NOVEL ROLES FOR HUMAN SETD1A HISTONE METHYLTRANSFERASE IN REGULATION OF CANONICAL WNT SIGNALING PATHWAY, CELLULAR PROLIFERATION, AND METASTASIS

By

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This dissertation is dedicated to the memory of my grandfather, Jack Salz, without whom I would most certainly be lost.

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LIST OF ABBREVIATIONS

3’UTR	Three prime Untranslated Region
Ash2L	Absent, Small, or Homeotic Discs 2-Like
CDH1	cadherin 1, type 1
COMPASS	Complex proteins associated with Set1
CRC	Colorectal Cancer
EMT	Epithlial-Mesenchymal Transition
Epigenetic	Greek: επί- over, above genetic
H3K4	Histone 3 Lysin 4
H3K4me1	Mono-methylation of Histone 3 Lysin 4
H3K4me2	Di-methylation of Histone 3 Lysin 4
H3K4me3	Tri-methylation of Histone 3 Lysin 4
HCFC1	Host Cell Factor C1
HDAC	Histone Deacetylase
HMT	Histone Methyltransferase
JARID1A	Jumonji, AT Rich Interactive Domain 1B
KD	Knock Down
LEF1	Lymphoid Enhancer-binding Factor 1
LSD1	Lysine-specific histone demethylase 1A
MET	Mesenchymal-Epithelial Transition
MLL	Mixed-Lineage Leukemia
MMP7	matrix metalloproteinase-7
MYC	Myelocytomatosis viral oncogene
PHD	Plant Homeo Domain
qRT-PCR	Quantitative Reverse-Transcription Polymerase Chain Reaction

RbBP5	Retinoblastoma Binding Protein 5
RNAP II	RNA Polymerase II
RT-PCR	Reverse-Transcription Polymerase Chain Reaction
SD	Standard Deviation
SETD1A	SET domain containing protein 1A
SETD1B	SET domain containing protein 1B
SNAI1/Snail	Snail Homolog 1
SNAI2/Slug	Snail Homolog 2
TAF3	TATA box binding protein (TBP)-Associated Factor 3
TAL1	T-cell acute lymphoblastic leukemia protein 1
TCF7L2/TCF4	Transcription Factor 7-Like 2 / T-Cell Specific Factor 7-Like 2
TERT	Telomerase reverse transcriptase
TFIID	Transcription Factor II D
TSS	Transcription Start Site
TWIST2	Twist Family Basic Helix-Loop-Helix Transcription Factor 2
VEGFA	Vascular endothelial growth factor A
WB	Western Blot
WDR5	WD Repeat-Containing Protein 5
Wnt	Wingless / homolog of int-1
Beta-catenin	Cadherin-associated protein beta 1

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Large and small-scale changes in epigenetic signatures are characteristic of all cancers. One such phenomenon is perturbation in methylation patterns of histone H3 lysin-4 (H3K4) during progression of cancer. The drivers and functions of such changes are not clear. H3K4 methylation is enacted by the COMPASS family of histone methyltransferases, which includes MLL1-4 and SETD1A/B. While SETD1A/B regulates promoter-associated H3K4me3 and affects global gene expression, MLLs are mainly responsible for enhancer- associated H3K4me1/2 and demonstrate only minor effect on global gene expression profile. The purpose of this study is to elucidate the role of human SETD1A (hSETD1A) in oncogenic functions and explore related underlying mechanisms.

Here, we report that hSETD1A, and hSETD1B in partial, are up-regulated in colorectal and breast cancer cells and specimens. Depletion of hSETD1A led to a dramatic decrease in bulk H3K4me3 in colorectal cancer cells and to retarded cellular proliferation, invasion, and migration in colorectal and/or breast cancer cells. In parallel, ablation of hSETD1A suppressed tumor growth and cell metastases in nude mice. To

identify genes affected by hSETD1A KD, we utilized a genome-wide microarray analysis approach, which revealed a significant decrease in expression levels of Wnt-signaling target genes. Mutations in this pathway are prevalent in many cancers, especially in colorectal cancer, and are critical for the manifestation of the disease. We found that hSETD1A interacts with β-catenin, the master regulator of the Wnt-signaling pathway, and that these interactions facilitate the localization of hSETD1A to Wnt/β-catenin responsive promoters, H3K4me3 enrichment, TAF3 occupancy, and subsequent increase in Wnt target genes' expression. These results were supported by the significant overlap in target genes of hSETD1A, TAF3, and β-catenin in colorectal cancer cells, and by the correlation between elevated levels of hSETD1A transcript and increased levels of Wnt target genes and promoter-H3K4me3 in colorectal tumors.

Finally, increased levels of hSETD1A and hSETD1B, but not MLLs, associated with poor survival in lymph-node positive breast cancer, suggesting that these enzymes could serve as good biomarkers. Taken together, this work provides novel biologic and mechanistic perspectives into the role of hSETD1A/B in cancer, and provides a potential candidate for cancer therapeutic applications.

CHAPTER 1
INTRODUCTION

Cancer Epigenetic

Eukaryotic DNA is a negatively charged molecule that is compacted by positively charged histones [1]. Together they form nucleosomes [1]. Each nucleosome molecule consists of a core particle containing 146 DNA base pairs wrapped around two tetramers, each consisted of histone H2A, H2B, H3, and H4, and a linker DNA containing histone H1[1]. Despite the need for DNA compaction, DNA must remain accessible to maintain functionality and allow for appropriate gene transcription and DNA replication to occur. One important mechanism impacting DNA accessibility is histone modifications [2-4]. Each histone contains unstructured amino terminal tail that protrudes out of the nucleosomal core [1]. The histone tails are prone to various post-translational modifications including acetylation, methylation, phosphorylation, sumoylation, ADP-ribosylation, ubiquitination, and proline isomerization [2-4]. These epigenetic marks, which are subjected to dynamic ‘reading’, ‘writing’, and ‘erasing’ by transcription regulators, act upon chromatin structure and regulate cellular functions, such as gene expression, splicing, DNA replication, DNA damage response, telomere length, and life span [2-4].

Cancer is a disorder driven by genomic instability [5]. It comprises six hallmarks which are acquired during progression: enhanced proliferation, inhibition of growth suppression, resistance to apoptosis, immortalization, angiogenesis, and metastasis [6]. Even prior to the awareness of important epigenetic modifications in cancer, conventional tumor biology focused on disruption in gene expression underlying tumor growth and metastasis. Perhaps the earliest link between epigenetic and cancer was

the discovery of DNA hypermethylation of tumor suppressor genes and hypomethylation of oncogenes [7-9]. Later on, post-translational modifications of histone were acknowledged to facilitate abnormal gene expression in cancer cells [2, 10, 11]. This includes dynamic small and large-scale changes in histone modifications affecting heterochromatin and euchromatin domains [11]. The absolute role of bulk changes in histone modifications in cancer cells is not entirely clear, but it seems to play a role in allowing for greater plasticity, rapid adaptation to new environments, and selection of cancer-favorite attributes [10-12]. These functions support phenotypic alterations in the context of both development and tumorigenesis [10, 11].

Mutations and altered expression of various epigenetic modifiers have been evident in cancer [2, 11]. These discoveries provide a potential mechanism for the chaotic chromatin landscape observed in cancer cells and portray epigenetic regulators as potential targets for cancer therapy. Additionally, identification of epigenetic changes associated with cancer progression early on could serve as a biomarker and enable better prognosis [13]. Together, understanding the epigenetic changes occurring in cancer cells and identifying their source provides powerful clinical applications including diagnosis, prognosis, and therapy [2, 13].

The COMPASS Family of Histone Methyltransferases

One important and highly studied histone modification is the methylation of histone H3 on lysine 4 (H3K4). This histone mark occurs in three states: mono-, di- and tri-methylation. It is mainly abundant at the transcription start site (TSS), enhancers, and gene body of active genes, and regulates transcription [14-18]. The first H3K4 methyltransferase was identified in *Saccharomyces cerevisiae* and was termed SETD1A/COMPASS [18-20]. SETD1A/COMPASS homologs were later identified in

higher eukaryotes (Figure 1-1) [18]. For example, three SETD1A/COMPASS homologs were found in *Drosophila melanogaster,* while six family members were found in *Homo Sapiens* [17, 18]. All family members are similar in their capacity to methylate H3K4, but different in their ability to Mono-, Di-, or Tri-methylate H3K4 (H3K4me1, H3K4me2, H3K4me3), and in their affinity to various loci [18, 21-24]. The human COMPASS family includes the SET1/COMPASS genes - *hSETD1A* and *hSETD1B,* the COMPASS-like genes- *MLL1* and *MLL2*, and the COMPASS-related genes - *MLL3* and *MLL4* (Figure 1-1) [18].

COMPASS HMTs operate as large enzymatic complexes, each containing several common subunits and few unique subunits, all of which are required for their enzymatic activity (Figure 1-1) [18]. The subunits Ash2L, RbBP5, WDR5, and DPY30 are present in all COMPASS complexes and are therefore perceived as core subunits (Figure 1-1) [18]. The subunits HCF1, Wdr82, and cxxc1, are unique to the hSETD1A/B complexes (Figure 1-1) [18]. In *Saccharomyces cerevisiae*, null mutants of anyone of the SETD1A/COMPASS subunits led to growth retardation and sensitivity to hydroxyurea [19].

The only yeast SET1/COMPASS HMT complex is unique in its ability to mono-, di-, and trimethylate H3K4 [19]. In eukaryotic cells, there is a division of labor among the trithorax members; SETD1 complexes regulate the bulk promoter-H3K4me3, while the MLL complexes regulate H3K4me1 and H3K4me2 at gene enhancer elements [18, 21-24]. Ablation of SETD1A proteins in mammalian cells heavily affected the genome wide expression profile while ablation of MLL proteins had only minor and localized effect on

gene expression. However, all COMPASS family members play an important role in normal development and various malignancies [18].

Roles for H3K4me3 in Transcription Activation

The importance of H3K4me3 as a prominent mark of active or ‘poised’ gene was first recognized when chromatin immunoprecipitation (ChIP) combined with rapid genome-wide sequencing techniques became available [14-16, 25]. Immunoprecipitation of eukaryotic chromatin with antibodies against H3K4me3 revealed specific enrichment of H3K4me3 at transcription start sites (TSS) of active genes [14-16, 25]. Other histone modifications, such as H3K27me3 and H3K9me2 were shown to act as repressive marks at heterochromatin regions [14]. Moreover, H3K4me3 was shown present, along with H3K27me3 at promoters of developmental genes in embryonic stem cells [26, 27]. These domains were named 'bivalent domains'.

As investigators soon realized, H3K4me3 not only decorated gene promoters but also played an active role in transcription activation by serving as a scaffold for transcription factors. For example, H3K4me3 can mediate the recruitment of the basal transcription machinery to gene promoters followed by transcription initiation [28]. The mechanism for H3K4me3-dependent transcription initiation was elucidated by showing that TAF3 (TATA box-binding protein-associated factor 3), component of the TFIID general transcription factor, interacts with H3K4me3 mark [28, 29]. This interaction was captured by NMR and revealed that the conserved plant homeodomain (PHD) finger of TAF3 recognizes the specific trimethylation state of H3K4 [30]. Association of TAF3 with H3K4me3 directs the TFIID complex to genes promoter orchestrate assembly of preinitiation complex (PIC) [28].

More insight into the relationship of H3K4me3 with transcriptional activity has come from the identification of several other proteins that recognize and bind H3K4me3, such as CHD1, BPTF, JMJD2A, RAG2, WDR5, and ING [31-34]. For example, the nucleosome remodeling factors, CHD1 and BPTF, interact with H3K4me3 and facilitate transcription initiation and elongation. CHD1 and BPTF recognize H3K4me3, but not naked H3, through their double chromodomains and PHD finger, respectively [31, 34]. It was later demonstrated that the interaction of CHD1 with H3K4me3 also facilitates spliceosome anchoring and pre-mRNA splicing on active genes, suggesting a novel role for H3K4me3 in pre-mRNA maturation [32]. Similar example is the binding of BPTF, a subunit of NURF, ATP-dependent chromatin-remodeling complex [34]. The recognition of H3K4me3 and H3K4me2 by WDR5 is an interesting case since WDR5 is a core subunit of all COMPASS HMT complexes [33]. The loss of WDR5 leads to a decrease in binding of COMPASS HMTs to H3K4me2 and to a global decrease in H3K4me3. Therefore, it was proposed that WDR5 participates in reading H3K4me2 prior to writing H3K4me3.

One important question remained to be answered is how H3K4me3 is regulated *in vivo*. Although the enzymes catalyzing H3K4me3 are largely known, how these enzymes are targeted to genes promoters is not fully understood. Nevertheless, it has been shown that H3K4 HMTs interact not only with transcription factors, but also with various histone modifications which affect the positioning of H3K4me3 to gene promoters. For example, transcription-couples H2B ubiquitylation and H3 acetylation were shown to cross talk with H3K4me3 and play an important role in its positioning [29, 35, 36]. Specific targeting of H3K4me3 HMTs to gene promoters by various

transcription factors was also reported. For example, USF1 transcription factor interacts with hSETD1A and targets it to the HOXB4 promoter [37]. Another example is the targeting of hSETD1A to the TERT promoter by β-catenin [38] and to p53 target genes by p300 and p53 [29]. The regulation of H3K4me3 is extremely complex, but further identification of H3K4me3 regulators will shed light on novel H3K4me3-dependent transcription mechanisms and enable to intervene with its functions.

Roles for H3K4me3 in Cancer

The profound role of H3K4me3 in transcription led investigators to explore possible roles of H3K4me3 in different diseases including cancer. Indeed, levels of H3K4me3 were shown to be globally and locally altered in different types of cancer and in different developmental stages of the disease, and serve as a predictor for recurrence [39-46]. In addition, H3K4me3 was also shown to be involved in cellular plasticity [45, 47]. A study demonstrated that TGFβ-dependent epithelial-mesenchymal transition (EMT) of hepatocytes led to a bulk increase in H3K4me3 and dramatic loss of H3K9me2 [45]. Hence, H3K4me3, along with other histone modifications, was suggested to serve as potential cancer prognosis marker [48]. Nevertheless, low levels of H3K4me3 were observed in some types of cancer. For example, H3K4me3 levels decrease in late stages of renal cell carcinoma [40]. Future studies are needed in order to determine if H3K4me3 mark is clinically relevant for patient assessment.

Despite limited publications regarding global changes in H3K4me3 levels, large number of studies shown position-specific alterations in H3K4me3 in cancer cells, especially at promoters of oncogenes and tumor suppressors, such as c-Myc and p53 [29, 49]. Understanding of H3K4me3 regulation in normal and cancer cells will provide opportunities for epigenetic interventions for cancer therapeutic.

Roles for SETD1A in Development and Cancer

The function of SETD1A is not as nearly characterized as those of MLL. The role of *MLL1* gene in leukemia is well established [50, 51]. The most prominent mechanism for *MLL1*-dependent leukemia is frequent chromosomal translocations [50, 51]. In contrast to MLLs, the role of SETD1A in development and cancer is not well understood. Nevertheless, recent work from our lab (unpublished) and other labs demonstrated that the SETD1A knockout (KO) mice are embryonic lethal at E11.5 [52]. These results present a non-redundant role for SETD1A in early development of mammals. Additional *In vitro* studies from our lab showed that ablation of SETD1A in mouse Embryonic Stem Cells (mESC) lead to a decrease in expression of mesoderm markers and inhibition of lineage differentiation [37], providing further explanation for the *SETD1A* KO phenotype. Few studies showed that hSETD1A impacts genes involved in cancer. For example, it regulate the *MYC* promoter through its binding to BORIS and BAT3 proteins [53], the TERT promoter through its binding to β-catenin [38], and the HOXB4 promoter by its binding to USF1 [37]. The true biological effect of those changes in gene expression is not well established. Another study demonstrated that intraperitoneal injection of hSETD1A antisense RNA into mice bearing colorectal tumor xenografts, leads to a decrease in tumor size [54]. However, those experiments lacked evidence for hSETD1A KD in the xenografts tumors and failed to explain the mechanism for antisense RNA systemic absorption.

The *hSETD1A* gene is often mutated in human cancers (http://www.sanger.ac.uk/cosmic) [55]. Interestingly, *hSETD1A* mutations are abundant in cancers of the endometrium (4.98%), large intestines (4.26%), and lungs (3.6%) [55].

In contrast, very small number or mutations in *hSETD1A* were identified in cancers of the liver, pancreas, cervix, prostate, kidney, or hematopoietic and lymphoid tissue [55]. The function of *hSETD1A* mutations is unknown, but their tendency to be missense and their absence from the methyltransferase domain, may suggest retention and even enhancement of the methyltrasferase activity of hSETD1A in cancer. Moreover, gain in *hSETD1A* gene copy number was detected in 45% (352/782) of breast cancer cases, 18.9% of CRC cases (92/486), and 18% of kidney cancer cases (54/300), while loss in gene copy was rare [55].

The interactions between hSETD1A and β-catenin are especially interesting [38]. β-catenin is the master regulator of the Wnt-signaling pathway, which has been strongly implicated in CRC and other types of cancer [56-61]. It was further shown that hSETD1A and H3K4me3 occupancy at the TERT promoter decrease in the absence of functional β-catenin [38]. However, an overall effect of hSETD1A on the Wnt-signaling pathway was not shown at the time.

Colorectal Cancer and the Wnt-signaling Pathway

Colorectal cancer is the cancer of the colon and rectum [62]. Conventional treatments, such as chemotherapy produce poor response rates [62-64]. However, detailed mapping of molecular changes initiating or escorting colon transformation yielded insights which contribute to targeted therapy [65]. One prominent molecular event characterizing most human CRCs is dysregulation of the Wnt-signaling pathway [57, 58, 60, 65-67]. Therefore, for decades, the Wnt pathway has been the focus of CRC research.

Colorectal Cancer Epidemiology

Colorectal cancer is the third most common cancer in both men and women, and the third leading cause of cancer related deaths in the USA [62, 64]. The American Cancer Society estimated 136,830 new cases and 50,310 deaths of CRC in 2014, in the USA [62].

Factors affecting incidence and mortality rates include age, diet, behaviors such as drinking and smoking, personal history of inflammatory bowel disease, ethnicity, and sex [62, 64]. Although most CRCs are sporadic, a small percentage of patients develops CRC as a result of genetic predisposition, such as familial adenomatous polyposis (FAP) and hereditary non-polyposis CRC (HNPCC) [56, 62, 64, 68, 69].

Colorectal cancer is a multi-stage disease (Figure 1-2). The progression of the disease is extremely slow, and painful symptoms such as rectal bleeding, occur only at late stages. Because it is often only in late stages that one become aware of the symptoms, regular screening is critical and early detection of CRC is the key for survival [64]. The recent decrease in CRC incidence and mortality indeed correspond to the increase in number of colonoscopies performed each year [64]. Determining the choice of treatment is heavily dependent on the cancer stage. Polypectomy remain the most common treatment for colorectal cancer (CRC), although it is only effective at early stages of adenomatous polyps [70, 71]. Chemotherapy is used at late stages of adenocarcinoma and metastasis [63]. Despite availability of conventional treatment, call for targeted therapy is urging, especially considering the impressive knowledge existing about the molecular changes that characterize different stages of the disease [63, 72].

The Wnt-signaling Pathway

The Wnt-signaling pathway is an evolutionarily conserved pathway that is required for proper development of all metazoans [58, 66]. It was named after the secreted Wnt glycoprotein ligands that activate it [58, 66, 73]. Misregulation of the Wnt pathway is linked to many human diseases, making this pathway one of the most intensely studies signaling pathways in academia and industry in the last 30 years [58, 73, 74]. One of the earliest events that led to the discovery of Wnt-signaling pathway was the identification of a wingless *Drosophila* that possessed mutation in the *wingless* locus [73]. Later, the *wingless* mouse homolog was discovered and named *wnt*. Interestingly, mutations in *wnt* induced mouse mammary tumors [75]. Experiments in *Xenopus* embryos and *Drosophila* further indicated that the Wnt pathway plays a critical role in axis formation [76]. These studies implicated the Wnt-signaling pathway in both development and carcinogenesis.

Because Wnt ligand activates cellular events of different contexts, a conscious distinction between canonical Wnt-signaling pathway and non-canonical Wnt-signaling pathways was made early on. Non-canonical Wnt-signaling pathways affect signaling in cytoplasm, and include the planar cell polarity (PCP) pathway and the Wnt/calcium pathway [77, 78]. The canonical Wnt-signaling pathway begins in the cytoplasm, but convey to the nucleus where it influences gene transcription [58].

In opposed to non-canonical Wnt-signaling pathways, activation and repression of the canonical Wnt-signaling pathway is dependent on the protein levels of its notorious master regulator and primary effector – β-catenin, a mammalian homolog of the *Drosophila* armadillo [58, 66]. Normally, cytoplasmic levels of β-catenin are kept low since it is bound by the “destruction complex” and targeted to degradation [58, 66, 79]

(Figure 1-3). This complex includes the Adenomatous Poliposis Coli (APC) tumor suppressor, GSK3β, CK1, Axin, and β-catein [58, 66]. GSK3β and CK1 kinases phosphorylate β-catenin, leading to its association with SCFβ-TRCP E3 ubiquitin ligase and to its degradation by the 26S proteosome [58, 66] (Figure 1-3). Traditional activation of the Wnt-signaling pathway relies on the association of extracellular Wnt ligand with the extracellular portion of its cognate receptors: G-protein coupled receptor - Frizzled, and the low-density lipoprotein receptor - LRP6 [58, 66]. Binding of Wnt ligand to Frizzled and LRP6 results in the recruitment of Axin and its associated "destruction complex" to the plasma membrane and phosphorylation of LRP6 by GSK3β and CK1 [58, 79] (Figure 1-3). Phosphorylated LRP6 has a higher affinity for Axin and further increase recruitment of Axin to the cell surface [58, 66]. This step creates a positive feedback loop and is known as the "Wnt pathway amplification step" [58, 66]. It prevents the phosphorylation of β-catenin in several ways [58, 66]. First, it occupies GSK3β and CK1, which instead of phosphorylating β-catenin are now phosphorylating LRP6 [58, 66]. Second, the redirection of the "destruction complex" from the cytoplasm to the plasma membrane sequesters it from binding to additional molecules of cytosolic β-catenin [58, 66, 79]. And third, once the destruction complex associates with LRP6, the phosphorylated cytoplasmic domain of LRP6 directly inhibits GSK3β activity, thereby blocking β-catenin phosphorylation and subsequent proteosomal degradation[58, 66, 79] (Figure 1-3).

As cytosolic β-catenin protein levels increase, β-catenin translocates from the cytoplasm into the nucleus independently of the classical Importin/RanGTPase importing system [80]. In the nucleus, β-catenin associates with the TCF/LEF family of

transcription factors and activates gene transcription [58, 66] (Figure 1-3). TCF/LEF directs β-catenin to genes that possess a specific consensus sequence [58, 66]. Those genes are known as β-catenin/Wnt target genes [58, 66]. One of the most studied Wnt target gene is the oncogne *MYC [58, 81]*. Activation of the Wnt-signaling pathway leads to an increase in the expression of *MYC* and other genes, and to an accelerated cellular proliferation [58, 81]. Other Wnt target genes, such as *SNAI1, SNAI2, TWIST, MMPs*, *VEGF*, and *CDH1* are involved in processes important for cellular plasticity, such as migration, invasion, and angiogenesis [58, 74]. Both cellular proliferation and motility are not only cancerous-related functions, but are also essential for embryogenesis and adult tissue maintenance [82-84]. Increased expression of SNAI1, SNAI2, and TWIST upon Wnt-signaling activation leads to a decrease in E-cadherin (*CDH1*) [85]. The repression of E-cadherin allows cells to detach from the tight epithelial junctions and migrate [85]. Additional important Wnt target genes are *OCT4*, *NANOG,* and *SOX2,* which are three developmental transcription factors supporting embryonic stem cell self-renewal [86].

Epigenetic Regulation of Wnt Target Genes

While the upstream events leading to activation of Wnt target genes are fairly understood, less is known about how transcription activation occurs. Notably, chromatin surrounding the promoters of Wnt target genes is heavily regulated by histone modifications [49, 87, 88]. Studies showed that targeted promoters bound by Tcf were H3 hyperacetylation and H3K4 hypertrimethylated, while nonresponsive promoters were devoid of active marks and enriched with the repressive mark H3K27me3 [89]. These observations were supported by studies showing that β-catenin interacts with hSETD1A, MLL1/2, and p300, which could explain the H3 acetylation and H3K4 methylation at Wnt

responsive promoters [38, 49, 90, 91]. In addition, it was more recently shown that wnt3a can stimulate interactions between the LEF1/TCF complex and SET8 HMT, resulting in the recruitment of SET8 to Wnt target genes and subsequent H4K20me1-mediated transcription activation [92].

The Role of Wnt-signaling Pathway in Colorectal Cancer

Constitutive activation of the Wnt-signaling pathway is a common feature of solid tumors, particularly colorectal tumors [57, 58, 60, 65-67]. Dysregulation of the Wnt pathway is largely attributed to somatic and genetic mutations in various components, most commonly in *APC, CTNNB1 (*β-catenin), and *AXIN2* [58, 60, 61]. Notably, germline mutations in the *APC* tumor suppressor are the direct cause for the familial adenomatous polyposis (FAP) syndrome, hereditary form of colon cancer, wherein patients develop thousands of polyps in the intestines [56, 93]. Similar to the FAP phenotype, heterozygous nonsense mutation in the *APC* gene resulted in polyps throughout the intestinal tract in hundred percent of Apc^{Min} mice [94, 95]. The majority of these mice do not survive beyond 120 days due to invasive colorectal carcinoma [94, 95]. Overall, more than 70% of all CRCs render germline or somatic mutations in *APC [61, 96-98]*. Inactivation of APC leads to a disruption of the "β-catenin destruction complex" and to activation of the Wnt pathway [58, 61, 79]. Mutations in *CTNNB1* are present in less than 10% of sporadic CRCs [58, 61, 99, 100]. Most mutations in *CTNNB1* are concentrated on exon 3 - the phosphorylation sites for GSK3β, affecting the stability of β-catenin [99, 101]. Deletion of the GSK3β phosphorylation site in the *CTNNB1* locus leads to a phenotype similar to the one observed in Apc^{Min} mice [59, 94, 95].

