Aus dem

University Hospital Schleswig-Holstein Medical Clinic 1

Direktor: Prof. Jens Marquardt

The Role of PRMT5-dependent Regulation of DKK1 in the Induction of Chemoresistance in Colorectal Cancer

Inauguraldissertation

Zum Erwerb des Doktorgrades

der Universität zu Lübeck

- Aus der Sektion Medizin vorgelegt von

Wafaa M. S. Abumustafa

Aus Sharjah, Vereinigte Arabische Emirate

Lübeck 2024

Berichterstatterin/Berichterstatter: Prof. Jens Marquardt

Berichterstatterin/Berichterstatter: Prof. Sven Perner.

Tag der mündlichen Prüfung: …………………………

Zum Druck genehmigt. Lübeck, den …………………………

Promotionskommission der Sektion Medizin

Table of Contents

Table of Contents iii

List of Figures ix

List of Tables xi

Abstract xii

Zusammenfassung xiii

Chapter I. Introduction 1

1.1. Colorectal cancer prevalence 1

1.2. Risk factors correlated with colorectal cancer 2

1.3. Colorectal cancer classification 3

1.4. Genetic alterations associated with CRC 6

1.5. Epigenetic alterations associated with colorectal cancer 9

1.5.1. DNA methylation in CRC 9

1.5.2. Histone modifications in CRC 10

1.6. Diagnosis of CRC 11

1.7. Treatment of CRC 12

1.7.1. Chemotherapy in CRC 13

1.7.2. Chemoresistance in CRC 15

1.7.3. Targeted therapy in CRC 16

1.7.4. Immunotherapy in CRC 16

Chapter II. Literature review 18

2. 1. The DKK protein family 18

2. 2. DKK protein family role in CRC 20

2. 3. DKKs regulation in CRC.....21

2. 4. Protein arginine N-methyltransferase 5 (PRMT5).....22

2. 5. The role of PRMT5 in CRC.....24

2. 6. Targeting PRMT5 in CRC.....26

2.6.1. PRMT5 regulation in CRC.....26

2.6.2. Arginine methyltransferase inhibitor 1 (AMI-1).....27

2.6.3. EPZ015666 (GSK3235025) and EPZ015866 (GSK591 or GSK3203591).....27

2.6.4. PR5-LL-CM01.....28

2.6.5. PRMT5 inhibitor compound 5 (CMP5).....29

2. 7. Statement of the problem.....29

Hypothesis, aims, and objectives.....30

2. 8. Opportunities for contributions to knowledge.....31

Chapter III. Methodology.....32

3.1. Work flow.....32

3.2. Cell lines.....33

3.3. Methods.....33

3.3.1. In silico analysis.....33

3.3.2. Cell culture.....33

3.3.3. RNA interference.....34

3.3.4. Polymerase chain reaction (PCR).....34

3.3.5. Western blot.....35

3.3.6. Resazurin....................35

3.3.7. Apoptosis assay....................35

3.3.8. Cell cycle assay....................35

3.3.9. Chromatin Immunoprecipitation (ChIP) assay....................36

3.3.10. Next-generation sequencing (NGS)....................36

3.3.11. Immunofluorescence assay....................37

3.3.12. Immunohistochemistry....................37

3.3.13. Statistical analysis....................38

Chapter IV. Results....................40

5.1. *DKK1* gene is differentially expressed in *KRAS* mutant CRC forms.....................40

5.2. PRMT5 expression is upregulated in CRC and correlated with pro-oncogenic pathways.....................50

5.3. DKK1 expression is regulated by PRMT5 in CRC.........57

5.4. PRMT5 inhibition reduces cancer cell viability in vitro.62

5.5. CMP5 and doxorubicin exert synergistic anti-tumor effects in KRAS mutant CRC cells.....................62

5.6. DKK1 inhibition sensitizes *KRAS* mutant CRC cells to the anti-tumor effects of doxorubicin via inhibiting the PI3K/AKT pathway.....................65

5.7. The oncogenic activity of DKK1 is WNT signaling pathway independent....................67

Chapter V. Discussion....................69

Chapter VI.....76

Conclusion 76

Conclusion and recommendations 76

Limitations of the study.....77

Future Perspectives.....77

References.....79

List of abbreviation

DKK	Dickkopf
PRMT5	Protein arginine N-methyltransferase 5
ChIP	Chromatin immunoprecipitation
CMP5	PRMT5 inhibitor compound 5
CRC	Colorectal cancer
IBD	inflammatory bowel disease
UC	ulcerative colitis
CD	Crohn's disease
APC	adenomatous polyposis coli
TNM	Tumor, node, metastasis
CMS	consensus molecular subgroups
CIN	Chromosomal Instability
MSI	Microsatellite Instability
CIMP	CpG Island Methylator Phenotype
SCNA	somatic copy number alterations
MMR	mismatch repair
STR	short tandem repeats
FAP	familial adenomatous polyps
SPS	Serrated polyposis syndrome
CS	Cowden syndrome
LFS	Li-Fraumeni syndrome
DNMT	DNA methyltransferases
HAT	histone acetyl transferases
HDAC	histone deacetyl transferases
HMT	histone methyl transferases
HDM	histone demethylases
LSD1	histone lysine-specific demethylase 1
FIT	fecal immunochemical test
5-FU	5-fluorouracil
PD-1	programmed death-1
CTLA-4	Cytotoxic T-lymphocyte associated protein 4
NACT	neoadjuvant chemotherapy
ROS	reactive oxygen species
ABC	ATP-binding cassette transporters
EGFR	epidermal growth factor receptor
CKAP4	Cytoskeleton-associated protein 4
TET1	Ten-eleven translocation methylcytosine dioxygenase 1
SAM	S-adenosylmethionine
FGFR3	factor receptor 3
NAA40	N-alpha-acetyltransferase 40
AMI-1	Arginine methyltransferase inhibitor 1
HTS	High throughput screening
TCGA	The Cancer Genome Atlas
COAD	colon adenocarcinoma
READ	rectum adenocarcinoma
siRNA	small interfering RNA
PCR	Polymerase chain reaction

NGS	Next-generation sequencing
RNAseq	RNA sequencing
IPA	QIAGEN Ingenuity Pathway Analysis
FDR	false discovery rate

List of Figures

Figure 1: CRC prevalence worldwide and in the UAE. 2

Figure 2: CRC consensus molecular subgroups (CMS). 5

Figure 3: CRC development pathways. 8

Figure 4: Summery of CRC treatment strategies. 13

Figure 5: Kaplan Meier graph of 1301 KRAS-WT and 1713 KRAS-MUT CRC cases..14

Figure 6: DKK protein family functional domains. 19

Figure 7: DKK1 implication in WNT/β-catenin and PI3K/AKT pathways. 21

Figure 8: The role of PRMT5 in CRC. 25

Figure 9: Experimental design work flow. 32

Figure 10: Differentially expressed DKK protein family in different cancers. 43

Figure 11: DKK gene family expression in COAD and READ. 44

Figure 12: DKK gene family expression in KRAS mutant COAD and READ. 47

Figure 13: DKK gene family expression correlation with KRAS expression in CRC 48

Figure 14: DKK1 gene expression in KRAS mutant versus normal CRC cell lines and correlation with CRC differentiation. 49

Figure 15: PRMT5 is overexpressed in CRC and associated with KRAS pathway activation. 53

Figure 16: PI3K signaling pathway is a potential target of PRMT5. 54

Figure 17: Differentially expressed genes in PI3K pathway upon PRMT5 knockdown in CRC cell lines. 57

Figure 18: PI3K signaling pathway is activated by PRMT5. 57

Figure 19: DKK gene family correlation with PRMT5. ……………………………..59

Figure 20: DKK gene family expression upon PRMT5 knockdown in CRC cell lines. 60

Figure 21: DKK1 is epigenetically regulated by PRMT5 in CRC.....................................61

Figure 22: PRMT5 inhibition reduces viability in vitro. A-b)..62

Figure 23: CMP5 and doxorubicin exert synergistic anti-tumor effects in KRAS mutant CRC cells...64

Figure 24: DKK1 inhibition sensitizes KRAS mutant CRC cells to the anti-tumor effects of doxorubicin...66

Figure 25: In silico analysis of protein networks associated with DKK1. 67

Figure 26: The oncogenic activity of DKK1 is Wnt signaling pathway independent......68

Figure 27: Graphical summary of our findings. 75

List of Tables

Table 1: TNM classification………………………………………………..…………….. 4

Table 2: Summary of the candidate drugs targeting PRMT5 in CRC……………………………………………………………………………………. 28

Table 3: CRC cell lines origine and mutational background…………………………. 33

Table 4: qPCR primers ……………………………………………………………….. 34

Table 5: Histopathological biomarkers of colon cancer patients & TMA…………………………………………………………………………………….. 38

Table 6: The association between DKK1 expression levels in CRC patients, TMA and the clinicopathological characteristics…………………………………………………. 49

Abstract

The Dickkopf family proteins (DKKs) are strong Wnt signaling antagonists and are believed to play a significant role in the development and progression of colorectal cancer (CRC). Recent work indicates that DKKs, mainly DKK1, are also associated with the induction of CRC chemoresistance. Additionally, the expression of DKK1 could be closely linked to the expression levels of protein arginine N-methyltransferase 5 (PRMT5) in different cancers, potentially suggesting a regulatory loop between DKK1 and PRMT5. However, whether PRMT5 is involved in regulating *DKK1* expression in CRC and whether such an axis could contribute to CRC chemoresistance has yet to be investigated. To address this issue, both in silico and in vitro approaches were employed to explore the relationship between PRMT5 and DKKs. Our data demonstrated that DKK1 expression is significantly upregulated in CRC clinical samples and particularly prominent in *KRAS*-mutated cancers. Furthermore, increased levels of *DKK1* were associated with the activation of PRMT5. Chromatin immunoprecipitation (ChIP) assay indicated an epigenetic role of PRMT5 in the upregulation of DKK1 via inducing H3R8 symmetric dimethylation. Consistently, knockdown of DKK1, as well as treatment of CRC cells with CMP5 (a PRMT5 inhibitor) in combination with doxorubicin, resulted in a synergistic anti-tumor effect in KRAS mutant, but not KRAS wild-type, CRC cells. Mechanistically, the role of DKK1 in CRC seems to be independent of WNT signaling but might be mediated by activation of the PI3K/AKT pathway. Taken together, these results suggest that PRMT5 regulates DKK1 expression in CRC and that inhibition of PRMT5 can effectively modulate DKK1 expression and significantly reduce CRC cell growth, KRAS-mutant CRC cells in particular.

Zusammenfassung

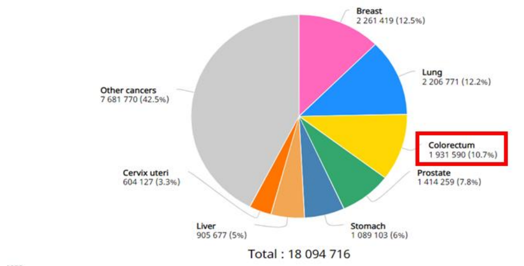
Proteine der Dickkopf-Familie (DKKs) sind starke Inhibitoren des Wnt-Signalweges . Verschiedene Studien zeigen, dass DKKs eine wichtige Rolle bei der Entstehung und dem Fortschreiten von Darmkrebs (CRC) spielen. Neuere Arbeiten haben zudem gezeigt, dass DKKs, hauptsächlich DKK1, auch mit der Induktion einer CRC-Chemoresistenz assoziiert sind. Darüber hinaus könnte die Expression von DKK1 eng mit den Expressionsniveaus des Proteins Arginin-N-Methyltransferase 5 (PRMT5) bei verschiedenen Krebsarten verknüpft sein, was auf möglichen einen Regelkreis zwischen DKK1 und PRMT5 hindeutet. Ob PRMT5 jedoch an der Regulierung der DKK1-Expression bei CRC beteiligt ist und ob eine solche Achse zur CRC-Chemoresistenz beitragen könnte, ist bislang unklar. Um dieses Problem zu adressieren, wurden sowohl In-silico- als auch In-vitro-Ansätze eingesetzt, um die Beziehung zwischen PRMT5 und DKKs zu untersuchen. Unsere Daten zeigen, dass die DKK1-Expression in CRC-Proben deutlich hochreguliert ist und bei KRAS-mutierten Krebsarten besonders ausgeprägt ist. Darüber hinaus waren erhöhte DKK1-Spiegel mit der Aktivierung von PRMT5 verbunden. Der Chromatin-Immunpräzipitationstest (ChIP) zeigte epigenetischen Einfluss von PRMT5 bei der Hochregulierung von DKK1 über die Induktion von H3R8-Dimethylierung. Konsequenterweise führte die Hemmungvon DKK1 sowie die Behandlung von CRC-Zellen mit CMP5 (einem PRMT5-Inhibitor) in Kombination mit Doxorubicin zu einer synergistischen Antitumorwirkung bei mutierten KRAS-CRC-Zellen, nicht jedoch bei KRAS-Wildtyp-CRC-Zellen. Mechanistisch gesehen scheint die Rolle von DKK1 bei CRC unabhängig von dem Einfluss auf den WNT-Signalweg zu sein, könnte aber durch die Aktivierung des PI3K/AKT-Signalwegs vermittelt werden. Zusammenfassend legen diese Ergebnisse nahe, dass PRMT5 die DKK1-Expression in CRC reguliert. Die Hemmung von PRMT5 die DKK1-Expression führt zu einer effektiven verminderungdes Wachstum von CRC-Zellen´und beeinflusst insbesondere KRAS-mutierte CRC-Zellen.

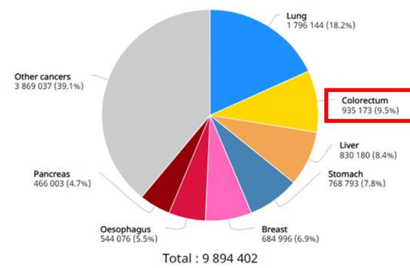
Chapter I.

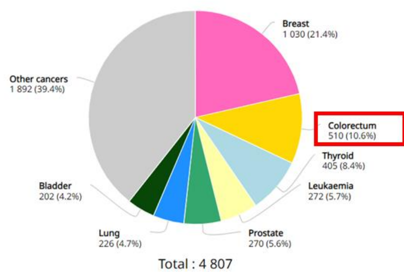
Introduction

1.1.Colorectal cancer prevalence

Colorectal cancer (CRC) is currently ranked as the third most common type of cancer worldwide accounting for 10% of new cancer cases in 2020 [1]. CRC incidence is increasing gradually and recent studies have predicted a 40% increase by 2030 [2]. CRC is also ranked as the second-highest in terms of mortality rates; it accounted for 9.4% of cancer-related deaths in 2020 (**Figure 1**) [3]. The International Agency for Research on Cancer (IARC) data indicated that approximately 1.9 million CRC cases and 935,000 deaths occurred in 2020 [3]. The vast majority of CRC is sporadic and usually diagnosed at the age of 50; 10% of the cases are diagnosed at less than 50 years of age and these are often familial. In general terms, 20% of CRC cases occur in individuals with a family history of the disease, though no known inherited syndrome is traceable among concerned family members [4]. According to GLOBOCAN records for 2020, CRC ranked second in incidence and mortality in the UAE [1]. Despite the employment of several newly discovered CRC biomarkers, in the majority of cases, the disease is still diagnosed at an advanced stage [5].







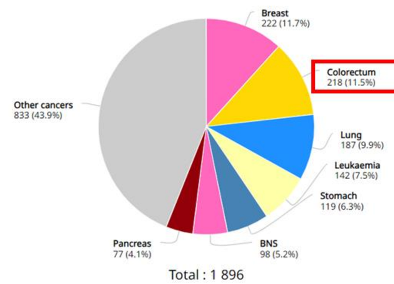


Figure 1: CRC prevalence worldwide and in the UAE. *A pie chart provided by GLOBOCAN for all cancer prevalence and mortality worldwide and in the UAE shows that colorectal cancer (marked with a red rectangular) is among the most three common and life-threatening cancers [1].*

1.2.Risk factors correlated with colorectal cancer

Multiple factors are implicated in increasing the risk of CRC incidence. Some of these factors are non-modifiable factors such as sex, age, race, medical history, and family history. However, some risk factors can be controlled to minimize this risk such as diet, obesity, smoking, and alcohol consumption [6]. For instance, males are more likely to develop CRC than females. CRC is more common in Blacks than in Caucasians [6]; it is also higher in people above the age of 50. Medical history could also increase the risk of CRC; for instance, the risk of CRC is twice as high in people with inflammatory bowel disease (IBD; ulcerative colitis (UC) or Crohn's disease (CD)) than those without IBD [7]. UC is a condition where inflammation is restricted to the mucosal layer of the colon while CD is a form of inflammation

that localizes to the digestive tract [8]. Although UC strongly correlates with an increased probability of CRC, the association between CRC and Crohn's disease is less clear [6, 9, 10]. Inherited mutations in the tumor suppressor gene adenomatous polyposis coli (APC) are among the key mutations involved in CRC initiation [11]. Furthermore, a high-fat diet, smoking, and alcohol consumption were previously associated with increased risk of CRC [6].

1.3.Colorectal cancer classification

CRC can be classified into different categories based on two criteria. Firstly, it can be categorized based on the tumor itself, lymph node involvement, and tumor metastases (TNM) staging system (**shown in table 1**) [12]. Secondly, it can be classified into four distinct consensus molecular subgroups (CMS) based on genetic and epigenetic variations [13]. To understand CMS classification, it is important to understand three main pathways in CRC which are Chromosomal Instability (CIN), Microsatellite Instability (MSI), and CpG Island Methylator Phenotype (CIMP) [14]. CIN is characterized by the loss of function of the tumor suppressor gene APC. It accounts for 85% of total CRC cases. CIN pathway shows a karyotypic heterogeneity from cell to cell due to the loss or gain of whole or vast segments of the chromosome during chromosome segregation in mitosis [14-16]. It is worth noting that some CRC cases show a phenomenon of loss or gain of 10 kb of DNA termed somatic copy number alterations (SCNAs) [17]. The MSI pathway usually involves a unique ‘loss of function mutation’ in mismatch repair (MMR) genes leading to a high mutational rate in the cell genome especially the short tandem repeats (STRs) or microsatellites as they are often called. Microsatellites are 1-6 base-pair that variably repeat themselves in different individuals, these STRs tend to be highly susceptible to sequence mutations [18]. The CIMP pathway is identified by global hypermethylation of cytosine/guanine-rich (CpG) islands of DNA [19]. In the hypermethylation state, a methyl group is added to the cytosine residues of CpG islands.

Cytosine hypermethylation occurring within CpG islands of the promoter region of a gene disrupts RNA transcription and downregulates the expression of the gene in question [19]. Therefore, the CIMP pathway is of particular significance in the context of tumor suppressor genes [13].

As noted earlier, CRC can also be divided into four molecular subtypes (CMS1-CMS4) based on the affected molecular pathway along with other factors including the patient's genetic background (**Figure 2**). CMS1 molecular subtype or MSI-immune, as it is often referred to, accounts for about 14% of cases and is characterized by high-MSI, CIMP presence, and low SCNA. CMS1 has a common mutation in the *BRAF* gene, it involves immune pathway activation including the JAK/STAT pathway, and usually occurs in the proximal colon. CMS2 or the canonical subtype, which accounts for 37% of cases, is known to be MSI-negative, CIN-positive, CIMP-negative, and SCNA-high. *APC* and *P53* mutations are key mutations in CMS2, and it is usually found in the distal colon or rectum. MSC3 or the metabolic subtype (13%) is MSI-negative, CIMP-low, SCNA intermediate. It is correlated with *KRAS* and *APC* mutations and usually localizes to the colorectum. MSC4 or the mesenchymal subtype (23%) is MSI-negative, CIN-low, and SCNA-high. It is also characterized by TGF-β and epithelial–mesenchymal transition pathway activation. The remaining 13% of CRC cases exhibit mixed features of the four molecular subtypes [13, 15, 20]. As a result of CRC heterogeneity, patients exhibit various responses to the treatment [21].

Table 1: TNM classification [12].

Primary tumor (T)	
TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma in situ: intraepithelial or invasion of the lamina propriaa
T1	Tumour invades submucosa
T2	Tumour invades muscularis propria
T3	Tumour invades through the muscularis propria into the subserosa, or into the non-peritonealized pericolic tissues
T4	Tumour directly invades other organs or structures and/or perforates the visceral peritoneum.b,c

Regional lymph nodes (N)	
NX	Regional nodes cannot be assessed
N0	No regional lymph node metastases
N1	Metastases in 1–3 regional lymph nodes
N2	Metastases in ≥4 regional lymph nodes
Distant metastases (M)	
MX	Presence of distant metastases cannot be assessed
M0	No distant metastases
M1	Distant metastases
Stage grouping	
Stage 0	Tis N0 M0: (carcinoma in situ)
Stage I	T1 N0 M0, T2 N0 M0; stage I equivalent to Dukes' A or MAC A or B1
Stage IIA	T3 N0 M0
Stage IIB	T4 N0 M0; stage II equivalent to Dukes' B or MAC B2 or B3
Stage III (A, B, C)	Any T1–2, N1, M0 (IIIA)any T3–4, N1 M0 (IIIB) any T N2 M0 (IIIC); stage III equivalent to Dukes' C or MAC C1–C3
Stage IV	any T, any N, M1

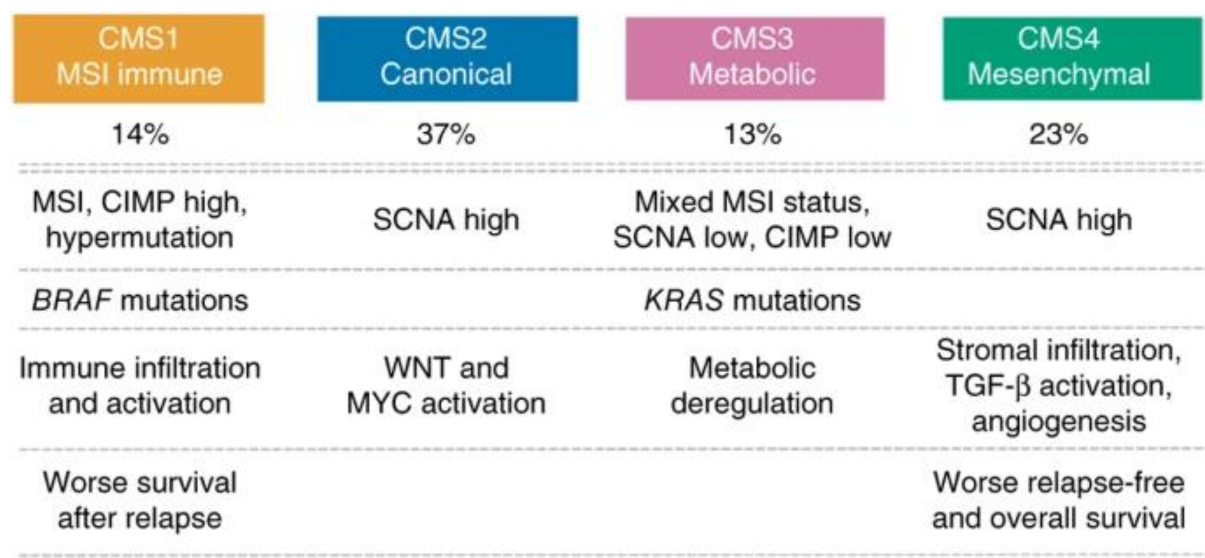


Figure 2: CRC consensus molecular subgroups (CMS). *Illustration of colorectal cancer CMS subtypes prevalence, key genetic and epigenetic alterations, activated pathway, and prognostic value. MSI, microsatellite instability; SCNA, somatic copy number alterations; CIMP, CpG island methylator phenotype. [13].*

1.4.Genetic alterations associated with CRC

CRC develops due to the accumulation of genetic and epigenetic alterations (**Figure 3**). It is well established that CRC tightly associates with genetic abnormalities and that familial adenomatous polyps (FAP) and hereditary non-polyposis CRC (HNPCC) are hereditary syndromes that are responsible for early-onset CRC [22]. Loss of function mutations in *APC* are found in FAP [11]. APC functions as a negative regulator of the canonical WNT signaling pathway [11], which is fundamental for the physiology of the intestine where it contributes to the maintenance and self-renewal of the epithelial stem in the intestinal crypts [23]. WNT signaling hyperactivity serves as a key player in CRC where it enhances cancer cell proliferation and self-renewal [24]. On the other hand, HNPCC, which is also known as lynch syndrome, is attributed to the loss of function of one or multiple genes involved in mismatch repair machinery in the cell including *MLH1, MSH2, MSH6*, and *PMS2* [25]. This machinery is responsible for the excision and replacement of mis-incorporated bases after DNA replication to minimize the mutational rate [26]. Serrated polyposis syndrome (SPS) is another genetic condition that was linked to early neoplastic lesions in the colon. However, it includes multiple defective genes due to mutations or epigenetic alterations and its pattern of inheritance remains controversial [27].

A few other familial syndromes that are associated with a higher risk of CRC and other cancers include Cowden syndrome (CS), Juvenile polyposis syndrome (JPS), and Li-Fraumeni syndrome (LFS) [28]. CS is characterized by loss of function mutations in the tumor suppressor gene *PTEN*, which is involved in regulating the phosphoinositide 3-kinase (PI3K) pathway; an important for cell survival and proliferation pathways [29]. JPS is distinguished by mutations that result in the dysfunction of the tumor suppressor gene *SMAD4* [28], which inhibits tumors by halting the cell cycle at the G1 phase [30]. A mutation that causes a defect in the master *P53*

tumor suppressor is a characteristic feature of LFS [28]. P53 is commonly known to induce cell cycle arrest and mediate cell death through apoptosis [31].

Somatic mutations in proto-oncogenes such as *KRAS, BRAF*, and *CTNNB1* play a significant role in CRC development [32]. For instance, around 30-40% of CRC cases exhibit *KRAS* mutations [33]. *KRAS* is a small GTPase protein that could be constitutively active in CRC cells due to the gain of function mutations [34]. While mutated *KRAS* loses the capacity to hydrolyze GTP [34], activated wild-type *KRAS* stimulates several pathways implicated in cancer including the RAF/MEK/ERK and the PI3K/AKT pathways [34]. The *BRAF* gene belongs to the RAF family and it produces a serine/threonine kinase protein that activates the MAP kinase/ERK signaling pathway, where ERK is the final effector protein to induce cell proliferation and tumor formation [32, 35]. *BRAF* shows an activating mutation in (V600C) in approximately 8-28% of CRC cases. *CTNNB1* gene encodes for the beta-catenin protein; 1% of CRC cases develop a gain of function mutation in *CTNNB1*, which leads to hyperactivity of the WNT signaling pathway. Many of these mutations have a predictive and prognostic value [32]. It must be noted that despite the significant appreciation of the genetic alterations that lead to and/or associate with CRC in recent years, much remains to be understood regarding the involvement of epigenetics in CRC. For instance, how epigenetic alterations influence disease onset, progression, and metastasis on the one hand and disease diagnosis and treatment on the other.

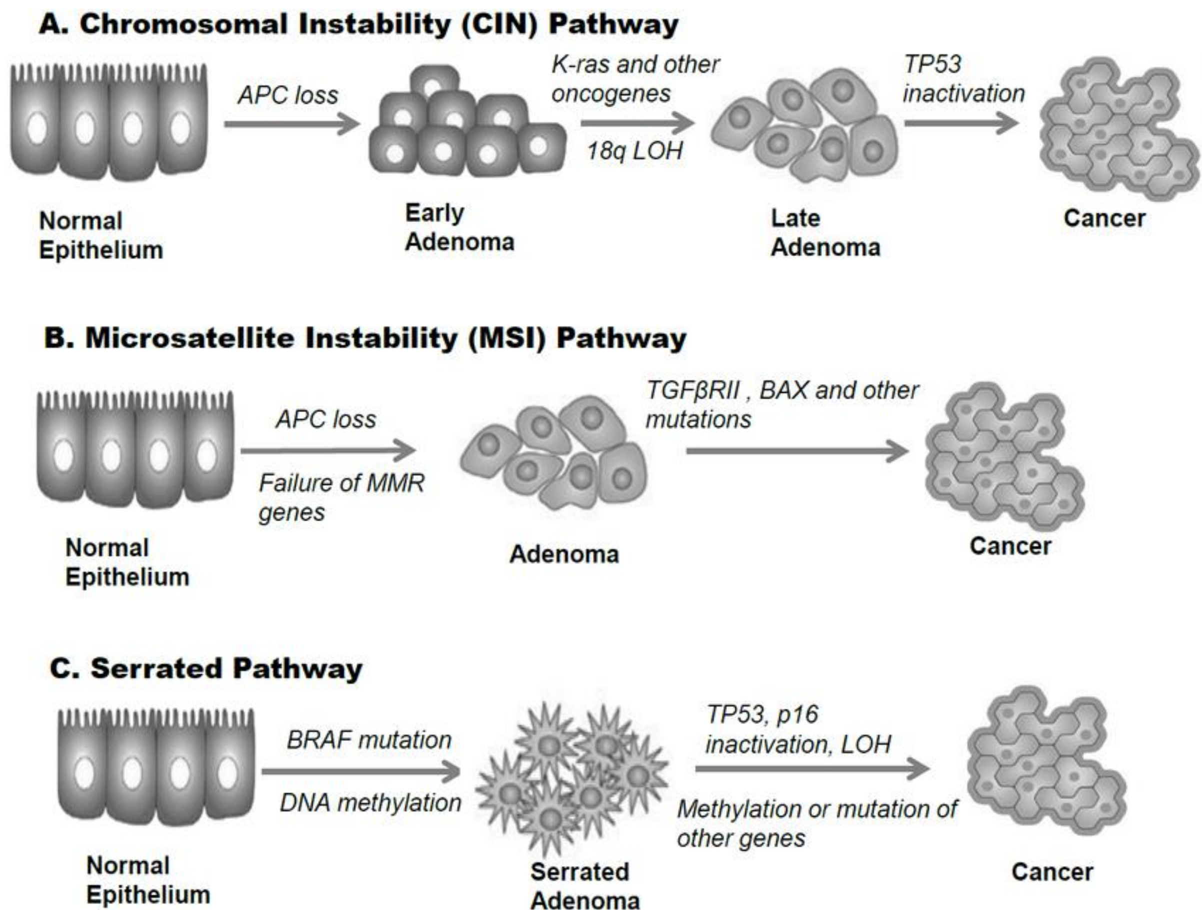


Figure 3: CRC development pathways. *describes genetic and epigenetic alterations involved in different CRC pathways including Chromosomal Instability (CIN), Microsomal Instability (MSI), and Serrated Pathway. The CIN pathway comprises inactivation mutations in the tumor suppressor gene APC, in addition to activation mutations in proto-oncogene KRAS, followed by loss of heterozygosity for chromosome 18 (18q), and deactivation of the tumor suppressor gene TP53, ultimately resulting in the development of invasive cancer. The MSI pathway is characterized by the absence of the APC gene and inactivation of mismatch repair (MMR) genes like MLH1, frequently caused by epigenetic silencing, which increases the mutational rate and eventually results in the evolution of microsatellite unstable invasive tumors. The Serrated Pathway includes DNA hypermethylation and a mutation in the oncogenic BRAF gene, leading to hyperactivation of MAPKs/ERKs signaling and enhanced*

cell proliferation, with subsequent gene methylation and loss of tumor suppressors such as TP53 and p16 playing a key role in colorectal cancer development [36].

