Role and Regulation of SATB1 in Colorectal Tumorigenesis and Progression

A Thesis

Submitted in partial fulfilment of requirements

Of the degree of

Doctor of Philosophy

By

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Dedicated to

My Parents for their love, encouragement, belief and support

CERTIFICATE

Certified that the work incorporated in the thesis entitled "**Role and Regulation of SATB1 in colorectal tumorigenesis and progression**" submitted by **Rafeeq Ahmad Mir** 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.

Sanjeev Galande
(Supervisor)

Date:

DECLARATION

I declare that this written submission represents my ideas in my own words and where others ideas have been included; I have adequately cited and referenced the original sources. I also declare that I have adhered to all principles of academic honesty and integrity and have not misrepresented or fabricated or falsified any idea/data/fact/source in my submission. I understand that violation of the above will be cause for disciplinary action by the Institute and can also evoke penal action from the sources which have thus not been properly cited or from whom proper permission has not been taken when needed.

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Abbreviations

APC	Adenomatous polyposis coli
β-TrCP	Beta-Transducin repeat Containing E3 ubiquitin Protein ligase
bp	base-pair
BRCA1	Breast cancer 1, early onset
BRCA2	Breast cancer 2, early onset
BURs	base unpairing regions
CD-	Clusters of Differentiation
CD	Cut repeat-containing domain
CDKN2A	Cyclin-Dependent Kinase Inhibitor 2A
ChIP	Chromatin immunoprecipitation
CSKα1	Casein kinase alpha 1
DACT3	Dishevelled-Binding Antagonist of β-catenin 3
DBTSS	Database for Transcription Start Site
DKK1	Dickkopf WNT Signaling Pathway Inhibitor1
DVLs	Dishevelled segment polarity protein
cDNA	Complementary Deoxyribonucleic acid
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
ECM	Extracellular Matrix
EDTA	Ethylene di-amine tetra-acetic acid
EMT	Epithelial-mesenchymal transition
ERBB	v-erb-b2 avian erythroblastic leukemia viral oncogene homolog
FAP	Familial adenomatous polyposis
FBS	Fetal Bovine Serum
FOXP3	Forkhead box P3
GFP	Green fluorescent protein
GSK3β	Glycogen synthase kinase3 beta
GTPase	Guanosine triphosphotase
h	hour
H3K27me$_3$	Histone H3 lysine 27 trimethylation
H3K4me$_3$	Histone H3 lysine 4 trimethylation
H3K9me$_3$	Histone H3 lysine 9 trimethylation
HAT	Histone acetyltransferases
HD	Homeodomain
HDAC	Histone deacetylase
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HMEC	Human mammary epithelial cells
HMG-1	High Mobility Group protein-1
HRP	Horseradish peroxidase
IB	Immunoblot

IgH	Immunoglobin heavy chain
IP	Immunoprecipitation
kb	Kilobase
KCl	Potassium chloride
kDa	Kilo Dalton
LEF1	Lymphoid Enhancer-Binding Factor 1
LRH1	Liver receptor homolog1
LRP6	Low density lipoprotein receptor-related protein 6
M	Molar
MARs/SARs	Matrix/Scaffold attachment regions
MBP	MAR-binding protein
MG132	carbobenzoxy-Leu-Leu-leucinal
MgCl2	Magnesium chloride
mg	milligram
MMP2	Matrix metallopeptidase 2
MMP11	Matrix metallopeptidase 11
MHC	Major histocompatibility complex
min	Minutes
miRNAs	MicroRNAs
ml	Millilitre
mM	Mill molar
ng	Nanogram
PBS	Phosphate Buffered Saline
PCAF	P300/CBP-associated factor
PCR	Polymerase Chain Reaction
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PML	Promyelocytic leukemia
RAS	Rat Sarcoma Viral Oncogene Homolog
RFP	Red fluorescent protein
RISC	RNA induced silencing complex
RNA	Ribonucleic acid
RPMI-1640	Rosewell Park Memorial Institute-1640
Runx	Runt-related transcription factor
SATB1	Special AT-rich Sequence Binding Protein 1
SATB2	Special AT-rich Sequence Binding Protein 2
SBS	SATB binding sequences
SCID	Severe combined immunodeficiency
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulphate- polyacrylamide gel electrophoresis
SFRP	Secreted frizzled protein
siRNA	Small interference RNA
SP1	Specific protein1
TCF7	Transcription Factor 7 (T-Cell Specific)
TCF7L1	Transcription Factor 7-Like 1 (T-Cell Specific)

TCF7L2	Transcription Factor 7-Like 2 (T-Cell Specific)
TERT	Telomerase reverse transcriptase
TGF-β	Transforming growth factor-β
Th	T-helper
TSS	Transcription Start Site
TRED	Transcriptional Regulatory Element Database
UTR	Untranslated region
Wnt	Wingless type protein

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ROLE AND REGULATION OF SATB1 IN COLORECTAL TUMORIGENESIS AND PROGRESSION

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Synopsis

Cancer development and progression is a complex multistage process, involves progressive changes in expression of myriad of genes. Cancer development is initiated by activation of oncogenes and inactivation of tumor suppressors which further lead to progressive accumulation of changes essential for tumor development. Various signaling pathways have been implicated in cancers which are hyperactivated either by inactivation or activation of key players in the pathway for example aberrant activation of Wnt signaling is mediated by loss of function mutation in tumor suppressor APC or by gain of function mutation in β-catenin (Valenta et al., 2012 ; MacDonald et al., 2009). Wnt signaling is critical for tissue development and homeostasis and its aberrant activation is deleterious and leads to consequences of disease and cancer (MacDonald et al., 2009). Aberrant Wnt signaling has been implicated in various cancers (Khalil et al., 2012 ; Cleary et al., 2014; Wang et al., 2009) and plays central role in colorectal cancer development and progression (Clevers, 2006 ; Najdi et al., 2011 ; Valenta et al., 2012).

The critical event in Wnt signaling is stabilization of β-catenin. The stabilization involves multiprotein complex consisting of APC, AXIN1/AXIN2, CSK1α and GSK3β. The pathway is mediated by binding of Wnt ligands to the receptors. In the absence of Wnt ligands, β-catenin is engaged in multiprotein complex that leads to phosphorylation by CSK1α followed by GSK3β. Phosphorylated β-catenin is recognized by β-TrCP ubiquitin ligase followed by proteosomal degradation (MacDonald et al., 2009). In the presence of Wnt ligand, β-catenin is disengaged from multiprotein complex thereby escaping the sequential phosphorylation by CSK1α and GSK3β and hence recognition by β-TrCP ubiquitin ligase. The non-phosphorylated β-catenin gets accumulated in the cytosol and translocates inside the nucleus interacts with TCF/LEF transcription factors to induce target gene expression (MacDonald et al., 2009). Aberrant Wnt signaling is considered as hallmark of colorectal tumorigenesis but recently epigenetic events in addition to genetic events also been implicated to modulate the outcome of Wnt signaling and play essential role in colorectal tumorigenesis. For example DACT3 plays antagonistic role in Wnt signaling activation and is epigenetically regulated to induce the colorectal tumorigenesis (Jiang et al., 2008). Similarly, cross talk of novel targets and hyperactivation of Wnt signaling has been reported to play stringent regulatory role in modulating the transcriptional

outcome of Wnt signaling and impact on colorectal tumorigenesis (Yook et al., 2006 ; Stemmer et al., 2008). Although the aberrant activation of Wnt signaling is critical for various cancers but recently various modulators have been shown to regulate Wnt signaling and they further suggest that in addition to genetic background additional layer of regulation can enhance Wnt signaling.

SATB1 (Special AT-rich DNA Binding protein 1) is a chromatin organizer which has been shown to regulate higher-order assembly of chromatin thereby allowing different machineries to activate or repress gene expression (Alvarez et al., 2000; Kohwi-Shigematsu et al., 2012; Kumar et al., 2007; Mir et al., 2012). SATB1 is a T–lineage-enriched regulator providing structural scaffold for regulation of genes essential for T cell development. Recently, chromatin organization has been implicated in regulating the large number of genes essential for tumorigenic transition. Chromatin organizer proteins provide structural framework to regulate multiple genes essential for various developmental processes and hence their misregulation can be deleterious. SATB1 was identified and cloned because of its ability to bind base unpaired regions (BURs) (Galande, 2002). SATB1 promotes chromatin structure and interacts with various factors to regulate nuclear integrity thereby regulating global gene expression. The expression of SATB1 was thought to be tissue-specific predominantly expressed in thymocytes and has been implicated in T cell development and differentiation. Because of global role of SATB1 in regulating gene expression, it was thought that aberrant expression may have deleterious effects. In 2008, Han et al., for the first time showed that aberrant expression of SATB1 reprograms gene expression to promote breast cancer development and metastasis (Han et al., 2008). The study showed that SATB1 is expressed in metastatic cells whereas it is not expressed in primary cells. The expression pattern of SATB1 correlates with poor prognosis of breast cancer patients. SATB1 provides the structural network of chromatin thereby differentially regulates tumor oncogenes and tumor suppressors during breast cancer tumorigenesis (Han et al., 2008). SATB1 expression in other cancers has been extensively studied (Mir et al., 2012 ; Kohwi-Shigematsu et al., 2012). SATB1 expression has been shown to be associated with glioma cancer (Chu et al., 2012), prostate cancer (Barboro et al., 2012), liver cancer (Tu et al., 2012) and recently shown to be a colorectal cancer poor prognosis marker (Nodin et al., 2012). Aberrant expression of SATB1 correlates with poor prognosis of colorectal cancer patients and

overall shorter survival rate. However, how SATB1 expression promotes colorectal tumorigenesis is not clear. Wnt signaling is a hallmark of colorectal tumorigenesis and recently Notani et al., demonstrated that SATB1 functionally overlaps with Wnt signaling during Th2 differentiation (Notani et al., 2010); thus raising an interesting possibility that SATB1 might overlap with Wnt signaling and promote molecular changes essential for colorectal tumorigenesis. Hyperexpression of SATB1 promotes molecular changes that are essential for tumorigenic transition. The expression of SATB1 increases during development of cancers that leads to subsequent accumulation of changes necessary for tumorigenesis. The regulation of SATB1 could thus be the key event for inducing tumorigenesis. Recently, Mcinnes et al. reported that FOXP3 and FOXP3 regulated microRNAs suppress SATB1 expression (Mcinnes et al., 2011). Similarly, cellular Prion protein has been shown to regulate SATB1 via Fyn-SP1 axis (Wang et al., 2012). However, these studies did not delineate the regulatory network and acquisitions of molecular changes responsible for inducing SATB1 expression.

In light of the above findings, I proposed to investigate:

1) Role of SATB1 in colorectal tumorigenesis.

2) Regulation of SATB1 in colorectal tumorigenesis- A novel role of Wnt signaling.

3) Interplay of SP1 and Wnt signaling in regulating chromatin organizer SATB1.

Accordingly, my thesis has three chapters as follows:

(1) Role of SATB1 in colorectal tumorigenesis.

Colorectal cancer development and progression is acquired by specific mutations that lead to aberrant activation of Wnt signaling. Wnt signaling promotes aggressive phenotype of colorectal cells to induce molecular changes essential for tumorigenic transition.

To elucidate the role of SATB1 in colorectal cancer, we analyzed the expression of SATB1 in 12 colorectal cancer cell lines and in primary colorectal cell line. The cell lines are classified according to aggressiveness and Duke's classification of cancers (Duke et al 1932; Leibovitz et al., 1976). The expression of SATB1 at protein level and at transcript level was higher in aggressive cell lines in comparison with primary

colorectal cells and with non- aggressive colorectal cancer cells. Thus, correlating SATB1 expression pattern with Duke's classification and aggressive phenotype of colorectal cancer cells. To examine physiological significance of higher expression of SATB1 in colorectal cancer cells, we analyzed the expression of SATB1 in colorectal cancer patient samples in comparison with matched normal adjacent tissues. The expression of SATB1 was higher in colorectal cancer tissue samples in comparison with adjacent normal samples. To investigate the higher expression of SATB1 is associated with aggressive phenotype, we stably knocked down SATB1 in aggressive HCT-15 cells and monitored the cell growth and proliferative potential of SATB1 depleted cells. Depletion of SATB1 reduced the cell growth as determined by colony assay and significantly reduced the proliferative potential of aggressive colorectal HCT-15 cells. Similarly, migratory potential essential for invasion was significantly reduced upon SATB1 depletion. SATB1 depletion further abolished the ability of colorectal cancer HCT-15 cells to form colonies in soft agar thereby restoring anchorage-dependant growth. These findings indicated that SATB1 supports anchorage independence and promotes cell growth and proliferative potential. To further delineate the role of SATB1 in colorectal tumorigenesis, we performed *in vivo* tumor growth assay. SATB1 depleted HCT-15 cells and control HCT-15 cells were injected subcutaneously in immunocompromised SCID mice and tumor growth was monitored for four weeks. Depletion of SATB1 reduced the potential of HCT-15 cells to induce tumors *in vivo*. The tumor volume measured for four weeks was significantly reduced upon SATB1 depletion in comparison with control HCT-15 cells. Tumor weight was significantly reduced upon SATB1 depletion. Furthermore, to investigate SATB1 is sufficient to induce tumorigenesis; we injected SATB1 overexpressing SW480 cells and control SW480 cells (Type B non aggressive cells) subcutaneously in SCID mice. SATB1 overexpression was sufficient to convert non-aggressive SW480 cells into aggressive cells and induced *in vivo* tumor growth. Thus, SATB1 is essential and is sufficient to potentiate non-aggressive cells to promote tumorigenesis.

Aberrant activation of Wnt signaling is a hallmark of colorectal cancer and provides essential changes to potentiate colorectal tumorigenesis. Since our findings established that SATB1 is involved in aggressive phenotype of colorectal cancer cells and promotes *in vivo* tumorigenesis, we thought to investigate whether SATB1 potentially modulates Wnt signaling and its downstream targets. To test this

hypothesis, we used TCF7L2-driven TOP/FOP Luciferase reporter assay in SATB1 overexpressing SW480 cells. SATB1 overexpression significantly augmented the reporter activity. Depletion of β-catenin and SATB1 in SATB1 overexpressing cells resulted in decrease in reporter activity indicating that SATB1 could affect the reporter activity through mediators of Wnt signaling. Next, we analyzed the expression of critical players of Wnt signaling in HCT-15 colorectal cells. Stable depletion of SATB1 resulted in decreased expression of β-catenin. Interestingly, depletion of SATB1 has drastically reduced the expression of TCF7L2 (important effector transcription factor of Wnt signaling) and DVLs. Similarly, SATB1 depletion reduced the expression of downstream targets AXIN2, c-Myc and TCF7. We also observed that SATB1 depletion increased the expression of SATB2 and Wnt signaling antagonist DKK1. SATB2 (Wang et al., 2009b) and DKK1 (Aguilera et al., 2006) are known to be downregulated in colorectal cancers. To further validate the role of SATB1 in Wnt signaling, we stably knocked down SATB1 in HCT116 cells and overexpressed transiently in primary colorectal cells CRL1790. Depletion of SATB1 in HCT116 colorectal cancer cells drastically reduced the expression of β-catenin, active β-catenin, DVLS, TCF7L2 and Wnt3A suggesting critical role of SATB1 in modulating Wnt signaling. Conversely, overexpression in CRL1790 induced the expression of β-catenin, DVLs and induced the expression of downstream targets. Depletion of SATB1 reduced the expression of downstream targets at transcript level also whereas overexpression induced their expression. To show that effect of SATB1 is not an off-target effect, we generated another stable cell line using a different siRNA sequence for SATB1 and also generated SATB1 knockout cell line using TALENs. Depletion of SATB1 in shSATB1 HCT116 cells and SATB1 knockout HCT116 cells resulted in drastic decrease in TCF7L2 and downstream targets AXIN2 and TCF7.

SATB1 regulates key players of Wnt signaling and its expression could be the key event to induce molecular changes essential for colorectal tumorigenesis. The conversion of epithelial phenotype to mesenchymal phenotype is acquired by inducing the expression of mesenchymal markers and reducing the expression of epithelial markers. To investigate whether SATB1 plays a role to induce such changes, we analyzed the expression of mesenchymal markers under SATB1 depletion. Depletion of SATB1 reduced the expression of N-cadherin, vimentin and LEF1 (mesenchymal markers) and increased the expression of E-cadherin (epithelial marker). Depletion of

SATB1 reduced the expression of cancer associated genes such as MMP2, MMP9, ERBB1 and ERBB2. Therefore data suggests that SATB1 regulates key molecular events essential for tumorigenic transition. Elevated levels of SATB1 could be key event during tumorigenic transition from primary cells to tumor cells. Since data suggest that SATB1 regulates key players of Wnt signaling and cancer associated genes, we wished to analyze whether SATB1 directly regulates the expression of downstream targets by directly binding to their promoters. To test this, ChIP assay was done for SATB1 occupancy in control HCT116 and SATB1 depleted HCT116 cells. However, since downstream targets are known to be regulated by TCF7L2/β-catenin complex and previously Notani et al. showed that SATB1 recruits β-catenin onto its target genes during TH2 differentiation, we analyzed the co-occupancy of SATB1 and β-catenin in control cells and in SATB1 depleted cells. The data suggests that SATB1 and β-catenin co-occupy the promoters of cancer associated genes such as MMP2 and Wnt signaling downstream targets such as c-Myc, AXIN2 and TCF7 in control HCT116 cells and depletion of SATB1 abolishes the occupancy of SATB1 and β-catenin. The reduced occupancy of SATB1 and β-catenin was also reflected in expression of downstream targets such that SATB1 depletion reduced their expression.

Collectively, this data establishes that SATB1 is very important player to promote colorectal tumorigenesis by modulating Wnt signaling through regulation of TCF7L2 and β-catenin. SATB1 also directly binds to promoters of downstream targets and regulates their expression, hence providing dual regulatory mechanism.

(2) Regulation of SATB1 in colorectal tumorigenesis-A novel role of Wnt signaling.

The chromatin organizer SATB1 reprograms global gene expression and thereby promotes cellular changes essential for tumorigenesis. In this study we show that SATB1 expression pattern correlates with colorectal cancer aggressiveness and elevated expression of SATB1 leads to dysregulation of cancer associated genes. SATB1 expression is elevated during tumorigenic transition from normal to cancer phenotype, however, molecular changes essential for inducing SATB1 expression are not fully elucidated. The regulatory pathways responsible for SATB1 regulation thereby deciding the fate of tumorigenic transition have remained elusive. In this study, we show that primary colorectal cell line CRL1790 virtually does not express SATB1. Similarly, β-catenin levels are significantly low. The expression of SATB1 increases with aggressiveness and Wnt signaling status of colorectal cancer cells, suggesting that β-catenin/TCF7L2 signaling could be involved in regulating chromatin organizer SATB1. To test this hypothesis, we induced Wnt signaling in primary colorectal CRL1790 cells by GSK3β depletion and alternatively by GSK3β kinase inhibitor CHIR. The treatment with CHIR and GSK3β depletion caused aberrant activation of Wnt signaling followed dramatic increase in SATB1 expression. The aberrant activation of Wnt signaling as indicated by stabilization of β-catenin induced the essential molecular changes to promote SATB1 expression at protein level. Similarly, the aberrant activation of Wnt signaling induced robust increase in SATB1 expression at transcript level and so as the expression of known downstream targets of Wnt signaling AXIN2, c-Myc and cyclinD1, thereby indicating that SATB1 is novel target of Wnt signaling. To further validate this, we tested SATB1 regulation using a different cellular model. We induced Wnt signaling by Wnt3A treatment in dose dependent manner in HeLa cells and alternatively overexpressed degradation resistant β-catenin (S37A β-catenin). The hyperactivation of Wnt signaling and β-catenin overexpression resulted in robust increase in SATB1 expression. Similarly time-dependent activation of Wnt signaling in HeLa cells by treatment with GSK3β inhibitor CHIR induced dramatic increase in SATB1 expression but no such change was observed in SATB2 expression indicating that Wnt signaling specifically induces SATB1 expression. To investigate whether SATB1 expression induced upon hyperactivation of Wnt signaling is dependent on β-catenin expression, we induced

Wnt signaling in CRL1790 by CHIR treatment and knocked down β-catenin in CHIR treated cells. The activation of Wnt signaling resulted in hyperexpression of β-catenin and SATB1 whereas depletion of β-catenin in CHIR treated cells reduced the expression of SATB1. These results indicated that hyperexpression of SATB1 induced by Wnt signaling is dependent on β-catenin expression. Activation of Wnt signaling leads to disengagement of β-catenin from the destruction complex consisting of AXIN1/AXIN2, APC, CSK1α and GSK3β. Stabilized β-catenin translocates to nucleus interacts with LEF/TCF transcription factors to induce target gene expression. We next analyzed the expression of SATB1 upom depletion of the major transcription factor TCF7L2 in β-catenin active mutant cell line HCT116 and APC mutant cell line HCT-15. Depletion of TCF7L2 reduced the expression of SATB1 as well as the known downstream targets AXIN2 and TCF7 indicating that SATB1 is a direct target of TCF7L2 signaling. To further validate the regulation, we knocked down β-catenin in β-catenin mutant HCT116 cells and APC mutant HCT-15 cells. Depletion of β-catenin reduced the expression of SATB1 and known target TCF7. Similar data was also observed in TNM breast cancer cell line MDA-MB231 upon β-catenin depletion. To further delineate the effect of Wnt signaling on SATB1 expression *in vivo*, we analyzed the expression of SATB1 in APC min mutant Zebra fish. The hyperactivation of Wnt signaling in APC min mutant Zebra fish resulted in robust increase in SATB1 expression. Thus, findings from *in vivo* and cellular models established that the cascade of TCF7L2/ β-catenin signaling induces and regulates SATB1 expression. The physiological importance of SATB1 regulation can be explained by the fact that SATB1 regulates cancer associated genes, potentiates changes essential for tumorigenesis and modulates Wnt signaling.

To delineate whether TCF7L2/β-catenin regulatory network is directly involved in inducing the expression of SATB1 by binding to its promoter during tumorigenic transition, we identified the *Satb1* promoter by *in silico* analysis using bioinformatics tools; transcription regulatory database and transcription start site databases and retrieved the *Satb1* promoter sequence (Zhao et al., 2005). We then analyzed the *Satb1* promoter and found multiple TCF7L2 consensus sequence motifs (A/T A/T CAAAG, CTTTGNN) (van de Wetering et al., 1997; Wagner et al., 2010; Frietze et al., 2012; Macdonald et al., 2009). Next we performed ChIP analysis for TCF7L2 and histone H3 lysine 4 trimethylation and established that TCF7L2 binds to *Satb1*

promoter along with histone activation mark. To gain further insight into regulation of SATB1, we analyzed the occupancy of β-catenin and TCF7L2 on *Satb1* promoter upon activation of Wnt signaling in primary colorectal cells CRL1790. Aberrant activation of Wnt signaling induced the β-catenin expression so as the occupancy of TCF7L2 and β-catenin on *Satb1* promoter. Activation also resulted in increased occupancy of histone activation mark thereby indicating that Wnt signaling induced the promoter activity of SATB1. Increased occupancy of β-catenin and TCF7L2 was also reflected in expression of SATB1 such that hyperactivation of Wnt signaling and increased enrichment of β-catenin and TCF7L2 on promoter induced SATB1 expression. Furthermore, we cloned the promoter sequence having multiple TCF7L2 consensus sites into PGL3 basic and analyzed the promoter Luciferase activity under β-catenin depletion. Depletion of β-catenin reduced the *Satb1* promoter activity significantly. Next, we analyzed the occupancy of β-catenin in HCT116 cells under β-catenin depletion. ChIP analysis revealed that depletion of β-catenin reduced the occupancy of β-catenin on *Satb1* promoter so as the expression of SATB1. Depletion of β-catenin also reduced its occupancy on known target *c-Myc* promoter, thereby indicating that SATB1 is direct and novel target of TCF7L2/β-catenin signaling.

The data so far provides evidences that SATB1 and β-catenin share positive feedback regulatory network. To prove there is regulatory feedback between SATB1 and β-catenin and find that SATB1 is required for Wnt signaling, we induced Wnt signaling in CRL1790 by CHIR treatment and knocked down SATB1 in CHIR treated cells. The activation of Wnt signaling by CHIR treatment induced the expression of β-catenin and resulted in robust increase in SATB1 expression. The effect of aberrant activation of Wnt signaling on β-catenin expression was abrogated upon SATB1 depletion in CHIR treated cells. Similar result was observed in HeLa cells that upon activation of Wnt signaling, expression of β-catenin were induced leading to increase in SATB1 expression. However, depletion of SATB1 was sufficient to reduce the levels of β-catenin even after activation of Wnt signaling. The data thus suggests that SATB1 is regulated by Wnt signaling and is required for Wnt signaling dependant regulation of β-catenin.

We further wished to investigate whether SATB1 is sufficient to rescue the effect of β-catenin depletion on downstream targets of Wnt signaling. We knocked down β-catenin and overexpressed GFP-SATB1 under β-catenin depletion in HCT116 cells

and analyzed the expression of SATB1 and known downstream targets of Wnt signaling. The depletion resulted in decreased expression of SATB1 and also that of the expression of known downstream targets. However, SATB1 re-expression was not sufficient to rescue the effect of β-catenin depletion on downstream targets of Wnt signaling. The data is consistent with the previous study from Galande laboratory that SATB1 drives the β-catenin-dependent gene expression (Notani et al., 2010). Since we showed that depletion of SATB1 reduces the expression of TCF7L2 and that of the expression of downstream targets, we wished to see whether TCF7L2 re-expression can rescue the expression of downstream targets. We knocked down SATB1 and ectopically expressed TCF7L2 in SATB1 depleted HCT116 cells. Depletion of SATB1 reduced the expression of TCF7L2 so as the expression of Wnt signaling downstream targets AXIN2 and TCF7, but forced expression was not sufficient to reinduce the expression of downstream targets.

To further gain insight into the mechanistic role of SATB1 and functional crosstalk with Wnt signaling, we ectopically expressed SATB1-DN (1-204) in HCT116 cells that acts as dominant negative for SATB1 function. The ectopic expression of SATB1-DN (1-204) resulted in dramatic decrease in TCF7L2 levels and the expression of downstream target AXIN2. The forced expression of TCF7L2 in SATB1-DN (1-204) HCT116 cells was not sufficient to re-induce the expression of AXIN2, similarly the expression of SATB1 (novel Wnt target) and TCF7 was reduced significantly whereas the re-expression of TCF7L2 in SATB1-DN (1-204) overexpressed cells, was not sufficient to reinduce the expression of TCF7 and SATB1. Thus, the data argues that SATB1 regulates the expression of TCF7L2 and both are essential for regulation of downstream targets. SATB1 has been shown to interact with β-catenin via its N-terminal domain (Notani et al., 2010). Thus, it is possible that sequestering effect of SATB1-DN (1-204) on β-catenin might be the reason that re-expression of TCF7L2 is not sufficient to re-induce the expression of downstream targets of Wnt signaling in SATB1-DN (1-204) over-expressing HCT116 cells. Together, this data argues that SATB1 regulates multiple events in Wnt signaling cascade and that elevated SATB1 expression might be the key event in colorectal tumorigenic transition.

(3) Interplay of SP1 and Wnt signaling in regulating chromatin organizer SATB1.

Specificity protein1 (SP1) is a member of specificity protein/ kruppel-like transcription factor family characterized by highly conserved zinc finger DNA binding domain (Li and Davie, 2010). SP1 regulates the expression of genes involved in cell cycle progression, cellular differentiation and oncogenesis. SP1 was earlier thought to be ubiquitously expressed and involved in transcription activation of housekeeping genes but recent studies have shown tissue specific role of SP1 and in regulating the expression of genes considered as hallmark of oncogenesis. Post-translational modifications have been shown to play critical role in switching the functions of SP1 (Chang and Hung, 2012). The studies have shown that posttranslational modifications alter the transcriptional activity, DNA-binding ability and stability of SP1.

Aberrant activation of Wnt signaling pathway has been implicated in various cancers promoted by mutations in the mediators of pathway (MacDonald et al., 2009). Recently epigenetic events have also been shown to modulate the outcome of Wnt signaling (Jiang et al., 2008). Similarly, crosstalk between novel targets and hyperactivation of Wnt signaling has been implicated in stringently regulating the outcome of Wnt signaling. SP1 has been shown to regulate the Wnt antagonistic factor (WIF1) (Liu et al., 2008). Authors have shown that Adiponectin regulates WIF1 expression by down regulating SP1. This was the first study to demonstrate the crosstalk of SP1 and Wnt signaling. Recently, it was shown that cellular Prion protein regulates the expression of SATB1 via Fyn-SP1 pathway. Authors demonstrate that SP1 binds to *satb1* promoter and directly regulates the expression of SATB1 (Wang et al., 2012) and our data for the first time establishes that TCF7L2/β-catenin signaling regulates SATB1 expression (Mir et al., 2014). Therefore based on observation by Wang et al and our earlier data, we thought that a regulatory network involving TCF7L2/β-catenin signaling and SP1 might be essential for SATB1 expression. To test this hypothesis, we analyzed the expression of SP1 in primary cell line CRL1790 and two colorectal cancer cell lines. The expression of SP1 correlated with SATB1 expression and Wnt signaling status of colorectal cancer cells. To elucidate further role of SP1 and β-catenin, we knocked down SP1 and β-catenin in metastatic cell line SW620. Depletion of SP1 and β-catenin results in downregulation of SATB1, however, knockdown of β-catenin exerted drastic effect on SP1 expression, indicating that SP1 may be downstream to TCF7L2/β-catenin. Next, to show that SP1 and TCF7L2/β-

catenin complex regulates SATB1 or to delineate whether SP1 is downstream to Wnt signaling, we induced Wnt signaling in CRL1790 by CHIR treatment and depleted SP1 in CHIR treated cells. Depletion of SP1 resulted in downregulation of SATB1 expression induced upon hyperactivation of Wnt signaling. Together, these findings suggest that SP1 is required for Wnt signaling-dependent regulation of SATB1. Activation of Wnt signaling also induced the expression of SP1. Hyperactivation of Wnt signaling drives the expression of downstream targets through TCF7L2/β-catenin complex and we thought to find whether SP1 also interacts with β-catenin and hence drives the expression of novel target SATB1. We analyzed the interaction of β-catenin with SP1. The co-immunoprecipitation data suggests that SP1 interacts with β-catenin in HeLa cells and COLO205 cells. Thus, together our data suggests that SP1 interacts with β-catenin and is required for Wnt signaling-dependent regulation of SATB1. Next, we analyzed the occupancy of TCF7L2, β-catenin and SP1 on *Satb1* promoter. ChIP data established that TCF7L2, β-catenin and SP1 bind to *Satb1* promoter to regulate its expression in HCT116 cells. To further delineate that binding of SP1 and β-catenin on *Sabt1* promoter is required for regulating SATB1 expression, we analyzed the expression of SATB1 and occupancy of SP1 and β-catenin on *Satb1* promoter under SP1 and β-catenin depletion in HCT116 cells. Depletion of SP1 caused marginal decrease in β-catenin expression but its occupancy was completely abolished. The ablation of β-catenin drastically reduced the expression of SP1 and also abolished the occupancy of SP1 and β-catenin on SATB1 promoter so as the expression of SATB1 was reduced indicating that SP1/β-catenin complex binds to *Satb1* promoter and regulates its expression.

Aberrant activation of Wnt signaling by GSK3β inhibition resulted in robust increase in SP1 expression and depletion of β-catenin in metastatic cell line SW620 abolished SP1 expression. These observations lead us to analyze the protein sequence of SP1 for presence of motif recognized by GSK3β. By sequence analysis we identified phospho-degron motif at C-terminus of SP1 that could be recognized and regulated by GSK3β. To elucidate the possible role of GSK3β in regulating SP1 stabilization, we overexpressed FLAG-SP1 in control 293T cells and GSK3β depleted cells. Depletion of GSK3β resulted in dramatic stabilization of ectopically expressed SP1, thus indicating the role of GSK3β recognized phosphodegron motif in stabilizing SP1 at protein level. Next, to delineate that stabilization of SP1 is β-catenin- dependent, we

ectopically overexpressed FLAG-SP1 in HCT116 control cells and β-catenin depleted cells. Depletion of β-catenin resulted in drastic decrease in ectopically expressed SP1. Thus, SP1 is stabilized at protein level by activation of Wnt signaling and depends on Wnt-dependent expression of β-catenin. Based on interaction data and stabilization data, it can be speculated that β-catenin interacts with SP1 and hence prevents the phosphodegron motif to be exposed for GSK3β mediated phosphorylation and degradation. Next to show that phosphodegron motif phosphorylated by GSK3β plays critical role in SP1 stabilization, we mutated the two serine residues in DSGAGS motif to alanine and also generated phosphodegron deletion construct of SP1. Mutation and phosphodegron deletion should render SP1 nonresponsive to GSK3β and should be stabilized even in the presence of active GSK3β. To test this we overexpressed wild-type SP1, phospho-mutant SP1 and delta-degron SP1 in 293T cells. The phospho-mutant and delta-degron SP1 were dramatically stabilized in comparison with the wild-type SP1, thus suggesting that GSK3β regulates SP1 by phosphorylation of phosphodegron motif followed by proteosomal degradation. To test that proteosomal degradation is involved in SP1 degradation; we overexpressed FLAG-SP1 and treated with MG132 proteosomal degradation pathway inhibitor. The MG132 treatment resulted in robust increase in SP1 stabilization at protein level. Further, we wished to find whether GSK3β physically interacts with SP1 and mediates its degradation. Co-immunoprecipitation analysis data established that GSK3β interacts with SP1. We further established that phospho-mutant interacts with GSK3β with lesser affinity and hence is stabilized in comparison with wild-type SP1. To further corroborate whether Wnt signaling cascade is pre-requirement to induce SP1 stabilization, we induced Wnt signaling in FLAG-SP1 expressing HEK293 by treatment with Wnt3A ligand for four hours. Wnt stimulation by Wnt3A treatment resulted in dramatic increase in the levels of ectopically expressed FLAG-SP1. This stabilization induced by Wnt3A was dependent on expression of β-catenin such that depletion of β-catenin resulted in the destabilization of FLAG-SP1. To delineate the further molecular mechanism of how Wnt3A signaling induces stabilization of SP1, we analyzed the interaction of SP1 with GSK3β and β-TrCP in Wnt3A induced HEK293 cells. The interaction data revealed that Wnt signaling impedes the interaction of SP1 with β-TrCP but not with GSK3β, thereby induces the stabilization of SP1. Since the findings indicated that SP1 follows the Wnt signaling pathway in same manner as that of β-catenin, we wished to determine whether β-catenin also requires SP1 for its stabilization. To prove this we

overexpressed FLAG β-catenin in control and SP1 depleted HCT116 cells. Depletion of SP1 resulted in dramatic decrease in the levels of ectopically expressed β-catenin. Further, Wnt signaling induced β-catenin levels were reduced upon SP1 depletion. Collectively, these findings establish that SP1 and β-catenin are mutually stabilized in Wnt signaling-dependent manner. This novel regulatory crosstalk is therefore required for TCF7L2/β-catenin signaling-dependent regulation of SATB1.

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Chapter 1

Role of SATB1 in colorectal tumorigenesis

1.1 Introduction

Cancer development is a complex multifactorial and multistage process involving programmed and progressive accumulation of changes governed by multiple genes for its initiation, progression and metastasis (Nowell, 2002; Yokota, 2000). This complex process starts with initiation of mutations that leads to the formation of tumor by unlimited and uncontrolled proliferation and thus giving malignant cells selective advantage to grow and acquire further mutations. The progressive accumulation of mutations that activate oncogenes and inactivate tumor suppressors leads to development of cancers (Kinzler and Vogelstein, 1996). After the first report of genetic alteration of *hras* gene in bladder cancer Reddy et al., 1982), several numbers of mutations have been identified in genes to promote tumorigenesis. Genes involved in cell proliferation, differentiation, cell death and in DNA repair have been implicated in cancers (Fishel et al., 1993; Leach et al., 1993; Parsons et al., 1993). Next generation DNA sequencing has revealed mutations in genes involved in growth signaling. It is now known that 40% of human melanoma cancers have mutation in B-RAF resulting in constitutive activation of MAP kinase signaling pathway (Davies and Samuels, 2010; Hanahan and Weinberg, 2011). Similarly, catalytic subunit of PI3 kinase is mutated in different cancers (Jiang and Liu, 2009; Yuan and Cantley, 2008). The genetic alterations have been shown to enhance the activity of proto-oncogenes for example point mutations in Ras oncogene commonly seen in lung, colon and pancreatic cancers. Mutation prevents the interaction of Ras with GTPase activating protein (GAP) and hence downstream signaling remains constitutively active (Downward, 2003). Similarly mutations in various tumor suppressors play essential role in cancer development, for example mutations in adenomatous polyposis coli (APC) are hallmark of colorectal cancers. In colorectal cancers other genes which have been shown to undergo mutation include β-catenin which causes it to escape proteosomal degradation (Ilyas et al., 1997; Morin et al., 1997). Germline mutations in APC account for 1% of all colorectal cancers. Most of the mutations in APC lead to truncated protein (Fearnhead et al., 2004). It has been observed that patients with mutation in 1309 codon of APC tend to have more colorectal adenomas and carcinoma in shorter time (Nugent et al., 1994). Axin1 which is involved in proteosomal

degradation of β-catenin is mutated in various colorectal cancers (Webster et al., 2000). Another study has shown that approximately 50% of human cancers have mutation in the tumor suppressor p53 (Soussi et al., 2006). Similarly mutation in TCF4 to more active form has been observed in colorectal cancers (Gayet et al., 2001). Thus, during colorectal cancer progression several proteins have been shown to undergo mutations which are important players in regulation of Wnt signaling. These mutations enhance the cancer cells to augment the Wnt signaling essential for cellular proliferation and growth.

Recent advances in cancer biology have led to the appreciation of the role of epigenetic regulatory mechanisms in the development of cancer (Baylin and Jones, 2011; Sandoval and Esteller, 2012). The epigenetic pathway in cancers is function of chromatin structure, DNA methylation, histone variants and modification, nucleosome remodelling (Sharma et al., 2009; You and Jones, 2012). Epigenetic regulatory mechanism has been shown to regulate genetic alteration for example hyper-methylation of tumor suppressor genes RB, BRAC1, BRAC2 and PTEN (Baylin and Jones, 2011). Similarly epigenetic regulatory role is observed in colorectal cancers wherein one allele of MLH1 and CDKN2A is mutated and other allele is silenced due to hypermethylation. The loss of MLH1 and CDKN2A causes defect in DNA repair and cell cycle regulation (Baylin and Ohm, 2006). The role of epigenetic regulation is also observed in silencing Wnt antagonistic protein SFRPs (secreted frizzled related protein) resulting in aberrant activation of Wnt signaling inducing cellular proliferation and hence providing proliferative and survival advantage to malignant cells which further leads to genetic alterations in other Wnt signaling mediators (Schepers and Clevers, 2012).

1.1.1 Colorectal cancer –Wnt signaling and molecular genetics.

Colorectal cancer is leading cause of deaths worldwide (Siegel et al., 2013). Colorectal cancer has been linked to various pathways most importantly with dysregulation of Wnt signaling that exerts paleotropic effects on cellular phenotypes during its development and progression. Cancers develop either due to activation of oncogenes or inactivation of tumor suppressor genes. Mutations in Colorectal cancer are mostly somatic in origin. Approximately 15-30% of colorectal cancers have inherited germline mutations in APC. Small fraction of mutations results in silencing of

APC while 95% of mutations are frame shift in origin resulting in truncation product of APC. Germline mutations in APC are root cause of FAP and FAP like syndromes characterized by development of thousands of adenomas. In addition to Germline mutations, 70-80 % colorectal cancers (sporadic colorectal cancers) have somatic mutation in APC. Tumor suppressor APC encodes a 300 KDa protein plays critical role in cell-cell adhesion, chromosomal segregation and apoptosis. The biological function of APC is attributed to its role in regulating β-catenin stabilization. About 80% of colorectal cancers have biallelic mutation in APC thereby leading to dysregulation of Wnt signaling pathway (Fearon, 2011). Recent studies have shown that 93% of colorectal cancers have mutation in Wnt signaling pathway including APC (Cancer Atlas, 2012).