The presence of mutations in Wnt-signaling components in human CRC and our ability to recapitulate disease state by genetic manipulations of wnt components in mouse models are the most prominent evidences for the importance of Wnt-signaling pathway in CRC [59-61, 94, 95, 98]. However, it is important to keep in mind that mutations in Wnt-signaling components are also present in other cancers [74]. The downstream effect of constitutively activated Wnt pathway is up-regulation in β-catenin protein levels and overexpression of its target oncogenes, such as c-Myc and others involved in self-renewal of the colonic epithelium [102-106]. These changes lead to hyperproliferation of undifferentiated intestinal cells and formation of intestinal polyps [102-106].

Regulation of Intestinal Stem Cells (ISC) By the Wnt-signaling Pathway

The Wnt-signaling pathway controls embryonic development and homeostasis of adult self-renewing intestinal tissues [102]. The lining of the intestine consists of several unique niches, each which is populated with different types of cells (Figure 1-4) [102]. The crypts are invaginations of the epithelium into the underlying connective tissue. At the bottom of the crypt, intestinal stem cells (ISC) proliferate to replenish sources of undifferentiated cells [102]. These dividing ISCs also differentiate and migrate from the very bottom of the crypts upwards to the surface of the lumen (villi) to renew the epithelium layer (Figure 1-4) [102].

It has been suggested that intestinal polyps rise from excessive proliferation of ISCs [102]. Studies identified the Wnt-signaling, Notch, and Eph/ephrin pathways as important in self renewal and differentiation of ISCs [102-106]. The Wnt-signaling pathway plays a critical role in maintaining the ISC population at the crypt bottom [103-

106]. The Wnt pathway is active in the crypt while inactive in the villus and mutations in Wnt componenets lead to irregular distribution of cell types along the crypt–villus axis and results in aberrant crypt foci [102-107]. While hyper-activated Wnt pathway results in enlarged crypts, hypo-activated Wnt pathway leads to disappearance of crypts and embryonic lethality [103-107]. Expansion of crypts and disappearance of villus upon activation of the Wnt pathway are some of the first transparent phenotypes prior to the development of intestinal polyps [102, 107].

Can We Win WNT?

Genetic and epigenetic alterations activating the Wnt-signaling pathway contribute to the development of variety of human cancers including CRC, pancreatic cancer, lung cancer, prostate cancer, leukemia, and others [57, 58, 60, 65-67]. Therefore, various approaches have been developed as an attempt to target different steps of the Wnt-signaling pathway and attenuate the transcriptional functions of β-catenin [57, 74]. Some approaches focus on upstream components such as Wnt3α, APC, GSK3β, Axin2, etc., while others approaches focus on downstream components such as β-catenin, TCF4, and other β-catenin partners [57, 74]. The advantage of inhibiting downstream components versus upstream components is the broader applicability of the therapeutic application to CRC cases, regardless of mutations type. For example, reactivation of APC will not be applicable for cases in which β-catenin is mutated, and attenuating wnt secretion by inhibition of porcupine will not be effective in cases of APC mutations. On the other hand, direct targeting of β-catenin will be, in theory, effective in most cases of CRC associated with misregulated Wnt pathway.

Although great efforts were made over the years to inhibit the Wnt-signaling pathway, there is currently no specific Wnt pathway inhibitor approved by the FDA [57, 74]. However, recently, four drugs targeting the Wnt-signaling have reached clinical trials, two which are biological inhibitors developed by OncoMed. Both inhibitors focus on targeting association of Wnt ligands with Frizzled receptors. One of them is the OMP-54F28 inhibitor, a fusion protein of the ligand-binding domain of Frizzled-8 and an immunoglobulin Fc region [108]. This fusion protein binds and sequesters soluble Wnt ligands, impairing their recognition by putative receptors. The other one is the OMP-18R5 inhibitor, a monoclonal antibody which targets the Frizzled receptors [109]. Another compound developed by Novartis is the LGK-974, which is a small molecule targeting porcupine, a critical enzyme facilitating the lipidation of secreted Wnt ligands [110]. Finally, PRI-724 (Prism Pharma), a small-molecule inhibitor targeting the interactions between β-catenin and CBP have recently entered phase I clinical trials for the treatment of solid tumors and leukemia [111]. Other approaches currently being developed are targeting the β-catenin-TCF4 interactions, the proteosome or E3/bTrcp ubiquitin ligase, and Axin2 by using compounds that bind and stabilize it [57, 74].

On another level, targeting epigenetic regulators of the Wnt-signaling pathway could also contribute to attenuation of the Wnt-signaling pathway. For example, inhibition of DNA methylation as aberrant CpG methylation of the APC promoter is observed in 30% of sporadic CRC cases [112]. Furthermore, inhibition of nuclear epigenetic modifiers, partners of β-catenin, could strategically be beneficial. Recent studies, including ours, have identified various epigenetic regulators that interact with β-catenin, such as Brg1, hSETD1A, MLL1/2, and P300, and activate Wnt putative

promoters [38, 49, 87, 90, 91]. For example, the PRI-724 inhibitor mentioned above prevents acetylation and activation of Wnt target genes[111].

Finally, the lack of true progress in inhibiting the Wnt-signaling pathway over the years is most likely due to the limited number of pathway-specific components that are also “druggable” enzymatic targets and due to toxicity pitfalls [57]. However, the success of few preclinical trials testing new Wnt pathway inhibitors, and recent studies identifying new Wnt pathway effectors, is encouraging.

Breast Cancer and Metastasis

Breast cancer is the most common cancer in women, and the second leading cause of cancer related deaths among women in the USA [113, 114]. The American Cancer Society estimated that 232,670 new cases of invasive breast cancer and 62,570 new cases of carcinoma in situ breast cancer will be diagnosed in women in the USA, in 2014. In addition, 2,360 new cases of breast cancer were estimated among men in the USA, in 2014. Approximately 40,000 women and 430 men are expected to die from breast cancer in 2014 alone, in the USA [113].

Breast cancer related deaths had slowly increased from1975 to1990 at a 0.4% annual increase rate [113]. This increase reflected changes in life span, delayed childbearing, reduced number of children, increased use of hormones, and increased obesity [113]. Death rates of breast cancer started to decline in 2001 due to the decreased use of menopausal hormones [113]. Survival rates of breast cancer are dependent on the location of the disease [113]. According to the National Cancer Institute, while there is 99-84% chance of five-year relative survival for localized and regional disease, there is only 24% chance of five-year relative survival for metastatic breast cancer. Therefore, early detection of breast cancer and prevention of metastasis

is critical for survival. Understanding of the reasons for breast cancer progression is crucial for developments of new interventions.

Like most cancers, breast cancer could be a result of genetic or somatic mutations. It is estimated that 5% to 10% of breast cancers are triggered by genetic mutations including mutations in *BRCA1* and *BRCA2*, *TP53*, *CHECK2*, *PTEN*, *SDHB*, *SDHD*, *KLLN*, and other genes [115]. Epigenetic is important for conveying genomic selection and adaptation and for sustention of a phenotype [10-12]. Understanding of the epigenetic changes that contribute to breast carcinogenesis will not only contribute to better diagnosis and prognosis, but also allow for new research and development of therapeutic revenues.

Molecular and Biological Basics of Metastasis

Metastatic cancer is defined as cancer that has spread from the primary tumor site to another location, to form secondary cancer [116, 117]. Virtually all cancers can metastasize. Metastasis-prone tissues are the bones, liver, lungs, and brain [117]. Tumor cells which populate and flourish in the primary or secondary tissue lead to organ dysfunction and death. Still, metastasis lies in the heart of therapeutic failure and mortality [116, 117]. Researchers have been trying to target metastasis by blocking certain functions important for dissemination of the primary tumor cells, such as migration, invasion and angiogenesis. These cellular processes play a critical role in enabling cells to detach from the primary tumor, disseminate through the blood stream, and establish new colonies [83, 85, 116, 117].

In order to metastasize, a cell needs to successfully complete serial of events in a temporal and spatial manner [83, 85, 116, 117]. Both molecular and morphological changes are required for such events to occur [83, 85, 116, 117]. First, primary tumor

cells need to detach from the primary tumor and invade into a nearby tissue (local invasion) (Figure 1-5) [83]. In order to disseminate to a distant location cells will migrate towards a nearby blood vessel and invade into the bloodstream (intravasation) (Figure 1-5) [83]. Circulating cells that survived in the blood will eventually reach small capillaries in which the blood stream conditions enable their movement arrest (Figure 1-5) [83]. Cells will then invade through the endothelial wall and migrate into the surrounding tissue (extravasation) (Figure 1-5) [83]. In order to form a secondary tumor, cells need to be capable of proliferating in the new environment (Figure 1-5) [83]. Once the secondary tumor reaches a certain size it becomes dependent on increased blood supply for oxygen and nutrients [118]. Therefore, it will be crucial for the tumor to stimulate the growth of new blood vessels to obtain sufficient blood supply (angiogenesis) [118, 119].

The cellular events enabling the dissemination of cells from their origin to other locations throughout the body are regulated by molecular events [83, 85, 116, 117]. For example, detachment of cells from the primary tumor is highly dependent on down-regulation of factors controlling cell-cell contact, such as E-cadherin [83]. Invasion is dependent on up-regulation of secreted proteases, such as Matrix metalloproteinases (MMPs) and Cathepsins, for degradation of the extracellular matrix [120, 121]. Directional cell migration is dependent on cell polarity, which is regulated by dynamic filaments [122]. The formation of new blood vessels (angiogenesis) is regulated by angiogenic proteins, such as vascular endothelial growth factors (VEGFs), Angiopoietins, and growth factors such as Fibroblast Growth Factors (FGFs) [118, 119]. The molecular and cellular changes characterizing metastatic cells are not exquisitely

regulated by the cell itself, but greatly dependent on the cellular environment in which the tumor resides in [123]. The tumor microenvironment plays a critical role in providing cancer cells with extracellular signals of growth, angiogenesis, invasion, and migration [123].

Epithelial-Mesenchymal Transition (EMT)

One of the initial changes a tumor cell has to go through in order to migrate is morphological [82-85]. There two morphologically different cell types which can interconvert: epithelial and mesenchymal [82-85]. Epithelial–mesenchymal transition (EMT) is a highly conserved process which was first recognized to be important in early development and cell differentiation [82, 84]. That is because mesenchymal features are necessary for migration, a function critical during development [82, 84]. Only later it was discovered to be a fundamental process in carcinoma progression and metastasis [82-85].

While epithelial cells are rounded, form adherent and tight junctions, and exhibit apical-basal polarity, mesenchymal cells are elongated and lack polarization [82, 84]. The molecular changes leading to alteration in morphogenesis are partially understood [83, 85]. The loss of E-cadherin expression is one of the major changes occurring during EMT [83, 85]. E-cadherin functions in establishing stable cell-cell contact through formation of adheren junctions along with β-catenin and alpha-catenin [83, 85]. Therefore the loss of E-cadherin allows cells to detach from one another and directly influences the acquisition of the mesenchymal phenotyp [83, 85]. The Wnt-signaling pathway also controls EMT since it tightly controls the expression of E-cadherin through regulation of Snail, Slug, and Twist, negative regulators of E-cadherin [84, 124, 125]. Activation of the Wnt-signaling pathway not only represses the expression of E-cadherin

but also sequesters β-catenin from adherens junctions thereby attenuating cell-cell contact [58, 66]. Another important positive regulator of EMT is TGFβ, which is also transcriptionally controlled by the Wnt-signaling pathway [83]. This cytokine is often used *in vitro* to induce EMT.

Role for Epigenetic in EMT

Tumor progression is dependent of genetic and epigenetic alterations. To effectively diagnose and treat advanced disease it is critical to improve our knowledge of genes that regulate metastasis and to identify novel biomarkers. Global changes in epigenetic marks are observed during EMT, but the function of such global changes and their source is largely unknown [126]. In contrast, modulations of epigenetics at specific gene loci, such as the E-cadherin promoter, have been studied for decades. For example, DNA methylation at the E-cadherin promoter was shown to repress transcription of E-cadherin and increase EMT [85, 127]. In fact, it is extremely difficult to reactivate the E-cadherin promoter by molecular manipulations after it has already gone through CpG island methylation. This demonstrates the important role of epigenetic modifications in transcription regulation of master regulators of EMT. Another example is the regulation of mesenchymal and epithelial genes, such as E-cadherin, HIF1α, vimentin, and N-cadherin by HDACs and SIRT1histone deacetylases [85, 127].

Induction of EMT by TGFβ1 supplementation was shown to globally increase active marks, such as H3K4me3 and H3K36me3, and decrease repressive marks, such as H3K9me2 [45]. Notably, it was recently demonstrated that H3K4 demethylase LSD1 regulates EMT and metastasis [45, 128, 129]. Ablation of LSD1 led to an increase in cell

invasion and migration *in vitro* and lung metastasis *in vivo* [128, 129]. These studies suggest the involvement of H3K4 methylation in stimulation of EMT.

Understanding of the epigenetic mechanisms related to metastasis is important and could offer novel approaches for innovative diagnosis and treatment of cancer patients. Future investigations and better understanding of the role of histone modifications during progression of cancer could greatly improve our therapeutic strategies.

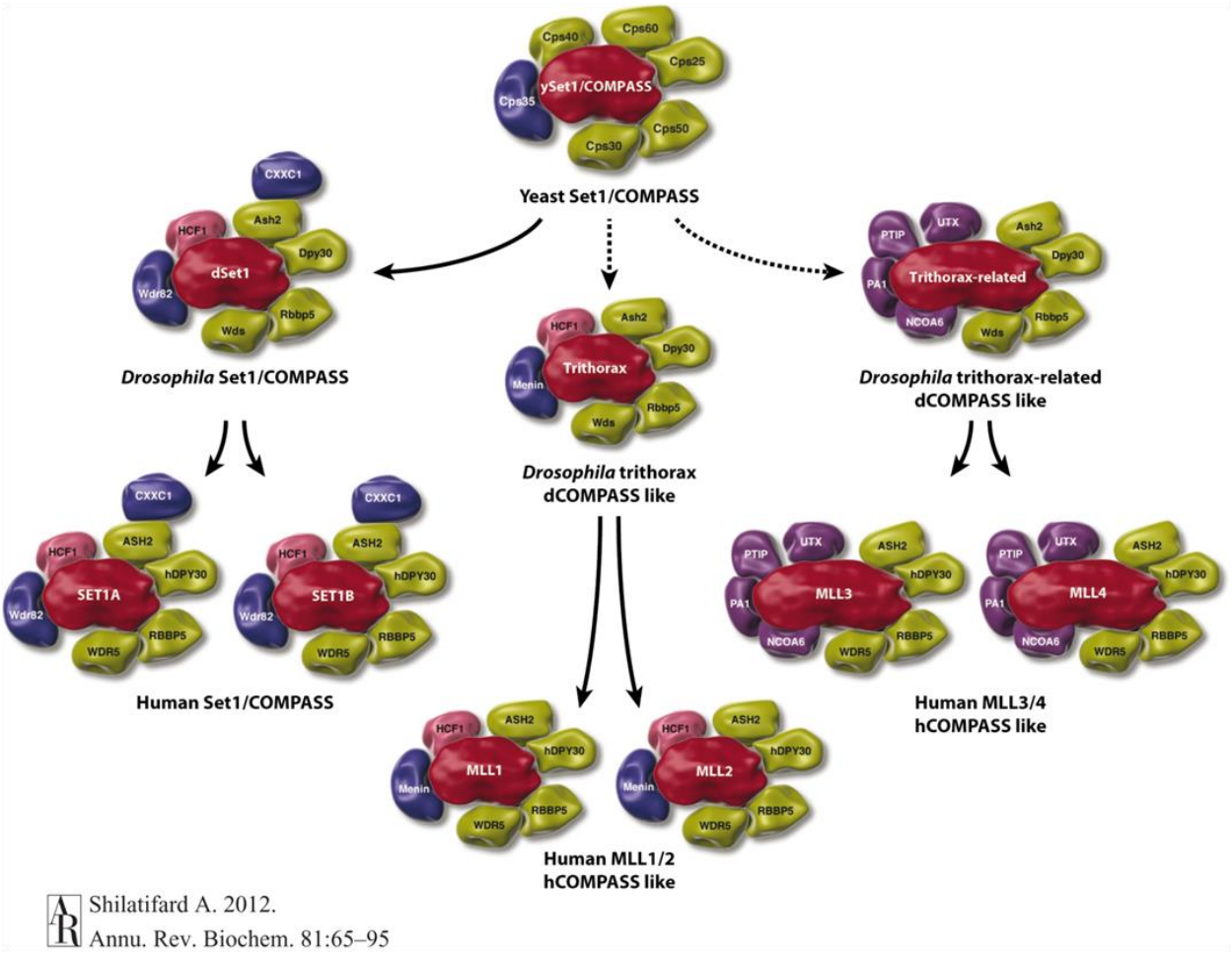


Figure 1-1. The COMPASS/Trithorax family of H3K4 methyltrasnferases [Adapted from Shilatifard, A. 2012. Annual Review of Biochemistry, Page 71, Figure 2]. [18].

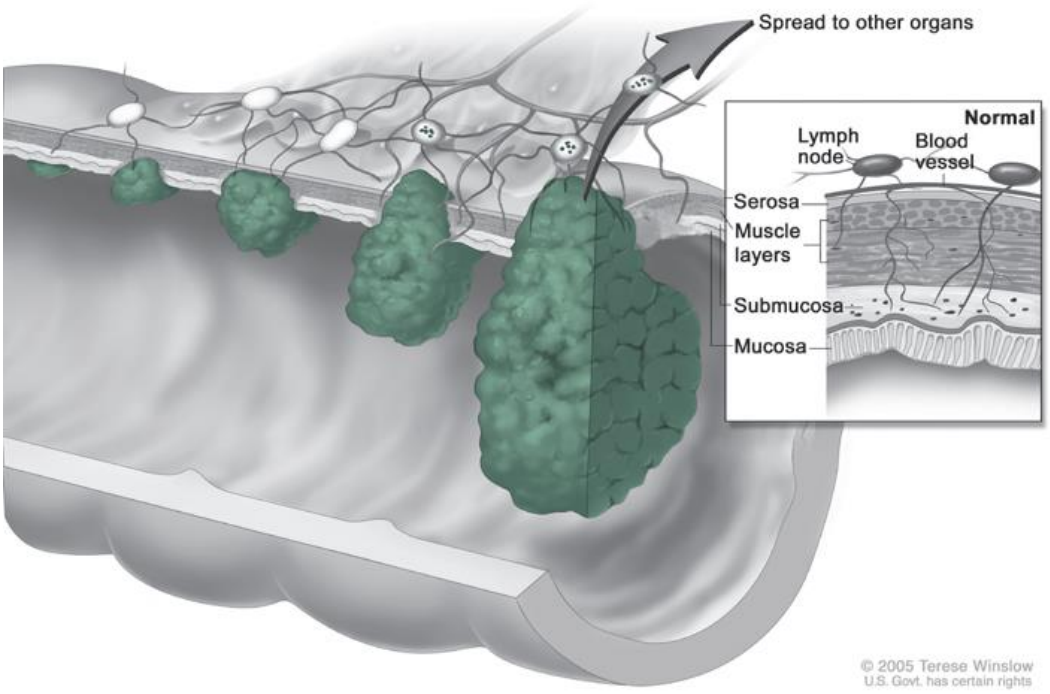


Figure 1-2. Multi-stage progression model of colorectal cancer. In situ: cancers that have not yet invaded the wall of the colon or rectum. Local: Cancers that have grown into the wall of the colon and rectum, but have not extended through the wall to invade nearby tissues. Regional: Cancers that have spread through the wall of the colon or rectum and have invaded nearby tissue, or that have spread to nearby lymph nodes. Distant: Cancers that have spread to other organs. [Adapted from The American Cancer Society, Colorectal Cancer Facts & Figures, 2011-2013, page 2, Figure 2].

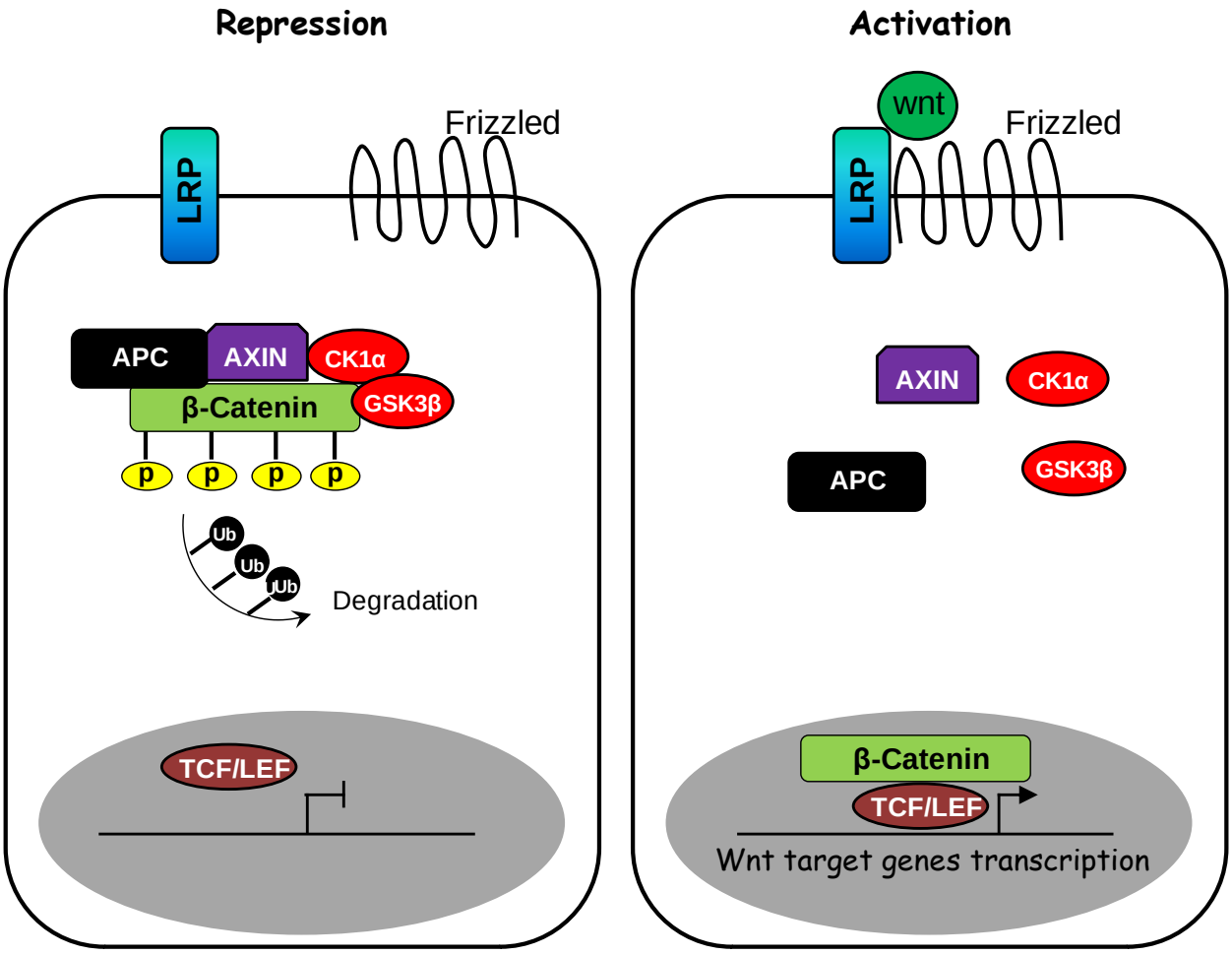


Figure 1-3. Schematic representation of canonical Wnt-signaling pathway. In the absence of wnt signal, β-catenin is bound by a destruction complex and targeted for phosphorylation, ubiqitination, and degradation by the 26S proteosome. Binding of Wnt ligand to a Frizzled/LRP-5/6 receptor complex leads to dissociation of β-catenin from the destruction complex, hypophosphorylation, and stabilization. β-catenin translocates into the nucleus where it interacts with TCF/LEF transcription factors and activates transcription of target genes.

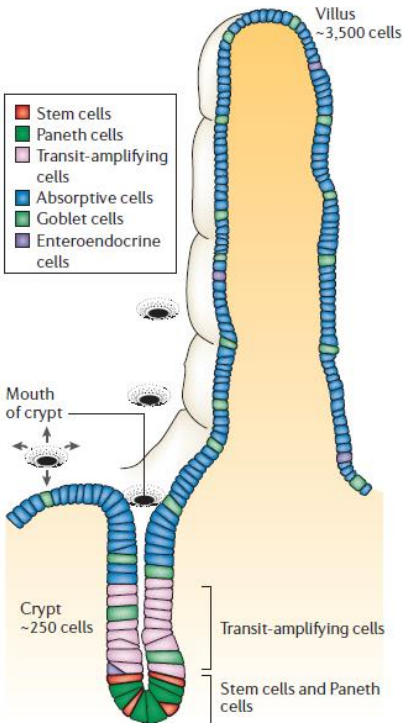


Figure 1-4. Cellular and structural organization at the intestinal crypt. Intestinal stem cells lie near the crypt base and differentiate upwards. Above the stem cells are transit-amplifying cells (dividing progenitors, some of them already partially differentiated); and above these, in the neck of the crypt and on the villus, lie post-mitotic differentiated cells (absorptive cells, goblet cells and enteroendocrine cells. [Adapted from Crosnier C. 2006. Nature reviews. Genetics, Page 350, Figure 1a] [102].