1.5.Epigenetic alterations associated with colorectal cancer

CRC evolves gradually and in a stepwise process where cells accumulate mutations and epigenetic alterations over time [37]. Accordingly, understanding the epigenetic modulation of molecular pathways that contribute to CRC development and progression is essential to gaining a deeper understanding of the disease itself and to the identification of novel disease biomarkers and therapeutic targets [38, 39]. Epigenetic alterations are heritable changes that alter the pattern of gene expression without affecting the DNA sequence; they include DNA methylation, histone modifications, and/or non-coding RNAs-mediated gene regulators [40].

1.5.1. DNA methylation in CRC

DNA methylation, which involves the addition of methyl groups to 5′ cytosine residues, tends to lead to transcriptional repression [40]. This process is catalyzed by DNA an enzyme family termed methyltransferase (DNMTs). In CRC, CpG island hypermethylation in the promoter region of tumor suppressor genes was usually linked to the inactivation of transcription. For instance, the *CDKN2* gene which is involved in cell cycle regulation showed a hypermethylation in the promoter region [41]. Never the less, hypomethylation in CpG island increased the expression of oncogenes in some CRC cases. For instance, hypomethylation of CpG islands was noticed in the promoter region of proto-oncogene *MYC* [42, 43]. DNA methylation is a well-studied feature of CRC that led to the discoveries of multiple diagnostic biomarkers. For instance, *SEP9* is a gene that encodes for septin 9 protein that is hypermethylated in CRC [44]. Septin 9 protein is involved in cytoskeletal remodeling during cytokinesis and it is considered as tumor suppressor [45]. *SEP9* hypermethylation test in peripheral blood commercially known as Epi ProColon is the first FDA-approved blood-based

diagnostic test for CRC. It has a high specificity of 97% and an acceptable specificity of 73% [38, 46]. DNA hypermethylation in CRC helped to identify new potential treatments including 5-azacitidine which is a DNMT inhibitor [47].

1.5.2. Histone modifications in CRC

Histones are basic proteins in which DNA is wrapped around to support chromatin structure formation [48]. The presence of extended lysine-arginine-rich tails in histones H2A, H2B, H3, and H4 makes them accessible for post-translational modifications including methylation, acetylation, and other such modifications [49]. Histone acetylation takes place on lysine residue usually leading to gene expression activation while histone methylation is induced on lysin or arginine residue resulting in increased or decreased gene expression [49, 50]. Histone acetylation reaction is catalyzed by enzymes called histone acetyl transferases (HATs) and reversed by histone deacetyl transferases (HDACs). Similarly, histone methyl transferases (HMTs) are enzymes that mediate histone methylation and histone dimethyl transferases and it is removed via histone demethylases (HDMs) [38]. previous studies highlighted the important role of histone modifications in CRC. For instance, CRC exhibits an elevated level of class I HDACs such as HDAC1 and HDAC2 [51-53]. HDAC1 low expression was linked to improved prognosis [54]. HDAC2 amplification was associated with adenoma progression to adenocarcinoma where a significantly higher expression level was found in adenoma [55]. CRC shows a global hyperacetylation of histone 3 in CRC tissue compared to its normal counterpart and was associated with poor overall survival [56, 57]. Some histone acetylation modification was proven to have a diagnostic and prognostic significance [58]. For instance, the significant increase in H3K27ac expression in CRC tissue made it a promising diagnostic biomarker [59]. Furthermore, H3K56ac increased expression was linked to improved prognosis [60]. Histone methylation is implicated in colorectal cancer. For example, a remarkable increase in H3K4 and H3K9 specific demethylase known as histone lysine-

specific demethylase 1 (LSD1/KDM1A) was detected in CRC [61]. Also, a histone methyltransferase termed SUV39H1 has an amplified expression in CRC. SUV39H1 is known to induce histone 3 methylation at lysin 9 residue [62]. H3K9 methylation has a high importance in CRC where H3K9me2 expression is increased in adenocarcinoma compared to adenoma and H3K9me3 elevation was reported metastatic CRC [62, 63]. LSD1 inhibitor (CBB1003) showed a cytotoxic effect on HCT116 colon cancer cells [64]. The majority of studies related to histone methylation focus on the methylation of lysine residue however minority discusses arginine residue methylation [58]. Despite the development of multiple drugs targeting epigenetic modifications, lack of specificity remains one of the major challenges in this field [38, 65]. More work on the role of histone arginine methyltransferases in CRC and other cancers could pave the way for the identification of novel therapeutic targets. H3R8 and H4R3 symmetric dimethylation induced by protein arginine N-methyltransferase 5 (PRMT5) was described as a therapeutic target in CRC [66, 67].

1.6.Diagnosis of CRC

Early diagnosis of CRC has a great impact on patient survival. Blood in the stool, changes in bowel habits, abdominal pain, fatigue, anemia-related symptoms, and weight loss are findings that invite further testing for the possibility of CRC. Multiple diagnostic methods are available to test for CRC including colonoscopy, capsule endoscopy, CT colonography, and fecal immunochemical test (FIT) [32]. Colonoscopy is the most commonly used method for CRC diagnosis, followed by specimen collection and immunohistochemistry testing, which is marked by high accuracy [68]. It is worth noting that there are multiple types of colonoscopies. Although high-definition white light endoscopy (hWLE) is a commonly used and highly efficiency colonoscopy imaging approach, chromoendoscopy has proved to be more efficient in detecting adenomas [69]. Capsule endoscopy could be an alternative method to

traditional colonoscopy as it allows for screening of the gastrointestinal tract using a small wireless camera [70]. Although colonoscopy is accurate, research is still ongoing to find less invasive diagnostic tools such as FIT, which is meant to identify hemoglobin in the stool [71, 72]. Furthermore, testing stool DNA for biomarkers such as *APC* and *KRAS* mutation has been shown to yield acceptable sensitivity and specificity in detecting colorectal cancer. However, identifying advanced colonic adenomas using stool DNA analysis is not without significant technical challenges [73].

1.7. Treatment of CRC

CRC treatment involves chemo-radiotherapy before and after surgical resection (**Figure 4**) [74]. There are multiple FDA-approved drugs for use in CRC patients including 5-fluorouracil (5-FU), oxaliplatin capecitabine, and irinotecan. 5-Fluorouracil, Leucovorin, Irinotecan (FOLFIRI), leucovorin calcium (folinic acid), fluorouracil, and oxaliplatin (FOLFOX), and capecitabine (Xeloda) and oxaliplatin (XELOX) are the therapeutic regimes that have been used as a CRC treatment [75, 76]. Furthermore, several therapeutic monoclonal antibodies have proven effective against CRC, especially when used as adjunctive therapies along with conventional chemotherapy. For instance, cetuximab, an epidermal growth factor receptor (EGFR) inhibitor, is effective in patients with wild-type *KRAS* CRC [77]. Pembrolizumab and Ipilimumab, immune checkpoint inhibitors of programmed death-1 (PD-1) receptor and Cytotoxic T-lymphocyte associated protein 4 (CTLA-4) protein are all FDA-approved drugs for the treatment of advanced CRC with high MSI-H index [78].

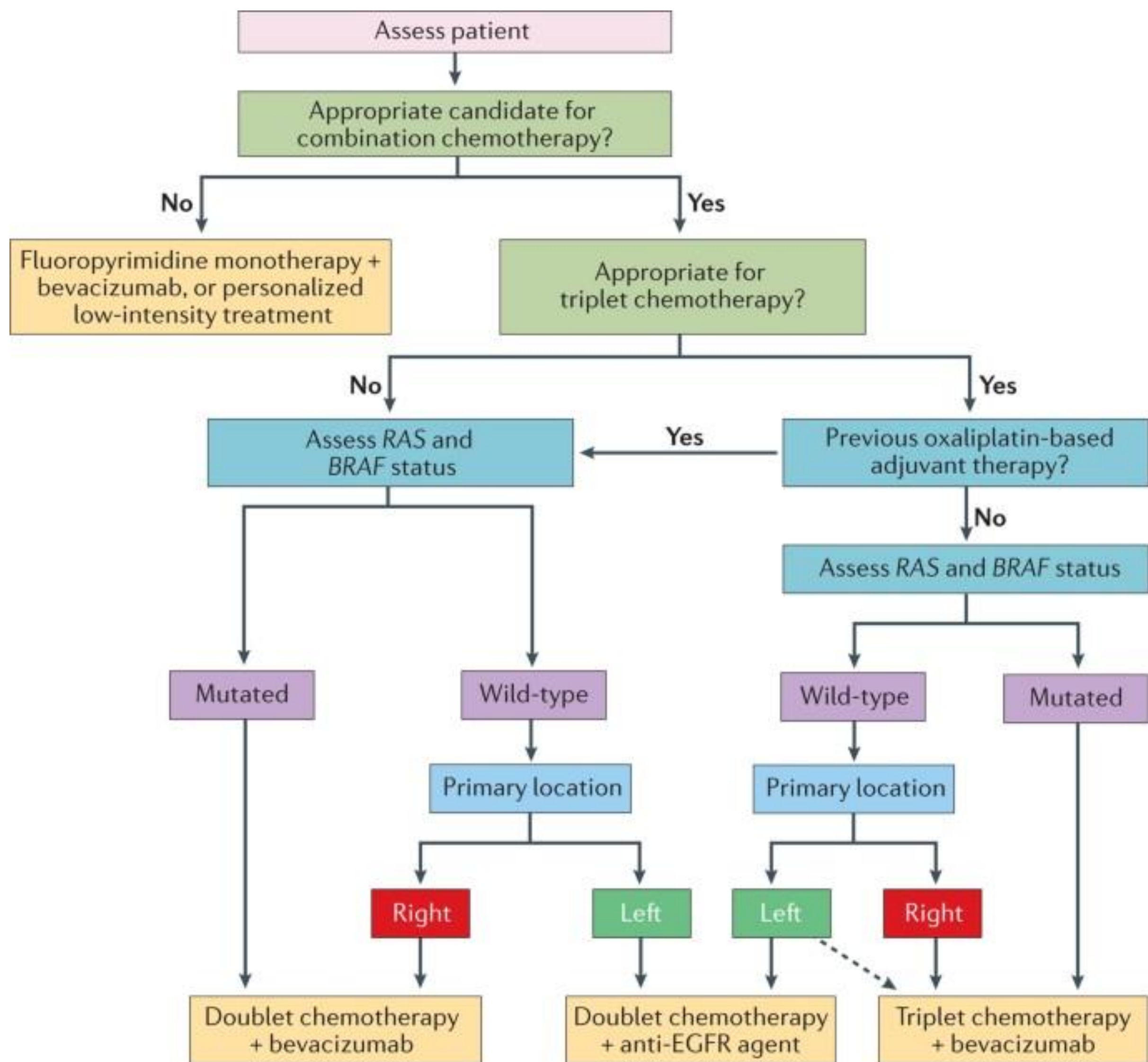


Figure 4: Summery of CRC treatment strategies. *CRC treatment approaches in consideration of molecular biomarkers [79].*

1.7.1. Chemotherapy in CRC

Chemotherapy showed the ultimate capability of enhancing CRC patient survival. Generally, chemotherapeutic agents can be divided into different categories based on the mechanism of action including alkylating agents, antimetabolites, topoisomerase inhibitors, and others [80]. Alkylating agents are anticancer drugs that induce cell death via DNA damage [81]. Antimetabolites are purine or pyrimidine analogs that obstruct DNA synthesis [82]. Topoisomerases are enzymes that function during DNA replication to release supercoil stress by inducing temporary single or double-strand breaks in the DNA while passing other single or double-strands through the brake. Topoisomerase class one is known to induce single-strand breaks while type two is responsible for double-strand breaks. therefore, topoisomerase inhibitors interfere with DNA replication machinery [83]. Approved CRC drugs include 5-fluorouracil (5-FU), oxaliplatin, irinotecan, leucovorin, and capecitabine each of which has its mechanism of action [84]. For example, 5-FU is pyrimidine analogs that function as an antimetabolite that inhibits thymidylate synthase which will consequently result in inhibition of DNA synthesis and cell death [85]. Oxaliplatin is an alkylating agent that creates DNA lesions via DNA crosslinking which will eventually halt DNA synthesis and exert a cytotoxic effect [86]. Irinotecan hinders DNA replication by creating a complex with topoisomerase I and DNA strand which results in the cell undergoing apoptosis. Leucovorin is used usually to boost 5-FU activity where it enhances active metabolite 5-FdUMP binding to thymidylate synthetase [87]. Capecitabine is another pyrimidine analog antimetabolite that has an active metabolite 5-fluorouridine triphosphate (5-FUTP) and FdUTP which compete with uridine triphosphate to inhibit RNA and DNA synthesis respectively [88]. Chemotherapy regimens successfully enhanced CRC patient survival. For instance, FOLFOX treatment plan for 6 months demonstrated a remarkable increase in disease free survival (73.3%) and overall survival (78.5%) [89]. It worth noting that neoadjuvant chemotherapy (NACT) a new strategy

of treatment of cancer that meant to introduce chemotherapy before operation. In CRC, NACT application using capecitabine and oxaliplatin combination (CAPOX) along with post-operative treatment demonstrated a promising result. Using CAPOX as NACT for locally advanced CRC is currently undergoing phase II clinical trial [90-92]. Although chemotherapy enhanced CRC patient survival, some cases with poor prognostic biomarkers such as *KRAS* mutations are still having less disease-free survival and overall survival (**Figure 5**) [93].

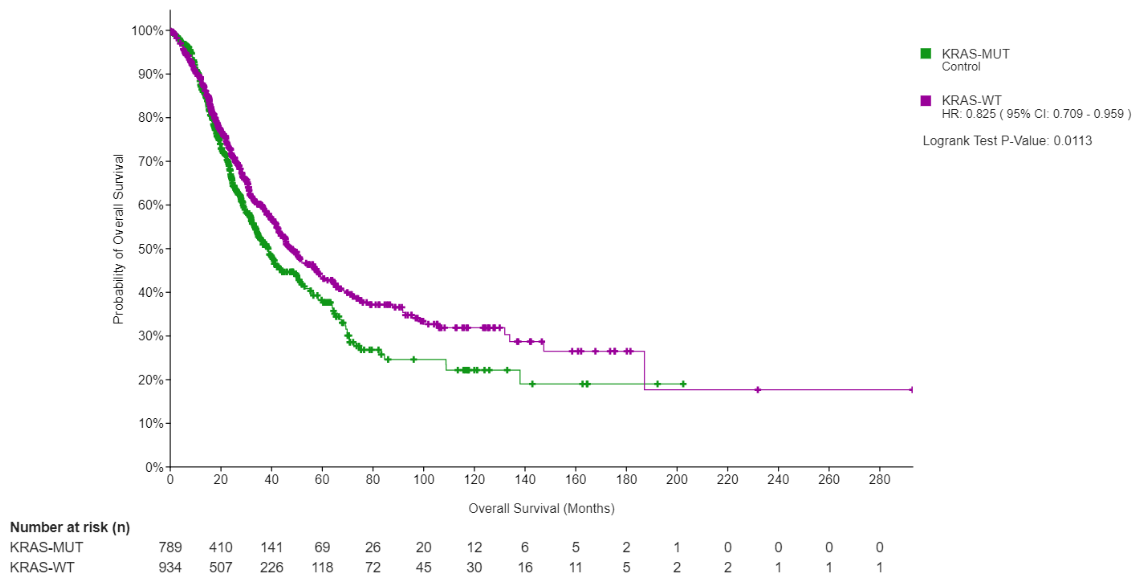


Figure 5: Kaplan Meier graph of 1301 KRAS-WT and 1713 KRAS-MUT CRC cases.

Illustrates poor prognosis of KRAS mutant CRC cases compared to KRAS wild-type counterpart. Data was generated using cBioPortal [94].

Doxorubicin is a golden standard in anticancer treatment [95, 96]; it acts on three arms to induce DNA damage where it intercalates into DNA, inhibits topoisomerase II, and produces reactive oxygen species (ROS) [97]. However, it is not widely used in CRC cases requiring high doses of the drug as this leads to cardiotoxic side effects due to ROS production and/or generation of doxorubicin cardiotoxic metabolites such as doxorubicinol [98-101].

Additionally, some CRC cases develop resistance to doxorubicin [102, 103]. Some studies have linked doxorubicin resistance to PI3K/AKT pathway hyperactivity and increased expression of ABC transporters such as BCRP and ABCG2; such ABC transporters were shown to enhance drug efflux in HT-29 CRC cells [104]. There were multiple attempts to use doxorubicin in combination with other treatments to reduce the dose and harm [105, 106]. However, to the best of our knowledge, none of these attempts passed the clinical trials. Although oxaliplatin, 5-FU, and irinotecan are the most commonly used drugs for CRC treatment, their high cost and toxicity remain major limitations [102]. Taking into consideration that doxorubicin is one of the most potent and cost-effective treatments in terms of anticancer therapy, identifying new combination treatments that enhance doxorubicin's utility in colorectal cancer would be of great benefit to cancer patients [105].

1.7.2. Chemoresistance in CRC

CRC cells can develop chemoresistance through a variety of mechanisms including the deletion of enzymes that activate the drug, increasing drug efflux, reducing drug uptake, modulating drug targets, and/or accelerating DNA repair mechanisms [107, 108]. For instance, a defect in orotate phosphoribosyl transferase (OPRT) which is involved in 5-FU metabolism was linked to 5-FU resistance [109, 110]. On the other hand, irinotecan resistance is developed due to increased drug efflux through ATP-binding cassette (ABC) transporters [111]. A mutation of topoisomerase I that inhibits drug binding is another mechanism of irinotecan resistance [112, 113]. Oxaliplatin resistance is developed due to cellular exportation by ABC transporter termed MRP2 [114, 115]. Furthermore, ATP7A and ATP7B ATPase transporters are involved in oxaliplatin efflux [116]. A reduction in choline transporter-like proteins-1 CTL1 was associated with reduced copper uptake which eventually attenuate oxaliplatin uptake [114, 117]. Excision repair cross-complementation group 1 (ERCC1) increase level was associated with oxaliplatin resistance [116]. It is worth noting that increased expression of pro-

apoptotic proteins (ex. Bax, PUMA, and Apaf1) and reduced expression of antiapoptotic proteins (ex. bcl-2) were found to contribute to oxaliplatin resistance in CRC [108]. Research is still ongoing to develop new strategies to overcome drug resistance and enhance the chemosensitivity of CRC cells.

1.7.3. Targeted therapy in CRC

Despite the fact that chemotherapy has been widely used for CRC treatment, lack of selectivity remains a major issue where it targets all rapidly dividing cells causing severe side effects. Unlike chemotherapy, targeted therapy specifically affects abnormal proteins in CRC [118]. For instance, epidermal growth factor receptor (EGFR), which is over-expressed in 60%-80% of CRC cases [119], activates multiple pro-survival pathways including RAS/RAF/MEK/ERK and AKT/PI3K/mTOR pathways [120]. Monoclonal antibodies targeting EGFR such as Cetuximab and panitumumab showed a promising result in treating CRC and were approved for wild-type *KRAS* metastasized CRC [84]. While Cetuximab is used either alone or in combination with FOLFIRI, panitumumab is in combination with FOLFOX. Such targeted therapies have been shown to enhance CRC patients overall survival [121]. However, mutations in downstream effectors of EGFR including BRAF (V600) and *KRAS* (G12) cause continues activations and hence remain an obstacle to using this approach in CRC [122]. Vemurafenib and Sotorasib (AMG 510) are dugs that target mutated *BRAF* (V600E) and *KRAS* (G12C) respectively [123]. Vemurafenib is currently in phase II clinical trials and Sotorasib (AMG 510) in phase I/II clinical trials [121, 124]. However, their efficacy in heterogenous tumors that contains mutant and wild type oncoproteins remains questionable [125].

1.7.4. Immunotherapy in CRC

Immunotherapy, as an emerging therapeutic approach in CRC, is focused on attenuating cancer cells' capability to evade the immune response by targeting immune check points [78].

Immune checkpoints are part of the immune system that prevent it from attacking healthy cells [126]. For instance, programmed death-ligand 1 (PD-L1) is a cell membrane protein that is expressed in healthy cells mainly during inflammation. It binds to programmed cell death protein 1 (PD-1) that is expressed on the surface of T-cells. PD-1 activation leads to a series of events including inhibition of PI3K/AKT and RAS/RAF/MEK pathways and consequently deactivation of T-cells. Cancer cells increase the expression of PD-L1 to avoid recognition of T-cells [127]. Pembrolizumab and nivolumab are a monoclonal antibody against PD-1 preventing it from binding to PD-L1 in tumor cells. Both of these modalities have received FDA approval for use in patients with metastatic MSI-H or MMR-defected CRC [84]. Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) is another protein that is expressed on cytotoxic T cells. CTLA-4 inhibits CD28 as a co-stimulatory molecule of T-cells from binding to CD80/86 on antigen-presenting cells (APCs), thus hindering T-cell activation [128]. Ipilimumab is a monoclonal antibody that inhibits CTLA-4 and consequently enhances T-cell activation. Low doses of Ipilimumab in combination with nivolumab have been approved for previously treated MSI-H and MMR-deficient metastasized CRC [84]. Nevertheless, immune checkpoint inhibitor treatments raise the risk of inflammatory and autoimmune disease side effects [129].

Chapter II.

Literature review

As described in the previous chapter, each of the currently available colorectal cancer treatments has limitations, hence the for new therapeutic strategies that could enhance CRC patient survival and quality of life. Dickkopf (DKK) protein family has garnered attention in CRC research due to its regulatory role in pro-oncogenic pathways such as the WNT signaling pathway. Recent studies have implicated DKKs in multiple cancer-related pathways. Therefore, it is of pivotal importance to investigate the oncogenic role of DKKs in the context of CRC.

2. 1. The DKK protein family

Dickkopf (DKK) is protein family that consist of four members termed DKK1, DKK2, DKK3, and DKK4 [130]. DKK's protein length ranges from 224-360 amino acids. All of them have a similar structural domain which are N-terminal cysteine-rich 1 (Cys1) and C-terminal cysteine-rich domain (Cys2) domains (**Figure 6**). DKK proteins get secreted from the cell where they can bind to the LRP5/6 co-receptor via the Cys2 domain. Cys1 is found to be functional only in DKK1 where it modulates this interaction Cys2/ LRP interaction. DKKs hinder LRP5/6 binding to frizzled receptor which is the main receptor responsible for WNT signaling pathway activation [131]. Furthermore, DKKs binding to the Kremen protein leads to the formation of a protein complex that triggers the internalization of LRP5/6. Interestingly, DKKs exert significant inhibitory effects against the canonical WNT signaling pathway [131]. Previous studies have also shown that DKK1 can enhance the activity of the non-canonical WNT signaling pathway [132]. DKKs play an essential role during embryo formation; for instance, DKK1 was reported to be involved in head and limb formation. Furthermore, DKKs

are involved in organogenesis and morphogenesis [133-136]. Research data on the role of DKKs in cancer remains controversial and contradictory. While some studies have suggested that DKKs act as tumor suppressors, other studies have shown that they often exert oncogenic activity [137]. For instance, DKK1 is described as an oncogene in lung cancer [138, 139]. Strikingly, it has a dual role in some cancers such as neuroblastoma where it is down-regulated indicating a tumor suppressor role however its expression was associated with chemoresistance [140, 141]. On the other hand, DKK2 plays an oncogenic role in CRC, though it was also correlated with better overall survival in ovarian cancer [142, 143]. It is worth noting that DKK3 has been described as a tumor suppressor in many cancers including those of the prostate, testicular, glioma, and breast [144-147]. However, it has been reported as an oncogene in oral squamous cell carcinoma [148]. DKK4 is the least studied member of the DKK protein family. It was tightly linked to tumor migration in multiple cancers including ovarian cancer [149]. Nevertheless, DKK4 inhibition in CRC and lung cancer was reported to improve chemosensitivity [150, 151].

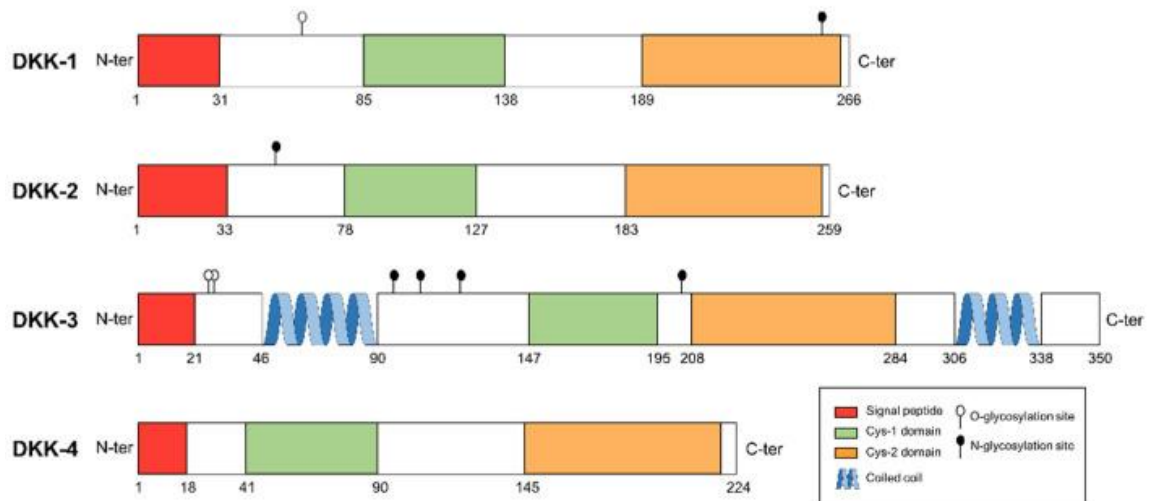


Figure 6: DKK protein family functional domains. *Cysteine-rich domain 1 (Cys1) and Cys2 domains are conserved in the DKK protein family that consists of four members. Signaling peptides are represented in red [137].*

2. 2. DKK protein family role in CRC

As alluded to earlier, DKKs 1-4 mainly act as WNT signaling pathway antagonists [152]. The canonical WNT signaling pathway plays a fundamental role in intestinal physiology, contributing to the self-renewal of epithelial stem cells within intestinal crypts [23]. WNT signaling serves as a key player in CRC [20, 21] and dysregulated WNT signaling pathway occurs in about 92% of sporadic CRC cases [153]. However, the role of the DKK proteins in cancer has been somewhat contradictory and has been implicated in pathways other than the WNT pathway. For example, DKK1 showed the capability of activating the PI3K/AKT pathway (**Figure 7**) [154]. DKK1 elevated expression was linked to cell differentiation in the SW480 CRC cell line [155]. Also, DKK2 has been shown to regulate stemness where it enhances tyrosine kinase Src activity and inhibits HNF4α1 in CRC organoids [142]. Furthermore, DKK2 expression influences the CRC tumor microenvironment where its inhibition increases the activity of natural killer cells and cytotoxic T cells *in vivo* [156]. Overexpressing DKK3 in LoVo CRC cells reduced cell invasion, reduced proliferation, and induced intrinsic apoptosis pathway [157]. DKK4 is over-expressed in CRC tissue associated with worse overall survival and correlated to metastasis. Furthermore, increased DKK4 expression was linked to WNT signaling-related gene mutations [158]. Conversely, DKK4 inhibition was reported to sensitize HCT116 and HT29 CRC cells to chemotherapy [150]. Also, there was evidence that DKK4 boosts the angiogenic potential of CRC cells [159]. Recent studies identified DKK1 as a biomarker of chemoresistance in CRC. DKK1 nuclear expression was associated with worse overall survival of CRC patients. Furthermore, nuclear DKK1 expression was associated with increased expression of genes implicated in chemotherapy detoxification in CRC tumors [160]. Furthermore, some studies proposed DKK3 as a potential neoangiogenesis, diagnosis and prognosis biomarker for CRC [161, 162].

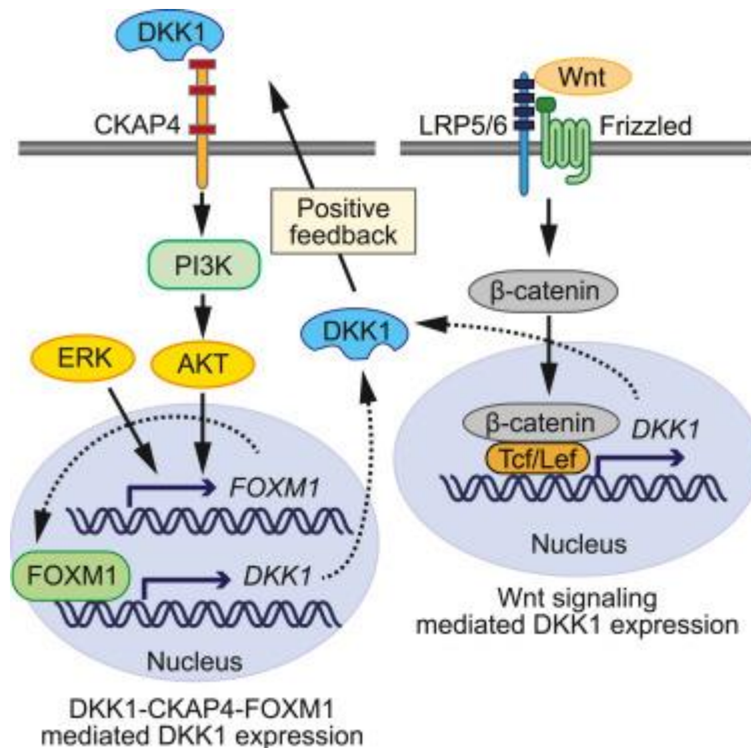


Figure 7: DKK1 implication in WNT/β-catenin and PI3K/AKT pathways.

Transcription factor TCF4 binding to the promoter region of the DKK1 gene in response to WNT signalling activation results in DKK1 upregulation. In cancer cells, ERK and AKT pathway activation induces transcription factor FOXM1 expression, which, in turn, stimulates DKK1 expression by binding to its enhancer region. This establishes a positive feedback loop between DKK1 and FOXM1 where secreted DKK1 activates the PI3K-AKT pathway via binding to the CKAP4 receptor [154].

2. 3. DKKs regulation in CRC

The emergence of evidence that implicates DKKs in CRC development raised questions about its regulatory mechanisms. *DKK1* and *DKK4* were found to be upregulated by β-catenin/TCF, the downstream effector of CRC [163, 164]. Furthermore, *DKK* expression is controlled by multiple epigenetic mechanisms in CRC such as DNA methylation, histone modifications, and non-coding RNA. For instance, the *DKK1* promoter was found to be hypermethylated in DLD-1, SW48, COLO 205, and SW480 CRC cell lines where its

expression was restored after treatment with the demethylating agent 5-aza. Also, *DKK1* promoter was found to be hypermethylated in CRC tissues [153]. Furthermore, *DKK1* and *DKK3* expression is downregulated in colon cancer-initiating cells by class I HDAC histone modulators [165]. Ten-eleven translocation methylcytosine dioxygenase 1 (TET1) is a protein involved in DNA demethylation. TET1 was found to bind to the promoter region of *DKK3* and *DKK4* and alter their expression in CACO2 and SW480 CRC cell lines [166]. miR-410 and miR-92a are microRNAs that play an oncogenic role in CRC by targeting DKK1 and DKK3 respectively [167, 168]. *DKK1* and *DKK3* expression is also controlled by a histone modulator called Protein arginine N-methyltransferase 5 (PRMT5) in breast cancer [169]. However, the role of PRMT5 in altering *DKK* expression has not yet been investigated.