The primary and critical event during activation of Wnt signaling is stabilization of β-catenin. Localization of β-catenin in cytoplasm and inside nucleus plays critical role in regulation of Wnt signaling. Cytoplasmic β-catenin is phosphorylated and regulated by multiprotein complex consisting of AXIN1/AXIN2, APC, CK1 and GSK3β. In absence of Wnt ligand, the multiprotein complex regulate the β-catenin levels (MacDonald et al., 2009). The multiprotein complex engages β-catenin and mediates sequential phosphorylation first by CK1at serine 45 and followed by GSK3β at threonine 41, serine 33 and serine 37. β-catenin phosphorylation at serine 33 and serine 37 is recognized by β-TrCP followed by proteosomal degradation (MacDonald et al., 2009). In presence of Wnt ligand, β-catenin is disengaged from multiprotein complex and is sequestered from GSK3β, hence escapes recognition by β-TrCP(Figure 1.1.1A). Recent study by Hans Clever group has showed that β-catenin remains bound to destruction complex and is phosphorylated in Wnt on condition but Wnt signaling prevents the ubiquitination by β-TrCP, thus phosphorylated β-catenin saturates the complex for further accumulation of β-catenin in complex thereby newly synthesized β-catenin escapes the engagement in the complex (Li et al., 2012). The dephosphorylated β-catenin accumulates in the cytosol, translocates into the nucleus and interacts with LEF/TCF family of transcription factors to induce target gene expression.

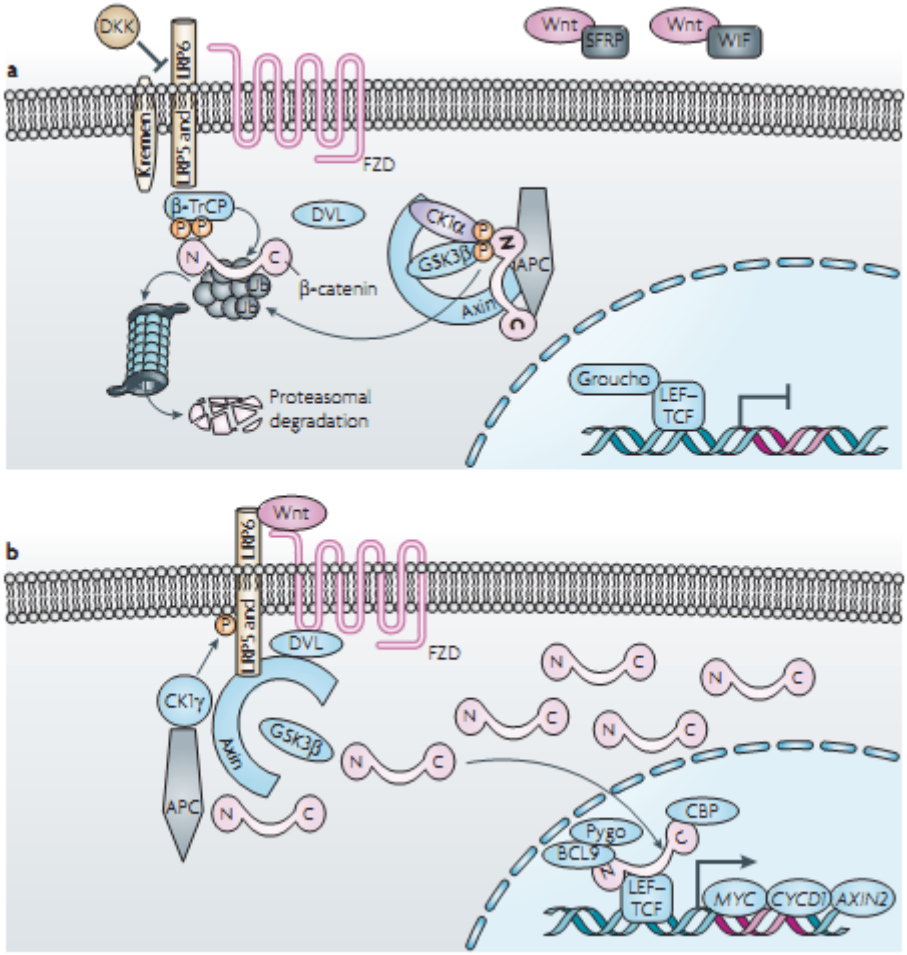


Figure 1.1.1A: Molecular changes during Wnt signaling (a) During Wnt-OFF condition frizzled and LRP receptors are inactivated by various Wnt antagonistic factors on cell membrane and inside cytosol, major driving factor β-catenin is engaged in destruction complex followed by sequential phosphorylation by CSK1α and GSK3β followed by recognition by β-TrCP ubiquitin ligase and proteosomal degradation. **(b)** During Wnt-ON condition Wnt ligands bind to LRP receptors that results in disengagement of β-catenin from Cytoplasmic destruction complex and recognition by β-TrCP. The stabilized β-catenin translocates inside nucleus interacts with TCF/LEF transcription factors to promote target gene expression (Reproduced from Klaus and Birchmeier, Nature Reviews Cancer, 2008).

The mutations or dysregulation that mediates the stabilization of β-catenin will result in aberrant activation of Wnt signaling pathway and will induce the changes associated with colorectal cancer such as mutations in APC destabilize the destruction complex required for regulation of β-catenin and for normal cellular homeostasis. Recent studies also have focused on the mutations in other components of pathway and demonstrated their critical role in colorectal tumorigenesis. For example small fractions

of colorectal cancers have mutation in N-terminus phosphorylation and ubiquitin motif making it non-responsive to destruction thereby driving aberrant activation of Wnt signaling and cellular phenotypes of colorectal cancers. Recent study conducted by cancer atlas group showed that 80% of colorectal cancers have mutation in β-catenin. Also mutations in AXIN1and AXIN2 critical for stabilization of β-catenin were observed by several studies. Similarly there were mutations in DKK family proteins, TCF7L2, SOX9 and overexpression of frizzled receptor FZD10 (Cancer and Atlas, 2012) Interestingly these mutations were observed in cancers harbouring APC mutations suggesting that aberrant activation of Wnt signaling in colorectal cancers is driven by multiple factors there by providing selective advantage for development and progression. Similarly mutations in oncogenes and tumor suppressors have been identified and predicted to be essential for initiation, progression and maintenance of colorectal cancers. Mutations in growth signaling genes such as KRAS have been linked to colorectal cancer. Approximately 40 % of colorectal cancers have mutation in KRAS (Fearon, 2011). Similarly mutation in P53 has been linked to colorectal adenomas to carcinoma transition. Selection of mutation at this point is not clear; probably to evade tumor cell stress mediated cell cycle arrest and apoptosis for continuous growth and subsequent acquisition of further changes (Fearon, 2011). Next generation sequencing has also found mutations in TGFβ signaling pathway in colorectal cancers. Thus various mutations acquired during tumorigenic transition provide selective advantage to colorectal cancer cells to survive and acquire further changes. Figure 1.1.1B summarizes the molecular changes acquired and associated with colorectal cancers. Most of the changes acquired result in aberrant activation of Wnt signaling. Thus suggesting changes that modulate Wnt signaling may be critical for colorectal tumorigenesis and also suggesting that additional layers of Wnt signaling players may be required to regulate this common theme. Recently epigenetic regulatory network has been linked with different cancers to differentially regulate oncogenes and tumor suppressors. For example Wnt antagonistic factor DKK1 has been shown to be inactivated by epigenetic changes in colorectal cancers. Thus providing mechanistic link between epigenetic modulation and Wnt signaling and providing enormous possibilities of similar such links may be required for pleotropic role of Wnt signaling in multiple cancers in context dependent manner.

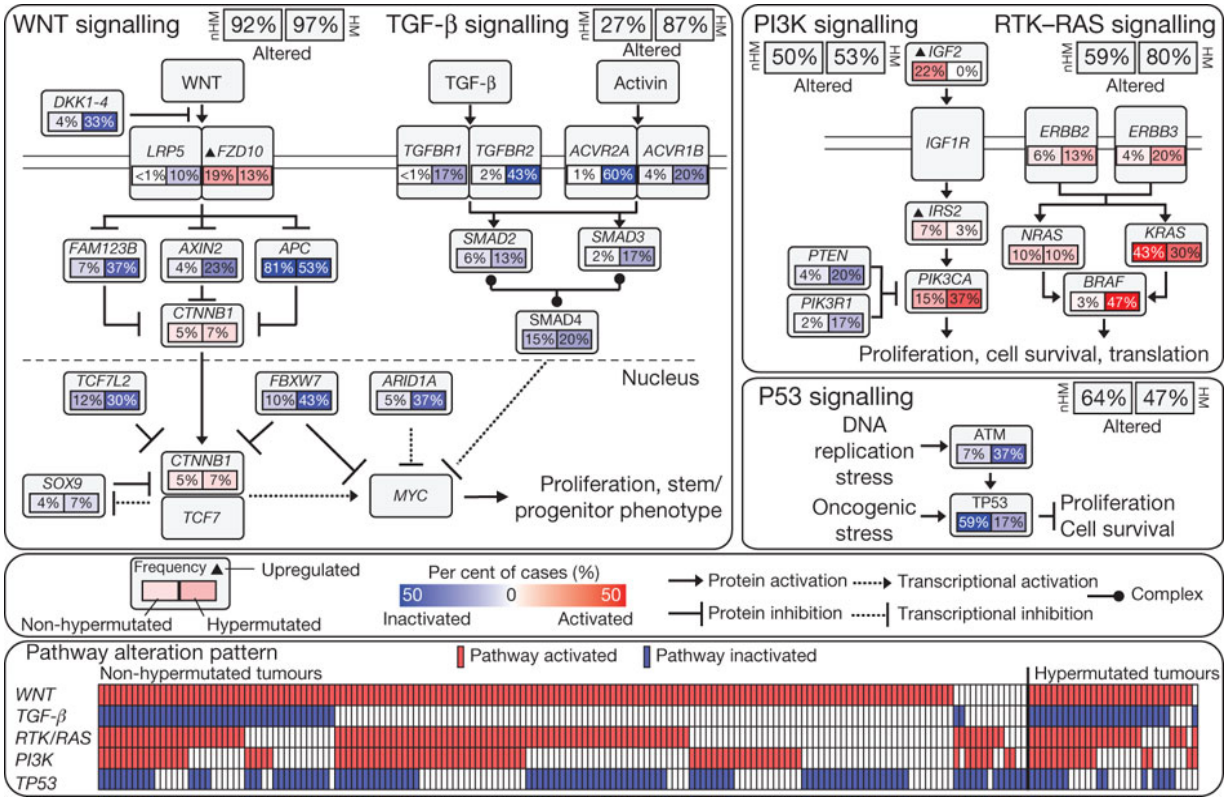


Figure 1.1.1B: The molecular changes associated with dysregulation of signaling pathways in colorectal cancer. The mutations acquired that hyperactivate the Wnt signaling pathway- major driving force for colorectal tumorigenesis**.** Similarly various growth signaling and P53 signaling pathways are also dysregulated to induce cellular changes required for colorectal tumorigenesis (Adapted from cancer Atlas 2012).

1.1.2 MicroRNAs in colorectal cancer-New pathway to tumorigenesis

MicroRNAs (miRNAs) are small non-coding RNAs which regulate the expression of genes by targeting mRNA by either mRNA degradation or translational repression (Bartel, 2009). MicroRNAs are transcribed by polymerase II into a long transcript called as pri-microRNA. Pri-microRNAs contain stem loop structures recognized by ribonuclease Drosha along with DGCR8 and processed into precursor microRNA by cropping. Precursor microRNA is further processed by DICER1 along with TARBP2 into double stranded mature microRNA. Mature miRNA is then transported into cytosol and after strand separation; guide strand is incorporated along with Argonaut into RNA induced silencing complex (RISC). The mature strand or guide strand in RISC is then targeted to target mRNAs to reduce gene expression either by translation inhibition or by mRNA degradation via binding to its 3'UTR seed sequence (Bartel, 2009). miRNAs have diverse range of targets including both tumor suppressors and oncogenes. Large number of microRNA is dysregulated during various cancers including breast cancer and colorectal cancer. Because of diverse targets and their role in modulating multiple cancer associated genes, microRNAs are considered as new drivers of tumorigenic transition and hallmark of cancers. Various functional studies have shown that miRNAs execute diverse roles by differentially regulating tumor oncogenes and tumor suppressors critical for tumorigenesis. For example miRNAs in cluster 17-92 suppress negative regulators of PI3 kinase pathway and pro-apoptotic members of BCL2 family that are known to influence cancer development (Mavrakis et al., 2010). Wnt signaling plays central and critical role in colorectal tumorigenesis and about 80% of colorectal cancers have biallelic inactivation of tumor suppressor protein APC (Cancer and Atlas, 2012). Recent study by Nagel et al shows that miRNAs are novel regulators of APC. The study shows that expression of miR135a and miR135b are upregulated colorectal adenomas and *in vitro* study suggests that miR135 reduces the levels of APC by translational inhibition(Nagel et al., 2008). miRNAs are also known to influence EGFR growth signaling (Chen et al., 2009; Johnson et al., 2005) and p53 pathways that are considered as drivers of colorectal tumorigenesis (Hermeking, 2007; Slaby et al., 2009). MicroRNAs discovery has given new dimension to the regulation of molecular changes that are critical for tumorigenesis. Molecular mechanism of regulation and

crosstalk with known mediators of tumorigenesis will be essential for therapeutic intervention in future.

1.1.3 SATB1 in tumorigenesis and metastasis

The biological events that regulate the multi-step transition from tumor initiation to metastasis have been described (Chambers et al., 2002). Tumor initiation involves the generation of unlimited proliferative potential in cells mediated by genomic alterations such as deletions and mutations, further accelerated by changes to tolerate cell division defects and genomic instability (Hanahan and Weinberg, 2011; Hanahan et al., 2000). The development of oncogenically transformed cells further leads to the acquisition of additional molecular changes that potentiate them to metastasize. Recent studies have identified chromatin organization as a key factor in regulating such events. Chromatin organizer proteins have been shown to regulate large number of genes during various developmental processes and diseases..

In 2008, Kohwi-Shigematsu and coworkers for the first time showed aberrant SATB1 expression in highly metastatic breast cancer cell lines while it is absent in normal mammary epithelial cells (Han et al., 2008). The authors examined 24 breast epithelial cell lines including normal epithelial cell line (HMEC), 5 immortalized derivatives, 13 non-metastatic cell lines and 5 metastatic cancer cell lines. The expression of SATB1 at transcript level and at protein level was detected in metastatic cancer cell lines only and hence correlating the expression pattern of SATB1 with aggressive phenotype. The expression of SATB1 was also analyzed using breast cancer tissue samples. The expression of SATB1 was found to be higher in aggressive poorly differentiated infiltrating ductal carcinomas, low level was detected in moderately differentiated tumor samples but no SATB1 was detected in normal adjacent tissues, thus providing compelling evidence that SATB1 is significantly associated with metastatic phenotype (Han et al., 2008). The prognostic significance of SATB1 expression was analyzed by Immunohistochemical analysis where nuclear staining using tissue microarray of 2197 breast cancer specimens with 5 year follow up data was determined. The tumor tissue was considered as positive for SATB1 expression based on nuclear staining. Kaplan Meier survival analysis showed correlation between higher SATB1 expression and shorter survival rate and multivariate analysis confirmed SATB1 is independent

prognostic marker for breast cancer. In establishing the role of SATB1 in breast cancer metastasis, knockdown of SATB1 in aggressive breast cancer cell line MDA-MB231 reversed the aggressive phenotype. Depletion of SATB1 reduced the proliferative potential of these cells, decreased their invasive capacity and anchorage dependent growth was restored. Knockdown of SATB1 depleted the potential of these to make undifferentiated tumors in mammary fat pads and their capacity to infiltrate to tissues was abolished. Conversely ectopic expression of SATB1 in non-aggressive cell line SKBR3 resulted in development of aggressive phenotype and enhanced their potential to make tumors, infiltrate tissues and metastasize to distant organs. The ability of SATB1 to convert the phenotype making non-aggressive cells to acquire metastatic potential raised the possibility that SATB1 might be inducing dynamic changes in the expression of metastasis associated genes that are essential for progressive accumulation of changes potentiating cells to migrate and metastasize to distant sites. Studies using microarray analysis and q-PCR expression analysis of cultured cells and mice xerographs revealed that SATB1 depletion causes dysregulation of more than 1000 genes (Han et al., 2008). SATB1 promotes breast cancer metastasis by reprogramming the expression of genes predominantly involved in cell-cell adhesion, extracellular matrix formation and cell cycle. The expression profile of genes regulated by SATB1 overlapped with 231 Rossetta poor prognosis genes (Veer et al., 2003). During metastatic progression different sets of genes are progressively expressed which are essential for initiation and propagation of metastasis (Nguyen and Massagué, 2007; Varki et al., 2009). Metastatic initiation involves the expression of genes such as SNAI1 and other EMT genes. Interestingly, SATB1 regulates these genes, which enable the transformed cell to invade surrounding tissues and also facilitate spreading of cancer cells. SATB1 also regulates metastasis progression genes such as MMP2, MMP3, MMP9 and TGFβ, which enhance the potential of cancer cells to infiltrate the distant organs. TGFβ is a prominent cytokine in tumor microenvironment (Bierie and Moses, 2006) and induces the expression of genes in breast cancer cells involved in disrupting epithelial cell contact. During tumor progression SATB1 downregulates the expression of tumor suppressor KAI1 (also known as CD82), which anchors tumor cells to endothelium and induces senescence (Bandyopadhyay et al., 2006). Similarly SATB1 regulates metastasis latency by downregulating KISS1 which has been shown to prevent reinitiation of growth of

cancer cells after infiltrating the distant organs (Nash et al., 2007). The gene expression profile thus suggests that SATB1 regulates key biological events such as metastasis initiation and metastasis progression by regulating expression of genes, which are critical for such processes. SATB1 seems to induce necessary cues for progression from primary tumor to metastasis. SATB1 directly regulates the expression of genes by binding to their promoters. SATB1 recruits histone modifying enzyme p300 on promoters of tumor oncogenes and maintains histone activation mark such as histone H3K9/14 acetylation mark and hence sustains their expression during breast cancer progression. In contrast, SATB1 recruits HDAC1 on promoters of tumor suppressor genes, depletes the activation marks and thereby reducing their expression (Han et al., 2008; Kohwi-Shigematsu et al., 2012).

The role of SATB1 in promoting breast cancer progression and metastasis has subsequently been supported by other independent studies. Multidrug resistant breast cancer cells exhibit higher expression of SATB1 and higher invasive potential than parental cell line (Li et al., 2009) SATB1 higher expression promoted multidrug resistance to the cells and depletion of SATB1 rendered the cells sensitive to various drugs (Li et al., 2009) Similarly, study by Patani et al also documented higher expression of SATB1 in advanced stage of breast cancer and expression positively correlated with increasing TNM stage (Patani et al., 2009). However, certain studies have contradicted the role of SATB1 in breast cancer (Hanker et al., 2011; Iorns et al., 2010). Based on transcript analysis of SATB1 expression in patient tumor data, Iorns et al. (2010) demonstrated that higher expression of SATB1 is not associated with poor prognosis of breast cancer. These authors also performed various in vitro and in vivo mice experiments and found no significant association of SATB1 in determining the aggressiveness of breast cancer metastatic cell lines. In response to this report, Han et al argued that tumor samples can be infiltrated with different immune cells such as activated T lymphocytes, fibroblasts and macrophages which also express SATB1 and will interfere with prognostic significance of SATB1. Therefore, Immunohistochemical or immunofluorescence analysis is very essential to score nuclear and/or sub-cellular levels of SATB1 in breast cancer tissue samples. Such studies will help in resolving the controversy surrounding the role of SATB1 in breast cancer progression and metastasis.

In the past two years the role of SATB1 in development of cancers of multiple tissues and organs has been extensively studied. SATB1 has been shown to promote various cancers including hepatocellular carcinoma (Huang et al., 2011; Kuo and Chao, 2010; Tu et al., 2012), Gastric cancer (Cheng et al., 2010; Lu et al., 2010),Bladder cancer (Han et al., 2013)], Colorectal cancer (Nodin et al., 2012; Zhang et al., 2013, 2014), Laryngeal squamous cell carcinoma (LSCC) (Zhao et al., 2010), Endometroid endometrial cancer (Mokhtar et al., 2011), Glioma cancer (Chu et al., 2012a) and Cutaneous Malignant Melanoma (CMM) (Chen et al., 2011). Immunohistochemical analysis showed that expression of SATB1 is prognostically and clinopathologically significant in CMM and gastric cancer (Chen et al., 2011; Cheng et al., 2010). Higher expression of SATB1 is associated with metastasis and Kaplar Meier analysis showed that patients with higher expression of SATB1 in gastric cancer have shorter survival rate in comparison with patients with lower expression of SATB1 [Cheng et al., 2010). Similarly, SATB1 expression was found to be higher in hepatoma tissue than adjacent non-cancerous tissue (Tu et al., 2012). SATB1 promotes the liver cancer development and metastasis by reprogramming the expression of genes particularly involved in cell cycle progression enhancing their proliferative potential (Tu et al., 2012). SATB1 potentially regulates the apoptotic pathway and thus makes liver cancer cells resistant to apoptosis. SATB1 induced the expression of EMT related genes particularly affected the expression of Snail1, Slug, Twist, Vimentin, E-cadherin, Zo1 and desmoplakin (Tu et al., 2012). In colorectal cancer SATB1 expression is significantly correlated with tumor invasion and metastasis (Meng et al., 2012; Nodin et al., 2012; Zhang et al., 2013, 2014). However, no study has been conducted to delineate the molecular mechanism of SATB1 during colorectal cancer tumorigenesis. Wnt signaling is significantly involved in colorectal cancer development and the functional overlap of SATB1 with Wnt signaling has been reported, albeit in another model system of Th2 differentiation (Notani et al., 2010a). These two findings raise the possibility that SATB1 might functionally overlap with Wnt signaling and promote colorectal cancer progression and metastasis.

Number of studies in breast cancer, gastric melanoma and liver cancer showed positive correlation of SATB1 with tumor progression. However, recent studies in non-small cell lung cancers (NSCLCs) revealed negative correlations. Zhou et al. showed lower expression of SATB1 at transcript level in NSCLCs in comparison with normal

tissues (Zhou et al., 2009) and another study showed that loss of SATB1 expression is marker of poor survival in lung cancer (Selinger et al., 2011). The expression of SATB1 is very critical in regulating the expression of large number of genes essential for T-cell development and differentiation (Alvarez et al., 2000; Burute et al., 2012; Mir et al., 2012; Notani et al., 2010). Aberrant expression of SATB1 in other cells leads to structural changes in chromatin, which is prerequisite for dynamic dysregulation of genes. The elevated expression of SATB1 enhances the potential of cancer cells to proliferate and acquire changes essential for metastasis. The molecular pathways that regulate the expression of SATB1 during cancer progression and metastasis are not yet studied to the finer details that are required to design strategies for therapeutic intervention. The critical role of SATB1 in various cancers and prognostic significance makes it a highly desired molecular target for cancer therapy.

1.2 Materials and Methods

1.2.1 Antibodies, reagents and Plasmids

SATB1, TCF7L2, TCF7, AXIN2 antibodies were obtained from Cell Signaling Technology. c-Myc, active β-catenin and vimentin antibodies were obtained from Millipore. β-catenin and E-cadherin antibodies were obtained from BD Biosciences. DVL2, DVL3 and MMP2 antibodies were from Santa Cruz Biotechnology. LEF1, N-cadherin, DKK1, SATB2, Wnt3a were obtained from Abcam. Actin and gamma-tubulin antibodies were obtained from Sigma. For ChIP analysis the anti-SATB1 was from Cell Signaling Technology. Pyrvinium pamoate and Puromycin were obtained from Sigma. G418 was procured from Roche. FLAG-SATB1 was used as described in Notani et al., 2010. For GFP fusion, SATB1 was subcloned from pCMV10-3XFLAG-SATB1. The siRNA sequences for sh1SATB1 and sh3SATB1 was designed using Dharmacon design center. The siRNA sequence for sh2SATB1 was used as described (Han et al., 2008). All shRNAs were cloned in pSUPER Puro vector (Oligoengine). TALEN constructs for knocking out SATB1 were custom designed and procured from Applied Biosystems – Life Technologies.

1.2.2 Colorectal tumor specimens

The study was approved by the Institutional Review Board (IRB) of the Tata Memorial Center, and the scientific research committee and human ethics committee of the Tata Memorial Hospital (TMH), Parel, Mumbai. Paired samples of from the colorectal cancer and adjacent normal tissues colon were obtained from patients during a routine diagnostic colonoscopy at TMH. Informed consent was provided by all patients prior to the procedure. None of the patients had received any prior systemic treatment for colorectal cancer. Tissues were snap frozen in liquid nitrogen and processed for protein extraction using RIPA buffer.

1.2.3 Cell culture, transfections and western blotting

SW480, SW1116, SW620, T84 and HCT116 cell lines were grown in DMEM with 10% FCS. COLO320, COLO205, COLO201, COLO741, HCT-15, HT-29, DLD1 cell lines were grown in RPMI with 10% FCS. CRL1790 were grown in MEM with 10% FCS. CRL1790, SW480, COLO201, COLO205, COLO320, SW620, HT-29 DLD1, HeLa and MDA-MB231 were obtained from American Type Culture Collection (ATCC). SW1116, T84, HCT116, HCT-15 were obtained from European Collection of Cell Cultures (ECACC) SIGMA. SW480 cells were seeded in 60 mm dishes for transfection to generate stable cell lines of 3XFLAG and 3XFLAG SATB1. Similarly HCT-15 cells were seeded for transfection to generate pSUPER shSATB1 stable cell lines. Forty eight hrs after transfection, cells were split and selected for puromycin resistance. After growing three weeks in antibiotic selection resistant colonies were screened and grown. Expression of SATB1 across different colorectal cancer cells, knockdown and overexpression of SATB1 were detected by immunoblotting and qPCR. For Immunoblotting 25 µg of lysate was loaded in each lane unless mentioned otherwise. For transfection with siRNA mediated depletion, cells were seeded and after 24 hours transfected with indicated siRNA and then harvested for immunoblotting and RNA extraction. The sequences of shRNA and siRNA used are listed in Table 1.2.3.

Table 1.2.3: siRNA/shRNA sequences used in this study

siRNA/shRNA	RNA interference sequence	Notes
SATB1#1(shRNA)	CTGGTAAACCTTCGGGCTA	Dharmacon design centre
SATB1#2 (shRNA)	GTCCACCTTGTCTTCTCTC	Han et al., 2008
GFP (shRNA)	GAAGCAGCACGACTTCTTC	
SATB1#3(shRNA)	GCTGAAAGAGACCGAATAT	Dharmacon design centre
SATB1	CCAGCAGTATGCAGTGAAT	(Eurogenetics)

1.2.4 RNA isolation & RT-PCRs

RNA was isolated using Trizol reagent (Invitrogen). Two µg of RNA was used for first strand cDNA synthesis using Superscript III (Invitrogen). The cDNA was then used for quantitative PCR analysis in triplicates using an ABI 7500 Fast real-time PCR System (Applied Biosystem) as described in Ordinario et al. (2012). The sequences of oligonucleotide primers used for real-time PCR and cloning are listed in Table 1.2.4.

Table 1.2.4: Sequences of primers used for qPCR analysis

qPCR Primer	Sequence	Notes
hSATB1 Forward	AGCAACAGGTTTCGACCAAC	TaqMan primer and probe (ABi)
hSATB1 Reverse	TCTGAAAGCAAGCCCTGAGT	
huMMP2 Forward	CGATAACCTGGATGCCGTC	
huMMP2 Reverse	GTCCTTCTCTAGTTCTCCAG	
huTCF7L2 Forward	ATGCTTCCATGTCCAGGTTC	
huTCF7L2 Reverse	CACTCTGGGACGATTCCTGT	
huGAPDH2 Forward	CTGCACCACCAACTGCTTAG	
huGAPDH2 Reverse	GTCTTCTGGGTGGCAGTGAT	
hu β-catenin Forward	AAGGTGTGGCGACATATGCA	
hu β-catenin Reverse	GTAATCTTGTGGCTTGTCCTCAGA	
huDKK1 Forward	GCGGCACTGATGAGTACTGC	
huDKK1 Reverse	GGCAGCACATAGCGTGACG	
huAXIN2 Forward	GTCTCCAAGCAGCTGAAGCC	
huAXIN2 Reverse	CCTCCATCACCGACTGGATC	
huCyclinD1 Forward	GCTCCTGTGCTGCGAAGTG	
huCyclinD1 Reverse	CATTTGAAGTAGGACACCGAGG	
huc-Myc Forward	GCCACAGCAAACCTCCTCAC	
huc-Myc Rverse	CTCTTGGCAGCAGGATAGTCC	

1.2.5 Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed as described (Karmodiya et al., 2012). Briefly, cells were cross-linked by addition of formaldehyde to 1% final concentration in media and incubated at room temperature for 10 min, neutralized with 125 mM Glycine. Cells were then subjected to sonication using Covaris sonicator to fragment chromatin to obtain 200–500 bp fragments. Sonicated chromatin was precleared with non-saturated beads. Precleared chromatin was incubated with specific antibodies and respective

IgG types were used as isotype controls. Next day, beads saturated with tRNA and BSA were added (40 µl packed beads) and incubated for 4 h on rocker to pull down the antibody-bound chromatin and were subjected to elution using buffer containing SDS and sodium bicarbonate. Eluted chromatin was de-crosslinked and protein was removed by treating with proteinase K. Purified immunoprecipitated chromatin was subjected to PCR amplification using specific primers. Input chromatin was used as a control. ChIP Primer details are listed in Table 1.2.5.

Table 1.2.5: Sequences of primers used for ChIP analysis

ChIP Primer	Sequence	Notes
huTCF7 F7 Forward	GAACCGAGTTGGGAGAGTCA	
huTCF7 F7 Reverse	TCCATCCCTGTGCCAATCAG	ChIP-qPCR
hu c-Myc Forward	CACAAGGGTCTCTGCTGACTCCCCC	Notani et al. 2010
hu c-Myc Reverse	GGA GGT TGC CTG CTC TCT GCC AGT C	
huTCF7 F8 Forward	CTGATTGGCACAGGGATGGA	
huTCF7 F8 Reverse	GACTTGGGCGTGGCTTAC	
huAXIN2 F7 Forward	TGTGCCTTATAAACGTTTTTGG	
huAXIN2 F7 Reverse	TGGCCATAAGACCCTCGTAG	
huAXIN2 F8Forward	CTACGAGGGTCTTATGGCCA	
huAXIN2 F8 Reverse	GCTGTCATAGGGCGGACTC	
huMMP2 Forward	GGTGCTTCCTTTAACATGCTAATGC	
huMMP2 Reverse	AGGGGCCCCGCGTTAGAGACGTT	

1.2.6 Proliferation and wound healing assay

Proliferation assay was performed for control and SATB1 knockdown stable cell lines using Cell Titre 96 AQ nonradioactive cell proliferation assay kit (Promega) as described (Zhang et al., 2004). For wound healing assay, control HCT-15 and SATB1 knockdown stable cell lines were seeded and grown to 80% confluency and wound was created by scraping the monolayer using 200 µl micropipette tip as essentially described (Liang et al., 2007). After 24 hours bright field images were acquired using Nikon Eclipse Ti microscope at 10X magnification. NIS elements BR imaging software (Nikon) was used for measurement of distance.

1.2.7 Colony formation and soft agar assay

The colony formation assay was performed essentially as described (Franken et al., 2006). Briefly, control HCT-15 and SATB1 knockdown cells were seeded in RPMI and 10% FBS (5000 cells) in 60 mm culture dish and incubated at 37°C for 14 days and then fixed with 3% PFA (in PBS) and stained with 0.05% crystal violet at room temperature for 2 h. Images of plates with colonies were acquired using digital camera.

For soft agar assay, cells (5 x 10^5) were re-suspended in DMEM containing 5% FBS with 0.3% agarose and layered on the top of 0.5% agarose in DMEM on 60 mm plates essentially as described (Ordinario et al., 2012). Cells were cultured for 14 days after which the colonies were stained with 0.05% crystal violet. Images of stained plates were acquired at 10X magnification using inverted microscope (AMG Evos).

1.2.8 *In vivo* tumor growth assay

1X10^6 SW480 cells expressing FLAG and FLAG-SATB1 were injected subcutaneously with Matrigel at 5 mg/ml in PBS in a volume of 200 µl in six NOD-SCID mice. Similarly 1X10^5 shcontrol HCT-15 cells and shSATB1 HCT-15 cells were injected. Tumor size was monitored for eight weeks in case of FLAG and FLAG-SATB1 stable cells and for four weeks in case of shcontrol and shSATB1 HCT-15 stable cells. Tumor growth was measured using Vernier caliper and tumor volume was calculated by using formula 0.5XLXW^2 where L is length and W is width. Mice were euthanized and tumor weight was calculated. For *in vivo* imaging, briefly 1x10^5 HCT-15 cells stably transfected with pSUPER puro-mcherry or pSUPER puro-shSATB1-mcherry and were injected subcutaneously into flanks of male NOD-SCID mice. *In vivo* fluorescence imaging was performed on cryogenically cooled IVIS system (Xenogen Corp.) using living image acquisition and analysis software. Images were acquired and analyzed qualitatively. All mice experiments were done according to guidelines of the animal ethics committee of experimental animal facility at the National Centre for Cell Science, Pune.

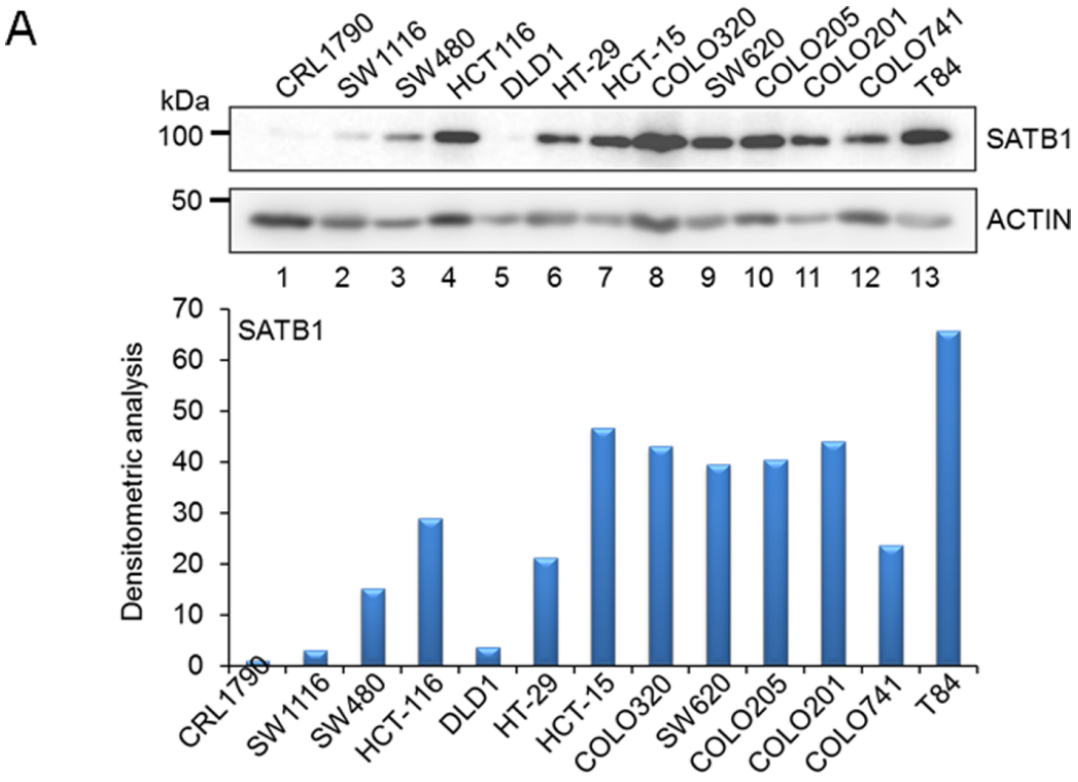
1.2.9 MicroRNA first strand synthesis and expression analysis

RNA for miRNAs expression analysis was extracted using Trizol according to manufacturer's instruction. The first strand synthesis was done using protocol developed by Varkonyi-gasic et al. Quantitative PCR was performed used Taqman based chemistry from Roche using universal probe as common for all microRNAs as described (Varkonyi-Gasic et al., 2007).

1.3 Results

1.3.1 Expression of SATB1 correlates with aggressive phenotype of colorectal cancer

To elucidate the role of SATB1 in colorectal cancer, we analyzed the expression of SATB1 in 12 colorectal cancer cell lines and in a primary colorectal cell line (CRL1790). These cell lines are classified according to Duke's classification of colorectal cancers (Dukes et al., 1932). The expression of SATB1 at protein level (Figure 1.3.1A) and at transcript level (Figure 1.3.1B) was higher in aggressive potentially metastatic cell lines (type C and D cell lines) in comparison with primary colorectal cell line (CRL1790), type A (SW1116) and type B cell line (SW480); thus correlating the expression of SATB1 with the aggressive phenotype of colorectal cancer cells as per the Duke's classification scheme (Leibovitz et al., 1976; Dukes et al., 1932). The aberrant activation of Wnt signaling is a hallmark of colorectal cancer development and various mutations in the mediators of Wnt signaling are involved in progressive development of colorectal cancers (Bienz and Clevers, 2000). To demonstrate that SATB1 expression correlates with cancer phenotype, we examined the expression of SATB1 in colorectal tissue samples. The expression level of SATB1 was significantly higher in at least 10 of 11 tumor samples in comparison with their matched normal adjacent tissues (Figure 1.3.1C). The TNM classification of colorectal tissue sample is listed in Table 1.3.1**.**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CRL1790
SW1116
SW480
HCT116
DLD1
HT-29
HCT-15
COLO320
SW620
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T84
kDa
100
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SATB1
ACTIN
1 2 3 4 5 6 7 8 9 10 11 12 13
SATB1
Densitometric analysis
70
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50
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CRL1790
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COLO320
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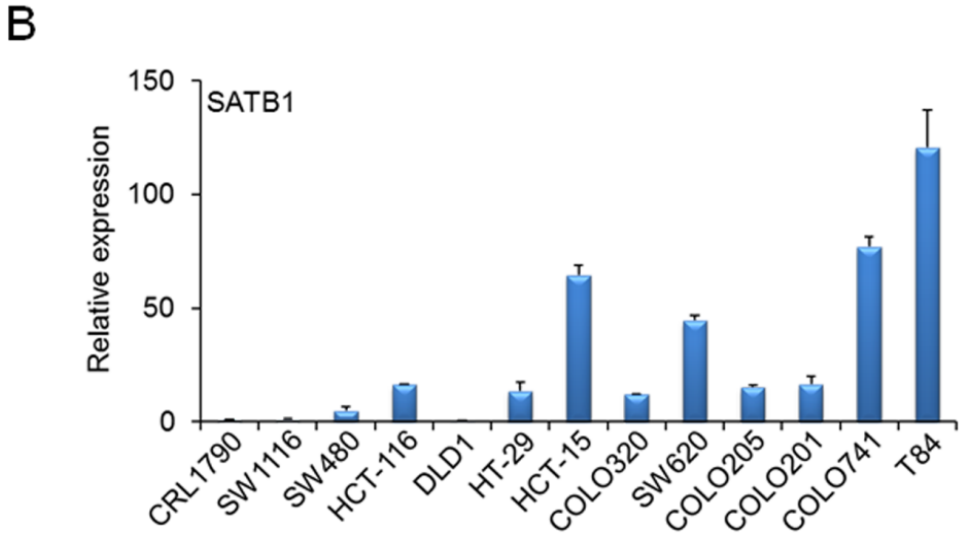
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SATB1
Relative expression
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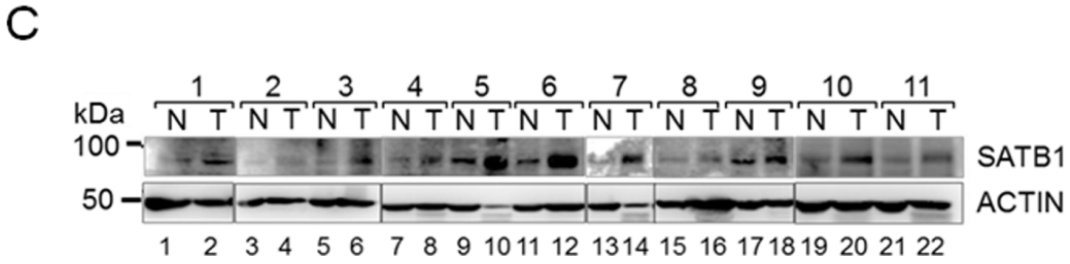
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Figure 1.3.1: Expression of SATB1 correlates with aggressive phenotype of colorectal cancer cell lines. **(A)** Immunoblot for expression of SATB1 in primary colorectal cell line (CRL1790), Type A (SW1116), Type B (SW480), Type C (HCT-15, HT-29, DLD1, COLO320), Type D and metastatic cell lines (COLO201, COLO205, COLO741 and T84). Densitometric analysis for expression of SATB1 in colorectal cancer cells normalized with Actin in comparison with CRL1790 primary cell line (lower panel). Expression of SATB1 correlates with Duke's classification and aggressive phenotype of colorectal cancers. **(B)** Relative mRNA expression levels of SATB1 in indicated colorectal cancer cell lines were determined by Taqman-qPCR. Actin was used as endogenous control. Data was normalized with that of primary cell line CRL1790. Error bars represent standard deviation calculated from triplicates. **(C)** Immunoblot for SATB1 expression in colorectal cancer tissue samples (T) in comparison with adjacent non-cancerous tissue (N) from the same patient determined by western blotting. Actin used as endogenous control.

