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ROLE OF PROTEIN ARGININE METHYLTRANSFERASE 5 (PRMT5) IN WNT/β-CATENIN PROLIFERATIVE SIGNALING IN BREAST CANCER

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ABSTRACT

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Title: Role of Protein Arginine Methyltransferase 5 (PRMT5) in WNT/β-CATENIN proliferative signaling in breast cancer

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Protein arginine methyltransferase 5 (PRMT5) activity is dysregulated in many aggressive cancers and its enhanced levels are associated with increased tumor growth and survival. In this study, we show that PRMT5 is overexpressed in breast cancer cell lines, and that it promotes WNT/β-CATENIN proliferative signaling through epigenetic silencing of pathway antagonists, *DKK1* and *DKK3*, by binding to their promoter and inducing symmetric dimethylation of H3R8 and H4R3, leading to enhanced expression of *c-MYC*, *CYCLIN D1* and *SURVIVIN*. Our findings also show that PRMT5 inhibition using compound 5 (CMP5), reduces PRMT5 recruitment and PRMT5-induced epigenetic marks in the promoter regions of *DKK1* and *DKK3*, which consequently results in reduced expression of *CYCLIN D1* and *SURVIVIN*. Furthermore, CMP5 treatment either alone or in combination with 5-Azacytidine and Trichostatin A restored expression of *DKK1* and *DKK3* in TNBCs. In addition, PRMT5 inhibition in TNBCs inhibited AKT/mTOR signaling by reducing phosphorylation of AKT and mTOR at Ser473 and Ser2448, respectively. These molecular changes were associated with reduced proliferation, migration and invasion of breast cancer cells, and induced their death.

DEDICATION

Dedicated to my family

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CHAPTER 1: INTRODUCTION

Post-translational modifications including methylation, phosphorylation, acetylation, ubiquitination and sumoylation play an important role in regulating the biological and cellular functions of various cytosolic and nuclear proteins (Karve & Cheema, 2011). Arginine methylation is one such post-translational modification carried out by a group of enzymes belonging to the protein arginine methyltransferase (PRMT) family. PRMTs are broadly categorized into three main classes. Type I PRMTs catalyze monomethylation and asymmetric dimethylation, while Type II PRMTs catalyze monomethylation and symmetric dimethylation of specific arginine residues. Type III PRMTs catalyze only monomethylation of arginine residues. Protein arginine methyltransferase 5 (PRMT5) is a type II PRMT enzyme that catalyzes symmetric dimethylation of specific arginine residues of target proteins (Branscombe et al., 2001). *In vitro* studies have shown that the PRMT5 enzymatic activity requires formation of a hetero-octameric complex with its co-factor, methylosome protein 50 (MEP-50), which governs substrate specificity and enzyme-substrate interaction with PRMT5 target proteins (Antonysamy et al., 2012; Burgos et al., 2015). In addition to MEP-50, PRMT5 is also known to form a complex with other proteins such as pICln and RIO kinase 1 (RioK1), which interact with PRMT5 and further regulates its substrate specificity (Guderian et al., 2011).

PRMT5 regulates transcription of target genes by directly binding to their promoter regions and inducing arginine methylation of promoter histones, which triggers chromatin remodeling. PRMT5-induced symmetrical dimethylation of H3R8 and H4R3

predominantly result in transcriptional repression of target genes, except in few cases where PRMT5-mediated histone methylation has been associated with transcriptional activation (LeBlanc et al., 2012; Pal et al., 2004; Zhang et al., 2015). Apart from histones, PRMT5 also methylates arginine residues of various non-histone proteins such as p53, E2F1, ribosomal protein S10 (RPS10), rapidly accelerated fibrosarcoma (RAF) protein kinase, and golgin (Shailesh et al., 2018).

Enhanced PRMT5 activity serves as one of the major drivers of cellular transformation and tumor development in various cancers. Elevated levels of PRMT5 have been detected in different types of cancers including mantle cell lymphoma (Pal et al., 2007), germ cell tumors (Eckert et al., 2008), epithelial ovarian cancer (Bao et al., 2013), metastatic melanoma (Nicholas et al., 2013), glioma (Han et al., 2014), colorectal cancers (Zhang et al., 2015) and breast cancer (Wu et al., 2017). Increased expression of PRMT5 accelerates G1 phase progression by elevating positive cell cycle regulators such as CDK4, CDK6, CYCLIN D1, CYCLIN D2 and CYCLIN E1, and inactivating retinoblastoma (RB) protein simultaneously (Wei et al., 2012). In addition, elevated levels of PRMT5 reduce the expression of several tumor suppressor genes such as *Suppressor of Tumorigenicity 7* (*ST7*) and *Protein Tyrosine Phosphatase Receptor-type O* (*PTPROt*), by binding to their promoter regions and inducing H3R8 and H4R3 symmetric methylation marks (Alinari et al., 2015; Pal et al., 2007).

The WNT/β-CATENIN pathway is a well-regulated signaling module that controls various biological processes during embryogenesis and adult homeostasis (Barker, 2008). Binding

of WNT ligand to its transmembrane receptor, Frizzled receptor, and co-receptor, low-density lipoprotein receptor-related protein 5/6 (LRP5/6), facilitates attachment of the dishevelled (DSH/DVL) protein to Frizzled receptor. Next, Frizzled-bound DSH/DVL recruits glycogen synthase kinase 3beta (GSK-3β) to the cell membrane, which phosphorylates LRP6 and promotes recruitment of Axis inhibitory protein 1 (AXIN1) to the cell membrane. The net outcome of these events is inactivation of cytosolic destruction complex composed of AXIN1, AXIN2, Protein-phosphatase-2A (PP2A), GSK-3β, Casein kinase1 (CK1) and Adenomatous Polyposis Coli (APC). In the absence of WNT ligand, the cytosolic destruction complex facilitates binding and phosphorylation of β-CATENIN, followed by ubiquitination and proteasomal degradation of phospho β-CATENIN (Clevers & Nusse, 2012; Zeng et al., 2005). However, binding of Wnt ligand to its receptor blocks formation of the cytosolic destruction complex and inhibits β-CATENIN degradation. Consequently, β-CATENIN levels rise in the cytosol, which then translocates to the nucleus, where it associates with transcription factors such as T-Cell Factor and Lymphoid Enhancer Factor (TCF/LEF), and promotes transcription of downstream target genes such as *c-MYC*, *CYCLIN D1* and *SURVIVIN*. (He et al., 1998; Tetsu & McCormick, 1999; Zhang et al., 2001)

Aberrant activation of the WNT/β-CATENIN pathway is frequently observed in cancer (Kahn, 2014), and is reported to be due to epigenetic silencing of pathway antagonists such as WIF1, DKK3 and SFRPs, in various cancers including glioblastoma (Lambiv et al., 2011), cervical cancer (Lee et al., 2008) and colorectal cancer (Suzuki et al., 2004). However, the role of PRMT5 in regulating WNT/β-CATENIN signaling was not studied

until recently, when our group showed that PRMT5 promotes WNT/β-CATENIN signaling in three different types of lymphoma cells by inhibiting expression of negative regulators of the pathway such as AXIN2 and WIF1 (Chung et al., 2019).

Breast cancer is the most commonly diagnosed cancer, and the primary cause of cancer-related deaths among women worldwide (Donepudi et al., 2014). Extensive studies in the last two decades have revealed that different types of genetic and epigenetic alterations are involved in breast cancer development; however, the role of PRMT5 in breast carcinogenesis remains underexplored. An early study by Scoumanne et al. (2009) showed that PRMT5 knockdown induces G1 cell cycle arrest in MCF7 cells, resulting in reduced cell proliferation. Another study showed that breast cancer patients with elevated levels of PRMT5 and Programmed Cell Death 4 (PDCD4) protein have poor survival rate when compared to patients with high PDCD4 levels and reduced PRMT5 levels (Powers et al., 2011). *In vitro* studies from the same group further confirmed that PRMT5 reduces the tumor suppressor activity of PDCD4 by directly binding and methylating it. A later study by Yang et al. (2015) revealed that PRMT5 levels are elevated in various breast cancer cells including MCF7, MCF-10A, MDA-MB-231, and clinical samples of ductal carcinoma, and that elevated PRMT5 levels positively correlate with increased mortality. More recently, Chiang et al. (2017) showed that PRMT5 levels are elevated in breast cancer stem cells (BCSCs), and that its knockdown reduces proliferation as well as self-renewal of BCSCs *in vitro* and *in vivo*. Although these findings have shown that PRMT5 plays an important role in breast carcinogenesis, the underlying mechanism through which PRMT5 promotes breast carcinogenesis is not studied extensively.

Based on these studies and our recent findings in lymphoma cells, we planned to investigate the role of PRMT5 in regulating WNT/β-CATENIN proliferative signaling in breast cancer. Our study objectives were

1) To determine whether PRMT5 activity is upregulated in various breast cancer cells with different aggressiveness

2) To determine if expression of WNT/β-CATENIN inhibitors and downstream target genes is altered

3) To determine whether PRMT5 can bind to the promoter region of WNT/β-CATENIN antagonists and repress their transcription

4) To assess whether PRMT5 inhibition reduces expression of WNT/β-CATENIN downstream target gene expression

5) To evaluate whether PRMT5 inhibition induces re-expression of the identified WNT/β-CATENIN antagonist genes

6) To determine whether PRMT5 inhibition reduces the growth characteristics of breast cancer cells including proliferation, migration and invasion, and, if its inhibition can also induce breast cancer cell death

ABBREVIATIONS

ADMA	Asymmetric dimethylarginine
AdoMet	S-adenosylmethionine
APC	Adenomatous Polyposis Coli
AXIN	Axis Inhibitory protein
CASP10	Caspase-10
CDH1	Cadherin 1
C/EBP	CCAAT Enhancer Binding Protein
CDK	Cyclin dependent kinase
CK1	Casein kinase 1
CTD1	Chromatin licensing and DNA replication factor
DIX	1
DKK	Dishevelled and Axin
DNMT3A	Dickkopf-related protein
eIF4E	DNA methyltransferase 3A
FZD	Eukaryotic elongation factor- 4E
GSK-3β	Frizzled receptor
H2A	Glycogen synthase kinase 3beta
H3	Histone 2A
H3R8	Histone 3
H4	Histone 3 Arginine 8

H4R3	Histone 4
HOXA9	Histone 4 arginine 3
HDAC	Homeobox A9
hSWI/SNF	Histone deacetylase
LEF	Human SWItch/Sucrose Non-Fermentable
LRP	Lymphoid enhancer factor
MEP-50	Low-density lipoprotein receptor-related protein
miR	Methylosome protein 50
MMA	microRNA
MMP	Monomethylarginine
OCT4	Matrix metalloproteinase
NF-κB p65	Octamer-binding transcription factor 4
NM23	Nuclear Factor-κB p65
PDCD4	Non-metastatic 23
PI3K	Programmed cell death 4
PRMT	Phosphoinositide 3-kinase
PRMT5	Protein arginine methyltransferase
PTPROt	Protein arginine methyltransferase 5
RB	Protein tyrosine phosphatase receptor-type O
RPS10	Retinoblastoma
SDMA	Ribosomal protein S10
SFRP	Symmetric dimethylarginine

SOX2	Secreted frizzled-related protein
ST7	SRY (Sex Determining Region Y)-Box 2
TCF	Suppressor of tumorigenicity 7
WIF1	T-cell factor
	Wnt inhibitory factor 1

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

In eukaryotes, post-translational modification of specific amino acid residues of proteins plays an important role in the regulation of various cellular processes such as DNA replication, transcription and translation (Karve & Cheema, 2011). Post-translation modifications of histone proteins include lysine acetylation, arginine and lysine methylation, serine and threonine phosphorylation, and sumoylation as well as ubiquitination of lysine residues. These modifications of histones are known to regulate gene expression by inducing chromatin remodeling that alters accessibility of DNA to various transcriptional activators and repressors (Bowman & Poirier, 2015).

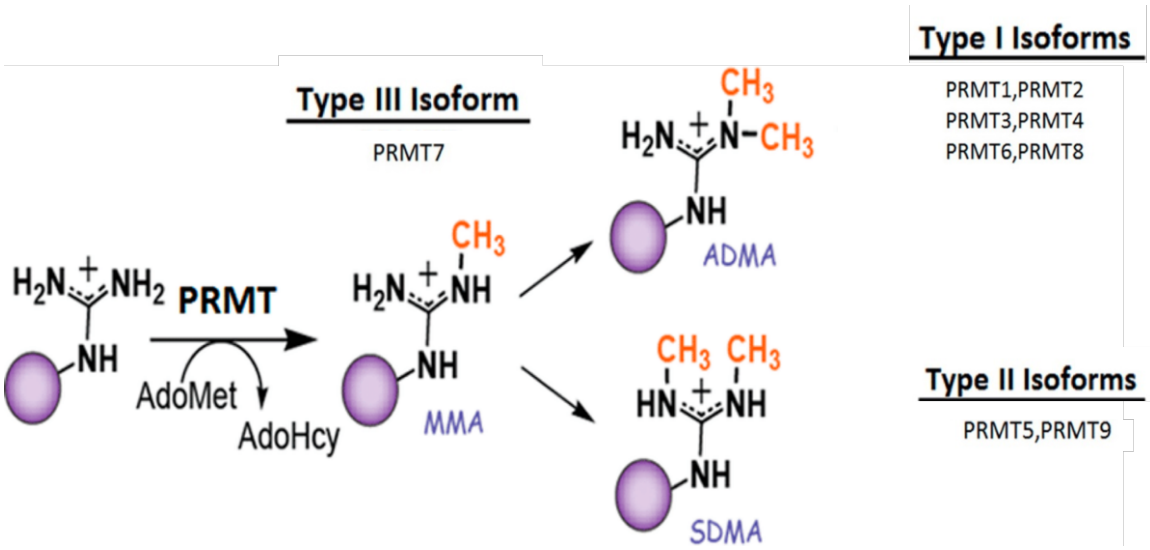


Figure 1: Arginine methylation carried out by PRMTs. Type I, II and III PRMTs induce methylation of the terminal guanidine nitrogen atom of arginine yielding

monomethylarginine (MMA). Type I PRMTs including PRMT1, PRMT2, PRMT3, PRMT4, PRMT6 and PRMT8 induce asymmetric dimethylation of arginine (ADMA). Type II PRMT enzymes (PRMT5 and PRMT9) catalyze symmetric dimethylation of arginine (SDMA), whereas Type III enzymes (PRMT7) catalyze formation of monomethylarginine (MMA) (Caceres et al., 2018).

Histone arginine methylation is an important post-translational modification that is carried out by enzymes belonging to the protein arginine methyltransferase (PRMT) family (Guccione & Richard, 2019). PRMTs catalyze transfer of a methyl group from S-adenosylmethionine (AdoMet) to the guanidino nitrogen of arginine, resulting in formation of methylarginine. Three types of methylarginine are found in mammals (Fig. 1). Monomethylarginine (MMA) is formed when a single methyl group is attached to one of the terminal nitrogen atoms of guanidine group, whereas asymmetric dimethylarginine (ω-NG, ω NG-dimethylarginine) is produced due to the addition of a second methyl group to the same terminal guanidino nitrogen. In contrast, attachment of the second methyl group to the other terminal nitrogen atom results in the formation of symmetric dimethylarginine (ω- NG, ω-N'G-dimethylarginine). Based on the type of methylarginine they produce, PRMTs are broadly classified into three groups: Type I PRMTs include PRMT 1, 2, 3, 4, 6 and 8, and catalyze monomethylation and asymmetric dimethylation of arginine residues; Type II PRMTs include PRMT5 and PRMT9 that catalyze formation of monomethyl and symmetric dimethylarginine; on the other hand, Type III PRMTs include PRMT7, which promotes only monomethylation (Shailesh et al., 2018).

2.2. Protein arginine methyltransferase (PRMT5)

PRMT5 is a Type II PRMT that was first identified as Jak binding protein 1(JBP1) with a molecular weight of 72.4 kDa in a study by Pollack et al. (1999), which focused on identifying novel Jak2 interacting proteins in human cells. Using an *in vitro* methylation assay, the authors of the same study demonstrated that JBP1 possesses methyltransferase activity and can methylate histones, H2A and H4, and myelin basic protein. Sequence homology showed that JBP1 is the human homolog of the Shk1 kinase binding protein1 (Skb1) gene of *Schizosaccaromyces pombe* and HSL7 (histone synthetic lethal 7) of *Saccharomyces cerevisiae*. A later study that analyzed the amino acid sequence of JBP1 showed that JBP1 has three conserved domains at its C-terminus, which are similar to those found in S-adenosyl-L-methionine-dependent protein arginine methyltransferases (PRMTs). This finding led the authors to propose that JBP1 is a PRMT family member, and therefore, named it as PRMT5. What further confirmed this notion is that endogenous JBP1/PRMT5 runs as a 72 kDa protein that can methylate arginine residues of myelin basic protein, histones, and the amino terminus of fibrillarin that is fused to glutathione S-transferase (GST) (Rho et al., 2001). At the same time, Branscombe et al. (2001) demonstrated that PRMT5 is a type II methyltransferase that can induce monomethylation and symmetric dimethylation of several methyl acceptor proteins such as myelin basic protein, bovine histone H2A, and a GST-fibrillarin (glutathione S-transferase-GAR).

2.2.1 Structure and enzymology

An early study that investigated the structure of PRMT5 by sedimentation analysis showed that PRMT5 exists as a homo-oligomer complex including dimer and tetramer *in vivo*. The

same study also demonstrated that several covalent interactions such as disulphide bond and non-covalent associations between C-terminal and N-terminal regions of different PRMT5 monomers induce homo-oligomerization of PRMT5 (Rho et al., 2001). The C-terminal domain of PRMT5 harboring methyltransferase activity is found to be evolutionarily conserved from yeast to human (Fig. 2) (Rho et al., 2001; Shailesh et al., 2018). The X-ray structure of human PRMT5 shows that it binds to MEP50 (Wdr77/ androgen receptor coactivator p44) to form a hetero-octomeric complex having a tetramer of PRMT5 and tetramer of MEP-50. A detailed structural analysis of the PRMT5/MEP50 complex revealed that PRMT5 has a triosephosphate isomerase (TIM) barrel domain in the N-terminal region, which interacts with the C-terminal catalytic domain of an adjacent PRMT5 monomer in the octomeric complex. The crystal structure also showed that the TIM barrel domain interacts with MEP50, and that the C-terminal catalytic domain contains a Rossmann fold region, which serves as the AdoMet binding site, and a β-sandwich domain in between C-and N-terminal domains that accomodates substrate binding (Antonysamy et al., 2012). Binding of MEP50 potentiates the histone methyltransferase activity of PRMT5 by enhancing its interaction with substrate (Antonysamy et al., 2012; Burgos et al., 2015). In addition, CYCLIN D1/CDK4-mediated phosphorylation of threonine 5 in MEP50 has been shown to further enhance PRMT5 enzymatic activity (Aggarwal et al., 2010). In contrast, phosphorylation of PRMT5 at tyrosines 297, 304 and 306 by the oncogenic Jak2 mutant protein (V617F) disrupts its association with MEP50 and reduces its histone methyltransferase activity (Liu et al., 2011).

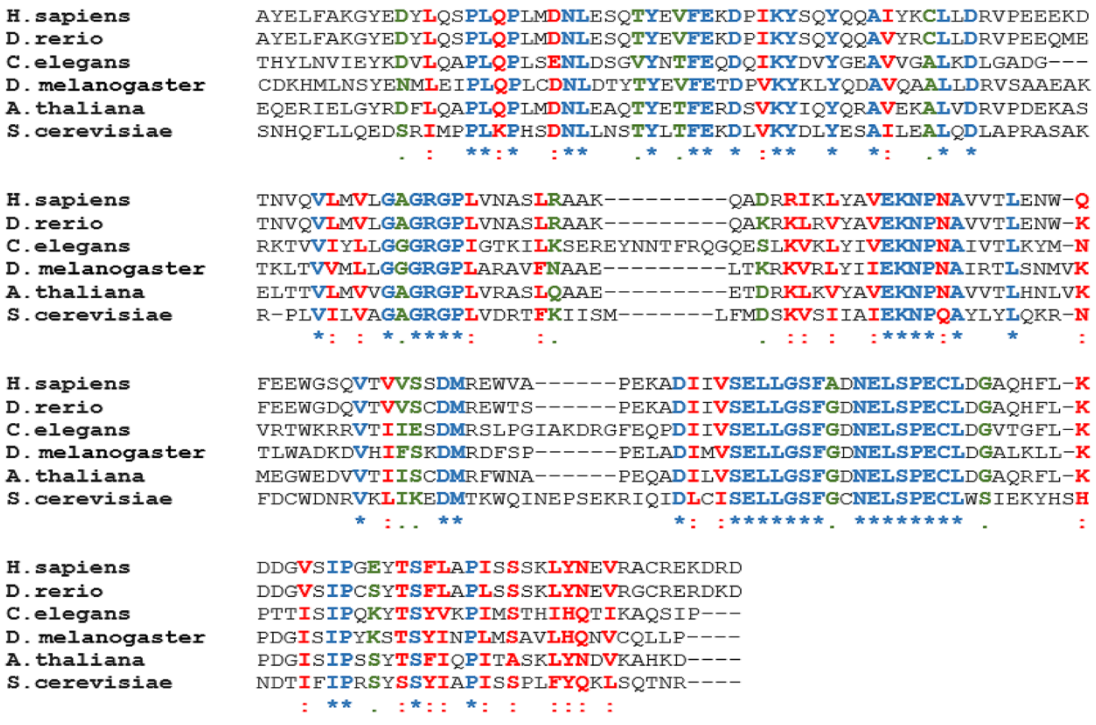


Figure 2: Catalytic domain of PRMT5 is highly conserved from yeast to human. Alignment of amino acid sequences from *H. sapiens* (296–493), *D. rerio* (292–489), *C. elegans* (348–554), *D. melanogaster* (272–466), *A. thaliana* (302–496), and *S. cerevisiae* (317–518) was done using the Clustal Omega software. The conserved sequences are highlighted using different colors and symbols (Blue star represents conserved identical residues, red colon represents conserved highly similar residues; and green bullet represents moderately similar residues) (Shailesh et al., 2018).

Apart from MEP-50, PRMT5 is also known to interact with various complexes such as the human SWI/SNF complex, and proteins like pICln, and RioK1, which increase its functional versatility. For example, in association with BRG1- and hBRM-based SWI/SNF

chromatin remodeling complexes, PRMT5 induces symmetric dimethylation of H3R8 and H4R3 in the promoter region of tumor suppressor genes including *ST7* and *NM23*, and represses their transcription (Pal et al., 2004). On the other hand, PRMT5 interacts with the Sm binding protein, PICln, and symmetrically methylates arginine residues of Sm family proteins, SMD1 and SMD3. Methylation of SMD proteins promotes their association with the SMN complex, which is a prerequisite step for formation of small nuclear ribonucleoprotein core (SnRP) during mRNA splicing (Brahms, et al., 2001; Friesen et al., 2001). In addition, PRMT5 can also bind to the cytosolic protein, RioK1 and promote recruitment and methylation of the RNA binding protein, nucleolin, which in turn favors its interaction with RNA during ribosome biogenesis (Guderian et al., 2011).

2.2.2. PRMT5 target proteins

PRMT5 is known to mediate methylation of histone proteins including histones, H3 and H4 in the promoter region of target genes, and regulate their transcription. For example, PRMT5-mediated methylation of H3R8 and H4R3 in the promoter region of tumor suppressor genes like *ST7* and *PTPROt* suppresses their transcription (Alinari et al., 2015; Pal et al., 2007). Apart from histone proteins, PRMT5 also methylates several non-histone proteins, which include transcriptional factors that regulate major cellular functions (Table 1). For example, during DNA damage PRMT5 associates with Strap (stress-responsive activator of p300) to methylate p53 and enhances its binding efficiency to the promoter region of its target gene, *Cyclin-Dependent Kinase Inhibitor 1A (CDKN1A)*, thereby leading to elevated expression of *p21*. The net outcome of these events is cell-cycle arrest. However, depletion of PRMT5 reduces occupancy of p53 on the *p21* promoter, which

Table 1: Proteins that are methylated by PRMT5

PRMT5 substrates	Biological role	Citations
Histones (H3R2, H3R8, H4R3), SPT5, FCP1, MBD2, KAP1, N-MYC PAX3, RNA polymerase II	Regulation of transcription	(Amente et al., 2005; di Caprio et al., 2015; Kwak et al., 2003; Pal et al., 2004; Park et al., 2015; Pesiridis et al., 2009; Tan & Nakielny, 2006; Tsai et al., 2013; Wu et al., 2015; Zhao et al., 2016)
p53, ASK1, PDCD4, CRN5	Regulation of apoptosis	(Chen et al., 2016; Fay et al., 2014; Jansson et al., 2008; Rastetter et al., 2015)
NF-κB/p65, HOXA9	Immune response regulation	(Bandyopadhyay et al., 2012; Harris et al., 2016)
cRAF, EGFR, RAF, E2F-1, FEN1, Androgen receptor, srGAP2, PDGFRα	Regulation of cell proliferation, migration, differentiation, and survival	(Andreu-Perez et al., 2011; Calabretta et al., 2018; Guo & Bao, 2010; Guo et al., 2010; Mounir et al., 2016; Zheng et al., 2013)

PRMT5 substrates	Biological role	Citations
FGF-2, RPS10, G3BP1	Regulation of Translation	(Bruns et al., 2009; Ren et al., 2010; Tsai et al., 2016)
MBP, GM130	Cellular integrity	(Branscombe et al., 2001; Zhou et al., 2010)
SmD3, CF 1(m)68	Post transcriptional regulation	(Martin et al., 2010; Pesiridis et al., 2009)
Piwi proteins, Rad9	Genome stability	(He et al., 2011; Vagin et al., 2009)
SHP, TDH, SREBP1	Metabolic regulation	(Han et al., 2013; Kanamaluru et al., 2011; L. Liu et al., 2016)
SHP, TDH, SREBP1	Metabolic regulation	(Han et al., 2013; Kanamaluru et al., 2011; L. Liu et al., 2016)

results in increased cell death due to elevated expression of apoptotic genes (Jansson et al., 2008).

PRMT5 methylates oncoprotein, N-MYC, and enhance the stability and oncogenic potential in neuroblastoma cells. Furthermore, inhibition of PRMT5 results in reduced levels of N-MYC protein leading to enhanced apoptosis of these cells (Park et al., 2015).

PRMT5-induced methylation of the NF-κB p65 subunit enhances its DNA binding activity and target gene expression (Wei et al., 2013). In contrast, PRMT5-dependent methylation of transcription factor E2F-1 targets it to ubiquitination, and hence reduces its ability to induce apoptosis of tumor cells during DNA damage (Cho et al., 2012).

Apart from transcription, PRMT5 also controls other cellular processes by methylating a variety of proteins such as ribosomal protein S10, golgin GM130, and rapidly accelerated fibrosarcoma (RAF) protein kinase. For example, PRMT5-dependent methylation of the N-terminal arginine residues of Golgi-associated protein, golgin GM130 is critical for the formation and adequate linking of Golgi ribbons in the Golgi apparatus (GA). However, PRMT5 knockdown results in fragmentation and aberrant tethering of GA ribbons, indicating that PRMT5 is involved in the maintenance of GA architecture (Zhou et al., 2010). PRMT5 interacts with and methylates the C-terminal arginine residues, Arg158 and Arg160 of RPS10 protein, an integral component of the 40S ribosomal subunit complex. Methylation of RPS10 protein is required for its localization into the granular component (GC) region of nucleolus, and effective interaction with another ribosome assembly protein, nucleophosmin/B23, thus favors an adequate assembly of 40S and 60S ribosome subunits, during ribosome biogenesis, resulting in efficient translation of mRNAs. However, studies using methylation mutant form of RSP10 showed the methylation deficient RSP10 is unable to localize to GC region and interact with B23, and fails to assemble into ribosomes. In addition, lack of methylation reduced the stability of the mutant protein, subjecting it for proteasomal degradation, highlighting the fact that PRMT5-mediated methylation is an essential event during ribosome biogenesis (Ren et al.,

2010). PRMT5 modulates RAS-ERK1/2 pathway activation by reducing the kinase activity of RAF family protein, CRAF that phosphorylates ERK1/2 proteins. Here, PRMT5-mediated methylation of R563 of CRAF protein reduces its stability, and hence, decreases its catalytic activity, resulting in diminished ERK1/2 signal amplitude (Andreu-Perez et al., 2011).