2. 4. Protein arginine N-methyltransferase 5 (PRMT5)

The Protein arginine N-methyltransferase 5 (PRMT5) enzyme is an essential epigenetic modulator that methylates several proteins including histones. PRMT5 is classified as a type II arginine methyltransferase which induces two patterns of methylations in terminal nitrogen atoms of the guanidyl group being omega-N monomethyl arginine (MMA) and symmetrical dimethylarginine (sDMA) [170, 171]. PRMT5 can also exert post-translational modulatory effects by transferring methyl groups from S-adenosylmethionine (SAM) to arginine in cellular proteins [172]. PRMT5 consists of four domains; namely, the TIM-barrel (protein-protein homodimerization), the Rossmann-fold domain (S-adenosyl-L-methionine binding sites), the β-barrel domain and the dimerization domain (arginine trapping site) [173-175]. It usually oligomerizes into homodimers or homotetramers and is characterized by its ability to bind several proteins [176]. PRMT5 tends to be generally complex with methylome protein 50 (MEB50) to promote its affinity to histones. PRMT5-induced methylation at histone sites H2AR3, H4R3, H3R2, and H3R8 regulates (suppresses or activates) the transcription of

multiple genes involved in cell growth, metabolism, survival, and death [177-179]. Accordingly, PRMT5 can greatly alter the pattern of cancer progression via the methylation and stabilization of multiple non-histone oncoproteins such as E2F1 in CRC and KLF4 [180, 181]. Moreover, PRMT5 can bind multiple protein partners aside from histones to direct it to different targets such as pICIn, RoK1, and CoPR5 [177].

Recent studies have classified PRMT5 as an oncogene, where it’s up-regulation in different cancer types, including CRC, correlates with cancer proliferation and invasion [182]. For instance, the transformation of the NIH3T3 cell line (derived from mouse embryonic fibroblast) post-PRMT5 overexpression demonstrated its role in tumorigenesis [183, 184]. Further, PRMT5 activates the transcription of several oncogenes such as the eukaryotic translation initiation factor 4E (eIF4E) in A549 lung cancer cells [185]. PRMT5-induced histone methylation also results in the down-regulation of Dickkopf WNT Signaling Pathway Inhibitor 1 and 3 (DKK1 and DKK3) genes in breast cancer, which impairs the negative regulation of WNT signaling. It is worth noting that WNT signaling serves as a key player in CRC [24, 169]. Therefore, PRMT5 became an attractive therapeutic target for multiple cancers and multiple PRMT5 inhibitors have been developed. from these inhibitors, GSK3326595 currently undergoing phase I/II clinical trials for solid tumors, myeloid leukemia (AML), and non-Hodgkin's lymphoma [186, 187]. PRMT5 inhibitors showed a synergistic effect in combination with different anticancer therapies. For instance, PRMT5 inhibitor (GSK3326595) helped to overcome palbociclib (CDK4 / 6 inhibitor) drug resistance in melanoma through P53 activation [188]. Mueller et al. provider further evidence showing that KrasG12D Tp53-null lung adenocarcinoma (LUAD) cell line develops resistance to PRMT5 inhibitor (EPZ015666), and becomes more sensitive to paclitaxel [189]. These observations suggest that PRMT5 inhibitors can serve as an important adjuvant therapy to overcome chemoresistance.

2. 5. The role of PRMT5 in CRC

PRMT5 contributes to the progression of CRC through different mechanisms, the most important of which is histone modification [66]. PRMT5 can enhance tumor cell growth and survival by upregulating several oncogenes such as eIF4E and the fibroblast growth factor receptor 3 (FGFR3). This occurs by PRMT5 binding to gene promoter regions and inducing a symmetrical dimethylarginine dimethylation of histones H4R3 and H3R8 [66]. EIF4E is an mRNA 5' cap-binding protein that facilitates protein synthesis. It is a downstream effector of the PI3K/AKT/mTORK pathway and is upregulated in human cancer [190]. Stimulation of the FGFR3 receptor activates PI3K/AKT/mTOR and RAS/RAF/MEK/ERK pathways as major players in tumorigenesis [191, 192]. Therefore, inhibiting PRMT5 may significantly disrupt these, and possibly other, pathways and thus halt tumor regression [66].

Previous work has suggested that PRMT5 can exert transcriptional suppression of crucial genes regulating cell cycling in CRC cells [193]. PRMT5 can significantly down-regulate cyclin-dependent kinase inhibitors such as *CDKN1B* (p27KIP1), *CDKN1A* (p21CIP1/WAF1), *CDKN2A* (p16INK4), *CDKN2C* (p18INK4C), *CDKN2B* (p15INK4b), and *CDKN3* [193]. *CDKN2B* gene is among the best-studied cyclin-dependent kinase inhibitor genes. PRMT5 was reported to induce H4R3me2s and H3R8me2s near the promoter of the *CDKN2B* gene with the help of an enhancer of zest homolog 2 (EZH2) [193]. EZH2 is part of the polycomb repressive complex 2 (PRC2) that comprises a suppressor of zeste 12 homolog (SUZ12), retinoblastoma binding protein 4/7 (RBBP4/7), and embryonic ectoderm development (EED) [194]. EZH2 acts as a transcriptional repressor by catalyzing tri-methylation of histone H3 at Lys 27 (H3K27me3) [195]. Simultaneous inhibition of PRMT5 and EZH2 precipitates a synergistic inhibitory effect in CRC both *in vitro* and *in vivo* [193].

PRMT5 was also reported to interact with histone transmethylase complex comprising the WD repeat domain 77 (WDR77) as a means of inducing H4R3 dimethylation in CRC cells

[196]. Sirtuin 7, a histone deacetylase targets WDR77 in CRC, attenuates the interaction between WDR77 and PRMT5, and reduces cell growth [196]. Although PRMT5 is commonly known to induce histone methylation, it can also induce symmetric dimethylation in 46 different proteins [197]. Collectively, these observations suggest that PRMT5 is one of the main drivers of histone modification and transcriptome profile alteration in CRC. Besides, PRMT5 plays multiple roles in CRC (**Figure 8**).

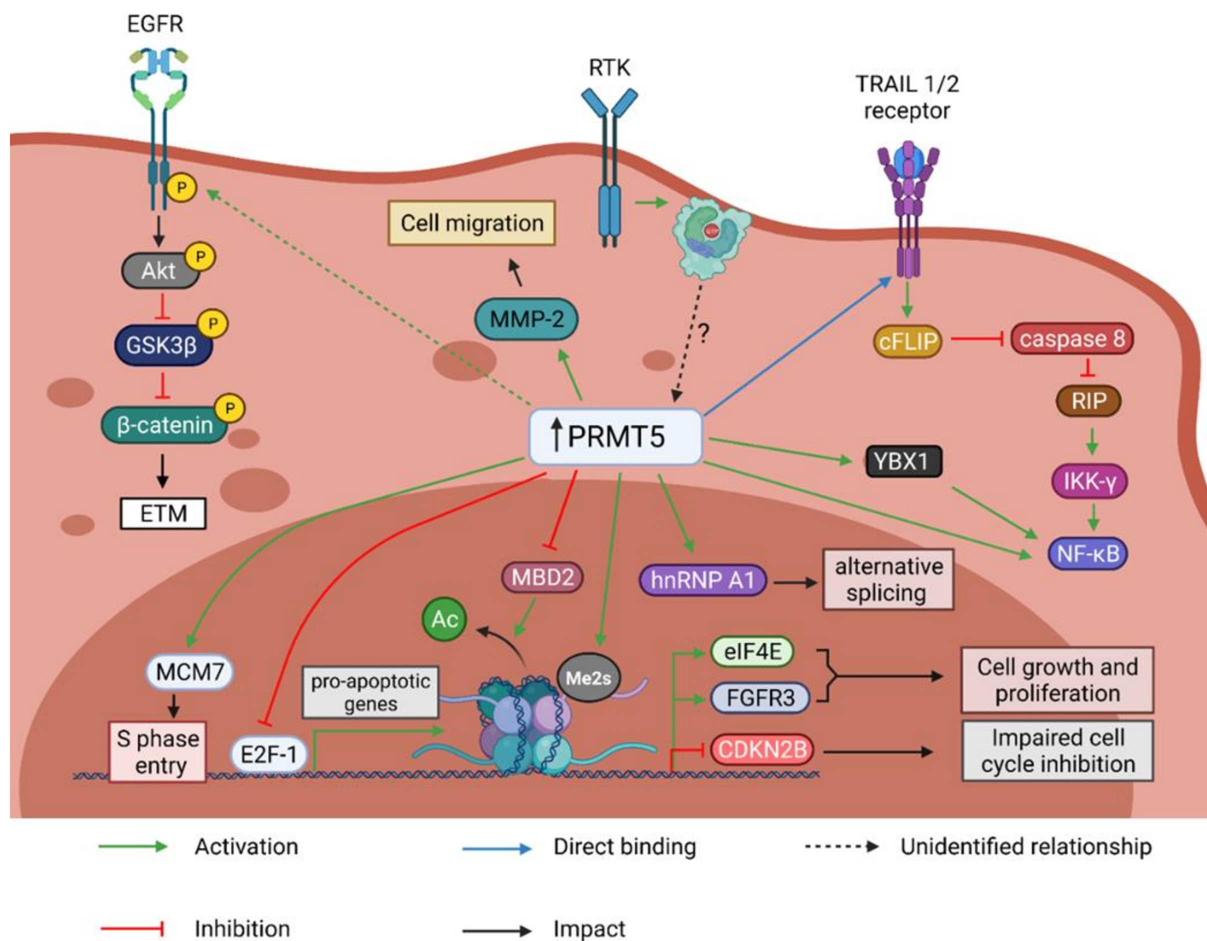


Figure 8: The role of PRMT5 in CRC. *PRMT5 induces histone symmetric di-methylation at arginine residue in the promoter region of multiple genes involved in CRC progression. It mediates transcriptional activation of oncogenes such as eIF4E and FGFR3 and promotes cell growth and proliferation. In addition, it suppresses the transcription of cell cycle inhibitors such as CDKN2B, which impairs the proper block of the cell cycle when needed.*

Furthermore, it activates multiple proteins implicated in CRC development through direct methylation at their arginine residue, such as the p65 subunit of NF-kB and YBX1 (NF-κB activator). PRMT5 functions to methylate hnRNP A1 protein involved in mRNA splicing machinery, resulting in alternative splicing in CRC. PRMT5 direct methylation can suppress the action of E2F-1, which promotes the transcription of pro-apoptotic genes. PRMT5-mediated methylation attenuates the function of MBD2 to catalyse histone deactivation; therefore, it restricts its transcriptional co-repressor function. PRMT5 activates MCM7 and MMP-2 which function to mediate S-phase entry and cell migration, respectively. Although PRMT5 up-regulation increased the activation phosphorylation of EGFR hence, stabilized β-catenin, which in turn mediates epithelial-mesenchymal transition (EMT). Mutated KRAS is correlated with PRMT5 over-expression. PRMT5-bound TRAIL1/2 receptor favours the NF-κB pathway over the death pathway where it tends to activate FLICE/caspase-8-inhibitory protein (cFLIP) which will restrict caspase-8 activity. Therefore, RIP protein is not cleaved by caspase-8. Stable RIP activates the NF-κB pathway [198].

2. 6. Targeting PRMT5 in CRC

2.6.1. PRMT5 regulation in CRC

Several regulatory mechanisms in CRC cells control PRMT5 expression and activity. For instance, NF-Y, Myc, and NF-κB are transcription factors that bind the *PRMT5* promoter to control its expression in several cancers [199-201]. However, the exact transcription factor responsible for PRMT5 up-regulation in CRC has yet to be identified [180]. At the epigenetic level, PRMT5 expression was found to be upregulated by N-alpha-acetyltransferase 40 (NAA40) [202]. NAA40 enzyme induces transportation of acetyl group to N-terminal residues of histones H4 and H2A. Besides, NAA40 was found to be upregulated in CRC cells [202]. At the functional level, phosphorylation of PRMT5 at serine 15 residue was found to enhance its

ability to catalyze NF-κB methylation. This phosphorylation event was thought to be mediated by protein kinase Cι (PKCι) [203]. PKCι was identified as an oncogenic isoform of protein kinase C that contributes to the malignancy of several types of cancer and was reported to be over-expressed in CRC cells [204]. Therefore, the elevated expression level of NAA40 and PKCι in CRC could be responsible for PRMT5 overexpression resulting in increased activity [202, 204].

2.6.2. Arginine methyltransferase inhibitor 1 (AMI-1)

AMI-1 is the first discovered small molecule that inhibits PRMT activity [205]. AMI-1 binds to a strategic site on several PRMTs, which allows it to simultaneously prevent SAM and arginine from binding to PRMTs [206]. Recent work has demonstrated that using AMI-1 can reduce the proliferation and migration of several CRC cell lines including HCT116 and SW480 [66]. Furthermore, AMI-1 was shown to reduce tumor growth *in vitro* [66]. Even though AMI-1 can inhibit the activity of multiple PRMTs *in vitro*, it showed selectivity to PRMT5 *in vivo* [66]. AMI-1 treatment for CRC xenograft demonstrates an increase in p53 and pro-apoptotic protein Bax and reduction in anti-apoptotic protein Bcl-2 [66]. AMI-1 could be a potential candidate therapy to suppress PRMT5 activity; however, clinical investigations on its therapeutic efficacy against CRC and other cancers are still lacking.

2.6.3. EPZ015666 (GSK3235025) and EPZ015866 (GSK591 or GSK3203591)

Comprehensive structure-activity relationship (SAR) analysis has identified some potent and selective PRMT5 inhibitors, namely EPZ015666 (IC_{50} 22 nM) and EPZ015866 (IC_{50} 4 nM), with the former showing superior pharmacokinetic properties [207, 208]. EPZ015666 binds to the target peptide-binding site and confers conformational change while also interacting with SAM [207]. To determine EPZ015666 selectivity, it was tested on 20 methyltransferase proteins, showing no activity up to 50 μM [207]. EPZ015666 was shown to

inhibit PRMT5 in *KRAS* mutant and wild-type CRC cell lines. Greater efficacy of this inhibitor was observed when used against *KRAS* mutant CRC cell lines both in terms of cell growth inhibition and cell cycle arrest at the G2 phase [209]. Furthermore, EPZ015866-mediated PRMT5 inhibition in HCT-116 cells reversed the alternative splicing phenomenon induced by PRMT5 resulting in cell growth reduction [210]. Given that *KRAS* mutant CRC cases have limited treatment options, the discovery of EPZ015666 may give a glimmer of hope to such patients [209]. Although EPZ015866 was more potent than EPZ015666, it was not tested *in vivo* studies due to its instability [208].

2.6.4. PR5-LL-CM01

AlphaLISA is a high throughput screening (HTS) technique that is used to identify inhibitors of epigenetic targets [211]. In 2017, Prabhu et al. developed an AlphaLISA technique specific for PRMT5 where 10,000 compounds were screened; among which, PR5-LL-CM01 showed an IC_{50} of 7.5 µM was selected. PR5-LL-CM01 specificity was further confirmed by the observation that it reacted with 10-fold higher IC_{50} against other PRMT family members [212]. The binding site of PR5-LL-CM01 is approximately similar to the EPZ015666 binding site in the presence of SAM [212]. However, in the absence of SAM, PR5-LL-CM01 binds to a SAM binding site. Interestingly, PR5-LL-CM01 was able to inhibit NF-κB activity in the HCT-29 CRC cell line with a concentration lower than that of EPZ015666 by about 10-15 folds, suggesting better antitumor efficacy. Furthermore, PR5-LL-CM01 showed significant efficacy *in vivo* [212].

Table 2: Summary of the candidate drugs targeting PRMT5 in CRC [198].

Candidate Drug	IC_{50}	Binding site	In vitro	In vivo	Outcome	Ref.
AMI-1	-	between SAM and arginine binding sites	HCT116, SW480	SW480 xenograft nude mice	Increases p53 and Bax expression Reduces Bcl-2 expression	[66]
EPZ015666	22 nM	peptide-binding site	HCT116, SW620, HT-29, and Caco-2	-	Selectively inhibits K-ras mutated cell line growth	[207, 209]

EPZ015866	4 nM	-	HCT-116	-	Alternative splicing	[208, 210]
PR5-LL-CM01	7.5 µM	peptide-binding site or SAM-binding site	HCT29	HT29 xenograft NOD scid gamma (NSG)	Inhibits NF-κB pathway	[212]

2.6.5. PRMT5 inhibitor compound 5 (CMP5)

1-(9-Ethyl-9H-carbazol-3-yl)-N-(pyridin-2-ylmethyl) methanamine a PRMT5 inhibitor compound 5 (CMP5) is a SAM binding domain inhibitor. It demonstrated activity against PRMT5 selectively whereas it exhibited no activity against PRMT1, PRMT4, or PRMT7 [213]. It showed activity against breast cancer cell lines *in vitro* [169]. Also, it was used for *in vivo* intracranial zebrafish model of glioma [214]. There are no reports about utilizing CMP5 against CRC yet.

2. 7. Statement of the problem

A significant percentage of CRC cases develop resistance to chemotherapy during therapy. KRAS mutant CRC cases in particular have limited options of targeted therapy where they don't respond to EGFR inhibitors. Also, KRAS mutant CRC patients show a worse prognosis than KRAS wild-type patients. Therefore, the development of new treatment strategies for KRAS mutant CRCs is urgently needed. The DKK protein family was shown to exert both oncogenic and anti-tumor activities depending on the specific member and the type of cancer. Additionally, DKK1 and DKK4 in particular were associated with chemosensitization in CRC. Until today, few studies have addressed the role of DKKs in CRC in general and in chemoresistance in particular. In this study, we aimed to investigate DKKs role in CRC, the molecular mechanisms underlying their expression, and the link between DKKs and PRMT5. It is worth emphasizing here that PRMT5 is a key epigenetic modulator that is typically over-expressed in a majority of CRC cases and has been strongly correlated with poor prognosis and reduced patient survival.

Hypothesis, aims, and objectives

Hypothesis: PRMT5 inhibition downregulate DKK1 in KRAS mutant CRC enhancing chemosensitivity.

Aims:

1- Identify differentially expressed DKK protein family members in *KRAS*-mutant colorectal cancer *in silico* and *in vitro.*

  a) Identify differentially expressed *DKKs* in CRC using TCGA datasets

  b) Examine differentially expressed *DKKs* in KRAS-wild type versus *KRAS* mutant CRC clinical case and cell lines *in vitro*.

2- Investigate the correlation between differentially expressed DKKs and PRMT5 *in silico, in vitro,* and patient tissues.

  a) Identify PRMT5 expression in CRC utilizing TCGA data sets and its correlated pathways in CRC cell lines by NGS using mRNA derived from PRMT5-silenced CRC cells.

  b) Identify DKKs that are significantly correlated with PRMT5 expression in CRC clinical samples using TCGA datasets

  c) Investigate differentially expressed DKKs expression upon PRMT5 knockdown in CRC cell lines using Q-PCR or NGS.

  d) Validate significant results using at the protein level western blot for PRMT5 knocked down cell lines and correlation analysis of protein expression in CRC tissues via immunohistochemistry

3- Test the synergistic effect between PRMT5 inhibitor (CMP5) and doxorubicin

  a) To test CRC cell lines (*KRAS*-mut vs *KRAS*-wt) proliferation upon treatment with CMP5, doxorubicin, or combination using resazurin and cell cycle assay.

b) Evaluate CRC cell lines (*KRAS*-mut vs *KRAS*-wt) apoptosis upon treatment with CMP5, doxorubicin, or combination via annexin/PI kit.

4- Determine the contribution of DKK1 in the synergistic effect on *KRAS*-mutant CRC cells *in vitro.*

a) Test whether DKK1 knockdown can sensitize *KRAS* mutant CRC cells to doxorubicin via resazurin, cell cycle assay, and apoptosis assay.

b) Identify DKK1 predicted protein-protein interactions *in silico*

c) Investigate DKK1 capacity to affect the WNT/β-catenin or the PI3K/AKT pathway when combined with doxorubicin using western blotting and immunofluorescence approaches in correlation in TCGA-derived data.

2. 8. Opportunities for contributions to knowledge

Our research aims to identify differentially expressed DKKs in CRC, especially the *KRAS* mutant cases, and to investigate the role of PRMT5 in regulating DKKs expression in CRC. The synergy between PRMT5 and doxorubicin will also be examined along with the ability of DKKs to modulate chemosensitization in CRC. These studies should provide further insight into the role of DKKs and PRMT5 in CRC, *KRAS* mutant CRC cases in particular. Furthermore, our study aims to investigate the molecular mechanisms by which DKKs cooperate in chemoresistance.

Chapter III.

Methodology

3.1.Work flow

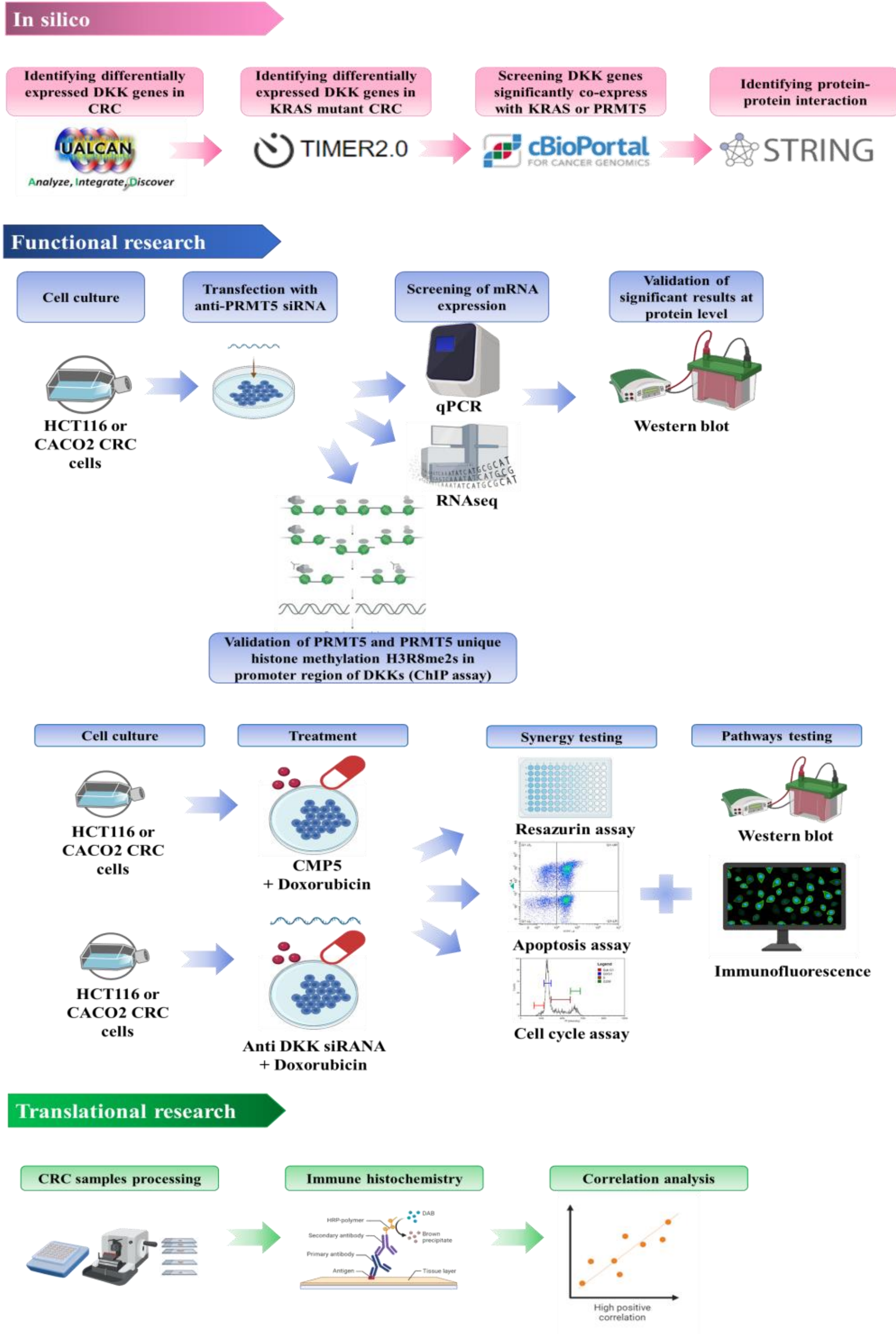


Figure 9: Experimental design work flow.

3.2.Cell lines

Table 3: CRC cell lines origine and mutational background

cell line	*Tumor Source*	*Histology*	*mutated protein*	*Gene Sequence*	*Protein Sequence*	*mutation type*
HCT 116	primary	Carcinoma	KRAS	c.38G>A	p.G13D	Missense
	primary	Carcinoma	PIK3CA	c.3140A>G	p.H1047R	Missense
	primary	Carcinoma	CTNNB1	c.133_135delTCT	p.S45del	
	primary	Carcinoma	CDKN2A	c.68_69insG	p.R24fs*20	
CACO2	primary	Adenocarcinoma	SMAD4	c.1051G>C	p.D351H	Missense
	primary	adenocarcinoma	TP53		C135F	Missense
	primary	Adenocarcinoma	TP53	c.610G>T	E204*	Nonsense
	primary	adenocarcinoma	APC	c.4099C>T	Q1367*	Nonsense

3.3.Methods

3.3.1. In silico analysis

In silico analyses were conducted using the UALCAN website (http://ualcan.path.uab.edu/analysis.html) to profile the expression of multiple genes in CRC patients as opposed to normal counterparts [215, 216]. Genes co-expressing was assessed using cBioPartal website data of 212 colorectal adenocarcinoma patients provided by (TCGA, Nature 2012) [94, 217-219]. The Spearman test was used to calculate the P value. TIMER2.0 was utilized to identify differentially expressed genes in *KRAS* mutant versus wild-type counterparts in 401 cases of COAD and 144 patients of READ [220, 221]. To assess DKK1 expression in CRC cell lines we obtained data on Genomic determinants of protein abundance variation in CRC cells utilizing the Expression Atlas tool (https://www.ebi.ac.uk/gxa/home) [222].

3.3.2. Cell culture

CRC cell lines HCT116 (*APC* wild type, *KRAS*, and *CTNNB1* mutant) and CACO2 (*KRAS* and *CTNNB1* wild type, *APC* mutant) were cultivated in RBMI-1640 (Sigma-Aldrich, USA) and EMEM medium respectively. The medium was supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, USA) and 1% Penicillin/Streptomycin (P/S) antibiotic (Sigma-

Aldrich, USA) in T75 cell-culture flasks were incubated in 37°C supplemented with 5% CO2 and humidity.

3.3.3. RNA interference

Silencer Select small interfering RNA (siRNA) against PRMT5 and DKK1 were supplied by Thermo Fisher Scientific (Waltham, MA, USA). Previous validation proved the 90% potency of the siRNA to inhibit the target gene. Lipofectamine 2000 or RNAi MAX with Opti-MEM Reduced Serum Medium provided by ThermoFisher were utilized to transfect patient-derived CRC cell lines, HCT116, CACO2, cell lines siRNA at a final concentration of 60 pmol or 100 pmol. Results were normalized to negative control transfected with siRNA (Thermo Fisher, catalog number: 4390843). RNA and protein isolation were performed. RNA was used for qPCR and whole transcriptome sequencing in section 1.4 and section 7.2 respectively.

3.3.4. Polymerase chain reaction (PCR)

HCT116 was treated with either control siRNA or two different concentrations of PRMT5 siRNA 30pmol or 60pmol for 24h. RNA extraction was performed after either 24h transfection termination. AllPrep DNA/RNA/Protein Mini Kit from QIAGEN was used for RNA extraction. cDNA synthesis was applied using the QuantiTect® Reverse Transcription kit from QIAGEN. GoTaq® qPCR Master Mix was utilized to apply real-time PCR using QuantStudio3.

Table 4: qPCR primers

Gene	Forward primer sequence	Reverse primer sequence
`DKK1	5’GATCATAGCACCTTGGATGGG3’	5’CTGATGACCGGAGACAAACAG3’
DKK3	ATGTGTGCAAGCCGACCTT	CCTCAGCGCCATCTCTTCA
DKK4	TGGACTTCAACAACATCAGGAG	GGTATTGCAGTCCGTGTCAG
GAPDH	5’CCAGGTGGTCTCCTCTGACTTC3’	5’ACATACCAGGAAATGAGCTTGACA3’

3.3.5. Western blot

Cultured cells were washed using PBS. Cells were lysed using 1x RIPA buffer with 0.1x protease inhibitor followed by sonication. protein lysates were collected and quantified using a protein assay kit (Bio-Rad, Irvine, CA, USA). Proteins were resolved in 12 or 10 % SDS polyacrylamide gels. Nitrocellulose membranes were used to blot the protein samples via a semi-dry transfer cell or wet transfer tank from Biorad. Membranes were blocked with 5% skim milk and then soaked with 1:1000 diluted primary antibodies at 4°C. The membranes then were probed with an Anti-rabbit IgG HRP-linked Antibody or anti-mouse IgG HRP-linked Antibody and ChemiDoc Imaging System (Bio-Rad, Irvine, CA, USA) was used for protein detection.

3.3.6. Resazurin

In brief, CRC cells were cultured in 96 well plates at a density of 5,000-7,000 cells per well and cultured at 37˚C with 5% CO2 for 24h. then, cells were treated with different concentrations of CMP5 and doxorubicin either individually or in combination for 24h. consequently, each well were incubated with 100 µL of 1x resazurin sodium salt (Sigma-Aldrich #R7017) for 4 h at 37°C. A plate reader was used to read the plate at florescence 530 nm excitation/ 600nm emission wavelength. Graphpad was used to identify the IC_{50}. The SynergyFinder website was utilized to determine the concentrations of the synergy.

3.3.7. Apoptosis assay

After treating cells with Doxorubicin, and/or CMP5 the cell pellets were collected and resuspended in 500 µL of Annexin-V (1X) Binding Buffer (Annexin V-FITC/PI Apoptosis Kit, Elabscience). Next, 5 µL of Annexin V-FITC and 5 µl of PI were added to the cells and incubated at room temperature for 15 min in the dark and then analyzed by flow cytometry (FACSAria III, BD Biosciences, USA).

3.3.8. Cell cycle assay

To analyze the cell cycle, cells were stained with Propidium Iodide from the Flow Cytometry Kit for Cell Cycle Analysis (ab139418). Briefly, cells were seeded in 6 well plates with 150,000 cells per well and cultured at 37˚C with 5% CO2 for 48h. HCT116 was treated with either control siRNA or two different concentrations of PRMT5 siRNA 30pmol or 60pmol for 48h and then it was subjected to staining. The detection was performed using a BD FACSAria™ Flow cytometer. Data were analyzed through FlowJo V.10 software.

3.3.9. Chromatin Immunoprecipitation (ChIP) assay

SimpleChIP Plus Enzymatic Chromatin IP Kit (Cell Signaling Technology) was used to conduct a ChIP assay by the supplier's instructions. Briefly, cells were subjected to 1% formaldehyde for 15 minutes at room temperature to achieve crosslinking. Micrococcal nuclease was used to digest the DNA into small fragments (around 150-1000 base pairs). Chopped chromatin was incubated with 2ug final concentration of primary antibody (PRMT5 or H3R8me2s). Histone 3 antibody was used as a positive control. Normal rabbit IgG was utilized as an internal negative control. Immune-precipitated chromatin was purified, and input chromatin and control-IP (IgG) were utilized for PCR amplification for DKK1 promoter region. DNA acrylamide gel electrophoresis was performed to visualize amplified DNA at the promoter region of DKK1. Primers designed for DKK1 promoter region forward 5'-CCGGATAATTCAACCCTTAC-3' and reverse 5'-GCAGACGACTTTAATAAATGC-3' were utilized to identify protein binding.