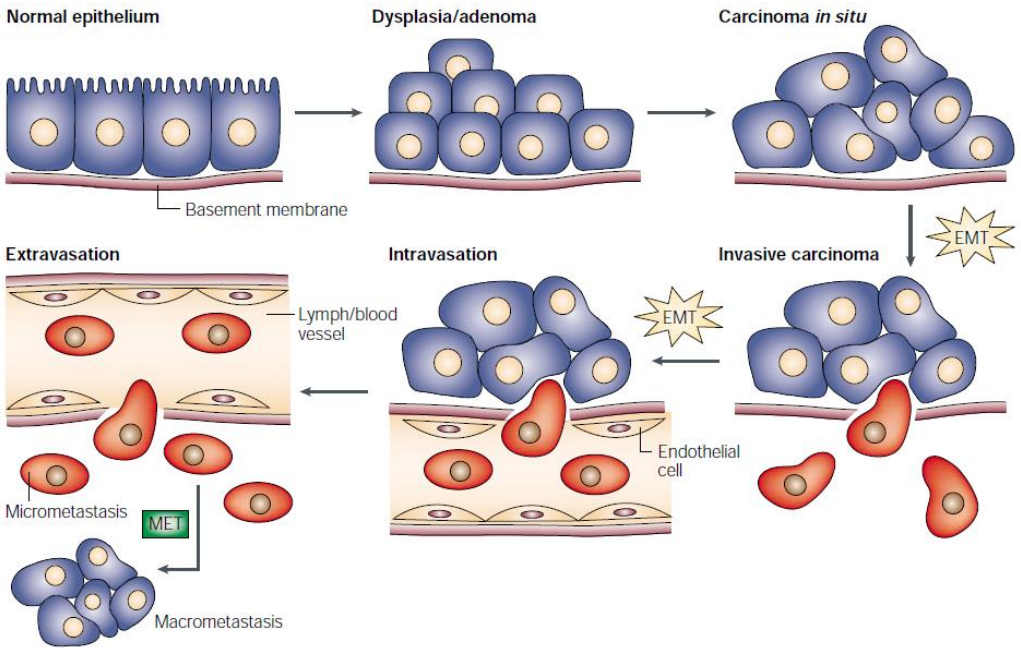


Figure 1-5. Schematic representation of the metastasis process. Cells which have gone through EMT can intravasate into lymph or blood vessels, and circulate until they extravasate through the endothelial layer and establish a secondary tumor locally (micrometastasis) or at distant organs (macrometastasis). [Adapted from Thiery JP. 2002. Nat Rev Cancer, Page 445, Figure 2] [83].

CHAPTER 2
MATERIALS AND METHODS

Cell Culture and Reagents

Human cell lines were purchased from American Type Culture Collection (ATCC) unless otherwise indicated. All cell lines were cultured at 37°C humidified atmosphere of 5% carbon dioxide (CO_2) in air. HEK293FT, Pheonix, HCT116, SW48, C2A, MDA-MB-231, MCF7, BT-549, and SUM-159 cell lines were maintained in Dulbecco' modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (Invitrogen). RKO and HT29 cells were maintained in MEM and RPMI, respectively, supplemented with 10% FBS and 1% penicillin–streptomycin. FHs int-74 cells, kindly provided by Dr. Maria Zajac-Kaye (University of Florida), were cultured in DMEM supplemented with 10% FBS, 1% penicillin–streptomycin, 10 μg/mL insulin (Sigma), and 30 ng /mL epidermal growth factor (EGF; Sigma). The human mammary epithelial cell lines MCF10A and HMEC were a kind gift from Dr. Jianrong Lu and Dr. Kevin Brown (University of Florida), respectively, and were cultured in DMEM–F-12 medium (Cellgro) supplemented with 5% horse serum (Sigma), 30 ng/mL EGF, 10 μg/mL insulin, and 0.5 μg/mL hydrocortisone (Sigma). Mouse Embryonic Fibroblasts (MEFs) over expressing the human Wnt3α gene (L Wnt-3A CRL-2647™) were purchased from ATTC and cultured for 4 days in DMEM supplemented with 10% FBS and 1% penicillin–streptomycin, according to the manufacturer instructions. Medium was then collected and replaced with fresh medium for additional 3 days of culturing. Conditioned medium from 4 days of culturing was mixed with the medium from the additional 3 days of culturing at 1:1 ratio to obtain Wnt3α-conditioned medium (WCM).

ShRNA-mediated Knock-Down

Viruses carrying specific shRNAs were generated by co-transfecting shRNA constructs along with viral packaging plasmids PMD2.G (envelope plasmid; Addgene) and PsPax2 (gag/pol packaging plasmid; Addgene) for lentiviruses, or packaging plasmids gag-pol and envelope protein (Oligoengine) for standard retroviruses. For virus propagation, plasmids were transfected into HEK293FT or Pheonix cells using the Calcium Phosphate Transfection method (Promega). Shortly, DNA plasmids (15 µg) were combined with packaging plasmids (8 µg of PsPax2 + 4 µg of PMD2.G or 2.5 µg of gag/pol + 2.5 of Env), 55 µL of 2M CaCl2, and H2O up to 500 µL. In a separate tube, HBS 2X (490 µL) was combined with pyrophosphate (10 µL). The CaCl2-DNA solution was added dropwise into the HBS solution while introducing air bubbles through the HBS solution. The solutions' mix was incubated at room temperature for 20 minutes and then added dropwise to the host cells (1-2 $x10^6$ cells / 10 cm dish). The medium was replaced with 8 mL fresh medium upon 24 hours. After additional 48 hours, media containing the virus particles was filtered and collected.

To generate stable knockdown (KD), $1x10^6$ cells were infected with 1-2 mL of the recombinant viruses in the presence of 4 µg/mL of polybrene infection reagent. Medium was replaced with fresh medium containing 1µg/mL puromycin (Calbiochem, 540222) upon 48-hours for stable selection. All stable KD cells were maintained in media containing 1 µg/mL puromycin.

Constructs used for hSETD1A KD in HCT116 and C2A cells were generated by subcloning shRNA oligonucleotides into pSuper.retro.puro vector following the manufacturer's instructions (Oligoengine). Constructs used for hSETD1A KD in other cell lines utilized lentiviral TRC vector harboring shRNAs targeting the hSETD1A gene

(Clone ID: TRCN0000152242) or CTNNB1 gene (TRCN0000003846) obtained from the UF Health Cancer Center shRNA library (Thermo Scientific). All shRNA sequences are listed in Table 2-1.

Antibodies

hSETD1A (A300-289A), Ash2L (A300-489A), MLL1 (A300-086A), TAF3 (A302-359A), and RbBP5 (A300-109A) antibodies were purchased from Bethyl Laboratories. β-catenin (C2206), β-actin (AC-15), and α-tubulin (T6199) antibodies from Sigma. TCF4 (sc-8631) and MYC (sc-40) antibodies from Santa Cruz Biotechnology. WDR5 (Ab56919), histone H3 (Ab1791), and H3K4me1 (Ab8895) antibodies from Abcam. H3K4me3 (MC315), H3K9me2 (05-768), H3K4me2 (07-030), and H3K27me3 (07-449) antibodies from Millipore. Snail antibodies (L70G2) from Cell Signaling. E-cadherin antibodies (610181) from BD Bioscience.

Western Immunoblotting

Protein samples were prepared as followed: cells ($1x10^6$) were collected by centrifugation at 1000 rpm for 3 minutes. Medium was aspirated and cell pellet was resuspend in ice-cold Phosphate-Buffered Saline (PBS). Cells were collected again by centrifugation at 1000 rpm for 3 minutes followed by aspiration of the PBS. Cells were lysed by resuspending cell pellet in RIPA buffer [140 mM NaCl, 0.5% Triton X-100, 1% sodium deoxycholate, 0.025% sodium azide,10 mM Tris, pH 8.0] containing 1 mM PMSF (Sigma, P7626), 1 mM DTT, 1 μg/mL Pepstatin A (Sigma, P5318), 1 μg/mL Leupeptin(Sigma, L2884), and 1 μg/mL Aprotinin(Sigma, A1153). Mixture was vortexed and sonicated (10 short bits). Sample was denatured by adding laemmli buffer and boiling at 95°C for 8 minutes.

Protein sample was separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Denatured samples (40-80 μg) were loaded onto polyacrylamide gel and ran at 100-120 V. Proteins were transferred to PVDF membranes at 350 milliamps for 3 hours using the Wet/Tank Blotting Systems (BioRad), according to the manufacturer's instructions. Membrane was blocked with 5% nonfat milk inTBST for 1 hour at room temperature and then incubated with primary antibody at 4°C overnight. Membrane was washed with 5% nonfat milk in TBST for 1 hour at room temperature, during which milk was replaced every 15 minutes. Membrane was then incubation with species-specific horseradish peroxidase-conjugated secondary antibodies in 5% nonfat milk in TBST for 1 hour at room temperature followed by 1 hour wash with TBST at room temperature, during which TBST was replaced every 15 minutes. Immunoreactive bands were visualized by using enhanced chemiluminescence (ECL; Pierce) and autoradiography. α-tubulin or β-actin were used as a loading control.

Co-immunoprecipitation

Nuclear extract was prepared as followed: cells (1-2x10^8) were collected and washed twice with PBS. Cell pellet was resuspended in 5 pcv of cold hypotonic buffer (10 mM HEPES PH 7.9, 1.5 mM $MgCl_2$, 10mM KCl] supplemented with protease inhibitors followed by centrifugation (3,000 rpm for 5 minutes at 4°C). Cell pellet was resuspended in 3 pcv hypotonic buffer and incubated on ice for 10 minutes. Cells were then homogenized with 10-18 up-and-down strokes using type B pestle and centrifuged (4,000 rpm for 15 minutes at 4°C). Nuclei pellet was resuspended in ½ pnv of low salt buffer [20 mM HEPES PH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 0.02M KCl, 0.2 mM EDTA]

supplemented with protease inhibitors, followed by dropwise addition of ½ pnv high-salt buffer [20 mM HEPES PH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 1.2M KCl, 0.2 mM EDTA] supplemented with protease inhibitors under continuous gentle mixing for 30 minutes at 4°C. Nuclear extract was centrifuged (20 minutes at maximum speed at 4°C) to remove nuclei debris and supernatant was dialyzed overnight against 50 volume of dialysis buffer [20 mM HEPES PH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA] supplemented with protease inhibitors.

Nuclear extract (0.7 mg) was precleared with 5 µg of immunoglobulin G (IgG) and Dynabeads® Protein A or G. Precleared nuclear extract was immunoprecipitated with antibodies against β-catenin (C2206) or components of the hSETD1A complex overnight at 4°C. Beads were thoroughly washed with RIPA and precipitants were eluted in laemmli buffer by boiling. Precipitated protein samples were subjected to SDS PAGE, and analyzed by Western blot (WB) analysis as described above.

Immunofluorescence

Immunofluorescence staining was carried out by the Molecular Pathology and Immunology Core at the University of Florida. Tissue samples were formalin fixed and subsequently paraffin embedded. Five micron sections were sequentially deparaffinized and rehydrated through graded ethanol. Optimal staining required 25 minutes of heat antigen retrieval in 10 mM Citrate buffer pH6.0. Sections were blocked with 2% horse serum in buffer for 1 hour, and then incubated with β-catenin antibodies (C2206 sigma) diluted at 1:1200 overnight at 4°C. Slides were washed with 1X PBS twice for 5 minutes at RT°C, incubated in donkey anti-rabbit alexafluor 488 diluted at 1:500 for 60 minutes at RT°C in dark, and mounted in Vectorshield with DAPI. Images were obtained using a

Leica DM2500 microscope equipped with an Optronics camera using magnifier software.

Immunohistochemistry

Immunohistochemistry staining was carried out by the Molecular Pathology and Immunology Core at University of Florida. Briefly, 5 microns serial sections were de-paraffinized and incubated with 3% H_2O_2/methanol to block endogenous peroxidase activity. Sections were treated by Citra (Biogenex, Fremont, CA) at 95°C for 30 minutes followed by blocking with Sniper (Biocare Medical, Walnut Creek, CA) to reduce non-specific background staining. Sections were incubated with rabbit anti-human hSETD1A (IHC-00171) at room temperature for 1 hour and then with Mach2 Gt x Rb HRP polymer (Biocare Medical, Walnut Creek, CA) for 30 minutes. Staining was visualized with DAB chromagen (Vector Laboratories, Burlingame, CA) and hematoxylin counterstain.

Chromatin Immunoprecipitation (ChIP)

Cells ($1x10^8$) were collected in 10 mL medium and cross-linked at room temperature with 1.5 mM Ethylene glycol sulfosuccinimidylsuccinate (EGS; Sigma, E3257) (30 minutes rotation) and 1% Formaldehyde (FisherSci, BP531) (10 minutes rotation). Cross-linking reaction was quenched by the addition of 0.125 M glycine (5 minutes rotation). Cells were pelleted (780 xg for 10 minutes) and washed twice with cold PBS. Chromatin from frozen tissue samples was prepared (30 μg tissue/ChIP) by slicing the tissue using a razor blade, cross-linking with formaldehyde and EGS as described above, homogenization, and filtration. Cells were then lysed with 3 mLs cell lysis buffer [10mM Tris-HCl, pH 8.0, 3mM $MgCl_2$, 0.4% NP-40] containing protease inhibitors, followed by centrifugation (5000 rpm for 5 minutes). Resuspension and centrifugation was repeated up to 3 times to completely lyse the cells. Nuclei were lysed with 1 mL of SDS-

lysis buffer [50 mM Tris-HCl, pH 8, 10 mM EDTA,1% SDS] containing protease inhibitors and incubated on ice for 10 minutes. Non-SDS lysis buffer (3 mLs) [50 mM Tris-HCl, pH 8, 10 mM EDTA] containing protease inhibitors was added to the nuclei lysate and the mixture was incubated on ice for additional 5 minutes. Chromatin was sonicated using Bioruptor TM UCD-200 (Diagenode) for 15-30 minutes to obtain DNA fragments of 200-500 bp. Cellular debris was removed by centrifugation for 30 minutes at maximum speed at 4°C and supernatant chromatin was subjected to IP.

Chromatin immunoprecipitation was performed as followed: chromatin was precleared by the addition of species- specific IgG (5 μg/ ChIP) for 2 hours at 4°C with rotation followed by the addition of protein A or G dynabeads. Precleared chromatin was precipitated overnight at 4°C with 10 μg IgG control or other antibodies. Chromatin was centrifuged for 30 minutes at maximum speed and supernatant was further incubated with protein A or G dynabeads for 2 hours at 4°C with rotation. Beads were then extensively washed and DNA was eluted from the beads with sodium bicarbonate solution [0.0084 g/mL NaHCO3, 1% SDS] followed by Phenol chloroform extraction and ethanol precipitation. Specific DNA loci were quantified by real-time qPCR. Relative fold enrichment of DNA fragments was determined by normalizing the quantification cycle value (Cq) of each IP with the Cq value of the input chromatin. The ChIP primers used are listed in Table 2-3.

Real-Time PCR

Total RNA was isolated from cells using the RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. Total RNA was isolated from tissues by trizol extraction (Ambion). Shortly, tissues were homogenized in 1 mL trizol. Chloroform (200 μL) was added to each sample and mixture was vortexed, incubated at room

temperature for 3 minutes, and centrifuged (12,000 x g for 15 minutes at 4°C) .The upper phase was collected and mixed with 500 µL 100% isopropanol. Mixture was incubated at room temperature for 10 minutes followed by centrifugation (12,000 x g for 10 minutes at 4°C). Supernatant was discarded and DNA pellet was washed with 75% ethanol followed by centrifugation (7,500 x g for 5 minutes). Supernatant was discarded and DNA pellet was dissolved in water. cDNA was synthesized from 2 µg mRNA using the Superscript II Reverse Transcriptase kit (Invitrogen) according to the manufacturer's instructions. cDNA was diluted 10 fold and Real-time PCR was performed on cDNA templates with a CFX real-time PCR detection system (Bio-Rad), using SYBR Green [10 µL SYBR Green, 4.4 µL H_2O, 0.3 µM primer mix, 5 µL cDNA]. PCR amplifications were performed in triplicate. GAPDH or β-actin or were used as an internal control. Primers sequences are listed in Table 2-2.

Growth Assays

Proliferation assay was performed by seeding cells in 10 mL medium at a density of 2 x 10^5 cells in 10 cm culture dishes. Cell number was determined by cell viability count every other day. Colony formation assays were performed by seeding cells in 6 cm culture dishes at a density of 1 x 10^3 cells per plate. After 2-3 weeks, colonies were stained with crystal violet and the number of colonies was determined. Soft agar colony formation assays were performed by seeding 1 x 10^4 cells in culture media containing 0.7% agarose medium on top of a base layer containing 0.5% agarose in medium. The number of colonies (>50 cells) in soft agar was determined after 3 weeks.

Migration Assay

shRNA-transfected MDA-MB-231 cells were harvested, resuspended (5×10^4) in 500 μL complete DMEM medium, and placed onto inserts containing 8-μm pore membranes (BD Biosciences). Inserts were placed inside wells (24-well plate) containing 700 uL of complete DMEM medium, serving as an attractant. After 24 h, nonmigrating cells remaining on top of the membranes were removed with a cotton swabs. Cells that were able to migrate through the membrane onto the underside surface of the membrane were fixed and stained with crystal violet and photos of the entire field were taken. Each experiment was carried out in 3-5 replicates and repeated at least twice.

Invasion Assay

This assay was performed as described for the migration assay, with the following modifications. MDA-MB-231 cells ($1x10^4$) were suspended in 500 μL serum free medium and seeded onto basement membrane matrix-coated invasion inserts (BD Biosciences). The insert is covered with a membrane containing 8-μm pores and is coated with a basement membrane matrix (Matrigel). Cells were fixed after 17 hours and the number of invading cells on the entire field was counted by light microscopy. Each experiment was carried out in 3-5 replicates and repeated at least twice.

Wound-Healing Assay

MDA-MB-231 or BT-549 cells were cultured to confluence or near confluence (>90%) in 6 cm tissue culture dish overnight. Using a sterile 1000 μL pipet tip, two perpendicular scratches were applied to the cells. Cells were then rinsed with 1X PBS which was then replaced with complete fresh DMEM medium. Pictures of the scratch at different point marks were taken and retaken after 16-72 hours.

Cell Cycle Analysis

HCT116 cells were grown to 60% confluence, trypsinized, and washed twice with cold PBS. Cells (1-2 x 10^6) were collected in in a 15 ml polypropylene tube and resuspended in 100 µL cold PBS. Cells were further fixed overnight at 4 °C in 1 mL of cold (-20 °C) 70% ethanol, which was added to the cells dropwise while vortexing. Cells were then treated with 20 µg/mL RNase A at 37 °C for 30 min, and stained with 20 µg/mL propidium iodide at room temperature for 90 min. Cell cycle was analyzed with a Becton Dickinson cell sorter.

Microarray Analysis

Ten micrograms of RNA were reverse transcribed to cDNA using the Applied Biosystems High Capacity cDNA Reverse Transcription Kit according to the manufacturer's instructions (Applied Biosystems). cDNA products were heated to 95^{o}C for 1min and treated with 100ng RNase A for 30min at 37^{o}C, and then purified using the Qiagen PCR purification kit according to the manufacturer's instructions. NimbleGen Human Gene Expression array was purchased from Roche Applied Sciences. cDNA samples were labeled, Cy3 hybridized, and processed at the FSU NimbleGen Microarray Facility at Florida State University. The well-characterized Wnt target genes analyzed were selected according to the Stanford University Wnt Home Page (http://www.stanford.edu/group/nusselab/cgi-bin/wnt/) and according to commercially available SABioscienses Wnt-signaling Targets PCR array. The expression data were analyzed using the bioconductor packages Oligo [130] and Limma [131]. Expression was background-corrected and quantile-normalized with RMA (robust multi-array) analysis, and the empirical Bayes approach in Limma package was applied to call differentially expressed genes. Genes with P-value (FDR adjusted) less than 0.005

were defined as differentially expressed. The P-value of over-representation in the Wnt-signaling pathway was calculated by a hypergeometric test. The CEO accession number for the microarray data set reported in this article is GSE52230.

Colorectal and Breast Patient Samples

Patients' samples were obtained from the Cooperative Human Tissue Network (CHTN) in the University of Alabama at Birmingham (IRB No. 031078 and 010294 University of Alabama). All samples were deidentified. The use of all human tissues for this study was approved by the Institutional Review Board (IRB-01) of the University of Florida (Non-Human Exempt IRB approval No. 24-2012). All procedures were performed according to the regulations and guidelines of the approved protocol.

Mouse Xenograft Model

The scramble (Scr) control or shSETD1A (C7) expressed HCT116 cells ($1x10^6$) were resuspended in 150 μL 50% Matrigel (BD Biosciences) and 1X PBS in 1:1 ratio. Cell suspension was injected subcutaneously using an insulin syringe into the flank of 5- to 6-week-old female athymic nude mice Tumors were harvested 16 days post injection and tumor volume was determined using the formula L*2*W*(π/6), L=shortest diameter, W=longest diameter. Dissected tumors were homogenized in RIPA lysis buffer (Chem Cruz) supplemented with protease inhibitors, tumor lysate was sonicated, and 80 μg protein samples were subjected to WB analysis as described above. Mice were purchased from Harlan Labs and housed in the Cancer and Genetics Research Center at the University of Florida. Animal handling and procedures were approved and performed according to the guidelines of the Institutional Animal Care and Use Committee of the University of Florida.

In vivo Metastasis

MDA-MB-231 cells that have been infected with lentiviruses carrying shRNA targeting firefly luciferase or hSETD1A were injected into the tail vein (1 x 10^6cells in 200 μL PBS) of 5-6 week-old female athymic nude mice (Harlan Laboratories). After 7-8 weeks, mice were sacrificed and lungs were dissected. Lungs were immediately fixed in bouin's solution for 24 hours and washed thoroughly with 70% ethanol. Lungs nodules were counted under a light microscopy. Mice were purchased from Harlan Labs and housed in the Cancer and Genetics Research Center at the University of Florida. Animal handling and procedures were approved and performed according to the guidelines of the Institutional Animal Care and Use Committee of the University of Florida.

Statistical Analysis

Quantitative results were expressed as means ± SD. Two tailed student's t-test was used for comparison between two different conditions. *, $p<0.05$; **, $p<0.01$, ***, $P<0.001$.

Table 2-1. Human shRNAs

Target Name	Primer Sequence	Vector	Cell line infected
hSETD1A	5’-GGAAAGAGCCATCGGAAAT-‘3	pSuper.retro.puro	HCT116, C2A
hSETD1A	5’-GACAACAACGAATGAAATA-‘3	pSuper.retro.puro	HCT116, C2A
hSETD1A	5’-CAACGACTCAAAGTATATA-‘3	pSuper.retro.puro	HCT116, C2A
hSETD1A	5’-TTCATTCGTTGTTGTCCTTTG-‘3	TRC	SW48, RKO,MDA-MBA-231
CTNNB1	5’-ATCAGCAGTCTCATTCCAAGC-‘3	TRC	HCT116
CTNNB1	5’-TTCAGACAATACAGCTAAAGG-‘3	TRC	HCT116

Table 2-2. RT-PCR Primer Sequences

Primer Name	Primer Sequence
MYC Fwd	5'-CACGAAACTTTGCCCATAGC-3'
MYC Rev	5'-AGCAGCTCGAATTTCTTCCA-3'
VEGFA Fwd	5'-GAGTACATCTTCAAGCCATC-3’
VEGFA Rev	5'-CATTTGTTGTGCTGTAGGAA-3'
MMP7 Fwd	5'-AAACTCCCGCGTCATAGAAAT-3'
MMP7 Rev	5'-TCCCTAGACTGCTACCATCCG-3'
TWIST2 Fwd	5'-AAACTCCCGCGTCATAGAAAT-3'
TWIST2 Rev	5'-TCCCTAGACTGCTACCATCCG-3'
SNAI1 Fwd	5′-ACCACTATGCCGCGCTCTT-3′
SNAI1 Rev	5′-GGTCGTAGGGCTGCTGGAA-3′
E-cadherin Fwd	5'-GGATGTGCTGGATGTGAATG-3'
E-cadherin Rev	5'-CACATCAGACAGGATCAGCAGAA-3'
TCF1 Fwd	5'-TCAGGGAAGCAGGAGCTG-3'
TCF1 Rev	5'-TTCTTGATGGTTGGCTTCTTG-3'
TCF7L2 Fwd	5'-ATGGAGGGCTCTTTAAGG-3'
TCF7L2 Rev	5'-AGGCGATAGTGGGTAATAC-3'
TGFβ1 Fwd	5'-CAACAATTCCTGGCGATACCT-3'
TGFβ1 Rev	5'-GCTAAGGCGAAAGCCCTCAAT-3'
TERT Fwd	5'-GCCTTCAAGAGCCACGTC-3'
TERT Rev	5'-CCACGAACTGTCGCATGT-3'
LEF1 Fwd	5’-ATGTCGTTGCTGAGTGTA-3’
LEF1 Rev	5’-TTGGACCTGTACCTGATG-3’
CLDN7 Fwd	5’-GGATGATGAGCTGCAAAATG-3’
CLDN7 Rev	5’-CACCAGGGAGACCACCATTA-3’
CCNA2 Fwd	5'-ACATTACAGATGATACCTACACCAAG-3'
CCNA2 Rev	5'-CTTTGTCCCGTGACTGTGTAGAGTG-3'
SETD1A Fwd	5'-AAGGTGTACCGCTATGAT-3'
SETD1A Rev	5'-CCAATATAGAACTCGTCCAG-3'
SETD1B Fwd	5'-ATGGCATGGACTGGCTTAAC-3'
SETD1B Rev	5'-CATCGTCCCGTTTCTTCTTC-3'
GAPDH Fwd	5' -CCACTCCTCCACCTTTGAC -3'
GAPDH Rev	5'-ACCCTGTTGCTGTAGCCA-3'
β-actin Fwd	5'-AGAAAATCTGGCACCACACC-3'
β-actin Rev	5'-AGAGGCGTACAGGGATAGCA-

Table 2-3. ChIP Primer Sequences

Primer Name	Primer Sequence
MYC TSS Fwd	5'-GCGTGGGGGAAAAGAAAA-3'
MYC TSS Rev	5'-GTCCAGACCCTCGCATTA-3'
MYC Enhancer Fwd	5'-GTGAATACACGTTTGCGGGTTAC-3′
MYC Enhancer Rev	5'-AGAGACCCTTGTGAAAAAAACCG-3′
MYC 3'UTR Fwd	5'-GTTTCTCTGTAAATATTGCCATT-3'
MYC 3'UTR Rev	5'-ACTAGGATTGAAATTCTGTGTA-3'
VEGFA +400 Fwd	5'-AAGTGAGTGACCTGCTTT-3'
VEGFA +400 Rev	5'-GGTGTCTGTCTGTCTGTC-3'
VEGFA TSS Fwd	5'-TTTAAAAGTCGGCTGGTAG-3'
VEGFA TSS Rev	5'-CGGATCAATGAATATCAAATTCC-3'
VEGFA 3'UTR Fwd	5'-TGAGTGGTTGACCTTCCTCC-3'
VEGFA 3'UTR Rev	5'-GATCCTGCCCTGTCTCTCTG-3'
MMP7 TSS Fwd	5'-TCCTGCCAATAACGATGTA-3'
MMP7 TSS Rev	5'-TCTTGGACCTATGGTTGATT-3
MMP7 3'UTR Fwd	5'-AACATCCATTCATTCATTCATTG-3'
MMP7 3'UTR Rev	5'-AAGACACAGTCACACCAT-3'
TWIST2 TSS Fwd	5'-CCTAGCTCCTGACAACTATT-3'
TWIST2 TSS Rev	5'-GCTGGAAAGGCTCTGATT-3'
TWIST2 3'UTR Fwd	5'-CTCTGGTGCTGACTCTATC-3'
TWIST2 3'UTR Rev	5'-TGTCCTCTTCCTCCTCTAA-3'
SNAI1 TSS Fwd	5'-CTAGCGAGTGGTTCTTCT-3'
SNAI1 TSS Rev	5'-TTCCTGACGAGGAAAGAG-3'
SNAI1 3'UTR Fwd	5' -TAATGGCTGTCACTTGTC- 3'
SNAI1 3'UTR Rev	5'-GAAATATAAATACCAGTGTACCTTT-3'
E-cadherin TSS Fwd	5'-AATCAGAACCGTGCAGGTCC-3'
E-cadherin TSS Rev	5'-ACAGGTGCTTTGCAGTTCCG-3'
E-cadherin 3'UTR ChIP-F	5'-CCAGGAGATGAAAGGGACAA-3'
E-cadherin 3'UTR ChIP-R	5'-GGATCACAGACTCCAGGTTTC-3'
Tal1 +70 Fwd	5'-GTGGCCACAAAGCAAGGAAT-3'
Tal1 +70 Rev	5'-TCTCTGGAATCTCCAAGGCAA-3'

CHAPTER 3
HUMAN SETD1A AND H3K4ME3 ARE UP-REGULATED IN COLORECTAL CANCER AND CONTROL CELLULAR GROWTH

Introductory Remarks

Colorectal cancer is the third most common cancer in both men and women, and the third leading cause of cancer related deaths in men and women, in the USA [62, 64]. The American Cancer Society estimated 136,830 new cases and 50,310 deaths of CRC in 2014, in the USA [62]. Despite availability of conventional treatment, calls for targeted therapies are urging.