2.2.3 Role of PRMT5 during embryonic life

PRMT5 plays an important role during embryonic development. In mice, maternally inherited cytosolic PRMT5 in the oocyte translocates into the nucleus to initiate cellular differentiation events, and its cellular localization fluctuates dynamically during different stages of embryonic development (Tee et al., 2010). In embryonic stem (ES) cells, cytoplasmic PRMT5 associates with STAT3 and suppresses transcription of ES cell differentiation genes, *FGF5*, *GATA6*, *LHX1*, *FOX2, HOXA3*, *HOXA7* and *HOXD9* to maintain embryonic pluripotency. Moreover, PRMT5 knockdown in ES cells leads to down-regulation of key pluripotency genes including *OCT4*, *NANOG* and *REX1*, whereas complete loss of PRMT5 results in aberrant growth and subsequent lethality in mice (Tee et al., 2010). Collectively, these results demonstrate that PRMT5 has an important role in maintaining pluripotency during embryonic life of mice.

The role played by PRMT5 during human embryonic development is not studied extensively; however, a study by Gkountela et al. (2014) demonstrated that depletion of PRMT5 in human ES cells does not alter the transcription of key pluripotent genes including *OCT4*, *SOX2*, and *NANOG*, indicating that PRMT5 does not play an important

role in regulating stem cell pluripotency in humans. Nevertheless, PRMT5 is important during embryonic life in humans as it promotes proliferation of ES cells during their self-renewal by inducing transcriptional repression of cyclin-dependent kinase inhibitor, $p57^{KIP2}$, which in turn promotes G1 to S progression.

2.2.4 Role of PRMT5 during neurogenesis

Various studies have shown that PRMT5 plays an essential role during neurogenesis. PRMT5 helps to maintain stemness of neural stem cells in the cerebral cortex of the mouse brain by associating with Schwann Cell Factor 1 (SC1)/PRDM4, a Positive Regulatory Domain (PRDM) family transcription factor that modulates cell cycle progression. Binding of PRMT5 with SC1 induces down-regulation of pro-mitotic genes including *CYCLIN B1* and *BUB1b*, which suppresses differentiation of neuronal stem cells. However, during neuronal stem cell differentiation, expression of SC1 and PRMT5 decreases with a concomitant increase in *CYCLIN B1* expression (Chittka et al., 2012). In addition, PRMT5 controls survival of neuronal stem cells and protects them from apoptosis. Depletion of PRMT5 in the central nervous system (CNS) of *Nestin-Cre* transgenic mice leads to abnormal CNS development ensued by postnatal lethality due to increased apoptosis of neural stem and progenitor cells (NPCs). In this scenario, PRMT5 depletion leads to reduced methylation of Sm proteins, resulting in abnormal splicing of mRNAs, especially for proteins involved in cell cycle progression of NPCs. *MDM4* mRNA is one such mRNA, which undergoes alternative splicing due to aberrant spliceosomal processing. The resulting *MDM4S* mRNA is short-lived and consequently contributes to p53 activation and increased cell death (Bezzi et al., 2013).

Furthermore, a work by Huang et al. (2011) showed that PRMT5 levels gradually increase during postnatal brain development, especially during active myelination of neurons. In their systematic approach, the authors showed that PRMT5 gets localized in the nuclear compartment of myelinating oligodendrocytes to promote their differentiation. In this situation, PRMT5 suppresses expression of transcription regulators that inhibit oligodendrocyte differentiation including inhibitors of differentiation/DNA binding (Id), *Id2* and *Id4* by conserving the CpG methylation status at their promoters. Furthermore, PRMT5 knockdown reduces expression of differentiation inducers such as *SOX10* and *NKX2.2*, indicating that PRMT5 positively controls their expression in oligodendrocyte progenitor cells to induce cell differentiation (Huang et al., 2011).

In agreement with these results, Scaglione et al. (2018) further confirmed that PRMT5 has a major role during differentiation and developmental myelination of oligodendrocytes. PRMT5 levels are upregulated and mostly localized to the cytosolic compartment in proliferating oligodendrocyte progenitor cells (OPCs) as compared to differentiating OPCs, where its levels are reduced and mostly restricted to the nuclear compartment. In mice, conditional ablation of PRMT5 in OPCs leads to reduction in number of oligodendrocytes and results in severe hypomyelination of the brain on post-natal day 14, followed by early mortality. Mechanistically, PRMT5 ablation leads to aberrant differentiation of OPCs, followed by an activation of p53-dependent apoptosis, which eliminates OPCs in the brain (Scaglione et al., 2018).

The mechanism by which PRMT5 regulates oligodendrocyte myelination and maturation

was further investigated by Calabretta et al. (2018). In this study, the authors showed that depletion of PRMT5 in *PRMT5*$^{FL/FL;\text{Olig2Cre}}$ mice results in abnormal brain development associated with marked hypomyelination and a reduced number of mature oligodendrocytes (OL), leading to early post-natal mortality. Further investigation to understand the molecular mechanism by which PRMT5 loss elicits these dramatic physiological changes showed that there is reduced expression and plasma membrane localization of PDGFRα in oligodendrocytes. PRMT5 operates by rescuing PDGFRα from ubiquitination through interaction with and methylation of R554 residue, which is located in the Casitas B-lineage Lymphoma (Cbl) E3 ubiquitin ligase binding site of the protein. Elevated levels of PDGFRα promote proliferation and self-renewal of OPCs. However, during oligodendrocyte differentiation, PRMT5 levels fall, reducing R554 symmetric methylation of PDGFRα. This results in exposition of Cbl E3 ligase binding site, and increased proteasomal degradation of PDGFRα, which consequently induces differentiation of OPCs. Collectively, these findings indicate that tight regulation of PRMT5 levels is crucial for normal development of the central nervous system.

2.2.5 Role of PRMT5 during germ cell development, hematopoiesis and immune response

During mouse embryonic development, PRMT5 protein levels rise in germ cells immediately following sex determination, and depletion of PRMT5 causes significant cell death and abnormal germline growth in testes. Furthermore, conditional PRMT5 knockdown in testes of PRMT5$^{\Delta/f;\text{STRA8-Cre}}$ transgenic mice results in downregulation of meiosis-associated genes, *STRA8*, *SPO11*, *RAD51*, *SEP1*, *SEP3*, *DME1*, and *REC8*,

leading to meiotic arrest and loss of germ cells during spermatogenesis. These findings show that PRMT5 plays a key role during spermatogenesis by regulating expression of meiosis-associated genes (Wang et al., 2015).

PRMT5 has shown to play a key role during adult-hematopoiesis. PRMT5 depletion in hematopoietic stem and progenitor cells (HSPCs) of 2-month-old *Mx1Cre*$^{+}$ *Prmt5*$^{fl/fl}$ mice leads to bone marrow aplasia, pancytopenia and reduced thymus mass within 15 days of the Cre induction. FACS analysis showed that PRMT5 depletion results in significant loss of hematopoietic progenitor cells, reduction in erythroid differentiation in bone marrow, and aberrant accumulation of thymocytes in thymus. Although PRMT5 loss induced initial transient expansion of hematopoietic stem cells, functional analysis indicated that these cells were functionally defective. Further analysis to understand the mechanism through which PRMT5 maintains HSPCs population showed that it promotes G1/S transition in these cells. PRMT5 also promotes cytokine-activated JAK/STAT and AKT/PI3K signaling in HSPCs by inducing the expression of cell surface cytokine receptors (Liu et al., 2015).

Work by Tsutsui et al. (2013) informed that PRMT5 is involved in regulating the immune system via modulation of expression of key genes of the immune response program. Under normal conditions, PRMT5 represses the expression of C/EBPβ target genes including *TNF-α* and *IL-2*, which are involved in acute phase immune response. In this instance, PRMT5 associates with its binding partner MEP-50 and cyclin dependent kinases, CDK8

and CDK9, and is recruited to the promoter of C/EBPβ target genes, where it suppresses their expression by promoting H4R3 symmetric dimethylation and DNMT3A recruitment. These studies indicate that PRMT5 regulates development of germ cells, population of hematopoietic stem and progenitor cell, and elicitation of immune response during adult life by regulating the expression of distinct sets of gene in each context.

2.2.6 PRMT5 and cancer

Several studies have shown that elevated PRMT5 activity promotes cellular transformation and enhanced tumor growth. PRMT5 expression is increased in a wide variety of cancer cells including glioblastoma, melanoma, non-small cell lung carcinoma, lymphoma and leukemia cells (Banasavadi-Siddegowda et al., 2017; Nicholas et al., 2013; Pal et al., 2007; Shilo et al., 2013). An early study by Pal et al. (2004) showed that PRMT5 overexpression induces transformation of NIH3T3 cells by directly suppressing the expression of tumor suppressor genes including *ST7* and *NM23*. A similar finding was reported by Wei et al. 2012, which showed that ectopic expression of PRMT5 induces enhanced growth of NIH3T3 cells by elevating the levels of positive cell cycle regulators including CYCLIN D1, CYCLIN D2, CYCLIN E1, CDK4 and CDK6, reducing expression of negative cell cycle regulator such as retinoblastoma (RB) proteins, and enhancing PI3K/AKT signaling, indicating that PRMT5 induces tumorigenicity by augmenting proliferative signals. Another study by Alinari et al. (2015) further supported the fact that PRMT5 induces cellular transformation by showing a rapid increase in PRMT5 protein level in 4 to 8 days after EBV infection of normal B lymphocytes, which coincides with cellular immortalization. Furthermore, our recent study showed that PRMT5 promotes growth and

proliferation of three different types of non-Hodgkin's lymphoma cells by activating WNT/β-CATENIN and AKT/GSK-3β proliferative signaling (Chung et al., 2019). Collectively, these studies imply that elevated expression of PRMT5 promotes transformation and growth of cancer cells by augmenting different proliferative signaling pathways.

Extensive studies that focused on understanding the molecular mechanism by which PRMT5 drives tumorigenic growth have shown that PRMT5 achieves enhanced growth of tumor cells via two mechanisms: 1) In the cytosol, PRMT5 methylates arginine residues of various transcription factors, which can translocate into the nucleus and control expression of their respective target genes, and 2) In the nucleus, PRMT5 regulates expression of its target genes by directly binding to their promoters and facilitating recruitment of transcriptional activators and repressors (Fig. 3)

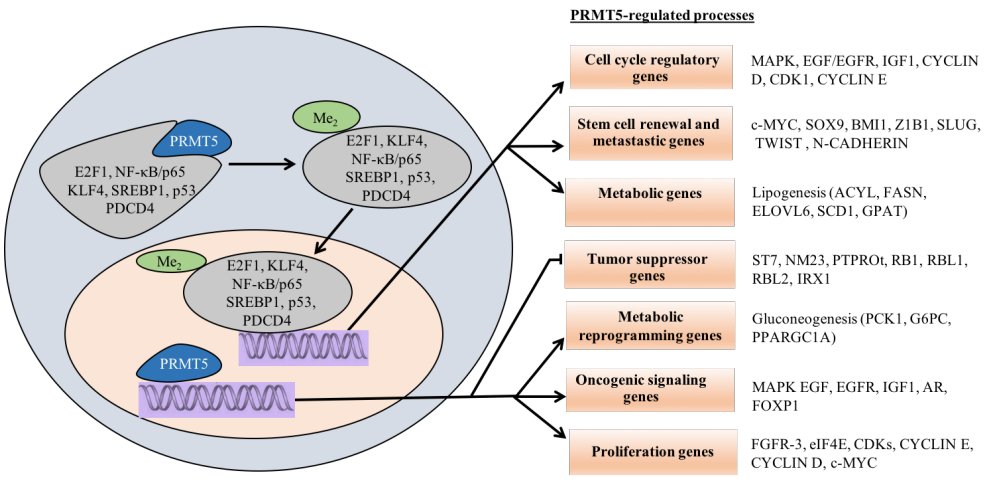


Figure 3: PRMT5 regulated processes.

Cytosolic PRMT5 induces arginine methylation of various transcription factors, which translocate into the nucleus and regulate expression of their respective target genes. Nuclear PRMT5 is also directly recruited to the promoter regions of specific target genes to enhance cellular proliferation and tumorigenesis (Shailesh et al., 2018).

PRMT5 suppresses expression of tumor suppressor genes including *Suppressor of Tumorigenicity 7* (*ST7*) and *Protein Tyrosine Phosphatase Receptor-type O* (*PTPRO*) by binding to their promoter and inducing H3R8 and H4R3 dimethylation in lymphoma and leukemia (Alinari et al., 2015; Wang et al., 2008). PRMT5 also induces transcriptional downregulation of RB family tumor suppressor genes including *RB1*, *RBL1* and *RBL2* through binding to their promoter and inducing symmetric methylation of H3R8 and H4R3 in chronic lymphocytic leukemia cells (Wang et al., 2008). However, only RBL2 protein levels are reduced in these cells compared to normal B cells. Further analysis showed that reduced expression of microRNAs that target 3′-UTR of RB1 and RBL1 mRNAs, resulted in enhanced translation and elevated protein expression (Wang et al., 2008). In a follow-up study, Chung and co-workers (2013) showed that PRMT5 is able to inactivate RB1 protein by inducing phosphorylation of the protein at multiple sites including Ser-780, Ser-795, Ser-807/Ser-811 through activation of the CYCLIN D1-CDK4/6 complex. The same study also showed that PRMT5-mediated inactivation of RB1 and RBL2 leads to induced expression of polycomb repressor complex proteins (PRC2), which eventually induce transcriptional repression of pro-apototic genes including *CASP10*, *DAP1*, *HOXA5* and *HRK* in three different types of non-Hodgkin lymphoma cells. However, shRNA-mediated

downregulation of PRMT5 induces apoptosis of these cells by inducing transcriptional derepression of RBL2 and dephosphorylation of RB1, which trigger reduced PRC2 expression and transcriptional derepression of pro-apototic genes (Chung et al., 2013).

In gastric cancer cells, PRMT5 promotes cell proliferation by suppressing the expression of a different tumor suppressor gene, *Iroquois Homeobox 1* (*IRX1*), by binding to its promoter region and recruiting DNMT3A, which consequently leads to promoter hypermethylation. PRMT5 knock-down induces expression of *IRX*1 and leads to reduced growth and metastasis of gastric cancer cells *in vitro* (Liu et al., 2018). A very recent work from our group has demonstrated that PRMT5 suppresses expression of tumor suppressors, AXIN2 and WIF1, which serve as inhibitors of WNT/β-CATENIN proliferative signaling in three different types of non-Hodgkin's lymphoma cells (Chung et al., 2019). PRMT5 binds to the promoter region of *AXIN2* and *WIF1*, and hypermethylates promoter H3R8 and H4R3 histones, leading to transcriptional repression. As a consequence, WNT/β-CATENIN proliferative signaling becomes aberrantly activated, leading to elevated expression of WNT/β-CATENIN downstream targets, *CYCLIN D1*, *c-MYC* and *SURVIVIN* (Chung et al., 2019).

In contrast to inducing transcriptional repression of tumor suppressor genes, PRMT5 can also promote transcriptional activation of oncogenes in several cancer cell types. PRMT5 increases transcription of proto-oncogenes including *eukaryotic elongation Initiation Factor-4E (eIF4E)* and *Fibroblast- derived Growth Factor Receptor-3 (FGFR3)*, by

directly binding and inducing H3R8 and H4R3 symmetric dimethylation marks in their promoter region in colorectal cancer cells. However, knockdown of PRMT5 in these cells resulted in reduced methylation of H3R8 and H4R3, and lowered expression *eIF4E* and *FGFR3*, which was associated with reduced cell growth (Zhang et al., 2015). Another study by Deng et al. (2017) showed that PRMT5 expression is elevated in prostate cancer and positively correlated with expression of androgen receptor (AR). Using immunoprecipitation assays, the authors showed that PRMT5 associates with SP1, and the BRG1-based hSWI/SNF remodeler, and is recruited to the promoter region of *AR* gene, where it induces H4R3 symmetric dimethylation and activates *AR* expression in LnCaP prostate cancer cells. In addition, inducible PRMT5 knockdown leads to reduced expression of *AR* and decreased cell growth in AR-positive cells *in vitro* and in mice xenograft tumors. Similar to the knockdown experiment, pharmacological inhibition of PRMT5 halted growth of AR-positive cells in an AR-dependent manner *in vitro*.

The role of PRMT5 in breast cancer stem cell (BCSCs) function was also investigated and results showed that elevated levels of PRMT5 upregulate expression of transcription factor, *forkhead box protein 1 (FOXP1)*, which is involved in normal and stem cell function. Here, PRMT5 binds to the promoter region of *FOXP1* and methylates H3R2, which serves as a recruitment epigenetic mark for the WDR5 subunit of SET1/MLL methyltransferase. Consequently, H3K4 becomes methylated and results in FOXP1 transcriptional activation. However, deactivation of PRMT5 enzymatic activity using GSK591 inhibitor or interrupting WDR5 and SET/MLL1 interaction leads to a substantial reduction in

methylated H3K4 level as well as *FOXP1* expression. The study also showed that PRMT5 knock-down diminishes the proliferative and self-renewing ability of breast cancer cells *in vitro* and *in vivo*. The study also indicated that tumors excised from NSG (NOD/Scid/IL-2Rγnull) mice injected with a PRMT5-specific shRNA had a less aggressive and more differentiated phenotype as compared to their control littermates. Similar to the results of PRMT5 knockdown, *FOXP1* knockdown also resulted in reduced growth of breast cancer cells *in vitro* as well as in xenograft tumors, indicating that PRMT5 promotes tumorigenicity of breast cancer by regulating expression of *FOXP1* (Chiang et al., 2017). These studies indicate that elevated levels of PRMT5 promotes cancer growth by altering expression of a variety of genes in a context dependent manner.

PRMT5-mediated methylation of several non-histone proteins has been shown to play an important role in carcinogenesis. A systematic study by Wan et al. (2015) showed that PRMT5 regulates genome stability and survival of cancer cells by methylating KLF4, a major transcription factor that controls the expression of genes involved in cell-cycle progression, genome-stability, cell adhesion, metabolism, apoptosis and stem cell renewal. The mechanism used by PRMT5 here is that it associates with KLF4 protein and methylates R374, R376 and R377 residues, which inhibits VHL/VBC E3 mediated ubiquitination of KLF4 leading to enhanced stability of the protein. When PRMT5 is depleted or KLF4 is mutated, its ubiquitination increases leading to a significant drop in its protein level. The same study also demonstrated that overexpression of KLF4 in breast cancer cells including MCF7 and MCF10A promotes expression of *CYCLIN D2*, *CYCLIN*

E1, *CDK1*, *MAPK*, *IGF1* and *EGF/EGFR* as well as genes involved in stem cell renewal and metastasis, which include *c-MYC*, *SOX9*, *Z1B1*, *BM1*, *SLUG*, *TWIST* and *E-CADHERIN*. A further investigation by the same group showed that PRMT5 and KLF4 protein expression is substantially elevated in clinical samples of aggressive breast tumor tissues compared to the adjacent normal tissues (Hu et al., 2015). These findings suggest that PRMT5 controls cell survival, metastasis and stem-cell renewal of cancer cells by modulating stability of KLF4 (Hu et al., 2015).

2.3 WNT/β-CATENIN signaling

2.3.1 Discovery of WNT protein

WNT1 was discovered by Nusse and Varmus in 1982 who named it INT-1, because this locus was the preferred site for insertion of DNA from Murine Mammary tumor virus in mouse bearing viral-induced mammary tumors (Nusse & Varmus, 1982). Later studies in fruit flies showed that wingless (*WG*), a gene that regulates polarity of embryo segments during larval growth is a *Drosophila* homolog of Int-1 (Rijsewijk et al., 1987). More studies confirmed that INT-1 is a member of the evolutionarily conserved gene family that is now referred to as WNT (wingless-type mouse mammary tumor virus integration site) gene family. 19 WNT genes have been identified in mammals including humans till date (Clevers & Nusse, 2012).

2.3.2 Canonical WNT/β-CATENIN signaling

WNT ligands belong to a large family of secretory proteins of 40 kDa in size that contain conserved cysteine-rich domains (Tanaka et al., 2002). Binding of WNT proteins to their cell membrane anchored receptors activates a group of downstream signaling modules

including the canonical WNT/β-CATENIN, noncanonical planar cell polarity (WNT/PCP), and WNT/Ca $^{2+}$pathways.

WNT/β-CATENIN signaling is an evolutionarily conserved pathway that plays a prime role during embryonic and adult life (Logan & Nusse, 2004). WNT/β-CATENIN regulates various processes during embryonic development such as differentiation of embryonic stem cells, formation embryonic body axis, and morphogenesis of various tissues that originate from different germ layers (Davidson et al., 2012; Lan et al., 2006; Petersen & Reddien, 2009). In adult life, WNT/β-CATENIN signaling is involved in maintenance of the stem cell niche and promotes their differentiation during tissue and organ regeneration (Chen et al., 2007; Fevr et al., 2007). Germline mutations of genes, which encode components of WNT/β-CATENIN signaling lead to abnormal embryonic development and several hereditary diseases, whereas somatic mutations and epigenetic alteration of these genes during adult life lead to the onset of cancer (Clevers, 2006).

Canonical WNT/β-CATENIN signaling is initiated by binding of WNT ligand to the membrane-bound Frizzled receptor (FZD), in cooperation with co-receptor, LDL receptor-related protein family receptors (LRP) called LRP5/6 (Bhanot et al., 1996; Tamai et al., 2000) (Fig. 4). Next, the C-terminal domain of the ligand bound Frizzled receptor directly interacts with cytoplasmic Dishevelled protein (DSH/DVL) and promotes recruitment of cytosolic glycogen synthase kinase 3beta (GSK-3β) to the plasma membrane (Wong et al., 2003).

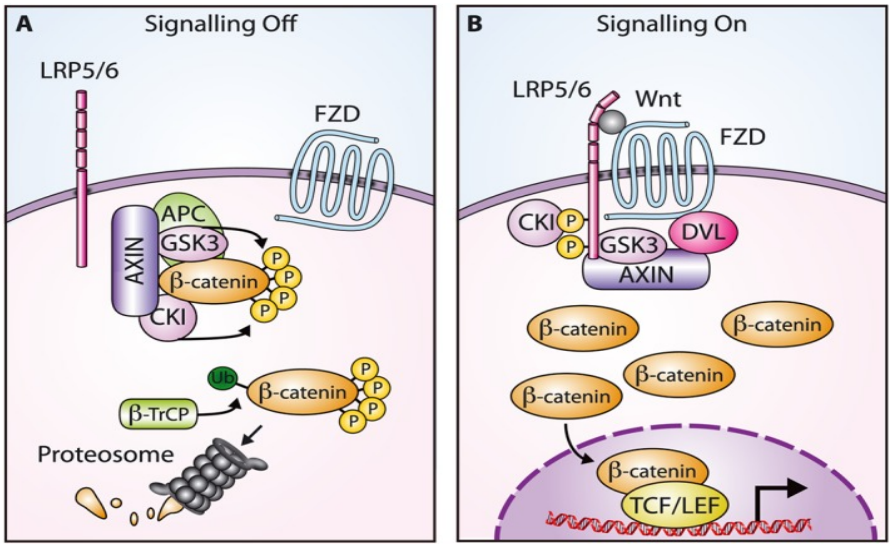


Figure 4: WNT/β-CATENIN signaling overview

A) In the absence of WNT ligand (signaling off), β-CATENIN binds to cytosolic destruction complex composed of AXIN, GSK-3β, APC and CK1, and is phosphorylated by CK1 and GSK-3β. Phosphorylated β-CATENIN subsequently undergoes degradation after its ubiquitination by the E3 ubiquitin ligase, β-TrCP. *B)* In the presence of WNT protein (signaling on), the WNT ligand binds to its receptor, FZD, which complexes with the LRP5/6 co-receptor. Ligand bound Frizzled receptor recruits DVL and promotes binding of GSK-3β and CK1, which phosphorylate LRP5/6 and favors AXIN recruitment. This prevents formation of the cytosolic destruction complex and phosphorylation of β-CATENIN, which in turn increases the levels of cytosolic β-CATENIN. Accumulated β-CATENIN then translocates into the nucleus, combines with transcription factors, TCF and LEF, and promotes WNT/β-CATENIN target gene transcription (Kagey & He, 2017).

The membrane-associated GSK-3β phosphorylates LRP5/6 and triggers casein kinase 1 (CK1) to induce further phosphorylation on LRP5/6. Frizzled associated DVL binds to Axis inhibitory proteins (AXIN1 and AXIN2) through the DIX domain and favors binding of AXINs to the cytosolic tail of phosphorylated LRP5/6 (Cliffe et al., 2003; Zeng et al., 2005). Consequently, levels of the cytosolic destruction complex, consisting of AXINs (AXIN1 and AXIN2), CK1, APC, and GSK-3β, decrease, which leads to reduced phosphorylation of β-CATENIN and prevents its degradation. Subsequently, cytosolic β-CATENIN levels rise and trigger its translocation into nucleus where it complexes with TCF (T-cell factor) and LEF (lymphoid enhancer factor) and binds to the promoter region of target genes, *c-MYC*, *CYCLIN D1*, and *SURVIVIN*. Upon binding, several chromatin remodelers such as BRG1 and CBP/p300 are recruited to induce transcription (Barker et al., 2001; Brunner et al., 1997; Kramps et al., 2002; Ma et al., 2005). In the absence of β-CATENIN, TCF and LEF form complex with transducing-like enhancer protein (TLE/Groucho) and recruit histone deacetylase enzymes to repress expression of target genes (Cavallo et al., 1998; Chen, et al., 1999; Kagey & He, 2017).

During WNT off state, the cytosolic destruction complex keeps the level of β-CATENIN low by binding and phosphorylating it (Kimelman & Xu, 2006). AXIN1 serves as a core scaffolding protein in the destruction complex that binds to other components through different domains. GSK-3β binds to the central domain of AXIN1 and phosphorylates AXIN1 at Thr609 and Ser614 residues, which in turn promotes attachment of β-CATENIN to AXIN1 (Jho et al.,1999). APC enhances AXIN1 multimerization by associating with it

through AXIN1:APC interaction domain (Pronobis et al., 2015). The second kinase, CK1 exhibits multiple interactions with various sites in the central domain of AXIN1 and catalyzes phosphorylation of destruction complex bound β-CATENIN at Ser45, that favors further phosphorylation of Ser33, Ser37 and Thr41 sites at N-terminal motif of β-CATENIN by GSK-3β (Amit et al., 2002; Hagen et al., 2002). Phosphorylation of β-CATENIN at Ser33 and Ser37 promotes binding of E3 ubiquitin ligase, β-TrCP, resulting in proteasomal degradation of β-CATENIN (Latres et al., 1999; Spiegelman et al., 2000).

2.4 Natural antagonists of the WNT/β-CATENIN pathway

WNT/β-CATENIN signaling is tightly regulated by several endogenous inhibitors that abrogate signal transduction through various mechanisms (Fig. 5). Secretory inhibitors like WNT inhibitory factor 1 (WIF1) and secreted frizzled-related proteins (SFRPs) can associate with WNT ligand directly and interrupt its binding to membrane receptors (Zhan et al., 2017). Dickkopf (DKK) family proteins are another type of secretory antagonists that bind to WNT co-receptor LRP5/6 to inhibit the association of ligand bound Frizzled receptor to LRP5/6 (Kawano & Kypta, 2003). Elevated levels of destruction complex components such as AXINs inactivate WNT/β-CATENIN signaling by inducing β-CATENIN destruction (Nakamura et al., 1998). Reduced levels of WNT/β-CATENIN antagonists have been reported in a variety of cancers including gastrointestinal cancer, leukemia, melanoma and breast cancer, and contribute to enhanced tumor growth by enhancing WNT/β-CATENIN activation (Zhan et al., 2017).

