Regulation of Wnt/Wg signaling pathway by chromatin organizer SATB1

Of the degree of

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By

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Dedicated to my family……

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Certified that the work incorporated in the thesis entitled "Regulation of Wnt/Wg signaling pathway by chromatin organizer SATB1" Submitted by RL Praveena 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

aa	Amino acid(s)
ACF	ATP-Utilizing Chromatin Assembly And Remodeling Factor Complex
ACF1	ATP-Dependent Chromatin Assembly Factor Large Subunit-1
AP	Anteroposterior boundary
ATF4	Activating Transcription Factor 4
ATP	Adenosine Triphosphate
Bcl2	B-Cell Leukemia/Lymphoma 2
BEAF	Boundary element associated factor
BIO	6-Bromoindirubin-3′-Oxime
β–ME	β–mercaptoethanol
BMP	Bone Morphogenetic Protein
BMSCs	Bone Marrow Stromal Cells
bp	base pair
β–ME	β–mercaptoethanol
BMP	Bone Morphogenetic Proteins
BURs	Base Unpairing Regions
CBP	CREB-Binding Protein
CD	Cut repeat containing Domain
Cdk	Cyclin-Dependent Kinase
CHD	Chromodomain Helicase DNA binding protein 1
ChIP	Chromatin Immunoprecipitation
ChIP-seq	Chip-Sequencing
CHRAC	Chromatin-Accessibility Complex
CK	Casein Kinase
CNS	Central Nervous System
CoIP	Co-Immunoprecipitation
CREB	cAMP Response Element Binding Protein
CSBS	Consensus SATB1 Binding Sequence
Ct	Threshold Cycle
CtBP1	C-terminal Binding Protein 1
CyO	Curly wings
DAPI	4',6-Diamidino-2-Phenylindole
Dkk1	Dickkopf Homolog 1
Dlg	Discs-Large septate junction protein
DMEM	Dulbecco'S Modified Eagle'S Medium
DNMT	DNA (cytosine-5)-Methyl Transferase
dpp	decapentaplegic
Dsh	Dishevelled
DV	Dorsoventral boundary
Dve	Defective proventriculus
Dvl	Dishevelled
DTT	Dithiothreitol
EDTA	Ethylene Di-amine Tetra-Acetic acid
EGFR	Epidermal growth factor
EGTA	Ethylene Glycol Tetra-Acetic acid
EMSA	Electrophoretic Mobility Shift Assay
ERK	Extracellular Receptor Kinases

FBS	Fetal Bovine Serum
FCS	Fetal Calf Serum
FGF	Fibroblast Growth Factor
FGFR	Fibroblast Growth Factor Receptor
FL	Full Length
Fz	Frizzled
GATA	Glutamyl-tRNA Amido Transferase Subunit-A
GMR	Glass multiple Reporter
GO	Gene Ontology
gp130	glycoprotein-130
GPCR	G-protein coupled Receptor
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
Hh	Hedgehog
HIV	Human Immunodeficiency Virus
HMG-1	High Mobility Group Protein-1
IB	Immunoblot
IFNγ	Interferon γ
IgH	Immunoglobin Heavy Chain
IL	Interleukin
IL-2Rα	Interleukin 2 Receptor alpha
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
mM	Milli molar
M	Molar
M2H	Mammalian Two-Hybrid
MAPK	Mitogen Activated Protein Kinase
MARs/SARs	Matrix Attachment Regions /Scaffold Attachment Regions
MBP	MAR-Binding Protein
MD	Matrix binding domain
MEME	Multiple EM for Motif Elicitation
mESCs	mouse Embryonic Stem Cells

mg	milligram
MHC	Major Histocompatibility Complex
min	minutes
miRNAs	microRNAs
u M	Micrometer/ micro molar
ml	millilitre
mM	milli Molar
Nkd	Naked cuticle
NLS	Nuclear Localization Sequence
NMTS	Nuclear Matrix Targeting Sequence
NuRD	Nucleosome Remodeling and Deacetylase Complex
OCT4	Octamer-binding transcription factor 4
PARP-1	Poly (ADP-Ribose) Polymerase-1
PBS	Phosphate Buffered Saline
PCAF	P300/CBP Associated Factor
PcG	Polycomb Group
PCR	Polymerase Chain Reaction
PD	Proximal distal axis
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
PRC	Polycomb Repressive Complex
PSD	Post Synaptic Density
p-TEFb	positive Transcription Elongation Factor b
PVDF	Polyvinylidene Difluoride
RNAi	RNA interference
SAF-A	Scaffold Attachment Factor-A
SATB1	Special AT-rich sequence Binding protein 1
SATB2	Special AT-rich sequence Binding protein 2
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis
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
TrxG	Trithorax Group
TSS	Transcription Start Site
UL	Upper Layer
UTR	Untranslated Region
vg	vestegial

Wg	Wingless
Wnt	Wingless type protein
ZO	Zonula occludens

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Synopsis

Introduction

Eukaryotic chromatin fibers are arranged into discreet independent loops of approximately 1-2 Mb, anchored to the nuclear matrix or chromosomal scaffold specific DNA sequences called scaffold/matrix-attachment regions (S/MARs). It has been postulated that such DNA regions form the base of chromosomal loops (Cockerill and Garrard, 1986) and help in compacting genomic DNA thereby playing a significant role in tissue specific gene expression (Galande, 2002). In general, MARs map to regions where the DNA is intrinsically curved or kinked (Bode et al., 2000) and has a propensity for base unpairing under extensive superhelical strain (Kohwi-Shigematsu and Kohwi, 1990). Although no consensus sequence has been identified yet, genomic segments identified as MARs/SARs are generally AT-rich. S/MARs are located in the introns of several large genes and also at borders of transcription units (Tikhonov et al., 2000). S/MAR elements have often been implicated in the regulation of gene expression. They are frequently found close to enhancers (Fernandez et al., 2001), can stimulate gene expression of heterologous reporter genes when integrated into the genome (Stief et al., 1989) and can regulate chromatin accessibility (Fernandez *et al.*, 2001).

A set of proteins called MAR-binding proteins (MARBPs) are known to bind and tether specific regions of genomic DNA to the matrix. MARBPs also dictate loop domain structure of chromatin, thereby affecting gene expression. These proteins exhibit a high binding specificity for the base-unpairing regions (BURs). A variety of MAR-binding proteins have been identified and characterized. These include- PARP-1 (Poly (ADP-Ribose) Polymerase-1), SAF-A (Scaffold Attachment Factor-A), Ku 70/86 (Autoantigen Ku), HMG-I(Y) (High Mobility Group Proteins) and nucleolin (Galande, 2002). Most of the MARBPs exist in a co-repressor/co-activator complex and regulate gene expression and therefore, any perturbation in the regulation and levels of MARBPs might lead to disease conditions, particularly those caused by abnormal cell proliferation.

Special AT-rich Binding Protein 1 (SATB1) is one such MAR/BUR-binding protein originally thought to be predominantly expressed in thymocytes (Dickinson et al., 1992) where it plays a significant role in T-cell development. Ablation of SATB1 is known to result in defective T-cell development in mice (Alvarez et al., 2000). During viral infections, SATB1 appears to be the primary target in T-cell (Kumar et al., 2005), suggesting a cell-type specific role of SATB1 as a regulator of genes. SATB1 has also shown to be an important silencing factor and plays a role in *Xist*-mediated gene silencing during X inactivation in mammals (Agrelo et al., 2009). Recent studies have established SATB1 as a key factor linking higher-order chromatin organization to regulation of genes (Cai et al., 2006). Studies from Galande laboratory have identified SATB1 as a mediator of Wnt/β-catenin signaling (Notani et al., 2010). Aberrant SATB1 expression potentiates structural and molecular changes that are associated with multiple cancers and increased SATB1 expression has been shown to be associated with poor prognosis (Han et al., 2008; Mir et al., 2012). SATB1 reprograms the expression of tumor growth and metastasis-associated genes to promote tumorigenesis and functionally overlaps with Wnt signaling critical for colorectal cancer tumorigenesis. SATB1 differentially regulates the positive and negative regulators of Wnt signaling and modulates the changes in their expression profiles critical for tumorigenic phenotype (Mir et al, 2015). Thus, SATB1 is a MARBP having a myriad regulatory functions.

SATB1 is composed of three characterized functional domains; central Cut repeat containing domain (CD) that was formerly referred to as the MAR-binding domain (MD), C-terminal homeodomain (HD) and N-terminal dimerization domain. SATB1 binds to the core-unwinding element within the BURs *via* its MAR-binding domain. The CUT domain contains two hydrophilic motifs spanning 9 amino acids at both N- and C-termini (Wang et al., 1995). The CUT domain along with the homeodomain comprises the DNA-binding domain *viz.* the MAR-binding domain (MD) of SATB1. The dimerization domain of SATB1 catalyzes its homodimerization essential for its DNA-binding activity thereby, affecting its role as a regulator of transcription. Caspase-6 mediated cleavage of SATB1 during T-cell apoptosis causes detachment of SATB1 from chromatin (Galande et al., 2001).

The dimerization domain of SATB1 was initially recognized to be a PDZ-like domain. Majority of the PDZ containing proteins are involved in recruiting proteins to form a network of larger supramolecular protein complexes (Fanning and Anderson, 1998, 1999). Approximately 30% of PDZ domains engage in PDZ-PDZ interactions (Chang et al., 2011). The predominance of PDZ domains in metazoans together with the scarcity of canonical PDZ domains in non-metazoans indicates a possibly critical function of PDZ domains in highly specialized scaffolding module probably evolved in response to the increased signaling needs of multicellular organisms (Harris and Lim, 2001).

The PDZ-like domain is also required for assembling SATB1 into a tetramer *in vitro*, a process essential for recognizing specific DNA sequences (such as multiple AT-rich DNA fragments) (Wang *et al*., 2012). SATB1 tetramer can simultaneously bind to two DNA segments (Wang *et al.*, 2014; Purbey et al., 2008) and thus, the tetramerization of SATB1 can lead to regulation of higher-order chromatin architecture by anchoring specialized DNA sequences in close proximity and recruiting various chromatin remodeling factors to coordinately regulate gene expression over long distances. This model is also consistent with the observations that SATB1 regulates the coordinated expression of genes located both at the 200-kb T-helper 2 cytokine locus (Cai et al., 2006) and at the 300-kb major histocompatibility Class I locus (Kumar et al., 2007), and that it reprograms chromatin organization and the transcriptional profiles of tumors to promote growth and metastasis (Han et al., 2008).

The N-terminal PDZ-like domain of SATB1 is also the site for post-translational modifications which regulate its interaction with other proteins, thereby acting as a molecular switch in regulating its transcriptional activity. SATB1 is phosphorylated at S185 by protein kinase C (PKC) and is acetylated at K136 by PCAF (p300/CBP associated factor) acetyltransferase. Phosphorylated SATB1 binds DNA with higher affinity and recruits HDAC1 to its target loci thereby leading to transcriptional repression. Unphosphorylated SATB1 is known to get acetylated by PCAF, leading to a decrease in DNA binding affinity. Thus, these two post-translational

modifications have contrasting effects on gene regulation by SATB1 at a global level (Pavan Kumar et al., 2006). SATB1 is also subject to other PTMs such as sumoylation and deacetylation which are required for its function (Tan et al., 2008). Most of the PTMs undergone by SATB1 reside within the PDZ-like domain.

The homeodomain (HD) and the cut domain (CD) of SATB1 work in concert and enhance sequence-specific binding of SATB1 to the consensus SATB1-binding sequence (CSBS) (Purbey et al., 2008). CSBS comprises of a palindromic sequence in which two identical AT-rich half-sites are arranged as inverted repeats flanking a central cytosine or guanine. As a PDZ-like domain and homeodomain containing protein, SATB1 provides a framework that mediates assembly of specific protein complexes like mSin3A, MTA-2, HDAC1 and HDAC2 and CHRAC/ACF complex subunits ACF1 and ISWI onto chromatin (Yasui et al., 2002). SATB1 serves as a molecular adaptor for several co-repressors and co-activators like HDAC1 (Kumar et al., 2005), CtBP (Purbey et al., 2009), p300/CBP (Jarman and Higgs, 1988; Wen et al., 2005) and β-catenin (Notani et al., 2010), thereby altering activity of the downstream promoters. SATB1 is known to change the chromatin loop organization in response to various signaling cues (Cai *et al.*, 2006; Kumar *et al.*, 2007; Kumar *et al.*, 2006). Thus, SATB1 functions as a global chromatin organizer involved in maintaining the higher-order chromatin architecture or 'loopscape' by tethering the MARs to nuclear matrix at fixed distances (Galande *et al.*, 2007).

Gene expression profiling upon overexpression of the N-terminal PDZ-like domain of SATB1 showed that a significant percentage of genes is deregulated. Of these, a majority are involved in key biological pathways. Some of the most significant hits were components of signaling pathways such as TGF-β pathway, MAPK pathway and the WNT/Wg pathway (Notani et al, 2011). At the cellular level, all developmental processes are ultimately controlled by the cooperative actions of different signal transduction pathways. Among them, Wnt signaling is

indispensable for orchestrating the complex cell behaviors that occur throughout development (Croce and McClay, 2008).

However, recent reports suggest that the N-terminal PDZ-like domain actually folds into a Ubiquitin-like domain (ULD) (Gly71 to Ser 172) and CUTL (His186 to Lys244) domains (Wang *et al.*, 2012). Detailed sequence alignments show that CUTL has the evolutionarily conserved amino acids involved in CUT1 DNA-binding. Recent reports based on the crystal structure of the N-terminal domain of SATB1 reveal that this domain resembles a ubiquitin-like domain (ULD) instead of the previously proposed PDZ-like domain (Wang et al., 2014). However, the N-terminal region of SATB1 that I used in my studies encompassed 1-204 amino acids residues which contain the PDZ-like (Galande et al., 2001) and ULD (Wang et al., 2012) domains, hence the conclusions will not change.

Wnt proteins transmit a myriad of intercellular signals crucial for the development and homeostasis of metazoan animals from *Hydra* (Almuedo-Castillo et al., 2012) to human and are conserved during the course of evolution. Development related processes including cellular proliferation, differentiation, motility, tissue maintenance (MacDonald et al., 2009), cell fate specification and maintenance of pluripotency (Tanaka et al., 2011) are regulated by the Wnt/Wg pathway. Therefore, abnormal Wnt signaling has been causally associated with multiple diseases including cancer (MacDonald et al., 2009) and has been implicated in degenerative diseases (Monroe et al., 2012) such as osteoporosis (Clevers and Nusse, 2012).

The complexity of Wnt/Wg signaling can be attributed to two main aspects first; both the ligands and receptors involved in Wnt signal transduction belong to large multi-gene families allowing for a variety of possible ligand-receptor interactions (Kikuchi et al., 2009). Depending upon the context, a particular Wnt signal may denote: cell proliferation or apoptosis; cell fate determination, differentiation, or stem cell

maintenance; a variety of changes in cell behavior; and/or coordinated interactions with its neighbors (Subramaniam et al., 2013; van Amerongen and Nusse, 2009). Second, Wnt-receptor interactions can elicit a variety of intracellular responses, best known of which is the canonical Wnt signaling pathway which results in the activation of β-catenin/TCF transcriptional complexes (Barker, 2008; Huang and He, 2008; Moon et al., 2004). A small yet dynamic pool of β-catenin rapidly shuttles between the cytoplasm and nucleus (MacDonald et al., 2009) and is responsible for transducing canonical Wnt signals from plasma membrane to the nucleus. Nuclear β-catenin is a hallmark of canonical Wnt signaling and regulates diverse cellular processes in multiple cell types including stem cells (Tanaka et al., 2011) and neurons (Misztal et al., 2011). Reports from our lab show that SATB1 interacts with β-catenin, the final effector of the canonical Wnt/Wg pathway (Notani et al., 2010).

Event(s) that occur in Wnt-responsive cells also depend critically on the ability of the upstream effector Dishevelled (Dsh/Dvl) to interpret distinct types of intracellular receptor-generated stimuli and transmit them to at least two distinct sets of effector molecules (Gao and Chen, 2010). Dishevelled is a common intracellular mediator of several pathways activated by Frizzled receptors (Boutros et al., 1998). It has been implicated in the regulation of cell fate decisions, cell polarity, and neuronal function. Data suggest that Dsh/Dvl proteins organize dynamic, pathway-specific subcellular signaling complexes that ensure correct information routing, signal amplification, and dynamic control through feedback regulation (Gao and Chen, 2010). However, the mechanism of Dishevelled action remains poorly understood, though it is known that Dishevelled over-expression mimics Wnt activation (Lee et al., 2008).

In *Drosophila*, a single Dishevelled isoform is expressed (Klingensmith et al., 1994) whereas, three isoforms of Dishevelled; Dvl1, Dvl2, and Dvl3 are expressed in mammalian system (Sussman et al., 1994). Three conserved domains provide the

major landmarks of Dvls: a DIshevelled and AXin (DIX) binding domain at the N-terminus; a PDZ domain in the middle region; and a Dishevelled, Egl-10, Pleckstrin (DEP) domain located between the PDZ domain and the C-terminus (Boutros and Mlodzik, 1999). The DIX domain enables the possible dimerization of Dishevelled with other members of the Dvl family as well as with Axin, which itself is a scaffold protein functioning downstream of β-catenin to organize the multiprotein complex responsible for degrading β-catenin (Kishida et al., 1999). As in case of SATB1, the PDZ domain of Dishevelled provides a docking site for a large number of proteins including protein kinases; casein kinase-1 (CK1) (Sakanaka et al., 1999), casein kinase-2 (CK2) (Peters et al., 1999) and p21-activated kinase (PAK) (Luo et al., 2002), phosphatases; serine/threonine protein phosphatase-2C family members (Strovel et al., 2000), adaptor proteins such as β-arrestin (Chen et al., 2001) and Frizzleds (Wong et al., 2003). The various isoforms of Dishevelled function cooperatively as well as uniquely with respect to mediation of Wnt3A stimulated canonical signaling. Since SATB1 also harbors a PDZ-like domain, it may be possible that it may interact with Dishevelled by means of PDZ- PDZ interactions, thereby modulating Wnt signaling.

Background

Studies from Galande laboratory have established that SATB1 mediates Wnt/β-catenin signaling by recruiting β-catenin to its genomic targets leading to drastic alteration of the transcriptional activity of the target genes. Here, I show that SATB1 interacts with another intermediary in the Wnt/β-catenin pathway - Dishevelled, which is a molecule present upstream to β-catenin in the Wnt/β-catenin pathway. In light of this finding, my objective was to elucidate the role of this interaction in the Wnt pathway.

In thymocytes, activation of the Wnt pathway is associated with increased deacetylation of SATB1. Deacetylated SATB1 exhibits higher affinity for DNA and recruits various cofactors such as β-catenin and p300 onto various SATB1 binding

sites in response to Wnt signal (Notani et al., 2010). Upon activation of Wnt/Wg signaling levels of c-myc increase presumably by direct recruitment of the SATB1-β-catenin complex to their upstream regulatory regions containing SATB1-binding sites. Moreover, the time-dependent increase in the occupancy of the SATB1-β-catenin complex is accompanied with an increase in H3K9 acetylation at the promoters, indicative of transcriptional activation (Notani et al., 2010). Collectively, these findings further confirm that recruitment of β-catenin drastically alters the fate of SATB1 regulated genes.

β-catenin is also known to interact with the Wnt effector, TCF and activate Wnt responsive genes. The cellular expression levels of TCF and SATB1 could play a decisive role in determining the outcome of their interaction with β-catenin. It is known that SATB1 competes with TCF for sequestering β-catenin. Since not all of SATB1 targets are Wnt targets and vice versa, the choice among these effector proteins as a partner of β-catenin could dictate the developmental fate of cells. The DNA binding sites for TCF and SATB1 are not similar, and therefore the competition is not at the level of DNA binding but at the level of protein-protein interactions (Notani et al., 2010).

Further, colorectal cancers which are characterized by mutations in APC (high levels of Wg signaling) are also associated with elevated expression of SATB1 (Mir et al, 2015). Thus, it can be summarized that SATB1 plays an important role in Wnt/Wg signaling.

In the light of all the above, the objectives of this study are:

1. To screen for proteins that interact with SATB1 via its N-terminal PDZ-like domain.

2. To study the significance of these interactions using the Wnt/Wg pathway as a read-out.
3. To test if SATB1 mediated regulation of the Wnt/Wg pathway is context-dependent.

Summary of the work done

1. Screening for various proteins that interact with SATB1 via its N-terminal PDZ-like domain.

PDZ domains are protein-protein recognition modules that play a central role in organizing diverse cell signaling assemblies. They typically span about 90 amino acid residues and were first identified as regions of sequence homology in diverse signaling proteins. PDZ domains of proteins are known to be involved in protein-protein interactions, be it either PDZ-PDZ interactions or PDZ-other domain interactions (Xia et al., 1997), thereby serving as an anchor for a number of multi-protein complexes. SATB1 is one the few nuclear proteins having a PDZ domain. Therefore, it may act as the nuclear end-point of the PDZ-mediated signal transduction pathway that originates from the cell surface, but this hypothesis needs to be verified experimentally. Hence, it would be interesting to study novel interacting partners of the PDZ-like domain of SATB1 and the functional significance of the same. The key points of consideration being, presence of a PDZ domain in these candidate proteins and the key role of these proteins in critical signaling pathways. Towards this end a few PDZ containing proteins involved in cell signaling such as neuronal NOS, Dishevelled-1 (Dvl-1), X-11β and CASK were shortlisted (using bioinformatic search). Mammalian two-hybrid assay was performed to monitor whether the PDZ domains of these candidate proteins interact with the N-terminal PDZ-like domain (1-204) of SATB1. Indeed, I found that the PDZ domains of CASK and Dvl-1 interact with that of SATB1. The next level was to verify the significance of such an interaction which was achieved by performing

co-immunoprecipitation assays. Of the tested PDZ proteins, CASK and Dvl-1 showed an interaction with full length SATB1 *in vivo*.

2. Analysis of effect of SATB1 expression on Wnt pathway

An increase in Dvl-1 transcript level was observed upon SATB1 over-expression and a concomitant increase in SATB1 transcript upon expression of Dishevelled. Thus, SATB1 and Dvl-1 reciprocally regulate each other at transcript level. SATB1 also upregulates the other two isoforms of Dishevelled, Dvl3 and Dvl2. Wnt responsive genes such as c-FOS (Fra-1), cyclinD1 (CCND1), Grem-2, c-myc and c-Jun are positively regulated by SATB1. SATB1 also upregulates the final effectors of the Wnt pathway, the two transcription factors TCF7L2 and CtBP1. Wnt responsive genes that negatively regulate the Wnt/Wg pathway like Nkd2, Dkk1 and Axin2 are repressed by SATB1. Transcript levels of sFRP4, a negative regulator of the Wnt/Wg pathway decreased upon SATB1 over-expression. Thus, in cell-line system, SATB1 acts as a positive regulator of the Wnt/Wg pathway. Furthermore, cross-talk exists between SATB1 and Wnt/Wg pathway as treatment with soluble Wnt3A ligand or over-expression of Dvl-1 leads to upregulation of SATB1 responsive genes BCL2, CHUK and ERBB2. This result corroborates with the previous data from reports wherein few genes were found to be differentially regulated by SATB1 and Wnt/Wg signaling (Cai et al., 2003; Ma et al., 2007; Li et al., 2007; Notani et al., 2010), suggesting a functional overlap between the two. SATB1 is therefore thought to act as a repressor in context- dependent manner.

3. Generation of flies transgenic for SATB1, SATB1 (1-204) and SATB1 (255-763).

To delineate whether SATB1 mediated positive regulation of the Wnt/Wg pathway is conserved or context-dependent, the approach of ectopic expression of SATB1 in *Drosophila* was used. Ectopic expression of human SATB1 in *Drosophila* tissues gave rise to multiple phenotypic defects - rough eyes, crumpled wings, wing venation and margin defects. Fly experiments suggest that the PDZ-like domain and the C-terminal domain SATB1 (255-763) are not responsible for generation of

a phenotype similar to the one generated upon ectopic expression of SATB1 suggesting that *in vivo* activity of SATB1 requires both the N-terminal PDZ-like domain and the C-terminal DNA-binding domain.

4. To study if human SATB1 can bind the *Drosophila* chromatin.

Putative SATB1-binding sites were identified in the *Drosophila* genome using the consensus SATB1-binding site (CSBS) as bait. Further, three prominent bands a few faint bands were observed upon immunostaining for SATB1 in the polytene spreads from salivary glands of flies ectopically expressing SATB1 in the salivary glands. This observation indicates that human SATB1 can bind to specific sites on fly chromatin, suggesting that SATB1 associated phenotypes might also be generated as a result of SATB1 binding to the fly chromatin, though the same needs to be confirmed further by ChIP-sequencing.

5. To study if SATB1 mediated positive regulation of the Wnt/Wg pathway is conserved across species using *Drosophila* as a model system.

Flies misexpressing *arm* or *dsh* under the control of *GMR-GAL4* driver exhibit a reduced eye phenotype. Expression of SATB1 in this background suppressed this small eye phenotype. These results suggest that SATB1 mediates Wnt signaling by interacting with *arm* and *dsh* at genetic level. Whereas, SATB1 (1-204) and SATB1 (255-763) did not suppress the small eye phenotype produced upon expression of *arm* suggesting that concerted action of both SATB1 (1-204) and SATB1 (255-763) region of SATB1 are required for SATB1 function *in vivo.* These results show that as opposed to the mammalian system, SATB1 is a negative regulator of the Wnt/Wg pathway in the fly system, and the PDZ-like domain by itself has no effect. This suggests that in an *in vivo* scenario, in addition to binding of SATB1 to its interacting partners, binding of SATB1 to DNA is required. Quantitative PCRs upon ectopic expression of SATB1 in the flies revealed that transcript levels of the negative regulators of the Wnt/Wg pathway such as *nkd* and

apc-2 increased upon SATB1 expression. However, *dsh* transcript did not reveal any appreciable change. Therefore, we conclude that SATB1 regulates the Wnt/Wg pathway in a context-dependent manner.

6. Verification of the significance of phenotypes produced upon SATB1 expression by expressing a putative *Drosophila* ortholog of SATB1

Based on structural prediction studies the fly ortholog of SATB1 is predicted to be *defective proventriculus* (*dve*) (Nakayama et al., 2005). The *Drosophila* homeobox domain containing protein, Defective proventriculus (DVE) is expressed in various tissues including the head primordium and functions as a transcription factor. Defective proventriculus contains an N-terminal domain homologous to the N-terminal region of SATB1 and two homeodomains. Like SATB1, it has propensity to bind A/T-rich regions of the genome (Nakagoshi et al., 1998; Purbey et al., 2008). Our results demonstrate that under the control of the *GMR-GAL4* driver *dve* phenocopies *SATB1*, as the resultant flies exhibit ommatidial fusion, bristle loss and also a slight reduction in the eye size is apparent. The fact that this phenotype is more intense than SATB1 could be attributed to endogenous levels of DVE protein. Furthermore, similar to *SATB1 dve* suppresses the small eye phenotype of *dsh* misexpression. The penetrance though is 100%. However, none of these results are sufficient to prove that *dve* is indeed the functional homolog of SATB1, for that complementation assays have to be performed to verify if SATB1 can rescue phenotype of the *dve* mutant flies. At the level of effect on WG protein, *dve* overexpression lead to restricting the Wg expression in the wing imaginal disc, SATB1 overexpression did not yield similar effect. Therefore, compensation of *dve* by SATB1 is not complete and hence it might have different functions as compared to SATB1.

Conclusion

Wnt signaling is one of the most well studied signaling pathways. In vertebrates, Wnt signaling specifies cell fate and controls growth in a variety of developmental

processes, including brain development, limb formation, axis specification and gastrulation. Most of the mechanistic insights into Wnt signaling have been derived from studies involving regulation and stabilization of β-catenin (a downstream effector), whereas the precise mechanism of function and regulation of Dishevelled (Dvl/Dsh), the upstream effector of Wnt signaling, is relatively less understood. The decision of canonical *vs* non-canonical signaling is made at the step of Dishevelled in the Wnt cascade. Reports from Galande lab have shown that SATB1 interacts with β-catenin - the final effector molecule of the Wnt/Wg signaling pathway, and competes with TCF7L2 for binding with β-catenin, thereby modulating the final output (Notani et al., 2010). However, it is still unclear whether the sole role of SATB1 in the Wnt/Wg pathway is the recruitment of β-catenin. Here, I present evidence that in mammalian system, SATB1 also interacts with Dishevelled (*Dvl/dsh*), an upstream component of the Wnt/Wg pathway. Experiments in cell-lines show that SATB1 activates expression of positive regulators of Wnt/Wg signaling (*fos, jun*) and represses the negative regulators of the Wnt/Wg pathway (*dkk, nkd*). To identify if SATB1 mediated regulation of Wnt/Wg signaling is a conserved phenomenon, it was decided to check the effect of SATB1 expression in a system where Wnt/Wg signaling is in place but SATB1 is absent. To address this question, transgenic flies ectopically expressing mammalian SATB1 and SATB1 deletion constructs were generated. Our results reveal that mammalian SATB1 suppresses the phenotypes associated with Wnt/Wg activation in flies whereas the N-terminal PDZ-like domain alone doesn't. These results suggest that SATB1 mediates Wnt/Wg signaling by genetically interacting with components of Wnt signaling such as Dishevelled (*dvl/dsh*) and β-catenin (*arm*). Further, these results also show that in contrast with the mammalian system, SATB1 acts as a negative regulator of the Wnt/Wg pathway in the fly model system, and the N-terminal PDZ-like domain by itself has no effect. This suggests that in an *in vivo* scenario, in addition to binding of SATB1 to its interacting partners, its DNA binding property is required. To corroborate our findings, we also showed that mammalian SATB1 binds to the *Drosophila* chromatin by polytene staining. Therefore, regulation by SATB1 is a context-dependent phenomenon. To establish that the negative

regulation of Wnt/Wg pathway by SATB1 in the fly system is a conserved feature, *defective proventriculus* (*dve*) - a putative fly homolog of SATB1 was used. Based on *in-silico* analysis, DVE is a structural homolog of SATB1 however, until now, functional homology has not been established. Towards this end, experiments were performed to analyze if *SATB1* and *dve* expressing flies exhibit similar phenotypes. Like mammalian SATB1, ectopic expression of fly *dve* lead to a rough eye phenotype. Overexpression of *dve* in the background of *dsh* misexpression resulted in suppression of Dsh misexpression phenotype. Although *dve* expression resulted in restricting Wg expression in the wing imaginal disc, ectopic expression of human *SATB1* did not yield such effect. Therefore, compensation of DVE by SATB1 is not complete and it might have few different functions. Hence, we hypothesize that DVE might be an ancestral molecule which evolved to give rise to SATB1 in the vertebrates.

I have therefore structured my thesis as follows:

Chapter 1 Review of Literature: This chapter serves as an introduction to SATB1, PDZ domains, Wnt signaling, and heterologous expression-all these constitute the central theme of the thesis.

The results are primarily presented in the following two chapters:

Chapter 2: Screening for putative interaction partners of SATB1 via its N-terminal PDZ-like domain and generation of SATB1 transgenic flies. This chapter documents all of the data generated using mammalian cell lines to monitor the effect of SATB1 on Wnt targets. To study if SATB1 mediated positive regulation of the Wnt/Wg pathway is conserved across species using *Drosophila* as a model system. I have documented here the generation of ectopic expression models in fly system.

Chapter 3: In vivo validation of SATB1 crosstalk with Wnt/Wg pathway using *Drosophila* as a model system. In this chapter, I present experimental verification of the significance of phenotypes produced upon SATB1 expression by expressing *dve*, a putative *Drosophila* ortholog of SATB1.

Additionally, several pieces of data that support some of the findings but might not be directly related to the main theme are presented in form of appendix.

Caveats

1. *Drosophila melanogaster* does not express SATB1 endogenously. In the current study, the fly system has simply been used as a 'test tube' to analyze the effect of SATB1 expression on the intermediaries of the Wnt pathway.
2. Motif search analysis using MEME only provides information about the putative binding sites of SATB1 on the fly chromatin. However, ChIP analyses need to be performed to ascertain the same *in vivo*.
3. Quantitative RT-PCRs were performed to monitor the expression of Wnt responsive genes upon ectopic expression of SATB1 in the eye disc. However, at tissue level, a great level of complexity exists and therefore might not provide a complete picture of the scenario inside the tissue.
4. SATB1 suppresses the phenotype of Dishevelled and activated armadillo expression, however, the same cannot be extrapolated to the Wnt/Wg pathway.
5. DVE is purported to be the structural homolog of SATB1, however, functionally they might have different roles.

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Chapter 1: Review of Literature

1.1. Introduction

Eukaryotic chromatin is segregated in to a series of discrete, topologically independent loop domains (Lebkowski and Laemmli, 1982; Rao et al., 2014) achieved by the periodic attachment of the genomic DNA to an insoluble intranuclear framework referred to as the nuclear matrix (Gasser and Laemmli, 1987; Ward and Coffey, 1991). Detailed analysis of the nuclear matrix demonstrated that it is composed of the nuclear lamina, hnRNP proteins (associated with transcription, transport and processing of hnRNA), an inner network comprising core filaments and a more diffuse portion (Bode et al., 2003). Chromosomal regions are tethered to the nuclear matrix in a cell type- and cell cycle-dependent manner (Britanova et al., 2005). These chromosomal segments structurally and functionally partition the genome into 50–200 kb regions (Linnemann et al., 2007), demarcating chromosomal domains of transcriptionally active genes as well as regions undergoing replication (Koina and Piper, 2005; Mizuguchi et al., 2014; Wilson and Coverley, 2013).

The Nuclear Matrix interacts with chromatin via specialized DNA sequences called the matrix attachment regions (MARs) or scaffold attachment regions (SARs). Although no consensus sequence has yet been ascertained, genomic segments identified as MARs/SARs are generally AT-rich and possess a unique property of unpairing under extensive superhelical strain (Frisch et al., 2002). MARs help in compacting genomic DNA and also perform a significant role in tissue specific gene expression (Galande, 2002), they have also been implicated in disease pathogenesis especially cancer (Barboro et al., 2012). Not all potential MARs are associated with the matrix or participate in the organization of chromatin loops. MAR-binding is a dynamic cell type- and/or cell cycle-dependent event which aids coordinated regulation of distant genes (Razin, 2001). Matrix associated proteins bind and tether specific regions of genomic DNA to the MARs, thereby dictating loop domain structure of chromatin, and hence gene expression. These proteins exhibit high binding specificity for the base unpairing regions (BURs), which are typically identified as smaller regions within MARs that exhibit high affinity to

isolated nuclear framework in vitro. 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), Bright (B cell regulator of IgH transcription), Ku 70/86 (Autoantigen Ku), HMG-I(Y) (High Mobility Group Proteins) and nucleolin (Albrethsen et al., 2009; Galande, 2002; Kaplan et al., 2001).

The architecture of the interphase nucleus is intimately linked to the structure of chromatin, the spatial arrangement of genes and gene clusters and the accessibility of regulatory DNA elements. The organization of the genome inside the nucleus is fairly complex and dynamic. Inside the nucleus, genomic DNA is hierarchically packaged by histone and non-histone proteins into chromatin. The dynamics of higher-order chromatin compaction plays a critical role in transcription and other biological processes inherent to DNA (Fudenberg and Mirny, 2012). In the context of chromatin, “higher-order structure” may be defined as an assembly of nucleosomes which acquires a reproducible conformation in 3D space. The fundamental unit of chromatin is the nucleosome, it comprises of 146 base pairs of DNA wrapped in 1.7 superhelical turns around an octamer of histone proteins. This nucleosomal array, a “beads-on-a-string” fiber with a diameter of 11-nm, represents the first level of chromatin organization (Woodcock et al., 1993; Wu et al., 2007). The nucleosome array is organized into a more condensed 30-nm chromatin fiber by the binding of the linker histone (H1 or H5), this is referred to as the second structural level of DNA organization. This 30nm fiber is thought to constitute the primary structure of chromatin (Li and Reinberg, 2011). However, the arrangement of linker DNA and nucleosomes within isolated 30 nm fibers has been difficult to study and remains controversial. Recent experiments incorporating sensitive techniques like small-angle X-ray scattering, electron spectroscopy and cryo-electron microscopy, have cast aspersions on the pervasiveness of the 30-nm fiber and, strongly argue against the presence of any fiber beyond the 10 fiber formed by nucleosomal arrays in majority of cellular genomes (Eltsov et al., 2008;

Maeshima et al., 2010). Arrangements resulting from interactions between nucleosomes give rise to secondary structures.

In a seminal study, Dekker and colleagues proposed a model of the local chromatin environment of human lymphoblast cells on a megabase scale as a fractal globule, a polymer conformation, wherein the chromatin partitions into adjacent regions with minimal interdigitation (Fudenberg and Mirny, 2012; Lieberman-Aiden et al., 2009). This model is consistent with the diffusion and binding properties caused by molecular crowding of chromatin binding proteins (Bancaud et al., 2009; Bancaud et al., 2012). Within an individual fractal globule, chromatin is organized into discrete domains. Hi-C analyses performed in mouse ES cells identified 2,200 topologically associating domains (TADs) in which the chromatin interacts locally (Dekker et al., 2013). These topological domains are enriched in housekeeping genes and SINE elements, and are separated by boundary regions having CTCF binding and enriched in H3K9me3 (a repressive mark) which are characteristics of insulator elements. These topological domains are conserved amongst species and cell types (Ciabrelli and Cavalli, 2014; Dixon et al., 2012). The fractal globules ultimately associate at the chromosome level to give rise to chromosome territories (Lieberman-Aiden et al., 2009), where each chromosome rather than being intertwined haphazardly, occupies its own distinct space and position within the nucleus.

The fractal globule model suggests an acceptable mechanism for the interaction of distant genomic sites located within the same chromosome or on different chromosomes, giving rise to chromosomal translocations. Bickmore and colleagues demonstrated that exons of mouse chromosome 2 predominantly localize at the surface of the chromosome territory (Boyle et al., 2011). This finding is consistent with the genic regions looping out of their chromosome territory, allowing for interactions with loci on other chromosomes. Chromosomal contacts are hierarchically arranged between domains wherein each domain behaves as a unit. Genome-wide modeling of contact density and domain clustering revealed that

inactive domains (enriched for H3K27me3 or HP1/H3K9me2 or, devoid of any detectable epigenetic enrichment) are condensed and are confined to modular chromosomal territories, whereas, active domains reach out of the territory forming remote intra- and interchromosomal contacts (Sexton et al., 2012).

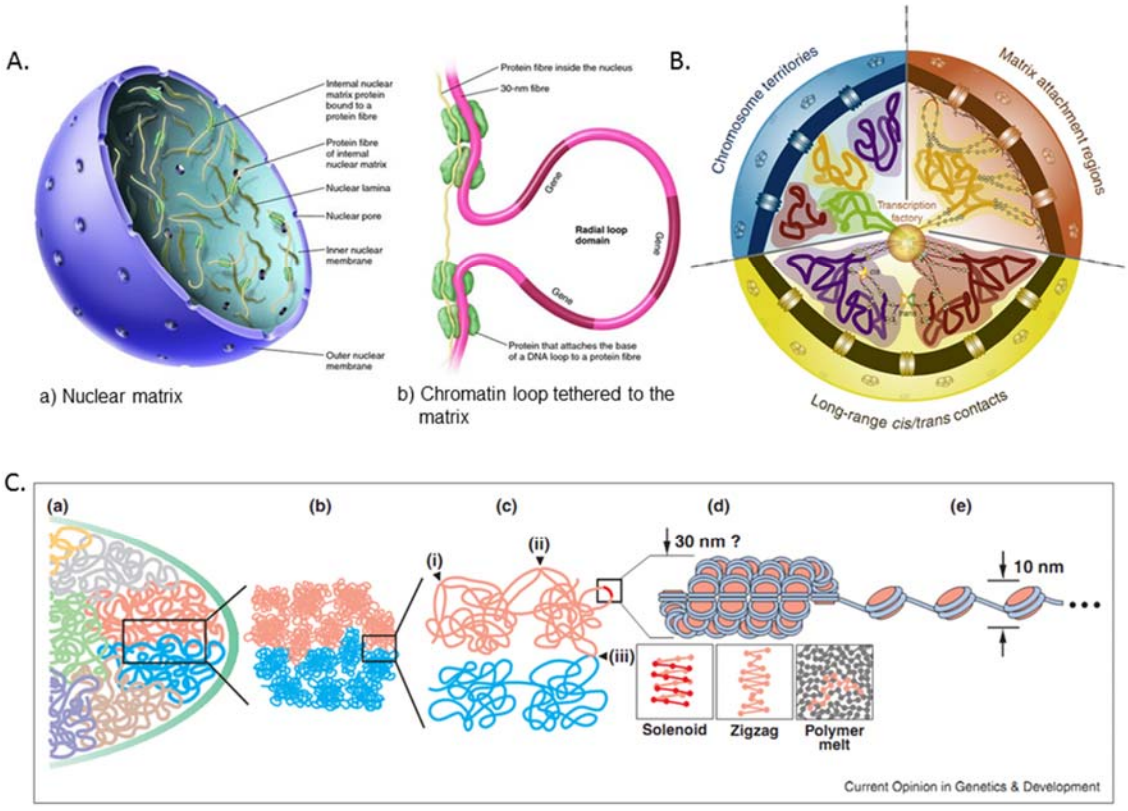

Figure 1.1.1 I Nuclear architecture and chromatin organization. A Cartoon depicting the arrangement of the matrix within the nucleus. Nuclear lamina is a collection of fibrous proteins lining the inner nuclear membrane. The nuclear matrix is composed of protein fibers and associated proteins (b) Radial chromatin loops are anchored to the nuclear matrix by means of these protein fibers. **B**, Schematic of a cross-section of a eukaryotic nucleus illustrating intrinsic aspects of genome organization. Nuclear pore complexes perforating the nuclear membrane are represented by ovals clustered together. Individual chromosome territories are indicated as thick lines highlighted in corresponding colors (*top left*). Brown webbing beneath the inner nuclear membrane represents the nuclear lamina, matrix attachment regions are depicted as red lines (*top right*). Yellow circles symbolize nucleosomes. Long-range interactions between chromatin segments from the same chromosome (*cis*) and between chromosomes (*trans*) are displayed in the bottom half of the cartoon. Proteins mediating physical contacts between chromosomes are illustrated as colored half-circles. For simplicity sake, a transcription factory comprising both *cis* and/or *trans* interactions is represented as a yellow circle at the center of the graphic. Reproduced from Ethier *et al*., 2012. **C, (a)** Inside a three dimensional space chromosomes

are organized into chromosome territories which might overlap. **(b)** Chromosome territories are comprised of fractal globules. Fractal globule is a compact polymer state that emerges during polymer condensation as a result of topological constraints which prevent one region of the chain from passing across another one. Fractal globules from adjacent chromosome territories can interdigitate. **(c)** Chromatin fibers interact (i) within a fractal globule (frequent), (ii) between fractal globules of the same chromosome territory (rare), or (iii) between adjacent chromosome territories (very rare). **(d)** Chromatin may organize into a 30 nm fiber with a solenoid zigzag, or polymer melt organization in which nucleosomes not present in continuum on the DNA strand interact within a chromatin region. **(e)** Chromatin is resolved as 10 nm 'beads on a string' fiber consisting of evenly spaced nucleosomes. Reproduced from Hubner et al., 2013.

