 resulted in the upregulation of various pluripotency associated genes and downregulation of various differentiation associated genes. We further show that Wnt signaling is activated upon RA mediated differentiation in NT2D1 cells. Interestingly, RA mediated differentiation could be mimicked by Wnt3a mediated Wnt activation in NT2D1 cells. siRNA mediated knockdown of SATB1 and SATB2 resulted in the downregulation of various Wnt responsive genes indicating that SATB1 and SATB2 may target Wnt responsive genes during differentiation in NT2D1 cells.

3. To study the roles of SATB1 and SATB2 in context of human embryonic stem cells.

We extended our studies of roles of SATB family proteins in human embryonic stem cells (hESCs). We preformed RA induced differentiation studies in two hESCs namely H9 and HS360. Unlike NT2D1 cells, we found that upon RA mediated differentiation SATB1 is upregulatedthroughout 7-day time-course of differentiation and SATB2 is transiently upregulated during first two days after RA treatment. However, SATB2 is then immediately downregulated during later days of differentiation in these human embryonic stem cells. We observed that activation of Wnt signaling using various Wnt signaling activators such as Wnt3A and BIO resulted in induction of differentiation of hESCs. On the other hand, inhibition of Wnt signaling using inhibitors like Dkk1 and XAV939 resulted in more stem cell like phenotype and prevented differentiation. Next, we performed gene expression profiling of H9 hES cells at various stages of differentiation. Gene expression profiling at 0 day, 3 day and 6 day of RA treatments revealed a number of genes that gets dysregulated upon differentiation. To correlate the expression profiling with that of SATB1 and SATB2 expression we performed the ChIP using SATB1 and SATB2 specific antibodies at similar stages of RA induced differentiation and performed genome-wide ChIP analysis using SOLiD 4 platform. ChIP-Seq analysis revealed novel categories of SATB1- and SATB2-bound targets involved in various biological pathways such as notch signaling, synaptic transmission, olfactory reception, sexual reproduction, development of primary male sexual characteristics and gonad development in addition to their previously reported roles in development of immunity and neuronal activities. We also derived consensus binding sequencesfor SATB1 and SATB2using ChIP-Seq data.

Chapter I

To study the functional and biochemical features of SATB family proteins

1.1 Introduction

Eukaryotic chromosomes are organized inside the nucleus in a three-dimensional assembly that involves topological attachments of chromatin loops to the nuclear matrix or scaffold; a proteinaceous structure first discovered by Berezney and Coffey as a nuclear component that resists salt extraction and digestion with DNase (Berezney and Coffey, 1974). Organization of the chromatin in form of such organized loops has significant implications in tissue specific regulation of gene expression (Schubeler D et al., 2000; Spector DL, 2003). Specific AT-rich DNA regions that exhibit high affinity to the nuclear matrix *in vitro* have been identified from various species and are called matrix attachment regions (MARs) or scaffold attachment regions (SARs). These AT-rich regions are represented by a specialized DNA context that contains a cluster of sequences where one strand consists exclusively of wellmixed As, Ts, and Cs, excluding Gs (ATC sequences). MARs harbor 100-150 bp 'core' sequences thatexhibit an intrinsic property to unwind under negative superhelical strain and are called base unpairing regions (BURs). MARs often exist in close proximity to transcriptionally active DNA or regulatory sequences including enhancers and therefore the MAR-binding proteins play a very important role in dictating cell type-specific gene expression (Fig. 1.1.1). Proteins which preferentially bind the MARs include SATB1 (Special AT-rich Sequence Binding Protein 1), PARP-1 (Poly (ADP-Ribose) Polymerase-1), SAF-A (Scaffold Attachment Factor-A), Ku 70/86 (Auto antigen Ku), HMG-I(Y) (High Mobility Group Proteins) and nucleolin (Galande, 2002) and a novel cell type-specific MAR-binding protein, SATB2 (Special AT-rich Sequence Binding Protein 2) (Dobreva et al., 2003).
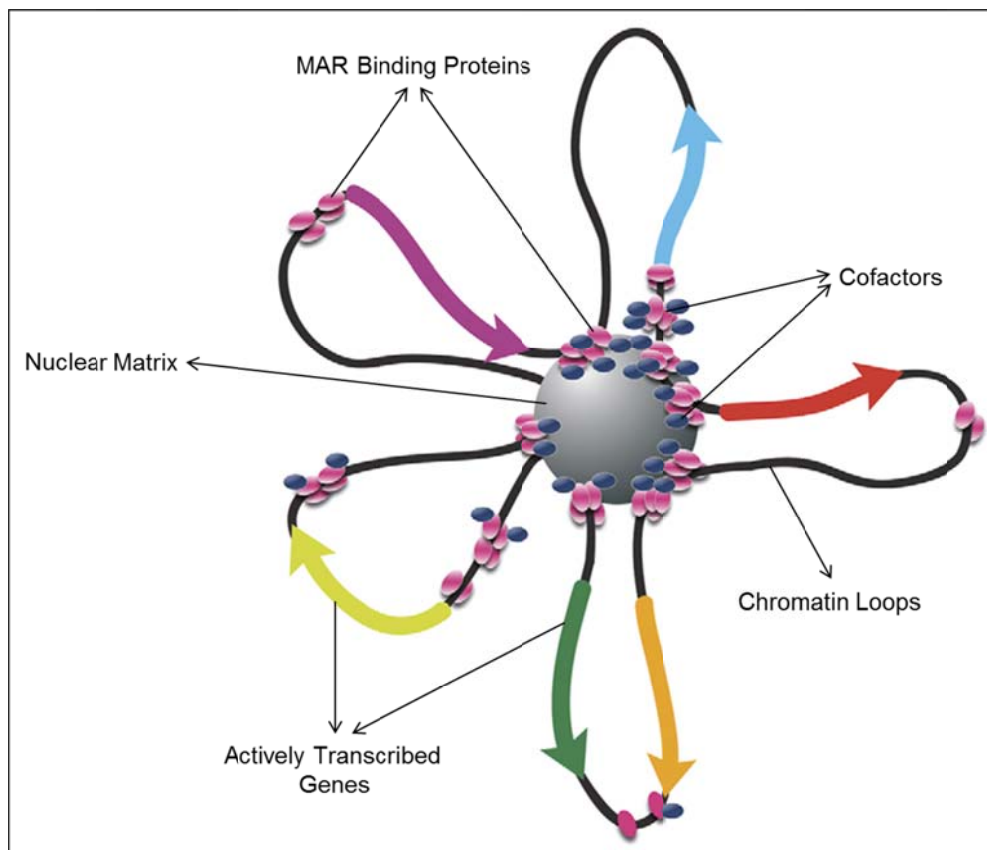
Figure1.1.1: Chromatinis organized into loop domains tethered to the nuclear matrix via matrix-attachment regions. A schematic representation of the chromatin loop domain structure or 'loopscape' of the human MHC-I locus. Within the nucleus, DNA is folded into complex higher-order structures resulting in formation of chromatin fibers and chromosome domains. The chromatin is looped out into 50-100 kb long DNA loop domains and these loops are tethered to the proteinaceous nuclear matrix (grey) via tethering of MAR sequences. Such compartmentalization of DNA into topologically independent loop domains is vital for efficient replication and transcription. MAR-binding proteins (pink) such as SATB1 help in the anchorage of the MAR sequences to the matrix and recruit various other interaxcting factors (cofactors) (blue) to facilitate coordinated transcription. DNA fiber is depicted as the solid line in loops and genes are shown by thick colored arrows (adapted from Galande et al., 2007).

Special AT-rich Sequence Binding (SATB) family of MAR-binding proteins includes SATB1 and SATB2 which regulate higher-order chromatin organization and gene expression by binding to MARs and presumably other regulatory regions in the genome. Human SATB1 is composed of 763 amino acid residues and harbors three well-characterized functional domains namely, N-terminal PDZ-like domain, middle CUT domain (CD) and C-terminal homeo domain (HD) (Fig. 1.1.2)(Dickinson et al., 1997). The PDZ-like domain of SATB1 isresponsible for its homodimerization and is required for its interaction with other proteins (Galande et al., 2001; Purbey et al., 2008). PDZ-like domain of SATB1, spanning amino acid residues 90-204,

undergoes post-translational modifications which act as a molecular switch regulating its transcriptional activity via modulating its association with other interacting partners (Pavan et al., 2006). However, recently solved crystal structure of the N-terminal domain of SATB1 revealed that this region of SATB1 does not fold into a typical PDZ-like domain structure; instead it resemblesthe ubiquitin domain and facilitates tetramer formation (Wang et al., 2012).
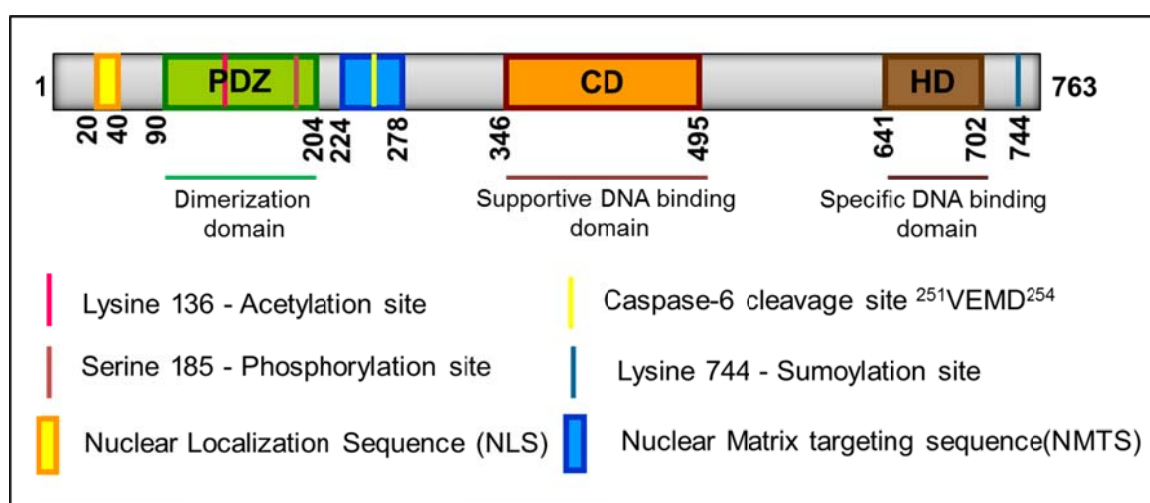
Figure 1.1.2: Schematic representation of functional domains and primary structure of human SATB1. SATB1 consists of 763 amino acids and many functionally characterized domains. The known functional domains of SATB1 are shown as various colored boxes along with amino acid position. Sites of known *in vivo* post-translational modifications of SATB1 are indicated as colored vertical lines. Proteins that interact with SATB1 via the PDZ-like domain are listed on top of PDZ region. N-terminal PDZ-like domain is required for homodimerization and its interaction with other PDZ domain containing proteins. Cut-domain (CD) and homeodomain (HD) contitute the C-terminal half and are required for DNA-binding. Post-translational modification sites (lysine 136, serine 185 and lysine 744), nuclear localization sequence (NLS), caspase-6 cleavage site and nuclear matrix targeting sequence (NMTS) are also depicted. The numbers below each domain indicate the amino acid residues. (Based on Dickinson et al., 1997; Seo et al., 2005; Nakayama et al., 2005;Pavan et al., 2006; Tan et al., 2008)

SATB1 shares 61% amino acid sequence homology with another member of SATB family protein, SATB2 (Fig. 1.1.3). Both proteins share common conserved domains: N-terminal PDZ-like domain, middle CUT domain (CD) and C-terminal homeo domain (HD). PDZ-like domain is shown to be important for dimerization of SATB1 (Galande et al., 2001), and may therefore have a similar function in case of SATB2. But at the same time, SATB1 differs from SATB2 in possessing a caspase-6 cleavage site, which is responsible for SATB1 cleavage during T-cell apoptosis. Additionally, SATB1 contains a glutamine rich region that is

not found in SATB2. Both proteins also differ in their post-translational modifications. SATB1 is known to be acetylated and phosphorylated (Pavan et al., 2006) whereas SATB2 is shown to be sumoylated (Dobreva et al., 2003), even though the possibility of other kinds of modifications in either protein cannot be ignored. SATB1 has also been shown to be sumoylated at lysine 744 (Tan et al., 2008). Sumo conjugation to SATB1 is important for targeting it to the PML bodies where it undergoes caspase-mediated cleavage during apoptotic induction (Tan et al., 2008).
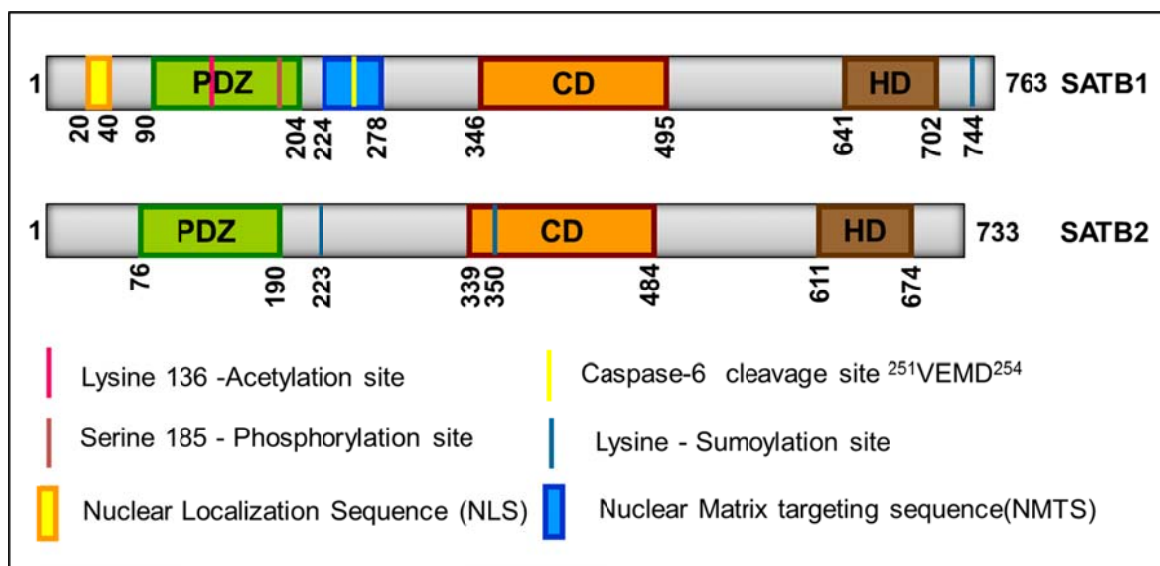
Figure 1.1.3: Comparison of various characterized domains of SATB1 and SATB2. SATB1 and SATB2 share 61% amino acid homology and common conserved domains also which include: N-terminal PDZ-like domain, middle CUT domain (CD) and C-terminal homeodomain (HD). Amino acid positions demarcating the domains are indicated. SATB1 harbors a caspase-6 cleavage site which is not present in SATB2. Apart from various well-characterized domains other important residues involved in various post-translational modifications are also depicted with colored vertical lines. Phosphorylation, acetylation and sumoylation are the major modifications reported for SATB1 whereas only sumoylation is reported for SATB2. The numbers below each domain indicate the amino acid residues. (Based on Dickinson et al., 1997; Seo et al., 2005; Nakayama et al., 2005; Dobreva et al., 2003; Pavan et al., 2006; Tan et al., 2008)

SATB1 was the first cell-type-restricted MAR-binding protein to be identified and is expressed predominantly in thymocytes. It was originally identified and cloned by virtue of its ability to bind to the BUR located within the 3' MAR of the IgH enhancer (Dickinson et al., 1992). SATB1 regulates higher-order chromatin organization, modification and gene transcription based on its ability to tether DNA elements and to act as a "landing platform" for several chromatin-remodeling complexes such as Sin3a, CHRAC, and ACF during T cell

development (Cai et al., 2003; Yasui et al., 2002). Gene knockout studies by Kohwi-Shigematsu group demonstrated that SATB1 acts as a global regulator of thymocyte differentiation and for the first timedemonstated that in SATB1-null mice thymocyte development is blocked at the CD4$^+$CD8$^+$ double-positive stage, many genes including *IL-2Rα* are upregulated (Alvarez et al., 2000). SATB1-bound targets remain active and are enriched for H3K9/K14ac as well as H3K4me, whereas in SATB1-null thymocytes these sites are heterochromatinized and are enriched for H3K9me (Cai et al., 2003). At least 2% of the genes including a proto-oncogene, cytokine receptor genes, and apoptosis-related genes were derepressed at inappropriate stages of T-cell development in SATB1-null mice. SATB1 is also known to bind the promoters of human *IL-2* and *IL-2Rα* genes and recruits HDAC1 *in vivo* resulting in their downregulation (Kumar et al., 2005). SATB1 regulates distant genes by selectively tethering BURs forming a distinguished 'cage-like' network in thymocytes (Cai et al., 2003). SATB1 also plays a role in positive regulation of genes as the c-myc locus, which contains a MAR region upstream of the transcription start site, is not properly upregulated in PMA-stimulated thymocytes from SATB1-deficient mice (Cai et al., 2003). SATB1 causes upregulation of ε-globin gene by selective binding in β-globin cluster as a complex with CREB-binding protein (CBP), thereby playing an important role in globin gene expression during early erythroid differentiation (Wen et al., 2005). However, the molecular mechanism responsible for the contrasting transcriptional activity of SATB1 has remained unclear. Previous work from our lab has shown that phosphorylation of SATB1 acts as a molecular switch regulating its transcriptional activity *in vivo* (Kumar et al., 2006). Furthermore, phosphorylation and dephosphorylation of SATB1 exerted opposing effects on the MAR-linked reporter activity. Phosphorylation of SATB1 at serine 185 by protein kinase C results in high affinity binding of SATB1 to DNA and recruitment of HDAC1 resulting into repression of downstream genes. Dephosphorylated SATB1 associates with PCAF and is acetylated by it at lysine 136 residue resulting in loss of its MAR binding ability resulting in derepression of its target genes. SATB1 network and the PML nuclear bodies intersect at the MHC class-I locus to regulate the coordinated expression of a subset of MHC-I genes (Kumar et al., 2007). Recent work from our lab shows that SATB1 coordinates Th2 lineage commitment by reprogramming gene expression through Wnt/beta-catenin signaling (Notani et al., 2010). Here we showed that during Th2 lineage commitment, Wnt signaling is upregulated, which results in deacetylation of SATB1 and thereby leading to increased DNA binding ability of SATB1. During this process SATB1 recruits β-catenin and p300 to its target sites, which results in

upregulation of Th2-specific transcription factors such as GATA3. SATB1 and GATA3 regulate *IL-5* transcription during human Th2 cell differentiation (Ahlfors et al., 2010). Recently, FOXP3 mediated downregulation of SATB1 is shown to be important for maintaining regulatory T cell (Treg) function (Beyer et al., 2011). SATB1 has shown to play important role in Xist-mediated gene silencing during X inactivation in mammals (Agrelo et al., 2009). SATB1 also plays important roles in various kinds of tumor progression and metastasis (Han et al., 2008; Meng et al., 2012). SATB1 is expressed at higher levels in breast cancer cell lines and RNAi mediated silencing of SATB1 in these cells leads to dysregulation of multiple genes. SATB1 leads to alteration in levels of specific epigenetic modifications at various target genes resulting into upregulation of metastatic genes and down regulation of tumor suppressor genes (Han et al., 2008). SATB1 promotes tumor growth by reprogramming chromatin organization in tumor cells. Based on these findings and similar findings in other cancers it was suggested that levels of SATB1 within cancer cells could have prognostic significance (Han et al., 2008; Mir et al., 2012). Interestingly, treatment with statins leads to downregulation of SATB1 in colon cancer cells and may presumably mediate the anti-cancer activity of Statins. Intriguingly, this regulation happens at the post-translational level since treatment with proteasome inhibitors reversed the statin-mediated down regulation of SATB1 (Lakshminarayana Reddy et al., 2010). A recent studyinvolving decoy DNA against SATB1 has highlighted importance of SATB1 as a drug target for the treatment of breast cancer where the invasive nature and metastatic capacity of a "triple negative" cancer cell line, which lacks expression of estrogen receptor, progesterone receptor and human epidermal growth factor receptor, was significantly decreased when it was treated with decoy DNA against SATB1 (Yamayoshi et al., 2011). A recent study has shown that SATB1 expression is lost in early stages of lung cancer and loss of SATB1 has been associated with poor prognosis in squamous cell carcinoma (Selinger et al., 2011). SATB1 was also found to be associated with decreased histone H3K9ac, a mark of active chromatin and increased histone H3K27me3, a mark of repressive chromatin (Selinger et al., 2011). SATB1 also has been associated with the development and progression of human glioma (Chu et al., 2012). FOXP3 and FOXP3 regulated microRNAs also have been implicated in regulating SATB1 expression and therefore tumor progression and metastasis (McInnes et al., 2012). All these reports have collectively established SATB1 as an important factor responsible for development and progression of various kinds of metastatic tumors suggesting that it is a potential target for cancer therapy (Mir et al., 2012).

SATB2 is second member of the SATB family of MAR-binding proteins. It was identified as a gene mutated in human patients with cleft palate (FitzPatrick et al., 2003). SATB2 was shown to bind MAR sequences flanking the enhancer of the endogenous immunoglobulin μ heavy chain (IgH) gene *in vivo*, and this binding correlated with an increase in the expression of a transfected rearranged μ wild-type gene, but not with a μ ΔMAR gene lacking the MAR sequences (Dobreva et al., 2003). Dobreva et al. demonstrated the role of SATB2 in craniofacial patterning and osteoblast differentiation (Dobreva et al., 2006). Specific roles of SATB2 in osteocyte differentiation and bone development have been discussed in section 2.1.2 of chapter II. RT-PCR analysis revealed high level of SATB2 expression in adult brain, moderate expression in fetal brain, and weak expression in adult liver, kidney, and spinal cord and in select brain regions, including amygdala, corpus callosum, caudate nucleus, and hippocampus. In all regions of the CNS, SATB2 expression peaked in differentiating neurons and decreased in mature cells suggesting its role in the control of neuronal differentiation. SATB2 has been shown to be involved in the differentiation and specification of callosal neurons in the neocortex and cerebral cortex in the brain by downregulating Ctip2 expression (Britanova et al., 2008; Alcamo et al., 2008) which have been discussion in greator details in section 2.1.3 of chapter II. SATB2 has been shown to interact with chromatin remodeling molecules in differentiating cortical brain neurons (Gyorgy et al., 2008). Like SATB1, SATB2 has also been shown to play important role in tumor progression and has been studied as a prognostic marker for various cancers (Patani et al., 2009; Wang et al., 2009b; Agrelo et al., 2009). Very recently it has been shown that SATB2 is expressed in erythroid cells and activates γ-globin genes by binding to MARs present in their promoters and recruiting histone acetylase PCAF (Zhou et al., 2012). Various microRNAs are known to play important role in tumor progression by regulating SATB2 expression (Aprelikova et al., 2010). Like SATB1, SATB2 is also known to undergo post-translational modifications. However, unlike SATB1, SATB2 is not acetylated at lysine 136 nor phosphorylated at serine 185 though it may possess some other acetylation or phosphorylation site(s). SATB2 has been shown to be sumoylated at lysines 233 and 350 (Dobreva et al., 2003). Mutation in sumoylation sites of SATB2 is shown to augment its activation potential and binding to MAR sequences *in vivo*.

SATB1 is expressed predominantly in thymocytes (Dickinson et al., 1992; Adams et al., 1993) but is also detected in the fetal brain (Adams et al., 1993). SATB1 is expressed in several regions of the CNS during development. SATB2 is also expressed in the developing CNS;

however, the expression patterns of SATB2 and SATB1 do not overlap. In the developing cerebral cortex, for example, SATB1 is expressed in the medial zone and piriform cortex whereas SATB2 is not expressed in these cells (Britanova et al., 2005). SATB2, in turn, is largely expressed in the superficial layers of the neocortex and subiculum. It has been reported that SATB1 knock-out mice have a neurological phenotype such as incomplete eye opening and the clasping reflex (Alvarez et al., 2000). Furthermore, both SATB1 and SATB2 can act as global regulators of cell differentiation in specific cell lineages within the developing CNS (Britanova et al., 2005). This mutually exclusive expression of SATB2 and SATB1 suggest that they control the expression of distinct subsets of genes at the level of chromatin in the developing CNS. Within the CNS some cells do not express either SATB1 or SATB2; however, there are reports where SATB1 and SATB2 have been shown to be expressed in the same tissues like at various stages of breast cancer development. Downregulation of SATB2 is associated with cancer progression whereas increase in SATB1 expression results in increased metastasis and cancer progression (Han et al., 2008; Wang et al., 2009b). It remains to be elucidated whether SATB1 and SATB2 can be substituted for one another or whether each acts on a unique set of targets. It also can be speculated that they might function in a coordinated manner or as a complex to regulate various genes. In the view of above discussion under this objective we set to study the comparative roles of SATB1 and SATB2 *in vivo* and the effect of various co-repressors and co-activators on the transcription regulatory activity of SATB1 and SATB2.

1.2 Materials and Methods

1.2.1 DNA constructs

Expression constructs for Flag-SATB1 and 6 X His-SATB1 has been described previously (Kumar et al., 2005, 2006). Full length SATB2 was cloned in p3XFlag-CMV10 (Sigma-Aldrich, St. Louis, MO, USA) and pTriEx 3 Neo vector (from Novagen/EMD Biosciences, CA, USA). N-terminal domains of SATB1 (1-254 aa) and SATB2 (1-240 aa), which harbor the PDZ domain of SATB1 and SATB2 respectively, as well as full-length proteins were subcloned in pACT and pBIND vectors (Promega Corp. WI, USA).

1.2.2 Cell culture and RNAi-mediated knockdowns

HEK-293T and SW480 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum and penicillin/streptomycin, at 37^oC under 5% CO_2 atmosphere. Knockdown experiments were performed using Lipofectamine RNAiMAX transfection reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer protocol. Briefly, SW480 cells were grown up to 60% confluency in 60 mm culture dishes at 37^oC in DMEM (Invitrogen) supplemented with 10% FBS (Fetal Bovine Serum, Invitrogen) and penicillin/streptomycin, under 5% CO_2 atmosphere. Twenty μl of 10μM siRNAs were transfected using Lipofectamine RNAiMAX as per manufacturer's instructions, in serum-free medium. The medium was supplemented with 10% fetal bovine serum 6 h post-transfection. The cells were allowed to grow for 48 h and harvested by scraping and used for RNA preparation and quantitative RT-PCR.

1.2.3 Antibodies and reagents

Anti-SATB1 (Cat. no. ab92307) and Anti-SATB2 (Cat. no. ab34735) antibodies used for coimmunoprecipitation and ChIP were purchased from Abcam (Cambridge, UK). Normal rabbit IgG (Cat. no. 12-370) and normal mouse IgG (Cat. no. 12-371) were purchased from Millipore/Upstate (Billerica, MA, USA). Anti-FLAG antibody was obtained from Sigma-Aldrich (St. Louis, MO, USA). Lipofectamine 2000 and Lipofectamine RNAiMAX transfection reagent were purchased from Invitrogen (Carlsbad, CA, USA). siRNAs for GFP (Cat. no. sc-45924), SATB1 (Cat. no. sc-36460) and SATB2 (Cat. no. sc-76456) were procured from SantaCruz Biotechnology (Santa Cruz, CA, USA). Steadylite plus reagent (Cat. no. 6016751) for reporter assays was procured from Perkin Elmer (Massachusetts, USA). Signal for immunoblot was detected using Visualizer™ Western Blot Detection Kit (Millipore/Upstate, Billerica, MA, USA, Cat. no. 64-202). Isopropyl-β-D-thiogalactopyranoside (IPTG) was procured from Sigma-Aldrich (St. Louis, MO, USA). Protein A/G Plus Ultralink resin (Cat. No. 53135) was obtained from Thermo Scientific (Rockford, IL, USA).

1.2.4 Protein expression and purification

Full length SATB1(1-763 aa) and full-length SATB2 (1-733 aa) cloned in pTriEx 3 Neo were expressed in BL21(DE3) strain of *E. coli* host (Novagen/Merck Biosciences, Philadelphia, USA) and were purified using Ni-NTA columns (Qiagen, Maryland, USA) as described in the instruction manual. The GST-1.6 (DNA binding C-terminal half of SATB1) and GST-1.5 (DNAbinding C-terminal half of SATB2), for EMSA, was expressed in XL-1 Blue strain of *E. coli* (Invitrogen, Carlsbad, CA, USA). Single colonies were inoculated in 5 ml of LB media supplemented with 50 μg/ml of ampicillin. Next day 1ml of culture was transferred to 500 ml of LB media supplemented with 100 μg/ml of ampicillin. Protein synthesis was induced at A_{600} by the addition of isopropyl-β-D-thiogalactopyranoside (Sigma-Aldrich, St. Louis, MO, USA) to a final concentration of 0.5 mM. Induction of protein expression was carried at $A_{600}$for 5 h with 0.5 mM IPTG. Cells were pelleted down by centrifugation (4000 X g, 5 min at 4°C) at the end of induction. Pellet was washed with PBS and resuspended in 50 ml of buffer A (50 mM Tris pH 8.0, 150 mM NaCl, 0.2% Triton-X 100) supplemented with 1X EDTA free complete protease inhibitor cocktail (Roche, Indianapolis, IN, USA) and 10 μg/ml of lysozyme. Cells were incubated on ice for 30 min and viscosity was reduced by sonication. The lysed samples were then centrifuged at 14000 X g at 4°C for 30 min and the supernatant was incubated with Glutathione Sepharose beads (GE Healthcare, Piscataway, NJ, USA) for 3h at 4°C. The beads were washed thrice (1000 X g, 5 min) with lysis buffer and the bound protein was eluted using 10 mM reduced glutathione (Sigma-Aldrich, St. Louis, MO, USA) in Buffer A (without Triton-X 100) and dialyzed twice against phosphate buffered saline (10 mM Tris, 138 mM NaCl). The protein quality in the eluentwas monitored by 12.5 % SDS-polyacrylamide gel electrophoresis (PAGE).

1.2.5 Electrophoretic mobility shift (EMSA) assay

Electrophoretic mobility shift assay (EMSA) was performed as described previously (Purbey et al., 2008). Full-length SATB1 and SATB2 were expressed and purified as 6XHis-tagged fusion proteins. Binding reactions were performed in a 10 μl total volume containing 2 μl of 5X C buffer (50 mM HEPES, pH7.9, 5 mM DTT, 250 mM KCl, 12.5 mM MgCl2, 50% glycerol), 0.5 μg of double- stranded poly dI-dC (1 mg/ml), 1 μg of BSA and

appropriate dilutions of recombinant protein. Samples were pre-incubated at room temperature, for 10 min prior to the addition of ^{32}P-labeled IgH-MAR probe (Purbey et al., 2008). After 15 min of addition of probes, the products of the binding reactions were resolved on 6% native PAGE gels at 150 volts for 1 h. The gels were dried under vacuum using gel-dryer (Bio-Rad, Hercules, CA), kept for exposure with X-ray film (Konica) and developed after 48h incubation at -80ºC.

1.2.6 Luciferase reporter assay

Luciferase assays were performed using LucLite reagent (Perkin Elmer, Massachusetts, USA) and luciferase activity was measured using TopCount NXT™ Microplate Scintillation and Luminescence Counter (Perkin Elmer, Massachusetts, USA). The IgH-MAR-Luc reporter constructs (Kumar et al., 2005; 2006) were used to score the effect of SATB1 and SATB2 along with HDAC1, CtBP1, PCAF and p300. All transfections were carried out using various combinations of constructs and the final amount of DNA was normalized by including pCDNA3.1 DNA. HEK-293T cells were seeded at 0.5 x 10^6 cells per well and transfected using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer protocol. Six h post-transfection cells were supplemented with complete media containing 10% FCS and were harvested after 48 h. Luciferase assay was performed using Steadylite plus reagent (Perkin Elmer, Massachusetts, USA) as per manufacturer's instructions and readings were obtained usingTopCount NXT™ Microplate Scintillation and Luminescence Counter (Perkin Elmer, Massachusetts, USA). The values were normalized with control (vector transfection) as reference andplotted using Sigmaplot 11 software.

1.2.7 Co-immunoprecipitation with SATB1 and SATB2 with CtBP1

Co-immunoprecipitation was performed to check *in vivo* interaction of SATB1 and SATB2 with the co-repressor protein CtBP1. For this HEK-293T cells were grown to 90% confluency in 90 mm culture dishes at 37ºC in DMEM medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10 % FBS Medium (Invitrogen, Carlsbad, CA, USA) and penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA), under 5% CO_2 atmosphere and

harvested by scraping to prepare whole cell lysate. The cell lysate was made in extraction buffer (0.42M NaCl, 1.5mM $MgCl_2$, 0.2 mM EDTA, 25mM HEPES, pH7.9, 25% glycerol, 1x EDTA free complete protease inhibitor cocktail) without dithiothreitol (DTT) (Dignam et al., 1983). All downstream processes were carried out under non-reducing conditions (i.e. in absence of dithiothreitol or β–mercaptoethanol in the lysis buffer and sample buffer). The lysate was diluted to a final   concentration of 1 μg/μl with 1X chilled PBS containing 1X EDTA-free complete protease inhibitor cocktail (Roche, Indianapolis, IN, USA). For each immunoprecipitation reaction, 500 μg of the lysate was precleared for 1 h at 4^oC on test tube rocker with 10 μl protein A/G Plus Ultralink resin (Thermo Scientific, Rockford, IL, USA). After preclearing, the beads were pelleted by centrifugation at 1000 X g, 5 min at 4^oC and the supernatant was transferred to a fresh tube. Precleared extract was then incubated with 1 μg each of IgG, SATB1, SATB2 and CtBP1 antibodies for 2-4h at 4^oC on test tube rocker. To this, 10 μl of protein A/G beads were added and incubation was further continued for 4 h. The protein-antibody complexes, bound to Protein A/G resin were recovered by centrifugation at 1000 X g for 5 min and washed five times with TSA buffer (2 mM Tris, pH 8.0, 140 mM NaCl, 0.025% sodium azide) containing 0.1 % Triton X-100. The complexes were eluted by incubating the beads in 30 μl Laemmli sample buffer (without DTT) at 37^oC for 5 min with intermittent mixing and eluate was resolved on a 10 % SDS-PAGE gel and transferred to PVDF membrane. Immunoblotting was performed usinganti-CtBP1 antibody. Immunoprecipitation with anti-CtBP1 and immunoblot with anti-CtBP1 served as control for co-immunoprecipitation.