Epigenetic modulations are prevalent in CRC including alteration in levels of DNA methylation and histone modifications [39, 132, 133]. It has been shown that genome-wide enhancer/promoter-associated H3K4 methylation profiles are significantly altered in CRC compared with the paired adjacent normal mucosa [39, 133]. The data suggest that specific H3K4 methyltransferases may play an important role in the development of colorectal tumors.

Human SETD1A and SETD1B are predominantly associated with transcribed regions through their interactions with Ser-5 phosphorylated RNA pol II [18]. These HMTs are mainly responsible for H3K4me3 and globally affect gene expression profile [18, 21-24]. Taken together, hSETD1A and hSETD1B are potential candidates for regulating bulk H3K4 methylation and cellular growth in CRC.

Results

hSETD1A and H3K4me3 are Up-regulated in Human Colorectal Cancer

To gain insights into the role of trithorax HMTs in CRC, we first examined the expression levels of hSETD1A, hSETD1B, and MLL1 in human CRC. Although MLL1 levels remained unchanged, hSETD1A levels markedly increased in all CRC cell lines

tested compared to the human normal intestinal cell line FHs Int-74 (Figure 3-1C). Moreover, the up-regulation of hSETD1A in CRC cells was accompanied by a global increase in H3K4me3 levels (Figure 3-1C). In addition, the mRNA levels of hSETD1A and hSETD1B were up-regulated in human CRC specimens compared with adjacent paired normal mucosa collected from 24 patients (Figure 3-1A and Figure 3-1B). More than 62% the CRC specimens showed 2-fold or more increase in hSETD1A transcript levels (15/24 patients; Figure 3-1A), whereas only 26% of patients showed 2-fold or more increase in hSETD1B expression (6/23 patients; Figure 3-1B). Immunohistochemistry staining further confirmed that hSETD1A was aberrantly expressed in colorectal tumors in comparison with adjacent normal mucosa (Figure 3-1D). Importantly, hSETD1A expression also positively correlated with a global increase in H3K4me3 levels in these colorectal tumors (Figure 3-1D bottom).

More interestingly, the distribution pattern of hSETD1A in normal mucosa indicated that hSETD1A was highly expressed in the mouse and human intestinal crypt bottoms, but under-expressed at the top of the crypts (Figure 3-1E). The intestinal crypt bottom is populated with highly proliferative and poorly differentiated intestinal stem cells while the crypt top (or villus) is occupied by differentiated enterocytes [102]. Several studies argue that early molecular changes in crypt bottoms drive the initiation of colorectal polyps [104, 105, 107]. The fact that hSETD1A levels were elevated in human CRC and in normal intestinal crypts implies that hSETD1A activity may be required for intestinal cell proliferation.

hSETD1A Impacts Proliferation of Colorectal Cancer Cells

To determine the effect of hSETD1A on cellular proliferation, hSETD1A was stably silenced in the CRC cell lines HCT116, SW48, C2A, and RKO using retrovirus or

lentivirus harboring shRNA specific for the *hSETD1A* gene (Figure 3-2 and Figure 3-3A). KD of hSETD1A in HCT116 cells led to a specific decrease in global H3K4me3 modification, but did not enact on other histone modifications, such as H3K4me1, H3K4me2, H3K27me3, and H3K9me2 (Figure 3-2). Most importantly, the KD of hSETD1A significantly reduced the proliferation rates of HCT116, SW48, C2A, and RKO cells (Figure 3-3A). In addition, hSETD1A KD HCT116 and SW48 cells displayed smaller colonies compared with the scramble control (Figure 3-3B). When grown on soft agar, the hSETD1A KD HCT116 cells displayed fewer and smaller colonies with approximately 45% less colonies in the hSETD1A KD cell pool, and averaged 70% less colonies in the two individual hSETD1A KD clones, compared with the scramble control (Figure 3-3C).

Furthermore, sub-G1 phase population or increase in cell death were not observed in hSETD1A KD HCT116 cells using fluorescence-activated cells sorting (FACS) analysis (Figure 3-4A) or Trypan blue exclusion dye, respectively (Figure 3-4B). These results exclude the possibility that apoptosis is accounted for the decrease in cellular proliferation in the hSETD1A KD cells. Cell cycle analysis rather revealed reduction in S-phase population (Figure 3-4A). Taken together, we concluded that hSETD1A and its associated H3K4me3 play an important role in regulating the proliferation of CRC cells, and were prompted to investigate the mechanism.

Closing Remarks

To investigate dysregulation of H3K4 methylation in human CRC we detected levels of HMTs such as, hSETD1A, hSETD1B, and MLL1 in human colorectal tumors and cell lines by qRT-PCR and WB analyses. hSETD1A was significantly up-regulated in CRC and its depletion resulted in reduced global H3K4me3 and decreased cellular

growth, but did not affect cell death. These results imply that increased levels of hSETD1A in CRC could enhance cellular growth.

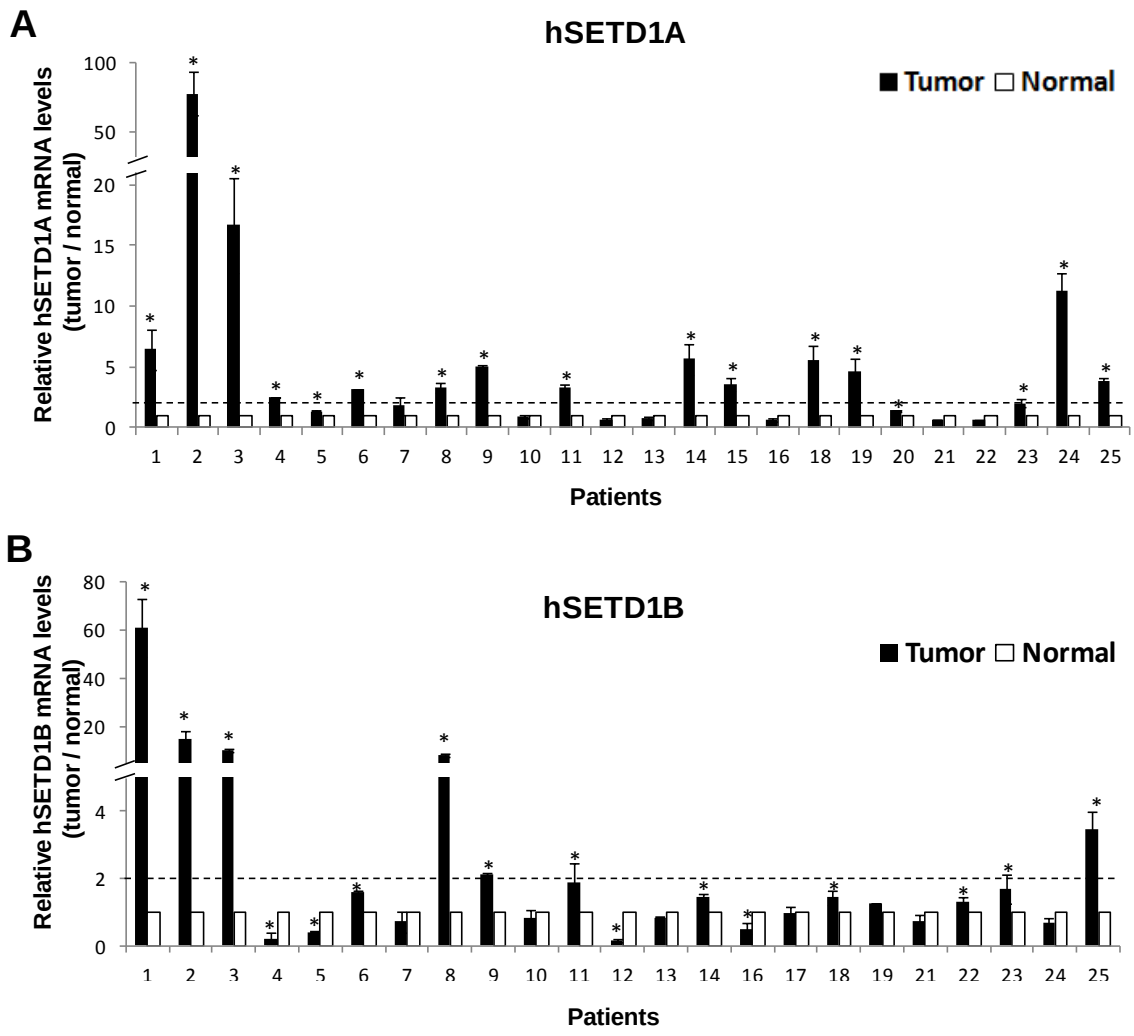


Figure 3-1. hSETD1A and H3K4me3 are up-regulated in human colorectal cancer. (A and B) hSETD1A (A) and hSETD1B (B) mRNA levels were up-regulated in human colorectal tumors compared with adjacent paired normal mucosa as detected by qRT-PCR. Shown are the mean ±SD of three replicates. *P < 0.05 by student t test. (C) hSETD1A and H3K4me3 were up-regulated in various human CRC cell lines compared with normal intestinal epithelial cell line FHs int-74 as detected by WB analysis. (D) Immunohistochemical (top) and WB (bottom) analyses revealed an increase in hSETD1A and H3K4me3 levels in human colorectal tumor compared with adjacent normal mucosa from patient #2. T = tumor, N = normal. (E) hSETD1A was highly expressed in normal intestinal crypts from mouse (left) and human (right). Red arrows indicate crypts and black arrows indicate crypt tops.

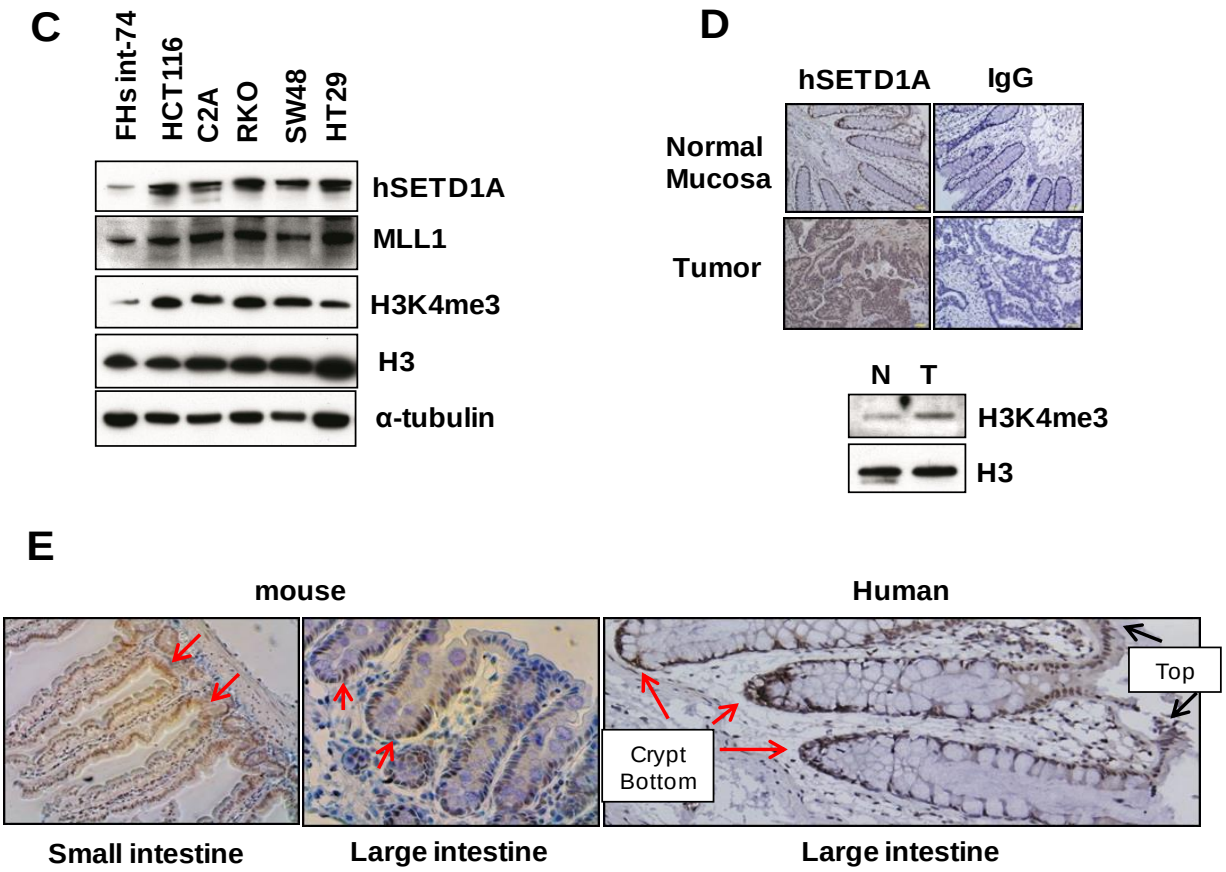


Figure 3-1. Continued

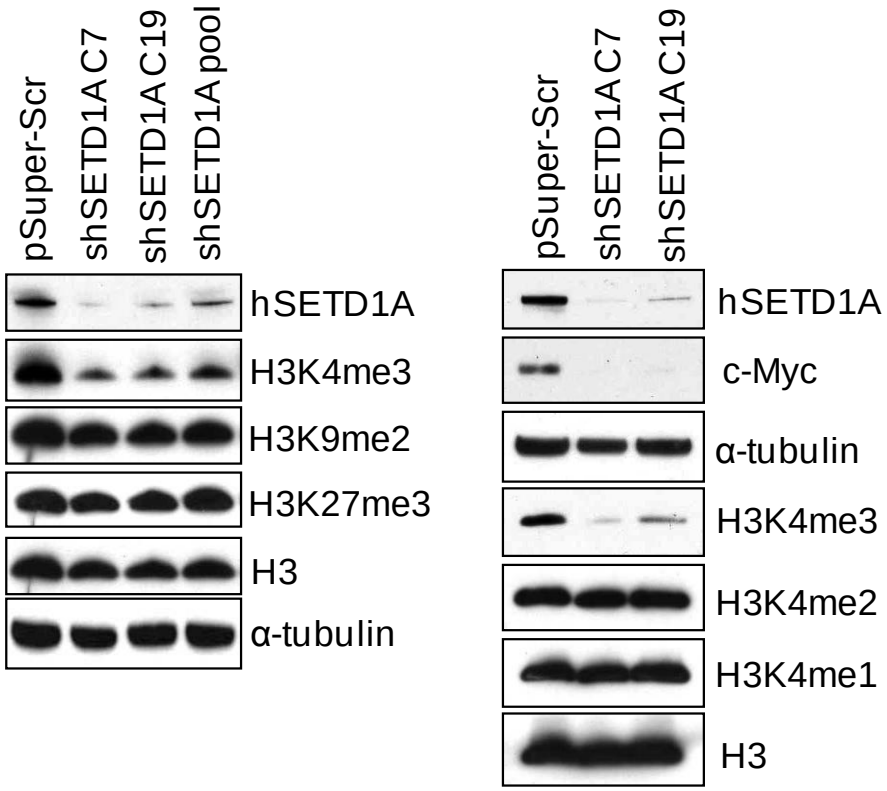


Figure 3-2. hSETD1A exclusively affects global H3K4me3. HCT116 cells were infected with a retrovirus harboring shRNA specific for hSETD1A or with scramble control. Two individual clones, C7 and C19, were selected from the hSETD1A KD cell pool. hSETD1A and histone modification levels were analyzed by WB analysis.

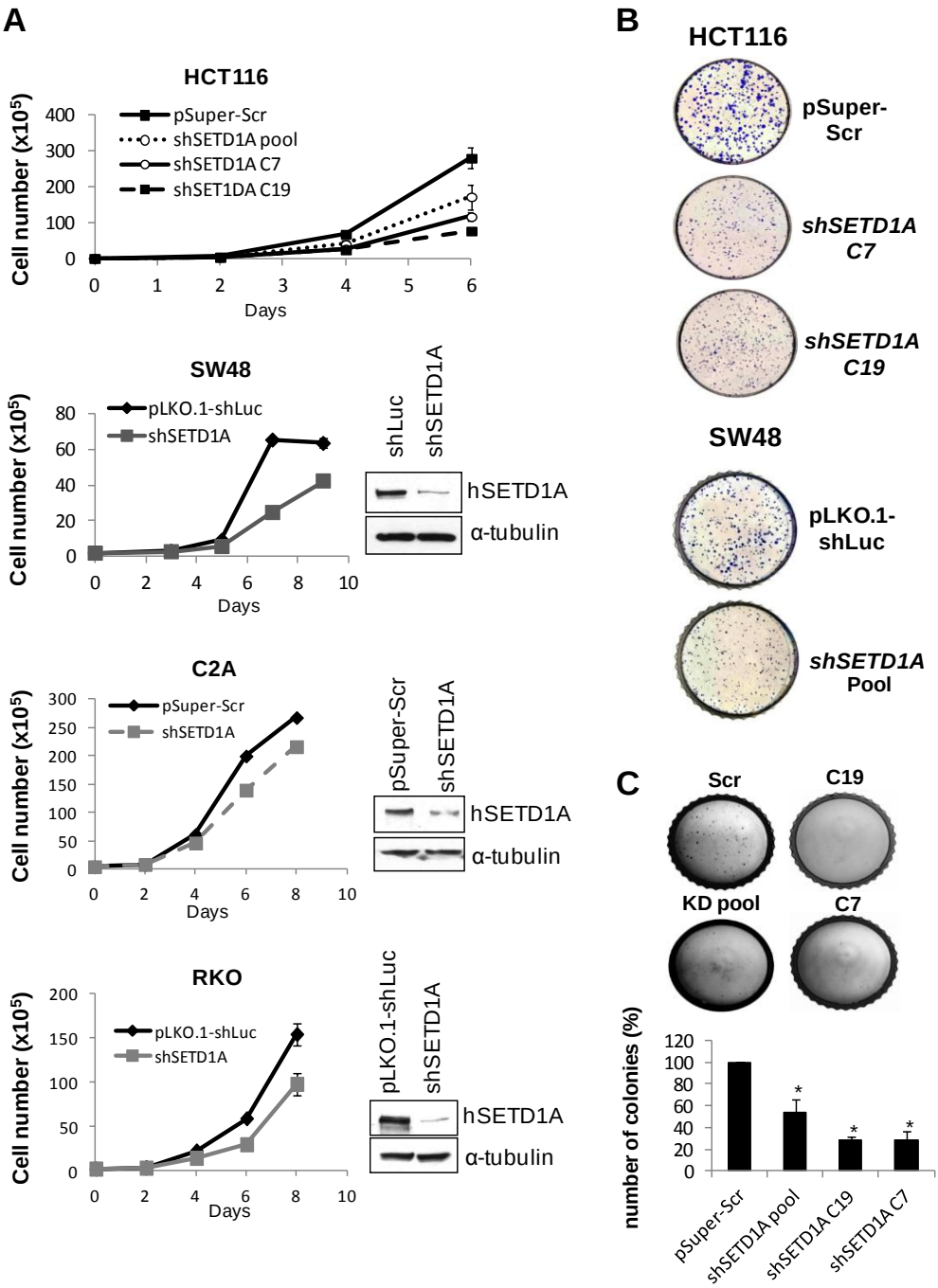


Figure 3-3. hSETD1A impacts cellular growth. (A) Depletion of hSETD1A in various CRC cell lines led to a decrease in cellular proliferation as measures by cell viability count. (B) Depletion hSETD1A in HCT116 and SW48 cells led to a decrease in colonies size under adherent conditions. (C) Depletion of hSETD1A in HCT116 led to a decrease in colonies size and number in soft agar, under anchorage-independent growth conditions. Shown are the mean ±SD of three independent experiments. *P < 0.05 by student t test.

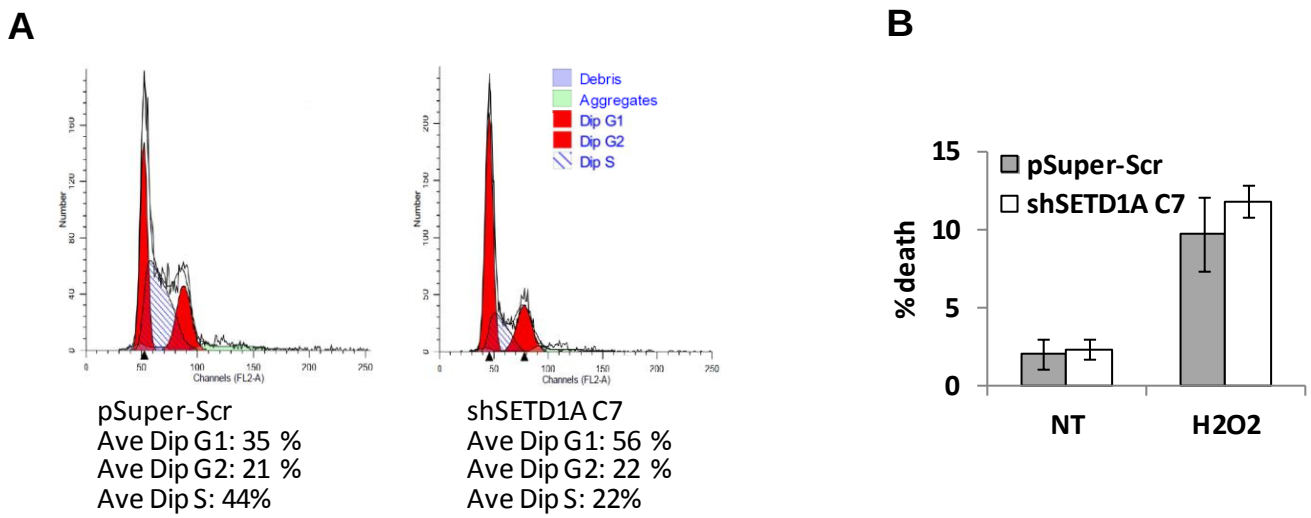


Figure 3-4. Depletion of hSETD1A is not associated with cell death. (A) Cell cycle analysis of shSETD1A and scramble control HCT116 cells was carried out using propidium iodide staining. Depletion of hSETD1A did not affect Sub G1 phase. (B) Depletion of hSETD1 did not affect cell death in the presence or absence of Hydrogen Peroxide (H2O2).

CHAPTER 4
HUMAN SETD1A REGULATES THE WNT-SIGNALING PATHWAY

Introductory Remarks

Constitutive activation of the Wnt-signaling pathway is a common feature of colorectal tumors [58]. The downstream effect of constitutively activated Wnt pathway is up-regulation of β-catenin protein levels and its target genes, such as c-Myc [58, 81]. The Wnt pathway is highly active in undifferentiated cells that reside at the bottom of intestinal crypts and regulates their self-renewal [103]. hSETD1A is not only over-expressed at the crypt bottom (Figure 3-1E), but also interacts with β-catenin, the master regulator of the Wnt-signaling pathway [38]. Therefore, we were prompted to investigate whether hSETD1A relates to the Wnt-signaling pathway.

Results

hSETD1A Regulates Transcription of Wnt Target Genes

To gain mechanistic insights into the role of hSETD1A in CRC, we carried out genome-wide microarray analysis in the scramble control and hSETD1A KD HCT116 cells. A total of 6,286 genes were differentially expressed (cutoff P-value of 0.005) in the hSETD1A KD cells compared with the scramble control cells (Figure 4-1A). Among them, 2,863 genes were up-regulated, whereas 3,423 genes were down-regulated upon loss of hSETD1A (Figure 4-1A). Interestingly, almost 1% of the total differentially expressed genes were Wnt-signaling target genes (56 of 6,286; 4-1A), representing approximately 50% (56 of 113) of the total known Wnt target genes. Further analysis revealed that the probability of these Wnt targets being affected by the hSETD1A KD was significantly higher than that of the random distribution of genome-wide hSETD1A-affected genes (FDR adjusted P-value = 4.67E-8; Figure 4-1B). Key Wnt-signaling

target genes involved in proliferation, invasion, migration, and angiogenesis were down-regulated in the hSETD1A-depleted cells (31/56), whereas target genes repressed by this pathway, such as E-cadherin (CDH1) and SOX9, were up-regulated (Figure 4-1C and Table 4-1). Up-regulation of other Wnt target genes could be an indirect effect of hSETD1A KD. The effect of hSETD1A silencing on the expression of selected Wnt target genes and two unaffected genes, CLDN7 and CCNA2, were further validated by qRT-PCR in HCT116 and SW48 cells (Figure 4-1C and Figure 4-1D). We also confirmed that the KD of hSETD1A in HCT116 cells reduced the protein levels of three important Wnt target genes; c-Myc (*MYC*), Snail (*SNAI1*), and TCF4 (*TCF7L2*) (Figure 4-2A). Thus, our data indicate that hSETD1A regulates a subset of Wnt-signaling target genes in CRC cells

hSETD1A Associates with β-catenin and Activates Wnt Target Genes

To elucidate the mechanism whereby hSETD1A controls transcription of Wnt target genes, we examined the effect of hSETD1A KD on the expression levels of β-catenin in HCT116 and SW48 cells. The KD of hSETD1A did not have an effect on the mRNA expression level (CTNNB1; Table 4-1) or protein level (Figs. 4-2A) of β-catenin. These results indicate that hSETD1A neither regulates β-catenin transcription nor β-catenin protein stability. Interestingly, recent studies demonstrated that hSETD1A can interact with β-catenin in the human embryonic kidney cells line HEK293 [38].