3.3.10. Next-generation sequencing (NGS)

HCT116 and CACO2 cell lines were subjected to whole transcriptome sequencing using Illumina HiSeq4000. The reads were mapped with a human genome reference sequence (GRCh37.82). Pathways analyses were conducted via the QIAGEN Ingenuity Pathway Analysis (IPA) online tool. –log (P value) analysis was performed to identify the most

significantly affected pathways. P values of less than or equal to 0.05 were considered statistically significant. A volcano plot was generated using the VolcaNoseR website.

3.3.11. Immunofluorescence assay

Cells were seeded in 8 wells of cell culture slides. After 24h transfection, cells were incubated for 48h. Consequently, were washed with PBS twice then cells were fixed using 4% formaldehyde for 20 mins in the dark and then washed with T-PBS. Cells were washed with PBS twice and then permeabilized with 0.1% Triton X for 15 minutes. Cells were incubated in 3% BSA for 1.5h and washed with T-PBS. Primary antibody was added in a final concentration of 1:200 and incubated for 1h then washed with T-PBS. A secondary antibody was added in a final concentration of 1:200 and incubated for 1h then washed with T-PBS. DAPI was added in a final concentration of 1:1000 and incubated for 20 minutes. Eventually, cells were embedded with a mounting medium.

3.3.12. Immunohistochemistry

Commercially available CRC tissue microarray (cat #: T8235722-5) containing 53 CRC cases and 3μm tissue of 37 CRC tissue sections was utilized to perform immunohistochemistry staining (total number 90 CRC cases). In brief, slides were incubated at 62°C to melt the wax, deparaffinized by xylene, and rehydrated by alcohol gradient concentration. Citrate and EDTA buffers and heat for 15-20 min were used for antigen retravel for PRMT5 and DKK1 antibodies respectively. Slides were washed with distilled water for 10 minutes and then incubated in 3% H_2O_2 for 30 minutes. Subsequently, slides were washed with distilled water once and with PBS three times. Protein-blocking reagents from the abcam IHC kit (cat #: ab64264) were placed on slides for 20 minutes. Slides were incubated in primary antibodies overnight with final concentrations of 1:1200 and 1:50 for PRMT5 and DKK1 primary antibodies respectively. Detection was done using the Abcam kit (cat #: ab64264). followed by hematoxylin staining

and dehydration by alcohol gradient and finally incubated in xylene before mounting. Slides were scored by two individual pathologists and an immunoreactive score (IRS) was given to slides relying on the intensity and quantity of stained cells The staining intensity included negative, weak, moderate, or strong, which was scored as 0, 1, 2, and 3, respectively. The percent positivity was scored as 0 (< 1%), 1 (1-50%), 2 (51-80%), and 3 (> 81%). The final immune-reactive scores were presented as the product of intensity and quantity (range 0–9) and then divided into negative/mild 1 (0-3), moderate 2 (4-6), and strong 3 (7-9) as described in table 2.

Table 5: Histopathological biomarkers of colon cancer patients & TMA

	Variables	**Total tissue sample = 37 n (%)**
PRTM5 Final Score	Negative/mild	8 (21.6%)
	Moderate	11 (29.7%)
	Severe	18 (48.6%)
DKK1 Final Score	Negative/ Mild	7 (18.9%)
	Moderate	17 (45.9%)
	Severe	13 (35.1%)
	Variables	**Total TMA sample = 53 n (%)**
PRTM5 Final Score	Negative/mild	34 (64.2%)
	Moderate	12 (22.6%)
	Severe	7 (13.2%)
DKK1 Final Score	Negative/Mild	15 (28.3%)
	Moderate	22 (41.5%)
	Severe	16 (30.2%)

3.3.13. Statistical analysis

A student t-test was used to calculate the significance of differences between two samples (control versus treated). A one-way ANOVA test was utilized to determine significance in sample groups more than two. Statistical tests were conducted using GraphPad Prism 8.4.2 were used for all statistical calculations. At least two independent biological replicates were analyzed for each experiment. Collective data were presented as mean ± SEM. P-value < 0.05 was considered a statistically significant difference. This was applied to all experiments. The correlation between PRMT5 and DKK1 biomarkers in tissue samples was done using Pearsons's Correlation test. The tests were two-tailed and the significance level was

set at p $value \leq 0.05$. Data were analyzed using IBM SPSS, version 23 for windows, Armonk, NY, USA.

Chapter IV.

Results

DKK protein family role in cancer is gaining increasing scientific and clinical attention due to its proposed oncogenic and chemoresistance activity in multiple cancer types including CRC. In this dissertation, we evaluated DKKs expression in *KRAS* mutant versus wild-type CRC using *in silico* and *in vitro* analysis. We evaluated the capability of PRMT5 to influence DKKs expression *in silico* and *in vitro* and it was validated in patient tissue *ex vivo*. We tested the capacity of PRMT5 inhibition in sensitizing CRC cell lines to doxorubicin *in vitro*. Also, we illustrated the role of DKK1 in reversing chemoresistance to doxorubicin in *KRAS* mutant cell lines by inhibiting the PI3K/AKT pathway *in vitro*.

5.1.*DKK1* gene is differentially expressed in *KRAS* mutant CRC forms.

To screen DKKs expression in different cancer types, TIMER 2.0 online tool was utilized. Our data highlighted a significant alteration in DKKs' expression in a wide range of cancers [220]. The expression of *DKK1* was found to be significantly upregulated in 7 cancers including colon adenocarcinoma (COAD), and was downregulated in 3 cancers **(Figure 10a).** The expression of *DKK2* was found to be significantly increased in 5 cancers including COAD and significantly reduced in 7 cancers **(Figure 10b)**. *DKK3* expression was elevated in 6 cancers and reduced in 9 cancers **(Figure 10c)**. *DKK4* expression was significantly upregulated in 7 cancers including COAD and significantly reduced in 5 cancers **(Figure 10D)**. To investigate *DKK* gene family expression in CRC, *in silico* analysis was employed using publicly available datasets from the TCGA [215]. Results demonstrated a significant upregulation of *DKK1* and *DKK4* in COAD and rectum adenocarcinoma (READ) (**Figure *11*d-h**). In addition, *DKK2* and *DKK3* levels were significantly increased in COAD but not READ

(**Figure *11*d-h**). Interestingly, *DKK1* and *DKK4* expression was also activated in *KRAS*-mutant CRC samples compared to *KRAS* wild type in COAD and READ (**Figure *12*a-b and g-h**), whereas no significant difference in the expression of *DKK2* or *DKK3* was observed (**Figure *12*c-f**). It is worth noting that *DKK1* expression was positively correlated with *KRAS* expression but not *DKK2, DKK3* or *DKK4* **(Figure *13*a-d).** Since DKK1 is the best-studied member of the DKK family and it shows a consistent association with *KRAS* it was carried out for the rest of the experiments. Consistently, DKK1 showed a significant upregulation in *KRAS* mutant CRC cells (COLO-678, GP5d, HCC2998, HCT-116, HCT-15, LS-1034, LS-123, LS-513 NCI-H747, RCM-1, SK-CO-1, SNU-175, SNU-C2B, SW1116, SW1463, SW620, SW837, SW948, T84) relative to wild-type counterparts (COLO-205, COLO-320-HSR, CW-2, CaR-1, DIFI, HT55, KM12, LS-411N, NCI-H508, NCI-H630, NCI-H716, RKO, SNU-C1, SNU-C5, SW48) using publicly available proteomics baseline data (**Figure 14a**). The results were confirmed via western blot using *KRAS* mutant HCT116 versus *KRAS* wild-type CACO2 **(Figure 14b**). Interestingly, table (6) highlights the increased expression of DKK1 protein in poorly differentiated CRC tissue compared to well-differentiated (**Figure 14c**).

a)

DKK1 Expression Level (log2 TPM)

b)

DKK2 Expression Level (log2 TPM)

c)

DKK3 Expression Level (log2 TPM)

d)

DKK4 Expression Level (log2 TPM)

Figure 10: Differentially expressed DKK protein family in different cancers. *a) analyzed TCGA data of differentially expressed DKK1 in different cancers using the TIMER 2.0 tool. b) analyzed TCGA data of differentially expressed DKK2 in different cancers using the TIMER 2.0 tool. b) analyzed TCGA data of differentially expressed DKK3 in different cancers using the TIMER 2.0 tool. c) analyzed TCGA data of differentially expressed DKK4 in different cancers using the TIMER 2.0 tool. The blue color represents normal samples while the red color represents tumor samples.*

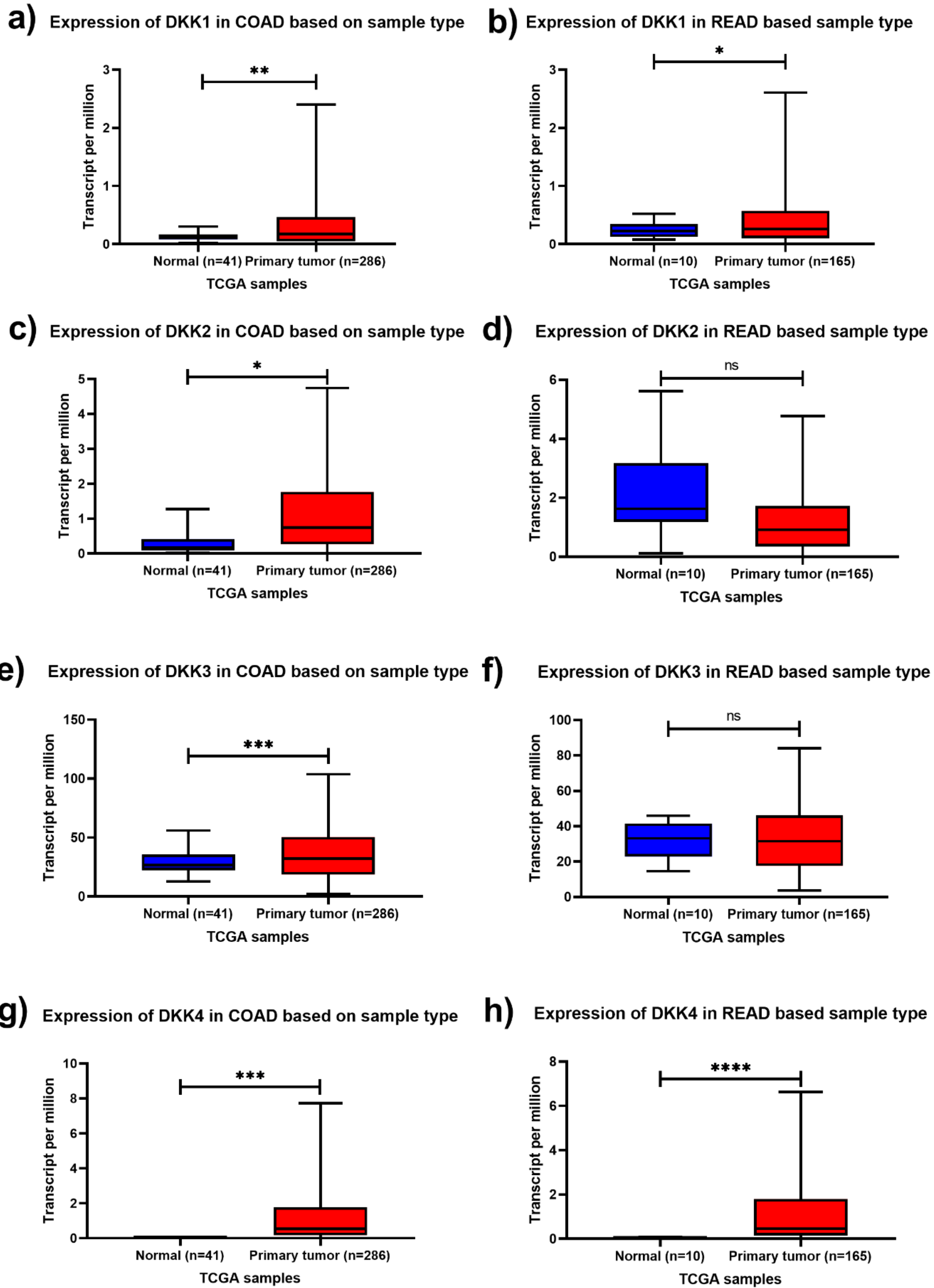
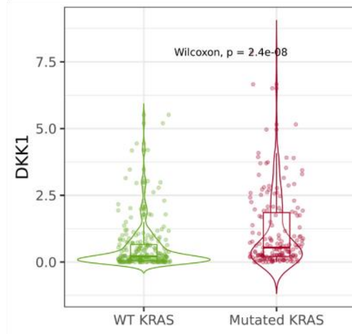


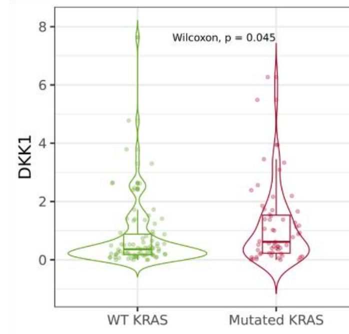
Figure 11: DKK gene family expression in COAD and READ. *a) DKK1 expression in COAD compared to normal tissues counterpart: using RNA seq dataset from TCGA data in*

U*ALCAN tool. b) DKK1 expression in READ compared to normal tissues counterpart: using RNA seq dataset from TCGA data in UALCAN tool. c) DKK2 expression in COAD compared to normal tissues counterpart: using RNA seq dataset from TCGA data in UALCAN tool. d) DKK2 expression in READ compared to normal tissues counterpart: using RNA seq dataset from TCGA data in UALCAN tool. e) DKK3 expression in COAD compared to normal tissues counterpart: using RNA seq dataset from TCGA data in UALCAN tool. f) DKK3 expression in READ compared to normal tissues counterpart: using RNA seq dataset from TCGA data in UALCAN tool. g) DKK4 expression in COAD compared to normal tissues counterpart: using RNA seq dataset from TCGA data in UALCAN tool. h) DKK4 expression in READ compared to normal tissues counterpart: using RNA seq dataset from TCGA data in UALCAN tool.*

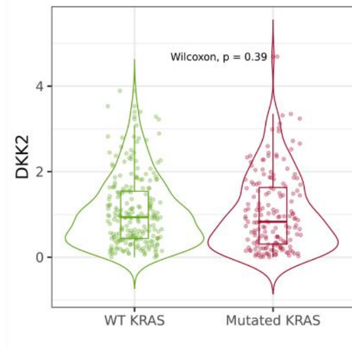
a) Differential DKK1 expression level in COAD (n=401)



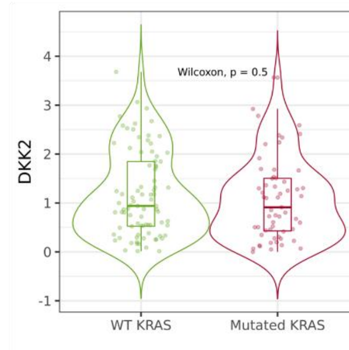
b) Differential DKK1 expression level in READ (n=144)



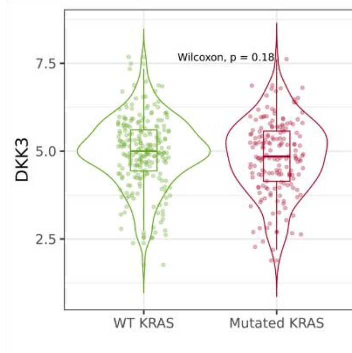
c) Differential DKK2 expression level in COAD (n=401)



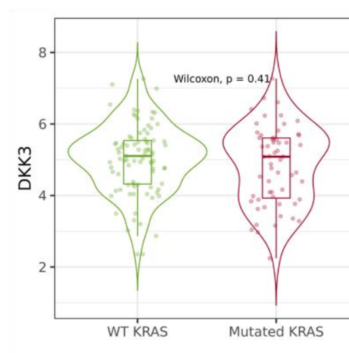
d) Differential DKK2 expression level in COAD (n=144)



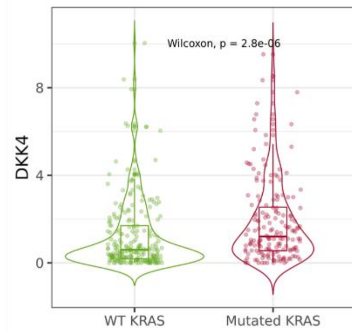
e) Differential DKK3 expression level in COAD (n=401)



f) Differential DKK3 expression level in READ (n=144)



g) Differential DKK4 expression level in COAD (n=401)



h) Differential DKK4 expression level in READ (n=144)

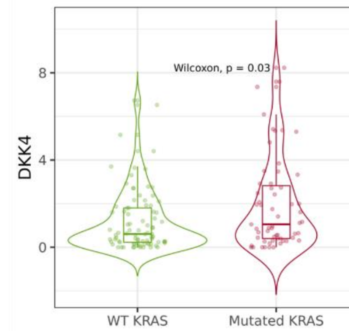


Figure 12: DKK gene family expression in KRAS mutant COAD and READ. *a) DKK1 expression in COAD clinical samples KRAS mutant versus wild type using RNA seq dataset, TCGA data, TIMER2.0 tool. b) DKK1 expression in READ clinical samples KRAS mutant versus wild type using RNA seq dataset, TCGA data, TIMER2.0 tool. c) DKK2 expression in COAD clinical samples KRAS mutant versus wild type using RNA seq dataset, TCGA data, TIMER2.0 tool. d) DKK2 expression in READ clinical samples KRAS mutant versus wild type using RNA seq dataset, TCGA data, TIMER2.0 tool. e) DKK3 expression in COAD clinical samples KEAS mutant versus wild type using RNA seq dataset, TCGA data, TIMER2.0 tool. f) DKK3 expression in READ clinical samples KRAS mutant versus wild type using RNA seq dataset, TCGA data, TIMER2.0 tool. g) DKK4 expression in COAD clinical samples KEAS mutant versus wild type using RNA seq dataset, TCGA data, TIMER2.0 tool. h) DKK4 expression in READ clinical samples KRAS mutant versus wild type using RNA seq dataset, TCGA data, TIMER2.0 tool.*

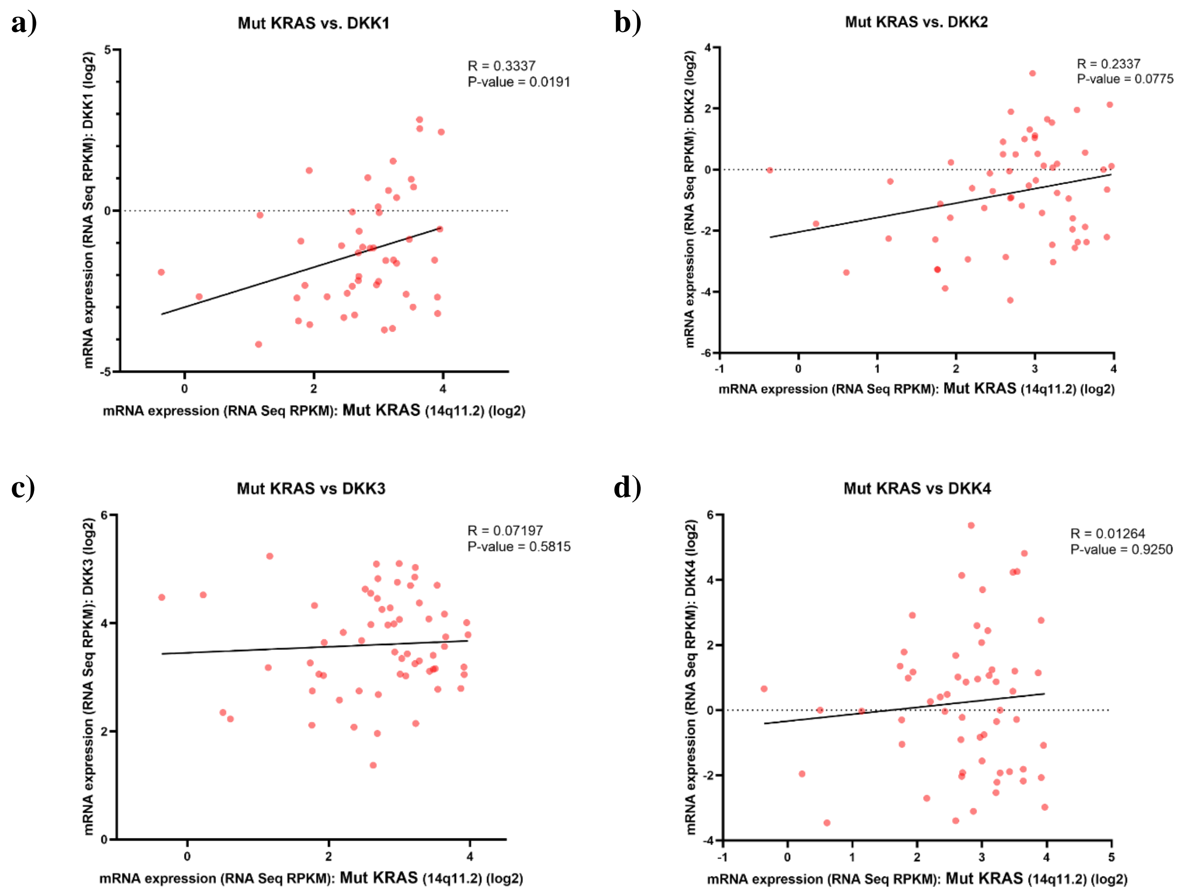


Figure 13: DKK gene family expression correlation with KRAS expression in CRC. *a) Spearman's rank correlation coefficient of PRMT5 versus DKK1 expression, cBioportal tool. b) Spearman's rank correlation coefficient of PRMT5 versus DKK2 expression, cBioportal tool. c) Spearman's rank correlation coefficient of PRMT5 versus DKK3 expression, cBioportal tool. d) Spearman's rank correlation coefficient of PRMT5 versus DKK4 expression, cBioportal tool.*

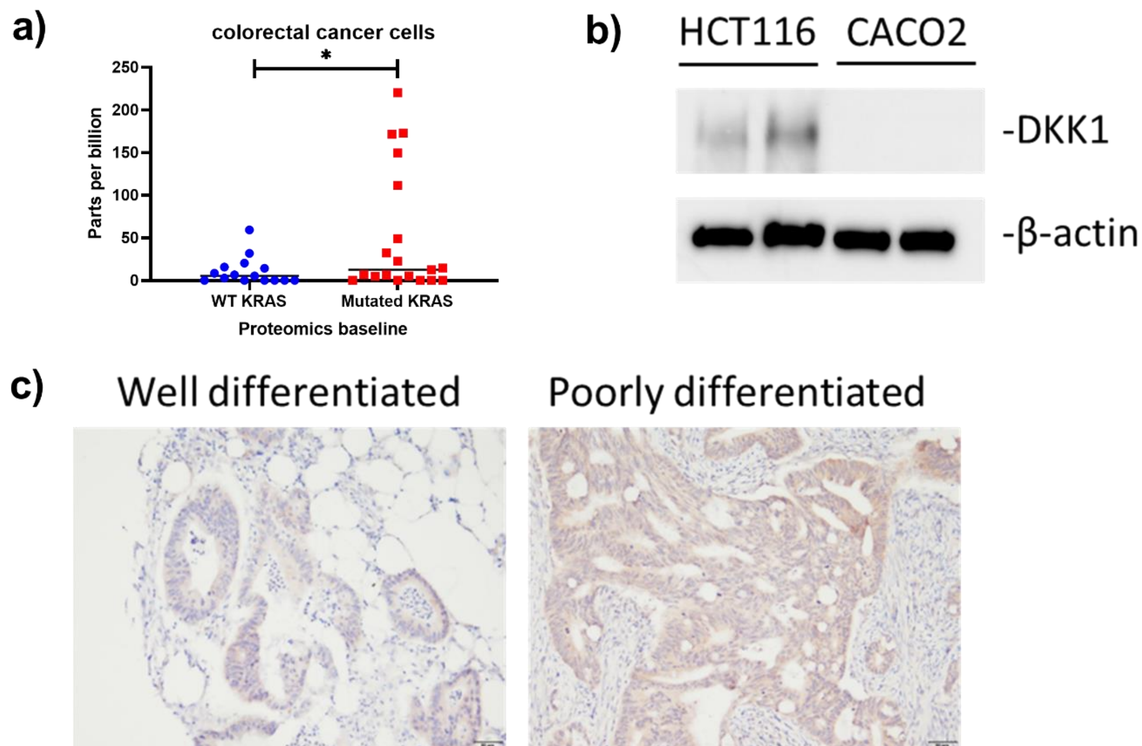


Figure 14: DKK1 gene expression in KRAS mutant versus normal CRC cell lines and correlation with CRC differentiation. *a) DKK1 expression in genomic determinants of protein abundance variation in CRC cells, expression atlas tool. b) Western blot of DKK1 basal expression in CRC cell. The P-value was calculated using a T-test. c) IHC analysis of DKK1 in well-differentiated and poorly differentiated CRC tissue samples.*

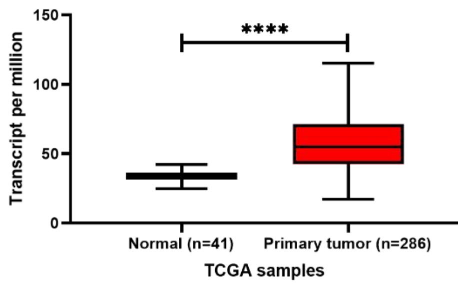
Table 6: The association between DKK1 expression levels in CRC patients, TMA, and the clinicopathological characteristics

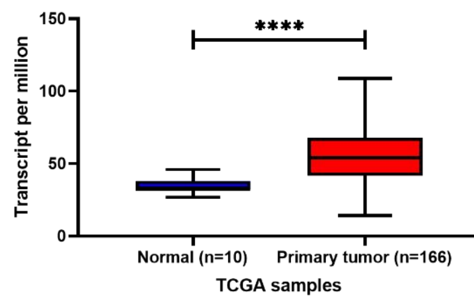
		DKK1 expression levels		
		Negative/Mild positive	**Moderate/strong positive**	**Total number of samples**
Tumor Grade: n (%)	***Well-differentiated***	7 (36.8%)	12 (63.2%)	19 (100%)
	Moderately differentiated	14 (21.5%)	51 (78.5%)	65 (100%)
	Poorly differentiated	1 (16.7%)	5 (83.3%)	6 (100%)

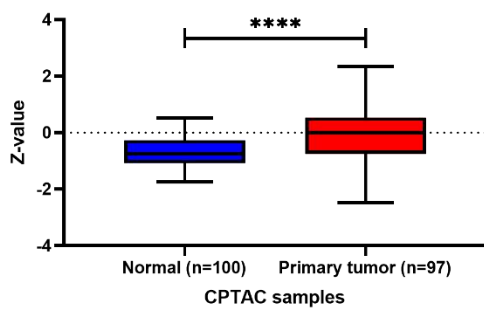
5.2. PRMT5 expression is upregulated in CRC and correlated with pro-oncogenic pathways.