1.1.1. SATB1: a brief prelude

1.1.1.1. Unique DNA-binding specificity of SATB1

Special AT-rich Binding Protein 1 (SATB1) is MAR/BUR binding protein thought to be predominantly expressed in thymocytes (Dickinson et al., 1992). SATB1 is a global chromatin organizer and transcription factor, involved in organization of the chromatin 'loopscape' and regulating its dynamic organization in response to physiological stimuli. At the genome-wide level, SATB1 plays a role in organization of transcriptionally poised chromatin (Galande et al., 2007).

The sequence context recognized by SATB1 consists of a well-mixed AT-rich sequence in which C's are exclusively found on one strand and G's on another (Dickinson et al., 1992). A high AT content is not sufficient to confer high affinity binding to SATB1; mutations within MARs which maintain the AT-richness but eliminate the unwinding property substantially reduce or abolish SATB1 binding (Dickinson et al., 1992; Nakagomi et al., 1994). SATB1 binds to the ATC sequences in the minor groove, virtually making no direct contact with DNA bases. It probably recognizes ATC sequences indirectly by the altered sugar-phosphate backbone determined by the ATC sequence context (Nakagomi et al., 1994). Most of the *in vivo* SATB1-binding sequences exhibit homology to autonomously replicating sequences (ARS) from the yeast *Saccharomyces cerevisiae*. A few are LINE 1 elements, satellite 2 sequences, and CpG island–containing DNA (de Belle et al.,

1998). To better understand SATB1 DNA binding preference, its optimal DNA-binding sequence was delineated by random oligonucleotide selection. The consensus sequence reads 'TATTAGTAATAA', where the underlined sequences emphasize the inverse palindromic arrangement of consensus elements which may pose critical ramifications for the recognition and high-affinity binding of such sequences by SATB1. Remarkably, the CSBS half-site is identical to the conserved element 'TAATA' known to be bound by homeodomains (HDs). Thus, SATB1 consensus sequence is an inverted palindrome. As it is known that SATB1 binds to DNA as a dimer, it might be that the DNA-binding regions of two SATB1 monomers bind the inverse palindromic consensus-binding element in an antiparallel fashion.

1.1.1.2. Functional domains of SATB1

SATB1 is composed of three characterized functional domains; central Cut repeat containing domain (CD) that was formerly referred to as the MAR-binding domain (MD), C-terminal homeodomain (HD) and N-terminal dimerization domain (PDZ-like). SATB1 binds to the core-unwinding element within the BURs via its MAR-binding domain. The PDZ-like domain mediates SATB1 homodimerization, essential for DNA binding and is homologous with many other PDZ domains. SATB1 is specifically cleaved by caspase 6-like protease after aspartate at the 251st position to produce a major fragment containing both CD and HD domains and a smaller fragment containing the PDZ-like domain. Once SATB1 is cleaved, even if the MAR-binding domain remains intact, it readily dissociates from chromatin *in vivo*, concomitant with the cleavage of its target sequences by apoptotic endonucleases (Galande et al., 2001). Caspase-6 mediated cleavage of SATB1 occurs post induction of apoptosis (Gotzmann et al., 2000; Galande et al., 2001), and also under non-apoptotic conditions where it is required for allowing stimulated B lymphocytes to enter the cell cycle (Olson et al., 2003). The PDZ-like domain of SATB1 participates in a variety of protein-protein interactions including HDAC1, β-catenin, CtBP and p300. In this report, we have used the acronym PDZ1 to describe the PDZ-like domain of SATB1.

In addition to the above-mentioned domains SATB1 harbors two additional targeting sequences. The region spanning amino acids (aa) 224 to 278 has been characterized as a nuclear matrix targeting sequence (NMTS) that is required for the transcription function of SATB1 (Seo et al., 2005). Furthermore, the stretch of amino acids from 20 to 40 targets SATB1 to the nucleus and hence constitutes its nuclear localization signal (NLS). This region is conserved among SATB family of proteins belonging to diverse species (Burglin and Cassata, 2002; Fuß and Hoch, 1998). The N-terminal 1-204 aa region of SATB1 harbors both the NLS (Nakayama et al., 2005) and the PDZ-like domain (Galande et al., 2001).

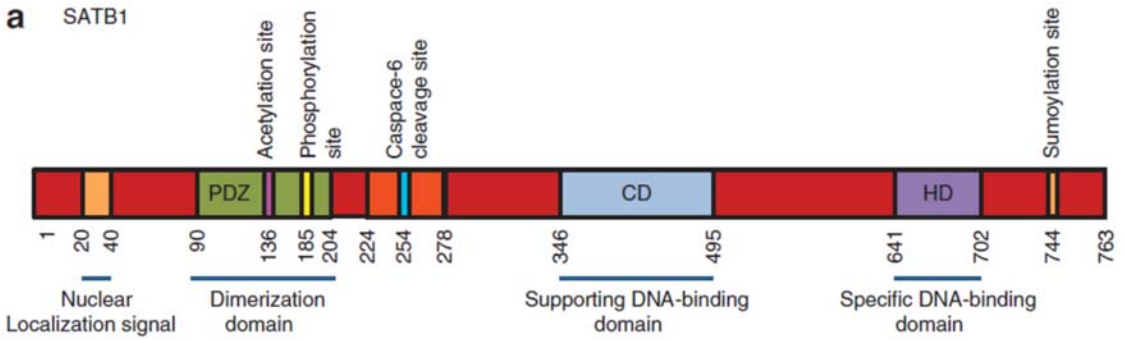

Figure 1.1.2 I Domain organization of SATB1. Graphic representation of human SATB1 protein (763 aa) depicting the functional domains and key amino acid (aa) residues. Vertical colored bars denote essential residues and horizontal blocks represent functional domains within the protein. The N-terminal PDZ-like domain (green, 90–204 aa) is required for dimerization and mediates interaction of SATB1 with HIV-Tat, HDAC, promyelocytic leukemia (PML), CtBP and β-catenin. Recent reports based on crystallization studies suggest that this N-terminal domain is actually a Ubiquitin like domain (ULD), involved in tetramerization of SATB1. This ULD-mediated SATB1 oligomerization may affect the DNA-binding affinity and stoichiometry for SATB1 (Wang et al., 2012). The Cut domain (CD, blue, 346–495 aa) is essential for DNA-binding and increases binding affinity. However, it is the homeodomain (HD, purple, 641–702 aa) which confers DNA-binding specificity. Reproduced from Burute et al., 2012.

The high-affinity binding of SATB1 to DNA is dimerization-dependent and the specificity of binding is mediated by the Homeodomain of SATB1 (HD) in conjunction with the Cut repeat containing domain of SATB1 (CD). The SATB1 CD binds major groove of DNA with low affinity and without much specificity whereas, the HD specifically binds through the minor groove of target DNA with high affinity.

This affinity is further increased many fold when both HD and CD domains are held together in dimeric form by the PDZ domain. Thus, the SATB1 dimer may form a clamp-like structure that wraps around the DNA helix by occupying both the major and minor grooves. This mode of binding is similar to that of LFB1/HNF1, wherein the DNA-independent dimerization domain is required to increase the DNA-binding affinity, but does not influence the dimer geometry. A single mutation in the HD consensus region (GTCATA or GTACTA) has detrimental effect toward its binding by SATB1. Additionally, the spacing between the two AT-rich half-sites is critical. The dyad symmetry could play a vital role in protein–DNA interaction and regulation of binding specificity (Purbey et al., 2008).

1.1.1.3. The PDZ domains

The PDZ domain was first recognized as a novel structural motif in the post-synaptic density protein PSD-95, the *Drosophila* Discs-Large septate junction protein Dlg-A and the epithelial tight junction protein Zonula occludens (ZO-1), therefore PDZ domains are now known by an acronym representing the first alphabet of each of these three PDZ harboring proteins (PDZ: PSD95/Dlg-A/ZO-1). The first draft of the human genome ranked the PDZ domain family as number 19 among the most abundant domain families (Kurakin et al., 2007). More than 400 different PDZ domains are currently estimated to exist in humans or in mice. The PDZ domain typically spans approximately 90 amino acid residues and folds into a compact globular fold in which the N and C-termini are in close proximity. PDZ domains are well represented in scaffold proteins, which are known to allow for organization of supra molecular complexes, a recurrent theme in several model systems (Fanning and Anderson, 1998, 1999). Examples of such PDZ-mediated supramolecular assemblies exist in photoreceptor cells of *Drosophila* and at mammalian synapses.

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The canonical PDZ domain consists of a core of β sandwich containing six β strands and two α helices. The interacting ligand binds in a hydrophobic cleft made by a β strand, α helix and a loop. The loop contains the conserved GLGF motif and interacts with terminal carboxylate group on the ligand (Figure 1.1.3). The ligand binds to the PDZ domain as an anti-parallel extension of the β sheet domain. The N and the C-terminal regions of the PDZ domain are often in close spatial proximity which possibly facilitates their insertion into existing protein scaffolds without causing major disruptions of the three dimensional architecture (Figure 1.1.3).

Individual PDZ domains display additional structural features that have a bearing on structure function relations of the PDZ protein. As per conservative, estimate up to 40% of the PDZ proteins have structured extensions at their termini which effect specificity of ligand binding (Songyang et al., 1997).

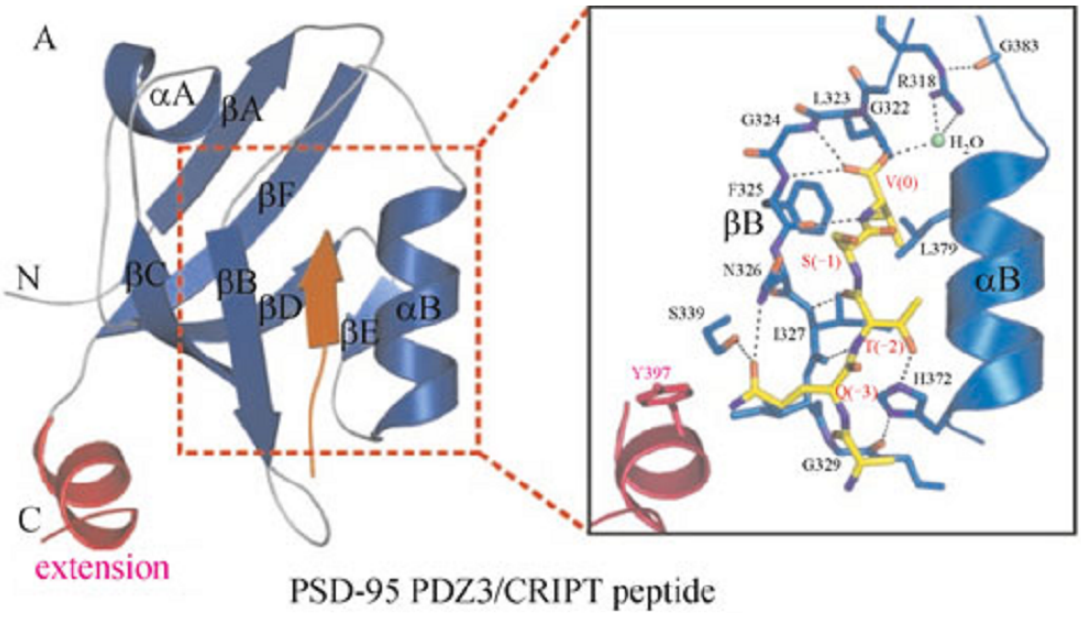

Figure 1.1.3 I A ribbon diagram depicting the three dimensional fold of peptide-bound PDZ domain. Ribbon representation of interaction between the third PDZ domain (PDZ3) of PSD-95 (corresponding to residues 309-393) bound to the CRIPT peptide (brown arrow), the inset depicts molecular details of the interaction (PDB ID:1BE9). Amino acids crucial for the interaction are in red. PDZ domain consists of six β-strands and two α-helices (labeled), and adopts the canonical PDZ fold that displays a binding groove for a peptide ligand (brown arrow). The peptide (brown arrow) inserts between the βB strand and αB helix structure of the complex and forms an antiparallel β sheet with βB. The connecting loop between βA and βB is involved in binding the peptide C-terminus and is therefore designated the carboxylate-binding loop. The extension of the PDZ domain which aids the interaction is also depicted (Red loop), Y397 present on the extension domain which is essential for the peptide PDZ interaction is depicted in red. Reproduced from (Wang et al., 2010).

At the level of primary sequence, there is a great deal of flexibility in PDZ domains, on an average 30% sequence identity is observed (Ho and Agard, 2010). However, the structural core is fairly conserved and relatively refractive to the effect of mutagenesis. Mutations in the peptide binding domain often result in alteration rather than loss of function allowing for rapid evolution of novel interaction networks (Ho and Agard, 2010). PDZ domains bind their ligands with modest affinities (Kd ~1 μM), which makes them appropriate for controlled interactions (Harris and Lim, 2001). Due to the great variation in binding selectivity of PDZ domains, it has been proposed that PDZ domains cannot be classified into distinct classes but rather lie along a continuum (Stiffler et al., 2007). PDZ domains recognize short specific

sequences, usually five residues long at the C-termini of their interacting partners (Niethammer et al., 1998) in a 'non-invasive' manner (Belotti et al., 2013). Nevertheless, they can also recognize internal sequences that structurally mimic a carboxy terminus (Lee and Zheng, 2010). PDZ domains can also bind membrane lipids (Ivarsson et al., 2013). Thus, enabling binding to practically any target protein. PDZ domain-ligand interactions are frequently rewired by C-terminal mutations in PDZ ligands during evolution. (Kim et al., 2012). PDZ-containing genes encoded in metazoan genomes vastly outnumber those in prokaryotes and fungi (Sakarya et al., 2010). The abundance of PDZ domains in metazoans together with the scarcity of canonical PDZ domains in non-metazoans indicates a possible critical function of this highly specialized scaffolding module in multicellular organization (Harris and Lim, 2001; Kurakin et al., 2007).

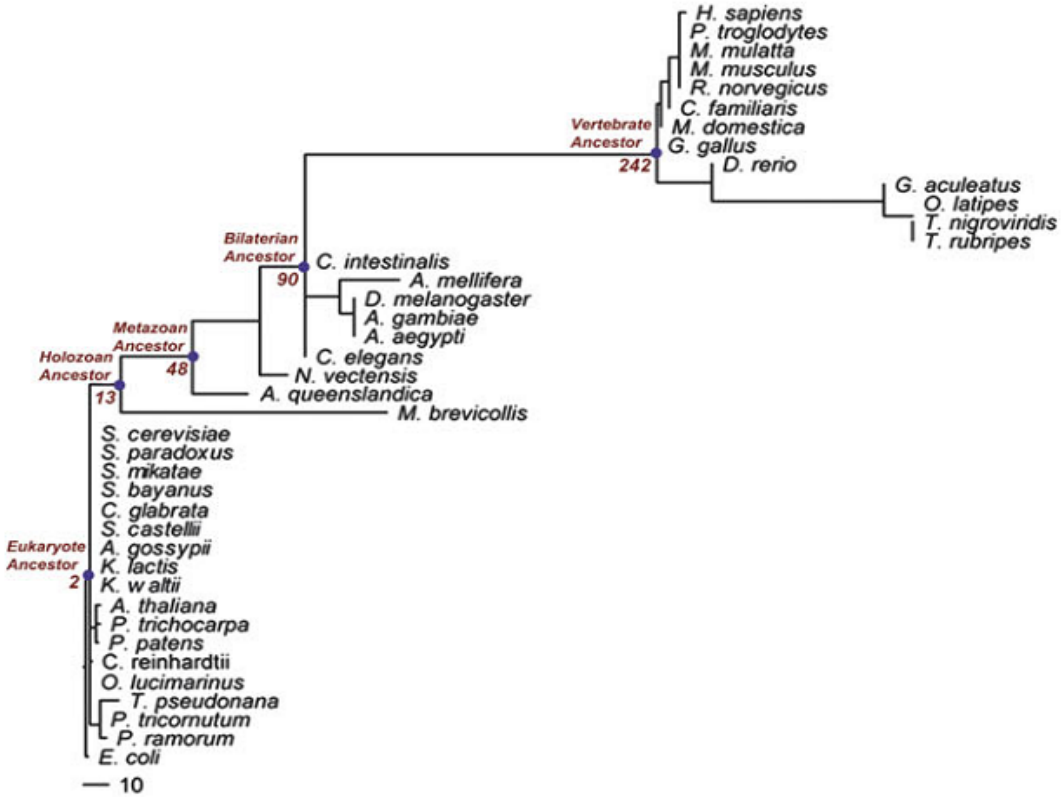

Figure 1.1.4 I PDZ domain expansion tree. Phylogenetic tree with number of gained PDZ domains shown as branch length. Scale bar corresponds to 10 absolute domain gains (gains minus losses). Reproduced from Sakarya et al., 2008.

PDZ domains are often found arranged in tandem arrays and/or associated with other protein interaction modules such as src homology 3 (SH3) and guanylate kinase-like (GK) domains (Kim and Sheng, 2004), thereby playing a pivotal role in organizing diverse cell signaling assemblies (Figure 1.1.5). As these domains are highly modular they could easily have been integrated into existing proteins without significant structural disruption through the course of evolution. On an average, a PDZ domain containing protein interacts with 17 partners and a PDZ ligand interacts with 3 PDZ proteins (Kim et al., 2012). The bias towards a larger number of PDZ ligands seems to suggest that there is a preferential expansion in the number of proteins that interact with PDZ. As a result of intrinsic promiscuity of PDZ domains, they function as an interface for interaction with multiple proteins, thereby serving as a scaffold for organization of multiprotein complexes. Most of the PDZ domain containing proteins are generally present in the cytosol or near the plasma membrane where they function as adaptor proteins for the establishment of signal transduction complexes (Kim and Sheng, 2004).

Protein-Protein interactions can also be mediated by binding of one PDZ Domain with another, forming heterodimers (Morales et al., 2007) or homodimers. Xu et al. showed that PDZ domains 3 and 4 from the *Drosophila* protein INAD could bind to each other and form homo-oligomers (Xu et al., 1998). Marfatia et al. demonstrated that the PDZ-containing protein hDlg forms oligomers in solution (Marfatia et al., 2000). There is widespread evidence implicating protein phosphorylation in disruption of PDZ-ligand interaction (Nourry et al., 2003; Zhang et al., 2011). Studies have demonstrated that the first two PDZ domains of NHERF/EBP50 oligomerize in phosphorylation-dependent manner (Fouassier et al., 2000). PDZ domain mediated homodimerization is also found in nature, one such protein which undergoes PDZ mediated homodimerization is the chromatin organizer SATB1, this is one of the few cases where the protein harboring the PDZ domain is predominantly localized inside the nucleus (Notani et al., 2010; Purbey et al., 2008).

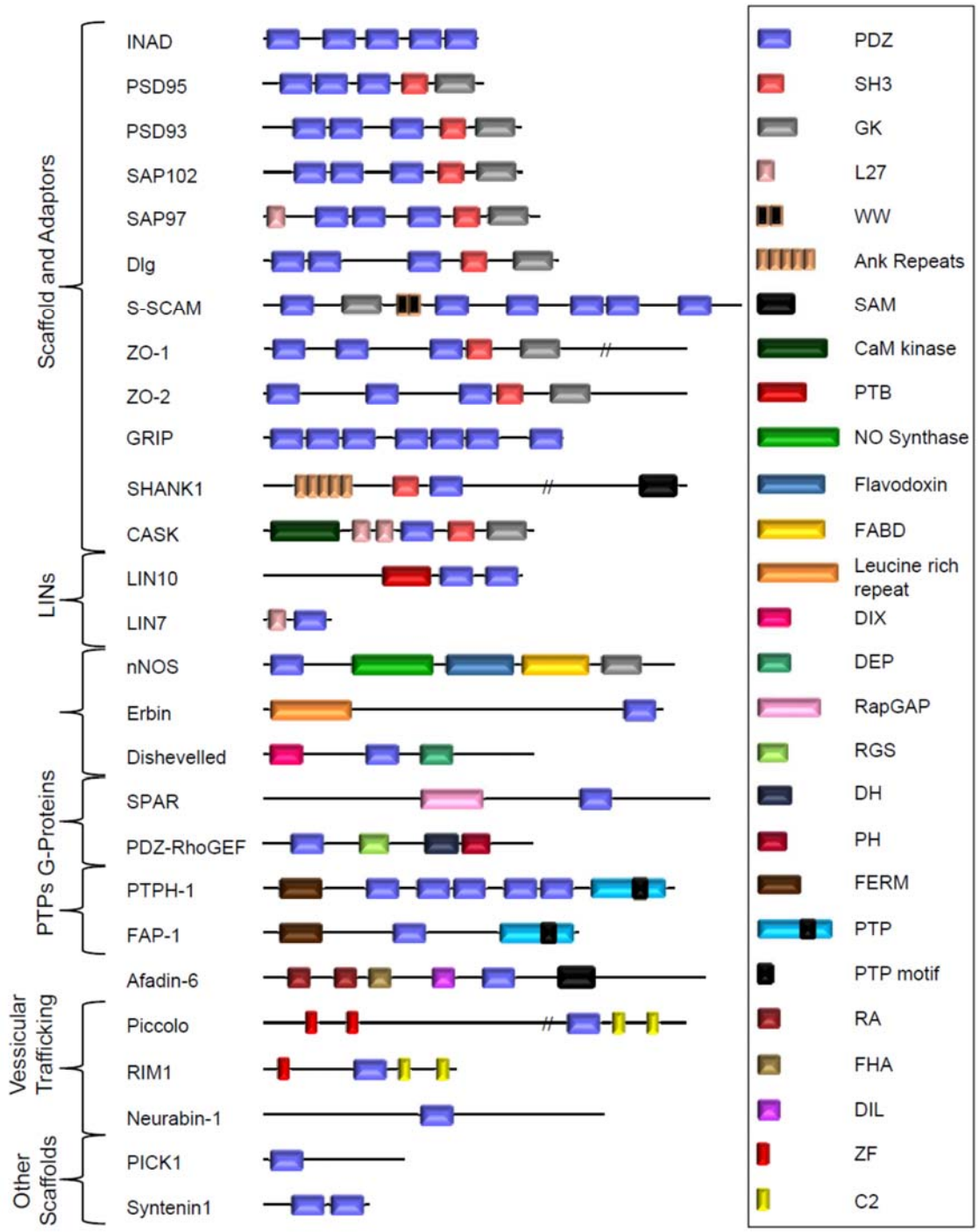

Figure 1.1.5 I Schematic diagram of PDZ containing proteins. PDZ domains are often found in scaffold/adaptor protein molecules as multiple tandem arrays and are generally associated with other modular protein-interaction domains. PDZ domains are depicted here as purple rectangles. Other domains are indicated: SH3, Src homology 3 domain; Ank, ankyrin repeats; GK, guanylate kinase-like domain; L27, domain initially found in LIN2 and LIN7; WW, domain with two conserved Trp (W) residues; SAM, sterile α motif; CaM kinase, calmodulin-dependent kinase (CaMK)-like domain; PTB, phosphotyrosine- binding domain; NO, nitric oxide; FABD, FAD-binding domain; DIX, present in Dishevelled and Axin; DEP,

found in Dishevelled, Egl-10 and Pleckstrin; RapGAP, RapGTPase-activating protein; RGS, Regulator of G-protein signaling; DH, Dbl homology domain; PH, Plekstrin homology domain; FERM, F for 4.1 protein, E for ezrin, R for radixin and M for moesin domain; PTP, Protein Tyrosine Phosphatase domain; RA, RAS association domain; FHA, forkhead-associated domain; DIL, dilute domain; ZF, Zinc Finger domain; C2, Calcium binding motif. Proteins: INAD; Inactivation no after potential D; PSD-95, postsynaptic density protein 95; PSD-93, postsynaptic density protein 93; SAP102, synapse-associated protein 102; SAP97, synapse-associated protein 97; Dlg, discs large; S-SCAM, synaptic scaffolding molecule; ZO-1/2, zona occludens protein 1/2; GRIP1, glutamate-receptor-interacting protein 1; SHANK1, SH3 and ankyrin repeat-containing protein 1; CASK, calcium/calmodulin-dependent serine protein kinase; LIN7, lin7 homologue; LIN10, lin10 homologue; nNOS, neuronal nitric oxide synthase; SPAR, spine-associated Rap-specific GTPase-activating protein; PTPH1, Protein Tyrosine Phosphatase H1; FAP-1, Fas-associated phosphatase-1; RIM1,Regulating synaptic membrane exocytosis protein 1; PICK1, protein interacting with C-kinase 1.

1.1.1.4. SATB1 and gene regulation

As a PDZ-like domain and homeodomain containing protein, SATB1 provides a framework that mediates assembly of specific protein complexes onto a discrete set of BURs and hence may act as an architectural protein on the nuclear matrix. These protein-protein interactions may lead to repression or activation of the gene loci to which the complex associates. Oligomerization of SATB1, mediated via its PDZ-like domain plays a key role in DNA-binding and has been implicated in affecting the gene regulatory function of SATB1.

Studies using gene clusters have revealed that SATB1 is involved in maintenance of the loop domain structure of chromatin (Cai et al., 2006; Galande et al., 2007; Kumar et al., 2007), which may further alter the accessibility of these sites to various transcription and recombination factors. There are two distinct mechanisms by which SATB1 regulates expression of genes. Primary regulation is via specific binding of SATB1 to promoters and upstream regions thereby, directly influencing the promoter activity. SATB1 directly regulates number of genes including IL-2, IL-2Rα and, globin, by recruiting either CBP (Wen et al., 2005) or HDAC1 (Pavan Kumar et al., 2006) thereby, leading to activation/repression of the respective promoters. Secondly, the context specific regulation of genes by SATB1 stems from its unique ability to bind MARs thereby regulating a large number of genes in a

coordinated manner by acting as a 'docking site' for several chromatin-remodeling complexes (Yasui et al., 2002). It regulates the transcriptional status of many genes by interacting with and, recruiting repressors and co-activators on to respective gene loci. SATB1 recruits the NURD complex subunits Mi-2, mSin1A, MTA-2, HDAC1 and HDAC2 and CHRAC/ACF complex subunits ACF1 and ISWI onto the chromatin (Yasui et al., 2002).

1.1.1.5. Post-translational modifications of SATB1

Another layer of gene regulation by SATB1 is by virtue of its post-translational modifications. SATB1 is phosphorylated by protein kinase C (PKC) and is acetylated by p300/CBP associated factor (PCAF) acetyltransferase (Kumar et al., 2006). Phosphorylation of SATB1 at S185 enhances its DNA-binding activity whereas acetylation at K136 decreases its IgH-MAR binding activity. Phosphorylation does not seem to be obligatory for the DNA-binding activity of SATB1, however, is required for its association with HDAC1 and HDAC2. The acetylated form of SATB1 exhibits reduced affinity for DNA as compared to the unmodified SATB1 (Figure 1.1.6). Dephosphorylated SATB1 might recruit various HATs and lead to gene activation in two ways. Recruitment of PCAF leads to acetylation of SATB1 and subsequent loss of DNA-binding activity leading to derepression of genes. CBP/p300 does not cause acetylation of SATB1, but acetylates histones in the vicinity and thereby resulting in activation of genes. Thus, phosphorylation influences interactions of SATB1, whereas acetylation affects the DNA-binding ability of SATB1 (Figure 1.1.6).

A distinct pattern is observed upon comparing the gene expression pattern of, SATB1 S185A to that of SATB1 K136A mutants, genes downregulated in wildtype and K136A are upregulated in S185A. Thus, these modifications affect the function of SATB1 in contrasting manner (Pavan Kumar et al., 2006), i.e. phosphorylation of SATB1 acts as a molecular switch regulating its transcriptional activity in vivo (Pavan Kumar et al., 2006). In Jurkat cells, endogenous SATB1 is phosphorylated

at serine and threonine, but not tyrosine residues (Pavan Kumar et al., 2006). SATB1 is phosphorylated at Serine-185 and Threonine-188 in vivo. T188A in conjunction with site directed mutagenesis at upstream serines such as S185, significantly increases SATB1 localization into PML nuclear bodies (PML NBs), indicating that T188 is a key phosphorylation site essential for regulating targeting of SATB1 to the PML NBs (Tan et al., 2010). Localization of target proteins at nuclear bodies is sumoylation-dependent (Shen et al., 2006). Sumoylation of PML is vital for proper formation of nuclear bodies (NB) and recruitment of nuclear body-associated proteins (Navascués et al. 2010).

SATB1 is known to undergo sumoylation in vivo. In a yeast two hybrid screen, Ubc9, SUMO-1, and protein inhibitor of activated STAT (PIAS) family members were found to interact with SATB1. All of these in concert enhance conjugation of the SUMO moiety to Lysine-744 of SATB1, culminating in caspase-6 mediated SATB1 cleavage in the PML NBs. As this highly regulated caspase mediated cleavage occurs in only a subset of SATB1, it might be involved in mediating cellular processes other than programmed cell death. Phosphorylation of SATB1 at Threonine-188 inhibits its interaction with PIAS1 thereby inhibiting SATB1 sumoylation, and subsequent caspase-6 mediated cleavage (Tan et al., 2010; Tan et al., 2008). Such tightly controlled proteolysis of SATB1 might be one of the modes of SATB1 mediated transcription regulation in cells of the immune system.

It can be speculated that SATB1 might participate in the recruitment of other PDZ-containing proteins or PDZ interacting proteins at the base of chromatin loops. Some of these interacting proteins may also constitute the nuclear matrix. Posttranslational modifications such as phosphorylation and ribosylation might further play a role in dictating the assembly and function of the molecular complexes at BURs (Galande, 2002).

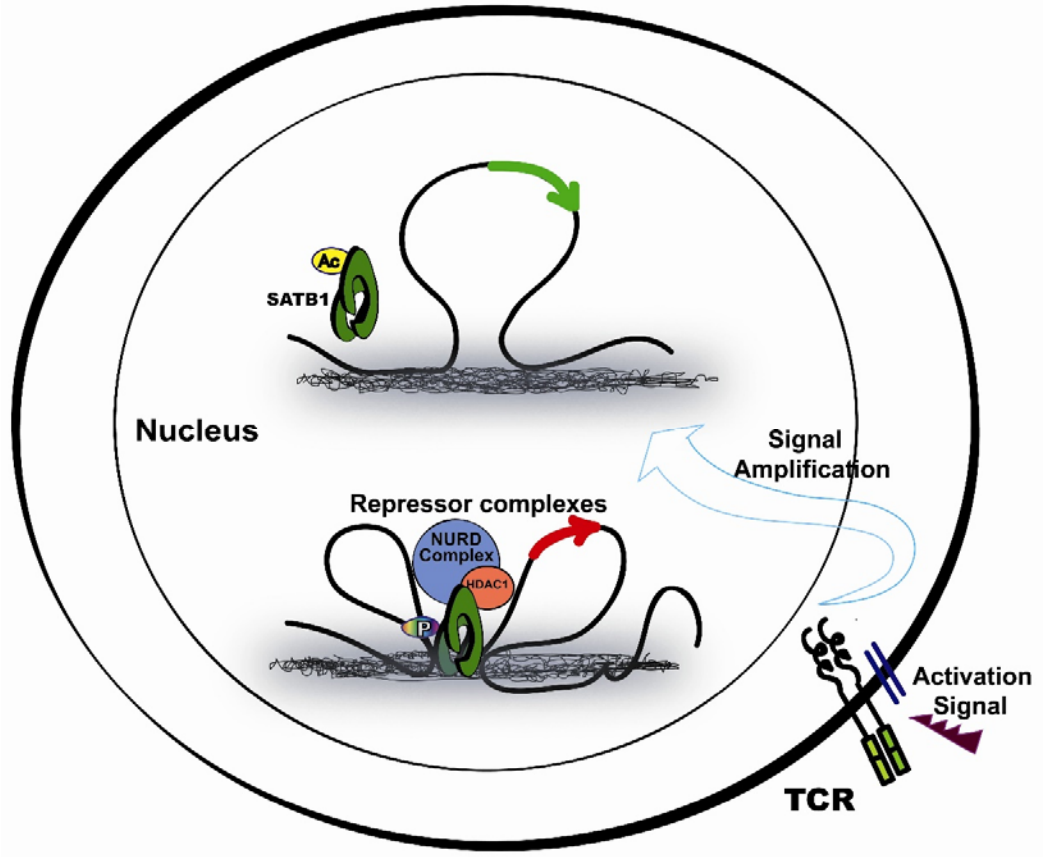

Figure 1.1.6 I Regulation of *IL-2* transcription by SATB1 in response to T cell activation stimuli. Post-translational modifications of SATB1 determine its association with co-repressor or co-activator complex and DNA binding activity and thereby regulate transcriptional activity of its target genes exemplified by *IL-2*. (**A**) In unactivated T cells SATB1 (green) is phosphorylated (red stars) by PKC and interacts with HDAC1 (blue), a component of NURD complex (brown), at the SBS (SATB1 binding site) within the *IL-2* promoter (red DNA wrapping orange nucleosomes) leading to repression of *IL- 2.* (**B**) In activated T-cells SATB1 (green) is acetylated (yellow stars) by PCAF as a result SATB1 loses its occupancy at the SBS within the *IL-2* promoter (DNA in red wrapped around nucleosomes in orange color) leading to derepression of *IL-2.* Reproduced from Purbey et al., 2009.

1.1.2. The N-terminal 1-204 amino acid harboring region of SATB1 acts as dominant negative effector towards its transcription activity

Gene expression profiling of control transfected (vector) with respect to overexpressed N-terminal PDZ-like region of SATB1 using microarrays revealed that out of the total 19000 genes analyzed, 600 were significantly upregulated in

case of PDZ-like domain overexpression. Pathway analysis of deregulated genes revealed that induced genes were found to be involved in various functions viz, extracellular attachment, cellular integrity and multiple signaling cascades such as TGFβ and Wnt signaling pathways (Figure 1.1.7). The gene expression profile unequivocally proved the role of SATB1's PDZ-like domain in global gene regulation presumably through its interaction with various cellular proteins. It was therefore of interest to study the role of SATB1 in cell signaling.

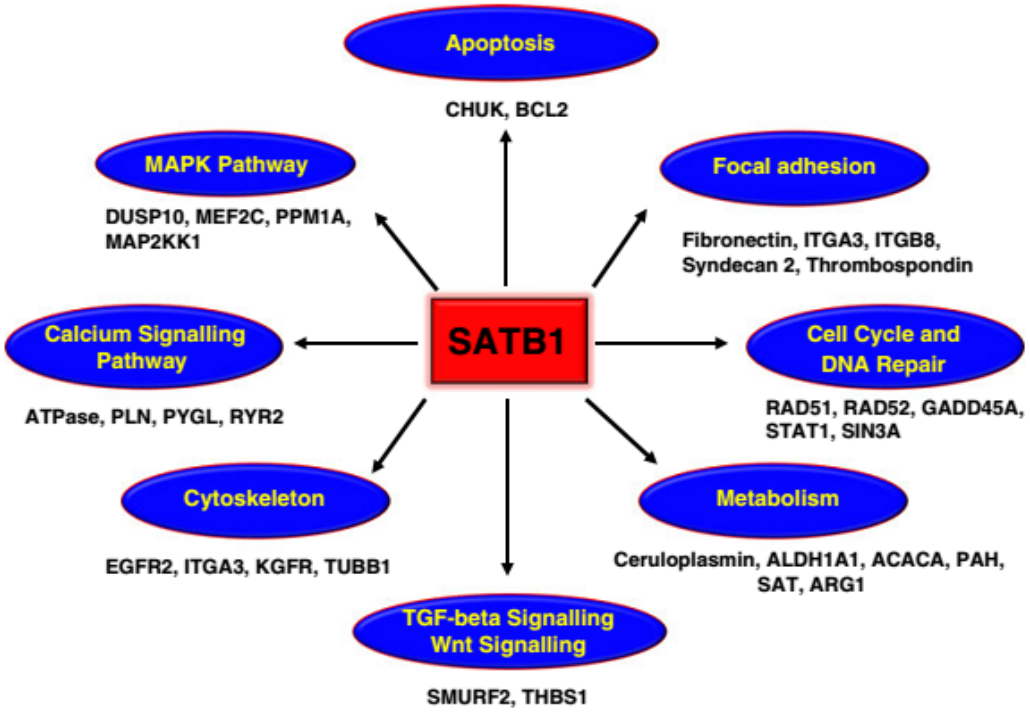

Figure 1.1.7 I SATB1 regulates genes involved in key biological pathways. Overexpression of the PDZ-like domain resulted in dysregulation of over 600 genes out of 19000. Genes that were significantly dysregulated were analyzed further using 'Pathways' and were found to be involved in different dynamic biological pathways as depicted above. Pathway analysis of gene expression profiling was performed as described in 'Materials and methods'. Only the most significantly affected pathways and their key genes are depicted. Reproduced from Notani et al., 2011.

1.1.3. Crystal structure of the N-terminal region of SATB1 reveals a ubiquitin-like domain fold which is required for its dimerization and tetramerization

Recent studies using X-ray crystallography revealed that the N-terminal region harboring the PDZ-like domain is not a typical PDZ domain but, is in effect folds into a ubiquitin-like domain (ULD) (Wang et al., 2014). The broad structure of this ubiquitin-like domain contains four antiparallel β-sheets (β1–β4) flanked by four α-helices (α1–α4), as opposed to the PDZ domain which consists of six β-strands and two α-helices (Wang et al., 2012). Even though the N-terminal domain of SATB1 does not fold like the conventional PDZ domains, our approach and logic of using the N-terminal domain for studying SATB1 mediated interactions would still work as we have already documented several protein interactions as well as dimerization/multimerization via the same domain. Hence, even if the N-terminal folds into a ULD in full-length SATB1 protein, our approach would still remain valid.

1.1.4. Signaling pathways: Wnt signaling

1.1.4.1. Signaling pathways: Evolution and study

The ability of an organism to survive depends on its capability to adapt and respond competently to external conditions. Towards this end, reliable signal transduction in addition to metabolic versatility and efficient replication is essential. The fact that multicellular metazoans possess much more elaborate regulatory and signaling organization as compared to unicellular organisms further underlines the abovementioned statement (King et al., 2008; Lim and Pawson, 2010; Manning et al., 2008; Putnam et al., 2007). Complex networks of signaling proteins process a large amount of information generating specific reactions in response to environmental cues. Upon perturbing cellular environments, signaling networks adapt accordingly. These signaling pathways are conserved across the animal kingdom and are involved in disease pathogenesis. Therefore, understanding of the evolutionary paths of cellular regulatory networks is important for the evolution of

animal complexity and for the understanding of development and the pathophysiology of complex diseases at a systemic level (Boran and Iyengar, 2010). Over the past several years, multiple families of signaling molecules such as the bone morphogenetic proteins (BMPs), the fibroblast growth factors (FGFs) and the Hedgehogs (Hh) have been identified and their signaling mechanisms elucidated. Surprisingly, studies have revealed that only a few classes of signaling pathways are sufficient to pattern a wide range of cells and morphologies. The specificity of these pathways is dependent on the cell's 'competence', the intensity of the signal and, the cross regulatory interactions with other signaling cascades (Pires-daSilva and Sommer, 2003).

The observation that the same set of pathways is used many times in development indicates that signaling systems are highly flexible in generating distinct responses in different tissues and species (Pires-daSilva and Sommer, 2003). From the aforementioned facts, it can be surmised that all biological processes are grounded on the utilization of a selected set of proteins for performing the many steps that underlie the final coherent set of events. Therefore, the present study was based on identification of various components of signaling pathways our protein of interest (SATB1) could interact with and the subsequent effects on the bigger picture. For this study, few candidate proteins involved in signal transduction process were selected and their interaction with SATB1 was tested.

Two fundamentally different approaches can be applied for identification of proteins involved in various pathways, biochemical and genetic. The biochemical approach involves isolation of proteins and characterization in vitro and then reintroducing the gene encoding the protein back into the cell to test its hypothesized function. In contrast, the genetic approach begins at organismal level wherein identity of proteins involved in a particular process can be divulged by first identifying the genes coding them. The genes are identified by their ability to disrupt the process in question when mutated. In other words, if mutation in a particular gene disrupts a given process, then the protein encoded by that gene must play a vital role in that

process. With this approach, much activity is spent in determining what type of protein is encoded by each mutated gene. For processes that can be studied in cell-free extracts, the biochemical approach is highly effective. However, for complex intertwined processes, such as signaling pathways or developmental events, in which many components of the entire organism are involved, genetics offers perhaps the only viable method for dissecting out the protein components. The genetic approach has been applied for examining gene/protein function in many organisms. However, the extent to which genetic methods and tools have been developed for *Drosophila melanogaster* far exceeds that for any other complex multicellular organism. Small size, prolific egg-laying, rapid reproduction and a short life cycle are only a few features that make large-scale genetic screens possible in reasonable time frames thereby making *Drosophila* an impressive model system. About 70% of the human disease gene repertoire and 80% of the signaling molecule pool has counterparts or homologs in *Drosophila*, hence results obtained from studying various disease paradigms in *Drosophila* can be extrapolated to humans (Jennings, 2011).

1.1.4.2. Signaling Cascades: Wnt signaling pathway

One of the most extensively studied signaling pathways is the Wnt/Wg signaling pathway. Comparative genomics data revealed that members of the Wnt signaling pathway are represented in all clades of metazoans, but not in plants, fungi, or unicellular eukaryotes. The Wnt signaling pathway is an invention of the first multicellular animals (Metazoa), as no single-cell organism (Protozoa) has a complete Wnt signaling pathway. No genes encoding for Wnt ligands or members of the Wnt secretion machinery have been found outside of the Metazoa. Nevertheless, Wnt pathway modules like CK1, GSK3 (Harwood et al., 1995), and an ortholog related to β-catenin called Aardvark have been identified in protozoans (Dickinson et al., 2011; Harwood, 2008). Sponges, one of the earliest branches of metazoa, contain several Wnt genes and components of the Wnt signaling pathway, such as Frizzled (Fz), Dickkopf (Dkk) and Dishevelled (Nichols et al.,

2006). Wnt signaling pathway is present in even the simplest free-living animals, the placozoans (primitive metazoans) (Srivastava et al., 2008) but, is absent in unicellular organisms. Therefore, the successful assembly of these modules is the driving force behind the formation of tissues and a signaling center at the point of transition from single-cell to multicellular organisms (Holstein, 2012; Petersen and Reddien, 2009a, b).

Conservation of the chromosomal order of Wnt genes (Nusse, 2001) is evident in many phyla, including Cnidaria, Tribolium (Bolognesi et al., 2008) and Amphioxus (Putnam et al., 2008). Most mammalian genomes, including the human genome, harbor 19 Wnt genes, falling into 12 conserved Wnt subfamilies. At least 11 of these subfamilies are present in the genome of a diploblastic sea anemone, *Nematostella* (Cnidaria). These genes are further expressed in a specific pattern along the axis of the developing embryos, emphasizing the crucial role for Wnt proteins in organismal patterning throughout the animal kingdom (Kusserow et al., 2005). The complexity of the Wnt family suggests a crucial function of Wnt genes in the diversification of eumetazoan body plans. Wnt signals are extremely pleiotropic in their activity, with consequences ranging from mitogenic stimulation to differentiation, changes in polarity and differential cell adhesion (Bauer and Willert, 2012; Delaunay et al., 2014; Niehrs and Acebron, 2012). The inception of a Wnt signaling center at the site of gastrulation is instrumental in the formation of a primary, anterior– posterior body axis, which can be traced throughout animal evolution (Arkell et al., 2013; Sepich et al., 2013). Hence, the Wnt pathway can be considered as a primordial signaling pathway during evolution. Because of the high degree of conservation in the pathway, insights gained through different systems can be applied to the understanding of the mechanism of ectopic Wnt activation in humans and the resultant outcomes. Wnt malfunction is implicated in various forms of disease, including cancer and degenerative diseases (Clevers and Nusse, 2012; Herr et al., 2012; Ring et al., 2014).