1.2.8 Co-immunoprecipitation of SATB1 for monitoring its homodimerization

To demonstrate that SATB1 interacts with other SATB1 molecules to form homodimer *in vivo* we performed co-immunoprecipitation assay using exogenously expressed recombinant GFP-SATB1 and Flag-SATB1. Towards this end, HEK-293T cells were grown upto 60% confluency in 90 mm culture dishes at 37^oC in DMEM medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS Medium (Invitrogen, Carlsbad, CA, USA) and penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA), under 5% CO_2 atmosphere. Ten micrograms of Cesium chloride density gradient-purified plasmids were transfectedusing Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) as per manufacturer's instructions in

serum-free medium. The medium was supplemented with 10% fetal bovine serum 6 h post-transfection. The cells were grown for 48 h and harvested by scraping and used for whole cell lysate preparation. The cell lysate was made in a extraction buffer (0.42M NaCl, 1.5 mM MgCl$_2$, 0.2 mM EDTA, 0.5mM DTT, 25 mM HEPES, pH 7.9, 25% glycerol, 1X EDTA free complete protease inhibitor cocktail) without dithiothreitol (DTT) (Dignam et al., 1983). All downstream processes were carried out under reducing condition. The lysate was diluted to a final concentration of 1 μg/μl with 1X chilled PBS containing 1X EDTA free complete protease inhibitor cocktail (Roche, Indianapolis, IN, USA). For each immunoprecipitation reaction, 500 μg of the lysate was precleared for 1 h at 4°C on test tube rocker with 10 μl protein A/G Plus Ultralink resin (Thermo Scientific, Rockford, IL, USA). After preclearing, beads were pelleted by centrifugation at 1000 X g, 5 min at 4°C and the supernatant was transferred to a fresh tube. Precleared extract was then incubated with 1 μg each of IgG and Flag antibodies for 2-4h at 4°C on test tube rocker. To this, 10 μl of protein A/G beads were added and incubation was continued further for 4 h. Protein-antibody complexes, bound to Protein A/G resin were recovered by centrifugation at 1000 X g for 5 min and washed five times with TSA buffer (2 mM Tris, pH 8.0, 140 mM NaCl, 0.025% sodium azide) containing 0.1 % Triton X-100. Complexes were eluted by boiling the beads in 30 μl Laemmli sample buffer (with DTT) at 95°C for 5 min with intermittent mixing and eluate was resolved on a 10% SDS-PAGE gel and transferred to PVDF membrane. Immunoblotting was performed using anti-GFP antibody.

1.2.9 Co-immunoprecipitation of SATB1 and SATB2

Co-immunoprecipitation assay was performed to check *in vivo* homodimerization of SATB1 as well as to show the interaction of SATB1 and SATB2. For this purpose HEK-293T cells were grown to 90% confluency in 90 mm culture dishes at 37°C in DMEM medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10 % FBS Medium (Invitrogen, Carlsbad, CA, USA) and penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA), under 5% CO2 atmosphere and harvested by scraping to prepare whole cell lysate. Cell lysate was made in extractionbuffer (0.42 M NaCl, 1.5 mM MgCl$_2$, 0.2 mM EDTA, 0.5 mM DTT, 25 mM HEPES, pH 7.9, 25% glycerol, 1x EDTA free complete protease inhibitors) (Dignam et al., 1983). All the downstream processes were carried out under reducing condition. The lysate was diluted to a

final concentration of 1 µg/µl with 1X chilled PBS containing 1X EDTA free complete protease inhibitor cocktail (Roche, Indianapolis, IN, USA). For each immunoprecipitation reaction, 500 µg of the lysate was precleared for 1 h at 4°C on test tube rocker with 10 µl protein A/G Plus Ultralink resin (Thermo Scientific, Rockford, IL, USA). After preclearing the beads were collected by centrifugation at 1000 X g, 5 min at 4°C and the supernatant was transferred to a fresh tube. Precleared extract was then incubated with 1 µg each of IgG, SATB1 and SATB2 antibodies for 2-4h at 4°Con test tube rocker. To this, 10 µl of protein A/G beads were added and incubation was further continued for 4 h. The protein-antibody complexes, bound to Protein A/G resin were recovered by centrifugation at 1000 X g for 5 min and washed five times with TSA buffer (2 mM Tris, pH 8.0, 140 mM NaCl, 0.025% sodium azide) containing 0.1% Triton X-100. The complexes were eluted by boiling the beads in 30 µl Laemmli sample buffer (with DTT) at 95°C for 5 min with intermittent mixing and eluate was resolved on a 10% SDS-PAGE gel and transferred to PVDF membrane. Immunoblotting was performed using anti-SATB1 antibody.

1.2.10 Mammalian two-hybrid assay

CheckMate mammalian two hybrid system (Promega Corp., USA) was used to score for protein-protein interactions. Cloning gene of interest in pBIND vector expresses it as GAL4 DNA-binding domain fusion protein and cloning in pACT fusion construct expresses it as VP-16 activation domain fusion protein. pBIND and pACT fusion constructs were transfected along with a reporter vector, which contains 4X GAL4 responsive element (pG5luc), and luciferase activity was compared with the control. Specifically, the N-terminal region of SATB1 (1-254 aa) and SATB2 (1-240), which harbors the respective PDZ-like domain and C-terminal region harboring CD+HD of SATB1 (255-763 aa) and SATB2 (241-733 aa) were subcloned separately in pACT and pBIND vectors (Promega Corp. USA). HEK-293T cells were seeded at 0.5×10^6 cells per well in a 24-well plate (BD Falcon) 24 h before transfection. Cells were transfected with pG5luc reporter vector (Promega Corp. USA) using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) along with either pBIND fusion construct and pACT empty vector in control and pBIND fusion construct with pACT fusion construct for the experimental set. DNA amount was kept constant up to 1.5µg (0.5µg of each DNA) in every well. Cells were supplemented with complete media containing 10% FCS, 6 h post-transfection and were

harvested after 48 h. Luciferase assay was performed using Steadylite plus reagent (Perkin Elmer, Massachusetts, USA) as per manufacturer's instructions and readings were recorded on TopCount NXT™ MicroplateScintillation and Luminescence Counter (Perkin Elmer, Massachusetts, USA). The values were normalized with respect to control (vector transfection) and plotted using Sigmaplot 11 software.

1.2.11 Quantitative RT-PCR

RNA was prepared from control and siRNA transfected cells using TRI reagent (Sigma-Aldrich Co., St. Louis, MO, USA). One µg of RNA was used for cDNA preparation per 20 µl of reaction. Quantitative RT-PCRs were carried out essentially as described (Kumar et al., 2005). The cDNA was used as template for the PCR with specific set of primers. Changes in threshold cycle (Ct) values were calculated as follows: $\Delta Ct = (Ct_{target\ genes} - Ct_{\beta-actin})$ for transcript analysis. These ΔCt values were used to calculate fold change using equation as relative fold change $= 2^{-(\Delta(\Delta Ct))}$ and plotted graph for the average fold values with standard deviation from three independent experimental samples in Sigma Plot.

1.2.12 Chromatin immunoprecipitation (ChIP) assay

ChIP was essentially performed as described previously (Jayani et al., 2010). HEK-293T cells were cross-linked for 10 min at 37°C by adding formaldehyde (to a final concentration of 1%) directly to the culture medium in the flask. Cross-linking was stopped by the addition of glycine to a final concentration of 125 mM. Cells were washed twice with ice-cold PBS and harvested using cell scraper and pelleted down. Subsequently, the cell pellet was washed with wash buffer I (0.25% Triton X-100, 10 mM EDTA, 0.5 mM EGTA, 10 mM HEPES pH 7.5, 1 mM PMSF, 10 mM sodium butyrate, 1X EDTA free complete protease inhibitor cocktail) and wash buffer II (0.2 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 10 mM HEPES pH 7.5, 1 mM PMSF, 10 mM sodium butyrate, 1XEDTA-free complete protease inhibitor cocktail). Pellet was resuspended in lysis buffer (150 mM NaCl, 25 mM Tris-HCl pH 7.5, 5 mM EDTA, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 1 mM PMSF, 10 mM sodium butyrate, 1XEDTA free complete protease inhibitor cocktail) and sonicated using Bioruptor

sonicator (Diagenode, Belgium) (8 min, 10 sec "ON", 10 sec "OFF"). The sonicated sample was centrifuged at 13000 X g for 10 min at 4°C and the supernatant was collected as soluble cross-linked chromatin. The chromatin was quantified and equal amount of chromatin was used for each ChIP. The volume of chromatin was normalized to 100µl by lysis buffer and then diluted five times before immunoprecipitation. The chromatin solution was then precleared by addition of 20 µl of protein A/G Sepharose beads cocktail (50% protein A/G beads slurry, 100 µg of salmon sperm DNA/ml, 500 µg of bovine serum albumin/ml) and kept on rocker at 4°Cfor 2 h. After centrifugation at 1000 X g and 4°C for 5 min, the supernatant was incubated separately with anti-SATB1, anti-SATB2 and normal rabbit IgG (as control) at 4°C on an end-to-end rocker for 8 h. Twenty µl of protein A/G-plus bead cocktail was added and rocking was continued for another 4 h. The beads were then harvested by centrifugation at 1000 X g at 4°C for 5 min and washed twice with RIPA buffer (Radio immunoprecipitation assay buffer; 150 mM NaCl, 0.1 % sodium dodecyl sulphate, 10 mM sodium phosphate, 1 % sodium deoxycholate, 2 mM EDTA, 0.2 mM sodium orthovandate and 1 % IGEPAL) and twice with TE (10 mM Tris, 1 mM EDTA). Chromatin-antibody complexes were eluted from the A/G Sepharose beads by addition of 2% SDS, 0.1 M NaHCO3 (freshly prepared), and 10 mM dithiothreitol to the pellet. Cross-linking was reversed by addition of 0.05 volume of 4 M NaCl and incubation of the eluted samples for 4 h at 65°C. After addition of 0.025 volume of 0.5 M EDTA and 0.05 volume of 1 M Tris-HCl (pH 6.5), proteinase K digestion was performed for 1 h at 45°C. DNA was recovered by phenol-chloroform-isoamylalcohol extraction followed by a chloroform-isoamylalcohol extraction and precipitated by addition of 0.1 volume of 3 M sodium acetate (pH 5.2), 20 µg of glycogen, and 2.5 volumes of ethanol. Precipitated DNA was dissolved in water, and was analyzed by PCR.

1.2.13 Sequential ChIP assay

ChIP was performed as described previously (Jayani et al., 2010) and above using anti-SATB1 and anti-SATB2 along with rabbit IgG as control. ChIP was performed exactly as above and chromatin-antibody complex was eluted by addition of 100µl ChIP elution buffer (2% SDS, 0.1 M NaHCO3 (freshly prepared), and 10 mM dithiothreitol) to the pellet. At this point, 10 µl was removed from each sample (except those not used for SeqChIP) for subsequent analysis of the first immunoprecipitation. Samples not used for SeqChIP were decrosslinked by addition of

0.05 volume of 4 M NaCl and incubation of the eluted samples for 4 h at 65°C. After addition of 0.025 volume of 0.5 M EDTA and 0.05 volume of 1 M Tris-HCl (pH 6.5), proteinase K digestion was performed for 1 h at 45°C. DNA was recovered by phenol-chloroform-isoamylalcohol extraction followed by a chloroform-isoamylalcohol extraction and precipitated by addition of 0.1 volume of 1 M sodium acetate (pH 5.2), 20 µg of glycogen, and 2.5 volumes of ethanol. Precipitated DNA was dissolved in water, and was analyzed by PCR. For SeqChIP, eluates (90 µl) were diluted five times with PBS and then specific antibody was added to each tube and kept for at 4°C on an end-to-end rocker for 8 h. Antibody used for first ChIP was also used as positive control for sequential ChIP. IgG was also used as a negative controlin SeqChIP. Twenty µl of protein A/G-plus bead cocktail was added and rocking was continued for another 4 h. Beads were then harvested by centrifugation at 1000 X g at 4°C for 5 min and were processed subsequently in same manner as for normal ChIP.

1.2.14 ChIP-Western assay

ChIP was performed using anti-SATB1 or anti-SATB2 along with rabbit IgG as control. Chromatin-antibody complex was washed twice with RIPA buffer and then twice with TE buffer. At this point, chromatin-antibody complex was treated with DNase1 (Invitrogen, Carlsbad, CA, USA) to remove free DNA if any. The complex was eluted by heating the beads at 90°C for 5 min in SDS elution buffer and resolved by loading onto 12.5% SDS-PAGE gel. Western blot analysis was performed using anti-SATB2 or anti-SATB1.

1.3 Results

1.3.1 Cloning of PDZ-like domains of SATB1 and SATB2 and full-length SATB1 and SATB2 in various vectors

For mammalian two hybrid assays, full length SATB1 and SATB2 as well as their PDZ-like domains were cloned as VP16 and GAL4 fusion consturcts in pACT and pBIND vectors respectively. Full-length SATB2 was cloned into3XFlag-CMV10 vector (Sigma-Aldrich Co., St. Louis, MO, USA).

1.3.2 SATB2 binds to IgH-MAR with higher affinity as compared to SATB1 *in vitro*

SATB1 and SATB2 both have been shown to bind IgH-MAR *in vitro* with high affinity (Dickinson et al., 1992; Dobreva et al., 2003) However, there is no report showing the relative binding of these two MAR-binding proteins to any MAR DNA. To study the comparative binding of SATB1 and SATB2 we purified full-length recombinant SATB1 and SATB2 proteins and performed EMSA using IgH-MAR as probe. Amount of SATB1 and SATB2 used for EMSA was normalized by densitometry of Coommassie brilliant blue stained gel. IgH-MAR DNA probe was radiolabeled through end filling using Klenow fragment of DNA polymerase using ^{32}P-dCTP and α^{32}P-dATP. Purified labeledprobe was incubated with increasing amount of recombinant full-length SATB1 and SATB2 and complexes were resolved on 6% native PAGE gels (Fig. 1.3.1). The autoradiogram revealed that incubation of SATB2 with the IgH-MAR probe led toincreasedretardation in gel mobility as compared to SATB1 at equimolar amounts, indicating that SATB2 might bind DNA with higher affinity than that of SATB1.
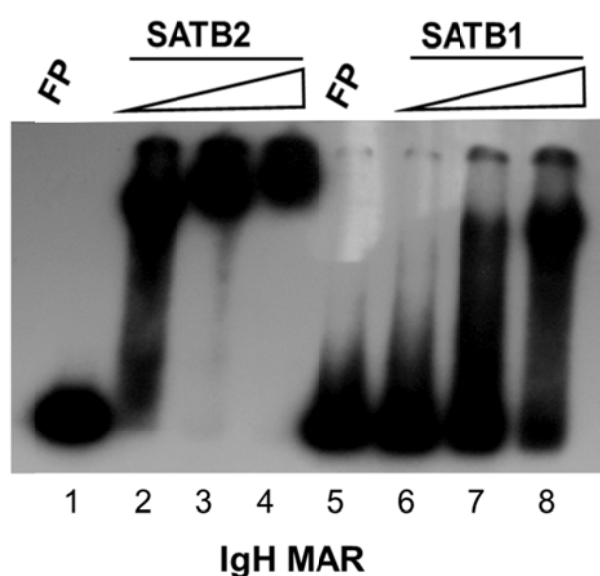
Figure 1.3.1: SATB2 binds to IgH-MAR with higher affinity as compared to SATB1 *in vitro*. EMSA was performed using IgH-MAR radiolabeled probe to check the relative binding of SATB1 and SATB2. Recombinant full-length SATB1 and SATB2 were expressed in *E. coli* and were purified using Ni-NTA beads for this assay. Radiolabeled IgH-MAR probe was incubated with increasing amount of SATB1 and SATB2 and then processed for running on 6% native PAGE gel followed by autoradiography. Lane 1 and lane 5 show free probe. Lanes 2-4 and lane 6-8 depict EMSA using increasing amounts of SATB2 and SATB1 respectively.

1.3.3 SATB1 and SATB2 bind and regulate IgH-MAR activity *in vivo*

After validating the binding of SATB1 and SATB2 *in vitro*, we next checked the comparative ability of these proteins to regulate IgH-MAR-linked reporter activity *in vivo*. For this purpose we cloned full-length SATB1 and SATB2 in pTriEx-3 neo vector and expressed them in HEK-293T cells along with pGL3 basic IgH-MAR-luc reporter construct. pCDNA 3.1 was used as normalizing vector control for this experiment (Fig. 1.3.2). We observed that SATB2 repressed IgH-MAR-linked reporter activity in amanner similar to that of SATB1.
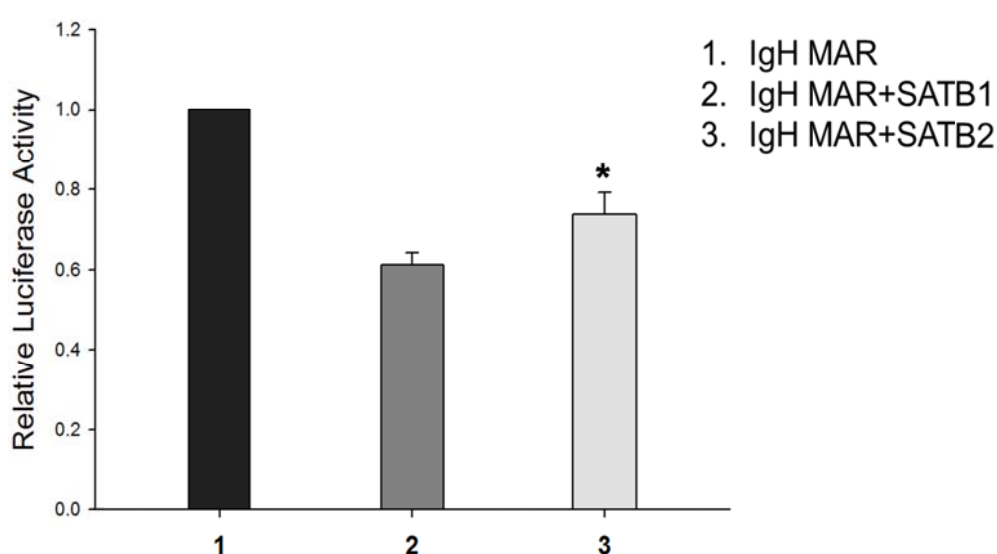
Figure 1.3.2: SATB2 binds and represses IgH-MAR-linked reporter activity like SATB1 *in vivo*. Reporter assay was performed in HEK-293T cells using pGL3 basic IgH-MAR-luc construct along with full-length SATB1 and SATB2. pCDNA 3.1 was used a normalization vector for transfection (bar 1). Cells were supplemented with 10% FCS, 6 h post-transfection and were harvested after 48 h. Luciferase assay was performed using Steadylite plus reagent as per manufacturer's instructions and luciferase activity was measured using TopCount NXT™ Microplate Scintillation and Luminescence Counter. The values were normalized with control (pCDNA 3.1 blank vector transfection) as reference and plotted using Sigmaplot 11 software. Individual vertical bar values represent average of technical triplicates. Error bars represent standard deviation calculated from triplicates. * Significant difference ($p < 0.05$).

1.3.4 Effect of various co-repressors and activators on SATB1 and SATB2 mediated transcriptional regulation

SATB1 has been shown to interact with various co-repressors and activators *in vivo* and *in vitro* leading to repression or activation of the target gene expression (Kumar et al., 2005 and

Pavan et al., 2006; Purbey et al., 2009). To monitor the effect of these co-repressors and activators *in vivo* on the MAR regulatory activity of SATB1 the luciferase reporter assay system was used earlier (Kumar et al., 2006; Purbey et al., 2008). To test whether and how these co-repressors and activators affect SATB2 mediated IgH-MAR regulation, we performed reporter assay in presence of various co-repressors and activators using IgH-MAR-luciferase reporter construct (Fig. 1.3.3). Coexpression of HDAC1, a co-repressor along with SATB1 or SATB2 resulted in further repression of MAR-linked reporter activity (Fig. 1.3.4, bar 3) whereas co-expression of various activators such as PCAF, p300 and SumoI resulted in de-repression of the MAR-linked reporter activity(Fig. 1.3.4, bars 4-6). This data suggests that these proteins effect SATB1 and SATB2 in a similar manner.
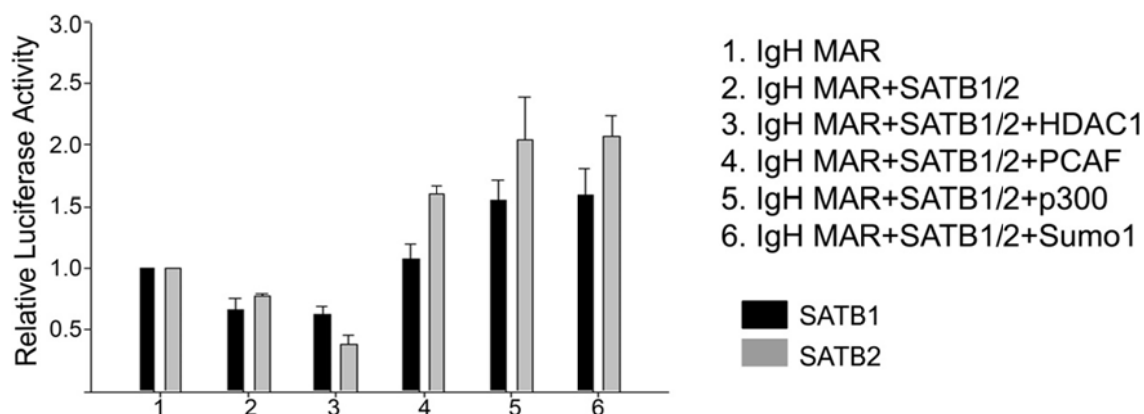
Figure 1.3.3: Effect of various co-repressors and activators on SATB1 and SATB2 mediated transcriptional regulation. Reporter assay was performed in HEK-293T cells using pGL3 basic IgH-MAR-luc construct along with full-length SATB1 and SATB2 in combination with HDAC1 co-repressor and activators such as PCAF, p300 and Sumo 1. pCDNA 3.1 was used as normalization vector for transfection (bar 1). Cells were supplemented with 10% FCS 6 h post-transfection and were harvested after 48 h. Luciferase assay was performed using Steadylite plus reagent as per manufacturer's instructions and TopCountNXT Microplate Scintillation counter. The values were normalized with control (pc DNA 3.1 blank vector transfection) as reference and plotted using Sigmaplot 11 software. Black bars represent the data from SATB1 transfected cells whereas grey bars represent the data from SATB2 transfected cells. Individual vertical bar values represent average of technical triplicates. Error bars represent standard deviation calculated from triplicates.

1.3.5 SATB1 interacts with CtBP1 whereas SATB2 does not interact with CtBP1 *in vivo*

SATB1 interacts with HDAC1 and CtBP1 co-repressors via its PDZ-like domain (Kumar et al, 2005; Purbey et al., 2009). SATB1 harbors the CtBP1 interaction motif "PVPLS" within its PDZ-like domain. Furthermore, interaction between SATB1 and CtBP1 depends on the

acetylation status of K136 residue of SATB1 (Purbey et al., 2009). SATB2 also contains a CtBP1 interaction motif "PLPLS" and this finding prompted us to test whether SATB2 interacts with CtBP1 as well. Towards this, we performed co-immunoprecipitation in HEK-293T cells using SATB1 as positive control. However, we failed to detect any interaction between SATB2 and CtBP1, whereas as expected SATB1 showed interaction with CtBP1 (Fig. 1.3.4 B). Purbey et al. have shown previously that acetylation of SATB1 at lysine residue at position 136 (K136) downstream to CtBP1 interaction motif is crucial for SATB1-CtBP1 interaction and this interaction is abolished when K136 gets acetylated. SATB2 contains the CtBP1 interaction motif "PLPLS"; however, it lacks the lysine upstream of this motif at the equivalent amino acid position. Interestingly, SATB2 harbors arginine residue upstream of the CtBP1 interaction motif which resembles the acetylation mutant version of SATB1 (Fig. 1.3.4 A).
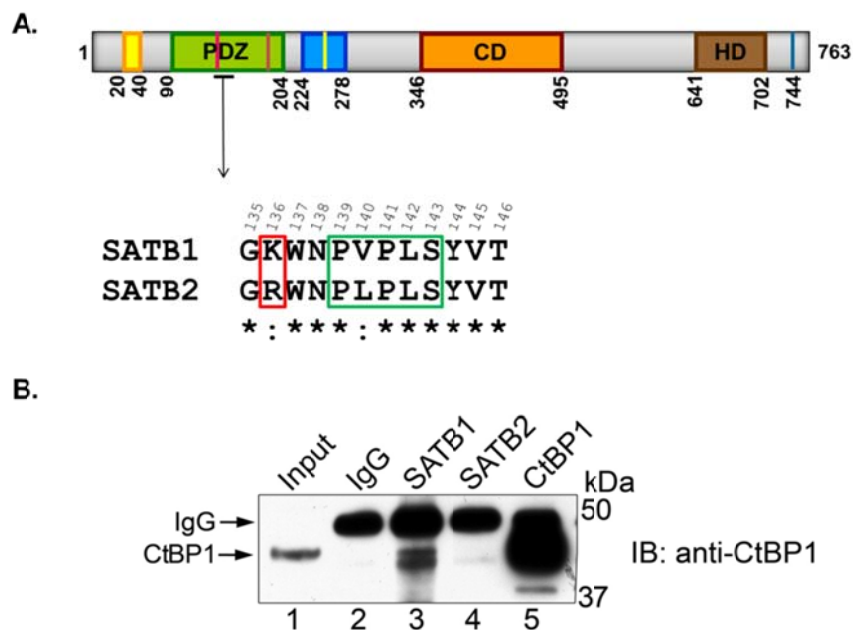
Figure 1.3.4: SATB1 interacts with CtBP1 whereas SATB2 does not interact with CtBP1 *in vivo*. (A) SATB1 acetylation site (K136) and CtBP1 interaction motif (PVPLS) has been enlarged from SATB1 schematic to show the residues present.Alignment of SATB1 and SATB2 protein sequence showed presence of CtBP1 interaction motif (shown in green box). Lysine at position 136 is not present at equivalent position in SATB2 (show in red box). (B) Co-immunoprecipitation was performed in HEK-293T cells to monitor the interaction of SATB1 and SATB2 with CtBP1. Immunoprecipitation was performed under non-reducing conditions using anti-SATB1 (lane 3), anti-SATB2 (lane 4). Normal anti-IgG (lane 2) and anti-CtBP1 were used as negative and positive controls respectively for immunoprecipitation. Immunoblot was performed using anti-CtBP1. Input as indicated (lane 1). Molecular mass standards (kDa) are indicated on right side. IB: immunoblot.

1.3.6 SATB1 forms homodimer *in-vivo*

Previous studies from Galande group had demonstrated that SATB1 forms homodimer via its PDZ-like domain and also interacts with other PDZ-containing proteins (Kumar et al., 2005; Pavan et al., 2006; Purbey et al., 2008 and 2009). However, another report (Tan et al., 2010) claimed that SATB1 fails to undergo *in vivo* dimerization by co-immunoprecipitaion using GFP- and Flag- tagged SATB1. To rule out their claim and validate ours, we used similar constructs for confirming SATB1 homodimerization *in vivo*. We exogenously expressed GFP-SATB1 and Flag- SATB1 in HEK-293T cells and performed co-immunoprecipitation using antibodies against the tag for immunoprecipitation. As expected, immunoprecipitation using anti-Flag antibody pulled down GFP-SATB1 (Fig. 1.3.6, lane 3). Thus, this data very convincingly shows that GFP-SATB1 and Flag-SATB1 interact and therefore once again established that SATB1 forms homodimer *in vivo* (Fig. 1.3.5).
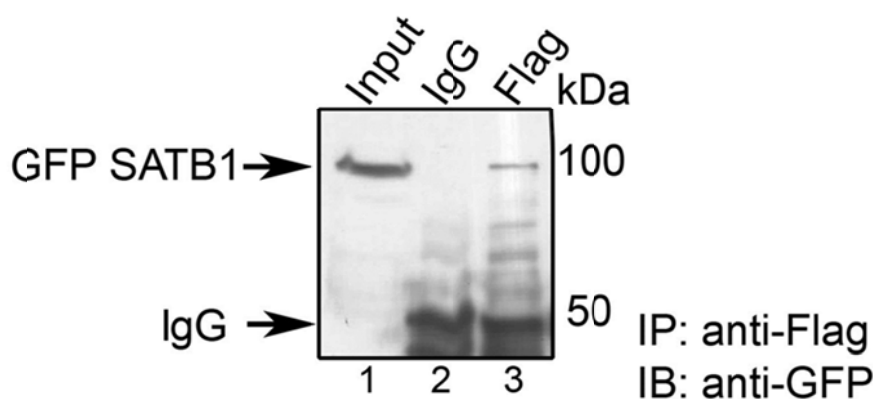
Figure 1.3.5: SATB1 forms homodimer *in vivo*. Co-immunoprecipitation was performed in HEK-293T cells to test the homodimerization of SATB1.GFP-SATB1 and Flag-SATB1 were overexpressed in HEK-293T cells. Whole cell lysate was prepared and immunoprecipitation was performed using anti-IgG and anti-Flag antibodies, as described in "Materials and Methods". The immunoprecipitated proteins were resolved on 7.5 % SDS-Polyacrylamide gel and transferred to PVDF membrane. Immunoblot was performed using anti-GFP. Normal anti-IgG (lane 2) was used as negative control for immunoprecipitation. Molecular mass standards (kDa) are indicated on right side. IP: immunoprecipitation, IB: immunoblot.

1.3.7 SATB1 interacts with SATB2 *in vivo*

SATB1 and SATB2 are PDZ domain containing proteins. SATB1 has been shown to interact with other PDZ containing proteins (Kumar et al., 2005; Pavan et al., 2006; Purbey et al., 2008 and 2009). To test whether SATB1 and SATB2 interact *in vivo* we used CheckMate™

mammalian two-hybrid system or M2H (Promega, Madison, WI, USA). M2H is a very well established technique for detecting protein-protein interaction(s) *in vivo*. Towards this, the PDZ-like domains of SATB1 and SATB2 (now onwards I shall refer to these as PDZ1 and PDZ2 respectively) were cloned into pBIND and pACT vectors to generate fusion proteins with the DNA-binding domain of GAL4 and the activation domain of VP16, respectively. The PDZ1 and PDZ2 cloned into pACT and pBIND vectors were transfected in HEK-293T cells along with the pG5luc reporter vector. The pG5luc vector contains five GAL4 binding sites upstream of a minimal TATA box, which in turn, is upstream of the firefly luciferase gene (luc+). pACT and pBIND vector DNAs were used for normalization of DNA amount in each well. Interaction between the two test proteins, as GAL4 and VP16 fusion constructs, results in an increase in firefly luciferase expression over the negative controls. Values from three independent experiments were normalized with vector control as reference and plotted (Fig. 1.3.6). When we transfected PDZ1 or PDZ2 alone as pACT or pBIND constructs we detected basal level of reporter activity. Upon cotransfection ofpACT-PDZ1 and pBIND-PDZ1 we observed enhanced reporter activity, which was detected in case of PDZ2 as well. Surprisingly, when we transfected pACT-PDZ1 along with pBIND-PDZ2 or vice versa, we observed significant increase in reporter activity as compared to that of PDZ1 or PDZ2 (Fig. 1.3.6, compare lanes 9 and 10 with 7 and 8)indicating that the two interact *in vivo* more strongly as heterodimer as compared to homodimer. Thus, it is likely that these two proteins can exist as heterodimer in cell types that express both of them *in vivo*.
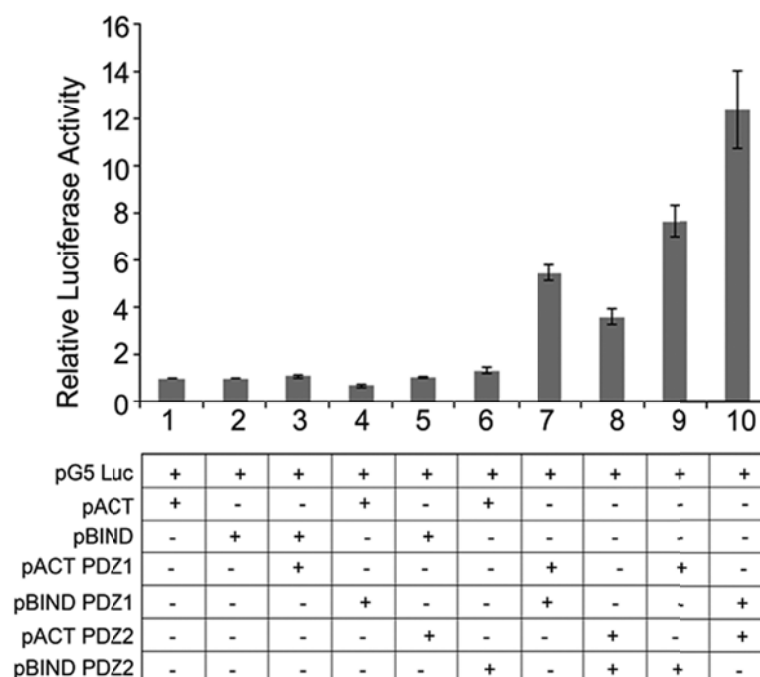
Figure 1.3.6: SATB1 interacts with SATB2 *in vivo***.** SATB1 interacts with SATB2 *via* its N-terminal PDZ containing domain. Mammalian two-hybrid assay was performed as described in 'Materials and Methods' to examine homo-domain or hetero-domain interactions of SATB1 and SATB2. Bars 1 and 2 indicate the relative luciferase activity of control (empty vector) transfections. Bars 3, 5 and bars 4, 6 indicate the relative luciferase activity of pACT PDZ1/PDZ2 and pBIND PDZ1/PDZ2 along with pACT and pBIND vector respectively. Bar 7 and 8 indicate the relative luciferase activity of pACT PDZ1/PDZ2 along with pBIND PDZ1/PDZ2 vector. Bar 9 and 10 indicate the relative luciferase activity of pACT PDZ1 along with pBIND PDZ2 and relative luciferase activity of pACT PDZ2 along with pBIND PDZ1 respectively. Reporter assay was performed in HEK-293T cells using pG5 luc construct along with indicated constructs. The values were normalized with control (blank vector transfection) as reference and plotted. Individual bar values represent average of three independent measurements. Error bars represent standard deviation calculated from triplicates.

