博士論文

Identification of FERM domain-containing protein 5 (FRMD5) as a novel target of β-catenin/TCF7L2 complex

(β-カテニン/ TCF7L2 複合体の新規標的遺伝子 FERM domain-containing protein 5 (FRMD5) の同定)

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Doctoral Dissertation

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β-カテニン/ TCF7L2 複合体の新規標的遺伝子 FERM domain-containing protein 5 (FRMD5) の同定

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Context

1. Abbreviation

APC: adenomatous polyposis coli

ChIP: chromatin immunoprecipitation

CCND1: cyclin D1

cMYC: v-myc avian myelocytomatosis viral oncogene homolog

CRC: colorectal cancer

ECM: extracellular matrix

EMT: epithelial-mesenchymal transition

FERM: four-point-one, ezrin, radixin, moesin domain

LEF: lymphoid enhancer factor

MAPK: mitogen-activated protein kinase

PI3K: phosphoinositide 3-kinase

TBE: TCF-binding element

TCF: T-cell factor

2. Abstract

Wnt signal pathway is highly conserved in evolution and plays an essential role in organ development. Molecular studies have unveiled that aberrant activation of Wnt signal is involved in human tumorigenesis. A number of genes regulated by β-catenin/TCF complex, a key transcriptional complex in the canonical Wnt signaling pathway, have been identified. Although the functional roles of several Wnt target genes such as *cMYC* and *Cyclin D1* have been already well characterized, there remain a set of the target genes whose role in tumorigenesis has not been clarified. To elucidate the precise molecular mechanisms in colorectal carcinogenesis, I searched genes regulated by β-catenin/TCF in HCT116, SW480 and LS174T colorectal cancer cells. Expression profiles of the three cell lines treated with control siRNA, β-catenin siRNAs or the dominant-negative form of TCF7L2 and the data of TCF7L2 ChIP-sequence identified FERM domain-containing protein 5 (FRMD5) as a novel target of β-catenin/TCF7L2. Furthermore, a reporter assay disclosed that a region in intron 1 transcriptionally regulated the expression of *FRMD5*. ChIP assay also corroborated that TCF7L2 associates with this region. These data suggested that FRMD5 is a novel direct target of the β-catenin/TCF7L2 complex. In addition, analysis of expression profile of HCT116 treated with FRMD5 siRNA revealed that its expression is associated with cell cycle

and regulation of extracellular matrix. These data may be helpful for the profound understanding of human carcinogenesis with abrogated Wnt signaling.

3. Introduction

Human colorectal carcinoma (CRC) is a major cause of mortality and morbidity worldwide. Several critical pathways including Wnt, RAS-MAPK, PI3K, and DNA mismatch-repair pathway have been proved being involved in the initiation and progression of CRC[1]. Among these pathways, aberrant activation of Wnt signaling is frequently observed in CRC, suggesting a critical event in colorectal carcinogenesis.

Wnt/β-catenin signaling, also known as canonical Wnt signaling, is a highly conserved pathway, and controls embryonic development and adult stem cell renewal through regulating the expression of downstream target genes. It has been shown that intestinal epithelium cells have high turnover rate and that Wnt/β-catenin signaling contributes to the tissue homeostasis through regulating the proliferation of intestinal stem cells[2]. Activity of Wnt signaling is strictly regulated at many levels. In normal condition, the expression of β-catenin, a key component of canonical Wnt signaling, is repressed by destruction complex consisting of APC, GSK-3β, AXIN1/2 and other factors. Upon the binding of Wnt ligands with Frizzled receptors, β-catenin is stabilized by the suppression of its phosphorylation, is released from destruction complex, and translocates into the nucleus. In the nucleus, β-catenin activates the transcriptional complex containing T-cell factor/lymphoid enhancer factor (TCF/LEF), which results in

the induced expression of target genes[3]. In CRC, mutations in the components of destruction complex or in the *β-catenin* gene itself lead to abnormal accumulation of β-catenin in the nucleus, and thereby overexpression of downstream target genes. Although previous studies have identified a number of Wnt target genes such as *cMYC* and *CCND1* that are associated with the proliferation of tumor cells[4,5], the entire downstream players have not been clarified. To understand comprehensive role of Wnt signaling in the development and progression of CRC, I have challenged in this study to identify novel target genes of Wnt/β-catenin signaling.