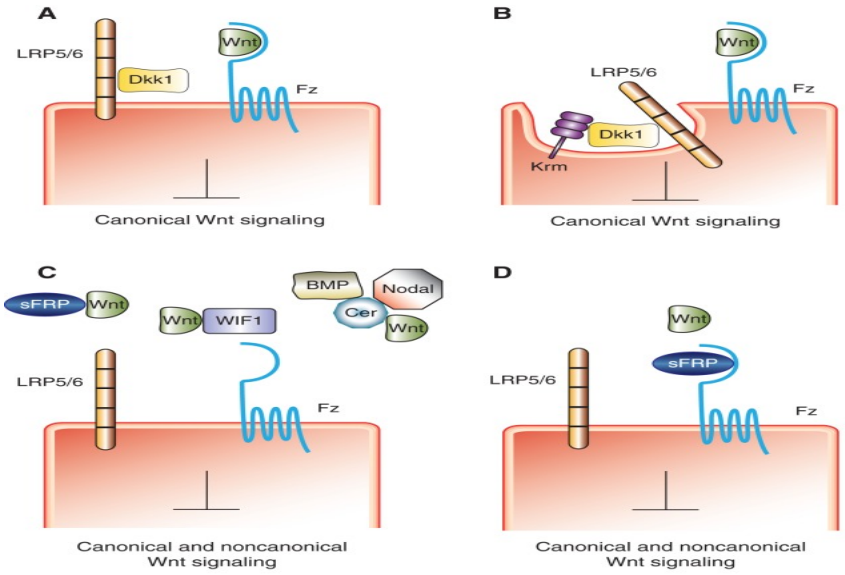


Figure 5. Antagonists of WNT pathway

(*A, B*) DKK1 can disrupt WNT signaling by binding to LRP5/6 and Kremen receptor in the plasma membrane and inducing endocytosis of LRP5/6. (*C, D*) SFRPs and WIF1 inhibit WNT signaling by binding to WNT ligand and disrupting binding of WNT and Frizzled receptor. SFRPs can also bind to Frizzled receptor and inhibit binding of WNT to Frizzled receptor (Cruciat & Niehrs, 2013).

2.4.1 Axis inhibitory (AXIN) proteins

The two isoforms of AXIN, AXIN1 and AXIN2, are known to work as scaffolding proteins in the cytosolic destruction complex and promote GSK-3β-mediated phosphorylation of β-CATENIN, which turns off WNT signaling. However, during WNT activation, both AXIN1 and AXIN2 dissociate from destruction complex, interact with Frizzled associated

DVL and bind to the cytoplasmic tail of phosphorylated LRP5/6. As a consequence, phosphorylation and subsequent degradation of β-CATENIN is inhibited, resulting in β-CATENIN accumulation in the cytoplasm, which can move into nucleus to promote transcription of WNT/β-CATENIN target genes (Gao et al., 2014). Hence, AXINs serve as endogenous antagonists of the WNT/β-CATENIN signaling cascade in the absence of WNT stimulation.

AXIN1 was initially discovered as a product of murine *Fused* gene locus, that regulates embryonic axis formation in the mouse embryo (Gluecksohn-Schoenheimer, 1949). Later, a study using Xenopus embryos showed that expression of AXIN1 inhibits secondary dorsal axis formation induced by WNT, DVL, and kinase-negative GSK-3β, indicating that AXIN1 has an inhibitory role in WNT/β-CATENIN signaling (Zeng et al., 1997). AXIN1 serves as a scaffolding protein in the cytosolic destruction complex by binding to various components of the WNT signaling cascade including APC, PP2A, GSK-3β, DVL and β-CATENIN through different domains. Binding of AXIN1 to GSK-3β facilitates β-CATENIN phosphorylation (Hinoi et al., 2000). On the other hand, association of AXIN1 with APC enhances its stability by promoting AXIN1 multimerization (Pronobis et al., 2015). AXIN1 interacts with CK1 through various sites in its central domain, catalyzes phosphorylation of destruction complex-bound β-CATENIN at Ser45, and promotes GSK-3β mediated phosphorylation of Ser33, Ser37 and Thr41 sites at N-terminal motif of β-CATENIN, which subsequently leads to proteasomal degradation of β-CATENIN (Amit et al., 2002; Hagen et al., 2002; Latres et al., 1999; Spiegelman et al., 2000).

Reduced expression of AXIN1 has been extensively linked with oncogenicity in various studies. Depletion of AXIN1 in *AXIN1*$^{fl/fl/Cre}$ mouse hepatocytes results in enhanced expression of WNT target genes, *c-MYC*, *AXIN2*, and *CYCLIN D1*, as well as a subset of genes that favor G2/M transition and cytokinesis. In addition, these mice show increased hepatocyte proliferation and develop liver tumors having features of hepatocellular carcinoma after 1 year, indicating a link between reduced expression of AXIN1 and hepatocarcinogenesis (Feng et al., 2012). Similarly, loss of endogenous AXIN1 expression due to genetic mutation or epigenetic modifications such as promoter hypermethylation and enhanced expression of microRNAs has been shown to activate WNT/β-CATENIN signaling and promote growth and metastasis of cancers such as non-small cell lung cancer, hepatocellular carcinoma, colorectal cancer and osteosarcoma (Chen et al., 2019; Picco et al., 2017; Salahshor & Woodgett, 2005; Xu et al., 2006).

Re-activation of AXIN1 has been shown to have a promising anti-tumor impact in various cancers. An early study by Satoh et al. (2000) showed that Adenovirus-mediated induction of AXIN1 in hepatocellular carcinoma and colorectal cancer cells, which have endogenously increased nuclear β-CATENIN levels due to mutations in AXIN1, reduced their growth and increased cell apoptosis. This implies that AXIN1 could serve as a viable therapeutic target in cancer treatment. Another recent independent study by Chen et al. (2019) showed that AXIN1 expression is downregulated in osteosarcoma due to elevated expression of its regulatory microRNA, miR-31-5p. However, reducing miR-31-5p

expression in osteosarcoma cell lines including HOS and U2OS results in AXIN1 upregulation and consequent downregulation of WNT/β-CATENIN signaling. Furthermore, these molecular changes were associated with reduced proliferation and metastasis of cancer cells *in vitro* and *in vivo*. Collectively, these findings indicate that modulation of AXIN1 expression may serve as a viable therapeutic strategy in cancer treatment

AXIN2 is another member of the AXIN family that shares structural similarity with AXIN1 by having several similar domains including the APC binding domain, β-CATENIN binding domain, and the DIX dimerization domain (Behrens et al., 1998). Functionally, like AXIN1, AXIN2 serves as a scaffolding protein in the destruction complex and promotes GSK-3β-mediated phosphorylation of β-CATENIN. However, *AXIN2*, unlike *AXIN1*, is a target gene of the WNT/β-CATENIN signaling cascade that is transcriptionally activated by β-CATENIN/TCF transcription factors during WNT activation. In addition, AXIN2 shows a weak interaction with DVL2 compared to AXIN1, and thus promotes degradation of cytosolic β-CATENIN, indicating that AXIN2 is a potent negative feedback repressor of WNT/β-CATENIN signaling (Leung et al., 2002).

Although, *AXIN2* is a target gene of the WNT/β-CATENIN pathway, its expression is reduced in a variety of cancers as a consequence of different epigenetic events such as promoter DNA hypermethylation and aberrant expression of microRNAs (miRs) that directly target the 3′-UTR region of *AXIN2* mRNA. As a result, WNT/β-CATENIN

signaling becomes hyperactivated in these cancers (Koinuma et al., 2006; Naghibalhossaini et al., 2012; Chen et al., 2019). For example, AXIN2 expression is reduced in colorectal cancer cells due to elevated levels of its direct functional target microRNAs, miR-103 and -107, which in turn favors β-CATENIN stabilization, resulting in prolonged activation of WNT/β-CATENIN pathway in these cells. As a consequence, these cells attain a stem-like phenotype with elevated expression of stem cell markers including *CD44* and *DCLK1*, indicating that miR-103/107 promote the stemness of colorectal cancer cells by downregulating AXIN2 expression (Chen et al., 2019).

In stark contrast, some studies have reported elevated expression of AXIN2 due to aberrant activation of WNT/β-CATENIN signaling in cancers such as hepatoblastoma and ameloblastoma. However, the mechanism through which elevated AXIN2 promotes oncogenicity of these cancers has not been studied yet (Koch et al., 2004; Wei et al., 2013; Wu et al., 2012). These findings indicate that AXIN2 exhibits a large variation in its expression pattern among different cancer subtypes, and careful investigation needs to be performed before targeting AXIN2 for cancer therapy.

2.4.2 Dickkopf (DKK) family proteins

The DKK family consists of a group of secretory proteins including DKK1-4, which are made of 255-350 amino acids and are endowed with two conserved cysteine-rich domains at the N- and C-terminal regions separated by a non-conserved linker region. DKK3 has an additional Soggy domain at N-terminal region (Krupnik et al., 1999). DKKs can antagonize WNT/β-CATENIN signaling through their direct interaction with the WNT co-receptor,

LRP5/6 and KREMEN proteins (Brott & Sokol, 2002; Krupnik et al., 1999; Mao et al., 2001). However, DKK2 and DKK3 proteins have been shown to activate WNT/β-CATENIN signaling in some cellular contexts (Nakamura et al., 2007; Xu et al., 2017).

DKK1 was first discovered as a secretory antagonist of WNT signaling that was required for the induction of head formation during Xenopus embryogenesis (Glinka et al., 1998). The human homologue of DKK1 was characterized by Fedi et al. (1999) in SK-LMS-1 leiomyosarcoma cells, and shown to be a 266 amino acid protein containing a signal peptide and two cysteine-rich domains. The same study also demonstrated that DKK1 when co-expressed with WNT2 in NIH3T3 cells could reverse the WNT2-mediated growth characteristics of these cells, and downregulate β-CATENIN levels, suggesting its antagonistic role in canonical WNT/β-CATENIN pathway (Fedi et al., 1999). Later studies that focused on investigating the mechanism of DKK1-mediated inhibition of WNT signaling revealed that DKK1 antagonizes WNT/β-CATENIN signaling by binding to its membrane receptor KREMEN2 and LRP5/6 to form a ternary complex that gets internalized and cleared by endocytosis, thus preventing association of WNT-bound Frizzled receptor with LRP5/6 co-receptor (Mao et al., 2002; Mao et al., 2001). *DKK1* is also a target gene of the WNT/β-CATENIN pathway that is upregulated upon WNT activation, suggesting its role as feedback repressor of WNT/β-CATENIN signaling (González-Sancho et al., 2005).

Although DKK1 is a target gene of WNT/β-CATENIN pathway, its expression is found to

be downregulated in neoplastic tissues as a consequence of various epigenetic events. For example, reduced expression of DKK1 due to promoter CpG hypermethylation is detected in the colon cancer cell lines and tumors of advanced stage colorectal cancers. However, exogenous expression of DKK1 in the colon cancer cell line DLD-1 reduces their growth and colony formation ability (Aguilera et al., 2006; González-Sancho et al., 2005). In gastric cancer, DKK1 is downregulated by its functional target miR-493, whereas forced expression of DKK1 in gastric cancer cells reverses miR-493 driven tumorigenicity by reducing growth, invasion and chemoresistance of these cells (Jia et al., 2016). Collectively, these studies show that DKK1 serves as a tumor suppressor gene and that restoration of DKK1 could also serve as a promising therapeutic strategy.

Enhanced accumulation of DKK1 as a consequence of aberrant WNT/β-CATENIN activation is observed in some cancers. Increased levels of DKK1 is observed in the serum and pancreas of pancreatic cancer patients as compared to the normal control group. Furthermore, patients with high serum DKK1 levels before surgery showed reduced post-surgical survival time compared to the patients with lower serum DKK1 level, indicating a potential oncogenic role of DKK1 in pancreatic cancer (Han et al., 2015). Similarly, elevated serum levels of DKK1 are associated with reduced survival time of multiple myeloma patients. However, depletion of DKK1 by siRNA or DKK1 neutralizing antibody (BHQ880) reduces the malignant behavior of multiple myeloma cells as evidenced by reduced proliferation and increased osteoblast differentiation and osteocalcin deposition (Feng et al., 2019). In hepatocellular carcinoma, elevated levels of DKK1 promote

metastatic events including migration and invasion. In this situation, DKK1 promotes the metastatic phenotype of cancer cells by enhancing β-CATENIN expression, thereby, inducing β-CATENIN target genes such as *c-MYC* and *MMP7*. Furthermore, DKK1 overexpression does not affect the levels of LRP6, highlighting the fact that DKK1-mediated upregulation of β-CATENIN is independent of canonical WNT signaling (Chen et al., 2013). These findings indicate that dysregulation in DKK1 expression plays an important role in carcinogenesis.

DKK2, was first identified as an activator of WNT signaling in *Xenopus* embryos as it upregulated expression of the WNT target gene, siamos, and stimulated WNT-mediated morphological changes such as axis duplication and microcephaly of embryos before and after midblastula transition, respectively. However, using Top-Flash reporter assay, the same study showed that DKK2 could inhibit WNT signaling similar to DKK1 in HEK293T cells, indicating that the role of DKK2 in WNT signaling is context-dependent (Wu et al., 2000). Later studies focused on understanding the mechanism by which DKK2 regulates WNT signaling showed that like DKK1, DKK2 forms a ternary complex with KREMEN2 and LRP6, and inhibits WNT signaling (Mao & Niehrs, 2003).

DKK2 has been shown to have tumor suppressor activity in a variety of cancers such as renal, ovarian, and breast cancers, where its expression is reduced due to aberrant epigenetic modifications (Hirata et al., 2009; Mu et al., 2017; Zhu et al., 2012). DKK2 is downregulated in renal cell carcinoma due to a combination of epigenetic modifications

such as increased methylation and dimethylation of CpG clusters and H3K9, respectively, reduced acetylation of H3 and H4 histones, and diminished H3K4 dimethylation in the promoter region. However, re-expression of DKK2 reduces proliferation and colony formation, induces G1 to S cell-cycle arrest, and enhances apoptosis of renal cancer cells *in vitro*. These changes are associated with reduced expression of WNT/β-CATENIN target gene, *CYCLIN D1*, as well as up- and down-regulation of BAX and BCL2 proteins respectively, indicating that DKK2 regulates multiple pathways in renal cell carcinoma (Hirata et al., 2009). A similar observation was reported in breast cancer, where *DKK2* was found to be downregulated due to promoter DNA hypermethylation. Here, exogenous expression of *DKK2* suppressed β-CATENIN levels and reduced transcription of WNT target genes, *CYCLIN D1* and *c-MYC*. In the same study, the authors also showed that DKK2 re-expression induces G0/G1 cell cycle arrest and inhibits proliferation, colony formation, and migration, which are all associated with increased apoptosis of breast cancer cells (Mu et al., 2017).

Elevated expression of microRNAs that directly target *DKK2* mRNA is also known to reduce *DKK2* expression in some cancers. A study investigating the biological role of miR-222 in the progression of glioma showed that miR-222 levels are abruptly elevated in glioma cell lines (U251, U87, SHG44 and A172) compared to normal human astrocyte cells (HA). Using Target scan algorithm, the authors found that miR-222 could target the 3'-UTR region of *DKK2* mRNA to reduce its stability. Furthermore, treating U251 and U87 cells with miR-222 antagomir resulted in DKK2 restoration, and reduced protein

levels of β-CATENIN and c-MYC in glioma cell lines, confirming the inhibitory role of miR-222 on DKK2 expression in glioma. Finally, siRNA mediated inhibition of miR-222 in U87 reduced malignancy of glioma cells as evidenced by reduced tumor growth *in vivo* (Li et al., 2013). Taken together, these findings indicate that DKK2 downregulation plays a key role in tumorigenesis, and that its restoration could serve as a potent inhibitor of growth of cancer cells with low DKK2 endogenous levels.

An early study by Mao et al. (2002) showed that unlike other DKKs, DKK3 is unable to bind to KREMEN and LRP co-receptors, indicating that DKK3 does not antagonize WNT/β-CATENIN signaling. Later, Caricasole et al. (2003) demonstrated that when DKK3 is cotransfected with LRP5 or LRP6, it can antagonize WNT7A-induced TCF-activity in rat pheochromocytoma cell line PC12. Extensive studies have confirmed the inhibitory role of various DKK3 in WNT/β-CATENIN signaling using a variety of approaches. For example, ectopic expression of DKK3 reduces the nuclear accumulation of β-CATENIN as well as expression of WNT target genes, *CYCLIN D1* and *c-MYC* in lung cancer cells (Yue et al., 2008). A recent study *in silico* has shown that DKK3 could interact with LRP and KREMEN with comparable binding energies to inhibit WNT/β-CATENIN signaling (Mohammadpour et al., 2016).

DKK3 is ubiquitously expressed in normal tissues, whereas its levels are found to be significantly downregulated in virtually all types of tumors (Zhang et al., 2010; Veeck & Dahl, 2012). Different studies focusing on understanding the molecular mechanism of

DKK3 downregulation in cancer cells have shown that promoter DNA hypermethylation is a common feature for its reduced expression (Veeck & Dahl, 2012). However, in some cancer subtypes such as renal cell carcinoma, rather than DNA hypermethylation, reduced trimethylation of H3K4 in the promoter region contributes to the downregulation of *DKK3*. Furthermore, exogenous expression of DKK3 reduces proliferation of renal carcinoma cells and induces their death by increasing expression of pro-apoptotic genes such as *p21*, *MDM-2* and *PUMA* (Ueno et al., 2011). In neuroblastoma, DKK3 is downregulated due to elevated levels of N-MYC, which promotes expression of microRNAs including miR-19b and miR-92a that directly bind to the 3′-UTR region on DKK3 mRNA and reduce its stability and translation (De Brouwer et al., 2012). These results suggest that a variety of mechanisms are involved in downregulation of DKK3 in cancer cells.

DKK4 is the smallest and least studied member of the DKK family protein that has been implicated in many types of cancers. Similar to DKK1, DKK4 can inhibit WNT induced secondary axis formation in Xenopus embryos, indicating its inhibitory role in WNT/β-CATENIN signaling (Krupnik et al., 1999). Interestingly, a study by Bazzi et al. (2007) has shown that DKK4 levels are upregulated upon activation of WNT/β-CATENIN signaling in 293T cells. By ChIP assay, the authors showed that β-CATENIN and LEF1 can bind to the promoter region of *DKK4* gene to induce its expression, indicating that *DKK4* is a target gene of WNT/β-CATENIN signaling, which can also serve as a negative feedback repressor of the pathway.

DKK4 shows differential expression patterns between different cancer subtypes, with some showing its upregulation while others having reduced DKK4 levels compared to their normal tissues. A study by Bahes et al. (2009) reported that the mRNA level of *DKK4* is reduced in the majority of colorectal cancer cell lines compared to the normal colon epithelial cells. In agreement with this, tumors from the majority of colorectal cancer patients show reduced *DKK4* mRNA expression compared to their respective adjacent normal colon tissues. Furthermore, exogenous expression of *DKK4* in HCT116 colorectal cancer cells inhibits basal as well as WINT3A-induced β-CATENIN/TCF activity. Functional analysis of DKK4 overexpressing HCT116 cells also showed that DKK4 reduces their colony forming ability, and induces G0/G1 cell cycle arrest. Treatment of colorectal cancer cells, DLD1, HTC116 and LoVo with histone deacetylase inhibitor, Trichostatin A, induced significant expression of DKK4, indicating that histone deacetylation is key in silencing DKK4 in colorectal cancer.

Another study investigating the functional role of DKK4 in liver cancer showed that its levels are reduced in a panel of hepatocellular carcinoma cell lines (Fatima et al., 2012). Similarly, immunohistochemical analysis of hepatocellular carcinoma clinical samples showed a reduced level of DKK4 and enhanced β-CATENIN accumulation in tumor tissues compared to adjacent normal tissues. Exogenous expression of DKK4 in hepatocellular carcinoma cell lines, PLC/PRF/5 and MHCC97L, reduced endogenous levels of β-CATENIN and inhibited β-CATENIN responsive luciferase activity, thereby reducing the expression of WNT/β-CATENIN downstream target gene, *CYCLIN D1*.

DKK4 overexpression also results in reduced tumor growth, migration, and colony formation. More interestingly, *in vivo* studies showed that tumors obtained from nude mice injected with DKK4 overexpressing MHCC97L cells were smaller in size and had reduced levels of β-CATENIN and CYCLIN D1 compared to control mice bearing MHCC97L cells with empty vector. These results indicate that DKK4 downregulation is an important event that leads to enhanced WNT/β-CATENIN activity in hepatocellular carcinoma (Fatima et al., 2012).

Upregulation of DKK4 is reported in various types of cancers including colon cancer, ovarian cancer and renal cancer, where it promotes tumor growth and metastasis (Hirata et al., 2011; Pendas-Franco et al., 2008; Wang et al., 2017). Since, DKK4 is known to inhibit WNT/β-CATENIN proliferative signaling, the mechanism by which elevated levels of DKK4 promote tumorigenesis of these cancers was not known. However, a study by Hirata et al. (2011) showed that DKK4 overexpression in renal cancer cell lines promotes their proliferation, migration and invasion *in vitro* and enhanced tumor growth *in vivo*. The results of Western blot studies showed that DKK4 overexpression reduces nuclear β-CATENIN levels as well as expression of β-CATENIN target genes, *c-MYC* and *CYCLIN D1*. However, these cells showed elevated levels of JNK, c-JUN, phospho-c-JUN and MMP-2 expression, indicating that DKK4 promotes tumorigenicity by upregulating β-CATENIN-independent pathways.

2.4.3 Secreted Frizzled-related proteins (SFRPs)

SFRPs are the largest family of secretory WNT antagonists that contains five glycoproteins

named SFRP1-5. SFRPs are approximately 300 amino acids in length with a signal peptide, a frizzled-like cysteine-rich domain having ten cysteine residues, which are conserved at the N-terminus, and a hydrophilic heparin-binding domain at the C-terminus. The cysteine-rich domain of SFRPs has close sequence homology with the extracellular cysteine-rich domain of WNT receptor, frizzled receptor. Therefore, they compete with Frizzled receptor to bind to WNT ligands and inhibit activation of WNT signaling (Keming Lin et al., 1997; Rattner et al., 1997). Furthermore, SFRPs can also bind to frizzled receptor due to the sequence homology in the cysteine-rich domains of both proteins and form a non-functional complex that inhibits binding of WNT ligand to the frizzled receptor and subsequent WNT activation (Bafico et al., 1999).

SFRP1 inhibits WNT signaling by binding to WNT ligands such as WNT1, WNT3a and WNT7a (Galli et al., 2006; Miao et al., 2018). Dysregulation of SFRP1 expression has been shown to play a critical role in promoting tumor growth in variety of cancers such as breast cancer, cervical cancer, epithelial ovarian cancer and gastric cancer (Chung et al., 2009; Chung et al., 2009; Qu et al., 2013; Shulewitz et al., 2006; Zhang et al., 2019). SFRP1 expression is reduced in various types of cancer due to transcriptional inactivation. For example, SFRP1 is downregulated in epithelial ovarian cancer cells as a result of enhanced promoter DNA methylation and its ectopic expression abrogates cell proliferation by reducing expression of β-CATENIN and its target genes *CYCLIN D1* and *c-MYC in vitro* and *in vivo* (Zhang et al., 2019).

As reported for other WNT/β-CATENIN antagonists, elevated expression of SFRP1 has also been reported in several cancer subtypes. For instance, a study by Qu et al. (2013) showed that SFRP1 levels are elevated in 54% of gastric cancer tissues compared to their matched control, and negatively correlates with patient's survival. To understand the role of SFRP1 in gastric cancer progression, the authors induced SFRP1 expression in gastric cancer cell line, SGC7901, which lacks endogenous expression of SFRP1. SFRP1 overexpression promoted growth and metastasis of these cells, and induced epithelial to mesenchymal transition by reducing the expression of epithelial cell markers such as α-CATENIN, β-CATENIN, γ-CATENIN and E-cadherin (ECAD), and enhancing the levels of mesenchymal cell markers such as α-smooth muscle actin (α-SMA), N-cadherin (NCAD), fibronectin and vimentin. Collectively, this evidence indicates that SFRP1 promotes WNT/β-CATENIN pathway when its levels are reduced in cancer cells, however, when overexpressed it promotes oncogenicity through different mechanisms, which are independent of the WNT/β-CATENIN signaling.

SFRP2 inhibits WNT/β-CATENIN pathway activation by binding to WNT ligands such as WNT3a and WNT16B (Sun et al., 2016; von Marschall & Fisher, 2010). SFRP2 is usually silenced in tumors due to various epigenetic events such as promoter DNA hypermethylation and microRNA-mediated SFRP2 mRNA degradation. SFRP2 promoter hypermethylation is reported in different cancers such as esophageal squamous cell carcinoma and breast cancer (Veeck et al., 2008; Qiu et al., 2019) and miRNA-mediated downregulation of SFRP2 is reported in hepatic cancer, colon cancer and triple-negative

breast cancer (Li et al., 2016; Liu et al., 2018; Tao et al., 2019). Most of these studies have shown that when SFRP2 is downregulated, it promotes tumorigenic growth of cancer cells by elevating WNT/β-CATENIN pathway. For example, in hepatocellular carcinoma, SFRP2 levels are suppressed due to elevated expression of miR-629-5p, which directly targets the 3′-UTR of *SFRP2* mRNA. By upregulating expression of miR-629-5p, the authors showed that miR-629-5p promotes proliferation and metastasis of hepatocellular carcinoma cells (Tao et al., 2019).

Previous studies have shown that restoration of SFRP2 could also serve as a potential antitumor strategy in cancers having reduced levels of SFRP2. A study by Chung et al. (2009) showed that re-expression of SFRP2 reduces proliferation, ability to form the colonies and invasiveness of colon cancer cell line, CaSki. These changes were associated with a significant reduction in expression of the WNT/β-CATENIN target genes, *c-MYC* and *CYCLIN D1*. Furthermore, SFRP2 restoration resulted in elevated expression of the epithelial marker, *E-CADHERIN*, and reduced expression of transcription factors, *SLUG*, *TWIST* and *SNAIL*, which regulate epithelial to mesenchymal transition (EMT). These findings indicate SFRP2 downregulation promotes tumorigenesis through different mechanisms and that its restoration could play a potential role in tumor management.

SFRP3 protein was identified as a membrane associated protein that had a chondrogenic role during bone morphogenesis. Later, many independent studies showed that SFRP3 can prevent WNT signaling by binding to WNT ligands such as WNT8, WNT1, WNT9 and

WNT5a (Leyns et al., 1997; Lin et al., 1997; Person et al., 2005; Liu et al., 2008). SFRP3 levels has been shown to be downregulated due to promoter hypermethylation in hepatocellular carcinoma cell lines (HCCs), HA22T, HepG2, Hep3B, and TONG, and clinical samples compared to their normal counterparts. Furthermore, treating HCC cells with DNA methyltransferase inhibitor, 5-aza-2′-deoxycytidine restored SFRP3 expression, further confirming the fact that SFRP3 downregulation in HCCs is caused by promoter DNA hypermethylation (Lin et al., 2014).

A recent investigation in a large cohort of lung adenocarcinoma patients showed that SFRP3 expression is reduced in tumor tissues as compared to normal lung tissue. Furthermore, pyrosequencing analysis indicated enhanced DNA methylation in *SFRP3* exon1 in tumor tissues compared to the normal lung tissues, with no significant difference in the methylation status of promoter DNA sequence between tumor and normal tissue. Treating lung adenocarcinoma cell lines, A549 and SK-LU-1, which lack endogenous expression of *SFRP3*, with 5-aza-2′-deoxycytidine alone or in combination with Trichostatin A reduced exon-1 methylation and restored *SFRP3* expression. This resulty confirms the fact that SFRP3 is downregulated in lung adenocarcinoma due to enhanced methylation in its exon-1 region (Schlensog et al., 2018).