1.3.8 SATB1 interacts with SATB2 *in vivo* to regulate transcription synergistically

After confirming that SATB1 and SATB2 interact via their PDZ-like domains, we next set out to find out the significance of this interaction towards transcription regulation *in vivo*. For this, we used pGL3 basic IgH-MAR-luciferase reporter construct and performed luciferase assay using full-length SATB1 and SATB2 constructs. We cotransfected this reporter construct along with increasing concentrations of SATB1, SATB2 or SATB1 and SATB2 together in HEK-293T cells (Fig. 1.3.7). Increasing amounts of SATB1 or SATB2 resulted in increased repression of reporter activity in a dose-dependent manner. However, the extent of repression was more evident when SATB1 and SATB2 were cotransfected together (Fig. 1.3.7, bars 7 and 8) suggesting that the heterodimer may have a great higher regulatory activity as compared to the homodimer.
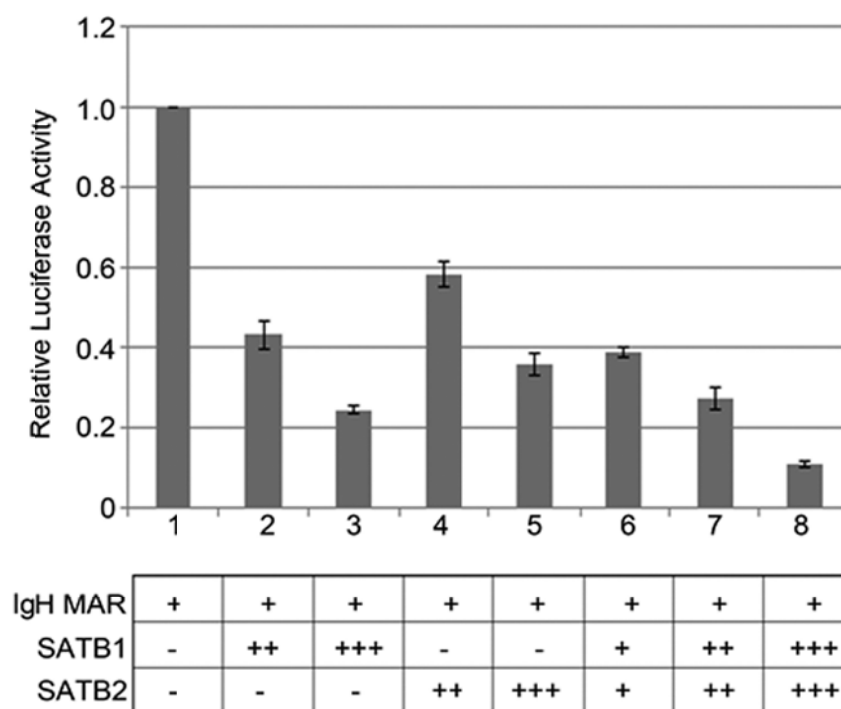
Figure 1.3.7: SATB1 interacts with SATB2 *in vivo* **to regulate transcription synergistically.** Reporter assay was performed in HEK-293T cells using pGL3 basic IgH-MAR-luc reporter construct along with full-length SATB1 and SATB2 alone or in combination in increasing amount as described in "Materials and Methods". pCDNA 3.1 was used for normalization of DNA amount in transfections (bar 1). Bars 2-3 and 3-4 indicate the relative luciferase activity of 1X and 2X amount of SATB1 or SATB2 along with pGL3 basic IgH-MAR-luc reporter respectively. Bars 7 and 8 indicate the relative luciferase activity of 1X and 2X amounts of SATB1 and SATB2 together along with pGL3 basic IgH-MAR-luc reporter respectively. The values were normalized with control (pCDNA 3.1 blank vector transfection) as reference and plotted. Individual values represent average of technical triplicates. Error bars represent standard deviation calculated from triplicates.

1.3.9 SATB1 interacts with SATB2 *in vivo* to from heterodimer

From the reporter assays it was apparent that SATB1 and SATB2 interact with each other and together regulate transcription. To further confirm the formation of heterodimer *in vivo* we performed co-immunoprecipitation of endogenous SATB1 and SATB2 using HEK-293T cells. SATB1 and SATB2 share significant homology at protein level so before proceeding for co-immunoprecipitation we validated the commercial SATB1 and SATB2 antibodies. We used cell lysates from Jurkat, HEK-293T and LN18 cells and probed the blot using 3 different dilutions of SATB1 and SATB2 antibodies. Jurkat is a T cell line which has been shown to express high level of SATB1 and low level of SATB2; HEK-293T is a kidney cell line which express moderate levels of both SATB1 and SATB2; LN-18 belongs to neuronal cell lineage which

express high SATB2 (Dobreva et al., 2003 and our unpublished data). SATB1 and SATB2 specific antibodies identified specific bands of SATB1 and SATB2 which were of correct molecular sizes and matched with previously reported expression profiles of these cell lines (Fig. 1.3.8 A). Next, weperformed pulldown using anti-SATB2 followed byimmunoblotting with anti-SATB1. Specific enrichment of SATB1 signal over IgG revealed the formation of a SATB1-SATB2 heterodimer *in vivo* (Fig. 1.3.8 B).
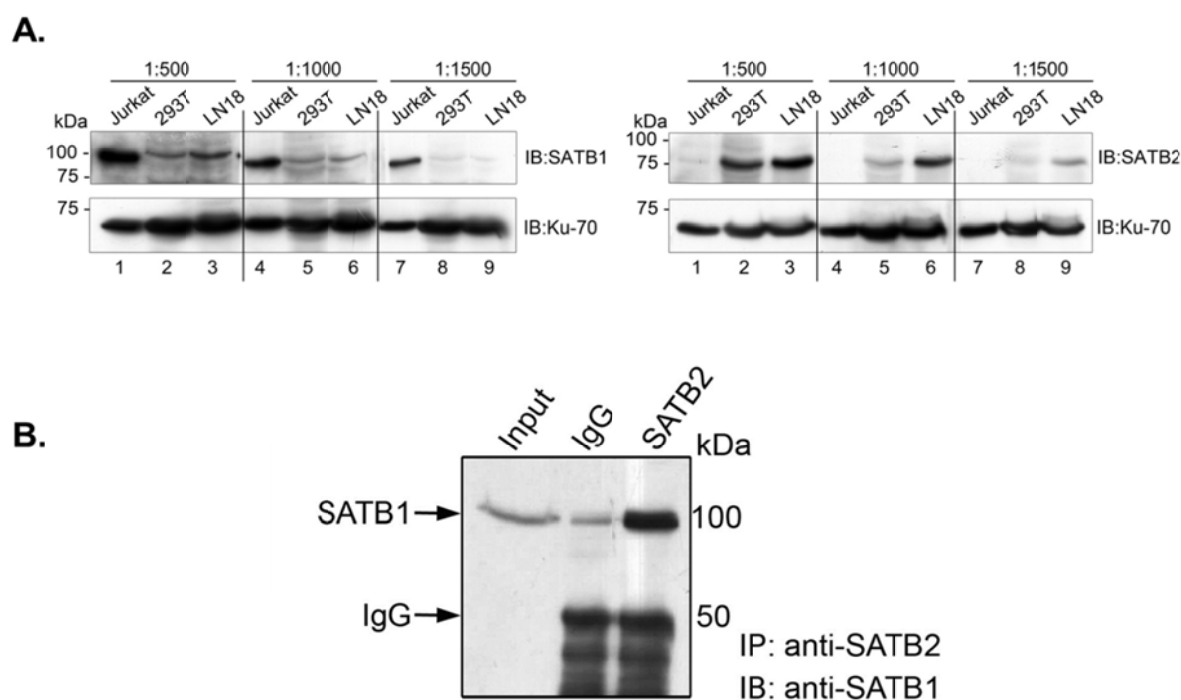
Figure 1.3.8: SATB1 interacts with SATB2 *in vivo* to from a heterodimer. (A) SATB1 and SATB2 antibodies were validated for their specificity and efficiency *via* western blotting using lysates from Jurkat, HEK-293T and LN18 cells. Ku-70 was used as loading control. **(B)** Co-immunoprecipitation was performed in HEK-293T cells to test the possibility whether SATB1 and SATB2 form a heterodimer *in vivo*.Whole cell lysate was prepared and immunoprecipitation was performed with anti-IgG and anti-SATB2 antibodies, as described in "Materials and Methods". The immune complexes were resolved on a 7.5 % SDS-Polyacrylamide gel and transferred to PVDF membrane. Immunoblot was performed using anti-SATB1 antibody that does not cross-react with SATB2. Normal anti-IgG (lane 2) was used as negative controls for immunoprecipitation experiment. Molecular mass standards (kDa) are indicated on right side. IP: immunoprecipitation, IB: immunoblot.

1.3.10 SATB1 and SATB2 reciprocally regulate each other's expression in cancer cell lines

SATB1 and SATB2 have been shown to play important roles in various kinds of tumor progression and metastasis (Han et al., 2008; Agrelo et al., 2009; Patani et al., 2009; Wang et al.,

2009; Meng et al., 2012). It has been shown that during cancer progression SATB2 is downregulated and SATB1 is upregulated. To test if SATB1 and SATB2 regulate each other's expression during cancer progression we performed knockdown of SATB1 in SW480, a colon cancer cell line. SATB1 knockdown resulted in upregulation of SATB2 expression and vice versa (Fig. 1.3.9). These results demonstrated that SATB1and SATB2 reciprocally regulate each other's expression.
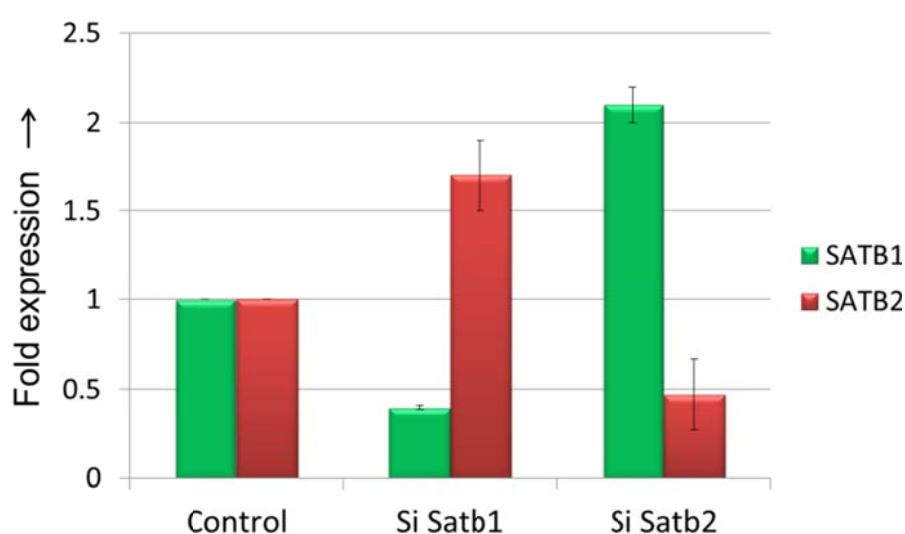
Figure 1.3.9: SATB1 and SATB2 reciprocally regulate their expression in cancer cell lines. SW480 cells were transfected with scrambled RNA (control) or with Si SATB1 or Si SATB2 RNA as described in "Materials and Methods". Forty eight hours post transfection, cells were harvested and RNA was extracted followed by cDNA synthesis. Real time RT-PCR was performed with cDNA synthesized from these samples. β-actin was used as internal control to normalize the values. Error bars indicate standard deviation calculated from triplicates. Fold change as compared to control is depicted on Y axis.

1.3.11 SATB1 and SATB2 recruit each other to regulate their expression reciprocally *in vivo*

Since SATB1 and SATB2 seem to regulate each other's expression we askedwhether these proteins bind to each other's promoter *in vivo*. To test this we scanned 2 kb upstream sequences of *Satb1* and *Satb2* genes for theconsensus SATB binding regionusing Genomatix software. We could indeed find two SATB binding sequences (SBS) on each of these promoters, which we called as proximal SBS and distal SBS starting from TSS (Fig. 1.3.10A). To test if

SATB1 and SATB2 bind to these SBS regions *in vivo,* we performed chromatin immunoprecipitation (ChIP) analysis.Cells were crosslinked using. The cells were then lysed and chromatinwassheared by sonication to generate chromatin enriched in 300 to 500bp fragments. These small chromatin fragments were pulled down using SATB1 and SATB2 specific antibodies. The pulldown chromatin fraction was purified and used as a template for PCR using specific primers against distal and proximal SBSs for promoters of *Satb1* and *Satb2*. PCR using specific primers against distal and proximal SBS in promoters of *Satb1* and *Satb2*revealed enrichment of immunoprecipitated DNAs in pulldown samples for both SATB1 and SATB2 over IgG, which served as negative control (Fig 1.3.10 B). By ChIP we could show that SATB1 and SATB2 both bind to their respective promoters as well as each other's promoter. However this experiment could not answer whether SATB1 and SATB2 bind to the same or different chromatin fragment. To address this question we performed ChIP experiment twice on the same chromatin one after the other, referred to as sequential ChIP. Sequential ChIP can distinguish whether two proteins are occupying the same or differentDNA fragment. Briefly, after doing the pulldown with the first antibody, eluted chromatin is subjected to a second round of ChIP (before decrosslinking) using the second antibody. Results were analyzed in the same manner as ChIP. Detection of signal in the second round of ChIP indicates that the two proteins remain bound to same DNA fragment *in vivo*. We performed sequential ChIP in HEK-293T cells to check if SATB1 and SATB2 targeted the same DNA region or different regions (Fig. 1.3.10 C). Sequential ChIP analysis revealed that SATB1 distal SBS is occupied primarily by SATB1 and SATB2 seems to get recruited to this site by SATB1. However on SATB1 and SATB2 proximal SBS sites, both SATB1 and SATB2 seem to co-occupy (Fig. 1.3.10 C; lane 6 and 7).
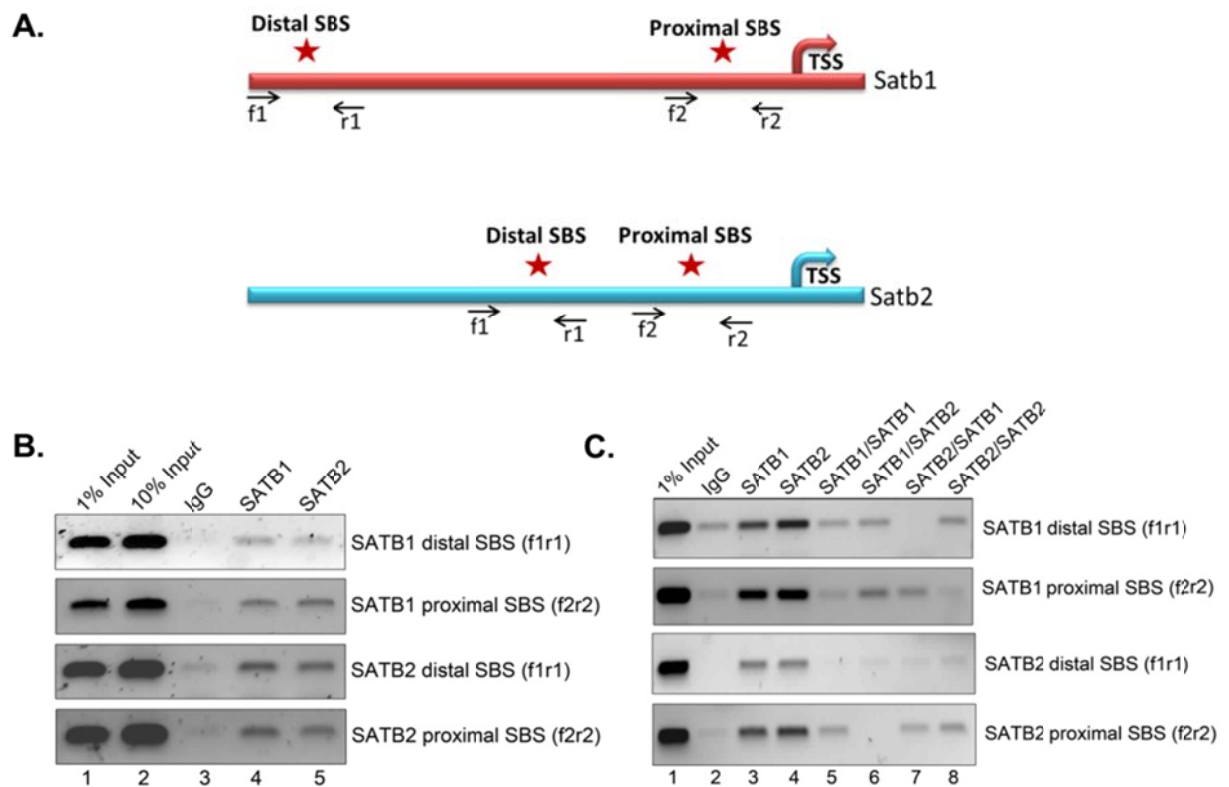
Figure 1.3.10: SATB1 and SATB2 recruit each other to regulate each other's expression *in vivo*. (A) Genomatix analysis of upstream region of SATB1 and SATB2 promoter. 2kb upstream sequence of SATB1 and SATB2 gene were analyzed for SATB binding sequence (SBS) using Genomatix software, which predicted presence of two strong SATB binding sequences. We named it as proximal and distal SBSs from TSS (shown as asterisks). We designed primers against these genomic regions for ChIP (primers and their positions are indicated as arrows). **(B)** ChIP was performed in HEK-293T cells to validate the binding of SATB1 and SATB2 on the software predicted SATB binding sequences (SBSs) using specific SATB1 and SATB2 antibodies as described in "Materials and Methods". IgG was used as a negative control. ChIP-PCR was performed using specific genomic primers designed for amplifying SBSs from promoters of *Satb1* and *Satb2*. Labels on the top of panels indicate antibodies used for ChIP. Lanes 1 and 2 indicate 1% and 10% inputs respectively. **(C)** Sequential ChIP depicting the co-occupancy of SATB1 and SATB2 on promoters of *Satb1* and *Satb2*. Sequential ChIP was performed using specific SATB1 and SATB2 antibodies as described in "Materials and Methods".Lane 1 indicates 1% input. In lanes 5, 6, 7 and 8 on top, first label from figure indicates the first antibody for ChIP whereas the second label indicates the second antibody used for SeqChIP. IgG was used as negative control.

1.3.12 SATB1 and SATB2 bind to their DNA target as SATB1:SATB2 heterodimeric complex

We have demonstrated in section 1.3.9 that SATB1 and SATB2 interact to form a heterodimer. From the knockdown and ChIP experiments, we observed that SATB1 and SATB2 regulate each other's expression by directly binding to respective promoter *in vivo*. Sequential ChIP revealed that SATB1 and SATB2 simultaneously bind to the various SBS sequences

(section 1.3.11); we next asked if the two proteins bind their target as a heterodimer. Towards this we performed ChIP-western analysis wherein we can detect whether the proteins of interest bind to same DNA sequence as a complex and not on different sites of the same DNA fragment. Here, the ChIP is performed essentially similar to normal ChIP using the antibody against first protein and chromatin-DNA complex is eluted from the beads, which is treated with DNaseI to remove any remaining DNA. The treated chromatin is then run on gel and immunoblot is performed using the antibody against the second protein. Thus, effectively only if the first protein which is bound to DNA and presumably interacts directly with second protein can be detected upon Western blotting whereas proteins which bind to same DNA sequence but don't interact directly fail to generate signal in Western blot analysis. To test whether SATB1 and SATB2 bind chromatin as a heterodimer we performed ChIP separately using SATB1 and SATB2 antibodies and resolved the treated fraction on gel and probed with anti-SATB2 antibody (Fig. 1.3.11). A specific band corresponding to SATB2 could be detected in anti-SATB1 pulldown fraction (Fig. 1.3.11, lane 2), which indicated that SATB1 and SATB2 occupy the target sequence as a heterodimeric complex. Anti-SATB2 pulldown fraction served as a positive control (Fig. 1.3.11, lane 3).
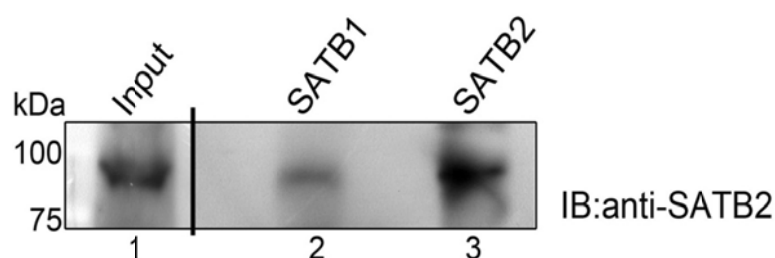
Figure 1.3.11: SATB1 and SATB2 bind to the target DNA as a heterodimeric complex. ChIP-Western was performed using specific SATB1 and SATB2 antibodies as described in "Materials and Methods".Lane 1 indicates 10% input. Labels on the top of panels indicate antibodies used for ChIP. Western was performed using anti-SATB2. Lane 3 shows positive control wherein ChIP was performed using SATB2 antibody followed by Western blotting with anti-SATB2. IB: immunoblot.

1.4 Discussion

SATB1 and SATB2 belong to Special AT-rich Sequence Binding (SATB) family of MAR-binding proteins involved in regulation of higher-order chromatin organization and gene expression. At the protein level, SATB1 and SATB2 share 61% sequence homology. Both share

three highly conserved functional domains namely N-terminal PDZ-like domain, central CUT repeat containing domain (CD) and C-terminal homeodomain (HD). N-terminal PDZ-like domain is required for dimerization of the protein and is indispensable for DNA binding activity. C-terminal half contains homeodomain (HD) which recognizes DNA specifically, whereas middle CUT domain (CD) enhances the binding affinity of HD. Both the proteins are known to interact with various co-activators and co-repressors. The PDZ-like domain of SATB1 has been shown to interact with histone modifiers HDAC1, PCAF, CBP, p300 (Kumar et al., 2005; Pavan et al., 2006), PML (Kumar et al., 2007), CtBP1 (Purbey et al., 2009) and β-catenin (Notani et al., 2010). SATB2 also has been shown to interact with various chromatin modifiers like HDAC1 and MTA2 (Gyorgy et al., 2008). SATB1 and SATB2 both are involved in regulation of chromatin architecture and gene expression in cell type specific manner. SATB1 is shown to be involved in regulation of T-cell development and differentiation (Alvarez et al., 2000; Kumar et al., 2006; Notani et al., 2010) whereas role of SATB2 has been discussed more specifically in regulation of the neuronal cell lineage (Britanova et al., 2006; Alcamo et al., 2008) and osteoblast differentiation (Dobreva et al., 2006; Hassan et al., 2010). Though SATB1 is also present in fetal brain as well as in some parts of adult brain, its role in the CNS has not been well studied. Over the past 4 years, many reports have been added to literature discussing the roles of SATB family proteins in progression of various kinds of tumors and metastasis (Han et al., 2008; Agrelo et al., 2009; Patani et al., 2009; Wang et al., 2009; Endo et al., 2012; McInnes et al., 2012; Mir et al., 2012; Chu et al., 2013; Shukla et al., 2013; Zhang et al., 2013).

There are no reports of any comparative study between SATB1 and SATB2 and they have not been studied together with respect to their roles in regulating gene expression. Here we attempt to study SATB family proteins in a comparative manner. Towards this, we first tested if they have similar MAR-binding potential *in vitro* using EMSA. We could show that equivalent amount of SATB2 binds with higher affinity to particular MAR sequence as compared to SATB1. Both SATB1 and SATB2 repressed MAR-linked luciferase gene expression *in vivo* to a similar extent. We have shown earlier the effect of various activators and co-repressor on the regulatory activity of SATB1 (Kumar et al., 2005; Pavan et al., 2006) however it was not known how these factors affect SATB2 mediated gene regulation. To test this, we cotransfected PCAF, p300, Sumo1 and HDAC1 along with SATB1 or SATB2. *In vivo* reporter assays indicated that all these activators and co-repressors interact in the same manner with both SATB2 and SATB1. SATB1 has been shown to regulate target gene expression as a part of multi-protein complex.

Purbey et al. showed that SATB1 interacts with a co-repressor CtBP1 *via* its PDZ-like domain in acetylation-dependent manner and represses target genes (Purbey et al., 2009). Sequence homology search revealed that SATB2 also harbors CtBP1 interaction motif in its PDZ-like domain at equivalent position. Therefore, we tried to detect the SATB2 and CtBP1 interaction *in vivo*; however we could not detect any such interaction.

SATB1 and SATB2 both harbor PDZ-like domain which is very important for interaction with other PDZ containing proteins. The dimerization property of SATB1 is primarily attributed to its PDZ-like domain (Galande et al., 2001; Purbey et al., 2008). However Tan et al. in 2010 claimed that SATB1 does not dimerize *in vivo*. Using *in vivo* coimmunoprecipitation of FLAG-SATB1 (FL) with EGFP-SATB1 (FL), and vice versa, they failed to detect *in vivo* SATB1 homodimerization. To establish homodimerization of SATB1 unequivocally, we performed the same experiment using identically tagged full-length SATB1 constructs. Our data argues very convincingly that SATB1 homodimerizes *in vivo*. As it is known that one PDZ containing protein interacts with other PDZ containing proteins, we asked whether SATB1 and SATB2 interact *in vivo* to form heterodimer. Using various techniques we demonstrated that SATB1 and SATB2 interact with each other forming heterodimer*in vivo*.

Roles of SATB family proteins have been discussed in progression of various types of cancer and their metastasis (Mir et al., 2012). SATB1 has been shown to express in metastatic cell lines whereas expression of SATB2 was reported in both malignant and non-malignant cells (Han et al., 2008). It was also shown that SATB1 expression increases while SATB2 expression decreases with tumor progression (Wang et al., 2009). Using colorectal cancer cell line SW480 we show that SATB1 and and SATB2 antagonize each other's expression *in vivo*. Some cells like HEK-293T express comparable level of SATB1 and SATB2 unlike cancer cell lines where SATB1/2 expression varies according to cancer progression. We propose transcriptional regulation by SATB1 and SATB2 varies in these two cell types. SATB1 and SATB2 synergistically regulate transcription of their target genes in the cells where they are coexpressed unlike in cancer cells where they are not expressed at the same time. Chromatin immunoprecipitation analysisrevealed that SATB1 and SATB2 bind and recruit each other to respective promoters *in vivo* and in turn regulate each other's expression. This reciprocal regulation of SATB1 and SATB2 can in turn affect their relative levels inside cells which might play important roles in regulating various pathways like cancer progression and cell differentiation. We also show that SATB1 and SATB2 bind to target genomic sequences as

homodimer as well as heterodimers. There are multiple targets which are regulated differentially by SATB1 and SATB2; however exhibit binding of SATB1 and SATB2 at the same time (Savarese et al., 2003; Asanoma et al., 2011). We showed that SATB1 and SATB2 interact and form homodimer as well as heterodimer (this work and Galande et al., 2001; Purbey et al., 2008). Interestingly, SATB1 and SATB2 differ with respect to their post-translational modifications and also with respect to their interaction partners including CtBP1. Thus, the composition of SATB dimers at various stages of cell development and differentiation may affect gene expression. We propose that SATB1 or SATB2 homodimers and SATB1/2 heterodimers mightplay differential roles in gene regulation. This is indeed an exciting possibility that requires further investigation and would provide important insights into coordinated gene expression during differentiation of embryonic stem cells.

Based on our results we propose that SATB family of proteins regulate the gene expressionby interacting with various kinds of activators and co-repressors. Nonetheless, they differ with respect to few interacting partners such as CtBP1, which might explain their differential roles in various biological processes. In addition to interaction with other proteins, they interact with each other as well to form homo- as well as heterodimers and regulate each other's expression *in vivo*. Reciprocal regulation of SATB1 and SATB2 is another exciting possibility pertaining to the biological activities of SATB family proteins. The possibility of dimerization and reciprocal regulation among SATB family proteins are two independent mechanisms by which different set of genes can be coordinately regulated in various cellular systems. Therefore, we evaluated the expression of SATB family proteins in undifferentiated pluripotent stem cells and during various time points upon differentiation. The results of these studies are presented in subsequent chapters.

1.5. References

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Chapter II

To study the roles of SATB1 and SATB2 in cellular context

2.1 Introduction

As discussed in the first chapter, various matrix binding proteins (MBPs) including SATB1 and SATB2 bind to these AT-rich MAR sequences and regulate higher-order chromatinorganizationin the form of topologically independent loop domains (Galande, 2002). Since binding of these MBPs depends primarily on such DNA sequences, the total number of their potential genomic sites is larger as compared to classical transcription factors which target specific genes and therefore are postulated to play significant role in global gene regulation. Cell type-specific gene expression is regulated by a number of different chromatin remodelers and transcription factors which bind to various DNA elements such as promoters and enhancers, resulting in subsequent activation or repression of gene expression. SATB1 and SATB2 have been shown to play important roles in development and differentiation of various cell types by regulating cell type-specific gene expression. For example, SATB1 has been shown to play important roles in development of various T cell subpopulations and cancer progression whereas role of SATB2 has been studied in regulation of differentiation of bone cells and neurons.Various aspects of regulation of cell differentiation by SATB family proteins will be discussed in detail in the following section.

2.1.1 Role of SATB family proteins in development and differentiation of T and B cells

SATB1 was initially cloned from a testis cDNA library (Dickinson et al., 1992), however its role was studied mainly as a T cell enriched global chromatin organizer presumably because of its abundant expression in thymus.Subsequently, SATB1 was shown to play important roles in

T cell development and differentiation by virtue of its ability to act as a global gene regulator (Alvarez et al., 2000; Yasui et al., 2002; Cai et al., 2003; Notani et al., 2010; Beyer et al., 2011; Burute et al., 2012). SATB1 knockout mice exhibit multiple defects in T cell development including reduced proliferation and arrest at double positive stage indicating important roles of SATB1 in T cell development (Alvarez et al., 2000). SATB1 is shown to play an important role in differentiation of Th2 cells by orchestrating expression of the Th2 cytokine locus including *IL-4*, *IL-5* and *IL-13* along with *GATA3* (Cai et al., 2006; Ahlfors et al., 2010; Notani et al., 2010). SATB1 in association with the PML oncoprotein organizes the MHC class I locus into distinct loop domains and facilitates the transcription of multiple genes in response to IFNγ treatment in T cells (Kumar et al., 2007). SATB1 also manifests its role in Th2 cell differentiation by regulating multiple genes in a Wnt dependent manner. SATB1 interacts and recruits CtBP1 and β-catenin at promoters of its target genes (Purbey et al., 2009; Notani et al., 2010). Increased expression of SATB1 is critical for Th2 cell differentiation (Ahlfors et al., 2010). However, SATB1 is downregulated during regulatory T cell (Treg) differentiation. Treg-specific transcription factor Foxp3 binds to the 3' UTR region of SATB1 and inhibits its expression specifically in Treg cells (Beyer et al., 2011). The other member of SATB family, SATB2 was discovered in 2003 by Dobreva et al. in pre-B cells as a MAR-binding protein where it was shown to bind intergenic MARs of the immunoglobulin μ heavy chain and activate transcription in sumoylation-dependent manner (Dobreva et al., 2003). After this not many reports were published depicting role of SATB2 in differentiation or development of any immune system related cell type until a recent report wherein Zhou et al. showed that SATB2 plays important role in upregulation of human γ globin gene expression from the β globin gene cluster during embryonic development. SATB2 coordinates induction of fetal γ globin expression by direct binding and recruitment of PCAF to the upstream MAR sequences on γ globin gene promoters during differentiation of human erythroleukemia K562 cells and human umbilical CD34$^+$ cells (Zhou et al., 2012). SATB2 level drops significantly in TER119$^+$ erythroid cells of 8.5, 9.0 dpc murine yolk sacs and is positively correlated with the decreasing expression of fetal γ globin gene expression; however SATB2 activates γ globin gene without activation of erythroid differentiation (Zhou et al., 2012). Interestingly, SATB1 also has been shown to express during early human adult erythroid progenitor cell differentiation where it binds and regulates the β globin gene cluster (Wen et al., 2005; Wang et al., 2009a; Gong et al., 2009). SATB1 induces expression of ε globin gene from the β globin gene cluster by binding and then recruiting CBP to

the hypersensitive site 2 (HS2) in the locus control region (LCR) on ε globin gene promoter (Wen et al., 2005). Erythroid differentiation of K562 cells by hemin simultaneously increases SATB2 and γ globin gene expression; and downregulates SATB1 and ε globin (Wen et al., 2005; Zhou et al., 2012). In conclusion, these studies indicate that SATB family proteins play important roles in differentiation of various T cell subtypes as well as B cell by regulating a number of important lineage specific genes *via* chromatin reorganization.