Four-point-one, ezrin, radixin, moesin (FERM) domains are present in a variety of proteins that have widely range of biological functions. Human genome encodes more than 30 FERM domain-containing proteins which include kindlin, talin, ezrin, radixin, moesin, protein-tyrosine phosphatase 1E (PTPE1), focal adhesion kinase (FAK), Janus kinases (JAKs), myosins (MYO7, MYO10, and MYO15), and FRMD proteins[6]. These proteins are classified by the similarity of FERM domains into three groups comprising, (i) kindlin and talin, (ii) ezrin-radixin-moesin (ERM) proteins, guanine nucleotide exchange factors (GEFs), kinases and phosphatases, and (iii) myosin and Krev interaction trapped (KRIT) proteins[6] involved in protein-protein or protein-lipid interactions. Among these proteins, ERM proteins are components that play an

important role in the regulated interaction between membrane protein and the cortical cytoskeleton, and signal transduction pathways[7]. FRMD proteins, members of FERM domain-containing proteins, are categorized into the group of ERM proteins, GEFs, kinases and phosphatases, according to the structural homology of their FERM domains. FRMD proteins include FRMD2, FRMDPD2, FRMD3, FRMD4A, FRMD4B, FRMD5, FRMD6 and FRMD7[10]. Among these members, it is reported that FRMD3 represses clonogenicity and promote apoptosis in HEK293, Hela, and A549 cells[11]. FRMD5 was reported to repress migration[12] and motility[13] of lung cancer cells. In addition, subsequent knockdown of FRMD5 showed increased tumor growth in mouse xenograft model of H1299[12]. These studies indicate that FRMD3 and FRMD5 play an important role in tumorigenesis.

In this study, I identified FRMD5 as a novel target of Wnt signaling. I show here the transcriptional regulation of FRMD5 as a downstream of β-catenin/TCF7L2 complex, its expression in colorectal cancer tissues, the association of its expression with clinical data, and its biological function in colon cancer cells. These data will be helpful for the comprehensive understanding of Wnt signaling in colonic epithelial cells and mechanisms underlying colorectal tumors.

4. Materials and methods

4.1 Cell culture

Human CRC cell lines, HCT116, HCT-15, SW480, DLD-1, LoVo, Caco-2, LS174T, and HT-29 were purchased from the American Type Culture Collection (Manassas, VA). All cells were grown in appropriate media (McCoy's 5a Medium Modified for HCT116 and HT-29; RPMI-1640 for HCT15 and DLD-1; Leibovitz's L-15 for SW480, F-12K for LoVo; EMEM for Caco-2 and LS174T) supplemented with 10% FBS (ThermoFisher, Waltham, MA), and antibiotic/antimycotic solution (Sigma, St. Louis, MO).

4.2 Genome-wide gene expression analysis and real-time PCR

Human *CTNNB1*-specific siRNA (ON-TARGETplus SMARTpool siRNA, L-003482-00) and control siRNA (ON-TARGETplus Non-targeting Pool #D-001810-10) were purchased (GE Dharmacon, Lafayette, CO). HCT116 and SW480 cells that show transactivated β-catenin/TCF7L2 complex due to either mutation in β-*catenin* or *APC*, respectively, were treated with the pooled β-catenin siRNA or control siRNA using Lipofectamine RNAiMAX (Life Technologies). RNA was extracted from the cells using RNeasy Plus mini Kit (Qiagen, Venlo, Niederlande), and subsequently

expression profiles were analyzed by SurePrint G3 Unrestricted Gene Expression 8x60K microarray (Agilent Technologies, Santa Clara, CA) according to the manufacturer's protocol. For validation, real-time PCR was performed using qPCR Kapa SYBR Fast ABI Prism™ Kit (Kapa Biosystems, Wilmington, MA) on StepOnePlus (ThermoFisher). cDNA was synthesized using Transcriptor First Strand cDNA Synthesis Kit (Roche diagnostics, Penzberg, Upper Bavaria, Germany), using RNA extracted from both HCT116 and SW480 cells treated with β-catenin siRNA (siβ-catenin #9 sense: 5'-GAUCCUAGCUAUCGUUCUU-3'; siβ-catenin #10 sense: 5'-UAAUGAGGACCUAUACUUA-3') or control siRNA. Primer sets used for the PCR are shown in Table S1.