Wnt signaling is one of the most extensively studied signaling pathways, it affects cell fate determination (e.g., the decision to proliferate or differentiate) and axis specification in all metazoan organisms. The interplay between these two processes allows a single cell with little discernible polarity to develop the complex morphologies prevalent throughout the animal kingdom (Sokol, 2015). A primitive rudimentary Wnt signaling pathway that regulates axis specification as well as stem cell proliferation is first observed in Cnidarians, thus dating these conserved \million years ago (Teo et al., 2006).

Wnt signaling is implicated in a variety of cellular processes, including proliferation, differentiation, survival, apoptosis and cell motility (Ring et al., 2014; Stamatakou and Salinas, 2014; van Amerongen and Nusse, 2009). Wnt signaling can be broadly demarcated into two categories; the β-catenin transcriptional activity-dependent canonical Wnt/Wg pathway and the non-canonical Wnt/Wg pathway independent of the transcriptional function of β-catenin (Bengoa-Vergniory and Kypya, 2015; Kohn and Moon, 2005; Niehrs, 2012). Recent studies have shown that canonical Wnt signaling virtually regulates all of the defined human adult stem cell systems, including skin, blood, intestine, and brain (Radtke and Clevers, 2005; Reya and Clevers, 2005; Van Camp et al., 2014). Aberrations of this pathway that lead to its constitutive activation are involved in many types of cancer (Clevers, 2006; Shimizu and Nakagawa, 2015). Most of the mechanistic insights into canonical Wnt signaling have been derived from studies detailing regulation and stabilization of β-catenin (Miller et al., 2013; Wantae et al., 2013).

Wnt growth factors are lipid-modified glycoproteins that belong to the Wnt family, which includes 19 members in mammals. These genes are expressed during very early stages of embryonic development (Kemp et al., 2005). Posttranslational modifications include palmitoylation (Willert et al., 2003) and N-linked glycosylation (Coudreuse and Korswagen, 2007; Tanaka et al., 2002). Glycosylation is essential for Wnt secretion, whereas palmitoylation is necessary for Wnt binding to its cognate receptor and hence its activity (Port and Basler, 2010; Takada et al., 2006).

The first Wnt gene was discovered by Roel Nusse and Harold Varmus in 1982 when they observed activation of Int1 (integration 1) in breast tumors of mice infected with mouse mammary tumor virus (MMTV) concomitant with the fact that Int was present near several MMTV integration sites (Nusse et al., 1984). The name 'Wnt' itself is derived from a fusion of Int (mouse) and Wg (wingless) in *Drosophila*, which is the best characterized Wnt gene (Klaus and Birchmeier, 2008). Wnts coordinate cell-to-cell interactions in many different cell types and are essential for embryonic development and tissue homeostasis (Angers and Moon, 2009; Clevers, 2006).

1.1.4.3. Wnt signaling pathway: *Drosophila* as model system

Much of the current understanding of Wnt biology was achieved through genetic screens and epistasis experiments performed in *Drosophila*. Aberrant Wnt signaling in flies does not induce tumors, but the loss or gain of *wg* function does create characteristic cell fate transformations that are easily detected. As the name itself suggests, wingless is required to pattern adult body structures including the wings. The first mutation was induced by X-irradiation (Sharma and Chopra, 1976), *wg1* mutation causes variable transformation of the wing or haltere into notum tissue. In wg loss of function mutants (*wg* embryos), denticles cover the entire ventral cuticle in contrast to wild type embryos which have denticles intermittently interspersed with naked cuticle (Siegfried et al., 1994). Mutations producing a phenotype similar to that of wingless are known as 'segment polarity' mutations (Nusslein-Volhard and Wieschaus, 1980; Siegfried et al., 1994).

Genetic epistasis experiments were performed by Wieschaus and Nusslein-Volhard (Jurgens et al., 1984; Nusslein-Volhard et al., 1984; Wieschaus et al., 1984) to narrow down on the downstream components. Few important genes such as *armadillo* (*arm*) and *dishevelled* (*dsh*) upon mutation exhibited a cuticle pattern similar to that observed upon *wg* mutation and the same phenotype was observed upon mutation of either *arm* or *dsh* in *wg* overexpression background, leading to a conclusion that both *arm* and *dsh* function downstream to *wg*. The opposite phenotype viz an excess specification of naked cuticle, was observed upon

mutations in *naked* or *zw3*, this phenotype was subsequently found to result from an excess of Wnt/ Wingless signaling activity (Bejsovec and Wieschaus, 1993; Noordermeer et al., 1992).

Decreased activity of *wg* in the *Drosophila* larva gives rise to absence of wings, phenotype in the adult fly (Figure. 1.1.8, middle panel), the same is phenocopied by *nkd* overexpression (Figure. 1.1.8, right-hand panel). As the phenotype of *Drosophila* with a *naked cuticle* (*nkd*) loss-of-function mutation resembles that of *wingless* (*wg*) gain-of function mutants, Nkd has been proposed to be an antagonist of Wg signalling (Depraetere, 2000).

Figure 1.1.8 I Decreased Wg activity and overexpression of Nkd produce similar adult phenotypes. Downregulation or mutation of winless (wg) results in loss of wing phenotype. The same is observed upon upregulation of naked cuticle (nkd/naked). Reproduced from Depraetere, 2000.

Embryos doubly mutant for *zw3* and *dsh* exhibited a zw3 phenotype viz, naked ventral cuticle whereas, *zw3 arm* double mutants exhibited a phenotype similar to arm indicating that *zw3* is downstream to *dsh* but upstream to *arm*. Thus, the hierarchical order of genes acting in the Wn/Wgt pathway was deduced from fly mutant phenotypes, in some cases before the molecular identity of the gene product was even known.

Table 1.1.1 | Basic genetic screen

Phenotype X: Bristles on embryo
Phenotype Y: Smooth embryo

A (Wg)	O/E						
B (Dsh)		O/E		O/E			K/O
C (GSK)					O/E	K/O	O/E
D (Arm)			O/E	K/O			
Phenotype	Phenotype	Phenotype	Phenotype	Phenotype	Phenotype	Phenotype	Phenotype
X	X	X	X	Y	Y	X	Y

Table 1.1.1 I Analysis of the Wnt/β-catenin signaling pathway. The intermediaries of the Wnt pathway can be both activators as well as the inhibitors of the pathway. Here, they have been designated alphabets viz. A=Wg, B=Dsh, C=Gsk, D=Arm. Phenotype X is observed upon activation of Wnt signaling, by over-expression of any of the positive regulators of the Wnt pathway (Wg, Dsh, Arm). Upon over-expression of any of the negative regulators of the Wnt pathway such as GSK3-β (zw3 in *Drosophila*), Phenotype Y is observed, knockdown of the same would lead to phenotype X. If an activator 'A' is expressed in the background of knockdown of 'D' and the phenotype observed is NOT X, then 'D' must be acting downstream to 'A' as overexpression of 'A' is unable to rescue the knockdown of 'B'. If a Wnt activator viz. 'B' is knocked down in the background of an inhibitor of Wnt viz. 'C' and the resultant phenotype is Y, then 'C' must be acting downstream to 'B'. *O/E- Overexpression; K/O- Knock out/ mutant.

1.1.4.4. Canonical Wnt/β-catenin signaling cascade

Wnt ligands couple the seven transmembrane domain receptor protein Frizzled (Fz) and the single-membrane-spanning low-density receptor-related protein 5/6 (LRP5/6) (MacDonald et al., 2009) to activate canonical Wnt/β-catenin signaling. Several lines of evidence suggest that trimeric G-proteins play an essential role in transmitting the Wnt signal (Ahumada et al., 2002; Katanaev et al., 2005), a possibility first indicated by the serpentine topology of Frizzled receptors, shared with all G-protein-coupled receptors (GPCRs). Binding of Wnt to Frizzled elicits a

complex cascade of molecular events, beginning with the recruitment of the intracellular protein Dishevelled (Dvl/Dsh) to the receptor: co-receptor complex, which in turn anchors the axin–GSK3 (glycogen synthase kinase 3) complex to the membrane, thereby promoting the initial phosphorylation of LRP6 (Zeng et al., 2008; Zeng et al., 2005). Further phosphorylation of LRP6 by casein kinase 1 (CK1ε) (Davidson et al., 2005) is associated with clustering of different proteins including LRP6, Dvl and Axin to form what is defined as the LRP6 signalosome (Bilic et al., 2007), an event that involves titration of components of the β-catenin destruction complex (axin, GSK3, Dvl, CK1, and APC) away from the destruction complex (Bienz and Clevers, 2003; Cadigan, 2002), resulting in stabilization of β-catenin and its translocation into the nucleus, where it associates with the lymphoid enhancer factor/T cell factor (LEF/TCF) to induce target gene expression (Clevers, 2006; MacDonald et al., 2009) (Figure 1.1.9, panel A). Most of the Wnt/Wg pathway studies have been performed at the level of β-catenin, the functioning of Dishevelled- the upstream effector is comparatively less well understood. Like SATB1, Dishevelled also contains a PDZ domain which aids in interaction of the protein with diverse proteins leading to efficient transduction of the Wnt signal (Mahindroo et al., 2008; Wong et al., 2003) (Details provided in Chapter 3). In this piece of work, interaction of SATB1 with Dishevelled- physical and genetic have been looked at.

β-catenin- a transcriptional activator

In the canonical Wnt/β-catenin signaling pathway, β-catenin, acts as a transcriptional activator by recruiting various transcriptional co-activators such as p300/CBP and TBP (Barker et al., 2001; Hecht et al., 2000) onto the Wnt responsive genomic targets via its C-terminus region. Both these proteins have Wnt target gene-selective roles as bimodal regulators that mediate activation and repression, this output is dependent upon the cell type and level of nuclear β-cat/Arm, reduction of CBP/p300 (Li et al., 2007; Mosimann et al., 2009). In a seminal study, the effect of the HAT activity of CBP on the chromatin of Wnt target genes in vivo was

analyzed. Upon Wnt activation, nucleosomes in the region surrounding the WREs (up to 30 kilobases) of the naked cuticle and Notum loci in *D. melanogaster* were rapidly acetylated and saturated after 5.5 hours in a CBP-dependent manner. Consequently, recruitment of TCF and β-catenin to a WRE swiftly induces extensive chromatin changes (Mosimann et al., 2009). The SWI2/SNF2 family protein BRG1 binds directly to β-catenin. Expression of an ATPase-deficient form of BRG1 has a dominant-negative effect on TCF/ β-catenin mediated transcription, suggesting that BRG1 directly regulates nucleosome arrangements at Wnt-responsive target genes (Barker et al., 2001). A myriad more factors are known to be recruited by the C-terminal region of β-catenin, including the Mediator component MED12, the PAF1 complex factor (Mosimann et al., 2009), Transformation/transcription domain-associated protein (TRRAP), a part of the TRRAP/p400/TIP60 and TRRAP/GCN5 (SAGA) complexes and, a histone methyltransferase (HMT) complex containing the Trithorax-family mixed lineage-leukemia (MLL1/MLL2) SET1-type proteins (Sierra et al., 2006). Thus, β-catenin can localize histone repositioning machineries on to its target gene loci (Mosimann et al., 2009). (Figure 1.1.9, panel D).

1.1.4.5. Non-canonical Wnt pathways

Multiple studies have shown that Wnt signal cascade may act independent of β-catenin, executing its effects in early development where calcium signaling or the JNK pathway maybe the central mediators (The planar cell polarity (PCP) pathway and Wnt-cGMP/Ca2+ signaling respectively (Liu et al., 2014; Semenov et al., 2007) (Figure 1.1.9, panels B and C, respectively). Many Wnt ligands also transduce the Wnt signaling cascade by binding to the orphan receptors, receptor tyrosine kinases, Ryk and Ror (Harris and Beckendorf, 2007; Xin et al., 2013). These receptors are implicated in Wnt-signal transduction in multiple species on account of their functional extracellular Wnt-binding domains (Green et al., 2014). While canonical Wnt signaling has been extensively dissected from the view-point of molecular biology and biochemistry, non-canonical Wnt signaling pathways have just started gaining focus. In vertebrates, it is known that canonical and non-

canonical pathways antagonize each other (Stoick-Cooper et al., 2007).

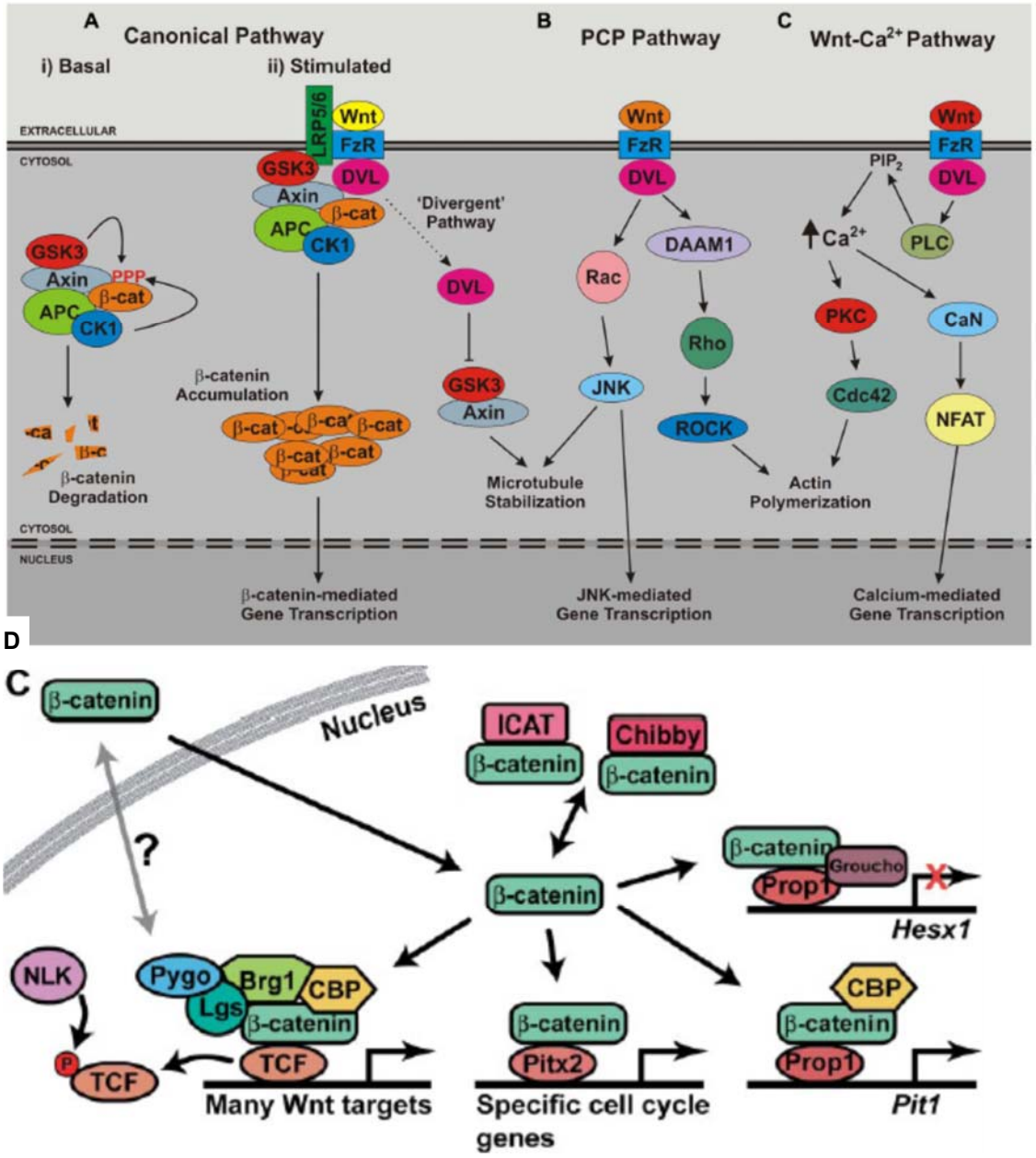

Figure 1.1.9 I A Working Model of the Wnt/β-Catenin Signaling Pathway. (A) Wnt/ β-catenin signaling pathway, in the absence of Wnt, β-catenin is phosphorylated by components of the degradation complex, CKI and GSK-3 and is subsequently degraded in a β-Trcp dependent manner. Wnt target genes are in repressed state due to the association of TCF with Groucho. Wnt stimulation results in phosphorylation of LRP and recruitment of Axin to the plasma membrane in a Dvl/Dsh dependent manner, leading to disruption of the Axin degradation complex and, inhibition of CKI/GSK-3 mediated β-catenin phosphorylation/degradation. β-catenin is stabilized and accumulates inside the nucleus where it replaces Groucho from TCF, and activates Wnt target genes. **(B) The planar cell polarity (PCP) pathway**, which signals through the Rho/ROCK (Park et al., 2006; Weber

et al., 2000) and Rac1/JNK signaling cascades (Qiu et al., 2011; Yamanaka et al., 2002). In vertebrates, PCP signaling regulates cell movements during gastrulation and neurulation (Kikuchi et al., 2009; Simons and Mlodzik, 2008; Wallingford and Harland, 2002) and is crucial for cardiogenesis (Eisenberg and Eisenberg, 1999; Pandur et al., 2002). (C) Wnt-cGMP/Ca2+ signaling, Wnt/Fz via the G protein activates PLC, generating DAG and IP3 leading to an increase in intracellular Ca2+ concentration. Ca2+ activates PKCα/Cdc42 (responsible for cell adhesion and tissue separation during vertebrate gastrulation) and CaMKII/NFAT (involved in ventral patterning in Xenopus) (Huang et al., 2011; Sheldahl et al., 2003). Adapted from Berwick and Harvey, 2013. (D) Emerging details of the complexity of nuclear β-catenin activity. In the classical canonical model, β-catenin forms a complex with TCF and the transcription factors Brg1 and CBP. Lgs and Pygo also bind to β-catenin, possibly driving its nuclear localization in addition to playing a direct role in transcriptional activation. Negative regulation of signaling is provided by NLK (Nemo-like kinase), which phosphorylates TCF, and ICAT (inhibitor of catenin) and Chibby, which are antagonists of β-catenin. In addition to TCF, two other DNA-binding proteins have been shown to associate with β-catenin: Pitx2 and Prop1 (center and right portion of panel). In the case of Prop1, β-catenin can act as a transcriptional activator or repressor of specific genes, depending on the co-factors present. The participation of any particular β-catenin complex in transcriptional regulation is highly cell type-dependent. Adapted from (Gordon and Nusse, 2006). APC, adenomatous polyposis coli; CaN, calcineurin; CK1, casein kinase 1; DAAM, dishevelled-associated activator of morphogenesis; DVL, dishevelled; FzR, frizzled receptor; GSK3, glycogen synthase kinase 3; JNK, c-Jun n-terminal kinase; LRP5/6, low-density lipoprotein; receptor-related protein 5/6; NFAT, nuclear factor of activated T cells; PKC, protein kinase C; PLC, phospholipase C; ROCK, Rho-associated protein kinase.

The different Wnt proteins have been frequently classified as “canonical” or “non-canonical” Wnts based on their capability to induce an ectopic axis in Xenopus embryos (McMahon and Moon, 1989) or, convergent extension and planar cell polarity movements (Heisenberg et al., 2000; Holstein, 2012). According to the ‘classical’ concept Wnt1, Wnt3a, Wnt8, and Wnt8b act in the canonical Wnt signaling pathway whereas Wnt4 and Wnt5a participate in the non-canonical Wnt signaling pathway. However, research has shown that such classification is oversimplified and it is difficult to classify Wnt ligands as ‘canonical’ or ‘non-canonical’, it has been observed previously that a single Wnt ligand can activate different signaling branches in the same cell; Wnt3a activates both canonical and non-canonical pathways in mouse ST2 cells (Tu et al., 2007). According to latest findings in the field, type of the Wnt signaling activated is dependent on the concentration of the Wnt ligand: low concentrations of Wnt3a trigger non-canonical Wnt signaling whereas high concentrations of Wnt3a activate Wnt/β-catenin signaling (Nalesso et al., 2011) (Figure 1.1.10, panel A). However, these findings

remain to be confirmed. Reports from Heasman group demonstrate that canonical Wnt signaling is favored by Wnt dimerization, which in turn might be a function of Wnt concentrations (Cha et al., 2009; Kestler and Kuhl, 2011).

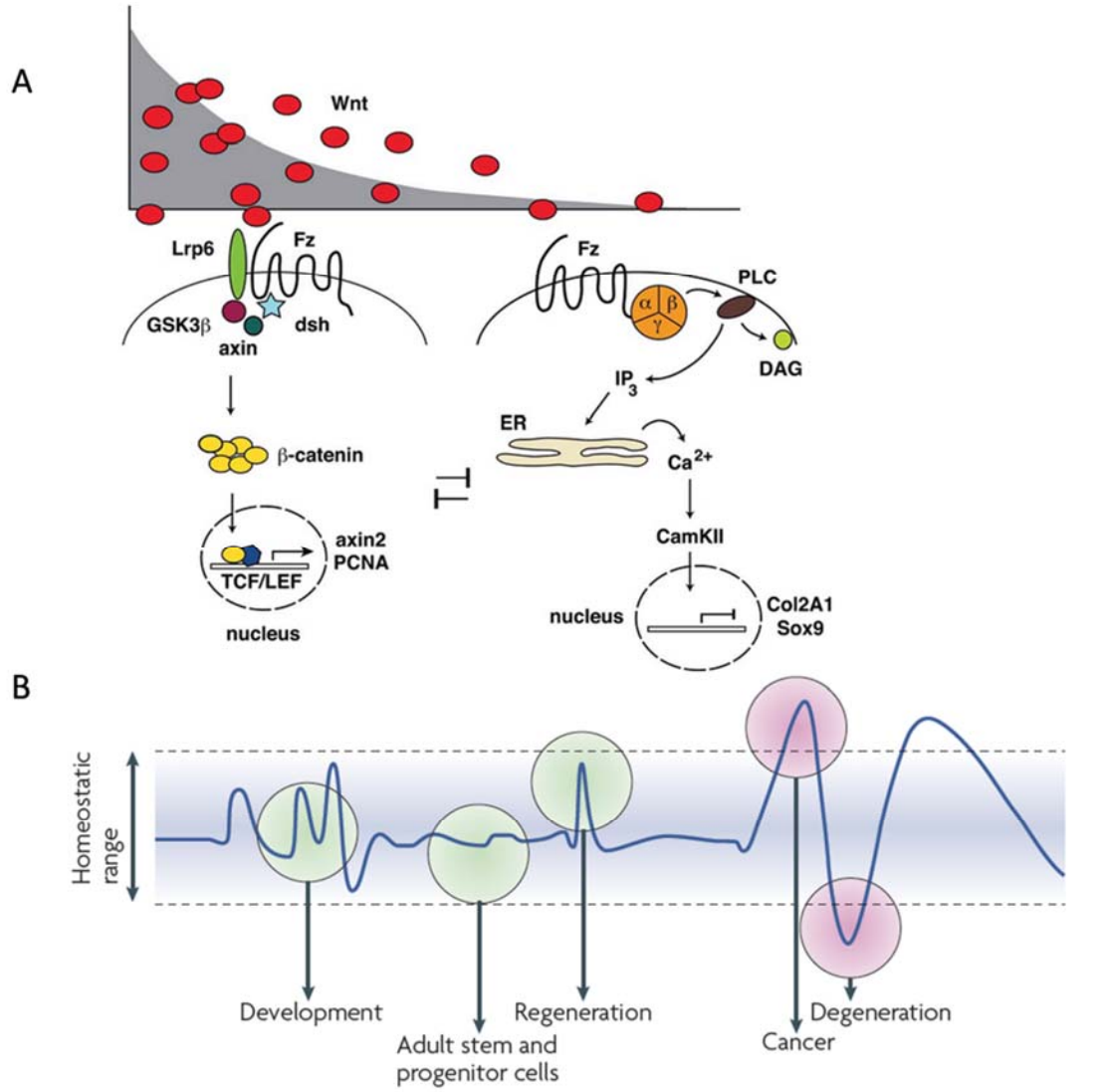

Figure 1.1.10 I Concentration-dependent activation of Wnt pathways. A, Wnt/β-catenin signaling is activated by high concentrations of Wnt ligands, resulting in the stabilization of β-catenin (left). Binding of Wnt protein results in LRP6/Fz heterodimer formation. Intracellular components of canonical Wnt signaling thereby are recruited to the receptor complex including disheveled (dsh), axin, and GSK3β. As a consequence, β-catenin accumulates in the cytoplasm, enters the nucleus, and interacts with transcription factors such as TCF/LEF, resulting in target gene activation. In contrast, Wnt/Ca2+ signaling is favored by lower concentrations of Wnt ligands (right). Wnt/Frizzled interaction results in a G protein (orange circle)- mediated activation of phospholipase Cβ (PLC) that generates diacylglycerol (DAG) and inositol-3,4,5-trisphosphate (IP3). IP3 production results in

release of calcium ions from the ER that in turn activate CamKII. Both pathways reciprocally inhibit each other. Reproduced from (Kestler and Kuhl, 2011). **B,** Wnt–β-catenin signaling in metazoans. During embryonic development, in adult stem and progenitor cells and during regeneration following acute injury, Wnt–β-catenin signaling is kept in a homeostatic range by an intricate web of regulatory proteins, thereby regulating cell fate, proliferation and stem cell self-renewal. However, when levels of signaling exceed this homeostatic range, diseases such as cancer can arise. Conversely, low levels of signaling activity probably underlie many degenerative conditions. Reproduced from Angers and Moon, 2009).

1.1.5. SATB1 and Wnt/Wg pathway- What is known uptill now

β-catenin, the final effector of the canonical Wnt/Wg pathway is known to interact with multiple proteins, one of them being the MAR-binding protein SATB1. This interaction is mediated via the N-terminal PDZ-like domain of SATB1. Reports from Galande laboratory have shown that SATB1 mediates Wnt/β-catenin signaling by recruiting β-catenin to its genomic targets thereby drastically altering the transcriptional activity of these target genes (Gattinoni et al., 2010; Notani et al., 2010). This facet of transcriptional activation is also shared by the HMG box containing TCF/LEF family transcription factors, which also require accessory factors for activating transcription (Hurlstone and Clevers, 2002). The HMG box mediates sequence-specific binding to a core consensus sequence AGATCAAAGGG (van de Wetering et al., 1991) as opposed to SATB1 which, binds to a 12-mer consensus sequence- TATTAGTAATAT resembling the HD consensus (Purbey et al., 2008). Given that SATB1 and TCF have disparate DNA recognition sites, it is unlikely that SATB1 competes for binding to TCF sites. Since, Wnt activation affects the SATB1 responsive IgH-MAR-linked reporter in vivo, the possibility of SATB1's effect on TCF binding sites cannot be ruled out. Many of the SATB1 regulated genes may also respond to Wnt signaling (Notani et al., 2010). The cellular protein levels of TCF and SATB1 play a decisive role in determining the outcome of their interaction with β-catenin as SATB1 competes with TCF for sequestering β-catenin. SATB1 interacts with β-catenin through its C-terminus whereas, TCF interacts via the arm repeats therefore competition is not driven by the site of interaction on β-catenin. Rather, the competition could be a function of

the post-translational modifications and associated partners of these proteins. Since not all SATB1 targets are Wnt responsive and vice versa, the choice among these effector proteins as a partner of β-catenin could dictate the potential outcome (Notani et al., 2010).

1.1.6. Heterologous expression

Having discussed in detail the current review of literature in the context of SATB1 mediated functions, the succeeding chapters would elaborate the strategy used to study the novel interacting partners of the N-terminal PDZ-like domain of SATB1 (physical as well as genetic). Quantitative RT-PCR assays were performed to study the regulation of Wnt responsive genes by SATB1 in cell lines. To better understand the effect of SATB1 on the Wnt/Wg pathway the approach of heterologous expression of human SATB1 in *Drosophila* using the *UAS-GAL4* system was taken.

Deciphering gene function is essential for understanding physiological processes. Expression of genes in heterologous organisms contributes towards the functional analysis of gene products. Heterologous expression system embarks upon the basic principles of protein expression and function which are conserved across species. This strategy is made feasible by the common descent of all organisms, and, conservation of developmental and metabolic pathways, and genetic material along the course of evolution. As previously mentioned, the same set of seven pathways comprising: Hedgehog (Hh), wingless related (Wnt), transforming growth factor-β (TGF-β), receptor tyrosine kinase (RTK), Notch, Janus kinase (JAK)/signal transducer and activator of transcription (STAT) and nuclear hormone pathway, are used repeatedly throughout the course of metazoan evolution, indicating that signaling modules are highly flexible in generating distinct responses in a tissue and species specific manner. Many components of these pathways are conserved, therefore the approach of heterologous expression of proteins in animal model systems is useful as well as advantageous (Pires-daSilva and Sommer, 2003).

Each model system has distinctive features that make it the model system of choice for a given study.

The fruit fly *Drosophila melanogaster* is a well-established genetic system used to model many human diseases such as epilepsy (Parker et al., 2011), Parkinson's disease (Whitworth, 2011), heritable cancer syndromes such as epithelial neoplasia (Khan et al., 2013) and metabolic disorders such as diabetes (Murillo-Maldonado et al., 2011). For decades, *Drosophila* has been used for studying the molecular and genetic functions of a wide range of viruses, and has provided seminal insights into the mechanisms of host antiviral immunity (Hughes et al., 2012). The genetic and cytological techniques available in *Drosophila* allow identification of novel genes and elucidation of signaling pathways involved in development.

Analyses of signaling pathways utilizing model organisms such as *Drosophila* and *C. elegans* have granted critical insights towards defining corresponding pathways in mammals which would not have been as feasible in mammalian species and humans. Components of these pathways are typically conserved between human and fly and carry out analogous if not identical functions (Adams et al., 2000). Even though there is a large order of difference in complexity between human and flies, the underlying cellular and biochemical processes are highly conserved. Approximately 61% of the human genes are conserved in *Drosophila* (Lander et al., 2001). Amongst the plethora of genetic tools available in the fly is the ability to misexpress heterologous genes in vivo. These studies are particularly useful for identifying potential human gene functions since they provide functional clues from abnormal phenotypes induced by ectopic expression of a particular gene.

In *Drosophila*, the overexpression phenotype often provides clues about gene function. These phenotypes serve as indications that the heterologous genes are capable of perturbing the function of one or more conserved signaling pathways in flies. Together with what can be learned from the *Drosophila* homologs, such

observations enable further investigation into the role and function of the human gene counterparts (Bhandari and Shashidhara, 2001; Herranz et al., 2012; Vonhoff et al., 2012). Human genes have been expressed in *Drosophila* by employing the *GAL4/UAS* binary system. Bhandari and colleagues undertook the approach of ectopic expression of human APC (hAPC) protein in *Drosophila*, for examining interactions between human APC, β-catenin and other components of Wnt signaling pathway. Expression of hAPC in the fly, induced phenotypes, which mimicked loss of Wg signaling. They also demonstrated that human APC was also able to suppress the degradation resistant Arm phenotypes (Bhandari and Shashidhara, 2001). The human sour-taste receptor, PKD2L1 (Polycystin-2L1) or bitter-taste receptors, T2R4 and T2R38 (Taste receptor, Type 2) were expressed in the fly gustatory receptor of fly, and behavioral assays were performed to assay if the transgene chemoreceptors are functional. It was confirmed that the changes observed in behavior are the direct result of the human transgenic receptors triggering signal transduction by using different types of receptors and ligands (Adachi et al., 2012).

For such studies, expression of the gene of interest in a particular tissue in a specific pattern is of paramount importance. The technique of targeting gene expression in a spatiotemporal fashion has proven to be one of the most powerful approaches for addressing gene function in vivo. In 1993, Brand and Perrimon devised a bimodal approach for directed gene expression *in vivo*. In this system, expression of the gene of interest, the responder, is regulated by the presence of a *UAS* element, in this case five tandemly arrayed GAL4 binding sites. As transcription of the responder requires the presence of GAL4, the absence of GAL4 in the responder lines maintains them in a transcriptionally silent state. To activate expression of the target gene, responder lines are mated to flies expressing *GAL4* in a particular pattern, termed the driver. The resulting progeny express the gene of interest in a pattern that reflects the pattern of expression of the respective *GAL4* driver. This bipartite approach, wherein, the responder and the driver, are maintained as separate parental lines, has numerous strengths.

For the present study, transgenic *Drosophila* expressing SATB1, SATB1 (1-204) and SATB1 (255-763) were generated. Most of these studies were focused at the level of two Wnt/Wg intermediaries- Dishevelled and armadillo. Further details of the same are provided in the subsequent chapters of this thesis.

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Chapter 2

Screening for putative interaction partners of SATB1 via its N-terminal PDZ-like domain and generation of SATB1 transgenic flies

2.1. **Introduction**

SATB1 is a genome organizer involved in regulation of multiple genes. It consists of an N-terminal PDZ-like signaling domain and a C-terminal DNA-binding region comprising CUT repeat containing and homeodomains (Dickinson et al., 1992). Due to the disparate functions of these domains, SATB1 acts like a modular protein wherein it's N-terminal half is involved in protein interactions while it's C-terminal half is involved in DNA-binding (Galande et al., 2001; Purbey et al., 2008). Wherein, the DNA-binding property of the C-terminal half is dependent upon SATB1 dimerization mediated by the N-terminal PDZ-like domain (Galande et al., 2001). The N-terminal PDZ-like domain of SATB1 acts as the principal interface for dimerization as well as for mediating protein interactions (Notani et al., 2010; Purbey et al., 2008). The PDZ domains of different proteins are also known to interact with each other in a heterotypic manner (Chang et al., 2011; Jelen et al., 2003). Most of the proteins which harbor a PDZ are cytosolic where they are involved in organizing multiprotein complexes (Kim and Sheng, 2004; Lee and Zheng, 2010). SATB1 is one of the few nuclear proteins which harbor a PDZ-like domain (Poulat et al., 1997) and might therefore, function as a scaffold for anchoring various proteins to genomic loci. SATB1 also harbors a nuclear matrix targeting sequence (NMTS) spanning residues 224 to 278, which aids SATB1 binding to the nuclear matrix. Deletion constructs lacking the NMTS exhibit greatly diminished localization to the nuclear matrix. Deletion of this nuclear matrix targeting sequence (NMTS) partially relieved SATB1-mediated transcriptional suppression of MMTV LTR-reporter plasmids (Seo et al., 2005). Much of what is known about SATB1 is restricted to its function inside the nucleus, however, very little is known about its cytosolic function if any. The role of SATB1 as a regulator of transcription is well studied (Close et al., 2012; Purbey et al., 2009; Skowronska-Krawczyk et al., 2014). However, the signaling cues in response to which SATB1 acts remain to be fully elucidated. Understanding the cross-talk between SATB1 and components of different signaling pathways will provide important insights about its function in specific contexts. Outcome of such studies would help identify pathways culminating in SATB1 mediated functions. From the previous Chapter, it

has been seen that global deregulation of genes occurs in response to change in the levels of SATB1, many of these genes are involved in signaling pathways such as TGF-β and Wnt/Wg signaling pathway (Notani et al., 2011). Further reports from our lab have demonstrated that SATB1 interacts with β-catenin, and in thymocytes SATB1 and β-catenin co-localize and regulate a common set of genes (Notani et al., 2010). These results indicate towards a possible cross-talk between SATB1 and the Wnt/Wg signaling pathway. As mentioned in Chapter 1, the PDZ-like domain is the main interface for interacting with proteins. The final effector of the canonical Wnt/Wg pathway- β-catenin also interacts with SATB1 by means of the PDZ-like domain (Notani et al., 2010). Reports suggest that N-terminal PDZ-like domain of SATB1 is dominant negative for SATB1 function in cell lines (Notani et al., 2011). Thus, studying the N-terminal based interactions becomes important for understanding the role SATB1 inside the cell. Therefore, one of the basic premises of this study was to identify PDZ domain containing proteins, involved in signal transduction cascades, which can also interact with SATB1 via its N-terminal PDZ-like domain thereby providing an insight into novel functions of SATB1. To screen for such novel interactors, mammalian two-hybrid (M2H) assay was used.

Protein interactomics is a growing area of research that aims to add sense to the wealth of genomic data generated by mapping “which protein associates with which other protein(s)” in the cellular proteome. Such an approach helps us understand the organization and function of proteins. Interaction mapping is particularly relevant as aberrations in protein interaction patterns rewire signaling networks and give rise to disease phenotypes (Taylor et al., 2009). Biochemical and genetic approaches have been applied for tabulating protein network maps in a variety of model organisms. The biochemical approach involves purification of protein complexes from lysates followed by identification of their constituents by mass spectrometry based methods (Kocher and Superti-Furga, 2007). In contrast, genetic methods are based on activation or repression of a reporter construct upon interaction of the genetically fused ‘bait’ and ‘prey’ proteins in vivo, and mostly generate binary interactions (Suter et al., 2008). High throughput binary mapping

efforts largely rely on the 'classic' yeast two-hybrid (Y2H) system (Yu et al., 2008). Mammalian two-hybrid assay system is a variation of the same, where the prey proteins are chosen using a biased approach.

Firstly, proteins harboring PDZ domains were selected by employing bioinformatic approach, the main requirement being that they should be involved signal transduction. The PDZ domains of the selected proteins were cloned into mammalian two hybrid vectors. Similar procedure was performed independently for the N-terminal PDZ-like domain of SATB1 (residues 1-204) and full length SATB1. The proteins selected were CASK, Dishevelled (Dvl/Dsh), Neuronal nitric oxide synthase (nNOS) and X-11β. CASK (calcium/calmodulin-dependent serine protein kinase) is a member of MAGUK family and was originally identified as an interaction partner of neurexins (Fairless et al., 2008; Hata et al., 1996). CASK has two regulatory roles at the postsynaptic site- one is to physically maintain spine morphology; the other is to regulate expression of the NR2b gene thereby controlling synaptic activity (Huang and Hsueh, 2009). Dishevelled is involved in propagating the Wnt/Wg signal, it acts as a switch between canonical and non-canonical Wnt/Wg pathways (Lee et al., 2008). Neuronal NOS is as an integral component for regulating synaptic transmission and intercellular signaling (Mungrue and Bredt, 2004). Inhibitors of NOS impair learning and produce amnesia in animal models (Bohme et al., 1993; Forstermann and Sessa, 2012). X11β is a member of X11 family of adaptor proteins, it is known to interact with the amyloid precursor protein (APP) (Lau et al., 2000).

Of these, CASK, neuronal NOS and Dishevelled (Dvl/Dsh) showed a positive interaction with the N-terminal PDZ-like domain of SATB1 in the mammalian two hybrid assay. Studies from Galande laboratory have shown that SATB1 co-localizes with the final effector of the Wnt/Wg cascade- β-catenin (arm). Further, and there are reports which show that a set of genes is co-regulated by SATB1 and Wnt/Wg signaling, whereas others are differentially regulated (Notani et al., 2010). These results, prompted investigation into the interaction of SATB1 with Dishevelled and the effect on the Wnt/Wg pathway. Towards this end, a physical

interaction with Dishevelled was established by means of co-immunoprecipitation assay. As SATB1 interacts with both Dishevelled and β-catenin, the effect of SATB1 expression on the Wnt/Wg cascade was analyzed by quantitative RT-PCRs. Various Wnt/Wg targets and Wnt regulators were monitored at transcript levels upon over-expression of SATB1 in cell-lines. Such studies established that SATB1 is a positive regulator of the Wnt/Wg pathway. To study if the positive correlation between SATB1 and Wnt/Wg pathway is a conserved phenomenon or if it is context dependent, it was decided to perform the study in a system which lacks endogenous SATB1 but, has a fully functional and well-studied Wnt/Wg pathway.

For this purpose, it was reasoned that the fruit fly *Drosophila melanogaster* would serve as an ideal model system as most signaling pathways are well characterized in the fly and sufficient tools are available for studying genetic interactions (Engstrom et al., 1997). Fly is also a system of choice for studying functions of heterologous genes (Cukier et al., 2008; Deshpande et al., 1997; Vonhoff et al., 2012). Therefore, the approach of ectopic expression of mammalian SATB1 in the fly was used. For the purpose of identifying which domains of SATB1 are essential for SATB1 function i.e. are responsible for generation of the phenotype, transgenic flies expressing SATB1 (1-204) (N-terminal PDZ-domain harboring region), and SATB1 (255-763) (C-terminal half containing the CUT and homeodomains) were generated. Full length SATB1 and SATB1 deletion constructs were ectopically expressed in specific fly tissues using various *GAL4* driver lines.

Expression of genes in heterologous organisms contributes towards the functional analysis of gene products. Heterologous expression system embarks upon the basic principles of protein expression and function(s) which are conserved across species. As mentioned in Chapter 1, the fruit fly *Drosophila melanogaster* is a well-established genetic system used to model many human diseases (Hughes et al., 2012; Parker et al., 2011; Khan 2013; Murillo-Maldonado et al., 2011; Whitworth 2011). Analyses of signaling pathways utilizing model organisms like the *Drosophila* and *C. elegans* have provided critical insights towards defining corresponding

pathways in mammals which would not have been as feasible in mammals in such a short time-span. Components of these pathways are conserved between human and fly and carry out analogous if not identical functions (Adams et al., 2000). Even though there is a large order of complexity between human and flies, the underlying cellular and biochemical processes are highly conserved. Approximately 61% of the human genes are conserved in *Drosophila* (Lander et al., 2001). The genetic and cytological techniques available in *Drosophila* allow identification of novel genes and elucidation of signaling pathways involved in development. Amongst the plethora of genetic tools available in the fly is the ability to misexpress heterologous genes in vivo. These studies are particularly useful for identifying potential human gene functions since they provide functional clues from abnormal phenotypes induced by ectopic expression of a particular gene. In *Drosophila*, the overexpression phenotype can often provide clues about the function of the gene. These phenotypes serve as indications that the heterologous genes are capable of perturbing the function of one or more conserved signaling pathways in flies. Together with what can be learned from the *Drosophila* homologs, such observations enable further investigation into the role and function of the human gene counterparts.

The *GAL4-UAS* system for inducible gene expression is used for performing such studies (Phelps and Brand, 1998). The key feature of this system lies in a separable activator element mediated by the *GAL4* gene whose expression is regulated by tissue-specific promoters and a *UAS-target* gene whose expression in turn is GAL4-dependent. Since *UAS* promoter sequences “CGGAGTACTGTCCTCC”, are absent in *D. melanogaster*, GAL4 would remain inactive in normal condition and therefore, targeted expression of transgenes would remain confined to specific tissues, while not otherwise affecting cells lacking GAL4 expression. Thus, transgene expression can be regulated both temporally and spatially during development by crossing flies carrying a particular *UAS-transgene* to different *GAL4* lines.

The *MS1096-GAL4* (Gullaud et al., 2003) is driven by the enhancer of the *beadex* (*bx*) gene locus which is expressed throughout the wing pouch where majority of growth occurs during wing development. Interference of one or more aspects of signaling required during this process can lead to various abnormal wing development phenotypes (Ratnaparkhi, 2013). Expression of some transgenes using *MS1096-GAL4* causes the loss or gain of wing venation while others bring about loss of tissues through apoptotic events leading to nicked or serrated margins in the adult wings (Katanayeva et al., 2010; Marygold et al., 2011).