To test whether hSETD1A collaborates with β-catenin in regulating Wnt target genes in CRC, co-immunoprecipitation assays were performed in HCT116 or SW48 nuclear extracts using antibodies against β-catenin or components of the hSETD1A complex. As expected, endogenous β-catenin interacted with hSETD1A and its components in both HCT116 and SW48 cells (Figure 4-2B and Figure 4-2C). In

addition, β-catenin was highly expressed in colorectal tumors and in normal crypt bottoms (Figure 4-2D), consistent with the role of the Wnt pathway in maintenance and regulation of intestinal crypt cells [103] and similar to the hSETD1A expression pattern observed (Figure 3-1E).

Thus, we investigated whether β-catenin and hSETD1A collaborate to regulate the expression of Wnt target genes. We first asked if hSETD1A and β-catenin complexes are both recruited to the same regions at promoters of the Wnt/β-catenin target genes. To that end, we performed ChIP assays using antibodies against the hSETD1A complex including hSETD1A, RbBP5, Ash2L, WDR5, and against the β-catenin complex including β-catenin and TCF4. Both hSETD1A and β-catenin complexes were enriched at known β-catenin-responsive elements at the MYC and VEGFA loci, compared with IgG and 3'UTR negative control region, in HCT116 cells (Figure 4-2E and Figure 4-2F). Moreover, the KD of hSETD1A in HCT116 and SW48 cells invoked a decrease in H3K4me3 enrichment at the TSSs of several Wnt/β-catenin target genes that are involved in cell proliferation, invasion, migration, and angiogenesis, such as *MYC*, *SNAI1*, *VEGFA*, *MMP7*, and *TWIST2*. In contrast, there was no significant change in H3K4me3 levels at the TSS of *CDH1* (Figure 4-3A and Figure 4-3B). Furthermore, β-catenin binding to its target genes was unaffected by hSETD1A KD in HCT116 cells (Figure 4-3C). These results suggest that recruitment of the hSETD1A complex and subsequent trimethylation of H3K4 at proximal promoters of Wnt/β-catenin target genes are required for β-catenin action.

Recruitment of hSETD1A by β-catenin is Critical for β-catenin-mediated Transcriptional Activation

We next sought to further examine the possibility that hSETD1A is recruited by β-catenin to Wnt target gene promoters. To test this, β-catenin was silenced in HCT116 cells using two independent lentiviral shRNAs (Figure 4-4A). As expected, the depletion of β-catenin invoked a decrease in expression of its target genes (Figure 4-4C) and impeded cellular proliferation (Figure 4-4B). Interestingly, H3K4me3 levels were also significantly decreased at the TSSs of Wnt/β-catenin target genes upon loss of β-catenin in HCT116 cells (Figure 4-4D). Moreover, induction of the Wnt-signaling pathway and β-catenin in cells (HEK293) that normally express low levels of β-catenin, resulted in enrichment of H3K4me3 at promoters of several Wnt target genes and to an increase in their expression levels (Figure 4-5).

This effect of β-catenin on H3K4me3 was not due to changes in hSETD1A levels, as β-catenin KD did not affect hSETD1A protein levels (Figure 4-4A). Since hSETD1A interacts with β-catenin (Figure 4-2B and Figure 4-2C), we further investigated whether β-catenin directs hSETD1A to these genomic loci by examining the effect of β-catenin KD on hSETD1A occupancy at promoters of selected Wnt/β-catenin target genes. The recruitment of hSETD1A to the proximal promoters of SNAI1, MYC, and VEGFA was significantly reduced upon loss of β-catenin, consistent with the observed decrease in H3K4me3 enrichment at those loci (Figure 4-4E). While the recruitment of hSETD1A to the Wnt/β-catenin-responsive promoters is dependent on β-catenin (Figure 4-4E), the occupancy of β-catenin at these promoters is independent of hSETD1A (Figure 4-3C). These results indicated that β-catenin recruits hSETD1A to promoters of the Wnt/β-

catenin target genes and relies, at least partially, on hSETD1A-mediated H3K4me3 for transcriptional activation.

A link between promoter associated H3K4me3 and transcriptional activation was proposed previously to rely on the binding of PHD domain containing TAF3 to H3K4me3 mark at active promoters [28-30]. Therefore, we examined whether binding of TAF3 to selected Wnt/β-catenin-targeted promoters was impaired by hSETD1A depletion. TAF3 occupancy at the TSSs of MYC, SNAI1, and VEGFA was decreased in the hSETD1A-depleted HCT116 cells compared with the scramble control (Figure 4-6A). Furthermore, we identified Wnt target genes that are coregulated by TAF3 and hSETD1A in HCT116 cells by comparing our microarray data set for hSETD1A KD in HCT116 cells with the TAF3 KD microarray data set in HCT116 cells obtained from NCBI GEO2R [28-30]. First, we found total of 991 genes that are coregulated by TAF3 and hSETD1AD1A [5605 (991) 2735] (Figure 4-6B). Secondly, hSETD1A and TAF3 shared 14 Wnt target genes including MYC, TCF7L2, SNAI2, and MMP7 (Figure 4-6C). These data support a novel mechanism for activation of the Wnt-signaling pathway, in which β-catenin activates transcription of Wnt target genes by recruiting the hSETD1A complex to deposit H3K4me3 marks and assembly of the preinitiation complex (PIC).

Levels of hSETD1A are Positively Correlated with Activation of Wnt Target Genes and Tumor Growth in Colorectal Cancer.

To examine whether levels of hSETD1A correlate with H3K4me3 levels at the TSSs of Wnt/β-catenin target genes in human CRC, we carried out H3K4me3 ChIP analyses in colorectal tumor tissues and adjacent normal mucosa from patient #1, which presented high levels of hSETD1A, and from patient #10, which did not show any change in hSETD1A levels compared to the normal mucosa. In opposed to the tumor

from patient #10, tumor from patient #1 presented high levels of hSETD1A and increased occupancy of H3K4me3 at TSSs of Wnt/β-catenin target genes (Figure 4-7A). Moreover, Wnt/β-catenin target genes were highly expressed in tumors presenting high levels of hSETD1A (patient #1–3), but not in other tumors (patients #7 and #10; Figure 4-7B). These results support our hypothesis that hSETD1A controls promoter H3K4me3 levels leading to the activation of Wnt/β-catenin target genes and subsequently Wnt pathway–mediated cellular proliferation.

Finally, to assess the role of hSETD1A and its mediated H3K4me3 in colorectal tumor growth, we employed a mouse xenograft model. The scramble control and hSETD1A KD HCT116 cells were injected subcutaneously into the flank of female athymic nude mice, and primary tumor volume was evaluated at 2 weeks postinjection. Consistent with the *in vitro* data, the KD of hSETD1A partially impaired primary tumor growth (Figure 4-7C). Variations in tumors size may have been attributed to fluctuations in hSETD1A protein levels in the absence of antibiotic selection *in vivo* (Figure 4-7D and Figure 4-7E). These results suggest that hSETD1A controls colorectal tumor growth in a dose-dependent manner.

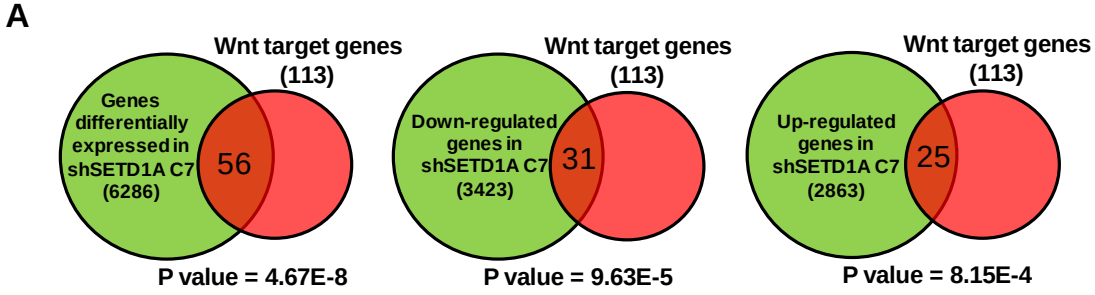
Closing Remarks

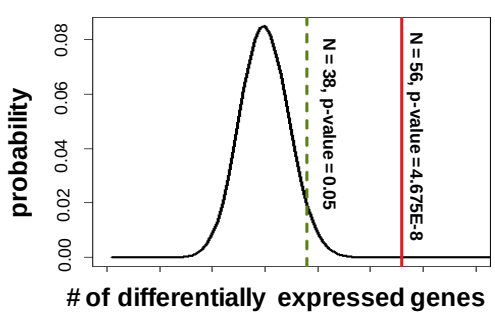
The Wnt-signaling pathway is controlled by levels of its master regulator - β-catenin [58]. This protein associates with promoters of Wnt target genes through its interactions with LEF/TCF transcription factors in the nucleus, but the question of how β-catenin activates Wnt target genes is not entirely clear [58]. Nevertheless, recent studies showed that β-catenin mediates histone modifications at targeted loci by recruiting various histone modifiers, such as p300, Brg1, MLL1, and MLL2 [38, 49, 87,

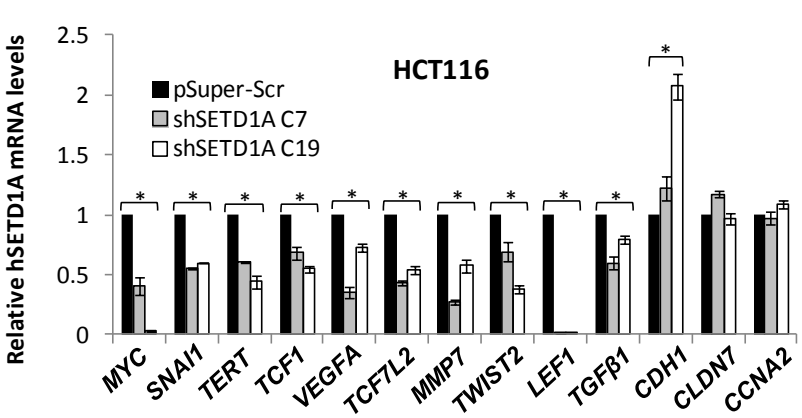
90, 91]. These perspectives suggest that activation of the Wnt pathway requires two steps: up-regulation in β-catenin and subsequent elevation in active histone marks. Ultimately, the increase in active marks at gene promoters serves as a nest for the basal transcription machinery and permits transcription.

Here we brought to light yet another epigenetic regulator – hSETD1A that interacts with β-catenin and controls H3K4me3 at TSSs (Figure 4-8). We showed that absence of either hSETD1A or β-catenin interferes with H3K4me3 at TSS of Wnt target genes and that transcription is down-regulated. Like other studies, we demonstrated that drained levels of H3K4me3 leads to a decrease in TAF3 recruitment to TSSs, which explains diminished transcription. But, our discovery is novel in that it shows that hSETD1A can mediate this decrease in H3K4me3 and TAF3. Finally, we demonstrated association between hSETD1A levels and expression of Wnt target genes in colorectal tumors from two patients. The relations between hSETD1A and the Wnt pathway could account for the decrease in cellular growth observed in the absence of hSETD1A.

Figure 4-1. hSETD1A regulates a subset of Wnt-signaling target genes. (A) Genome-wide microarray analysis of HCT116 cells depleted of hSETD1A revealed a decrease in the expression levels of Wnt target genes compared to the scramble control cells. Venn diagram shows the overlap between total differentially expressed genes in the hSETD1A KD and Wnt-signaling target genes. The P-value (FDR adjusted) < 0.005. (B) Statistical analysis was performed using a hypergeometric distribution test comparing the distribution of Wnt target genes among the total hSETD1A target genes to the random distribution of hSETD1A target genes. The probability of Wnt target genes that were affected by the hSETD1A KD (N=56; red line) is significantly higher than that of the randomly distributed hSETD1A target genes (black curve). The green dashed line indicates the P-value=0.05 cutoff expected if the number of representative Wnt target genes would have been N=38. (C and D) validation of microarray analysis by qRT-PCR in HCT116 cells (C) and SW48 cells (D). Wnt target genes expression is normalized to GAPDH and relative to the scramble control. Shown are the mean ±SD of two or three independent repeats. *P < 0.05 by student t test.


A
Wnt target genes (113)
Genes differentially expressed in shSETD1A C7 (6286)
56
P value = 4.67E-8
Wnt target genes (113)
Down-regulated genes in shSETD1A C7 (3423)
31
P value = 9.63E-5
Wnt target genes (113)
Up-regulated genes in shSETD1A C7 (2863)
25
P value = 8.15E-4


B
probability
0.00
0.02
0.04
0.06
0.08
N = 38, p-value = 0.05
N = 56, p-value = 4.675E-8
# of differentially expressed genes


C
Relative hSETD1A mRNA levels
HCT116
pSuper-Scr
shSETD1A C7
shSETD1A C19
MYC
SNAI1
TERT
TCF1
VEGFA
TCF7L2
MMP7
TWIST2
LEF1
TGFβ1
CDH1
CLDN7
CCNA2

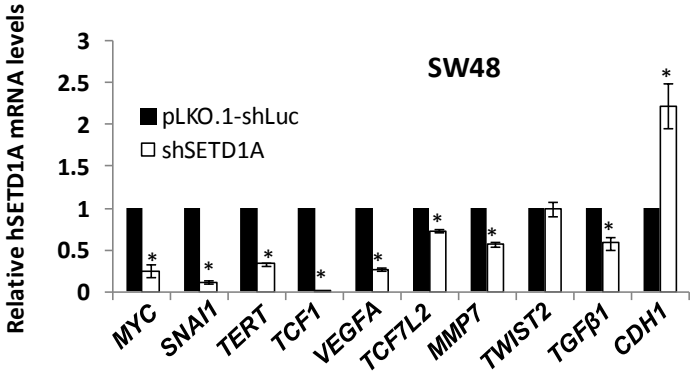

D
Relative hSETD1A mRNA levels
SW48
pLKO.1-shLuc
shSETD1A
MYC
SNAI1
TERT
TCF1
VEGFA
TCF7L2
MMP7
TWIST2
TGFβ1
CDH1

Table 4-1. Expression Profile of Wnt target genes in hSETD1A KD HCT116 cells

Down-regulated (p-value<0.005)		Up-regulated (p-value< 0.005)		Unaffected (p -value>0.005)			
Gene name	P value (FDR)	Gene name	P value (FDR)	Gene name	P value (FDR)	Gene name	P value (FDR)
GDNF	4.77E-03	NRP1	7.99E-06	GDF5	1.26E-01	MYCBP	5.18E-03
TCF7L1	3.54E-03	FGF18	1.16E-05	CD44	1.37E-01	EGR1	6.40E-03
SIX1	2.81E-03	RUNX2	1.80E-05	T	2.06E-01	WISP2	6.75E-03
FGF7	2.69E-03	AHR	2.71E-05	DAB2	2.27E-01	ETS2	7.02E-03
SOX2	2.10E-03	DKK1	3.52E-05	SOX17	2.57E-01	TLE1	7.76E-03
TWIST1	2.03E-03	PTGS2	6.07E-05	CUBN	3.65E-01	BIRC5	9.00E-03
MET	2.00E-03	ANTXR1	8.47E-05	NANOG	4.18E-01	HIF1A	9.65E-03
ABCB1	1.74E-03	EPHB3	1.05E-04	FGF4	5.06E-01	SMO	7.02E-03
NRCAM	1.67E-03	IGF2	1.25E-04	MYCN	5.27E-01	LRP1	6.36E-03
VEGF	1.67E-03	KLF5	1.43E-04	WNT3A	5.69E-01	EFNB1	5.71E-03
MMP2	1.38E-03	CYR61	1.53E-04	MAP3K3	6.61E-01	BTRC	5.03E-03
NTRK2	1.35E-03	PPAP2B	1.56E-04	CTNNB1	6.36E-01	CACNA2D3	8.00E-02
JUN	1.28E-03	GJA1	2.40E-04	PTCH	4.30E-01	GREM1	7.97E-02
LBH	1.10E-03	FGF9	2.48E-04	PLAUR	2.64E-01	ID2	6.11E-02
POU5F1	1.07E-03	CTGF	2.87E-04	CCND3	2.12E-01	IGF1	5.75E-02
MYC	1.05E-03	CCND1	4.38E-04	BMP4	1.93E-01	CDON	3.78E-02
WISP1	9.56E-04	CLDN1	6.54E-04	LGR5	1.64E-01	PITX2	1.50E-02
TERT	8.13E-04	CDH1	7.48E-04	FN1	1.45E-01	EDN1	1.44E-02
SALL4	8.03E-04	WNT9A	1.07E-03	DPP10	1.44E-01	ATOH1	1.38E-02
SNAI2	5.41E-04	AXIN2	1.20E-03	IL6	1.40E-01	TNFSF11	1.35E-02
FOSL1	4.70E-04	PDGFRA	1.26E-03	SFRP2	1.13E-01	CEBPD	1.31E-02
SNAI1	4.28E-04	IRS1	1.37E-03	DLK1	3.91E-02	FZD7	1.23E-02
WNT5A	2.97E-04	FZD2	1.71E-03	MMP9	4.51E-02	GAST	1.21E-02
MMP7	2.43E-04	SOX9	3.35E-03	TWIST2	5.08E-02	TGFB3	1.09E-02
TIAM1	2.38E-04	FST	4.28E-03	EPHB2	6.37E-02	NF1	1.24E-02
TCF7L2	1.66E-04			FGF20	6.75E-02	TGFB1	1.57E-02
CCND2	1.62E-04			PPARD	7.12E-02	TNFRSF11B	1.71E-02
CDKN2A	8.46E-05			EGFR	3.87E-02	BGLAP	1.76E-02
LEF1	3.67E-05					TCF7	2.20E-02
ANGPTL4	2.05E-05						
JAG1	1.94E-05						
hSETD1A	7.99E-06						

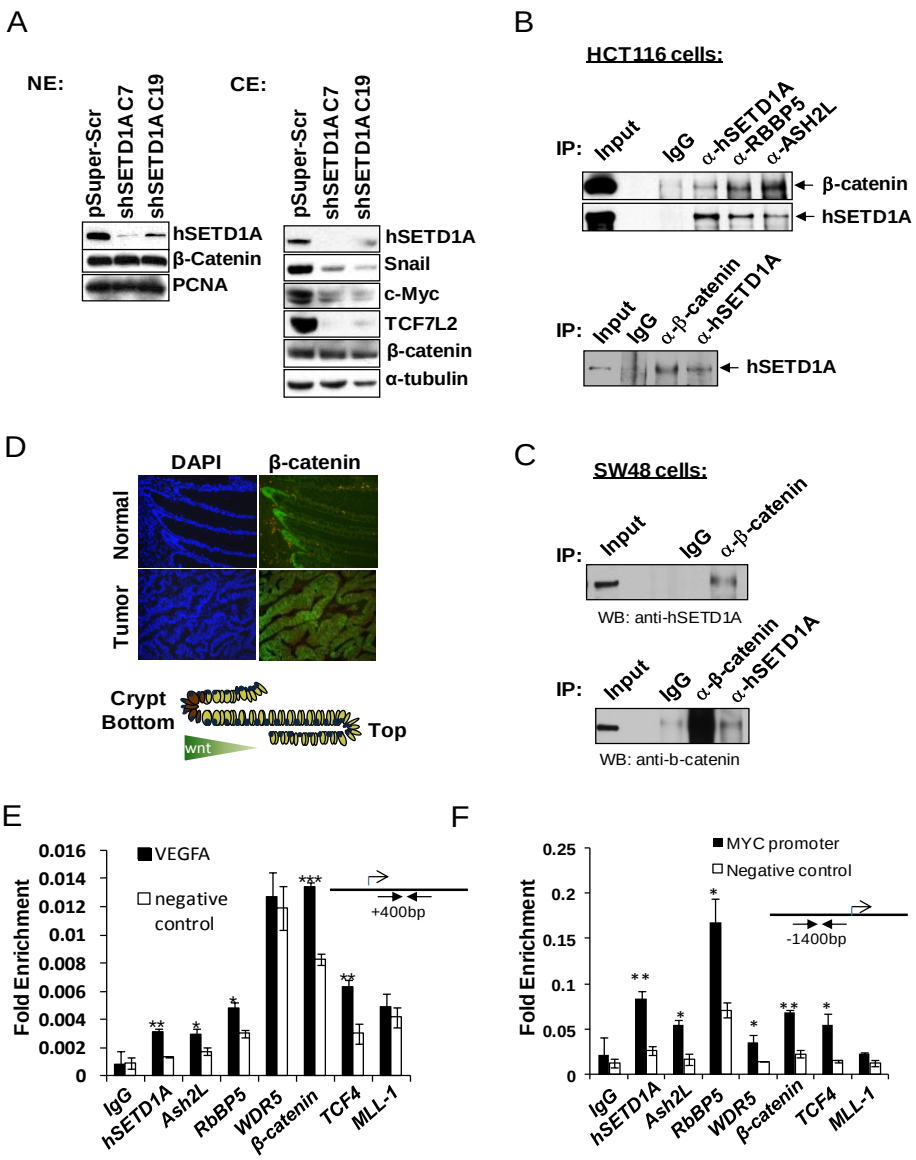


Figure 4-2. hSETD1A colocalizes with β-catenin.(A) Protein levels of Wnt target genes, but not β-catenin, are decreased in hSETD1A KD HCT116 cells. (B and C) co-IP assays revealed interactions between hSETD1A and β-catenin in HCT116 cells (B) and in SW48 cells (C). (D) IF staining of colorectal tumor and paired normal mucosa from patient #2 indicate high expression of β-catenin in the intestinal crypt bottoms. (E and F) hSETD1A complex colocalizes with the β-catenin complex at β-catenin-responsive elements in the VEGFA loci (F) and MYC loci (E), in HCT116 cells. ChIP analysis was performed using antibodies against hSETD1A complex (hSETD1A, Ash2L, WDR5, and RbBP5) and β-catenin complex (β-catenin and TCF4) to determine their binding to responsive elements compared with a negative control region located 70 Kb downstream of the TAL1 promoter and relative to IgG, in HCT116.

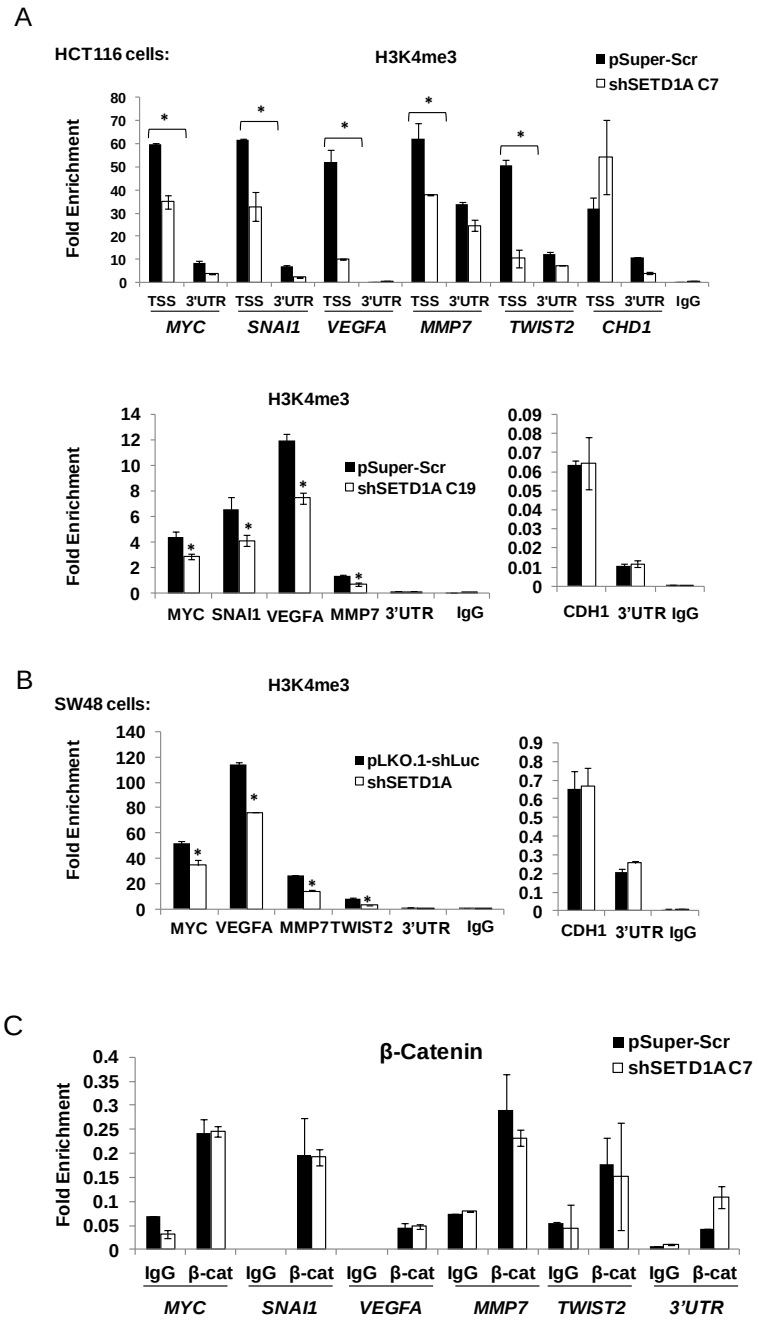


Figure 4-3. hSETD1A mediates promoter-H3K4me3 at Wnt target loci. (A and B) H3K4me3 ChIP in HCT116 cells depleted of hSETD1A, C7 and C19 clones. (C) H3K4me3 ChIP in SW48 cells depleted of hSETD1A, cell pool. (D) β-catenin ChIP in HCT116 cells depleted of hSETD1A, clone C7. Promoter-H3K4me3 (A and B), but not β-catenin (C), diminished in the absence of hSETD1A, compared with a 3'UTR negative control region and relative to IgG. Shown are the mean ±SD of two or three independent experiments. *P < 0.05, by student t-test.