2.1.2 Role of SATB family proteins in differentiation of osteocytes and bone development

Role of SATB family proteins, specifically of SATB2 has been studied in detail in osteoblast differentiation during bone development. SATB2 expression is first detected in mouse in the rhombomere region at E8.5 followed by its expression in first branchial arch at E9.0 and then in medial parts of remaining four branchial arches that form future facial structures of the adult (Dobreva et al., 2006). As expected from its expression patterns, SATB2 knockout embryos exhibited several craniofacial defects in skeletal elements including cleft palate and osteoblast differentiation (Dobreva et al., 2006). SATB2 has been shown to regulate bone development by inhibiting multiple genes such as*Lhx7* and several Hox genes including *Hoxa2* and *Hoxb2*. *Hoxa2* plays an important role in patterning and antagonizing bone formation. *Hoxa2* is upregulated in neural crest cells via activation of an upstream enhancer element, whereas binding of SATB2 to a different enhancer element downstream of the gene suppresses *Hoxa2* expression.SATB2 also binds directly to three osteoblast-specific elements on bone sialoprotein (Bsp) promoter and upregulates Bsp expression during differentiation of mesenchymal progenitor stem cells into bone forming osteoblast cells. During this process SATB2 interacts with Runx2 and ATF4 and stimulates Osteocalcin (Ocn) gene expression (Dobreva et al., 2006). Subsequently, SATB2 was shown to stimulate differentiation of adult stem cells, induced pluripotent stem cells (iPSCs) and various osteoblast like cells into bone forming osteoblast lineage cells by regulating the expression of a group of osteogenic transcription factors such as Runx2, ATF4, BSP, OCN and SOX2 (Zhang et al., 2011; Ye et al., 2011; Kim et al., 2012). Overexpression of SATB2 in dental follicle cells (DFCs) and bone marrow stromal cells (BMSCs) resulted in upregulation of various bone matrix proteins, osteogenic transcription factors and also VEGF,a major angiogenic factor (Zhang et al., 2011).

Enhanced expression of these osteogenic and angiogenic factors resulted in improved angiogenesis and healing during bone regeneration which indicate the role of SATB2 in promoting osteogenic differentiation and bone tissue regeneration from adult stem cells (Zhang et al., 2011). Post-transcriptional regulation of SATB2 by various microRNAs (miRNAs) such as miR-34b/c and miR cluster 23a~27a~24-2 has been demonstrated to play important role in the bone development and terminal maturation (Hassan et al., 2010; Wei et al., 2012a) (Fig. 2.1.1). Oxidative stress is known to exert negative effects on osteoblast differentiation and skeletal development. Recently, Wei et al. showed that oxidative stress in human osteoblast results in induction of SATB2 expression, which protects them from apoptotic injury (Wei et al., 2012b). SATB2 has been shown to be of evolutionary significance for the development vertebrate jaws; and differential regulation of SATB2 has important consequences on the development and evolution of various jaw modules in gnathostomes (Depew and Compagnucci, 2008; Fish et al., 2011). The role of SATB2 in jaw development can be attributed towards its specific and major role in development and differentiation of osteocytes.
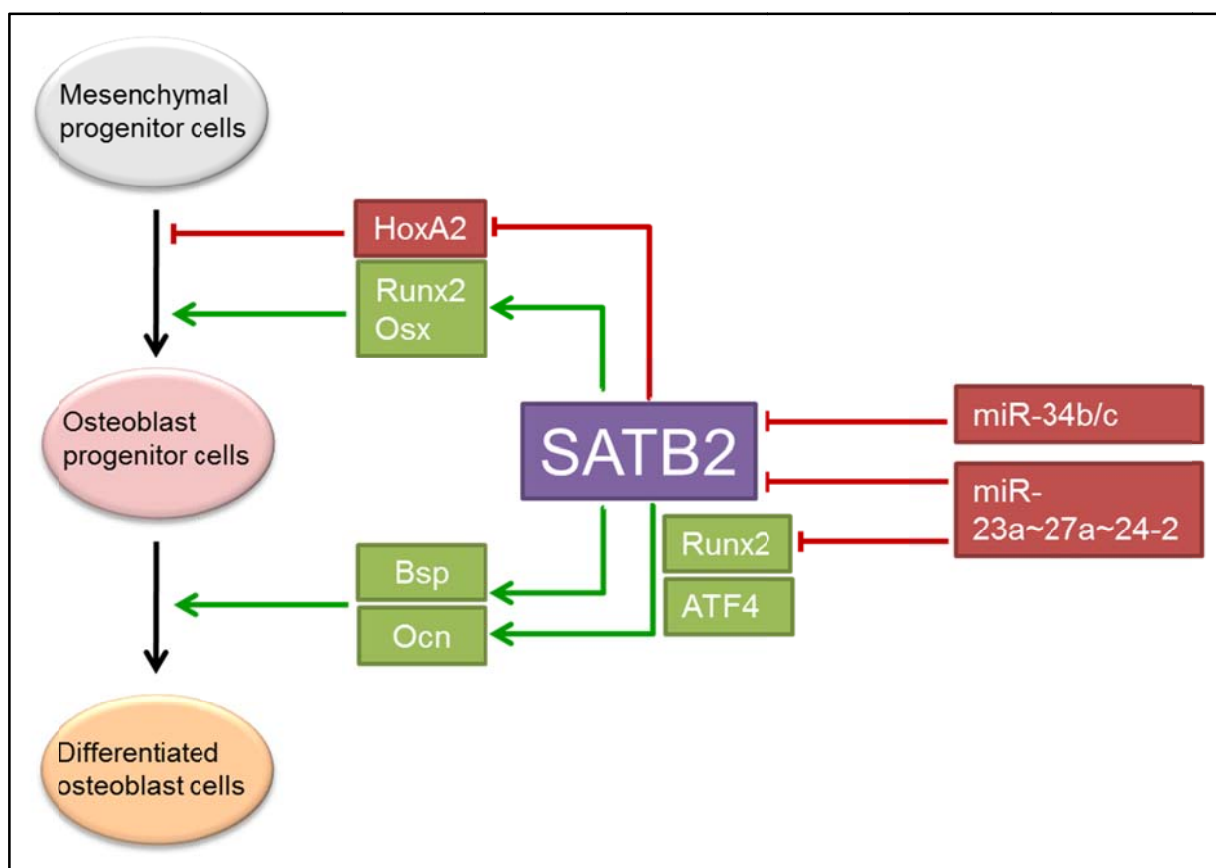
Figure 2.1.1: Role of SATB2 in regulation of development and differentiation of osteoblasts. HoxA2 is expressed by mesenchymal progenitor stem cells and inhibits differentiation into osteoblast progenitor cells. SATB2 promotes differentiation of these mesenchymal progenitor cells into osteoblast progenitor cells by repressing HoxA2 and stimulating Runx2 and Osx expression. SATB2 promotes terminal osteogenic differentiation and maturation through activation of Bsp and Ocn (the latter through in cooperation with Runx2 and ATF4). Level of SATB2 is tightly regulated by various microRNAs during the process of osteogenesis. (Based on Dobreva et al., 2006; Hassan et al., 2010; Zhang et al., 2011; Wei et al., 2012a)

2.1.3 Role of SATB family proteins in differentiation of neurons and brain development

In the mouse brain, expression of SATB1 and SATB2 can be detected as early as embryonic day 13.5 (E13.5) and E11.5 respectively (FitzPatrick et al., 2003; Britanova et al., 2005). SATB family proteins have been demonstrated to play important roles in neuronal differentiation during brain development especially in the development of cerebral cortex and coordinate the expression of multiple genes by binding to AT-rich sequences (Britanova et al., 2005; Szemes et al., 2006; Alcamo et al., 2008; Britanova et al., 2008; Gyorgy et al., 2008; Balamotis et al., 2012; Baranek et al., 2012). SATB1 and SATB2 are expressed in a non-

overlapping fashion in developing brain and may act as global regulators of cell differentiation in specific cell lineages within the developing CNS (Britanova et al., 2005; Baranek et al., 2012). In the developing brain SATB2 expression was at maximum level in differentiating neurons and decreased upon differentiation in mature neurons (Britanova et al., 2005). Unlike SATB2, SATB1 expression was mostly observed in postnatal brain and was found to be expressed exclusively in a subset of mature neurons in mouse brain but not in astrocytes and glial cells (Huang et al., 2011; Balamotis et al., 2012). Though SATB family proteins are expressed in brain during E11.5 to E 13.5, SATB2 null mice do not survive past postnatal day 0 (P0). SATB1 null mice survive until 2.5 to 3 postnatal weeks, indicating an essential role of SATB2 during embryonic development. Postnatal increase in SATB1 expression in cerebral cortex plays a major role in regulation of multiple immediate early genes (IEGs). IEGs are important class of genes, which include regulatory transcription factors (RTFs) (e.g., Fos, Jun, and Egr) which regulate many downstream genes as well as many effector IEGs (e.g., Arc and Bdnf) which directly modulate neural morphogenesis and plasticity (Andreasson and Kaufmann, 2002; Tzingounis and Nicoll, 2006; Miyashita et al., 2008). SATB1 binds to genomic loci of various IEGs and regulates proper timing and temporal expression pattern of these genes (Balamotis et al., 2012). In addition, SATB1 also regulates other neuronal genessuch as genes involved in synaptogenesis (Sst, Areg, and Thbs1) and stress, fear/anxiety response or hyperactive behavior (Th, Crh, Atf3, Penk1, and Tacr1), and the Ca^{2+} binding and damage/inflammation response (Calb1, Calb2, Calr, NoxA, S100a8, S100a9, Tnf, and Il6) (Balamotis et al., 2012). SATB1 expression has a major impact on synapse formation in the cerebral cortex and amygdala during postnatal brain development, loss of SATB1 results in reduced cortical dendritic spine density in neurons (Balamotis et al., 2012). Despite regulating important genes involved in synapse formations and various other functions in cerebral cortex, SATB1 null mice do not show any morphological abnormality in the cerebral cortex (Fig. 2.1.2).
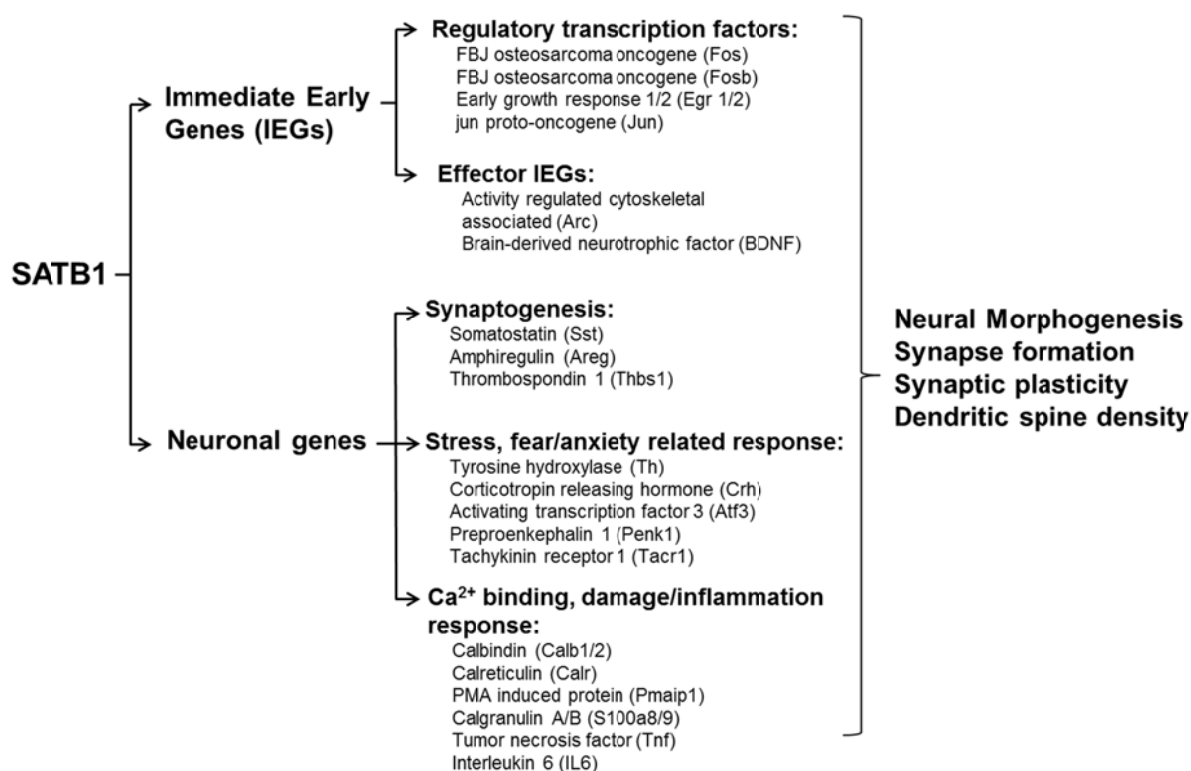
Figure 2.1.2: Role of SATB1 in development and differentiation of neurons. SATB1 is expressed at high level in post-natal brain and plays important roles in regulating important class of genes including immediate early genes and various other neuronal genes. Collectively, it plays important roles in regulation of synapse formations and plasticity by regulating dendritic spine density.(Based on Balamotis et al., 2012).

In contrast, SATB2 has been shown to play an important role in maintaining normal cerebral cortical layer morphology by regulating the differentiation and migration of upper layer neurons in neocortex (Alcamo et al., 2008; Britanova et al., 2008). Neocortex is a six-layered structure of molecularly and functionally distinct projection neurons, which is formed by differentiation of proliferating neural progenitor stem cells of germinal centers of the dorsal telencephalon. Neurons of upper layers (UL; layers 2 and 3) form corticocortical connections (include projections to the contralateral hemisphere across the corpus callosum); neurons in deep layers (DL; layers 5 and 6) form subcortical projections (to the spinal cord, pons, midbrain, and thalamus) and neurons in layers 1 and 4 extend axons locally within the cortex (Butt et al., 2005; Wonders and Anderson, 2006). Majority of the UL neurons form projections towards corpus callosum and are characterized by expression of SATB2, Svet1, Cux1/2 and Ski.The DL neurons form subcortical projections, express Fezf2, Sox5 and Ctip2 and are devoid of SATB2, though in

some layers a few neurons exclusively express one of them or sometimes more than two together (Alcamo et al., 2008; Britanova et al., 2008; Chen et al., 2008; Baranek et al., 2012). SATB2 expression is very critical for determining identity of UL callosal projection neurons to form corpus callosum and in absence of SATB2 these neurons upregulate Ctip2 expression and failed to migrate to the superficial cortical plate (CP) and instead project their axons towards subcortical parts of cortex like DL neurons. SATB2 controls the differentiation of UL neurons to form corpus callosum by activating UL specific genes (e.g. Sip1 and Tbr1) and downregulating DL associated genes (e.g. Ctip2 and Nurr1). Ectopic expression of SATB2 in DL neurons results in downregulation of Ctip2 and impairment of formation corticospinal tract (Alcamo et al., 2008; Britanova et al., 2008). SATB2 expression is critical for differentiating UL neurons to form corpus callosum and is enhanced specifically in axons of projection neurons forming callosal callosum by a specific enhancer known as AS021 SINE locus (Tashiro et al., 2011). SATB2 mediated specification of UL neurons is mainly achieved by virtue of its direct binding to MAR regulatory region of Ctip2 locus where it recruits HDAC1 and MTA2, components of the NuRD complex,causing efficient repression of Ctip2 expression in UL neurons (Alcamo et al., 2008; Britanova et al., 2008; Gyorgy et al., 2008). However, recently a proto-oncogeneSki was shown to play an important role in SATB2 mediated knockdown of Ctip2 (Baranek et al., 2012). It was shown that in UL neurons SATB2 and Ski are coexpressed and Ski facilitates recruitment of HDAC1 to *Ctip2* locus and thereby formation of an active NuRD complex consisting of HDAC1 and MTA2 along with SATB2 resulting in suppression of *Ctip2*. In contrast, DL neurons do not coexpress Ski and SATB2 and therefore an active NuRD complex is not formed at *Ctip2* locus (Baranek et al., 2012) (Fig. 2.1.3). Thus, SATB2 regulates the differentiation of UL neurons to form callosal projections.
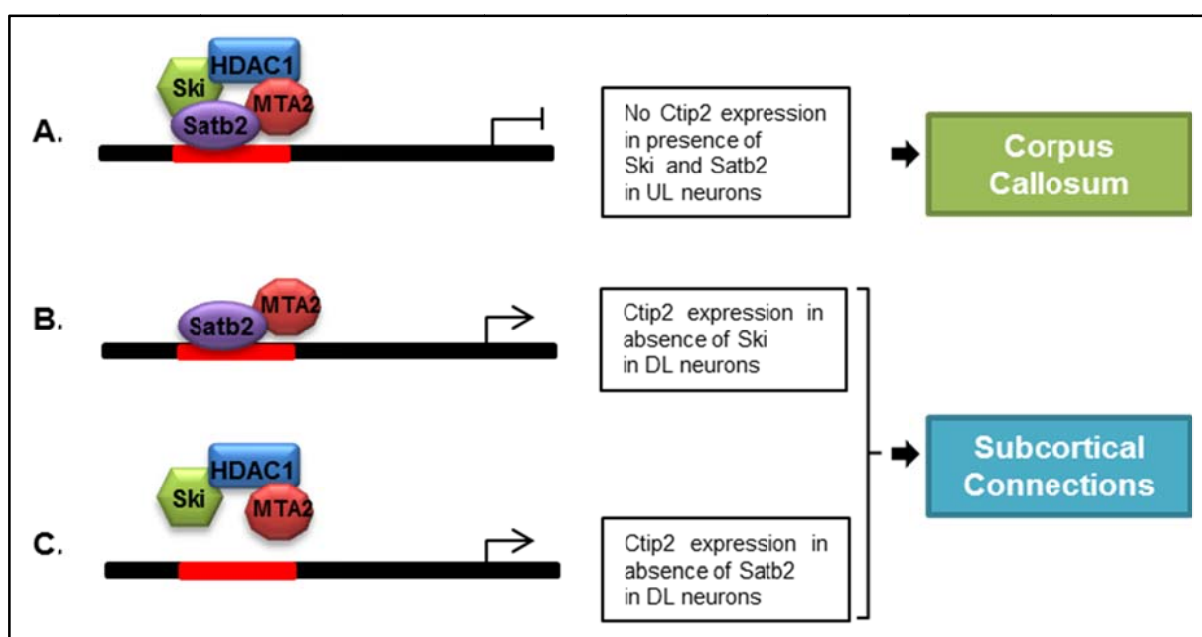
Figure 2.1.3: Role of SATB2 in development and differentiation of upper layer neurons. SATB2 coexpresses along with Ski at high level in UL neurons of neocortex where it binds to MARs at *Ctip2* promoter and recruits Ski and MTA2. Ski further recruits HDAC1 and facilitates the formation of active NuRD complex on *Ctip2* promoter and efficiently represses Ctip2 expression in UL neurons in absence of which they differentiate and form corpus callosum (A). In DL neurons SATB2 and Ski do not coexpress, due to which active NuRD complex is not formed on *Ctip2* promoter and it is expressed and induces formation of subcortical connections (B and C). (Based on Alcamo et al., 2008; Britanova et al., 2008; Baranek et al., 2012; Gyorgy et al., 2008)

On the basis of above introduction it is evident that SATB family proteins play important regulatory roles in differentiation and development of a wide variety of cells and tissues. Majority of their roles have been studied in great detail in T cells, neurons and osteocytes; and there are few recent reports which documented roles of SATB family proteins in stem cell differentiation (Savarese et al., 2009; Agrelo et al., 2009; Asanoma et al., 2012; Nechanitzky et al., 2012). We were interested in studying the roles of SATB family proteins towardsdirecting the differentiation of various cell lines especially into neurons. We screened a number of cell lines from various lineages and found that SATB1 and SATB2 are expressed at comparable levels in various neuronal cell lines. We set out to dissect the roles of SATB family proteins in regulation of pluripotency and differentiation of pluripotent human embryonic stem cells (hESCs). However, as it is very challenging to work with hESCs, we started our experiments using a relatively simpler and easier stem cell model called NT2D1. NT2D1 is an embryonic testicular teratocarcinoma cell line and has been well established as a model cell line to study

hESCs (Andrews, 1984; Lee and Andrews, 1986). We used NT2D1 to study the roles of SATB family proteinstowards regulation of pluripotency and differentiation. We found that SATB family proteins regulate various stem cells as well as pluripotency associated genes. We also showed that Wnt signaling effectors areactivated during process of differentiation. At the molecular level, we showed that SATB family proteins play important roles in regulating Wnt responsive genes during this process. We then extended this study using hESCs which will be discussed in detail in chapter III.

2.2 Materials and Methods

2.2.1 Antibodies and reagents

Anti-SATB1 (Cat. no. 3050S) was purchased from Cell Signaling Technology (Beverly, Massachusetts) and anti-SATB2 (Cat. no. ab51502) was purchased from Abcam (Cambridge, UK). Anti-OCT4 and anti-SOX2 were purchased from SantaCruz Biotechnologies (Santa Cruz, CA, USA). Anti-Nanog was purchased from R&D Systems (Minneapolis, USA). Anti-tubulin was purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-β-Catenin and anti-e-cadherin were purchased from BD Biosciences (Franklin Lakes, NJ, USA). Wnt3a and Dkk1 were obtained from R&D Systems (Minneapolis, USA). All-trans-retinoic acid, BIO and XAV939 were obtained from Sigma-Aldrich (St. Louis, MO, USA). Lipofectamine RNAiMAX transfection reagent was purchased from Invitrogen (Carlsbad, CA, USA). siRNAs against GFP (Cat. no. sc-45924), SATB1 (Cat. no. sc-36460), SATB2 (Cat. no. sc-76456) were procured from SantaCruz Biotechnologies (Santa Cruz, CA, USA). Immunoblotsignal was detected using Visualizer^{TM} Western Blot Detection Kit (Cat.no. 64-202; Millipore/Upstate, Billerica, MA, USA).

2.2.2 Cell lines

HEK-293T, LN18, SKNMC and U373MG cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine

serum and penicillin/streptomycin, at 37°C under 5% CO2 atmosphere. These cells were passaged using 0.05% trypsin (Gibco, Carlsbad, CA, USA). Jurkat and JM1 cell lines were maintained in RPMI (RPMI-1640, Gibco, Carlsbad, CA, USA) medium, supplemented with 10% fetal bovine serum and penicillin/streptomycin, at 37°C under 5% CO_2 atmosphere. Embryonic carcinoma cell line NT2D1 were obtained as a kind gift from Dr. Peter Andrews and were grown in Dulbecco's Modified Eagle's Medium with no sodium pyruvate, high glucose (DMEM, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum, 2mM L-glutamine (200 mM stock) and penicillin/streptomycin, at 37°C under 5% CO_2 atmosphere. NT2D1 cells were passaged by gentle scraping and trypsin was not used for passaging.

2.2.3 Isolation of mouse tissues and RNA extraction

Various tissues were isolated from BALB/c mice and kept submerged in RNAlater solution (Invitrogen, Carlsbad, CA, USA) after dissecting them out.Tissues were then stored frozen at -80°C for later processing. RNA was isolated by homogenizing the tissue pieces in TRI reagent (Sigma-Aldrich, St. Louis, MO, USA) according to manufacturer's protocol.

2.2.4 Differentiation experiments in NT2D1

All-trans-retinoic acid (RA)-induced differentiation series were performed in 6-well plates for NT2D1 and hES cell lines. RA was reconstituted at a concentration of 5mg/ml in DMSO (Sigma-Aldrich, St. Louis, MO, USA) and stored in dark at -80 ^{o}C as 10 μl aliquots to avoid repeated freeze thaw cycle. For differentiation experiments, NT2D1 cells were harvested using 0.05% Trypsin, 0.15×10^6 cells were seeded in each well of 6-well plate and allowed to grow for 24 h. Next day, 0 day or control cells were harvested, RA (Sigma-Aldrich, St. Louis, MO, USA) was added to a final concentration of 13.7 μM to the remaining wells and cells were maintained in RA upto 7 days, with media replacement (containing freshly thawed RA) every day. At indicated days, cells were washed once with chilled 1X PBS and harvested for RNA and protein extraction.

2.2.5 RNAi mediated knockdowns

Knockdowns were performed using Lipofectamine RNAiMAX transfection reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer protocol. Briefly, NT2D1 cells were grown in Dulbecco's Modified Eagle's Medium with no sodium pyruvate, high glucose (DMEM, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum, 2mM L-glutamine (200 mM stock) and penicillin/streptomycin, at 37°C under 5% CO_2 atmosphere. Twenty μl of 10μM siRNAs were transfected using Lipofectamine RNAiMAX reagent as per manufacturer's instructions in serum-free medium. The medium was supplementedwith10% fetal bovine serum 6 h post-transfection. The cells were allowed to grow for 48 h and harvested by scrapping and used for RNA preparation and real-time RT-PCR.

2.2.6 Real-time quantitative RT-PCR

RNA was prepared from control and siRNA transfected cells using TRI reagent (Sigma-Aldrich, St. Louis, MO, USA). One μg of RNA was used for cDNA preparation per 20 μl of reaction. Real- time RT-PCRs were carried out essentially as described (Kumar et al., 2005). The cDNA was used as template for the PCR amplification with gene-specific set of primers. The changes in threshold cycle (Ct) values were calculated as follows: $\Delta Ct = (Ct_{target\ genes} - Ct_{\beta-actin})$ for transcript analysis. These ΔCt values were used to calculate fold change using equation as relative fold change $= 2^{-(\Delta(\Delta Ct))}$ and plotted graph for the average fold values with standard deviation from three independent experimental samples using Sigma Plot version 11.

2.2.7 Western blotting

Cells were lysed in lysis buffer (50mM Tris-HCl, pH7.5, 150mM NaCl, 0.5% Triton X-100, 5% glycerol, 1% SDS, 1mM Na_3VO_4, 10mM NaF, 1mM PMSF). Protein concentrations were determined using Bradford assay kit (Bio-Rad, Hercules, CA). Ten μg of protein lysate was boiled with 6X SDS sample buffer (0.5M Tris-HCl pH6.8, 28% glycerol, 9% SDS, 5% 2-mercaptoethanol, 0.01% bromophenol blue) and electrophoresed on a 12.5% SDS-Polyacrylamide gel and transferred onto a PVDF membrane. Membranes were incubated

overnight at 4°C with primary antibodies and then incubated with horseradish peroxidase conjugated secondary antibodies. The signals were developed using Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA) and detected using ImageQuant LAS 4000(GE Healthcare, Piscataway, NJ, USA) according to manufacturer's instructions.

2.3 Results

2.3.1 SATB1 and SATB2 are expressed differentially in various mouse tissues

Expression of any gene varies in different tissues and the level of particular gene's expression can give an indication of tissue specific roles of that gene. For example, SATB1 expression is very high in thymus and it has been shown to play important roles in development and differentiation of various T-cell subpopulations (Alvarez et al., 2000; Pavan et al., 2006; Notani et al., 2010). SATB1 expression is also significantly high in various tumors and it has been shown to play an important role in cancer progression and metastasis (Han et al., 2008; Meng et al., 2012, Chu et al., 2012). Similarly, important roles of SATB2 have been discussed in brain and bone development where it is expressed in considerable amount (Britanova et al., 2006; Alcamo et al., 2008; Britanova et al., 2008, Dobreva et al.; 2006, Hassan et al., 2010). To get an idea of relative expression of SATB family proteins across various tissues, we performed SATB1 and SATB2 expression profiling across various mouse tissues. As expected, SATB1 level was maximum in thymus whereas SATB2 was maximally expressed in brain (Fig. 2.3.1). SATB1 and SATB2 expressed in equivalent amount in kidney.
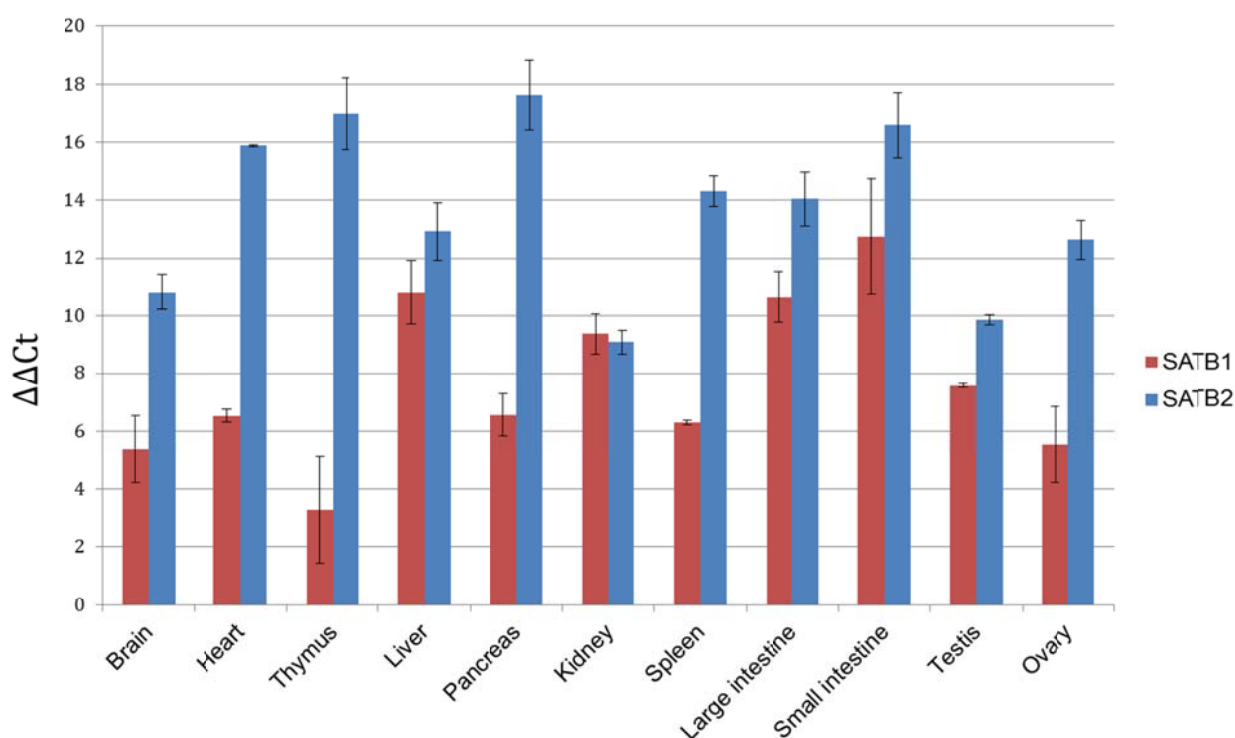
Figure 2.3.1: SATB1 and SATB2 are expressed differentially in various mouse tissues. Various mouse tissues were dissected and subjected for RNA extraction followed by cDNA synthesis. Real time RT-PCR was performed with cDNA synthesized from these samples. β-actin was used as internal control to normalize the values. Error bars indicate standard deviation calculated from triplicates. ΔΔCt value for each tissue is depicted on Y axis.