The *Drosophila* eye is a holistic model system in itself. This makes it a particularly useful organ to observe for phenotypic consequences, whether caused by loss-of-function mutations or overexpression of a gene in the eye. Eye development proceeds in an ordered fashion wherein cells within the eye imaginal discs are specified, recruited, and differentiated in a sequential manner eventually leading to the highly organized structure of the adult fly eye (Papayannopoulos et al., 1998; Pignoni et al., 1997; Voas and Rebay, 2004). Perturbations of signaling pathways regulating cell proliferation, cell death, or differentiation, can lead to abnormal eye development. The *GMR-GAL4* driver, for example, is expressed mainly in the post-mitotic cells that are undergoing differentiation in the eye imaginal disc (Freeman, 1996). Genes interfering with this developmental aspect will give rise to a rough eye phenotype when expressed under the control of this driver.

Certain problems exist with respect to heterologous expression of a human gene in *Drosophila*. There are many challenges: the difficulty of defining genetic and non-genetic factors, the difficulty of understanding the interactions amongst these and the intervening multiple stages between expression of a gene and manifestation of a particular phenotype or behavior. Expression of SATB1 in fly gives an indication towards its role in a certain pathway but no comments can be made whether it is direct or indirect. As SATB1 is a transcription factor, manifestation of a phenotype upon ectopic expression is expected but whether this phenotype is the result of

SATB1 binding to the *Drosophila* chromatin needs to be verified. This was achieved by performing immunostaining for SATB1 on the polytene spreads.

Polytene chromosomes are giant chromosomes present in salivary glands of *Drosophila* larvae. In dividing diploid cells, the DNA synthesis phase (S phase) is followed by mitosis (M phase) but in certain cases, the S phase is not followed by the M phase, resulting in repeated cycles of DNA synthesis thereby, giving rise to polyploidy. Polyploid chromosomes are multi-stranded and exhibit a banded pattern reproducible from individual to individual. This phenomenon of recurrent DNA duplication cycle without subsequent mitosis is called endoreduplication, this phenomenon is common in as endocyclic cells/tissues. Fat bodies and salivary glands are prominent examples of such tissues in *Drosophila*. After each replication cycle, the DNA remains aligned resulting in greatly enlarged chromosomes providing a unique opportunity to correlate chromatin morphology with the localization of specific proteins. Consequently, there has been a high level of interest in defining the factors occupying different loci. An important tool for such studies is the immunostaining of polytene chromosomes using antibodies against proteins of interest. To observe if human SATB1 can bind genomic loci in the fly, Multiple EM for Motif Elicitation analysis (MEME) was performed to identify the putative binding sites for SATB1 in the *Drosophila* genome. Simultaneously, SATB1 binding to *Drosophila* chromatin was examined by performing immunostaining for SATB1 on polytene chromosome spreads upon ectopic expression of human SATB1 in the salivary glands.

A point to remember at all times is that the fly system is simply being used as a test-tube, no physiological significance is being assigned to the protein at any given point of time. Even though, the fly system lacks endogenous SATB1, the binding partners of SATB1 are bound to be present.

2.2. Materials and Methods

2.2.1. Cell lines and reagents

HEK293 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, under 5% CO_2 atmosphere. pTriEx-3 Neo vector was procured from Novagen/EMD Biosciences (CA, USA). Cells were transfected with pTriEx SATB1 and pTriEx Dishevelled-1 (Dvl-1) constructs using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA). DNA to Lipofectamine ratio was used as per manufacturer's instructions. Recombinant human Wnt3A was procured from R&D Systems (Minneapolis, MN).

2.2.2. Mammalian two-hybrid assay

Two-hybrid systems are extremely powerful ways of detecting functional protein-protein interactions in vivo. CheckMate mammalian two hybrid system (Promega Corp., USA) was utilized to score for protein-protein interactions. It comprises of three vectors: the pBIND vector, containing the yeast GAL4 DNA binding domain (DBD) upstream to the multiple cloning site, the pACT vector having the herpes simplex virus VP16 activation domain upstream of the multiple cloning region and the pG5luc reporter vector containing five GAL4 binding sites upstream of a minimal TATA box, which in turn is upstream of the firefly luciferase gene. Association of the DNA-binding domain and the transcriptional activation domain via interaction between the proteins results in transcriptional activation of the firefly luciferase reporter gene. The cDNA sequences encoding potential interactive proteins were cloned into the pBIND and pACT vectors to generate GAL4 and VP16 fusion proteins respectively. The pGAL4 and pVP16 fusion constructs were transfected along with the pG5luc reporter vector into mammalian cells. Specifically, the N-terminal region of SATB1 (1-204 aa) harboring the PDZ-like domain of SATB1 and the PDZ domains of the prey proteins i.e. Dvl-PDZ, CASK-PDZ, nNOS-PDZ and X11-PDZ were subcloned into pACT and pBIND vectors (Promega Corp. USA). Twenty-four hours prior to transfection, 5 X 10^4 HEK293 cells were seeded per well

in a 24-well plate (BD Falcon). Transfections were performed using pG5luc reporter vector along with pBIND fusion construct and pACT empty vector in control and pBIND fusion construct with pACT fusion construct for the experimental set. Total DNA per well was kept constant at 1.5 µg (0.5 µg of each DNA). Cells were harvested 48 h post-transfection. Luciferase assay was performed using Steadylite reagent (Perkin Elmer, Shelton, CT, USA). Luciferase counts were recorded on TopCount NXT™ Microplate Scintillation and Luminescence Counter (Perkin Elmer, Massachusetts, USA). Values were normalized with respect to control (vector transfection) and plotted as relative fold change units.

2.2.3. Transfections and Luciferase reporter assays

Binding of SATB1 to its binding sites downstream of the immunoglobulin µ heavy chain (IgH) gene enhancer, or the IL2Rα Promoter (IL2Rα P) is known to repress the downstream genes. Therefore, IgH-MAR-Luc and IL2Rα P-Luc constructs having SATB1 binding sites (SBS) were used as reporter constructs, to score for the effect of protein binding on promoter activity (Kumar et al., 2005; Kumar et al., 2006). HEK293 cells were grown up to 70% confluence in 24-well plates at 37^{o}C in DMEM (Dulbecco's Modified Eagle's Medium, (Life Technologies, Carlsbad, CA, USA) supplemented with 10 % FBS (Fetal Bovine Serum, Life Technologies) under 5% CO_2 atmosphere. DNA was transfected using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA), as per manufacturer's instructions, in serum-free medium. The medium was supplemented with 10 % fetal bovine serum and 0.5 µL/ml enhancer solution (Perkin Elmer, Shelton, CT, USA), 6 h post-transfection. Transactivation assays were performed 48 h post-transfection. Cells were harvested, washed with buffer (1X PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$) and resuspended in 25 µL of buffer (1X PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$), equal volume of Steadylite Plus reagent (Perkin Elmer, Shelton, CT, USA) was added and the resultant chemiluminescence was measured on TopCountNXT^{TM} Microplate Scintillation Counter (Perkin Elmer, Waltham, MA, USA). Fold changes were calculated by normalizing the transfected sample values to the vector control values.

2.2.4. Co-immunoprecipitation assay

HEK293 cells were grown to 70% confluency in 90 mm culture dishes. Ten micrograms of purified plasmid was transfected per 90 mm culture dish. Transfections were performed as per the manufacturer's instructions, and cells harvested 48 h post-transfection. Cells were lysed in RIPA buffer (150 mM NaCl, 0.1 % sodium dodecyl sulphate, 10 mM sodium phosphate, 1 % sodium deoxycholate, 2 mM EDTA [pH 8.0], 1X EDTA free complete protease inhibitors). The lysate was diluted to a final concentration of 1 µg/µL using 1X chilled PBS containing 1X EDTA free complete protease inhibitor cocktail (Roche, Indianapolis, IN, USA). For each immunoprecipitation reaction, 600 µg of the lysate was pre-cleared for 1 h at 4^{o}C on an end-to-end rocker with 10 µL protein A/G Plus UltraLink resin (Thermo Scientific, Rockford, IL, USA). Post pre-clearing the supernatant was collected by centrifugation at 1000 X g, 5 min at 4^{o}C. Pre-cleared extract was incubated with 1 µg of antibody for 4 h at 4^{o}C on an end- to-end rocker. This complex was then immunoprecipitated using 10 µL of protein A/G beads. These protein A/G resin bound protein-antibody complexes were recovered by centrifugation at 1000 X g for 5 min. Beads were washed five times with PBS containing 0.1% Triton X-100. The complexes were eluted by boiling the beads in 30 µL Laemmli sample buffer (with DTT) at 95^{o}C for 5 min with intermittent mixing and eluate was resolved on a 10% SDS-PAGE gel and transferred to PVDF membrane (Millipore). Immunoblotting was performed using an antibody against the second protein, followed by incubation with horseradish peroxidase (HRP)-conjugated anti-IgG antibody. Signal for immunoblot was detected using VisualizerTM Western Blot Detection Kit (Millipore/Upstate, Billerica, MA, USA).

2.2.5. RNA extraction and quantitative PCR

Total cellular RNA was extracted using TRIzol reagent (Life Technologies). Isolated RNA was then subjected to DNase treatment (Promega) as per the manufacturer's instructions. RNA was further purified using Acid Phenol: $CHCl_3$, followed by

ethanol precipitation. One µg of purified RNA was reverse transcribed into first strand cDNA using ImProm-II™ Reverse Transcription System (Promega). The resulting cDNA was subjected to qPCR using Power Sybr reagent (ABI), essentially as described previously (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: $\Delta Ct = (Ct_{target\ genes} - Ct_{internal\ control})$ 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.

2.2.6. Fly culture and stocks

All experiments were carried out at 25°C on standard cornmeal/molasses/yeast/agar medium (corn-flour 75 g, sugar 80 g, yeast 24 g, agar 10 g and malt 60 g per 1000 ml) Propionic acid (5 ml/l), ortho-phosphoric acid (1 ml/l) and p-methyl benzoate (5% solution/l) were added prior to pouring the media into vials and bottles. All cultures were transferred to a new vial every three weeks to avoid overcrowding.

w^{1118} was used as the background strain for performing micro-injections. Canton-S strains (CS) of *Drosophila* melanogaster was used as control. Double balancer stock (CyO/Pin; TM2/TM6) was used for balancing transgenic flies.

GAL4 drivers:
MS1096-GAL4 (Capdevila and Guerrero, 1994), a strong driver which drives expression in the entire wing pouch, and exhibits a faint and variable expression in the notum; *GMR-GAL4* (Freeman, 1996), an eye specific GAL4 whose expression is restricted to the presumptive photoreceptor cells posterior to the morphogenetic furrow in the eye imaginal disc; and *Sgs3-GAL4* (BL6870; w[1118]; P{w[+mC]=Sgs3-GAL4.PD}TP1) expressed in the salivary glands of wandering third instar larvae were used.

2.2.7. Generation of transgenic flies

Full-length SATB1, FLAG-SATB1 (1-204), and SATB1 (255-763) were cloned into the pUAST expression vector (Brand and Perrimon, 1993). Constructs were purified using Cesium Chloride gradient method. Three days prior to injection, cages for w^{1118} flies were set up and food plates changed daily so as to get the flies acclimatized to the new environment and maximize the number of eggs laid.

On the day of injection plates were changed every 40-60 min and embryos collected, washed under running water, and were dechorionated using 50% bleach. Excess bleach was removed by washing with water and embryos blotted dry. Dechorionated embryos were placed over 2% agarose containing bromophenol blue and aligned in an anterior to posterior fashion viz all posterior ends oriented in the same direction. Properly aligned embryos were then transferred onto a coverslip containing glue, these coverslips were placed on slides, layered with clove oil and dessicated for 5-7 min.

Injection mixture was prepared using a total of 4 µg of purified construct, one unit of transposase was used per microgram of DNA. Embryos were injected with the injection mixture at the posterior end. Injected embryos were incubated in a humidified chamber. Larvae emerging from the embryos were collected and added to fresh food vials. These vials were incubated at 18°C, flies emerging from these larvae were collected and crossed with w^{1118}. The progeny from this cross were screened for red eye color. These red-eyed flies are the flies transgenic for the given gene (Figure 2.2.1).

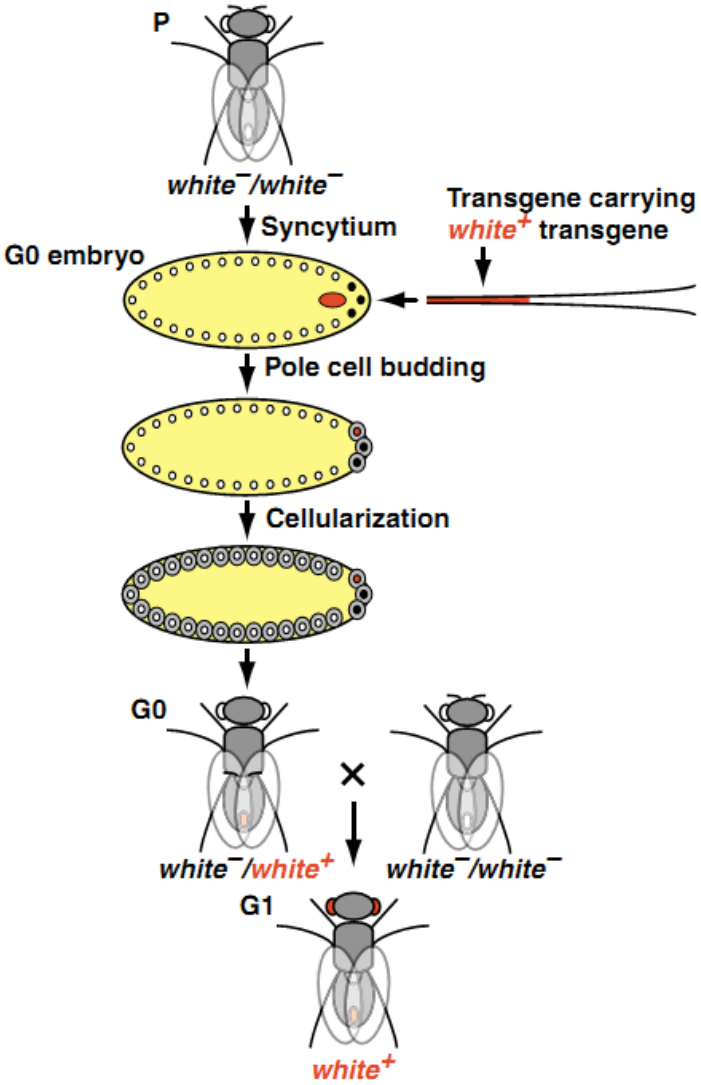

Figure 2.2.1 I *Drosophila* transgenesis. Transgene containing a *white*⁺ reporter (red) is injected into generation zero *Drosophila* embryos (G0) which are less than 1 h old. The early developmental stages of *Drosophila* are characterized by the presence of syncytial embryos created as a result of rapid karyokinesis events that occur without the accompanying cytokinesis. The transgenic DNA must be taken up into the pole cells (black) that are fated to become germ cells, for germ line transmission to occur. Transgenic DNA integrated into a pole cell (red pole cell) can be transmitted from one generation (G0) to the next (G1 progeny). The resulting integration events are identified using an appropriate marker, such as white⁺ which when expressed in the background of a mutant white strain gives rise to red eyed flies which contain the transgene. (P): parental generation. Reproduced from Venken and Bellen, 2007.

2.2.8. Balancing of transgenic flies

Flies carrying the transgene were then balanced using the scheme depicted in Figure 2.2.2.

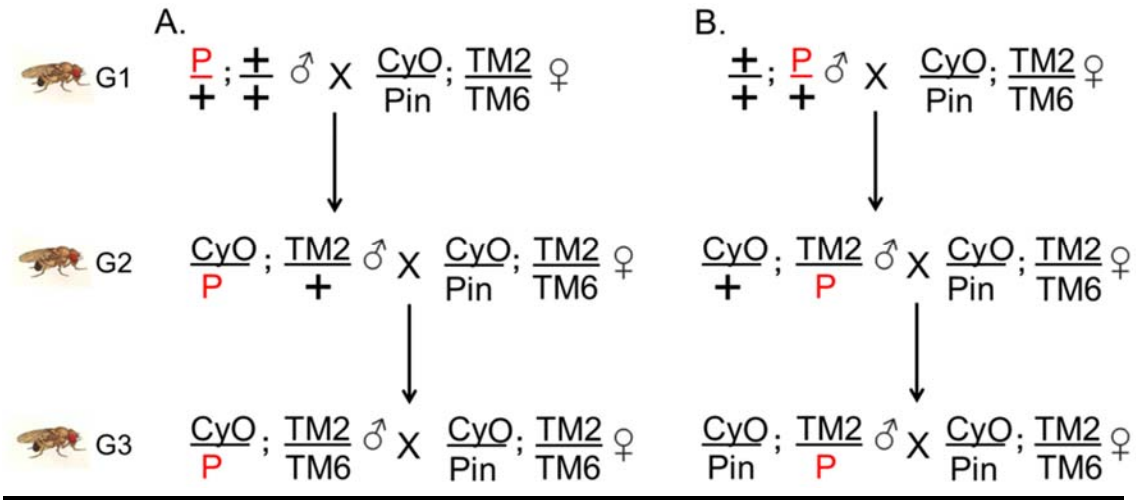

Figure 2.2.2 I Balancing of transgene in *Drosophila*. Transgenic (G1) generation flies having red eyes as a consequence of possessing the *white*$^{+}$ reporter gene are characterized to assess the identity of the chromosome into which the transgene has integrated. The transgenic element, **'P'** can be present on any of the four chromosomes of *Drosophila*. For identifying the chromosome on which transgene integration has occurred, crosses are set up with double balancer fly stock. In the case study depicted by the schematic on top, it can be assumed that the transgene **'P'** has integrated into either chromosome II or chromosome III. Few of the (G2) progeny would have red eyes as they contain the transgene. In case **A**, the transgene **'P'** has integrated into chromosome II, in (G3) generation, flies having red eyed flies would have a completely balanced third chromosome *viz.* TM2/TM6, but flies having a balanced chromosome II *viz.* CyO/Pin would have only white eyes. In case **B**, the transgene **'P'** integration has occurred on chromosome III, in (G3) generation, red eyes would be observed only in flies having a completely balanced chromosome II *viz.* CyO/Pin, but none of the flies having a balanced chromosome III *viz.* TM2/TM6 would have red eyes. *If red eyes are observed in only the female progeny of a cross between the red eyed males of the (G1) generation and double balancer females, it can be inferred that transgene integration has occurred on the X-chromosome.

2.2.9. Motif search analysis using MEME

MEME (Multiple EM for Motif Elicitation) is one of the most widely used tools for searching for novel 'signals' in sets of biological sequences. The MEME algorithm identifies and characterizes shared motifs in a set of unaligned sequences. MEME

works by searching for repeated, ungapped sequence patterns that occur in the DNA or protein sequences provided by the user. Users can perform MEME searches via the web server hosted by the National Biomedical Computation Resource (http://meme.nbcr.net) and several mirror sites. Through the same web server, users can also access the Motif Alignment and Search Tool to search sequence databases for matches to motifs encoded in several popular formats.

2.2.10. Preparation of polytene chromosome spreads

Polytene spreads were prepared as per the specifications of protocol from the Cavalli laboratory (Lavrov et al., 2004). Briefly, third instar larvae were collected and the salivary glands dissected in solution 1 (0.1 % Triton X-100 in 1 X PBS pH 7.5), care was taken to remove fat body cells and finish dissection within 20 min. Glands were transferred onto a poly-L-lysine coated slide. Here, a drop of freshly prepared solution 2 (3.7% Paraformaldehyde, 1% Triton X-100 in 1 X PBS pH 7.5) was added and the glands fixed for 30 secs, these were then moved to freshly made solution 3 (3.7% Paraformaldehyde and 50% acetic acid in 1 X PBS pH 7.5) and incubated for 30 sec. Glands were then washed with 1 X PBS to remove excess fixative. A coverslip was placed over the salivary glands. Using a pencil, uniform pressure was applied on the salivary glands, avoiding lateral movement of the coverslip. Excess liquid was blotted off and the slide frozen in liquid nitrogen. The coverslip was flicked off using a needle. Slides were washed twice with 1 X PBS, 15 min each, and processed for immunostaining.

2.2.11. Immunostaining of polytene chromosome

Slides were washed 2 X 15 min in 1 X PBS, blocked at room temperature for 1 h in blocking solution (3% BSA, 0.2% (w/v) NP40, 0.2% Tween 20, 10% non-fat dry milk in 1 X PBS pH 7.5). Polytene spreads were incubated at room temperature for 4 h with 50 µL of SATB1 antibody (In house) (1:30 dilution in blocking solution) inside a humidified chamber. Slides were washed twice with wash buffer (400 mM NaCl, 0.2 % NP40, 0.2% Tween 20 in 10 mM Phosphate buffer pH 7.5). Slides were rinsed

using 1 X PBS and incubated for 1 h at room temperature with 50 µL of fluorescently labeled secondary antibody (Molecular Probes, Invitrogen) prepared in blocking solution at a dilution of 1:200. Slides were washed for 15 min with wash buffer. DAPI (SIGMA) was used at a working concentration of 1 µg/ml, to stain the DNA. Slides were washed twice for 15 min in wash solution and once using 1 X PBS for 10 min. A drop of Mounting medium (DAKO-cytomation) was placed over the polytene spread and a coverslip was carefully placed over the same. Excess liquid was blotted away and the slide acquired for imaging.

2.2.12. Immunostaining of imaginal discs

Imaginal discs were dissected in 1 X PBS, fixed using 4% Paraformaldehyde prepared in 1X PBS containing 0.1 % Triton X-100 (1X PBST) for 10 min at room temperature. Discs were given three 1 min rinses with 1X PBST followed by three 10 min washes with the same. Imaginal discs were blocked for 2 h at room temperature in blocking solution (0.5% BSA, 2%FBS in 1X PBST pH 7.4). Samples were incubated over-night with primary antibody was prepared in blocking buffer minus TritonX-100. Discs were washed twice with blocking buffer and incubated for 2 h at room temperature with fluorescently labeled secondary antibody (Molecular Probes, Invitrogen) prepared in blocking solution without FBS. Two 10 min washes with 1X PBST were performed, followed by two 10 min washes with 1X PBS. Discs were incubated for 10 min with DAPI (SIGMA) which was used at a working concentration of 1 µg/ml. Two 10 min washes were performed using 1X PBS, followed by mounting of imaginal discs in mounting medium (DAKO-cytomation).

2.3. Results

2.3.1. The N-terminal PDZ-like domain of SATB1 acts as dominant negative for SATB1 function

Since the N-terminal PDZ-like domain of SATB1 mediates multiple protein interactions and is also responsible for SATB1 dimerization which is a prerequisite for binding of SATB1 to DNA (Galande et al., 2001; Purbey et al., 2008), it was decided to analyze the effect of the N-terminal PDZ-like domain on SATB1 function. Luciferase reporter assay was performed for assessing the regulatory activity of the N-terminal PDZ-like domain on SATB1 function. The heptameric IgH-MAR and the 555 bp promoter region of IL-2RαP, which contain SATB1-binding sites (SBS) (Kumar et al. 2005; Purbey et al. 2008), were cloned upstream of the luciferase gene in the promoterless reporter vector pGL3basic. SATB1 and its N-terminal (1–204) region were transiently overexpressed in HEK293 cells. Since the N-terminal (1–204) region lacks DNA binding activity, it was hypothesized that it would fail to recruit SATB1 interacting partners onto its genomic target sites and hence would fail to repress genes repressed by SATB1. As expected, SATB1 overexpression repressed luciferase activity (Figure 2.3.1, graphs A and B, bar 2). SBS-linked reporter activity increased upon expression of the N-terminal PDZ-like domain (Figure 2.3.1, graphs A and B, bar 3 versus bar 1) as opposed to the decrease observed upon SATB1 expression. This phenomenon could be attributed to the titration of SATB1-bound repressor complexes by molecules of the overexpressed PDZ- like domain. Furthermore, when SATB1 and its N-terminal PDZ-like domain (PDZ1) were co-expressed in HEK293 cells, de-repression of MAR-linked luciferase activity was observed (Figure 2.3.1, graph A, bar 4). The de-repression observed was lower than that observed with overexpression of PDZ1 alone, but nevertheless was significant and sufficient to confirm the dominant negative effect. Thus, these results demonstrate that in HEK293 cells, the N-terminal PDZ-like domain acts in a dominant negative fashion by abolishing the transcriptional repressor function of SATB1 (Published in Notani et al., 2011).

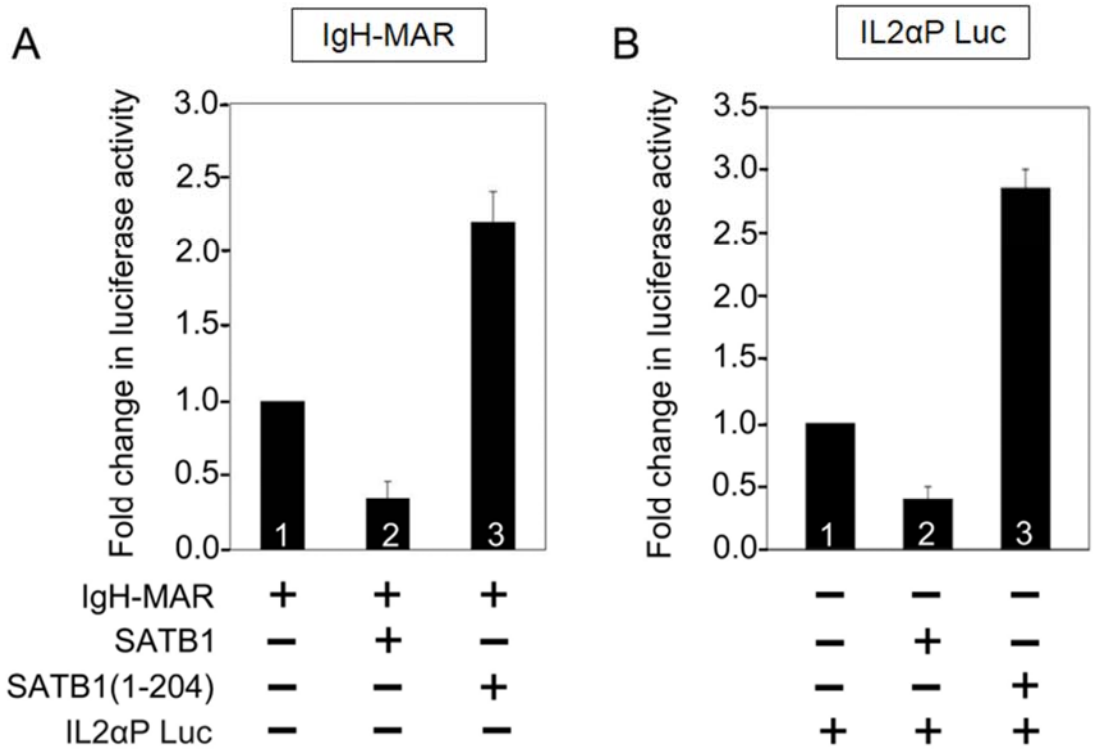

Figure 2.3.1 I The PDZ-like domain induces SBS-linked reporter gene expression. Luciferase reporter assay was performed using the **(A)** IgH-MAR-Luc and **(B)** IL-2RαP-Luc reporter constructs. Equal amounts of SATB1 and SATB1 (1–204) constructs and IgH-MAR luciferase reporter construct were transfected in the indicated combinations in HEK293 cells. Cells were harvested 48 h post transfection and the luciferase activity measured. Relative Luciferase activity is expressed as fold increase or decrease with respect to the control, which was set to 1. Relative luciferase units are represented as fold activity with respect to the reporter alone. Expression of the PDZ-like domain leads to de-repression of MAR-linked reporter activity, as opposed to the repression observed by expression of SATB1. Expression of both full length SATB1 and the PDZ-like domain together lead to de-repression of MAR activity, though this de-repression was less than the one observed upon expression of the PDZ-like domain alone (graph A, bar 4 versus bar 3). Each error bar depicts the standard deviation calculated from triplicates.

2.3.2. The N-terminal PDZ-like domain of SATB1 interacts with PDZ domains of nNOS, CASK and Dishevelled-1

To identify novel interacting partners of SATB1, bioinformatics approach was taken and proteins involved in signal transduction and containing a PDZ domain were chosen. The nucleotide sequence of the PDZ-like domain of SATB1 was used for performing BLAST analysis and proteins involved in various signaling cascades were then selected. Four proteins- CASK, nNOS, Dishevelled-1 (Dvl-1/Dsh), and

X11β were selected. The PDZ domain of each of these proteins was cloned into the mammalian two-hybrid (M2H) system vector, pBIND. HEK293 cells were co-transfected using pBIND-PDZ fusion construct and the pACT-SATB1 (1-204) along with the reporter vector pG5luc (Figure 2.3.2).

A two-fold increase in reporter activity was observed in cells co-transfected with the PDZ domain of neuronal NOS (nNOS), and the SATB1 (1-204) which harbors the PDZ-like domain (Figure 2.3.2, graph A, bar 2), it was therefore concluded that the PDZ domain of neuronal NOS functionally interacts with the N-terminal PDZ-like domain of SATB1. Transfection of HEK293 cells with the PDZ domain of Dishevelled-1 (Dvl-1) together with the N-terminal PDZ-like domain of SATB1 resulted in a four-fold increase in reporter activity with respect to control, suggesting that the PDZ domain of Dishevelled-1 (Dvl-1) also interacts with the N-terminal PDZ-like domain of SATB1 in vivo (Figure 2.3.2, graph B, bar 2). A five-fold increase in reporter activity was observed upon transfecting HEK293 cells with the PDZ domain of CASK and the N-terminal (1-204) aa region of SATB1, (Figure 2.3.2, graph C, bar 2), suggesting that the PDZ domain of the neuronal NOS protein might be interacting with the PDZ-like domain of SATB1. Upon transfecting HEK293 cells with PDZ domain of X11 and the PDZ-like domain of SATB1, a decrease in reporter activity was observed (Figure 2.3.2, graph D, bar 2). Based on the results of mammalian two hybrid assay, neuronal nNOS, CASK and Dishevelled-1 (Dvl-1) were identified as the novel interactors of the MAR binding protein, SATB1. Out of these three, Dishevelled is an intermediary of the Wnt/Wg signaling pathway which is crucial for proper development of an organism (Nusse, 2005). It was decided to follow up the functional interaction of Dishevelled with SATB1.

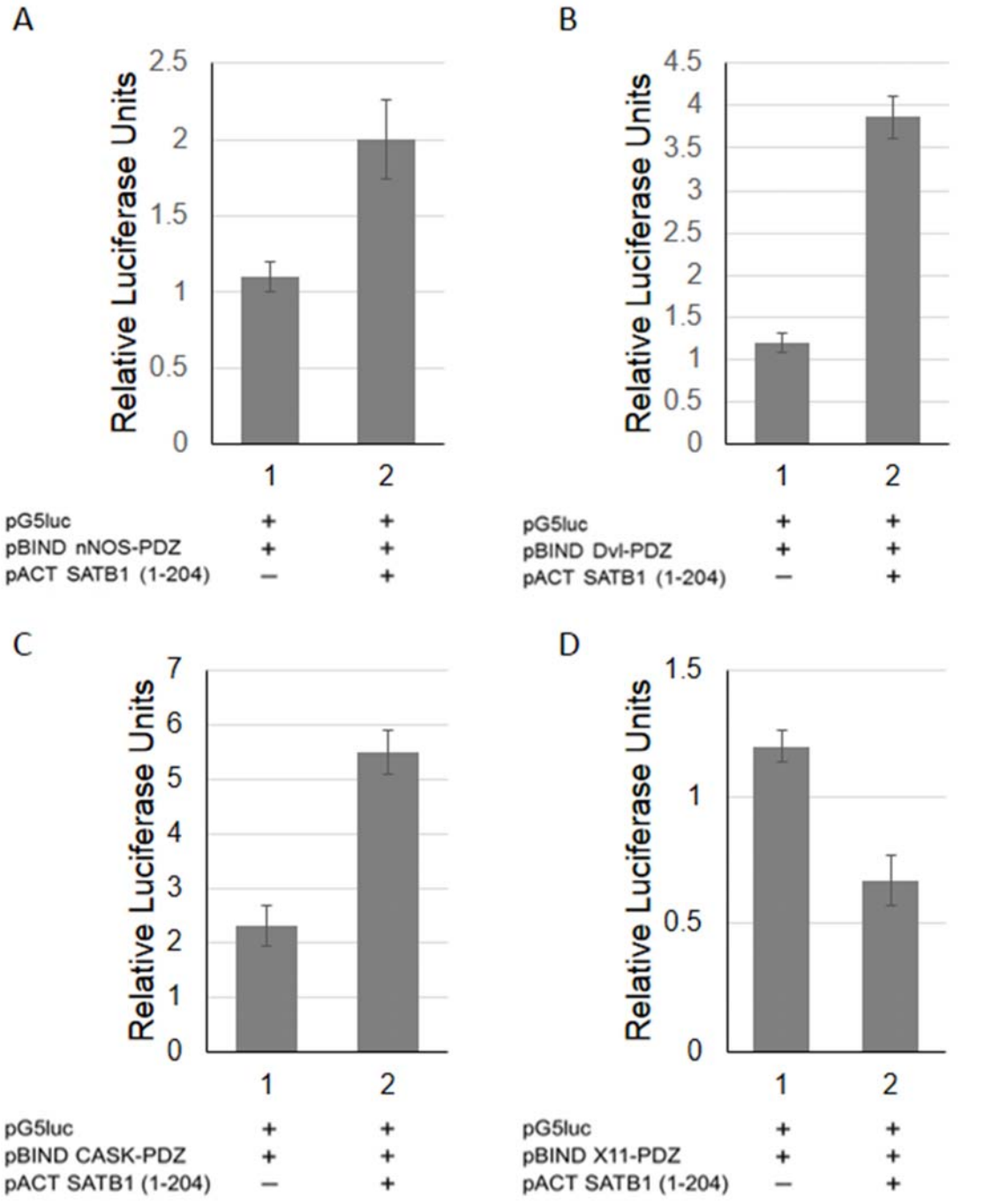

Figure 2.3.2 l The N-terminal (1-204) region of SATB1 interacts with the PDZ domains of neuronal NOS, Dishevelled (Dvl-1) and CASK. (A) A two-fold increase in reporter activity was observed upon co-transfecting the PDZ domain of neuronal Nitric oxide synthase (nNOS) and SATB1 (1-204), which is the PDZ-like domain of SATB1 (graph A, bar 2). **(B)** Four-fold increase in reporter activity was observed upon expression of Dvl-PDZ along with the PDZ-like domain of SATB1 (graph B, bar 2). **(C)** The PDZ domain of CASK increased the reporter activity of the pG5luc reporter (graph C, bar 1). Reporter activity was further increased upon transfecting the PDZ domain of CASK along with the N-terminal (1-204) region of SATB1. **(D)** Reporter activity decreased four fold upon

transfecting the PDZ domain of X11 along with the N-terminal (1-204) region of SATB1 (graph D, bar 2 versus bar 1). Error bars indicate standard deviation calculated from triplicates. Luciferase activity is expressed as relative luciferase units on Y axis.

2.3.3. Dishevelled-1 functionally and physically interacts with SATB1

To, verify if the PDZ domain of Dishevelled functionally interacts with full-length SATB1, one more round of Mammalian two-hybrid assay was performed, this time using full-length SATB1 as bait. Transfection of cells with pBIND-Dvl-PDZ and pACT-SATB1 (1-204) (pACT-PDZ1) constructs resulted in a four-fold increase in reporter activity of the test with respect to the control, suggesting that the PDZ domain of Dishevelled-1 (Dvl-1) functionally interacts with the N-terminal PDZ-like domain of SATB1 *in vivo* (Figure 2.3.3, graph A, bar 4). Whereas, a 2.5-fold increase in reporter values was observed upon transfecting cells with pBIND-Dvl-PDZ and pACT-SATB1 full length, suggesting that the PDZ domain of Dishevelled-1 interacts with full length SATB1 via its PDZ-like domain (Figure 2.3.3, graph A, bar 3). For studying if SATB1 and Dishevelled (Dvl-1) physically interact *in vivo*, co-immunoprecipitation assay was performed. HEK293 cells were transfected with pTriEx-SATB1 construct, cells were harvested 48 h post-transfection and lysed in RIPA Buffer. Anti-Dvl-1 antibody was used to immunoprecipitate Dishevelled-1, followed by immunoblotting with anti-SATB1 antibody to probe if SATB1 immunoprecipitates along with Dishevelled-1. A band corresponding to that of the molecular weight of SATB1 was observed upon immunoprecipitation with anti-Dvl-1 antibody, indicating that Dvl-1 and SATB1 are the part of the same complex *in vivo* (Figure 2.3.3, B). A band corresponding to the molecular weight of SATB1 was observed in the positive control where the pull down was performed using anti-SATB1 antibody and probed with the same (Figure 2.3.3, B).

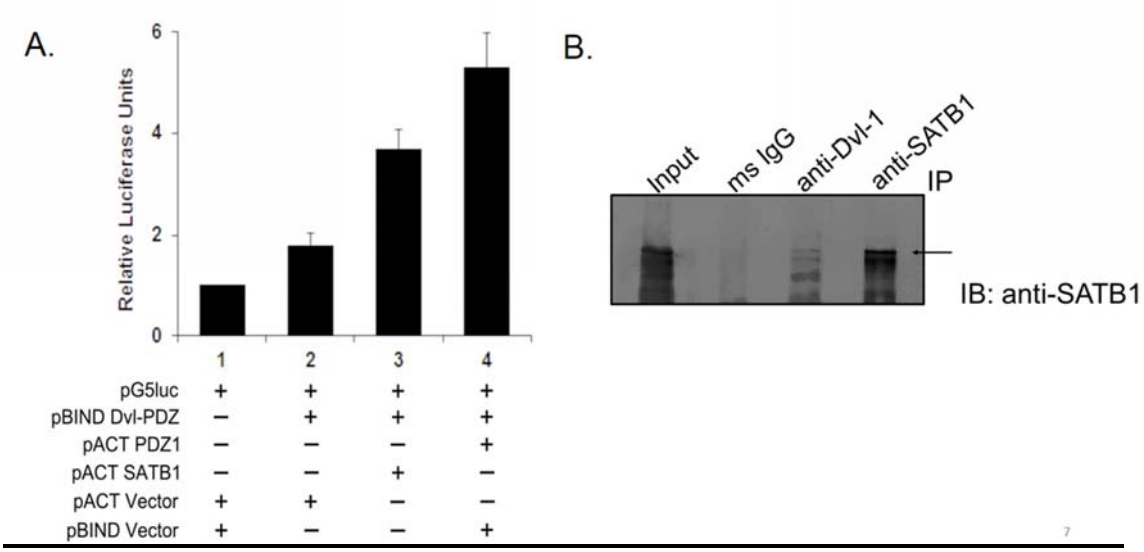

Figure 2.3.3 I SATB1 physically and functionally interacts with Dishevelled-1 (Dvl). A, Mammalian two-hybrid assay: Mammalian two-hybrid assay was performed as described in 'Materials and Methods'. Co-transfection of Dvl-PDZ along with full length SATB1 resulted in 4-fold increase in reporter activity with respect to control (bar 3 versus bar 1). Whereas, reporter activity upon co-transfection of Dvl-PDZ and SATB1 (1-204) (PDZ1) resulted a 5-fold increase in reporter activity with respect to control (bar 4 versus bar 1). Thus, the PDZ domain of Dishevelled-1 interacts more strongly with the PDZ-like domain of SATB1 (PDZ1) as compared to full-length SATB1. **B**, **Co-immunoprecipitation assay to score for the interaction of SATB1 and Dishevelled-1.** His-tagged SATB1 was expressed in HEK293 cells, cells were lysed in RIPA buffer, a total of 600 µg of lysate was used for the experiment. Pull-down was performed using Dvl-1 antibody. Protein/Antibody complex was eluted in Laemmli Buffer, eluents were resolved on a 10% reducing gel, immunoblotting was performed using anti-SATB1 antibody. A band corresponding to the molecular weight of SATB1 is observed in case of immunoprecipitation with anti-Dvl-1 antibody. Immunoprecipitation using SATB1 antibody which was used as a positive control, also showed a band of the same size.

2.3.4. SATB1 and Dvl-1 reciprocally regulate each other at transcript level

As SATB1 is observed to physically interact with two crucial intermediaries of the Wnt/Wg signaling cascade- Dishevelled and β-catenin (Notani et al., 2010), it would be interesting to study the effect of SATB1 expression on genes regulated by the Wnt/Wg signal and which in turn regulate Wnt/Wg signaling. Towards this end, HEK293 cells were subjected to the following treatments: a) treatment with Wnt3A; b) overexpression of SATB1; c) overexpression of Dishevelled-1. Quantitative RT-PCR data showed a 3.8-fold increase in Dvl-1 transcript upon SATB1 over-expression and a concomitant four-fold increase in SATB1 transcript upon over-expression of Dishevelled-1 (Figure 2.3.4, graphs A. and B. respectively). Hence, it

can be inferred that SATB1 and Dvl-1 regulate each other at transcript level. SATB1 also upregulated cellular transcript levels of Dvl3 and Dvl2 isoforms of Dishevelled (Figure 2.3.4, graphs C. and D. respectively). Since Dishevelled mimics Wnt activation (Lee et al., 2008), SATB1 mediated upregulation of Dishevelled might be effectively leading to activation of the Wnt/Wg pathway. Treatment with Wnt3A or over-expression of Dishevelled-1 (Dvl1) led to upregulation of SATB1, this maybe because SATB1 might be indirectly regulated by the Wnt/Wg pathway, but more in depth experiments need to be performed for better understanding the reason underlying this observation.

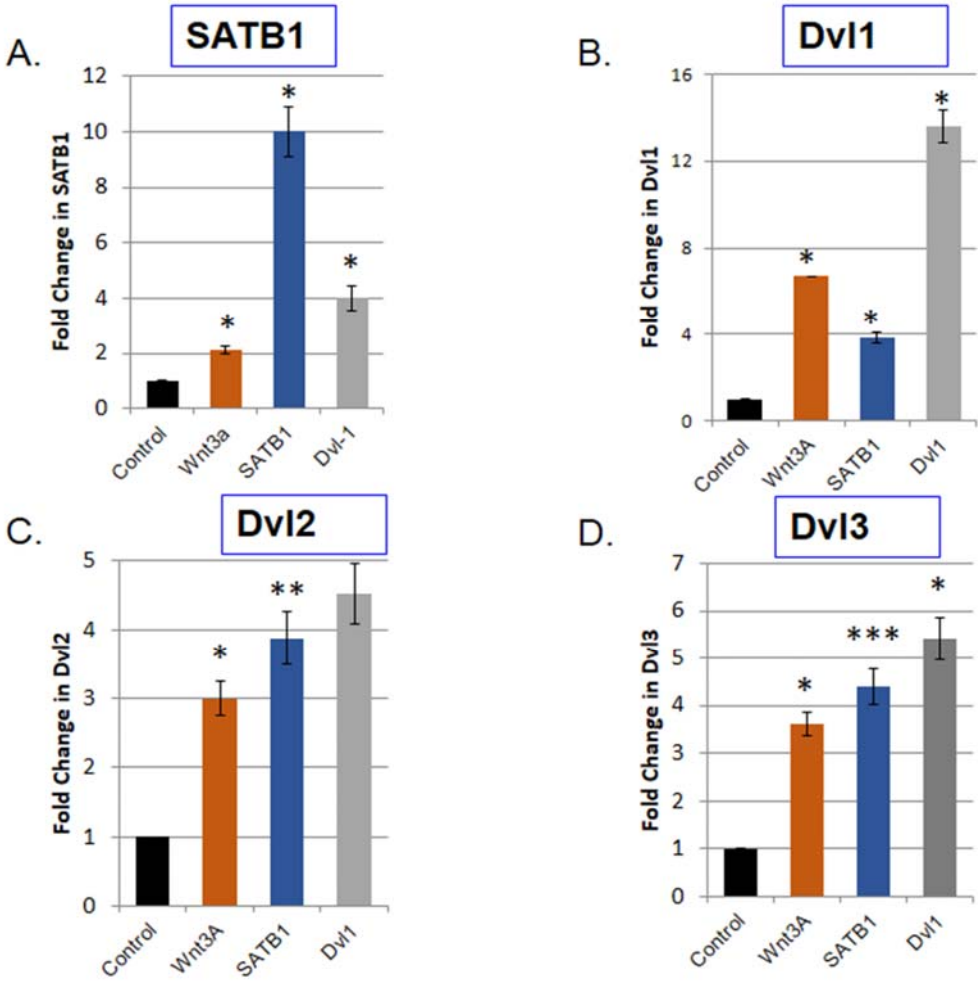

Figure 2.3.4 I SATB1 and Dishevelled reciprocally regulate each other. Upon overexpressing SATB1 in HEK293 cells transcripts of all three isoforms of Dishevelled, Dvl1 (graph B, blue bar), Dvl2 (graph C, blue bar), and Dvl3 (graph D, blue bar) are upregulated**.** A similar increase in transcript levels of all three isoforms of Dishevelled is observed upon Wnt3A treatment (red bar in graphs B, C and D, respectively). Dishevelled-

1 positively regulates SATB1 expression at transcript level (graph A). All RNAs were treated with DNase prior to conversion into cDNA. 18S RNA was used as an internal control for normalizing the C_T values. Fold Change was calculated as described in 'Materials and methods'. Error bars indicate standard deviation calculated from triplicates. Fold change as compared to control is depicted on Y axis.