Table 1.3.1: TNM classification of Patients' tumor samples

Tumor Sample	Staging	T-Stage	N-Stage	M-Stage
T1	II	T3	N0	M0
T2	III	T3	N1	M0
T3	III	T2	N1	M1
T4	III	T3	N2	M0
T5	III	T2	N1	M0
T6	IV	T2	N2	M1
T7	III B	T3	N2	M0
T8	III A	T2	N1	M0
T9	NA	T0	N0	M0
T10	II	T4	N0	M0
T11	IV	T2	N2	M1

1.3.2 SATB1 promotes cellular proliferation and migration

To investigate the role of SATB1 in aggressive phenotype of colorectal cancer cells *in vitro*, we knocked down SATB1 using two shRNAs (sh1 and sh2) in the aggressive cell line HCT-15. Both shRNAs independently decreased the expression of SATB1 at transcript level and at protein level (Figure 1.3.2A). Intriguingly, SATB1 depletion reduced cell growth determined by colony formation assay in SATB1 knockdown cells in comparison with control cells (Figure 1.3.2B). Further, SATB1 depletion decreased the proliferative potential of the aggressive cell line HCT-15. The proliferative potential of control cells was higher than that of SATB1 knockdown cells (Figure 1.3.2C). Next, we performed wound-healing assay to evaluate the effect of SATB1 depletion on migratory potential of HCT-15 cells. SATB1 depletion reduced the migration of HCT-15 shSATB1 cells in comparison with control cells (Figure 1.3.2D). The distance migrated by control cells was higher than SATB1 knockdown cells. Thus SATB1 knockdown reduced the proliferative capacity and migratory potential of aggressive colorectal cell line HCT-15. Depletion of SATB1 in HCT-15 cells reduced their ability to form colonies in soft agar thereby restoring their anchorage-dependent growth (Figure 1.3.2E). Collectively, these results suggest that SATB1 expression promotes anchorage independence and induces cellular proliferation.

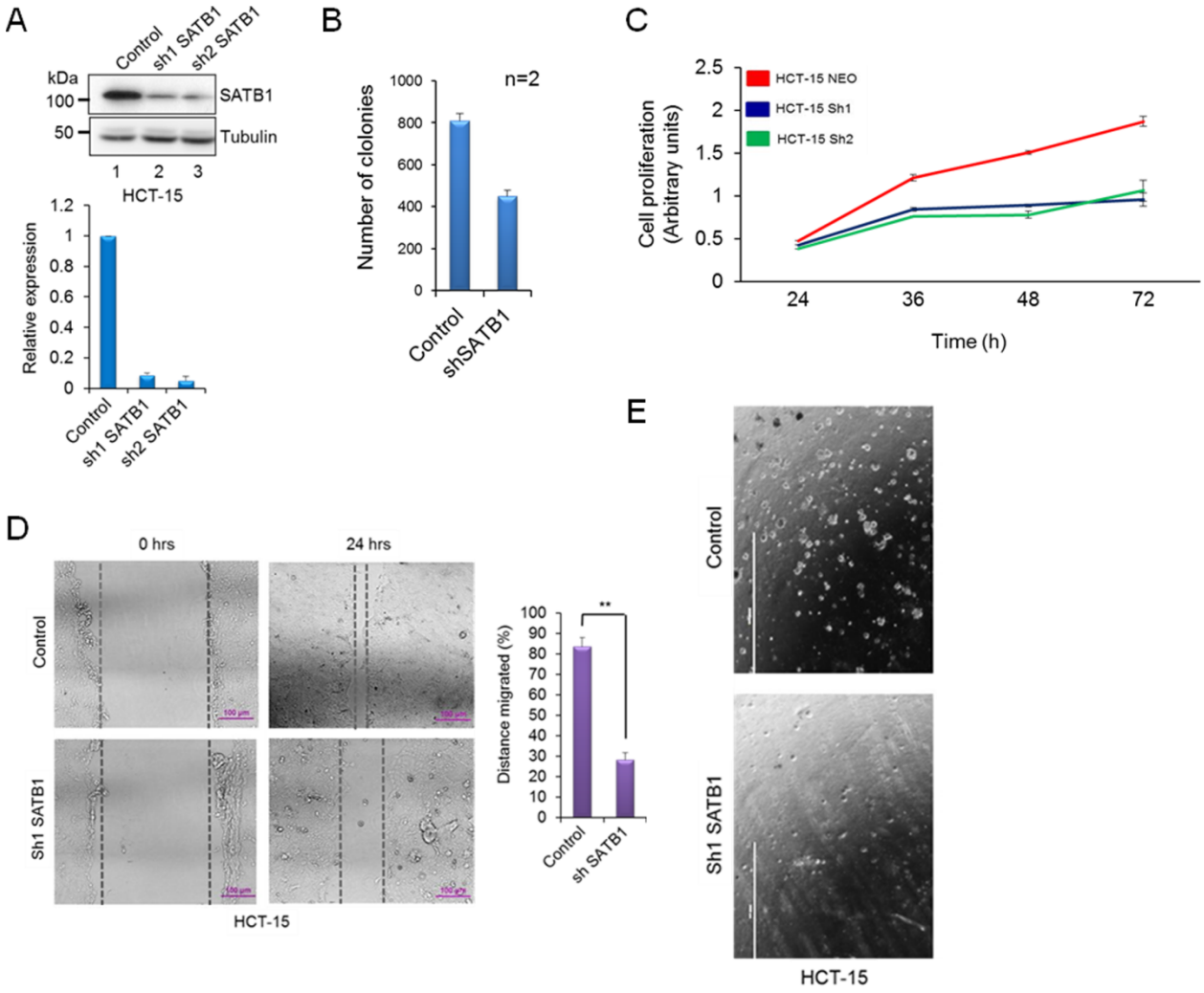


Figure 1.3.2: SATB1 promotes cellular proliferation and migration (A) Decreased expression of SATB1 in sh1 and sh2 HCT-15 stable cells in comparison with control cells determined by Immunoblotting (upper panel) and by qPCR (lower panel). Actin was used as loading control for Immunoblotting and qPCR. **(B)** SATB1 depletion reduced cell growth of SATB1 knockdown cells in comparison with control cells as determined by colony assay. The assay was done in four different 60mm dishes for control and for shSATB1 cells. **(C)** Proliferation assay in control HCT-15 and shSATB1 knockdown stable cells using non-radioactive proliferation assay kit. Depletion of SATB1 reduced the proliferative potential of SATB1 knockdown cells in comparison with control cells. **(D)** Depletion of SATB1 reduced the migratory potential of SATB1 knockdown cells in comparison with control cells. Phase contrast images of HCT-15 control cells and shSATB1 cells subject to wound healing assay in serum free condition. Images were taken at 0 h and 24 h after wounding. Graphical representation of distance migrated by control cells and shSATB1 cells (right panel). **(E)** HCT-15 and SATB1 knockdown cells were plated on soft agar and grown for 14 days. Representative images are shown.

1.3.3 SATB1 depletion reverses the tumorigenic growth *in vivo*

To further investigate the potential role of SATB1 in colorectal tumorigenesis, we performed *in vivo* tumor growth assay. We injected control HCT-15 cells and HCT-15:shSATB1 cells subcutaneously in six weeks old immunocompromised mice (SCID) and monitored the tumor growth over four weeks. Immunoblot analysis confirmed decreased expression of SATB1 upon silencing using shSATB1 construct (Figure 1.3.3A). Quantitative RT-PCR analysis confirmed decrease in expression at transcript level (Figure 1.3.3B). Depletion of SATB1 reduced the potential of HCT-15 cells to develop tumors *in vivo* (Figure 1.3.3C). To monitor the effect of SATB1 depletion on tumor growth we measured tumor volume every week for four weeks in control mice and in mice injected with SATB1 knockdown cells. Depletion of SATB1 resulted in tumor regression significantly in SATB1 knockdown cells in comparison with control cells (Figure 1.3.3D). After four weeks mice were sacrificed and tumor weight was measured. Depletion of SATB1 significantly reduced the tumor weight (Figure 1.3.3E). To monitor *in vivo* tumor growth by *in vivo* imaging in live mice, we developed mcherry expressing control and shSATB1 cell lines. *In vivo* imaging analysis revealed increased tumor growth within two weeks after subcutaneous injection in mice with control HCT-15 cells and tumor regression in mice injected with SATB1 silenced HCT-15 cells (Figure 1.3.3F).

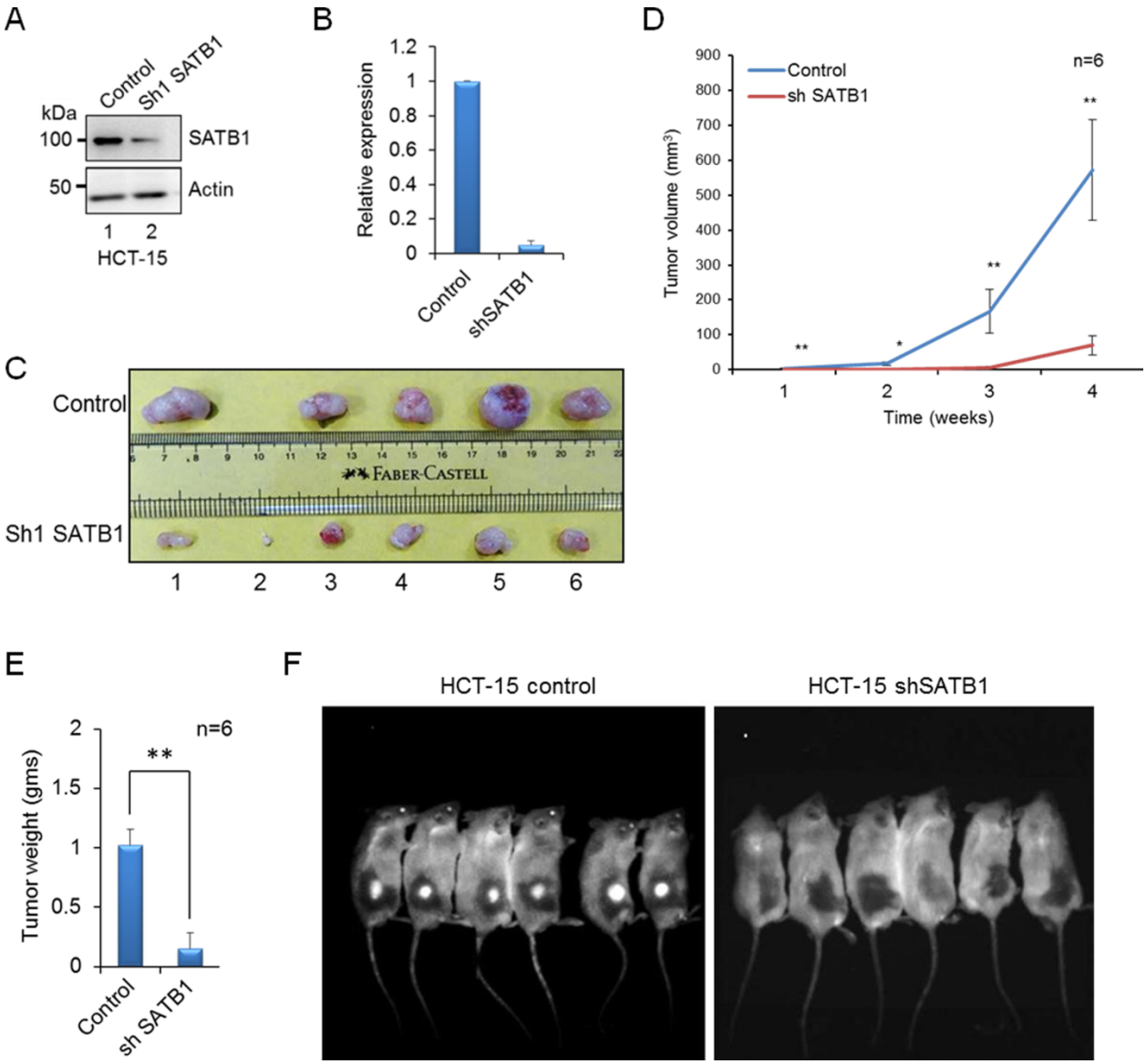


Figure 1.3.3: SATB1 depletion reverses the tumorigenic growth *in vivo.* (A) Immunoblot showing expression of SATB1 in knockdown cells in comparison with control cells. **(B)** Taqman qPCR showing expression of SATB1 at transcript in knockdown stable HCT-15 cells **(C)** Six weeks old SCID mice were injected subcutaneously with HCT-15 control cells and shSATB1 cells and tumors were monitored for 4 weeks. Depletion of SATB1 reduced the tumor growth in shSATB1 mice in comparison with control mice. **(D)** Graphical representation of tumor volume monitored for four weeks. Error bars represent S.E.M. **(E)** Tumor weight for control mice and for shSATB1 mice. Error bars represent S.E.M. **(F)** *In vivo* imaging reveals burden of tumor in control mice (panel on left side) in comparison with shSATB1 mice (panel on the right). Six mice were used for each experiment. *p<0.05, **p<0.01.

1.3.4 Overexpression of SATB1 induces tumors in vivo

To investigate whether SATB1 is sufficient to induce tumorigenesis *in vivo*, we generated SATB1 overexpression stable cells in SW480 cells, a type B cell line. Immunoblot analysis confirmed the overexpression of FLAG-tagged SATB1 in these cells and not in FLAG vector transfected control cells at protein level (Figure 1.3.4A) and by quantitative PCR at transcript level (Figure 1.3.4B). SATB1 overexpression and control vector stable cells were injected subcutaneously in SCID mice. Injection of control SW480 cells subcutaneously in SCID mice did not result in significant tumor formation *in vivo* whereas injection of SATB1 overexpressing SW480 cells induced tumors in all mice (Figure 1.3.4C). Thus, increased expression of SATB1 in SW480 cells induced their potential to promote *in vivo* tumorigenesis. The tumor volume and tumor weight showed significant increase upon SATB1 overexpression (Figure 1.3.4D and Figure 1.3.4E). Thus SATB1 is sufficient to transform the non-aggressive colorectal cells to promote tumorigenesis *in vivo*.

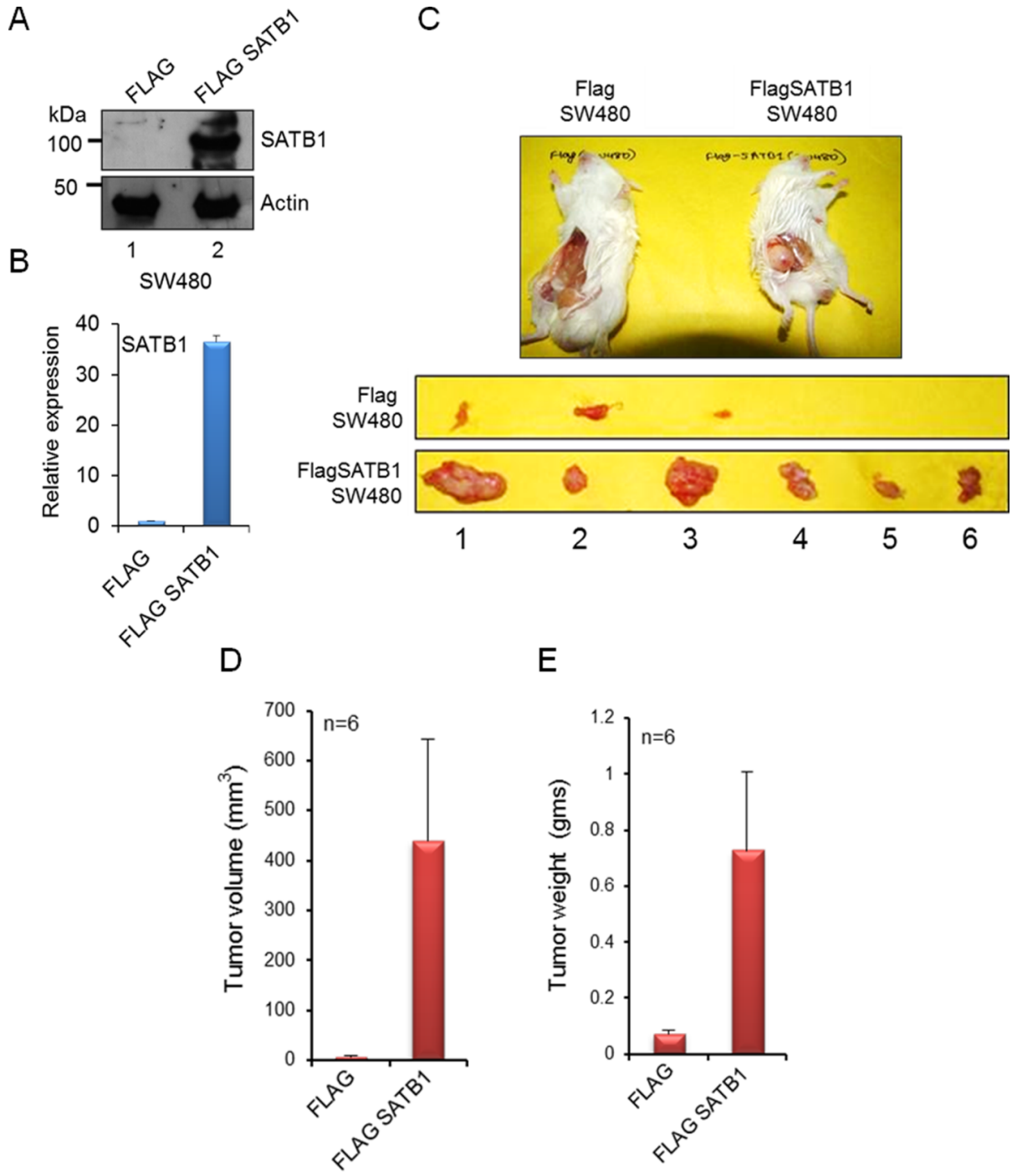


Figure 1.3.4: SATB1 promotes tumorigenesis *in vivo*.(A) Immunoblot showing expression of SATB1 in FLAG SW480 cells and FLAG SATB1 SW480 cells. **(B)** Taqman-qPCR showing relative levels of SATB1 at transcript level in control cells and in SATB1 overexpressing cells. Actin was used as endogenous control for Immunoblot and qPCR. **(C)** Six week old SCID mice were subcutaneously injected with FLAG SW480 cells and FLAG-SATB1 SW480 cells and tumor growth was monitored for 8 weeks. Overexpression of SATB1 in SW480 cells induced the tumorigenesis in comparison with control. **(D)** Tumor volume and **(E)** Tumor weight measured after 8 weeks in control mice injected subcutaneously with FLAG SW480 cells and mice injected with FLAG-SATB1 SW480 cells. Error bars represent S.E.M.

1.3.5 SATB1 modulates Wnt signaling in colorectal cancer cells

Aberrant Wnt signaling is a hallmark of colorectal cancers - it provides the essential molecular changes to potentiate colorectal cells to undergo tumorigenic transition (Bienz and Clevers, 2000) . Since our results suggested that SATB1 is involved in promoting the aggressive phenotype of colorectal cancer cells and *in vivo* tumorigenesis, we thought to investigate whether SATB1 potentially modulates Wnt signaling. We addressed the role of SATB1 in Wnt signaling by analyzing the expression of mediators and downstream targets of Wnt signaling upon SATB1 depletion. SATB1 depletion decreased the expression of β-catenin, c-Myc, DVL2 and DVL3 in SATB1 knockdown cells in comparison with control cells (Figure1.3.5A). Interestingly, SATB1 depletion also resulted in decrease of expression of TCF7L2 and Wnt downstream targets AXIN2 and TCF7 at protein level, thus providing evidence that SATB1 regulates the expression of very essential players of Wnt signaling (Figure 1.3.5B). Downregulation of SATB2 expression has been shown to be associated with poor colorectal cancer prognosis (Wang et al., 2009). We therefore monitored the expression of SATB2 and observed that SATB1 depletion increased the expression of SATB2 (Figure 1.3.5B). Thus, SATB1 depletion results in the downregulation of key players of Wnt signaling and also induces SATB2 expression.

To further validate the effect SATB1 on Wnt signaling and assess whether SATB1 is sufficient to modulate Wnt signaling, we developed SATB1 silenced stable cell line in HCT-116 and overexpressed SATB1 transiently in primary cell line CRL1790. β-catenin is a major downstream player in Wnt signaling and TCF7L2 drives the effects of Wnt signaling by binding to Wnt response elements in promoters of downstream targets. Depletion of SATB1 resulted in marked reduction in the levels of β-catenin, active β-catenin, DVL2, DVL3 and Wnt3A and had drastic effect on expression of TCF7L1 and TCF7L2 in HCT-116 cells (Figure 1.3.5C). Similarly siRNA mediated knockdown of SATB1 resulted in decreased expression of β-catenin, DVL2, DVL3 and expression of TCF7L2 (Figure 1.3.5D). Furthermore, knockdown using a different shRNA for SATB1 and knockout of SATB1 using TALENs targeted against SATB1 in HCT116 resulted in drastic downregulation of TCF7L2 and downstream targets AXIN2 and TCF7 (Figure 1.3.5E). We next monitored the expression of downstream targets of Wnt signaling such as c-Myc, AXIN2, DKK1 and cyclinD1 at transcript level. The depletion of SATB1 reduced the expression of Wnt signaling targets (Figure 1.3.5F).

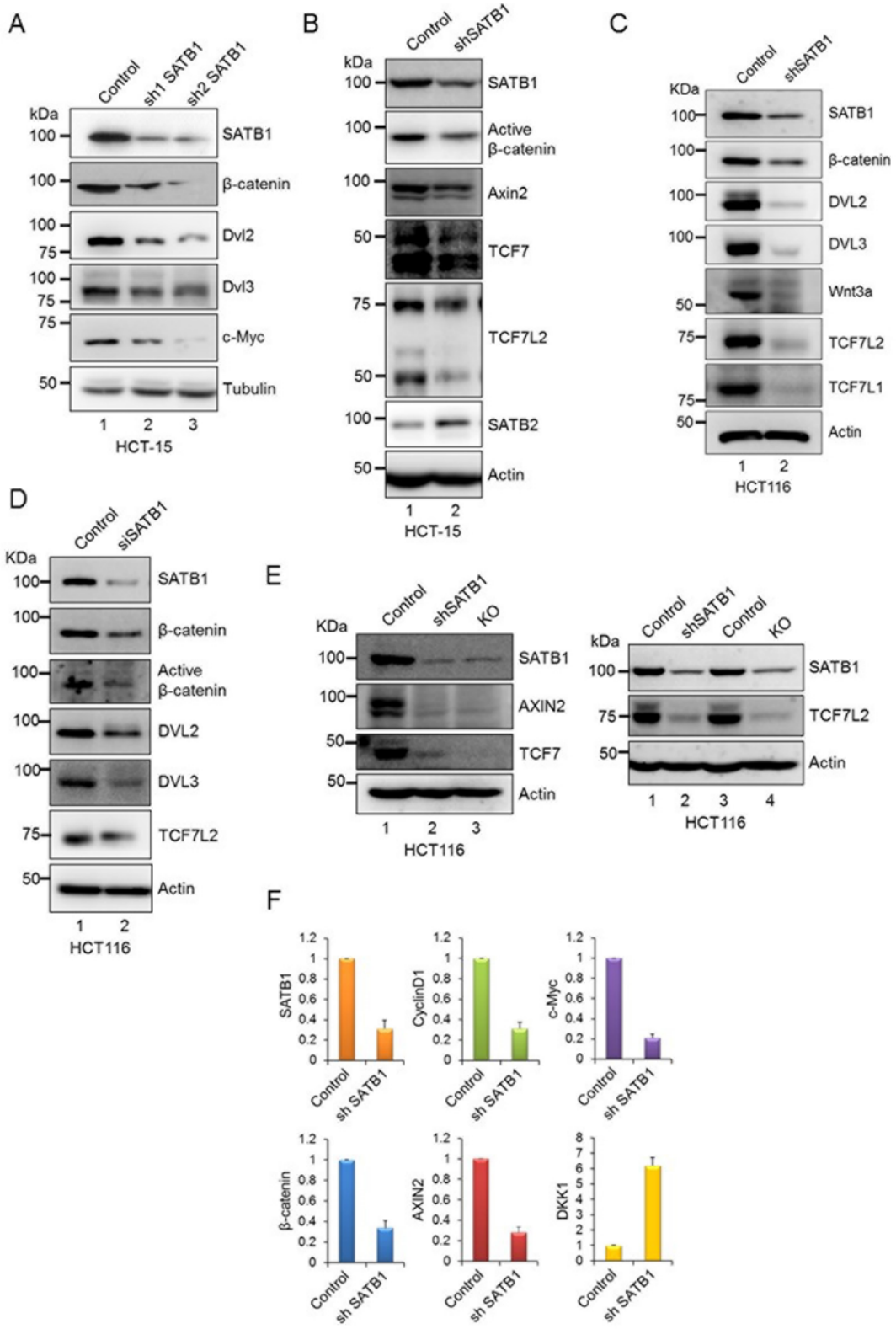


Figure 1.3.5: SATB1 modulates Wnt signaling in colorectal cancer cells (A) Immunoblot showing decreased expression of SATB1 in HCT-15 sh1 and sh2 SATB1 knockdown stable cells in comparison with control HCT-15 cells. Knockdown of SATB1 reduced the expression of DVL2, DVL3, β-catenin and c-Myc in comparison

with control cells. γ-tubulin used as loading control **(B)** Immunoblot for expression of active β-catenin, TCF7, TCF7L2 and SATB2 under SATB1 knockdown. Actin used as loading control. **(C)** Immunoblot for expression of SATB1 in control and shSATB1 HCT116 cells. Depletion of SATB1 reduced the expression of β-catenin, DVL2, DVL3, Wnt3A and TCF7L2. **(D**) Immunoblot showing expression of SATB1, β-catenin, active β-catenin, DVL2, DVL3 and TCF7L2 in control cells and in siSATB1-HCT116 cells. Actin was used as endogenous control. **(E)** SATB1 knockout cells (KO) were generated using TALEN technology. However, after transfection of the TALEN construct the cells were not subjected to selection of clones (as described in Sanjana et al 2012) and hence the polyclonal mixture of cells yielded only partial depletion in SATB1 levels. Expression of SATB1, AXIN2 and TCF7 in SATB1 knockout and under shRNA mediated stable knockdown of SATB1 was monitored by Immunoblot analysis (left panel). Right panel represents Immunoblot showing expression of TCF7L2 and SATB1 under SATB1 knockdown and knockout conditions. **(F)** Relative expression of Wnt signaling downstream targets upon SATB1 knockdown in HCT116 determined by qPCR.

1.3.6 SATB1 expression induces changes associated with Wnt/β-catenin signaling

Next we wished to analyze the potential changes driven by SATB1 expression in primary colorectal cell line CRL1790. The overexpression of SATB1 in primary cell line induced the expression of β-catenin (Figure 1.3.6A). The overexpression of SATB1 also induced the expression of Wnt signaling downstream target AXIN2 and abolished the expression of DKK1 (Figure 1.3.6A). DKK1 negatively regulates Wnt signaling and has been shown to be downregulated in colorectal cancers (González-Sancho et al., 2005; Aguilera et al., 2006). The increased expression of SATB1 also induced the expression of Wnt signaling mediators such as DVL2, DVL3 and downstream targets such as c-Myc (Figure 1.3.6B). Next we wished to see whether SATB1 expression induces the Wnt signaling changes at transcript level. The increased expression of SATB1 induced the expression of AXIN2, c-Myc and CyclinD1 at transcript level (Figure 1.3.6C). Thus increased SATB1 expression seems to be an essential event during colorectal tumorigenesis and plays critical role in Wnt signaling. Collectively, these results suggest that SATB1 regulates key players of Wnt signaling and increase in its expression could serve as a critical molecular event for induction of colorectal tumorigenesis.

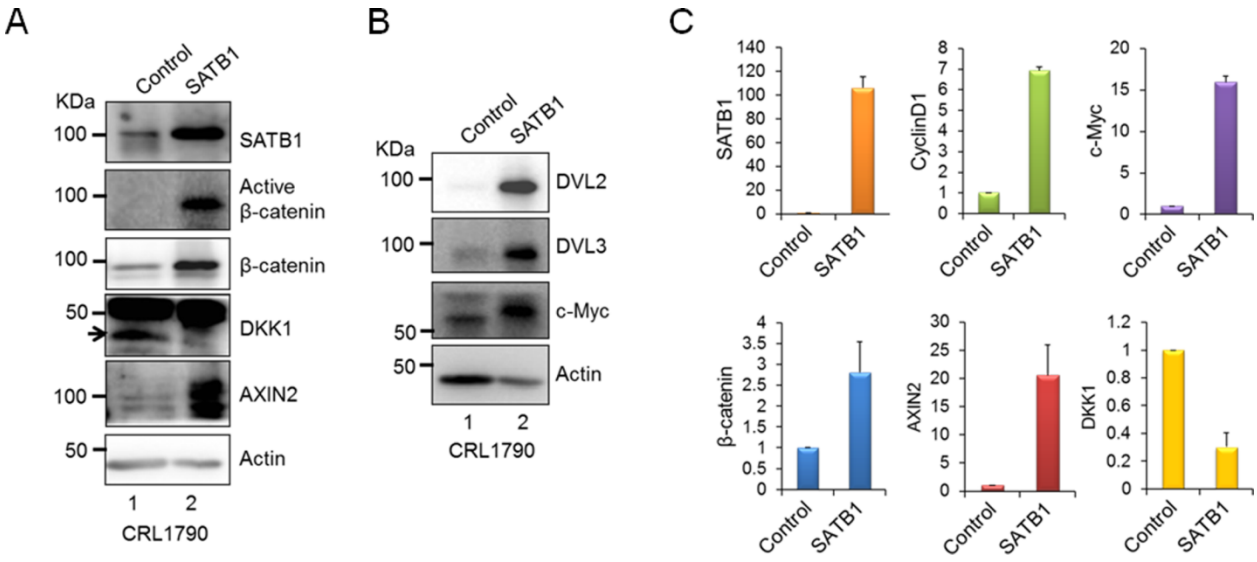
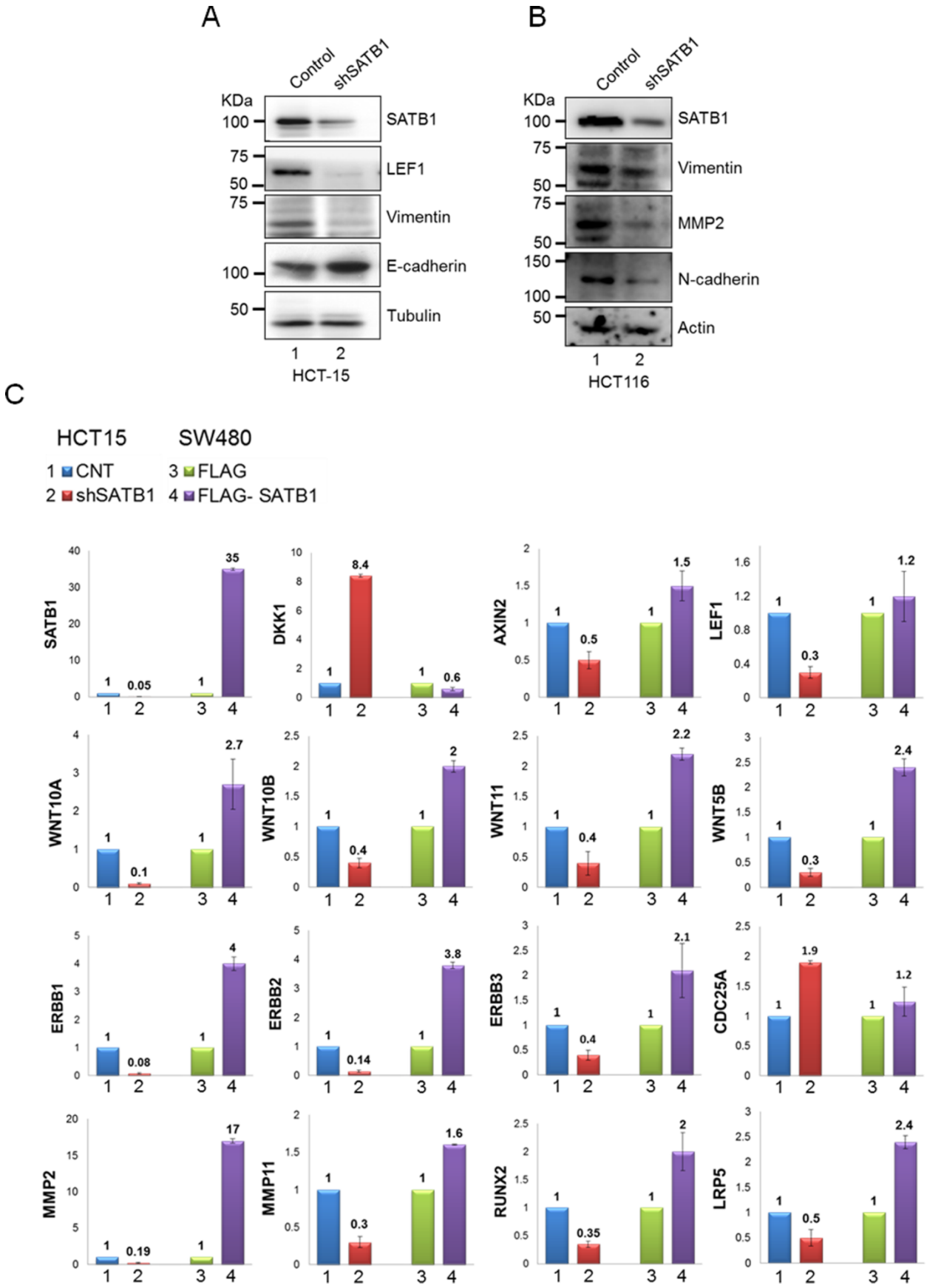


Figure 1.3.6: SATB1 expression induces changes associated with Wnt/β-catenin signaling (A) Immunoblot for expression of SATB1 in CRL1790 transfected with FLAG and FLAG SATB1. Expression of β-catenin and AXIN2 was induced upon SATB1 overexpression. Expression of DKK1 was reduced upon SATB1 overexpression. Actin was used as loading control. **(B)** Immunoblot showing increase in expression of DVL2, DVL3 and c-Myc upon overexpression of SATB1 in CRL1790 colorectal primary cells. Actin was used loading control. **(C)** Relative expression of Wnt signaling downstream targets under SATB1 overexpression in CRL1790. Data was normalized using GAPDH2 as endogenous control (error bar represents standard deviation from triplicates).

1.3.7 SATB1 is required for maintenance of mesenchymal phenotype and regulation of cancer associated genes in colorectal cancer cells

To investigate whether SATB1 regulates the molecular changes essential for tumor progression, we analyzed the expression of EMT markers. Depletion of SATB1 decreased the expression of markers essential for mesenchymal phenotype such as LEF1 and Vimentin while induced the expression of E-Cadherin essential for epithelial phenotype (Figure 1.3.7A). Similarly, the knockdown of SATB1 in HCT116 resulted in decreased expression of MMP2, vimentin and N-cadherin (Figure 1.3.7B). SATB1 expression has been shown to be critical event and essential for switching the molecular signatures to induce breast cancer tumorigenesis and metastasis (Han et al., 2008). We next analyzed the expression of various cancer-associated genes and Wnt signaling mediators under SATB1 knockdown and overexpression conditions quantitatively using customized Taqman low density gene array (TLDA). SATB1

depletion reduced the expression of epidermal growth signaling genes such as ERBB2, ERBB3, and matrix metalloproteases such as MMP2, MMP11 which degrade ECM and are involved in tumor invasion, whereas SATB1 overexpression induced the expression of these genes (Figure 1.3.7C). SATB1 also seems to be essential for expression of Wnt ligands (Figure 1.3.7C). The results of quantitative transcript profiling suggested that SATB1 negatively regulates the Wnt signaling antagonist DKK1 (Figure 1.3.6A, Figure 1.3.7C). Collectively, these results indicate that SATB1 is essential for regulating key Wnt signaling events during colorectal cancer development. SATB1 hyperexpression presumably leads to accumulation of molecular changes including EMT that are essential to induce the tumorigenic phenotype of colorectal cells. SATB1 depletion reverses the mesenchymal phenotype of colorectal cancer cells and cancer associated gene expression profile.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Control
shSATB1
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100
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LEF1
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Vimentin
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Control
shSATB1
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SATB1
75
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Vimentin
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MMP2
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N-cadherin
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Actin
1 2
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HCT15
SW480
1 CNT
2 shSATB1
3 FLAG
4 FLAG- SATB1
SATB1
DKK1
AXIN2
LEF1
WNT10A
WNT10B
WNT11
WNT5B
ERBB1
ERBB2
ERBB3
CDC25A
MMP2
MMP11
RUNX2
LRP5

Figure 1.3.7: SATB1 is required for maintenance of mesenchymal phenotype and regulation of cancer associated genes in colorectal cancer cells (A) Immunoblot for expression of SATB1, Vimentin, LEF1 and E-cadherin in control HCT-15 cells in comparison with shSATB1 HCT-15 cells. **(B)** Immunoblot for expression of SATB1, Vimentin, MMP2 and N-cadherin in control HCT116 cells in comparison with sh SATB1 HCT116 cells. **(C)** Relative expression levels of cancer associated genes and various effectors of Wnt signaling under SATB1 knockdown in HCT-15 CRC and upon overexpression in SW480 CRC were evaluated by quantitative PCRs using Taqman low density arrays (TLDA). 18S RNA and Actin were used as endogenous controls. The box provides the key for all bars and y-axis represents fold change in gene expression normalized with the controls (Bars 1 and 3 respectively). Names of genes are indicated on the left side of each graph.

1.3.8 SATB1 regulates oncogenic microRNAs in colorectal cancer

MicroRNAs are small non-coding RNAs which regulate the expression of genes by targeting mRNA by either mRNA degradation or translational repression (Lujambio and Lowe, 2012). MicroRNAs have diverse range of targets including both tumor suppressors and oncogenes. Large number of microRNA is dysregulated during various cancers including breast cancer and colorectal cancer. Recently role of diverse microRNAs has been implicated in various stages of cancer development and progression. Also crosstalk of various pathways during colorectal cancer development with microRNAs has been studied recently (Liu and Chen, 2010). MicroRNAs has been shown to play critical role in regulation of APC and hence essential for progression of colorectal cancer. Similarly various microRNAs have been implicated in pathways that are considered as drivers of colorectal tumorigenesis (Liu and Chen, 2010). Recent study by Yang et al has shown that colorectal cancer associated microRNA miR31 negatively regulates SATB2 by binding to its 3'UTR (Yang et al., 2013). SATB2 has been known to be downregulated in colorectal cancers and essential for good prognosis of colorectal patients (Wang et al., 2009). Our study for the first time shows SATB1 negatively regulates SATB2 and hence raises the possibility that SATB1 might be exerting its diverse effects on downstream targets through microRNAs regulation.

To delineate the diverse roles of SATB1 in colorectal cancer development and possible mechanism of regulation of SATB1 target genes, we analyzed the expression of known oncogenic microRNAs in colorectal cancer cells (Liu and Chen, 2010) under SATB1 depletion in HCT-15 and upon SATB1 overexpression in SW480 cells.