For the identification of genes regulated by FRMD5, HCT116 cells treated with two different FRMD5 siRNAs (siFRMD5 #2 sense: 5'-CUUACAUCCUUCAAGCGGA-3', siFRMD5 #3 sense: 5'-GACAGAUAGCAAUGAGCGA-3') or control siRNA (ON-TARGETplus Non-targeting Pool #D-001810-10, GE Dharmacon). RNA extracted from the cells was used for the gene expression profile analysis with SurePrint G3 Unrestricted Gene Expression 8x60K microarray (Agilent Technologies). For the validation of microarray data, real-time PCR was performed with a set of primers shown in Table S1. Gene set

enrichment analysis was performed using MSigDB (Molecular signatures database, http://www.broadinstitute.org/gsea/msigdb/index.jsp) with gene sets derived from Canonical pathways, BioCarta, KEGG, and Reactome.

4.3 Chromatin immunoprecipitation-sequencing (ChIP-seq)

HCT116 cells were cross-linked with 1% formaldehyde for 10 min at room temperature and quenched with 0.4 M glycine. Chromatin extracts were sheared by micrococcal nuclease digestion, and subsequently protein-DNA complexes were immunoprecipitated with 10 μg of anti-TCF7L2 antibody (#05-511, EMD Millipore, Billerica, MA). Normal mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA) was used as a negative control. The precipitated protein-DNA complexes were purified by conventional DNA extraction methods, and the DNAs were subjected to next generation sequencing or quantitative PCR analysis. The next generation sequencing was carried out by Ion Proton System according to the manufacturer's protocol (ThermoFisher), and the data were analyzed using Hotelling Observer, a shape-based peak caller in CLC Genomics Workbench version 6.0 (Qiagen). In the calling process, peaks were primarily filtered by reads>49, normalized difference<0.33, and FDR<0.1%. Peaks located in the loci or flanking region (5' and 3') of genes were further selected for the screening.

ChIP-qPCR was performed using a set of primers (forward primer, 5’-CGCCAGATGCTCAACTAGAA-3’ and reverse primer; 5’-CAATCTCTTGCTGCCACAAA-3’). Primer sets of intron 1 and an intergenic region of *RNF43* were used as a positive and a negative control, respectively[14]

4.4 Luciferase reporter assay and site-directed mutagenesis

Reporter plasmids containing a putative promoter region of *FRMD5* were constructed by cloning its 5’ flanking region (-1255 to +112) into the *Xho*I and *Bgl*II restriction enzyme sites of pGL4.14 (Promega, Madison, WI). Reporter plasmids containing a putative enhancer region of *FRMD5* were constructed by cloning a region within *FRMD5* intron 1 (hg19-chr15:44,449,571-44,450,548) into *Kpn*I and *Xho*I restriction enzyme sites of pGL4.23 (Promega). The putative promoter and enhancer regions were amplified by PCR using genomic DNA from healthy volunteers as a template with a set of primers (forward: 5’-CCGCTCGAGCAGCATTAATGTTCTATGTT-3’, and reverse: 5’-GGAAGATCTCCAGGCACCTGCACCAT-3’), and another set (forward: 5’-CGGGGATCCGACAGGGCTTAAGGTCACAAC-3’, and reverse: 5’-CCGCTCGACTGGACCTTCAGACTGCTCTT-3’), respectively. Mutant reporter

plasmids were prepared by the substitution from CTTTCA to CGCTCA in the putative TCF7L2 binding sites using the reporter plasmid as a template with a set of primers (forward: 5'-TGGATTTTCCTTTTCGCTCATCTCCTGAATTG-3', and reverse: 5'-CAATTCAGGAGATGAGCGAAAAGGAAAATCCA-3') and Pfu DNA polymerase (Agilent Technologies). CRC cells seeded on 12-well plates were transfected with the reporter plasmids together with pRL-Null (Promega) using FuGENE 6 reagent (Promega). The cells were harvested 48 hours after transfection, and reporter activities were measured by dual luciferase system (TOYO B-Net, Tokyo). For the knockdown of β-catenin, cells were transfected with 10 nM of *CTNNB1* siRNA (siβ-catenin #9 and siβ-catenin #10, respectively) at 6 hours after seeding, and incubated for an additional 48 hours.