SFRP4 is the largest and distinct member with least structural homology with other SFRP glycoproteins of the family, which has been shown to inhibit WNT/β-CATENIN signaling by binding to WNT ligands such as WNT7a and WNT3a (Carmon & Loose, 2008; Perumal

et al., 2017). Accumulating evidence has shown that SFRP4 expression is reduced in cancers of the brain, pancreas and colon due to promoter hypermethylation (Qi, et al., 2006; Bu et al., 2008; Schiefer et al., 2014). Like other SFRPs, SFRP4 expression is also suppressed as a consequence of elevated expression of its functional target microRNAs in some cancer subtypes. For example, in pancreatic cancer, elevated levels of miR-135-5p directly target SFRP4 to reduce its expression. By modulating expression of miR-135-5p in pancreatic cancer cells, the authors demonstrated that miR-135-5p-mediated suppression of SFRP4 promotes proliferation and migration of pancreatic cancer cells through increased expression of β-CATENIN (Han et al., 2017).

Mounting evidence shows that re-expression of SFRP4 has a potential antitumorigenic effect in a variety of cancers. Exogenous expression of SFRP4 in prostate cancer cell lines, LNCaP and PC3, changes their morphology into a more epithelioid appearance and reduces their proliferation. In addition, SFRP4 induction also reduces the metastasis of these cells by enhancing membrane localization of E-Cadherin, N-Cadherin and β-CATENIN without affecting their total protein content (Horvath et al., 2007). These findings strongly imply that dysregulation of SFRP4 plays a key role in tumor progression and its re-expression might be a promising approach to treat cancer patients with reduced SFRP4 levels

SFRP5 interacts with WNT5a ligand to disrupt its binding to Frizzled receptor, and hence, it antagonizes WNT/β-CATENIN signaling (Cho et al., 2018). SFRP5 expression is reduced in different cancers due to epigenetic inactivation, and leads to enhanced

oncogenicity of cancer cells by activating WNT/β-CATENIN signaling. Furthermore, SFRP5 downregulation often contributes to resistance to chemotherapeutic drugs and poor survival in many cancers. For example, an investigation by Veeck et al. (2008) showed that SFRP5 expression was substantially reduced in most breast cancer cells and clinical samples due to enhanced promoter DNA methylation. Survival analysis of breast cancer patients in the same study showed that increased SFRP5 promoter methylation is positively correlated with reduced overall survival rate.

In another study using a panel of ovarian cancer cells, it was shown that the hypermethylation of SFRP5 promoter results in its downregulation in ovarian cancer. The authors also showed that exogenous expression of SFRP5 reduces anchorage-dependent growth and invasiveness of these cells *in vitro* and tumor growth *in vivo*. Simultaneously, the study revealed that the tumor suppressing effects of SFRP5 in ovarian cancer cells were associated with reduced nuclear accumulation of β-CATENIN, which concomitantly led to reduced expression of β-CATENIN target genes, *c-MYC* and *CYCLIN D1*. Additionally, SFRP5 re-expression also promoted sensitivity to chemotherapeutic drugs such as Taxol and cisplatin in ovarian cancer cells *in vitro*. Clinical investigation of ovarian cancer patients who were under chemotherapy showed that enhanced methylation of SFRP5 is associated with reduced response to chemotherapy (Su et al., 2010). These results imply that reduced levels of SFRP5 promote tumorigenicity in different cancers, and that re-expression of SFRP5 may serve as a viable therapeutic intervention against cancer cells.

2.4.4. WNT inhibitory factor (WIF) 1

WIF1 was first purified as a secretory protein capable of binding to Drosophila Wingless and Xenopus WNT8 ligands, and inhibiting WNT signaling (Hsieh et al., 1999). Structurally, WIF1 is a 379 amino acid protein that has an N-terminal signal peptide, followed by a highly conserved WIF1 domain of 55 amino acids, five Epidermal Growth Factor (EGF)-like domains, and a C-terminal hydrophilic tail made of 44 amino acids. Crystal structure analysis indicates that the N-terminal WIF1 domain and EGF-like domains facilitate binding of WNT ligand to WIF1 protein (Malinauskas et al., 2011).

Silencing of the *WIF1* gene due to different epigenetic modifications has been reported in breast, prostate, bladder and lung cancers (Wissmann et al., 2003). Methylation-specific PCR studies in various cancers such as lung cancer and endometrial cancer have revealed that enhanced CpG methylation in the functional promoter region results in silencing of *WIF1* in these cancer types (Mazieres et al., 2004; Reguart et al., 2004; Deng et al., 2017). However, exogenous expression of *WIF1* or treatment with DNA demethylating agents such as 5-aza-2′-deoxycytidine restores WIF1 expression and reduces cell proliferation by down-regulating WNT/β-CATENIN signaling (Deng et al., 2017).

Dysregulation of the microRNA program is mechanism by which WIF1 expression is downregulated in some cancer subtypes. A mechanistic study by Tan et al. (2013) showed that reduced expression of microRNAs belonging to the miR-29 family (miR-29a, miR-29b and miR-29c) indirectly regulates expression of WIF-1 in non-small-cell lung cancer (NSCLC). Their systematic study using NSCLC cells, A549 and H1299, revealed that

reduced levels of miR-29s lead to upregulation of their direct targets, DNA methyltransferases, DNMT3A and -3B, which induce WIF1 promoter methylation. In contrast, exogenous expression of miR-29s elevates expression of *WIF1* in these cells, which subsequently reduces β-CATENIN levels. These changes were associated with reduced tumorigenicity as shown by reduced proliferation and enhanced apoptosis of these cells (Tan et al., 2013). In osteosarcoma, *WIF1* gene is silenced by microRNA, miR-552-5p, which directly binds to the *WIF1* 3′-UTR, and decreases its mRNA instability. Knockdown of miR-552-5p reduces expression of the WNT target gene, *CYCLIN D1*, and suppresses proliferation, migration and invasion of osteosarcoma cell lines, MG63 and U2OS. Taken together, these results indicated that miR-552-5p promotes tumorigenecity of osteosarcoma cells by inhibiting WIF1 expression (Cai et al., 2019). These studies showed that restoration of WIF1 expression reduces tumorigenesis.

2.5 WNT/β-CATENIN target genes

Activation of WNT signaling promotes nuclear localization of β-CATENIN and its association with TCF and LEF1 transcription factors. Once bound to the promoter region, β-CATENIN/TCF recruits several co-activators such as histone acetyltransferases, p300 and CREB-binding protein (CBP), leading to transcriptional activation of target genes, *CYCLIN D1*, *c-MYC* and *SURVIVIN* (He et al., 1998; Tetsu & McCormick, 1999; Ma et al., 2005; Kimelman & Xu, 2006). Most of the WNT target genes are known to be oncogenes that promote a wide range of cellular events such as proliferation, transformation, immortalization and metastasis. Several WNT target genes including

SURVIVIN are also known to inhibit apoptosis (He et al., 1998; Tetsu & McCormick, 1999; Kimelman & Xu, 2006; Ma et al., 2005) .

2.5.1. CYCLIN D1

CYCLIN D1 is one of the first D-type CYCLINs involved in regulating G1 to S transition in response to external mitogenic stimuli such as serum (Baldin et al., 1993). During G1 stage, CYCLIN D1 binds to cyclin dependent kinases such as CDK4 and CDK6 to induce phosphorylation of the retinoblastoma (RB) protein, and triggers a second wave of phosphorylation by the CYCLIN E/CDK2 complex. As a consequence, RB loses its ability to bind to E2F, which then initiates transcription of genes involved in DNA synthesis, thus leading to G1 to S transition (Lundberg & Weinberg, 1998). In addition to RB, CYCLIN D1/CDK4/6 can also induce phosphorylation of several transcription factors that regulate cell differentiation. For example, in early embryonic development, CYCLIN D1/CDK4 activation inhibits differentiation of cardiomyocytes by directly phosphorylating Ser105 of GATA binding protein 4 (GATA4), which is a key regulator of cardiomyocyte differentiation, and subjecting it for proteasomal degradation. However, during the later stages of cardiac development, CYCLIN D1 levels are kept low by the chromatin remodeler, jumonji/Jarid2 protein, which binds to the *CYCLIN D1* promoter and reduces its expression by inducing H3K9 methylation. This eventually results in increased GATA4 levels and subsequent differentiation of cardiomyocytes (Nakajima et al., 2011; Shirato et al., 2009; Toyoda et al., 2003). These findings indicate that elevated levels of CYCLIN D1 are associated with cell proliferation and lack of differentiation.

WNT activation can induce CYCLIN D1 expression by promoting binding of β-CATENIN/TCF complex to the promoter region of *CYCLIN D1* gene and activating its transcription, indicating *CYCLIN D1* is a target gene of WNT/β-CATENIN signaling (Shtutman et al., 1999). Abundant accumulation of CYCLIN D1 due to abnormal activation of WNT/β-CATENIN signaling is implicated in different types of cancer (Nishida et al., 1994; Rousseau et al., 2001; Sewify et al., 2014; Liu et al., 2018) . Elevated CYCLIN D1 promotes oncogenicity by interacting with and modulating function of several proteins in addition to RB family of proteins. For example, CYCLIN D1/CDK4 complex phosphorylates MEP50, which is known to be a PRMT5 binding partner, and enhances the methyltransferase activity of PRMT5. ChIP analysis of CYCLIN D1, CDK4 and PRMT5 co-transfected HeLa cells indicated that the PRMT5/CYCLIN D1/CDK4 complex binds to the *CUL4A/B* promoter and induces H3R8 and H4R3 methylation leading to its transcriptional repression (Aggarwal et al., 2010). Cullins are known to inhibit DNA replication by promoting ubiquitination of the chromatin licensing and DNA replication factor 1 (CTD1) (Hu, et al., 2004). Low levels of CUL4A/B favors CTD1 stabilization and favors DNA re-replication resulting in cellular transformation (Aggarwal et al., 2010). In addition, the CYCLIN D1/CDK4 complex is also known to phosphorylate major transcription factors such as FOXM1, and enhance its stability, thereby promoting transcription of its target genes, which are involved in cell proliferation, migration, survival, DNA damage response, and angiogenesis to promote tumor growth (Anders et al., 2011). These studies show that CYCLIN D1 promotes malignant phenotypes in different cancers by modulating functions of various proteins that are involved in tumor growth, in addition to cell cycle progression.

2.5.2 c-MYC

c-MYC belongs to the MYC oncogene family, which comprises N-MYC and L-MYC. *c-MYC* was identified as a target gene of WNT/β-CATENIN signaling while screening differentially expressed mRNAs after exogenously expressing the *APC* gene in the colorectal cancer cell line HT29. The study also revealed the existence of two β-CATENIN/TCF4 binding sites in the *c-MYC* promoter region, which can be activated upon APC repression or β-CATENIN activation, indicating that transcription of *c-MYC* can be directly induced in response to WNT/β-CATENIN signaling activation (He et al., 1998). Transcriptionally activated c-MYC can further enhance WNT/β-CATENIN signaling by directly binding to the promoters of WNT pathway antagonists, *DKK1* and *SFRP1* and repressing their transcription, indicating a positive feedback role of c-MYC in WNT/β-CATENIN signaling (Cowling et al., 2007).

c-MYC is referred as a super transcription factor because it regulates transcription of 15% of genes of the entire genome. Target genes regulated by c-MYC are involved in different cellular programs such as mRNA translation, ribosome biogenesis, cell-cycle progression, metabolism and mitochondrial function, indicating that c-MYC can regulate a wide spectrum of biological functions in the cell (Dang et al., 2006). c-MYC interacts with its binding partner, MAX, and binds to the E-box sequence of target genes (Luscher & Eisenman, 1990). After binding to E-box, MYC-MAX can interact with variety of transcriptional co-activators such as TRRAP, which can promote recruitment of histone acetyltransferases (HATs), including TIP60 and GCN5, and induce transcriptional

activation of target genes (Frank et al., 2003). c-MYC can also induce transcriptional repression of several target genes by directly binding to transcription factors such as Miz1, and promote the recruitment of DNAMT3a to induce promoter hypermethylation (Brenner et al., 2005).

Enhanced expression of c-MYC is reported in 50% of cancers (Morton & Sansom, 2013). A variety of mechanisms such as chromosomal translocation, gene amplification, activation of the enhancer region of *c-MYC* gene and aberrant activation of WNT/β-CATENIN signaling promote c-MYC expression in cancer cells (Meyer & Penn, 2008; Chung et al., 2019). c-MYC has shown to play multiple roles in cancer development and growth. c-MYC promotes G1/S transition of estrogen-dependent breast cancer cells by suppressing expression of the CDK inhibitor, p21, and activating CYCLIN E/CDK2 kinase activity, resulting in enhanced cell proliferation (Prall et al., 1998; Mukherjee & Conrad, 2005). Elevated expression of c-MYC induces mesenchymal phenotype in mammary epithelial cells by reducing E-cadherin protein levels, indicating that c-MYC plays an important role in epithelial to mesenchymal transition of cells (Cowling & Cole, 2007). Increased levels of c-MYC also promote tumor angiogenesis by elevating expression of vascular endothelial growth factor (VEGF). Mechanistically, c-MYC promotes VEGF expression by binding to the promoter region of miR-9 and activating its transcription, which in turn can directly target the 3′-UTR of E-CADHERIN (CDH1) mRNA and reduce its translation. Reduced levels of E-CADHERIN promotes cytosolic and nuclear localization of extracellular β-CATENIN, and consequently activates β-CATENIN

signaling in cancer cells and promotes VEGF expression (Ma et al., 2010). These studies indicate that c-MYC serves as a key proto-oncogene in cancer by regulating several mechanisms that control tumor growth.

2.5.3 SURVIVIN

SURVIVIN (BIRC5) is a 15kb gene that codes for a protein of 142 amino acids with a predicted molecular weight of 16.2kDa. SURVIVIN is the smallest member of the inhibitor of apoptosis protein (IAP) family, which can inhibits apoptosis by suppressing enzymatic activities of terminal effectors of the Caspase pathway, Caspase 7 and Caspase 3, in response to external apoptotic stimulus (Tamm et al., 1998). In addition, during pro-metaphase, SURVIVIN regulates microtubule stability and formation of the mitotic spindle. Inhibition of SURVIVIN reduces microtubule density and leads to short mitotic spindles (Giodini et al., 2002). SURVIVIN expression is completely suppressed in normal adult tissues whereas its levels are dramatically upregulated in cancer cells where it is associated with poor prognosis and reduced survival (Altieri, 2003). Since *SURVIVIN* is a well-known WNT/β-CATENIN target gene, aberrant activation of WNT signaling cascade in tumor cells serves as one of the cues for elevated expression of *SURVIVIN* (Chung et al., 2019).

SURVIVIN promotes oncogenicity by enhancing proliferation, metastasis and angiogenesis of tumor cells, and inhibiting their apoptosis. In hepatocellular carcinoma, elevated levels of SURVIVIN are known to release inhibition of the CYCLIND1/CDK4 complex by directly interacting with CDK4 and releasing p21 inhibitor, which results in

G1 to S phase progression (Ito et al., 2000). SURVIVIN promotes metastasis of melanoma cancer cells into lungs by enhancing expression of the cell adhesion molecule receptor, α_5 integrin, through activation of the PI3K/AKT pathway (McKenzie et al., 2013). Furthermore, activation of the PI3K/AKT pathway as a result of SURVIVIN upregulation is also shown to enhance β-CATENIN/TCF activity in the promoter region of vascular endothelial growth factor (VEGF), and promote its expression. Elevated VEGF expression in turn stimulates growth of endothelial cells, and induces angiogenesis (Fernández et al., 2014).

2.6 Breast cancer

Breast cancer is the second leading cause of death among women affecting nearly 1.5 million females worldwide every year, and causing the highest number of cancer-related deaths in women (Donepudi et al., 2014). Triple-negative breast cancer (TNBC) is the most aggressive sub-type of all breast cancers, accounting for 10-20% of total breast cancer incidence that associates with enhanced morbidity (Perou, 2010). TNBCs show aggressive clinical pathologies such as higher-grade tumors with large size and have extensive metastasis into lymph node and distant organs. TNBC cells lack estrogen, progesterone and HER2 receptors, which are usually targeted in other breast cancer subtypes where they are expressed. In the absence of specific druggable molecular targets, conventional chemotherapy and surgery are currently the mainstay treatment for TNBC patients. In addition, TNBCs show reduced prognosis compared to other subtypes of breast cancer, as evidenced by an enhanced risk of disease recurrence and reduced survival rate (Jitariu et

al., 2017). Hence, there is an urgent need for the development of a novel therapeutic strategy for treating these aggressive breast cancer subtypes.

2.7 WNT/β-CATENIN signaling activation in breast cancer: Possible role of PRMT5

Investigation of the underlying signaling cascades, which are dysregulated in TNBCs will help to develop novel treatment strategies. Dysregulation of the WNT/β-CATENIN signaling pathway is often reported in TNBCs, and serves as a major oncogenic driver in these cancers. Upregulation of WNT/β-CATENIN signaling is seen in many aggressive and invasive breast cancer types, and it is found to promote drug resistance in these cancers (Prosperi & Goss, 2010; King et al., 2012; Loh et al., 2013). Furthermore, elevated WNT signaling in TNBC patients promotes invasion and metastasis of cancer to the lung and liver (Dey et al., 2013). Enhanced expression of β-CATENIN target genes, *c-MYC* and *CYCLIN D1*, drives transformation, proliferation and invasion of mammary epithelial cells *in vitro* and *in vivo* (Wang et al., 2007). Unlike other cancers, genetic mutations of WNT/β-CATENIN signaling components are rare in TNBC cells, whereas epigenetic modifications are frequently observed (Klarmann et al., 2008; Mukherjee et al., 2012). Reduced expression of WNT/β-CATENIN antagonists such as *APC*, *CDH1*, *SFRP1* and *SFRP2* due to promoter hypermethylation is reported in many breast cancer samples (Jin et al., 2001; Mukherjee et al., 2012).

More strikingly, epigenetic silencing of *WIF1* due to promoter hypermethylation is much frequently observed in breast cancer, and also serves as an early trigger in the development

of hereditary breast cancers (Ai et al., 2006; Alvarez et al., 2013). A more recent study indicates that WIF1 expression is significantly repressed in breast cancer stem cells, which in turn promotes their self-renewal (Wang et al., 2015). *AXIN1* mRNA levels are found to be repressed in many breast cancer clinical samples and MDA-MB-231 cell lines (Zhang et al., 2012). Promoter hypermethylation of *DKK3* is also seen in many breast cancer samples (Xiang et al., 2013). However, upstream epigenetic events leading to transcriptional repression of these negative regulators have not been studied extensively.

A recent study from our group showed that PRMT5 upregulates WNT/β-CATENIN proliferative signaling in lymphoma by suppressing expression of pathway antagonists, *AXIN2* and *WIF1* (Chung et al., 2019). However, the role played by PRMT5 in breast cancer onset and progression is poorly understood. It has been demonstrated that PRMT5 plays a crucial role in MCF7 cancer cell proliferation (Scoumanne et al., 2009). PRMT5 can also associate with Programmed Cell Death 4 (PDCD4) and reduce its tumor suppressor activity in MCF7 cells. Moreover, patients overexpressing both PRMT5 and PDCD4 show poor survival rate when compared to those expressing high PCD4 levels and low levels of PRMT5 (Powers et al., 2011). A very recent study indicated that PRMT5 levels are upregulated in MCF-7, MDA-MB-231 and MCF-10A cell lines as well as in clinical samples of ductal carcinoma (Yang et al., 2015). High levels of PRMT5 have been detected in clinical samples of triple-negative breast cancer (Hu et al., 2015). PRMT5 has also been shown to be upregulated by the tumor necrosis factor receptor-associated 4 (TRAF4) in different breast cancer cell lines including MCF7 and MDA-MB-231, and that there is positive correlation between TRAF4 and PRMT5 protein expression in breast

cancer tissues and cell lines (Yang et al., 2015). These findings indicate that PRMT5 plays an important role in breast tumorigenesis. However, further investigation is required to confirm the role of PRMT5 in driving breast tumorigenesis. Based on these studies and our recent findings, which clearly show that PRMT5 controls WNT/β-CATENIN and AKT/GSK-3β signaling through epigenetic silencing of pathway antagonists, AXIN2 and WIF1, **we hypothesized that this mechanism of PRMT5-mediated activation of proliferative signaling might be conserved in all aggressive tumors including breast cancer. We also hypothesized that PRMT5 inhibition might thwart growth and proliferation of breast cancer cells via re-expression of WNT/β-CATENIN antagonists and downregulation of downstream target genes including *CYCLIN D1*, *c-MYC* and *SURVIVIN* (Fig. 6).**

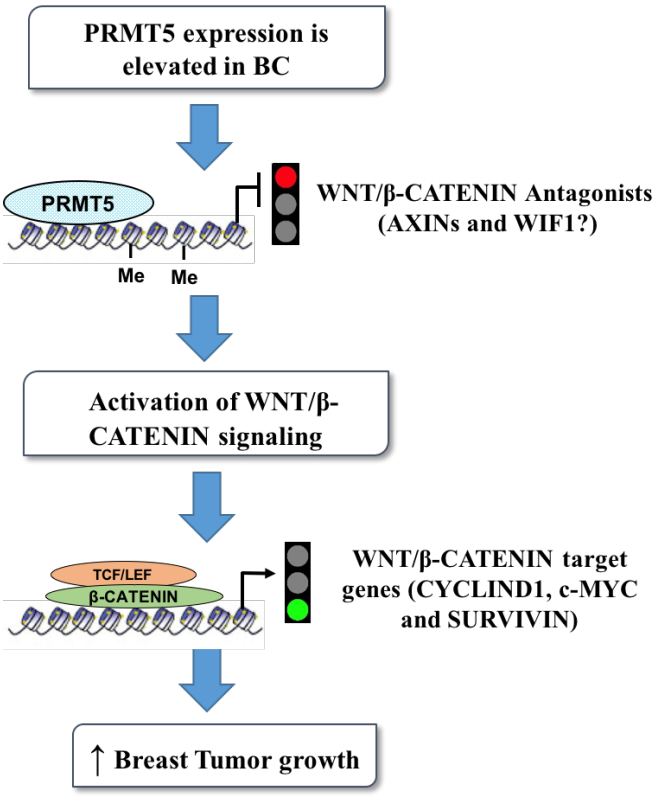


Figure 6: Working hypothesis

PRMT5 expression is elevated in breast cancer and that it suppresses the transcription of pathway antagonists, *AXIN2* and *WIF1* by directly binding to their promoter region and inducing H3R8 and H4R3 symmetrical methylation. As a result, transcription of WNT/β-CATENIN downstream target genes such as *CYCLIN D1*, *c-MYC* and *SURVIVIN* is enhanced, which in turn promotes the growth of breast cancer cells.

CHAPTER 3. MATERIALS AND METHODS

3.1 Cell culture

MCF7 breast cancer cells, which were originally isolated from metastatic pleural effusion of an adenocarcinoma patient and express the estrogen receptor, were purchased from ATCC (ATCC HTB-22^{TM}). HCC1937 triple negative breast cancer cells, which were isolated from the mammary duct of a grade 3 primary ductal carcinoma patient, were purchased from ATCC (ATCC CRL-2336^{TM}). BT549 triple negative breast cancer cells were originally isolated from the mammary gland of a ductal carcinoma patient, and were also purchased from ATCC (ATCC HTB-122^{TM}).

Human mammary epithelial cells (HMECs) were grown in mammary epithelial cell basal medium (American type culture collection) supplemented with rH-insulin (5 μg/ml), L-Glutamine (6 mM), Epinephrine (1 μM), Apo-transferrin (5 μg/ml), rH-TGF-α (5 ng/ml), pituitary extract (0.4%) and hydrocortisone (100 ng/ml). Human breast cancer cell lines HCC1937 and BT549 cells were cultured in RPMI 1640 medium (Gibco, Life Technologies Inc.) supplemented with 10% fetal bovine serum (FBS). BT549 cells were supplemented with insulin (0.023 U/ml). MCF7 cells were cultured in DMEM medium (Gibco, Life Technologies Inc.) supplemented with 10% FBS. All the cells were grown at 37°C, in a humidified CO_2 incubator supplied with 5% CO_2.

3.2 Real Time PCR

Total RNA was extracted using TRIzol reagent (Invitogen^{TM}). Briefly, TNBC cells from

a T75 flask were removed using 750 µl of TRIzol reagent, transferred to an eppendorf tube and incubated at room temperature for 5 min. Next, 300 µl of chloroform was added and samples were mixed vigorously, and incubated at room temperature for 10 min. Samples were then centrifuged at 4°C for 15 min before the upper layer containing RNA was transferred to a new eppendorf tube containing 500 µl of isopropanol. After incubation at room temperature for 10 min, the reactions were centrifuged at 4°C for 15 min. The RNA pellet was rinsed with 75% ethanol, air-dried before resuspension in 20 µl of nuclease-free water. Next, mRNA was converted into cDNA using High-Capacity cDNA Reverse Transcription Kit as per manufacturer's instructions (Applied Biosystems^{TM}). Briefly, 2 µg of total RNA was reverse transcribed in a 20-µl reaction mixture containing 2.5 µM random primers, 100 mM dNTP Mix, and Taqman reverse transcription reagents. The reverse transcription was carried out by first incubating the reaction mixture at 25°C for 10 min, which was followed by an incubation at 37°C for 2 hrs. Finally, the reaction was terminated by increasing the temperature to 85°C for 5 min, followed by incubation at 4°C. The cDNA synthesized was stored at -20°C until further use. To measure the mRNA levels of WNT target genes and antagonists, real time PCR was carried out in a 20-µl reaction containing 2 µl of cDNA, 10 µl of 2X TaqMan^{TM} Universal PCR Master Mix, 1.6 µl each of the reverse and forward primers (20 picomole/µl), 0.2 µl of Universal probe, and 6.2 µl of RNase free water. The primer and probe sets used are listed in Table 2. β-ACTIN was used as an internal control to normalize expression of tested genes.