Depletion of SATB1 in HCT-15 colorectal aggressive cells reduced the expression of oncogenic microRNAs (oncomiRs) such as miR21, miR31 and miR10b whereas SATB1 overexpression in SW480 nonaggressive colorectal cancer cells induced their expression (Figure 1.3.8A). We also analyzed the expression of tumor suppressor microRNAs under SATB1 depletion and overexpression but no change was observed in their expression (Figure 1.3.8B). Thus, suggesting that SATB1 specifically regulates oncogenic microRNAs essential for colorectal cancer development and progression. Next we wished to check expression the expression of miR31 target SATB2 under SATB1 depletion. Depletion of SATB1 HCT-15 colorectal cells drastically upregulated the SATB2 expression (Figure1.3.8C). Thus data suggests that SATB1 is essential for induction of miR31 and also reduces the expression of its target. To test that SATB1 exerts its regulation on SATB2 via regulation of miR31, we depleted SATB1 and upregulated miR31 to see whether it can rescue the effect of SATB1 depletion. The depletion of SATB1 induced the expression of SATB2 and downregulated the expression of miR31 whereas re-expression of miR31 was sufficient to recapitulate or phenocopy the effect of SATB1 expression on SATB2 regulation (Figure 1.3.8D and E). We also checked the expression of SATB2 under inhibition of miR31 using specific inhibitors. The inhibition of miR31 was sufficient to induce the expression of SATB2, indicating that SATB 1 mediates the regulation of SATB2 via miR31 expression. Next, we analyzed the expression of downstream target of miR9 under SATB1 depletion in colo320 cells. The depletion of SATB1 reduced the expression of miR9 whereas resulted in increase in expression of E-cadherin (Figure1.3.8F). Further we show that SATB1 regulates miR10b expression in colorectal cancer cells, we wished to see whether SATB1 directly binds to its promoter. Transfac analysis predicted the binding of SATB1 on miR10b promoter and ChIP was performed in control SW480 cells and in SATB1 overexpressing cells. ChIP analysis reveals the binding of SATB1 upon overexpression and this binding to miR10b promoter was reflected in upregulation of miR10b expression (Figure 1.3.8G).

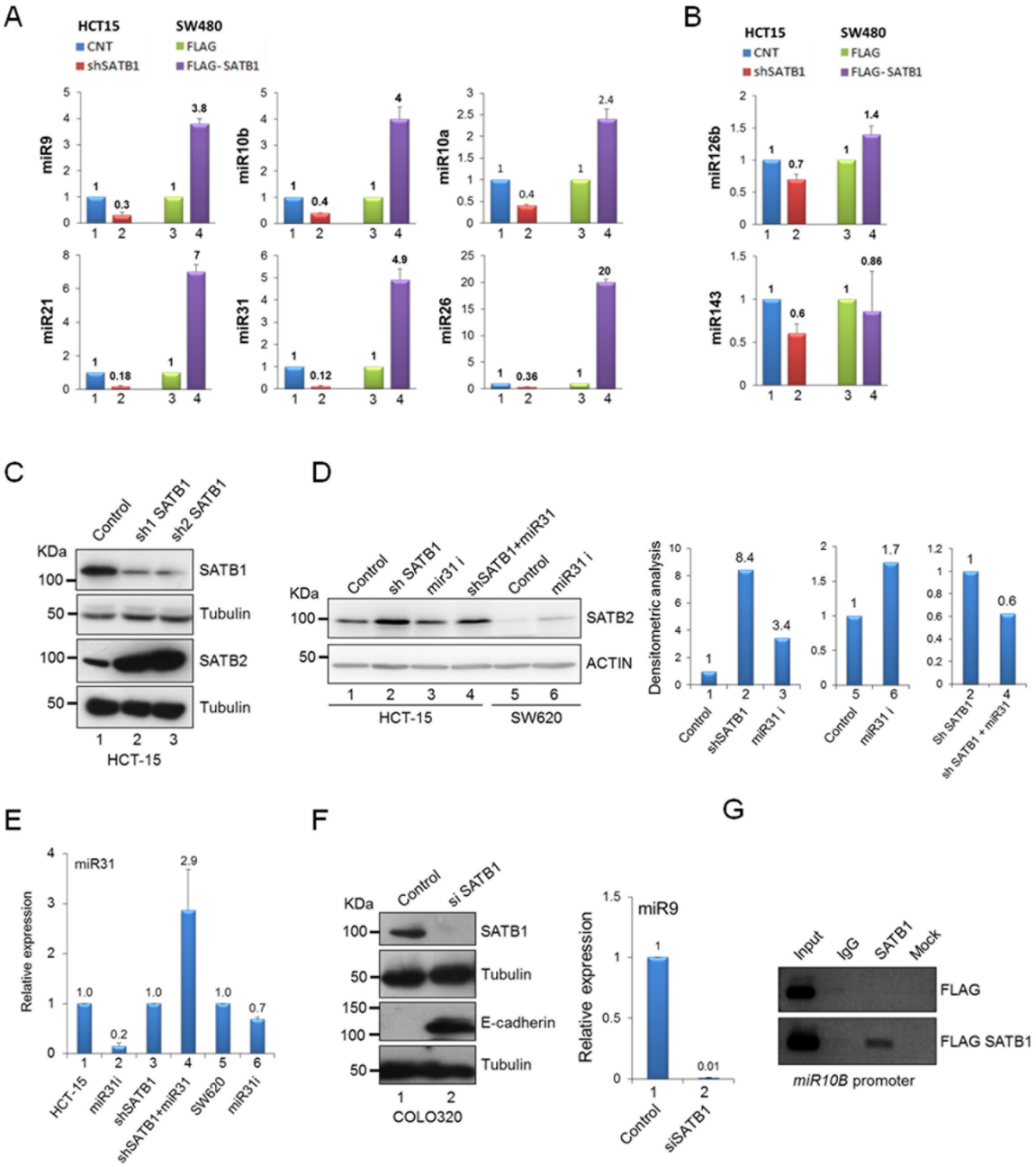

Figure 1.3.8: SATB1 regulates oncogenic microRNAs in colorectal cancer. (A) Taqman gene specific qPCR for expression analysis of oncomiRs under SATB1 depletion in HCT-15 aggressive cells and upon overexpression of SATB1 in SW480 cells. U6 was used as endogenous control. (B) Taqman gene specific qPCR for expression analysis of tumor suppressor miRs such as miR143 and miR145 under SATB1 depletion in HCT-15 aggressive cells and upon overexpression of SATB1 in SW480 cells. U6 was used as endogenous control. Error bar represents standard deviation of three replicates. (C) Immunoblot showing expression of SATB1 and SATB2 in control HCT15 cells and SATB1 knockdown HCT-15 sh1 and sh2 cells. Tubulin was used as endogenous control. (D) Immunoblot for SATB2 in SATB1 depleted cells (compare lane 1 with lane 2), upon overexpression of miR31 in SATB1 depleted cells (compare lane lane 2 and lane 3), upon transfection with miR31

inhibitor (lane 4) in HCT-15 cells and upon transfection of miR31 inhibitor in SW620 (lane 5 and lane 6). Actin used as endogenous control. Right panel showing densitometry of Immunoblot for Figure 1.3.8 D left panel. (E) Taqman gene specific qPCR for miR31 in HCT-15 cells under SATB1 depletion, upon miR31 overexpression in HCT-15 SATB1 knockdown cells and under miR31 inhibition in HCT-15 and SW620 cells. U6 was used as endogenous control for qPCR. (F) Left panel: Immunoblot for expression of SATB1 and E-cadherin in control and SATB1 knockdown COLO 320 cells. Tubulin was used as endogenous control. Right panel: Taqman qPCR for expression of miR9 in control and SATB1 depleted COLO 320 colorectal cancer cells. (G) ChIP-PCR for enrichment of SATB1 on miR10B promoter in FLAG SW480 and FLAG-SATB1 SW480 cells. ChIP was performed using anti-SATB1. IgG was used as isotype control.

1.3.9 SATB1 regulates β-catenin/TCF7L2 mediated transcription

Since the data here suggests that SATB1 regulates the expression of critical players of Wnt signaling, we wished to monitor the regulation of downstream targets of Wnt signaling and analyzed whether SATB1 directly binds to the promoters of cancer-associated genes and Wnt signaling downstream targets. In continuation with effects of SATB1 depletion on regulation of TCF7L2 and β-catenin and regulation of downstream targets of Wnt signaling (Figures 1.3.6B, 1.3.6C); we wished to analyze the occupancy of SATB1 on promoters of the downstream targets of Wnt signaling and cancer-associated genes. However, since downstream targets of Wnt signaling are regulated by TCF7L2/β-catenin, we wished to analyze the occupancy of β-catenin on promoters of the downstream targets of Wnt signaling in control cells and SATB1 knockdown cells. Previously it was shown that SATB1 interacts with β-catenin and recruits it to c-Myc promoter during Th2 differentiation (Notani et al., 2010). Consistent with this since colorectal cancer cell lines have aberrant activation of Wnt signaling, we analyzed the co-occupancy of SATB1 and β-catenin on promoters of the downstream targets of Wnt signaling in control and SATB1 knockdown cells by ChIP assay. ChIP analysis revealed that SATB1 binds to the promoters of *c-Myc* and *MMP2* and the occupancy of SATB1 on *c-Myc* and *MMP2* promoters was abrogated upon SATB1 depletion (Figure 1.3.9A). Interestingly, under these conditions the occupancy of β-catenin on these promoters was also abrogated (Figure 1.3.9A). Similarly, ChIP analysis further reveals that SATB1and β-catenin bind to *Tcf7* and *Axin2* promoters and the occupancy of SATB1 and β-catenin on *Tcf7* and *Axin2* promoters was abrogated upon SATB1 depletion (Figure 1.3.9B and 1.3.9C). Next we wished to determine whether SATB1binds to *Tcf7l2* promoter and regulates its

expression. The ChIP analysis confirms that SATB1 binds to *Tcf7l2* promoter along with β-catenin and occupancy of both SATB1 and β-catenin is lost upon SATB1 depletion (Figure 1.3.9D) Next we wished to see whether loss of SATB1 occupancy on promoters of Wnt responsive is responsible for their decreased expression, we analyze the expression of Wnt responsive genes in SATB1 knockdown cells. The reduced occupancy of SATB1 was reflected in the decreased expression of these downstream targets of Wnt signaling and expression of TCF7L2 (Figure 1.3.9E). Thus these results suggest that SATB1 is an important player in promoting colorectal tumorigenesis by modulating Wnt signaling through regulation of TCF7L2 and β-catenin. Additionally, SATB1 directly binds to the promoters of downstream targets of Wnt signaling and therefore governs the outcome of Wnt signaling in this newly discovered manner.

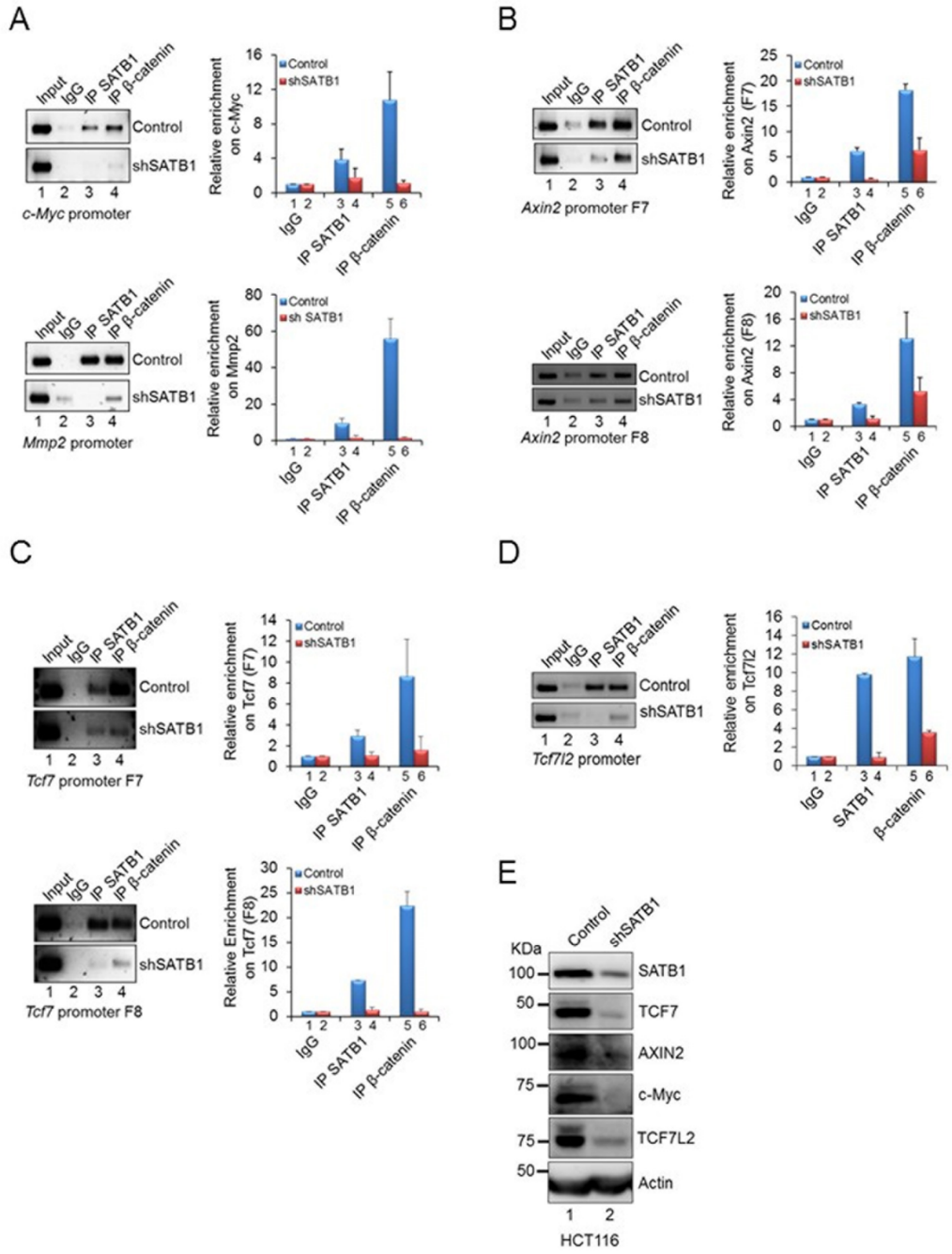

Figure 1.3.9: SATB1 regulates β-catenin/TCF7L2 mediated transcription (A) ChIP- PCR to show occupancy of SATB1 and β-catenin on promoters of *Mmp2* and *c-Myc* (left panel) and ChIP qPCR for relative enrichment (right panel). **(B and C)** ChIP-PCR to show occupancy of SATB1 and β-catenin on promoters of *Axin2* and *Tcf7* (left panel) and ChIP-qPCR for relative enrichment (right panel). ChIP was performed in control HCT116 cells and in shSATB1 HCT116 cells using antibodies against IgG, β-catenin and SATB1 followed by ChIP-PCR and ChIP-qPCR using primers corresponding to the promoters of *Mmp2*, *c-Myc*, *Axin2* and *Tcf7*. **(D)** ChIP-PCR to show occupancy of SATB1 and β-catenin on promoter of *Tcf7L2* (left panel) and ChIP-qPCR for relative enrichment (right panel) **(E)** Immunoblot showing expression of AXIN2, TCF7,TCF7L2 and c-Myc under SATB1 depletion in HCT116 cells. Actin was used as loading control.

1.4 Discussion

Aberrant activation of Wnt/ β-catenin signaling is a major driving force in colorectal cancer, but in addition to genetic mutations in Wnt/ β-catenin signaling, epigenetic events have also been shown to be involved in regulation of Wnt signaling e.g. Wnt signaling factor 1, DKK1 (Aguilera et al., 2006; González-Sancho et al., 2005) and DACT3 have been reported to involve epigenetic changes in colorectal cancer development (Jiang et al., 2008). The regulatory networks that potentially modulate and functionally overlap with Wnt signaling have opened new dimensions to the role of Wnt signaling in cancer development. The novel targets of Wnt signaling identified recently have been shown to modulate Wnt signaling and potentiate the cellular changes essential for tumor progression (Stemmer et al., 2008; Yook et al., 2006). Snail1 has been shown to be regulated by AXIN – GSK3β axis in Wnt-dependent manner (Yook et al., 2006). Snail1 functionally overlaps with Wnt signaling and modulates transcriptional outcome of Wnt signaling (Stemmer et al., 2008). A relevant example of cooperative action and crosstalk of Wnt signaling with nuclear receptor LRH1 has recently been studied (Botrugno et al., 2004; Wagner et al., 2010). The novel targets of Wnt signaling have also been shown to promote tumorigenesis (Li et al., 2011), hence may be essential for providing stringent regulatory network and augment Wnt signaling to induce tumorigenesis.

The chromatin organizer SATB1 regulates structural framework of chromatin thereby potentially regulates multitude of genes essential for development and disease (Mir *et al*, 2012; Kohwi-Shigematsu et al., 2012). Recently, elevated expression of SATB1 has been implicated in multiple cancers. Aberrant hyperexpression of SATB1 has been implicated in reprogramming expression of multiple genes to promote breast tumorigenesis. Similarly, aberrant expression of SATB1 has been implicated in liver cancer (Chu et al., 2012b), glioma (Chu et al., 2012b), melanoma (Chen et al., 2011), ovarian cancer (Xiang et al., 2012), prostate cancer (Barboro et al., 2012; Shukla et al., 2013) and hyperexpression has been shown to be poor prognostic marker for disease outcome (Kohwi-Shigematsu et al., 2012; Mir et al., 2012). Recent studies also have provided evidence that SATB1 expression is associated with colorectal tumorigenic phenotype and also correlated elevated expression of SATB1 in colorectal

cancer progression and prognosis (Meng et al., 2012; Nodin et al., 2012a; Zhang et al., 2014). However, the molecular mechanism how SATB1 expression is induced and molecular changes essential for tumorigenic transition remain elusive. SATB2, close homologue of SATB1, has been shown to be downregulated in colorectal cancers and loss of its expression is significantly associated with poor prognosis (Wang et al., 2009). Recent report by Nodin et al. demonstrated that colorectal tumor patients exhibiting higher expression of SATB1 and lower expression of SATB2 have lower survival rate and poor prognosis (Nodin et al., 2012a). The mechanism that governs the differential expression of SATB1 and SATB2 and their functional outcome remains largely unclear. An interesting possibility is that SATB family proteins might regulate each other's expression. Knockdown of SATB2 results in upregulation of SATB1 (Wang et al., 2009; Singh and Galande, manuscript under preparation). Conversely, SATB1 knockdown results in upregulation of SATB2 (this study) suggesting that both members of SATB family might be involved in a feedback regulation loop. The molecular mechanism of regulation of SATB2 by SATB1 in colorectal cancer could act as a key event during colorectal cancer progression.

In this study we show that expression of SATB1 is elevated in colorectal metastatic cell line in comparison with primary colorectal cells and pattern of expression correlates with Duke's classification of cancers. The recently reported findings (Nodin et al., 2012a; Zhang et al., 2014) and those from this study indicate that SATB1 is upregulated in colorectal tumors in comparison with matched non-tumor tissues. Furthermore, the levels of SATB1 are higher in patients with high grade or poorly differentiated tumors. Elevated expression of SATB1 in a cohort of colorectal tumors is significantly associated with poor prognosis and overall shorter survival of colorectal patients (Nodin et al., 2012a; Zhang et al., 2014). These findings establish that hyperexpression of SATB1 is associated with aggressiveness of the colorectal cancer and expression of SATB1 is elevated during progression of colorectal cancer. Furthermore, we show that depletion of SATB1 from aggressive type C cell line HCT-15 resulted in reduction of proliferation, migratory potential, restored anchorage dependence and resulted in reduction of tumorigenic potential of these cells, thereby regression of tumors *in vivo.* Conversely, the ectopic expression in non-aggressive cell line SW480 induced their potential to promote tumorigenesis. These results firmly

establish the role of SATB1 in colorectal cancer tumorigenesis. SATB1 potentiates the cellular changes to convert non-aggressive into aggressive phenotype and thus raises the possibility that SATB1 might be involved in regulating the expression of genes associated with colorectal tumorigenesis. Wnt signaling is considered as hallmark for colorectal tumorigenesis and is responsible for progressive accumulation of subsequent cellular changes that mediate colorectal tumorigenesis (Valenta et al., 2012; Clevers, 2006b). Since SATB1 expression is elevated in metastatic cell lines and is essential for tumorigenesis, we investigated the mechanistic role of SATB1 in colorectal tumorigenesis and established that SATB1 plays critical role in regulating the expression of important players of Wnt signaling. Depletion of SATB1 resulted in downregulation of β-catenin, DVLs and downstream targets of Wnt signaling whereas overexpression in primary cell line resulted in dramatic increase in the expression of these genes. β-catenin plays central role in colorectal tumorigenesis and SATB1 seems to play a critical role in regulating β-catenin and so as the downstream targets of Wnt signaling. We show that depletion of SATB1 in aggressive cell lines reduced the expression of downstream targets of Wnt signaling such as AXIN2, c-Myc and TCF7. Interestingly, the depletion of SATB1 had drastic effects on expression of TCF7L1 and TCF7L2 whereas Immunoblot analysis demonstrated that SATB1 negatively regulates SATB2 and antagonistic factor of Wnt signaling such as DKK1. Recent studies have shown that DKK1 (González-Sancho et al., 2005; Aguilera et al., 2006) and SATB2 (Wang et al., 2009) are downregulated during colorectal tumorigenesis and are essential for good prognosis. We further show that SATB1 regulates oncomiRs that are critical for colorectal tumorigenesis. We also delineated the molecular mechanism of SATB2 regulation by SATB1. We show that SATB1 induces the expression of miR31 and SATB2 is downstream target of miR31, hence SATB1 exerts its regulation on SATB2 via miR31expression. Thus, our data provides compelling evidences that SATB1 differentially regulates the positive and negative regulators of Wnt signaling and modulates the changes in expression profiles critical for tumorigenic phenotype.

SATB1 has emerged as a key factor linking higher-order chromatin organization with regulation of genes inside the nucleus (Cai et al., 2006; Galande et al., 2007). Using ChIP assays, we established that SATB1 directly binds to promoters of MMP2, AXIN2,

TCF7, TCF7L2 and c-Myc along with the occupancy of β-catenin. These data confirmed the direct role of SATB1 in regulating critical players of Wnt signaling and downstream targets of Wnt signaling. Based on these findings, we also propose that SATB1 directly exerts its effect on the outcome of Wnt signaling in two ways: First, it directly regulates the expression of TCF7L2, β-catenin and DVls and secondly, it directly binds to the promoters of Wnt target genes and regulates their expression.

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Chapter 2

Regulation of SATB1 in colorectal tumorigenesis- A novel role of Wnt signaling

2.1 Introduction

During development and progression of cancers, the activation of an oncogene or inactivation of a tumor suppressor often plays dominant role and therefore regulation of such molecular events is very important. The expression pattern of chromatin organizer SATB1 correlates with aggressiveness and progression of cancers (Al-Sohaily et al., 2011; Chu et al., 2012; Frömberg et al., 2014; Han et al., 2008; Lu et al., 2010; Meng et al., 2012). The elevated expression of SATB1 leads to dysregulation of large set of cancer- associated genes thereby disrupting the normal homeostasis of cells (Han et al., 2008). SATB1 expression accumulates the molecular events that facilitate the development of tumors and further lead to metastasis. Here we show that SATB1 modulate the cellular changes critical for Wnt signaling pathway- considered as hallmark of colorectal cancers. SATB1 exhibits dual role such that in one hand regulates the expression of key players of Wnt pathway and in other hand regulates downstream targets of Wnt signaling. Further promotes tumor growth *in vivo.* Thus molecular changes driven by SATB1 are critical for cellular phenotype of colorectal cancers and tumorigenic transition.

Global gene regulator SATB1 promotes tumorigenic transformation by reprogramming the expression of large set of cancer associated genes which are involved in inducing molecular pathological events contributing towards the transition from normal to tumor phenotype. SATB1 mediates changes not only at transcription level but also at chromatin architectural level. Recent study also linked SATB1 nuclear matrix interaction with prostate cancer progression (Barboro et al., 2012). Thus the structural and functional roles of SATB1 are linked to decide the cellular phenotype essential for tumorigenesis.

2.1.1 SATB1 in chromatin organization and gene regulation

The structure and organization of chromatin plays an important role in spatial arrangement of genes inside the nucleus thereby allowing different machineries to activate or silence the transcription of genes regulated by various epigenetic events. These chromatin events have been addressed to be tissue specific and essential factors have been identified to regulate these events inside the nucleus. One such factor identified is cell-type specific, the special AT-rich binding protein 1 (SATB1).

SATB1 is a genome organizer and global regulator predominantly expressed in thymocytes (Dickinson et al 1992). SATB1 is essential for the expression of large number of genes involved in T-cell development and maturation (Alvarez et al., 2000; Kumar et al., 2006). Ablation of SATB1 results in dysregulation of numerous genes and arrest of T-cell development. SATB1 binds to special AT rich sequences called as base unpaired regions. These BURs are elements of matrix attachment region (MAR). The well characterized MBP special AT-rich binding protein 1 (SATB1) promotes chromatin organization by periodic tethering of MAR to matrix sites resulting in cage like network thereby regulating the expression of genes (Cai et al., 2003). The unique binding property of SATB1 to AT-rich sequences provides structural framework for higher order chromatin organization. SATB1 plays dynamic role in regulating gene expression, some of these properties are attributed by direct regulation of gene expression by binding to their promoters while others are by promoting the higher order organization. SATB1 acts as docking site for various chromatin remodelers (Yasui et al., 2002). Chromatin organization, differential and cell specific expression of SATB1 is dependent on its interaction partners. SATB1 is a multi-domain protein consists of N-terminal domain, middle CUT domain (CD) and C-terminal Homeodomain (HD). The N-terminal domain is shown to be essential for homodimerization of SATB1 (Galande et al., 2001) and its posttranslational modification acts as molecular switch to regulate its functional role (Kumar et al., 2006) but it is not known whether any posttranslational modification is also responsible for its structural role. SATB1 interacts with other factors essential for nuclear integrity during chromatin organization for example Kumar et al. showed that SATB1 interacts with PML and forms a unique regulatory complex thereby regulating global gene expression by establishing distant chromatin loop architecture (Kumar et al., 2007). Recent study by Rosenfeld group has demonstrated another layer of regulatory network mediated by SATB1 with interaction partners partly mediated by β-catenin. The study showed that SATB1 and β-catenin are required for tethering event of pit1 to matrin3 rich nuclear architecture for activation transcriptional program. Thus from these studies SATB1 is a very critical player in organizing nuclear structural framework to establish the chromatin architecture to regulate global gene expression. SATB1 is T-lineage enriched protein and it was thought its expression in other cell types may be deleterious as its expression may cause global chromatin disorganization and hence will lead to dysregulation of genes. In 2008 Han et al. showed that SATB1 promotes

breast cancer development and metastasis by reprogramming global gene expression (Han et al., 2008). SATB1 in breast cancer provides structural platform to facilitate the regulatory and epigenetic network to regulate gene expression and thereby inducing tumorigenesis . To further support that structural organization of chromatin mediated by SATB1 can be crucial for its functional role in cancers, recently Barboro et al documented that binding of SATB1 to MARs is more in aggressive prostate cancer cell lines (PC3) in comparison with less aggressive cell line LNCaP, although the expression of SATB1 is higher in less aggressive cell line (Barboro et al., 2012). This study thus provides evidences that structural organization of chromatin by SATB1 may be more important for prostate cancer aggressive phenotype than its expression. The study by Barboro et al raises question of whether posttranslational modifications are important in regulating structural role of SATB1. The study by Kumar et al gives significant importance to this study that Phosphorylated SATB1 interacts with DNA and acts as molecular switch to regulate gene expression. It will be interesting to study whether the posttranslational modifications of SATB1 provides structural platform for chromatin organization and are thus important for reorganization of chromatin structure during progression of cancers.

It is potentially very interesting to decipher how SATB1 promotes changes in chromatin and nuclear structure that leads to dysregulation of large number of genes during cancer progression. Another interesting aspect to be investigated is how SATB1 regulates structural integrity of chromatin thereby regulating tumor suppressors and oncogenes differentially and molecular events that maintain the normal structural balance of chromatin and whether SATB1 plays any role in such events. It is not known whether any cancer type has mutation in SATB1 that enhances functional and structural properties of SATB1. Chromatin organizer proteins provide structural network to regulate large number of genes and their dysregulation may be deleterious. Hence their aberrant expression may lead to accumulation of molecular events that are responsible for tumorigenesis. Special AT-rich binding protein 1 (SATB1) was cloned because of its ability to specifically bind base unpairing regions (BURs) and its expression was thought to be cell type specific, predominantly expressed in thymocytes (Dikinson et al 1992). However, subsequent studies revealed that SATB1 is expressed in multiple different types of tissues and cell types (Fessing et al., 2011; Kumar et al., 2006).

2.1.2 SATB1 regulation-Novel link to tumorigenesis

The studies so far have provided evidences that SATB1 expression increases during development and progression of cancers and molecular mechanisms that induce this expression during such transition from normal to cancer phenotype remains elusive and could be the key event. The regulatory events governing SATB1 expression were investigated in a recent study in breast cancer by McInnes et al (McInnes et al., 2012) This study showed that FOXP3 and FOXP3 regulated microRNAs miR7 and miR155 regulate the expression of SATB1. These authors showed that upon ectopic expression of FOXP3 in aggressive breast cancer cell line BT549 resulted in downregulation of SATB1 and upregulation of mirR7 and miR155. FOXP3 binds to the promoter of *Satb1*, miR7 and miR155 and downregulates SATB1 whereas miR7 and miR155 are upregulated. These miRs then bind to the 3'UTR of SATB1 and further decrease its expression (Mcinnes et al., 2011). Another study using Treg maturation and development showed that FOXP3 and FOXP3 regulated miRs miR7 and miR155 regulate the expression of SATB1 (Beyer et al., 2011). Suppression of SATB1 expression is very important for Treg development. FOXP3 thus regulates normal homeostasis and tissue architecture by downregulating the expression of key mediators of tumorigenic transition such as SATB1. The functional association between SATB1 and FOXP3 is summarized in Fig 2.1.2. Ablation of FOXP3 thus provides the adequate and sufficient environment for accumulation of various favourable changes that induce SATB1 expression to promote tumorigenesis. However, the molecular events or cellular pathways that co-ordinately regulate such changes are not completely understood. In particular, the molecular pathways responsible for differential expression of SATB1 and FOXP3 thereby dictating the fate of tumorigenic transition remain to be investigated. In another study cellular Prion protein (PrPc) has been shown to induce the expression of SATB1 in colorectal cancer SW480 cells (Wang et al., 2012). Similarly depletion of PrPc resulted in downregulation of SATB1. The study further delineated that PrPc regulates the SATB1 expression through Fyn-SP1 pathway. Promoter analysis reveals binding site for SP1 on *Satb1* promoter and ChIP analysis further showed that the transcription factor Specific protein1 (SP1) binds to the *Satb1* promoter and regulates its expression. Recently another study has shown that Plakoglobin (γ-catenin) regulates SATB1

expression in breast cancer. Authors further demonstrated that Plakoglobin binds to SATB1 promoter along with p53 to regulate its expression (Aktary and Pasdar, 2013). However, the signaling pathways or molecular changes that induce SATB1 expression are not clear.

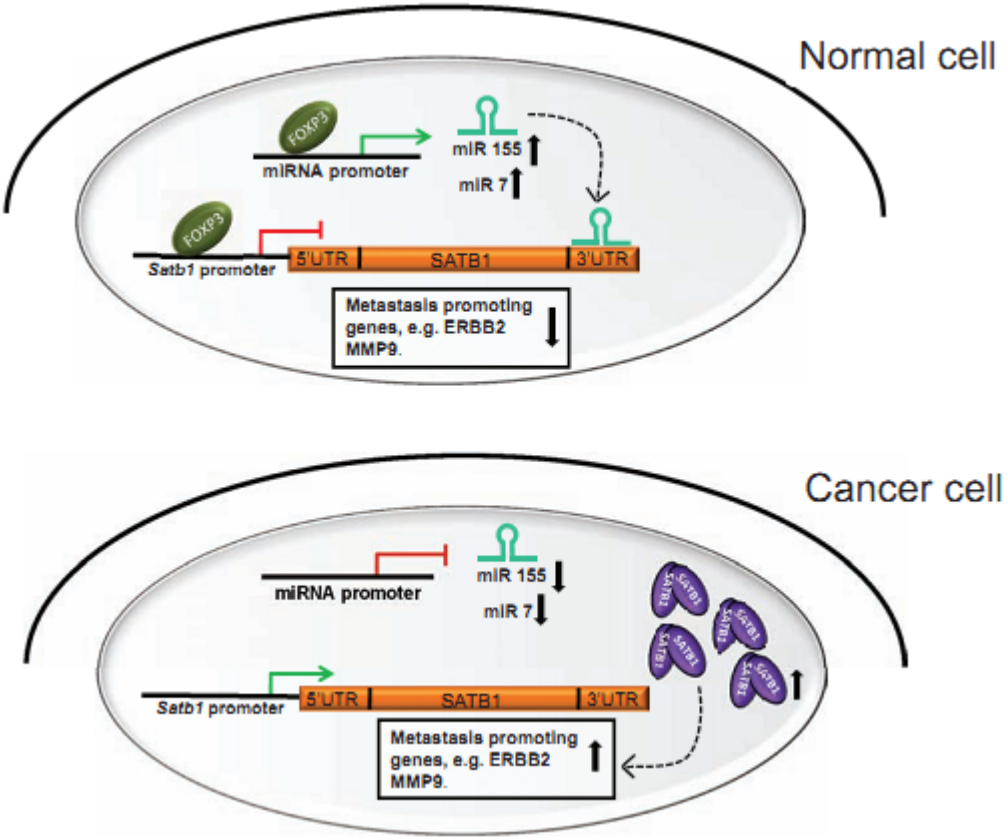


Figure 2.1.2: FOXP3 and FOXP3 regulated miRs negatively regulate SATB1 expression. In normal cells, higher expression of FOXP3 leads to its binding to *Satb1* promoter to repress its expression. FOXP3 also binds to promoters of microRNAs to induce their expression which intern bind to 3'UTR of SATB1 to cause further repression of SATB1 expression. During tumorigenesis, ablation of FOXP3 leads to dysregulation of cellular homeostasis followed by tumorigenesis mediated by hyperexpression of SATB1 (Reproduced from Mir et al., 2012).

2.1.3 Therapeutic approach-SATB1 as molecular target

The chromatin organizer SATB1 regulates large repertoire of genes essential for development and disease. Recently, hyperexpression of SATB1 has been implicated in poor prognosis and shorter survival in multiple cancers including the Wnt signaling

driven colorectal cancer. Studies until now have shown that SATB1 expression is induced during progression of cancers. However, the factors and mechanisms(s) that regulate SATB1 expression have remained elusive**.** Here, we demonstrate that elevated expression of chromatin organizer SATB1 is necessary and sufficient to promote colorectal tumorigenesis. Most importantly, SATB1 modulates Wnt signaling and governs the outcome of Wnt signaling in two modes such that first it regulates TCF7L2, Dvls and β-catenin in Wnt signaling-dependent manner. Secondly, SATB1 binds directly to promoters of multiple Wnt responsive genes and regulates their expression. The present study provides a mechanistic link between regulation of expression of chromatin organizer SATB1 and regulation of Wnt signaling. Additionally, this novel mechanistic insight has opened new avenues towards development of therapeutic strategies to control cancers driven by hyperexpression of SATB1 and Wnt signaling. Due to multiple lines of evidence pointing at the critical role of SATB1 in tumorigenesis, SATB1 is considered as molecular target for cancer therapy. Two studies have recently documented the molecular targeting of SATB1 for therapeutic approach in cancers (Reddy et al., 2010; Yamayoshi et al., 2011). Han et al. have demonstrated that SATB1 promotes metastasis in triple negative (TN) breast cancer cell line MDA-MB231. TN breast cancer is most advance and aggressive form of breast cancer with poor prognosis. Yamayoshi et al used DNA decoy strategy to deplete the functional role of SATB1 in TN MDA-MB231 breast cancer cell line (Yamayoshi et al., 2011). DNA Decoys are double-stranded oligonucleotide DNAs recognized by DNA-binding proteins, hence trapping them and depleting their function [158-160]. Transfection of SATB1 DNA decoy significantly reduced proliferative capacity of MDA-MB231 cells and drastically abolished their invasive and metastatic potential. This effect is very specific to SATB1 expressing cells because no effect was observed in MCF10A that do not express SATB1 (Yamayoshi et al., 2011). It is known that phosphorylation of SATB1 is essential for binding of SATB1 to DNA (Kumar et al., 2006) and authors argued that SATB1 DNA decoy traps the phosphorylated form of SATB1 and hence depleting its functional role in breast cancer cells. In another study, the mevalonate pathway inhibitors statins were used to target SATB1 in colorectal cancer (Reddy et al., 2010). Statin inhibits 3 hydoxy 3 methylglutaryl coenzyme A (HMGCoA) reductase which catalyses the formation of mevalonic acid. Various studies have documented anti-tumorigenic effect of statin in cancers (Freed-Pastor et al., 2012; Hindler et al., 2006; Katz, 2005; Larsson, 1996). However, studies on the

molecular mechanisms of anti-tumor activity of statins have been far from clear. Statin treatment in cancer cells downregulates the expression of SATB1 at protein level. Interestingly, treating colorectal cancer with mevalonate, the enzymatic product of HMGcoA reductase, rescued the expression of SATB1 (Reddy et al., 2010). Treatment with the proteasome inhibitors lactacystine and MG-132 inhibited the statin-mediated downregulation of SATB1, suggesting that the regulation occurs at post-translational level. The mevalonate pathway thus seems to be essential for signaling pathway(s) that might play crucial role in regulating the expression of SATB1 posttranslationally. The therapeutic approaches used to target SATB1 do not provide detailed mechanistic views on how precisely these two diverse agents - DNA decoy and statins, regulate SATB1 expression and its functional role in cancer development. It will be interesting to test whether statin and decoy DNAs will result in regressing SATB1-derived tumors using *in vivo* mouse models.

2.2 Materials and Methods

2.2.1 Antibodies and reagents

SATB1, TCF7L2, TCF7, AXIN2 antibodies were obtained from Cell Signaling. c-Myc, active β-catenin and vimentin antibodies were obtained from Millipore. β-catenin and E-cadherin antibodies were obtained from BD Biosciences. DVL2, DVL3 and MMP2 antibodies were from Santa Cruz Biotechnology. LEF1, N-cadherin, DKK1, SATB2, Wnt3A were obtained from Abcam. Actin and gamma-tubulin antibodies were obtained from Sigma. For ChIP analysis SATB1 and β-catenin (XP) antibodies from Cell Signaling and histone H3 lysine 4 trimethylation, H3 lysine 9 trimetylation and H3 lysine 27 trimethylation antibodies from Upstate were used. Polyclonal antibody for Zebra fish SATB1 was raised in house in rabbit using standard procedures. Recombinant Wnt3a and DKK1 proteins were obtained from R&D systems. Pyrvinium Pamoate was obtained from Sigma. Puromycin was obtained from Sigma and G418 was from Roche. Simvastatin was obtained from Sigma.

2.2.2 Plasmids

TCF7L2 was cloned in pCMV9-3XFLAG. FLAG SATB1 was used as in (Notani et al., 2010). For GFP fusion, SATB1 was subcloned from pCMV10-3XFLAG-SATB1. The N-terminal SATB1-DN (1-204) was cloned in pCMV10-3XFLAG (SIGMA). SATB1 promoter sequence having multiple TCF7L2 binding was cloned in pGL3 basic vector (Promega). The siRNA sequences for sh1SATB1 and sh3SATB1 were designed using Dharmacon design center. The siRNA sequence for sh2SATB1 was used as described (Han et al., 2008). All shRNAs were cloned in pSUPER Puro vector (Oligoengine).