4.5 Western blotting

Total protein was extracted from cultured cells using radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS) supplemented with a Protease Inhibitor Cocktail Set III (Calbiochem, San Diego, CA). Protein concentration was determined by BCA Protein Assay Kit (ThermoScientific, Rockford, IL, USA). Protein (30-50 μg/lane) was

separated by 10% SDS-PAGE and transferred to nylon and PVDF transfer membranes. Primary antibodies used for western blotting were anti-FRMD5 (HPA011746, Sigma), anti-E-Cadherin (ab1416, Abcam), anti-Histone H3 (#39164, Active Motif, Carlsbad, California), anti-β-tubulin (T4026, Sigma), anti-lamin B (sc-6216, Santa Cruz) and anti-β-actin (a5441, Sigma) antibodies. Horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG (GE Healthcare, Buckinghamshire, UK) served as the secondary antibody for the ECL Detection System (GE Healthcare).

4.6 Cell cycle analysis

HCT116, DLD-1, LS174T and HCT-15 cells treated with/without siFRMD5 #2 and siFRMD5 #3 were harvested by trypsinization, fixed with 70% ethanol, and stored at -20˚C until use. The cells were rehydrated with PBS, treated with ribonuclease A (2 mg/ml) at 37˚C for 30 min, and incubated with propidium iodide (20 μg/ml) in PBS at room temperature for 30 min. After filtration with nylon mesh, the cells were applied for FACS analysis (FACSCalibur, Becton,Dickinson, Franklin Lakes, NJ).

4.7 Transwell migration assay

HCT116 cells were transfected with siFRMD5 or control siRNA, and maintained

in McCoy's 5a Medium Modified medium with 10% FBS for an additional 24 hours. Then the cells were starved in medium without FBS for 24 hours. Transwell migration assay was performed using Transwell chambers (Corning, NY) with 8.0 μm pore size. Approximately 1.5 × 105 cells were seeded on the upper surface of the Transwell membranes, incubated in FBS (-) McCoy's 5a Medium Modified medium without FBS for 24h at 37 °C. The lower surface of the Transwell membrane was immersed to the medium with 10% FBS. The Transwell membrane was then fixed with methanol for 2 min and stained by hematoxylin solution for 10min. Four microscopic fields were randomly chosen for the analysis.

4.8 Statistical anlysis

Statistical analysis was carried out by R software (https://www.r-project.org/). The unpaired Student's t-test and Dunnett's test were used to determine the significance of experimental data.

5. Results

5.1 Screening of novel target genes of the Wnt/β-catenin signaling

5.1.1 Integrated analysis of expression microarray and TCF7L2 ChIP-seq

In order to find novel target genes of the β-catenin/TCF7L2 complex, expression profile analysis was performed using HCT116 and SW480 cells that have increased transcriptional activity of β-catenin/TCF7L2 complex due to either mutation in *β-catenin* or *APC*, respectively. RNA was extracted from the cells treated with pooled β-catenin or control siRNA. I selected genes whose expression levels were decreased in the cells treated with β-catenin siRNA to less than two-thirds of the cells with control siRNA, and identified a total of 2671 and 2886 genes in HCT116 and SW480 cells, respectively. Additional analysis of public microarray data (GSE46465) discovered a total of 1118 genes whose expression levels were decreased to less than two-thirds by a dominant-negative form of TCF7L2 (dnTCF7L2) in LS174T cells. These data consequently identified a total of 134 genes that were commonly down-regulated by the suppression of β-catenin or TCF7L2 (Figure 1A).