2.3.5. SATB1 positively regulates Wnt responsive genes

SATB1 positively regulates Wnt responsive transcriptional factors- TCF7L2 and CtBP (Figure 2.3.5, graphs A and B). Similar upregulation is observed upon Dishevelled-1 overexpression or Wnt3A treatment. Transcriptional activation of Wnt responsive genes such as c-FOS (Fra-1), cyclinD1 (CCND1), c-myc and c-Jun increased upon overexpression of SATB1 (Figure 2.3.5, graphs C, D, E and F, respectively). Treatment with Wnt3A treatment for 3 h served as a positive control. Similar to Wnt3A treatment, overexpression of Dishevelled-1 also led to increase in transcript levels of Wnt responsive genes.

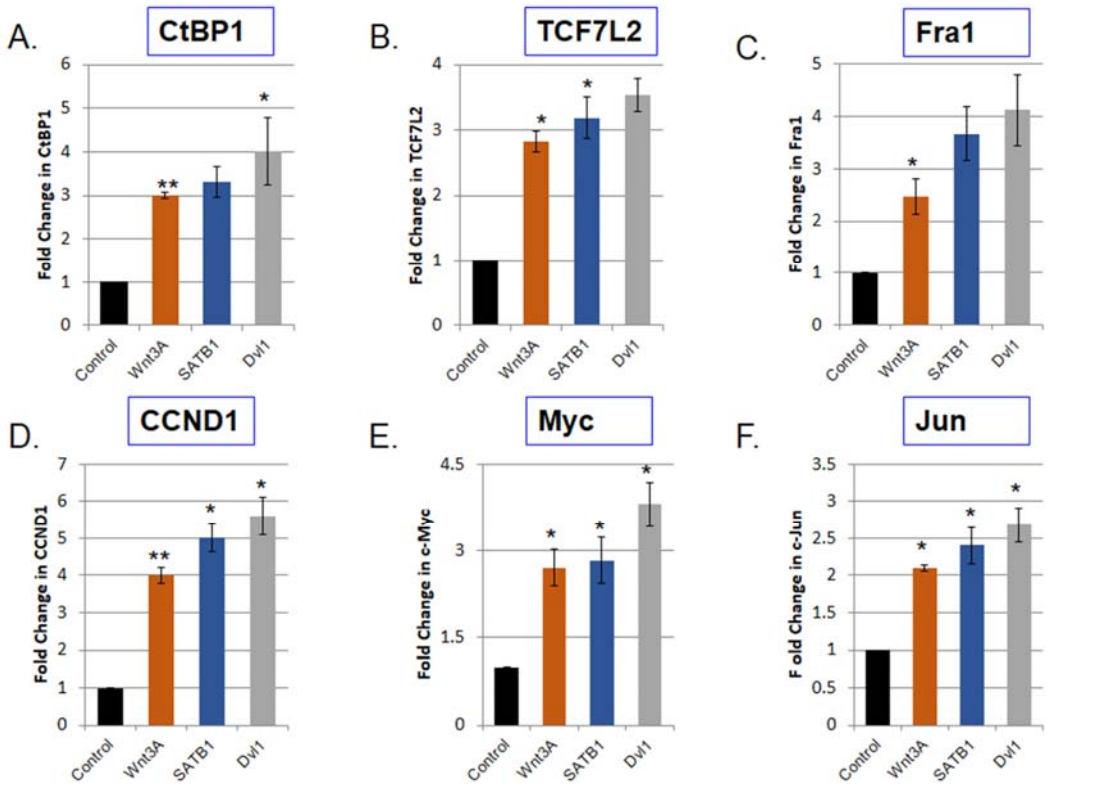

Figure 2.3.5 I SATB1 positively regulates Wnt responsive genes. Using quantitative RT-PCRs, effect of SATB1 overexpression was observed on the transcript levels of Wnt responsive genes. **(A)** CtBP and (**B)** TCF7L2 which are transcription regulators known to

be regulated by the Wnt/Wg pathway are positively regulated by both SATB1 and Wnt activation brought about by either Wnt3A treatment or overexpression of Dishevelled. Bonafide Wnt responsive genes which are known to be upregulated in response to Wnt activation such as, **(C)** Fra1, **(D)** CCND1, **(E)** Myc, and **(F)** Jun are also upregulated upon SATB1 over-expression. 18S RNA was used as internal control for normalizing the C_T values. Error bars indicate standard deviation calculated from triplicates. Fold change as compared to control is depicted on Y axis.

2.3.6. SATB1 regulates negative regulators of the Wnt/Wg pathway

SATB1 overexpression led to repression of Wnt/Wg pathway antagonists- Nkd2, Dkk1 and sFRP (Figure 2.3.6, all blue bars). Over-expression of Dishevelled-1 resulted in repression of Nkd, Axin2 and sFRP transcripts, though the degree of repression of the transcript is very slight (Figure 2.3.6, all grey bars). Treatment with Wnt3A for 3 h didn't cause much change in the transcript level of the same (Figure 2.3.6, orange bars), this was expected as these genes are known to be expressed at latter time points in response to Wnt3A stimulation thereby, leading to feed-back inhibition of Wnt/Wg signaling.

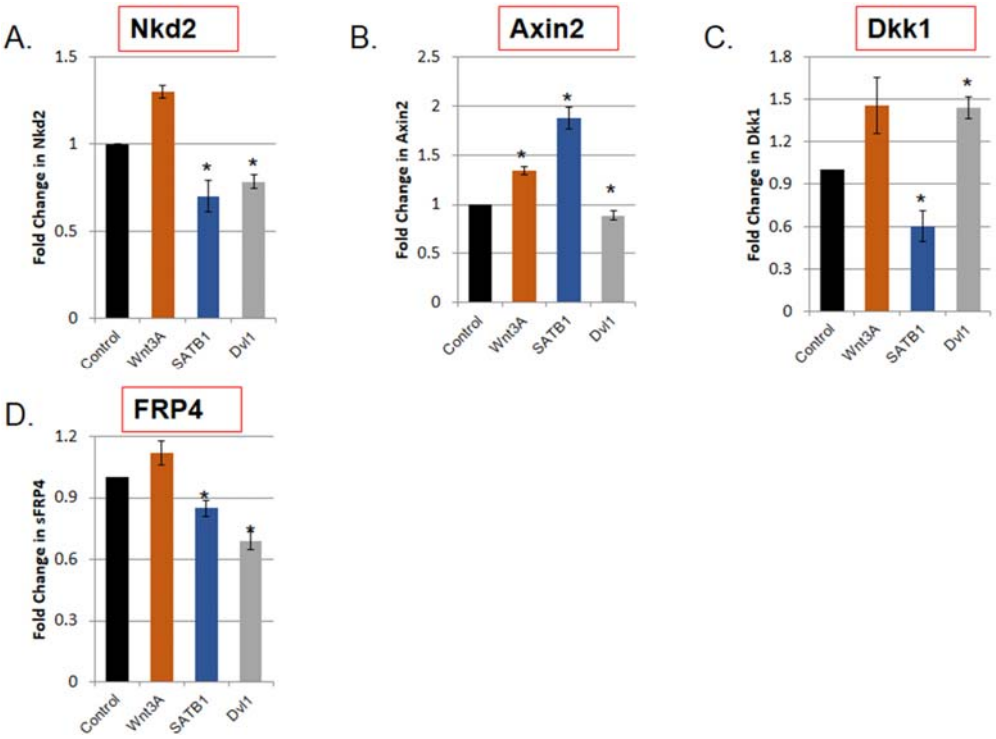

Figure 2.3.6 I SATB1 downregulates negative regulators of Wnt/Wg pathway. Quantitative RT-PCRs were performed to study the effect of SATB1 expression on negative

regulators of the Wnt/Wg signaling pathway (A) Nkd2, (C) Dkk1 and (D) FRP4 which are negative regulators of Wnt are downregulated upon SATB1 over-expression, Dishevelled-1overexpression leads to downregulation of Nkd2 and FRP4. Wnt3A treatment doesn't show any significant effect on the expression levels of these negative regulators. (B) SATB1 activates expression of Axin2 which is a negative regulator of Wnt along with being a Wnt responsive gene. 18S RNA was used as internal control for normalizing the C_T values. Error bars indicate standard deviation calculated from triplicates. Fold change as compared to control is depicted on Y axis.

2.3.7. Cross-talk between Wnt/Wg pathway and SATB1

SATB1 regulated genes like BCL2 and ERBB2 are also upregulated in response to Wnt activation by either Wnt3A treatment or overexpression of Dishevelled-1 (Figure 2.3.7, graphs A and C). Whereas, CHUK is differentially regulated by SATB1 and the Wnt/Wg signaling (Figure 2.3.7, graph B). This result is indicative of cross-talk between SATB1 and Wnt/Wg pathway. Some genes are co-regulated (BCL2, ERBB2) whereas others are regulated differentially (CHUK). This result validates previous reports from Galande lab where some genes known to be regulated by SATB1 are also responsive to Wnt/Wg signaling (Notani et al., 2010).

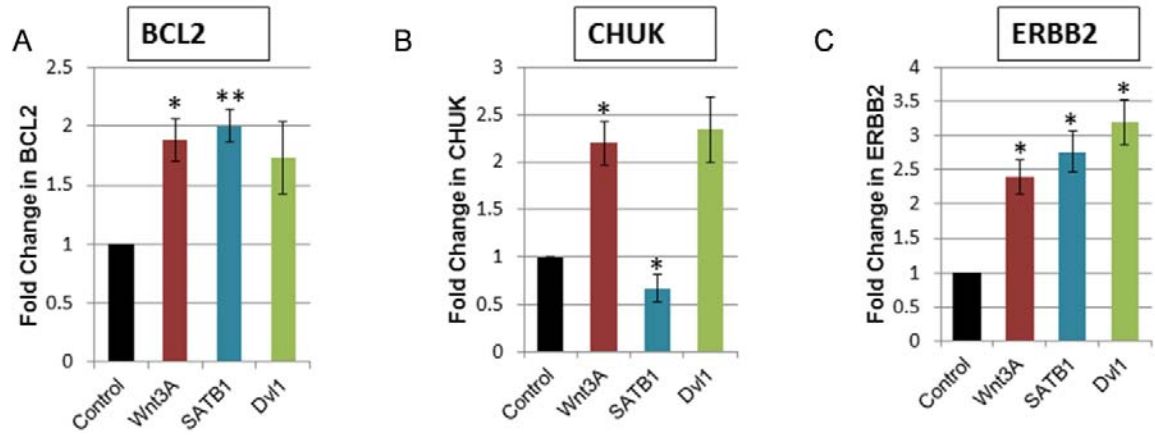

Figure 2.3.7 I Crosstalk between Wnt signaling pathway and SATB1. Quantitative PCR was performed to check if genes regulated by SATB1 are also responsive to Wnt signaling. **(A)**, **(C)**, BCL2 and ERBB2, both of which are positively upregulated upon Wnt activation viz. Wnt3A treatment or over-expression of Dishevelled whereas CHUK which is repressed upon SATB1 overexpression is downregulated upon Wnt activation **(B)**. 18S RNA was used as internal control for normalizing the C_T values. Error bars indicate standard deviation calculated from triplicates. Fold change as compared to control is depicted on Y axis.

2.3.8. Ectopic expression of SATB1 in *Drosophila* eye

The fly eye is a very convenient model system to study the effect of a particular protein in the fly system since phenotypes are easy to assay and infer. Ectopic expression of SATB1 under the control of *GMR-GAL4* driver in the fly eye results in flies having reduced size with slightly rough eyes. SEM analysis of such eyes revealed a severely distorted morphology wherein fusion of ommatidia is observed as opposed to the compact hexagonal arrangement of ommatidia observed in wildtype flies, leading to a rough eye phenotype. The positioning, the regular pattern of bristle orientation as well as the bristles number is also disrupted. Bristles are fewer, haphazardly distributed and randomly oriented (Figure 2.3.8). This phenotype is evenly spread out all over the fly eye and is not restricted to any particular part of the fly eye. The penetrance of this phenotype is 100%. One possible scenario to explain generation of a phenotype is that expression of SATB1 in the fly eye leads to sequestration of proteins a subset of which may be involved in eye development, thereby leading to such a drastic defect in the eye.

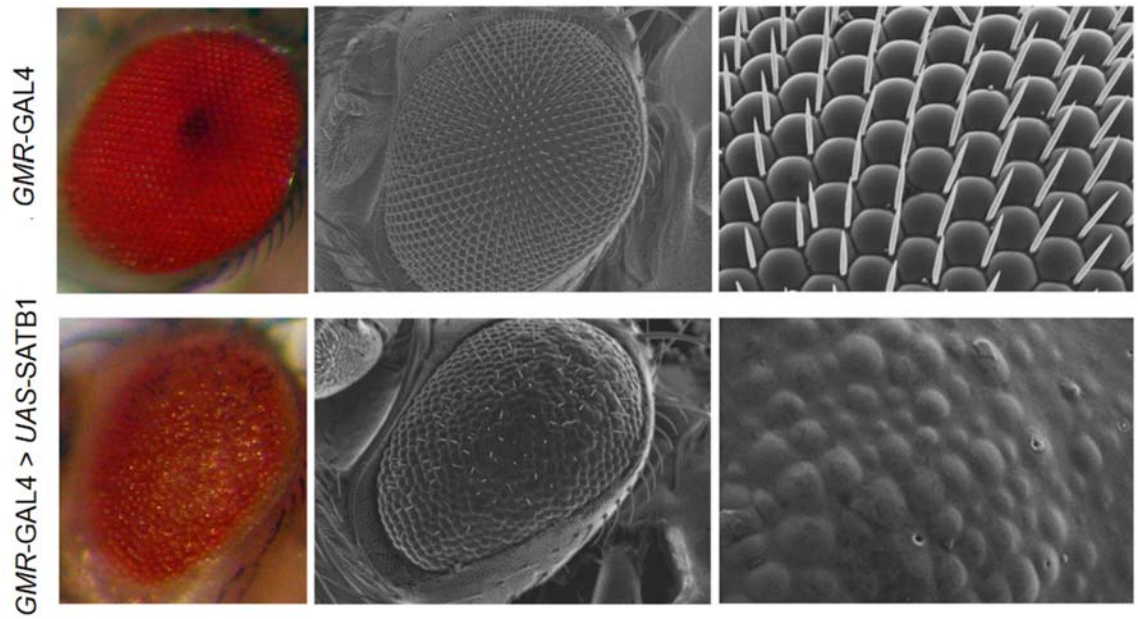

Figure 2.3.8 I SEM images depicting fusion of ommatidia as a result of ectopic expression of full length SATB1. Ectopic expression of SATB1 in the eye leads to generation of a rough eye phenotype in flies. These flies have a slightly smaller eye as compared to the normal wildtype. SEM analysis reveals that ommatidia lose their widltype hexagonal shape and are slightly rounded, thus the compact arrangement of ommatidia is lost. Fusion of ommatidia is observed which causes the fly eye to appear rough. Bristles normally present at alternate vertices of the ommatidia in the wildtype are now randomly arranged, some regions of the eye completely lack bristles. In wildtype flies, all bristles are oriented towards a particular direction, this feature is absent from SATB1 expressing flies

wherein the bristles are oriented randomly, the overall bristle number is also less as compared to the wildtype flies. The penetrance of this phenotype is 100%, n=110. Flies were mounted onto the SEM platform without processing of any kind and images acquired at magnifications of 100X and 400X respectively.

2.3.9. Validation of SATB1 expression in the eye disc

SATB1 was ectopically expressed in the fly eye using *GMR-GAL4* driver, immunostaining for SATB1 was performed in the eye imaginal discs to validate the same. Staining for SATB1 was observed to be restricted to the posterior part of the eye imaginal disc, which correlates with the expression domain of *GMR-GAL4.* Further, the pattern of expression of SATB1 in the eye imaginal disc of *Drosophila* is the same as that observed in case of cell lines i.e. ectopically expressed SATB1 is localized entirely inside the nucleus and is enriched in DAPI poor regions whereas it is totally excluded from DAPI rich heterochromatic regions (Figure 2.3.9).

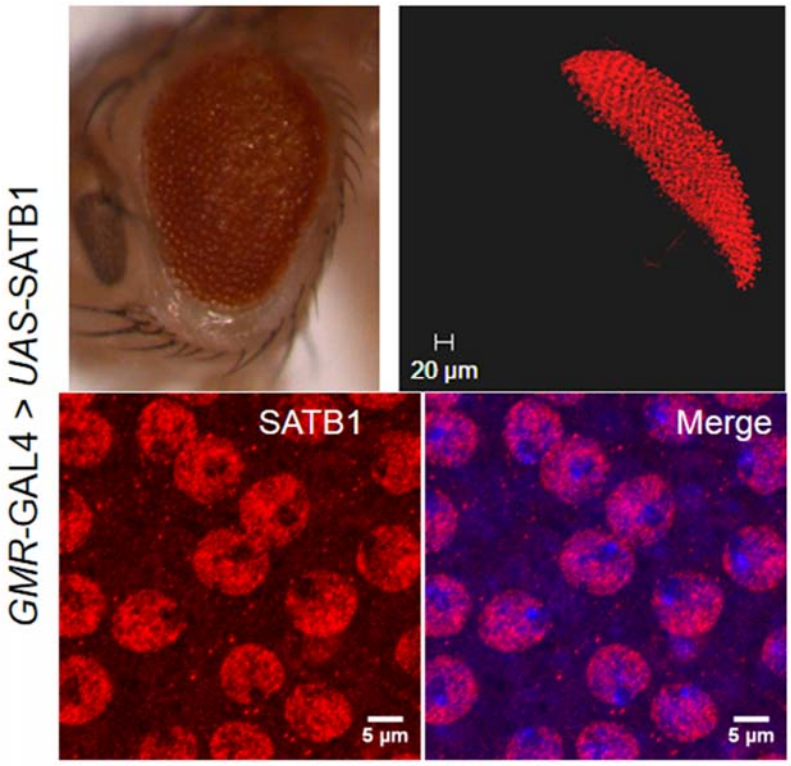

Figure 2.3.9 l Subcellular localization of SATB1 is similar in mammalian cell lines and cells of the eye imaginal discs. Ectopically expressed human SATB1 under the control of *GMR-GAL4* driver gives rise to rough eye characterized by ommatidial fusion and bristle loss. Immunostaining for ectopically expressed SATB1 in the eye imaginal disc revealed that SATB1 is localized in the region posterior to the morphogenetic furrow (top panel).

Higher magnification image reveals that SATB1 resides entirely inside the nucleus, is enriched in DAPI poor regions, and is excluded from highly condensed heterochromatic regions (bottom panel) as typically observed in mouse thymocytes (Galande et al., 2007).

2.3.10. Wing-specific ectopic expression SATB1 leads to distorted wing phenotype

When human SATB1 was ectopically expressed in the *Drosophila* wing under the control of *MS1096-GAL4*, majority of the flies were flightless flies having crumpled wings (Figure 2.3.10).

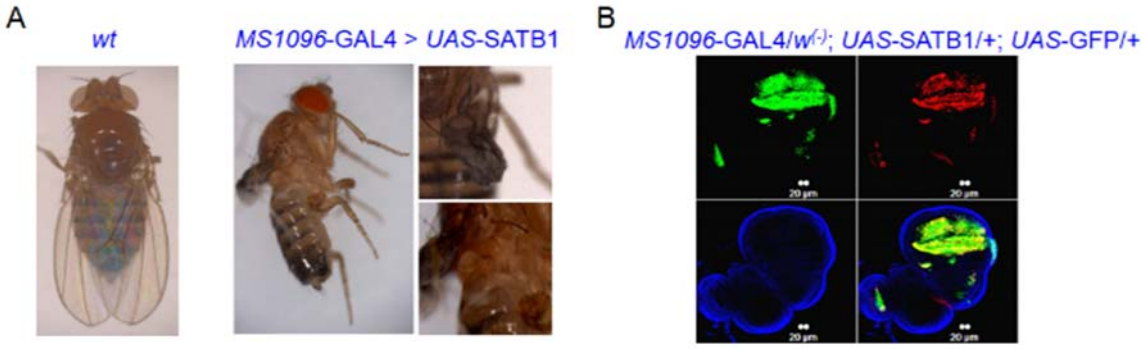

Figure 2.3.10 l Wing –specific ectopic expression of full-length SATB1 in *Drosophila*. A. Ectopic expression of human SATB1 in the fly wing under the control of *MS1096-GAL4* led to distorted wings, and rendered the organism flightless (Right panel) whereas, wildtype flies had normal wings (Left panel). The penetrance of this phenotype was 80%, n=132. **B.** Validation of SATB1 staining in the wing imaginal disc. SATB1 (red) localizes inside the wing pouch where the GAL4 is known to express. GAL4 expression pattern was tracked by GFP staining.

2.3.11. Ectopic expression of full length SATB1 gives rise to venation defects in the wings

Ectopic expression of SATB1 full-length under the control of the strong *MS1096-GAL4* mostly gives rise to flies with crumpled wing but, a few flies exhibit mild venation defects, like vein fusion. Venation defects are observed in case of disruption multiple signaling pathways (Figure 2.3.11, panel A). The most common signaling pathways responsible for this phenotype are EGFR and BMP pathways. But, little is known about the role of the Wnt/Wg signaling in regulating wing venation. Of the 132 flies screened, this phenotype was observed in 30 flies and

the intensity and extent of the venation pattern differed from fly to fly. Ectopic expression of SATB1 (1-204) region which harbors the N-terminal PDZ-like domain in the fly wing using *MS1096-GAL4*, did not give rise to a phenotype (Figure 2.3.11, panel B).

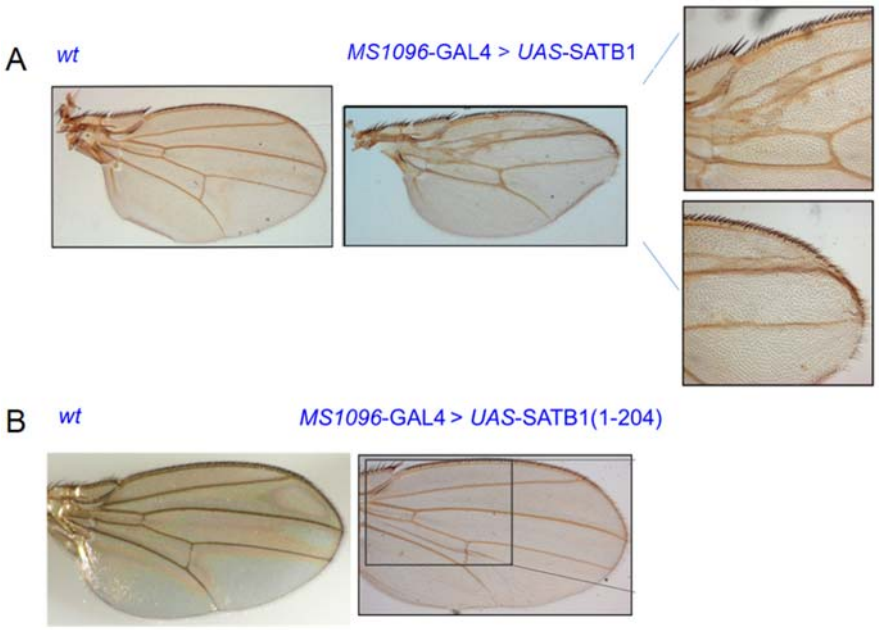

Figure 2.3.11 I Pattern of full-length SATB1 and SATB1 (1-204) expression in fly wing. **A.** Ectopic expression of human SATB1 under the control of *MS1096-GAL4* driver causes venation defects such as fusion of L2 and L3 and some wing blade defects. **B.** Ectopic expression of the N-terminal (1-204) region in the wing did not give rise to any phenotype. *The boxed region in B highlights a wing fold and not a phenotype.

2.3.12. Human SATB1 can bind the *Drosophila* genome

Since expression of SATB1 resulted in a visible phenotype which was backed by validation of protein expression by immunostaining, the next question was if the phenotype is the result of SATB1 binding to *Drosophila* chromatin and the subsequent changes. Therefore, it was decided to screen for any putative SATB1 binding sites in the *Drosophila* genome. MEME analysis was performed to score for sequences similar to the consensus SATB1 binding site (CSBS) in the *Drosophila* genome, twenty-two significant hits, distributed equally over chromosome II and chromosome III (Figure. 2.3.12) were observed. A subset of the genes like Charc-

14 and Homothorax (Figure 2.3.12) having the SATB1 consensus binding site are involved in chromatin remodeling and might therefore be indirectly responsible for SATB1 mediated phenotypic changes. Although, this analysis by no means suggests that the protein binds to the chromatin in vivo. This study just brings to fore the fact that certain loci in the *Drosophila* genome can be bound by human SATB1.

Bioinformatic analysis of putative SATB1 binding sites in *D. melanogaster* genome

Chromosome	start	stop	score	p-value	q-value	matched sequence	Gene	Function
chr2L	2446260	2446274	20.3	1.13E-07	0.778	CCAATTAGTAATAAA	Decapentaplagic	Development
chr3R	10583595	10583581	21.9	3.69E-08	0.604	CCAATTACTAATAAC	Homothorax	Development
chr2L	8437902	8437888	20.3	1.15E-07	0.778	CCTATTCGTAATCAC	Chrac-14	Chromatin remodeling
chr3R	17507940	17507954	21	6.65E-08	0.618	CCAATTCGTAATAAC	Down syndrome cell adhesion molecule 3	Cell adhesion
chr2L	12649645	12649631	22.6	1.64E-08	0.648	CCTATTAGTAATAAA	RNA and export factor binding protein 2 (*ref2*)	RNA Export
chr2R	9115437	9115451	21.6	4.71E-08	0.574	CTAATTAGTAATAAC	Phosphorylated adaptor for RNA export (*phax*)	RNA Export
chr3L	5707345	5707359	22	2.83E-08	0.596	CCTATTTGTAATAAC	Still life (*sif*)	Cytoskeletal organization
chr3R	355583	355597	20.9	7.34E-08	0.618	CCTATAACTAATAAC	Myosin 81F	Motor activity
chr3R	25675306	25675320	20.7	8.42E-08	0.618	CCTATTCGTAATAAA	Igr3	GPCR pathway
chr2L	2132094	2132080	20.6	9.99E-08	0.778	CTTATTACTAAAAAC	Glycogen phosphorylase (*glyP*)	Glycogen catabolic process
chr3R	26417202	26417188	21.6	4.71E-08	0.604	CTTTTTAGTAATAAC	CG5432	Glycolysis
chr3R	17425231	17425245	22.6	1.64E-08	0.42	CCTATTAGTAATAAA	CG5873	Response to oxidative stress
chr2R	14095239	14095225	19.7	1.74E-07	0.902	CCAATTTGTAATAAC	Prosap	Scaffold protein (PSD)
chr2R	15176434	15176420	20.3	1.13E-07	0.902	CCTTTTAGTAATAAA	igloo (*igl*)	Neuronal phosphoprotein
chr3L	15855480	15855466	20.7	8.42E-08	0.806	CTTATTTGTAATAAC	pHCl	Chloride channel
chr3R	22847768	22847782	22.6	1.64E-08	0.42	CCTATTAGTAATAAA	CG4704	Unknown
chr2R	19690061	19690047	19.7	1.74E-07	0.902	CCAATTTGTAATAAC	CG15126	Unknown
chr2L	15360959	15360945	20.3	1.13E-07	0.778	CCTTTTAGTAATAAA	CG15258	Unknown

Figure 2.3.12 I Human SATB1 has binding sites in *Drosophila* genome. MEME analysis identified 22 putative SATB1 binding sites in flies, these sites were highly significant and spread across chromosomes 2 and 3. These binding sites were present in genes involved in varied functions such as chromatin remodeling, metabolic and developmental pathways.

2.3.13. Human SATB1 binds *Drosophila* chromatin

To verify if ectopically expressed human SATB1 binds to *Drosophila* chromatin *in vivo*, SATB1 was ectopically expressed in the salivary glands under the control of *Sgs3-GAL4*. Upon performing immunostaining for SATB1 in these polytene

spreads, four intense bands corresponding to SATB1 were observed, all of these are present in the vicinity of the chromocenter. However, detailed analysis of the same needs to be performed to identify the precise loci where it binds. Immunostaining for SATB1 in spreads from CS flies was used as control. The *Drosophila* boundary element associated MAR-binding protein BEAF was used as a positive control for staining (Appendix A3). Bands corresponding to characteristic pattern of BEAF binding were observed, confirming that the staining method was optimal.

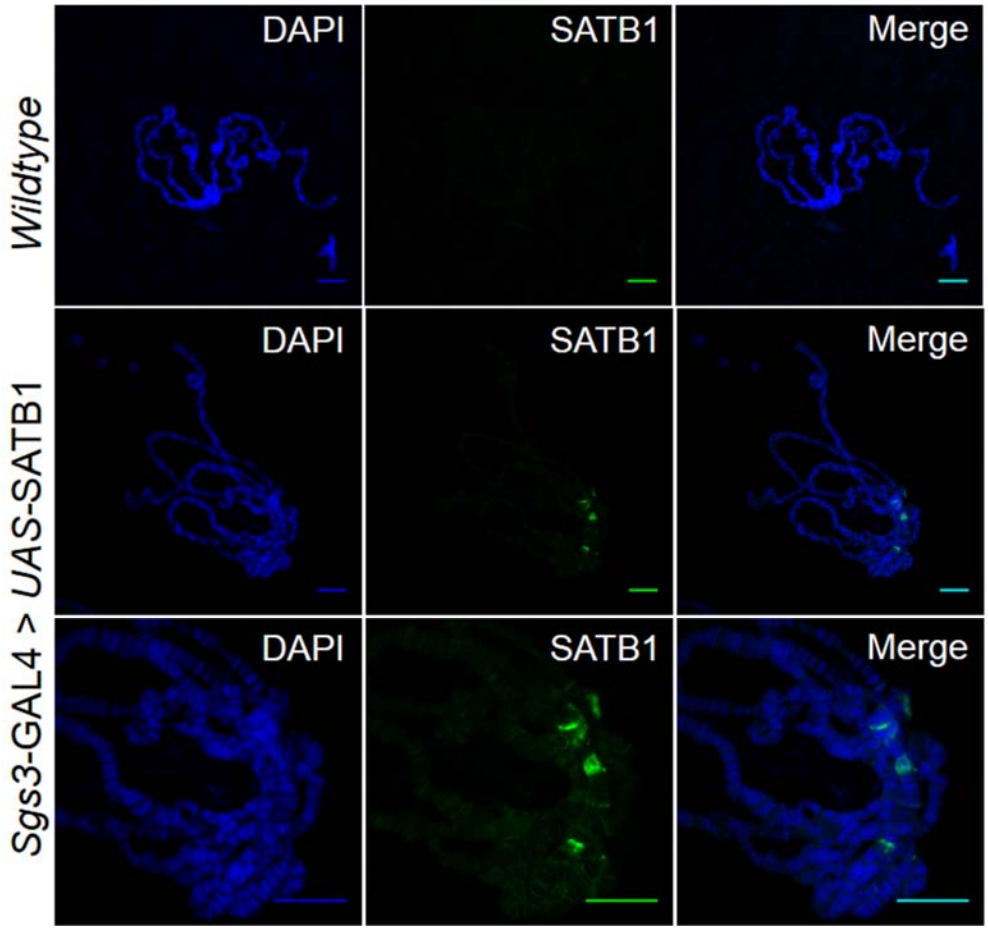

Figure 2.3.13 I Human SATB1 binds *Drosophila* chromatin. Polytene spreads were prepared from larvae expressing human SATB1 in the salivary glands under the control of *Sgs3-GAL4*. Bands corresponding to SATB1 (green) were observed in case of *Sgs3*-GAL4 > *UAS*-SATB1 but, were totally absent in control spreads. Images were acquired at 40X magnification. Lower panel depicts the staining for SATB1 at 100X magnification. All the scale bars represent a distance of 20 µm.

2.4. Discussion

Signaling pathways allow cells to respond to their environment by regulating expression of target gene. The transcriptional control function of each pathway is carried out by one or more signal-regulated transcription factors, which bind to specific regulatory elements called signaling pathway response elements (SPREs) and recruit co-activators and chromatin-remodeling machinery, thereby regulating target gene expression. Several developmental control pathways harness a transcriptional switch, whereby target genes are activated in the presence of an external signal but, repressed in the absence of the same. In this way, cells are transcriptionally activated in response to environmental cues thereby, modulating their gene expression (Guasconi and Puri, 2009; Natoli, 2009).

Lauffenburger and co-workers devised a terminology wherein, any signaling network can be regarded as comprising three informational layers: the ‘cue’ (extracellular ligands that activate the network), the ‘signal’ (intracellular proteins which transduce the message) and the ‘response’ (transcriptional program that effects a phenotype) (Janes et al., 2004). It is worth noting that a particular signaling pathway can modulate very different responses in different cell types (Clevers, 2006; Massague et al., 2005). How the same extracellular cues generate varied cell type-specific responses remains poorly understood. Signal-regulated transcription factors share two functional characteristics, activator-insufficiency and cooperative activation which, together with default repression, may explain signaling pathways’ ability to maintain stringent control of target gene expression.

Overexpression of transcription factors that respond to developmental cues has been linked to cancer development and progression. Disproportionate expression of these transcription factors is thought to reestablish developmental programs out of context, thereby, contributing to tumor formation and progression. Special AT-rich sequence binding protein 1 (SATB1) is a nuclear factor that functions as a global chromatin organizer regulating chromatin structure and gene expression

(Galande et al., 2007). SATB1 has been found to be abnormally expressed in various types of cancer and has been proposed to function as an oncogene which promotes malignancy (Chu et al., 2012; Han et al., 2008). SATB1 provides a key link between DNA loop organization, chromatin modification/remodeling and association of transcription factors at matrix attachment regions (MARs), these two properties are vested by virtue of the C-terminal DNA-binding domain and the N-terminal protein interacting region respectively. The N-terminal half of SATB1 contains a PDZ-like domain which is known to be dominant negative for SATB1 function. As, much of the functional information pertaining to SATB1 is with respect to its function as a transcription factor it would be interesting to study novel cellular functions of SATB1. One step towards this end, would be to identify new molecular entities which interact with SATB1. In order to identify novel functional interacting partners of SATB1, Mammalian two hybrid assay system was used. As our interest lie in identifying functional interactions, a biased approach resulting in a transcriptional output was used instead of using a genome-wide proteomics based approach. Dishevelled-1, neuronal NOS and CASK were identified as being able to functionally interact with the N-terminal PDZ-like domain of SATB1.

One of these candidate proteins, Dishevelled (Dvl) is an upstream regulator of the Wnt/Wg signaling pathway (Figure 2.4.1), which is known to be involved in governing crucial biological processes including development, cell fate, proliferation, cell-cell communication, organogenesis and stem cell renewal (Angers and Moon, 2009; Fuerer et al., 2008). In response to Wnt/Wg signal Dishevelled gets localized to LRP6 signalosome where it is required for phosphorylation of LRP6, a step essential for signal transduction by Wnt (Bilic et al., 2007). Dishevelled is known to be the branchpoint at which Wnt/Wg pathway bifurcates into canonical (β-catenin dependent) and non-canonical (β-catenin independent) pathways (Katoh, 2005; Schlessinger et al., 2009). As overexpression of Dishevelled mimics Wnt treatment (Lee et al., 2008), any perturbance in the levels of Dishevelled is manifest in form of abnormalities (Gordon and Nusse, 2006). But still until date, very little is known about the function of Dishevelled.

Therefore, Dishevelled is an interesting molecule to study for getting a better understanding of the Wnt/Wg pathway. Thereby making the analysis of interaction of Dishevelled-1 with full-length SATB1 and the subsequent outcome, an interesting question to study.

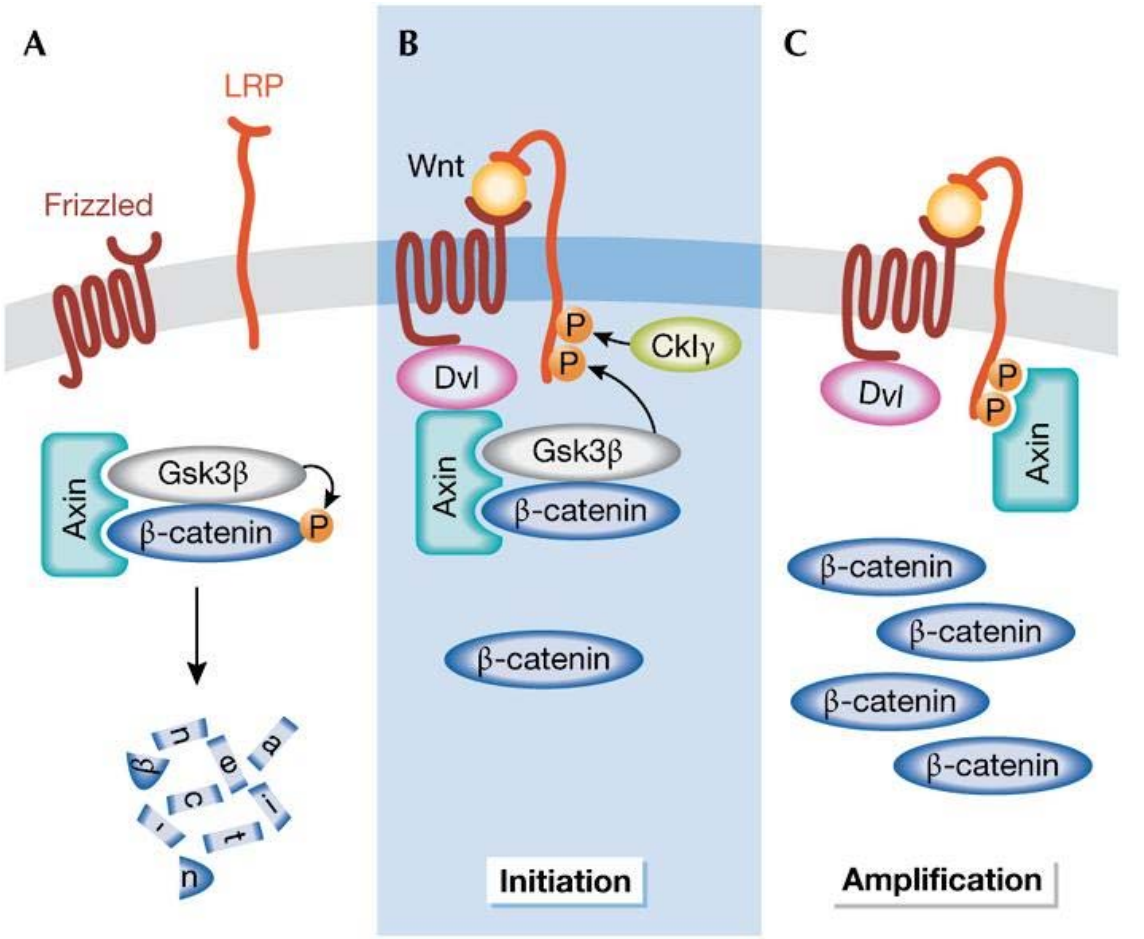

Figure 2.4.1 I Model for the activation of the Wnt/β-catenin pathway. (**A**) In the absence of a Wnt signal, β-catenin is phosphorylated and targeted for proteasome-mediated degradation by a destruction complex that contains axin and Gsk3β among other proteins. (**B**) On binding of Wnt to the receptors Fz and LRP, Dvl binds to Fz and recruits the destruction complex through interaction with axin. Subsequently, Gsk3β phosphorylates critical sites on LRP, which, together with residues phosphorylated by Ckl γ, act as docking sites for axin. (**C**) Binding of axin to LRP leads to inhibition of the destruction complex and stabilization of β-catenin. Ckl γ, casein kinase Iγ; Dvl, Dishevelled; Fz, Frizzled; Gsk3β, glycogen synthase kinase 3β. Reproduced from Fuerer et al., 2008.

Evidence of physical interaction between SATB1 and Dishevelled was established by co-immunoprecipitation. Therefore, Dishevelled is a component of SATB1 complex *in vivo*. Initially, Dishevelled was known to be a cytosolic protein, but of

late, there are reports supporting the presence of Dishevelled inside the nucleus in response to Wnt activation. Reports from Sokol lab have identified the presence of a nuclear export signal, mutation in which leads to nuclear accumulation of Dishevelled. This nuclear localization is essential for Dishevelled function and transduction of canonical Wnt/Wg signaling (Itoh et al., 2005). Even though Dishevelled doesn't have a DNA binding domain it is known to bind and regulate Wnt responsive genes. Towards this end, Dishevelled is known to independently associate with both c-Jun and β-catenin inside the nucleus, which in turn bind TCF4. Disruption of either of these interactions leads to suppression of canonical Wnt signaling stimulated gene activation. Knockdown of Dvl diminishes the association of β-catenin–TCF4 complex on promoters of Wnt target genes (e.g. *c-myc* promoter) *in vivo*, thereby leading to altered expression levels of Wnt responsive genes (Gan et al., 2008). This might be because Dishevelled contains multiple domains crucial for mediating protein interactions like the DIX and the PDZ domains, by virtue of which, nuclear Dvl functions as a scaffold for recruiting multiple transcription activators onto the genomic loci. Loss of Dishevelled might therefore result in disruption of this complex leading to deregulation of certain genes. In some cancers, Dvl- β-catenin complex is known to be recruited onto the promoters of Wnt responsive genes even in the absence of Wnt activation, thereby leading to expression of these genes (Pethe et al., 2011).