2.2.3 Cell culture, transfections and western blotting

SW480, SW1116, SW620, T84 and HCT116 cell lines were grown in DMEM with 10% FCS. COLO320, COLO205, COLO201, COLO741, HCT-15, HT-29, DLD1 cell lines were grown in RPMI with 10% FCS. CRL1790 were grown in MEM with 10% FCS. CRL1790, SW480, COLO201, COLO205, COLO320, SW620, HT-29 DLD1, HeLa and MDA-MB231 were obtained from American Type Culture Collection (ATCC). SW1116, T84, HCT116, HCT-15 were obtained from European Collection of Cell Cultures

(ECACC) SIGMA. For Immunoblotting 25 µg of lysate was loaded in each lane unless mentioned otherwise. For transfection with siRNA mediated depletion, cells were seeded and after 24 hours transfected with indicated siRNA and then harvested for immunoblotting and RNA extraction. The sequences of siRNA used are listed in Table 2.2.3.

Table 2.2.3: siRNA/shRNA sequences used in this study.

GSK3β	CCCAAATGTCAAACTACCA	Azzolin et al., 2012
β-catenin#1	GTAGCTGATATTGATGGAC	Equimolar mix of 1 and 2 was used (Azzolin et al., 2012)
β-catenin#2	GCTTGGAATGAGACTGCTG	
TCF7L2#1	AGAGAAGAGCAAGCGAAATAC	Equimolar mix of 1 and 2 was used (Azzolin et al., 2012)
TCF7L2#2	TATCGAGTTCATTGGTCAATA	

2.2.4 Biochemical assays

To activate Wnt signaling CRL1790 cells were treated with CHIR (3 µM) and BIO (1 µM) for 48 h and harvested for protein and RNA. Alternatively, cells were transfected with siGSK3β and harvested after 48 h. HeLa cells were treated with 3 µM CHIR 99021 (GSK3β kinase inhibitor) and with different concentrations of Wnta3a as mentioned in figure legends. Similarly Pyrvinium Pamoate (PP) was used at concentration of 100 nM and harvested after 48 h. For knockdown of SATB1 and β-catenin under CHIR and Wnt3A (in HeLa cells), cells were first transfected with control shRNA, shSATB1 and shβ-catenin and after 12 h treated with CHIR for 48 h in case of CRL1790 cells, whereas HeLa cells were treated with CHIR and Wnt3a for 6 h after 42 h of transfection. For Statin treatment cells were seeded and treated with 25 µM Simvastatin after 24h and harvested for protein extraction after 48h.

2.2.5 Reporter Assays

Luciferase assay was performed in HCT116 cells. HCT116 cells were first transfected with siGFP and siβ-catenin and after 24 h transfected with pGL3 basic control and SATB1 promoter reporter constructs essentially as described (Azzolin et al., 2012). All reporter assays were performed in triplicates.

2.2.6 RNA isolation & RT-PCRs

RNA was isolated using Trizol reagent (Invitrogen). Two µg of RNA was used for first strand cDNA synthesis using Superscript III (Invitrogen). The cDNA was then used for quantitative PCR analysis in triplicates using an ABI 7500 Fast real-time PCR System (Applied Biosystem) as described in Ordinario et al. (2012). The sequences of oligonucleotide primers used for real-time PCR are listed in Table 2.2.6.

. **Table 2.2.6: Sequences of primers used for qPCR analysis.**

qPCR Primer	Sequence	Notes
SATB1 DANIO	CGCTGGTACAAACATTTCAAGAAG	
SATB1 DANIO	GACCGTCCATCTCAGCTAACG	
hSATB1 Forward	AGCAACAGGTTTCGACCAAC	TaqMan primer and probe (ABi)
hSATB1 Reverse	TCTGAAAGCAAGCCCTGAGT	
huMMP2 Forward	CGATAACCTG GATGCCGTC	
huMMP2 Reverse	GTCCTTCTCTAGTTCTCCAG	
huTCF7L2 Forward	ATGCTTCCATGTCCAGGTTC	
huTCF7L2 Reverse	CACTCTGGGACGATTCCTGT	
huGAPDH2 Forward	CTGCACCACCAACTGCTTAG	
huGAPDH2 Reverse	GTCTTCTGGGTGGCAGTGAT	
hu β-catenin Forward	AAGGTGTGGCGACATATGCA	
hu β-catenin Reverse	GTAATCTTGTGGCTTGTCCTCAGA	
huDKK1 Forward	GCGGCACTGATGAGTACTGC	
huDKK1 Reverse	GGCAGCACATAGCGTGACG	
huAXIN2 Forward	GTCTCCAAGCAGCTGAAGCC	
huAXIN2 Reverse	CCTCCATCACCGACTGGATC	
huCyclinD1 Forward	GCTCCTGTGCTGCGAAGTG	
huCyclinD1 Reverse	CATTTGAAGTAGGACACCGAGG	
huc-Myc Forward	GCCACAGCAAACCTCCTCAC	
huc-Myc Rverse	CTCTTGGCAGCAGGATAGTCC	

2.2.7 Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed as described (Karmodiya et al., 2012). Briefly, cells were cross-linked by addition of formaldehyde to 1% final concentration in media and incubated at room temperature for 10 min, neutralized with 125 mM Glycine. Cells

were then subjected to sonication using Covaris sonicator to fragment chromatin to obtain 200–500 bp fragments. Sonicated chromatin was precleared with non-saturated beads. Precleared chromatin was incubated with specific antibodies and respective IgG types were used as isotype controls. Next day, beads saturated with tRNA and BSA were added (40 µl packed beads) and incubated for 4 h on rocker to pull down the antibody-bound chromatin and were subjected to elution using buffer containing SDS and sodium bicarbonate. Eluted chromatin was de-crosslinked and protein was removed by treating with proteinase K. Purified immunoprecipitated chromatin was subjected to PCR amplification using specific primers. Input chromatin was used as a control. ChIP primer details are listed in Table 2.2.7.

Table 2.2.7: Sequences of primers used for ChIP analysis.

ChIP Primer	Sequence	Notes
hu c-Myc Forward	CACAAGGGTCTCTGCTGACTCCCCC	Notani et al. 2010
hu c-Myc Reverse	GGA GGT TGC CTG CTC TCT GCC AGT C	
huSATB1 Forward	TGGAATGTTTAAGTTTTTAATGGAGTT	ChIP-PCR
huSATB1 Reverse	TCAGTGGAATATGTGACTAGGTATTGA	
huSATB1 Forward	GTGGGGCTCATTGATTGATT	ChIP-qPCR
huSATB1 Reverse	TGATGTTTCAAAGGGGTCTTG	

2.2.8 Expression analysis in Zebra fish

Heterozygous and APC min mutant zebra fish embryos were harvested 48 h post fertilization for RNA extraction and protein extraction using RIPA buffer. For zebra fish maintenance and experimentation, the guidelines recommended by committee for the purpose of control and supervision of experiments on animals (CPCSEA), Government of India, were followed. Sequences of primer used for qPCR are listed in Table 2.2.8.

Table 2.2.8: Sequences of primers used for qPCR analysis in Zebra fish

qPCR Primer	Sequence	Notes
SATB1 DANIO Forward	CGCTGGTACAAACATTTCAAGAAG	
SATB1 DANIO Reverse	GACCGTCCATCTCAGCTAACG	

2.3 Results

2.3.1 TCF7L2/β-catenin signaling regulates SATB1 expression in colorectal cancer

SATB1 has been shown to be involved in the development and progression of multiple types of cancers. SATB1 orchestrates the molecular changes essential for tumorigenesis (Barboro et al., 2012; Han et al., 2008; Mir et al., 2012; Nodin et al., 2012; Tu et al., 2012). Our results have demonstrated for the first time that the molecular mechanism of the regulatory role of SATB1 in colorectal tumorigenesis by modulating the Wnt signaling. However, the molecular changes that are responsible for inducing the expression of SATB1 are not clear. The regulatory pathways that may regulate SATB1 expression are elusive. In this study we have shown that primary colorectal cells either do not express SATB1 or express it at very low levels. Furthermore, colorectal cancer cell lines exhibit aberrant or hyperactivated Wnt signaling, suggesting that TCF7L2/β-catenin signaling could be involved in the regulation of SATB1 expression. To test this hypothesis, we activated Wnt signaling in primary colorectal cell line (CRL1790) using the GSK3β inhibitor CHIR (Azzolin et al., 2012) and by knocking down GSK3β using RNA interference. CHIR treatment and GSK3β depletion resulted in robust increase in β-catenin expression indicating that Wnt signaling in CRL1790 was activated (Figure 2.3.1A). Further, the increased expression of β-catenin upon activation of Wnt signaling resulted in robust increase in SATB1 expression (Figure 2.3.1A). Next we investigated whether Wnt signaling regulates SATB1 expression at transcript level. The quantitative RT-PCR analysis showed that CHIR treatment and GSK3β knockdown resulted in 15-20-fold increase in SATB1 expression at transcript level. The known downstream targets of Wnt signaling AXIN2 and c-Myc also showed similar effect on expression upon activation of Wnt signaling, indicating that SATB1 is a direct target of Wnt signaling (Figure 2.3.1B). To further validate the regulation of SATB1 by Wnt signaling we employed HeLa cells which express very low levels of SATB1. We induced Wnt signaling in HeLa cells in dose-dependent manner using Wnt3A and separately overexpressed degradation resistant form of β-catenin (S37A β-catenin). The dose-dependent activation of Wnt signaling upon Wnt3A treatment and subsequent stabilization of β-catenin resulted in induction of SATB1 expression (Figure 2.3.1C). We also induced Wnt signaling by

treating HeLa cells with GSK3β inhibitor (CHIR) in time-dependent manner. The activation resulted in robust increase in expression of SATB1, but not that of SATB2, indicating that Wnt signaling specifically induces the SATB1 expression (Figure 2.3.1D). Similar induction in SATB1 expression was observed upon treatment of CRL1790 primary colorectal cells with BIO treatment at transcript level (Figure 2.3.1E). Activation of Wnt signaling cascade leads to disengagement of β-catenin from the destruction complex consisting of APC, AXIN1, GSK3β and casein kinase1α; hence β-catenin is stabilized at protein level and then translocates inside the nucleus. Nuclear β-catenin subsequently interacts with TCF7L2 and contributes to the upregulation of downstream targets of Wnt signaling. We next analyzed the expression of SATB1 under depletion of TCF7L2, the major transcription factor of Wnt signaling, in β-catenin active mutant HCT116 cells and APC null HCT-15 cells. The depletion of TCF7L2 resulted in downregulation of SATB1 at protein level in HCT116 and HCT-15 cells (Figure 2.2.1F, Figure 2.3.1G) and downregulation of known targets of Wnt signaling such as AXIN2 and TCF7 (Figure 2.3.1F, Figure 2.3.1G). Further, depletion of TCF7L2 reduced the expression of SATB1 at transcript level (Figure 2.3.1H), suggesting that SATB1 is a direct target of Wnt signaling.

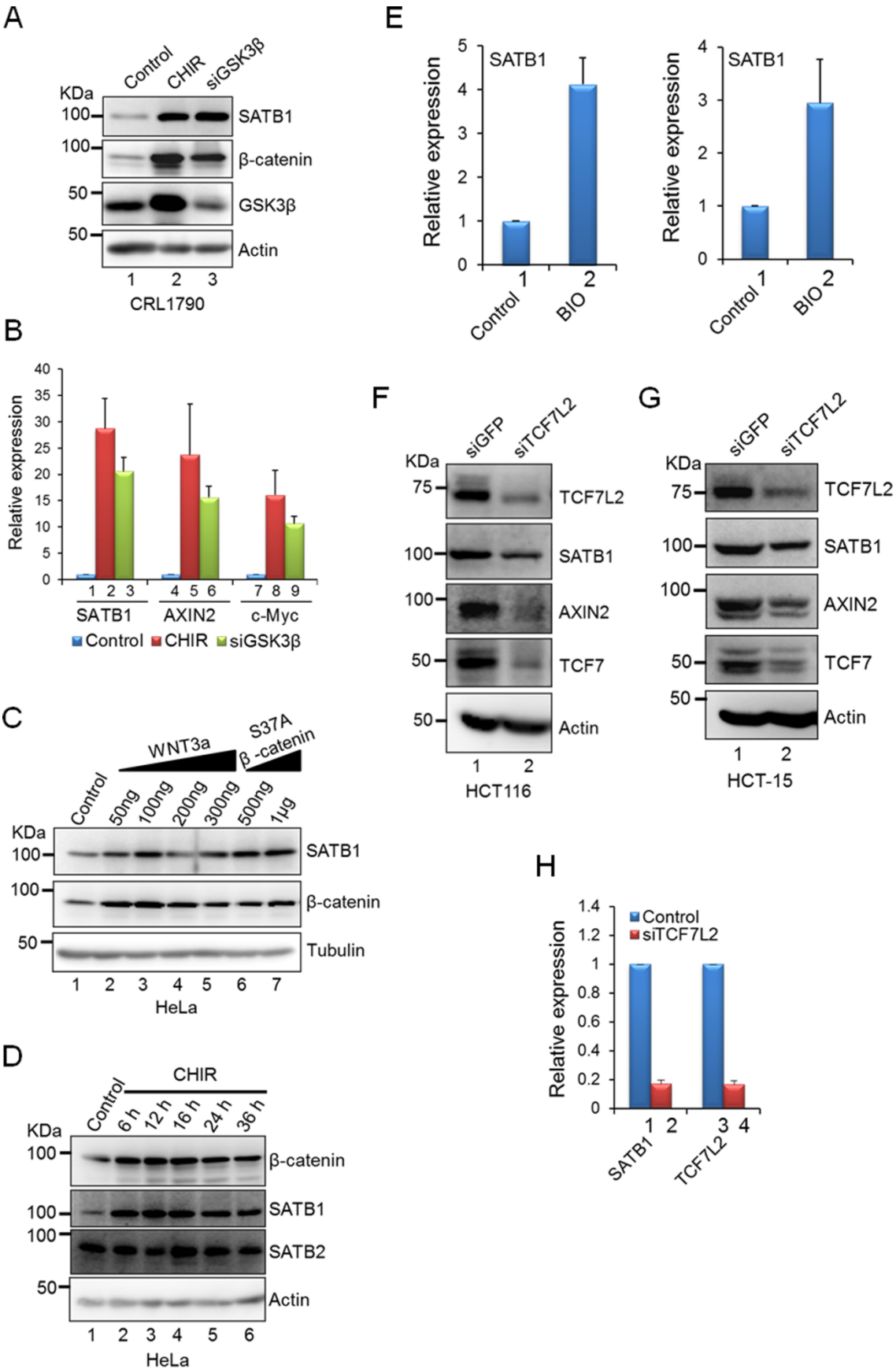

A
Control
CHIR
siGSK3β
KDa
100
SATB1
100
β-catenin
50
GSK3β
50
Actin
1 2 3
CRL1790
B
Relative expression
SATB1
AXIN2
c-Myc
Control
CHIR
siGSK3β
C
WNT3a
S37A β-catenin
Control
50ng
100ng
200ng
300ng
500ng
1μg
SATB1
β-catenin
Tubulin
HeLa
D
CHIR
Control
6 h
12 h
16 h
24 h
36 h
β-catenin
SATB1
SATB2
Actin
HeLa
E
Relative expression
SATB1
Control
BIO
F
siGFP
siTCF7L2
KDa
75
TCF7L2
100
SATB1
100
AXIN2
50
TCF7
50
Actin
HCT116
G
HCT-15
H
Control
siTCF7L2
SATB1
TCF7L2

Figure 2.3.1: TCF7L2/β-catenin signaling regulates SATB1 expression in colorectal cancer (A) CRL1790 cells were treated with CHIR (3 μM) for 48 h and/or transfected with siGSK3β to induce Wnt signaling. Immunoblot showing expression of β-catenin, SATB1 and GSK3β upon CHIR treatment and GSK3β knockdown. Actin was used as loading control. **(B)** Relative mRNA levels of SATB1, c-Myc and AXIN2 under CHIR treatment and GSK3β knockdown in CRL1790 primary cells as determined by qPCR. GAPDH2 was used as endogenous control (error bar represents standard deviation from triplicates). (**C)** Immunoblot for expression of β-catenin and SATB1 under Wnt3A treatment for 6 h and upon overexpression of mutant S37A β-catenin in dose dependant manner in HeLa cells. **(D)** Immunoblot showing expression of SATB1, β-catenin and SATB2 in time-dependent manner under CHIR treatment in HeLa cells. **(E)** Relative mRNA levels of SATB1 under GSK3β inhibitor BIO in CR1790 primary cells. Data for two biological replicates. GAPDH was used as an endogenous control. **(F)** Immunoblot showing expression of SATB1, AXIN2, TCF7 and TCF7L2 in siGFP control HCT116 in comparison with siTCF7L2 HCT116 cells. Actin was used as loading control. **(G)** Immunoblot showing expression of SATB1, AXIN2 and TCF7 in control and TCF7L2 knockdown HCT-15 cells. Actin was used as endogenous control. **(H)** Relative mRNA levels of SATB1 under depletion of TCF7L2. Expression levels were normalized with that of the control cells.

2.3.2 β-catenin is required for Wnt signaling dependent regulation of SATB1

To further delineate the role of Wnt/β-catenin signaling in regulation of SATB1 expression, we silenced β-catenin expression using siRNA in the metastatic cell lines HCT-15 and HCT-116. Depletion of β-catenin by siRNA mediated knockdown resulted in downregulation of SATB1 and TCF7 (Figure2.3.2A, Figure 2.3.2B). Further, expression of SATB1 was reduced at transcript level upon β-catenin depletion in HCT116 colorectal cancer cells (Figure 2.3.2C). Furthermore, depletion of β-catenin by siRNA mediated knockdown or induction of its degradation by treatment with Pyrvinium Pamoate (PP), activator of casein kinase 1α mediated phosphorylation of β-catenin (Thorne et al., 2010), in SW620 (Figure 2.3.2D) and COLO201 colorectal cancer cells (Figure 2.3.2E) resulted in decrease in SATB1 expression. To test whether the same regulatory network is involved in other cancers we used a breast cancer cell line model. Knockdown of β-catenin in aggressive breast cancer cell line MDA-MB-231 resulted in robust decrease in SATB1 expression (Figure 2.3.2 F). Similarly the treatment of MDA-MB-231 cells with Wnt antagonistic DKK1 resulted in robust decrease in expression of SATB1 (Figure 2.3.2G). To evaluate whether increased expression of SATB1 by hyperactivation of Wnt signaling is β-catenin dependent, we induced Wnt signaling in primary colorectal cell line CRL1790 by CHIR

treatment and knocked down β-catenin in CHIR treated cells. SATB1 expression induced upon CHIR treatment was abrogated by β-catenin knockdown (Figure 2.3.2H), thereby indicating that SATB1 expression induced upon activation of Wnt signaling is β-catenin dependent. Further, we wished to monitor whether SATB1 expression is first induced or β-catenin expression. To test this we treated HeLa cells with CHIR in dose dependent manner from half an hour to 6 hr. The activation of Wnt signaling by CHIR treatment resulted in induction of β-catenin expression followed by SATB1 expression (Figure 2.3.2I).Thereby further confirming that SATB1 requires β-catenin for Wnt signaling dependent upregulation. To further monitor the effect on SATB1 expression upon hyperactivation of Wnt signaling *in vivo*, we analyzed the expression of SATB1 in min (APC) mutant zebra fish in comparison with the wild type zebra fish post 48 hours of fertilization. The hyperactivation of Wnt signaling *in vivo* elevated SATB1 expression at protein level (Figure 2.3.2J) and at transcript level (Figure 2.3.2K), providing conclusive evidence that TCF7L2/β-catenin signaling cascade regulates SATB1 expression.

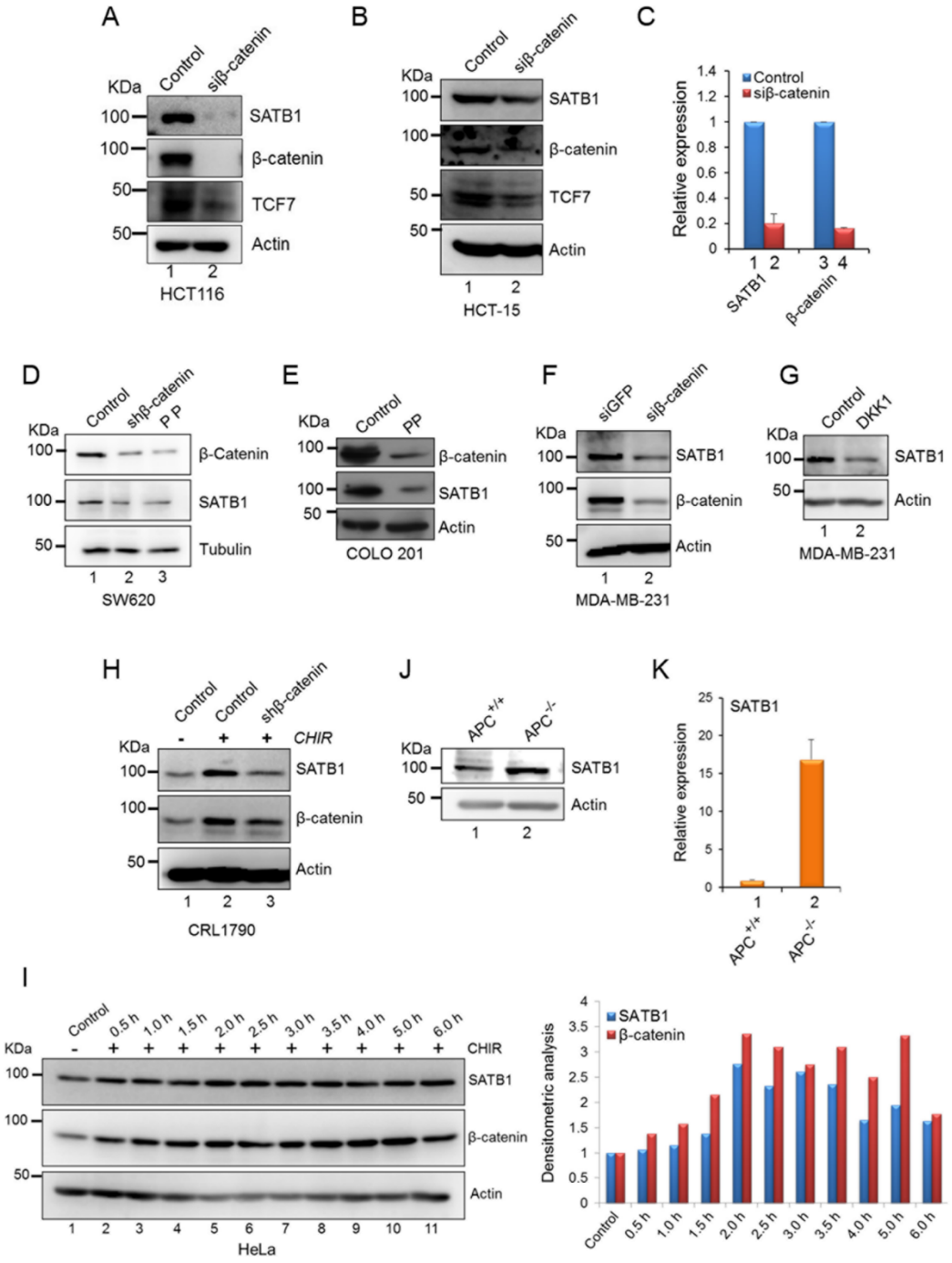


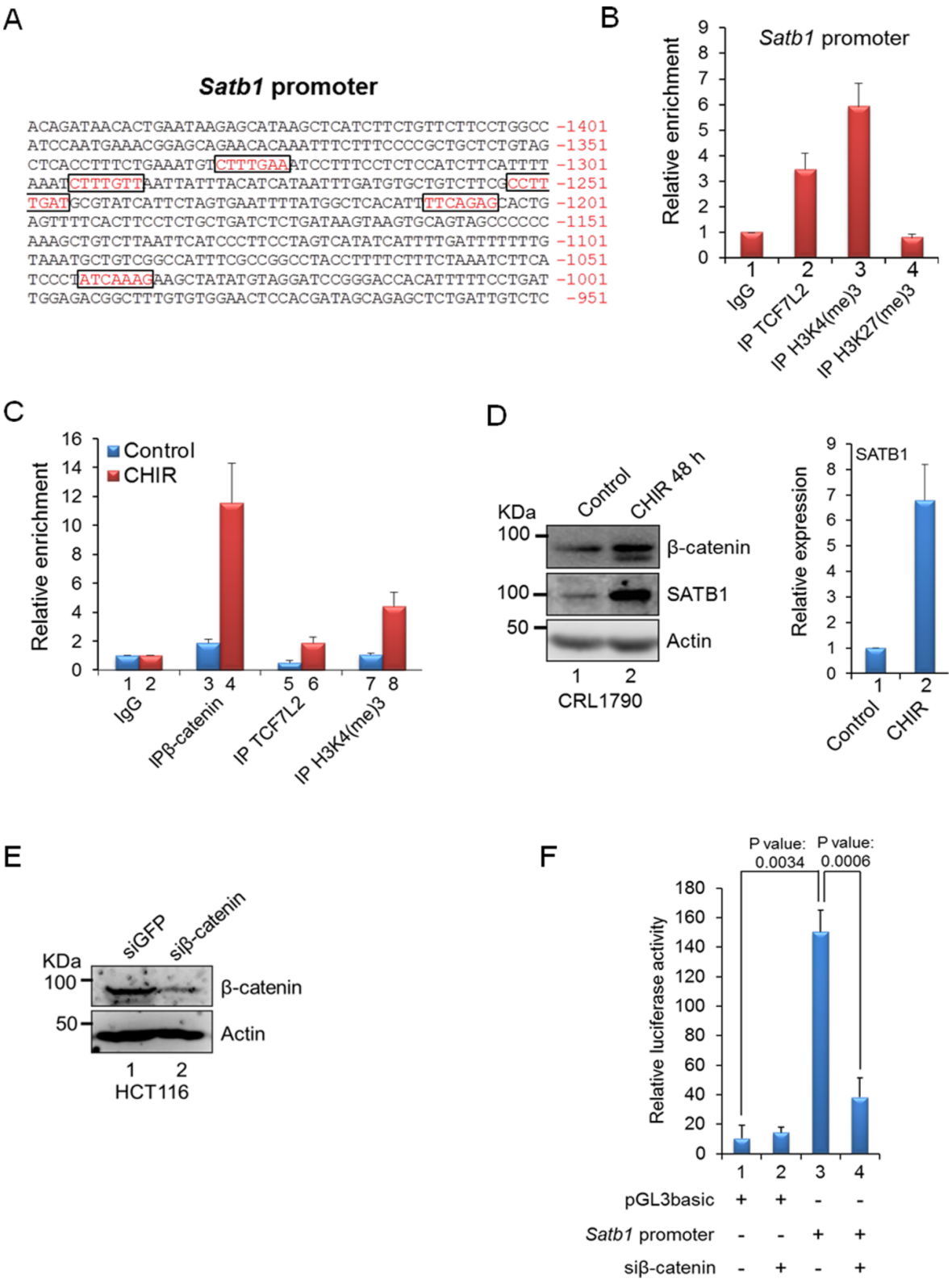
Figure 2.3.2: β-catenin is required Wnt signaling dependent regulation of SATB1. (A) Immunoblot for expression of SATB1, TCF7 and β-catenin in siGFP control HCT116 in comparison with siβ-catenin HCT116 cells. **(B)** Immunoblot showing expression of SATB1 and TCF7 in control and β-catenin knockdown HCT-15 cells. **(C)** Relative mRNA levels of SATB1 under depletion of β-catenin. Expression levels were normalized with that of the control cells.**(D)** Immunoblot showing decrease

in expression of SATB1 under β-catenin depletion in SW620 metastatic colorectal cells and upon treatment of Wnt signaling inhibitor Pyrvinium Pamoate (PP, 100 nM). **(E)** Immunoblot showing decrease in expression of SATB1 in COLO 201 cells upon degradation of β-catenin by treatment with PP. **(F)** Immunoblot for expression of SATB1 and β-catenin upon β-catenin depletion in MDA-MB-231 cells. **(G)** Immunoblot showing expression of SATB1 upon treatment with Wnt antagonistic DKK1 in MDA-MB231 breast cancer cells. Cells were treated with 100 ng DKK1 for 48 h. **(H)** Immunoblot for expression of SATB1 and β-catenin upon CHIR treatment in CRL1790 cells and reduction in expression of SATB1 upon depletion of β-catenin in CHIR treated cells (compare lane 1 and 2, lane 2 and 3). **(I)** Immunoblot for expression of SATB1 in HeLa cells in time-dependent manner from 0.5 h to 6 h.**(J)** Immunoblot for expression of SATB1 in heterozygous and APC min mutant Zebra fish (48 h post fertilization). **(K)** Relative mRNA levels of SATB1 in heterozygous and APC min mutant Zebra fish (48 h post fertilization). Actin was used as endogenous control for Immunoblot and for qPCR.

2.3.3 TCF7L2/β-catenin complex binds to *Satb1* promoter and directly regulates SATB1 expression

To delineate whether TCF7L2/β-catenin regulatory network is directly involved in inducing the expression of SATB1 by binding to its promoter during tumorigenic transition, we identified *Satb1* promoter by *in silico* analysis using bioinformatics tools; transcription regulatory database and transcription start site databases and retrieved the *Satb1* promoter sequence (Zhao et al., 2005). We then analyzed the *Satb1* promoter and found multiple TCF7L2 consensus sequence motifs (A/T A/T CAAAG, CTTTGNN) (van de Wetering et al., 1997; Wagner et al., 2010; Frietze et al., 2012; MacDonald et al., 2009) (Figure 2.3.3A). Next we performed ChIP using antibodies for TCF7L2, Histone H3K4$(me)_3$ and Histone H3K27$(me)_3$. The ChIP analysis revealed that TCF7L2 is enriched on *Satb1* promoter along with increased enrichment of activation associated histone mark H3K4(me)3 but not the repression associated histone mark H3K27(me)3 (Figure 2.3.3B). Together, these results suggest that TCF7L2/β-catenin signaling directly regulates SATB1 expression. To gain further insight into regulation of SATB1 by regulatory complex of TCF7L2 and β-catenin, we analyzed the occupancy of TCF7L2 and β-catenin on *Satb1* promoter upon activation of Wnt signaling in primary colorectal cell line (CRL1790). Cells were treated with CHIR for 48 h and then harvested for immunoblotting and ChIP analysis. The hyperactivation of Wnt signaling induced the expression of β-catenin and induced occupancy of TCF7L2 and β-catenin on *Satb1* promoter along with enrichment of the

histone activation mark H3K4(me)3 (Figure 2.3.3C) and thereby presumably inducing the expression of SATB1 at protein and transcript level (Figure 2.3.3D). Next we analyzed activity of *Satb1* promoter upon knockdown of β-catenin in HCT116 cells. We cloned 381 bp of human *Satb1* promoter in a luciferase reporter vector and transfected it in HCT-116 cells. The depletion of β-catenin (Figure 2.3.3E) resulted in about 5-fold reduction in *Satb1* promoter activity (Figure 2.3.3F). Furthermore, we also analyzed the occupancy of β-catenin on *Satb1* promoter upon β-catenin depletion. The knockdown of β-catenin resulted in decreased enrichment of β-catenin on *Satb1* promoter and also the known Wnt target *c-Myc* promoter (Figure 2.3.3G). ChIP-qPCR analysis further revealed that the enrichment of β-catenin along with a histone activation mark is lost upon β-catenin depletion whereas enrichment of a repressive histone mark is induced (Figure 2.3.3H). The decreased occupancy of β-catenin on *Satb1* promoter was also reflected in expression of SATB1 upon β-catenin knockdown. The depletion of β-catenin resulted in decrease in *Satb1* expression in comparison with control (Figure 2.3.3I). These results provide evidence in favor of the argument that TCF7L2/β-catenin signaling positively regulates SATB1 expression. Proposed model showing changes associated with regulation and activation of SATB1 expression (Figure 2.3.3J).


A
Satb1 promoter
ACAGATAACACTGAATAAGAGCATAAGCTCATCTTCTGTTCTTCCTGGCC -1401
ATCCAATGAAACGGAGCAGAACACAAATTTCTTTCCCCGCTGCTCTGTAG -1351
CTCACCTTTCTGAAATGTCTTTGAAATCCTTTCCTCTCCATCTTCATTTT -1301
AAATCTTTGTTAATTATTTACATCATAATTTGATGTGCTGTCTTCGCCTT -1251
TGATGCGTATCATTCTAGTGAATTTTATGGCTCACATTTTCAGAGCACTG -1201
AGTTTTCACTTCCTCTGCTGATCTCTGATAAGTAAGTGCAGTAGCCCCCC -1151
AAAGCTGTCTTAATTCATCCCTTCCTAGTCATATCATTTTGATTTTTTTG -1101
TAAATGCTGTCGGCCATTTCGCCGGCCTACCTTTTCTTTCTAAATCTTCA -1051
TCCCTATCAAAGAAGCTATATGTAGGATCCGGGACCACATTTTTCCTGAT -1001
TGGAGACGGCTTTGTGTGGAACTCCACGATAGCAGAGCTCTGATTGTCTC -951
B
Satb1 promoter
Relative enrichment
IgG
IP TCF7L2
IP H3K4(me)3
IP H3K27(me)3
C
Control
CHIR
Relative enrichment
IgG
IPβ-catenin
IP TCF7L2
IP H3K4(me)3
D
KDa
Control
CHIR 48 h
β-catenin
SATB1
Actin
CRL1790
SATB1
Relative expression
Control
CHIR
E
siGFP
siβ-catenin
KDa
β-catenin
Actin
HCT116
F
P value: 0.0034
P value: 0.0006
Relative luciferase activity
pGL3basic
Satb1 promoter
siβ-catenin

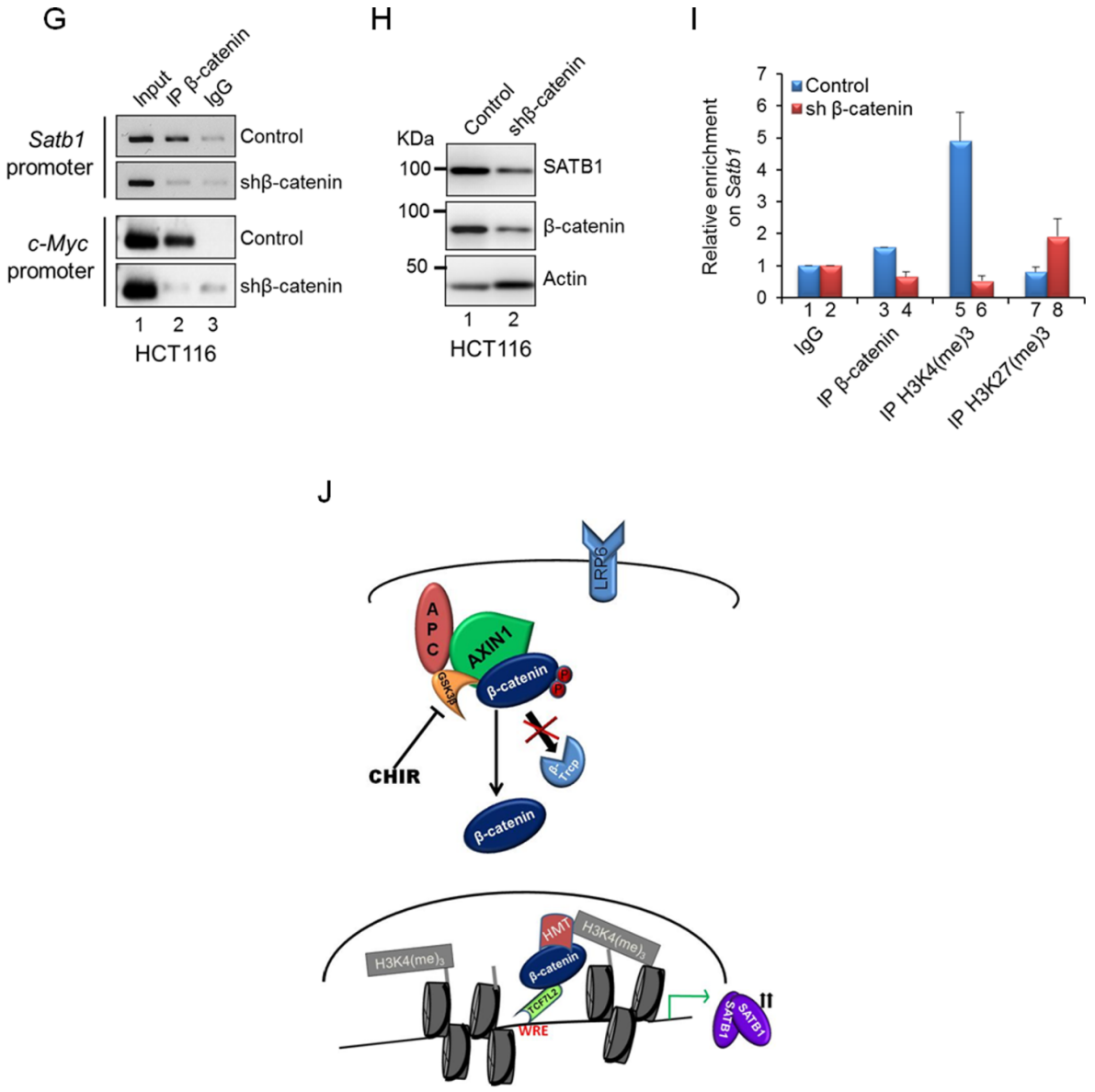


Figure 2.3.3: TCF7L2/β-catenin complex binds to *Satb1* promoter and directly regulates SATB1. **(A)** *In silico* analysis of Satb1 promoter sequence showing multiple TCF7L2 consensus sites upstream to TSS. Promoter sequence was retrieved by using bioinformatics tools: TRED and DBTSS. The TCF7L2 binding sequences are boxed. **(B)** ChIP Assay showing occupancy of TCF7L2 and Histone H3 lysine activation and repressor marks on *S*atb1 promoter. ChIP was performed using antibodies to TCF7L2, Histone H3K4$(me)_3$ and H3K27$(me)_3$ followed by ChIP-qPCR using primers flanking the TCF7L2 binding site on *Satb1* promoter (Figure A). ChIP using rabbit IgG served as control. **(C)** ChIP showing the increase in the occupancy of β-catenin and TCF7L2 along with a Histone H3 activation mark on *Satb1* promoter upon CHIR treatment. CRL1790 primary cells were treated with CHIR (3 μM) for 48 h followed by ChIP using antibodies to TCF7L2, β-catenin, histone H3K4$(me)_3$. CHIR treatment induced the expression of β-catenin so did the occupancy of TCF7L2 and β-catenin along with enrichment of H3K4$(me)_3$ on *Satb1* promoter. **(D)** Left panel: Immunoblot showing increased expression of SATB1 and β-catenin upon CHIR treatment in CRL1790 cells used for ChIP assay in Figure 2.2.3 C. Right panel: Relative mRNA levels of SATB1 upon CHIR treatment in CRL1790 cells used for ChIP assay in Figure 2.2.3 C. **(E)** Immunoblot showing decrease in expression of β-catenin upon β-catenin knockdown used for SATB1 promoter reporter assay in Figure 2.2.3F. Actin used as endogenous control. **(F)** *Satb1* promoter-driven

luciferase reporter assay in control cells and in β-catenin knockdown cells. The HCT116 cells were transfected with control siRNA and siβ-catenin. After 24 h, cells were transfected with pGL3 basic vector and pGL3-*Satb1* promoter construct in both control cells and β-catenin knockdown cells. The experiment was performed in triplicates and error bars depict standard deviation. **(G)** ChIP-PCR for occupancy of β-catenin on *Satb1* promoter and *c-Myc* promoter upon depletion of β-catenin in HCT116 cells. **(H)** Immunoblot showing decrease in SATB1 expression upon β-catenin depletion in HCT116 cells used in ChIP assay. **(I)** ChIP-qPCR showing relative enrichment of β-catenin and H3K4$(me)_3$ and not of H3K27$(me)_3$ on *Satb1* promoter in control cells. In β-catenin knockdown cells, the occupancy profile for these three is reversed. **(J)** Model showing changes associated with Wnt-dependant regulation of SATB1. Activation of Wnt signaling induces the stabilization and nuclear transport followed by binding TCF7L2/β-catenin complex on *Satb1* promoter. The binding of complex recruits the Histone activation marks thereby promoting the expression of SATB1.