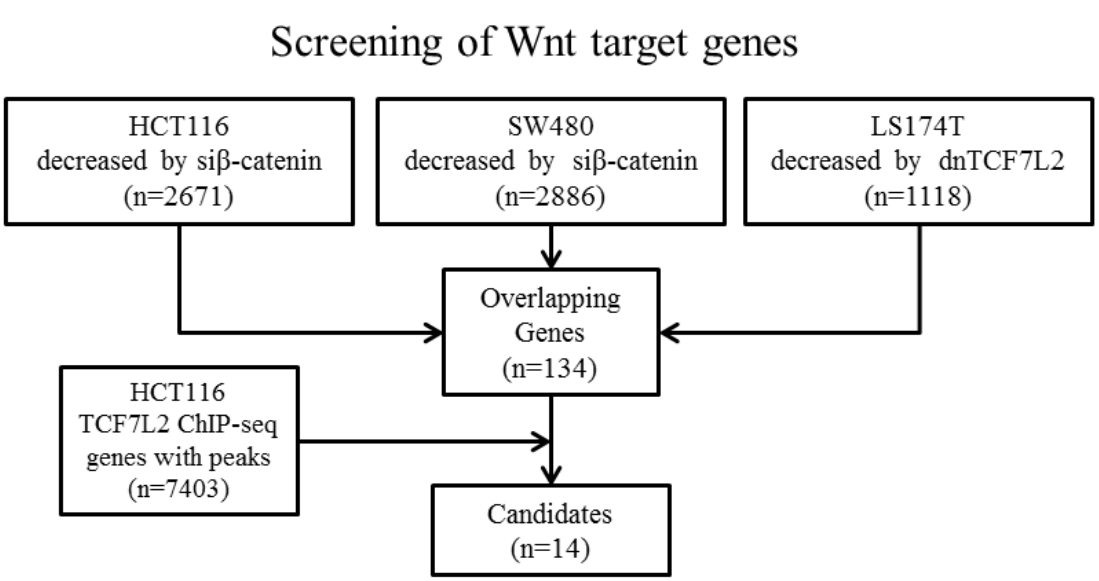
To identify genes that are directly regulated by the β-catenin/TCF7L2 complex, I carried out chromatin immunoprecipitation with high-throughput sequencing (ChIP-seq) of TCF7L2 in HCT116 cells. As a result, I identified a total of 7403 peaks that have

significantly increased read-counts compared with the background in the genome. Among the 134 candidate genes, 14 genes harbored at least one peak of the 7403 within a region between 10 kb upstream and downstream of the gene (Figure 1A).