Table 2. List of real time RT-PCR primers and probe sets

Gene	RT primer sets and probe
PRMT5	forward, 5′-CCAGAGCCTTGGAAGCA-3′
	reverse, 5′- CTGATGGGCAAGGGGAAT-3′
	probe 68
CCND1	forward, 5′-GAAGATCGTCGCCACCT-3′
	reverse, 5′-GACCTCCTCCTCGCACTT-3′
	probe 67
c-MYC	forward, 5′-ACCAGCTGGAGATGGTGA-3′
	reverse, 5′-CGGGTCGCAGATGAAACT-3′
	probe 52
SURVIVIN	forward, 5′ GCCCAGTGTTTCTTCTG-3′
	reverse, 5′-CCGGACGAATGCTTTTT-3′
	probe 11
AXIN1	forward, 5′-AGCTCTCCGAGACAGAGA-3′
	reverse, 5′-CAACGATGCTGTCACACG-3′
	probe 6
AXIN2	forward, 5′-CTAGGAGTGCGTTCATGG-3′
	reverse, 5′-GGGACGTAGTGCAAAGC-3′
	probe 6
WIF-1	forward, 5′-AAGCCAGCCTCATACATG-3′
	reverse, 5'-AAGTGAAGGCGTGTGCT-3′
	probe 40
DKK1	forward, 5′-CAGGCGTGCAAATCTGTCT-3′
	reverse 5′-GATTTTGATCAGAAGACACAC-3′
	probe 4

Gene	RT primer sets and probe
DKK2	Forward, 5′-GGCAGTAAGAAGGGCA-3′ reverse, 5′-CCTCCCAACTTCACACTC-3′ probe 43
DKK3	forward, 5′-CACATCTGTGGGAGACGAA-3′ reverse, 5′-CCCACAGTCCTCGTCGAT-3′ probe 29
DKK4	forward, 5′-AGGAGGTGCCAGCGAGA-3′ reverse, 5′- CATCTTCCATCGTAGTACA-3′ probe 37
SFRP1	forward, 5′- GGGCCAATGCTTATACC -3′ reverse, 5′- GAATCTACCATCCTTTCT -3′ probe 90
SFRP2	forward, 5′-TAGCAGCGACCACCTC-3′ reverse, 5'-GCAGGCTTCACATACC-3′ probe 83
SFRP3	forward, 5′-GATCCAAGGAAGCGGTGA-3′ reverse, 5′-GTGGACACAAGGATCTGG-3′ probe 52
SFRP4	forward, 5′-CCTGAAGCCATCGTCAC-3′ reverse, 5′-ATCATGTCTGGTGTGATGT-3′ probe 88
SFRP5	forward, 5′-CTCAGGGTCTCAGAAAG-3′ reverse, 5′-GAGCCCCTCCACCTTT-3′ probe 52
β-ACTIN	forward, 5′-AGCTACGAGCTGCCTGAC-3′ reverse, 5′-GGCTGGAAGAGTGCCTCA-3′ probe 9

3.3 Western blot analysis

Whole cell extracts were prepared in radioimmune precipitation assay (RIPA) buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 0.5 mM DTT, 0.5 mM PMSF and 2.5 mM Roche protease inhibitor cocktail). The extracts were subjected to Western blot analysis as described previously (Chung et al., 2013). Briefly, 20 to 40 μg of total protein were separated using 7-12% SDS-PAGE, and transferred to PVDF membrane. The membrane containing transferred proteins was blocked by incubating with 5% BSA containing 0.05% Tween-20 and incubated overnight at 4°C with primary antibody to detect CYCLIN D1 (Abcam, ab134175), c-MYC (Abcam, ab62928), SURVIVIN (Abcam, ab76424), α-TUBULIN (Abcam, ab4074), DKK1 (Abcam, ab109416), DKK3 (Abcam, ab186409), β-ACTIN (Cell Signaling Technology, 4970), CYCLIN D3 (Cell Signaling Technology, DCS22) and PRMT5 (Thermo Fisher, MA1-25470). After incubation with primary antibody, the membrane was treated with HRP-conjugated goat anti-mouse (Amersham Biosciences, NA931) or anti-rabbit (Amersham Biosciences, NA934V) secondary antibody. Next, proteins were visualized using the ECL detection kit (Amersham, RPN2209) in a Western blot imager (Flurochem E system, proteinsimple, California, USA).

3.4 Chromatin immunoprecipitation (ChIP) assay

Chromatin immunoprecipitation was carried out as described previously (Chung et al., 2013). Cross-linked chromatin was resuspended in ChIP lysis buffer (100 mM Tris-HCl [pH 8.6], 15 mM NaCl, 60 mM KCl, 1 mM $CaCl_2$, 3 mM $MgCl_2$) supplemented with

protease inhibitors, Aprotinin (10 μg/ml), PMSF (100 mM), Pepstatin (2.25 μg/ml), and Leupeptin (10 μg/ml), and fragmented using Q55 sonicator (Qsonica, USA). Sonicated chromatin was further digested by micrococcal nuclease (MNase) (0.6 Units) treatment at 37°C for 20 min. MNase-treated chromatin was analyzed by agarose gel electrophoresis to ensure that DNA fragment sizes did not exceed 500 bp. To evaluate PRMT5 recruitment as well as PRMT5-induced H3R8 and H4R3 symmetric methylation marks, chromatin was immunoprecipitated overnight at 4°C using either pre-immune or immune antibodies against PRMT5, H3(Me_2)R8 and H4(Me_2)R3 in the presence of protein A-Sepharose beads, which were pre-blocked with sheared salmon sperm DNA (0.2 mg/ml) and BSA (0.5 mg/ml). The retained complexes were washed successively with mixed micelle buffer (20 mM Tris-HCl [pH 8.1], 50 mM NaCl, 5 mM EDTA, 5% w/v sucrose, 0.2% Triton X-100, 0.2% SDS), buffer 250 (50 mM HEPES [pH 7.5], 0.1% sodium deoxycholate, 250 mM NaCl, 1 mM EDTA, 0.2% Triton X-100), and wash buffer (10 mM Tris-HCl [pH 8], 150 mM LiCl, 1 mM EDTA, 0.5% sodium deoxycholate, 0.25% NP-40,). Next, chromatin was eluted with 200 μl of elution buffer (50 mM Tris-HCl [pH 8.0], 10 mM EDTA, 1% SDS) at 65°C for 10 min, and cross-links were reversed by incubating the samples at 65°C overnight. Chromatin was then incubated with proteinase K (5 μg/ml), yeast tRNA (0.5 μg/ml) and TE [pH 7.6] at 37°C for 2 hrs before 50 μl of 4 M LiCl was added, and DNA was purified by phenol and chloroform extraction. Purified DNA was resuspended in 30 μl of Tris-EDTA (pH 8.0) and 3 μl of RNase A (10 mg/ml). To amplify promoter sequences of PRMT5 target genes, approximately 6 μl of eluted DNA was used in a 20-μl real time PCR reaction mixture containing the primer pairs and specific Roche Applied Science

universal probes listed in table 3.

Table 3. List of real ChIP primers and probe sets

Gene	ChIP Primer sets and probe
DKK1	forward 5′-CCGGATAATTCAACCCTTAC-3′ reverse 5′-GCAGACGACTTTAATAAATGC-3′ probe 18
DKK3	forward: 5′-AGGAGCTGCAAGTGCGTTAT-3′ reverse: 5′-GGCCTTAGTCTGCCGTGAT-3′ probe 19
RBL2	forward 5′-TCCAGGCGATGAGGAAGT-3′ reverse 5′-TGACGTGTGAAGGTTTCG-3′ probe 64

3.5 Transwell migration and invasion assays

Boyden migration assay was carried out using a 24-well plate with polycarbonate transwell inserts of pore size 8.0 μm (BD, Falcon). Boyden invasion assay was conducted using the BD Bio-Coat Matrigel system. Approximately $3x10^4$ cells resuspended in 500 μl serum free medium with or without PRMT5 inhibitor were added into the upper well of Boyden chamber. The lower chamber was filled with 1.3 ml of culture medium containing serum for 24 or 48 hrs. After incubation, cells that migrated to the lower surface of the upper chamber were fixed by treating them first with 4% formaldehyde, followed by absolute methanol, and then cells were stained with 1% Crystal violet. Images from 4 random fields

were captured using an inverted microscope, and cells were counted using ImageJ software.

3.6 Proliferation assay

Approximately $2x10^4$ cells were seeded into a 24-well plate, and 24 hrs later, cells were treated with or without PRMT5 inhibitor for different time intervals. Next, cells were collected, resuspended in 20-µl solution containing equal volume of culture medium and Trypan blue dye, and counted in order to determine the number of viable cells.

3.7 Flow Cytometry

Approximately $6x10^4$ cells were seeded in a 6-well plate prior to treatment with or without inhibitor for 24 or 48 hrs. Next, cells were harvested, washed and resuspended in 100 µl of 1X Annexin binding buffer containing 5 µl Annexin V-FITC and 5 µl PI, and then incubated for 15 min in the dark at room temperature. Stained cells were analyzed with BD LSRFortessa flow cytometer using FACS Diva software (Becton Dickinson, USA).

3.8 ELISA Assay

Levels of secreted antagonists of the WNT/β-CATENIN pathway were detected by ELISA technique using DuoSet ELISA kit (R&D system, USA). Essentially 100 µl of capture antibody was added to 96-well culture plate, and incubated at room temperature overnight. Next, the antibody solution was removed, and wells were washed twice with 400 µl of wash buffer (0.005% Tween-20 in filter sterilized 1X PBS). Wells were blocked by

incubating with 300 µl of blocking buffer (1% BSA in filter sterilized 1X PBS) for 1 hr at room temperature. Blocked wells were washed twice with wash buffer before adding 100 µl of either specific standard protein or sample solution. Wells were sealed with adhesive plastic strips, incubated at room temperature for 2 hr before washing twice with wash buffer. Next, 100 µl of Streptavidin-HRP A was added to each well and incubated in the dark for 20 min at room temperature. Wells were then washed, and 100 µl of Substrate solution containing 1:1 mixture of color reagent A (H_2O_2) and color reagent B (Tetramethylbenzidine) was added before incubating at room temperature in the dark for 20 min. After incubation, 50 µl of stop solution (2N H_2SO_4) was added, and the optical density of each well was read at 450 nm (TECAN, Infinite 200PRO, Switzerland).

3.9 Statistical analysis

The real-time RT-PCR and ChIP experiments were performed at least two times using different biological replicates, and the data obtained were represented as mean ± SD. Statistical validation of the data obtained from multiple samples within different groups was performed by two-way ANOVA analysis using GraphPad Prism 7 software, and p values ≤ 0.05 were considered as statistically significant.

CHAPTER 4: RESULTS

4.1 PRMT5 levels are upregulated in breast cancer cell lines

Previous studies have shown that PRMT5 levels are elevated in a wide variety of cancer cells including glioblastoma, melanoma, non-small cell lung carcinoma, lymphoma and leukemia cells (Pal et al., 2007; Nicholas et al., 2013; Shilo et al., 2013; Banasavadi-Siddegowda et al., 2017). Enhanced PRMT5 expression has also been shown in breast cancer cell lines as well as clinical samples of ductal carcinoma (Yang et al., 2015). In light of these findings, we investigated PRMT5 expression in HER2 positive breast cancer cell line, MCF7, and TNBC cell lines, HCC1937 and BT549 (Fig. 7).

The mRNA expression of *PRMT5* in breast cancer cells was investigated by RT-PCR as described in methodology section. Real time RT-PCR revealed that PRMT5 mRNA levels were enhanced by 7-fold ($p < 10^{-3}$) in MCF7, 9.9-fold ($p < 10^{-3}$) in HCC1937 and 3.7-fold ($p < 10^{-3}$) in BT549 cells compared to normal human mammary epithelial cells (HMECs) (Fig. 7*A*).

Next, the expression of PRMT5 protein in breast cancer cells and normal breast epithelial cells was analyzed by Western blotting as explained in section methodology section (Chapter 3.3). In accord with this result, Western blot analysis showed that PRMT5 protein expression was increased in breast cancer cell lines compared to normal HMECs (Fig. 7*B*). These results show that PRMT5 levels are upregulated in breast cancer cells.

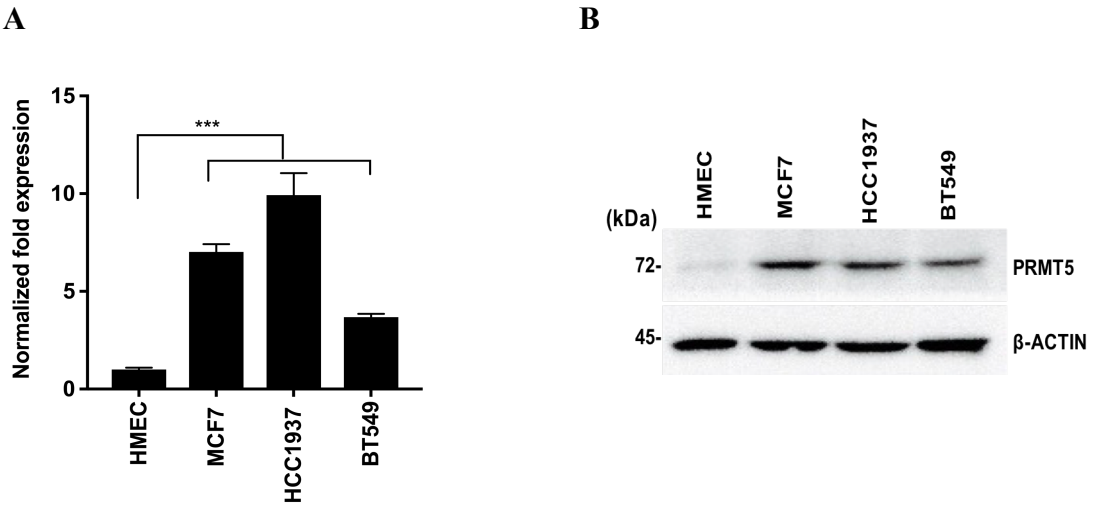


Figure 7. Expression of PRMT5 is elevated in breast cancer cells.

(*A*) mRNA level of PRMT5 was analyzed by real time RT-PCR using 2 µg of total RNA extracted from either from normal human mammary epithelial cells (HMECs) or breast cancer cells, MCF7, HCC1937 and BT549. The real time RT-PCR experiments were performed two times with three technical replicates, using gene specific primers and probe sets, and the results are represented as mean ± SD. *β-ACTIN* was used as an internal control. (*B*) RIPA extracts (20 µg) from either normal HMECs or breast cancer cells were analyzed by SDS-PAGE to detect proteins by Western blotting using the indicated antibodies. β-ACTIN was used as loading control. *** indicates p values < 10^{-3}.

4.2 WNT/β-CATENIN signaling is elevated in breast cancer

Aberrant activation of WNT/β-CATENIN signaling is frequently reported in wide range of cancers including breast cancer (Prosperi & Goss, 2010; King et al., 2012; Loh et al., 2013). Enhanced activation of WNT/β-CATENIN pathway results in increased expression

of downstream target genes such as *CYCLIN D1*, *c-MYC* and *SURVIVIV* (Barker et al., 2001; Kramps et al., 2002; H. Ma et al., 2005). Our recent work using aggressive lymphoma cell lines has shown that PRMT5 promotes WNT/β-CATENIN proliferative signaling by transcriptionally repressing expression of pathway antagonists, *AXIN2* and *WIF1*. Consequently, expression of WNT/β-CATENIN target genes such as *CYCLIN D1*, *c-MYC* and *SURVIVIN* increases (Chung et al., 2019). To investigate whether elevated levels of PRMT5 regulate WNT/β-CATENIN proliferative signaling in breast cancer, we checked expression of all three WNT/β-CATENIN pathway target genes in different breast cancer cell lines (Fig. 8).

Analysis of mRNA expression showed that *CYCLIN D1* was increased by 2.5-fold ($p < 10^{-3}$) in MCF7, and 2.4-fold ($p < 10^{-3}$) in HCC1937 compared to control HMECs (Fig. 8*A*). In agreement with work by Lin et al. (2000), which showed previously that *CYCLIN D1* is not expressed in BT549 cells, we were unable to detect *CYCLIN D1* mRNA in BT549 cells (Fig. 8*A*). An initial study by Bartkova et al. (1998) investigated the abundance of D type CYCLINs in human diploid cells and tumor cell lines, and showed that CYCLIN D3 is the most widely expressed D-type CYCLIN in most of human diploid cells as well as cancer cells including BT549. Therefore, we measured *CYCLIN D3* mRNA levels in breast cancer cells. We found that *CYCLIN D3* mRNA levels were increased by 2.4-fold ($p < 10^{-3}$) in MCF7, 2-fold ($p < 10^{-3}$) in HCC1937 cells, and 1.8-fold ($p < 10^{-3}$) in BT549 cells (Fig. 8*A*). Furthermore, we found that *c-MYC* mRNA levels were elevated by 4.3 to 6-fold ($p < 10^{-3}$) in breast cancer cells. Similarly, *SURVIVIN* mRNA was enhanced 12.9 to 49.3-fold ($p < 10^{-3}$) in all three breast cancer cell lines compared to normal HMECs (Fig. 8*A*).

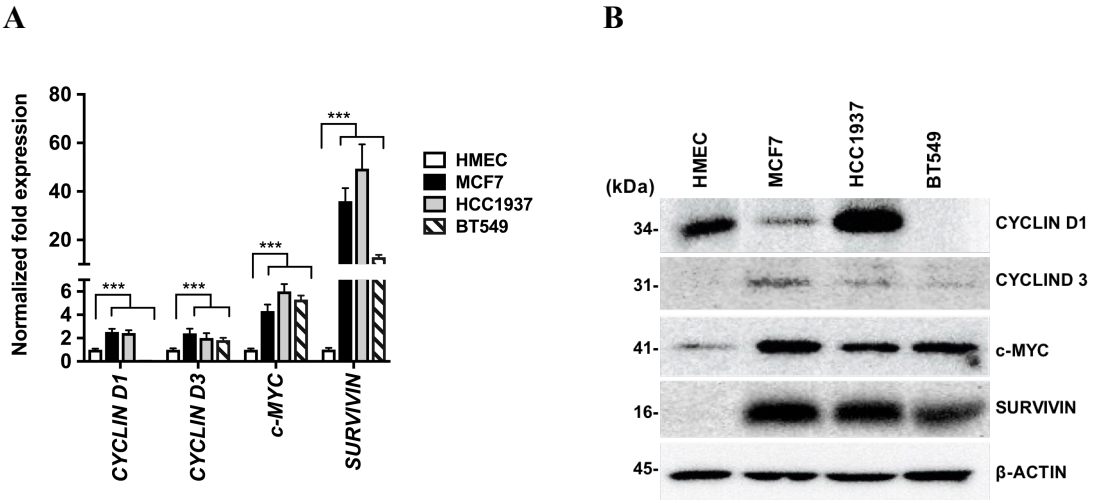


Figure 8. Expression of WNT/β-CATENIN target genes is elevated in breast cancer cells

(*A*) mRNA levels of WNT/β-CATENIN target genes including *CYCLIN D1*, *CYCLIN D3*, *c-MYC* and *SURVIVIN* were analyzed by real time RT-PCR using 2 µg of total RNA extracted either from normal human mammary epithelial cells (HMECs) or breast cancer cells, MCF7, HCC1937 and BT549. The real time RT-PCR experiments were performed two times with three technical replicates, using gene specific primers and probe sets, and the results are represented as mean ± SD. *β-ACTIN* was used as an internal control. (*B*) RIPA extracts (20 µg) from either normal HMECs or breast cancer cells were analyzed by SDS-PAGE to detect proteins by Western blotting using the indicated antibodies. β-ACTIN was used as loading control. *** indicates p values < 10^{-3}.

Having found that mRNA levels of WNT/β-CATENIN target genes are increased in transformed breast cancer cell lines, we checked c-MYC, SURVIVIN, and CYCLIN D1

and 3 protein levels (Fig. 8*B*). Western blot analysis showed that CYCLIN D1 expression is significantly upregulated in HCC1937, compared to MCF7 and BT549 where its expression was either reduced or not detected, respectively. Although, MCF7 cells had elevated expression of CYCLIN D1 mRNA (2.5-fold ($p < 10^{-3}$)) compared to HMEC, its protein expression was reduced, which might be due to posttranscriptional regulatory mechanism such as increased expression of inhibitory microRNA that target CYCLIN D1 mRNA in MCF7 cells. In contrast, CYCLIN D3 protein expression was increased in all breast cancer cells examined compared to normal HMECs. Similarly, the protein levels of c-MYC and SURVIVIN were elevated in all three types of breast cancer cells (Fig. 8*B*). Together, these findings indicate that expression of target genes of WNT/β-CATENIN pathway is enhanced in breast cancer cell lines.

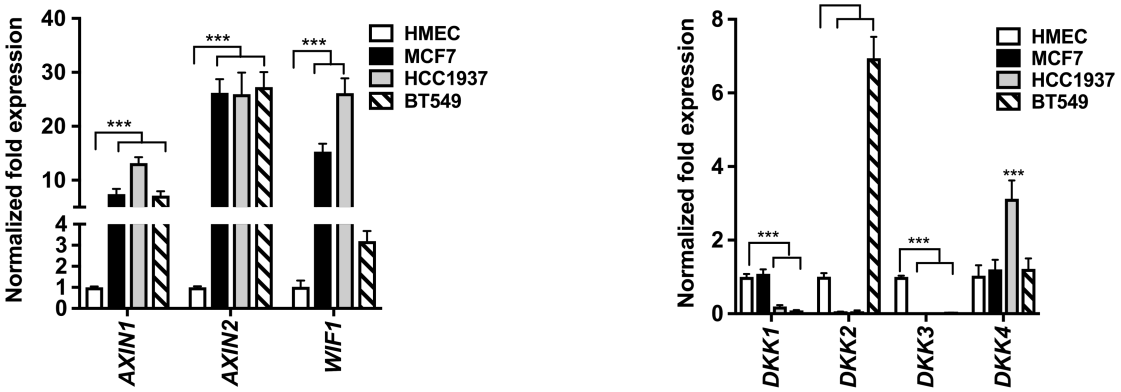
4.3 PRMT5 promotes WNT/β-CATENIN activation through repression of DKK1 and DKK3

WNT/β-CATENIN pathway is regulated by various natural endogenous inhibitors such as AXIN1, AXIN2, WIF1, DKK1-4, SFRP1-5, which antagonize the signaling by either disrupting the binding of WNT ligands to their membrane receptors or forming a cytosolic β-CATENIN destruction complex (Zhan et al., 2017). Extensive studies have reported that expression of WNT/β-CATENIN antagonists is reduced in breast cancer due to various epigenetic modifications that lead to enhanced WNT/β-CATENIN signaling (Prosperi & Goss, 2010; King et al., 2012; Loh et al., 2013). Our recent study indicates that PRMT5 suppresses the expression of antagonists of WNT/β-CATENIN module, *AXIN2* and *WIF1*

in aggressive lymphoma cells leading to enhanced activation of WNT/β-CATENIN signaling cascade (Chung et al., 2019). These findings prompted us to investigate the role of PRMT5 in WNT/β-CATENIN signaling by examining expression of pathway antagonists including AXIN1, AXIN2, WIF1, DKK1-4 and SFRP1-5 in breast cancer cell lines (Fig. 9).

The expression of WNT/β-CATENIN antagonists was analyzed by real time RT-PCR. In contrast to our results in the different lymphoma cell types, we found that *AXIN1*, *AXIN2* and *WIF1* mRNA levels were enhanced in transformed breast cancer cells (Fig. 9*A*). mRNA levels of *AXIN1* were increased by 7.1 to13.2-fold ($p < 10^{-3}$), while *AXIN2* mRNA levels were augmented by 25.9 to 27.3-fold, ($p < 10^{-3}$) in MCF7, HCC1937, and BT549 cells compared to normal HMECs. Similarly, *WIF1* also showed increased mRNA levels in MCF7 cells (15.3-fold, $p < 10^{-3}$), HCC1937 (26.1-fold, $p < 10^{-3}$) and BT549 cells (3.2-fold, $p = 0.075$) compared to normal HMECs (Fig. 9*A*).

A **B**



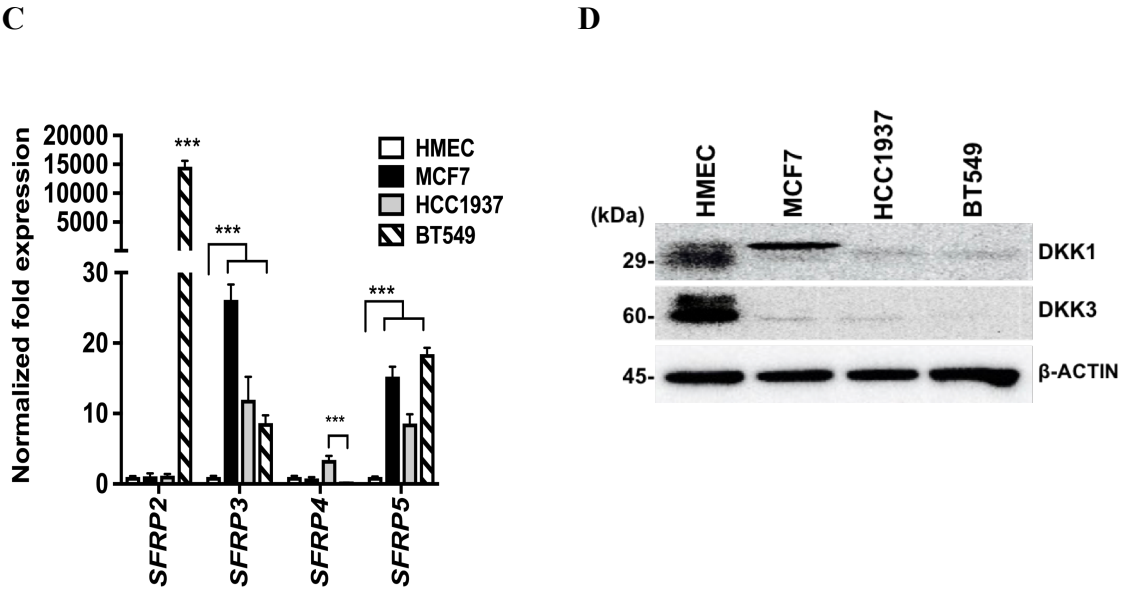


Figure 9: Expression of WNT/β-CATENIN antagonists, DKK1 and DKK3, is downregulated in breast cancer cells.

Relative mRNA expression of WNT/β-CATENIN antagonists *AXIN1*, *AXIN2*, and *WIF1* (*A*), *DKK1-4* (*B*), and *SFRP2-5* (*C*) in normal HMECs or MCF7, HCC1937 and BT549 cells were determined by real time RT-PCR using 2 µg of total RNA. The experiment was performed twice, and *β-ACTIN* was used as internal control. The results are represented as mean ± SD. (*D*) DKK1 and DKK3 protein levels of were analyzed by Western blotting using 20 µg of RIPA extracts from normal HMECs or the indicated breast cancer cells. β-ACTIN was used as loading control. *** indicates p values $<10^{-3}$.

Next, we assessed the mRNA expression of the Dickkopf gene family members, DKK1-4, and we found that *DKK1* mRNA levels were unaffected in MCF7 cells; however, they were substantially decreased in HCC1937 cells (5-fold, $p < 10^{-3}$) and BT549 cells (11.1-fold, p

$< 10^{-3}$) (Fig. 9*B*). *DKK2* mRNA expression was reduced in MCF7 and HCC1937 cells by 21.3-fold ($p < 10^{-3}$) and 21.6-fold ($p < 10^{-3}$), respectively. In contrast, DKK2 mRNA levels were elevated by 6.9-fold ($p < 10^{-3}$) in BT549. *DKK3* mRNA was undetectable in MCF7 and HCC1937 cells, and was reduced by 38.6-fold ($p < 10^{-3}$) in BT549 cells. Expression of *DKK4* mRNA was unaffected in MCF7 and BT549 cells compared to normal HMECs, and was upregulated by 2.4-fold ($p < 10^{-3}$) in HCC1937 cells (Fig. 9*B*).

When we examined expression of secreted frizzled-related proteins (SFRPs), we found that SFRP1 mRNA was not expressed in neither normal HMECs or breast cancer cells, while *SFRP2* mRNA was unaffected in MCF7 and HCC1937 cells compared to control HMECs (Fig. 9*C*). This was not the case in BT549 cells, which showed elevated levels of *SFRP2* mRNA (14,609-fold, $p < 10^{-3}$). Two other members of the SFRP family, *SFRP3* and *SFRP5*, showed substantial increase (8.6 to 26.1-fold, $p < 10^{-3}$) in their mRNA levels in breast cancer cells compared to normal HMECs. Expression of *SFRP4* mRNA in MCF7 cells did not differ from normal HMECs; however, its level was elevated in HCC1937 (3.4-fold, $p < 10^{-3}$) and decreased in BT549 cells (6.5-fold, $p < 10^{-3}$) (Fig. 9*C*).

Since both *DKK1* and *DKK3* showed significant reduction in their mRNA levels in both TNBCs, HCC1937 and BT549, we evaluated their protein levels in both normal and transformed breast cells. In accord with our RT-PCR results, Western blot analysis showed that both DKK1 and DKK3 proteins were suppressed in HCC1937 and BT549 compared to normal HMECs (Fig. 9*D*). In contrast to TNBCs, MCF7 cells had detectable expression of DKK1 protein, which showed altered mobility by SDS-PAGE compared to normal

HMECs, which may be due to DKK1 posttranslational modification by either methylation or phosphorylation. In addition, similar to TNBCs, DKK3 protein was significantly suppressed in MCF7 cells compared to normal HMECs (Fig. 9*D*). These findings suggested that expression of WNT/β-CATENIN pathway antagonists, DKK1 and DKK3, is altered in breast cancer cells, and suggest that PRMT5 might be involved in their regulation.