2.3.4 Regulation of SATB1- A molecular approach to cancer therapy

Colorectal cancer is one of the leading causes of cancer related death worldwide. Recently, in addition to genetic changes, epigenetic events have also been implicated in colorectal tumorigenesis. Wnt signaling is a hallmark of colorectal cancer development and progression. Aberrant activation of Wnt signaling is the major driving force in colorectal cancers. Novel targets and modulators of Wnt signaling have opened new avenues for therapeutic intervention in colorectal cancer. Aberrant expression of SATB1 potentiates molecular changes that are critical for tumorigenesis and poor prognosis and overall shorter survival of patients. Recently statins have been established as drugs for treatment of cancers but precise mechanism of action is far from clear. The study by Reddy et al has shown that Statin degrade SATB1 in mevalonate pathway dependent manner. We wished to delineate the molecular mechanism of action and analyzed the expression of SATB1 under Statin treatment in SW620 (Figure 2.3.4A) and COLO205 (Figure 2.3.4B) colorectal cancer aggressive cells. The treatment of Statin reduced the levels of SATB1 in both cell lines. Thus, indicating that Statin targets SATB1 hyper expressing cells. Further since colorectal cancer is driven by aberrant activation of Wnt signaling and Wnt signaling is required for SATB1 expression, we wished to see whether Statin also modulates Wnt signaling. We checked the expression of β-catenin under Statin treatment. The results suggest that Statin drastically reduces the expression of both SATB1 and β-catenin in HCT-15 colorectal cancer cells (Figure2.3.4C) and in DLD1 colorectal cancer cells (Figure 2.3.4D). Recently study by Taelman et al. has shown that multiple proteins have

putative GSK3β recognizing sequence including SATB1 (Taelman et al., 2010) and since β-catenin has canonical GSK3β recognizing sequence, we thought that Statin could be exerting its effects on SATB1 and β-catenin via GSK3β pathway. To test this hypothesis we checked the expression of inactive form of GSK3β under Statin treatment (Figure 2.3.4E). The Statin treatment reduced the amount of inactive GSK3β but no change was observed in total GSK3β, thus suggesting that Statin could be inducing the activation of GSK3β thereby promoting proteosomal degradation of SATB1 and β-catenin. To further prove that Statin induces the GSK3β activity, we depleted GSK3β in Statin treated cells and analyzed the expression of SATB1 and β-catenin. The depletion of GSK3β was not sufficient to rescue the effect of Statin on SATB1 and β-catenin expression (Figure 2.3.4F). Thus data suggests that Statin mediates the regulation of SATB1 and β-catenin via disturbing the mevalonate pathway. The effect on activation of GSK3β could be because of decrease in expression of SATB1 known to modulate the Wnt signaling pathway.

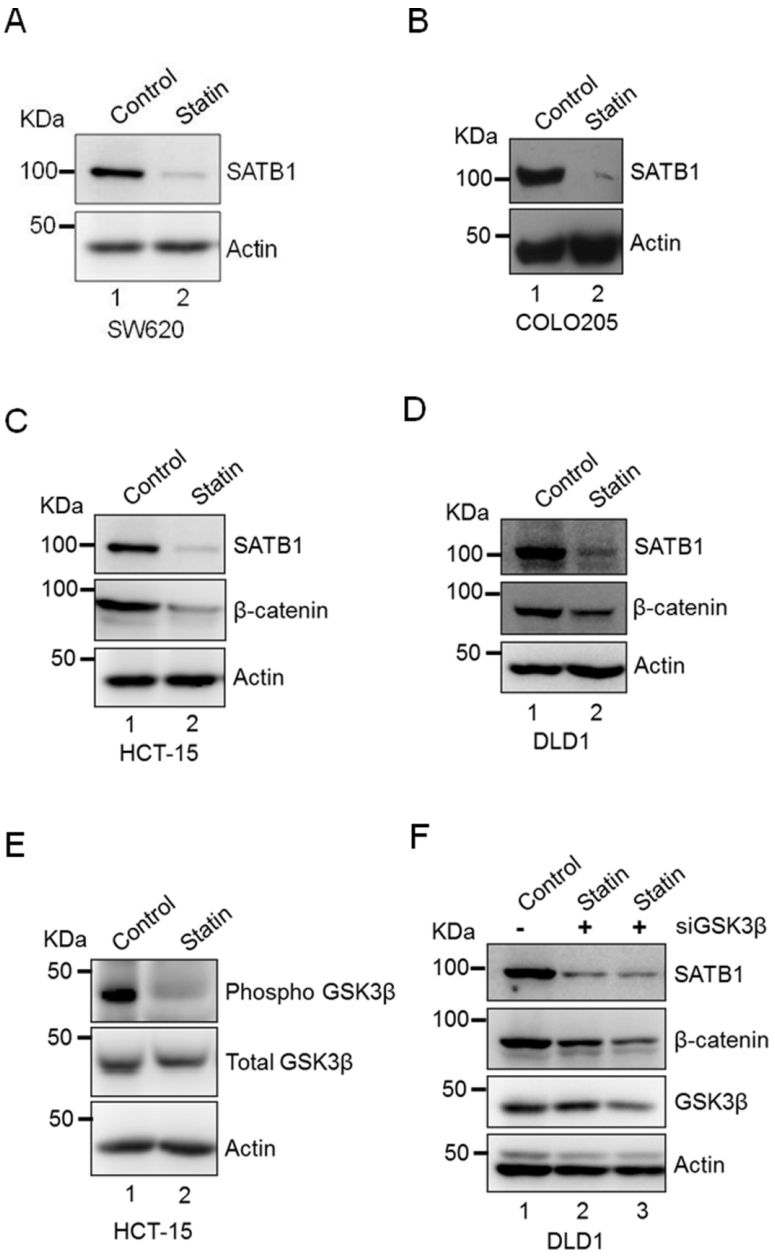


Figure 2.3.4 Regulation of SATB1: Anti-cholesterol drug mediated degradation of SATB1 and β-catenin (A) Immunoblot showing expression of SATB1 under Simvastatin treatment in SW620 cells. Actin was used as endogenous control. **(B)** Immunoblot showing expression of SATB1 under Simvastatin treatment in COLO205 cells. Actin used as endogenous control. Cells were treated with 25μM Simvastatin for 48 h. **(C)** Immunoblot showing expression of SATB1 and β-catenin under Simvastatin in HCT-15 cells. Actin was used as endogenous control. **(D)** Immunoblot showing expression of SATB1 and β-catenin under Simvastatin in DLD1 cells. Actin used as endogenous control. **(E)** Immunoblot showing expression of phospho-GSK3β (inactive GSK3β) and total GSK3β under Simvastatin treatment. Actin was used as endogenous control. **(F)** Immunoblot for expression of SATB1 and β-catenin under Simvastatin treatment and under depletion of GSK3β in Simvastatin treated cells. Cells were transfected with siGFP and siGSK3β first and after 12 h were then treated with simvastatin or DMSO as vehicle control. Actin was used as endogenous control.

2.3.5 SATB1 and TCF7L2/β-catenin signaling share positive feedback regulatory network.

The results described above provide multiple evidences that SATB1 and β-catenin share positive feedback regulatory network. To unequivocally establish the regulatory feedback between SATB1 and β-catenin and test whether SATB1 is indeed required for Wnt signaling, we induced Wnt signaling in CRL1790 by CHIR treatment and knocked down SATB1 in CHIR treated cells. The activation of Wnt signaling by CHIR treatment induced the expression of β-catenin and resulted in robust increase in SATB1 expression (Figure 2.3.5A). The effect of aberrant activation of Wnt signaling on β-catenin expression was abrogated upon SATB1 depletion in CHIR treated cells (Figure 2.3.5A). Similarly, upon activation of Wnt signaling in HeLa cells, expression of β-catenin was induced leading to increase in SATB1 expression at protein level and at transcript level. Depletion of SATB1 was sufficient to lower the levels of β-catenin even after activation of Wnt signaling (Figure 2.3.5B and Figure 2.3.5C). These results therefore indicate that SATB1 is regulated by Wnt signaling and is required for Wnt signaling-dependent regulation of β-catenin.

We further wished to investigate whether SATB1 is sufficient to rescue the effect of β-catenin depletion on downstream targets of Wnt signaling. Towards this, we knocked down β-catenin and overexpressed GFP-SATB1 in HCT116 cells and analyzed the expression of SATB1 and known downstream targets of Wnt signaling. The depletion of β-catenin resulted in decreased expression of SATB1 and that of known downstream targets. However, re-expression of SATB1 was not sufficient to rescue the effect of β-catenin depletion on expression of the downstream targets of Wnt signaling (Figure 2.3.5D). These results are consistent with the previous study demonstrating that SATB1 drives the β-catenin- dependent gene expression (Notani et al., 2010). We show that depletion of SATB1 downregulates the expression of TCF7L2 and that of its downstream targets. Next, we wished to monitor whether TCF7L2 re-expression can rescue the expression of the downstream targets of Wnt signaling. We knocked down SATB1 and ectopically expressed TCF7L2 in SATB1 depleted cells. Depletion of SATB1 resulted in decreased expression of TCF7L2 as well as the downstream targets of Wnt signaling AXIN2 and TCF7. However, forced expression of TCF7L2 was not sufficient to re-induce the expression of downstream

targets (Figure 2.3.5E). To further address whether changes mediated by SATB1 expression are dependent on TCF7L2 expression and can be reversed by TCF7L2 depletion, we overexpressed SATB1 in SW480 control cells and TCF7L2 depleted cells to segregate the effect of SATB1 on TCF7L2 and on downstream targets. The overexpression of SATB1 resulted in increase in expression of Wnt responsive gene AXIN2 and so as the expression of Wnt signaling mediator DVL2, but depletion of TCF7L2 was not sufficient to reverse the changes mediated by SATB1 overexpression (Figure 2.3.5F) Thus indicating that SATB1 exerts dual effect on Wnt signaling outcome, such that in hand regulates Wnt signaling intermediates and in the other hand regulates the expression of Wnt responsive genes. To further gain insight into the mechanistic role of SATB1 and functional crosstalk with Wnt signaling, we ectopically expressed the N-terminal 204 amino acid region of SATB1 in HCT116 cells that acts as dominant negative for SATB1 (Notani et al., 2011). The ectopic expression of the dominant negative form of SATB1 (SATB1-DN(1-204)) resulted in dramatic decrease in TCF7L2 and the expression of downstream target AXIN2. The forced expression of TCF7L2 in SATB1-DN(1-204) HCT116 cells was not sufficient to re-induce the expression of AXIN2 (Figure 2.3.5G), similarly the expression of SATB1 (novel Wnt target) and TCF7 was reduced significantly whereas the re-expression of TCF7L2 in SATB1-DN(1-204) overexpressed cells, was not sufficient to reinduce the expression of TCF7 and SATB1 (Figure 2.3.4G). Thus, these results strongly argue that SATB1 regulates the expression of TCF7L2 and both are essential for regulation of the downstream targets of Wnt signaling. SATB1 has been shown to interact with β-catenin via its N-terminal domain (Notani et al., 2010). Thus, it is plausible that the sequestering effect of SATB1's N-terminal domain on β-catenin might be the reason that re-expression of TCF7L2 is not sufficient to re-induce the expression of downstream targets of Wnt signaling. These data suggest that SATB1 regulates multiple events in the Wnt signaling cascade and regulation of SATB1 expression is an important determinant of colorectal tumorigenic transition.

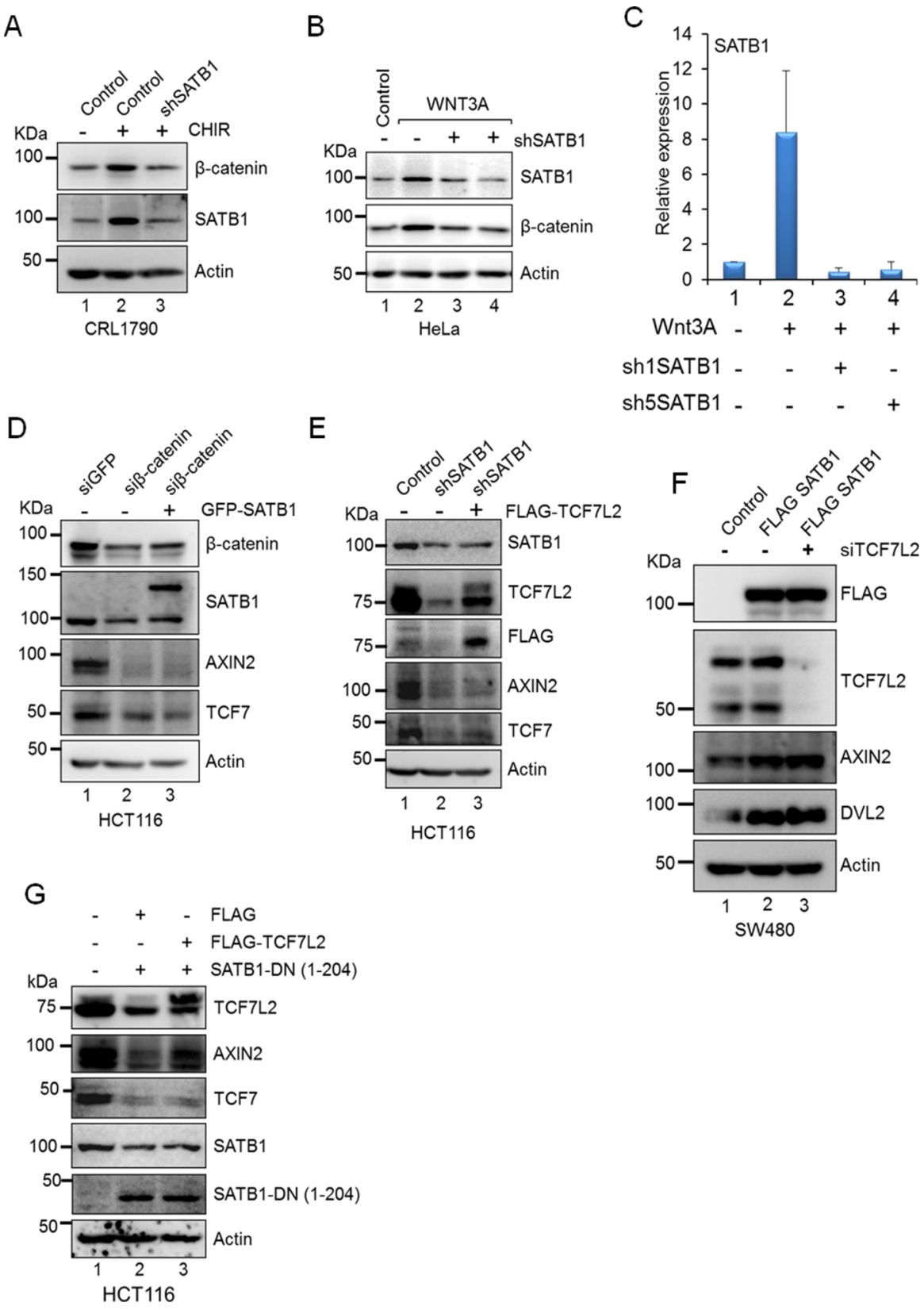


Figure 2.3.5: SATB1 and TCF7L2/β-catenin signaling share positive feedback regulatory network. (A) Immunoblot showing increase in expression of SATB1 and β-catenin upon CHIR treatment in CRL1790 cells and reduction in expression of β-catenin upon depletion of SATB1 in CHIR treated cells (compare lane 1 and lane 2;

lane 2 and lane 3). **(B)** Immunoblot showing increase in expression of SATB1 and β-catenin upon WNT3a treatment in HeLa cells and reduction in β-catenin upon SATB1 depletion in WNT3a treated cells (Compare lane 1 and lane 2; lanes 2, 3 and 4). **(C)** Increase in expression of SATB1 at transcript level upon WNT3A treatment and decrease upon SATB1 knockdown in Wnt3a treated HeLa cells. **(D)** Immunoblot showing expression of SATB1 and β-catenin under β-catenin depletion and under overexpression of SATB1 in β-catenin depleted HCT116 cells. HCT116 cells were transfected with siβ-catenin in combination of GFP and GFP-SATB1. **(E)** Immunoblot showing expression of SATB1, TCF7L2, AXIN2 and TCF7 in SATB1 depleted HCT116 cells transfected with FLAG and FLAG-TCF7L2. Actin was used as endogenous control and FLAG antibody to show overexpression of TCF7L2. **(F)** Immunoblot showing expression of SATB1, TCF7L2, AXIN2 and DVL2 in SATB1-SW480 cells transfected with siGFP and siTCF7L2. Actin used as endogenous control. **(G)** Immunoblot showing expression of TCF7L2, AXIN2, TCF7 and SATB1 in HCT116 cells transfected with SATB1-DN (1-204) (dominant negative) in combination with FLAG vector and FLAG-TCF7L2.

2.4 Discussion

The chromatin organizer SATB1 regulates large repertoire of genes essential for development and disease. Recently, hyperexpression of SATB1 has been implicated in poor prognosis and shorter survival in multiple cancers including the Wnt signaling driven colorectal cancer. In this study we have presented a novel regulatory network of SATB1 expression and Wnt signaling in colorectal cancer. Our results demonstrate for the first time that expression of SATB1 is induced by Wnt signaling which in turn is required for Wnt-dependent regulation of downstream targets of Wnt signaling.

SATB1- A novel target of Wnt signaling

Our data strongly suggests that induction of SATB1 expression could be the key molecular event during this transition from normal to cancer phenotype. This study sheds light on the role of Wnt signaling towards induction and regulation of SATB1. The molecular events or signaling pathways that induce SATB1 expression had not been elucidated until now. The expression of SATB1 is virtually undetectable in primary colorectal cell line CRL1790 and increases with aggressive and hyperactivated Wnt signaling phenotype of colorectal cancer cells. Thus, we reasoned that hyperactivated Wnt signaling could be responsible for induction and regulation of SATB1 expression. We analyzed the expression of SATB1 upon hyperactivation of Wnt signaling in colorectal primary cell line and established that Wnt signaling regulates SATB1 expression and this is dependent on β-catenin expression. Aberrant SATB1 expression was induced in primary colorectal cells upon activation of Wnt signaling at protein and at transcript level. Using other cellular model systems and *in vivo* model of APC mutant zebra fish, we could observe the robust increase in expression of SATB1 upon hyperactivation of Wnt signaling. Our data suggests that Wnt signaling could be the primary event leading to upregulation of SATB1 expression during tumorigenic transition in β-catenin-dependent manner.

To further characterize the direct regulation of SATB1 by TCF7L2/β-catenin signaling cascade, we analyzed the *Satb1* promoter and found multiple TCF7L2 binding motifs (CTTTGNN) (MacDonald et al., 2009; Frietze et al., 2012). ChIP analysis determined that TCF7L2 binds to *Satb1* promoter, promotes histone modifications such as histone H3 lysine 4 trimethylation and thereby regulates SATB1 expression. Similarly, hyperactivation of Wnt signaling induced β-catenin stabilization so as the occupancy

of TCF7L2 and β-catenin on *Satb1* promoter and thereby inducing SATB1 expression. Our data suggests that direct binding of TCF7L2/β-catenin complex to *Satb1* promoter induces SATB1 expression in colorectal cancer cells. Further, silencing of TCF7L2 and β-catenin downregulated SATB1 as well as known downstream targets of Wnt signaling, corroborating that SATB1 expression is regulated by TCF7L2/β-catenin signaling. Recent investigation of regulation of SATB1 in mouse CD4 T cells has shown that expression of SATB1 is governed by TCF1 (Gottimukkala and Galande et al., submitted). Together, these findings unequivocally document the role of TCF family proteins in regulation of chromatin organizer SATB1. The physiological importance of this regulation can be addressed by the fact that SATB1 expression promotes colorectal tumorigenesis, cellular proliferation and modulates Wnt signaling. Our data further establishes that SATB1 is required for Wnt signaling-dependent expression of β-catenin. Thus, SATB1 plays a critical role in modulating Wnt signaling and colorectal tumorigenesis. This is also supported by recent report by Nodin et al which demonstrated that SATB1 expression correlates with overexpression of β-catenin in colorectal tumors (Nodin et al., 2012a).

The data presented here suggests that SATB1 shares feedback regulatory loop with TCF7L2/β-catenin signaling that is very critical for colorectal tumorigenesis and cellular outcome of Wnt signaling. This is further explained by the fact that increased expression of β-catenin upon hyperactivation of Wnt signaling was reduced upon SATB1 depletion, thus suggesting that SATB1 is required for Wnt signaling-dependent regulation of β-catenin. Furthermore, we observed that SATB1 depletion led to concomitant decrease in TCF7L2 and Wnt target gene expression such as AXIN2 and TCF7 and investigated whether SATB1 mediates the regulation of Wnt target genes via TCF7L2 regulation. Surprisingly, we found that re-expression of TCF7L2 in SATB1 depleted cells was not sufficient to re-induce the expression of Wnt target genes, thus corroborating that SATB1 is a critical player essential for regulation of TCF7L2 and Wnt target genes. This is further strengthened by ChIP analysis which suggests that SATB1 directly binds to the promoters of Wnt target genes and hence regulates their expression. SATB1 is known to interact with various cofactors via its N-terminal domain. The N-terminal 204 amino acid region is essential for functional switching of SATB1 and acts as dominant negative for its function. SATB1 interacts with β-catenin during differentiation of T helper type 2 cells (Notani et al., 2010). The present study

further delineates whether crosstalk of SATB1 via its N-terminal domain can be essential for regulation of TCF7L2 and Wnt target genes in colorectal cancer cells. We found that overexpression of the dominant negative version of SATB1 (1-204) dramatically reduced the expression of TCF7L2 and its target genes, thus establishing that SATB1 crosstalk with cofactors is essential for outcome of Wnt signaling. Further re-expression of TCF7L2 is not sufficient to reinduce the expression of its target genes, thus suggesting that SATB1 independently regulates expression of TCF7L2 and its target genes. Previous data (Notani et al., 2010a) and data presented here therefore suggest that SATB1 might be interact with β-catenin and thereby mediating the changes associated with TCF7L2/β-catenin signaling. We also propose that the two complexes of SATB1/β-catenin and TCF7L2/β-catenin might occupy the promoters of Wnt target genes and regulate their expression (see model depicted in Figure 2.4.1). Thus our data suggests that SATB1 regulates TCF7L2 and both are essential for coordinated regulation of downstream Wnt signaling targets. It would be very interesting to delineate whether SATB1 can regulate downstream targets of Wnt signaling independent of β-catenin. Our data further demonstrates that depletion of β-catenin and inhibition of Wnt signaling by DKK1 in aggressive breast cancer cells (MDA-MB-231) reduced the expression of SATB1. Elucidation of the mechanistic role of SATB1 in colorectal tumorigenesis and its regulation by Wnt signaling provides enormous therapeutic possibilities. Having said this we elucidated this possibility to use anti-cholesterol drug Statin long known for its anti-cancerous role to target SATB1 and its possible role in modulating Wnt signaling. The data here suggests that Statin induces robust decrease in SATB1 and β-catenin in multiple colorectal cancer cells. Also induce activation of GSK3β activity, thereby hinting at that Statin mediated dysregulation of mevalonate pathway could be inducing activity of GSK3β to promote degradation of SATB1 and β-catenin, but subsequent experiments role out this possibility. Thus, targeting the regulation of SATB1 could provide new therapeutic approach towards various cancers.

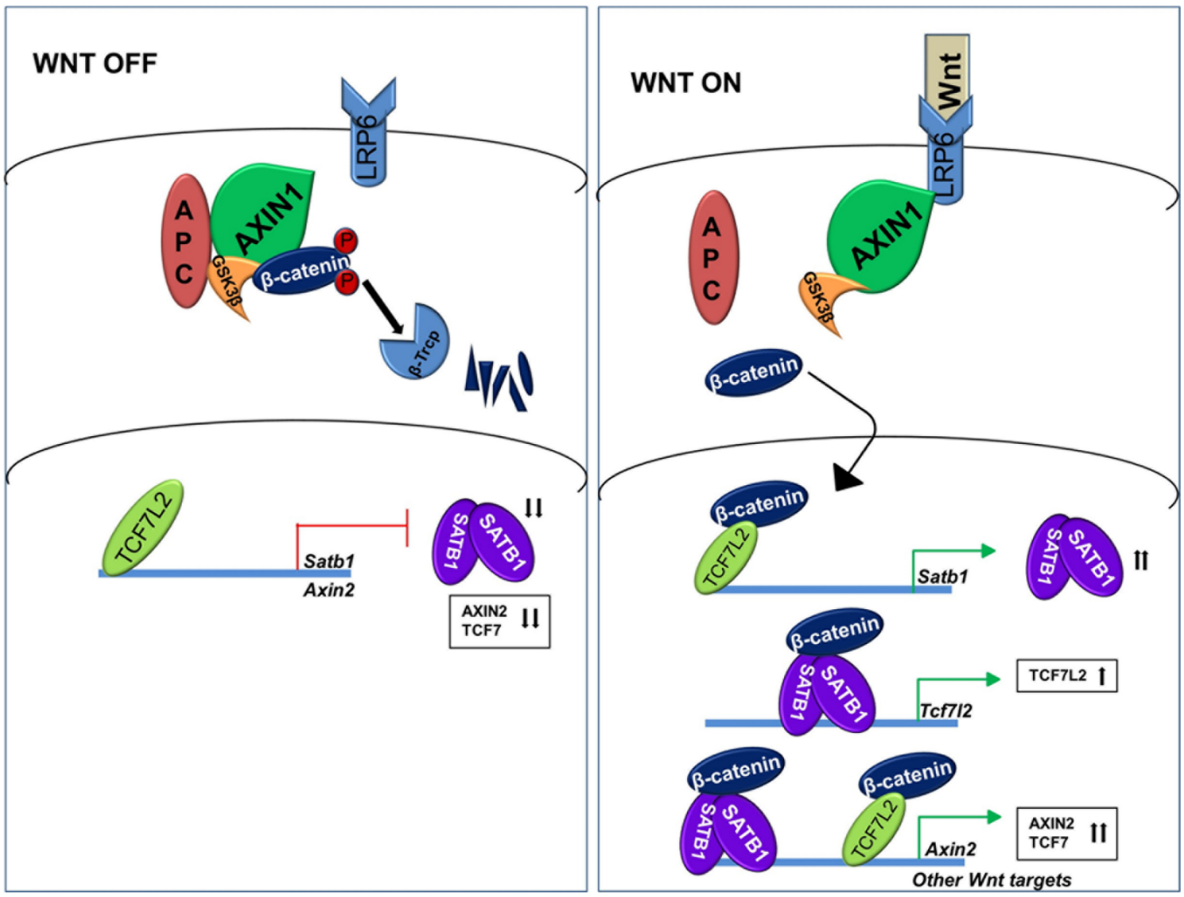


Figure 2.4.1: Model depicting the proposed molecular mechanism for SATB1's role and regulation. In Wnt-OFF state (Left), the levels of β-catenin are low and therefore the expression of Wnt responsive genes and SATB1 is reduced. In Wnt-ON state (Right), β-catenin levels increase. Subsequent to nuclear accumulation of β-catenin, the TCF7L2/ β-catenin complex binds to *Satb1* promoter thereby inducing its expression. The TCF7L2/β-catenin and SATB1/β-catenin complexes then bind to Wnt responsive genes to induce their expression.

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Chapter 3

Interplay of SP1 and Wnt signaling in regulating chromatin organizer SATB1

3.1 Introduction

Specificity protein 1 (SP1), a member of Kruppel family proteins containing C2H2 zinc finger domain (Black et al., 2001) was earlier thought to be a general transcription factor required for regulation of housekeeping genes. However, recent studies demonstrate SP1 is critical for cell specific regulation of genes required for hematopoietic development and essential for initiation and progression of cancers. Recent study by Gilmour et al. (2014) has shown that there are 12000 sites for SP1 genome-wide and SP1 is not only required for basal transcription but also required for induction and regulation of genes (Gilmour et al., 2014). SP1 is a member of SP family proteins classified into two groups-SP1-4 and SP4-9. SP1-4 have similar domain organization. SP1 has two N-terminal transactivation domains A and B, characterized by Glutamine-rich regions. The C-terminal domain of SP1 consists of highly conserved three zinc fingers required for highly specific DNA-binding activity (Kadonaga et al., 1986; Nagaoka et al., 2001). SP1 specifically binds to GC consensus site (Kadonaga et al., 1986; Nagaoka et al., 2001). Figure 3.1 summarizes the domain structure of SP1 and sites of post-translational modifications. Transactivation domain of SP1 undergoes posttranslational modifications. The D domain of SP1 is a multimerization domain mediated by DNA bound SP1 and unbound SP1 that leads to tetramerization (Mastrangelo et al., 1991; Su et al., 1991). Tetramerized SP1 mediates interaction of proximal promoter with enhancer elements thereby promotes super-activation of promoters (Mastrangelo et al., 1991; Su et al., 1991). SP1 is highly conserved in mammalian species. DNA-binding domain of SP1 is conserved in reptiles, fish and birds. SP1 is also conserved in Drosophila and shares homology with Drosophila button head protein (Estella et al., 2003; Wimmer et al., 1996). SP1 knockout is embryonic lethal since it is critical for embryonic development and plays essential role in cellular differentiation and development (Krüger et al., 2007; Marin et al., 1997). Recent study by Gilmour et al. showed that SP1 is required for terminal differentiation during embryonic development (Gilmour et al., 2014). Also SP1 specifically regulates Hox genes and is essential for regulation of genes involved in hematopoietic specification (Gilmour et al., 2014).

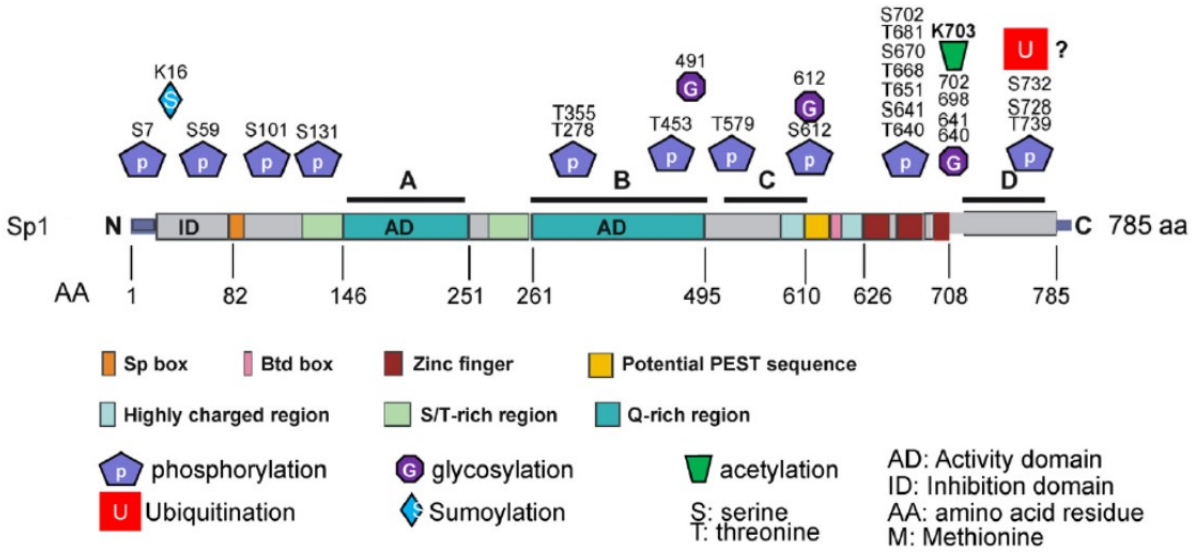

Figure 3.1: Schematic for domain organization and sites of modification on SP1. SP1 has two N-terminal transactivation domains A and B, characterized by Glutamine-rich regions. The C-terminal domain of SP1 consists of three highly conserved zinc fingers required for highly specific DNA-binding activity. The 'D' domain of SP1 is a multimerization domain mediated by DNA bound SP1 and unbound SP1 that leads to tetramerization. SP1 undergo phosphorylation, acetylation, glycosylation, ubiquitination and sumoylation (Adapted from Li et al., 2010).

3.1.1 SP1 in Cancer

Recent studies have provided contrasting evidences that SP1 not only is involved in regulating the expression of housekeeping genes but it also regulates myriad of genes essential for development and differentiation. SP1 expression has been shown to influence and regulate genes considered as hallmark of cancers (Beishline and Azizkhan-Clifford, 2015). SP1 expression is linked to number of cancers including breast, colorectal, pancreatic and lung cancers (Hsu and Hung, 2012; Jiang et al., 2008a; Wang et al., 2007). SP1 expression correlates with invasive and metastasis potential of cancers (Beishline and Azizkhan-Clifford, 2015). SP1 expression has also been shown to be associated with patient survival and higher expression correlates with poor prognosis. Higher expression in gastric cancer is shown to be associated with poor prognosis (Wang et al., 2003; Yao et al., 2004). Similarly, higher SP1 expression is correlated with overall poor survival and prognosis in breast, colorectal lung and thyroid cancers (Guan et al., 2012; Hsu and Hung, 2012; Wang et al., 2007).

To maintain independent proliferative signal, cancer cells undergo upregulation of genes required for this transition. For example, expression of growth signaling molecules is upregulated. SP1 has been shown to regulate the expression of growth signaling molecules; e.g. expression of insulin like growth factor is directly regulated by SP1 (Okamoto et al., 2001; Pollak, 2012; Werner et al., 1990) in coordination with insulin receptor-binding protein. Thus higher SP1 levels seem to induce molecular changes in cancer cells and crosstalk with various factors to maintain their self-proliferative signals. Another phenotype of cancer cells is to acquire replicative immortality (Hanahan and Weinberg, 2011). SP1 has been shown to regulate the expression of MDM2, key regulator of p53 activity in cancers, thereby inducing the inactivity of p53 and hence tumorigenic development and progression (Bond et al., 2004; Knappskog et al., 2011). Additionally, SP1 is involved in regulating the catalytic active domain of telomerase, thereby directly influencing replicative immortality of cancers (Kyo et al., 2000; Takakura et al., 1999; Wick et al., 1999). The human TERT gene which encodes catalytic subunit of human telomerase has been shown to have SP1 binding consensus site and lacks canonical TATA box (Kyo et al., 2000; Takakura et al., 1999; Wick et al., 1999). Furthermore, the TERT transcriptional activity is regulated by SP1 (Kyo et al., 2000). Comprehensive characterization suggests that SP1 plays role in cancer development and regulation of genes required during inhibition of growth suppression thereby prevents cells undergo quiescence. For example SP1 is directly involved in regulation of cyclin-dependent kinase inhibitor-p21(Abbas and Dutta, 2009). The regulation of p21 by SP1 was shown for the first time in leukemic cells (Biggs et al., 1996). Cancer cells activate angiogenic pathway to sustain oxygen and nutrient availability. SP1 has been implicated in regulating angiogenic factors, e.g. regulation of vascular endothelial growth factor (VEGF). Characterization of VEGF promoter revealed number of SP1- binding sites (Pages and Pouyssegur, 2005). Thus, SP1 expression is required for expression of molecular signatures critical for survival of cancer cells and progression of cancers. Higher expression of SP1 promotes tumor cell invasion and metastasis in various tumor models. SP1 has been shown to regulate extracellular matrix remodelers such as MMP2 (Qin et al., 1999). SP1 is required to recruit BRG1 component SWI/SNF complex on MMP2 promoter to induce transcriptional activation of MMP2. Further, crosstalk of p16 and SP1 is also essential for expression of MMP2 (Ma et al., 2004). Thus, SP1 is involved in regulating multiple factors that participate in cell migration

and metastasis. SP1 regulates the expression of molecular players considered as hallmarks of cancers (Beishline and Azizkhan-Clifford, 2015) and therefore regulation of SP1 could be one of the key events in deciding the fate of tumorigenic transition.

3.1.2 Role of SP1 in chromatin remodelling - Global perspective

SP1 induces transcriptional super-activation by tetramerization through its D domain (Doetzlhofer et al., 1999; Li et al., 2004; Porter et al., 1996). This mode of SP1 organization mediates synergistic activation or repression of genes. Further, tetrameric aggregation of SP1 has been shown to induce DNA looping between enhancer and promoter regions of genes such as the human heme oxygenase-1 gene (Deshane et al., 2010). The tetrameric structure on DNA provides docking site for transcription factors, transcriptional regulators and chromatin remodelers (Li et al., 2004), thus providing essential platform to activate or repress gene expression. SP1 interacts with SWI/SNF family proteins, recruits and remodels chromatin to promote accessibility for transcription machinery to initiate transcription (Chen et al., 1994; Lu et al., 2003). SP1 can differentially recruit repressor complexes such as SIN3A, HDAC1/HADAC2 to promote gene repression (Zhang and Dufau, 2003). Despite these intense efforts, the molecular mechanisms governing differential roles of SP1 in repression and activation of gene expression is not clear. The signaling pathways that might be involved in governing this could be interesting to pursue in future. Furthermore, SP1 has been shown to interact with acetyltransferases such as p300 or CBP to induce histone acetylation thereby promoting gene activation (Nunes et al., 2010). Interaction of SP1 with histone chaperons such as TAF1α has been shown to inhibit its DNA-binding activity (Kadam and Emerson, 2003). Another aspect of SP1's influence on chromatin is that it prevents spreading of heterochromatin. SP1 binds to nucleosomal DNA and blocks spreading of heterochromatin to neighbouring euchromatin (Kadam and Emerson, 2003). This is also established by preventing methylation of CPG island bound by SP1 (Brandeis et al., 1994; Macleod et al., 1994). SP1 also promotes repression independent of HDACs, however through DNMT1, to repress MAZ (Myc Associated Zing finger). Thus, SP1 is critical for influencing chromatin architecture to induce or repress gene expression. The signaling pathways that differentiate or segregate these two events remain to be elucidated. Whether the post-translational

modifications of SP1 could influence any of these two aspects is also not clear. Regulation of SP1 could be the plausible mechanism to establish this regulatory segregation of gene activation and gene suppression.

3.1.3 Regulation of SP1

SP1 was earlier thought to be ubiquitously expressed and involved in transcriptional regulation of housekeeping genes. However, recent growing evidences suggest tissue-specific role of SP1 in regulating genes considered as hallmarks of cancers and genes required during development and differentiation (Beishline and Azizkhan-Clifford, 2015; Gilmour et al., 2014). Considering the specific and dynamic role of SP1, its regulation could be a critical event to modulate cellular changes acquired during tumorigenic transition. SP1 is heavily modified by all types of modifications such as O-linked glycosylation, acetylation, phosphorylation, ubiquitination and sumoylation and is critical for switching SP1 functions. Large number of modifications discovered so for have not established any connection with signaling pathways or cellular context under which such modifications are acquired. Number of signaling kinases have been implicated in SP1 posttranslational modification that influence SP1 mode of action, DNA binding, stability and transactivation. Phosphorylation at Serine 59 is linked to SP1 stability and DNA-binding (De Borja et al., 2001; Haidweger et al., 2001; Iwahori et al., 2008; Kim and Lim, 2009; Spengler et al., 2008; Vicart et al., 2006; Wang et al., 2011). Similarly phosphorylation at Thr453 and Thr739 increases SP1 interaction with PDGFR-α promoter in ERK- dependent manner (Bonello and Khachigian, 2004). Moreover, phosphorylation at Thr 579 also reduces the interaction of SP1 with DNA (Armstrong et al., 1997) without changing the levels of total SP1. O-Glycosylation of SP1 was the earliest study to show that starvation regulates stability and induces degradation of SP1 (Han and Kudlow, 1997). The degradation mediated by glucose starvation was induced by N-terminus cleavage dependent on first seven amino acids. This process was shown to be proteasome pathway dependent (Su et al., 2000). Further SP1 was shown to undergo proteosome-dependent degradation in prostate cancer after treatment with Thiazolidinediones (Wei et al., 2009). Treatment with Thiazolidinediones was shown to mimic glucose starvation and induced the upregulation of beta transducin β-TrCP ubiquitin ligase. β-TrCP presumably interacts

with DSG motif at c-terminus of SP1 upon treatment with Thiazolidinediones thereby mediating subsequent degradation. Evidences further suggest that degradation can be mediated by phosphorylation by GSK3β at Ser-728 and Ser-732 and by ERK at Thr-739 (Wei et al., 2009). Despite evidences of proteosomal degradation, it is not clear how ubiquitination in cellular context takes place and secondly which residues are ubiquitinated. Further, the signaling pathways regulating SP1 phosphorylation and thereby its stability are not fully elucidated. SP1 is also regulated by SUMOylation at N-terminus (Wang et al., 2008). Sumo modified SP1 is recognized by Sumo-dependent RNF4 ubiquitin ligase (Wang et al., 2011). RNF4 binds to both C-terminus and Sumo modified N-terminus and induces SP1 degradation (Wang et al., 2011). Phosphorylation also influences the stability of SP1, e.g. study by Chuang et al. has demonstrated that phosphorylation at threonine 728 and threonine 739 prevents ubiquitination mediated degradation of SP1 and enhances its stability (Chuang et al., 2008).