2.3.2 SATB1 and SATB2 express differentially in various cell lines

SATB1 and SATB2 show differential expression across various tissues (Dobreva et al., 2006 and our data). Next, we screened various cell lines derived from various tissues for expression pattern of SATB family proteins. We screened Jurkat; a T cell line and JM1; a B cell line for expression of SATB1 and SATB2 (Fig. 2.3.2 A). Quantitative transcript profiling revealed that SATB1 is expressed higher in Jurkat cells as compared to JM1 and this expression pattern was reversed in case of SATB2. We also screened a few lineage specific cell lines where we used Jurkat; a T-cell line, HEK-293T; a kidney cell line and LN18; a neuronal cell line. We observed that SATB1 is expressed more in the T cell line while expression of SATB2 was higher in LN18 (Fig. 2.3.2 B). Next, we screened a number of neuronal cell lines namely SK-N-MC, LN18 and U-373-MG which showed variable levels of SATB1 and SATB2 expression (Fig. 2.3.3 C). These results showed that different cell lines express variable amount of SATB1 and

SATB2, however we observed that various neuronal cell lines express comparable level of SATB1 and SATB2.
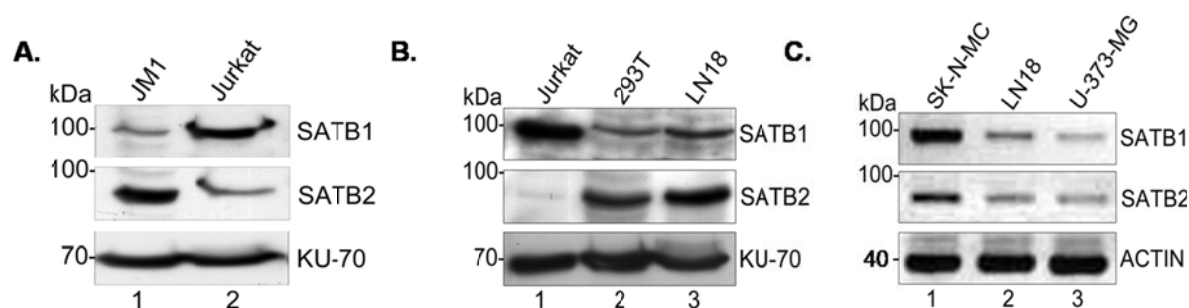
Figure 2.3.2: SATB1 and SATB2 are differentially expressed in various cell lines. Cell lines of various lineages were used to study the expression profiling of SATB family protein by western blotting. **(A)** Expression of SATB1 (panel 1) and SATB2 (panel 2) in a B cell line (JM1) and a T cell line (Jurkat). **(B)** Expression of SATB1 (panel 1) and SATB2 (panel 2) in a T cell line (Jurkat), a kidney cell line (HEK-293T) and a neuronal cell line (LN18). **(C)** Expression of SATB1 (panel 1) and SATB2 (panel 2) in various neuronal cell lines; SK-N-MC, LN18 and U-373-MG. First panel in each figure shows SATB1 expression and second panel shows SATB2 expression in the indicated cell line on the top of each figure. KU-70 and ACTIN (last panel in each figure) were used as loading controls. Molecular mass standards (kDa) are indicated on left side.

2.3.3 RA-induced differentiation of NT2D1 results in upregulation of SATB1 and SATB2

It was known from earlier studies that SATB2 is involved in neuronal development and differentiation in mouse brain. From section 2.3.2 also it was indicated that cells from different neuronal lineage express different, though comparable levels of SATB1 and SATB2. So we asked if differential level of SATB1 and SATB2 contributed towards the neuronal differentiation in human cell lines. To delineate the role of SATB1 and SATB2 in neuronal differentiation we used a well-established embryonic carcinoma cell line NTERA-2 clone D1 or "NT2D1" which is used as a model for studying human embryonic stem cells and can be differentiated into neurons upon retinoic acid (RA) differentiation (Andrews, 1984; Lee and Andrews, 1986) (Fig. 2.3.3).
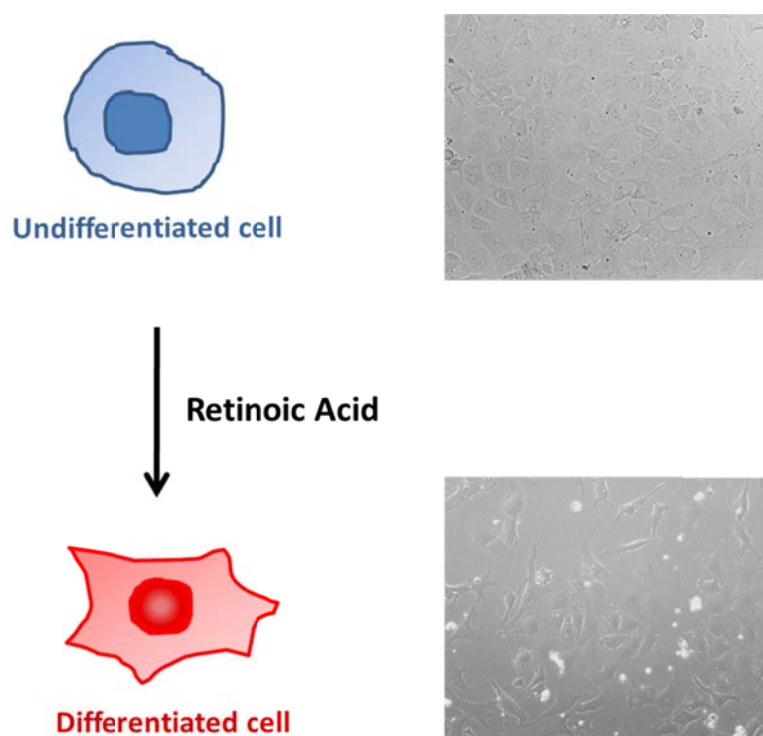
Figure 2.3.3: NT2D1; an embryonic carcinoma cell model for Retinoic Acid (RA) induced neuronal differentiation. The NTERA-2 cl.D1 or NT2D1 cell line is a pluripotent human testicular embryonal carcinoma cell line derived by cloning the NTERA-2 cell line. NT2D1 responds best to passage in clumps by scraping; prolonged passaging using trypsin can alter the phenotype of the cells. In general, human EC cells benefit from being cultured at high density maintaining stable and undifferentiated phenotype. This clone differentiates along neuroectodermal lineages after exposure to retinoic acid (RA). To induce differentiation, the cells should be trypsinized and seeded at a density 1 x 10^6 cells per 75 sq. cm. in medium containing 13.7uM all-trans-retinoic acid (RA). Prolonged RA induced differentiation of NT2D1 cells results into morphological changes where cells become flat, elongated and develop neurons like projections.

We performed differentiation series experiment in NT2D1 using retinoic acid up to 7 days. At the end of differentiation series cells were analyzed for SATB1 and SATB2 expression at transcript as well as protein level. SATB1 and SATB2 expression increased during differentiation at transcript level (Fig. 2.3.4 A). Upon differentiation expression of OCT4, a marker for pluripotency decreases while PAX6, a marker of differentiation increases indicating a successful differentiation series. We validated the expression profiles of SATB1 and SATB2 at protein level also by western blotting and we could see that SATB1 expression increases upon differentiation while SATB2 expression is reduced in initial days but then immediately increases during later time points (Fig. 2.3.4 B). Expression of pluripotency associated genes OCT4, SOX2 and NANOG decreases upon differentiation validating successful differentiation.
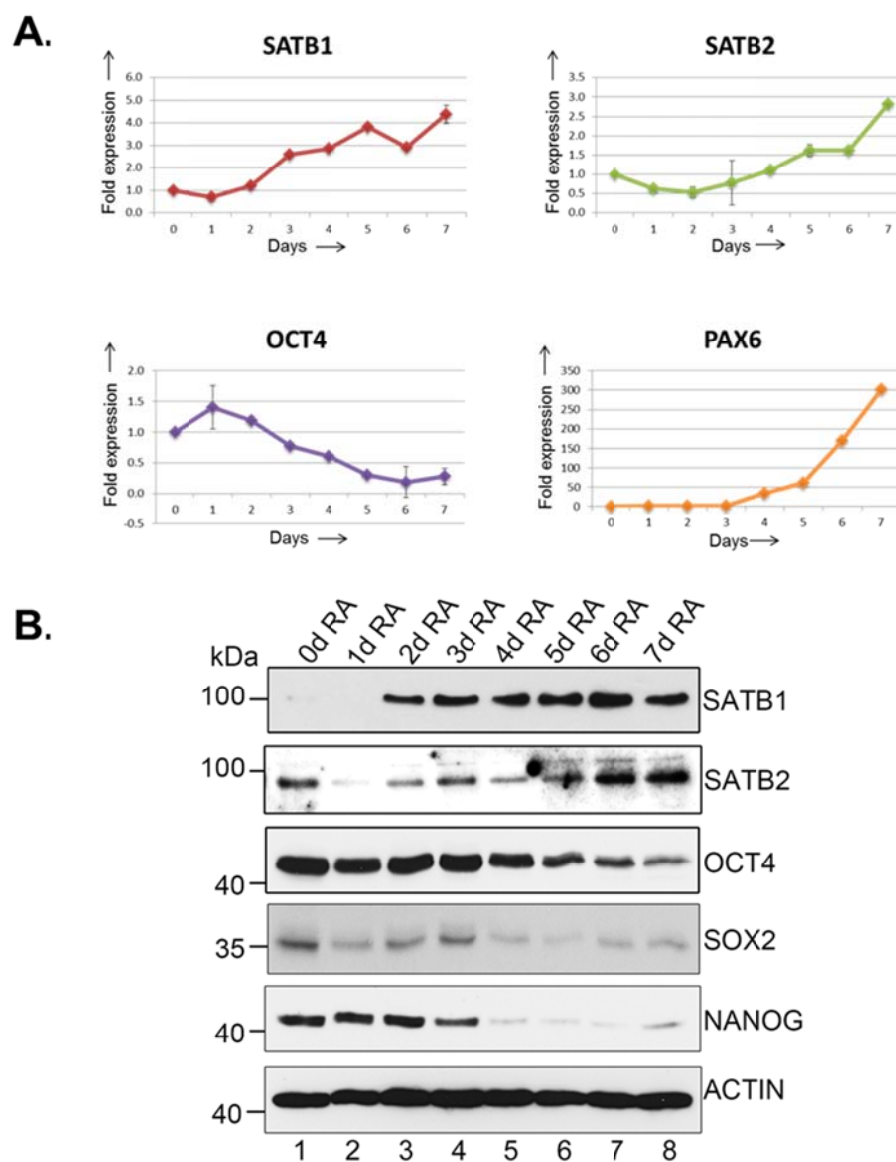
Figure 2.3.4: RA-induced differentiation of NT2D1 results in upregulation of SATB gene expression. NT2D1 cells were differentiated using all-trans-retinoic acid as described in "Materials and Methods" section. Zero day or control cells were harvested at the beginning of differentiation and the RA was added to remaining cells. Cells were harvested at indicated days and frozen immediately. At the end of differentiation series, RNA and protein extraction was performed as described in "Materials and Methods". (**A**)cDNA was made from the RNA isolated from each day sample and it was used to perform real time RT-PCR using gene specific primers. Fold expression was calculated using ΔΔCt method as described in "Materials and Methods". EF1a expression was used as endogenous control for normalization. (**B**) Lysates from the same differentiation series was used to detect the expression of SATB1 and SATB2 at protein level. For this 10 µg lysate from each day was loaded on 12.5% SDS-PAGE gel and transferred to a PVDF membrane. The membrane was horizontally cut in to two pieces. Upper part was probed with anti-SATB1 and anti-SATB2 respectively after stripping. Lower part was immunoblotted with antibodies against OCT4, followed by SOX2 and NANOG and then ACTIN (lower panels) after stripping. ACTIN served as loading control. Days of differentiation have been indicated on top. Molecular mass standards (kDa) are indicated on left side.

2.3.4 SATB1 and SATB2 regulate genes involved in maintenance of self-renewal and pluripotency

Differentiation series performed in NT2D1 cells indicated that the level of SATB family protein changes during differentiation. This prompted us to ask if SATB1 and SATB2 have any role in regulating differentiation. To address this question, we silenced expression of SATB1 and SATB2 in NT2D1 cells by siRNA mediated knockdown. We validated the knockdown of SATB1 and SATB2 by PCR (Fig. 2.3.5 A). We analyzed the effect of SATB1 and SATB2 knockdown on the expression of OCT4, SOX2 and NANOG; the master regulators of pluripotency. NANOG and OCT4 expression increased while SOX2 expression decreased upon SATB1/SATB2 knockdown (Fig. 2.3.5 B).
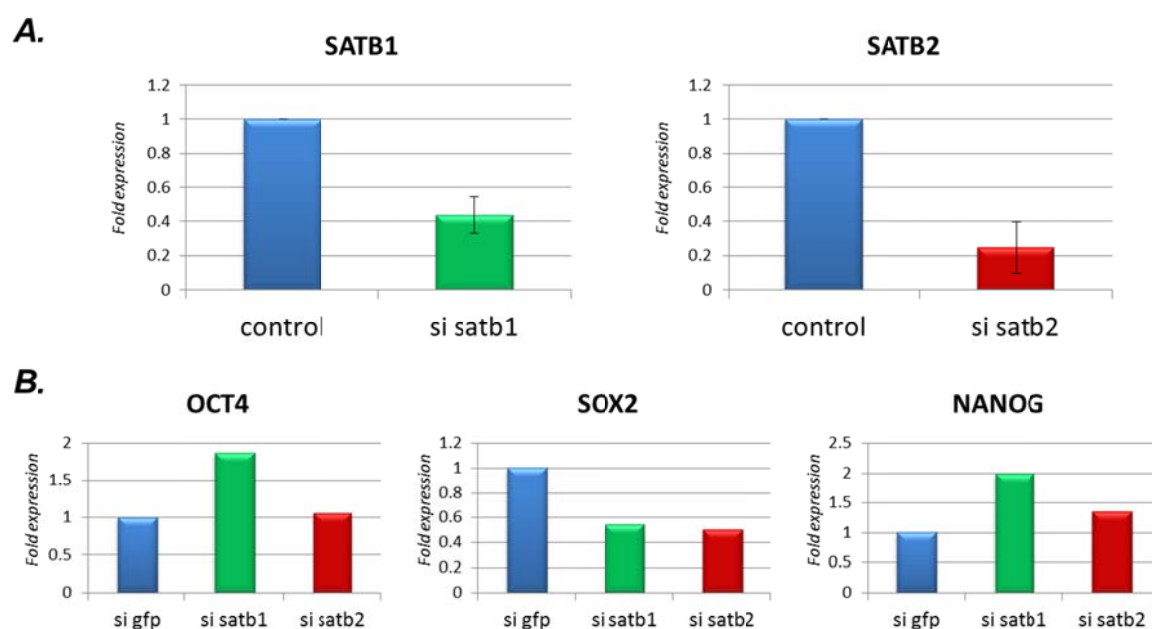
Figure 2.3.5: Knockdown of SATB1 and SATB2 results in upregulation of genes involved in maintenance of self-renewal and pluripotency. (**A**) NT2D1 cells were transfected with scrambled RNA (control) or with siSATB1 or siSATB2 RNA as described in "Materials and Methods". Forty eight hours post transfection, cells were harvested and RNA was extracted followed by cDNA synthesis. Real time RT-PCR was performed with cDNA synthesized from these samples. EF1a was used as internal control to normalize the values. Fold change was calculated using ΔΔCt method and is depicted as compared to control on Y axis. (**B**) Expression of Oct4, Sox2 and Nanog in the SATB1 or SATB2 knockdown NT2D1 cells. EF1a was used as internal control to normalize the values. Fold change was calculated using ΔΔCt method and is depicted as compared to control on Y axis.

To study the effect of knockdown of SATB1 and SATB2 on various genes involved in pluripotency and differentiation we used low density Taqman arrays and performed the taqman

probe based quantitative PCR. The expression of various genes was normalized with respect to endogenous EF1α expression. A subset of genes associated with stemness and pluripotency or differentiation were selected for the analysis. It was observed that genes associated with stemness and pluripotecy was upregulated upon knockdown of SATB1 and SATB2 while genes associated with differnetaiton were downregukated (Fig. 2.3.6).
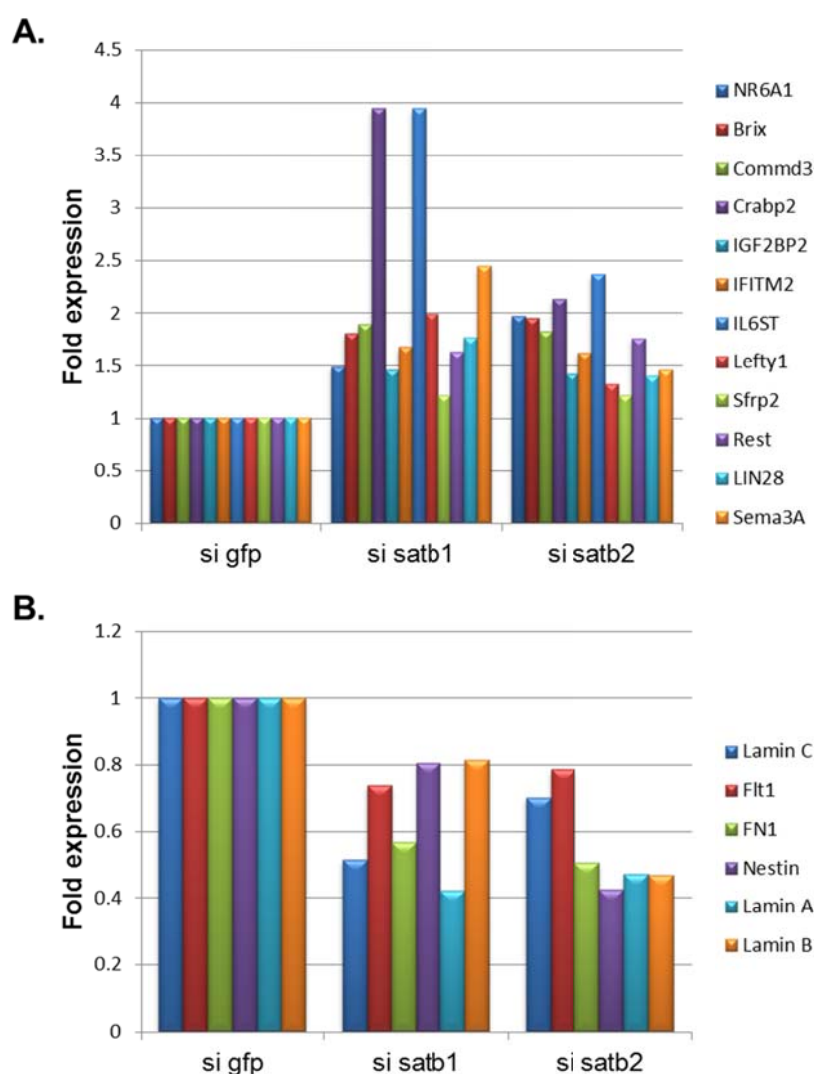
Figure 2.3.6: SATB1 and SATB2 regulate genes involved in self-renewal and differentiation. NT2D1 cells were transfected with scrambled RNA (control) or with siSATB1 or siSATB2 RNA as described in "Materials and Methods". Forty eight hours post transfection, cells were harvested and RNA was extracted followed by cDNA synthesis. Real time RT-PCR was performed with cDNA synthesized from SATB1 or SATB2 knockdown NT2D1 cells. EF1a was used as internal control to normalize the values. Fold change was calculated using ΔΔCt method and is depicted as compared to control on Y axis. EF1a was used as internal control to normalize the values. Fold change was calculated using ΔΔCt method and is depicted as compared to control on Y axis. Expression of various genes associated with self-renewal or pluripotency **(A)** and genes involved in differentiation **(B)** were analyzed in the NT2D1 cells where SATB1 and SATB2 expression were downregulated by siRNA mediated knockdown.

2.3.5 Genes involved in Wnt signaling are upregulated during RA induced differentiation

A number of studies have suggested that Wnt signaling is involved in the maintenance of pluripotent state of mouse and human embryonic stem cells (mESCs and hESCs) (Sato et al., 2004; Hao et al., 2006; Takao et al., 2007; ten Berge et al., 2011). Furthermore, activation of Wnt signaling promoted self-renewal while inhibition of the Wnt pathway resulted in multi-lineage differentiation. Marson et al. showed that activation of Wnt signaling promotes reprogramming of somatic cells to a pluripotent state (Marson et al., 2008). To test the role of Wnt signaling in NT2D1 differentiation we monitored the expression of various Wnt responsive genes in a differentiation dependent manner. We differentiated NT2D1 cells for 3 and 6 days using RA. Various Wnt responsive genes were analyzed by quantitative RT-PCR (Fig. 2.3.7). We detected increased expression of a number of Wnt responsive genes during RA induced differentiation. DKK1; which is an antagonistof Wnt signaling, is downregulated during differentiation. These results indicate that Wnt signaling is upregulated during RA mediated differentiation of NT2D1 cells.
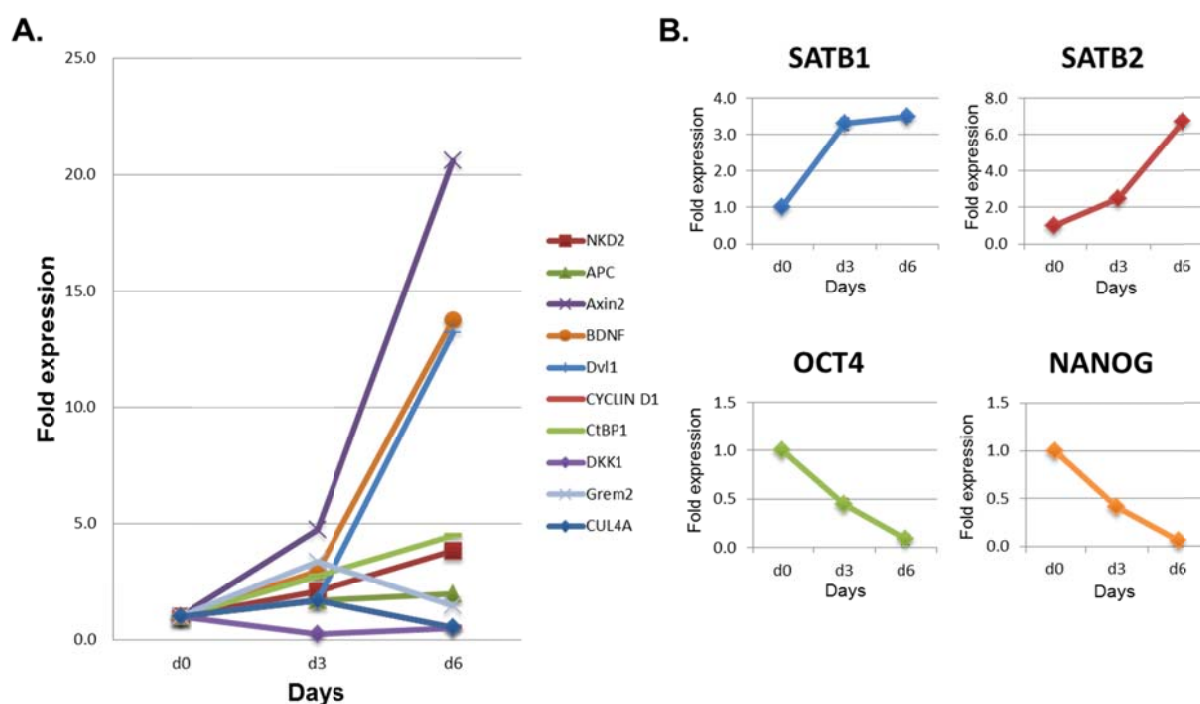
Figure 2.3.7: Genes involved in Wnt signaling are upregulated during RA induced differentiation of NT2D1 cells. NT2D1 cells were differentiated with RA as described in "Materials and Methods. Cells were harvested at indicated timepoints and RNA was extracted followed by cDNA synthesis. Real time RT-PCR was performed with cDNA synthesized from differentiated NT2D1 cells. EF1a was used as internal control to normalize the values. Fold change was calculated using ΔΔCt method and is depicted as compared to control on Y axis. EF1a was used as internal control to normalize the values. Fold change was calculated using ΔΔCt method and is depicted as compared to control on Y axis. **(A)** Expression of various Wnt responsive genes (indicated on left) was analyzed in differentiated NT2D1 cells at 3 and 6 day of differentiation. **(B)** Expression of SATB1 and SATB2 at day 3 and day 6 after RA induced differentiation. OCT4 and NANOG expression were also analyzed to validate the differentiation.

2.3.6 Induction of Wnt signaling results into differentiation

The differentiation series study indicated that Wnt signaling is upregulated during RA induced differentiation of NT2D1 cells. Next, we asked what will happen if Wnt signaling is ectopically activated in these cells. Wnt signaling was induced in NT2D1 cells by treating them with soluble Wnt3a ligand. Cells were harvested at various intervals and expression of pluripotency genes OCT4, SOX2 and NANOGwas analyzed at protein level (Fig. 2.3.8 A). We observed that induction of Wnt signaling in NT2D1 cells resulted in downregulation of pluripotency genes OCT4, SOX2 and NANOGin a time-dependent manner. We also validated these results by activating Wnt signalling using 6-bromoindirubin-3′-oxime (BIO), a specific pharmacological inhibitor of glycogen synthase kinase-3 (GSK-3). Similar to Wnt3a, BIO

treatment of NT2D1 cells also resulted in downregulation of pluripotency genes in a time-dependent manner (Fig. 2.3.8 B). Induction of Wnt signaling was confirmed by increased expression of two Wnt signaling associated genes, β-Catenin and e-Cadherin. These results indicate that induction of Wnt signaling promotes differentiation.
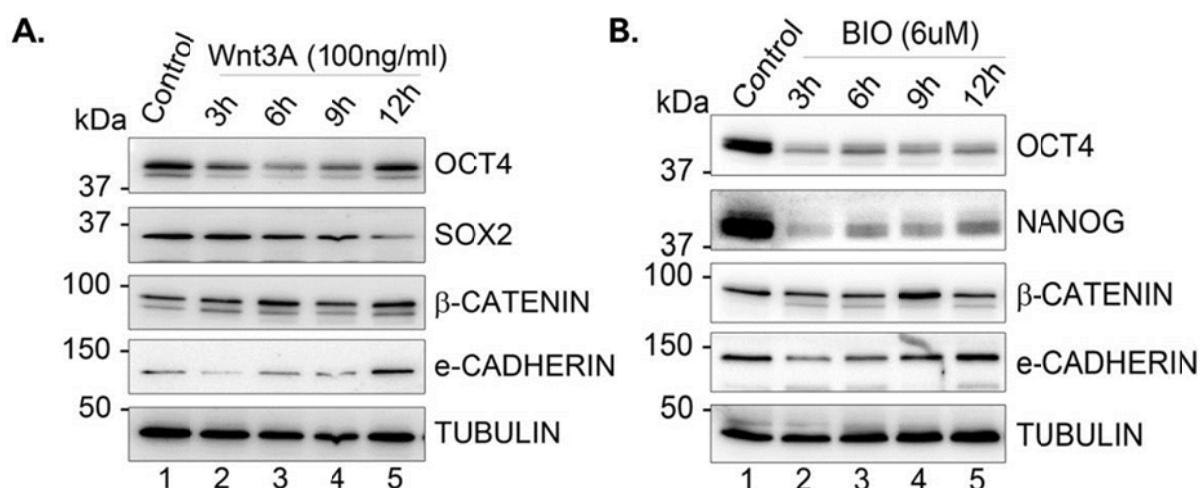
Figure 2.3.8: Induction of Wnt signaling results into differentiation of NT2D1 cells. Wnt signaling was induced in NT2D1 cells by soluble Wnt3a ligand **(A)** or BIO **(B).** NT2D1 cells were harvested at various time points (indicated in hours on the top of panel) after addition of Wnt3a or BIO and immunoblot analysis was performed using specific antibodies to analyze the expression of pluripotency-associated genes. For this 10ug lysate from each time point was loaded on 12.5% SDS-polyacrylamide gel and transferred to a PVDF membrane. The membrane was horizontally cut in to two pieces. Upper part was probed with anti-b-CATENIN and anti-e-CADHERIN respectively after stripping. Lower part was immunoblotted with antibodies against OCT4, followed by SOX2 and NANOG and then Actin (lower panels) after stripping. Immunoblot using anti-actin served as loading control. Levels of β-catenin and e-cadherin increased during Wnt induction indicating active Wnt signaling. Immunoblot using anti-tubulin was used as loading control. Time points of induction in hoursare indicated on top. Molecular mass standards (kDa) are indicated on left side.

2.3.7 Induction of Wnt signaling mimics RA induced differentiation

It was clear from the above experiments that Wnt signaling gets upregulated during differentiation and induction of Wnt signaling leads to differentiation. To compare differentiation mediated by Wnt signaling induction and RA treatment, we treated NT2D1 cells with Wnt3A and RA for 2 days and then compared the expression of SATB1, SATB2, OCT4, SOX2 and NANOG (Fig 2.3.9). We observed that expression of SATB1, SATB2 increases whereas that of OCT4, SOX2 and NANOG decreases upon Wnt activation, which was comparable to retinoic

acid induced differentiation. These results indicated that Wnt signaling mimics RA induced differentiation.
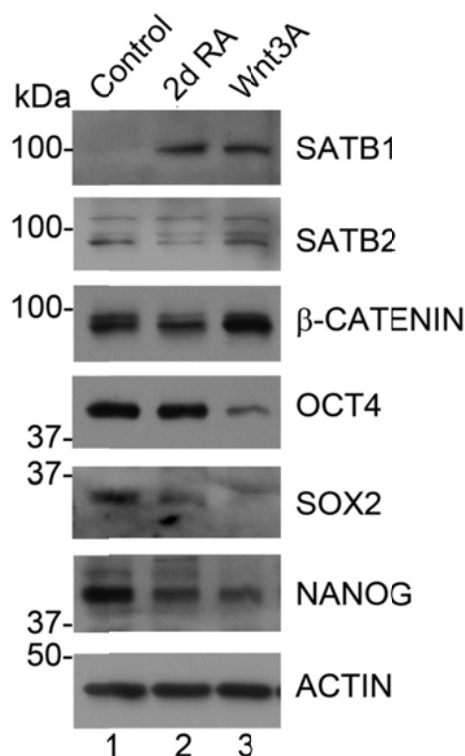
Figure 2.3.9: Induction of Wnt signaling mimics RA induced differentiation of NT2D1 cells. To compare the differentiation of NT2D1 cells by activation of Wnt signaling to RA induced differentiation we treated NT2D1 cells with Wnt3A and performed the western to check the expression of SATB1, SATB2 along with OCT4 and NANOG and compared the result to that of RA induced differentiation **of** NT2D1 cells that were treated with Wnt3a or RA for two days followed by immunoblot analysis. Ten μg lysate was loaded on 12.5% SDS-Polyacrylamide gel and transferred to a PVDF membrane. The membrane was horizontally cut into two pieces. Upper part was probed with anti-SATB1, anti-SATB2 and anti-β-catenin and anti-e-cadherin respectively after stripping. Lower part was immunoblotted with antibodies against OCT4, followed by SOX2 and NANOG and then ACTIN (lower panels) after stripping successively. Immunoblot using anti-Actin served as loading control. Molecular mass standards (kDa) are indicated on left side.

2.3.8 SATB1 and SATB2 regulate the expression of Wnt signaling responsive genes

SATB1 has been shown to regulate Wnt responsive genes in a Wnt signaling-dependent manner during Th2 cell differentiation (Notani et al., 2010). From the above experiments it was clearly evident that expression of SATB1 and SATB2 increased during RA induced differentiation and the same effect can be mimicked by inducing Wnt signaling in undifferentiated NT2D1 cells. We asked if SATB family target Wnt responsive genes during RA

induced differentiation. To address this question we transiently knocked down SATB1 and SATB2 expression in NT2D1 cells and analyzed the expression of Wnt responsive genes. Knockdown of SATB1 and SATB2 resulted in the decreased expression of Wnt responsive genes and therefore indicating that these genes might be targets of SATB family proteins during differentiation (Fig. 2.3.9)
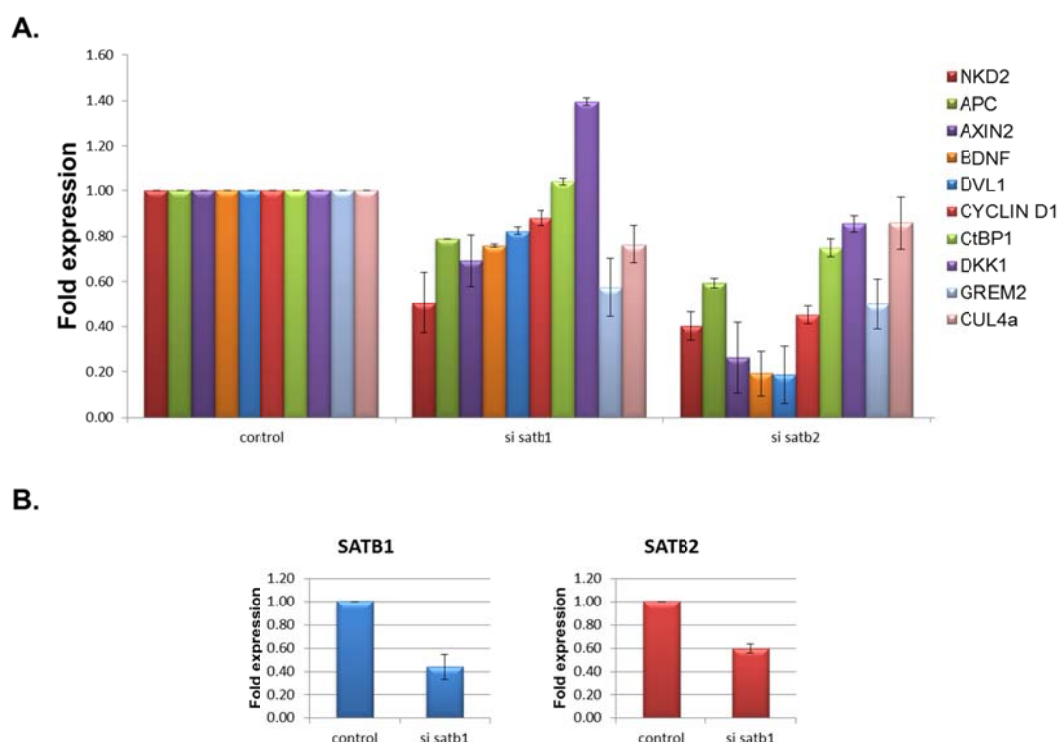
Figure 2.3.10: Knockdown of SATB1 and SATB2 result in the downregulation of Wnt responsive genes. NT2D1 cells were transfected with scrambled RNA (control) or with Si SATB1 or Si SATB2 RNA as described in "Materials and Methods". Forty eight hours post transfection, cells were harvested and RNA was extracted followed by cDNA synthesis. Real time RT-PCR was performed with cDNA synthesized from SATB1 or SATB2 knockdown NT2D1 cells. EF1a was used as internal control to normalize the values. Fold change was calculated using $\Delta\Delta Ct$ method and is depicted as compared to control on Y axis **(A)** Expression of various Wnt responsive genes was analyzed in NT2D1 cells wherein SATB1 and SATB2 expression was downregulated. (B) Real time RT-PCR showing the knockdown of SATB1 and SATB2.