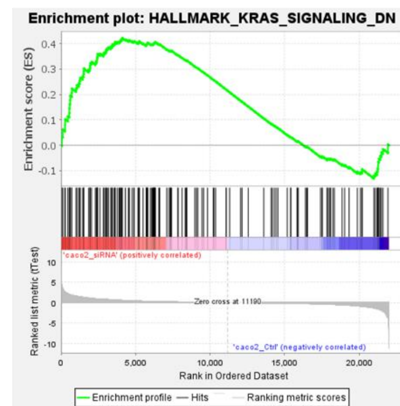
To investigate *PRMT5* gene expression at the mRNA and protein levels in CRC, *in silico* analysis was performed using publicly available datasets from the TCGA and CPTAC. Our data demonstrate increased expression of PRMT5 in COAD as well as READ at the mRNA level **(Figure 15a-b)**. Furthermore, analyzing proteomics data of CPTAC confirmed PRMT5 upregulation in colon cancer **(Figure 15c)**. To identify oncogenic pathways associated with PRMT5 expression in CRC, whole transcriptome NGS analysis of *KRAS* wild type CACO2 as well as *KRAS* mutant HCT116 upon PRMT5 knockdown was performed. Strikingly, GSEA analysis revealed that PRMT5 knockdown in *KRAS* wild-type CACO2 CRC cell line was significantly (Nominal p-value = 0.014, FDR = 0.21, Normalized Enrichment Score NES = 1.4) associated with the enrichment of KRAS_SIGNALING_DN gene set which includes genes down-regulated by KRAS activation **(Figure 15d)**. Consistently, IPA analysis of the upstream effect illustrates a predicted inhibition of *KRAS* based on the altered downstream genes **(Figure 15e)**. The affected genes downstream KRAS upon PRMT5 silencing in CACO2 cell lines includes *DKK1, ABCC5, ACLY, ACVR1, ADH4, AGT, AIMP2, AKR1B10, ANK1, ANXA8/ANXA8L1, ARHGDIB, ASPH, ATF2, ATP1B1, AXL, B3GALNT2, B4GALT5, B4GAT1, BAK1, BSG, BZW2, CAMK2G, CCDC125, CCDC80, CCP110, CDA, CDK4, CDK7, CDKN2C, CEACAM1, CELSR2, CFTR, CLIC5, CLU, COL6A1, CPOX, CRYAB, CSF2RA, CX3CL1, CXADR, CYP2J2, DDAH1, DENND2B, DNMT1, DUSP4, DUSP5, DUSP6, EDEM1, EIF3I, EIF5, EIF5A, EMP2, ENO1, ENO2, ENOPH1, EPCAM, EPHA2, EXOSC5, EXT2, F3, FBLN2, FLOT2, FOXM1, GADD45A, GALNT11, GAPDH, GPC3, HEXIM1, HIRA, HPCAL4, ID1, IFITM2, IFITM3, IGFBP2, IL11, IP6K2, ITM2B, IVNS1ABP, LAMC2, LAMP2, LBH, LCN2, LDHA, LEF1, LIF, LRP8, LTBP4, LYAR, MACC1, MAN2A1, MAPK12, MAPK14, MAPK3, MCAM, MECR, MET, MICB, MMP2, MST1R, MTHFD1, NCEH1, NCL,*

NDRG4, NFKBIB, NGFR, NOS2, NR4A1, NRAS, NT5E, NUDC, OAS1, OSTM1, P3H3, PAIP2, PALD1, PALLD, PCDH1, PCNP, PECR, PFKL, PIP4K2B, PKM, PLEKHO2, PPA1, PRC1, PRPF19, PSMB8, PTGS2, QKI, RAB27A, RAI14, RANBP1, RBBP7, RHOB, RNH1, RPN2, RXYLT1, S100A4, SCMH1, SERPINE1, SERPINE2, SKP2, SLC25A6, SLC26A2, SLC3A2, SLC7A1, SNX16, SOD3, SORT1, SQOR, SREBF1, SRPK2, ST3GAL4, ST3GAL6, STAT3, STAT5A, SUN1, TAP1, TCP1, TEAD2, TGFB1, TGFBR1, TIMP2, TLN1, TLR2, TMEM259, TNFRSF10B, TP53, TUBA4A, VAPA, VPS35, XIAP, YAP1, YES1, ZEB1, ZPR1 **(Figure 15e)**. Furthermore, IPA pathway analysis exhibited a significant alteration of the PI3K/AKT pathway in both *KRAS* mutant HCT116 and *KRAS* wild-type CACO2 **(Figure 16a-b)**. However, a deeper look at the altered pathway in both cell lines illustrates the predicted inhibition of AKT protein in the HCT116 cell line which will lead to inhibition of cell growth and survival, While in CACO2 there was a predicted activation of AKT which enhances cell growth and survival activation. HCT116 showed alteration in *GSK3A, IL6ST, MTOR,* and *PIK3CB* genes while CACO2 exhibit differential expression in *AKT3, EIF4EBP1, ELP1, GSK3A, HSP90AB1, IL1R1, IL27RA, IL6ST, INPP5K, ITGA2B, ITGAV, ITGB4, MAP2K2, MAPK1, MAPK3, MAPK8IP1, NFKBIB, NRAS, OCRL, PDPK1, PIK3CB, PIK3CD, PIK3R1, PIK3R3, PPM1L, PPP2CB, PTGS2, PTPA, RAP1A, RRAS, TP53, YWHAB, YWHAH,* and *YWHAZ* genes. **(Figure 17a-b)**. To confirm the results, western blot analysis was done to evaluate PI3K/AKT pathway activity in vitro in both cell lines upon PRMT5 silencing. Our results highlighted a reduced expression of AKT as well as AKT phosphorylation in the HCT116 cell line while no change was observed in the CACO2 cell line **(Figure 18)**.

a) 

b) 

c) 

d) 

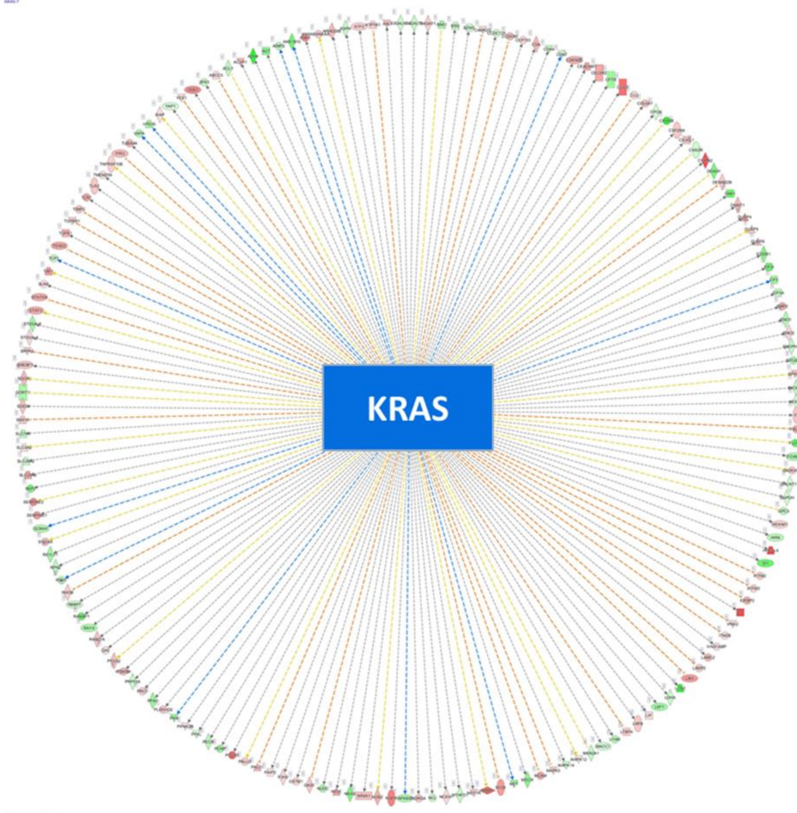
e) 

Figure 15: PRMT5 is overexpressed in CRC and associated with KRAS pathway activation. *(a-b) PRMT5 expression in CRC clinical samples compared to normal tissue counterpart using RNA seq dataset, TCGA data, UALCAN tool. PRMT5 expression in CRC clinical samples compared to normal tissue counterpart using proteomics dataset, CPTAC data, UALCAN tool. d) RNAseq data GSEA analysis of PRMT5 knocked down the CACO2 cell line versus control. e) RNAseq data IPA upstream analysis of PRMT5 knocked down CACO2 cell line versus control.*

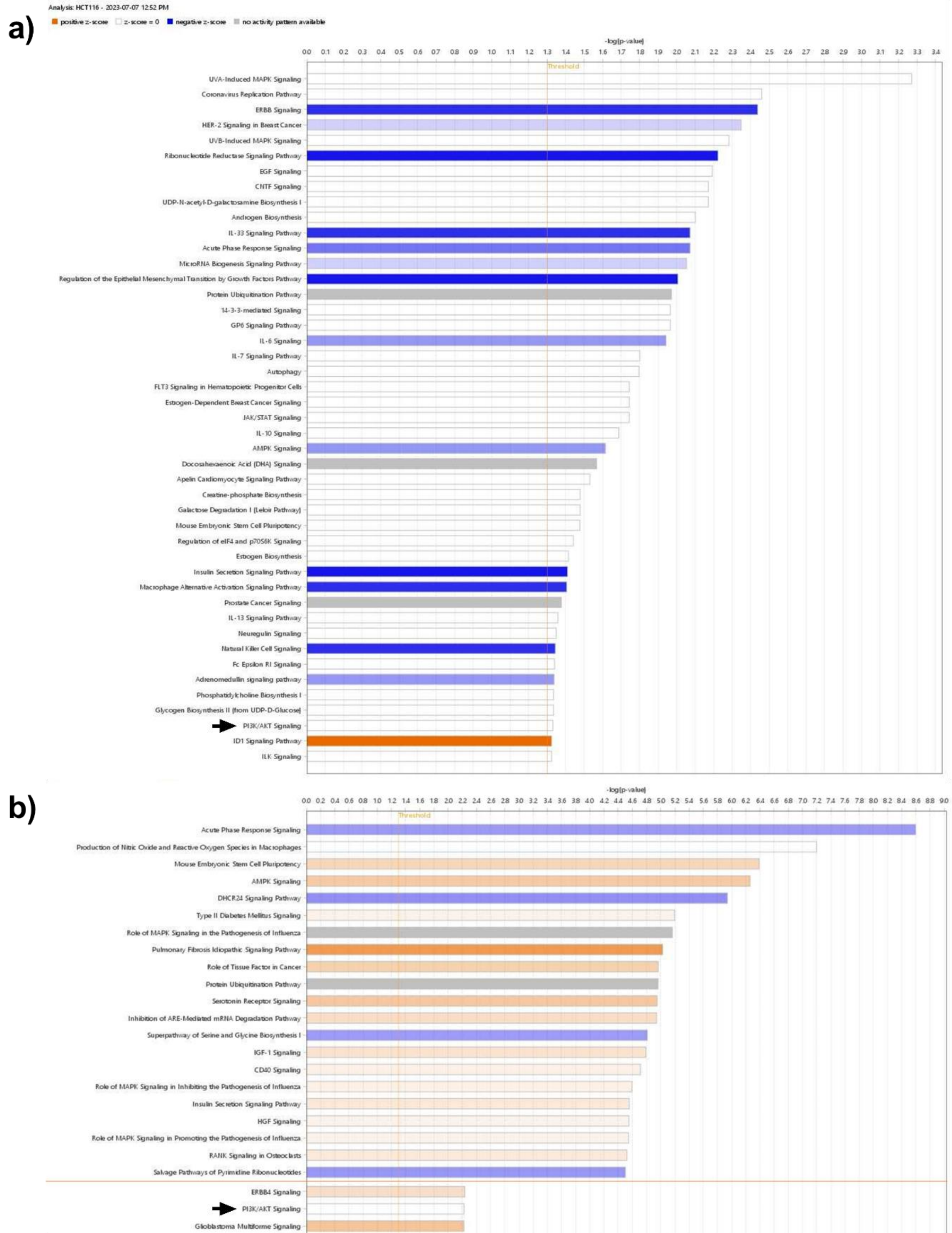
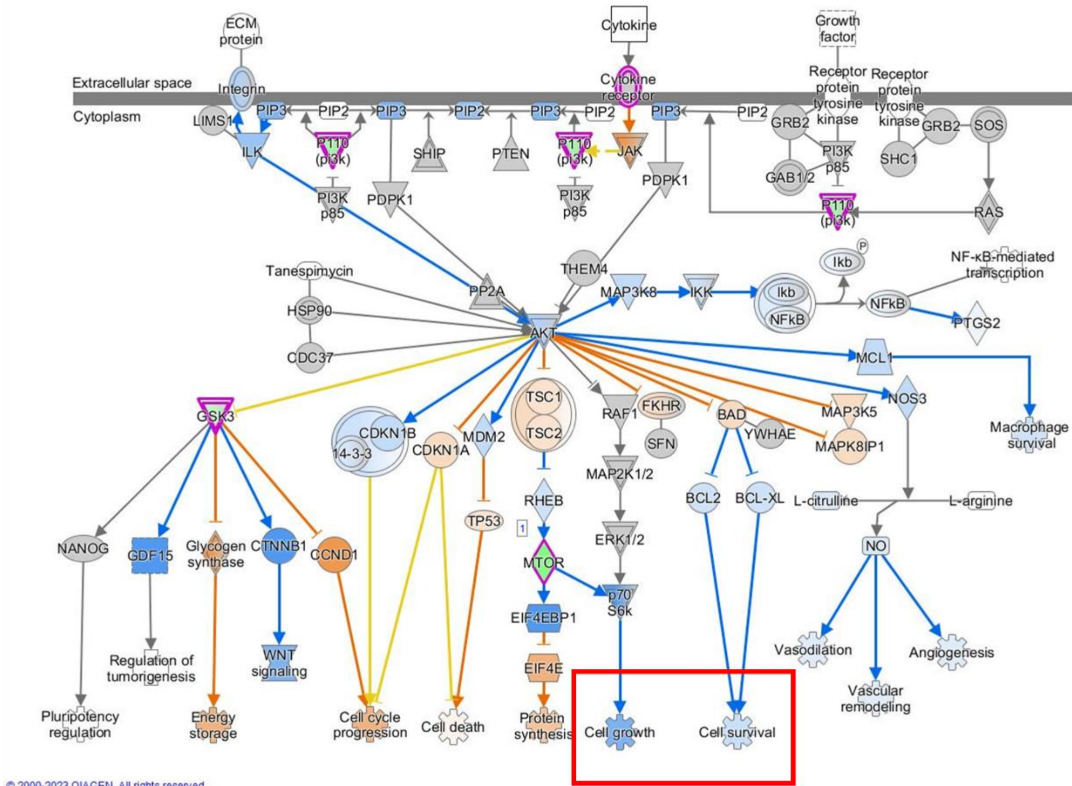


Figure 16: PI3K signaling pathway is a potential target of PRMT5. *The HCT116 and CACO2 cell lines were transfected with ctrl, or PRMT5 siRNA for 24h and then incubated for 48h. a) pathway enrichment analysis of PRMT5 knockdown HCT116 cell line using IPA. b)*

pathway enrichment analysis of PRMT5 knockdown CACO2 cell line using IPA. The red line indicates hidden data.

a)



b)

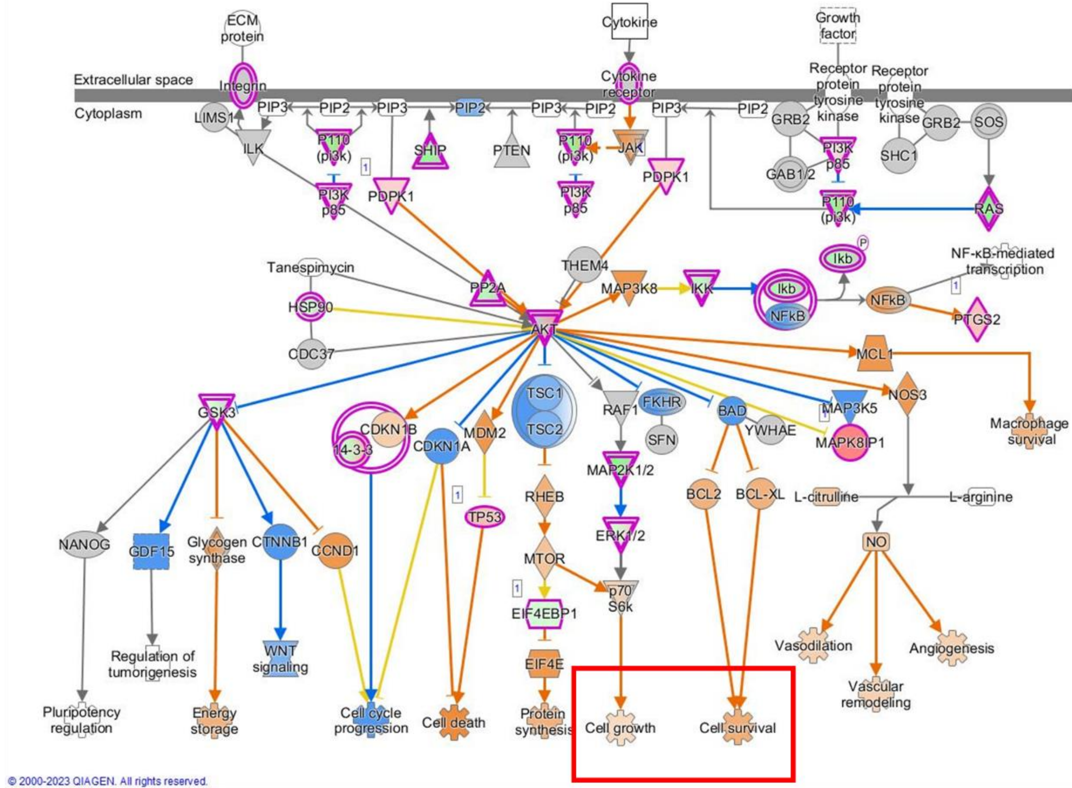


Figure 17: Differentially expressed genes in PI3K pathway upon PRMT5 knockdown in CRC cell lines. *a) IPA visual diagram of the PI3K pathway illustrates the location and predicted changes in the pathway molecules in the HCT116 cell line upon PRMT5 knockdown. b) IPA visual diagram of the PI3K pathway illustrates the location and predicted changes in the pathway molecules in the CACO cell line upon PRMT5 knockdown. Differentially expressed genes are marked purple. Green color refers to decreased expression while red color refers to increased expression. Molecular activity variations are highlighted orange for activation and blue for inhibition.*

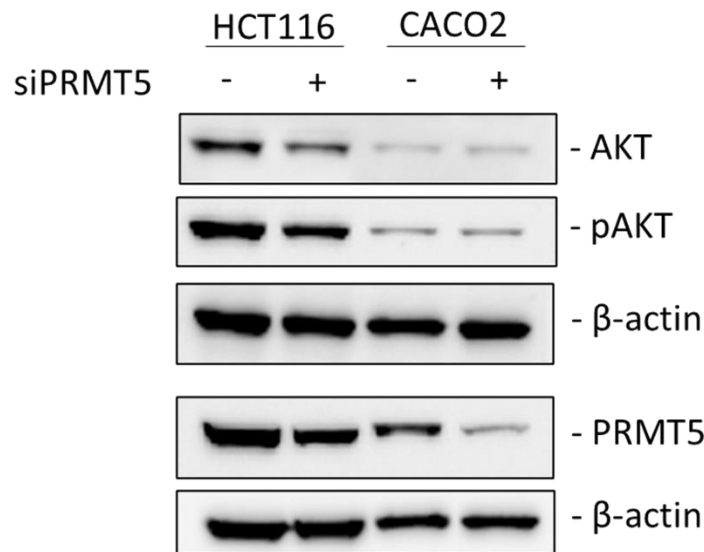


Figure 18: PI3K signaling pathway is activated by PRMT5. *The HCT116 cell line was transfected with ctrl, PRMT5 siRNA for 24h and then incubated for 48h. Western blot of AKT, pAKT, DKK1, and PRMT5 expression upon DKK1 knockdown and/or doxorubicin treatment. β-actin was used as a housekeeping control.*

5.3.DKK1 expression is regulated by PRMT5 in CRC.

To evaluate a putative association between PRMT5 and DKK genes, TCGA data analysis involving 212 CRC samples was performed. The results revealed a positive correlation between

PRMT5 and DKK1 but not DKK2, DKK3, or DKK4 (**Figure 19a-d**). To confirm the regulatory role of PRMT5 on selected DKK family members, the expression of several *DKK* genes was evaluated in PRMT5-silenced HCT116 and CACO2 cells. Silencing of PRMT5 in HCT116 cells reduced DKK1 expression at the mRNA level **(Figure 20a)**. However, PRMT5 silencing did not alter the expression of DKK3 or DKK4 (**Figure 20b-c**). Although DKK1 is not expressed on the protein level in *KRAS* wild-type CACO2, the gene expression at the mRNA level was significantly downregulated upon PRMT5 knockdown **(Figure 20d)**. It is worth noting that DKK2 was not expressed at the mRNA level either in the control or in the treated cells. Since DKK1 was the only gene of the DKK protein family that is associated with PRMT5 expression it will be taken into consideration in the following experiments. To validate the PRMT5 regulatory effect on DKK1, a western blot was used to detect DKK1 expression upon PRMT5 knockdown. The results show reduced DKK1 expression upon PRMT5 knockdown in the HCT116 cell line **(Figure 21a)**. A significant positive correlation between PRMT5 and DKK1 at the protein level was proven in CRC patient biopsies (**Figure 21b-c**). The transcriptional regulation of *DKK1* by PRMT5, the ChIP assay was performed to pull down PRMT5 and H3R8me2s followed by *DKK1* promoter region enrichment. There was a remarkable reduction in *DKK1* promoter region enrichment, likely suggesting a DNA interaction between DKK1 and PRMT5. Reduced *DKK1* promoter enrichment upon H3R8me2s pull-down indicates an epigenetic role of PRMT5 in regulating DKK1 expression (**Figure 21d**).

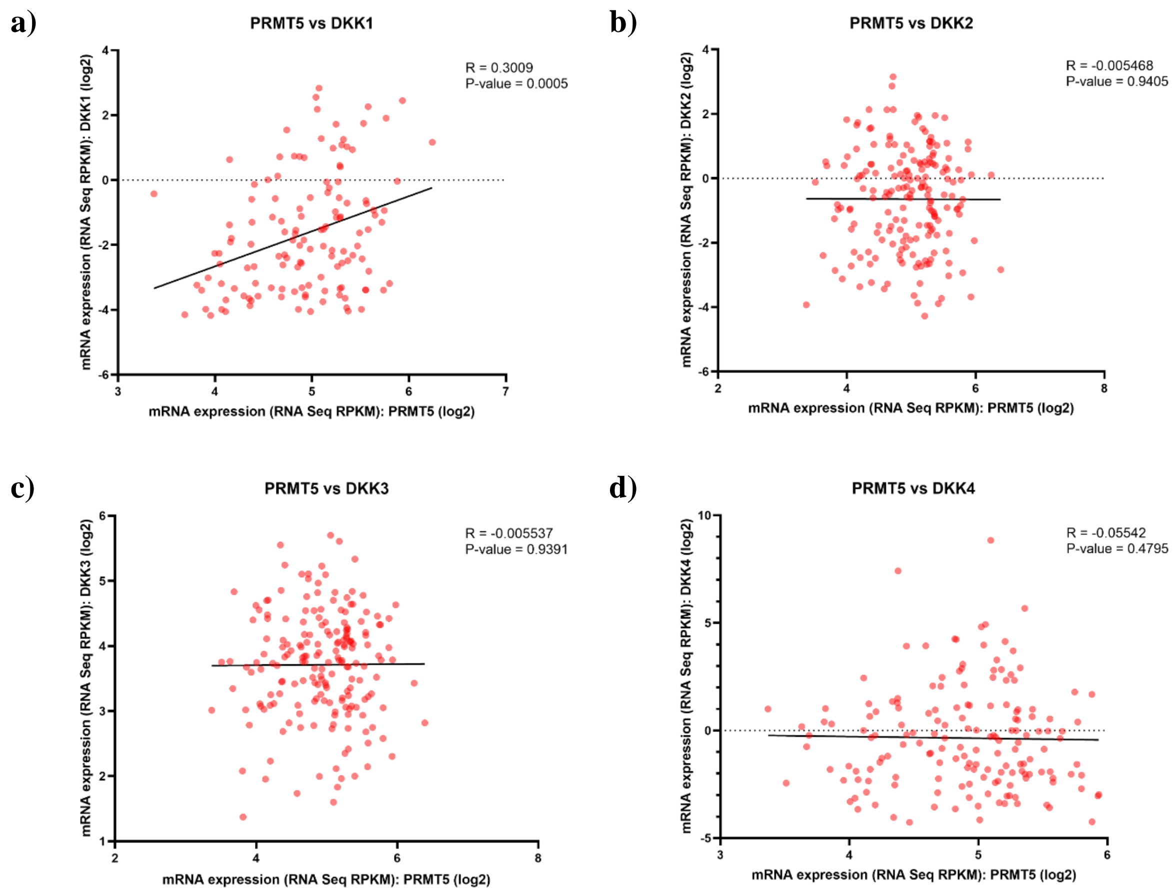


Figure 19: DKK gene family correlation with PRMT5. *a) Spearman's rank correlation coefficient of PRMT5 versus DKK1 expression, cBioportal tool. b) Spearman's rank correlation coefficient of PRMT5 versus DKK2 expression, cBioportal tool. c) Spearman's rank correlation coefficient of PRMT5 versus DKK3 expression, cBioportal tool. d) Spearman's rank correlation coefficient of PRMT5 versus DKK4 expression, cBioportal tool.*

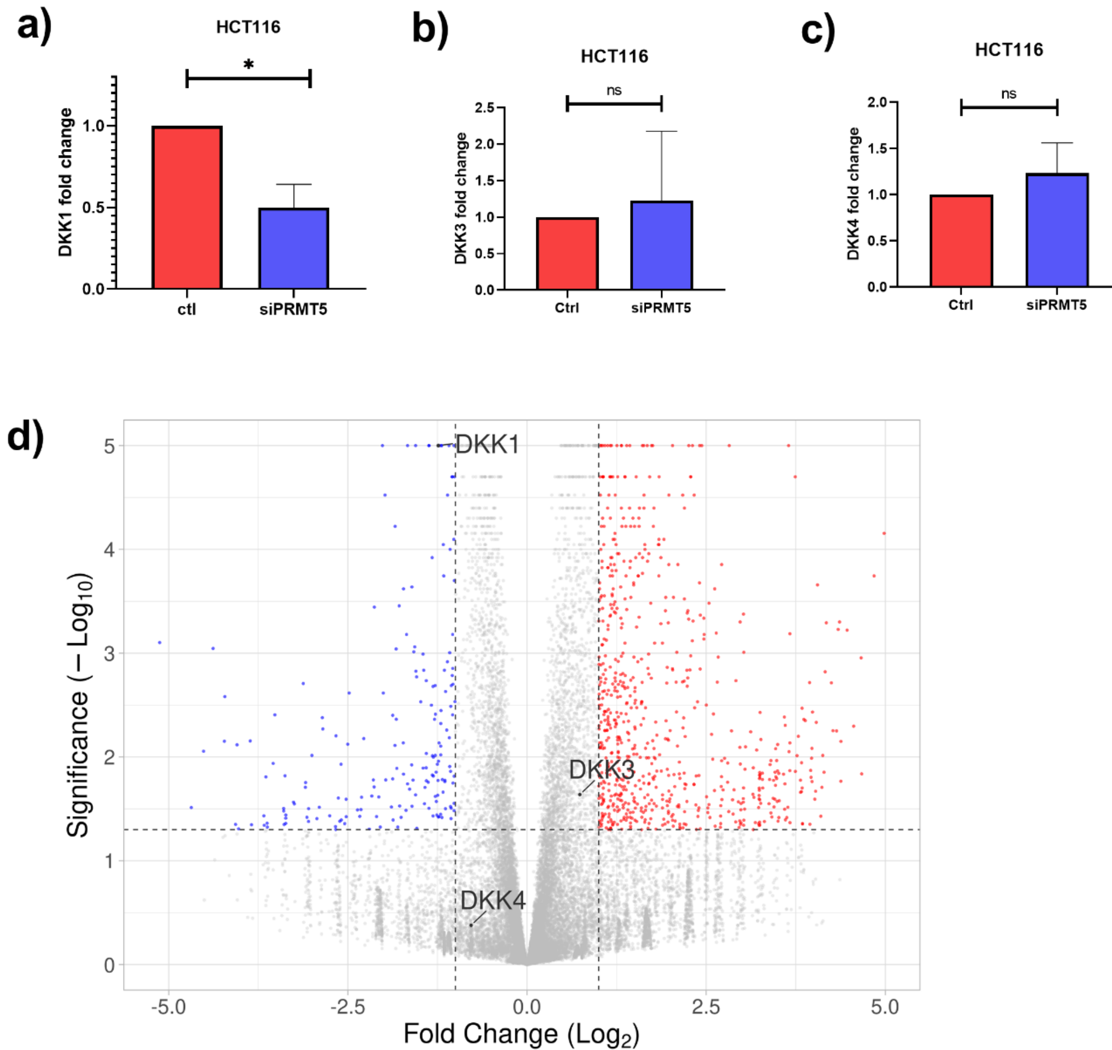


Figure 20: DKK gene family expression upon PRMT5 knockdown in CRC cell lines. *a-c) The HCT116 cell line was transfected with ctrl siRNA or PRMT5 siRNA for 24h and then incubated for 48h. a) DKK1 fold change. b) DKK3 fold change. c) DKK4 fold change. f) Volcano plot of differentially expressed genes in PRMT5 knocked down CACO2 cell lines compared to control. Red dots represent upregulated genes, blue dots represent downregulated genes, and gray dots represent genes that were not differentially expressed (P < 0.05, |logFC|>1.0).*

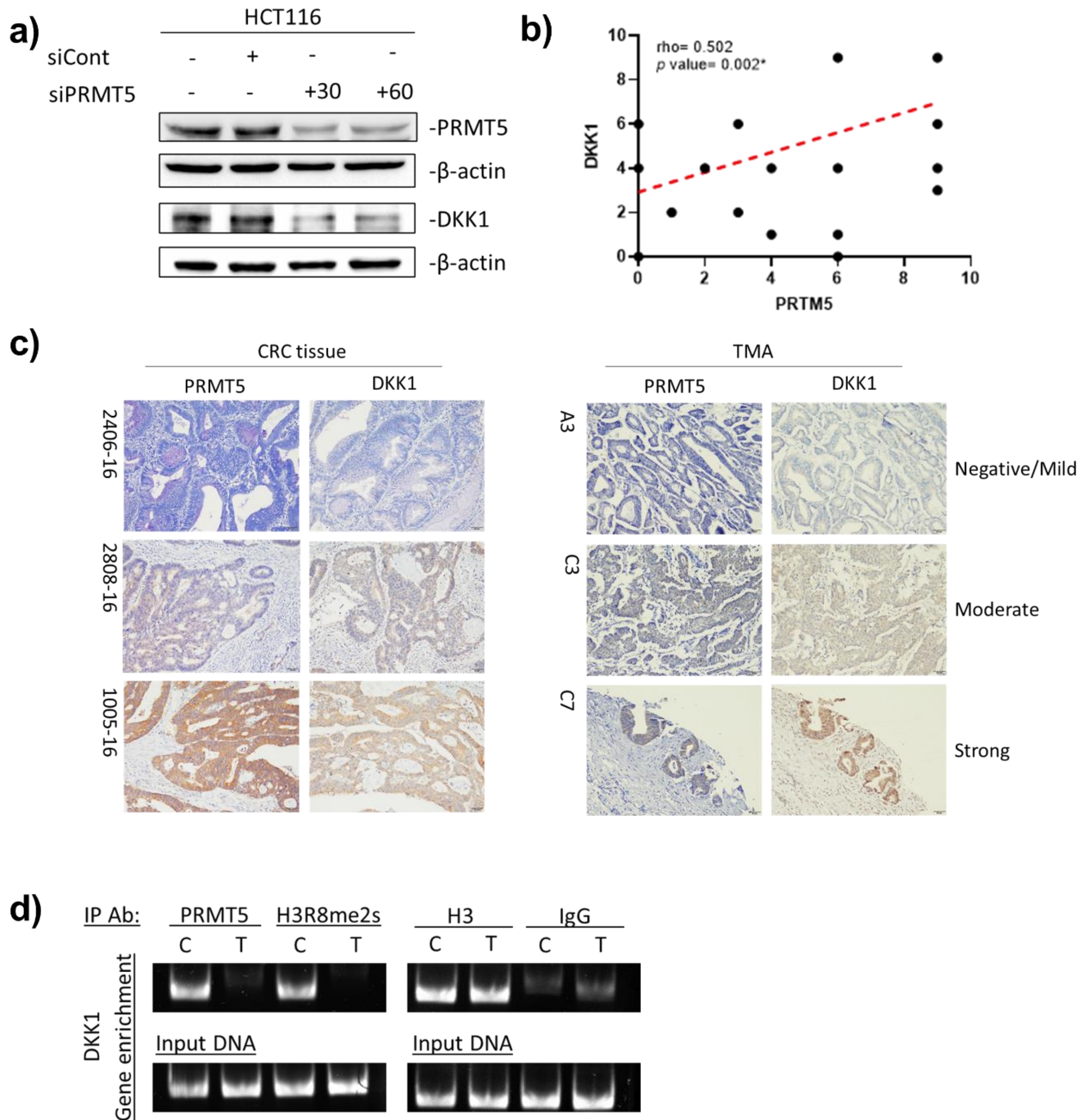


Figure 21: DKK1 is epigenetically regulated by PRMT5 in CRC. *a) The HCT116 cell line was transfected with ctrl siRNA or PRMT5 siRNA for 24h and then incubated for 48hthen protein lysate was collected for western blot assessing DKK1 expression. b-c) IHC analysis of PRMT5 and DKK1 proteins co-expression in CRC tissue samples. d) ChIP assay of DKK1 promoter enrichment in PRMT5 knocked down HCT116 (T) versus control (C). *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.*

5.4.PRMT5 inhibition reduces cancer cell viability in vitro.

To evaluate the effect of PRMT5 inhibition on CRC cell viability, HCT116 and CACO2 cells were treated with multiple concentrations of the PRMT5 inhibitor CMP5. HCT116 cells were treated with 0, 20, 30, 50, 70, and 100 µM of CMP5 for 24h and CACO2 cells were treated with 0, 5, 10, 20, 40, 60, 80, and 100 µM of CMP5 for 24h. Cell viability was measured by resazurin assay. CMP5 reduced cell viability by up to 50% in HCT116 and CACO2 at 57.59 µM and 72.85 µM respectively (**Figure *22*a-b**). This indicates that PRMT5 is a promising target in all CRC cell lines, but predominantly in *KRAS* mutants.