Aberrant activation of Wnt signaling has been implicated in various cancers mediated by mutations in various regulators of this pathway. Recently, epigenetic events in addition to genetic changes have also been shown to be critical for outcome of Wnt signaling and progression of cancers (Jiang et al., 2008b). Similarly crosstalk between novel targets and hyperactivation of Wnt signaling has been implicated in regulating the fate of Wnt signaling (Mir et al., 2015). Interestingly, both SP1 and β-catenin possess the regulatory phosphodegron motif and interact with β-TrCP and thereby hinting at possible overlap of regulation of β-catenin and SP1. Multiple studies also reveal overlap of regulation and function. For example, SP1 negatively regulates Wnt antagonistic factor WIF1 (Liu et al., 2008). Similarly both SP1 (Wang et al., 2012) and β-catenin/Wnt signaling (Mir et al 2015) regulate SATB1 expression. Thus, based on these studies, there is possibility of SP1 and β-catenin are regulated by common pathway and hence regulating the novel target SATB1 and other Wnt target genes.

The chromatin organizer SATB1 has been shown to reprogram global gene expression to promote tumorigenesis (Han et al., 2008; Kohwi-Shigematsu et al., 2012; Mir et al., 2012). SATB1 hyperexpression is required for tumorigenic transition and regulation of SATB1 could be critical event during this transition. Earlier studies have shown that cellular prion accelerates the colorectal cancer metastasis via Fyn SP1-SATB1 axis (Wang et al., 2012). The study has demonstrated that cellular prion

induce colorectal tumorigenesis by upregulating SATB1 expression. Further, Wang et al. demonstrate that Prion-induced expression of SATB1 requires SP1. Furthermore, SP1 binds to SATB1 promoter and regulate its expression. However, it is not clear whether SP1 expression is also induced by the cellular prion pathway. The question that remains to be answered is what changes are acquired to induce the binding of SP1 to *Satb1* promoter. Cellular prion expression has been shown to influence PI3K/Akt, cAMP/PKA, PKC, Fyn, and Erk1/2 pathways. However, whether these pathways are required to induce the SP1 binding to *Satb1* promoter is not clear. Our study has shown that in colorectal cancer cells SATB1 expression is induced and regulated by TCF7L2/β-catenin signaling cascade. Also P13K/AKT pathway has been shown to crosstalk with Wnt signaling (Naito et al., 2005). Thus based on the common role of SP1 and Wnt/β-catenin signaling in regulating SATB1, we investigated whether β-catenin and SP1 follow the same pathway for their regulation and whether SP1/β-catenin signaling cascade could be involved in the regulation of chromatin organizer SATB1. In this study we demonstrate that SP1 stability is regulated by Wnt/β-catenin signaling cascade. We also demonstrate that SP1 is required for Wnt signaling-dependent stabilization of β-catenin to regulate TCF7L2/ β-catenin signaling-dependent regulation of SATB1.

3.2 Materials and Methods

3.2.1 Antibodies, reagents and plasmids

SATB1, TCF7L2, TCF7, SP1, GSK3β, β-TrCP, AXIN1 and AXIN2 antibodies were obtained from Cell Signaling Technology. β-catenin antibody was obtained from BD Transduction Laboratories. β-catenin for IP in HeLa was used from Santa Cruz. Actin and gamma-tubulin antibodies were obtained from Sigma. Secondary HRP conjugated antibodies were obtained from BioRad. FLAG-SP1 was cloned in pCMV9 3XFLAG. Mutant phosphodegron was generated by site directed mutagenesis and primers were designed as mentioned in Quickchange primer designer tool. Sequences were verified by sequencing. Phosphodegron delta was cloned in pCMV9 3XFLAG. 3XFLAG β-catenin and HA S37A β-catenin were used as described (Notani et al., 2010). The siRNA sequences for shSP1 and shβ-catenin was designed using Dharmacon design center. The siRNA sequence for siSP1, siβ-catenin and siGSK3β were procured from Dharmacon. All shRNAs were cloned in pSUPER Puro vector (Oligoengine). Recombinant Wnt3A protein was obtained from R&D systems. Pyrvinium Pamoate was obtained from Sigma.

3.2.2 Reporter assays

Luciferase assay was performed in HEK293 cells. HEK293 cells were first transfected with siGFP and siSP1 and after 24 h transfected with pGL3 basic control and TOP/FOP reporter constructs followed by CHIR treatment after 42 h post second transfection essentially as described (Azzolin et al., 2012). All reporter assays were performed in triplicates.

3.2.3 Cell culture, transfections and western blotting

SW480, SW620 and HCT116 cell lines were grown in DMEM with 10% FCS. CRL1790 were grown in MEM with 10% FCS. CRL1790, SW480, SW620 and HeLa were obtained from American Type Culture Collection (ATCC). HCT116 was obtained from European Collection of Cell Cultures (ECACC) SIGMA. For Immunoblotting 25 µg of lysate was loaded in each lane unless mentioned otherwise. For transfection with siRNA mediated depletion, cells were seeded and after 24 hours transfected with

indicated siRNA and then harvested for immunoblotting and RNA extraction. The sequences of shRNA and siRNA used are listed in Table 3.2.1.

Table 3.2.1 siRNA/shRNA sequences used in this study.

siRNA/shRNA	RNA interference sequence	Notes
GFP (shRNA)	GAAGCAGCACGACTTCTTC	
GSK3β	CCCAAATGTCAAACTACCA	Azzolin et al., 2012
β-catenin#1	GTAGCTGATATTGATGGAC	Equimolar mix of 1 and 2 was used (Azzolin et al., 2012)
β-catenin#2	GCTTGGAATGAGACTGCTG	
TCF7L2#1	AGAGAAGAGCAAGCGAAATAC	Equimolar mix of 1 and 2 was used (Azzolin et al., 2012)
TCF7L2#2	TATCGAGTTCATTGGTCAATA	
SP1#1	GCCAATAGCTACTCAACTA	Equimolar mix of 1,2 and 3 was used
SP1#2	GAAGGGAGGCCCAGGTGTA	
SP1#3	GGGCAGACCTTTACAACTC	

3.2.4 Biochemical assays

To activate Wnt signaling CRL1790 cells were treated with CHIR (3 μM) and BIO (1 μM) for 48 h and harvested for protein and RNA. For stability experiments cells were first transfected with siRNA and after 24 h transfected with plasmids and/or treated with Wnt3A (100ng/ml) or (CHIR 3μM/ml) as mentioned in figure legends. Similarly Pyrvinium Pamoate (PP) was used at concentration of 100 nM and cells were harvested after 48 h. For knockdown of SP1 and β-catenin under CHIR and Wnt3A, cells were first transfected with control siRNA, siSP1 and siβ-catenin and after 12 h treated with CHIR for 48 h in case of CRL1790 cells, whereas other cells were treated with CHIR and Wnt3a for 6 h after 42 h of transfection.

3.2.5 RNA isolation & RT-PCR

RNA was isolated using Trizol reagent (Invitrogen). Two μg of RNA was used for first strand cDNA synthesis using Superscript III (Invitrogen). The cDNA was then used for quantitative PCR analysis in triplicates using an ABI 7500 Fast real-time PCR System (Applied Biosystems) as described (Ordinario et al., 2012). The sequences of oligonucleotide primers used for real-time PCR are listed in Table 3.2.2.

Table 3.2.2 Sequence of Primers used for qPCR.

qPCR Primer	Sequence	Notes
hSATB1 Forward	AGCAACAGGTTTCGACCAAC	TaqMan primer and probe (ABi)
hSATB1 Reverse	TCTGAAAGCAAGCCCTGAGT	
huGAPDH2 Forward	CTGCACCACCAACTGCTTAG	
huGAPDH2 Reverse	GTCTTCTGGGTGGCAGTGAT	

3.2.6 Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed as described (Karmodiya et al., 2012). Briefly, cells were cross-linked by addition of formaldehyde to 1% final concentration in media and incubated at room temperature for 10 min, neutralized with 125 mM Glycine. Cells were then subjected to sonication using Covaris sonicator to fragment chromatin to obtain 200–500 bp fragments. Sonicated chromatin was precleared with non-saturated beads. Precleared chromatin was incubated with specific antibodies and respective IgG types were used as isotype controls. Next day, beads saturated with tRNA and BSA were added (40 µl packed beads) and incubated for 4 h on rocker to pull down the antibody-bound chromatin and were subjected to elution using buffer containing SDS and sodium bicarbonate. Eluted chromatin was de-crosslinked and protein was removed by treating with proteinase K. Purified immunoprecipitated chromatin was subjected to PCR amplification using specific primers. Input chromatin was used as a control. ChIP primer details are listed in Table 3.2.3.

Table 3.2.3 Sequnces of Primers used for ChIP-PCR

ChIP Primer	Sequence	Notes
huSATB1 Forward	TGGAATGTTTAAGTTTTTAATGGAGTT	ChIP-PCR
huSATB1 Reverse	TCAGTGGAATATGTGACTAGGTATTGA	
huSATB1 Forward	GTGGGGCTCATTGATTGATT	ChIP-qPCR
huSATB1 Reverse	TGATGTTTCAAAGGGGTCTTG	

3.2.7 Immunoprecipitation and co- immunoprecipitation

Immunoprecipitation and co- immunoprecipitation were done as essentially mentioned in (Cordenonsi et al., 2011). Briefly cells were harvested and lysed in lysis buffer- 20 mM HEPES (pH 7.8), 400 mM KCl, 5% Glycerol, 5 mM EDTA, 0.4% NP40,

phosphatase and protease inhibitors, lysates were sonicated and cleared by centrifugation. Before immunoprecipitation, lysates were diluted to 20 mM HEPES (pH 7.8), 50 mM KCl, 5% Glycerol, 2.5 mM MgCl2, 0.05% NP40 and incubated with the appropriate protein G-Dyna beads bound antibodies for four hours at 4°C (1/8th to the lysis buffer). After three washes in binding buffer, co-purified proteins were analyzed by immunoblotting by using the ExactaCruz reagents (Santa Cruz biotechnology) as secondary antibodies to reduce the background from IgG. Beads used for IP were pre-coated with antibody and then washed twice with binding buffer.

3.2.8 GST pull-down

For GST pull-down, beads with purified proteins GST and GST-β-catenin were incubated with lysate in binding buffer (25 mM HEPES (pH 7.9), 0.4 M KCl, 0.4% NP40, 5 mM EDTA, 1 mM DTT, 10% glycerol with protease inhibitors) for one hour. After four washes with binding buffer, interaction of endogenous protein from cell lysate with purified proteins was determined by immunoblotting. For GST Pull-downs with purified proteins FLAG-SP1 expressing cells, FLAG-SP1 was first immunoprecipitated using FLAG antibody. After immunoprecipitation, FLAG-SP1 was eluted from beads using the FLAG peptide, followed by incubation with GST and GST-β-catenin in binding buffer for one hour. After four washes with binding buffer, interaction complexes were resolved by SDS-PAGE and determined by immunoblotting.

3.3 Results

3.3.1 SP1 expression correlates with aggressiveness of colorectal cancer cells and Wnt signaling is required for SP1 stabilization

To elucidate the regulation of SP1, we analyzed the expression of SP1 in aggressive colorectal cancer cells in comparison with primary cell line CRL1790. The data shows the levels of SP1 are higher in colorectal cancer cells HCT116 and SW620 in comparison with primary cell line CRL1790 (Figure 3.3.1A). The expression pattern of SP1 indicates that tumor derived cells have higher expression and correlates with expression of β-catenin (Figure 3.3.1A). The levels of SP1 are higher in colorectal cancer cells harbouring aberrantly active Wnt signaling, thus we thought Wnt signaling could be regulating SP1 expression. Further, SP1 is heavily regulated by posttranslational modifications especially phosphorylation which is critical for its stability. A recent study by Wang et al. has shown that treatment of Thiazolidinediones mimics glucose starvation in prostate cancer cells and thereby induces beta transducin β-TrCP expression and promotes degradation of SP1 but signaling pathway or molecular mechanism for SP1 stabilization is far from clear. The C-terminus has putative phospho-degron motif recognized by GSK3β (Figure 3.3.1B). To test whether stimulation of Wnt signaling could be involved in stabilization of SP1, we treated primary colorectal cells CRL1790 expressing lower levels of SP1 with GSK3β inhibitor. The inactivation of GSK3β induced the higher levels of SP1 so as the known target β-catenin (Figure 3.3.1C). Next, to prove that stimulation of Wnt signaling cascade in HEK293 cells can induce the SP1 stabilization at protein level, we treated mCherry-SP1 expressing cells with GSK3β inhibitor CHIR. The treatment of HEK293 cells induced the levels of cherry tagged SP1 and also levels of β-catenin and endogenous SP1 (Figure 3.3.1D, compare lane 2 with lane 3). To further corroborate, we depleted GSK3β in FLAG-SP1 expressing HEK293 cells. Depletion of GSK3β induced the levels of FLAG tagged SP1 (Figure 3.3.1E, compare lane 2 with lane 3), thus demonstrating that activity of GSK3β is critical in regulating the stability of SP1. Since HEK293 cells have intact destruction complex and respond to Wnt stimulation, we sought to use these cells to determine whether Wnt stimulation can induce stability of SP1. To test this we treated HEK293 FLAG-SP1 expressing cells with Wnt3A in time-dependent manner (scheme for hypothesis presented in Figure 3.3.1E). The treatment of FLAG-SP1 HEK 293 cells induced the robust stability of SP1 at protein

level as determined by levels of FLAG tag in comparison with control (Figure 3.3.1F compare lane 2 with lane 3 and lane 4). The Wnt stimulation also induced the stability of known target β-catenin indicating the activation of Wnt signaling cascade. This analysis revealed that aberrant activation is required to induce the stability of SP1 and thus could be reason that Wnt driven colorectal cancer cells express higher levels of SP1. Further to investigate that Wnt stimulation induces stability of SP1 through inhibition of proteosomal pathway, we treated FLAG-SP1 expressing HEK293 cells with proteosomal pathway inhibitor MG132. The inhibition of pathway induced the stability of SP1 (Figure 3.3.1G), indicating that proteosomal pathway is involved in destabilization of SP1 in HEK293 cells to keep the levels of SP1 low. Moreover, to investigate that phosphorylation of serine residues in phospho-degron motif is responsible for GSK3β mediated degradation we mutated both the serine residues and also alternatively deleted phosphor-degron containing C-terminus. Mutation and deletion drastically induced the levels of ectopically expressed FLAG-SP1 in HEK293 cells phenocopying the effect of Wnt stimulation and GSK3β inhibition (Figure 3.3.1H).

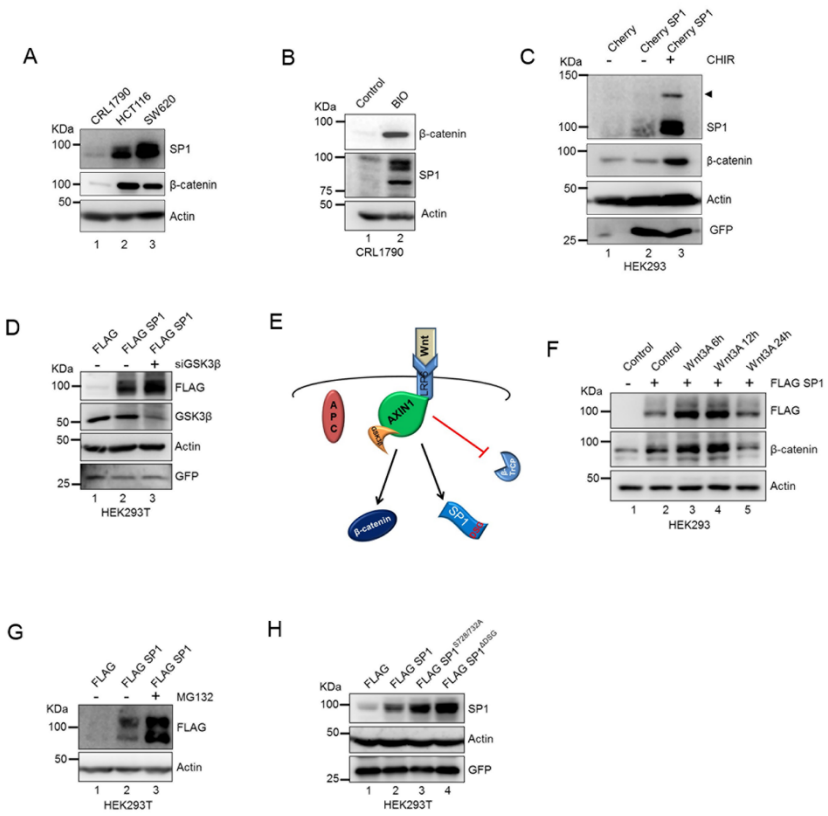

Figure 3.3.1: Wnt/β-catenin signaling induces SP1 stabilization (A) Immunoblot for expression of SP1 and β-catenin in CRL1790, HCT116 and SW620 Wnt signaling driven colorectal cancer cells. SP1 showing higher expression in Wnt signaling driven colorectal cancer cells in comparison with primary cell line CRL1790 (Compare lane 1,2 and lane3). Actin was used as endogenous control. **(B)** Immunoblot for expression of SP1 in CRL1790 in control cells and BIO (GSK3β inhibitor) treated cells. Bio treated cells show higher SP1 expression in comparison with control. Actin was used as endogenous control. **(C)** Immunoblot for FLAG-SP1, GSK3β in sicontrol and in siGSK3β HEK293 cells. Depletion of GSK3β increasing the expression/ stability of FLAG tagged SP1 in comparison with sicontrol FLAG-SP1 expressing cells. GFP used as transfection control. Actin was used as endogenous control. **(D)** Immunoblot for Cherry SP1 and β-catenin in control and CHIR treated HEK293 cells. Treatment of CHIR increases the Cherry tagged SP1 and β-catenin in comparison with control. Actin used as endogenous control. GFP was used as transfection control. **(E)** Schematic prediction of Wnt signaling in SP1 stabilization. **(F)** Immunoblot for FLAG-SP1 and β-catenin in control and Wnt3A treatment in time-dependent manner. HEK293 cells were transfected with FLAG and FLAG-SP1 and FLAG-SP1 transfected cells were treated with recombinant Wnt3A for 6 h-24 h. FLAG-SP1 stability increased in Wnt3A treated cells in comparison with non-treated cells. The levels of β-catenin increased upon Wnt3A treatment indicating the activation of Wnt signaling. Actin was used as endogenous control. **(G)** Immunoblot for FLAG-SP1 in control and MG132 (proteasome pathway inhibitor) treated cells. HEK293 cell were transfected with FLAG-SP1 and treated with DMSO and MG132 for 4 h. MG132 treated cells showed higher levels of FLAG-SP1 in comparison with DMSO treated cells. **(H)** Immunoblot for FLAG-SP1, FLAG-SP1 mutant and FLAG-SP1 delta phosphodegron in HEK293 cells.

HEK293 cells were transfected with FLAG, FLAG-SP1, FLAG-SP1 mutant and FLAG-SP1 delta phosphodegron. Actin used as endogenous control. GFP was used as transfection control.

3.3.2 SP1 interacts with β-catenin in colorectal cancer cells

We show that SP1 stability is induced by Wnt signaling stimulation. Also that Wnt signaling stimulation by Wnt3A or CHIR treatment induces stabilization of β-catenin. Further both SP1 and β-catenin degradation is mediated by GSK3β in WNT OFF condition. Thus this correlation prompted us to investigate whether both physically interact with each other and are part of same Wnt signaling cascade. To determine whether SP1 physically interacts with β-catenin, we immunoprecipitated endogenous SP1 and β-catenin from colorectal cancer HCT-15 cells and interaction was determined by western blotting. The data reveals that SP1 interacts with β-catenin (Figure 3.3.2A, lane 3 and lane 4). Further, similar interaction was observed in COLO205 wherein immunoprecipitation with β-catenin pulled down SP1 (Figure 3.3.2B). To further confirm the SP1 interaction with β-catenin, we overexpressed mutant S37A β-catenin in HeLa cells and immunoprecipitated with β-catenin antibody. The analysis reveals that SP1 physically interacts with β-catenin (Figure 3.3.2C). To further corroborate that SP1 directly interacts with β-catenin, we performed *in vitro* GST pull-down assays using HCT116 lysate. The data reveals that GST-β-catenin interacts with endogenous SP1 *in vitro* (Figure 3.3.2D left panel lane 3). Similar result was observed upon GST pull-down assay using lysate from FLAG-SP1 expressing HEK293 cells (Figure 3.3.2E left panel, lane 3). Thus data confirms that SP1 physically interacts with β-catenin. Since our data suggests that SP1 has putative phosphodegron motif and GSK3β induces the degradation of SP1 under physiological condition and follows the Wnt signaling pathway for its stabilization similar to β-catenin, we wished to see whether SP1 interacts with GSK3β in WNT-OFF state in HEK293T cells. We overexpressed FLAG-SP1 in HEK293T cells and performed immunoprecipitation using anti-FLAG antibody and interaction was monitored by western blotting. The co-immunoprecipitation data suggests that SP1 physically interacts with GSK3β in WNT-OFF state (Figure 3.3.2F). HEK293T cells possess functional destruction complex consisting of AXIN1, APC and GSK3β and since we show that SP1 follows the Wnt signaling pathway for its stabilization, we sought to

determine whether SP1 interacts with AXIN1 and is part of destruction complex under WNT OFF state of HEK293T cells. We overexpressed FLAG-SP1 in HEK293T cells and did co-immunoprecipitation using FLAG antibody and association of SP1 with AXIN1 was determined by western blotting. The data reveals that SP1 physically interacts with AXIN1 in WNT-OFF state (Figure 3.3.2G). Thus data suggests that SP1 follows the Wnt signaling pathway for stabilization and interacts with components of cytoplasmic destruction complex.

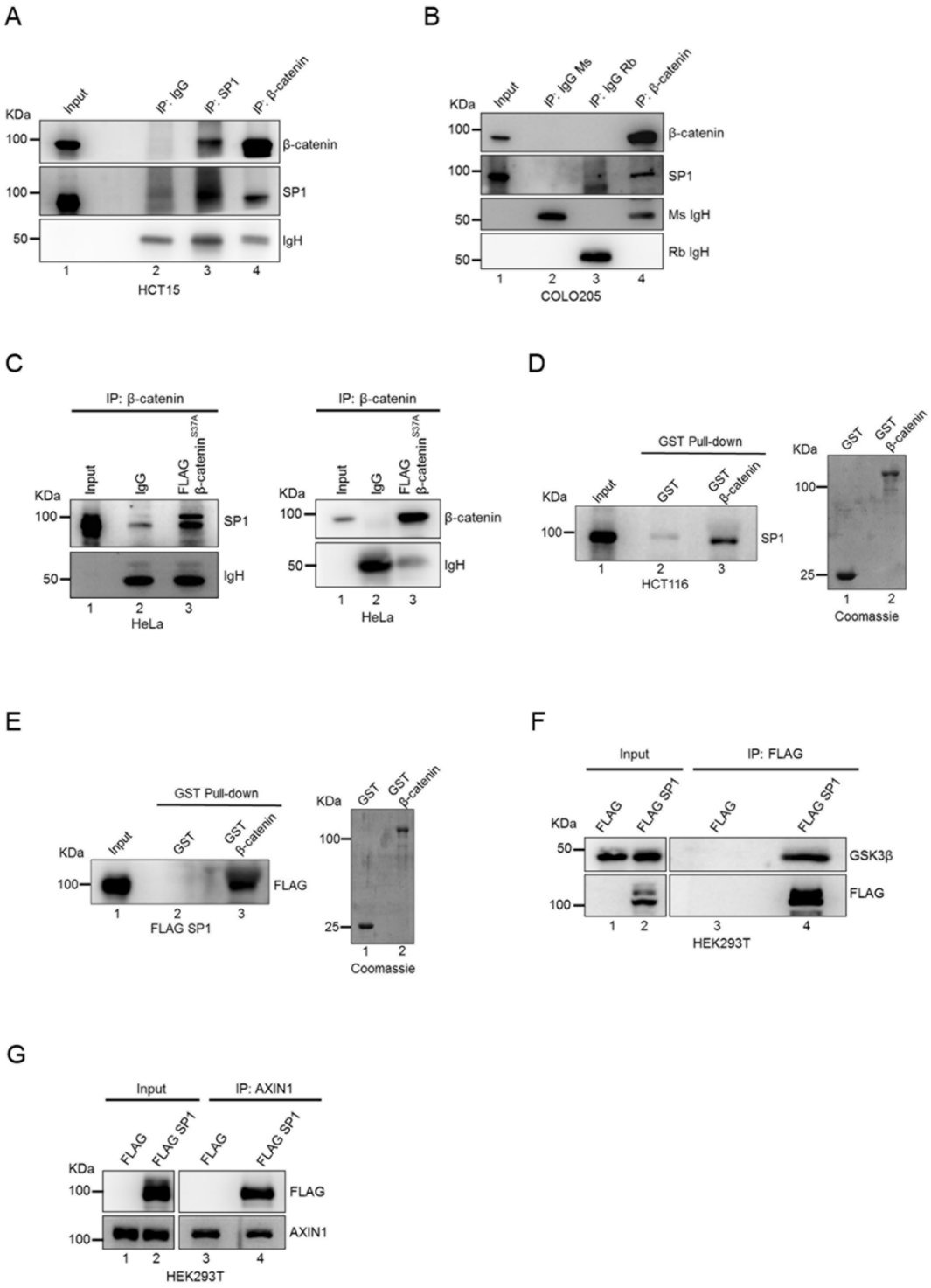

Figure 3.3.2: SP1 interacts with β-catenin in colorectal cancer cells (A) Immunoblot for endogenous SP1 and β-catenin co-immunoprecipitation with β-catenin immunoprecipitation and SP1 immunoprecipitation respectively from HCT-15 lysates. Immunoprecipitation (IP) was done using antibody against β-catenin and SP1. IP with IgG was used as negative control. **(B)** Immunoblot for endogenous SP1 co-immunoprecipitated with endogenous β-catenin from colorectal cancer cell line COLO205. IP with respective IgG Isotype was used as negative control. **(C)**

Immunoblot for endogenous SP1 co-immunoprecipitated with FLAG-S37A β-catenin using β-catenin antibody from HeLa lysate. FLAG-S37A β-catenin was overexpressed in HeLa cells and IP was performed with anti-β-catenin. IP with specific IgG Isotype was used as negative control. **(D)** Left panel: Immunoblot for endogenous SP1 from HCT116 lysate pull-down by GST tagged β-catenin immobilized on glutathione resin. GST protein used as negative control**.** Right panel: coomassie brilliant blue stained gel for GST and GST-β-catenin**. (E)** Left panel: Immunoblot for FLAG from HEK293T FLAG-SP1 expressing cells pull-down by GST β-catenin immobilized on glutathione resin. GST protein used as negative control. Right panel: coomassie for GST and GST-β-catenin. **(F)** Immunoblot for endogenous GSK3β co-immunoprecipitated with FLAG-SP1 from HEK293T lysate. IP was performed using FLAG antibody in FLAG-HEK293T and FLAG-SP1 HEK293T cells. Lysate from FLAG-HEK293T cells was used as negative control. **(G)** Immunoblot for FLAG-SP1 co-immunoprecipitated with endogenous AXIN1 from HEK293T lysate. IP was done using AXIN1 antibody in FLAG-HEK293T and FLAG-SP1 HEK293T cells. Lysate from FLAG-HEK293T cells was used as negative control.

3.3.3 Expression of β-catenin is required for Wnt signaling induced stabilization of SP1

The core event of Wnt signaling is stabilization of β-catenin. Since our data establishes that Wnt signaling via inactivation of GSK3β induces the stabilization of SP1 and we also show that SP1 interacts with β-catenin in Wnt signaling driven colorectal cancer cells, we wished to investigate whether Wnt stimulation induces SP1 stabilization through β-catenin. To prove this, we analyzed the expression of SP1 under β-catenin depletion in β-catenin mutant HCT116 cells. Depletion of β-catenin resulted in drastic decrease in SP1 expression at protein level (Figure 3.3.3A), but no change was observed at transcript level (Figure 3.2.2B). Similarly overexpression of β-catenin in SW480 cells induced the expression of SP1 (Figure 3.3.3C) but β-catenin overexpression did not upregulate the expression of SP1 at transcript level (Figure 3.3.3D). Thus this analysis revealed that β-catenin is required for stability of SP1 at protein level. Next we sought to determine that Wnt signaling induced SP1 stability requires β-catenin (see schematic in Figure 3.3.3E), we stimulated the FLAG-SP1 expressing cells with Wnt3A and depleted β-catenin. Activation of Wnt signaling by Wnt3A treatment induced the stability of ectopically expressed FLAG tagged SP1 (Figure 3.3.3F compare lane 1 and lane 2). Depletion of β-catenin in FLAG-SP1 expressing cells drastically reduced the stability of SP1 even after stimulation by

Wnt3A (Figure 3.3.3F, compare lane 2 and lane 3). Thereby data reveals that β-catenin is required for inducing Wnt signaling dependent SP1 stabilization and further provides link that Wnt signaling could be common route for stabilization of β-catenin and SP1. To further corroborate the role of β-catenin in stabilization of SP1, we analyzed the stability of SP1 in colorectal cancer cells having Wnt signaling pathway constitutively in ON state. We overexpressed FLAG-SP1 in sicontrol HCT116 and siβ-catenin HCT116 cells. Depletion of β-catenin in FLAG-SP1 expressing HCT116 cells drastically reduced the levels of ectopically expressed FLAG-SP1 in comparison with sicontrol FLAG-SP1 expressing cells (Figure 3.3.3G, compare lane 2 and lane 3, Experiment in duplicates). GFP used as transfection control. Conversely, to demonstrate that β-catenin can induce SP1 stability, we overexpressed FLAG-SP1 in control HEK293 cells and in HA-S37A β-catenin (mutant β-catenin non-responsive to GSK3β mediated regulation and therefore constitutively active) expressing cells. The overexpression of β-catenin robustly induced the SP1 stability in comparison with Vector control FLAG-SP1 expressing cells (Figure 3.3.3H, compare lanes 2 and 3). Since our data suggests that GSK3β induces degradation under WNT-OFF state and WNT-ON state induces stabilization, also overexpression of β-catenin reverses the effect of GSK3β on stabilization of SP1 even in WNT-OFF state. This prompted us to investigate whether depletion of β-catenin induces degradation of SP1 via GSK3β activation. To prove this we overexpressed FLAG-SP1 in HEK293 cells and depleted β-catenin and alternatively in combination of GSK3β and β-catenin depletion. Depletion of β-catenin reduced the levels of ectopically expressed FLAG-SP1 in comparison with sicontrol (Figure 3.3.3I, compare lane 2 with lane 3). In contrast, depletion of GSK3β in β-catenin depleted FLAG-SP1 expressing HEK293 cells rescued the ectopic levels of FLAG-SP1 (Figure 3.3.3I, compare lane 3 with lane 4). Further, to understand this crosstalk, we analyzed the SP1 stability under β-catenin degradation using Pyrvinium Pamoate (PP) and also inhibited GSK3β activity in PP treated FLAG-SP1 expressing cells using CHIR. PP treatment induces the degradation of β-catenin so as that of FLAG tagged SP1 (Figure 3.3.3J, compare lane 2 and lane 3), whereas inhibition of GSK3β by CHIR restored the levels of FLAG-SP1 (Figure 3.3.3J, compare lane 3 and lane 4). Thus above analysis reveals that β-catenin is essential to prevent the degradation of SP1 mediated by GSKβ activity. Interestingly, HCT116 cells possess functional destruction complex, however, with a mutation in β-catenin to evade the effect of destruction complex on levels β-catenin

and maintain higher levels of β-catenin. This could be the reason that SP1 levels are higher in HCT116 cells even though having active GSK3β and functional destruction complex. These findings therefore establish mechanistic link for requirement of β-catenin to maintain stabilization of SP1. Notably, the degradation of β-catenin also reduced the levels of the known Wnt-responsive gene TCF7, however rescue of SP1 expression by CHIR treatment did not reinduce the expression of TCF7 and β-catenin.

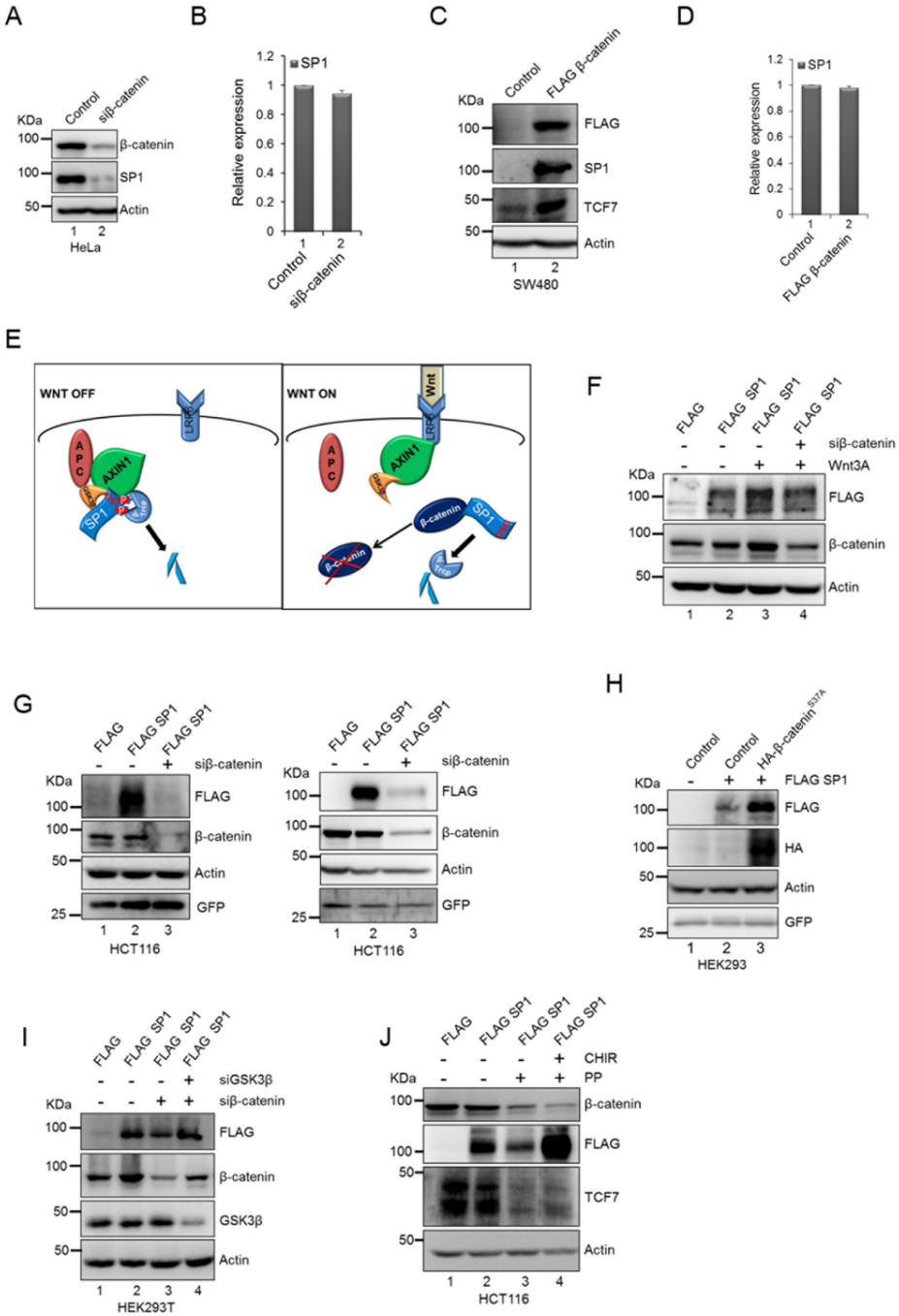

Figure 3.3.3: Expression of β-catenin is required for Wnt signaling induced stabilization of SP1. (A) Immunoblot for expression of β-catenin and SP1 in sicontrol

HCT116 cells and siβ-catenin HCT116 cells. Depletion of β-catenin reduces the expression of SP1 at protein level. Actin was used as endogenous control. **(B)** Relative transcript level of SP1 in sicontrol and siβ-catenin. Depletion of β-catenin did not decrease the expression of SP1 at transcript level. GAPDH2 used as endogenous control. Error bar represents SD for triplicates. **(C)** Immunoblot for expression of SP1, TCF7 and FLAG β-catenin in control SW480 cells and FLAG β-catenin SW480 cells. Overexpression of β-catenin induces the expression of SP1. Actin was used as endogenous control. **(D)** Relative transcript level of SP1 in control SW80 and FLAG β-catenin SW480 cells. Overexpression of β-catenin did not induce the SP1 expression at transcript level. GAPDH2 used as endogenous control. Error bar represents SD for triplicates. **(E)** Schematic prediction of Wnt signaling dependent role of β-catenin in stabilization of SP1. **(F)** Immunoblot for ectopically expressed FLAG-SP1 using FLAG antibody and for β-catenin in FLAG-SP1 sicontrol HEK293 cells and FLAG-SP1 Wnt3A treated and siβ-catenin FLAG-SP1 Wnt3A treated HEK293 cells. Wnt3A treatment induced the stability of FLAG-SP1. HEK293 cells were transfected with sicontrol and siβ-catenin. After 24h transfected with FLAG and FLAG-SP1 as indicated in lanes. After 42h, cells were treated with Wnt3A for 6h. Actin was used as endogenous control. **(G)** Immunoblot for ectopically expressed FLAG-SP1 using FLAG antibody and β-catenin in sicontrol HCT116 cells and siβ-catenin HCT116 cells. Depletion of β-catenin decreased the stability of FLAG-SP1. Actin was used as endogenous control. Data for two independent experiments is presented. **(H)** Immunoblot for ectopically expressed FLAG-SP1 using FLAG antibody and for HA β-catenin using HA antibody in control and HA β-catenin expressing HEK293 cells. HEK293 cells were co-transfected FLAG and HA vector, FLAG-SP1 and HA vector, FLAG-SP1 and HA β-catenin. Overexpression of HA β-catenin increases the stability of FLAG-SP1 in comparison with control. GFP used as transfection control. Actin was used as endogenous control. **(I)** Immunoblot for FLAG-SP1 using FLAG antibody in sicontrol FLAG-SP1 HEK293 cells, siβ-catenin FLAG-SP1 and siβ-catenin, siGSK3β, FLAG-SP1 transfected HEK293T cells. HEK293T cells were transfected with sicontrol, siβ-catenin and siβ-catenin siGSK3β. After 24h transfected with FLAG and FLAG-SP1 as indicated in figure. Depletion of β-catenin reduced the stability of FLAG-SP1 while depletion of GSK3β upon β-catenin knockdown rescued the stability of ectopically expressed FLAG-SP1. Actin was used as endogenous control. **(J)** Immunoblot for FLAG-SP1 using FLAG antibody, β-catenin and TCF7 in control FLAG-SP1 HCT116 cells, PP (Pyrvinium Pamoate) treated FLAG- SP1 HCT116 cells and PP FLAG-SP1 HCT116 cells treated with CHIR. HCT116 cells were transfected with FLAG and FLAG-SP1 and treated with PP for 48h and PP in combination with CHIR (for 6h). PP treatment reduced the stability of β-catenin and FLAG-SP1 while CHIR treatment rescued the effect of PP on stability of FLAG-SP1, but did not rescue the expression of Wnt responsive gene TCF7. Actin was used as endogenous control.