As SATB1 is a nuclear protein and Dishevelled (Dvl) is a predominantly cytosolic protein, the location of cellular compartment where they interact becomes important. Immunofluorscence assays were performed multiple times to identify the same but, due to unavailability of a good antibody we were unable to do so. With the aforementioned antibody, even in the 'WNT OFF' condition, most of the Dishevelled protein was observed to localize inside the nucleus. Thus, results until now, highlight that SATB1 functionally and physically interacts with Dishevelled, the upstream effector of the Wnt/Wg pathway. Previous reports from our lab show, that in thymocytes, SATB1 also binds and recruits β-catenin, the final effector of the Wnt/Wg pathway, onto the promoters of Wnt responsive genes, ultimately leading

to altered transcriptional activity of the target Wnt responsive genes in response to Wnt signaling (Notani et al., 2010). It is also known that SATB1 competes with TCF4 for binding to β-catenin (Gattinoni et al., 2010; Notani et al., 2010). As, SATB1 physically associates with two of the components of the Wnt/Wg signaling pathway, it was logical to analyze if SATB1 expression has any effect on Wnt signaling by looking at Wnt responsive genes at transcript level upon SATB1 expression.

Transcript analysis for checking levels of Wnt responsive genes was performed in HEK293 cells overexpressing SATB1, it was observed that SATB1 over-expression led to upregulation of various bonafide Wnt responsive genes such as CtBP1, TCF7L2, c-fos (Fra-1), c-jun, c-myc and cyclin D1 (CCND4). These results mimicked those observed upon 3 h of Wnt3A stimulation. Transcript levels of Dvl-1 were also enhanced upon SATB1 expression, as mentioned previously, Dishevelled over-expression mimics Wnt activation, therefore SATB1 over-expression indirectly activates the Wnt pathway. Overexpression of SATB1 also led to repression of Wnt antagonistic genes such as Nkd, Dkk and SFRP4 at transcript level.

Study of Wnt3A-mediated stimulation of HEK293 cells revealed two phases of transcriptional regulation: 1) an early phase in which early Wnt responsive genes such as c-myc and CCND1 are upregulated, and signaling antagonists such as Dkk, Nkd and Axin are held in check (0-3 h), and 2) a later phase in which many of these same antagonists are upregulated (3-24 h), attenuating signaling (Gujral and MacBeath, 2010), but in the current study not much difference was observed with respect to control in the transcript levels of the antagonists upon treatment of cells with Wnt3A for 3h, although overexpression of Dishevelled-1 did lead to a slight decrease in transcripts of Nkd2 and SFRP. Reports suggest that some genes such as c-Myc and Bcl-2 are regulated by both SATB1 as well as Wnt/β-catenin pathway (Cai et al., 2003; Li et al., 2007; Ma et al., 2007), suggesting a functional overlap between the two. A subset of such genes was chosen to validate the current observations. It was observed that ERBB2 and Bcl-2 are co-regulated by SATB1

and Wnt signaling whereas, CHUK is differentially regulated. This result corroborates previous reports from our lab which demonstrate that certain genes are co-regulated whereas, others are differentially regulated by SATB1 and Wnt (Notani et al., 2010). Our results show that SATB1 activates expression of positive regulators of the Wnt pathway such as Dishevelled(s) and Wnt responsive genes, and represses the negative regulators of the Wnt pathway such as Nkd. These results suggest that in cell lines, SATB1 is a positive regulator of the Wnt/Wg pathway in cell lines. Position of SATB1 with respect to the hierarchy of the Wnt/Wg signaling pathway in a biological system remains to be verified *in vivo*. It would be interesting to study, if this positive regulation of the Wnt/Wg pathway by SATB1 is a conserved phenomenon or if it is context dependent (organism). To identify if the positive regulation of the Wnt/Wg pathway is a conserved phenomenon or if it is context dependent, it was decided to use the approach of ectopic expression of mammalian SATB1 in a system that lacks SATB1 and has a fully functional and well-characterized Wnt/Wg pathway. Towards this end *Drosophila melanogaster* was chosen as a model system. *D. melanogaster*, is a versatile system for studying protein functionality and majority of the signaling pathways have been genetically dissected using this model system. The fly system contains multiple proteins which harbor a homeodomain, and few proteins which harbor the Ubiquitin like domain (ULD) but, none of the fly proteins have a domain architecture similar to SATB1. Even though the fly system lacks endogenous SATB1, it contains conserved homologs of various known SATB1 interacting factors such as Arm and Dsh, and most of the components of the chromatin machinery known to associate with SATB1 in the cellular context. All the above mentioned facts make the Drosophila model a choice for conducting these type of studies.

Transgenic flies expressing full-length mammalian SATB1, SATB1 (1-204), dominant negative for SATB1 function, and SATB1 (255-763) were generated by microinjection. Multiple fly lines were generated for each construct, each of these fly lines exhibited a marked difference with respect to the expressivity of the

phenotype in question. This can be attributed to the fact that the fly transgenics have the exogenous DNA integrated at different genetic loci, thus position effect has a role to play in the generation of the phenotype. Depending upon where the integration of DNA has occurred, the level of protein expression would vary and hence the phenotype. Ectopic expression of SATB1 in the *Drosophila* eye gave rise to a rough eye phenotype with a high degree of ommatidial fusion and bristle loss. Since majority of ectopically expressed transcription factors give rise to a rough eye phenotype, SATB1 was ectopically expressed in the fly wing using *MS1096-GAL4.* Flies ectopically expressing mammalian SATB1 had crumpled wings, a few exhibited wing venation defects like fusion of L2 and L3. Flies homozygous for mutant *wingless (wg)* exhibit a phenotype wherein wings are frequently missing and duplicated notum structures are generated at the expense of the lost wing (Morata and Lawrence, 1977). Hence, the observed crumpled wing phenotype might not be due to disruption in the wingless pathway. In the early *Drosophila* wing disc, cell fate decisions assign cells to compartments (dorsal or ventral and anterior or posterior) and demarcate the prospective wing from the body wall (notum). Demarcation of the early wing disc into presumptive wing and body wall is defined by the action of two secreted signaling molecules, Wingless and Vein *(vn)*, a secreted neuregulin-like molecule that activates EGFR signaling pathway (Schnepp et al., 1996); *wg*, a pro-wing gene, is required to repress *vn* expression, which at high levels antagonizes wing development. These results are corroborated by the observation that loss of *wg* results in the spread of *vn* expression and the resultant EGFR activity leads to loss of wing and generation of a notum duplication phenotype. Antagonistic action of Wnt/Wg and EGFR signaling has also been documented in case of segmental patterning of the embryo (Szuts et al., 1997) and in development of the head and wing pouch of third instar larva (Amin et al., 1999; Wessells et al., 1999), suggesting such a relationship between these pathways may be a common theme in a number of cell fate choices. This suggests that the mechanisms by which *wg* and *vn* specify alternate cell fates in the early wing disc, wing, or notum are antagonistic.

During wing development, *wingless* functions at several different stages, reflecting the sequential compartmentalization of the wing disc. In the hierarchy of wing development *wingless (wg)* activity is vital for the establishment of the wing as a distinct entity from the body wall or notum, for the formation of the ventral compartment and finally, to define the wing margin, which forms at the dorsal–ventral compartment boundary and has a characteristic bristles pattern (Treisman et al., 1997). Whereas, EGF-receptor (EGFR) signaling plays a fundamental role in directing cells towards a notum fate by antagonizing wing development, and by activating notum-specifying genes. EGFR signaling is also involved in directing cells to become a part of the dorsal compartment by inducing the dorsal selector gene, *apterous* (Wang et al., 2000).

The stereotyped pattern of longitudinal vein (L2 to L5) positioning of the along the A/P axis is a read-out of the positional information provided by Hh and Dpp signaling gradients. The formation of wing veins requires expression of *rhomboid* (*rho*) gene which encodes a membrane protease involved in epidermal growth factor receptor (EGFR) signaling. Flies homozygous for mutant rho have missing veins and ectopic expression of rho in the course of wing development leads to the formation of extra veins (Crozatier et al., 2004). Venation defects are known to occur upon activation of Egfr, BMP or Wg signaling or by reduction in Notch signaling or Thickveins (*tkv*) function. Irrespective of how ectopic venation is initiated, at some point or the other during the course of development ectopic veins activate 1) Egfr signaling by expressing *rhomboid* and *Star*, 2) BMP signaling by expressing *dpp*, and 3) Notch signaling via the expression of *Delta* and *Serrate*. Crosstalk between diverse signaling pathways at genetic level is known, therefore, a candidate cannot be assigned to a pathway on the basis of venation pattern alone (Blair, 2007; Sotillos and De Celis, 2005). On the basis of the phenotype observed upon SATB1 expression in the wing it can be surmised that SATB1 might be involved in the EGFR pathway.

To elucidate which function of SATB1 viz. the protein interaction (PDZ-like domain) or the DNA binding function (C-terminal CUT and Homeodomain) is essential for SATB1 function *in vivo*, *UAS* lines expressing SATB1 (1-204) and SATB1 (255-763) were generated. Flies expressing the N-terminal (1-204) region in the wing exhibited a phenotype similar to wildtype. This proves that in an *in vivo* system the N-terminal PDZ-like domain of SATB1 is insufficient to confer a phenotype, the DNA binding property of SATB1 also plays a significant role in SATB1 function. Another possibility which answers the lack of phenotype maybe at the level of expression of SATB1 (1-204) *in vivo.*

Validation of SATB1 expression in the fly system was performed by immunostaining for SATB1 upon expression in the eye and wing imaginal discs (data for SATB1 expression in wing imaginal discs in appendix). Misexpressed SATB1 localized inside the nuclei and excluded the DAPI rich regions, this staining pattern strongly correlates with the staining pattern of SATB1 in cells lines viz. the protein entirely resides within the nucleus and excludes the DAPI rich regions (Notani et al., 2010). Thus, it is established that SATB1 is indeed being expressed in flies and the localization of the protein is similar to what is observed in mammalian cells. To validate if the phenotype observed upon SATB1 expression is indeed due to binding of SATB1 to the *Drosophila* genome or the result of the misexpressed protein sequestering endogenous fly proteins, polytene staining was performed. Four bands corresponding to SATB1 were observed in the polytene spreads, but the identity of these loci still needs to be determined. These bands were located near the chromocenter of the polytene chromosome, which is known to be highly heterochromatinized with a central block of heterochromatin, surrounded by a large number of smaller, interconnected blocks which further continue into the euchromatic chromosome arms (Lakhotia and Jacob, 1974). Simultaneously, 21 putative SATB1 binding sites were identified in the *Drosophila* genome by employing MEME analysis using the consensus SATB1 binding site (CSBS) as bait (Purbey et al., 2008). These loci were represented on both Chromosome II and chromosome III. Majority of these loci are implicated in chromatin remodeling, a few

are involved in developmental pathways, and a few in metabolic pathways. It still needs to be verified if SATB1 binds to these loci in an *in vivo* scenario.

Since, this study has been performed by ectopically expressing a mammalian protein in the fly, it would be interesting to study if an endogenous fly protein homologous to our protein of interest would function in a similar manner. To, this day none of the fly proteins have been observed to have a domain architecture similar to SATB1 i.e. a PDZ-like domain, CUT domain and a homeodomain. But, there are reports that identify Defective proventriculus (DVE), a protein involved in the development of proventriculus as the closest homolog of vertebrate SATB1 in fly, based on protein structure (Burglin and Cassata, 2002; Fuss and Hoch, 1998). As opposed to sequence level homology, these reports are based on *in-silico* structural prediction analysis. However, functional homology if any has not been established until now.

The next chapter deals with analysis of genetic interaction of SATB1 with the Wnt/Wg pathway, in order to analyze if the positive regulation of the Wnt/Wg pathway is a conserved phenomenon using *Drosophila melanogaster* as a model system.

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Chapter 3

In vivo validation of SATB1 crosstalk with Wnt/Wg pathway using *Drosophila melanogaster* as a model system

3.1. Introduction

In the previous chapter, it was demonstrated that SATB1 physically and functionally interacts with Dishevelled-1, an upstream effector of the Wnt/Wg signaling pathway in mammalian cells. A positive correlation between the Wnt/Wg pathway and SATB1 was also established by means of quantitative RT-PCRs in HEK293 cells. The next line of inquiry was whether this positive correlation between SATB1 levels and the Wnt/Wg pathway is specific to mammalian systems or is it a conserved phenomenon. Towards this end, ectopic expression of SATB1 in a system which lacks endogenous SATB1 and has a simpler Wnt/Wg pathway would prove insightful. Therefore, the system of ectopic expression in *Drosophila* was used, as majority of studies pertaining to cell signaling have been performed in *Drosophila* melanogaster, and it is the model system of choice for studying signaling pathways. The plethora of genetic screens and tools available for analyzing signaling pathways in flies, far exceed those developed for studying the same in other complex multi-cellular organisms, thereby making it the model system of choice for conducting such studies. Small size, prolific egg-laying, rapid reproduction and a short life cycle further make large-scale genetic screens in reasonable time frames possible, thereby making *Drosophila* an impressive model system (Jennings, 2011). The current study is focused on two Wnt intermediaries, Dishevelled (Dvl/Dsh) and β-catenin/armadillo (arm), whose physical and functional interaction with SATB1 has been established by us previously (Chapter 2 and published data).

The phosphoprotein Dishevelled, is the upstream effector of the Wnt/Wg signaling, it relays the Wnt signal from the receptor-co-receptor complex to the downstream effectors (Habas and Dawid, 2005). Genetic experiments in early *Drosophila* embryos first placed Dishevelled (Dsh) in the canonical Wnt/Wg signaling pathway governing segment polarity (Couso et al., 1994; Noordermeer et al., 1994; Riggleman et al., 1990; Siegfried et al., 1994). Subsequent experiments in *Drosophila* demonstrated that Dishevelled also governs the Planar Cell Polarity (PCP) in the wing, legs and abdomen (Krasnow et al., 1995; Theisen et al., 1994).

Additional experiments in the fly demonstrated that Dishevelled is positioned at the branchpoint between the canonical WNT and PCP signaling pathways (Axelrod et al., 1998; Boutros et al., 1998). In D. melanogaster, knockdown of Dishevelled using siRNA interferes with Wnt-dependent phosphorylation of LRP6 (Bilic et al., 2007), a similar phenomenon is observed in mice (Zeng et al., 2008). The first vertebrate homologs of Dishevelled- Dvl1 (Sussman et al., 1994) and Xdsh (Sokol et al., 1995), were identified in mouse and Xenopus, respectively. Two additional mouse homologues of Dishevelled- Dvl2 and Dvl3 were identified thereafter (Klingensmith et al., 1996; Tsang et al., 1996). Three homologues of Dishevelled- Dvl1, 2 and 3 have been identified in humans (Pizzuti et al., 1996; Semenov and Snyder, 1997).

A high degree of conservation exists between the vertebrate and *Drosophila* Dishevelled (referred to as Dvl and Dsh, respectively), at both structural and functional levels (Klingensmith et al., 1996; Rothbacher et al., 1995). Vertebrate Dishevelled, like its fly counterpart also signals via a PCP cascade, controlling cell polarity during convergent extension cell movements that drive gastrulation (Heisenberg et al., 2000; Tada and Smith, 2000; Wallingford et al., 2000). Overexpression of any one particular Dishevelled isoform is capable of promoting Lef/Tcf-sensitive transcriptional activation in the absence of Wnt3A stimulation, i.e. overexpression of Dvl1, Dvl2, or Dvl3 is Wnt mimetic. Though, it is known that these three isoforms of Dishevelled function cooperatively as well as uniquely with respect to mediation of the canonical Wnt signaling pathway (Lee et al., 2008).

Dishevelled proteins possess three structurally conserved domains, an N-terminal DIX (Dishevelled, Axin) domain, which mediates Dvl self-association, leading to formation of multimerized receptor complexes at the membrane, which provide a high local concentration of binding sites for Wnt signaling proteins (Bilic et al., 2007; Schwarz-Romond et al., 2007); a central PDZ domain (Postsynaptic density 95, Discs Large, Zonula occludens-1), approximately 90 amino acid long which directly interacts with the Frizzled receptor (Wong et al., 2003), and a DEP (Dvl, Egl-10, Pleckstrin) domain which is critical for the membrane recruitment of Dishevelled

during Wnt-mediated signaling (Pan et al., 2004; Simons et al., 2009; Tauriello et al., 2012) and clathrin mediated endocytosis of Frizzled4 and Dvl2 upon Wnt activation (Yu et al., 2007; Yu et al., 2010) (Figure 3.1.1). In addition to these three domains, Dishevelled contains a basic region that precedes the N-terminus of the PDZ domain as well as a proline-rich region located between the PDZ and DEP domains that contains an SH3 binding motif. Both these regions are conserved in most Dishevelled orthologs and are implicated in mediating protein–protein interaction and/or phosphorylation (Wang and Malbon, 2012). A fourth conserved domain called the DSV or Dishevelled domain has recently been reported, its functional significance is not known (Dillman et al., 2013) (Figure 3.1.1).

By virtue of the interactions mediated by these various domains Dishevelled can interact with a wide range of partner proteins, while retaining the essential ability of distinguishing between suitable partners i.e. it provides a multivalent protein scaffold essential for various cellular functions. Through these numerous interactions, Dishevelled facilitates dissemination of the Wnt signal, leading to the activation of β-catenin and T-cell factor (TCF) dependent transcription of developmental genes and genes associated with tumorigenesis (Gao and Chen, 2010; Uematsu et al., 2003; Wharton, 2003).

Dishevelled plays a dual role in both canonical and non-canonical signaling: one as a common component of the shared machinery involved in co-receptor activation, and another downstream and specific for each pathway. Depending on the domains involved, Dishevelled can propagate either canonical or non-canonical Wnt pathways (Wallingford and Habas, 2005) but, the exact mechanism by which it causes the cascade to toggle between canonical and non-canonical forms is not known.

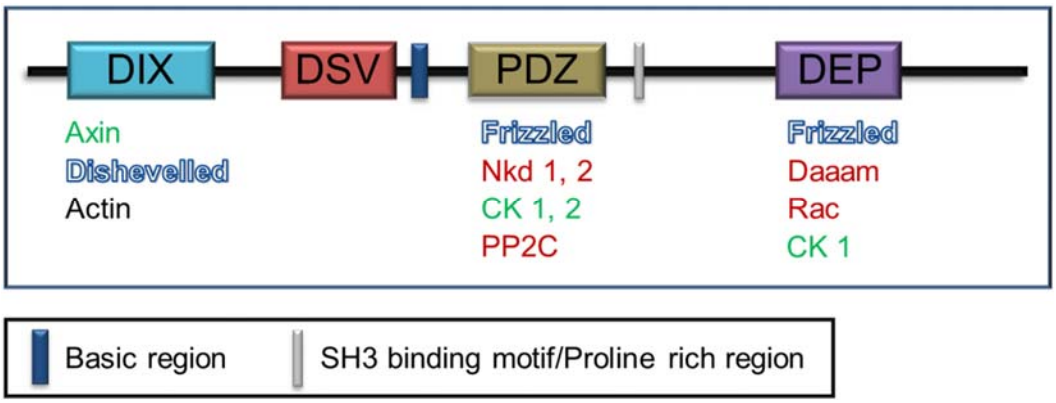

Figure 3.1.1 I Domain organization of Dishevelled (Dvl) protein. Dishevelled proteins are approximately 700 residues long, harboring conserved DIX, basic and serine/threonine-rich region, PDZ, proline-rich region, DEP domain, and a novel domain known as the DSV domain has been identified recently. Details of the same have been provided in the above text. Few of the interactors of the respective domains have been depicted in the cartoon. Intermediaries of the canonical Wnt/β-catenin pathway are depicted in green; proteins involved in the planar cell polarity pathway (PCP) are shown in red; proteins involved in both canonical and non-canonical pathways are stenciled in black; proteins involved in non-canonical Wnt pathways other than PCP are depicted in black viz. actin. Key: Nkd 1, 2, Naked cuticle 1& 2; CK 1, 2, Casein Kinase 1& 2; PP2C, Protein phosphatase 2C; Daam, Dishevelled Associated Activator of Morphogenesis; Rac, Ras-related C3 botulinum toxin substrate (GTPase)

β-catenin is a pivotal molecule of the Wnt/Wg signaling cascade involved in orchestration of organismal development, a process which is the sum total of the molecule's dual roles- cellular adhesion and signaling. Thus, there are two cellular pools of β-catenin- one involved in cadherin mediated cell adhesion and another involved in transducing the Wnt/Wg signal (Lyashenko et al., 2011). In the absence of Wnt activation, Armadillo (the fly homolog of β-catenin) is phosphorylated by Zeste white-3 kinase (Zw3/GSK-3β) and targeted towards proteasomal degradation. In response to Wnt/Wg signal, Armadillo (β-catenin) gets stabilized and enters the nucleus where it functions as a transcription activator by recruiting various transcriptional co-activators such as ISWI-containing NURF complex onto the Wnt responsive genomic targets (Song et al., 2009). β-catenin is known to interact with multiple proteins (Mosimann et al., 2009), one such important interacting partner of β-catenin is the MAR-binding protein SATB1 (Notani et al., 2010), described in detail in Chapter 1 of this thesis. In T-cell lineages SATB1 is generally known to function as a repressor. SATB1 upon binding β-catenin recruits

it to SATB1's genomic targets so that genes formerly repressed by SATB1 are upregulated upon receiving the Wnt signal (Notani et al., 2010). Inside the nucleus β-catenin is known to function as a transactivator with the transactivation function residing inside the C-terminal. A LEF-1-β-catenin fusion lacking the transactivation domain of β-catenin is impaired in signaling while fusion of just the C-terminal of β-catenin to the DNA-binding domain of LEF-1 is sufficient for successful transduction of Wnt/Wg signaling (Vleminckx et al., 1999). It is this C-terminal transactivation domain of β-catenin which serves as an interface for interaction with SATB1 (Notani et al., 2010).

To identify if a genetic interaction exists between SATB1 and intermediaries of the Wnt/Wg pathway, and to analyze if the positive correlation between SATB1 levels and the Wnt/Wg pathway observed in cell lines (Chapter 2) is a conserved feature, the approach of ectopic expression of SATB1 in a system lacking endogenous SATB1 i.e. *Drosophila* was used. Tissue specific expression of full length SATB1 or specific domains of SATB1 was induced using the *UAS-GAL4* system, described in detail in Chapter 2 of this thesis. To identify the domain(s) of SATB1 responsible for generation of the SATB1 misexpression phenotypes i.e. rough eyes/wing venation defects, flies transgenic for the N-terminal PDZ-like domain (FLAG-SATB1 (1-204), and the C-terminal DNA-binding region (SATB1 (255-763)) were generated. These were then ectopically expressed in a tissue-specific manner using *GAL4* driver lines, and the phenotypes observed. Further, each of these constructs was expressed in the background of *arm* or *dsh* misexpression to examine if ectopic expression of SATB1 or its truncations aids or antagonizes the over-expression phenotype of Arm^{S10} or Dsh. In order to examine if the phenotypes generated upon ectopic expression of SATB1 in the fly are significant, it was decided to express a putative homolog of SATB1 in flies. To date none of the known fly proteins have been identified to have a domain organization similar to SATB1. Defective proventriculus (DVE), a homeodomain containing protein is purported to be the putative homolog of SATB proteins in flies (Burglin and Cassata, 2002; FitzPatrick et al., 2003). The gene for coding for DVE protein was identified on the

basis of the observation that larvae homozygous for *dve*[1] die during the first instar stage of larval development due to an inability to ingest food. This is caused due to defects in morphogenesis of the proventriculus, which is a valve like structure that regulates the passage of food from the larval foregut to the midgut (Nakagoshi et al., 1998). Both DVE and SATB belong to the CUT superclass of homeobox genes and have an evolutionarily conserved COMPASS domain (Bürglin and Cassata, 2002). The DVE protein consists of two different domains, a PDZ-like domain which shows significant homology to the N-terminal PDZ-like domain of SATB1 (also characterized as a COMPASS domain), and two homeodomains (Fuß and Hoch, 1998) (Figure 3.1.2). In nature, COMPASS (CMP) domains are followed by two homeodomains as is the case of DVE; or a homeodomain and a cut domain as is observed in case of SATB proteins. Only two CUT domain containing proteins are present in *Drosophila*, and none of them have a COMPASS domain i.e. they do not belong to the SATB1 family of CUT superclass proteins (Burglin and Cassata, 2002).

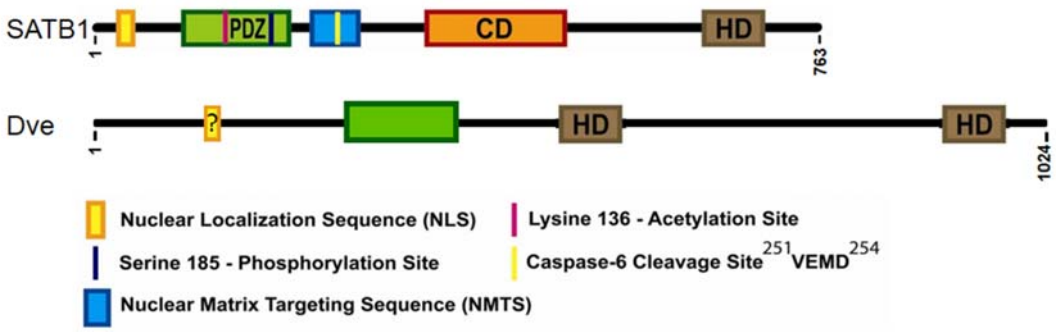

Figure 3.1.2 I Domain organization of *Drosophila* Defective proventriculus protein (DVE) in comparison to SATB1. Top panel. SATB1 domain architecture. The N-terminal region of SATB1 harbors a Nuclear Localization signal (NLS) spanning amino acids (20-40) (yellow box) and a PDZ-like domain region spanning amino acids (90-204). This region is also characterized as COMPASS domain (green box). SATB1 is known to be regulated by post-translational modifications- phosphorylation and acetylation at Serine 185 and Lysine 136 (Pink bar and blue bar respectively). Both of these reside within the PDZ-like domain. SATB1 is targeted to the matrix by virtue of Nuclear matrix targeting sequence (NMTS) spanning amino acids (224 to 278) (blue box). SATB1 contains a Caspase-6 cleavage site at amino acid 255, cleavage of SATB1 at this position interferes with SATB1 dimerization and subsequent DNA-binding. The C-terminal region of SATB1 spanning amino acids 2555-763 harbors a CUT domain (orange box) and a Homeodomain both (brown box) of which are involved in binding to DNA. The homeodomain of SATB1 exhibits very little similarity with the typical homeodomains. Though the consensus DNA binding site of SATB1 is similar to DNA elements bound by Homeodomain proteins.

Bottom Panel. Defective proventriculus (DVE) protein domain architecture. DVE harbors a conserved PDZ-like domain (green box) which exhibits a high degree of similarity with the PDZ-like domain of SATB1. It also contains two homeodomains (brown boxes), though they exhibit very little similarity with SATB1 at the level of homeodomains. Even though, a nuclear localization signal (yellow box) hasn't been identified in DVE, the protein is known to localize inside the nucleus. Another point at which DVE differs from SATB proteins is that any Nuclear Matrix targeting sequence (NMTS) has not been identified yet, in DVE. Two isoforms of DVE, isoform A and isoform B are present in *Drosophila*, here for simplicity sake only isoform A has been shown.

A few similarities exist between the DVE and SATB1 proteins, the same are enumerated in Table 3.1.1. At the level of cellular localization, these two proteins show some dissimilarities but both of them are known to bind A/T rich regions and regulate transcription (Nakagoshi et al., 1998; Notani et al., 2010; Purbey et al., 2008). But of date, functional homology between these two proteins has not been established. One of the aims for this study is to probe for the same.

Table: 3.1.1

Function	SATB1	DVE
Transcription factor	Yes	Yes
A/T region binding	Yes	Yes
Nuclear Matrix binding	Yes	Unknown
Subcellular localization	Nuclear	Nuclear and cytosolic

Table 3.1.1 I A comparative analysis of SATB and DVE proteins.

3.2. Materials and Methods

3.2.1. Fly stocks

The following fly stocks were used in the current study, all the crosses. All stocks were maintained at 25^{o}C.

GAL4 driver lines:
dpp-GAL4$^{40.6}$ driver (Morimura et al., 1996), was used to drive expression along the anteroposterior boundary (AP) in wing imaginal discs; *vg-GAL4* driver (Simmonds et al., 1995), was used to drive expression along the dorso-ventral (DV) compartment boundary of the wing imaginal discs; *GMR-GAL4* (Freeman, 1996) was used to drive transgene expression in the eye.

UAS lines for ectopic expression:
UAS-dve (BL7086)*; UAS-arm.S10,* the Arm^{S10} mutant has a deletion of 54 amino acids in its N-terminal domain by virtue of which, it remains constitutively active (Pai et al., 1997); *UAS-dsh* (Penton et al., 2002). *UAS-SATB1, UAS-FLAG-SATB1 (1-204), UAS-SATB1 (255-763)* were generated in house (refer to Chapter 2).

3.2.2. Immunostaining of imaginal discs

Larvae were collected in a cavity block, washed and dissected in 1XPBS. To access the imaginal discs, a cut was made at the posterior 2/3 part of the larvae slight pressure was applied to expose the contents of the larval gut. This excess was removed and the remaining 1/3rd was turned inside out with the help of a pair of needles. The exposed imaginal discs attached to the inverted cuticle were fixed using 4% Paraformaldehyde prepared in 1X PBS containing 0.1 % Triton X-100 (1X PBST) for 10 min at room temperature. Discs were given three 1 min rinses with 1X PBST followed by three 10 min washes with the same. Wing discs were blocked for 2 h at room temperature in blocking solution (0.5% BSA, 2%FBS in 1X PBST pH 7.4). Samples were incubated over-night with primary antibody was prepared in blocking buffer minus Triton X-100. Discs were washed twice with blocking buffer

and incubated for 2 h at room temperature with fluorescently labeled secondary antibody (Molecular Probes, Invitrogen) prepared in blocking solution without FBS. Two 15 min washes with 1X PBST were performed, followed by two 10 min washes with 1X PBS. Discs were incubated for 10 min with DAPI (SIGMA) which was used at a working concentration of 1 µg/ml. Two 10 min washes were performed using 1X PBS. The desired imaginal discs were detached and mounted in mounting medium (DAKO-cytomation).

3.3. Results

3.3.1. Expression of SATB1 deletion constructs in the fly eye

Wild-type *Drosophila* eyes are characterized by 750 ommatidia that develop during the third larval instar stage as the morphogenetic furrow progresses from posterior to anterior in the eye disc (Bate and Arias, 1993). The *GMR* promoter is expressed posterior to the morphogenetic furrow in developing photoreceptor cells (Moses and Rubin, 1991). Ectopic expression of SATB1 in the fly eye under the control of *GMR-GAL4* leads to generation of a rough eye phenotype (Chapter 2). In order to narrow down the domain/function responsible for generating the SATB1 misexpression phenotype in the fly, SATB1 and its various domains were expressed in the *Drosophila* eye using *GMR-GAL4*. The N-terminal (1-204) domain is required for interaction with proteins whereas the C-terminal (255-763) region is involved in DNA-binding (Purbey et al., 2008). As mentioned previously ectopic expression of full-length SATB1 in the eye results in flies having rough eye phenotype, characterized by loss of ommatidial structure, fusion of ommatidia, and improper arrangement of inter-ommatidial bristles (Figure 3.3.1 Panel ii vs Panel i). Ectopic expression of SATB1 (1-204) in the developing eye using the *GMR-GAL4* results in a slightly rough eyed phenotype in flies, this rough eyed phenotype is not spread uniformly across the eye, but is restricted to a few random areas on the eye. The same was documented by SEM which revealed fusion of ommatidia at a few places with concomitant disruption in the bristle pattern, but reduction in

eye size was not observed (Figure 3.3.1, Panel iii). The intensity of this phenotype was not as prominent as that observed in case of SATB1 expression (Figure 3.3.1, Panel iii vs Panel ii). Penetrance of this phenotype is 70%. Ectopic expression of SATB1 (255-763) in the developing eye using the *GMR-GAL4* driver doesn't have any phenotypic effect on the eye, all flies exhibited the wild type phenotype. SEM analysis of these fly eyes revealed that ommatidia are compactly packed in the usual hexagonal pattern and individual ommatidia are separated by uniformly spaced bristles (Figure 3.3.1, Panel iv). Penetrance of this phenotype is 100%. This is in concordance with our hypothesis that in the absence of the N-terminal PDZ-like domain mediated dimerization, SATB1 (255-763) would not bind to DNA and bring about any changes which would then manifest in a phenotype. Thus, the (255-763) region acts as a negative control.

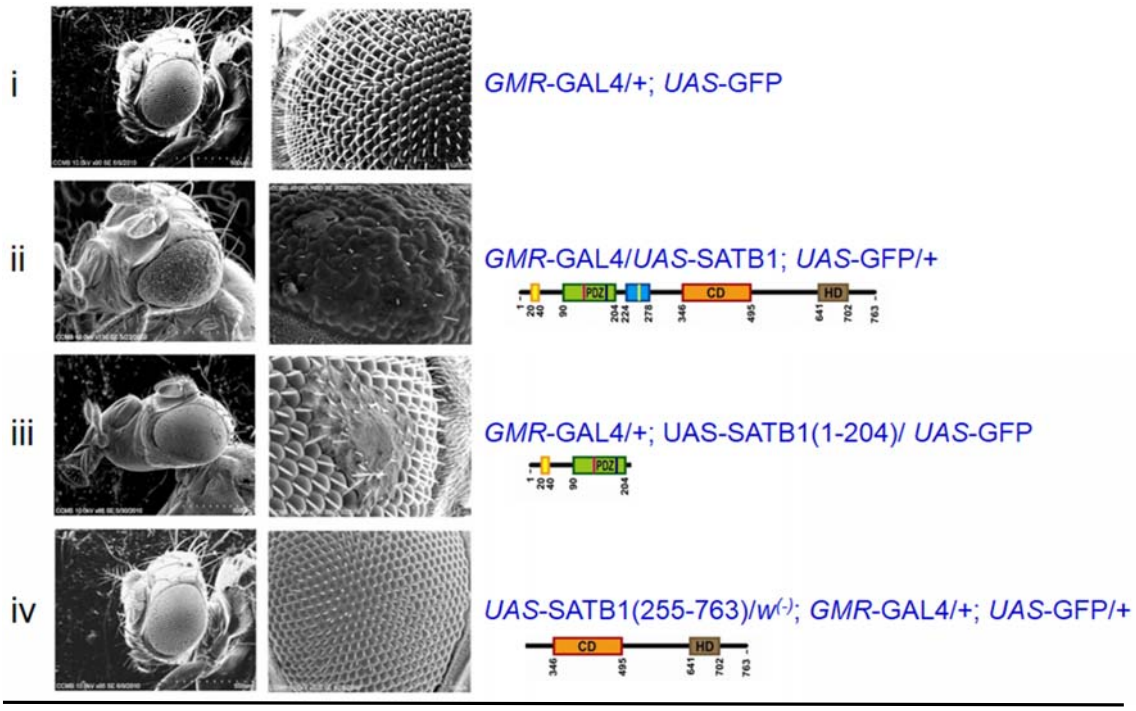

Figure 3.3.1 I SEM images of expression of SATB1 and SATB1 deletion constructs under the control of *GMR-GAL4*. Ectopic expression of SATB1 and SATB1 truncations under the regulation of *GMR-GAL4*. **(i)** control *GMR-GAL4*/+. **(ii)** Eyes ectopically expressing SATB1. **(iii)** Eyes ectopically expressing the N-terminal (1-204) region. **(iv)** Eyes ectopically expressing the (255-763) region of SATB1. At all stages *UAS-GFP* was used as control. Details of the experiment provided in text above and the genotype mentioned in the figure.

3.3.2. SATB1 suppresses the small eye phenotype of Dsh misexpression

Elevated levels of *dsh* in the eye, result in ommatidial degeneration and cell death leading to generation of flies having severely reduced eyes. (Zhang et al., 2015). SEM analysis of such eyes revealed very small and glossy eyes with complete fusion of ommatidia and very few bristles (Figure 3.3.2, Panel iii vs Panel i). To verify if SATB1 genetically interacts with Dishevelled, SATB1 was expressed in the background of Dishevelled over-expression. Upon expressing SATB1 in this small eye background, it was observed that SATB1 suppressed the Dsh misexpression phenotype. These flies had larger eyes as compared to flies ectopically expressing Dsh, and they also had rough eyes (Figure 3.3.2, Panel iv vs Panel iii). The penetrance of this phenotype was 30%. This experiment was performed thrice with similar results, expression of SATB1 in the background of GFP expression was used as control. On the basis of the observed suppression of the small eye phenotype of Dishevelled (Dsh) it can be inferred that SATB1 might be acting downstream to *dsh*.

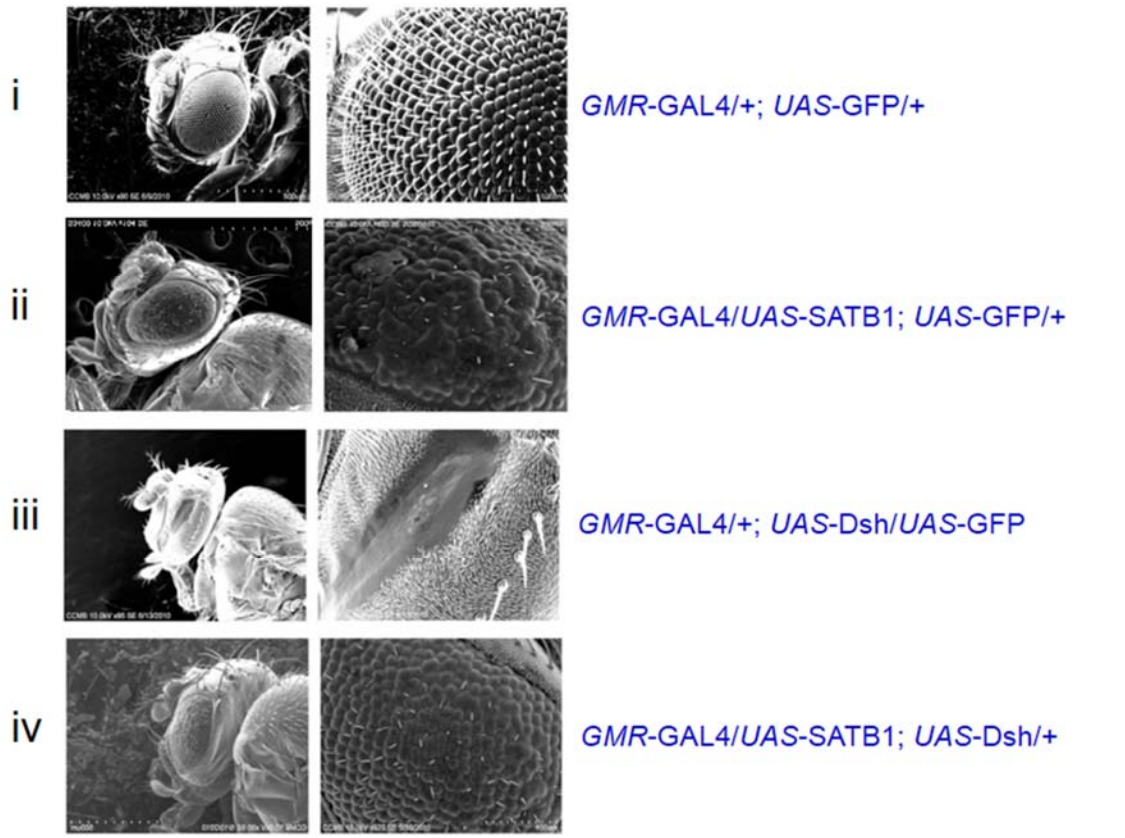

Figure 3.3.2 I Suppression of small eye phenotype of Dsh over-expression by human SATB1. (i) control *GMR-GAL4*/+. **(ii)** Fly eyes ectopically expressing SATB1. **(iii)** Fly eyes ectopically expressing Dsh. **(iv)** Fly eyes expressing SATB1 in the background

of Dsh misexpression. At all stages *UAS-GFP* was used as control. Details of the experiment are provided in text above and the genotype mentioned in the figure.

3.3.3. SATB1 N-terminal region (1-204) harboring the PDZ-like domain does not suppress the small eye phenotype of Dsh overexpression

According to our cell line data, SATB1 physically and functionally interacts with Dishevelled *via* its PDZ-like domain (Chapter 2) which is also dominant negative for SATB1 function (Notani et al., 2011). Expression of SATB1 (1-204) in the eye resulted in a slightly rough eyed phenotype characterized by ommatidia fusion along with disruption in bristle pattern in certain regions (Figure 3.3.3, Panel ii). As mentioned above, ectopic expression of Dishevelled in the eye causes severe reduction in the eye size with complete fusion of ommatidia and loss of bristles (Figure 3.3.3, Panel iii). This GMR/Dsh background was used to screen whether SATB1 (1-204) expression could suppress the small eye phenotype. It was observed that SATB1 (1-204) did not suppress the Dsh misexpression phenotype, these flies had completely fused ommatidia and small eyes, the same as *dsh* misexpression flies (Figure 3.3.3, Panel iv). Indicating that *dsh* doesn't genetically interact with the (1-204) region of SATB1 *in vivo*.

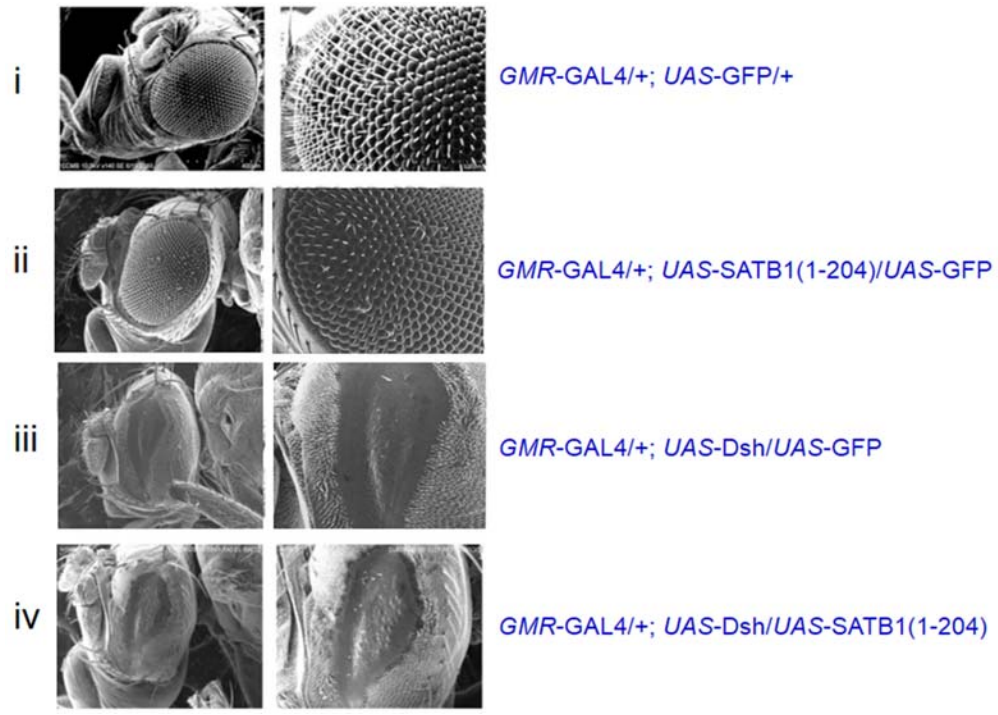

Figure 3.3.3 I The N-terminal PDZ-like domain does not suppress the small eye phenotype of Dsh misexpression. (i) control *GMR-GAL4*/+. **(ii)** Fly eyes ectopically

expressing SATB1 (1-204). **(iii)** Fly eyes ectopically expressing Dsh. **(iv)** Fly eyes expressing SATB1 (1-204) in the background of Dsh overexpression. At all stages *UAS-GFP* was used as control. Details of the experiment are provided in text above and the genotype indicated in the figure.