2.4 Discussion

Eukaryotic gene expression is regulated by various transcription factors and global gene regulators which impart cell type specific gene expression patterns. SATB family proteins are one such class of MAR-binding proteins which bind to special AT-rich DNA sequence and

coordinate expression of multiple genes in different tissues in cell type specific manner (Cai et al., 2003; Dobreva et al., 2006; Kumar et al., 2007; Alcamo et al., 2008; Britanova et al., 2008; Balamotis et al., 2012; Zhou et al., 2012). SATB1 and SATB2 have been studied in many different cell and tissue types and have been shown to be expressed at varying levels however there are no reports mentioning the relative expression in same cell or tissue types except in brain where they have been shown to express in distinct and mutually exclusive manner (Britanova et al., 2005; Balamotis et al., 2012). To obtain an insight into the relative expression of SATB1 and SATB2 in various cell and tissue types, we analyzed the expression levels of SATB1 and SATB2 in various cell and mice tissue types. We observed that SATB1 expression is highest in thymus while SATB2 expression is the maximum in brain. We also screened few cell lines of diverse origins and analyzed SATB1 and SATB2 expression in them. Jurkat, a T lymphoblastic cell line exhibits high expression of SATB1 at protein level whereas SATB2 expression was very low. JM1, a B cell line exhibits high SATB2 and low SATB1 expression. 293T, a kidney cell line expresses both SATB1 and SATB2 to similar extent. On contrary, most of the neuronal cell lines that we screened express comparable level of SATB1 and SATB2, which indicates that differential expression of SATB1 and SATB2 could be attributed todifferent subpopulations of neuronal lineage and the same has been supported by previous studies as well (Britanova et al., 2005; Huang et al., 2011; Baranek et al., 2012).

As it was reported earlier that neurons from various regions of the brain exhibit different levels of SATB1 and SATB2 expression, we asked if SATB family proteins play any roles in the neuronal differentiation. For this we used a human embryonic carcinoma cell line, NT2D1 which is a type of human testicular carcinoma cell line. Human teratocarcinoma cell lines were first isolated as xenografts in hamster cheek pouches (Pierce et al., 1957) after which many subsequent lines established *in vitro* notably TERA1, TERA2 (Fogh and Trempe, 1975) and SuSa (Hogan et al., 1977). One of the important carcinoma cell lines, TERA2 was established by Peter Andrews in 1980 which exhibited pluripotent capabilities and could be differentiated *in vitro* (Andrews et al., 1980), subsequently another clone of TERA2 called as NTERA2 cl. D1 (NT2D1) was established from a xenograft tumor in a nude mouse (Andrews et al., 1984). TERA2 and its sub clone line NTERA2 are pluripotent and unlike stem cells they do not require feeder cells to grow which makes them relatively easy system to work with as compared to hESCs (Andrews et al., 1984). NTERA2 has been shown to be capable of

differentiatingextensively in distinct cell populations including functional neurons in response to retinoic acid treatment (Andrews et al., 1984; Lee and Andrews, 1986; Pleasure et al., 1992; Pleasure and Lee, 1993; Squires et al., 1996; Przyborski et al., 2000). All these properties of NT2D1 make it a highly efficient and easy model for studying basic principles of pluripotency and differentiation and can be used as a replacement for human embryonic stem cells (hESCs) which are relatively more difficult to maintain and require specific culture conditions such as feeder cells, defined media and various growth factors.

We therefore used NT2D1 cells as a model system to study roles of SATB family proteins during RA induced differentiation. We observed that SATB1 and SATB2 are induced during differentiation with SATB1 showing more induction as compared to SATB2. We also analyzed expression of various pluripotency genes such as OCT4, SOX2 and NANOG, which were downregulated upon differentiation. To delineate the role of SATB family proteins during differentiation, we knocked down SATB1 and SATB2 in NT2D1 cells and analyzed the expression levels of various genes associated with pluripotency or differentiation. Expression of pluripotency and stem cell related genes such as OCT4, NANOG, NR6A1, BRIX, COMMD3, CRABP2, IL6ST LEFTY1, LIN28, REST and SEMA3A was increased upon SATB1 or SATB2 knockdown. On other hand, SATB1 and SATB2 knockdown resulted in downregulation of genes namely Flt1, FN1, Nestin and Lamins which are involved in differentiation. These findings indicate that SATB1 and SATB2 are important in downregulating genes involved in maintaining stemness or pluripotency and upregulate genes associated with differentiation. This goes hand in hand with the expression profile of SATB1 and SATB2 which get upregulated during differentiation. We propose that the SATB family proteins play important role in coordinating NT2D1 differentiation by regulating expression of genes associated with differentiation.

There are many different developmental pathways involved in regulating stemness and differentiation of hESCs (Noggle et al., 2005; Liu et al., 2007; Feng et al., 2009). Multiple studies have reported and discussedthe role of Wnt signaling in the maintenance of hESCs however contradictory views exists on whether Wnt signaling inhibits or promotes differentiation of hESCs. Sato et al. showed that Wnt signaling is active in undifferentiated stem cells and is downregulated upon differentiation; they also showed that cells could be maintained in undifferentiated condition by the addition of BIO in culture medium which inhibits GSK3β (Sato et al., 2004). Marson et al. showed that somatic cells can be reprogrammed to

induced pluripotent cells (iPSCs) by Wnt3A mediated activation of Wnt signaling (Marson et al., 2008). However contrasting studies show that activation of Wnt signaling by BIO or Wnt3A may promote but is not sufficient to maintain the cells in pluripotent state over many passages (Dravid et al., 2005; Cai et al., 2007). Activation of Wnt signaling by inhibiting GSK3β in hESCs promotes differentiation towards primitive streak, endodermal and mesodermal lineage (Nakanishi et al., 2009; Bone et al., 2011). There are also many studies which show that Wnt signaling is important and promote neurogenesis from neural stem cell culture (Muroyama et al., 2004; Hirsch et al., 2007). A very recent report shows that OCT4 represses Wnt signaling in undifferentiated hES cells which gets activated upon differentiation due to loss of OCT4 (Davidson et al., 2012). Taken together, role of Wnt signaling has been a controversial and open question. To monitor the role of Wnt signaling in NT2D1 differentiation we first check the status of Wnt signaling in our differentiation series. We observed that various Wnt responsive genes are upregulated during RA induced differentiation. Activation of Wnt signaling by soluble Wnt3A or BIO resulted in downregulation of various pluripotency markers including OCT4, SOX2 and NANOG. SATB1 has been shown to interact with β-catenin and coordinate gene expression in a Wnt-dependent manner (Purbey et al., 2009; Notani et al., 2010). Interestingly, activation of Wnt signaling also induced SATB1 and SATB2 expression and therefore mimicked RA induced differentiation. Knockdown of SATB1 and SATB2 resulted in downregulation of Wnt responsive genes indicating that SATB family proteins may target Wnt responsive genes during differentiation. In conclusion, Wnt signaling is induced during differentiation and activation of Wnt signaling in undifferentiated cells promotes differentiation. SATB family proteins are induced upon Wnt signaling and may regulate Wnt responsive genes during differentiation (Fig. 2.4.1).
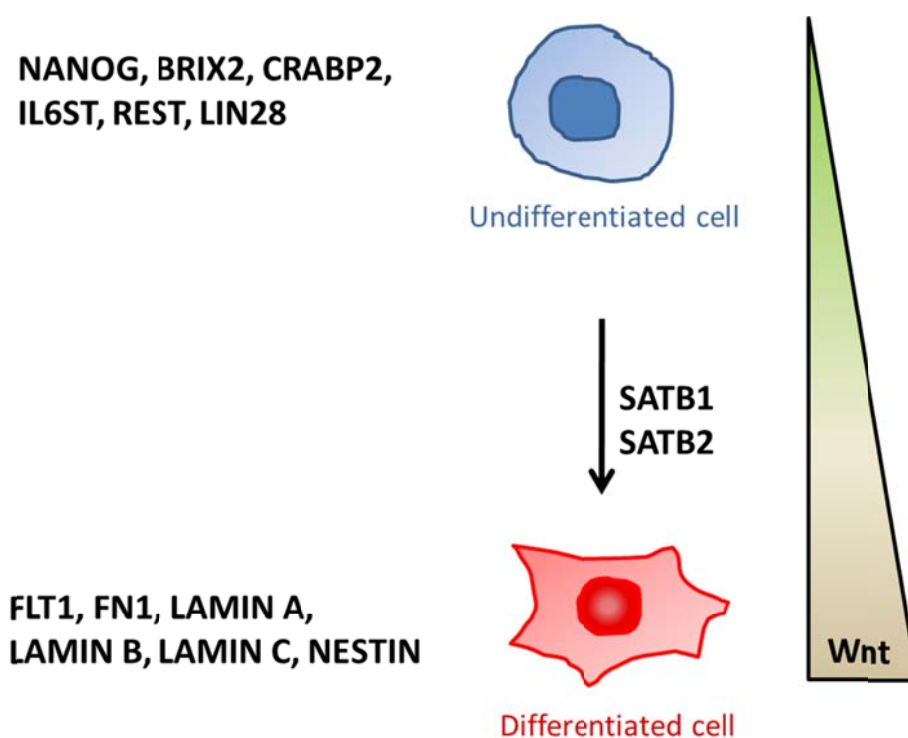
Figure 2.4.1: Model depicting RA-induced differentiation of NT2D1 cells. RA treatment of NT2D1 cells results in differentiation, which is accompanied by upregulation of SATB family proteins along with increased expression of various Wnt responsive genes. Differentiation results in downregulation of many pluripotency associated genes and upregulation of genes involved in differentiation. Knockdown of SATB1 and SATB2 results in impairment of differentiation and induction of genes involved in pluripotency and stemness. Induction of Wnt signaling by Wnt3A or BIO treatment to the cells mimics RA signaling and results in enhanced differentiation.

Our data show that SATB family proteins are expressed in a variety of cell types and tissues at different levels. In NT2D1 and hES cells a critical balance of SATB1 and SATB2 is crucial for maintaining pluripotency and inducing differentiation. RA-induced differentiation results in upregulation of Wnt responsive genes, SATB1, SATB2 and downregulation of genes associated with pluripotency. We show that Wnt signaling is not important for maintaining cells in pluripotent state and activation of Wnt signaling results in differentiation. Activation of Wnt signaling by Wnt3A and BIO results in downregulation of master regulators of pluripotency including OCT4, SOX2 and NANOG and upregulation of SATB1 and SATB2 in a manner similar to RA-induced differentiation. SATB family proteins play important role in regulating many Wnt responsive genes and also regulate the expression of multiple genes associated with pluripotency and differentiation. Taken together, we conclude that SATB1 and SATB2 interact with Wnt signaling and promote NT2D1 differentiation by upregulating Wnt signaling. We

report novel findings implicating role of SATB family proteins in regulation ofdifferentiationvia regulation of Wnt signaling.

2.5. References

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Chapter III

To study the roles of SATB1 and SATB2 in context of human embryonic stem cells

3.1 Introduction

Stem cells are special types of cells characterized by their unique feature of self-renewal and potency as they can divide indefinitely to give rise to more stem cells and also can differentiate into variety of specific cell types. In mammals, stem cells can be isolated from the inner cell mass (ICM) of preimplantation embryos known as embryonic stem cells.Some stem cells are also found in certain tissues of adult organisms which are termed as adult stem cells. Embryonic stem cells were first established from the inner cell mass (ICM) of mouse blastocysts in 1981 (Evans and Kaufman, 1981) and after a long time in 1998 Thomson and colleague isolated first human embryonic stem cells (hESCs) (Thomson et al., 1998). Establishment of hES cell lines (Thomson et al., 1998; Reubinoff et al., 2000) and development of induced pluripotent stem (iPSCs) through the reprogramming of adult human cells (Takahashi, K. & Yamanaka, S., 2006; Takahashi et al., 2007; Yu et al., 2007) developed new hopes in the field of regenerative medicines and treatment of degenerative diseases (Pera and Trounson, 2004; Liew et al., 2005). Detailed understanding of the various pathways and molecular mechanisms regulating self-renewal and pluripotency as well as differentiation of ES and iPS cells into various differentiated cell lineages is very important for developing develop stem cell based therapies and regenerative medicines. A number of signaling pathways along with multiple stem cell factors have been implicated in regulation of pluripotency and induction of differentiation of mESCs as well as hESCs which will be discussed in brief in the following section.

3.1.1 Transcription factors, cofactors and regulatory networks involved in self-renewal and pluripotency

Of the various transcription factors implicated in regulating pluripotency OCT4, SOX2 and NANOG are the most important factors that regulate pluripotency and form a core regulatory network in both mES and hES cells (Boiani and Scholer, 2005; Boyer et al., 2005; Loh et al., 2006; Chambers et al., 2007; Masui et al., 2007; Niwa, 2009). OCT4 is a POU domain containing transcription factor and its precise level of expression is very important for maintaining self-renewal conditions for example, decreased expression of OCT4 causes differentiation of stem cells into trophoectoderm while overexpression results in induction of primitive endoderm and mesoderm (Yeom et al., 1996; Niwa, 2001). SOX2 is a HMG-family protein that co-occupies regulatory regions of multiple genes with OCT4 and is required for maintenance of pluripotent state of stem cells (Ambrosetti et al., 2000; Avilion et al., 2003; Masui et al., 2007). NANOG is also a HMG domain containing protein playing an important role in regulating stemness along with OCT4 and SOX2 and disruption of NANOG results in differentiation towards endodermal lineage (Nichols et al., 1998; Chambers et al., 2003; Mitsui et al., 2003; Chambers and Smith, 2004; Chambers et al., 2007). Heterodimeric OCT4/SOX2 complex regulates expression of OCT4, SOX2 and NANOG (Kuroda et al., 2005; Rodda et al., 2005). These core factors co-occupy and repress many lineage-specific genes while activating the expression of other genes involved in maintaining pluripotency (Boyer et al., 2005; Loh et al., 2006; Rao and Orkin, 2006). OCT4, SOX2 and NANOG form interconnected autoregulatory loops among them to regulate various pluripotency and differentiation associated genes. Loss of these core factors induces rapid induction of genes in stem cells, which results in differentiation towards various cell lineages indicating that these genes are poised for activation. These core pluripotency factors facilitate recruitment of various histone modifying enzymes and activator complexes such as trithorax group (TrxG) proteins. TrxG proteins catalyze H3K4me4 modification and attract further histone modifiers such as Tip60-p400 complex and chromatin remodelers (esBAF and members of CHD family) to induce open chromatin formation for active transcription of pluripotency and self-renewal associated genes (Fazzio et al., 2008; Gaspar-Maia et al., 2009; Ho and Crabtree, 2010). OCT4 maintains the differentiation associated genes in a poised state by recruiting SetDB1 to the promoters of these genes which marks them with H3K9me3 modification and recruit and polycomb group (PcG) proteins which further catalyze

ubiquitination of histone H2A lysine 119 (H2AK119ub) and trimethylation of histone H3 lysine 27 (H3K27me3) keeping these genes ''poised'' for activation during differentiation (Boyer et al., 2006; Bilodeau et al., 2009; Yeap et al., 2009). c-Myc is one of the important Yamanaka factors in addition to OCT4, SOX2 and NANOG (Takahashi and Yamanaka, 2006) and has been recently shown to prevent lineage-specific differentiation through direct repression of GATA6 expression (Smith et al., 2010; Varlakhanova et al., 2010). c-Myc interacts with and recruits histone acetyltransferases (HATs; GCN5, p300), various chromatin remodeling complexes, histone deacetylases (HDACs) and histone demethylases (Lin et al., 2009) which induces global histone acetylation allowing core pluripotency factors to bind their specific targets. OCT4, SOX2 and NANOG interact with various cofactors to facilitate recruitment of RNA polymerase II onto the promoters of various pluripotency-associated genes (Kagey et al., 2010). c-Myc recruits p-TEFb (a cyclin dependent kinase) to these sites facilitating active transcription of these genes along with core pluripotency factors (Jaenisch and Young, 2008; Rahl et al., 2010). In addition to OCT4, SOX2, NANOG, and c-Myc there are many other factors and cofactors which are involved in maintaining pluripotency, some of which are transcription factors such as Tcf3, Smad1, Stat3, Esrrb, Sall4, Tbx3, Zfx, Ronin, Klf2, Klf4, Klf5, and PRDM14 (Chen et al., 2008;Kim et al., 2008). Transcription cofactors such as cohesin and mediators facilitate active transcription in concert with core pluripotency factors through physical association of enhancers with core promoters by loop formation (Kagey et al., 2010). Corepressorssuch as Dax1, Cnot3, and Trim28 have also been implicated in regulating pluripotency, loss of their expression results in differentiation towards trophotectoderm and primitive ectoderm lineage (Fazzio et al., 2008; Hu et al., 2009; Sun et al., 2009) (summarized in Fig. 3.1.1).
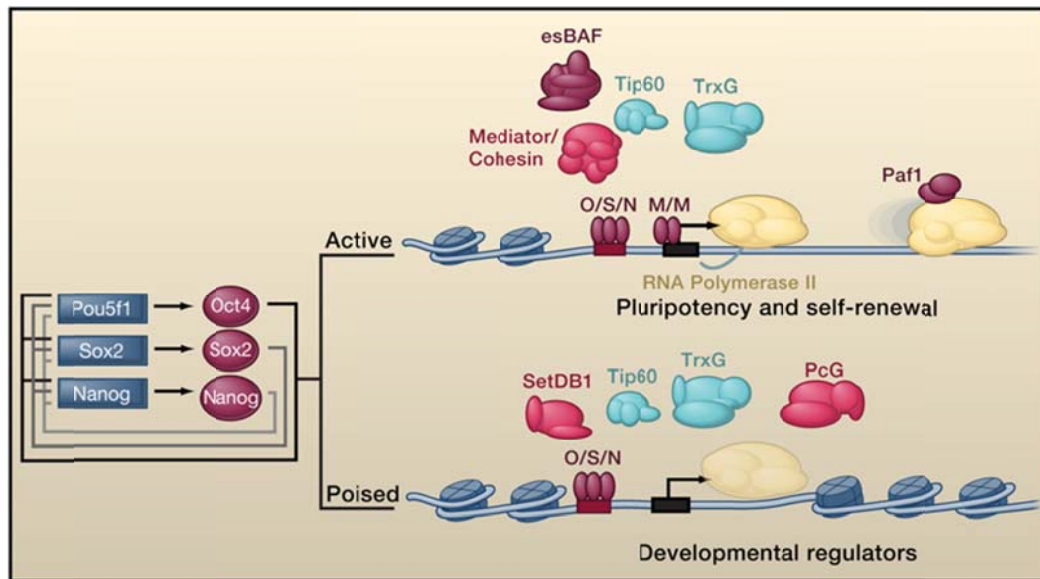
Figure 3.1.1: Transcription factors and autoregulatory interconnected loop of core pluripotency factors. OCT4 and SOX2 interact and collaborate with NANOG to regulate their own promoters, forming an interconnected autoregulatory loop. The Pou5f (OCT4), SOX2, and NANOG genes are represented as blue boxes and proteins as red balloons. These core transcription factors occupy and facilitate the recruitment of various transcription factors and coactivators to activate the expression of genes involved in maintenance of pluripotency and self-renewal. On other hand they occupy the promoters of various lineage specific genes and recruit various corepressors to keep them poised for expression (Reproduced from Young, 2011).

3.1.2 Epigenetics and bivalent chromatin status of embryonic stem cells

Stem cellidentity is maintained by core pluripotency factors and various other transcription factors and chromatin regulators which regulate expression of genes in the pluripotent state. The interplay between self-renewal and differentiation is achieved by various histone modifiers and chromatin remodelers which govern the epigenetic landscape of stem cells. These factors keep the pluripotency associated genes active while maintaining the differentiation-promoting genes in a permissive or poised state by means of various epigenetic modifications. Polycomb group of proteins (PcG) play a very important role in stem cell self-renewal and pluripotency (Pasini et al., 2004; Pasini et al., 2008). PcG are repressive multimeric proteins complex namely polycomb repressive complex 1 and 2 (PRC1 and PRC2) which bind and repress most of the genes promoting stem cell differentiation, keeping them poised for derepression upon differentiation. PRC2 catalyzes H3K27me3 modification (Azuara et al., 2006; Bernstein et al., 2006; Boyer et al., 2006; Landeira et al., 2010; Lee et al., 2006; Li et al., 2010) which acts as docking site for PRC1 which then catalyzes H2AK119Ub1 (Min et al., 2003; de

Napoles et al., 2004) resulting in repression by attenuation of RNA pol II activity (Stock et al., 2007). Genome-wide analysis of PcG bound regions show large regions of the repressive mark of H3K27me3 but at the same time are enriched for the activation mark of H3K4me3 around the transcriptional start site (Bernstein et al., 2006; Guenther et al., 2007; Mikkelsen et al., 2007; Ku et al., 2008). These genomic regions marked with both active and repressive histone marks at the same time are termed as "bivalent domains". Genome-wide ChIP studies revealed that about 50% of bivalent domains coincide with binding sites of at least one of the core pluripotent factor indicating that these genes are poised for activation upon differentiation (Bernstein et al., 2006). Upon differentiation, these pluripotency factors are downregulated resulting in derepression of the poised genes in acoordinated manner (Fig. 3.1.2).
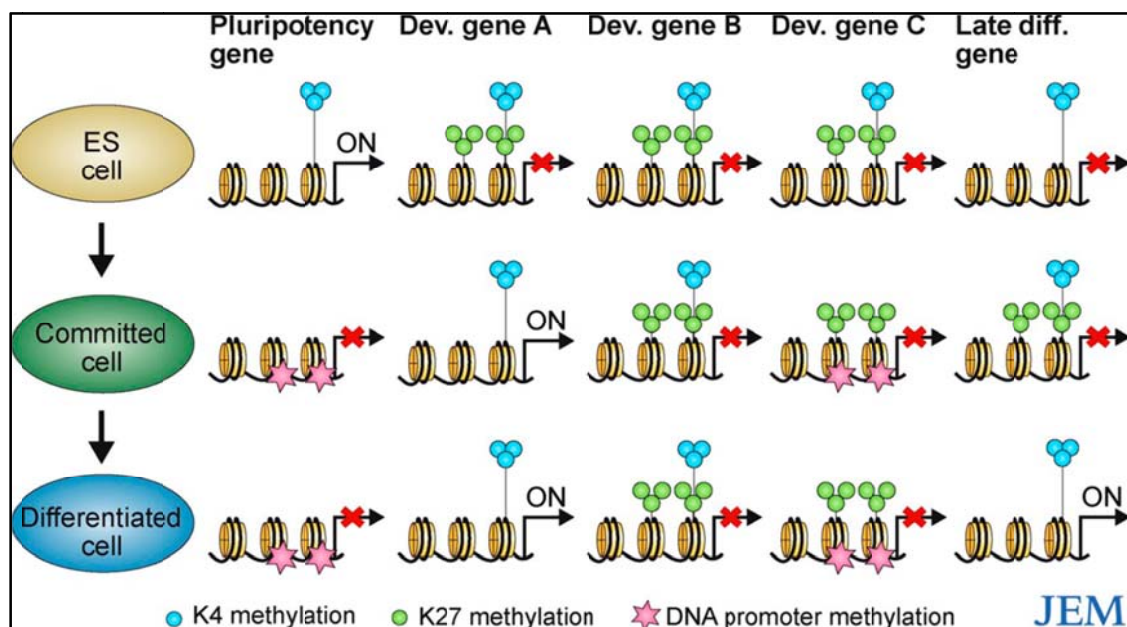
Figure 3.1.2: Epigenetic regulation of gene expression via PcG gropup of proteins. In stem cells early differentiation and development associated genes (Dev. A, B, and C) are kept in poised state by bivalent histone marks, whereas late differentiation genes are not marked by H3K27me3, but not expressed. Pluripotency genes such as OCT4 are methylated at H3K4 and expressed. Differentiation signals activate lineage specific genes that lose the repressive H3K27me3 mark (Dev. A). However, many genes preserve the bivalent domains and are not expressed (Dev. B and C); a few of these genes (e.g., those that are selectively expressed in other somatic cell lineages) also gain promoter DNA methylation during lineage commitment to ensure silencing (Dev. C). Late differentiation genes become marked by H3K27 in a manner dependent on the particular committed cell type, resulting in the formation of new bivalent domains that may be resolved in more mature differentiated cells (Reproduced from Christophersen and Helin, 2010).

Silencing of certain genes is also achieved by DNA methylation of CpG islands by various DNMTs (DNA methyltransferases). DNMTs have been shown to play important role

during embryonic development however they are not required for maintenance of pluripotency (Okano et al., 1999). TKO cells lacking all major DNMTs can be maintained in pluripotent state in culture. However they show lower differentiation potential as compared to wild type ESCs. In addition to this defect in the differentiation potential, after differentiation into EB bodies these TKO cells rapidly revert to expression profiles typical of undifferentiated ESCs unlike EB bodies derived from wild type ESCs, under pluripotency promoting conditions. Thus, while DNA methylation is dispensable for the pluripotency and initial activation of differentiation programs, it seems to be crucial for permanently restricting the developmental fate during differentiation. A recent study reveals that DNMT3b specifiaclly contributes in initiation of differentiation by downregulating stem cell proliferation (Umehara et al., 2013). During stem cell differentiation a majority of promoters maintain their methylation levels whereasa few genes exhibit small changes in DNA methylation at their promoter regions (Fouse et al., 2008; Meissner et al., 2008). Some promoters of stem cell genes get demethylated during reprogramming to enable reacquisition of pluripotency (Simonsson and Gurdon, 2004; Takahashi and Yamanaka, 2006).

3.1.3 Signaling pathways involved in regulation of pluripotency

Though mES and hES cells are characterized by the expression of core pluripotency factors they also show differences in expression of some surface markers as well as genes and pathways associated with pluripotency and differentiation. In addition to the differences in various surface markers and gene expression, mES and hES cells also differ in terms of their growth requirements. For example mES cells can be maintained in presence of LIF and serum whereashES cells require FGF2 to proliferate in an undifferentiated state.

In the following section we will discuss aboutvarious signaling pathways that have been implicated in regulation of pluripotency and differentiation of mESCs and hESCs (Figs. 3.1.3 and 3.1.4). These signaling pathways include LIF and JAK-STAT signaling, MAPK-ERK signaling, PI3K signaling, FGF signaling, TGFβ signaling and Wnt signaling.

LIF signaling acts via leukemia inhibitory factor (LIF) which is secreted by the feeder cells (mitotically inactivated embryonic fibroblast cells) used for maintaining mES cells in undifferentiated state (Smith et al., 1988; Williams et al., 1988). LIF is a member of the IL-6

cytokine family and signals through LIFR-gp130 heterodimer receptor on the cell surface, which dimerizes and gets phosphorylated upon binding of LIF to its receptor LIFR (Gearing et al., 1991; Davis et al., 1993). LIFR-gp130 activation results in recruitment of Janus-associated tyrosine kinase (JAKs; JAK1, JAK2 and Tyk2) and signal transducer and activation of transcription (STATs; STAT1 and STAT3) class of transcription factors to LIFR-gp130 receptors. This results in phosphorylation and subsequent dimerization of STATs by JAKs, which are then translocated inside the nucleus and activate many important genes responsible for maintaining pluripotency in mES cells (Boeuf et al., 1997; Niwa et al., 1998; Matsuda et al., 1999). One such important target of LIF-STAT3 signaling is c-myc, which promotes self-renewal and inhibits differentiation (Cartwright et al., 2005). Interestingly, LIF alone is unable to maintain mES cells in undifferentiated state in serum free conditonindicating that some component from the serum augments LIF in maintaining self-renewing conditions in culture. This factor was later discovered as one of the bone morphogenetic proteins (BMPs) (Ying et al., 2003). LIF and JAK-STAT signaling pathway is one of the most important signaling pathways involved in mES cells self-renewal however these pathways do not operate in undifferentiated hES cells (Humphrey et al., 2004; Sato et al., 2004). STATs remain phosphorylated and active in undifferentiated mES cells but the same is not observed in undifferentiated hES cells (Sato et al., 2004). Moreover, even stimulation of LIF and JAK-STAT pathway fail to maintain hESs in pluripotent state (Sato et al., 2004).

Mitogen-activated protein kinase/extracellular receptor kinases (MAPK/ERK) signaling pathway is active in both mES as well as hES cells but acts in acontrasting manner in these two type of cells. Burdon et al. demonstrated that differentiated mES cells exhibit high ERK activity and inhibition of ERK activation enhanced mES cell self-renewal (Burdon et al., 1999). BMP4 cooperates with LIF signaling to maintain cells in self-renewal state by inhibiting ERK activation in undifferentiated mES cells indicating that inhibiting ERK signaling might help to maintain cells in pluripotent state over multiple passages (Burdon et al., 1999;Qi et al., 2004;Lodge et al., 2005). Based on this finding Ying and colleagues modified the traditional way of culturing mES cells in "2i" culture medium to a much better and efficient "3i" culture medium which is defined by the presence of three inhibitors including the fibroblast growth factor receptor (FGFR) inhibitor, PD184352; the Erk cascade inhibitor, SU5402 and the glycogen synthase kinase-3 (GSK3) inhibitor, CHIR99021 (Ying et al., 2008). In contrast to mES cells, undifferentiated hES

cells have high basal ERK activity as compared to differentiated hES cells (Li et al., 2007). In undifferentiated hES cells ERK signaling acts downstream to FGF signaling which is important for maintaining cells in self-renewal state (Dvorak and Hampl, 2005; Kang et al., 2005; Levenstein et al., 2006). ERK signaling cooperates with the upstream PI3K/AKT signaling pathway to maintainhES cells in pluripotent state (Li et al., 2007).
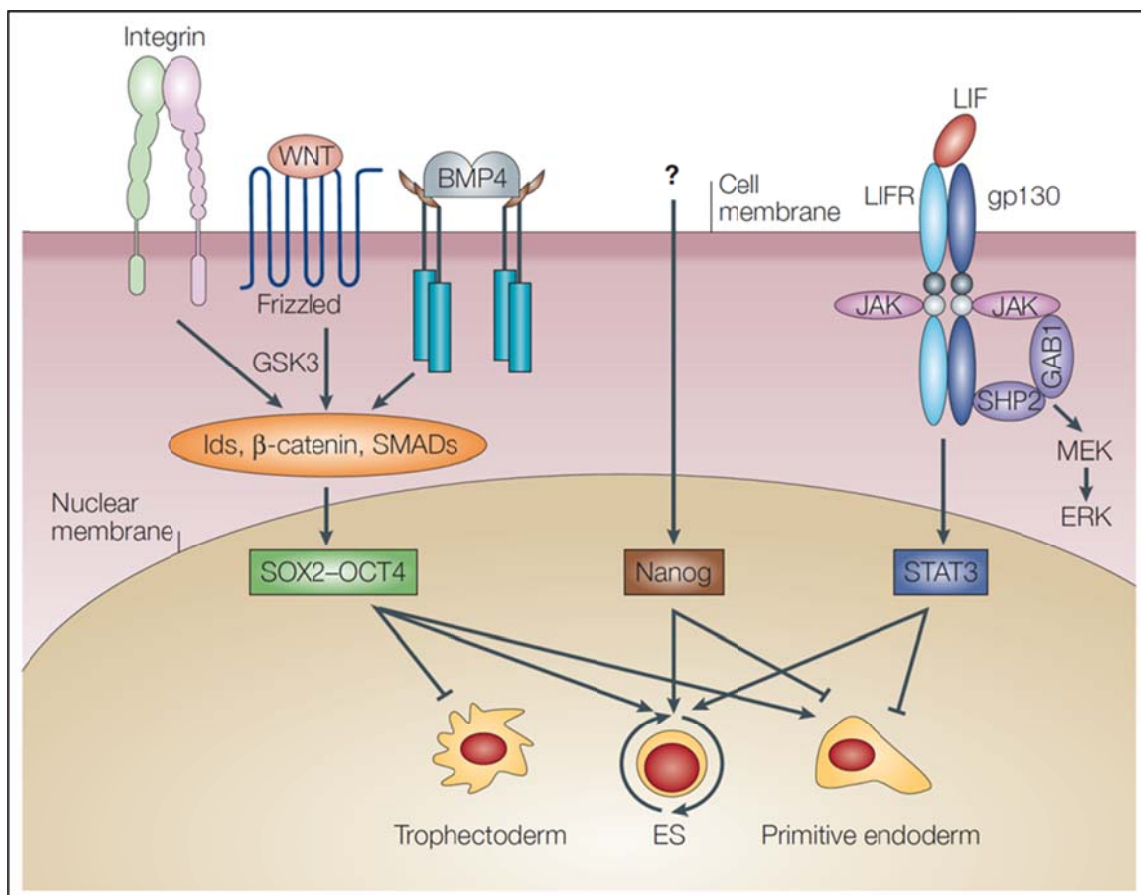
Figure 3.1.3: Summary of various signaling pathways involved in maintaining pluripotency in mES cells. Various signaling pathways operate via interaction with activation of ligand binding to cell surface receptors and converge in nuclear compartment to regulate various pluripotency and differentiation associated genes via core pluripotency factors. BMP4, bone morphogenetic protein-4; GAB1, GRB2-associated binding protein-1; gp130, glycoprotein-130; GSK3, glycogen synthase kinase-3; Id, inhibitor of differentiation, JAK, janus kinase; LIF, Leukemia inhibiting factor; MEK, mitogen-activated protein kinase (MAPK) and extracellular signal regulated kinase (ERK) protein kinase; SMAD, similar to mothers against decapentaplegic homologue; SHP2, SH2-domain-containing protein tyrosine phosphate-2; STAT3, as signal transducer and activator of transcription-3; TIK, antiphosphotyrosine immunoreactive kinase; WNT, wingless type protein (see text for details; reproduced from Michele Boiani and Hans R. Schöler, 2006).