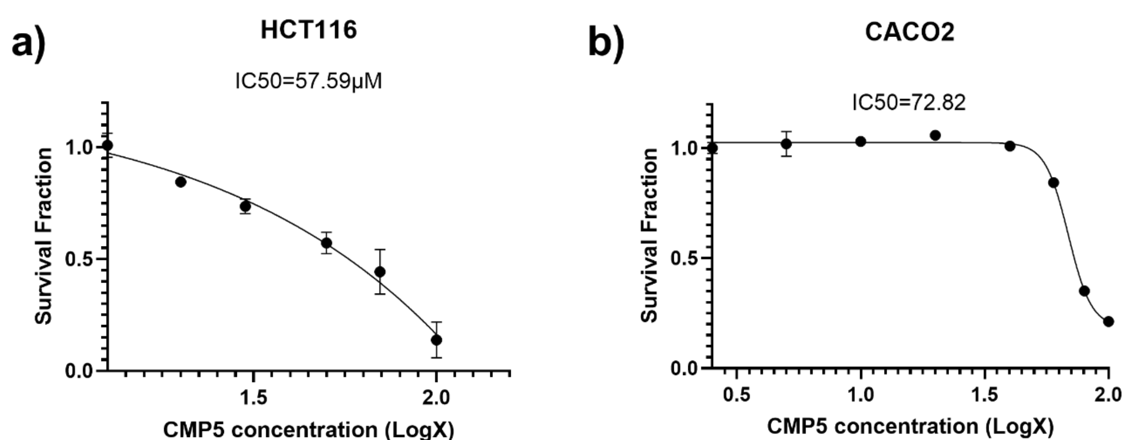


Figure 22: PRMT5 inhibition reduces viability in vitro. A-b) *Resazurin proliferation assay of CRC cell line treated with a range of concentrations of doxorubicin for 24 hours. IC50 was calculated using GraphPad Prism 8.0.*

5.5.CMP5 and doxorubicin exert synergistic anti-tumor effects in KRAS mutant CRC cells.

To evaluate whether CMP5 and doxorubicin exert additive or synergistic effects on CRC, HCT116, and CACO2 were treated with CMP5 and doxorubicin individually and in combination and assessed by functional assays. The HCT116 cells were treated with

doxorubicin at 0, 0.1, 2, 5, and 10 μM and with CMP5 at 0, 10, 20, 60, and 100 μM. CACO2 cells were treated with doxorubicin at 0, 0.1, 0.5, 1, and 2 μM. The average of the highest synergy scores was 25.48 and 10.7 in HCT116 and CACO2 cells respectively (**Figure 23a-b**). A significant increase ($P<0.01$) in the number of apoptotic HCT116 cells in the combination treatment (0.5 μM doxorubicin plus 60 μM CMP5) relative to single agent treatments was evident (**Figure 23c**). No significant difference in the number of apoptotic CACO2 cells treated with combination treatment (0.1 μM doxorubicin plus 60 μM CMP5) (**Figure 23d**) as compared with the single agent treatments. Cell cycle analysis of HCT116 showed a significant increase in sub-G1 ($P<0.01$) following combination treatment relative to controls (**Figure 23e**). No significant changes in cell cycling were observed in CACO2 cell lines treated with the combination treatment relative to controls (**Figure 23f**). which indicates a link between KRAS mutation and chemosensitivity. Strikingly, doxorubicin treatment increased DKK1 expression level in HCT116 while CMP5 helped to reduce this effect (**Figure 23g**). PRMT5 expression level was not affected by the treatment in HCT116 and CACO2 cell lines (**Figure 23g-h**).

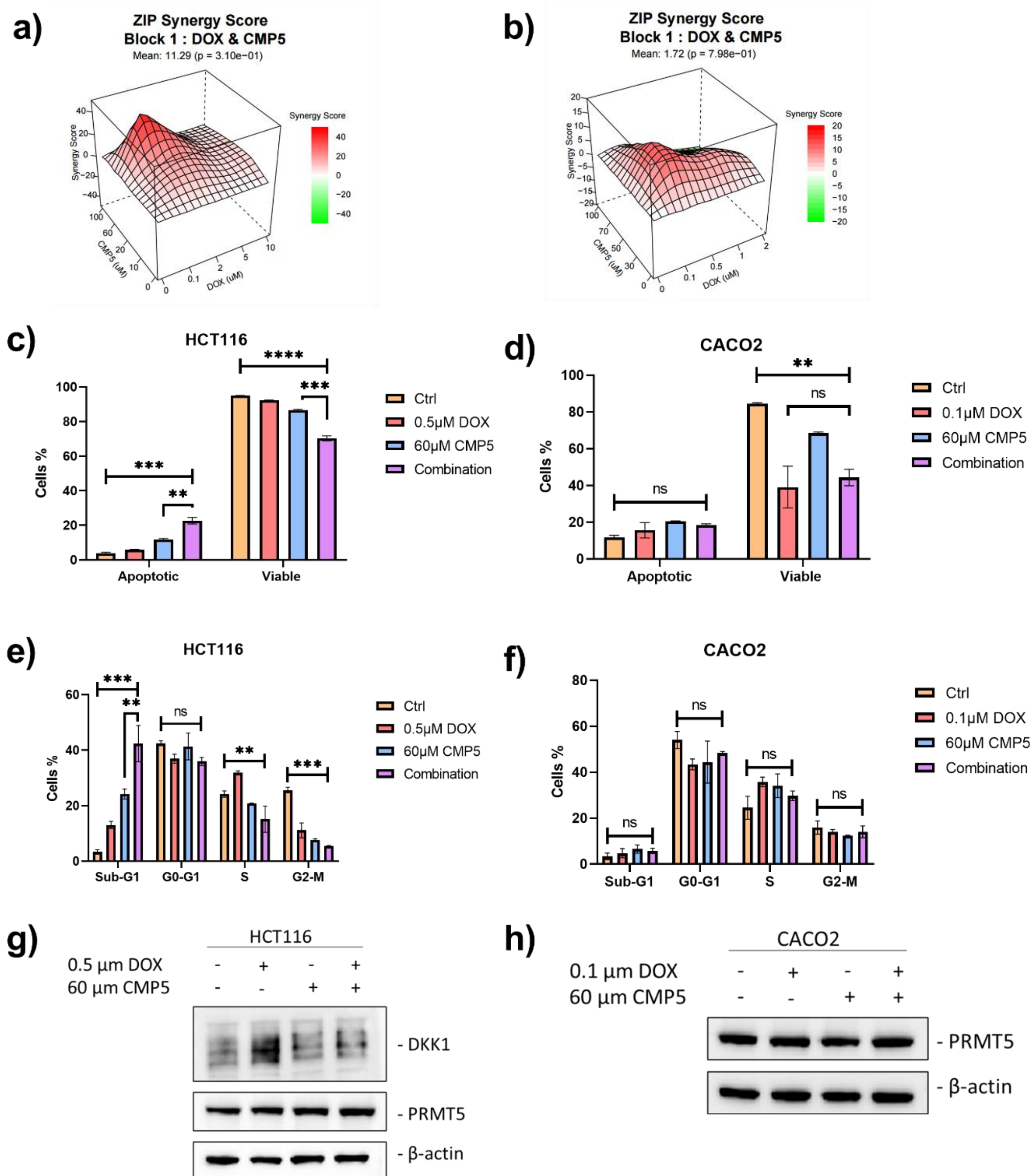


Figure 23: CMP5 and doxorubicin exert synergistic anti-tumor effects in KRAS mutant CRC cells. *a) resazurin proliferation assay of HCT116 cell line treated with a range of concentrations of doxorubicin (x-axis) and CMP5 (y-axis) for 24h and synergy was calculated using the SynergyFinder website (z-axis). a) resazurin proliferation assay of CACO2 cell line treated with a range of concentrations of doxorubicin (x-axis) and CMP5 (y-axis) for 24h and synergy was calculated using SynergyFinder website (z-axis). c-*

*d) HCT116 and CACO2 were treated with doxorubicin and/or CMP5 and after 24 h cells were collected for apoptosis assay. c-f) HCT116 and CACO2 were treated with doxorubicin and/or CMP5 and after 24 h cells were collected for cell cycle assay. c-f) HCT116 and CACO2 were treated with doxorubicin and/or CMP5 and after 24 h protein lysate was collected for western blot. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.*

5.6. DKK1 inhibition sensitizes *KRAS* mutant CRC cells to the anti-tumor effects of doxorubicin via inhibiting the PI3K/AKT pathway.

To assess the role of DKK1 in the previously noted synergism between CMP5 and doxorubicin, DKK1 was depleted in HCT116 cell lines by siRNA silencing followed by treatment with different concentrations of doxorubicin. A significant reduction in cell viability (**Figure 24a**) and a significant increase in the percentage of apoptotic cells (**Figure 24b**) was observed in DKK1-silenced doxorubicin (0.5,1 and 2 µM)-treated cells relative to controls. However, DKK1 silencing plus doxorubicin treatment did not lead to any significant changes in HCT116 cell cycling (**Figure 24c**). To assess the involvement of DKK1 in AKT activation, a combination treatment of doxorubicin and siDKK1 was performed. The results confirmed the increase in DKK1 expression level upon doxorubicin treatment along with increased phosphorylation level of AKT. However, in combination treatment reduced DKK1 expression and AKT phosphorylation were observed (**Figure 24b**). It is worth noting that *in silico* investigation demonstrated the capability of DKK1 to interact with CKAP and EGFR both of which are involved in PI3K pathway activation (**Figure 25**).

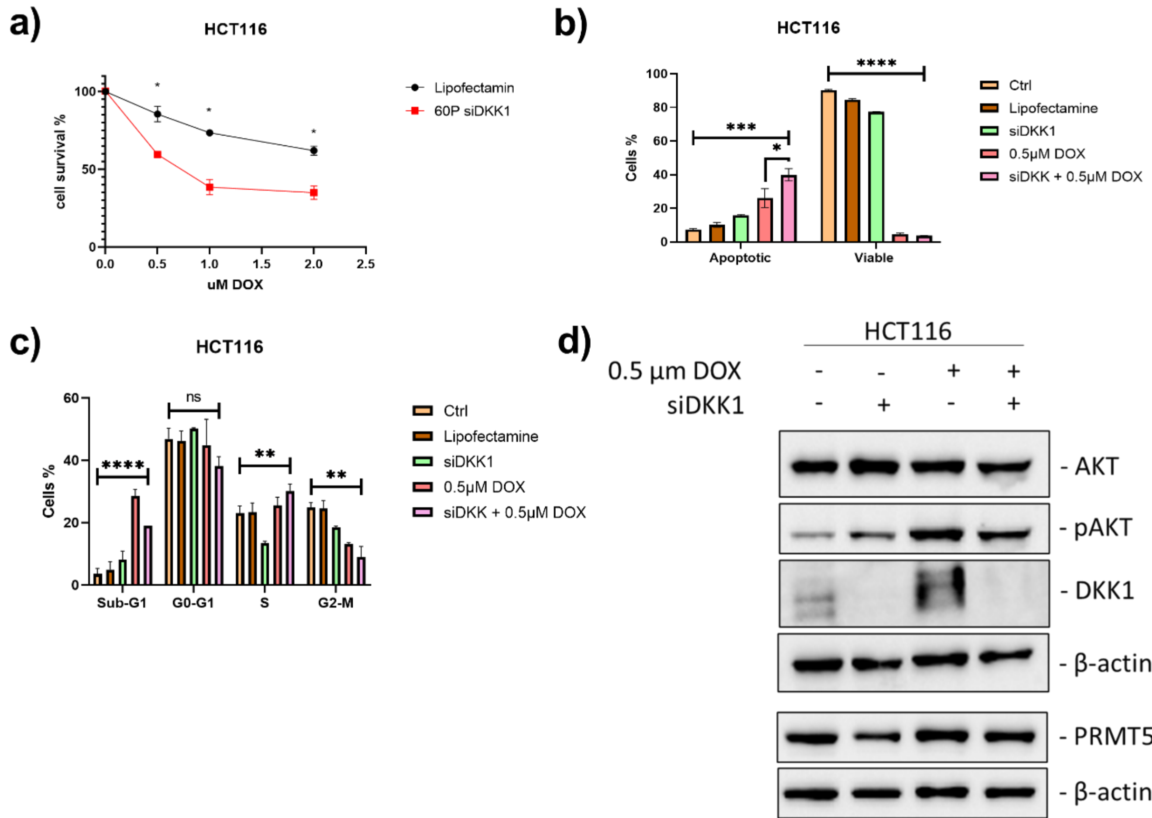


Figure 24: DKK1 inhibition sensitizes KRAS mutant CRC cells to the anti-tumor effects of doxorubicin. *a-c) The HCT116 cell line was transfected with DKK1 si-RNA for 24h and then treated with doxorubicin for 48h. a) resazurin cell proliferation assay. P- value was calculated using the T-test. b) apoptosis assay. P- value was calculated using one one-way ANOVA test. c) cell cycle assay. The P-value was calculated using a one-way ANOVA test. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.*

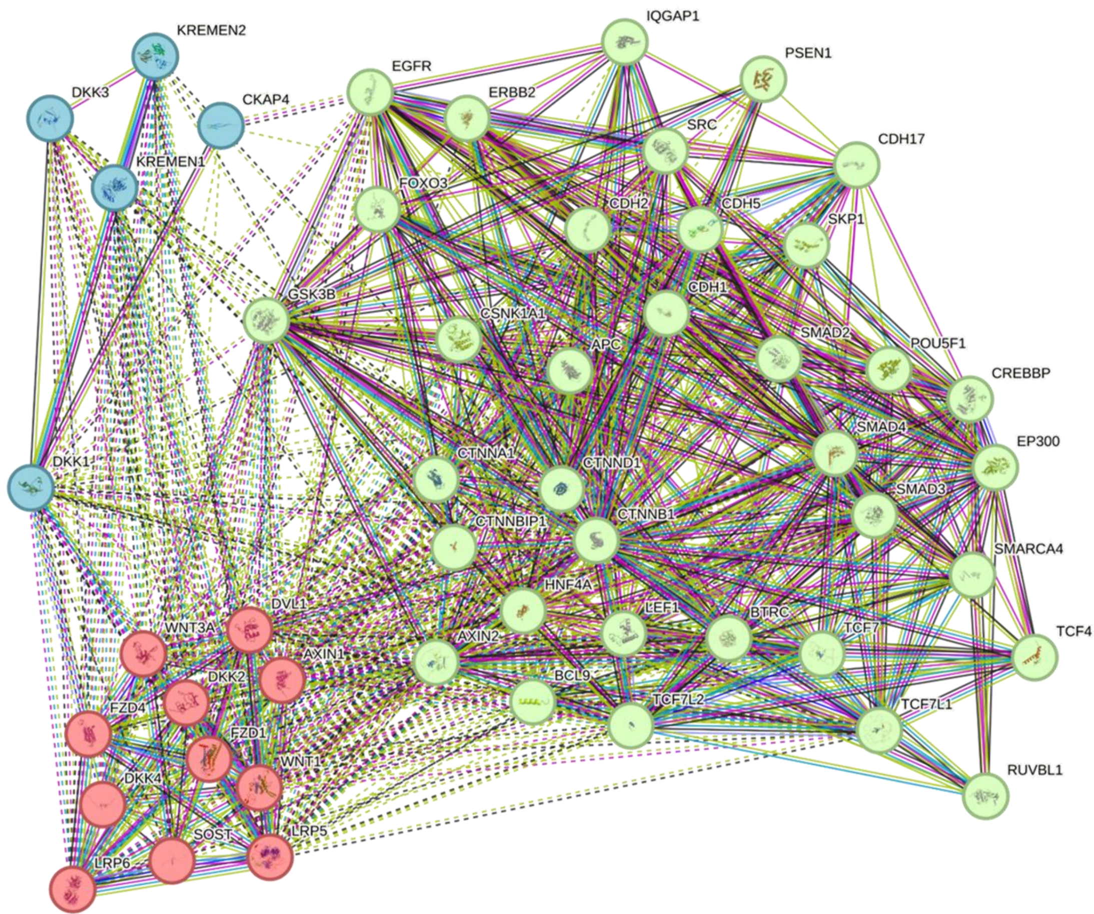


Figure 25: In silico analysis of protein networks associated with DKK1. *STRING database was used to predict protein-protein interactions with DKK1.*

5.7.The oncogenic activity of DKK1 is WNT signaling pathway independent

Since DKK1 is well known to be a WNT signaling antagonist. The role of DKK1 in the WNT signaling pathway, beta-catenin expression, and localization were assessed in DKK1-silenced HCT116 cells. Beta-catenin protein expression was not altered in DKK1-silenced cells (**Figure 26a**). Furthermore, beta-catenin is mainly localized to the cell membrane, with no translocation of the beta-catenin protein upon PRMT5 or DKK1 silencing (**Figure 26b**). To further validate these findings, DKK1 and beta-catenin gene co-expression was assessed using

TCGA patient samples. The *in-silico* analysis reveals no significant correlation between DKK1 and beta-catenin gene expression (**Figure 26c**).

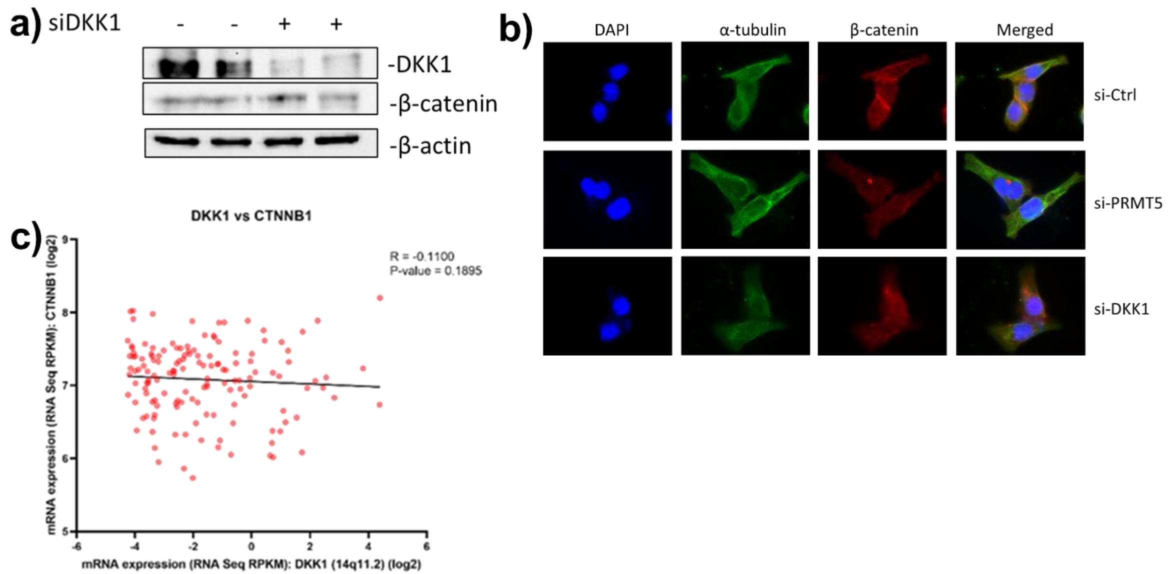


Figure 26: The oncogenic activity of DKK1 is Wnt signaling pathway independent. *a-b) The HCT116 cell line was transfected with ctrl, PRMT5, or DKK1 siRNA for 24h and then incubated for 48h. a) western blot of DKK1 expression and β-catenin expression upon DKK1 knockdown. β-actin was used as a housekeeping control. b) Immunofluorescence of DAPI blue, α-tubulin green, and β-catenin red.*

Chapter V.

Discussion

The DKK protein family has gained a lot of attention due to its role in cancer either as an oncogene or as a tumor suppressor. Our data proposed DKK1 as an indicator of chemoresistance in CRC. DKK1 is significantly upregulated in the majority of the cancers where it is differentially expressed compared to other DKKs. It showed upregulation in colorectal cancer and was associated with dedifferentiation in CRC tissue samples. In CRC cells, it was established that DKK1 expression is reduced in CRC due to DNA hypermethylation at the promoter region. However, the study included CRC cell lines and 5 patient samples only [153]. Logically, it was considered a tumor suppressor due to its function as a WNT signaling pathway inhibitor [163]. However, it was revealed that its expression is associated with other oncogenic pathways including the PI3K/AKT pathway [223]. We found that it is overexpressed due to PRMT5-induced histone methylation. This could be explained by the fact that cancer is heterogeneous where different tumors or tumor cells might behave differently and express different genetic and epigenetic alterations [224]. We suggest that DKK1 might have a dual role in colorectal cancer by acting as a tumor suppressor and chemoresistance inducer.

KRAS, a small GTPase protein, remains constitutively active in CRC cells due to gain-of-function mutations [34]. Activated KRAS triggers several cancer-related pathways, including RAF/MEK/ERK and PI3K/AKT [34]. Around 40% of CRC patients carry a mutation in *KRAS* [225]. Interestingly, we observed significant differential expression of DKK1 and DKK3 between HCT116 and CACO2 cell lines. *In silico* data further confirmed that DKK1, but not DKK3, is significantly upregulated in *KRAS* mutant CRC cases compared to their wild-type *KRAS* counterparts. Also, Previous studies have reported higher PRMT5 expression in

KRAS mutant CRC cell lines compared to KRAS wild-type cell lines [209]. In our study, we described the positive relationship between PRMT5 and DKK1. Furthermore, the upregulation of micro-RNA (miR-410) is thought to be responsible for DKK1 downregulation in CRC cell lines. Inhibition of miR-410 could restore the expression of DKK1 in the SW480 cell line and disturb WNT signaling activity [167]. However, our *in-silico* search showed upregulation of DKK1 in CRC patient samples. It is worth noting that, the DKK1 promoter region was hypermethylated in many CRC cell lines but not HCT116 [226].

PI3K/AKT pathway is a well-identified oncogenic pathway where it controls cell proliferation and apoptosis. phosphatidylinositol-3-kinase (PI3K) and serine/threonine protein kinase B (PKB/AKT) are the two key proteins in this pathway [227]. PI3K/AKT pathway is known to be hyper-activated in CRC. Furthermore, its inhibition showed a promising result in the treatment of colorectal cancer [228]. *KRAS* protein binds and activates PI3K therefore, PI3K/AKT pathway inhibition is thought to be a promising therapeutic approach to CRC [229]. PRMT5 protein is upregulated in CRC and was associated with the activation of the PI3K/AKT pathway [230]. previous discoveries have illustrated the relationship between PRMT5 to RAS/RAF/MEK/ERK and PI3K/AKT [231]. Our NGS data revealed that PRMT5 inhibition was associated with the predicted inhibition of *KRAS* in the *KRAS* wild-type CACO2 cell line. Strikingly, PRMT5 expression was associated with RAF and ERK inhibition in cervical cancer cells. however, it was linked to ERK activation in hepatocellular carcinoma cells [231]. Also, NGS data provided evidence of PRMT5's implication in altering the PI3K/AKT pathway in both *KRAS* wild-type CACO2 and *KRAS* mutant HCT116 cell lines. It was reported in previous studies that PRMT5 can epigenetically enhance the expression of the FGFR3 receptor which activates the PI3K/AKT pathway in SW480 cell lines [66] However, our NGS results describe the involvement of PRMT5 in altering the mRNA expression of multiple genes within the pathway. It was established in the literature that PRMT5 is positively correlated with PI3K

activity and negatively correlated to PTEN in human embryonic kidneys, glioblastoma neutrospheres, and gastric cancer [231]. We found that in the HCT116 cell line, PRMT5 knockdown resulted in a significant reduction in the PIK3CB gene which encodes for the PI3K isoform. Furthermore, it significantly reduced MTOR gene expression. Interestingly, western blot validation highlighted PI3K/AKT pathway inhibition in HCT116, and no change in the activity was observed in the CACO2 cell line. It is worth noting that despite its antagonist role in WNT signaling, DKK1 exhibits several pro-oncogenic activities. For instance, in lung cancer, DKK1 activates the PI3K pathway through interaction with CKAP4 receptor [232]. These facts raise the question of whether DKK1 plays a role in PRMT5/PI3K/AKT pathway cross-talk.

Prior research has suggested that PRMT5 enhance the expression of DKK1 in breast cancer, however, our study reveals that PRMT5 activates DKK1 expression in CRC [169]. Usually, PRMT5 acts as an epigenetic inhibitor of gene expression by methylating histones. however, there was multiple evidence that it can upregulate gene expression via regulating transcription factors [233]. Interestingly, PRMT5 upregulation was associated with KRAS mutation in CRC cell lines [209]. We highlighted the coexpression of PRMT5 and DKK1 in CRC via in silico analysis as well as IHC of CRC tissue. Among other DKKs PRMT5 knock down was significantly correlated with DKK1 down regulation. It is worth noting that our screening did not include DKK2 due to its absence in HCT116 and CACO2 as a result of promoter hypermethylation that was reported previously by Sato, et. al. [226]. Also, this study shed light on the role of PRMT5-induced histone methylation H3R8me2s in activating *DKK1* expression in the HCT116 cell line. Although it is most commonly described as a transcription suppressor, H3R8me2s methylation was linked to *FGFR3* and *eIF4E* gene expression activation in CRC. The contradictory role of H3R8me2s could be due to cross-talk between histone modifications or influencing the recruitment of transcription-related protein complexes

[180]. Global H3R8me2s increase was observed in leukemia and lymphoma cells and correlated to PRMT5 expression [234].

We showed that PRMT5 inhibitor (CMP5) has a higher IC50 in KRAS wild-type CACO2 than *KRAS* mutant HCT116. Our study revealed that blocking PRMT5-mediated regulation of *DKK1* enhances chemosensitization in HCT116 cells. This supports the hypothesis that PRMT5 upregulates *DKK1* expression which boosts CRC cell's chemoresistance. PRMT5 inhibitors (CMP5) have demonstrated a synergistic effect with doxorubicin. It significantly reduced cell proliferation and increased apoptosis in HCT116. Perouse studies showed that PRMT5 inhibitors are more potent against KRAS mutant cell lines and induce G2 cell cycle arrest. our data further emphasizes this fact [209]. Despite the significant anticancer potential of doxorubicin, its use in treating CRC is limited due to its cardiotoxicity [105]. However, combining PRMT5 inhibitors with doxorubicin treatment could potentially allow for a reduction in effective doxorubicin dosage and improved tolerability. Doxorubicin primarily operates by inducing DNA damage and oxidative stress in sensitive target cells [235]. PRMT5 inhibitors have also been shown to enhance DNA damage in breast and ovarian cancer [236]. Notably, inhibition of N-alpha-acetyltransferase 40 (NAA40), a PRMT5 activator protein, was reported to sensitize CRC cells to 5-FU treatment and induce DNA damage [202, 237]. CMP5 was described as a SAM-binding domain inhibitor of PRMT5 [214]. This explains that the PRMT5 level was not influenced by the treatment. However, downstream effector DKK1 was reduced. Previous studies have shown that PRMT5 expression is reduced upon CMP5 treatment in breast cancer cells. However, in the previous study have treated the cells for 72h, and in this study, we treated the cells for 24h [169]. Hence, we assume that a long treatment period with the inhibitor might induce drug degradation. The efficiency and the safety of the combination must be tested *in vivo.*

Although it was commonly known to hinder tumor progression, the nuclear expression of DKK1 has been identified as a chemoresistant marker in CRC [160]. DKK1 has also been implicated in upregulating the expression of genes responsible for drug detoxification such as *ALDH1A1, REPS2,* and *AKR1C3* [160]. Our study further reinforces the role of DKK1 in chemoresistance, as DKK1 inhibition sensitized HCT116 cell lines to doxorubicin treatment. However, determination of DKK1 localization in HCT116 and other CRC cell lines will have a great benefit. Furthermore, it is important to confirm the results using a specific small molecule inhibitor against DKK1. Interestingly, our results demonstrated an increase in DKK1 expression level upon treatment with doxorubicin as well as increased activity of AKT pathway. Previous studies have described an increase in AKT phosphorylation upon treatment with doxorubicin in the MCF7 breast cancer cell line [238]. Therefore, we suggest that doxorubicin treatment increases the positive feedback loop of DKK1 expression and PI3K/AKT pathway activity, however, DKK1 inhibition will dampen this effect and enhance the activity of doxorubicin especially that PI3K/AKT pathway activity was associated to doxorubicin resistance in CRC [104]. Previous work has described the role of DKK1 inhibitor (WAY-262611) in enhancing the efficacy of sorafenib via inhibiting PI3K/AKT and WNT signaling pathways in hepatocellular carcinoma cell lines [239]. Nevertheless, our work shows that DKK1 role in sensitizing CRC cells to doxorubicin is correlated to PI3K/AKT pathway inhibition but independent from WNT signaling activity. consistent with our data previous study showed that DKK1 inhibition alone did not greatly influence apoptosis nor influenced cell cycle in HEP3B however it significantly boosted sorafenib cytotoxic effect [239]. Our data illustrates the role of DKK1 silencing in enhancing doxorubicin activity against the HCT116 cell line additionally, we observed a reduction in the G2/M phase correlated to DKK1 inhibition in the HCT116 cell line which was also previously observed in the HUH-7 hepatocellular carcinoma cell line [239]. Furthermore, in silico protein-protein interaction illustration showed

that DKK1 binds CKAP4 receptor as well as EGFR receptors where CKAP4 is highly expressed in the colon and rectum while EGFR expression is low to medium in the colon and rectum according to The Human Protein Atlas [240].

Although WNT signaling inactivation post DKK1 inhibition was a key player in sensitizing hepatocellular carcinoma cells to sorafenib, our in-vitro investigation illustrated that DKK1 expression did not influence WNT signaling pathway activity in the HCT116 cell line [239]. This could be due to the fact that DKK1 antagonizes WNT signaling via binding to low-density lipoprotein receptor-related protein (LRP5/6) upstream beta-catenin complex [241, 242]. However, HCT116 contains a heterogenous mutation resulting in the deletion of a serine residue at codon 45 and prevents the degradation of beta-catenin regardless of the upstream signals [243, 244]. S45 phosphorylation flag beta-catenin for further phosphorylation by GSK-3 followed by ubiquitylation and degradation [245].

Collectively, our findings propose PRMT5 inhibitors as a promising adjuvant therapy for *KRAS* mutant and DKK1-expressing CRC patients. It highlights the role of DKK1 expression in the chemosensitization of CRC cell lines. Further studies exploring the downstream effectors of DKK1 are pivotal. Also, it is necessary to apply the current knowledge about DKK1 in a large-scale study investigating DKK1 as a diagnostic and prognostic marker. Our findings pave the way for more research discussing the role of DKK1 in CRC progression and treatment.