3.3.4. The DNA-binding domain of SATB1 by itself does not suppress the phenotype produced upon Dishevelled misexpression

Reports from Galande lab show that in the absence of PDZ domain mediated dimerization, the DNA-binding domain of SATB1 does not bind DNA (Galande et al., 2001; Purbey et al., 2008). SATB1 (255-763) was expressed in the background of Dsh overexpression, to study if the DNA-binding domain can suppress the small eye phenotype produced by ectopic expression of Dsh. SEM analysis revealed that the DNA binding domain of SATB1 does not suppress the small eye phenotype of Dsh misexpression (Figure 3.3.4, Panel iv vs iii), indicating that in an *in vivo* scenario, Dsh requires both the N-terminal PDZ-like domain in addition to the DNA binding domain for interacting with full-length SATB1.

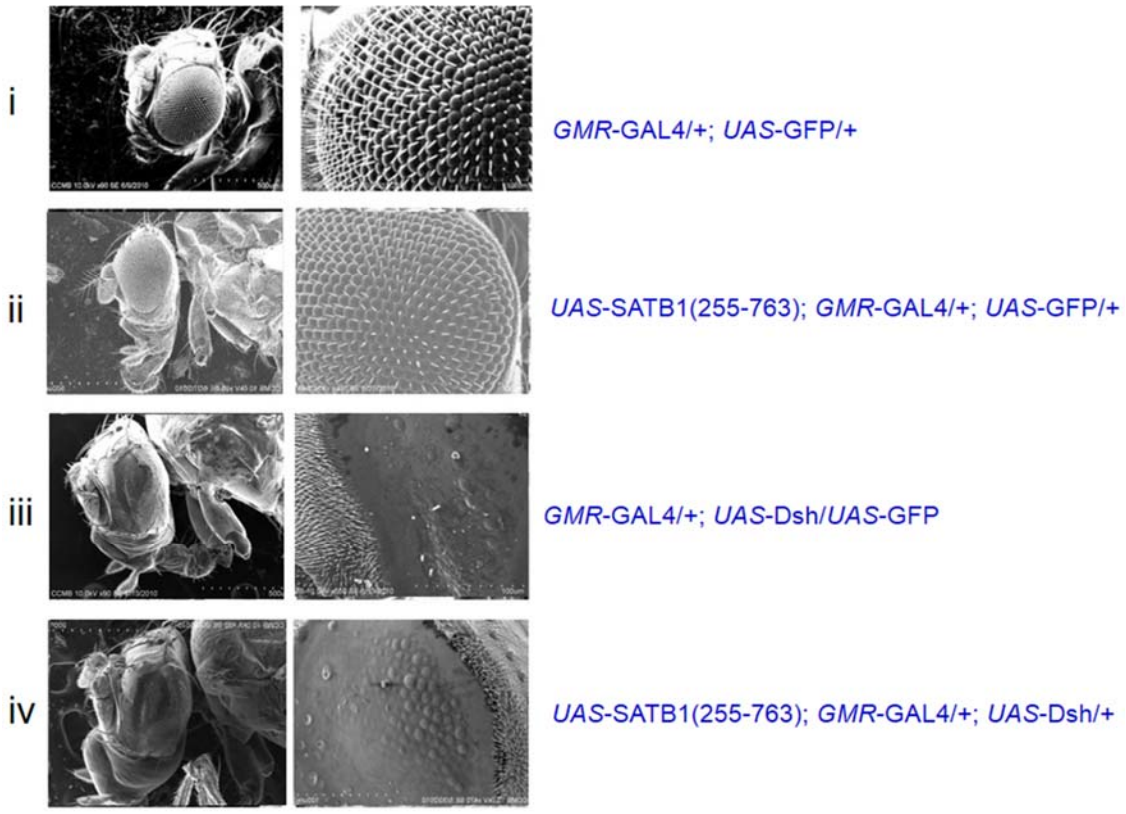

Figure 3.3.4 I Ectopic expression of the DNA-binding domain of SATB1 does not suppress the small eye phenotype generated upon misexpression of Dsh. (i) control *GMR-GAL4*/+. **(ii)** Fly eyes ectopically expressing SATB1 (255-763). **(iii)** Fly eyes

ectopically expressing Dsh. **(iv)** Fly eyes expressing SATB1 (255-763) in the background of Dsh misexpression. At all stages *UAS-GFP* was used as control. Details of the experiment are provided in text above and the genotype mentioned in the figure.

3.3.5. SATB1 suppresses the small eye phenotype of activated Arm

Wingless signaling pathway regulates the expression of eye specification genes; *eyes absent*, *sine oculis* and *dachshund* thereby controlling the final size of the eye field. Activation of the Wingless signaling pathway is sufficient to change the fate of eye cells as it is known to respecify eye cells into a variety of fates, most notably the head cuticle (Baonza and Freeman, 2002). Armadillo is the final effector of the Wnt/Wg signaling, it gets stabilized in response to Wnt/Wg activation effectively leading to expression of Wnt/Wg responsive genes. As mentioned in materials and methods, Arm^{S10} is a mutant which is constitutively expressed as a result of stabilization of Arm protein. Ectopic expression of activated arm (Arm^{S10}) mimics activation of the Wingless signaling cascade (Pai et al., 1997; Sanders et al., 2009). Experiments performed in cell-lines reveal that SATB1 physically interacts with β-Catenin (armadillo), the downstream effector of the Wnt/Wg pathway (Notani et al., 2010). To verify if SATB1 interacts with *arm* at a genetic level in *Drosophila*, SATB1 was expressed in the background of constitutive expression of arm (Arm^{S10}). Misexpression of Armadillo (Arm^{S10}) using the eye specific *GMR-GAL4* driver resulted in drastic reduction of the eye size. SEM analysis of such eyes revealed completely fused ommatidia along with bristle loss leading to generation of a glossy eye (Figure 3.3.5, Panel III vs I). Upon ectopically expressing SATB1 in this small eye background, it was observed that SATB1 suppresses the small eye arm phenotype of activated arm (Arm^{S10}) misexpression, these flies had larger eyes as compared to flies expressing activated arm, they exhibited incomplete ommatidial fusion and hence, a rough eye phenotype in a manner similar to SATB1 over-expression (Figure 3.3.5, Panel IV vs Panel III). The penetrance of this phenotype was 20%, the experiment was performed three times with similar results. Control crossed were set up using *UAS-GFP.* This result is in agreement with the hypothesis that a cross-talk would be present between human SATB1 and the fly complement of the Wnt/Wg pathway. But, from the aforementioned results it is also clear that in the fly system the context of SATB1 action is different from the one observed in cell-lines, where it is known to aid/activate the Wnt/Wg pathway. Thus, the effect of SATB1 on the outcome of the Wnt/Wg pathway is context dependent. It would be interesting to study the reason underlying this differential functioning of SATB1 in flies and cell-lines.

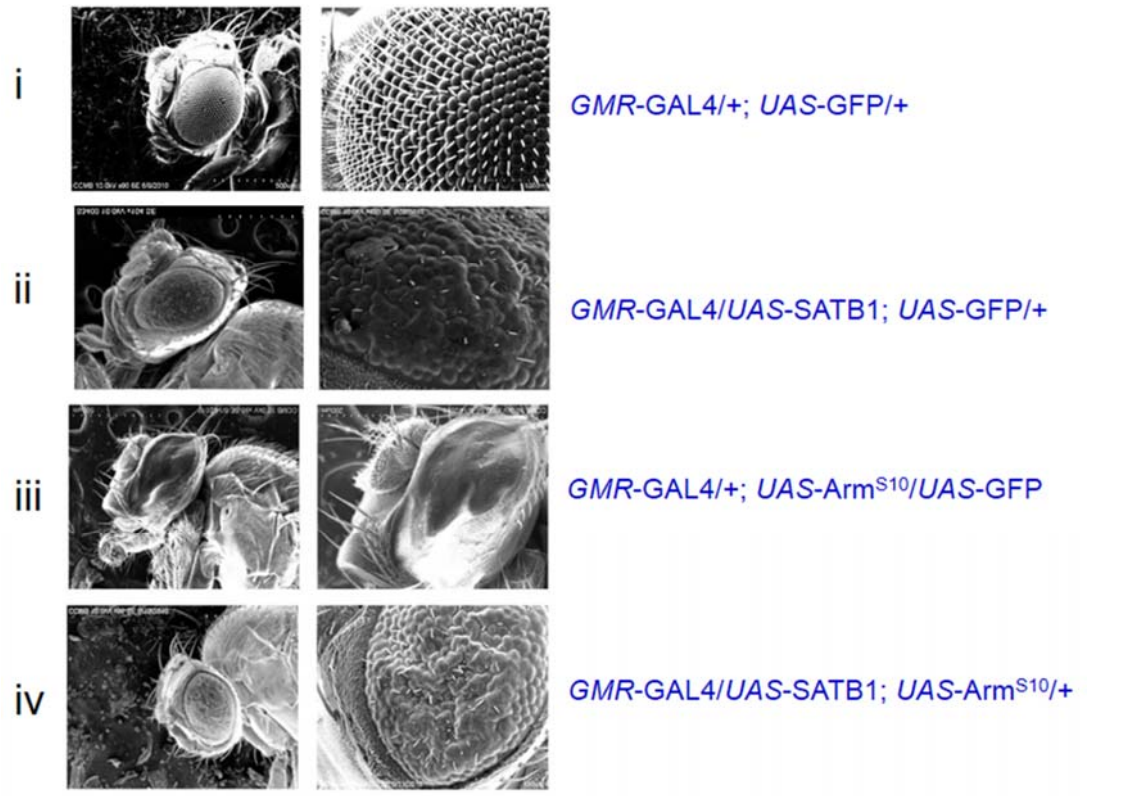

Figure 3.3.5 I Suppression of small eye phenotype of activated arm expression by SATB1. (I) control *GMR-GAL4*/+. **(II)** Fly eyes ectopically expressing SATB1. **(III)** Fly eyes ectopically expressing Arm^{S10}. **(IV)** Fly eyes expressing SATB1 in the background of Arm^{S10} expression. At all stages *UAS-GFP* was used as control. Details of the experiment are provided in text above and the genotype mentioned in the figure.

3.3.6. Expression of N-terminal PDZ-like domain of SATB1 by itself does not suppress the phenotype produced upon over-expression of activated arm

Reports from Galande laboratory show that the N-terminal PDZ-like domain of SATB1 interacts with the C-terminal transactivation domain of β-catenin (Notani et al., 2010). So it was decided to analyze the effect of ectopic expression of the SATB1 (1-204) region harboring the PDZ-like domain on the phenotype generated upon constitutive expression of Arm protein. As mentioned previously, SEM analysis of fly eyes ectopically expressing SATB1 (1-204) showed fusion of ommatidia and disruption in bristle pattern (Figure 3.3.6). Upon expression of SATB1 (1-204) in the background of constitutive arm expression in the eye, it was observed that SATB1 (1-204) fails to suppress the Arm^{S10} phenotype (Figure 3.3.6,

Panel IV vs III). Indicating that in an in vivo system, armadillo does not genetically interact with the N-terminal (1-204) region of SATB1. This might be another point of difference with respect to cell-line data but, the same needs to be checked by immunostaining whether SATB1 and Arm protein interact *in vivo.*

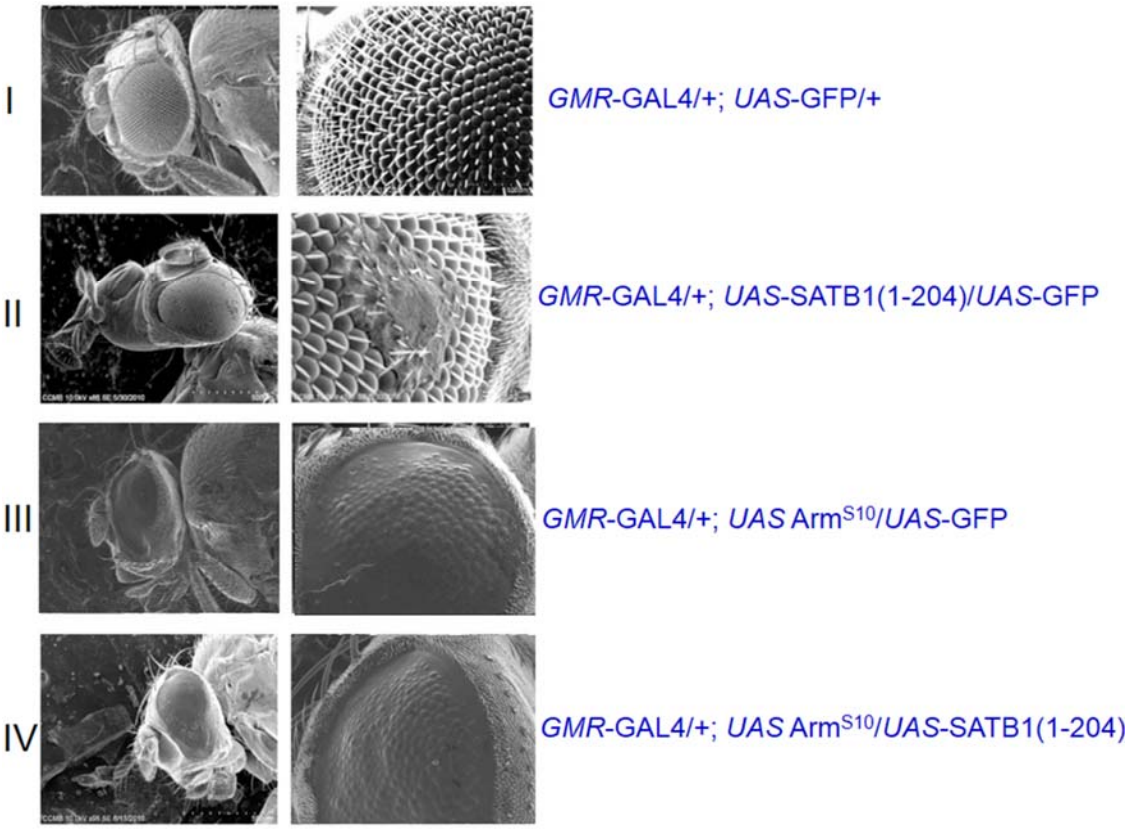

Figure 3.3.6 I The N-terminal PDZ-like domain of SATB1 fails to suppress the small eye phenotype of Arm^{S10}. (I) control *GMR-GAL4*/+. **(II)** Fly eyes ectopically expressing SATB1 (1-204). **(III)** Fly eyes ectopically expressing Arm^{S10}. **(IV)** Fly eyes expressing SATB1 (1-204) in the background of Arm^{S10} misexpression. At all stages *UAS-GFP* was used as control. Details of the experiment are provided in text above and the genotype mentioned in the figure.

3.3.7. Ectopic expression of the DNA-binding domain of SATB1 by itself does not suppress the phenotype produced upon expression of activated arm

As mentioned previously, ectopic expression of SATB1 (255-763) region which harbors the DNA-binding domain resulted in a phenotype similar to the wildtype in flies, the same was documented by SEM (Figure 3.3.7, Panel II). When this region

was ectopically expressed in the background of constitutive expression of Arm, it was observed that the DNA-binding domain of SATB1 by itself did not suppress the small eye phenotype of activated arm (Figure 3.3.7, Panel IV vs III), indicating that similar to *dsh*, *arm* requires both the N-terminal PDZ-like domain and the DNA-binding domain for interacting with full-length SATB1, in an *in vivo* scenario.

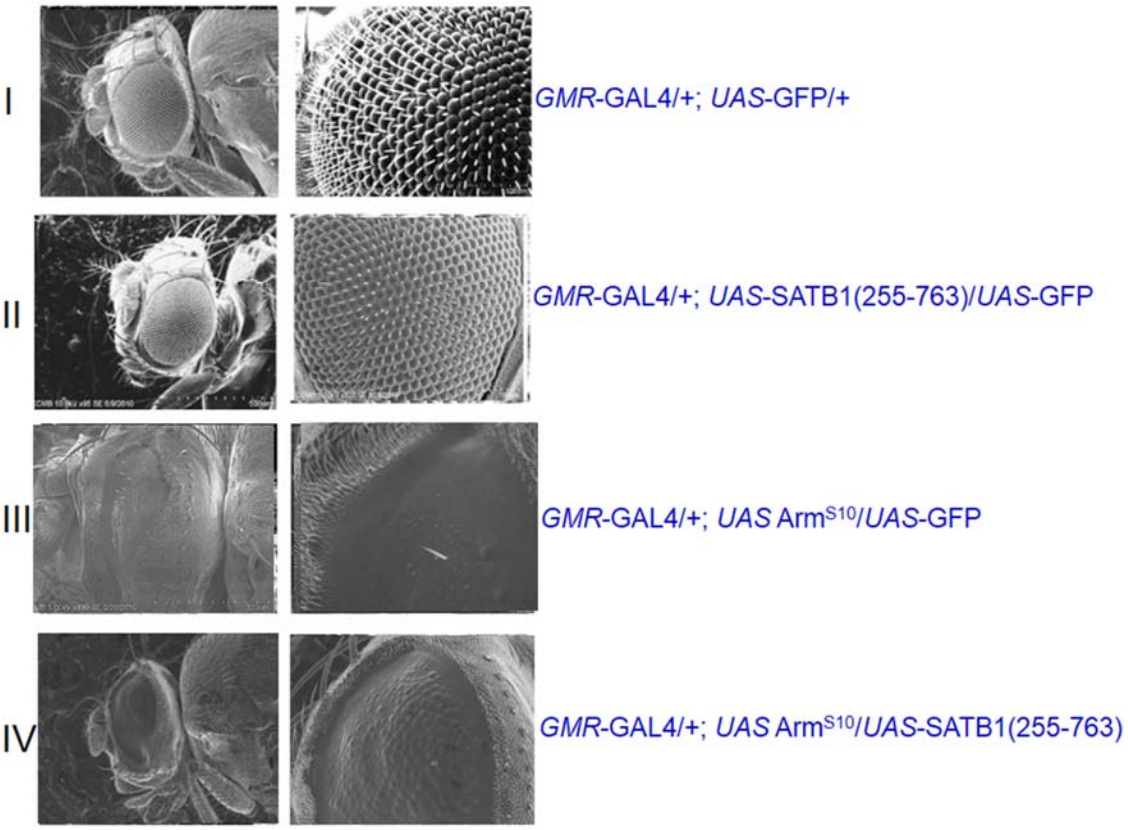

Figure 3.3.7 I Ectopic expression of the DNA-binding domain of SATB1 does not suppress the small eye phenotype of activated arm. (I) control *GMR-GAL4*/+. **(II)** Fly eyes ectopically expressing SATB1 (255-763). **(III)** Fly eyes ectopically expressing Arm^{S10}. **(IV)** Fly eyes expressing SATB1 (255-763) in the background of Arm^{S10} expression. At all stages *UAS-GFP* was used as control. Details of the experiment are provided in text above and the genotype mentioned in the figure.

3.3.8. SATB1 induces expression of Wnt/Wg antagonists at transcript level

In cell lines, SATB1 activates expression of the Wnt responsive genes such as, TCF7L2, CtBP, c-FOS (Fra-1), cyclinD1 (CCND1), c-myc and c-Jun (Chapter 2). SATB1 expression also led to upregulation of Dishevelled (*dsh*)- a Wnt/Wg activator. Thus, in cell-lines SATB1 acts as positive regulator of the Wnt/Wg

pathway. However, from the aforementioned fly experiments it can be gathered that SATB1 antagonizes the Wnt/Wg pathway in the fly system. To verify the same at molecular level, transcript levels of Wnt responsive genes and Wnt antagonists was quantified by qRT-PCR analysis upon ectopic expression of SATB1. Towards this end, SATB1 was ectopically expressed in the eye, and transcript levels of Wnt responsive genes such as dsh, stripe (*sr*), *axin*, *nemo*, *nkd*, *apc1*, and *apc2* were quantified. Of these, *dally*, *axin*, *nkd*, *apc1* and *apc2* are known to antagonize the Wnt/Wg pathway, *stripe* and *nemo* are Wnt readouts, and *dsh* mimics Wnt activation (Lee et al., 2008).

It was observed that transcript levels of genes which antagonize the Wnt/Wg pathway such as *dally*, *apc-2*, and *nkd* were upregulated, and levels of Wnt regulated genes such as stripe were downregulated (Figure 3.3.8). Further, in contrast to results from cell-line experiments, where a significant increase in dsh transcript is observed in response to Wnt activation, here a very slight increase of 1.5 fold is observed. This decrease in *dsh* levels can be attributed to an increase in the levels of *nkd*, which is known to antagonize the Wnt/Wg pathway by inhibiting *dsh*. As *dsh* over-expression, activates Wnt signaling (Lee et al., 2008), this would effectively have a bearing on the outcome of Wnt/Wg signaling. Taken together, the increased transcript levels of Wnt/Wg pathway antagonists such as *apc2*, *nkd* and *dally*, along with very mild increase in *dsh* transcript might be responsible for the suppression of Wnt activation phenotypes (overexpression of Dsh/constitutive expression of arm) upon ectopic expression of SATB1. But, the high degree of complexity at tissue level should be taken into consideration.

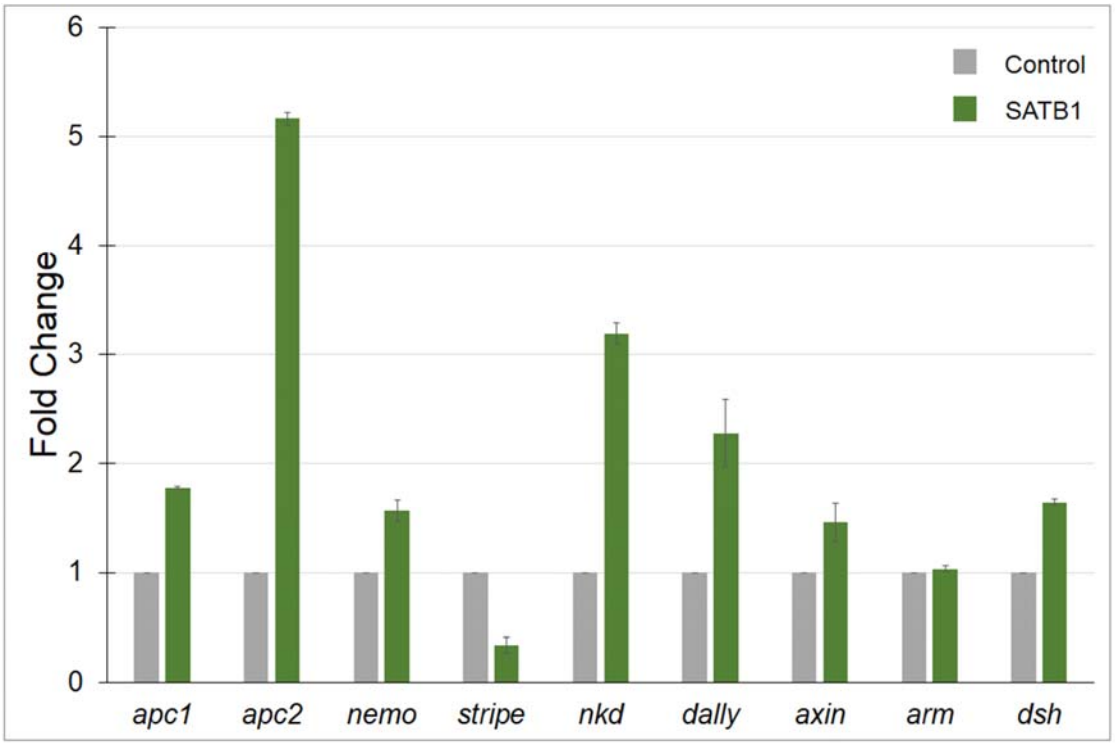

Figure 3.3.8 I SATB1 upregulates the antagonists of the Wnt pathway. Quantitative RT-PCRs were performed to study the effect of SATB1 expression on molecules involved in/or having a cross-talk with the Wnt/Wg signaling pathway. Grey bars represent control discs. Green bars represent SATB1 overexpression discs. Ectopic expression of SATB1 led to an increase in the transcript levels of Wnt antagonists such as *nkd*, *dally*, and *apc-2*. Wnt read-out genes- *nemo* and *dsh* show very little increase in transcript levels. It is known that *arm* is stabilized at the level of protein in response to the Wnt/Wg activation but no change is observed at transcript level. Similar result is observed in here, wherein there is no change in *arm* transcript levels with respect to the control. Details of the same are provided in the above text. Rp49 was used as an internal control for normalizing the C_T values. Error bars indicate standard deviation calculated from triplicates. Fold change as compared to control is depicted on Y axis.

3.3.9. SATB1 binding sites are present upstream of *nkd* and *apc-2*

As a first towards studying the mechanism underlying the upregulation of *nkd* and *apc-2* upon ectopic expression of SATB1, the regions upstream to the TSS were analyzed for the presence of consensus SATB1 binding sites (Purbey et al., 2008). Motif search analysis was performed using MEME. Putative SATB1 binding sites were observed in the upstream regions of both genes (Figure 3.3.9). Therefore, it can be hypothesized that ectopically expressed SATB1 can bind to the regulatory regions of these genes, thereby leading to regulation of the genes. But, binding of

the protein to these genomic loci in vivo, needs to be verified by chromatin immunoprecipitation assay.

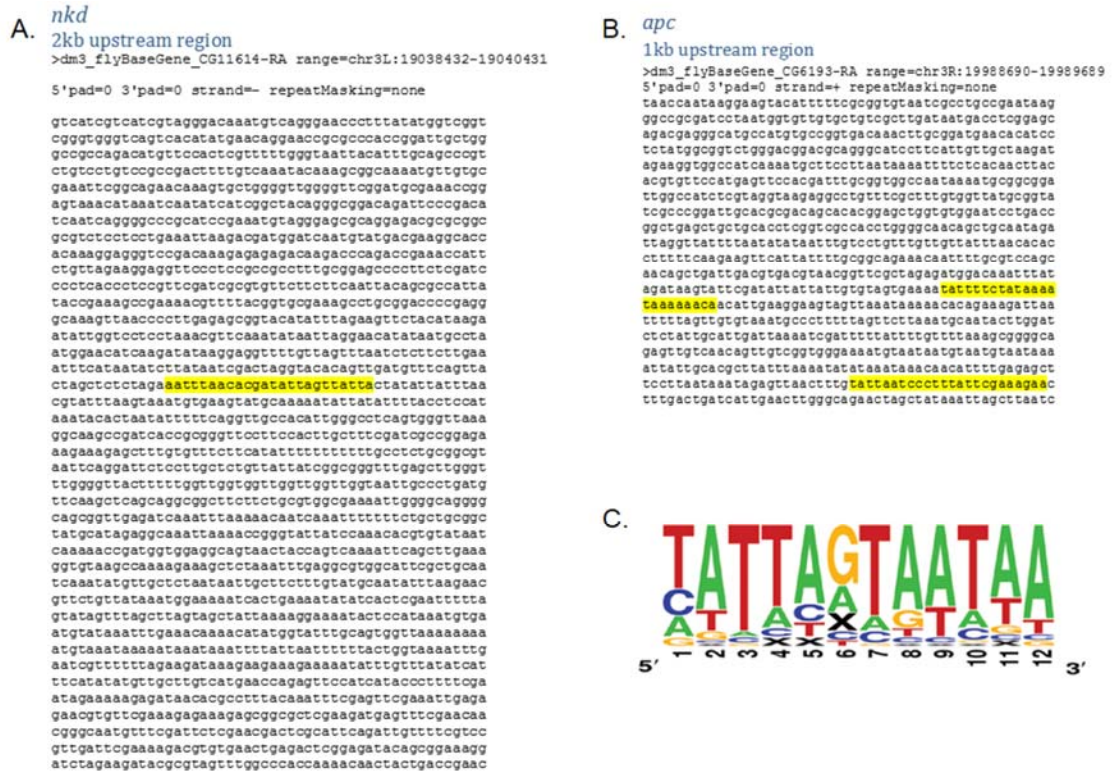

Figure 3.3.9 I SATB1-binding sites are present upstream to the TSS of *naked cuticle* (*nkd*) and *apc-2*. The sequences upstream sequences of *naked cuticle* and *apc-2* genes were analyzed for the presence of consensus SATB1 binding sites (CSBS) by MEME analysis. CSBS **(C)** was identified in the region upstream to the TSS of *nkd* **(A)** and *apc-2* **(B)** genes (highlighted in yellow). The upstream regulatory region of *apc-2* gene shows presence of two CSBS sites **(B)**. Hence, mammalian SATB1 can potentially bind these sequences in vivo.

3.3.10. The putative fly homolog of SATB1, *defective proventriculus* (*dve*) gives rise to a rough eye phenotype

The putative homolog of SATB1 in flies based on in silico structural analysis, is *dve*. DVE protein exhibits 62% similarity to mammalian SATB1 at the level of the N-terminal PDZ-like domain of SATB1 (also predicted to be a COMPASS domain) (Fuß and Hoch, 1998). DVE is usually expressed in the midgut, the leg disc, the wing imaginal disc, and the eye antennal disc (Kiritooshi et al., 2014; Kölzer et al., 2003; Nakagawa et al., 2011; Shirai et al., 2007). When *dve* was ectopically

expressed in the fly eye, a rough eye phenotype observed. The observation that this phenotype was much stronger than the one observed upon SATB1 expression can be attributed to the basal level of DVE present in the tissue. SEM analysis of the same revealed a rough eye with complete ommatidial fusion and very few bristles, as opposed to SATB1 expressing flies which exhibited ommatidial fusion to a lesser degree and randomly oriented bristles (Figure 3.3.10).

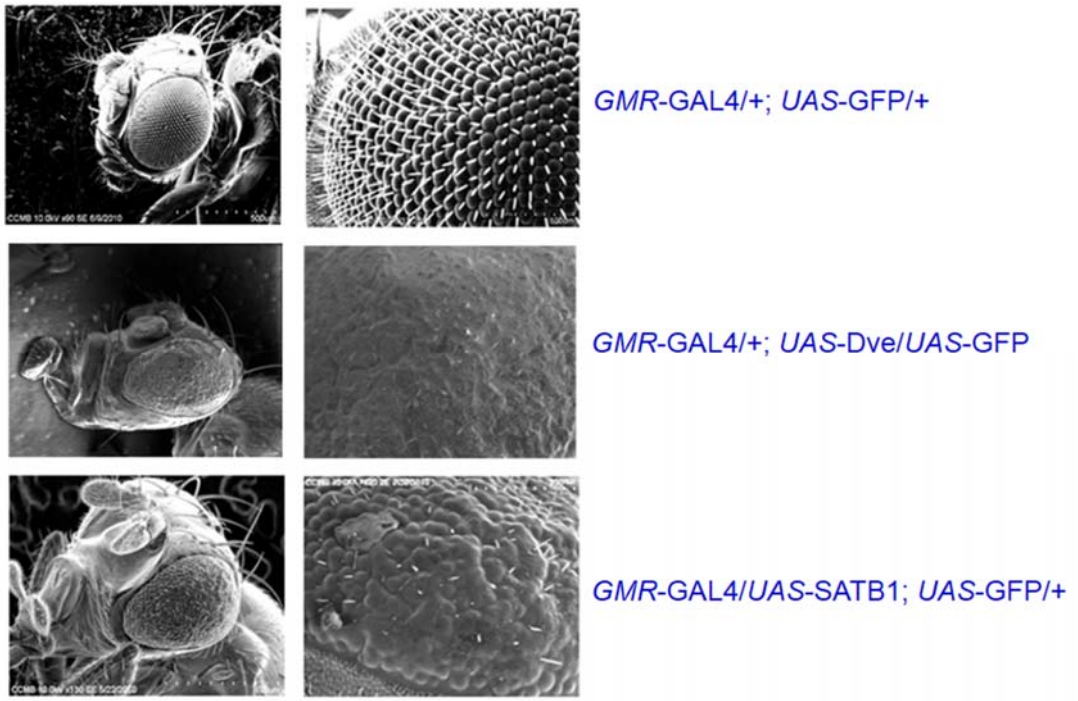

Figure 3.3.10 I SATB1 and DVE give rise to similar eye phenotypes. (Top panel) control *GMR-GAL4*/+. (**Middle panel)** Fly eyes ectopically expressing SATB1. **(Bottom panel)** Fly eyes ectopically expressing *dve*. At all stages *UAS-GFP* was used as control. Details of the experiment are provided in text above and the genotype mentioned in the figure.

3.3.11. DVE suppresses the small eye phenotype of Dsh over-expression

To verify if like SATB1, DVE can suppress the small eye phenotype, *dve* was expressed in the background of *dsh* over-expression. SEM analysis of eyes misexpressing Dsh revealed very small and glossy eyes with complete fusion of ommatidia and bristle loss (Figure 3.3.11, Panel iii vs Panel i). Ectopic expression of *dve* in the fly eye gives rise to a rough eye phenotype with very few or no bristles. When *dve* was expressed in the background of *dsh* mis-expression, all the progeny

exhibited a DVE like phenotype. These flies had larger eyes as compared to flies over-expressing Dsh, and they also had rough eyes (Figure 3.3.11, Panel iv versus Panel iii). The penetrance of this phenotype was 100%. Thus, in a manner similar to *SATB1*, *dve* suppresses the *dsh* over-expression phenotype. The disparity in penetrance can be attributed to basal level of DVE protein in the fly eye.

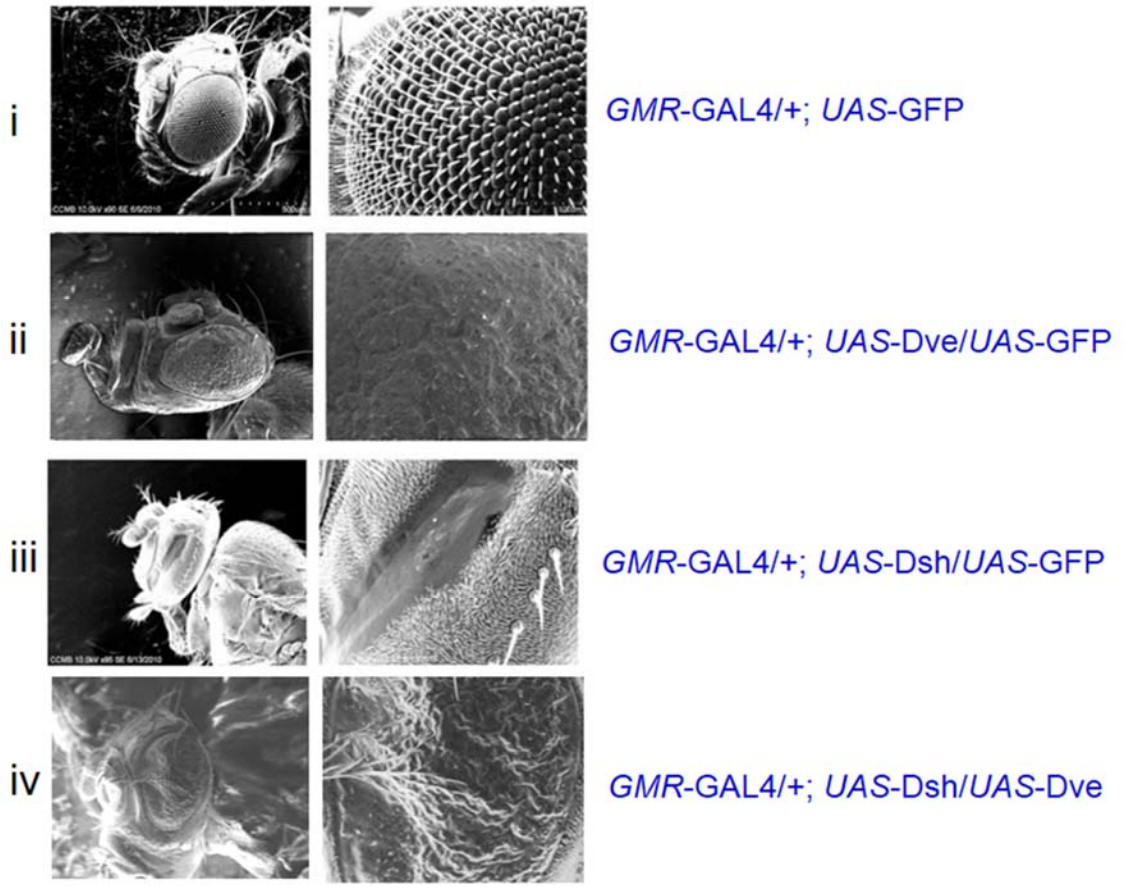

Figure 3.3.11 I DVE suppresses the small eye phenotype of Dsh over-expression. (i) control *GMR-GAL4*/+. **(ii)** Fly eyes ectopically expressing DVE. **(iii)** Fly eyes ectopically expressing Dsh. **(iv)** Fly eyes expressing DVE in the background of Dsh overexpression. At all stages *UAS-GFP* was used as control. Details of the experiment are provided in text above and the genotype mentioned in the figure.

3.3.12. Ectopic expression of DVE restricts Wg expression whereas that of SATB1 does not lead to the same effect

In the wing imaginal disc, *dve* is involved in pattern formation along the proximal distal axis (PD). In addition to its role in patterning of the PD axis, DVE is also required for proliferation of the wing pouch cells. Expression of *dve* is initiated during the early phase of the third instar larval stage, this expression is dependent on Dpp and Wg signals. In the wing imaginal disc from the latter phases of third instar larvae, DVE expressed in the entire wing pouch with the exclusion of the dorso-ventral (DV) boundary. This is achieved through the combined activity of Wg and nubbin (Kölzer et al., 2003).

In the wing disc, *wg* is expressed in the notum and along the DV boundary. When *vg-GAL4* was used to ectopically express *dve* along the DV boundary of the wing imaginal disc, the pattern of Wg staining along the DV boundary was severely disrupted. This finding correlates with previously published data (Kölzer et al., 2003). But, ectopic expression of SATB1 along the DV boundary, did not affect the staining pattern of Wg protein (Figure 3.3.12). To conclusively verify the same, it was decided to duplicate the same experiment using a different *GAL4* driver. Towards this end, it was decided to use a *GAL4* driver line which expressed along the anteroposterior (AP) boundary of the wing imaginal disc, so as to be able to properly visualize any interruptions in the expression pattern of Wg protein along the DV boundary much more easily.

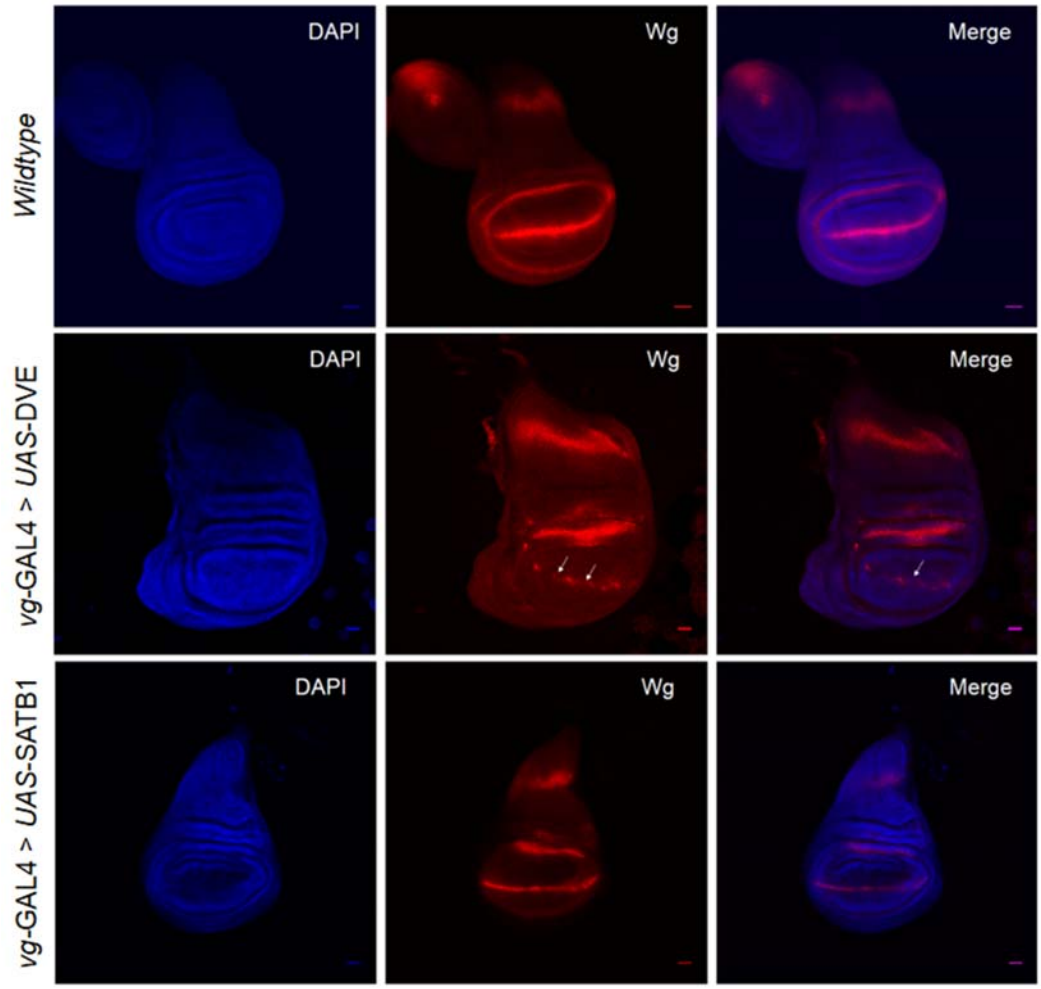

Figure 3.3.12 I DVE and SATB1 affect Wg expression differently in the wing imaginal disc. *vg-GAL4* was used to drive all expressions. Top Panel, wildtype imaginal discs, Wg (red) is expressed in the notum, the hinge, and along the DV boundary. **Middle panel,** ectopic expression of DVE along the DV boundary resulted in disrupted Wg signal (marked by white arrows). **Bottom panel,** expression of SATB1 along the DV boundary did not have any effect on the pattern of Wg staining. Images were acquired at a magnification of 25X. Scale bars represent 20 µm.

3.3.13. Ectopic expression of human SATB1 does not restrict Wg expression

Since the interruption in the Wg pattern along the dorsoventral (DV) boundary of the wing imaginal disc was imperceptible, expression of SATB1 was analyzed upon ectopic expression using *vg-GAL4* driver. It was observed that SATB1 expression was high in the hinge region as compared to the DV boundary, where *vg-GAL4* is usually expressed (Appendix A2). To circumvent this issue and to achieve a

confirmatory result, SATB1 was ectopically expressed in the wing disc using the *dpp-GAL4,* which expresses along the anteroposterior (AP) boundary of the wing disc, bisecting the dorsoventral (DV) boundary. Upon expression of SATB1 in the AP boundary, no interruption was observed in the staining pattern of Wg along the DV boundary (Figure 3.3.13). Thus, expression of SATB1 does not affect the levels of Wg protein, however it might be affecting the downstream players of the Wnt/Wg signaling cascade. Thus, even though DVE and SATB1 phenocopy each other, the molecular basis and the molecular players responsible for the same might be different.