Phosphoinositide 3-kinase (PI3K)/AKT signaling has been proposed to promote pluripotency and survival of mES cells by inhibiting MAPK pathway (Paling et al., 2004). PI3K/Akt signaling is activated in mES by exogenous factors such as insulin and LIF or by endogenously expressed ES cell expressed Ras (ERas) (Takahashi et al., 2003; Takahashi et al., 2005). Specific inhibition of PI3K activity results in differentiation of mES in presence of LIF (Paling et al., 2004) whereas expression of myristoylated Akt (active form of Akt) maintains mES cells in an undifferentiated state even in the absence of LIF (Watanabe et al., 2006). In hES cells PI3K/Akt signaling crosstalk with activin (McLean et al., 2007) and SMADs to promote self-renewal by repressing Erk and Wnt signaling (Singh et al., 2012). Absence of PI3K/Akt signaling activates Erk and Wnt pathways and reduces GSK3β activity resulting in increased β-catenin and SMAD2/3 activity which induce differentiation (Singh et al., 2012).

Transforming growth factor-β (TGF-β) pathway is another important signaling pathway, which plays important role in regulatingpluripotency in both mES and hES cells. The TGF-β superfamily contains more than 40 structurally related signaling proteins including TGF-β proteins, activin and nodal, growth differentiation factors (GDFs) and bone morphogenetic proteins (BMPs). The TGF-β signaling molecules exert their effect by binding to heteromeric complex of serine/threonine kinase receptors known as TGF-β type I (for TGF-β and Actvin A) and type II receptors (for BMPs) (Valdimarsdottir and Mummery, 2005). Activin/Nodal branch of TGF-β signaling pathway seems to be important for maintaining the pluripotent state of both mES and hES cells. In mouse embryos Nodal signaling playsan important role in maintaining epiblast stem cell pluripotency and prevents precocious neural differentiation (Camus et al., 2006; Mesnard et al., 2006). Similarly, inhibition of Nodal pathway in hES cells leads to decreased stem cell self-renewal and loss of expression of the pluripotency regulators OCT4 and NANOG (James et al., 2005; Vallier et al., 2005). Activin A, secreted by mouse embryonic feeders (MEFs), maintains undifferentiated hES culture while Nodal is produced by undifferentiated hES cells themselves (Sato et al., 2003; Beattie et al., 2005). Activin/Nodal-mediated signaling activates transcription factors SMAD2 and/or SMAD3 which further induce pluripotency related gene expression (Xu et al., 2008; Vallier et al., 2009). BMP4 signaling, the other branch of TGF-βsignaling pathway, behaves differentially in mES and hES cells. BPM4 signaling acts in combination with LIF-STAT3 signaling and activate JAK/STAT pathway to maintain mES cells in undifferentiated state in absence of serum (Ying et al., 2003). BMP4

signaling along with LIF induces phosphorylation of SMAD1, SMAD5 and SMAD8 which then upregulate expression of inhibitor of differentiation (Id) protein resulting in inhibition of differentiation (Ying et al., 2003; Gerrard et al., 2005). BMP4 signaling in combination with LIF also blocks MAPK signaling to maintain mES cells in undifferentiated state (Qi et al., 2004). However, in absence of LIF activation of BMP4 signaling promotes differentiation (Ying et al., 2003). In contrast to its role in mES cells, BMP4 signaling induces differentiation in hES towards various neuronal and trophoblast or primitive endoderm lineages (Xu et al., 2002; Itsykson et al., 2005). Inhibiting BMP4 signaling by inhibitors such as Noggin promotes self-renewal of hES in culture in presence of FGF (Pera et al., 2004; Xu et al., 2005). GDF3 is another member of TGF-β signaling pathway which acts differentially in mES and hES cells. GDF3 is expressed at high level in undifferentiated hES cells (and decreases rapidly upon differentiation) where it induces Nodal signaling and inhibits BMP4 signaling thereby promoting pluripotency (Sato et al., 2003). However in mES cells, reduction in GDF3 levelis associated with maintainence of pluripotency and inhibition of differentiation (Chen et al., 2006; Levine and Brivanlou, 2006).
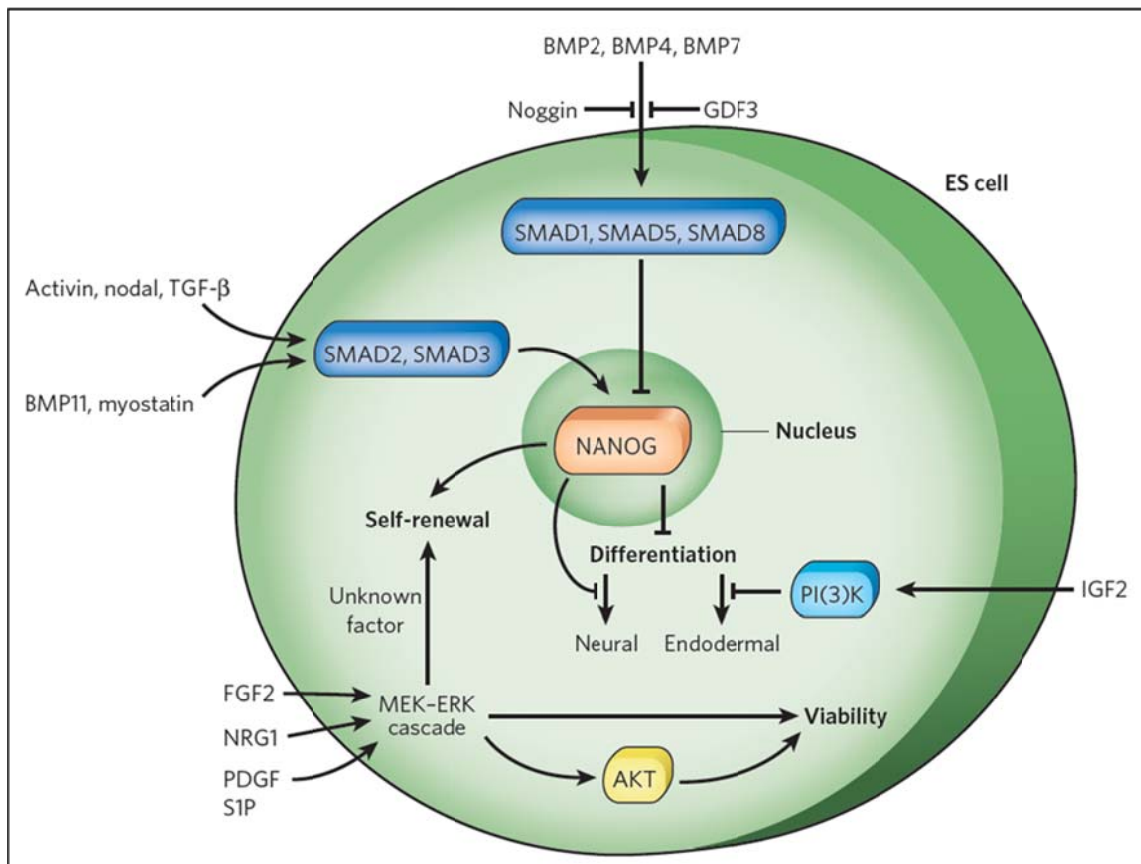
Figure 3.1.4: Summary of various signaling pathways involved in maintaining pluripotency in hES cells. Various signaling pathways converge on NANOG to regulate various pluripotency and differentiation associated genes. BMPs, bone morphogenetic proteins; GDFs, growth differentiation factors; NRG1, neuregulin 1; PDGF, platelet-derived growth factor; S1P, sphingosine 1-phosphate; TGF-β, transforming growth factor-β. (see text for details; reproduced from Martin F. Pera & Patrick P. L. Tam, 2010).

Wnt signaling is one of the recently implicated but the most controversial signaling pathways to be involved in regulating pluripotency. The canonical Wnt pathway is activated upon binding of the WNT protein to the Frizzled receptor present on the cell surface resulting in inhibition of glycogen-synthase kinase-3-beta (GSK3β) which otherwise leads to degradation of β-catenin by ubiquitnation. Inhibition of GSK3β results in stabilization and subsequent nuclear accumulation of β-catenin which results in the expression of target genes. Recent studies have implicated role of Wnt signaling in maintainance of hESCs; however, contradictory reports also exist that question whether Wnt signaling inhibits or promotes differentiation in hESCs. Many reports demonstrated that Wnt signaling acts in combination with LIF/STAT3 signaling to promote pluripotency in mES cells (Hao et al., 2006; Ogawa et al., 2006; ten Berge et al., 2011).

In undifferentiated mES cells β-catenin physically associates with OCT-3/4 and up-regulates NANOG in an OCT-3/4-dependent manner (Takao et al., 2007). Sato et al. showed that Wnt signaling is active in undifferentiated mES as well as hES cells and gets downregulated upon differentiation. Further, they also showed that cells could be maintained in an undifferentiated state by the addition of BIO in culture medium which inhibits GSK3β (Sato et al., 2004). Marson et al. showed that somatic cells can be reprogramed to induced pluripotent cells (iPSCs) by Wnt3A mediated activation of Wnt signaling (Marson et al., 2008). However, other studies claimedcontrasting results showing that though activation of Wnt signaling by BIO or Wnt3A could promote but is not sufficient to maintain the cells in pluripotent state over multiple passages (Dravid et al., 2005; Cai et al., 2007). Activation of Wnt signaling by inhibition of GSK3β in hESCs promotes differentiation towards primitive streak, endodermal and mesodermal lineage (Nakanishi et al., 2009; Bone et al., 2011). A few studies also show that Wnt signaling is important and promotes neurogenesis from mouse neural stem cell culture (Muroyama et al., 2004; Hirsch et al., 2007). A recent report from Moon group claimed that Wnt signaling is repressed by OCT4 in undifferentiated hES cells and is activated upon differentiation when OCT4 level is reduced (Davidson et al., 2012). All of the above studies indicate that the role of Wnt signaling in ES cell maintenance and differentiation is not fully clear and requires further investigation.

3.1.4 SATB family proteins and pluripotency

SATB1 and SATB2 are MAR-binding proteins that act as global gene regulators by directly binding to chromatin and regulate target gene expression *via* recruitment of various corepressors and activators. Few recent reports highlight the importance of roles of SATB family proteins in regulating mouse embryonic stem cell differentiation (Savarese et al., 2009; Agrelo et al., 2009; Asanoma et al., 2012; Nechanitzky et al., 2012). Savarese and colleagues reported for first time that SATB family proteins are expressed in mouse embryonic stem cells (mESCs) and play important and antagonistic roles in regulating pluripotency by targeting various self-renewal and differentiated associated genes. They reported that SATB1-deficient mESCs showed impaired differentiation along with increased levels of NANOG and reduced expression of Nestin and Bcl-2 even after 6 days of retinoic acid (RA) mediated differentiation. SATB2 was

upregulated in SATB1 deficient mESCs which exhibited high NANOG expression. It was shown that both SATB1 and SATB2 could bind to NANOG promoter. Overexpression of SATB2 specifically inhibited RA induced silencing of NANOG and none of the other pluripotency associated genes indicating that SATB2 specifically upregulates NANOG expression and favors self-renewal (Savarese et al., 2009). Contrary to this, another study by Agrelo et al. showed that though SATB family proteins are expressed in undifferentiated mESCs, SATB1 expression is lost completely after 3 days of RA induced differentiation whereas SATB2 shifts to a lower molecular weight; and claim levels of SATB family protein play important roles in regulating Xist mediated X inactivation (Agrelo et al., 2009). However, a recent study from Grosschedl's group showed that SATB1 expression increases during differentiation, they also claim that SATB family proteins are dispensable for X inactivation (Nechanitzky et al., 2012). SATB family proteins have been also shown to regulate stemness of trophoblast stem cells (Asanoma et al., 2012). SATB proteins are expressed at high levels in trophoblast stem cells where they activate self-renewal associated genes such as Eomes and CDX2, and rapidly decrease upon differentiation (Asanoma et al., 2012).

On the basis of above compilation of literature it is evident that SATB family proteins play important regulatory roles in differentiation and development of a wide variety of cells and tissues. The roles of SATB family proteins have been well studied in great detail in diverse cells such as T cells, neurons and osteocytes; however there are very few reports which have investigated role of the SATB family proteins in stem cell differentiation. To gain further insight into roles of SATB family proteins we chose hES cell lines over mES cell lines as observations from mES cell lines cannot be extrapolated to hES cells due to multiple reasons as discussed above. First, we studied role of SATB1 and SATB2 using a well characterized cell line model of hES cells called NT2D1, an embryonic testicular teratocarcinoma cell line (presented in chapter II) and then extended the same in hESCs with genome-wide gene expression analyses. Next, we performed ChIP-seq analysis to monitor genome-wide occupancy of SATB1 and SATB2 in undifferentiated and differentiated hES cells.

3.2 Materials and Methods

3.2.1 Antibodies and reagents

Anti-SATB1 (Cat. no. ab92307) and Anti-SATB2 (Cat. no. ab34735) antibodies used for and ChIP were purchased from Abcam (Cambridge, UK). For westerns anti-SATB1 (Cat. no. 3050S) was purchased from Cell Signaling Technology (Beverly, Massachusetts) and anti-SATB2 (Cat. no. ab51502) were purchased from Abcam (Cambridge, UK). Anti-OCT4 and anti-SOX2 were purchased from SantaCruz Biotechnologies (Santa Cruz, CA, USA). Anti-NANOG was purchased from R&D Systems (Minneapolis, USA). Anti-tubulin was purchased from Sigma-Aldrich (St. Louis, MO, USA). Wnt3a and Dkk1 were obtained from R&D Systems (Minneapolis, USA). All-trans-retinoic acid, BIO and XAV939 were obtained from Sigma-Aldrich (St. Louis, MO, USA). Lipofectamine RNAiMAX transfection reagent was purchased from Invitrogen (Carlsbad, CA, USA). siRNAs against GFP (Cat. no. sc-45924), SATB1 (Cat. no. sc-36460), SATB2 (Cat. no. sc-76456) were procured from SantaCruz Biotechnologies (Santa Cruz, CA, USA). Immunoblotssignalswere detected using Visualizer™ Western Blot Detection Kit (Millipore/Upstate, Billerica, MA, USA, Cat. no. 64-202).

3.2.2 Human embryonic stem cell culture

Human embryonic stem cell lines (hESC) HS360 were obtained from Outi Hovatta (Karolinska Institutet, Sweden) and H9 was purchased from WiCell Research Institute (Madison, WI, US). Cells were maintained on 0.1% gelatin-coated (Sigma-Aldrich, St. Louis, MO, USA) plates on mitomycin C inactivated human foreskin fibroblasts (ATCC). The ES culture media consisted of DMEM-F12 (StemCell Technologies Inc, Vancouver, Canada) supplemented with 20% serum Replacement, 2 mM glutamax, and 1% non-essential amino acids, 50 U/ml penicillin-streptomycin, 0.1 mM 2-mercaptoethanol (all from Invitrogen, Carlsbad, CA, USA) and 4 ng/ml basic fibroblast growth factor (R&D Systems, Minneapolis, USA). In feeder-free culture conditions, the cells were cultured on Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) coated plates and maintained in mTeSR1 media (StemCell Technologies Inc, Vancouver, Canada). Cells were passaged using type IV collagenase (Invitrogen, Carlsbad, CA, USA).

3.2.3 Differentiation experiments in hES cell lines

For RA induced differentiation in hESCs, cells were harvested using type IV collagenase (Invitrogen, Carlsbad, CA, USA) and plated on Matrigel coated 6-well plate containing mTeSR1 media (StemCell Technologies Inc, Vancouver, Canada) in feeder free conditions. One confluent 100mm dish was used to seed 1.5 X 6-well plate. Cells were allowed to grow for 1-2 days before addition of RA. To start the differentiation series, mTeSR1 media was replaced with ES media without FGF and was supplemented with 13.7 μM of all-trans-retinoic acid (Sigma-Aldrich, St. Louis, MO, USA). Spent media was daily replacedwith fresh ES media (without FGF) containing 13.7μM RA. At indicated days, cells were washed once with chilled 1X PBS and harvested for RNA and protein extraction. Control cells were harvested on the 0 day itself at the beginning of differentiation series.

3.2.4 RNA extraction and real time PCR

RNA was prepared from hES cells using TRI reagent Sigma-Aldrich (St. Louis, MO, USA). One μg of RNA was used for cDNA preparation per 20 μl of reaction after DNaseI (Invitrogen, Carlsbad, CA, USA) treatment according to manufacturer protocol. The cDNA was used as template for the PCR with specific set of primers and taqman probes. Changes in threshold cycle (Ct) values were calculated as follows: $\Delta Ct = (Ct_{target\ genes} - Ct_{b-actin})$ for transcript analysis. These ΔCt values were used to calculate fold change using equation as relative fold change $= 2^{-(\Delta(\Delta Ct))}$ and plotted graph for the average fold values with standard deviation from three independent experimental samples in Sigma Plot.

3.2.5 Western blotting

Cells were lysed in lysis buffer (50mM Tris-HCl pH7.5, 150mM NaCl, 0.5% Triton X-100, 5% glycerol, 1% SDS, 1mM Na_3VO_4, 10mM NaF, and 1mM PMSF). Protein concentrations were determined with Bradford Assay (Bio-Rad, Hercules, CA). Ten-15μgof protein in lysate was boiled with 6X SDS sample buffer (0.5M Tris-HCl pH6.8, 28% glycerol, 9% SDS, 5% 2-mercaptoethanol, 0.01% bromophenol blue) and lysates were electrophoresed on

a 12.5% SDS-polyacrylamide gel and transferred onto a PVDF membrane. Membranes were incubated overnight at 4°C with primary antibodies. All secondary antibodies were horseradish peroxidase-conjugated and for detection SuperSignal West Pico Chemiluminescent Substrate from Thermo Scientific (Rockford, IL, USA) or Immobilon Western Chemiluminescent HRP Substrate from Millipore (Billerica, MA, USA) were used.

3.2.6 Microarray experiment and data analysis

Total RNA was isolated from control (0 day), 3 day and 6 day RA treated H9 hES cells using TRI reagent (Sigma-Aldrich, St. Louis, MO, USA) and the RNA quality was ascertained using Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) prior to microarrayhybridization.Microarray hybridization was carried out at Genotypic Technology, Bangalore, India. RNA was purified using Qiagen's RNeasy minikit and T7 promoter based-linear amplification labeling method was used to generate labeled complementary RNA (One-Color Microarray-Based Gene Expression Analysis) using Agilent's Quick-Amp labeling Kit. Gene expression human 8 X 60k slides were used for hybridization using Agilent's in situ hybridization kit. Data analysis was performed using GeneSpring GX version 11.5 and Microsoft Excel.

3.2.7 Next generation sequencing and data analysis

ChIP-seq was performed in-house using SOLiD 4 platform and ChIP-seq data were mapped using Lifescope 2.5.1 software.Mapped read data were used as an input to establish list of loci using MACS version 2.0 (Using default parameters) (Zhang et al., 2008). Input DNA file was used as a control in all peak detection analyses. To calculate a single enrichment value for a binding site, tag density is defined as the number of tags present or overlapping in user-defined window around the reference site. The algorithm for signal enrichment calculation was used as per described in Zhang et al., 2008.

3.2.8 Visualization of ChIP regions over the genome

The distribution of SATB1 and SATB2 target sites over genome was calculated using the CEAS software (Shin et al., 2009). Briefly, CEAS estimates the relative enrichment level of ChIP regions over each chromosome with respect to the whole genome [Further details available at http://liulab.dfci.harvard.edu/CEAS/usermanual.html and (Shin et al., 2009)]. X-axis of the plot represents the actual chromosome sizes whereas Y-axis represents the peak height. Line graph was plotted to illustrate the distribution of peak heights (or scores). Distribution of gene ontology was performed using David Bioinformatics tools (http://david.abcc.ncifcrf.gov/home.jsp).

3.3 Results

3.3.1 Expression of SATB1 and SATB2 changes upon RA induced differentiation of human embryonic stem cells (hESCs)

After studying the roles of SATB family proteins we next moved to study their roles in human embryonic stem cell lines. hESCs were grown on feeder cells for regular culture and passaged on feeder plates by collagenase treatments. For differentiation experiments hESCs colonies were harvested with collagenase or accutase and seeded on matrigel coated plates avoiding minimum feeder cells contamination (Fig. 3.3.1).
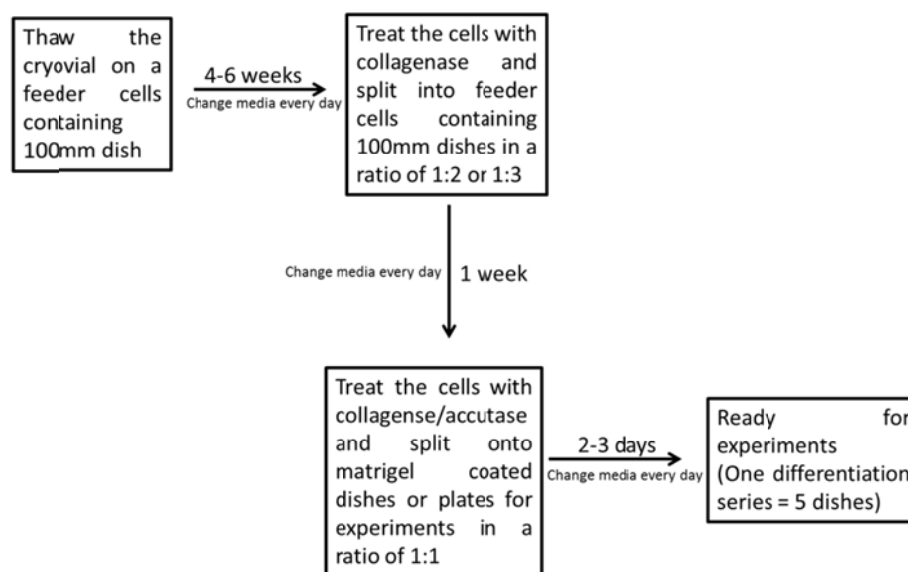
Figure 3.3.1: Work flow of hESCs culture. hESCs were cultured on feeders cells as described in "Materials and Methods". For differentiation experiments hESCs colonies were transferred to matrigel coated plates to avoid contamination of feeder cells in their culture.

We performed RA differentiation series in two hESC lines namely H9 and HS360 as mentioned in Materials and Methods. The hESC colonies were harvested with collagenase and grown on matrigel-coated plates in hES media containing 13.7μM RA and without FGF upto 7 days. The differentiation was very evident with the clear morphological change in the colony size and appearance. The differentiated colonies looked bigger, flat and translucent; also could be easily recognized by presence of multiple embryoid bodies. Media werereplaced daily with fresh media containing RA upto 7 days and cells were harvested at regular intervals of 24h for RNA and lysate preparation. Expression of SATB1 and SATB2 was analyzed at transcript as well as protein level in the 7 day long differentiation series in H9 and HS360 hESCs (Fig. 3.3.2). SATB1 was expressed at low level in undifferentiated hES cells and was upregulatedupon RA induced differentiation in both H9 as well as HS360 hESCs. In contrast, SATB2 was expressed at relatively higher level in undifferentiated hES cells and its level decreased significantly upon differentiation. We also monitored expression of the master regulators of pluripotency OCT4, SOX2 and NANOG, which decreased following the differentiation. Based on this differentiation series it was clear that SATB1 and SATB2 both express in hES cells; however, SATB1 is further induced while SATB2 is downregulated upon differentiation.
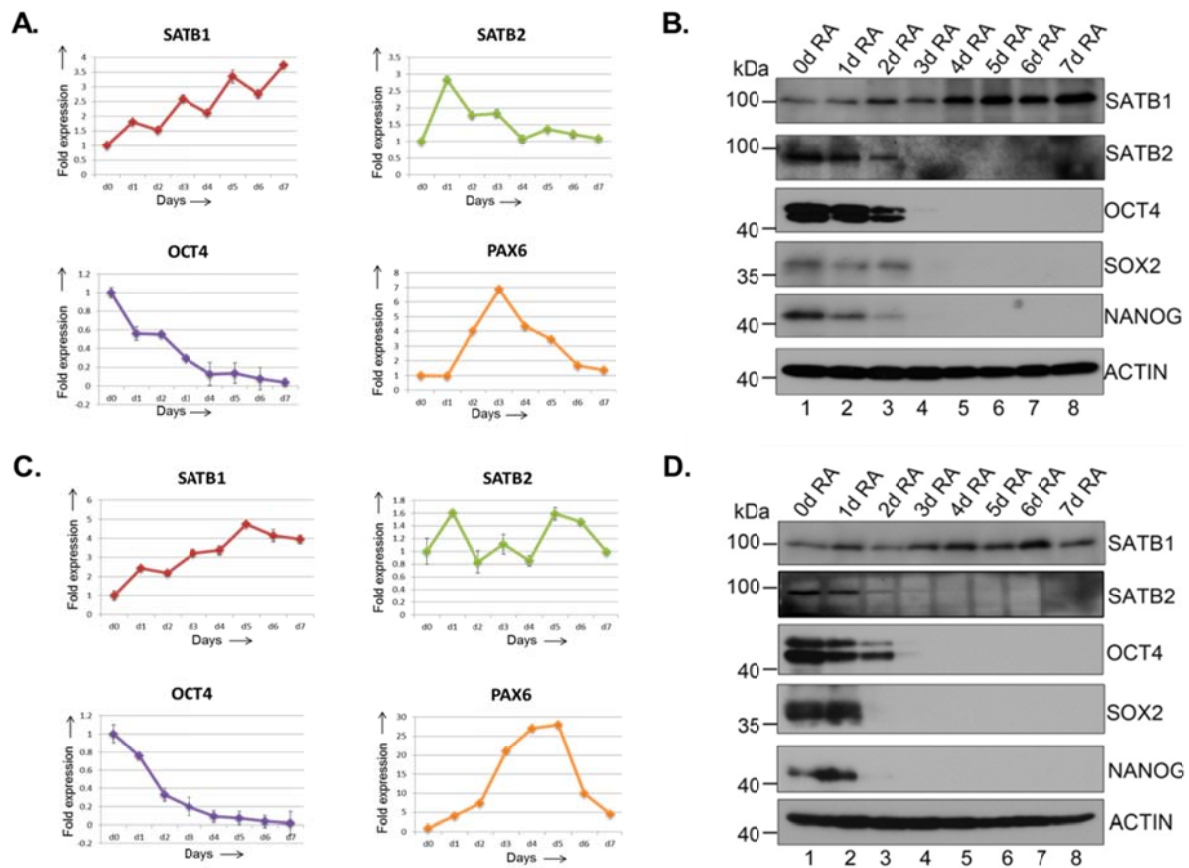
Figure 3.3.2: RA induced differentiation of hESCs results in upregulation of SATB1 and downregulation of SATB2. H9 (A and B) and HS360 (C and D) hES cells were differentiated using all-trans-retinoic acid as described in "Materials and Methods" section. Zero day or control cells were grown in mTeSR1 media and harvested at the beginning of differentiation. Differentiation series was carried out in presence of RA in hES culture media without any FGF. Cells were harvested at indicated days and frozen immediately or process for transcript and protein analysis. **(A and C)** cDNA was prepared from the RNA from each day sample and was used to perform real time RT-PCR using gene specific primers and Taqman probes. Fold expression was calculated using ΔΔCt method as described in "Materials and Methods". EF1α expression was used as endogenous control for normalization of expression data. **(B and D)** Lysates from the same differentiation series was used to detect the expression of SATB1 and SATB2 at protein level. Ten μg lysate from each day was loaded on 12.5% SDS-PAGE gel and transferred to a PVDF membrane. The membrane was horizontally cut into two pieces. Upper part was probed with anti-SATB1 and anti-SATB2 respectively after stripping. Lower part was immunoblotted with antibodies against OCT4, followed by SOX2 and NANOG and then ACTIN (lower panels) after stripping. ACTIN served as loading control. Days of differentiation have been indicated on top. Molecular mass standards (kDa) are indicated on left side.

3.3.2 Activation of Wnt signaling results in differentiation while inhibition of Wnt signaling keeps cells in pluripotent state

We showed earlier that Wnt signaling cascade is induced during RA mediated differentiation of NT2D1 human embryonic carcinoma cells (Chapter II). We also showed that

activation of Wnt signaling mimics RA induced differentiation in NT2D1 cells. Role of Wnt signaling has been controversial in regulating pluripotency of hESCs. To test the importance of Wnt signaling in regulating pluripotency of H9 hESCs we activated and inhibited Wnt signaling by using pharmacological agents and soluble recombinant proteins(Table 3.3.1).

S. No.	Reagent	Supplier and Cat. No.	Stock Conc.	Working Conc.	Target	Inhibitor/activator of the target	Effect on signaling	Reference
1	BIO	Sigma (BIO_B1686)	10mM	6uM	GSK3-β	Inhibitor	Activates	Sato et al., 2004
2	XAV939	Sigma (XAV939_X3004)	10mM	6uM	Tankyrase 1/2	Inhibitor	Inhibits	Huang et al., 2009
3	Wnt3A	R&D (5036-WNP_CF_wnt)	50ng/ul	100ng	Frizzled and LRP5/6 receptor	Activator	Activates	Mikels and Nusse, 2006
4	DKK1	R&D (5439-DK)	50ng/ul	100ng	LRP and Kremen receptor	Inhibitor	Inhibits	Logan and Nusse, 2004

Table 3.3.1: Various pharmacological agents and recombinant proteins were used to activate or inhibit Wnt signaling. Table showing detailed information of various small molecules and recombinant proteins used to activate or inhibit Wnt signaling.

We activated Wnt signaling by treating H9 hESCs with 100 ng Wnt3A or 6 μM BIO for 24h and 48h respectively. The H9 colonies where the Wnt signaling was induced exhibited multiple differentiated colonies marked with appearance of many embryoid bodies and flattened cells as compared to untreated cells (Fig. 3.3.3 A).
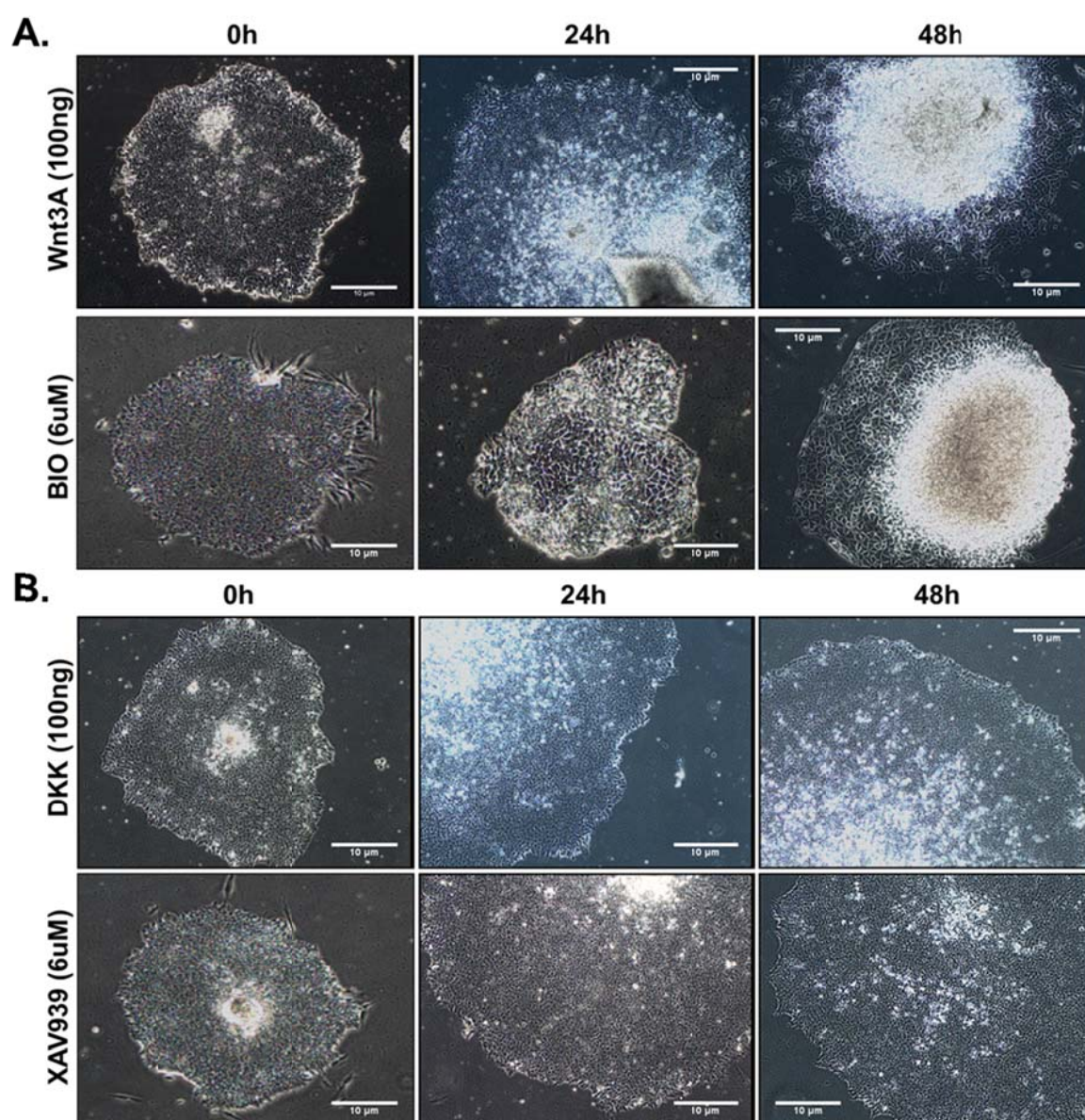
Figure 3.3.3: Effect of Wnt signaling in H9 hES cells. H9 cells were treated with Wnt signaling activators Wnt3A or BIO and Wnt signaling inhibitors DKK or XAV939 and cells were observed under a bright field microscope (10X magnification, scale bar 10 µM). hES colonies showed morphological changes upon these treatments after 24 h and 48 h respectively. (A) Cells treated with Wnt signaling activators showed induced differentiation marked with appearance of multiple embryoid bodies and flat differentiated colonies. (B) Cells treated with Wnt signaling inhibitors retained similar morphology as compared to control cells even after 48 h of treatments.