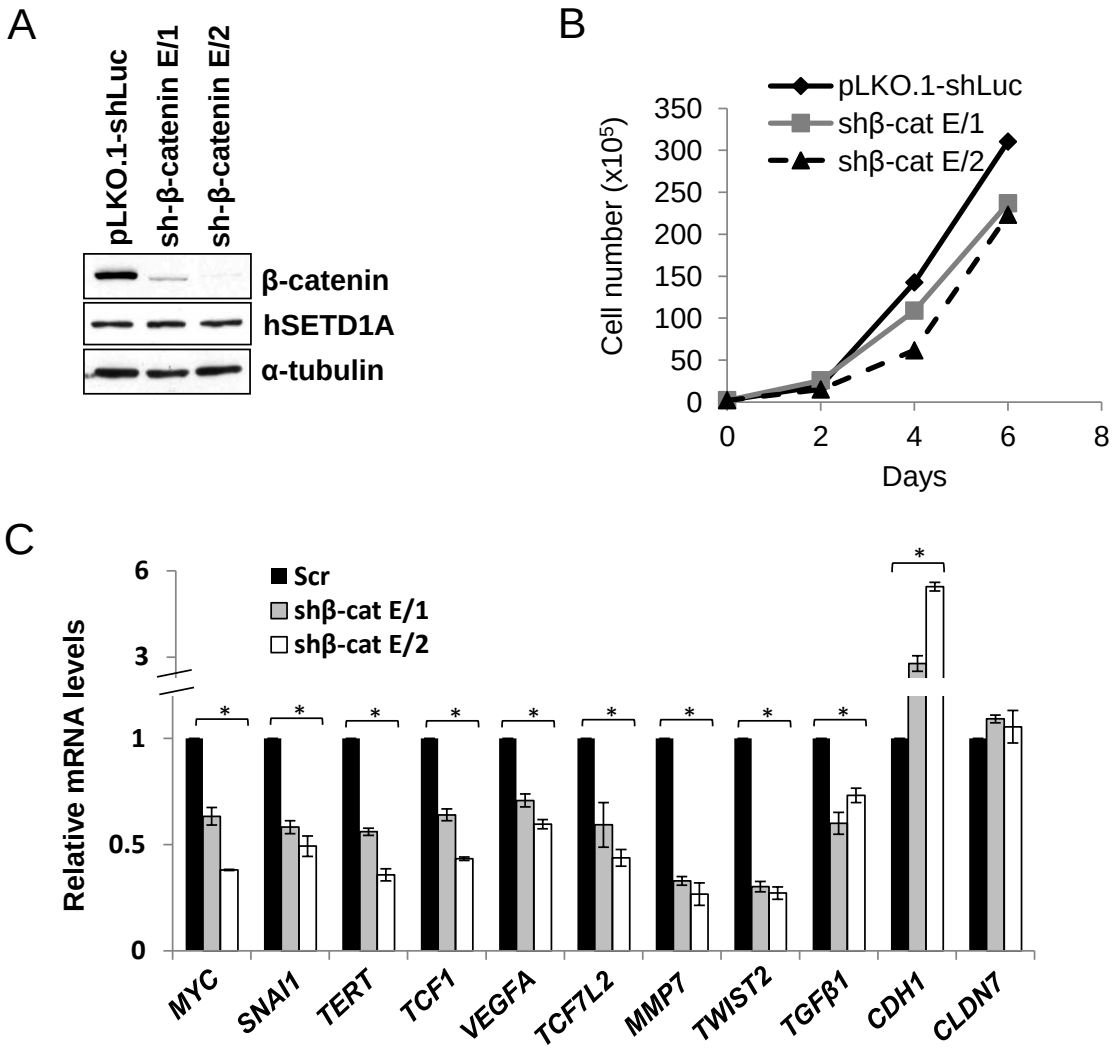


Figure 4-4. β-catenin recruits hSETD1A to regulate Wnt target gene expression. β-catenin was knocked down in HCT116 cells using two independent lentiviral shRNAs, E/1 and E/2. (A) Depletion of β-catenin did not affect hSETD1A protein levels compared to the shLuciferase (shLuc) control, as detected by WB analysis. Depletion of β-catenin led to a decrease in cellular proliferation (B) and to a decrease in expression of Wnt target gene, as detected by qRT-PCR (C). Wnt target genes expression is normalized to GAPDH and relative to the shLuciferase control. KD of β-catenin resulted in reduced occupancy of H3K4me3 (D) and hSETD1A (E) at TSSs of the Wnt target genes, compared to 3'UTR and TAL1+70 negative control regions, as detected by ChIP analyses. Shown are the mean ±SD of three replicates. *P < 0.05, by student t-test

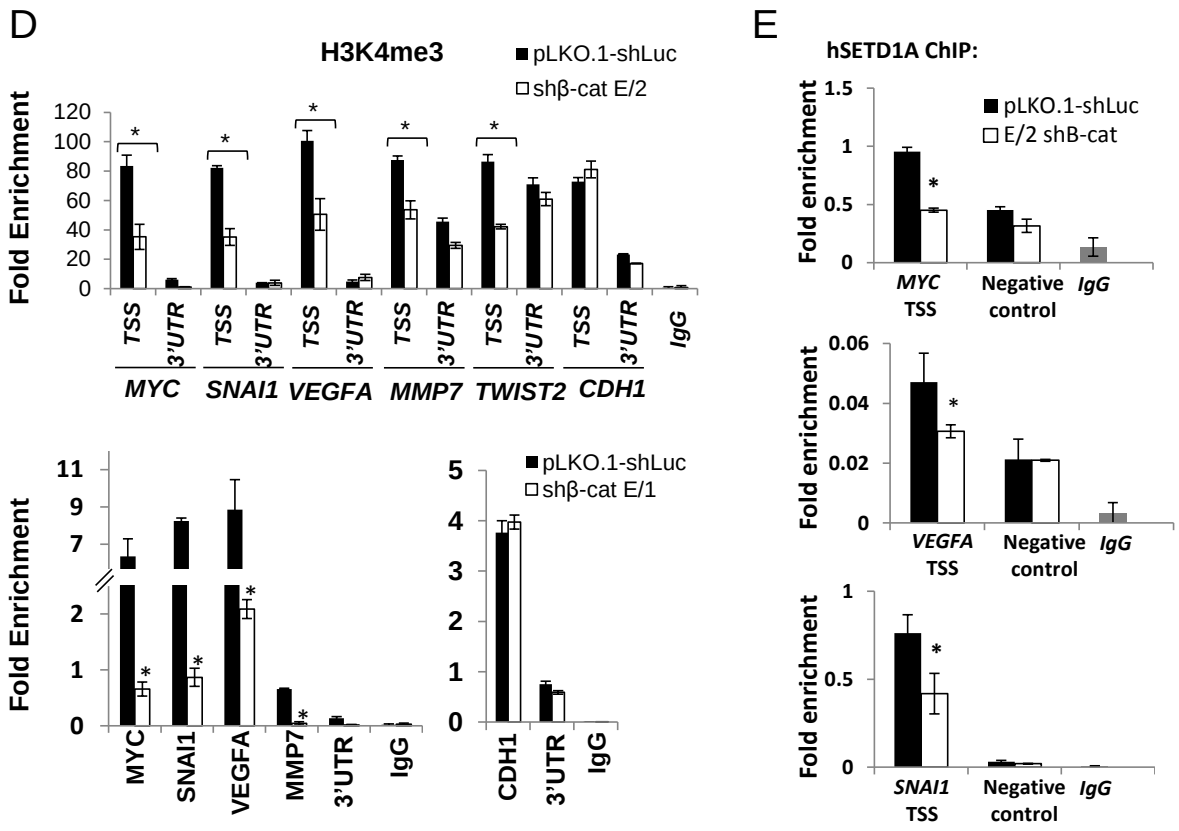


Figure 4-4. Continued.

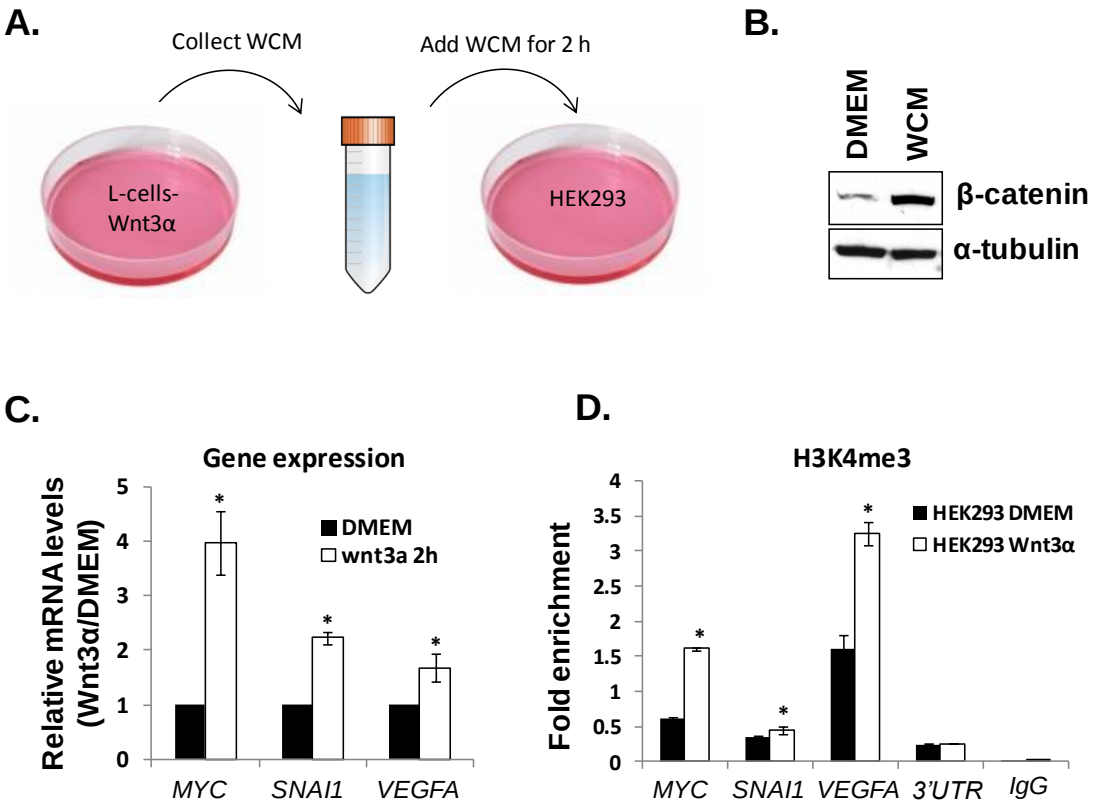
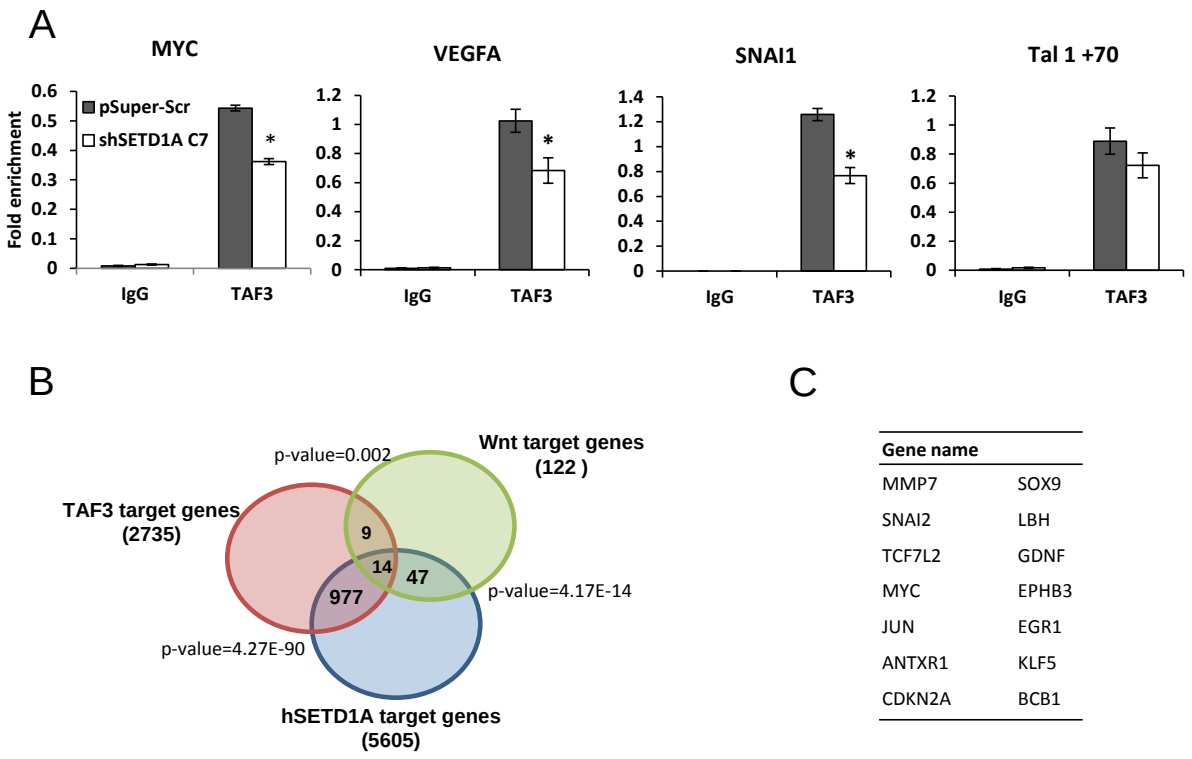


Figure 4-5. Induced β-catenin levels lead to an increase in H3K4me3. (A) Experimental design. MEF cells over expressing the human Wnt3α gene (L-cells) were cultured and Wnt3α-conditioned medium (WCM) was then collected. HEK293 cells, which express low levels of β-catenin were cultured in WCM and harvested upon 2 hours. (B) WB analysis of β-catenin levels in HEK293 cells upon 2 hours of WCM treatments. qRT-PCR analysis of Wnt target genes (C) and promoter-H3K4me3 ChIP (D) in HEK293 cells upon 2 hours of WCM treatments. H3K4me3 was enriched at promoters of Wnt target genes, and Wnt targets expression levels were elevated upon induction of β-catenin. Shown are the mean ±SD of three replicates. *P < 0.05, by student t-test



Gene name	
MMP7	SOX9
SNAI2	LBH
TCF7L2	GDNF
MYC	EPHB3
JUN	EGR1
ANTXR1	KLF5
CDKN2A	BCB1

Figure 4-6. TAF3 and hSETD1A co-regulate Wnt target genes. (A) TAF3 ChIP in hSETD1A KD HCT116 cells. TAF3 occupancy decreases at TSSs of several Wnt target genes in the absence of hSETD1A and H3K4me3. Shown are the mean ±SD of three replicates. *P < 0.05, by student t-test. (B) Venn diagram presenting the overlapping among target genes of hSETD1A, Wnt, and TAF3 in HCT116 cells. TAF3 was silenced in HCT116 cells. TAF3 microarray data was analyzed with the online software NCBI GEO2R [134] using the data set GSE43542 [29] to call the differentially expressed genes with the cutoff p-value (FDR adjusted) of 0.05. Cutoff p-value used for the shSETD1A microarray was 0.005 (FDR adjusted). Genes that appear in multiple arrays were identified with GeneWeaver. TAF3 target genes significantly overlapped with hSETD1A target genes (p-value=4.27E-90) and with Wnt target genes (p-value=0.002). (C) Shown are Wnt target genes that regulated by both hSETD1A and TAF3.

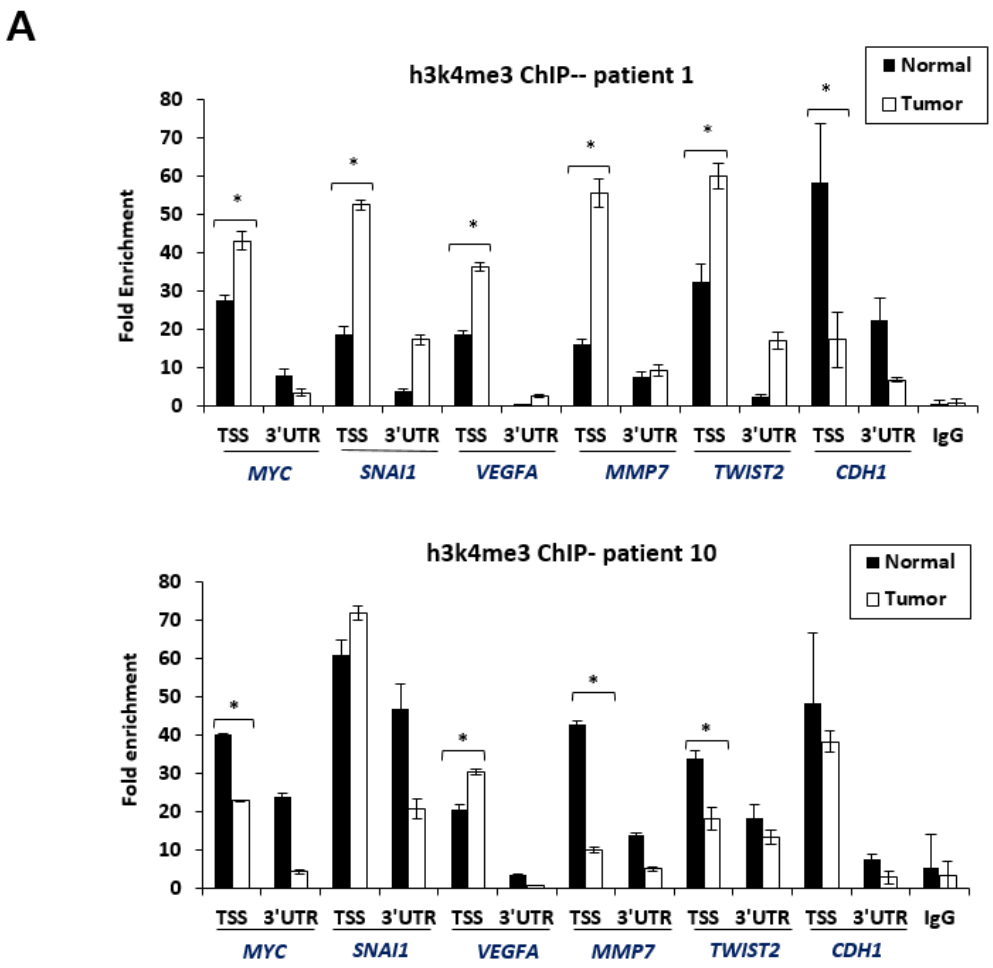
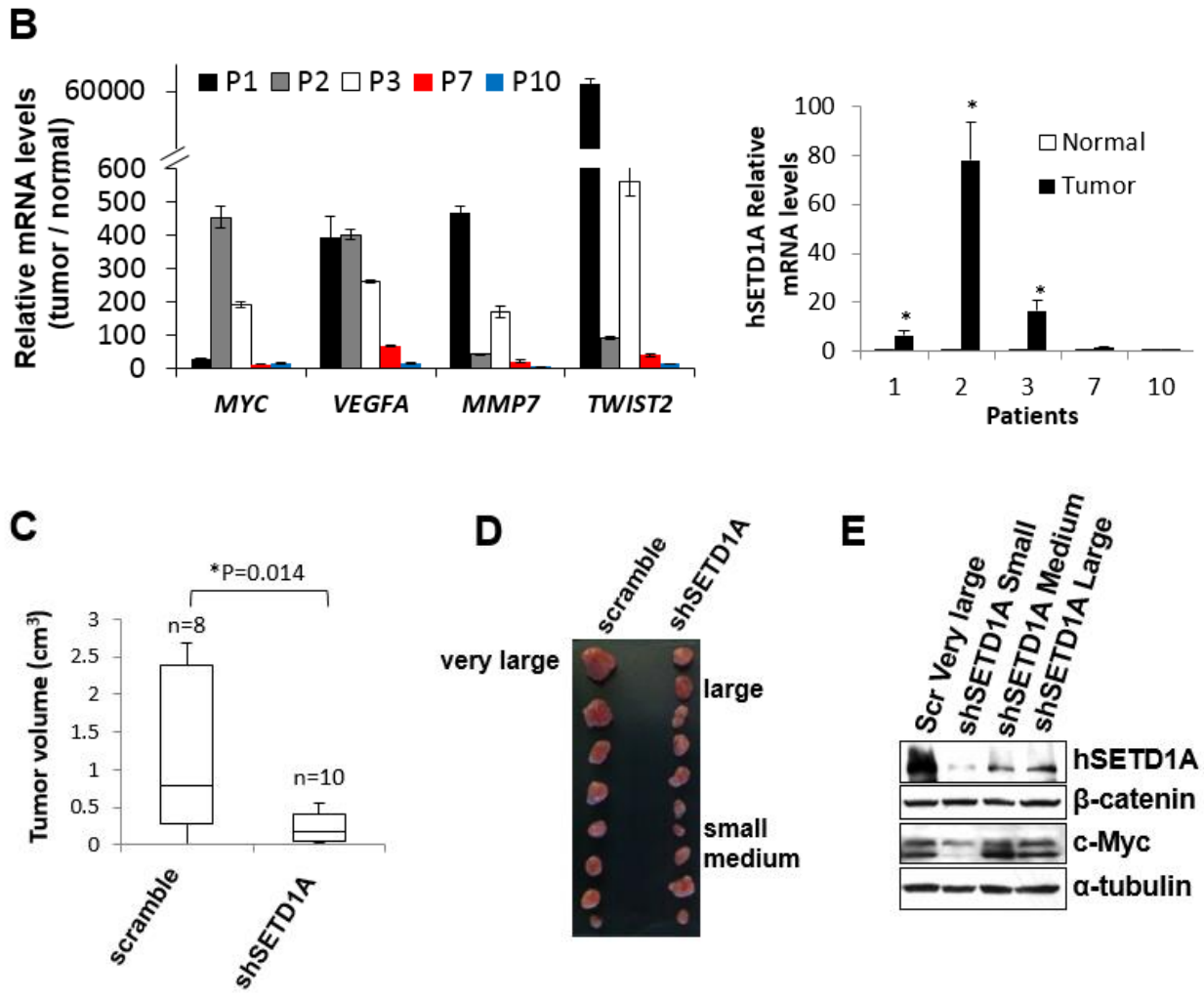


Figure 4-7. hSETD1A levels associate with H3K4me3 and Wnt target gene expression in human colorectal tumors. (A) H3K4me3 ChIP assays in human colorectal tumors and normal mucosa from two patients; patient # 1, which exhibit high levels of hSETD1A, and patient #10, which did not show change in hSETD1A levels compared with the paired normal mucosa. In oppose to colorectal tumor from patient #10, the colorectal tumor from patient # 1 revealed enrichment of H3K4me3 at TSSs of Wnt target genes compared with the matching normal mucosa. (B) Wnt target genes are highly active in human colorectal tumors in which hSETD1A is highly expressed (patients #1–3), but are less active in tumors in which hSETD1A levels are unchanged compared with the normal mucosa (patients #7 and #10). Levels (mRNA) of Wnt target were determined by qRT-PCR and normalized to β-actin. Shown are the mean ±SD of three replicates. *P < 0.05, by student t-test. (C) Mouse xenograft model. shSETD1A (C7) or Scramble control HCT116 cells ($1x10^6$) were injected s.c. in 50% Matrigel into the flank of 5-6 week old females athymic nude mice. Mice were sacrificed at 2 weeks and tumor volume was measured. (D and E) hSETD1A, c-MYC, and β-catenin protein levels were analyzed by WB in cellular extract from tumors of different sizes. hSETD1A levels positively corresponded to tumor xenograft sizes.


B
Relative mRNA levels (tumor / normal)
60000
600
500
400
300
200
100
0
P1 P2 P3 P7 P10
MYC
VEGFA
MMP7
TWIST2
hSETD1A Relative mRNA levels
100
80
60
40
20
0
Normal
Tumor
1
2
3
7
10
Patients
C
*P=0.014
Tumor volume (cm³)
3
2.5
2
1.5
1
0.5
0
n=8
n=10
scramble
shSETD1A
D
scramble
shSETD1A
very large
large
small
medium
E
Scr Very large
shSETD1A Small
shSETD1A Medium
shSETD1A Large
hSETD1A
β-catenin
c-Myc
α-tubulin

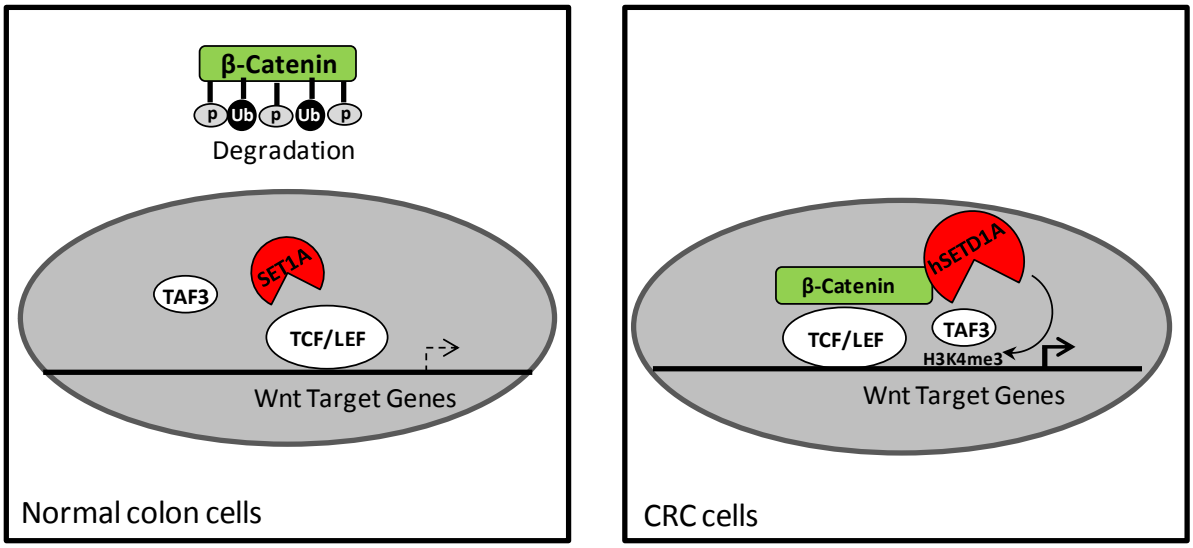


Figure 4 -8. A working model illustrating the cooperative role of hSETD1A and β-catenin in regulation of Wnt target genes in colorectal cancer. In normal colon cells, β-catenin levels remain low due to its phosphorylation and subsequent degradation. In colorectal cancer, both hSETD1A and β-catenin levels are up-regulated. Localization of β-catenin to the nucleus enables its interactions with hSETD1A HMT. These interactions facilitate promoter-H3K4me3, recruitment of TAF3 (and transcription machinery), activation of Wnt/β-catenin target genes, and cellular growth.