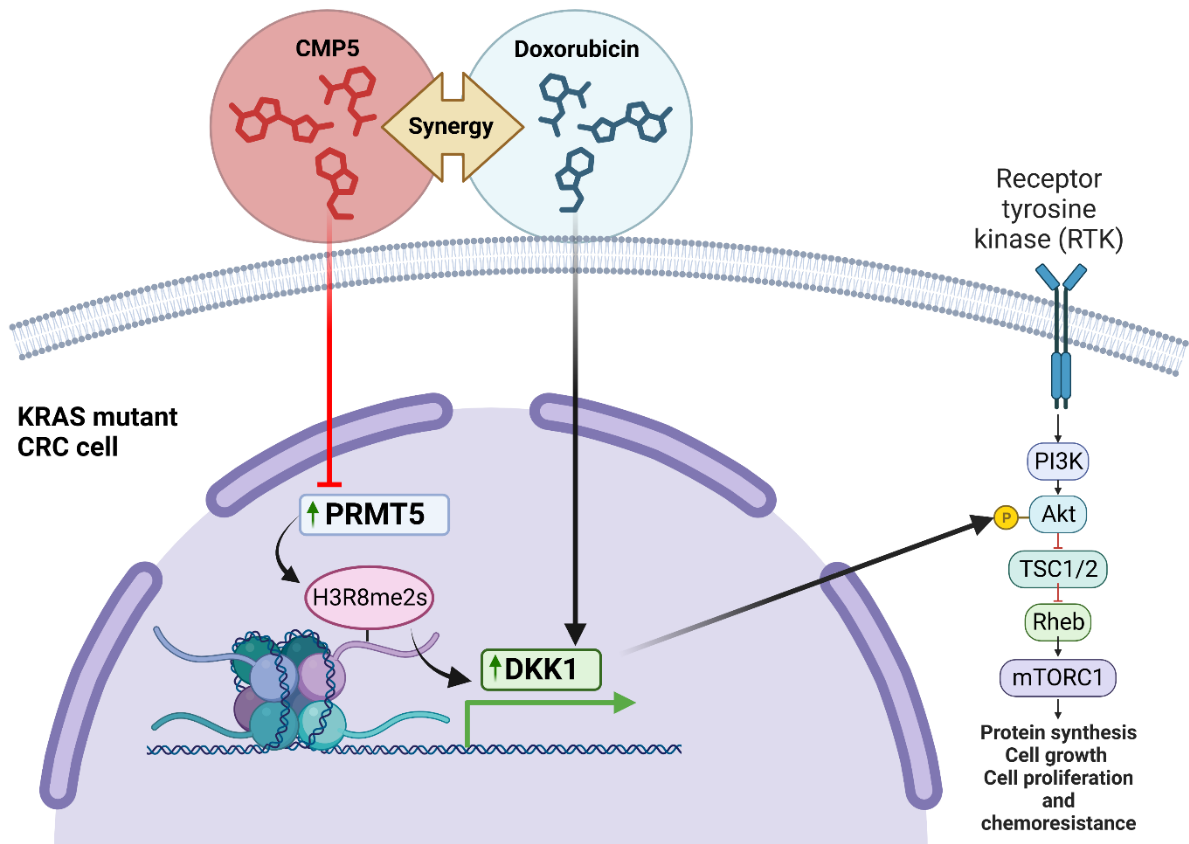


Figure 27: Graphical summary of our findings. *Our data shows that PRMT5 epigenetically upregulates the expression of DKK1 in KRAS mutant cell line HCT116. DKK1 increases PI3K/AKT pathway activity. Furthermore, doxorubicin treatment increases DKK1 expression as well as PI3K/AKT pathway activity. inhibiting PRMT5 helps to reduce DKK1 expression and PI3K/AKT pathway activity therefore enhancing HCT116 cell line response to doxorubicin.*

Chapter VI.

Conclusion

Conclusion and recommendations

To conclude, we highlighted the synergistic effect between PRMT5 inhibitor (CMP5) and doxorubicin in HCT116, an HCT116 *KRAS* mutant cell line. We found that DKK1 is overexpressed in *KRAS* versus wild-type CRC cell lines and patients. We showed that DKK1 overexpression is regulated by PRMT5 in CRC. Furthermore, we proved that DKK1 upregulation did not influence WNT signaling activity in the HCT116 cell line. These results support the hypothesis that PRMT5 is implicated in chemoresistance by regulating DKK1. Our study supports previous studies, which discussed PRMT5 as an anticancer treatment. It brings to light its capability as a chemosensitizer in CRC. However, the role of DKK1 in CRC is a contradictory topic, where it acts as a WNT signaling antagonist. Our findings describe the link between DKK1 expression and chemosensitivity in CRC patients. Our results suggest PRMT5 inhibitors combined with doxorubicin as an alternative treatment for CRC where it regulates DKK1. This treatment gives a glimmer of hope for late-stage cases by reducing chemotherapy doses and, therefore, the side effects. Indeed, animal studies are necessary to investigate the safety of the combination treatment. Furthermore, our study raises the necessity of investigating the alternative roles of the DKK protein family in CRC, rather than regulating WNT signaling. In summary, our results highlight the role of PRMT5 in chemosensitization via regulating the *DKK1* expression in CRC cell lines, emphasizing the urgency of further investigations of the role of DKK1 in CRC.

Limitations of the study

In our study, we have used two cell lines each of which resample *KRAS* wild type or *KRAS* mutant. Indeed, applying our experiments on a larger number of cells from both groups will provide more confirmatory results. Although we identified DKK1's role in activating the PI3K pathway in CRC, it might exert other functions implicated in chemoresistance. Furthermore, we have tested the role of DKK1 in chemosensitization using doxorubicin only. Applying the experiments with other chemotherapies would further emphasize this role. A more generalized study of DKK1's role in CRC using whole proteome analysis will strengthen our understanding of the topic. Also, assessing DKK1 localization might give us a hint about its mechanism. DKK1 expression was screened in less than 100 patient samples. Expanding the study to a larger scale population will strengthen our hypothesis. Furthermore, linking DKK1 expression to patients who are receiving chemotherapy was not provided due to the limited patient data. Also, it would have been a beneficial addition to look at DKK1 localization or secretion in CRC cells using immunofluorescence and enzyme-linked immunosorbent assay (ELISA).

Future Perspectives

To expand the knowledge on this topic, screening CMP5 and doxorubicin using different approaches such as CRC organoids and animal models is mandatory. Indeed, organoids are better at mimicking the tumor in a 3D structure than cell lines in a 2D structure. Also, testing the combination in the animals will provide a better idea about the safety and the toxicity of the combination which will pave the way to clinical trials. Furthermore, assessing the expression of DKK1 in *KRAS*-mutant versus *KRAS*-wild type CRC tissues at the protein level using IHC. Studding the direct correlation between KRAS mutation and DKK1 expression via transfecting KRAS wild type CACO2 with KRAS mutant plasmid. In addition,

to investigate the DKK1 role in CRC, it is important to evaluate the proteins that interact with it in CRC using immunoprecipitation technique followed by proteomics analysis.

References

1. *GLOBOCAN*. 2020; Available from: https://gco.iarc.fr/today/data/factsheets/populations/784-united-arab-emirates-fact-sheets.pdf.
2. Høydahl, Ø., et al., *Long-term trends in colorectal cancer: incidence, localization, and presentation.* BMC Cancer, 2020. **20**(1): p. 1077.
3. Sung, H., et al., *Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries.* CA Cancer J Clin, 2021. **4**(10): p. 21660.
4. Saran Lotfollahzadeh, S.K., Andrea Tsoris, Alejandro Recio-Boiles, Hani M. Babiker, *Rectal Cancer.* StatPearls, 2023.
5. Andrew, A.S., et al., *Risk Factors for Diagnosis of Colorectal Cancer at a Late Stage: a Population-Based Study.* J Gen Intern Med, 2018. **33**(12): p. 2100-2105.
6. Ionescu, V.A., et al., *Colorectal Cancer: From Risk Factors to Oncogenesis.* Medicina (Kaunas), 2023. **59**(9).
7. Hossain, M.S., et al., *Colorectal Cancer: A Review of Carcinogenesis, Global Epidemiology, Current Challenges, Risk Factors, Preventive and Treatment Strategies.* Cancers (Basel), 2022. **14**(7).
8. Seyedian, S.S., F. Nokhostin, and M.D. Malamir, *A review of the diagnosis, prevention, and treatment methods of inflammatory bowel disease.* J Med Life, 2019. **12**(2): p. 113-122.
9. Olén, O., et al., *Colorectal cancer in ulcerative colitis: a Scandinavian population-based cohort study.* Lancet, 2020. **395**(10218): p. 123-131.
10. Yashiro, M., *Ulcerative colitis-associated colorectal cancer.* World J Gastroenterol, 2014. **20**(44): p. 16389-97.
11. Hankey, W., W.L. Frankel, and J. Groden, *Functions of the APC tumor suppressor protein dependent and independent of canonical WNT signaling: implications for therapeutic targeting.* Cancer Metastasis Rev, 2018. **37**(1): p. 159-172.
12. Labianca, R., et al., *Primary colon cancer: ESMO Clinical Practice Guidelines for diagnosis, adjuvant treatment and follow-up.* Annals of oncology : official journal of the European Society for Medical Oncology, 2010. **70**(21).
13. Guinney, J., et al., *The consensus molecular subtypes of colorectal cancer.* Nature Medicine, 2015. **21**(11): p. 1350-1356.
14. Pino, M.S. and D.C. Chung, *The chromosomal instability pathway in colon cancer.* Gastroenterology, 2010. **138**(6): p. 2059-72.
15. Singh, M.P., et al., *Molecular subtypes of colorectal cancer: An emerging therapeutic opportunity for personalized medicine.* Genes Dis, 2021. **8**(2): p. 133-145.
16. Bakhoum, S.F. and L.C. Cantley, *The Multifaceted Role of Chromosomal Instability in Cancer and Its Microenvironment.* Cell, 2018. **174**(6): p. 1347-1360.
17. Harbers, L., et al., *Somatic Copy Number Alterations in Human Cancers: An Analysis of Publicly Available Data From The Cancer Genome Atlas.* Front Oncol, 2021. **11**: p. 700568.
18. Nojadeh, J.N., S. Behrouz Sharif, and E. Sakhinia, *Microsatellite instability in colorectal cancer.* Excli j, 2018. **17**: p. 159-168.
19. Nazemalhosseini Mojarad, E., et al., *The CpG island methylator phenotype (CIMP) in colorectal cancer.* Gastroenterol Hepatol Bed Bench, 2013. **6**(3): p. 120-8.

20. Inamura, K., *Colorectal Cancers: An Update on Their Molecular Pathology.* Cancers, 2018. **10**(1).
21. Sveen, A., S. Kopetz, and R.A. Lothe, *Biomarker-guided therapy for colorectal cancer: strength in complexity.* Nature reviews. Clinical oncology, 2020. **17**(1): p. 11-32.
22. Giglia, M.D. and D.I. Chu, *Familial Colorectal Cancer: Understanding the Alphabet Soup.* Clin Colon Rectal Surg, 2016. **29**(3): p. 185-95.
23. Schatoff, E.M., B.I. Leach, and L.E. Dow, *Wnt Signaling and Colorectal Cancer.* Current colorectal cancer reports, 2017. **13**(2): p. 101-110.
24. Bienz, M. and H. Clevers, *Linking colorectal cancer to Wnt signaling.* Cell, 2000. **103**(2): p. 311-20.
25. Duraturo, F., et al., *Genetics, diagnosis and treatment of Lynch syndrome: Old lessons and current challenges.* Oncol Lett, 2019. **17**(3): p. 3048-3054.
26. Alhmoud, J.F., et al., *DNA Damage/Repair Management in Cancers.* Cancers (Basel), 2020. **12**(4).
27. Ashburn, J.H., T.P. Plesec, and M.F. Kalady, *Serrated Polyps and Serrated Polyposis Syndrome.* Clin Colon Rectal Surg, 2016. **29**(4): p. 336-344.
28. Rebuzzi, F., P. Ulivi, and G. Tedaldi, *Genetic Predisposition to Colorectal Cancer: How Many and Which Genes to Test?* Int J Mol Sci, 2023. **24**(3).
29. Chalhoub, N. and S.J. Baker, *PTEN and the PI3-kinase pathway in cancer.* Annu Rev Pathol, 2009. **4**: p. 127-50.
30. Zhao, M., L. Mishra, and C.X. Deng, *The role of TGF-β/SMAD4 signaling in cancer.* Int J Biol Sci, 2018. **14**(2): p. 111-123.
31. Ozaki, T. and A. Nakagawara, *Role of p53 in Cell Death and Human Cancers.* Cancers (Basel), 2011. **3**(1): p. 994-1013.
32. Kuipers, E.J., et al., *Colorectal cancer.* Nat Rev Dis Primers, 2015. **1**: p. 15065.
33. Dinu, D., et al., *Prognostic significance of KRAS gene mutations in colorectal cancer--preliminary study.* J Med Life, 2014. **7**(4): p. 581-7.
34. Ternet, C. and C. Kiel, *Signaling pathways in intestinal homeostasis and colorectal cancer: KRAS at centre stage.* Cell communication and signaling : CCS, 2021. **19**(1): p. 31-31.
35. Hussain, M.R., et al., *BRAF gene: From human cancers to developmental syndromes.* Saudi J Biol Sci, 2015. **22**(4): p. 359-73.
36. Mundade, R., et al., *Genetic pathways, prevention, and treatment of sporadic colorectal cancer.* Oncoscience, 2014. **1**(6): p. 400-6.
37. Testa, U., E. Pelosi, and G. Castelli, *Colorectal cancer: genetic abnormalities, tumor progression, tumor heterogeneity, clonal evolution and tumor-initiating cells.* Med Sci (Basel), 2018. **6**(2).
38. Jung, G., et al., *Epigenetics of colorectal cancer: biomarker and therapeutic potential.* Nat Rev Gastroenterol Hepatol, 2020. **17**(2): p. 111-130.
39. Rawla, P., T. Sunkara, and A. Barsouk, *Epidemiology of colorectal cancer: incidence, mortality, survival, and risk factors.* Prz Gastroenterol, 2019. **14**(2): p. 89-103.
40. Kanwal, R. and S. Gupta, *Epigenetic modifications in cancer.* Clinical genetics, 2012. **81**(4): p. 303-311.
41. Bihl, M.P., et al., *Characterization of CDKN2A(p16) methylation and impact in colorectal cancer: systematic analysis using pyrosequencing.* J Transl Med, 2012. **10**: p. 173.
42. Jung, G., et al., *Epigenetics of colorectal cancer: biomarker and therapeutic potential.* Nature Reviews Gastroenterology & Hepatology, 2020. **17**(2): p. 111-130.

43. Luo, J., et al., *S-adenosylmethionine inhibits the growth of cancer cells by reversing the hypomethylation status of c-myc and H-ras in human gastric cancer and colon cancer.* Int J Biol Sci, 2010. **6**(7): p. 784-95.
44. Leon Arellano, M., et al., *Analysis of Septin 9 Gene Hypermethylation as Follow-Up Biomarker of Colorectal Cancer Patients after Curative Surgery.* Diagnostics (Basel), 2022. **12**(4).
45. Sun, J., et al., *Structure and function of Septin 9 and its role in human malignant tumors.* World J Gastrointest Oncol, 2020. **12**(6): p. 619-631.
46. He, N., et al., *The Pathological Features of Colorectal Cancer Determine the Detection Performance on Blood ctDNA.* Technol Cancer Res Treat, 2018. **17**: p. 1533033818791794.
47. Overman, M.J., et al., *Phase I/II study of azacitidine and capecitabine/oxaliplatin (CAPOX) in refractory CIMP-high metastatic colorectal cancer: evaluation of circulating methylated vimentin.* Oncotarget, 2016. **7**(41): p. 67495-67506.
48. Henikoff, S. and M.M. Smith, *Histone variants and epigenetics.* Cold Spring Harb Perspect Biol, 2015. **7**(1): p. a019364.
49. Chi, P., C.D. Allis, and G.G. Wang, *Covalent histone modifications--miswritten, misinterpreted and mis-erased in human cancers.* Nat Rev Cancer, 2010. **10**(7): p. 457-69.
50. Lee, H.T., et al., *The Key Role of DNA Methylation and Histone Acetylation in Epigenetics of Atherosclerosis.* J Lipid Atheroscler, 2020. **9**(3): p. 419-434.
51. Yang, H., et al., *Overexpression of histone deacetylases in cancer cells is controlled by interplay of transcription factors and epigenetic modulators.* Faseb j, 2014. **28**(10): p. 4265-79.
52. Lutz, L., et al., *Histone modifiers and marks define heterogeneous groups of colorectal carcinomas and affect responses to HDAC inhibitors in vitro.* Am J Cancer Res, 2016. **6**(3): p. 664-76.
53. Ye, P., et al., *Histone deacetylase 2 regulates doxorubicin (Dox) sensitivity of colorectal cancer cells by targeting ABCB1 transcription.* Cancer Chemother Pharmacol, 2016. **77**(3): p. 613-21.
54. Cao, L.L., et al., *The expression of histone deacetylase HDAC1 correlates with the progression and prognosis of gastrointestinal malignancy.* Oncotarget, 2017. **8**(24): p. 39241-39253.
55. Ashktorab, H., et al., *Global histone H4 acetylation and HDAC2 expression in colon adenoma and carcinoma.* Dig Dis Sci, 2009. **54**(10): p. 2109-17.
56. Liu, Y., et al., *Oncogene Ras/phosphatidylinositol 3-kinase signaling targets histone H3 acetylation at lysine 56.* J Biol Chem, 2012. **287**(49): p. 41469-80.
57. Elsheikh, S.E., et al., *Global histone modifications in breast cancer correlate with tumor phenotypes, prognostic factors, and patient outcome.* Cancer Res, 2009. **69**(9): p. 3802-9.
58. Qin, J., et al., *Histone Modifications and their Role in Colorectal Cancer (Review).* Pathol Oncol Res, 2020. **26**(4): p. 2023-2033.
59. Sun, W.J., et al., *Histone acetyltransferases and deacetylases: molecular and clinical implications to gastrointestinal carcinogenesis.* Acta Biochim Biophys Sin (Shanghai), 2012. **44**(1): p. 80-91.
60. Benard, A., et al., *Nuclear expression of histone deacetylases and their histone modifications predicts clinical outcome in colorectal cancer.* Histopathology, 2015. **66**(2): p. 270-82.
61. Ding, J., et al., *LSD1-mediated epigenetic modification contributes to proliferation and metastasis of colon cancer.* Br J Cancer, 2013. **109**(4): p. 994-1003.

62. Yokoyama, Y., et al., *Cancer-associated upregulation of histone H3 lysine 9 trimethylation promotes cell motility in vitro and drives tumor formation in vivo.* Cancer Sci, 2013. **104**(7): p. 889-95.
63. Nakazawa, T., et al., *Global histone modification of histone H3 in colorectal cancer and its precursor lesions.* Hum Pathol, 2012. **43**(6): p. 834-42.
64. Hsu, H.C., et al., *CBB1003, a lysine-specific demethylase 1 inhibitor, suppresses colorectal cancer cells growth through down-regulation of leucine-rich repeat-containing G-protein-coupled receptor 5 expression.* J Cancer Res Clin Oncol, 2015. **141**(1): p. 11-21.
65. Kelly, T.K., D.D. De Carvalho, and P.A. Jones, *Epigenetic modifications as therapeutic targets.* Nat Biotechnol, 2010. **28**(10): p. 1069-78.
66. Zhang, B., et al., *Targeting protein arginine methyltransferase 5 inhibits colorectal cancer growth by decreasing arginine methylation of eIF4E and FGFR3.* Oncotarget, 2015. **6**(26): p. 22799-22811.
67. Huang, T., et al., *Targeting histone methylation for colorectal cancer.* Therap Adv Gastroenterol, 2017. **10**(1): p. 114-131.
68. *Screening methods for early detection of colorectal cancers and polyps: summary of evidence-based analyses.* Ont Health Technol Assess Ser, 2009. **9**(6): p. 1-65.
69. Nagorni, A., G. Bjelakovic, and B. Petrovic, *Narrow band imaging versus conventional white light colonoscopy for the detection of colorectal polyps.* Cochrane Database Syst Rev, 2012. **1**: p. Cd008361.
70. Mehedi, I.M., et al., *Intelligent Wireless Capsule Endoscopy for the Diagnosis of Gastrointestinal Diseases.* Diagnostics (Basel), 2023. **13**(8).
71. Song, D., et al., *Application and development of noninvasive biomarkers for colorectal cancer screening: a systematic review.* Int J Surg, 2023. **109**(4): p. 925-935.
72. Doubeni, C.A., et al., *Fecal Immunochemical Test (FIT) for Colon Cancer Screening: Variable Performance with Ambient Temperature.* J Am Board Fam Med, 2016. **29**(6): p. 672-681.
73. Ahlquist, D.A., et al., *Stool DNA and occult blood testing for screen detection of colorectal neoplasia.* Ann Intern Med, 2008. **149**(7): p. 441-50, w81.
74. Housini, M., et al., *Colorectal cancer: Genetic alterations, novel biomarkers, current therapeutic strategies and clinical trials.* Gene, 2024. **892**: p. 147857.
75. Goodwin, R.A. and T.R. Asmis, *Overview of systemic therapy for colorectal cancer.* Clin Colon Rectal Surg, 2009. **22**(4): p. 251-6.
76. Van Cutsem, E., et al., *ESMO consensus guidelines for the management of patients with metastatic colorectal cancer.* Ann Oncol, 2016. **27**(8): p. 1386-422.
77. Van Cutsem, E., et al., *Cetuximab plus irinotecan, fluorouracil, and leucovorin as first-line treatment for metastatic colorectal cancer: updated analysis of overall survival according to tumor KRAS and BRAF mutation status.* J Clin Oncol, 2011. **29**(15): p. 2011-9.
78. Ganesh, K., et al., *Immunotherapy in colorectal cancer: rationale, challenges and potential.* Nat Rev Gastroenterol Hepatol, 2019. **16**(6): p. 361-375.
79. Cremolini, C., et al., *First-line therapy for mCRC — the influence of primary tumour location on the therapeutic algorithm.* Nature Reviews Clinical Oncology, 2017. **14**(2): p. 113-113.
80. Bukowski, K., M. Kciuk, and R. Kontek, *Mechanisms of Multidrug Resistance in Cancer Chemotherapy.* Int J Mol Sci, 2020. **21**(9).
81. Damia, G. and M. D'Incalci, *Mechanisms of resistance to alkylating agents.* Cytotechnology, 1998. **27**(1-3): p. 165-73.

82. Lansiaux, A., *[Antimetabolites].* Bull Cancer, 2011. **98**(11): p. 1263-74.
83. Baker, N.M., R. Rajan, and A. Mondragón, *Structural studies of type I topoisomerases.* Nucleic Acids Res, 2009. **37**(3): p. 693-701.
84. Kumar, A., et al., *Current and emerging therapeutic approaches for colorectal cancer: A comprehensive review.* World J Gastrointest Surg, 2023. **15**(4): p. 495-519.
85. Parker, W.B., *Enzymology of purine and pyrimidine antimetabolites used in the treatment of cancer.* Chem Rev, 2009. **109**(7): p. 2880-93.
86. Mehmood, R.K., *Review of Cisplatin and oxaliplatin in current immunogenic and monoclonal antibody treatments.* Oncol Rev, 2014. **8**(2): p. 256.
87. Hammond, W.A., A. Swaika, and K. Mody, *Pharmacologic resistance in colorectal cancer: a review.* Ther Adv Med Oncol, 2016. **8**(1): p. 57-84.
88. Jiménez-Fonseca, P., et al., *Gemcitabine plus capecitabine (Gem-Cape) biweekly in chemorefractory metastatic colorectal cancer.* Clin Transl Oncol, 2015. **17**(5): p. 384-92.
89. André, T., et al., *Oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment for colon cancer.* N Engl J Med, 2004. **350**(23): p. 2343-51.
90. Van Cutsem, E., et al., *Expert opinion on management of gastric and gastro-oesophageal junction adenocarcinoma on behalf of the European Organisation for Research and Treatment of Cancer (EORTC)-gastrointestinal cancer group.* Eur J Cancer, 2008. **44**(2): p. 182-94.
91. Arredondo, J., et al., *Mid-term oncologic outcome of a novel approach for locally advanced colon cancer with neoadjuvant chemotherapy and surgery.* Clin Transl Oncol, 2017. **19**(3): p. 379-385.
92. Arredondo, J., et al., *Preliminary outcome of a treatment strategy based on perioperative chemotherapy and surgery in patients with locally advanced colon cancer.* Colorectal Dis, 2013. **15**(5): p. 552-7.
93. Taieb, J., et al., *Prognostic Value of BRAF and KRAS Mutations in MSI and MSS Stage III Colon Cancer.* J Natl Cancer Inst, 2017. **109**(5).
94. *cBioPorta*. 2020]; Available from: https://www.cbioportal.org/.
95. Peter, S., et al., *Doxorubicin-Based Hybrid Compounds as Potential Anticancer Agents: A Review.* Molecules, 2022. **27**(14).
96. Asensio-López, M.C., et al., *Doxorubicin-induced oxidative stress: The protective effect of nicorandil on HL-1 cardiomyocytes.* PLoS One, 2017. **12**(2): p. e0172803.
97. Roychoudhury, S., et al., *Molecular avenues in targeted doxorubicin cancer therapy.* Future Oncol, 2020. **16**(11): p. 687-700.
98. Minotti, G., et al., *Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity.* Pharmacol Rev, 2004. **56**(2): p. 185-229.
99. Doroshow, J.H., *Effect of anthracycline antibiotics on oxygen radical formation in rat heart.* Cancer Res, 1983. **43**(2): p. 460-72.
100. Doroshow, J.H., G.Y. Locker, and C.E. Myers, *Enzymatic defenses of the mouse heart against reactive oxygen metabolites: alterations produced by doxorubicin.* J Clin Invest, 1980. **65**(1): p. 128-35.
101. Olson, R.D., et al., *Doxorubicin cardiotoxicity may be caused by its metabolite, doxorubicinol.* Proc Natl Acad Sci U S A, 1988. **85**(10): p. 3585-9.
102. Ewer, M.S. and S.M. Ewer, *Cardiotoxicity of anticancer treatments.* Nat Rev Cardiol, 2015. **12**(9): p. 547-58.
103. Hu, T., et al., *Mechanisms of drug resistance in colon cancer and its therapeutic strategies.* World J Gastroenterol, 2016. **22**(30): p. 6876-89.

104. Jung, K.A., B.H. Choi, and M.K. Kwak, *The c-MET/PI3K signaling is associated with cancer resistance to doxorubicin and photodynamic therapy by elevating BCRP/ABCG2 expression.* Mol Pharmacol, 2015. **87**(3): p. 465-76.
105. Sonowal, H., et al., *Aldose reductase inhibitor increases doxorubicin-sensitivity of colon cancer cells and decreases cardiotoxicity.* Sci Rep, 2017. **7**(1): p. 3182.
106. Weinländer, G., et al., *Treatment of advanced colorectal cancer with doxorubicin combined with two potential multidrug-resistance-reversing agents: high-dose oral tamoxifen and dexverapamil.* J Cancer Res Clin Oncol, 1997. **123**(8): p. 452-5.
107. Wang, Q., et al., *Drug Resistance in Colorectal Cancer: From Mechanism to Clinic.* Cancers (Basel), 2022. **14**(12).
108. Peters, G.J., *Chapter 1 - Drug resistance in colorectal cancer: General aspects*, in *Drug Resistance in Colorectal Cancer: Molecular Mechanisms and Therapeutic Strategies*, C.H. Cho and T. Hu, Editors. 2020, Academic Press. p. 1-33.
109. Peters, G.J., et al., *Fluctuations in phosphoribosyl pyrophosphate levels in monolayer tumor cell lines: Effects of drugs.* FEBS Letters, 1984. **170**(2): p. 277-280.
110. Reyes, P. and T.C. Hall, *Synthesis of 5-fluorouridine 5'-phosphate by a pyrimidine phosphoribosyltransferase of mammalian origin. II. Correlation between the tumor levels of the enzyme and the 5-fluorouracil-promoted increase in survival of tumor-bearing mice.* Biochem Pharmacol, 1969. **18**(10): p. 2587-90.
111. Beretta, G.L., et al., *Camptothecin resistance in cancer: insights into the molecular mechanisms of a DNA-damaging drug.* Curr Med Chem, 2013. **20**(12): p. 1541-65.
112. Cuya, S.M., M.A. Bjornsti, and R. van Waardenburg, *DNA topoisomerase-targeting chemotherapeutics: what's new?* Cancer Chemother Pharmacol, 2017. **80**(1): p. 1-14.
113. Gmeiner, W.H., *Entrapment of DNA topoisomerase-DNA complexes by nucleotide/nucleoside analogs.* Cancer Drug Resist, 2019. **2**(4): p. 994-1001.
114. Perego, P. and J. Robert, *Oxaliplatin in the era of personalized medicine: from mechanistic studies to clinical efficacy.* Cancer Chemother Pharmacol, 2016. **77**(1): p. 5-18.
115. Taniguchi, K., et al., *A human canalicular multispecific organic anion transporter (cMOAT) gene is overexpressed in cisplatin-resistant human cancer cell lines with decreased drug accumulation.* Cancer Res, 1996. **56**(18): p. 4124-9.
116. Noordhuis, P., et al., *Coexisting Molecular Determinants of Acquired Oxaliplatin Resistance in Human Colorectal and Ovarian Cancer Cell Lines.* Int J Mol Sci, 2019. **20**(15).
117. Hershberger, P.A., et al., *Cisplatin potentiates 1,25-dihydroxyvitamin D3-induced apoptosis in association with increased mitogen-activated protein kinase kinase kinase 1 (MEKK-1) expression.* Mol Cancer Ther, 2002. **1**(10): p. 821-9.
118. Shuel, S.L., *Targeted cancer therapies: Clinical pearls for primary care.* Can Fam Physician, 2022. **68**(7): p. 515-518.
119. Pabla, B., M. Bissonnette, and V.J. Konda, *Colon cancer and the epidermal growth factor receptor: Current treatment paradigms, the importance of diet, and the role of chemoprevention.* World J Clin Oncol, 2015. **6**(5): p. 133-41.
120. Wee, P. and Z. Wang, *Epidermal Growth Factor Receptor Cell Proliferation Signaling Pathways.* Cancers (Basel), 2017. **9**(5).
121. Xie, Y.-H., Y.-X. Chen, and J.-Y. Fang, *Comprehensive review of targeted therapy for colorectal cancer.* Signal Transduction and Targeted Therapy, 2020. **5**(1): p. 22.
122. Arcila, M., et al., *Detection of KRAS and BRAF mutations in colorectal carcinoma roles for high-sensitivity locked nucleic acid-PCR sequencing and broad-spectrum mass spectrometry genotyping.* J Mol Diagn, 2011. **13**(1): p. 64-73.

123. Taieb, J. and C. Gallois, *Adjuvant Chemotherapy for Stage III Colon Cancer.* Cancers (Basel), 2020. **12**(9).
124. *A Phase 1/2, Study Evaluating the Safety, Tolerability, PK, and Efficacy of AMG 510 in Subjects With Solid Tumors With a Specific KRAS Mutation*. 2018; Available from: https://clinicaltrials.gov/ct2/show/NCT03600883
125. Canon, J., et al., *The clinical KRAS(G12C) inhibitor AMG 510 drives anti-tumour immunity.* Nature, 2019. **575**(7781): p. 217-223.
126. Pardoll, D.M., *The blockade of immune checkpoints in cancer immunotherapy.* Nat Rev Cancer, 2012. **12**(4): p. 252-64.
127. Ai, L., A. Xu, and J. Xu, *Roles of PD-1/PD-L1 Pathway: Signaling, Cancer, and Beyond.* Adv Exp Med Biol, 2020. **1248**: p. 33-59.
128. Willsmore, Z.N., et al., *Combined anti-PD-1 and anti-CTLA-4 checkpoint blockade: Treatment of melanoma and immune mechanisms of action.* Eur J Immunol, 2021. **51**(3): p. 544-556.
129. Carlsen, L., K.E. Huntington, and W.S. El-Deiry, *Immunotherapy for Colorectal Cancer: Mechanisms and Predictive Biomarkers.* Cancers (Basel), 2022. **14**(4).
130. Hartung, N., et al., *Paracrine and autocrine regulation of gene expression by Wnt-inhibitor Dickkopf in wild-type and mutant hepatocytes.* BMC Syst Biol, 2017. **11**(1): p. 98.
131. Niehrs, C., *Function and biological roles of the Dickkopf family of Wnt modulators.* Oncogene, 2006. **25**(57): p. 7469-81.
132. Pandur, P., et al., *Wnt-11 activation of a non-canonical Wnt signalling pathway is required for cardiogenesis.* Nature, 2002. **418**(6898): p. 636-41.
133. Glinka, A., et al., *Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction.* Nature, 1998. **391**(6665): p. 357-62.
134. Monaghan, A.P., et al., *Dickkopf genes are co-ordinately expressed in mesodermal lineages.* Mech Dev, 1999. **87**(1-2): p. 45-56.
135. MacDonald, B.T., M. Adamska, and M.H. Meisler, *Hypomorphic expression of Dkk1 in the doubleridge mouse: dose dependence and compensatory interactions with Lrp6.* Development, 2004. **131**(11): p. 2543-52.
136. Pinho, S. and C. Niehrs, *Dkk3 is required for TGF-beta signaling during Xenopus mesoderm induction.* Differentiation, 2007. **75**(10): p. 957-67.
137. Giralt, I., et al., *Dickkopf Proteins and Their Role in Cancer: A Family of Wnt Antagonists with a Dual Role.* Pharmaceuticals (Basel), 2021. **14**(8).
138. Sheng, S.L., et al., *Clinical significance and prognostic value of serum Dickkopf-1 concentrations in patients with lung cancer.* Clin Chem, 2009. **55**(9): p. 1656-64.
139. Qiao, R., et al., *Serum dickkopf-1 as a clinical and prognostic factor in non-small cell lung cancer patients with bone metastases.* Oncotarget, 2017. **8**(45): p. 79469-79479.
140. Koppen, A., et al., *Dickkopf-1 is down-regulated by MYCN and inhibits neuroblastoma cell proliferation.* Cancer Lett, 2007. **256**(2): p. 218-28.
141. Granchi, D., et al., *Neuroblastoma and bone metastases: clinical significance and prognostic value of Dickkopf 1 plasma levels.* Bone, 2011. **48**(1): p. 152-9.
142. Shin, J.H., et al., *Dickkopf-2 regulates the stem cell marker LGR5 in colorectal cancer via HNF4α1.* iScience, 2021. **24**(5): p. 102411.
143. Fraungruber, P., et al., *G Protein-Coupled Estrogen Receptor Correlates With Dkk2 Expression and Has Prognostic Impact in Ovarian Cancer Patients.* Front Endocrinol (Lausanne), 2021. **12**: p. 564002.
144. Tsuji, T., et al., *Antiproliferative activity of REIC/Dkk-3 and its significant down-regulation in non-small-cell lung carcinomas.* Biochem Biophys Res Commun, 2001. **289**(1): p. 257-63.