When H9 hESCs were treated with inhibitors of Wnt signaling such as DKK1 (100 ng) or XAV939 (6 µM), the number of differentiated colonies remained unchanged as compared to control cells and colony morphology was similar to that of control undifferentiated colonies even

after 48 h of treatment (Fig. 3.3.3 B). These results suggest that induction of Wnt signaling results in differentiation while cells can be maintained in undifferentiated culture by addition of Wnt signalinginhibitors to the culture media.

3.3.3 Gene expression profiling upon RA induced differentiation of human embryonic stem cells (hESCs)

RA treatment induces differentiation in hESCs; however it is not known how the global gene expression profile changes upon differentiation. To gain insight about the genes affected upon differentiation we differentiated H9 hES cells for 3 days and 6 days and then isolated total RNA. Differentiation was confirmed by analyzing the expression of pluripotency markers namely OCT4, SOX2 and NANOG, which are downregulated upon differentiation. Total RNA from control undifferentiated, 3 days and 6 days differentiated H9 hESCs was hybridized to human 60 K array using single color labeling and separate hybridizations were performed.Analysis of microarray data revealed large number of dysregulated genes upon differentiation. Gene expression profiling revealed a total of 2804 genes upregulated and 2832 genes downregulated upon differentiation. We then specifically focused on genes associated with maintenance of pluripotency and differentiation and found out a number of genes affected; top 20 genes from which have been depicted in Fig. 3.3.4 (B and C). Expression of multiple genes associated with pluripotency such as Eomes, NANOG were downregulated whereas genes associated with differentiation such as GATA2, FOXA1, GDF15 were upregulated (Fig. 3.3.4).

A.

Samples	Up	Down
H9 3d RA	2804	2832
H9 6d RA		

B.

GeneName	Fold Change		
	H9 Control	_H9 3d RA	H9 6d RA
SYNE1	0.000	12.367	1.148
FOXA1	0.000	4.369	7.427
LOC389333	0.000	3.244	2.170
HOXB5	0.000	2.976	4.253
GATA2	0.000	2.904	2.046
IFI16	0.000	2.894	2.200
FLT3	0.000	2.804	1.558
MAL	0.000	2.623	3.259
NTRK2	0.000	2.511	3.018
SEMA3E	0.000	2.408	1.243
MLPH	0.000	2.215	1.102
SEMA4G	0.000	2.151	1.597
SLIT1	0.000	2.114	1.715
GDF15	0.000	2.094	0.819
MITF	0.000	2.016	2.967
ROBO2	0.000	1.932	1.882
CAND1	0.000	1.851	1.290
MGP	0.000	1.824	1.142
SEMA6D	0.000	1.807	1.777
NTRK3	0.000	1.703	1.320

C.

GeneName	Fold Change		
	H9 Control	_H9 3d RA	H9 6d RA
EOMES	0.000	-5.886	-4.968
MSGN1	0.000	-5.341	-2.527
GDF3	0.000	-4.956	-8.049
IRGM	0.000	-3.282	-3.438
LECT1	0.000	-3.095	-4.237
RHOH	0.000	-3.023	-3.015
VAX1	0.000	-2.993	-2.494
PAX8	0.000	-2.991	-2.176
SFRP2	0.000	-2.952	-3.506
NANOG	0.000	-2.880	-3.293
DLX2	0.000	-2.829	-1.693
FIGF	0.000	-2.671	-2.359
DAPL1	0.000	-2.569	-2.124
TLL2	0.000	-2.559	-3.332
DMRTB1	0.000	-2.517	-2.730
ZAP70	0.000	-2.500	-3.469
DAZL	0.000	-2.490	-4.051
PCSK9	0.000	-2.440	-2.724
SIM1	0.000	-2.416	-1.841
GPM6B	0.000	-2.378	-2.993

Figure 3.3.4: Differential gene expression profiling of H9 hES cells differentiated using RA treatment. H9 hES cells were differentiated for 3 days (3d RA) and 6 days (6d RA) using all-trans-retinoic acid as described in "Materials and Methods" section. Zero day or control cells were grown in mTeSR1 media and harvested at the beginning of differentiation. Differentiation series was carried out in presence of RA in hES culture media without any FGF. RNA was extracted and gene expression profiling was performed using microarrays (60 K) as described in "Materials and Methods" section. (A) Total number of dysregulated genes upon RA induced differentiation as compared to control. (B) List of top 20 upregulated and (C) List of top 20 downregulated genes associated with pluripotency and differentiation. Fold change has been shown as compared to control or undifferentiated cells.

3.3.4 Genome-wide occupancy analysis upon RA induced differentiation of human embryonic stem cells (hESCs) revealed a number of genomic targets bound by SATB1 and SATB2

SATB1 and SATB2 bind directly to the DNA and recruit many other components of chromatin remodeling machineries to their genomic targets (Cai et al., 2003; Yasui et al., 2002; Kumar et al., 2005; Gyorgy et al., 2008; Zhou et al., 2012). To obtain insight into the repertoire

of genomic sites targeted by SATB1 and SATB2, we performed ChIP-Seq analysis in H9 hESCs at various timepoints of differentiation which included 0 day or control cells, 3days and 6 days RA treated cells. ChIP-Seq analysis revealed a number of genomic loci as well as promoters bound by SATB1 and SATB2 *in vivo* at various stages of differentiation as well as in control cells (Fig 3.3.5 A). A comparison of total SATB1- and SATB2-bound genomic sites revealed that SATB1-bound sites were significantly decreased at day 3 of RA treatment (Fig. 3.3.5 B). We compared SATB1- and SATB2-bound sites independently at various stages of differentiation, which indicated that SATB1 and SATB2 bind to multiple targets in stage-specific manner (Fig. 3.3.5 C and D). Gene ontology analysis of SATB1-bound sequences revealed enrichment of specific GO terms including neuronal activities, immunity and defense indicating important roles of SATB1 in regulating genes involved in these processes. Likewise, SATB2-bound targets were involved in notch signaling, proteolysis, neuronal activities, synaptic transmission and ligand mediated signaling.
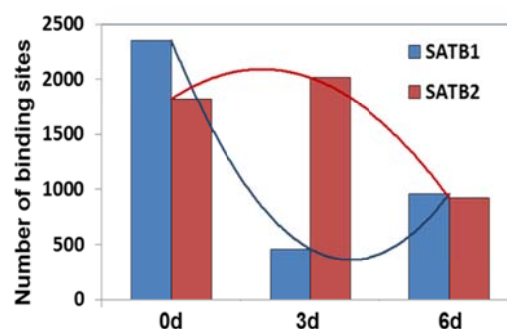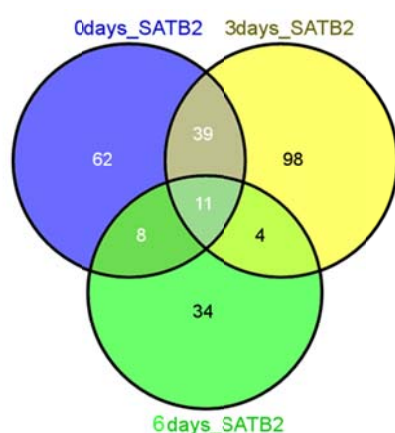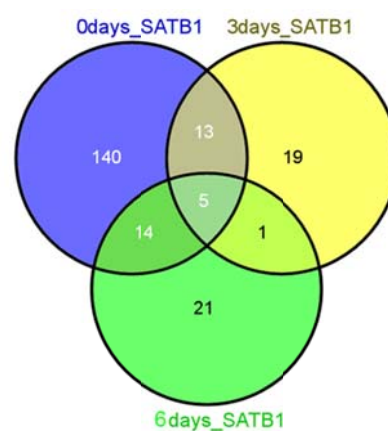
Figure 3.3.5: Genome-wide occupancy analysis of SATB1 and SATB2 from H9 hES cells differentiated using RA treatment. ChIP-Seq was performed using chromatin from 0 day, 3 day and 6 day RA treated cells and data was analyzed as described in "Materials and Methods" section. Analysis revealed a number of genomic targets including promoters bound by SATB1 and SATB2 at all 3 stage of differentiation (A). SATB1-bound sites were significant decreased upon day 3 of RA induced differentiation (B). Venn diagram showing specific and common genomic targets (around +/- 5kb from TSS) bound by SATB1 (C) or SATB2 (D) at all the 3 stages.

3.3.5 SATB1- and SATB2-bound sites are overrepresented on X and Y chromosomes

We analyzed the distribution of SATB1- and SATB2-bound sites around TSS and found that it was distributed widely at upstream and downstream of TSS indicating that both of them have genome-wide binding sites and not specific only to TSS or immediate upstream of TSS (Fig. 3.3.6).
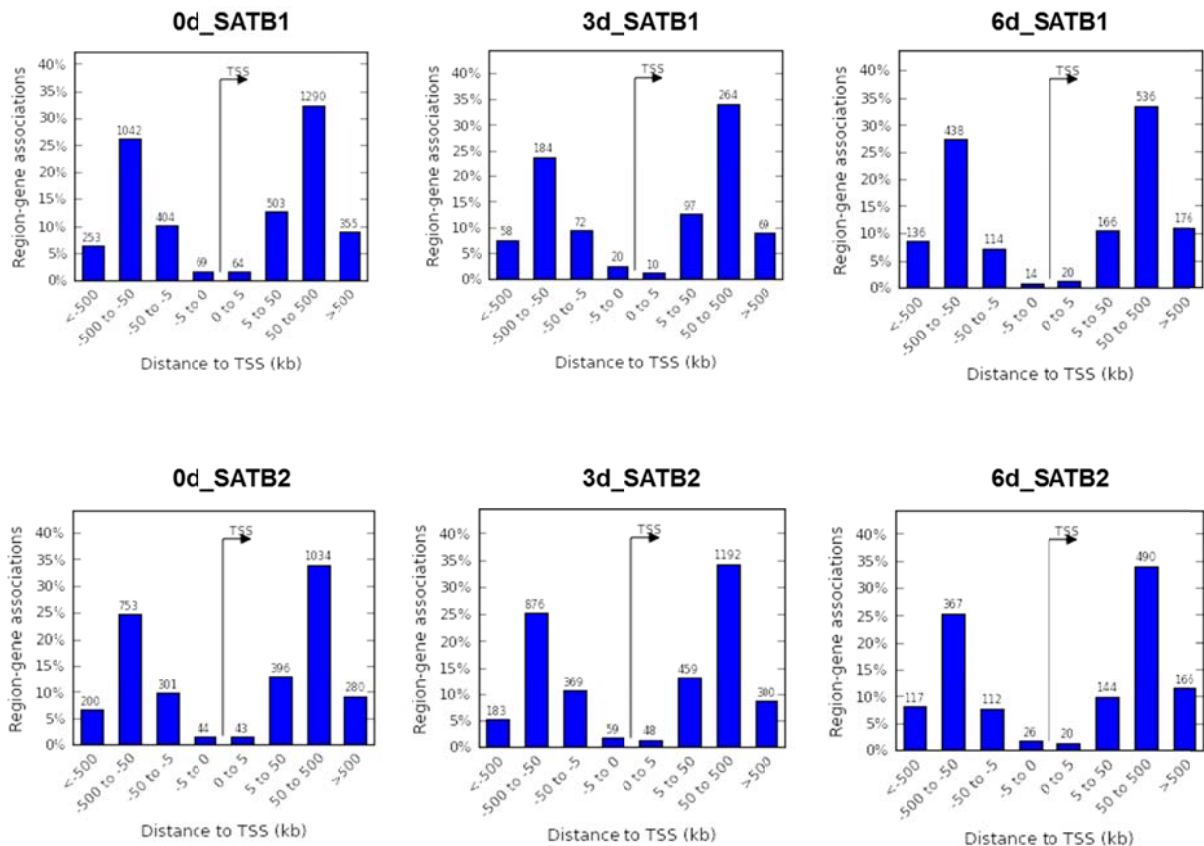
Figure 3.3.6: Genome-wide occupancy analysis of global SATB1- and SATB2-bound sequences around TSS in H9 hES cells differentiated using RA treatment.. ChIP-Seq was performed from 0 day, 3 day and 6 day RA treated cells and data was analyzed as described in "Materials and Methods" section. The data revealed that SATB1 and SATB2 bind to multiple genomic loci, which are distributed around TSSs across the genome. GO term analysis of these SATB1- and SATB2-bound sequences revealed majority of the processes involved in sexual reproduction.

Interestingly, when we analyzed the gene ontology of biological processes associated with these genome-wide SATB1- and SATB2-bound loci, we came across a number of biological processes majority of which were involved in sexual reproduction for example gamete production, development of primary male sexual characteristics, male sex differentiation, male gonad development and sex determination. This analysis prompted us to search for SATB1 and SATB2 targets across different chromosomes. Interestingly, corroborating the GO term data we found a large number of SATB1- and SATB2-bound sites enriched on chromosome X and Y (Fig. 3.3.7). SATB1- and SATB2-bound sites were overrepresented on X and Y chromosomes than autosomes indicating that it might have prominent role in sex-linked pathways (Fig. 3.3.7). These data indicate that SATB1 and SATB2 might play hitherto unidentified role in reproductive

functions. Specifically, SATB1 and SATB2 might be involved in regulation of gamete production, development of male sexual features including gonad development.
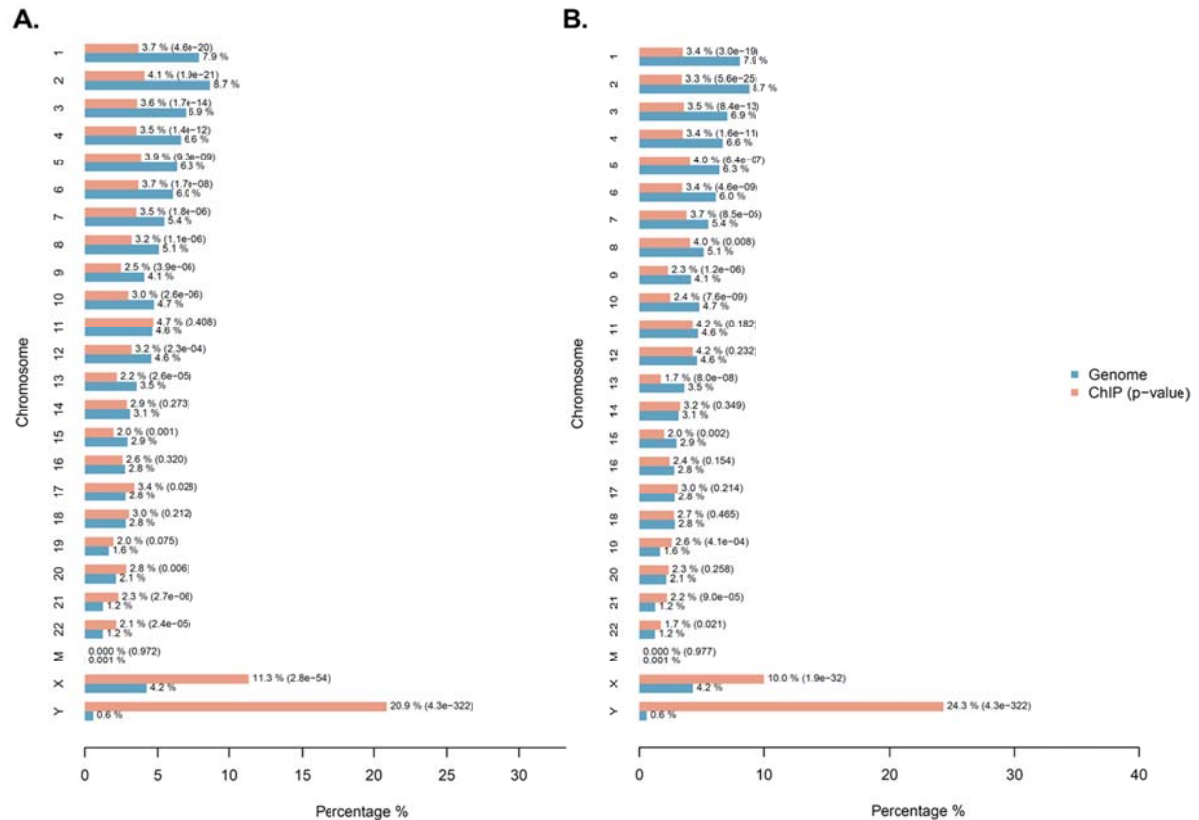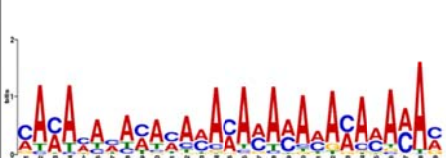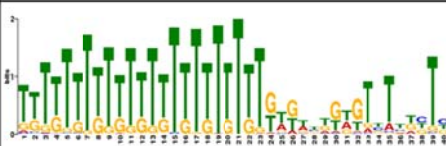
Figure 3.3.7: Visualization of ChIP regions over the genome for SATB1 and SATB2 enrichment revealed specific enrichment of SATB1 and SATB2 on X and Y chromosomes. Relative enrichment level of ChIP regions over each chromosome with respect to the whole genome for (A) SATB1 and (B) SATB2 occupancy was analyzed using CEAS software. The X-axis of the plot represents the actual chromosome sizes whereas y-axis represents the peak height. The line graph illustrates the distribution of peak heights (or scores).

3.3.6 Identification of consensus sequences for SATB1 and SATB2 specific and common target sites

SATB1 and SATB2 are two closely related MAR-binding proteins which regulate gene expression and higher order chromatin structure. In order to gainfurther insights into the DNA sequence(s) bound by these two proteins *in vivo* we screened SATB1 and SATB2 bound genomic regions for consensus and also looked for known transcription factor (TF) binding motif using JASPAR database (Portales-Casamar et al., 2010). SATB1 and SATB2 specific binding sites were selected and co-occurrence of each other was checked on these sites (Fig. 3.3.8).

Summit of the binding site generated using MACS version 2.0 (Using default parameters) (Zhang et al., 2008) was considered as center of the peak. As computational restrictions of MEME allowed only a limited number of base pairs to be analyzed at a time, 100-200 highest (SATB1 and/or SATB2) occupancy peaks were analyzed. Hundredbp DNA sequences around the center of the peaks were extracted using Fetch Sequence tool from Galaxy web server (https://main.g2.bx.psu.edu/). De novo motif discovery was performed using motif-based sequence analysis tools, MEME (Bailey et al., 2009). The consensus sequence was compared to known motif databases such as JASPAR. We first assessed which motifs were enriched with SATB1 and SATB2 specific and common target regions. The unbiased de novo motif finder MEME identified ATCT repeat as consensus sequence of SATB1 specific target sites with an E value of $6.8e^{-182}$. GATA3 and PBX1 transcription factors motifs were enriched on SATB1 consensus sequence. In similar way, unbiased de novo motif finder MEME identified A/T repeat sequence in the center of the all the SATB2 specific target sites (203/203). Motif analysis of common target sites of SATB1 and SATB2 revealed a consensus sequence which was more similar to that of SATB2. We also observed the GO term associated with SATB1 and SATB2. SATB1 motif was mainly found associated with pathways such as sequence-specific DNA binding, signal transduction, G-protein coupled receptor protein signaling pathway, calcium ion binding while SATB2 motif showed novel functions associated with olfaction (Summarized in Fig. 3.3.8).

Protein	Consensus Motif	Occurrence (E-value)	Associated GO terms	Associated transcription factor
SATB1		42 (6.8e-182)	• Sequence-specific DNA binding • Signal transduction • G-protein coupled receptor protein signaling pathway integral to plasma membrane • calcium ion binding	GATA3 PBX1
SATB2		203 (1.1e-596)	• Olfactory receptor activity • Sensory perception of smell • G-protein coupled receptor protein signaling pathway	ZBTB3 MET31
SATB1 and SATB2		166 (9.8e-1179)	• Olfactory receptor activity • Sensory perception of smell	SRF AZF1

Figure 3.3.8: Consensus sequence binding sites for SATB1 and SATB2 specific and common target sites. SATB1 and/or SATB2 bound DNA sequences were analyzed for presence of consensus motif (Column 2). GO pathways associated with found motif are shown in column 4. Consensus motifs found for SATB1 and/or SATB2 exhibited co-occurrences with motif of other transcription factors also which are showed in column 5.

3.4 Discussion

SATB family proteins have been known to play important roles in development and differentiation of cells of various lineages (Cai et al., 2003; Dobreva et al., 2006; Kumar et al., 2007; Alcamo et al., 2008; Britanova et al., 2008; Balamotis et al., 2012; Zhou et al., 2012). However, the roles of SATB1 and SATB2 in regulating pluripotency and differentiation have been controversialas discussed in few recent reports (Agrelo et al., 2009; Savarese et al., 2009; Asanoma et al., 2012; Nechanitzky et al., 2012). Savarese et al. showed that SATB1 and SATB2 are expressed in undifferentiated mES cells. SATB1 induces differentiation by negatively regulating NANOG expression whereas SATB2 promotes pluripotency by upregulating NANOG expression (Savarese et al., 2009). However, Agrelo et al. claimed that SATB1 is expressed in undifferentiated mES cells whereas SATB2 is expressed predominantly in differentiated mES

cells (Agrelo et al., 2009). All of these results were based on experiments performed in using mES cells, which behave very differently from hES cells (see introduction for details). Moreover, these results are contradictory and do not provide clear insights into role of SATB family proteins in regulating pluripotency and differentiation. To elucidate the role of SATB family proteins in hES cells, we used retinoic acid (RA) induced differentiation series in two hES cell lines namely H9 and HS360. RA differentiation series in both H9 and HS360 cells revealed changes in expression of SATB family proteins at both transcript and protein level. Both hES cell lines exhibited high expression of SATB2 in undifferentiated state, which is lost upon differentiation. In contrast, SATB1 is expressed at low level in undifferentiated cells and is induced upon differentiation. We could detect simultaneous expression of SATB1 and SATB2 at onset of differentiation. We propose that SATB1 and SATB2 might form heterodimer during this window, which may play important role in differential regulation of genes involved in onset of differentiation. It is worth mentioning here that SATB1/2 expression pattern in hES varies significantly from NT2D1 cells (Chapter II). NT2D1 cells exhibit moderate expression of SATB1 and SATB2 and both are upregulated upon differentiation. However, undifferentiated hES cells exhibit differential expression of SATB1 and SATB2. Unlike NT2D1 cells, hES cells show enhanced SATB1 expression and reduced SATB2 level upon differentiation. This difference of SATB1/2 expression could be attributed to origin of NT2D1, which is a human testicular carcinoma cell line and does not represent a true pluripotent human embryonic stem cell.

Role of Wnt signaling in regulating self-renewal and differentiation has been debatable as elaborated in chapter II and published literature (Sato et al., 2004; Dravid et al., 2005; Cai et al., 2007; Bone et al., 2011). We observed that activation of Wnt signaling in hESCs resulted in induced differentiation whereas inhibition of Wnt signaling maintained more undifferentiated colonies in culture. These experiments suggest that Wnt signaling is not important for maintaining pluripotency and rather it is involved in differentiation. Here, we would also like to suggest that inhibition of Wnt signaling could promotemaintenance of ES cells in a self-renewing pluripotent state. Our observation is supported by a recent report by Davidson et al. wherein the authors claim that Wnt signaling is activated during differentiation of hES cells. Furthermore, they demonstrated that OCT4 keeps Wnt signaling in repressed state in undifferentiated hES cells and this repression is lost upon differentiation when OCT4 level is

reduced (Davidson et al., 2012). Interestingly, SATB1 has been shown to interact with β-catenin and coordinate gene expression in Wnt-dependent manner in T cells (Purbey et al., 2009; Notani et al., 2010). Similarly, SATB1 may crosstalk with Wnt signaling pathway during hES cell differentiation and play important role to regulate gene expression in Wnt dependent manner.

Gene expression profiling revealed that a large number of genes were deregulated during differentiation. These genes are involved in importantbiologicalprocesses such as cancer, metabolic pathways, axon guidance, neuroactive ligand-receptor interaction, cytokine-cytokine receptor interaction, focal adhesion, olfactory transduction, retinol metabolism etc. The dysregulated genes alsoincluded those involved in many signaling pathways like Wnt signaling, TGF-β signaling, MAPK signaling, Notch signaling etc.

Genome-wide occupancy analysis by ChIP-Seq at similar stages of differentiation revealed multiple genomic loci bound by SATB1 and SATB2. SATB1 and SATB2 seem to have differential binding at various stage of differentiation and might play role in differential gene expression during differentiation. Occupancy of SATB1 and SATB2 was observed at promoters of multiple genes involved in notch signaling, neuronal activities, synaptic transmission, immunity and defense and ligand mediated signaling. Surprisingly, gene ontology (GO) analysis of all SATB1- and SATB2-bound sequences (irrespective of promoters) revealed multiple sexual reproduction associated pathways. Corroborating this, SATB1 and SATB2 ChIP-Seq revealed significantly enriched peaks on X and Y chromosomes as compared to other chromosomes. We also analyzed the genomic region bound by SATB1 and SATB2 for presence of consensus motif, which revealed specific motifs for SATB1 or SATB2 alone as well as SATB1 and SATB2 together. Motif analysis identified repeat ATCT as SATB1 consensus binding motif which was also enriched for GATA3 and PBX1. Interestingly, GATA factors have been shown to play important role in differentiation of mES cells (Fujikura et al., 2002) and have been shown to be positively regulated by SATB1 upon Wnt mediated differentiation of Th2 thymocytes (Notani et al., 2010). Based on the motif search analysis and these reports we hypothesize that SATB1 and GATA3 might coordinate gene expression during hES differentiation by targeting a common DNA binding motif. SATB2 motif analysis revealed an A-rich motif which was also found to be enriched in case of ZBTB3 and MET31.

Collectively, we show that the two members of the SATB family chromatin organizer proteins exhibit contrasting expression profiles during differentiation of human ES cells. SATB1 is upregulated whereas SATB2 is downregulatedduring RA induceddifferentiation of hES cells. Furthermore, activation of Wnt signaling induces differentiation whereas inhibition of Wnt signaling maintains hES cells in undifferentiated state. Gene expression profiling and ChIP-Seq data analysis revealed many novel targets of SATB1 and SATB2 upon differentiation. One among such class are genes involved in sexual reproduction and male gonad development which is positively correlated with the highest enrichment of SATB1 and SATB2 on X and Y chromosomes. These set of findings provide novel insights into the regulation of gene expression in hES cells especially during differentiation. Our data provide further insights into the differential targets of SATB1 and SATB2 and also throw light on the differential roles of these to global regulators towards maintenance of pluripotency and induction of differentiation. Our data also suggests that NT2D1 cells do not represent a true model system for studying properties of hES cells. Finally, our data also provides further evidence towards the role of Wnt signaling in differentiation of hES cells.

3.5. References

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Appendices

Appendix 1:

List of oligonucleotides used for RT-PCR (Sections 1.3.10, 2.3.1, 2.3.3, 2.3.4, 2.3.5 and 3.3.1).

Description	Sequence (5' to 3')	No. of nucleotides	GC%	Tm	Product size (bp)
hSATB1 F	ACCAGTGGGTACGCGATGA	19	57.9	65.3	72
hSATB1 R	TGTTAAAAGCCACACGTGCAA	21	42.9	67	
hSATB2 F	CCAGAGCACATTAGCCAAAGA	21	47.6	64.9	67
hSATB2 R	TGTGCTATTTACAATGGATGAAATC	25	32	66	
hEF1a F	CTGAACCATCCAGGCCAAAT	20	50	66.3	59
hEF1a R	GCCGTGTGGCAATCCAAT	18	55.6	65.6	
hOct4 F	AGCAAAACCCGGAGGAGT	18	55.6	63.1	114
hOct4 R	CCACATCGGCCTGTGTATATC	21	52.4	64.5	
hSox2 F	TGCTGCCTCTTTAAGACTAGGAC	23	48	59	75
hSox2 R	CCTGGGGCTCAAACTTCTCT	20	55	60	
hNanog F	CCTGAACCTCAGCTACAAACAG	22	50	63.8	94
hNanog R	GCTATTCTTCGGCCAGTTGT	20	50	63.8	
hPAX6 F	GGCACACACACATTAACACACTT	23	43	59	71
hPAX6 R	GGTGTGTGAGAGCAATTCTCAG	22	50	59	
hNKD2 F	GTGGCTGACAGGAGGTTGTC	20	60	60.72	93
hNKD2 R	GTTCTCGTCCACGCAGTAGG	20	60	60.83	
hAPC F	GTTGCCAGGCAGACATCC	18	60	60	125
hAPC R	CGGCCAGGAGACTGTATAGG	20	60	60	
hAxin2 F	GTCTCCAAGCAGCTGAAGCC	20	60	61.3	118
hAxin2 R	CCTCCATCACCGACTGGATC	20	60	60.9	
hBDNF1 F	AGGGTTGCAGGTCCACAC	18	61.11	59.5	98
hBDNF1 R	CGTGGAGGTACACAGCACAG	20	60	60.37	
hDvl1 F	CGAGCTTGAGTCCAGCAGC	19	63.2	60	90 bp
hDvl1 R	CTGGATGAGGTGCTCTGCTC	20	60	59	
hCyclin D1 F	GCTCCTGTGCTGCGAAGTG	19	63.2	60.9	129
hCyclin D1 R	CATTTGAAGTAGGACACCGAGG	22	50	60.9	
h CtBP1 F	GGGACTGCACAGTGGAGATG	20	60	60	149
h CtBP1 R	GTCCTCCCTGGTGAGAGTGA	20	60	60	
hDKK1 F	GCGGCACTGATGAGTACTGC	20	60	60	114
hDKK1 R	GGCAGCACATAGCGTGACG	19	63	62	
hGREM2 F	GGAGAGATGGCAGCACCAG	19	63.2	59.4	100
hGREM2 R	GCGCTTGCACCAGTCACTC	19	63.2	60.7	
hCul4A F	CAGGTGTCCCTCTTCCAGAC	20	60	59.68	125
hCul4A R	AGGGACTGCAGCGTTCTG	18	61.11	60.14	

Appendix 2:

List of oligonucleotides used for ChIP-PCR (Sections 1.3.11).

Description	Sequence (5' to 3')	No. of nucleotides	GC%	Tm	Product size (bp)
hSATB1 distal SBS f1	AGGCACGAAAAATCATCCAC	20	45	59.94	187
hSATB1 distal SBS r1	TTTTCCTCTGATTGCTGCAC	20	45	59	
hSATB1 proximal SBS f2	TTATGTCCCTGGTTTCTGCTG	21	47.62	60.12	263
hSATB1 proximal SBS r2	GCCTGACATCTGCTTCTTCC	20	55	59.96	
hSATB2 distal SBS f1	GAGGGATACAAAACGAAACCAA	22	40.91	60.22	265
hSATB2 distal SBS r1	TATGTCCGGTGTGGGTTTTT	20	45	60.09	
hSATB2 proximal SBS f2	GGGTGCAGAATCTGAAAACAA	21	42.86	60.1	220
hSATB2 proximal SBS r2	CAACAGCATGATTTGCAGGA	20	45	60.81	

Publications

Research papers published:

1. Purbey PK, **Singh S**, Kumar PP, Mehta S, Ganesh KN, Mitra D, Galande S. PDZ domain-mediated dimerization and homeodomain-directed specificity are required for high-affinity DNA binding by SATB1. *Nucleic Acids Res*. 2008, 36:2107-22.

2. Purbey PK, **Singh S**, Notani D, Kumar PP, Limaye AS, Galande S. Acetylation-dependent interaction of SATB1 and CtBP1 mediates transcriptional repression by SATB1. *Mol Cell Biol*. 2009, 29:1321-37.

Manuscriptsubmitted:

Singh S, Purbey PK, Galande S. Special AT-rich Binding proteins SATB1 and SATB2 interact in vivo to form heterodimer and regulate gene expression.

Manuscript under preparation:

Singh S and Galande S. Role of SATB family proteins in gene regulation during retinoic acid mediated differentiation of NT2D1, an embryonic carcinoma cell line.

Singh S, Karmodiya K, Jangid R, Rahkonen N, Lund R, Lahesmaa Rand Galande S. Differential occupancy of SATB1 and SATB2 on their genomic targets during human embryonic stem cell differentiation.