4.4 PRMT5 epigenetically silences DKK1 and DKK3 by binding to their promoter and inducing H3R8 and H4R3 symmetric dimethylation

In light of a recent work, which showed that PRMT5 represses transcription of antagonists of WNT/β-CATENIN pathway, *AXIN2* and *WIF1* by directly binding to their promoter region and inducing H3R8 and H4R3 symmetric methylation (Chung et al., 2019), we planned to investigate the role of PRMT5 in transcriptional repression of WNT/β-CATENIN antagonists, *DKK1* and *DKK3* in breast cancer cells (Fig. 10).

To analyze recruitment of PRMT5 and enrichment of PRMT5-induced epigenetic marks in the promoter region of *DKK1* and *DKK3*, chromatin immunoprecipitation (ChIP) experiments were carried out as discussed in Chapter 3.4. Cross-linked chromatin from MCF7, HCC1937 and BT549 cells was immunoprecipitated with either pre-immune, anti-PRMT5, anti-H3(Me_2)R8 or anti-H4(Me_2)R3. Real time PCR amplification of the immunoprecipitated DNA showed that there was no significant enrichment in PRMT5 recruitment or H3(Me_2)R8 and H4(Me_2)R3 epigenetic marks at the *DKK1* promoter in

MCF7 cells (Fig. 10*A*). In contrast, there was a 5.8-fold ($p < 10^{-3}$) increase in PRMT5 binding to the promoter region of *DKK3*, which was accompanied by enhanced symmetric methylation of H3R8 (6.8-fold, $p < 10^{-3}$) and H4R3 (2.5-fold, $p < 10^{-3}$) in MCF7 cells (Fig. 10*A*).

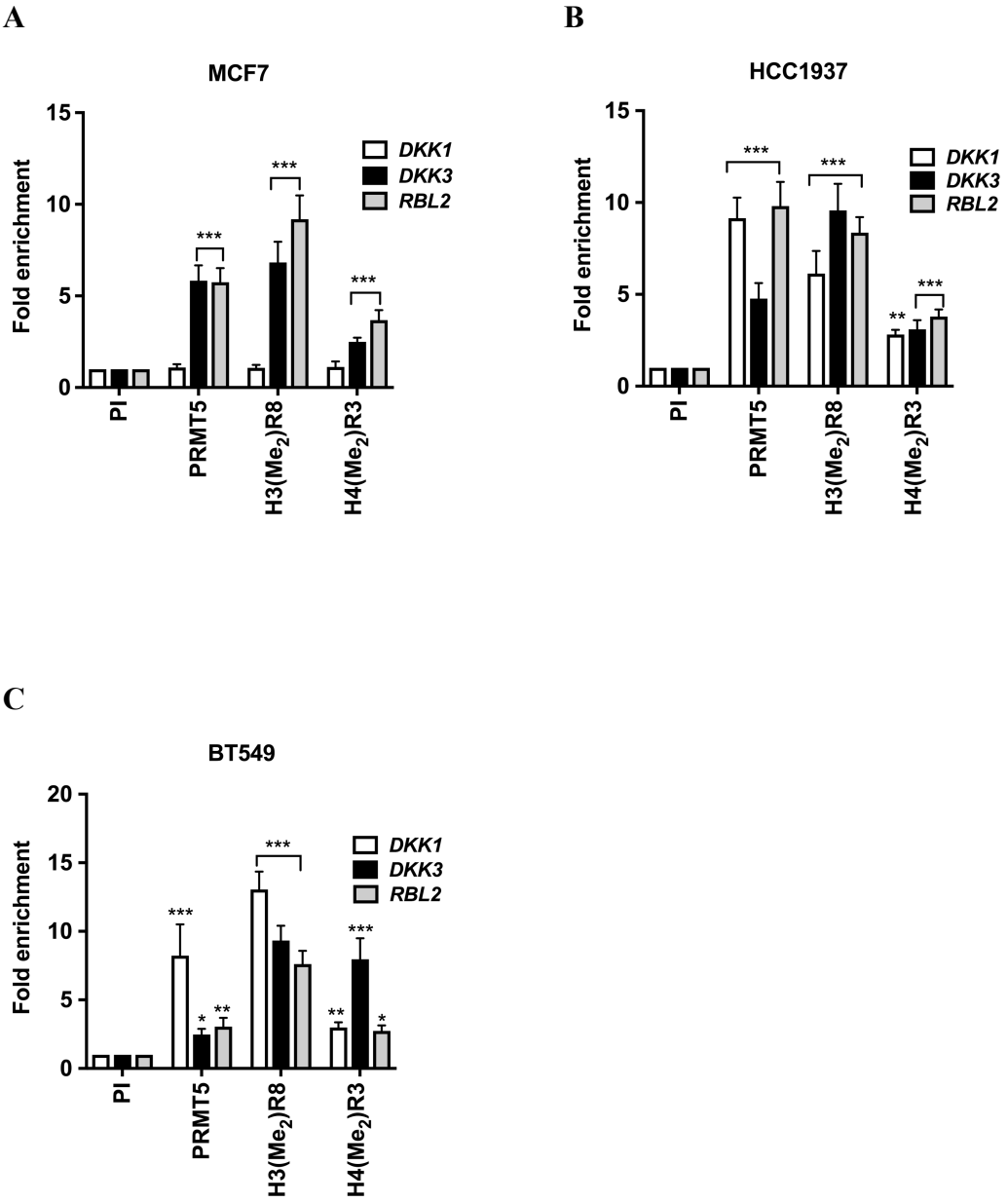


Figure 10: PRMT5 epigenetically suppresses expression of WNT/β-CATENIN antagonists, DKK1 and DKK3 in breast cancer cells.

ChIP assays were conducted using cross-linked chromatin from either MCF7 (*A*), HCC1937 (*B*) or BT549 (*C*) cells. Immunoprecipitation of nucleoprotein complexes was performed using pre-immune (PI), anti-PRMT5, anti-H3(Me_2)R8 or anti-H4(Me_2)R3 antibodies, and the purified DNA was used to detect the promoter sequences of *DKK1* and *DKK3* by real time PCR. ChIP assays were repeated two times with three technical replicates. The results are represented as mean ± SD. *** indicates p values < 10^{-3}, ** indicates p values < 10^{-2} and * indicates p value < 10^{-1}.

ChIP assay in HCC1937 cells showed enhanced PRMT5 recruitment (9.2-fold, $p < 10^{-3}$) and elevated methylation of H3R8 (6.1-fold, $p < 10^{-3}$) and H4R3 (2.8-fold, $p = 0.001$) at the *DKK1* promoter (Fig. 10*B*). Similarly, PRMT5 recruitment and enrichment of its epigenetic marks were enhanced by 3.1 to 9.6-fold, ($p < 10^{-3}$) in the promoter region of *DKK3* (Fig. 10*B*). PRMT5 recruitment in the promoter region of *DKK1* was enriched by 8.2-fold ($p < 10^{-3}$), whereas PRMT5 binding to the promoter region of *DKK3* was increased by 2.5-fold ($p = 0.038$) in BT549 cells (Fig. 10*C*). Consistent with these results, elevated levels of H3(Me_2)R8 (13.1-fold, $p < 10^{-3}$) and H4(Me_2)R3 (3-fold, $p = 0.003$) were detected in the promoter region of *DKK1*. Furthermore, symmetric methylation of H3R8 (9.3-fold, $p < 10^{-3}$) and H4R3 (8-fold, $p < 10^{-3}$) was also detected in the promoter region of *DKK3* (Fig. 10*C*). These results indicate that PRMT5 regulates WNT/β-CATENIN proliferative signaling through epigenetic transcriptional repression of pathway antagonists, *DKK1* and

DKK3, in breast cancer cell lines.

4.5 PRMT5 inhibition reduces viability of breast cancer cells

Elevated PRMT5 activity promotes cell proliferation in different types of cancers, whereas its inhibition is shown to have potential antitumor effect. Previous study by our group has shown that PRMT5 inhibition using first class small molecule inhibitor, CMP5, reduces the viability of diffuse large B-cell lymphoma (DLBCL) cells (LC_{50} value of 20-30 μM) (Alinari et al., 2015) *in vitro*. Having found that PRMT5 levels are upregulated in breast cancer cells, we planned to investigate the impact of PRMT5 inhibition on proliferation of breast cancer cells *in vitro* (Fig. 11).

To assess the effect of PRMT5 inhibition on viability of breast cancer cells, we incubated HMEC, MCF7, HCC1937 and BT549 cells with increasing amounts of CMP5 (2, 4, 8 and 16 μM) for either 24, 48 or 72 hrs. Trypan blue dye exclusion indicated that CMP5 had limited cytotoxicity on HMECs even at higher concentrations at any given time intervals. (Fig. 11*A*). Furthermore, treatment with lower concentrations of CMP5 (2 and 4 μM) did not reduce breast cancer cell viability significantly at any of the tested time intervals (Fig 11). However, treatment with 8 μM of CMP5 caused a reduction in MCF7 cell viability by 11.4% ($p < 10^{-3}$), 39% ($p < 10^{-3}$) and 61.2% ($p < 10^{-3}$) after 24, 48 and 72 hrs, respectively, compared to control DMSO treated cells (Fig. 11*B*). Treatment with 16 μM of CMP5 showed significant decrease in viability by 18% ($p < 10^{-3}$), 46.9% ($p < 10^{-3}$), and 69.1% ($p < 10^{-3}$) after 24, 48 and 72 hrs, respectively. Based on these results, we found that

CMP5 had a LC_{50} value of 15.46 μM at 48 hrs for MCF7 cells (Fig. 11*B*). Similarly, incubating HCC1937 cells with 8 μM of CMP5 for 24, 48 and 72 hrs time intervals reduced cell viability by 11.5% ($p < 10^{-3}$), 72.2% ($p < 10^{-3}$), and 88.5% ($p < 10^{-3}$), respectively. HCC1937 cell viability was substantially reduced by 82% ($p < 10^{-3}$) after 24 hrs treatment with 16μM of CMP5, and all cells were dead (0% viability, $p < 10^{-3}$) after 48 and 72 hrs treatment. Data analysis showed that CMP5 had a LC_{50} of 11.78 μM after 24 hrs treatment for HCC1937 cells (Fig. 11*C*).

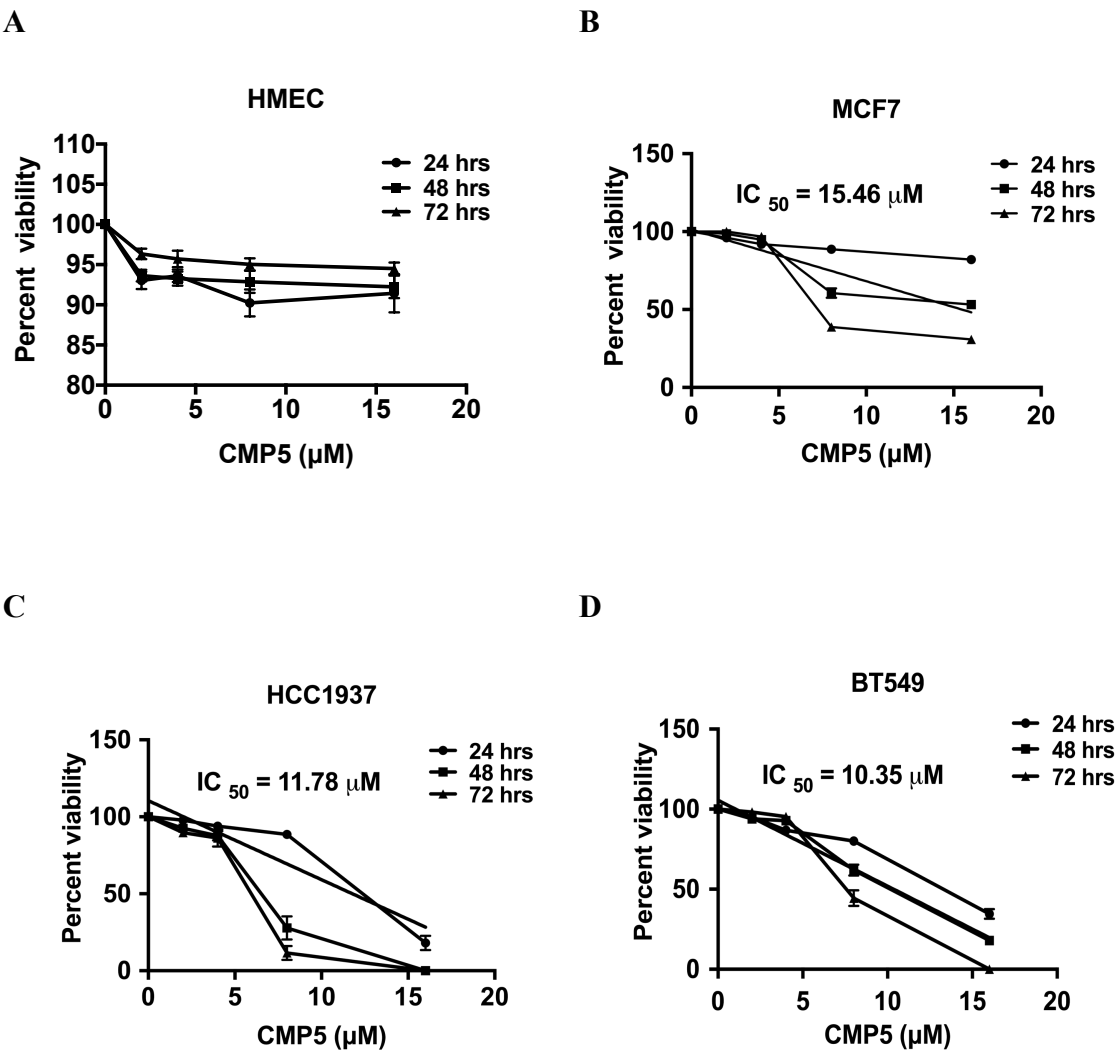


Figure 11. PRMT5 inhibition reduces the viability of breast cancer cells

Normal HMEC (*A*), MCF7 (*B*), HCC1937 (*C*) and BT549 (*D*) were incubated with different concentrations of CMP5 for 24, 48 and 72 hrs. Cell viability was measured by Trypan blue dye exclusion assay.

Treating BT549 cells with 8 μM of CMP5 reduced their viability by 20% ($p < 10^{-3}$), 38% ($p < 10^{-3}$), and 55.6% ($p < 10^{-3}$) after 24, 48 and 72hrs post-treatment, respectively. In addition, treatment with 16 μM of CMP5 reduced viability of BT549 cells by 65.5% ($p < 10^{-3}$), and 82% ($p < 10^{-3}$) after 24 and 48 hrs, respectively. Treatment for 72 hrs with 16μM of CMP5 resulted in complete loss of cell viability. When we plotted the results, we found that CMP5 had a LC_{50} of 10.35 μM at 48 hrs for BT549 cells (Fig. 11*D*). Taken together, these findings indicate that PRMT5 inhibition selectively reduces the proliferation of breast tumor cells that overexpress PRMT5 without inducing any significant toxicity to normal breast epithelial cells.

4.6 Inhibition of PRMT5 derepresses *DKK1* and *DKK3* in BT549 cells

Our recent study showed that inhibiting PRMT5 either using specific small molecule inhibitor, CMP5, or shRNA induces transcriptional transcriptional derepression of pathway antagonists AXIN2 and WIF1 in lymphoma cells. Based on this study, and our results, which indicate that PRMT5 protein levels are inversely correlated with WNT/β-CATENIN antagonists, we wanted to assess the effect of inhibition of PRMT5 on expression of *DKK1* and *DKK3* in TNBCs (Fig. 12).

HCC1937 and BT549 cells were treated with increasing concentrations of CMP5 for 24 or 48 hrs, respectively, and total RNA was analyzed. Real time RT-PCR data indicated that transcription of *DKK1* was induced 4 to 4.3-fold ($p < 10^{-2}$) at lower concentrations of CMP5 (3 μM to 9 μM), and was significantly upregulated by 24-fold ($p < 10^{-3}$) at 12 μM in BT549 cells (Fig. 12*A*). Similarly, *DKK3* mRNA levels were upregulated by 5.6-fold ($p < 10^{-3}$) in the presence of 12 μM of CMP5 in BT549 cells (Fig. 12*A*). The mRNA expression of *DKK1* and *DKK3* were unaffected in HCC1937 cells (Fig. 12*B*), implying that alternative mechanisms might be involved in their regulation. As control, the expression of PRMT5 mRNA was also analyzed, and were found to be unaffected in the presence of CMP5 (Fig. 12*A* and *B*). To determine the biological significance of *DKK1* and *DKK3* transcriptional derepression, we measured the levels of DKK1 and DKK3 proteins in BT549 cells treated with CMP5 (Fig. 12*C*). ELISA analysis showed that both DKK1 and DKK3 were derepressed by 2 and 1.4-fold ($p < 10^{-3}$), respectively (Fig. 12*C*). Consistent with the real time RT-PCR results, levels of DKK1 and DKK3 proteins were unchanged in HCC1937 cells (Fig. 12*D*).

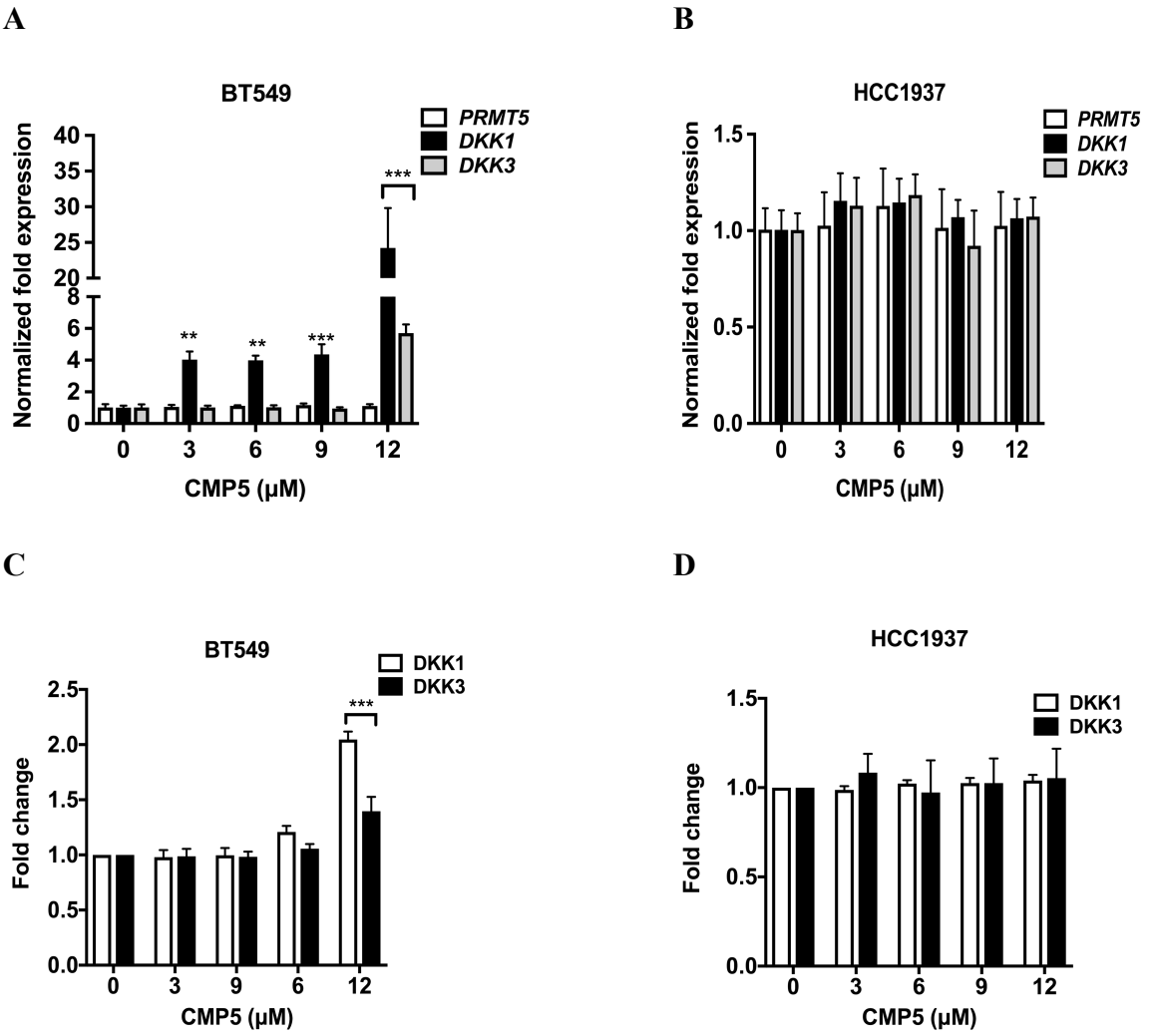


Figure 12: PRMT5 inhibition induces transcriptional derepression of WNT/β-CATENIN antagonists DKK1 and DKK3 in BT549 cells

mRNA levels of *DKK1* and *DKK3* were ditermined by real time RT-PCR using 2μg of total RNA extracted from BT549 (*A*) and HCC1937 (*B*) treated with increasing amounts of CMP5 (0, 3, 6, 9, 12 μM) for 48 hrs and 24 hrs, respectively. Protein levels of DKK1 and DKK3 were analyzed by ELISA after collecting the culture media from either DMSO- or CMP5-treated BT549 (*C*) and HCC1937 (*D*) cells for 24 and 48 hrs, respectively. The

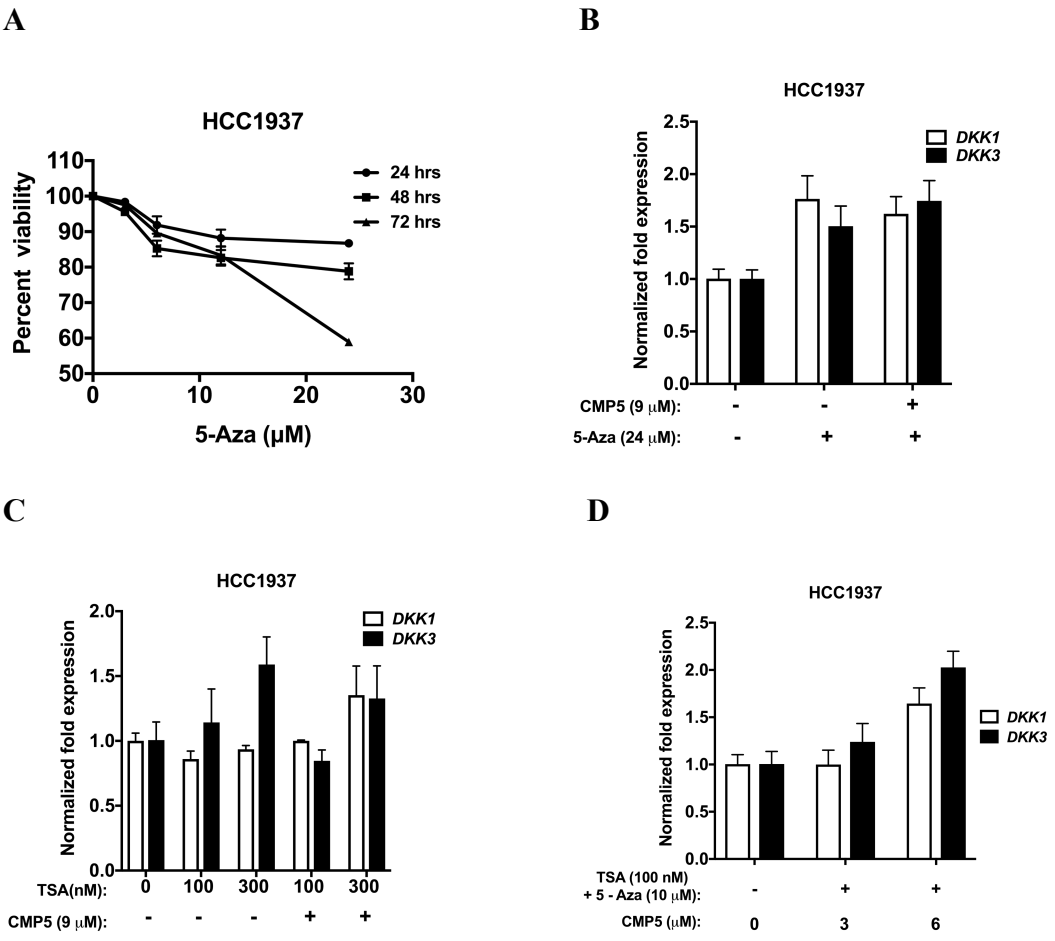
results are represented as mean ± SD. *** indicates p values < 10^{-3} and ** indicates p values < 10^{-2}.

4.7 Inhibition of PRMT5 in combination with HDACs and DNMT3A derepresses *DKK1* and *DKK3* in HCC1937 cells

Since PRMT5 inhibition could not restore expression of *DKK1* and *DKK3* in HCC1937 cells (Fig. 12*B* and *D*), we investigated other epigenetic mechanisms that could be involved in their transcriptional regulation. PRMT5 has previously been shown to work in concert with DNA methyltransferase 3A (DNMT3A) (Zhao et al., 2009; Liu et al., 2018). Therefore, we reasoned that PRMT5 might be promoting DNA methylation of *DKK1* and *DKK3* promoters in HCC1937 cells (Fig. 13).

To address whether DNA methylation is involved in transcriptional regulation of *DKK1* and *DKK3*, we treated HCC1937 cells with the DNA methyltransferase inhibitor, 5-Azacytidine (5-Aza). HCC1937 cells were incubated with increasing amounts of 5-Aza for either 24, 48 or 72 hrs, and viability was determined by Trypan blue dye exclusion assay, which showed that even a higher concentration of 5-Aza (24 μM) does not have significant cytotoxicity on HCC1937 cells (Fig. 13*A*). Next, we examined the effect of 5-Aza (24 μM) alone or in combination with suboptimal amount of CMP5 (9 μM), on transcriptional derepression of *DKK1* and *DKK3* in HCC1937 cells. Treatment of HCC1937 cells with 24 μM of 5-Azacytidine for 48 hrs did not impact transcription of *DKK1* (1.75-fold, $p < 10^{-3}$) and *DKK3* (1.5-fold, $p < 10^{-3}$). Furthermore, sequential

incubation of HCC1937 cells with 24 μM of 5-Aza first for 24 hrs, followed by addition of 9 μM CMP5 for another 24 hrs also did not induce significant transcriptional derepression of *DKK1* (1.6-fold, $p < 10^{-3}$) and *DKK3* (1.7-fold, $p < 10^{-3}$) (Fig. 13*B*).