CHAPTER 5
HUMAN SETD1A REGULATES METASTASIS IN BREAST CANCER

Introductory Remarks

Breast cancer is the most common cancer in women, and the second leading cause of cancer related deaths among women in the USA [113, 114]. According to the National Cancer Institute, while there is 99-84% chance five-year relative survival for localized and regional disease, there is only 24% chance five-year relative survival for metastatic breast cancer [113]. As metastasis lies in the heart of therapeutic failure and mortality [117], understanding and preventing metastasis is critical for intervention and survival.

Metastatic cancer cells are cells that in addition to have acquired uncontrollably proliferative characteristics have also acquired excessive motility, and are highly adaptive and resistant to new environments [83, 85, 116, 117]. Alterations in epigenetic modifications are important for conveying genomic adaptation, cells plasticity, and sustention of a phenotype [10-12]. Understanding of the epigenetic changes that occur during progression of breast cancer is important not only in making decisions regarding diagnosis and prognosis but also in treatments strategies.

Epigenetic regulators are often mutated or over-expressed in cancer [11], and vast epigenetic changes, such as increase in global H3K4me3, have been observed during induced EMT switch [45, 127]. Gain in hSETD1A gene copy number was detected in 45% (352/782) of breast cancer, the highest frequency of hSETD1A gain in gene copy among all cancers cases (http://www.sanger.ac.uk/cosmic) [55]. Therefore, we investigated whether hSETD1A could affect the metastatic phenotype in breast cancer.

Results

hSETD1A and hSETD1B are Up-regulated in Breast Cancer

hSETD1A is up-regulated in colorectal carcinoma and controls cellular proliferation [135]. However, the role of hSETD1A in metastasis has not been studied. H3K4me3 has been shown to increase during EMT [45], suggesting dysregulation of HMTs or/and Histone demethylases. In fact, it was recently demonstrated that H3K4 demethylase LSD1 regulates EMT and metastasis in breast cancer [45, 128, 129]. Ablation of LSD1 in MDA-MB-231 cells invoked an increase in cell invasion and migration, *in vitro,* and lung metastasis *in vivo*. Yet, the involvements of H3K4 HMTs in breast cancer metastasis in unknown.

First, gain in hSETD1A gene copy number was detected in 45% (352/782) of breast cancer cases compared with only 6.8% loss in gene copy (53/782) (Table 5-1). This frequency of hSETD1A gain of gene copy is the highest among all cancers. On the other hand, loss in gene copy of hSETD1B (19.3%), MLL1 (49.4%), MLL2 (16.5%), MLL3 (22.0%), was more prevalent than gain in copy number, in breast cancer. MLL4 gene copy was lost in 11.4% and gained in 18.8% of breast cancer samples (http://www.sanger.ac.uk/cosmic) [55]. Together, this data suggest an increase in hSETD1A expression and a unique role for hSETD1A in breast cancer. To examine this hypothesis we detected protein levels of hSETD1A in breast cancer cell lines compared with the mammary epithelial cell lines MCF10A and HMEC. hSETD1A and H3K4me3 levels were elevated in breast cancer cell lines including MCF7, MDA-MB-231, BT-459, and SUM159 (Figure 5-1A). In addition, mRNA levels of both hSETD1A and hSETD1B were detected by qRT-PCR in primary and metastatic breast tumors compared with normal breast samples. hSETD1A and hSETD1B levels were elevated in metastatic

breast tumors compared with primary breast tumors and normal breast samples (Figure 5-1B). However, hSETD1A levels in primary breast tumors were not significantly elevated compared with normal breast tissues (Figure 5-1B).

Increased Levels of hSETD1A and hSETD1B Are Associated with Poor Survival in Lymph-node Positive Breast Cancer

Since hSETD1A and hSETD1B were up-regulated in metastatic breast tumors, we examined the relations between their expression levels and clinical outcomes in breast cancer. Kaplan-Meier survival curves of breast cancer patients who were lymph-node positive or lymph-node negative were plotted and grouped by low/high levels of hSETD1A or hSETD1B (http://www.kmplot.com [136] (Figure 5-2A and Figure 5-2B). Interestingly, increased levels of hSETD1A or hSETD1B were associated with poor survival in lymph-node positive but not in lymph-node negative breast cancer. On the other hand, levels of other COMPASS members, such as MLL1, MLL2, or MLL4, were not associated with survival of lymph-node positive or lymph-node negative breast cancer patients (Figure 5-2A and Figure 5-2B). This is consistent with the accumulated knowledge about MLLs, implicating them mainly in hematologic malignancies. It also coincides with the overall agreement that dysregulation of MLLs in leukemia is largely through gene rearrangements rather than abnormal transcriptional [50, 51].

The association of hSETD1A and hSETD1B with survival in breast cancer suggests that they could serve as good biomarkers for progression of breast cancer in patients who showed metastasis to the sentinel lymph-node (Figure 5-2A). The fact that hSETD1A and hSETD1B were not associated with survival in lymph-node negative breast cancer suggests that the significance of their expression levels is most likely relevant to metastasis (Figure 5-2B).

Perturbed hSETD1A Reduces Metastasis

The role of hSETD1A in invasion and migration of breast cancer cells was investigated *in vitro* and *in vivo*. hSETD1A was stably knocked down in MDA-MB-231 and BT-459 cells using a lentiviral shRNA (Figure 5-3A). These cells express low levels of E-cadherin (Figure 5-1A) and are therefore highly migratory and invasive. Interestingly, the hSETD1A KD MDA-MB-231 cells showed morphological changes suggesting Mesenchymal-Epithelial Transition (MET). For example, mesenchymal MDA-MD-231 cells became round shaped and smaller, characteristics of epithelial cells (Figure 5-3B). We next assessed the effect of hSETD1A KD on cell migration using wound healing assay (Figure 5-3C) and transwell migration assay (Figure 5-3D). hSETD1A KD cells shown a decrease in their migratory capacity compared to the control cells in both assays (Figure 5-3C and Figure 5-3D). In addition, hSETD1A KD MDA-MB-231cells showed decreased invasion by a transwell invasion assay (Figure 5-3E). These results indicated that hSETD1A is important not only for cell motility but also for invasion through matrigel which imitates the surrounding tissue. The KD of hSETD1A had only minor effect on cellular proliferation of MDA-MD-231and BT-549 cells (Figure 5-3F).

Finally, we also tested the ability of the hSETD1A KD cells to metastesize *in vivo.* MDA-MD-231-shSETD1A or MDA-MD-231-shLuciferase cells (1 x10^6) were injected into the tail vein of nude mice and metastasis in the lungs was assessed after 7-8 weeks. We found a significant decrease in the number of lung nodules detected in mice which were injected with the MDA-MD-231-shSETD1A cells compared with mice that were injected with the MDA-MD-231-shuciferase cells (Figure 5-4). Therefore, we speculated that the up-regulation in hSETD1A observed in advanced breast cancer

(Figure 5-1A) could serve as a regulatory mechanism by which hSETD1A stimulates metastasis (Figure 5-4) and associate with survival of lymph-node positive breast cancer patients (Fig 5-2A). In other words, the increased levels of hSETD1A in primary breast tumors could account for the ability of the tumors to expand their territories and metastasize, thereby affecting clinical outcomes. Nevertheless, the molecular events controlled by hSETD1A in breast cancer are still not clear.

Closing Remarks

Piling evidences for alterations in histone modification patterns in EMT and metastasis have led to investigations of histone modifier enzymes during progression of cancer [45, 85, 92, 126-129]. Altered H3K4me3 (Figure 5-1A) and *hSETD1A* gene duplications in breast cancer (Table 5-1) [55] have prompted us to inspect the biological functions of hSETD1A and its relative hSETD1B in breast cancer. In summary, we found that these enzymes are up-regulated in breast cancer cell lines and in metastatic human breast cancer samples compared with normal mammary cells (Figure 5-1). We further discovered association between increased levels of hSETD1A and hSETD1B and poor survival in lymph-node positive breast cancer (Figure 5-2). Moreover, depletion of hSETD1A in mesenchymal breast cancer cells invoked a decrease in metastasis (Figure 5-4). However, gene Loci affected by hSETD1A KD have not been identified in this study and therefore the molecular mechanism by which hSETD1A control metastasis is currently not clear. One interesting observation is that the hSETD1A KD in MDA-MD-231 cells did not affect global H3K4me3 as it did in HCT116 cells. The reason for such difference between cell lines is unknown but could be extremely valuable in understanding the importance of this enzyme in different cell contexts.

We previously demonstrated that hSETD1A regulates the Wnt-signaling pathway in CRC [135]. This is also a plausible mechanism in breast cancer, as the Wnt-signaling pathway surely regulates cell invasion and migration. Nevertheless, further investigation of how hSETD1A and hSETD1B regulate breast cancer metastasis is needed before we can effectively target them. Yet, it seems that those enzymes might serve as good markers for clinical outcomes in patients who were positive for breast metastasis in the sentinel lymph-node.

Table 5-1. *hSETD1A* gene copy analysis in various types of human cancers

Tissue	Gain in Copy Number (%)	Loss in Copy Number (%)
Breast	45	6.8
Lung	22.1	15.1
Large intestine	18.9	4.1
Kidney	18	1.3
NS	13.6	4.5
Ovary	10.6	30.3
Endometrium	10.2	6.1

(http://www.sanger.ac.uk/cosmic [55])

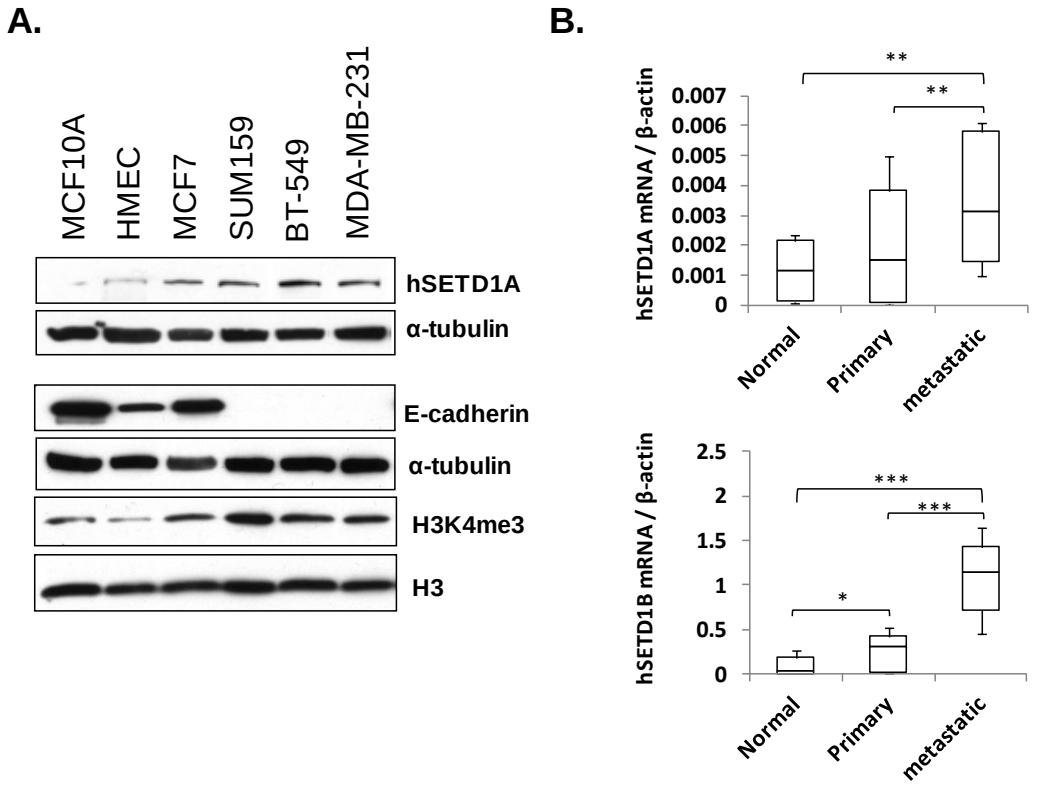
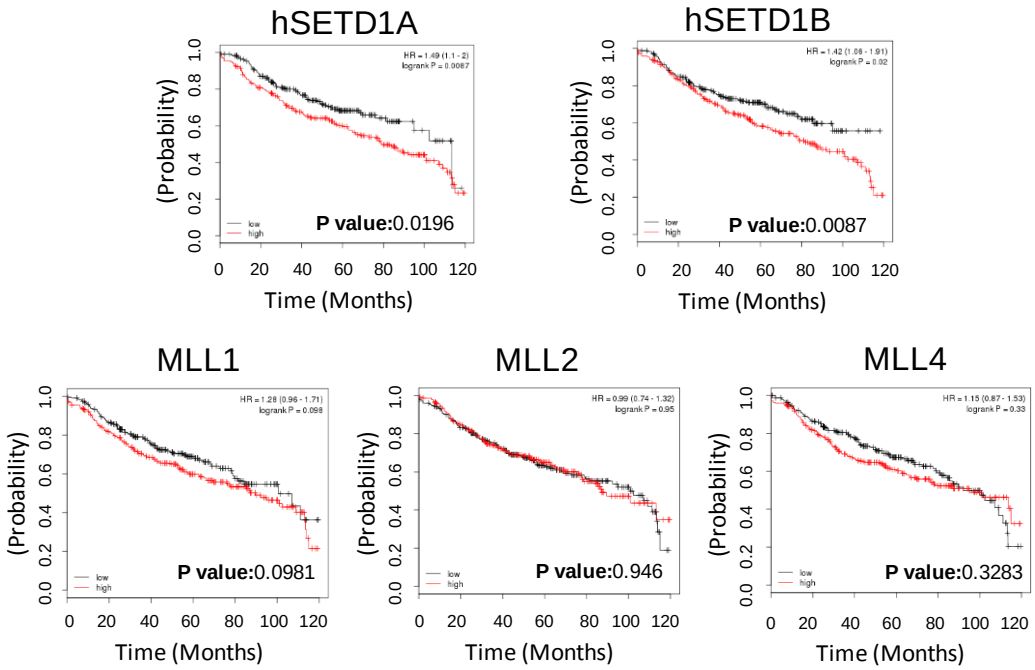


Figure 5-1. hSETD1A, hSETD1B, and H3K4me3 are up-regulated in breast cancer.(A) Cell extract were prepared for WB from mammary epithelial cell lines: MCF10A and HMEC, and the breast cancer cell lines: SUM159, MCF7, BT-549, and MDA-MB231. Levels of hSETD1A and H3K4me3 were up-regulated in the breast cancer cells compared with normal mammary epithelial cells. E-cadherin expression was lower in SUM159, BT-549, and MDA-MB231, indicating a pro-mesenchymal phenotype. (B) hSETD1A and hSETD1B mRNA levels were detected by qRT-PCR in normal breast tissues and in primary and metastatic breast cancer samples (n=6 each). hSETD1A and hSETD1B expression levels were both up-regulated in metastatic breast cancer compared to normal beast and primary breast cancer samples. Shown are the mean ±SD of three replicates. *P < 0.05, **P < 0.01, ***P < 0.001 by student t-test.

A Lymph –node positive breast cancer n=488



B Lymph –node negative breast cancer n=1120

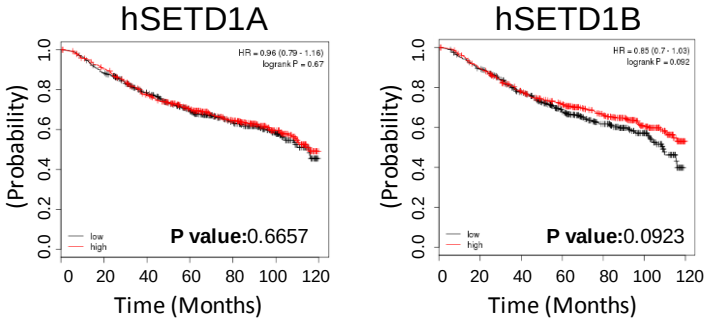


Figure 5-2. hSETD1A and hSETD1B levels predict clinical outcomes for lymph-node positive breast cancer patients. Shown are Kaplan–Meier plots of lymph-node positive (A) and lymph-node negative (B) cohorts. Patients were separated into two groups with mRNA levels higher or lower than the median levels of the indicated gene. Hazard ratio with 95% confidence intervals and logrank P value were calculated. Microarray analysis used the Affymetrix HG-U133A, HG-U133 Plus 2.0, and HG-U133A 2.0 (www.kmplot.com; 2012 version [136]). Increased levels of hSETD1A (Aff ID 213202_at) and hSETD1B (Aff ID 213153_at), but not levels of MLLs, was associated with poor survival of lymph-node positive but not lymph-node negative breast cancer patients.

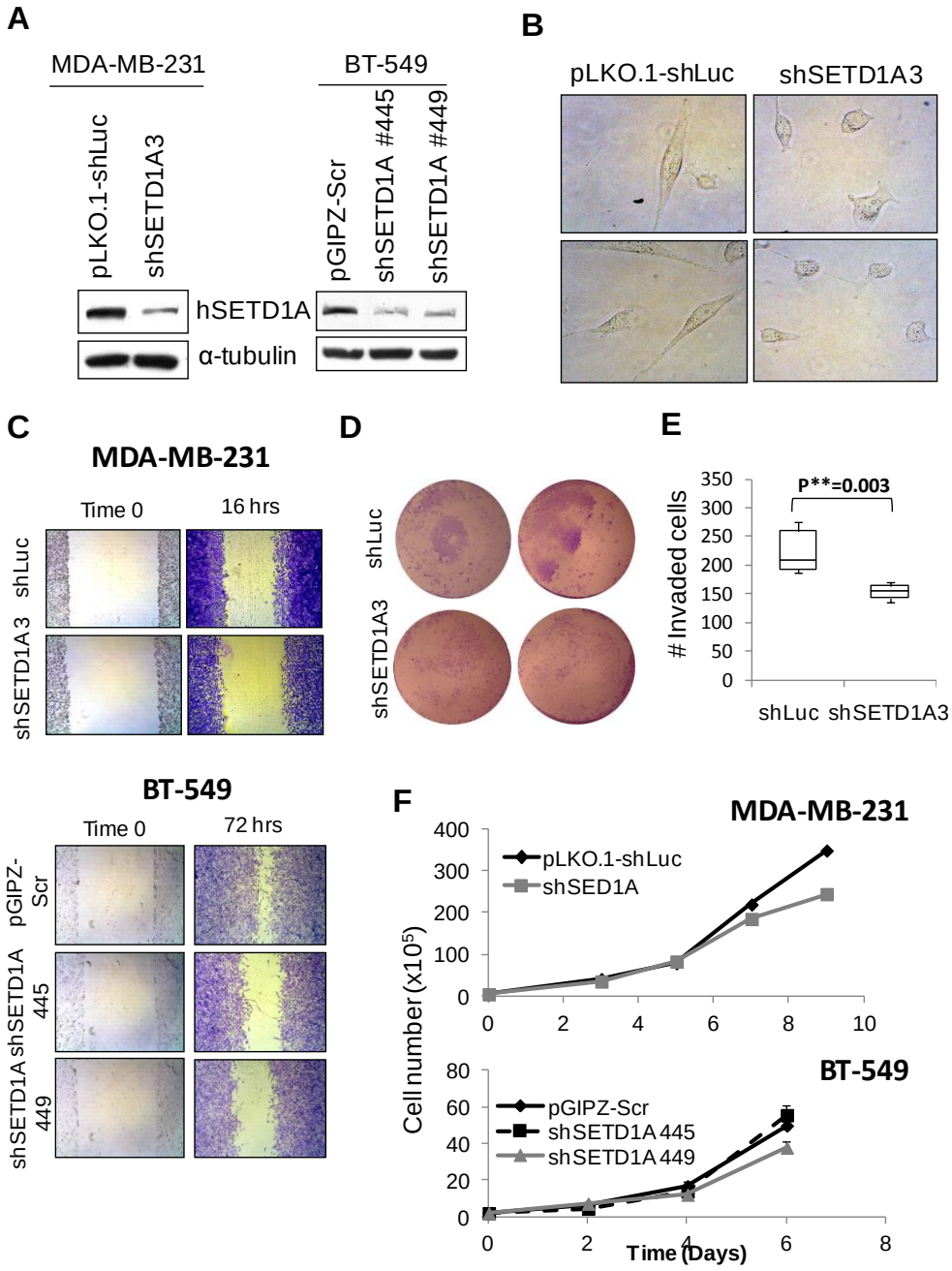


Figure 5-3. Depletion of hSETD1A reduces cell invasion and migration. (A) Lentivirus-mediated KD of hSETD1A in breast cancer cell lines MDA-MD-231and BT-549. (B) hSETD1A KD MDA-MD-231 cells invoked morphological changes resembling Mesenchymal-Epithelial Transition (MET). The effect of hSETD1A KD on cell migration was assessed by wound healing assay (C) and transwell migration assay (D). (E) The impact of hSETD1A KD on cell invasion was assessed by transwell invasion assay. MDA-MD-231 cells ($1x10^4$) were seeded and invaded cells were counted 17 hours later. Shown are the mean ±SD of five independent repeats. **P < 0.01by student t-test. (F) Proliferation assay.

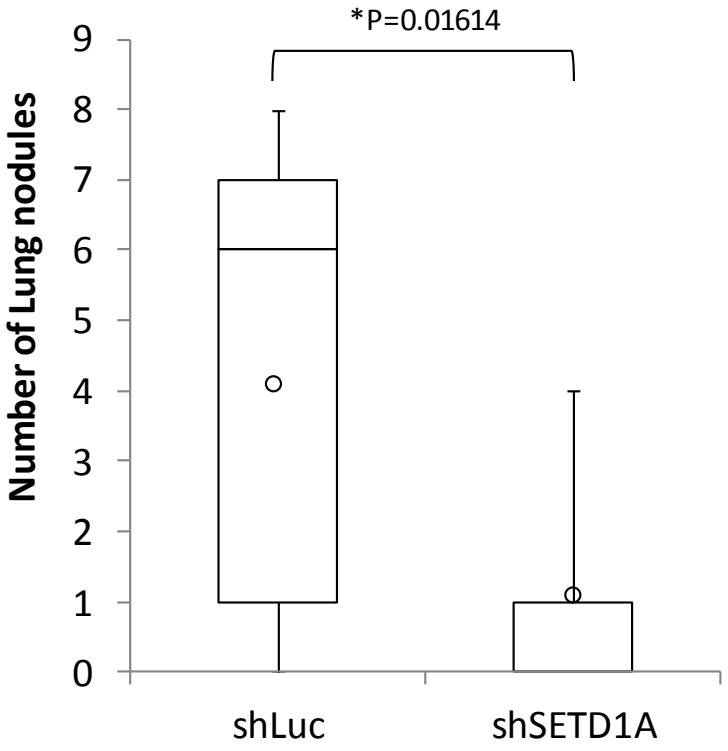


Figure 5-4. KD of hSETD1A reduces metastasis in mice. MDA-MD-231-shSETD1A or MDA-MD-231-shLuciferase cells ($1x10^6$) in 1X PBS were injected into the tail vein of female athymic nude mice. Mice were sacrificed 7-8 weeks later and lung nodules were counted. *P < 0.05 by student t-test. shLuc ; n=7, shSETD1A; n=9.

CHAPTER 6
SUMMARY AND DISCUSSION

Significance

Understanding the reasons for the epigenetic turmoil observed in cancer is critical for targeting specific epigenetic regulators and rebooting epigenetic. The goal of this dissertation was to elucidate the roles of hSETD1 HMTs and H3K4me3 in cancer-related functions, in solid tumors. The rationale for this work was the observations of up-regulation in H3K4me3, hSETD1A, and hSETD1B in cancer and the frequent mutations of hSETD1A/B in CRC and breast cancer (Figure 3-1 and Figure 5-1) [39-46, 55]. The H3K4me3 mark was suggested to play a role in transcription activation by recruiting transcription regulators to gene promoters [28-34]. Hence, misregulation of hSETD1A/B could change normal gene expression patterns and affect cellular function. We therefore proposed a model in which the HMTs hSETD1A and hSETD1B play pro-oncogenic roles in CRC and breast cancer [135].

Here, we showed that hSETD1A and hSETD1B are overexpresses in 62% and 26% of the human colorectal carcinomas examined, respectively [135]. These results led us to focus our efforts on hSETD1A. We uncover an important pro-proliferative role for hSETD1A in CRC and identified a possible link between this function and the Wnt-signaling pathway [135]. Dysfunction of the Wnt pathway plays an essential role in the manifestation and progression of most CRCs [65]. We found that hSETD1A regulates proximal promoters of Wnt target genes through its interactions with β-catenin, a centered player which conveys the Wnt signal [135]. Such interactions have been previously identified in HEK293 cells, but the overall meaning of these interactions was not elucidated [38]. Similarly, interactions between β-catenin and relatives of hSETD1A,

such as MLL1 and MLL2 were previously identified, and were shown to play a role in activating the c-MYC enhancer in CRC [49]. The possibility that MLLs play a similar role to hSETD1A in regulating other Wnt target genes has not been explored.