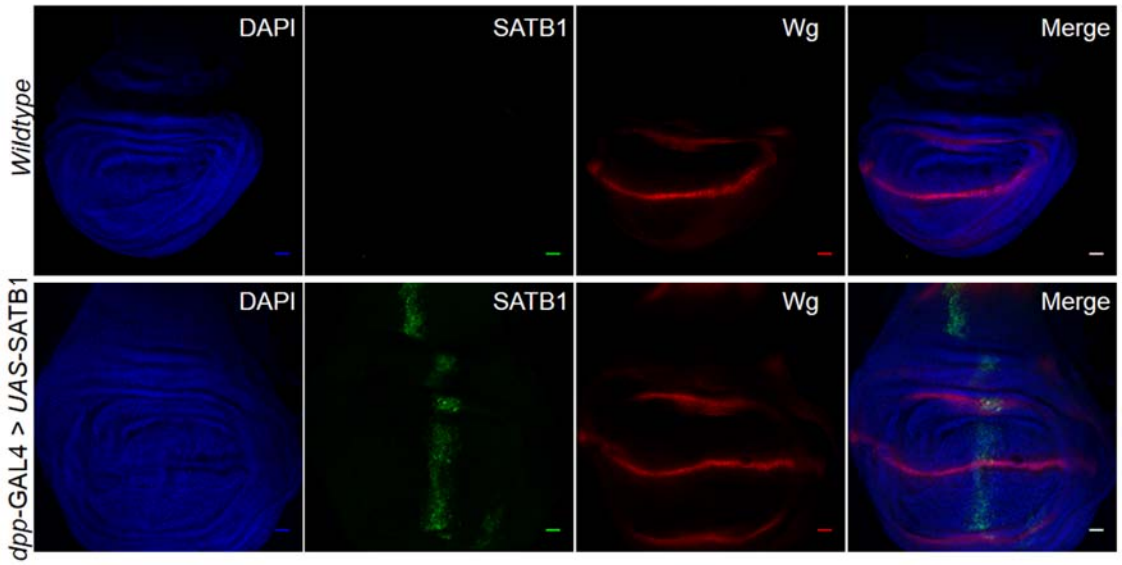

Figure 3.3.13 I Ectopic expression of SATB1 does not affect the expression pattern of Wg protein. Top panel, in the wildtype wing imaginal discs, Wg (red) is expressed along the DV boundary and SATB1 (green) is not present. **Bottom panel,** ectopic expression of SATB1 (green) along the AP boundary using *dpp-GAL4* did not have any effect on the pattern of Wg staining (red). Images were acquired at a magnification of 25X, the scale bar represents 20 µm.

3.4. Discussion

The compound eye of *Drosophila melanogaster* contains an array of hexagonally packed ommatidia or simple eyes, numbering approximately 800. Each ommatidium comprises 8 photoreceptor neurons (R cells, designated R1–R8) and a set of accessory cells comprising four non-neuronal lens-secreting cone cells seven pigment cells and a sensory bristle. This pattern develops from an undifferentiated field of pluripotent cells, the eye imaginal disc. The eight photoreceptors differentiate in a sequential order: R8, R2 and R5, R3 and R4, R1 and R6, and finally R7 (Bonini et al., 1993; Friedrich, 2003; Jang et al., 2003). The cells are not clonally related, ruling out cell lineage based determination of cell fate, but is consistent with a recruitment mechanism. The cone cells are added soon after the photoreceptors and the three classes of pigment cells in two waves later in pupal development. The establishment of cellular identity in the developing eye imaginal disc occurs through a series signals from neighboring cells to undifferentiated cells, inducing them towards a specific fate (Freeman, 1996). The typical organized structure of an ommatidium results from spatially restricted cell death depending on the balance between epidermal growth factor receptor (EGFR) and Notch signaling (Brachmann and Cagan, 2003; Doroquez and Rebay, 2006). *Drosophila* eye development is a well-documented process which proceeds in a stepwise manner wherein cells within the eye imaginal disc are specified, recruited and differentiate in a sequential order contributing to the highly precise structure of the adult fly eye. The development of the fly eye is amenable to classical and molecular genetic analysis and the phenotypes are easily identifiable. Hence, the *Drosophila* eye is a useful model to screen for phenotypic consequences, caused as a result of loss-of-function mutations or overexpression of a particular gene. Perturbations in signaling pathways involved in the process of eye development can lead to abnormal eye development. Studies over the past decade have utilized this system to gain insights into the cellular and molecular strategies regulating development.

Rough eye phenotype is a commonly observed phenotype wherein the regular arrangement of the ommatidia gets disrupted, this phenotype is generated in response to any defect in cell-fate determination. A rough eye phenotype is expressed if the normal order of cell recruitment for assembling photoreceptor is disrupted. The severity of this defect is reflected in the number of ommatidia affected. The *GMR-GAL4* driver mainly expresses in post-mitotic cells undergoing differentiation in the eye imaginal disc. Genes that participate in this aspect of development will lead to generation of a rough eye phenotype when misexpressed using this driver. Heterologous expression of the human genomic organizer SATB1 in *Drosophila* under the control of *GMR-GAL4* driver led to generation of a rough eye phenotype wherein the ommatidia are fused and the bristles are improperly organized. On the other hand, ectopic expression of individual domains of SATB1 like SATB1 (1-204) and SATB1 (255-763) (which lack the DNA binding domain and the N-terminal PDZ-like domain, respectively) give rise to mild phenotypes like fused ommatidia restricted to certain regions or mildly disrupted bristle positioning respectively. The difference in phenotypes between SATB1 and its deletion mutant SATB1 (1-204) cannot be attributed to their different sub-cellular localization as it is known that the SATB1 nuclear localization signal (NLS) resides in the N-terminal region and therefore, SATB1 (1-204) would also localize inside the nucleus. Also, there is a marked difference in the penetrance of various phenotypes, this may be attributed to the position effect which comes into play as a result of the exogenous DNA getting integrated at various positions in the *Drosophila* genome.

Experiments performed in cell lines pointed towards a cross-talk between the Wnt/Wg signaling pathway and SATB1. To validate the same in an in vivo scenario, SATB1 was overexpressed in the Wnt overexpression background in *Drosophila*. To mimic Wnt activation in flies, *UAS-dsh* or *UASarm.S10* flies were used both of which activate of Wnt signaling. Ectopic expression of Wnt pathway intermediaries like dsh or arm in the fly eye leads to generation of a small eye phenotype where all the ommatidia have completely fused together to give rise to a glassy eye having loss of bristles. Ectopic expression of SATB1 in the background of Wnt activation

(ectopic expression of Dsh/constitutive active Arm) resulted in partial suppression of the small eye phenotype, but majority of the flies had small eyes. The plausible reasons for suppression of the small eye phenotype by SATB1 maybe as follows, a) Since, flies lack endogenous SATB1, ectopic expression may lead to sequestration of various proteins a subset of which might be involved in the Wnt signaling pathway, thereby causing downregulation of Wnt signaling; b) Many molecules mediating or having a cross-talk with Wnt signaling, present in fly might be absent in higher organisms therefore, when SATB1 is expressed in such a scenario novel interactions might be generated; c) The physiological concentration of SATB1 upon expression might be so high that the result is skewed to give out such an output; d) Expression of SATB1 in the fly system might be activating genes which negatively regulate the Wnt/Wg pathway. But, at no point can we conclude with absolute certainty if SATB1 is indeed acting downstream or upstream to the Wnt/Wg pathway, on the basis of the current data.

Thus, suppression of the Wnt activation phenotype fly experiments points towards a negative correlation between SATB1 and the Wnt/Wg pathway, which is diametrically opposite to what was observed in the mammalian cell-line system. To identify if ectopic expression of SATB1 has any effect on Wnt regulated genes in the fly, quantitative PCRs were performed using eye imaginal discs to estimate transcript levels of Wnt responsive genes, and Wnt regulator genes upon ectopic expression of SATB1. It was observed that SATB1 upregulated the antagonists of the Wnt/Wg pathway, such as *nkd*, *dally* and *apc-2* while no significant effect was observed on positive regulators like *dsh*. This is interesting as, in the mammalian system, over-expression of SATB1 is always associated with upregulation of the *dsh* transcript. Thus, in the fly system, SATB1 negatively regulates the Wnt/Wg pathway by activating the transcription of the antagonists of the pathway. But, a caveat of conducting a tissue level analysis would be inherent complexity of the sample. Therefore, this may not provide us with an accurate and exact depiction of the status of gene expression. Presence of consensus SATB1 binding motifs in the promoter regions of these genes was established by performing MEME analysis, but in vivo binding analyses are yet to be conducted.

In order to establish that, the negative regulation of Wnt/Wg pathway by the SATB family proteins in the fly system is a conserved phenomenon, the paradigm of ectopic expression of *defective proventriculus* (*dve*) a putative fly homolog of SATB1 was used. Study of a putative ortholog of SATB1 in flies would indicate if the phenotype observed upon SATB1 expression is a genuine read-out. On the basis of in-silico analysis, *dve* was found to be a structural homolog of SATB1, but until now, functional homology has not been established. The homeobox containing gene defective proventriculus (dve) is expressed in various tissues including the head primordium and functions as a transcription factor. Notch target genes, wg, *cut* (*ct*), and *dap-2*, are repressed by DVE at the dorso-ventral and segment boundaries of wing and leg discs, respectively (Kölzer et al., 2003; Nakagoshi et al., 2002; Shirai et al., 2007). In photoreceptor (PR) cells, DVE is expressed in R1–R6 and yR7 thereby repressing rhodopsin 3 (Johnston et al., 2011; Yorimitsu et al., 2011). Loss of *dve* results in de-repression of Rhodopsins in outer PRs, and leads to a wide distribution of expression levels.

DVE protein was identified as being necessary for midgut development, homozygous mutants die in first instar stage of larval development due to problems in digesting food. In dve mutants, two distinct parts of the midgut, the proventriculus and middle midgut, are abnormally organized. The Wg signaling regulates dve expression during proventriculus development. On the other hand, in the middle midgut, dve is a downstream target of Dpp and defines the functional specificity of copper cells along with another Dpp target gene, labial. Thus, the dve gene acts under the two distinct extracellular signals at distant parts of the midgut primordia (Nakagoshi et al., 1998). Defective proventriculus contains an N-terminal domain homologous to the N-terminal region of SATB1 and two homeodomains, like SATB1 it has propensity to bind A/T rich regions of the genome (Nakagoshi et al., 1998). Experiments were performed to analyze if flies ectopically expressing *dve* exhibit phenotypes similar to ones observed upon SATB1 expression. Similar to mammalian SATB1, ectopic expression of fly *dve* resulted in a rough eye phenotype, though intensity of this phenotype was stronger than that observed upon expression of SATB1. Expression of DVE in the background of ectopic

expression of Dishevelled resulted in all the progeny having a DVE misexpression phenotype (rough eye). At molecular level, DVE expression resulted in restricting Wingless (Wg) expression to the wing imaginal disc, however SATB1 expression did not yield such effect. This was established by using two separate wing specific GAL4 drivers, *vg-GAL4* and *dpp-GAL4.* Therefore, even though DVE and SATB1 give rise to similar phenotypes, they might have different functions. Incidentally, the *C. elegans dve* (*dve-1*), is known to function in a manner similar to human SATB1 in determining lifespan (Zhang et al., 2009). Therefore, we hypothesize that *Drosophila* DVE might be an ancestral molecule which evolved to give rise to SATB1 in the vertebrates. This is hypothesis is backed by studies by Burglin and Cassata which proposed that SATB1 and DVE both belong to the COMPASS/CUT family of proteins. The initial COMPASS/CUT gene gave rise to the SATB family genes in vertebrates. They mention that it is unclear if SATB family genes were gained by the vertebrate lineages or lost from other phyla (Burglin and Cassata, 2002). It would be interesting to study if DVE is indeed an ancestral form of SATB1. An added point of interest would be to identify if there is an overlap in the consensus binding sites of these two proteins. Analysis of transformation of SATB1/DVE along the evolutionary landscape would prove to be an interesting case study in itself.

3.5. References

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Perspectives

Special AT-rich binding protein 1 (SATB1) is a global chromatin organizer and transcription factor that links higher-order chromatin architecture to gene regulation. In addition to its key role in gene regulation, it is also known to interact genetically/physically with components of multiple signaling pathways. One of these pathways is the Wnt/Wg signaling pathway. The role played by SATB1 in the Wnt pathway in higher organisms is well cataloged, and is further attested by the positive co-relation between SATB1 levels and aggressiveness of multiple types of cancers. Studies from the Galande laboratory have shown that SATB1 interacts with β-catenin - the final effector molecule of the Wnt/Wg signaling pathway, and is known to compete with TCF7L2 for binding with β-catenin, thereby modulating the final outcome of the Wnt/Wg cascade. However, it is still unclear whether the recruitment of β-catenin id the sole role of SATB1 in the Wnt/Wg pathway. Here, evidence is presented that, in mammalian cells, SATB1 also interacts physically and functionally with Dishevelled (*Dvl/dsh*), an upstream component of the Wnt/Wg pathway; a known Wnt mimetic. Experiments using cell-lines revealed that SATB1 upregulates positive regulators of Wnt/Wg signaling (*dsh*), Wnt responsive genes (*fos, jun*) and downregulates the negative regulators of the Wnt/Wg pathway (*dkk, nkd*). Thus, collectively these results demonstrate SATB1 is a positive regulator of the Wnt/Wg pathway. However, in mouse thymocytes it was shown that genes that are typically repressed by SATB1 are upregulated upon Wnt activation. Therefore, it was important to understand whether regulation of Wnt/Wg pathway by SATB1 is a context-dependent phenomenon. We chose to introduce SATB1 in a model system that lacks endogenous SATB1 but has a well-characterized Wnt/Wg pathway in place. To address this question, the strategy of ectopic expression of mammalian SATB1 and its deletion constructs was employed. The insect Drosophila not only has a well-characterized Wnt/Wg cascade, it also expresses conserved homologs of various known SATB1 interacting factors such as Arm and Dsh, as well as most of the components of the chromatin machinery. For the current

study development of the Drosophila eye and wing were used as model systems. Ectopic expression of mammalian SATB1 in the fly eye results in a rough eye phenotype characterized by incomplete ommatidial fusion. Immunostaining of polytene chromosomes in salivary glands ectopically expressing SATB1 established that mammalian SATB1 binds to Drosophila chromatin. Therefore, the phenotypes observed upon SATB1 expression cannot simply be attributed to sequestration of endogenous fly proteins by over-expressed SATB1. Activation of the Wingless signaling pathway is known to re-specify eye cells into a variety of fates, most notably the head cuticle. Therefore, over-expression of the Wnt/Wg intermediaries, viz. Dishevelled and constitutively active Armadillo in the developing eye results in 'small eye' phenotype. Results from this study revealed that mammalian SATB1 suppresses this 'small eye phenotype' associated with Wnt/Wg activation in flies. Had SATB1 been a positive regulator of the Wnt/Wg pathway in this system, it should have exacerbated the small eye phenotype instead of suppressing it. These results suggest that as opposed to the mammalian system, SATB1 is a negative regulator of the Wnt/Wg pathway in the fly system. To dissect this further, the transcript levels of various Wnt/Wg pathway related genes were analyzed upon ectopic expression of SATB1. It was observed that in the fly system ectopic expression of SATB1 resulted in activation of the negative regulators of the Wnt/Wg pathway such as *nkd*, *apc-2* and *dally*. Using in silico analysis, SATB1-binding sites were identified in the upstream regions of these genes. However, surprisingly, no appreciable change was observed in the transcript levels of the positive regulator- *dishevelled*, whose transcript levels increase dramatically in response to SATB1 over-expression in cell-lines. Therefore, we argue that regulation by SATB1 is a context-dependent phenomenon. Next, we asked the question if any fly protein similar to SATB1 would result in similar phenotypes. As mentioned above, *Drosophila* does not express an obvious homolog of human SATB1. However, it was reported that Defective proventriculus (DVE), a protein involved in the development of proventriculus is a protein bearing most resemblance to vertebrate SATB1, based on phylogenetic analysis of protein structure. Further, based on *in-silico* analysis, among the fly proteins, DVE is

closest to mammalian SATB1 in terms of its domain architecture. However, until now functional similarity between these two evolutionarily distant proteins had not been established. Towards this end, we tested whether flies expressing SATB1 or DVE in their eyes exhibit similar phenotypes. Interestingly, over-expression of DVE in the developing fly eye phenocopied mammalian SATB1. However, where ectopic expression of SATB1 in the background of Wnt activation resulted in suppression of the Wnt activation phenotype, ectopic expression of DVE in this background resulted in complete phenotypic rescue. Thus, where DVE was able to limit expression of WG in the wing imaginal disc, SATB1 failed to do so. These results suggested that at gross phenotypic level these proteins might behave similarly but at the molecular level they might exhibit some non-overlapping functions. These functional differences can perhaps be attributed to differences in their domain architecture. While DVE harbors an N-terminal PDZ-like domain similar to SATB1, it lacks the MAR-binding domain containing Cut repeats and a Homeodomain and instead harbors two homeodomains. It is known that *Drosophila* DVE is unable to bind to the nuclear matrix whereas MAR-binding activity is a hallmark of the mammalian SATB1. These observations prompt us to hypothesize that DVE might be an ancestral molecule which evolved to give rise to SATB1 in the vertebrates.

Key Findings:

- SATB1 physically and functionally interacts with Dishevelled - the upstream effector of the Wnt/Wg pathway.
- SATB1 is a positive regulator of the Wnt/Wg pathway in cell-lines but a negative regulator in the fly context. Therefore, regulation of Wnt/Wg pathway by SATB1 is context-dependent.
- Mammalian SATB1 can bind Drosophila chromatin and multiple consensus SATB1-binding sites occur in the fly genome.
- Ectopic expression of mammalian SATB1 activates transcription of the Wnt antagonists *nkd, apc-2* and *dally* in the fly system.

- Defective Proventriculus (DVE), the fly protein closest to SATB1 in terms of domain architecture, phenocopies SATB1 but exhibits different functions at molecular level.

Open Questions:

- Whether Dve really represents the ancestral homolog of Human SATB1? How the evolution of SATB-like molecules must have progressed in intermediate organisms such as echinoderms, hemichordates and chordates?

- Since multiple SATB1-binding sites occur in the fly genome, is there any fly DNA-binding protein (e.g. DVE) that specifically binds to them and regulates downstream genes?

- Does the interactome of Dve in the fly have any overlap with that of SATB1 in the mammalian system? The similarities and differences in the interacting partners of these two proteins might explain the differences in their effects at phenotypic and molecular level. This might shed light on the context-specific effects.

- Does DVE interact with ARM and regulate Wnt/Wg pathway targets?

- Does the difference in domain architecture (presence or absence of a MAR-binding domain) have any bearing on the difference in functions of SATB1 and DVE? Is the MAR-binding property such a crucial feature so as to impart drastic differences in the molecular functioning of these proteins?

APPENDIX

APPENDIX A1

Validation of SATB1 expression in the *Drosophila* wing imaginal disc

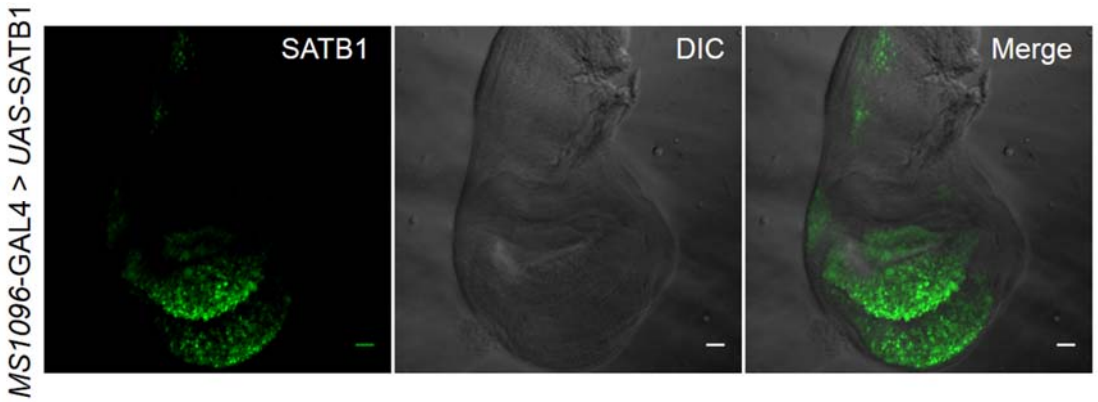

Figure A1 I Validation of SATB1 expression. Ectopically expressed human SATB1 (green) under the control of *MS1096-GAL4* driver is localized in the wing pouch and some areas in the notum. These are the regions where *MS1096-GAL4* is known to express. Image was acquired at 25X magnification; the scale bars represent 20 µm. Immunostaining was performed as per the protocols described in chapter 2 and chapter3 of this thesis.

APPENDIX A2

Expression of SATB1 using *vg-GAL4* driver and its effect on pattern of Wg protein expression

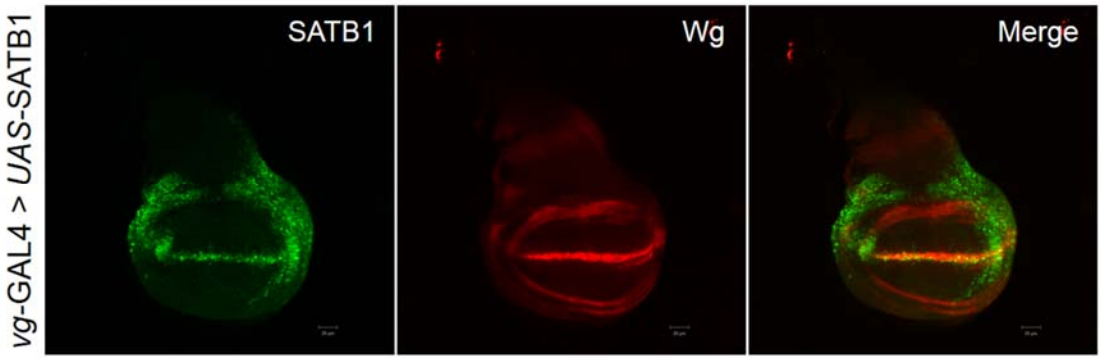

Figure A2 I Pattern of ectopic expression of human SATB1 in wing discs. Ectopically expressed human SATB1 (green) under the control of *vg-GAL4* driver is localized along the DV boundary, but majority of the expression is along the margins of the disc and the hinge region. The level of SATB1 expression along the DV boundary is less in comparison to the expression in the hinge region. Wg (red) is expressed predominantly along the DV boundary hinge and notum. Expression of human SATB1 along the DV boundary has no effect on the level of Wg protein. Images were acquired at 25X magnification; the scale bars represent 20 µm. Immunostaining was performed as per the protocols described in chapter2 and chapter3 of this thesis.

APPENDIX A3

Immunostaining for the Matrix binding protein BEAF in polytene spreads- a positive control for SATB1 staining

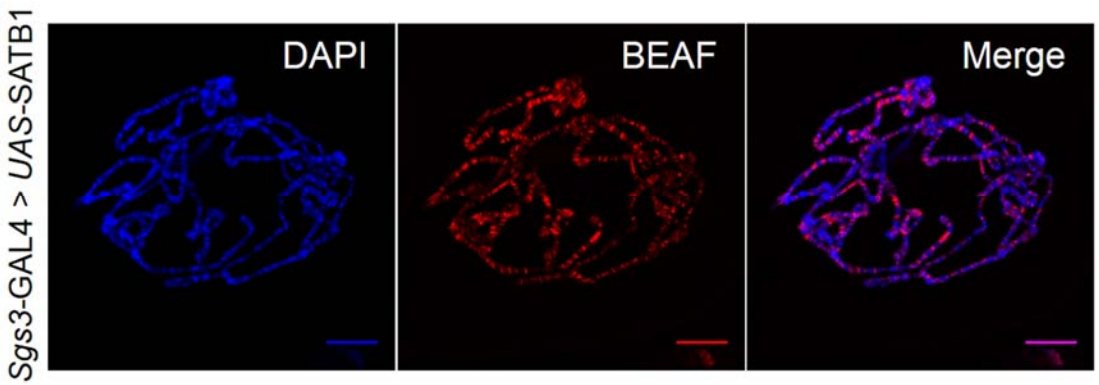

Figure A3 I Staining for the *Drosophila* Matrix binding protein BEAF. Polytene spreads were prepared from salivary glands of third instar larvae expressing human SATB1 under the control of *Sgs3-GAL4*. Staining for BEAF, a Boundary associated MAR binding protein was used as a positive control for SATB1 immunostaining (described in chapter2 Figure 2.3.13). Image was acquired at 63X magnification; the scale bar is equivalent to 20 µm.

APPENDIX B

SATB1 physically interacts with CASK

B1 Full-length CASK interacts with the N-terminal PDZ-like domain of SATB1 in vitro

Bead bound GST-tagged truncations of SATB1 were prepared and separated by gel electrophoresis and the amount of protein normalized by densitometry (Figure B1, panel D). [35S]-methionine labeled full-length CASK was synthesized by in vitro translation (Figure B1, panel E), 50μl of this labeled product was incubated with each truncation. Bead bound complex was washed and eluted using serially increasing salt concentrations viz. 400mM, 800mM and 1M respectively. Eluents were separated on SDS-PAGE and the gel dried and exposed. A band corresponding to the molecular weight of CASK was observed in almost all truncations upon elution with PBS containing 400mM NaCl (Figure B1, panel A). But, as the elutions proceeded towards more stringent conditions viz. elutions with PBS containing 800mM and 1M NaCl respectively eluates showed lowering amounts of SATB1 (255-763) viz. s255 and increasing amounts of SATB1 (1-254) (Figure B1, panels B and C versus panel A), Sharper and more intense bands were observed in case of GST 1-254 truncation as one proceeded towards more stringent conditions. Faint bands corresponding to the molecular weight of CASK were observed in GST 90-204 and GST S255 truncations. These results show that affinity of interaction of CASK is more for the (1-254) aa region of SATB1 which happens to represent the N-terminal PDZ-like domain but, the C-terminal DNA binding (255-763) region also plays a role in governing this interaction. Perhaps, in an in vivo scenario, both of these domains are involved in mediating interaction of SATB1 with full length CASK.

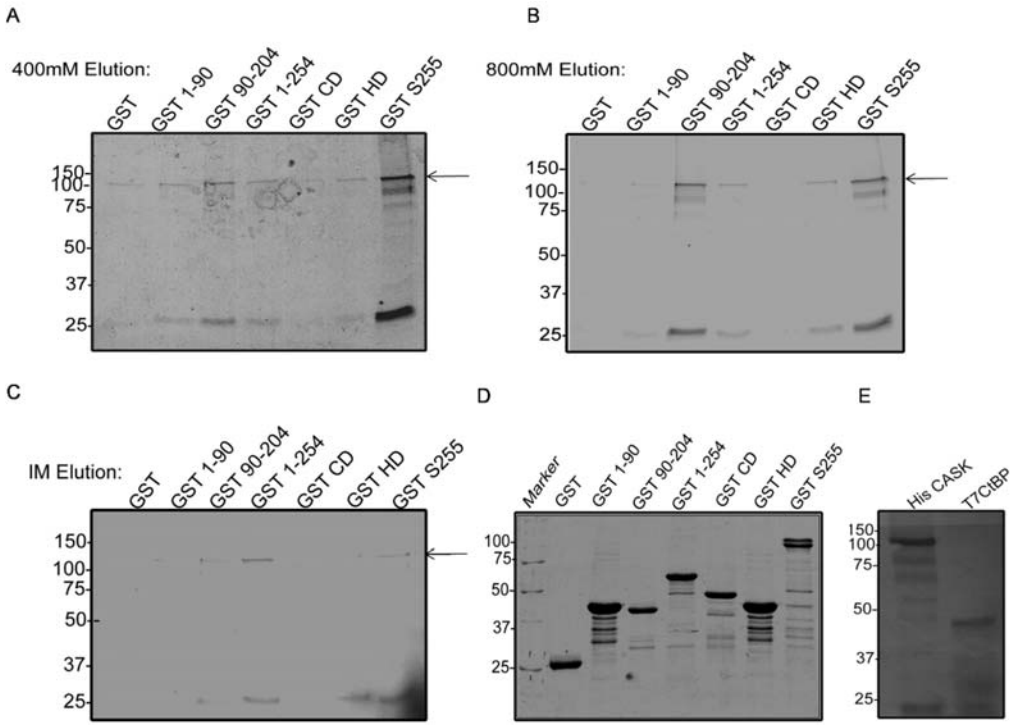

Figure B1 I Binding of full-length CASK to N-terminal of SATB1 (1-254) is highly specific. Forty μl of each eluent was run on a 10% gel and the gel exposed to X-ray film. The autoradiographs were developed 8 h post exposure. (A) Elutions using 400 mM NaCl, band corresponding to full length CASK is observed in all deletion constructs, (B) Elutions using 800mM NaCl, a band corresponding to full-length CASK is observed in case of GST 90-204 and GST-S255, (C) Elutions using 1M NaCl, a band corresponding to full-length CASK is observed in GST 1-254 even upon eluting with such a high salt concentration, (D) Bead bound GST tagged truncations of SATB1 protein run on a 12.5% reducing gel, stained with Coomassie Brilliant Blue R-250, (E) [35S]methionine labeled full-length CASK separated on a 7.5% gel, [35S]methionine labeled T7CtBP was used as a positive control, 4 μl of each labeled protein was used for monitoring quality.

B2 CASK interacts with the PDZ-like domain of SATB1 in vitro

Full-length 6X His-tagged CASK was purified and the imidazole removed by using PD-10 columns followed by elution in 20 mM Tris-HCl pH 7.4 containing 50 mM NaCl. Equal amounts of bead bound GST 1-254 and GST S255 (SATB1 (255-763)) were incubated with 250 μl of purified CASK, bead bound GST was used as control. After the completion of incubation beads were washed and the proteins eluted using 10 mM reduced glutathione prepared in 50 mM Tris-HCl pH 8.0 containing 150 mM NaCl. Immunoblotting was performed using anti-CASK

antibody. A band corresponding to that of full-length CASK was observed in case of both GST 1-254, but the band for CASK in case of GST-S255 was very faint as compared to the band in case of GST 1-254, indicative of a stronger existing interaction between the N-terminal PDZ-like domain of SATB1 and the full length CASK protein (Figure B2, panel B). This implies that in an in vivo scenario, SATB1 is capable of sequestering the CASK protein.

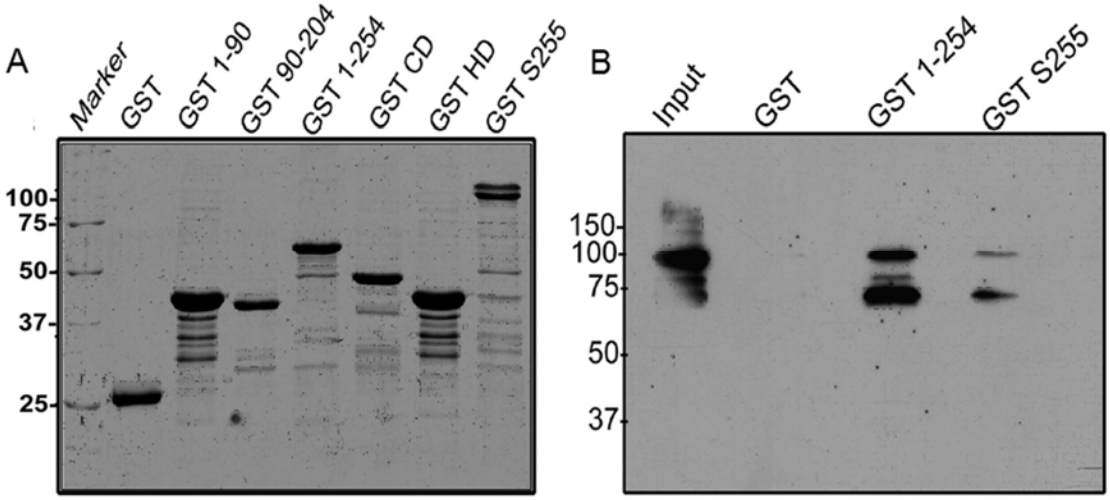

Figure B2 I GST-Pull downs using purified full-length CASK: (A) Gel picture depicting the quality of affinity purified recombinant proteins used for pull-down, (B) After incubating purified full length CASK with the bead bound GST truncations, beads were washed and eluted. Eluents were run on a 7.5% reducing gel, 10 μl of purified CASK was loaded as input. Western blotting was performed using anti-CASK antibody, band corresponding to that of full-length CASK is observed in case of both GST 1-254 and GST S255, but the band for CASK in case of GST S255 is very faint as compared to the band for CASK in case of GST 1-254, no band was observed in GST control.

B3 CASK associates with SATB1 in vivo

Brain lysate was prepared in RIPA Buffer, estimated by Bradford assay, 600 μg of this lysate was used for the purpose of co-immunoprecipitation. After pre-clearing the supernatant was distributed into three tubes. One μg each antibody viz. Rabbit IgG, anti-SATB1 (S255) antibody (generated in house) and anti-CASK antibody were added into each tube and incubated. Antibody bound protein complexes were pulled-down using protein A/G beads. Beads were washed thrice to remove any

non-specific binding and then eluted in 2X reducing Laemmeli buffer. These eluents were loaded onto a 10% reducing gel and then transferred onto a PVDF membrane. Immunoblotting was performed using anti-CASK antibody to check if anti-SATB1 antibody had pulled-down complex of CASK along with SATB1. A band corresponding to that of the molecular weight of CASK was observed upon pull down withanti-SATB1 antibody, indicating that CASK and SATB1 are the part of the same complex in vivo. However, a faint band for CASK was also repeatedly observed in case of IgG, this was therefore treated as background. A strong band corresponding to the molecular weight of CASK was observed in the positive control where the pull down was performed using anti- CASK antibody and probed with the same (Figure B3).

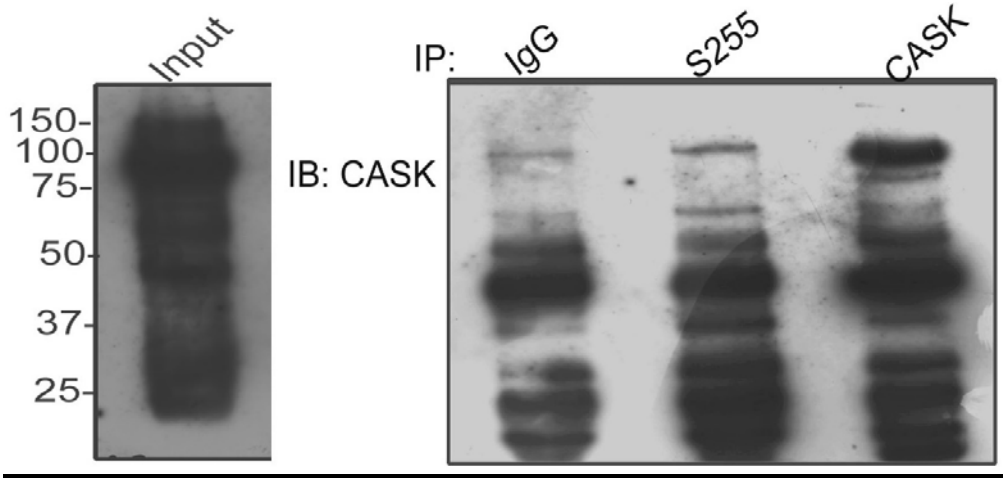

Figure B3 I Co-immunoprecipitation assay to check for the interaction of SATB1 and CASK in brain lysate. Pull-down was performed using SATB1 antibody (S255). Protein/Antibody complex was eluted in Laemmeli Buffer, eluents were resolved on a 10% reducing gel, immunoblotting was performed using anti-CASK antibody. A band corresponding to the molecular weight of CASK is observed in case of pull-down using anti-SATB1 antibody (S255). Pull-down using CASK antibody was used as a positive control.

APPENDIX C

List of oligonucleotides used for quantitative RT-PCRs described in Section 2.3.4.

Description	Sequence	Nucleotides	%G-C	Tm	Expected size (bp)
h SATB1 EI-J F	GCTAGGAAGAGGAAGGCTTG	20 bp	55	61.8	
h SATB1 EI-J R	CACAGAAAACTGGCAGCATG	20 bp	50	61.9	119
h Dvl1 EI-J F	GCTACTTCACCGTCCCAC	18 bp	61.6	61.6	
h Dvl1 EI-J R	GCCTCTTCCAGCTCGTAG	18 bp	61.1	61.1	112
h Dvl3 EI-J F	ATCCGCCATACCGTCAAC	18 bp	55.6	61.6	
h Dvl3 EI-J R	CACTGGAGCCATCGTGATC	19 bp	57.9	62	112
h Dvl2 EI-J F	AAGTCTATGGATCAGGATTTCGG	23 bp	43.5	61.9	
h Dvl2 EI-J R	GGATTATCTGAGGACACCAGC	21 bp	52.4	61.8	113

List of oligonucleotides used for quantitative RT-PCRs in described Section 2.3.5.

Description	Sequence	Nucleotides	%G-C	Tm	Expected size (bp)
h Jun EI J F	GACCTTCTATGACGATGCCC	20 bp	55	61.8	
h Jun EI J R	AGGGTCATGCTCTGTTTCAG	20 bp	50	61.9	99
h c-myc EI-J F	TTCGGGTAGTGGAAAACCAG	20 bp	50	61.8	
h c-myc EI-J R	AGTAGAAATACGGCTGCACC	20 bp	50	61.9	108
h TCF7L2 EI-J F	CGACAGACTTTATGGTGCAAAC	22 bp	45.5	59.5	
h TCF7L2 EI-J R	GAGGCGAATCTAGTAAGCTTCC	22 bp	50	59.7	118
h CTBP1 F	GGGACTGCACAGTGGAGATG	20 bp	60	60	
h CTBP1 R	GTCCTCCCTGGTGAGAGTGA	20 bp	60	60	149
h CCND1 F	GCTCCTGTGCTGCGAAGTG	19 bp	63.2	60.9	
h CCND1 R	CATTTGAAGTAGGACACCGAGG	22 bp	50	60.9	129
h Fra1 EI-J F	CAAGTGCAGGAACCGGAG	18 bp	61.1	62.2	
h Fra1 EI-J R	TGCAGCCCAGATTTCTCATC	20 bp	50	62.3	84

*The prefix `h' in front of the gene name indicates that the gene is of human origin

*EI-J denotes that the primer pair is designed against exon-intron junction

List of oligonucleotides used for quantitative RT-PCRs described in Section 2.3.6.

Description	Sequence	Nucleotides	%G-C	Tm	Expected size (bp)
h Dkk1 EI-J F	TGCAAATCTGTCTCGCCTG	19 bp	52.6	62.4	
h Dkk1 EI-J R	AGCTTTCAGTGATGGTTTCCTC	22 bp	45.5	62.7	150
h Nkd2 EI-J F	AGGTTGTCTGCACACGTC	18 bp	55.6	61.9	
h Nkd2 EI-J R	GTAGTTCTCAATCCCGGCG	19 bp	57.9	62	129
h Axin2 EI-J F	GTGTCTCTACCTCATTTCCCG	21 bp	52.4	61.7	
h Axin2 EI-J R	CTTTTCCAGCCTCGAGATCAG	21 bp	52.4	62.2	102
h SFRP4 EI-J F	TGACTGTAAACGCCTAAGCC	20 bp	50	62.1	
h SFRP4 EI-J R	GCACAGCTTTTATTTTGGCATG	22 bp	40.9	61.6	116

List of oligonucleotides used for quantitative RT-PCRs described in Section 2.3.7.

Description	Sequence	Nucleotides	%G-C	Tm	Expected size (bp)
h Bcl2 F	GACAACATCGCCCTGTGGATG	21 bp	57.1	61.6	
h Bcl2 R	TCTTCAGAGACAGCCAGGAGA	21 bp	52.3	59.95	145
h ERBB2 F	AGTGTGAACCAGAAGGCCAA	20 bp	50	60.7	
h ERBB2 R	TCTGAATGGGTCGCTTTTGTTC	22 bp	45	63	300
h CHUK F	CCGGTCCCTTGTAGGATCCAGTC	23 bp	60	60	
h CHUK R	GGGGACAGTGAACAAGTGACAACTC	25 bp	52	60	270

List of oligonucleotides for internal controls in quantitative RT-PCRs in Sections 2.3.5. - 2.3.7.

Description	Sequence	Nucleotides	%G-C	Tm	Expected size (bp)
h 18s RNA F	AACGGCTACCACATCCAAG	19 bp	52.6	61.9	
h 18s RNA R	ATTCCAATTACAGGGCCTCG	20 bp	50	62	115
h actin EI-J F	GTCTTCCCCTCCATCGTG	18 bp	61.1	61.1	
h actin EI-J R	GTACTTCAGGGTGAGGATGC	20 bp	55	61.7	120

*The prefix `h' in front of the gene name indicates that the gene is of human origin

*EI-J denotes that the primer pair is designed against exon-intron junction

List of oligonucleotides used for quantitative RT-PCRs described in Section 3.3.9.

Description	Sequence	Nucleotides	%G-C	Tm	Expected size (bp)
dRp49 F RpL32 PB (CDS)	GCTAAGCTGTCGCACAAATG	20 bp	50	59.64	
dRp49 R RpL32 PB (CDS)	GTTCGATCCGTAACCGATGT	20 bp	50	59.82	105 bp
d apc1 F	CAATTGTGGACCTTAGAACATCAG	24 bp	41.7	60.6	
d apc1 R	GAGACATAGGCTGGTTTCTTGC	22 bp	50	60.8	100 bp
d apc2 F	CAAGAACAGATTCCACGTGGAG	22 bp	50	61.4	
d apc2 R	GCATTAGAGTCGTCCATCATGC	22 bp	50	61.5	90 bp
d Nemo F	GATCCGGATAAGCGCATCTC	20 bp	55	61.4	
d Nemo R	GTGAAGCAGCATTTGCACATG	21 bp	47.6	61.4	101 bp
d Stripe F	TGCACCACCAATTCATCACTC	21 bp	47.6	60.1	
d Stripe R	AGTTGCTGAACCTGCAGTATGTC	23 bp	47.8	60.7	108 bp
d Nkd F	GGAAGTGGAACAAAGGCGAC	20 bp	57.9	60.2	
d Nkd R	GGCGTATTCTGTTCCGTGC	19 bp	50	59.5	113 bp
d Dally F	TCTCGTGACGATCACTCCTG	20 bp	55	59.98	
d Dally F	CGGGATTATATCGCTGGCTA	20 bp	50	60.04	97 bp
d Axin F	CGACTACATTCGAACGAGCAC	21 bp	52.4	60	
d Axin R	GCTCTTTCCTCCAGGTCTCG	20 bp	60	60.4	107 bp
d Arm F	ACGAGGAGATGGAGGGAGAT	20 bp	55	60.03	
d Arm R	CGTCCACTTGGTCTTGTGTG	20 bp	55	60.19	81 bp
d Dsh F	GGTGCTGAACAAGCAGAACA	20 bp	50	60.1	
d Dsh R	ATTGAAGCAGGGCAGTATGG	20 bp	50	60.03	115 bp

*The prefix `d' in front of the gene name indicates that the gene is of *Drosophila* origin

LIST OF PUBLICATIONS

- Notani, D., **Ramanujam, P.L.**, Kumar, P.P., Gottimukkala, K.P., Kumar-Sinha, C., and Galande, S. (2011). N-terminal PDZ-like domain of chromatin organizer SATB1 contributes towards its function as transcription regulator. Journal of Biosciences *36*, 461-469.

- Jayani, R.S., **Ramanujam, P.L.**, and Galande, S. (2010). Studying histone modifications and their genomic functions by employing chromatin immunoprecipitation and immunoblotting**.** Methods in Cell Biology *98*, 35-56.