3.3.4 Wnt signaling stimulation impedes interaction of SP1 with β-TrCP and not with GSK3β

Wnt stimulation induces stabilization of SP1 in β-catenin-dependent manner. Also β-catenin prevents degradation of SP1 mediated by GSK3β. These results prompted us

to study the molecular mechanism of Wnt signaling induced SP1 stabilization. An earlier study by Wang et al. has shown that treatment of Thiazolidinediones in prostate cancer induces degradation of SP1 via induced expression of β-TrCP. We therefore sought to determine whether Wnt signaling impedes the interaction of SP1 with GSK3β and β-TrCP in HEK293 cells. To prove this we treated FLAG-SP1 expressing cells with Wnt3A. Treatment with Wnt3A robustly stabilize the FLAG-SP1 (Figure 3.3.4A compare lane 2 and lane 3). Notably overexpression of SP1 also induced the expression of β-catenin (Figure 3.3.4A compare lane 1 and lane 2). Similarly Wnt3A treatment further increased the β-catenin expression indicating the activation of Wnt signaling (Figure 3.3.4A compare lane 2 and lane 3). Next, to prove what leads to increase in stability (see scheme Figure 3.3.4B), we immunoprecipitated β-TrCP in FLAG-SP1 expressing cells upon Wnt stimulation and interaction of SP1 with β-TrCP upon Wnt3A treatment was determined by western blotting. The co-immunoprecipitation analysis reveals that Wnt stimulation impedes the interaction of SP1 with β-TrCP (Figure 3.3.4B, compare lane 3 and lane 4 in IP blot), thereby inducing the stability of SP1. To determine whether Wnt stimulation also abrogates the SP1 interaction with GSK3β, we immunoprecipitated FLAG-SP1 expressing cells with FLAG antibody in control and Wnt3A treated cells. The co-immunoprecipitation data reveals that Wnt stimulation did not impede the SP1 interaction with GSk3β (Figure 3.3.4C compare lane 2 and lane 3). Thus indicating that Wnt stimulation only prevents interaction of β-TrCP, not GSK3β with SP1, thereby evading the recognition by proteosomal pathway post ubiquitination by β-TrCP. To further corroborate the role of aberrant activation of Wnt signaling in SP1 stabilization, we inactivated GSK3β by CHIR treatment and analyzed the interaction of SP1 with GSK3β. Co-immunoprecipitation analysis revealed that CHIR treatment although results in robust increase in SP1 stabilization but did not impede the interaction of SP1 with GSK3β (Figure 3.3.4D compare lane 2 and lane 3). Further, we wished to investigate the interaction of GSK3β with SP1 and its phosphodegron mutant. The co-immunoprecipitation was performed by overexpressing wild-type FLAG-SP1 and mutant FLAG-SP1 in HEK293T cells. Co-immunoprecipitation analysis suggested that mutant SP1 interacts with lesser affinity with GSK3β in comparison with the wild-type SP1 (Figure 3.3.4E compare lane 2 and lane 3). These results indicate that SP1 remains bound to GSK3β even after inactivation of GSK3β activity but loses interaction with β-TrCP. Moreover the rate limiting step in stabilization of SP1 under

Wnt stimulation is interaction with β-TrCP. The data provides a novel link that in WNT-ON cells, Wnt stimulation prevents interaction of β-TrCP with SP1 thereby inducing its stability where as in WNT-OFF cells GSK3β phosphorylation of serine residues in Phosphodegron motif allows recognition by β-TrCP and subsequent degradation by proteosomal pathway.

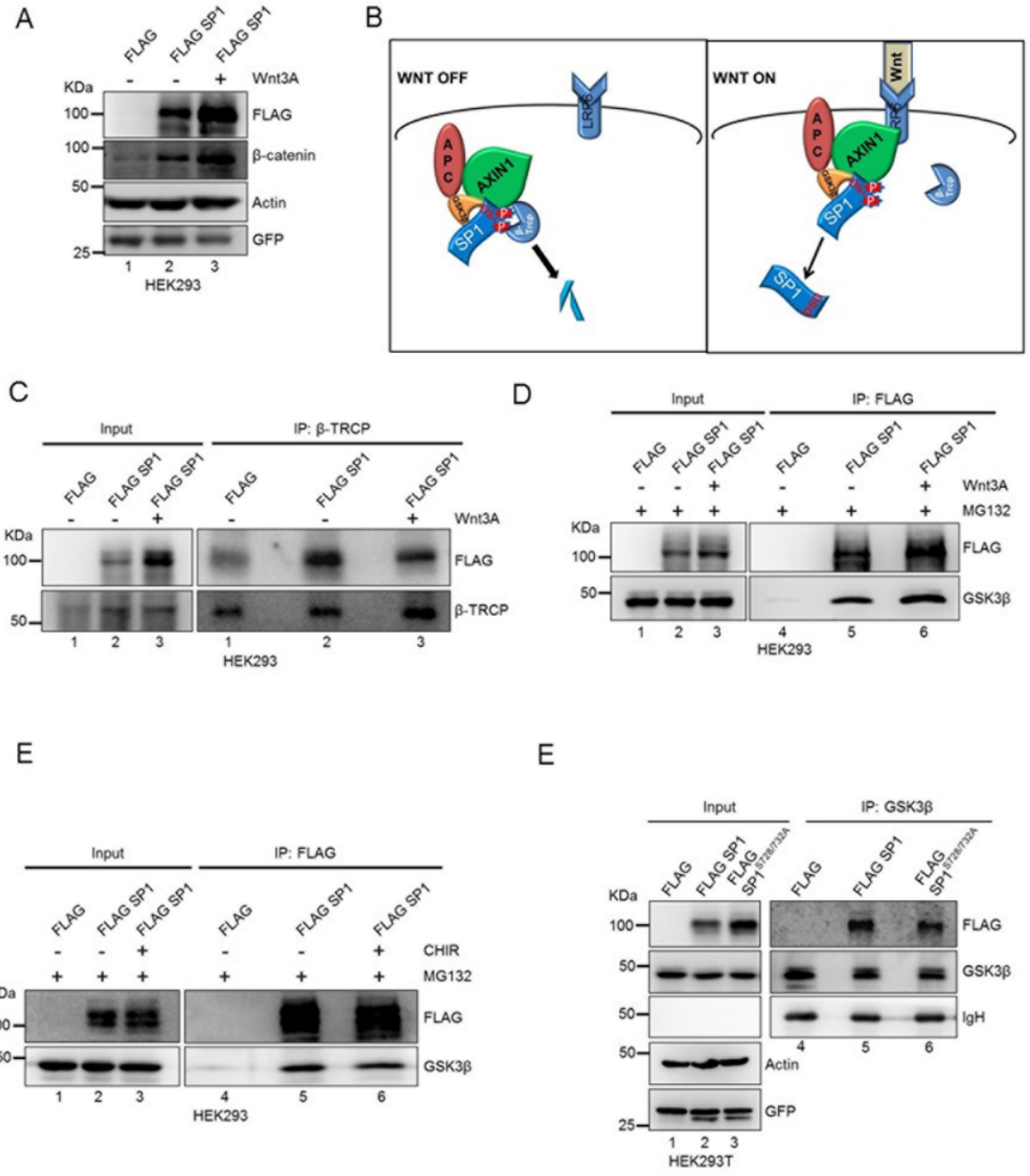

Figure 3.3.4: Wnt signaling stimulation abrogates SP1's interaction with β-TrCP and not with GSK3β. (A) Immunoblot for FLAG-SP1 in control and Wnt3A treated HEK293 cells. HEK293 cells were transfected with FLAG and FLAG-SP1. After 42h

FLAG-SP1 HEK293 cells were treated with Wnt3A for 6h. Wnt3A treatment induced the stability of FLAG-SP1 determined by FLAG antibody. Actin was used as endogenous control. GFP used as transfection control. **(B)** Schematic for prediction of Wnt- dependent stabilization of SP1 in β-TrCP-dependent manner **(C)** Immunoblot for FLAG-SP1 co-immunoprecipitation with endogenous β-TrCP from HEK293 lysates transfected with FLAG and FLAG-SP1. IP with β-TrCP was done in FLAG, FLAG-SP1 control and FLAG-SP1 Wnt3A treated HEK293 cells. Wnt3A treatment impedes the interaction of SP1 with β-TrCP. IP from FLAG HEK293 cells used as negative control. **(D)** Immunoblot for endogenous GSK3β co-immunoprecipitation with FLAG-SP1 from HEK293 lysates transfected with FLAG and FLAG-SP1. IP with FLAG was done in FLAG, FLAG-SP1 control and FLAG-SP1 Wnt3A treated HEK293 cells. HEK293 cells were treated with 10 μM MG132 along with Wnt3A for 6 h. Wnt3A treatment did not impede interaction of SP1 with GSK3β. IP with FLAG in FLAG HEK293 cells used as negative control. **(D)** Immunoblot for endogenous GSK3β co-immunoprecipitation with FLAG-SP1 from HEK293 lysates transfected with FLAG and FLAG-SP1. IP with FLAG was done in FLAG, FLAG-SP1 control and FLAG-SP1 CHIR treated HEK293 cells. HEK293 cells were treated with 10 μM MG132 along with 3 μM CHIR for 6h. CHIR treatment did not impede interaction of SP1 with GSK3β. IP with FLAG in FLAG HEK293 cells used as negative control. **(E)** Immunoblot for endogenous GSK3β co-immunoprecipitation with FLAG-SP1 and FLAG-SP1 Phosphodegron mutant from HEK293 cells transfected with FLAG, FLAG-SP1 and FLAG-SP1 Mutant. IP was done using FLAG antibody in FLAG, FLAG-SP1 and FLAG-SP1 mutant. SP1 phosphodegron shows inefficient interaction with GSK3β.

3.3.5 SP1 is required for Wnt signaling dependent stabilization of β-catenin and regulation of Wnt responsive genes

Our data suggests that SP1 and β-catenin are regulated by Wnt signaling in GSK3β dependent manner. Further, we show that β-catenin associates with SP1 and is required for its stabilization. The common pathway regulating SP1 and β-catenin and also data showing increased expression of β-catenin upon SP1 overexpression (Figure 3.3.5A) prompted us to investigate whether SP1 is required to stabilize β-catenin (schematically represented in Figure 3.3.5B). Intriguingly, overexpression of SP1 in SW480 colorectal cancer cells upregulated expression of β-catenin and so as the expression of common target SATB1. Next, to establish that SP1 is required for Wnt signaling-dependent stimulation of β-catenin, we treated sicontrol HEK293 cells and siSP1 HEK293 cells with Wnt3A for 6 h. The Wnt stimulation induced the stability of both SP1 and β-catenin (Figure 3.3.5C, compare lane 1 with lane 2), whereas depletion of SP1 in Wnt stimulated HEK293 cells was sufficient to reduce the levels of β-catenin so as the expression of known Wnt responsive gene TCF7 (Figure 3.3.5C,

compare lane 2 and lane 3). These results indicated that cellular changes acquired upon Wnt stimulation especially β-catenin expression requires SP1 expression. To delineate that SP1 is required to stabilize β-catenin at protein level, we investigated the stability of ectopically expressed FLAG β-catenin in HeLa cells upon SP1 depletion. We overexpressed FLAG β-catenin in sicontrol and siSP1 cells. Overexpression of β-catenin resulted in robust increase in SP1 expression (Figure 3.3.5D compare lane1 and lane2), whereas depletion of SP1 reduced the levels of ectopically expressed FLAG β-catenin (Figure 3.3.5D compare lane 2and lane 3 FLAG blot). Similar data was observed in HEK293 cells such that overexpression of FLAG β-catenin induced the stability of SP1 whereas SP1 depletion drastically reduced the levels of ectopically expressed FLAG β-catenin (Figure 3.3.5E compare lane 2 and lane 3). Next we investigated to see whether SP1 is sufficient to stabilize β-catenin, we overexpressed HA tagged β-catenin in control HEK293 cells and mutant SP1 (stable and resistant) expressing HEK293 cells. The overexpression of mutant SP1 induced the levels of ectopically expressed HA β-catenin in comparison with control (Figure 3.3.5G compare lane 2 and lane 3). To further understand SP1 is required for β-catenin stabilization, we generated a β-catenin stable HEK293 cell line and induced stabilization of β-catenin by treating these cells with CHIR (GSK3β inhibitor). Inactivation of GSK3β induced the stability of FLAG β-catenin so as the stability of SP1 (Figure 3.3.5H compare lane 1 and lane 2) whereas the depletion of SP1 in FLAG β-catenin expressing cells reduced the levels of ectopically expressed FLAG β-catenin (Figure 3.3.5H compare lane 2 and lane 3). The activation of Wnt signaling resulted in upregulation of the Wnt responsive genes AXIN2 and TCF7 whereas SP1 depletion was sufficient to reduce their expression significantly. To test whether SP1 depletion mediated degradation of FLAG tagged β-catenin at protein level occurs through proteosomal pathway, we treated CHIR treated FLAG β-catenin SP1 depleted cells with proteosomal pathway inhibitor MG132 (Lane 4). The MG132 treatment rescued the stabilization of FLAG β-catenin (Figure 3.3.5H compare lanes 3 and 4), thus indicating that SP1 is required for β-catenin stabilization in GSK3β-dependent manner and SP1 depletion induces the cellular events as induced under WNT-OFF state by reversing the effect of GSK3β inactivation. Interestingly, the rescue of β-catenin stabilization did not re-induce the expression of Wnt responsive genes, suggesting the role of SP1 in regulation of Wnt responsive genes. This is further confirmed by TOP/FOP reporter activity in CHIR treated SP1 depleted cells. The CHIR

treatment induced the reporter activity (Figure 3.3.5I compare lane 1 and lane 2). The depletion of SP1 in CHIR treated cells was sufficient to reduce the TOP/FOP reporter activity significantly (Figure 3.3.5I compare lane 2 and lane 3). Further depletion of SP1 reduced the basal level of β-catenin in WNT OFF condition (Figure 3.3.5J). These results establish a novel mechanistic role of SP1 in regulating stabilization of β-catenin and regulation of Wnt responsive genes. The SP1 and β-catenin association seems to be critical for mutual stabilization. Further both follow the Wnt signaling pathway for stabilization and regulation of Wnt responsive genes.

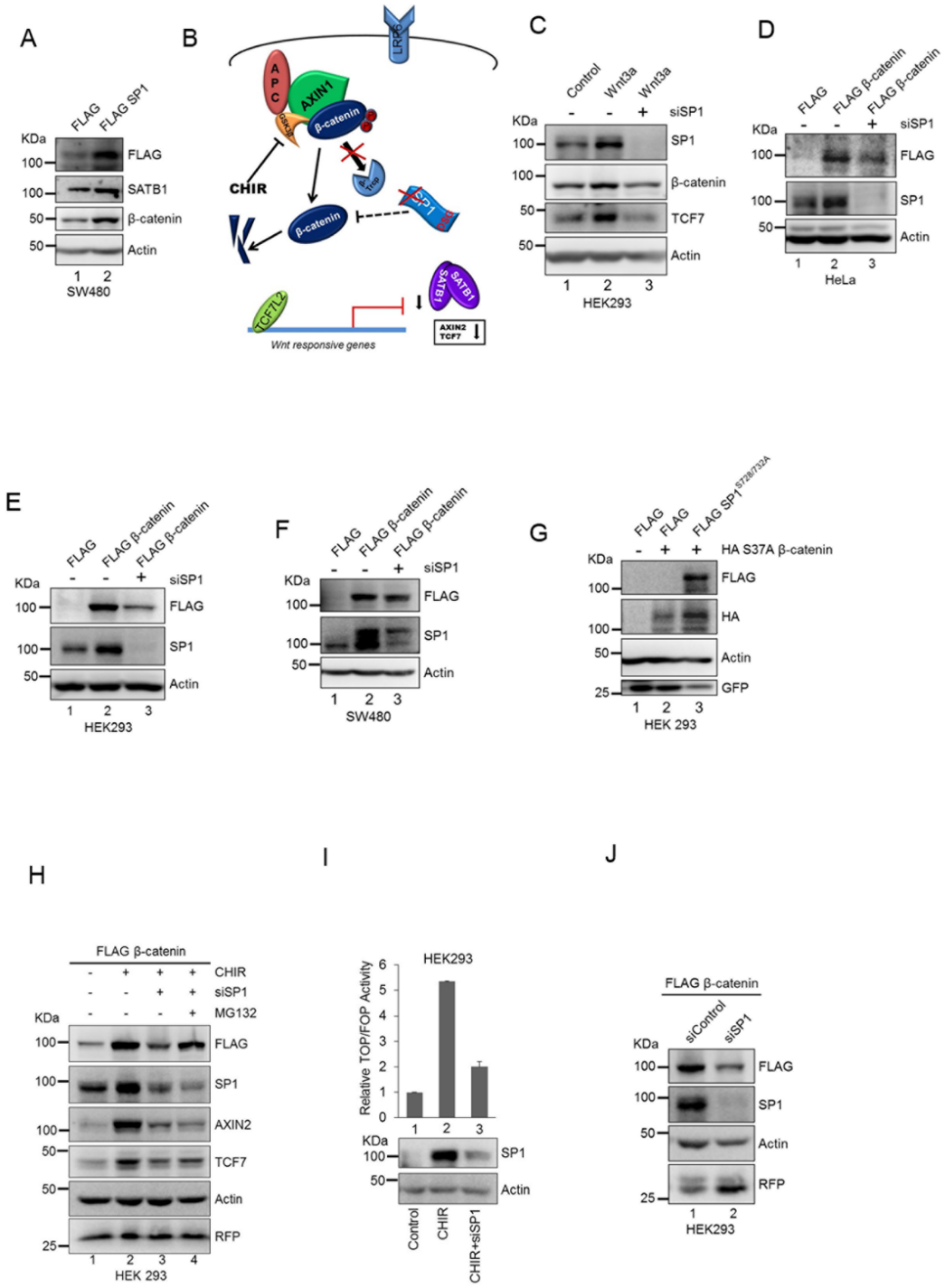

Figure 3.3.5: SP1 is required for Wnt signaling-dependent stabilization of β-catenin and regulation of Wnt responsive genes. (A) Immunoblot for expression of β-catenin, SATB1 and TCF7 in control SW480 and FLAG-SP1 SW480 cells. Overexpression of SP1 induced the expression of β-catenin and the known Wnt responsive genes. Actin was used as endogenous control. **(B)** Schematic showing SP1 dependent stabilization of β-catenin **(C)** Immunoblot for expression of SP1 and β-catenin upon Wnt3A treatment in control and SP1 depleted HEK293 cells. HEK293 cells were transfected with siGFP and siSP1, treated with Wnt3A after 42 h for 6 h. Wnt3A treatment induces the stability of SP1 and β-catenin while SP1 depletion

destabilizes the β-catenin levels. Actin was used as endogenous control. **(D)** Immunoblot for ectopically expressed FLAG β-catenin using FLAG antibody and for SP1 in FLAG-β-catenin sicontrol HeLa cells and FLAG-β-catenin siSP1 cells. Depletion of SP1 decreased the stability of ectopically expressed FLAG β-catenin in comparison with sicontrol. Actin was used as endogenous control. **(E)** Immunoblot for ectopically expressed FLAG-β-catenin using FLAG antibody and for SP1 in FLAG-β-catenin sicontrol HEK293 cells and FLAG-β-catenin siSP1 cells. Depletion of SP1 decreased the stability of ectopically expressed FLAG-β-catenin in comparison with sicontrol. Actin was used as endogenous control. **(F)** Immunoblot for ectopically expressed FLAG β-catenin using FLAG antibody and for SP1 in FLAG-β-catenin sicontrol SW480 cells and FLAG β-catenin siSP1 cells. Depletion of SP1 decreased the stability of ectopically expressed FLAG β-catenin in comparison with sicontrol. Actin was used as endogenous control. **(G)** Immunoblot for ectopically expressed HA-β-catenin using HA antibody and for FLAG-SP1 mutant using FLAG antibody in HA-β-catenin control HEK293 cells and HA-β-catenin FLAG-SP1 mutant cells. Overexpression of FLAG-SP1 mutant induced the stability of ectopically expressed HA-β-catenin in comparison with HA-control. Actin was used as endogenous control. **(H)** Immunoblot for ectopically expressed FLAG β-catenin using FLAG antibody, AXIN2, TCF7 and SP1. Stable FLAG-β-catenin HEK293 cells were treated with CHIR in sicontrol, siSP1 and siSP1 MG132 HEK293 cells. CHIR treatment induced stability of SP1 and FLAG-β-catenin, While Depletion of SP1 reduced the stability of FLAG-β-catenin. MG132 treatment re-induced the FLAG β-catenin stability but not that of Wnt responsive genes. Actin was used as endogenous control. RFP used as transfection control. **(I)** Graphical representation of TOP/FOP reporter activity under CHIR treatment in sicontrol and siSP1 HEK293 cells. Below graph, Immunoblot for reporter assay. **(J)** Immunoblot for ectopically expressed FLAG-β-catenin using FLAG antibody and for SP1. Depletion of SP1 reduced the basal levels of FLAG-β-catenin in Wnt OFF state. Actin was used as endogenous loading control and RFP was used as transfection control.

3.3.6 SP1 is required for Wnt/ β-catenin signaling dependent regulation of chromatin organizer SATB1

Above results confirm that SP1 is not only critical for stabilization of β-catenin but is also required for the regulation of its downstream targets. Next, we wished to delineate whether SP1 is required for Wnt/β-catenin-dependent regulation of SATB1. To test this hypothesis we induced Wnt signaling in CRL1790 by treatment with GSK3-β CHIR and depleted SP1 in CHIR treated cells. The CHIR treatment induced the expression of β-catenin so as the levels of SATB1 (Figure 3.3.6A) as shown in our recent study (Mir et al 2015). The hyperactivation of Wnt signaling resulting in robust increase in SATB1 was reduced upon SP1 depletion. Furthermore, CHIR treatment resulted in robust increase in SP1 expression (Figure 3.3.6A compare lane 1 and lane 2).

Additionally, depletion of SP1 resulted in decrease in expression of β-catenin (Figure 3.3.6A lane 3). These findings suggested that higher expression of SP1 is required to maintain higher levels of β-catenin and SATB1 induced upon hyperactivation of Wnt signaling. Next, we analyzed the expression of SATB1 at transcript level upon depletion of SP1 in CHIR treated cells. The depletion of SP1 resulted in decreased expression of SATB1 at transcript level induced upon activation of Wnt signaling (Figure 3.3.6B), thus indicating that transcriptional activation of SATB1 upon hyper-activation of Wnt signaling by CHIR treatment in the primary cell line CRL1790 requires expression of SP1. To further confirm the role of SP1 in regulation of SATB1, we overexpressed β-catenin and depleted SP1 in β-catenin expressing HeLa cells. Overexpression of β-catenin induced the expression of SATB1 and the stability of SP1 (Figure 3.3.6C, compare lane 2 and lane 3) whereas depletion of SP1 resulted in decrease in SATB1 expression and β-catenin stabilization (Figure 3.3.6C, compare lane 2 and lane 3). Further, we wished to investigate whether β-catenin is required for SP1 regulated SATB1. To test this we overexpressed SP1 in HeLa cells and depleted β-catenin in SP1 expressing cells. Overexpression of SP1 induced the expression of SATB1 so as the expression of β-catenin (Figure 3.3.6D compare lane 2 and lane 3). In our earlier study we have shown that TCF7L2/β-catenin complex binds to *Satb1* promoter and regulate its expression (Mir et al., 2015). Here we show that SP1 interacts with β-catenin and is required for Wnt/β-catenin signaling-dependent regulation of SATB1. This prompted us to investigate the presence of SP1/ β-catenin complex and TCF7L2/β-catenin complex on *Satb1* promoter. We performed ChIP assay in HCT116 cells to monitor the co-occupancy of TCF7L2, SP1 and β-catenin along with histone activation and repression marks. The ChIP analysis revealed enrichment of SP1, TCF7L2 and β-catenin along with the histone activation mark H3K4(me)3, thereby indicating transcriptional activation of SATB1 (Figure 3.3.6E). To further understand regulation of SATB1 involves direct binding of SP1 and β-catenin on *Satb1* promoter, we performed ChIP assay in control HCT116 and β-catenin depleted and SP1 depleted HCT116 cells. ChIP analysis revealed co-occupancy of SP1 and β-catenin on *Satb1* promoter whereas depletion of SP1 and β-catenin resulted in loss of occupancy (Figure 3.3.6F, compare lane 3 and lane 4). The loss of SP1 and β-catenin on *Satb1* promoter is reflected in SATB1 expression (Figure 3.3.6G compare lanes 1, 2, and 3). Depletion of β-catenin leads to complete abrogation of the expression of SP1, thus resulting in complete loss of its occupancy. Similarly, the

depletion of SP1 and β-catenin depletion resulted in decrease in β-catenin expression and complete loss of occupancy in both cases, indicating that presence of SP1 is essential for occupancy of β-catenin on *Satb1* promoter. Thus data suggests that SP1 and β-catenin are critical for mutual stabilization in Wnt-dependent manner and undergo common route to degradation under WNT-OFF condition. The physiological importance of SP1 and β-catenin could be attributed to their association and requirement for regulation of Wnt responsive genes.

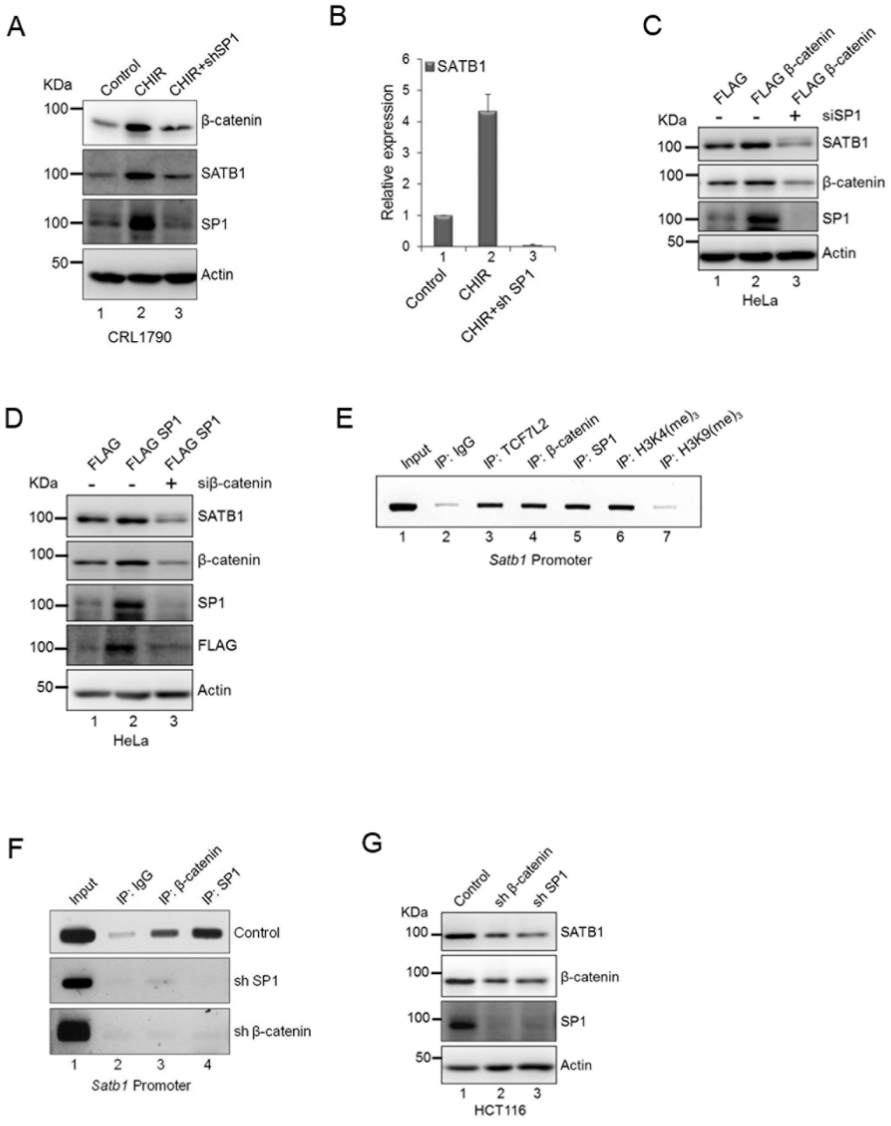

Figure 3.3.6: SP1 is required for Wnt/ β-catenin signaling dependent regulation of chromatin organizer SATB1. (A) Immunoblot for expression of SATB1, SP1 and β-catenin upon CHIR treatment in sicontrol and siSP1 in CRL1790. CHIR induced the expression of SATB1, SP1 and β-catenin, while SP1 depletion reduced their levels. Actin was used as endogenous control. **(B)** Quantitative PCR for SATB1 upon CHIR treatment in sicontrol and siSP1 CRL1790 cells. Standard deviation is from triplicates.

GAPDH2 is used to normalize the CT values. **(C)** Immunoblot for expression of SATB1, SP1 and β-catenin under FLAG-β-catenin in sicontrol and siSP1 in HeLa cells. Overexpression of β-catenin induces the expression of SATB1 and stability of SP1 whereas SP1 depletion reduced the SATB1 levels. Actin was used as endogenous control. **(D)** Immunoblot for expression of SATB1, SP1 and β-catenin under FLAG-SP1 in sicontrol and siβ-catenin in HeLa cells. Overexpression increased the SATB1 expression and so as that of β-catenin, while β-catenin depletion reduced the stability of SP1 and expression of SATB1 as determined by western bloting. Actin was used as endogenous control. **(E)** Binding of SP1, TCF7L2, β-catenin, $H3K4(me)_3$, $H3K9(me)_3$ on *Satb1* promoter determined by ChIP analysis by ChIP-PCR using primers specific for *Satb1* promoter. **(F)** Binding of SP1 and β-catenin, on *Satb1* promoter in shcontrol, shSP1 and shβ-catenin HCT116 cells determined by ChIP analysis by ChIP-PCR using primers specific for *Satb1* promoter. Upon depletion of β-catenin and SP1, occupancy of SP1 and β-catenin is lost on *Satb1* promoter. (G) Immunoblot for expression of SATB1, SP1 and β-catenin in shcontrol, shSP1 and shβ-catenin used for ChIP analysis in Figure 3.3.6F. Actin used as endogenous control.

3.4 Discussion

Here we present a novel cross talk of SP1 and β-catenin stabilization as a part of Wnt signaling cascade (model depicted in Figure 3.4.1).

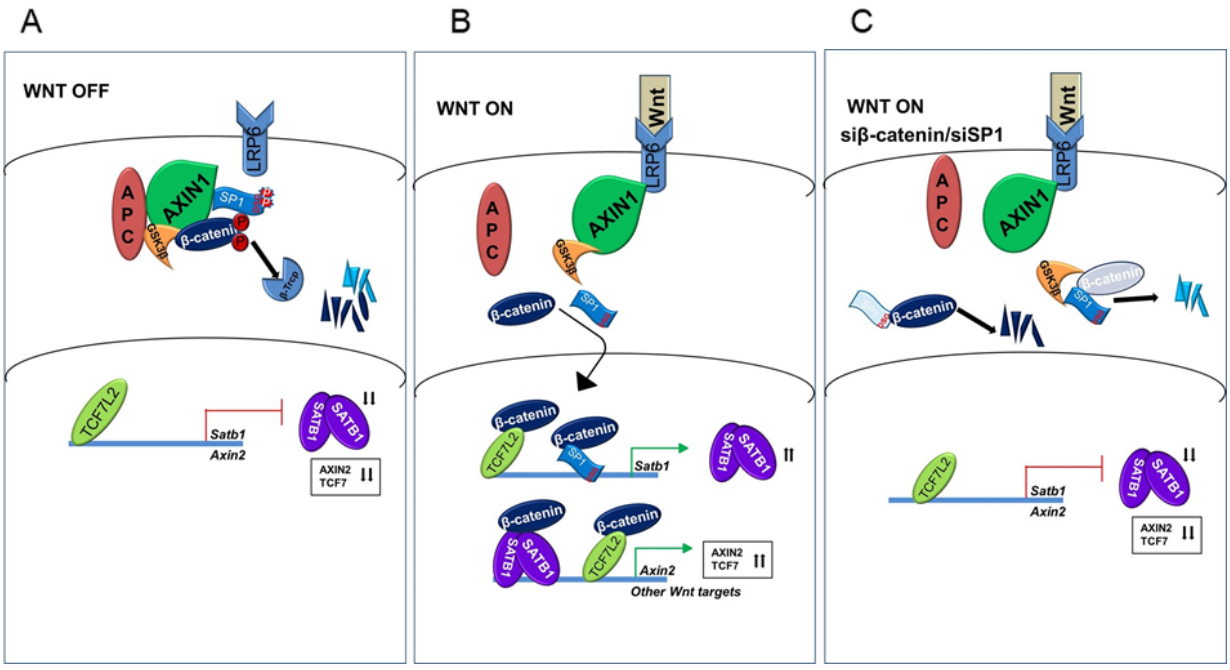

Figure 3.4.1 Wnt signaling mediated stabilization of SP1, and SP1 and β-catenin mutual stabilization (A) In WNT-OFF state, SP1 and β-catenin are degraded in GSK3β-dependent manner. Decreased expression of SP1 and β-catenin is reflected in reduced expression of Wnt responsive genes. **(B)** In WNT-ON state, both SP1 and β-catenin are stabilized. Increased expression of SP1 and β-catenin induce the expression of SATB1 and SATB1/β-catenin and TCF7L2/β-catenin complexes induce Wnt responsive genes. **(C)** In WNT-ON state, depletion of β-catenin induces the degradation of SP1 and depletion of SP1 induces the degradation of β-catenin, thereby reversing the Wnt induced cellular phenotype of increased expression of Wnt responsive genes.

We show that Wnt stimulation induces the simultaneous stabilization of SP1 and β-catenin. Further, using various biochemical inhibitors of downstream effectors we show that Wnt signaling induces SP1 stabilization. By showing that SP1 and β-catenin follow similar events upon activation, we investigated to find whether SP1 is integral part of β-catenin destruction complex. Indeed, HEK293 cells have intact destruction complex and SP1 is part of this in Wnt-OFF state. The results demonstrate that SP1 interacts with AXIN1 and GSK3β in Wnt-OFF state. Moreover, we investigated the interaction of SP1 with β-catenin. Our findings reveal that in colorectal cancer cells harbouring constitutively active Wnt signaling, SP1 interacts with β-catenin.

β-catenin promotes Wnt signaling dependent stabilization of SP1

β-catenin stabilization is central theme to Wnt signaling. Stimulation of Wnt signaling in our study resulted in stabilization of both SP1 and β-catenin. We investigated to find whether β-catenin is requirement for this stabilization. Depletion of β-catenin reduced the levels of SP1 at protein level but no such change was observed at transcript level. Similarly the β-catenin overexpression induced the expression of SP1 at protein level but no change at transcript level. Thus data reveals that β-catenin promotes the changes required to stabilize SP1. Next, we determined whether Wnt signaling mediated SP1 stabilization depends on β-catenin availability. The Wnt stimulation increases SP1 and β-catenin stability whereas depletion of β-catenin even in Wnt ON state destabilizes SP1 at protein level. Similarly stability assay under constitutively active Wnt signaling in colorectal cancer cells suggests that depletion of β-catenin induces the degradation of SP1. Moreover, stable β-catenin even in Wnt-OFF state stabilizes SP1, thereby mimicking changes acquired upon Wnt stimulation. Our study provides unique function of β-catenin stability apart from inducing the transcriptional activation of Wnt responsive genes, inducing the stability of SP1. The availability of β-catenin is critical to maintain the stability of SP1.

GSK3β mediated SP1 degradation and role of β-catenin

In this study we shed light on requirement of GSK3β in SP1 degradation in the absence of β-catenin or in the Wnt-OFF state of cells. We propose that in the absence of β-catenin or in the Wnt-OFF state, GSK3β engages SP1 for phosphorylation in phosphodegron motif which is subsequently ubiquitinated by E3 ubiquitin ligase followed by proteasome mediated degradation. The degradation of SP1 upon β-catenin depletion can be reversed by depleting GSK3β or inactivating its kinase activity. The importance of β-catenin can be understood by that depletion in colorectal cancer cells harbouring constitutively active Wnt signaling induces destabilization of SP1 that is reversed by inactivating kinase activity of GSK3β. To determine how Wnt signaling induces the stabilization of SP1, we analyzed the interaction of SP1 with E3 ubiquitin ligase upon Wnt stimulation. The data reveals that Wnt stimulation impedes the interaction of SP1 with β-TrCP, thereby increase the stabilization of SP1. But Wnt stimulation did not impede interaction of SP1 with GSK3β. Similarly, inactivation of GSK3β kinase activity by CHIR treatment did not impede its interaction with SP1. The results presented here point towards similar regulatory mechanism upon Wnt stimulation as recently shown for β-catenin stabilization (Li et al., 2012). We also show that in Wnt-OFF state SP1 remains bound to AXIN1 and GSK3β. Therefore, we have provided multiple evidences that SP1 and β-catenin follow common route in the Wnt signaling pathway.

SP1 promotes β-catenin stabilization

Our study shows that upon Wnt stimulation, both SP1 and β-catenin are stabilized. Here we investigated whether Wnt stimulated SP1 is required for stabilization of β-catenin. We provide evidences that SP1 stability is critical for β-catenin stability. To maintain stabilization of β-catenin induced upon Wnt stimulation, SP1 stability is also induced such that depletion potentiates the degradation of β-catenin. Moreover stabilized SP1 such as mutant SP1 (non-responsive to GSK3β mediated degradation) stabilizes β-catenin thereby mimicking the Wnt stimulation in Wnt OFF state. Also CHIR induced β-catenin stability was reduced upon SP1 depletion. We also provide evidences that effect of SP1 depletion on β-catenin stability can be reversed by inhibiting proteosomal degradation pathway, thus indicating that SP1 ablation

potentiates the cellular changes to induce β-catenin degradation. Further, although we can see rescue of β-catenin stabilization by inhibiting proteosomal pathway upon SP1 depletion but this did not rescue the expression of Wnt responsive genes. Based on these findings we argue that Wnt-dependent transcriptional regulation of Wnt responsive genes requires SP1. The loss of SP1 expression or stability initiates the similar events as acquired upon β-catenin depletion. Therefore our study provides new paradigm of Wnt signaling that consists of mutual stabilization of SP1 and β-catenin, and this is critical for expession of Wnt responsive genes.

SP1 and Wnt outcome

In present study, we provide evidences that SP1 and β-catenin mutually stabilize each other. This novel crosstalk could be key for regulation of Wnt responsive genes. The destabilization of β-catenin by SP1 depletion reduces the expression of Wnt responsive genes but rescuing the β-catenin stabilization does not re-induce the expression of Wnt responsive genes. Therefore this duality of SP1 function such that on one hand is required for β-catenin stabilization and on the other hand it is required for regulation of Wnt responsive genes. Similarly, β-catenin also exhibits dual role in stabilizing SP1 and regulating Wnt responsive genes. We further extended our understanding in delineating the co-regulatory role of SP1 and β-catenin in regulation of common target SATB1. Wnt stimulation results in stabilization of SP1 and β-catenin, and transcriptional activation of SATB1. However, depletion of SP1 reduces the hyper-expression of SATB1 induced upon Wnt stimulation. To further characterize the direct role of SP1 and β-catenin in regulating SATB1, we analyzed the occupancy of SP1, TCF7L2 and β-catenin on *Satb1* promoter along with histone activation and repression marks. We establish that SP1, TCF7L2 and β-catenin bind to *Satb1* promoter and induce its transcriptional activation as evidenced by occupancy of the histone activation mark H3K4(me)3. Thus, this new paradigm of Wnt signaling mediated stabilization of SP1 and β-catenin, and their dependence for mutual stabilization could be critical for multiple developmental programs. Additionally this mechanism could also be involved in driving tumorigenic potential as explained by the novel role of the SP1/ β-catenin complex in regulation of chromatin organizer-SATB1 which is known for its oncogenic role in various cancers. The dependence of SP1 and β-catenin for mutual stabilization can be targeted for therapeutic intervention in cancers driven by SP1/ β-catenin signaling cascade.

3.5 References

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Publications

A. Published

- **Mir, R**., Pradhan, S.J., and Galande, S. (2012). Chromatin organizer SATB1 as a novel molecular target for cancer therapy. *Curr. Drug Targets* 13: 1603–1615.

- **Mir, R**., Pradhan, S.J., Patil, P., Mulherkar, R., and Galande, S. (2015). Wnt / β -catenin signaling regulated SATB1 promotes colorectal cancer tumorigenesis and progression. Oncogene Jul 13. doi: 10.1038/onc.2015.232.

B. Manuscripts under preparation

- **Mir, R.** and Galande, S. (2015). Interplay of SP1 and β-catenin in Wnt signaling-dependent regulation of the chromatin organizer SATB1.

- **Mir, R.** and Galande, S. (2015). Signaling mechanism of Statin mediated regulation of SATB1 and β-catenin.