While H3K4me3 occupies proximal promoters, H3K4me1is rather enriched at enhancers [14, 25]. Interestingly, epigenomic analysis of H3K4me1 indicated changes in enhancer-associated H3K4me1 in CRC [39]. It was further suggested that enhancer-associated H3K4me1 can drive a specific transcription program to promote colon carcinogenesis [39]. This common role for H3K4me1 and H3K4me3 in activating transcription of CRC related genes is interesting, considering the different mechanisms utilized by MLLs and hSETD1A/B to activate gene transcription; while both MLLs and hSETD1A/B can activate gene transcription, MLLs do so through regulation of enhancer-H3K4me1 while hSETD1A/B do so through regulation of proximal promoter-H3K4me3 [18, 21-24]. In our context, this could imply a clever mechanism whereby β-catenin amplifies transcription of Wnt target genes: recruitment of hSETD1A to proximal promoters complemented by the recruitment of MLLs to enhancers of Wnt target genes. This concept could be readily tested by ChIP-seq of COMPASS members and H3K4 methylation states in CRC cells.

β-catenin act as a platform to recruit histone-modifying enzymes such as hSETD1A, CBP/p300, and MLLs [38, 49, 90, 91]. It was further proposed that the β-catenin/TCF/LEF complex mediates the assembly of mediator and TFIID complexes that are required for activation of Wnt target genes [137]. It was suggested that TFIID is recruited to these loci in Drosophila through the binding of TAF4 to Pygopus, a protein which associates with the β-catenin/TCF/LEF complex [137]. Nevertheless, we

proposed another mechanism wherein hSETD1A mediates H3K4me3 at Wnt targeted promoters upon activation on the Wnt pathway followed by direct binding of TAF3 to H3K4me3 [135]. The H3K4me3 modification was previously shown to interact with the PHD domain of TAF3, a TFIID subunit [28-30]. Our data demonstrated that TAF3 and hSETD1A share a large number of target genes including 14 Wnt target genes, and that hSETD1A-dependent H3K4me3 is critical for the recruitment of TAF3 to Wnt/β-catenin targeted promoters in HCT116 cells [135].

Another interesting finding of this dissertation is that hSETD1A is highly expressed and colocalized with β-catenin at intestinal crypts, where proliferating ISCs reside and where the Wnt-signaling pathway is constitutively active [135]. Activation of the Wnt-signaling pathway is a key event in maintenance of ISC self-renewal [102-106]. It serves as a master switch between the proliferation and differentiation of crypt cells, hence involved in intestinal malignancies [102-106]. Thus, aberrant expression of hSETD1A in crypt bottoms may cooperate with β-catenin in activating Wnt-signaling target genes and initiating oncogenic programs.

Based on our results, we depicted a model demonstrating collaborative relations between hSETD1A and β-catenin to activate transcription program that could lead to the development of CRC [135]. In this model, β-catenin and hSETD1A are up-regulated and engage in the nucleus. β-catenin recruits hSETD1A to Wnt putative promoters to confer H3K4me3 [135]. This allows for PIC assembly, activation of Wnt target genes, and subsequent promotion of cellular growth. Transcriptional activation of Wnt target genes is the last and most important step of the Wnt-signaling pathway [58]. Identifying and targeting β-catenin partners, such as hSETD1A, could be an effective therapeutic

approach for inhibiting the Wnt-signaling pathway in CRC [57, 74]. Such targeting strategies showed effective, for example in the case of inhibitors of the interactions between CBP/P300 and β-catenin [111].

In addition to our intention to study the effect of hSETD1A/B on colorectal tumor growth, we were also intrigued to understand their roles in metastasis. Unfortunately, the study of metastatic CRC *in vitro* is difficult since most CRC cell lines are epithelial. However, we found evidences for misregulation of hSETD1A and/or hSETD1B in other cancers, especially in breast cancer (Table 5) [55]. Gain in copy number of the *hSETD1A* gene was present in 45% of breast cancers (Table 5-1) [55]. Consistent with that, we showed that hSETD1A and hSETD1B are overexpressed in breast cancer specimens and cell lines (Figure 5-1). Fortunately, there are many mesenchymal breast cancer cell lines available, and in fact, breast cancer is a model disease for the study of metastasis. Hence, we further characterize hSETD1A, and hSETD1B in partial, in breast cancer metastasis.

Increased levels of both hSETD1A and hSETD1B were associated with worse outcomes in lymph-node positive breast cancer (Figure 5-2). There was no clear association between levels of hSETD1A and survival in cohorts grouped by levels of human epidermal growth factor receptor 2 (HER2), progesterone receptor (PR), or estrogen receptors (ER) [136]. Nevertheless, increased hSETD1B levels were associated with poor survival of PR/ER double negative cohorts [136]. Together, these results support that hSETD1 enzymes could serve as good biomarkers for prognosis in breast cancer patients who already diagnosed with at least lymphatic metastasis. It is noteworthy that there was even stronger association in lung cancer [138]. Increased

levels of hSETD1A (P value = 1.3e-06) was associated with 10-year poor outcomes for lung cancer patients [138]. On the other hand, the exact opposite association was observed for hSETD1B in lung cancer – increased levels of hSETD1B (P value = 3e-07) were associated with favored outcomes [138]. This differential association of hSETD1A and hSETD1B levels with survival in lung cancer, although not directly relevant to the findings of this current work, is interesting because it suggests that those enzymes are not entirely redundant, and emphasizes the need to carefully characterize them independently. Despite statistical significant relations between hSETD1A/B levels and breast cancer outcomes (hSETD1A; *P <0.02, hSETD1B; **P<0.009), it should be noted that they were not dramatically significant (Figure 5-2). In fact, it is likely that the large number of samples that were used for this analysis could have influenced the statistical test (too much statistical power) [136]. While it is difficult to comment on the clinical relevance and significance of these results, they at least support a role for hSETD1A and hSETD1B in advanced breast cancer. There was no association between COMPASS core components, such as RbBP5 and Ash2L and survival of lymph-node positive breast cancer [136]. However HCF1, component of both hSETD1A/B and MLL1/2 complexes, and WDR82, component unique to the hSETD1A/B complexes, showed association with lymph-node positive breast cancer [136].

Metastasis-relevant functions such as migration and invasion were affected by the depletion of hSETD1A in MDA-MD-231 or BT-549 cells (Figure 5-3). Moreover, depletion of hSETD1A impeded metastasis of MDA-MD-231 to the mouse lungs (Figure 5-4). Based on these results we concluded that hSETD1A impacts metastasis, but the

mechanism was not elucidated. Of note is the fact that in contrast to what was observed in HCT116 cells, depletion of hSETD1A in MDA-MB-231 cells did not affect global H3K4me3 (data not shown). This could be related to the efficiency of the KD or more interestingly - to the cell type. The effect of hSETD1B on metastasis has not been investigated due to technical difficulties in obtaining KD and for the lack of commercial antibodies.

In summary, versatile roles for hSETD1A and hSETD1B in solid tumors were identified here for the first time. These results indicate that different COMPASS family members, although structurally similar, can carry out different functions, some which are more prominent in blood cancers and others which are selective for solid cancers. Inhibition studies of SETD1A/B complementing the results obtained here are necessary for determining clinical relevance and feasibility.

Clinical Relevance

The Wnt/β-catenin signaling pathway was first characterized in the 1970s in *Drosophila melanogaster* [73]. It was later recognized that constitutive activation of the Wnt/β-catenin signaling pathway is a hallmark of numerous malignancies in human, especially gastrointestinal cancers [57, 58, 60, 65-67]. Traditionally, abnormal signaling of this pathway was attributed to genetic or somatic mutations in APC, Axin, and β-catenin, but as layers of complexity of this pathway were revealed it became clear that there are many other mechanisms affecting this signaling [58].

Despite the many years of research and attempts to develop attractive inhibitors for this pathway, to date there are no therapies meant to attenuate the Wnt pathway in clinic [57, 74]. Although *in vitro* manipulations of the Wnt pathway were promising in theory, in practice it has been difficult to obtain specific and effective therapeutics [57,

74]. There are several reasons for that: first, the Wnt-signaling pathway is essential for normal development and regeneration, particularly that of intestinal tissue. Therefore, blockage of this pathway is accompanied by unfavorable toxicities and side effects. Second, potential target Wnt components are also involved in other cellular processes, thereby reducing the specificity of the candidate inhibitors. Third, the small number of enzymatic components in this pathway makes it difficult to target.

Although in recent years there has been some progress in targeting the Wnt pathway, the benefit for CRC is questionable as upstream antagonists may not be effective in cases where the downstream components have been mutated. For example, inhibitors of the Wnt putative receptors LRP and Frizzled, or porcupine (PORCN) are probably going to be ineffective in cases of APC truncations. These insights clearly support a rationale for targeting the pathway downstream of the destruction complex. However, inhibitors targeting the obvious binding of β-catenin and TCF downstream were so far only shown useful in cell-based assays [139-141]. Nevertheless, small-molecule inhibitor (PRI-724 ;Prism Pharma) targeting the interactions between β-catenin and CBP/P300 and preventing transcription of Wnt target genes, has recently entered phase I clinical trials for the treatment of solid tumors and leukemia [111]. So far, data from 18 patients with solid tumors demonstrated an acceptable safety profile. These encouraging results suggest that targeting the interactions of β-catenin with other coactivators in the form of histone modifying enzymes, for example hSETD1A, could also be beneficial.

The findings of this dissertation not only identified hSETD1A as a novel binding partner of β-catenin in CRC cells but also showed that hSETD1A is up-regulated in CRC

[135]. Therefore, theoretically, several therapeutic strategies could be applied: specific inhibition of hSETD1A or inhibition of the interactions between hSETD1A and β-catenin. Nevertheless, inhibition of hSETD1A activity might carry out off-target effects as revealed by the large number of hSETD1A target genes in HCT116 cells (Figure 4-1A). In addition, specific inhibition of the hSETD1A SET domain might be difficult due to its high similarity to other COMPASS members. Since KD of hSETD1A only partially inhibited the Wnt-signaling pathway, toxicities associated with blocking the Wnt pathway would likely be avoided. The successes of PRI-724 inhibitor in the phase I clinical trials could have been attributed to the very same reason.

The impact of DNA and histone methylation events on the advanced disease is poorly understood compared with our understanding of initial oncogenic events. Metastasis is a hallmark of advanced cancer and the number one reason for therapeutic failure and death [6, 116, 117]. Metastatic cells require down-regulation of genes related to cell-cell adhesion and up-regulation of genes related to cell motility, angiogenesis, and matrix digestion [83, 85, 116, 117]. Epigenetic changes can greatly impact these characteristics [127]. Therefore, identification of epigenetic regulatory mechanisms is important for potential therapeutic avenues. Misregulation of a large number of histone-modifying enzymes has been established in advanced cancer. For examples, the polycomb-group protein histone-lysine N-methyltransferase EZH2 is overexpressed in many cancers and its expression correlates with tumor grade [142, 143]. The Heterochromatin protein 1 (HP1) is down-regulated in metastatic breast cancer and controls cell invasion [144]. LSD1, while promoting metastasis of colon cancer cells [145], suppresses metastasis of breast cancer [128, 129]. The potential reversibility of

epigenetic events makes their regulators an attractive biomarkers and therapeutic targets of the metastatic disease.

The effect of hSETD1A on metastasis and its association with lymph-node positive breast cancer, found here, are other layers demonstrating the importance of this enzyme in cancer. Whether hSETD1A also affects CRC metastasis is currently unknown, although likely. The Wnt-signaling pathway controls many genes related to metastasis and angiogenesis [58].Therefore, as a regulator of the Wnt-signaling pathway, hSETD1A is expected to affect metastasis. The question of how hSETD1A affects metastasis is yet to be answered. We currently do not have evidence that the enzymatic activity of hSETD1A is related to its function in metastasis and we do not know which genes are affected by it. Understanding of the molecular events regulated by hSETD1A during EMT and metastasis in breast cancer could be a key for an effective therapeutic.

The identification of critical biomarkers in cancer has led to advances in cancer detection, prognosis, and treatment. hSETD1A and hSETD1B levels were associated with clinical outcomes in lymph-node positive but not lymph-node negative breast cancer (Figure 5-2). These results suggest that the targeting of hSETD1A in breast cancer will perhaps only be beneficial to patients who already showed signs of metastasis. While ER or PR positive breast cancer patients benefit from endocrine therapy such as tamoxifen, and HER2 positive breast cancer patients greatly benefit from trastuzamab, triple negative patients (negative for PR, ER, and HER2), representing 10-20% of breast cancers, are not currently a match for any targeted therapies [146-148]. hSETD1A levels were not associated with survival in cohorts

defined by ER, PR, or HER2 levels suggesting an independent mechanism [136]. On the other hand, hSETD1B levels associated with poor 10-year survival of PR and ER negative breast cancer patients (P value = 0.034; n=185) [136]. These results indicate that PR and ER negative patients who showed lower levels of hSETD1B, have better chances to survive breast cancer. Although hSETD1A and hSETD1B levels associated with clinical outcomes these results require validation. There were many important patients' characteristics that were not taken into account using the www.kmplot.com database [136]. For example, age at diagnosis, genetic predispositions such as BRCA1/2 mutations, pre or post-menopausal, and childbearing [113]. These characteristics play a critical role in prognosis of breast cancer patients and should be considered when characterizing biomarkers.

Future Direction

The findings of this study present a novel role for hSETD1A in solid tumors and call for further research. Future studies elucidating the effect of hSETD1A *in vivo* are critical, for example, testing of the capacity of hSETD1A conditional KO to reduce the burden of colorectal polyps in mouse models, such as the Apc^{Min} mouse model [94, 95]. In addition, the effect of hSETD1A on the development of breast cancer and metastasis needs to be further investigated *in vivo*. While we utilized tail injection model to study metastasis of MDA-MB-231-shSETD1A cells, an orthotopic model might better recapitulate the natural habitat and microenvironment of breast cancer cells [149]. That is implantation of breast cancer cells in the mammary fat pad, leading to the development of a primary tumor and metastasis to distant sites. Other mouse models could be utilized for genetic manipulations to rigorously study the effect of various genes on the development and progression of breast cancer [149]. For example, the MMTV-

Neu mouse model, in which the neu (Erbb2) oncogene was inserted under the transcriptional control of the mouse mammary tumor virus (MMTV) promoter, wherein mice develop mammary tumors at 29 weeks [150]. Crossing these mammary tumor-prone mice with hSETD1A conditional KO strain would allow for testing the effect of hSETD1A KO on the onset and progression of breast cancer. Nevertheless, it is somewhat difficult to predict which mouse model would be most suitable as the molecular mechanism of how hSETD1A affect breast cancer metastasis is currently unknown. Therefore, *in vitro* studies of gene expression profiles of hSETD1A/B KD in breast cancer cell lines should be applied first.

In addition, the utilization of hSETD1A/B as biomarkers in breast cancer should be further studied while considering tumor heterogeneity; examining the association of hSETD1A/B with survival of patients bearing breast tumors of different classifications, such as histological type, grade, lymph node status, and the presence of predictive markers such as ER/PR/HER2 [113]. In addition, other patient characteristics, such as pre/post-menopausal, childbearing age, and genetic predispositions such as BRCA1 and BRCA2 mutations, should also be considered [113]. Complementary studies examining the impact of hSETD1A/B KD in different classifications of breast cancer cell lines would be useful in further understanding the relations of hSETD1A/B to already known biomarkers. For example, ER^{-} PR^{-} $HER2^{+}$ (SKBR3), ER^{-} PR^{-} $HER2^{-}$ (BT549, MDA-MB-231, MDA-MB-468), Luminal A $ER^{+}PR^{+/-}HER^{2-}$ (MCF-7, T47D, SUM185), Luminal B $ER^{+}PR^{+/-}HER^{2+}$ (BT474, ZR-75) [151].

Full hSETD1A KO was shown by our lab and others to be embryonic lethal, indicating that hSETD1A is essential for normal development [52]. Therefore,

investigating the possible functions of hSETD1A in normal development of intestine and breast tissues is an important one. To that end conditional KO of hSETD1A could be generated. Moreover, the effect of hSETD1A on normal intestinal tissue should be carefully studied considering our important findings revealing that hSETD1A is highly expressed at the intestinal crypt [135]. These results suggest a regulatory role for hSETD1A in self-renewal or/and differentiation of intestinal stem cells and regeneration of intestinal tissue. First, the expression of hSETD1A in specific type of crypt cells should be more comprehensively studied. Second, hSETD1A-dependent mechanisms regulating self-renewal, migration, and differentiation of cells in the crypt, dependently or independently of the Wnt-signaling pathway, should be examined. The normal human intestinal epithelial crypt-like (HIEC) cell line and the villus-like primary cultures of differentiated enterocytes (PCDE) could be resourceful for studying the former *in vitro* [152].

Increasing evidences indicate that β-catenin mediates activation of Wnt target genes by recruiting epigenetic modifying enzymes [38, 49, 87, 90, 91]. This idea could be more carefully tested though induction of β-catenin followed by ChIP-seq analyses of various histone modifications. Understanding of the role of histone modifications in activation of the Wnt-signaling pathway could lead to the discovery of novel targets in the form of histone modifying enzymes.

Finally, we discovered a link between the Long non-coding RNA (lncRNA) ‘Hemolinc’, first discovered in our lab, and breast cancer. This lncRNA is expressed from the HOXB locus and serves a transcription enhancer for HOXB genes. Hemolinc interacts with the hSETD1A complex and affect many target genes other than the

HOXB genes, for example, genes associated with EMT. Therefore we investigated the expression levels of hemolinc in breast cancer cell lines and found that compared with the human mammary epithelial cell lines MCF10A and HMEC, it is over expressed in the mesenchymal breast cancer cell lines MDA-MB-231 and SUM159. These preliminary results could suggest that the hemolinc regulates EMT, perhaps through interactions with hSETD1A. Nevertheless, further research is required to answer the question of whether or not Hemolinc affects progression of breast cancer and how.

APPENDIX
THE LONG NON-CODING RNA HEMOLINC IS UP-REGULATED IN MESENCHYMAL BREAST CANCER CELLS

Long non-coding RNAs (lncRNAs) are diverse class of transcribed RNA molecules of 200 or more nucleotides that do not encode protein. However, like proteins they are expressed in a spatial and temporal manner and can regulate variety of cellular processes, including gene transcription. A significant number of lncRNAs have been shown to collaborate with epigenetic regulators to modify gene transcription [153].

The long-noncoding RNAs (lincRNAs) '*Hemolinc*' was characterized in our lab and was shown to transcribed from the *HOXB* locus (Deng et. al.). *HoxB*-associated *Hemolinc* governs transcription activation of anterior *HoxB* genes and promotes hematopoietic differentiation of mouse embryonic stem cells (mESC). Importantly, *Hemolinc* associates with the conserved SET domain of the Setd1a/MLL1TrxG complexes, in the erythroleukemia cell line K562 and in embryoid bodies (EB).

Epithelial plasticity is a common theme during embryonic development and cancer progression [82, 83, 85]. Like cancer cells, embryonic stem cells undergo EMT during development in order to migrate and travel long distances to form tissues and organs. Because *Hemolinc* regulates stem cell differentiation and interacts with the EMT-related hSETD1A, we investigated the potential link between *Hemolinc* and EMT. This idea was further supported by the changes in expression of EMT-related genes upon KD of *Hemolinc* in mESC. Therefore we analyzed the expression levels of *Hemolinc* in breast cancer cell lines in which hSETD1A was elevated and compared it with human mammary epithelial cell lines. *Hemolinc* expression increased significantly in the triple negative mesenchymal breast cancer cell lines MDA-MB-231 and BT-549, but not in the epithelial breast cancer cell line MCF7 or in the human mammary

epithelial cell lines MCF10A or HMEC (Figure A-1A). Interestingly, the expression of *Hemolinc* was low in the HER2 positive mesenchymal breast cancer cell line SUM159 cells *(*Figure A-1A). These results imply a role for *Hemolinc* in EMT.

We further examined the expression of *Hemolinc* in normal human breast tissues and in primary and metastatic breast cancer samples. There was no association between *Hemolinc* expression levels and progression of breast cancer (Figure A-1B). However, it is difficult to generalize these results considering the large variation in breast tumor subtypes and the small number of samples we tested (n=6-7 each category).

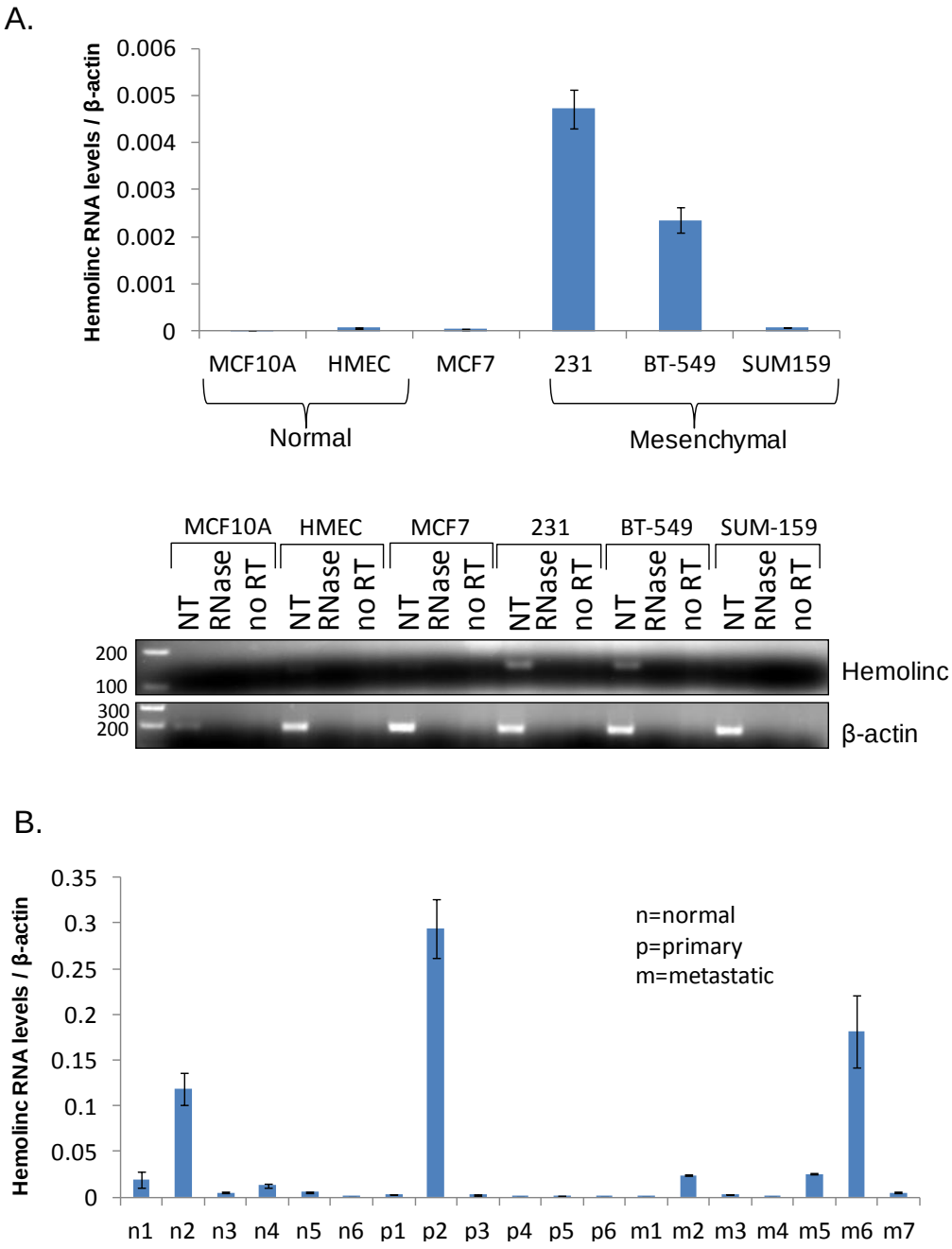


Figure A-1. Hemolinc expression in breast cancer. (A) Expression of hemolinc in normal and breast cancer cell lines by RT-PCR. Hemolinc is over expressed in the mesenchymal breast cancer cell lines MDA-MB-231 and SUM159. The bottom figure shows the appropriate controls; No treatment (NT), treatment of RNA with RNase A prior to reverse transcription (RNase), Reverse transcription in the absence of RT polymerase (no RT). β-actin is shown as a control gene. (B) Expression of Hemolinc in normal breast, breast carcinoma, and metastatic breast samples by RT-PCR.

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BIOGRAPHICAL SKETCH

Tal Hila Salz was born (1984) in Israel, to Nili and David Salz. She holds an American and Israeli dual citizenship. Tal attended Mevo HaGalil, the private regional elementary school (1991-1997), located at kibbutz Ayelet HaShahar, and graduated from the Har Vagai private junior and senior regional high school (1997-2002). She joined the women professional basketball league at the age of 15 (1998-2008) and was recruited to the youth, U-20, and adult Israeli national team. She was the captain of U-20 Israeli national basketball team (age 18-21), with she has travelled to the youth Olympic games in Spain and played the Euro league. Tal has also completed a mandatory army service (2002-2004) in which she was awarded a prestigious athlete status that allowed her to continue playing professional basketball under minimal service conditions.

Tal graduated from the University of Bar-Ilan in Ramat-Gan, Israel, with a Bachelor of Science degree in Biotechnology (2005-2008). She then retired from basketball and relocated to the United States to work as a scientist at the laboratory of Dr. Karl Drlica at the Public Health Research Institute Center, University of Medicine and Dentistry of New Jersey (UMDNJ). She co-authored one scientific paper (*Cell Rep., 2013*), and a book (*Antibiotic Discovery and Development, 2009*) discussing Fluoroquinolone resistance. In 2009, Tal was admitted to the PhD program in Biomedical Sciences at the University of Florida, Gainesville, Fl. She joined the laboratory of Dr. Suming Huang and completed two curriculums; Biochemistry and Molecular Biology and Clinical and Translational Science. At the laboratory of Dr. Huang Tal studied epigenetic regulation in cancer and co-authored several scientific papers

(*PLoS Genet. 2013, Leukemia.* 2013) and one first author paper (*Cancer Res. 2014*). During her graduate years she was awarded several internal incentive and travelling awards, as well as a training grant in clinical and translational science TL1. She was also invited to speak at an international symposium on Cancer epigenetic (Keystone Symposia, Santa Fe, NM, 2013) and part took in poster sessions in other international and internal meetings. Tal received her doctoral degree in May 2014.