145. Abarzua, F., et al., *Adenovirus-mediated overexpression of REIC/Dkk-3 selectively induces apoptosis in human prostate cancer cells through activation of c-Jun-NH2-kinase.* Cancer Res, 2005. **65**(21): p. 9617-22.
146. Tanimoto, R., et al., *REIC/Dkk-3 as a potential gene therapeutic agent against human testicular cancer.* Int J Mol Med, 2007. **19**(3): p. 363-8.
147. Sakaguchi, M., et al., *Overexpression of REIC/Dkk-3 in normal fibroblasts suppresses tumor growth via induction of interleukin-7.* J Biol Chem, 2009. **284**(21): p. 14236-44.
148. Fujii, M., et al., *Dickkopf (Dkk)-3 and β-catenin expressions increased in the transition from normal oral mucosal to oral squamous cell carcinoma.* J Mol Histol, 2011. **42**(6): p. 499-504.
149. Wang, S., H. Wei, and S. Zhang, *Dickkopf-4 is frequently overexpressed in epithelial ovarian carcinoma and promotes tumor invasion.* BMC Cancer, 2017. **17**(1): p. 455.
150. He, S., et al., *DKK4 enhances resistance to chemotherapeutics 5-Fu and YN968D1 in colorectal cancer cells.* Oncol Lett, 2017. **13**(2): p. 587-592.
151. Yang, X., et al., *DKK4-knockdown enhances chemosensitivity of A549/DTX cells to docetaxel.* Acta Biochim Biophys Sin (Shanghai), 2017. **49**(10): p. 899-906.
152. Zhu, G., et al., *Expression and Role of Dickkopf-1 (Dkk1) in Tumors: From the Cells to the Patients.* Cancer Manag Res, 2021. **13**: p. 659-675.
153. Aguilera, O., et al., *Epigenetic inactivation of the Wnt antagonist DICKKOPF-1 (DKK-1) gene in human colorectal cancer.* Oncogene, 2006. **25**(29): p. 4116-21.
154. Kikuchi, A., S. Matsumoto, and R. Sada, *Dickkopf signaling, beyond Wnt-mediated biology.* Seminars in Cell & Developmental Biology, 2022. **125**: p. 55-65.
155. Aguilera, O., et al., *The Wnt antagonist DICKKOPF-1 gene is induced by 1alpha,25-dihydroxyvitamin D3 associated to the differentiation of human colon cancer cells.* Carcinogenesis, 2007. **28**(9): p. 1877-84.
156. Xiao, Q., et al., *DKK2 imparts tumor immunity evasion through β-catenin-independent suppression of cytotoxic immune-cell activation.* Nat Med, 2018. **24**(3): p. 262-270.
157. Yang, Z.R., et al., *Overexpression of Dickkopf-3 induces apoptosis through mitochondrial pathway in human colon cancer.* World J Gastroenterol, 2012. **18**(14): p. 1590-601.
158. Tsukui, Y., et al., *Dickkopf-4 gene expression is associated with differentiation and lymph node metastasis in colorectal cancer.* JGH Open, 2019. **3**(5): p. 409-416.
159. Pendás-Franco, N., et al., *DICKKOPF-4 is induced by TCF/β-catenin and upregulated in human colon cancer, promotes tumour cell invasion and angiogenesis and is repressed by 1α,25-dihydroxyvitamin D3.* Oncogene, 2008. **27**(32): p. 4467-4477.
160. Aguilera, Ó., et al., *Nuclear DICKKOPF-1 as a biomarker of chemoresistance and poor clinical outcome in colorectal cancer.* Oncotarget, 2015. **6**(8): p. 5903-17.
161. Zitt, M., et al., *Dickkopf-3 as a new potential marker for neoangiogenesis in colorectal cancer: expression in cancer tissue and adjacent non-cancerous tissue.* Dis Markers, 2008. **24**(2): p. 101-9.
162. Safari, E., G. Mosayebi, and S. Khorram, *Dkk-3 as a potential biomarker for diagnosis and prognosis of colorectal cancer.* Med J Islam Repub Iran, 2018. **32**: p. 86.
163. González-Sancho, J.M., et al., *The Wnt antagonist DICKKOPF-1 gene is a downstream target of beta-catenin/TCF and is downregulated in human colon cancer.* Oncogene, 2005. **24**(6): p. 1098-103.

164. Pendás-Franco, N., et al., *DICKKOPF-4 is induced by TCF/beta-catenin and upregulated in human colon cancer, promotes tumour cell invasion and angiogenesis and is repressed by 1alpha,25-dihydroxyvitamin D3.* Oncogene, 2008. **27**(32): p. 4467-77.
165. Sikandar, S., et al., *The class I HDAC inhibitor MGCD0103 induces cell cycle arrest and apoptosis in colon cancer initiating cells by upregulating Dickkopf-1 and non-canonical Wnt signaling.* Oncotarget, 2010. **1**(7): p. 596-605.
166. Neri, F., et al., *TET1 is a tumour suppressor that inhibits colon cancer growth by derepressing inhibitors of the WNT pathway.* Oncogene, 2015. **34**(32): p. 4168-76.
167. Wang, W., et al., *miR-410 acts as an oncogene in colorectal cancer cells by targeting dickkopf-related protein 1 via the Wnt/β-catenin signaling pathway.* Oncol Lett, 2019. **17**(1): p. 807-814.
168. Yamada, N., et al., *Role of Intracellular and Extracellular MicroRNA-92a in Colorectal Cancer.* Transl Oncol, 2013. **6**(4): p. 482-92.
169. Shailesh, H., K.S. Siveen, and S. Sif, *Protein arginine methyltransferase 5 (PRMT5) activates WNT/β-catenin signalling in breast cancer cells via epigenetic silencing of DKK1 and DKK3.* J Cell Mol Med, 2021. **25**(3): p. 1583-1600.
170. Yang, Y. and M.T. Bedford, *Protein arginine methyltransferases and cancer.* Nat Rev Cancer, 2013. **13**(1): p. 37-50.
171. Tewary, S.K., Y.G. Zheng, and M.-C. Ho, *Protein arginine methyltransferases: insights into the enzyme structure and mechanism at the atomic level.* Cellular and molecular life sciences : CMLS, 2019. **76**(15): p. 2917-2932.
172. Al-Hamashi, A.A., K. Diaz, and R. Huang, *Non-Histone Arginine Methylation by Protein Arginine Methyltransferases.* Curr Protein Pept Sci, 2020. **21**(7): p. 699-712.
173. Sun, L., et al., *Structural insights into protein arginine symmetric dimethylation by PRMT5.* Proceedings of the National Academy of Sciences of the United States of America, 2011. **108**(51): p. 20538-20543.
174. Antonysamy, S., et al., *Crystal structure of the human PRMT5:MEP50 complex.* Proc Natl Acad Sci U S A, 2012. **109**(44): p. 17960-5.
175. Cheng, X., R.E. Collins, and X. Zhang, *Structural and sequence motifs of protein (histone) methylation enzymes.* Annual review of biophysics and biomolecular structure, 2005. **34**: p. 267-294.
176. Rho, J., et al., *Prmt5, which forms distinct homo-oligomers, is a member of the protein-arginine methyltransferase family.* The Journal of biological chemistry, 2001. **276**(14): p. 11393-401.
177. Stopa, N., J.E. Krebs, and D. Shechter, *The PRMT5 arginine methyltransferase: many roles in development, cancer and beyond.* Cellular and molecular life sciences : CMLS, 2015. **72**(11): p. 2041-59.
178. Shailesh, H., et al., *Protein arginine methyltransferase 5 (PRMT5) dysregulation in cancer.* Oncotarget, 2018. **9**(94): p. 36705-36718.
179. Muhammad, J.S., et al., *Estrogen-induced epigenetic silencing of FTH1 and TFRC genes reduces liver cancer cell growth and survival.* Epigenetics, 2020. **15**(12): p. 1302-1318.
180. Kim, H. and Z.A. Ronai, *PRMT5 function and targeting in cancer.* Cell Stress, 2020. **4**(8): p. 199-215.
181. Hu, D., et al., *Interplay between arginine methylation and ubiquitylation regulates KLF4-mediated genome stability and carcinogenesis.* Nat Commun, 2015. **6**: p. 8419.
182. Xiao, W., et al., *Role of protein arginine methyltransferase 5 in human cancers.* Biomed Pharmacother, 2019. **114**: p. 108790.

183. Pal, S., et al., *Human SWI/SNF-associated PRMT5 methylates histone H3 arginine 8 and negatively regulates expression of ST7 and NM23 tumor suppressor genes.* Mol Cell Biol, 2004. **24**(21): p. 9630-45.
184. Dastagir, K., et al., *Murine embryonic fibroblast cell lines differentiate into three mesenchymal lineages to different extents: new models to investigate differentiation processes.* Cell Reprogram, 2014. **16**(4): p. 241-52.
185. Wang, Q., et al., *Identification of a Novel Protein Arginine Methyltransferase 5 Inhibitor in Non-small Cell Lung Cancer by Structure-Based Virtual Screening.* Front Pharmacol, 2018. **9**(173).
186. *An Open-label, Dose Escalation Study to Investigate the Safety, Pharmacokinetics, Pharmacodynamics and Clinical Activity of GSK3326595 in Participants With Solid Tumors and Non-Hodgkin's Lymphoma.* https://ClinicalTrials.gov/show/NCT02783300.
187. *Study to Investigate the Safety and Clinical Activity of GSK3326595 and Other Agents to Treat Myelodysplastic Syndrome (MDS) and Acute Myeloid Leukemia (AML).* https://ClinicalTrials.gov/show/NCT03614728.
188. AbuHammad, S., et al., *Regulation of PRMT5-MDM4 axis is critical in the response to CDK4/6 inhibitors in melanoma.* Proc Natl Acad Sci U S A, 2019. **116**(36): p. 17990-18000.
189. Mueller, H.S., et al., *Acquired resistance to PRMT5 inhibition induces concomitant collateral sensitivity to paclitaxel.* Proc Natl Acad Sci U S A, 2021. **118**(34).
190. Musa, J., et al., *Eukaryotic initiation factor 4E-binding protein 1 (4E-BP1): a master regulator of mRNA translation involved in tumorigenesis.* Oncogene, 2016. **35**(36): p. 4675-4688.
191. Kang, S., et al., *FGFR3 activates RSK2 to mediate hematopoietic transformation through tyrosine phosphorylation of RSK2 and activation of the MEK/ERK pathway.* Cancer Cell, 2007. **12**(3): p. 201-14.
192. Hafner, C., et al., *FGFR3 mutation affects cell growth, apoptosis and attachment in keratinocytes.* Exp Cell Res, 2010. **316**(12): p. 2008-16.
193. Yang, L., et al., *PRMT5 functionally associates with EZH2 to promote colorectal cancer progression through epigenetically repressing CDKN2B expression.* Theranostics, 2021. **11**(8): p. 3742-3759.
194. Hansen, K.H., et al., *A model for transmission of the H3K27me3 epigenetic mark.* Nature Cell Biology, 2008. **10**(11): p. 1291-1300.
195. Cao, R., et al., *Role of histone H3 lysine 27 methylation in Polycomb-group silencing.* Science, 2002. **298**(5595): p. 1039-43.
196. Qi, H., et al., *Sirtuin 7-mediated deacetylation of WD repeat domain 77 (WDR77) suppresses cancer cell growth by reducing WDR77/PRMT5 transmethylase complex activity.* The Journal of biological chemistry, 2018. **293**(46): p. 17769-17779.
197. Lim, Y., et al., *A Comprehensive Analysis of Symmetric Arginine Dimethylation in Colorectal Cancer Tissues Using Immunoaffinity Enrichment and Mass Spectrometry.* Proteomics, 2020: p. e1900367.
198. Abumustafa, W., et al., *Protein arginine N-methyltransferase 5 in colorectal carcinoma: Insights into mechanisms of pathogenesis and therapeutic strategies.* Biomedicine & Pharmacotherapy, 2022. **145**: p. 112368.
199. Zhang, H.T., et al., *Transcriptional activation of PRMT5 by NF-Y is required for cell growth and negatively regulated by the PKC/c-Fos signaling in prostate cancer cells.* Biochim Biophys Acta, 2014. **1839**(11): p. 1330-40.
200. Koh, C.M., et al., *MYC regulates the core pre-mRNA splicing machinery as an essential step in lymphomagenesis.* Nature, 2015. **523**(7558): p. 96-100.

201. Zhu, F., et al., *PRMT5 is upregulated by B-cell receptor signaling and forms a positive-feedback loop with PI3K/AKT in lymphoma cells.* Leukemia, 2019. **33**(12): p. 2898-2911.
202. Demetriadou, C., et al., *NAA40 contributes to colorectal cancer growth by controlling PRMT5 expression.* Cell death & disease, 2019. **10**(3): p. 019-1487.
203. Hartley, A.V., et al., *Regulation of a PRMT5/NF-κB Axis by Phosphorylation of PRMT5 at Serine 15 in Colorectal Cancer.* Int J Mol Sci, 2020. **21**(10).
204. Murray, N.R., et al., *Protein kinase Ciota is required for Ras transformation and colon carcinogenesis in vivo.* The Journal of cell biology, 2004. **164**(6): p. 797-802.
205. Hwang, J.W., et al., *Protein arginine methyltransferases: promising targets for cancer therapy.* Exp Mol Med, 2021. **53**(5): p. 788-808.
206. Castellano, S., et al., *Design, synthesis and biological evaluation of carboxy analogues of arginine methyltransferase inhibitor 1 (AMI-1).* ChemMedChem, 2010. **5**(3): p. 398-414.
207. Chan-Penebre, E., et al., *A selective inhibitor of PRMT5 with in vivo and in vitro potency in MCL models.* Nat Chem Biol, 2015. **11**(6): p. 432-7.
208. Duncan, K.W., et al., *Structure and Property Guided Design in the Identification of PRMT5 Tool Compound EPZ015666.* ACS Med Chem Lett, 2016. **7**(2): p. 162-6.
209. Shifteh, D., et al., *Protein Arginine Methyltransferase 5 as a Therapeutic Target for KRAS Mutated Colorectal Cancer.* Cancers, 2020. **12**(8).
210. Li, W.J., et al., *Profiling PRMT methylome reveals roles of hnRNPA1 arginine methylation in RNA splicing and cell growth.* Nat Commun, 2021. **12**(1): p. 1946.
211. Prabhu, L., et al., *Development of an AlphaLISA high throughput technique to screen for small molecule inhibitors targeting protein arginine methyltransferases.* Molecular bioSystems, 2017. **13**(12): p. 2509-2520.
212. Prabhu, L., et al., *Adapting AlphaLISA high throughput screen to discover a novel small-molecule inhibitor targeting protein arginine methyltransferase 5 in pancreatic and colorectal cancers.* Oncotarget, 2017. **8**(25): p. 39963-39977.
213. Alinari, L., et al., *Selective inhibition of protein arginine methyltransferase 5 blocks initiation and maintenance of B-cell transformation.* Blood, 2015. **125**(16): p. 2530-2543.
214. Banasavadi-Siddegowda, Y.K., et al., *PRMT5 as a druggable target for glioblastoma therapy.* Neuro Oncol, 2018. **20**(6): p. 753-763.
215. Chandrashekar, D.S., et al., *UALCAN: A Portal for Facilitating Tumor Subgroup Gene Expression and Survival Analyses.* Neoplasia, 2017. **19**(8): p. 649-658.
216. Chandrashekar, D.S., et al., *UALCAN: An update to the integrated cancer data analysis platform.* Neoplasia, 2022. **25**: p. 18-27.
217. Cerami, E., et al., *The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data.* Cancer Discov, 2012. **2**(5): p. 401-4.
218. de Bruijn, I., et al., *Analysis and Visualization of Longitudinal Genomic and Clinical Data from the AACR Project GENIE Biopharma Collaborative in cBioPortal.* Cancer Res, 2023.
219. Gao, J., et al., *Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal.* Sci Signal, 2013. **6**(269): p. pl1.
220. Li, T., et al., *TIMER: A Web Server for Comprehensive Analysis of Tumor-Infiltrating Immune Cells.* Cancer Res, 2017. **77**(21): p. e108-e110.
221. Li, T., et al., *TIMER2.0 for analysis of tumor-infiltrating immune cells.* Nucleic Acids Res, 2020. **48**(W1): p. W509-w514.
222. Edkins, S., et al., *Recurrent KRAS codon 146 mutations in human colorectal cancer.* Cancer Biol Ther, 2006. **5**(8): p. 928-32.

223. Kikuchi, A., K. Fumoto, and H. Kimura, *The Dickkopf1-cytoskeleton-associated protein 4 axis creates a novel signalling pathway and may represent a molecular target for cancer therapy.* Br J Pharmacol, 2017. **174**(24): p. 4651-4665.
224. Molinari, C., et al., *Heterogeneity in Colorectal Cancer: A Challenge for Personalized Medicine?* International journal of molecular sciences, 2018. **19**(12): p. 3733.
225. Zhu, G., et al., *Role of oncogenic KRAS in the prognosis, diagnosis and treatment of colorectal cancer.* Molecular Cancer, 2021. **20**(1): p. 143.
226. Sato, H., et al., *Frequent epigenetic inactivation of DICKKOPF family genes in human gastrointestinal tumors.* Carcinogenesis, 2007. **28**(12): p. 2459-66.
227. Rascio, F., et al., *The Pathogenic Role of PI3K/AKT Pathway in Cancer Onset and Drug Resistance: An Updated Review.* Cancers (Basel), 2021. **13**(16).
228. Narayanankutty, A., *PI3K/ Akt/ mTOR Pathway as a Therapeutic Target for Colorectal Cancer: A Review of Preclinical and Clinical Evidence.* Curr Drug Targets, 2019. **20**(12): p. 1217-1226.
229. Ebi, H., et al., *Receptor tyrosine kinases exert dominant control over PI3K signaling in human KRAS mutant colorectal cancers.* J Clin Invest, 2011. **121**(11): p. 4311-21.
230. Yan, Y., et al., *PRMT5 regulates colorectal cancer cell growth and EMT via EGFR/Akt/GSK3β signaling cascades.* Aging (Albany NY), 2021. **13**(3): p. 4468-4481.
231. Sapir, T., et al., *Protein Arginine Methyltransferase 5 (PRMT5) and the ERK1/2 & PI3K Pathways: A Case for PRMT5 Inhibition and Combination Therapies in Cancer.* Mol Cancer Res, 2021. **19**(3): p. 388-394.
232. Kimura, H., et al., *CKAP4 is a Dickkopf1 receptor and is involved in tumor progression.* J Clin Invest, 2016. **126**(7): p. 2689-705.
233. Litzler, L.C., et al., *PRMT5 is essential for B cell development and germinal center dynamics.* Nat Commun, 2019. **10**(1): p. 22.
234. Wang, L., S. Pal, and S. Sif, *Protein arginine methyltransferase 5 suppresses the transcription of the RB family of tumor suppressors in leukemia and lymphoma cells.* Mol Cell Biol, 2008. **28**(20): p. 6262-77.
235. Thorn, C.F., et al., *Doxorubicin pathways: pharmacodynamics and adverse effects.* Pharmacogenet Genomics, 2011. **21**(7): p. 440-6.
236. O'Brien, S., et al., *Inhibiting PRMT5 induces DNA damage and increases anti-proliferative activity of Niraparib, a PARP inhibitor, in models of breast and ovarian cancer.* BMC Cancer, 2023. **23**(1): p. 775.
237. De Angelis, P.M., et al., *Cellular response to 5-fluorouracil (5-FU) in 5-FU-resistant colon cancer cell lines during treatment and recovery.* Mol Cancer, 2006. **5**: p. 20.
238. Li, X., et al., *Differential responses to doxorubicin-induced phosphorylation and activation of Akt in human breast cancer cells.* Breast Cancer Res, 2005. **7**(5): p. R589-97.
239. Seo, S.H., et al., *Inhibition of Dickkopf-1 enhances the anti-tumor efficacy of sorafenib via inhibition of the PI3K/Akt and Wnt/β-catenin pathways in hepatocellular carcinoma.* Cell Communication and Signaling, 2023. **21**(1): p. 339.
240. *The Human Protein Atlas*. [cited 2024; Available from: https://www.proteinatlas.org/.
241. Bourhis, E., et al., *Reconstitution of a frizzled8.Wnt3a.LRP6 signaling complex reveals multiple Wnt and Dkk1 binding sites on LRP6.* J Biol Chem, 2010. **285**(12): p. 9172-9.
242. Ahn, V.E., et al., *Structural basis of Wnt signaling inhibition by Dickkopf binding to LRP5/6.* Dev Cell, 2011. **21**(5): p. 862-73.

243. Sparks, A.B., et al., *Mutational analysis of the APC/beta-catenin/Tcf pathway in colorectal cancer.* Cancer Res, 1998. **58**(6): p. 1130-4.
244. Kaler, P., L. Augenlicht, and L. Klampfer, *Activating mutations in β-catenin in colon cancer cells alter their interaction with macrophages; the role of snail.* PLoS One, 2012. **7**(9): p. e45462.
245. Maher, M.T., et al., *Beta-catenin phosphorylated at serine 45 is spatially uncoupled from beta-catenin phosphorylated in the GSK3 domain: implications for signaling.* PLoS One, 2010. **5**(4): p. e10184.

Author’s Biographical Sketch

Wafaa M. S. Abumustafa, a Ph.D. student in the molecular medicine and translational program at, the University of Sharjah since 2020 and at the Luebeck-Sharjah dual Ph.D. program in Molecular Medicine since 2021. During my Ph.D., I published a scientific review in the biomedicine and pharmacotherapy journal impact factor 7.5. Furthermore, I participated in three publications. I completed my master’s degree in molecular medicine and translational research at the University of Sharjah in 2020. I completed my Bachelor's degree in biotechnology in 2018 with an excellent GPA score from the American University of Ras Al Khaimah. I have experience as a research assistant for 6 years at the University of Sharjah.

Publications

- Abi Zamer B, Rah B, Jayakumar MN, **Abumustafa W**, Hamad M, Muhammad JS. DNA methylation-mediated epigenetic regulation of oncogenic RPS2 as a novel therapeutic target and biomarker in hepatocellular carcinoma. Biochem Biophys Res Commun. 2023 Dec 28;696:149453. doi: 10.1016/j.bbrc.2023.149453. Epub ahead of print. PMID: 38181486.**Abumustafa, W**., Zamer, B. A., Khalil, B. A., Hamad, M., Maghazachi, A. A., & Muhammad, J. S. (2022). Protein arginine N-methyltransferase 5 in colorectal carcinoma: Insights into mechanisms of pathogenesis and therapeutic strategies. Biomedicine & Pharmacotherapy, 145, 112368. doi: https://doi.org/10.1016/j.biopha.2021.112368Abi Zamer B, **Abumustafa W**, Hamad M, Maghazachi AA, Muhammad JS*. “Genetic Mutations and Non-Coding RNA-Based

Epigenetic Alterations Mediating the Warburg Effect in Colorectal Carcinogenesis". Biology 2021: 10(9): 847. https://doi.org/10.3390/biology10090847.

- **Wafaa Abumustafa,** Darko Castven, Diana Becker, Shahenaz Shaban Salih, Shaista Manzoor, Batoul Abi Zamer, Iman Talaat, Mawieh Hamad, Jens Uwe Marquardt, Jibran Sualeh Muhammad, "Uncovering the Role of Protein Arginine N-Methyltransferase in Hepatocellular Carcinoma: Insights into HIF1α Signaling and Ras-Related Nuclear Protein as Promising Biomarker" submitted.
- **Wafaa Abumustafa,** Darko Castven, Diana Becker, Shahenaz Shaban Salih, Shaista Manzoor, Batoul Abi Zamer, Iman Talaat, Mawieh Hamad, Jens Uwe Marquardt, Jibran Sualeh Muhammad, "Inhibition of PRMT5-mediated regulation of DKK1 sensitizes colorectal cancer cells to chemotherapy" in progress.

Honors and Awards

- Best abstract (1st place) in the category of youth-university students, the 5th Combined Gulf Cancer Conference Title: "Protein arginine N-methyltransferase 5 (PRMT5) inhibitors sensitize colorectal cancer cell lines to doxorubicin" **Wafaa Abumustafa**, Azzam A. Maghazachi, Mawieh Hamad, Jens Uwe Marquardt, Jibran Sualeh Muhammad. 23rd Nov 2022, Sharjah https://www.focp.ae/our-programs/the-5th-combined-gulf-cancer-conference-2022/
- 3rd place award for poster presentations at the 9th Emirati-German Congress in Medicine and Dental Medicine Title: The role of protein arginine N-methyltransferase 5 (PRMT5) as an epigenetic modulator in colorectal carcinoma **Wafaa Abumustafa**, Mawieh Hamad, Azzam A. Maghazachi, Jens Uwe Marquardt, Jibran Sualeh Muhammad. 22nd Nov 2022, Sharjah https://www.sharjah.ac.ae/en/Media/Conferences/9egcm/Pages/default.aspx

- The highest CGPA, 4th year BS in biotechnology at American University of Ras Al Khaimah Ras Al Khaimah
- The highest CGPA, 3rd year BS in biotechnology at American University of Ras Al Khaimah Ras Al Khaimah
- The highest CGPA, 2nd year BS in biotechnology at American University of Ras Al Khaimah Ras Al Khaimah

Participation in scientific and social events

- Oral presentation in “Fight colon cancer” event at the College of Medicine, University of Sharjah
- Organized “A Gateway to Research” event at the College of Medicine, University of Sharjah
- Participated in cervical cancer awareness day event organized by Abdullah bin Omran Hospital for Obstetrics and Gynecology in Ras Al Khaimah
- Joined research promotion committee at the Research Institute of Medical & Health Sciences (RIMHS) at the University of Sharjah
- Attended Joint Annual Meeting German Society for Gastroenterology, Digestive and metabolic diseases (DGVS) in Hamburg, Germany
- Poster presentation in the 3rd Forum for Women in Research "Quwa: Women Empowerment for Global Impact"
- Attended A journey of discovery into the CRISPR-Cas genome engineering technology event, Lecture, and Q&A with Nobel Prize laureate Emmanuelle Charpentier
- Poster presentation at the 8th Emirati-German conference
- Teaching assistant in The National Ambassadors Program Estedad 2021 "Epigenetics in Cancer Development"
- certificate in Central Labs Online Safety Training University of Sharjah

- Master thesis defense presentation “Investigation of APOBEC gene family mutations and their implication in the heterogeneity of colorectal and breast cancer” at the University of Sharjah
- Oral presentation for the senior project “Investigating bacteria and carbohydrates in different brands of cow milk in UAE” at the American University of Ras Al Khaimah
- Participated in world autism awareness day with Ras Al Khaimah autism center poster presentation at the think science fair in Dubai Title: plants as a cancer therapy

Declaration

I hereby declare that the work presented in this thesis has not been submitted for any other degree or professional qualification and that it is the result of my own independent work.

I also declare that there is no conflict of interest.

Any figures or tables that have been published before should be reproduced or get copyright.

I declare that based on the similarity check the total similarity is not more than 15%, excluding the bibliography (references) and quotes

Wafaa M. S. Abumustafa

Full Name Goes Here (Candidate)

15/Jan/2024

Date

Acknowledgments

My deep appreciation to my supervisor Dr. Jibran Sualeh Muhammad for his limitless support and for expanding my knowledge in a lot of aspects. Also, I would like to express my gratitude to my co-supervisor Prof. Mawieh Hamad for directing me to the right path with his valuable advice. I would also like to acknowledge with much appreciation my supervisor from the University of Lübeck Prof. Jens Marquardt for his boundless guidance and support. I would like to extend my sincere gratitude to my co-supervisor Hauke Busch and my mentor from Lübeck University Dr. Darko Castven for his continued support and help in NGS analysis. My appreciation also goes to Ms. Monika Herr and all Prof. Jens Marquardt group members for their continuous help and support. of My gratitude also goes to Dr. Iman Talaat for providing colorectal cancer biopsies and pathological scoring thanks to Ms. Shahenaz Nour for her help in immunohistochemistry. My acknowledgement goes to Prof. Rafat El-Awady for providing antibodies. My thanks to Dr. Noha Mousaad Elemam for helping with orders and providing lab materials. My gratitude also goes to Dr. Shaista Manzoor and Ms. Jasmin Shafarin for their help in molecular biology techniques.

In addition, I would like to thank His Highness Sheikh Doctor Sultan Bin Mohammad Al-Qassimi for granting me a scholarship to do my Ph.D. in Molecular medicine and translational research, and for his continuous support to the youth and encouragement towards gaining knowledge. I would also like to thank Prof. Qutayba Hamid the dean of the College of Medicine for hosting the Ph.D. program and Prof. Eman Abu-Gharbieh for coordinating the program. My gratitude goes to Prof. Rafat El-Awady the dean of the college of graduate Studies for accommodating our needs. My acknowledgement goes to Prof. Rabih Halwani the director of the research institute of medical & health sciences for providing lab facilities. My gratitude also goes to Prof. Jennifer Hundt, Prof. Hauke Busch and Dr. Marieke Hoehn for organizing Lübeck-Sharjah Dual Degree Program and their continunse support.

My appreciation also goes to my friends and colleagues and the department faculty and staff for making my time at the University of Sharjah a great experience. Finally, my heartfelt gratitude is extended to my parents for their encouragement.

- **Ethical approval**

To use the Colorectal cancer paraffin-embedded tissue blocks we received ethical approval from the University of Sharjah research ethics committee on 9th Jan 2024. Reference number: REC-23-11-30-01-PG. Title of the research: Inhibition of PRMT5-mediated regulation of DKK1 sensitizes colorectal cancer cells to chemotherapy