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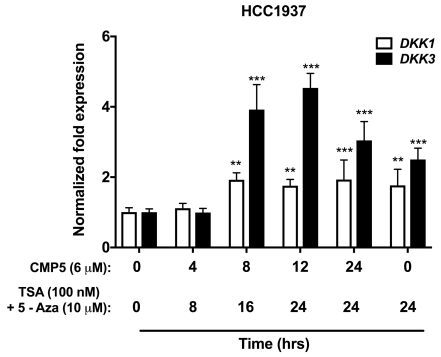


Figure 13: PRMT5 inhibition in combination with HDACs and DNMT3A induces transcriptional derepression of DKK1 and DKK3 in HCC1937 cells

(*A*) HCC1937 cells were incubated with different concentrations of 5-Azacytidine for 24, 48 and 72 hrs. Cell viability was measured by Trypan blue dye exclusion assay, and data represented in the graph is from three technical replicates. (*B*) mRNA levels of *DKK1* and *DKK3* were analyzed by real time RT-PCR using 2μg of total RNA extracted from HCC1937 cells treated with 5-Azacytidine (24 μM) alone or in combination with CMP5 (9 μM) for 24 hrs. (*C*) mRNA levels of *DKK1* and *DKK3* were determined by real time RT-PCR using 2μg of RNA extracted from HCC1937 cells treated with TSA (100 nM and 300 nM) alone or in combination with CMP5 (9 μM) for 24 hrs. (*D*) mRNA levels of DKK1 and DKK3 were determined by real time RT-PCR using 2μg of total RNA extracted from HCC1937 cells treated with suboptimal concentration of TSA (100 nM) and 5-Azacytidine (10 μM) for first 24 hrs, followed by CMP5 (6 μM) incubation for next 24 hrs (*E*) mRNA levels of DKK1 and DKK3 were evaluated by real time RT-PCR using 2μg of RNA extracted from HCC1937 cells treated with suboptimal concentration of CMP5 (6 μM) in

combination with TSA (100 nM) and 5-Azacytidine (10 μM) for the indicated time intervals. All the experiments were repeated two times with three technical replicates. The results are represented as mean ± SD. *** indicates *p* values < 10^{-3} and ** indicates p values < 10^{-2}.

Deacetylation of histone lysine residues by histone deacetylase enzymes (HDAC) has been shown to be essential for transcriptional silencing of genes (Gallinari et al., 2007). An earlier study by our group showed that PRMT5 can associate with mSin3A/histone deacetylase 2 (HDAC2) to form a transcriptional repression complex with the BRG1-based hSWI/SNF chromatin remodelers (Pal et al., 2004). In addition, we have previously shown that PRMT5 associates with an NF-κB transcriptional repressor complex containing HDAC3, which promotes deactylation of H3K14 lysine, and triggers gene silencing in lymphoma cells (Alinari et al., 2015). To investigate whether histone deacetylation is involved in transcriptional repression of WNT antagonists, *DKK1* and *DKK3*, we treated HCC1937 cells with Trichostatin A (TSA). Real time RT-PCR analysis showed that treatment with either 100 nM or 300 nM of TSA for 24 hrs had no effect on *DKK1* and *DKK3* transcription in HCC1937 cells. Similarly, combinatorial treatment with 9 μM of CMP5 and either 100 or 300 nM of TSA for 24 hrs also failed to induce transcriptional activation of WNT antagonists, *DKK1* and *DKK3* in these cells (Fig. 13*C*).

Previous studies have shown that simultaneous inhibition of histone deacetylase enzyme and DNA methyltransferase enzyme results in restoration of epigenetically silenced genes

(Bartoli et al., 2003; Tang et al., 2006). Therefore, we investigated the effect of combinatorial treatment of TSA and 5-Aza in inducing transcriptional derepression of *DKK1* and *DKK3* in HCC1937 cells. We incubated HCC1937 cells with a combination of suboptimal doses of TSA (100 nM) and 5-Aza (10 μM) for 24 hrs (Fig. 13*E*). Real time RT-PCR data showed that this treatment increased mRNA level of *DKK1* and *DKK3* by 1.8-fold (p=0.006) and 2.5-fold ($p < 10^{-3}$), respectively (Fig. 13*E*). Since histone lysine deacetylation, histone arginine methylation, and DNA methylation can all work together to induce gene silencing, we hypothesized that combinatorial treatment with inhibitors against HDAC, PRMT5 and DNMT3A might enhance transcriptional derepression of *DKK1* and *DKK3* in HCC1937 cells as compared to treatment with CMP5 in the presence of either TSA or 5-Aza. First, we treated HCC1937 cells with 100 nM of TSA and 10 μM of 5-Aza for 24 hrs, followed by incubation with 9 μM of CMP5 for an additional 24 hrs. The results of this study showed that mRNA level of *DKK1* and *DKK3* by 1.6-fold and 2-fold, respectively (Fig. 13*D*), suggesting that combinational treatment with all three inhibitors was not able to induce further expression of *DKK1* and *DKK3* in these cells.

Since, PRMT5-induced H4R3 histone methylation is known to direct binding of DNMT3A, and induce gene silencing (Zhao et al., 2009), we tested the effect of an initial treatment with CMP5, followed by addition of TSA and 5-Aza in HCC1937 cells. We treated HCC1937 cells with 6 μM of CMP5 for 4 hrs, and then added 100 nM of TSA and 10 μM of 5-Aza for an additional 8 hrs. Results from this experiment showed that *DKK1* and *DKK3* expression was not affected (Fig. 13*E*). Next, we treated HCC1937 cells with CMP5 (6 μM) for 8 hrs, followed by an incubation with TSA (100 nM) and 5-Aza (10 μM)

for another16 hrs. Real time RT-PCR analysis showed that the mRNA levels of *DKK1* were enhanced by 1.9-fold ($p = 0.023$) and those of *DKK3* were elevated by 3.9-fold ($p < 10^{-3}$) (Fig. 13*E*). Furthermore, when we increased time of incubation with CMP5 to 12 hrs, followed by incubation with TSA and 5-Aza for an additional 24 hrs, *DKK1* transcriptional derepression remained at 1.8-fold ($p = 0.0063$), whereas *DKK3* mRNA was derepressed by 4.5-fold ($p < 10^{-3}$) (Fig. 13*E*). Similarly, treating CMP5 for 24 hrs and then incubating with TSA and 5-Aza for next 24 hrs resulted in derepression of mRNA levels of *DKK1* and *DKK3* by 1.9-fold ($p = 0.0055$) and 3-fold ($p < 10^{-3}$), respectively (Fig. 13*E*). Collectively, these results indicate that PRMT5 is an important epigenetic enzyme, which is involved either alone or in combination with other epigenetic enzymes in silencing WNT pathway antagonists, *DKK1* and *DKK3* in TNBC cells.

4.8 Inhibition of PRMT5 downregulates WNT/β-CATENIN target genes breast cancer cells

Having found that PRMT5 inhibition can lead to transcriptional derepression of *DKK1* and *DKK3*, we wished to evaluate expression of WNT/β-CATENIN target genes, *c-MYC*, *CYCLIN D1* and *SURVIVIN* (Fig. 14).

HCC1937 treatment with 12 μM of CMP5 for 24 hrs triggered reduction in mRNA expression of *CYCLIN D1* and *SURVIVIN* by 1.8-fold ($p < 10^{-3}$) and 2.8-fold ($p < 10^{-3}$), respectively (Fig. 14*A*). Similar repression was seen in BT549 cells, which showed reduced expression of *CYCLIN D3* (1.6-fold, $p < 10^{-3}$) and *SURVIVIN* mRNAs (3.6-fold, $p < 10^{-3}$), after 48 hrs incubation with 12 μM of CMP5 (Fig. 14*B*). Since *c-MYC* is a known

target gene of the WNT signaling pathway, we expected that its mRNA levels would be reduced upon PRMT5 inhibition. Surprisingly, *c-MYC* mRNA levels were upregulated by 3.4-fold ($p < 10^{-3}$) upon treatment of HCC1937 cells with 9 μM and 12 μM of CMP5 for 24 hrs (Fig. 14*A*). Similar results were observed with BT549 cells, which showed an upregulation of mRNA levels of *c-MYC* by 2.2-fold ($p < 10^{-3}$) and 3.7-fold ($p < 10^{-3}$) after treatment with 9 μM and 12 μM of CMP5 for 48 hrs, respectively (Fig. 14*B*).

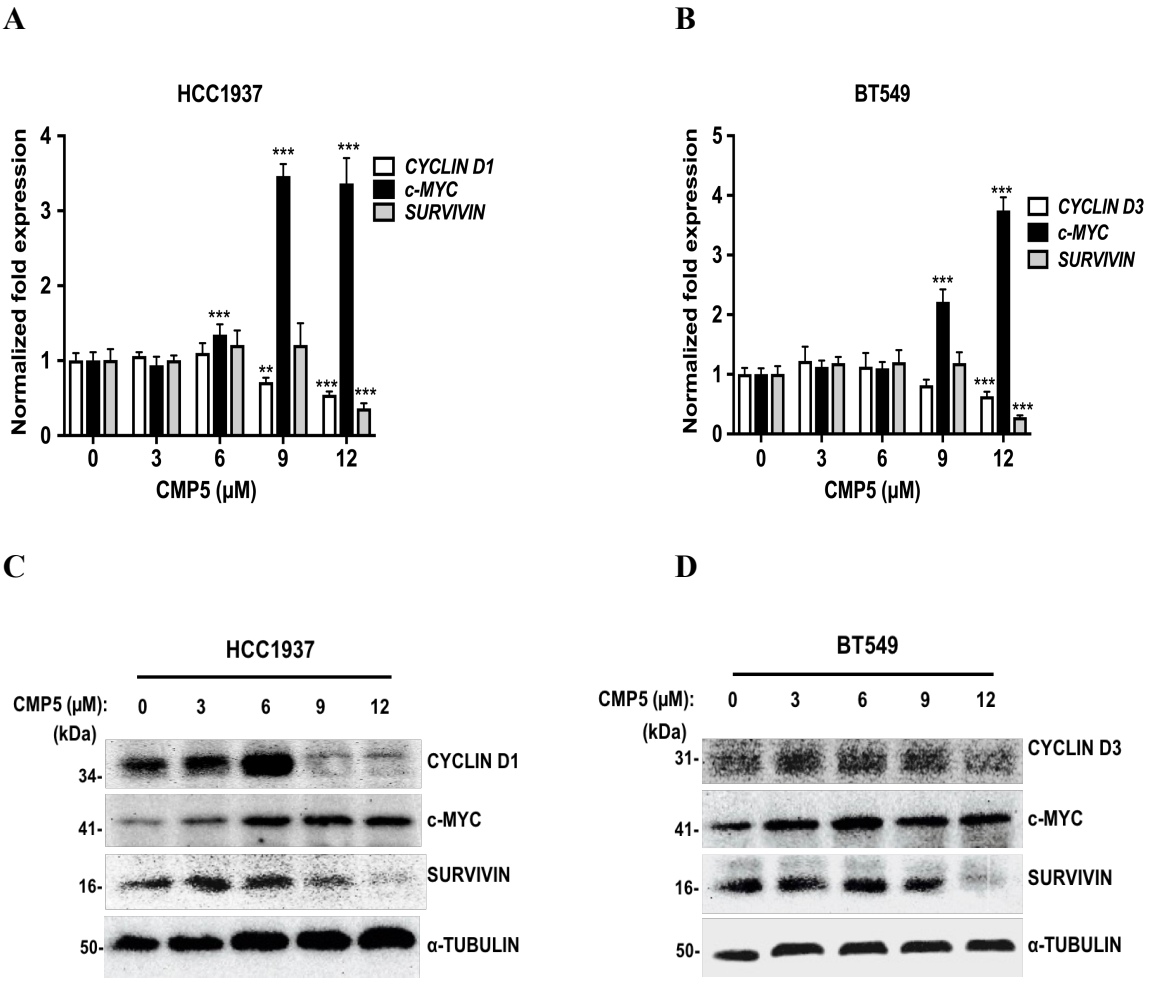


Figure 14: PRMT5 inhibition downregulates expression of CYCLIN D1 and

SURVIVIN in TNBC cells.

HCC1937 (*A*) and BT549 (*B*) TNBC cells were treated with increasing amounts of CMP5 (0, 3, 6, 9, 12 μM) and total RNA was extracted 24 hrs and 48 hrs post-treatment, respectively. Expression of WNT/β-CATENIN target genes were analyzed by real time RT-PCR using gene specific primers and probe sets. The experiment was repeated two times with three technical replicates and *β-ACTIN* was used as an internal control. The data shown in the graph represent the mean for each concentration ± SD. Approximately 20 μg of RIPA extracts from either treated or non-treated HCC1937 (*C*) and BT549 (*D*) cells were analyzed by immunoblotting using the indicated antibodies. α-TUBULIN was detected to show equal loading. *** indicates *p* values < 10^{-3}, and ** indicates p value < 10^{-2}.

Next, we examined the impact of PRMT5 inhibition on protein expression of WNT/β-CATENIN target genes in TNBCs. In accord with the RT-PCR data, CYCLIN D1 and SURVIVIN protein levels decreased in HCC1937 cells after treatment with either 9 or 12 μM of CMP5 for 24 hrs (Fig. 14*C*). BT549 cells also showed significant reduction in CYCLIN D3 and SURVIVIN protein expression after 48 hrs incubation with 12 μM of CMP5 (Fig. 14*D*). In stark contrast, the levels of c-MYC protein showed an increase with either 9 or 12μM of CMP5 treatment in both TNBCs (Fig. 14*C* and *D*). These results indicate that through its ability to regulate WNT/β-CATENIN signaling, PRMT5 controls expression of key survival and cell cycle regulators including CYCLIN D1, CYCLIN D3 and SURVIVIN in TNBC cells.

4.9 PRMT5 inhibition alters its recruitment and H3R8 and H4R3 symmetric methylation in the promoter region of *DKK1* and *DKK3*

In our recent study, we have seen that CMP5 treatment reduces PRMT5 recruitment as well as PRMT5-induced epigenetic marks on the promoter region of WNT pathway antagonists, AXIN2 and WIF1 in lymphoma (Chung et al., 2019). In light of these findings, we investigated the impact of inhibition of PRMT5 on recruitment of PRMT5 as well as methylation of H3R8 and H4R3 in the promoter regions of DKK1 and DKK3 in TNBCs (Fig. 15)

Treatment of BT549 with PRMT5 inhibitor resulted in *DKK1* and *DKK3* transcriptional derepression (Fig. 12*A*). Therefore, we conducted ChIP experiments to monitor PRMT5 recruitment as well as methylation of H3R8 and H4R3 in the promoter region of *DKK1* and *DKK3* in the presence and absence of CMP5 (Fig. 15). ChIP analysis revealed that PRMT5 recruitment was increased by 7.4-fold ($p < 10^{-3}$) and 2.3-fold ($p < 10^{-3}$) in the promoter regions of *DKK1* and *DKK3*, respectively, in control DMSO-treated BT549 cells. Consistent with these results, symmetric methylation of H3R8 was enriched by 9.6 ($p < 10^{-3}$) and 7.1-fold ($p < 10^{-3}$) in the promoter of *DKK1* and *DKK3*, respectively. Similarly, methylation of H4R3 histones in the promoter region of *DKK1* and *DKK3* was increased by 2.6 to 6.3-fold ($p < 10^{-3}$). When BT549 cells were treated with CMP5, there was a 3.4-fold ($p < 10^{-3}$) decrease in PRMT5, which was accompanied by a 2.6-fold ($p < 10^{-3}$) and 1.5-fold ($p = 0.072$) reduced methylation of H3R8 and H4R3, respectively in the *DKK1* promoter (Fig. 15*A* and *B*). In addition, BT549 cells showed that CMP5 treatment resulted

in a decrease of PRMT5 binding by 2.6-fold ($p < 10^{-3}$), and H3R8 and H4R3 methylation by 2-fold, ($p < 10^{-3}$) and 6.6-fold ($p < 1\ 0^{-3}$) respectively, in the *DKK3* promoter region (Fig. 15*A* and *B*).

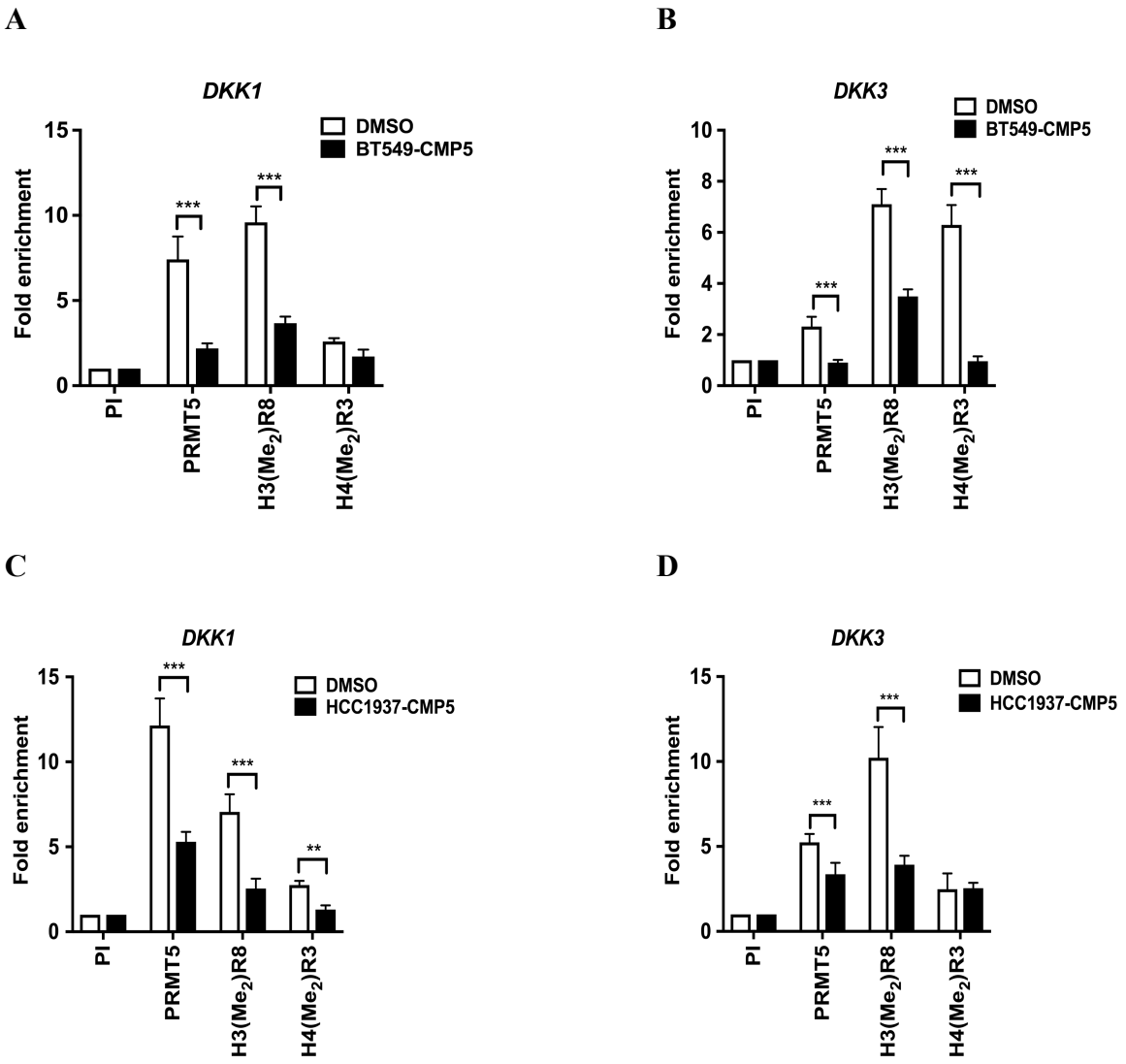


Figure 15: PRMT5 inhibition alters its recruitment and symmetric dimethylation of histones, H3R8 and H4R3 on promoter regions of DKK1 and DKK3

ChIP assay was performed to detect recruitment of PRMT5 and enrichment of its epigenetic marks in the promoter region of WNT/β-CATENIN antagonists as described in Fig. 10. Chromatin from either DMSO- or CMP5-treated BT549 (*A* and *B*) and HCC1937 (*C* and *D*) cells was immunoprecipitated using PI or the indicated immune antibodies. *DKK1* and *DKK3* promoter sequences were detected by real time PCR using specific primers and probe sets. The experiment was repeated two times with three technical replicates, and data in each graph represent the mean ± SD. *** indicates p values < 10^{-3}.

ChIP assay in DMSO-treated HCC1937 cells showed that PRMT5 recruitment was enriched in the promoter region of *DKK1* and *DKK3* by 12.1 ($p < 10^{-3}$) and 5.2-fold ($p < 10^{-3}$), respectively. Consistent with this result, symmetric methylation of H3R8 was enhanced by 7.06 to 10.2-fold ($p < 10^{-3}$) on both promoters, respectively. Similarly, symmetric methylation of H4R3 was increased by 2.8 and 2.5-fold ($p < 10^{-3}$) on *DKK1* and *DKK3* promoters, respectively. Treatment of HCC1937 cells with CMP5 reduced enrichment of PRMT5 by 2.3-fold ($p < 10^{-3}$) and decreased methylation of H3R8 and H4R3 by 2.8-fold ($p < 10^{-3}$) and 2.1-fold ($p = 0.007$) at the *DKK1* promoter, respectively (Fig. 15*C* and *D*). Similarly, treatment with CMP5 reduced PRMT5 binding by 1.6-fold ($p < 10^{-3}$) and symmetric methylation of H3R8 by 2.6-fold ($p < 10^{-3}$) at the *DKK3* promoter in HCC1937 cells. However, there was no reduction in symmetric methylation of H4R3 at the *DKK3* promoter upon treatment of HCC1937 cells with CMP5 (Fig. 15*C* and *D*). These results indicate that PRMT5 regulates proliferative WNT/β-CATENIN signaling through direct epigenetic repression of pathway antagonists, *DKK1* and *DKK3*.

4.10 PRMT5 modulates AKT/mTOR signaling in breast cancer in breast cancer

Hyperactivation of AKT/mTOR signaling due to multiple genetic aberrations and epigenetic dysregulations has been reported in various cancers. Furthermore, we have recently shown that PRMT5-mediated activation of AKT/GSK-3β signaling indirectly stimulates WNT/β-CATENIN proliferative signaling in lymphoma (Chung et al., 2019). We wanted to investigate whether PRMT5-meiated epigenetic dysregulation promote AKT/mTOR signaling in breast cancer (Fig. 16).

Western blot analysis showed that incubation with 12 μM CMP5 reduced the phosphorylation of AKT (S473) and mTOR (S2448) significantly in HCC1937 and BT549 cells after 24 hrs and 48 hrs, respectively. However, the levels of total AKT and mTOR remained unaffected (Fig. 16*A* and *B*). These findings indicated that in addition to WNT/β-CATENIN proliferative signaling, PRMT5 also affects AKT/mTOR signaling in breast cancer.

A **B**

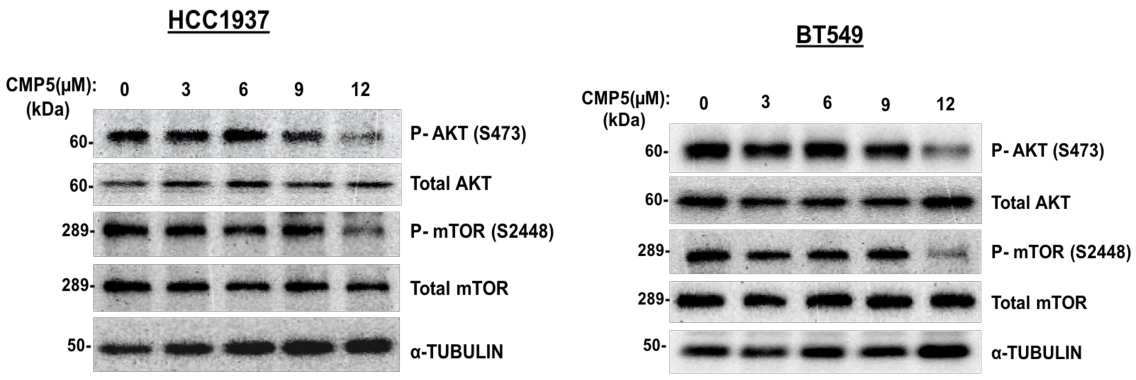


Figure 16: PRMT5 inhibition downregulates AKT/mTOR signaling in breast cancer

RIPA extracts (20 μg) from HCC1937 (*A*) and BT549 (*B*) cells treated with PRMT5 inhibitor, CMP5 (0, 3, 6, 9, 12 μM) for 24 hrs and 48 hrs, respectively were analyzed by Western blotting to evaluate the levels of phospho-AKT (S473) and total AKT as well as phospho-mTOR (S2448) and total mTOR. α-TUBULIN was detected to represent equal loading.

4.11 PRMT5 is required for migration and invasion of TNBC cells

Enhanced migration is an intrinsic property of cancer cells, which also helps metastasis, a hallmark of triple negative breast cancers. Having found that CMP5 inhibits the proliferation of breast cancer cells we planned to discover the effect of PRMT5 inhibition on breast cancer cell migration and invasion *in vitro* by migration assay and invasion assay, respectively as described in methodology section (Fig. 17).

Treatment of MCF7, HCC1937 and BT549 with CMP5 at their respective LC_{50} dose, showed that migration of MCF7 and HCC1937 cells was completely inhibited compared to control DMSO-treated cells. However, migration of BT549 cells was reduced by 58.6% ($p < 10^{-3}$) (Fig. 17*A* and *B*). To assess if PRMT5 inhibition can impact invasiveness of breast cancer cells, we utilized Boyden invasion assay. Our findings showed that PRMT5 inhibition using the respective LC_{50} completely abolished invasion by MCF7, HCC1937 and BT549 (Fig. 17*C* and *D*).

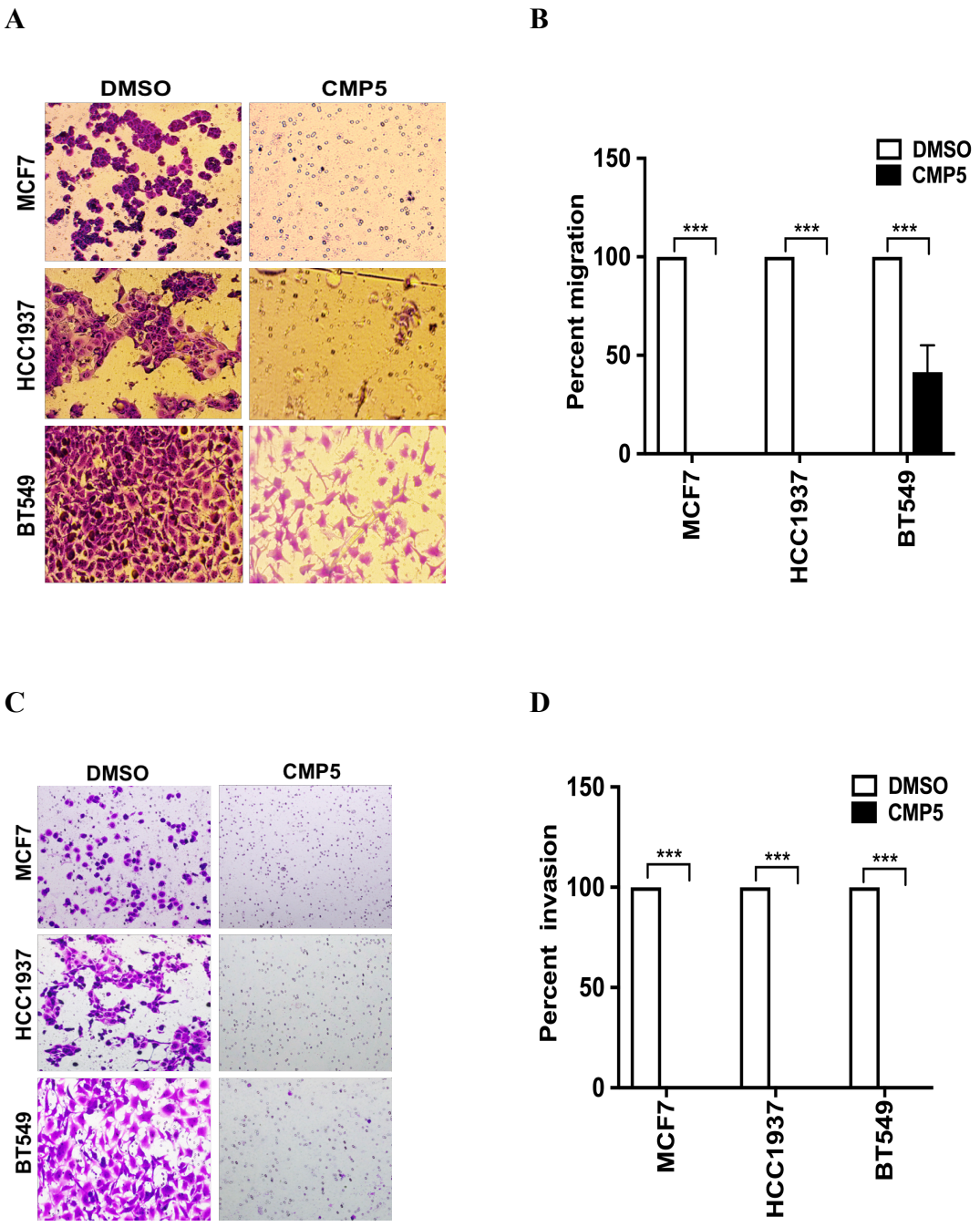


Figure 17: PRMT5 inhibition reduces migration, invasion and induces apoptosis of breast cancer cells.

(*A*) Migration of breast cancer cells was evaluated using Matrigel coated Boyden chamber. Breast cancer cells were treated with either DMSO or CMP5 for 48 hrs (MCF7 and BT549) or 24 hrs (HCC1937) as described in Materials and Methods. The migrated cells were stained with crystal violet and the photomicrographs were captured using an inverted microscope with a magnification of 100X. (*B*) The percentage of migrated cells was determined using ImageJ software, and the migration capacity was normalized to that of DMSO treated cells. (*C*) Invasion of breast cancer cells was assessed using modified Boyden chamber. MCF7, HCC1937 and BT549 cells were treated with either DMSO or CMP5 for 24 hrs or 48 hrs as described in Materials and Methods section. The percentage of invading cells was determined after staining with crystal violet using an inverted microscope with a magnification of 100X. (*D*) Membrane-associated invading cells were quantified using ImageJ software, and the percent invasion was determined in comparison to DMSO-treated cells.

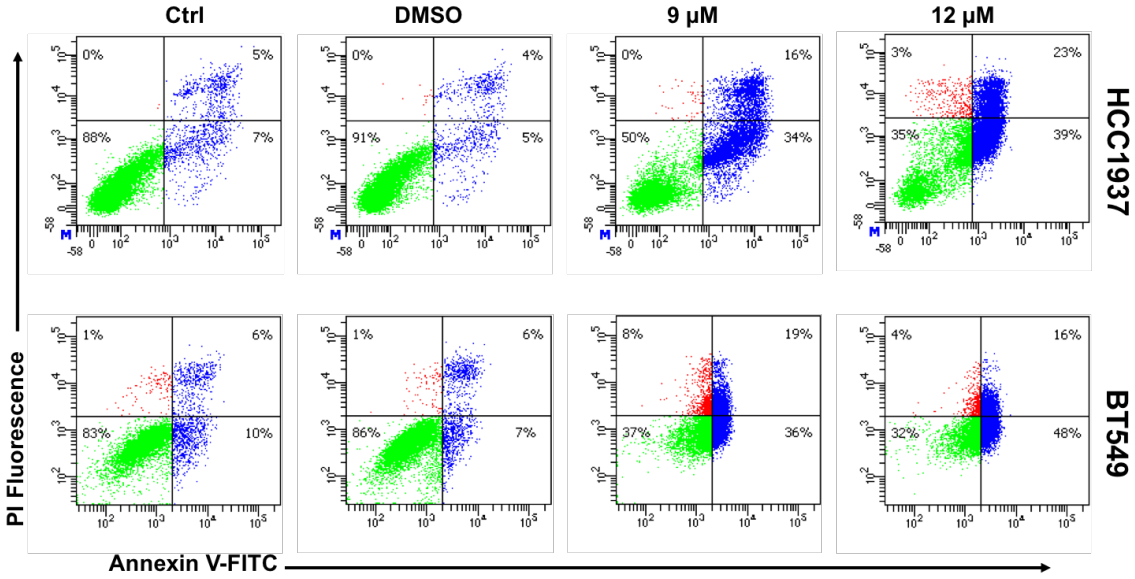
4.12 PRMT5 inhibition induces death of TNBC cells

Our findings showed that upon incubation with PRMT5 inhibitor, CMP5, breast cancer cells become less viable (Fig. 11). In order to determine how CMP5 reduced viability of breast cancer cells, we treated them with 9 μM and 12 μM of CMP5 and analyzed ANNEXIN V positive cells using FACS analysis (Fig. 18).

Treatment of HCC1937 cells with 9 μM and 12 μM of CMP5 for 24 hrs, using four technical replicates from two biological replicates, showed an average of 46.2% ($p < 10^{-3}$)

and 57.1% ($p < 10^{-3}$) cell death, respectively (Fig. 18*A* and *B*). Similarly, incubating BT549 cells with 9 µM and 12 µM of CMP5 for 48 hrs resulted in an average of 58.7% ($p < 10^{-3}$) and 60.3% ($p < 10^{-3}$) cell death (Fig, 18*A* and *B*). Collectively, these results show that PRMT5 regulates the growth characteristics of breast cancer cells and that its inhibition can arrest growth and induce death of aggressive TNBC cells.

A



B

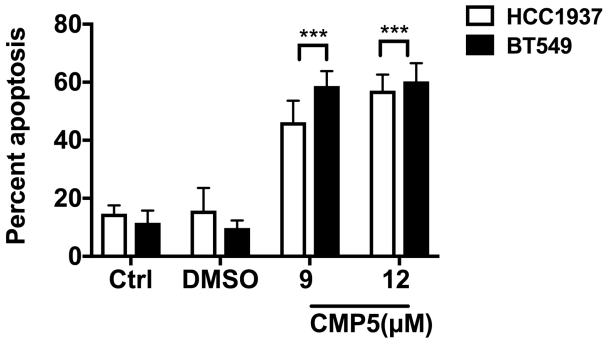


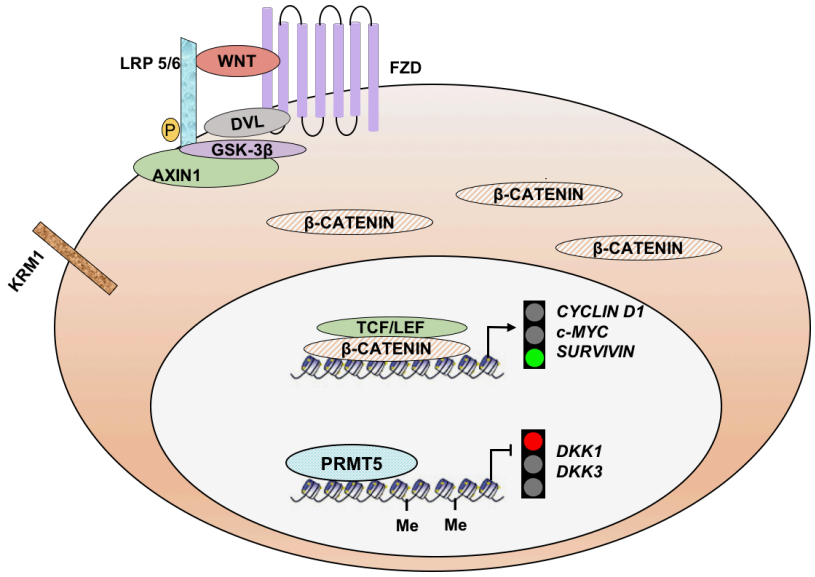
Figure 18. PRMT5 inhibition induces apoptosis of breast cancer cells

(*A*) HCC1937 and BT549 cells were treated with different concentrations of CMP5 as indicated, and the number of dead cells was measured by FACS analysis after staining cells with ANNEXIN V and propidium iodide (PI). (*B*) The average result from the two biological replicates used in *E* is represented as mean ± SD. All experiments were carried out twice in duplicates, and the data is represented as mean ± SD. *** indicates p values < 10^{-3}.

CHAPTER 5: DISCUSSION

Studies on epigenetic modifications that regulate proliferative and death signaling pathways have become center stage for therapeutic intervention. PRMT5 is now considered a key epigenetic modifying enzyme that governs major cellular process including cell proliferation and apoptosis. We have recently reported that enhanced expression of PRMT5 upregulates WNT/β-CATENIN proliferative signaling by transcriptionally repressing pathway antagonists, *AXIN2* and *WIF1*, in three different types of non-Hodgkin's lymphoma cells (Chung et al., 2019). In the current study, we show that PRMT5 levels are upregulated in different types of breast cancer cell lines compared to normal human mammary epithelial cells. Our investigation revealed that PRMT5 promotes growth of breast cancer cells *in vitro* by promoting WNT/β-CATENIN signaling via suppression of expression of WNT antagonists, DKK1 and DKK3, thereby leading to enhanced expression of WNT/β-CATENIN target genes including *c-MYC*, *CYCLIN D1* and *SURVIVIN*. More specifically, our experiments show that PRMT5 inhibition using the specific inhibitor, CMP5, in breast cancer cells reduces expression of WNT/β-CATENIN target genes, *CYCLIN D1* and *SURVIVIN*, and induces expression of pathway antagonists, DKK1 and DKK3 (Fig. 19). Our findings also show significant reduction in cell proliferation, migration and invasion in response to PRMT5 inhibition. These changes in the growth characteristics of breast cancer cells are also accompanied by induced cell death.

PRMT5 activity on



PRMT5 activity off

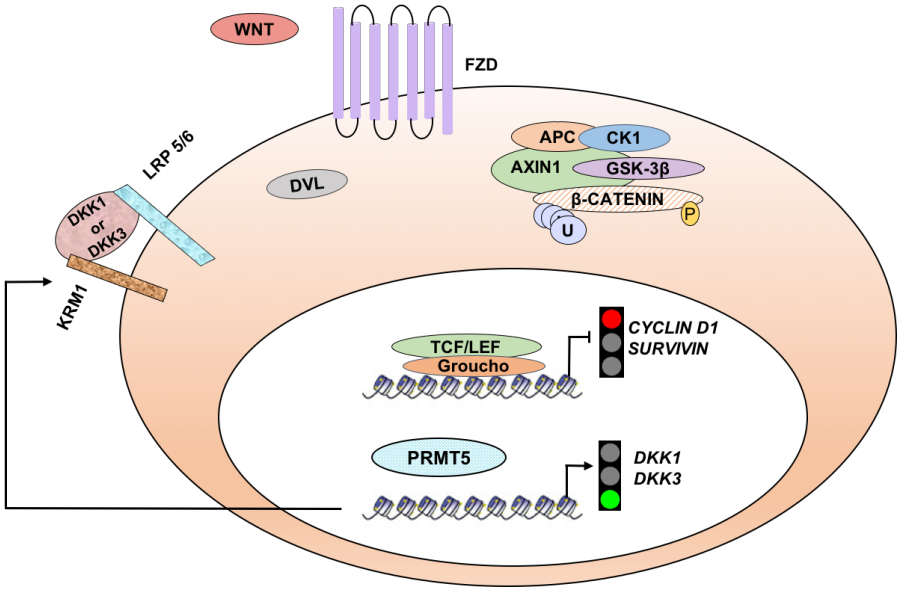


Figure 19. Model for PRMT5-mediated upregulation of WNT/β-CATENIN signaling in breast cancer

PRMT5 is recruited to the promoter region of WNT/β-CATENIN pathway antagonists, *DKK1* and *DKK3*, and methylates H3R8 and H4R3 leading to their transcriptional repression. In the absence of DKK1 and DKK3, WNT ligand binds to its receptor, Frizzled receptor (FZD), which complexes with LRP5/6 co-receptor. Ligand bound Frizzled receptor recruits Dishevelled (DVL) and promotes binding of GSK-3β, which phosphorylates LRP5/6 and favors AXIN1 recruitment. Consequently, the cytosolic destruction complex formation and β-CATENIN phosphorylation are prevented, which in turn increases cytosolic β-CATENIN level. Accumulated β-CATENIN translocates to the nucleus, where it complexes with TCF/LEF and activates transcription of target genes of WNT signaling, *CYCLIN D1*, *c-MYC* and *SURVIVIN* (PRMT5 On). PRMT5 inhibition results in transcriptional derepression of *DKK1* and *DKK3*, which are excreted and bind to LRP5/6 through interaction with KREMEN1 (KRM1), thereby form a ternary complex that is endocytosed and degraded. In the absence of LRP5/6, WNT signaling is inhibited, resulting in β-CATENIN degradation. During β-CATENIN unavailability, TCF and LEF associate with transcriptional co-repressors, Groucho to suppress transcription of target genes of WNT pathway, *CYCLIN D1* and *SURVIVIN* (PRMT5 Off). These molecular changes are associated with reduced proliferation, migration and invasion, and increased death of breast cancer cells.

PRMT5 is associated with abnormal proliferation of breast cancer cells, and it was shown that PRMT5 promotes growth of MCF7 cells by increasing expression of the translation factor, eIF4E (Scoumanne et al., 2009). In another study, elevated levels of PRMT5 were shown to reduce the tumor-suppressor activity of PDCD4 in primary breast cancer tumors

by methylating its N-terminal arginine residues, resulting in reduced patient survival (Powers et al., 2011) . A more recent study indicated that PRMT5 levels are upregulated in MCF7, MDA-MB-231 and MCF-10A cell lines as well as in clinical samples of ductal carcinoma (Yang et al., 2015). In line with these findings, our investigation showed that PRMT5 levels are upregulated in three different types of breast cancer cell lines including MCF7, HCC1937 and BT549 compared to normal human mammary epithelial cells (Fig. 7). Dysregulation of WNT/β-CATENIN signaling in breast cancer serves as a major oncogenic driver, and its upregulation has been implicated in many aggressive and invasive breast cancer types, and correlates with acquired drug resistance (Prosperi & Goss, 2010; King et al., 2012; Loh et al., 2013). Aberrant activation of WNT/β-CATENIN signaling leads to enhanced expression of downstream target genes. Our study showed that levels of WNT/β-CATENIN target genes *CYCLIN D1*, *c-MYC* and *SURVIVIN* are elevated in breast cancer cells (Fig. 8).

Epigenetic silencing of WNT/β-CATENIN antagonists is frequently observed in breast cancer, and reduced expression of *APC*, *CDH1*, *SFRP1* and *SFRP2* due to promoter hypermethylation has been reported in many breast cancer samples (Klarmann et al., 2008). More strikingly, *WIF1* promoter hypermethylation has been frequently observed in breast cancer, and also serves as an early trigger in the development of hereditary breast cancers (Ai et al., 2006; Alvarez et al., 2013). A previous study indicated that WIF1 expression is significantly repressed in breast cancer stem cells, which in turn promotes their self-renewal (Wang et al., 2015). *AXIN1* mRNA levels were also found to be repressed in MDA-MB-231 cells and many breast cancer clinical samples (Zhang et al., 2012). Furthermore,

DKK1 and *DKK3* promoter hypermethylation has been documented in many breast cancer samples (Suzuki et al., 2008; Xiang et al., 2013). We have examined expression of a panel of WNT/β-CATENIN pathway antagonists in breast cancer cell lines, and found that expression of DKK1 and DKK3 is significantly reduced in triple negative breast cancer cell lines (Fig. 9). In light of our findings, which showed that PRMT5 is overexpressed in breast cancer cells, we checked if it was involved in inducing transcriptional repression of *DKK1* and *DKK3* in breast cancer. ChIP analysis showed that PRMT5 binds to the promoter region of WNT antagonists, *DKK1* and *DKK3*, and induces symmetric methylation of H3R8 and H4R3, thereby causing their transcriptional repression (Fig. 10). To evaluate the contribution of PRMT5 to transcriptional regulation of *DKK1* and *DKK3*, we inhibited its activity and monitored expression of both WNT/β-CATENIN antagonists. We found that PRMT5 inhibition brings about *DKK1* and *DKK3* transcriptional derepression, which correlates with reduced expression of WNT/β-CATENIN target genes *CYCLIN D1* and *SURVIVIN* in TNBCs (Fig. 12 and 14). However, *c-MYC* showed enhanced expression in response to PRMT5 inhibition. A prior study by Evan et al. (1992) showed that when expressed in the absence of proliferation signals, c-MYC induces cell death. In agreement with this result, a later study by Murphy et al. (2008) showed that distinct c-MYC expression levels regulate proliferation and apoptosis. While low levels of c-MYC protein promote cell proliferation, its elevated expression activates the ARF/p53 tumor suppressor pathway, which leads to cell death (Murphy et al., 2008). In our study, we found that PRMT5 inhibition reduces viability of breast cancer cells and induces their death (Fig. 11 and 18). Therefore, it is conceivable that CMP5 treatment induces breast cancer cell death through decreased expression of CYCLIN D1 and SURVIVIN, and

enhanced c-MYC expression. Further studies focusing on the mechanism by which c-MYC induction is achieved are needed.

We have shown that PRMT5 epigenetically induces transcriptional repression of *DKK1* and *DKK3* in TNBCs, and that its inhibition restores expression of both WNT/β-CATENIN antagonists in BT549 cells. Surprisingly, CMP5 treatment did not induce transcriptional derepression of *DKK1* and *DKK3* in HCC1937 cells, indicating that there are other epigenetic modifications involved in their regulation (Fig. 12*B*). The fact that PRMT5 is known to induce transcriptional repression through interaction with DNMT3A (Liu et al., 2018), we thought that both PRMT5 and DNMT3A might be playing a similar role in silencing *DKK1* and *DKK3* in HCC1937 cells. However, treatment of HCC1937 cells with the DNA methyltransferase inhibitor, 5-Azacytidine either alone or in combination with CMP5 did not trigger *DKK1* and *DKK3* derepression more than 1.6 to1.7-fold (Fig. 13*B*). PRMT5 is also known to associate with histone deacetylases 2 and 3 in large multisubunit transcriptional repressor complexes (Pal et al., 2003; Pal et al., 2004; Alinari et al., 2015) Therefore, we reasoned that reduced expression of *DKK1* and *DKK3* could be due to enhanced hypoacetylation of *DKK1* and *DKK3* promoter histones in HCC1937 cells. Treatment of HCC1937 cells with TSA alone or in combination with CMP5 did not induce *DKK1* and *DKK3* transcriptional derepression (Fig. 13*C*). However, combinatorial treatment of HCC1937 cells with CMP5, 5-Aza and TSA resulted in increased transcriptional derepression of *DKK1* and *DKK3*, indicating that histone lysine deacetylation, histone arginine methylation and DNA methylation work in concert to epigenetically silence DKK1 and DKK3 (Fig. 13*E*).

To further understand the role of PRMT5 in regulating other pathways that promote breast cancer growth, we investigated the effect of major growth promoting signaling cascade, PI3K/AKT/mTOR pathway in breast cancer. The PI3K/AKT/mTOR signaling cascade regulates the gene expression program that regulate cell growth and survival. Aberrant activation of PI3K/AKT/mTOR pathway is frequently associated with many cancer types including breast cancer. External cues such as growth factors can activate Phosphoinositide 3-kinase (PI3K) resulting in phosphorylation of AKT at T308 and S473 by PDK1 and mTORC2, respectively. Phosphorylated AKT can activate several downstream substrates, including TSC2 (Tuberous Sclerosis Complex 2), and prevents the formation of TSC1-mTORC1 kinase complex, resulting in activation of mTORC1. Activated mTORC1 phosphorylates its downstream target proteins including the eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1) at its multiple sites including Ser6, Thr37/46, and Thr70. Phosphorylation of 4E-BP1 results in releasing of eukaryotic initiation translation factor 4E (eIF4E) from 4E-BP1- eIF4E complex. eIF4E can then form an active eIF4F complex, resulting in stimulation of cap-dependent translation. mTORC1 is also a direct target of activated AKT which phosphorylates at Ser 2448 (Sekulic et al., 2000). An initial study by Wei et al. (2012) showed that PRMT5 overexpression elevates phosphorylation of PI3K p85 (Tyr458) and AKT (Ser308 and Ser473) and reduces phosphorylation of negative regulator of AKT, PTEN (Ser380). Consequently, the phosphorylation of AKT downstream effector, mTOR (Ser 2448), and expression of eIF4E was elevated, indicating that PRMT5 can activate PI3K/AKT/mTOR signaling. In

addition, a recent study by Zhang et al. (2019) showed that PRMT5 can directly interact with AKT and promote its phosphorylation at Thr308 and Ser473 in lung cancer cells. In line with these findings, the current study showed tha inhibition of PRMT5 reduces phosphorylation of AKT and mTOR at Ser 473 and Ser 2448 respectively in both HCC1937 and BT549 cells (Fig. 16), further confirming the role of PRMT5 in AKT/mTOR pathway.

Metastasis is a common characteristics of all aggressive breast tumors, and contributes to poor prognosis and increased mortality (Redig & McAllister, 2013). A previous study by Dey et al. (2013) showed that enhanced activation of WNT/β-CATENIN signaling is positively correlated with increased metastatic potential and overall worse prognosis of breast cancer patients, highlighting the role of WNT/β-CATENIN activation in breast cancer metastasis. Furthermore, inhibition of WNT/β-CATENIN activation has been shown to reduce proliferation and migration of BT549 cells, and induce their death *in vitro* (Bilir et al., 2013). Since our study showed that PRMT5 inhibition reduces WNT/β-CATENIN signaling in breast cancer cells, we examined the effect of PRMT5 inhibition on migration and invasion of breast cancer cells. Our results showed significant reduction in breast cancer cell migration and invasion in response to PRMT5 inhibition (Fig. 17*A-D*). Our results also showed that PRMT5 inhibition using CMP5 LC_{50} concentration induced death of TNBCs as measured by Annexin V/PI staining (Fig. 18*A*and *B*).

Taken together, our work shows that PRMT5 levels are upregulated in breast cancer cell

lines, and that it is involved in epigenetic silencing of a different set of WNT antagonists compared to lymphoma cells. In aggressive lymphomas, PRMT5 epigenetically inhibits expression of WNT antagonists, AXIN2 and WIF1 (Chung et al., 2019). AXIN2 serve as a scaffolding protein in the cytosolic destruction complex, which induces β-CATENIN degradation (Hinoi et al., 2000), whereas, WIF1 inactivates WNT/β-CATENIN signaling by directly binding to WNT ligand and inhibiting its interaction with the FZD receptor (Hsieh et al., 1999; Malinauskas et al., 2011). In breast cancer cells, PRMT5 suppresses expression of DKK1, which antagonizes WNT/β-CATENIN signaling by binding to its cognate membrane receptor, KREMEN2, and promoting cell surface removal of LRP5/6 by endocytosis, thereby preventing dimerization of WNT-bound FZD with LRP5/6 co-receptor (Mao et al., 2001; Mao et al., 2002). DKK3 is also epigenetically suppressed by PRMT5; however, the mechanism by which DKK3 down-regulates WNT signaling remains unclear. Regardless, it appears that PRMT5 targets various WNT/β-CATENIN pathway antagonists that operate at different levels, highlighting the complexity and diversity of its mechanism of action in the different cell types. The net outcome of PRMT5-induced epigenetic silencing is to promote growth and survival of cancer cell, which renders its ideal for therapeutic intervention, because its selective inhibition is marked by decreased expression of pro-survival proteins, *CYCLIN D1* and *SURVIVIN*. These molecular changes trigger reduced proliferation, migration, and invasion, and increased cell death, which are all desired attributes for killing tumor cells.

CHAPTER 6: CONCLUSION AND FUTURE WORK

CONCLUSION

Protein arginine methyltransferase 5 (PRMT5) activity is dysregulated in many aggressive cancers and its enhanced levels are associated with increased tumor growth and survival. The current study was focused on investigating the role of PRMT5 in promoting breast tumorigenesis. The results of the study showed that PRMT5 is overexpressed in breast cancer cell lines of varying aggressiveness, and that it promotes their growth by activating WNT/β-CATENIN proliferative signaling through epigenetic silencing of pathway antagonists, *DKK1* and *DKK3*, leading to enhanced expression of *c-MYC*, *CYCLIN D1* and *SURVIVIN*. Through chromatin immunoprecipitation (ChIP) studies, it was confirmed that PRMT5 binds to the promoter region of WNT antagonists, *DKK1* and *DKK3*, and induces symmetric dimethylation of H3R8 and H4R3 histones. Furthermore, PRMT5 inhibition using a specific small molecule inhibitor, compound 5 (CMP5), reduced PRMT5 recruitment as well as methylation of H3R8 and H4R3 histones in the promoter regions of *DKK1* and *DKK3*, which consequently results in reduced expression *CYCLIN D1* and *SURVIVIN*. In addition, CMP5 treatment either alone or in combination with 5-Azacytidine and Trichostatin A restored expression of *DKK1* and *DKK3* in TNBCs. PRMT5 inhibition also altered the growth characteristics of TNBC cells including proliferation, migration and invasion, and induced their death.

PROSPECTIVE

The current study compared the expression of PRMT5, WNT/β-CATENIN downstream target genes and pathway antagonists in three different breast cancer cell lines versus normal mammary epithelial cells (HMECs). The results of the study indicated that there is positive correlation between PRMT5 expression and that of WNT/β-CATENIN target genes, *CYCLIN D1*, *c-MYC* and *SURVIVIN*, and that there is an inverse correlation between PRMT5 and WNT/β-CATENIN antagonists, *DKK1* and *DKK3,* expression in breast cancer cells (Chapter 4.1, 4.2 and 4.3). Since breast cancer is a heterogeneous disease, it is important to screen more breast cancer cell lines as well as clinical samples to determine and validate the biological relevance of our findings. In lymphoma, PRMT5 levels are increased due to increased translation and reduced expression of miRs 92 and 96 (Pal et al., 2007). However, the current study revealed that expression of PRMT5 is elevated due to enhanced gene transcription in breast cancer. The mechanism by which PRMT5 transcription is increased in breast cancer is at present unknown, and more work is required to address this question. Hence, we plan to perform ChIP experiments using antibodies against transcriptional activation marks including H3K9ac, H3K27ac and H3K4me3 to decipher the mechanism of transcriptional activation of PRMT5 in breast cancer.

In the current study it has been demonstrated that PRMT5 inhibition reduces proliferation, migration and invasion of breast cancer cells and induces their death *in vitro* (Chapter 4.5, 4.11 and 4.12). Therefore, it is important to characterize the role of PRMT5 overexpression and impact of its inhibition *in* vivo using TNBC xenografts in SCID mice. This would

further validate the therapeutic significance of targeting PRMT5 for breast cancer treatment.

The mechanism by which PRMT5 activates WNT/β-CATENIN signaling in breast cancer was investigated in the current study, which showed that PRM5 epigenetically suppresses expression of WNT pathway antagonists, *DKK1* and *DKK3,* by binding to their promoters and inducing symmetric methylation of H3R8 and H4R3 histones (Chapter 4.3 and 4.4). DKKs antagonize WNT/β-CATENIN signaling by binding to the membrane receptor, KREMEN1, and promoting cell surface removal of LRP5/6 by endocytosis, thereby preventing dimerization of WNT-bound FZD with LRP5/6 co-receptor (Mao et al., 2002; Mao et al., 2001). Hence, it would be interesting to design peptide mimicks corresponding to the biologically active DKK1 and DKK3 domains, and to test their efficacy in reducing breast cancer growth and metastasis. We also plan to investigate the impact of stable re-expression of DKK1 and DKK3 using tet-inducible lentiviral expression vector on WNT/β-CATENIN signaling and to assess their influence on the growth characteristics of TNBCs *in vitro* and *in vivo*.

PRMT5 inhibition also resulted in reduced expression of WNT/β-CATENIN downstream target genes, *CYCLIN D1* and *SURVIVIN*. However, c-MYC expression was increased in both HCC1937 and BT549 cells. It would be interesting to study the mechanism by which PRMT5 regulates c-MYC expression in breast cancer cells. Furthermore, we plan to conduct differential gene expression studies using RNA-Seq analysis before and after

CMP5 treatment. We also plan to conduct proteomic analysis to investigate the differential expression of proteins before and after CMP5 treatment in TNBCs. These studies will help us identify PRMT5-regulated networks and pathways involved in breast cancer tumorigenesis.

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