5.1.2 Validation of novel candidate genes of Wnt signaling

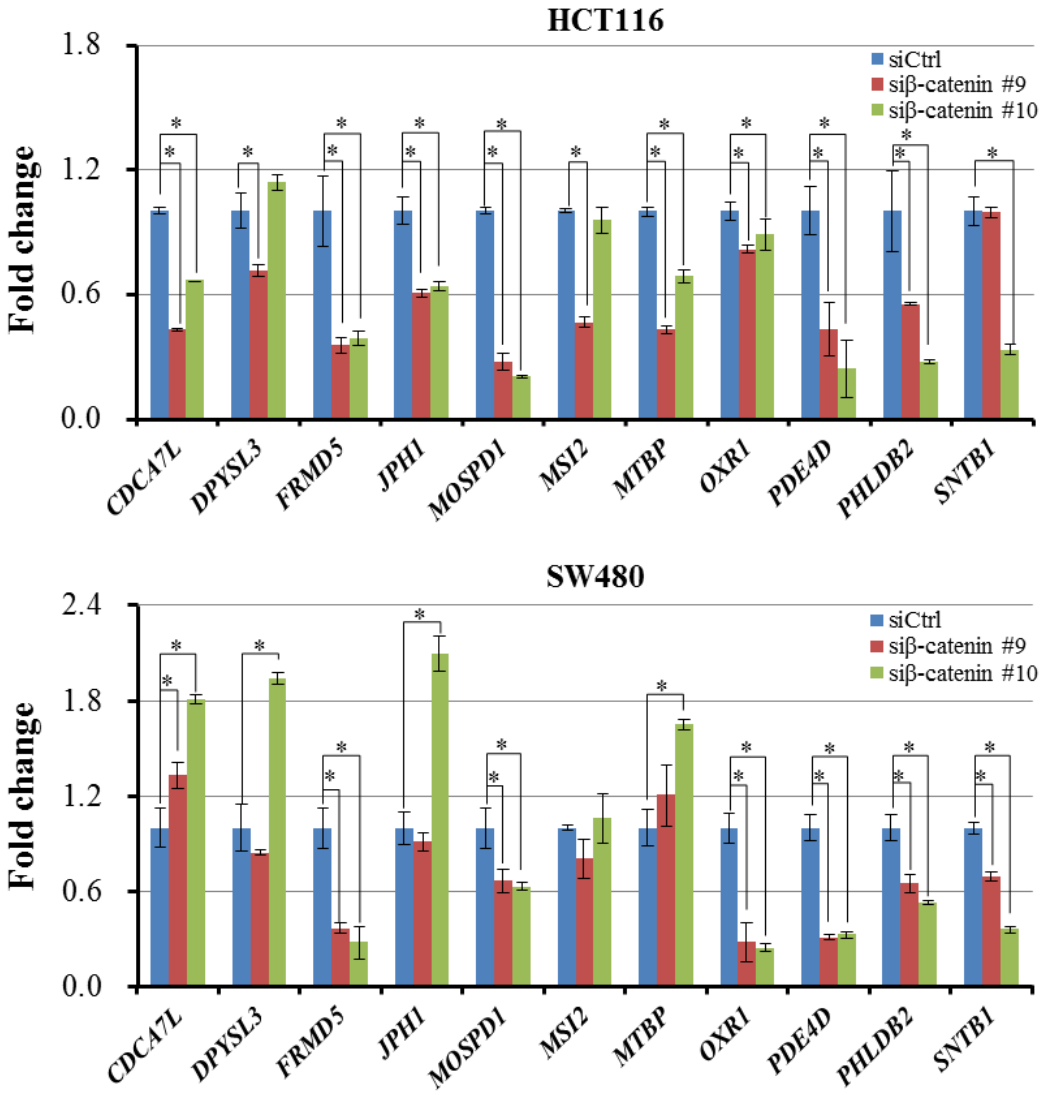
Notably, the 14 genes included *AXIN2*, *RNF43*, and *cMYC*, the well-known direct targets of the complex. I treated HCT116 and SW480 with two independent siRNAs of β-catenin and tested whether these 11 novel candidates are down-regulated by β-catenin siRNAs. Quantitative PCR analysis revealed that among the 11 candidate genes, expression of *PDE4D (Phosphodiesterase 4D)*, *PHLDB2* (*Pleckstrin Homology-Like Domain, Family B, Member 2*), *MOSPD1* (*Motile Sperm Domain Containing 1*), and *FRMD5* (*FERM domain-containing protein 5*) was significantly decreased with the two siRNAs in both cell lines. Expression of *JPH1 (Junctophilin 1)*, *CDCA7L (Cell Division Cycle Associated 7 Like)*, and *MTBP (MDM2 Binding Protein)* was decreased with the two siRNAs in HCT116 but not in SW480 (Figure 1B). Since the role of FRMD5 in colorectal carcinogenesis had not been studied, I focused on FRMD5 in this study. Decrease of FRMD5 protein in response to β-catenin siRNA was additionally confirmed in HCT116 and DLD-1 cells by western blot analysis (Figure 1C).

(A)



Gene Symbol	Fold change in microarray data		
	HCT116 with β-catenin siRNA	SW480 with β-catenin siRNA	LS174T with dnTCF7L2
AXIN2[#]	-19.3	-4.8	-5.0
CDCA7L	-1.9	-1.6	-2.0
DPYSL3	-1.6	-1.5	-2.1
FRMD5	-2.7	-1.8	-2.0
JPH1	-2.5	-2.3	-1.7
MOSPD1	-2.0	-1.9	-1.7
MSI2	-5.3	-1.9	-1.8
MTBP	-2.2	-1.7	-1.7
MYC[#]	-1.5	-4.1	-2.3
OXR1	-1.8	-8.6	-1.6
PDE4D	-1.6	-3.7	-1.6
PHLDB2	-2.1	-2.1	-2.7
RNF43[#]	-5.4	-1.8	-2.7
SNTB1	-2.4	-2.2	-1.8

(B)


HCT116
siCtrl
siβ-catenin #9
siβ-catenin #10
Fold change
1.8
1.2
0.6
0.0
CDCA7L
DPYSL3
FRMD5
JPH1
MOSPD1
MSI2
MTBP
OXR1
PDE4D
PHLDB2
SNTB1
SW480
siCtrl
siβ-catenin #9
siβ-catenin #10
Fold change
2.4
1.8
1.2
0.6
0.0
CDCA7L
DPYSL3
FRMD5
JPH1
MOSPD1
MSI2
MTBP
OXR1
PDE4D
PHLDB2
SNTB1

(C)

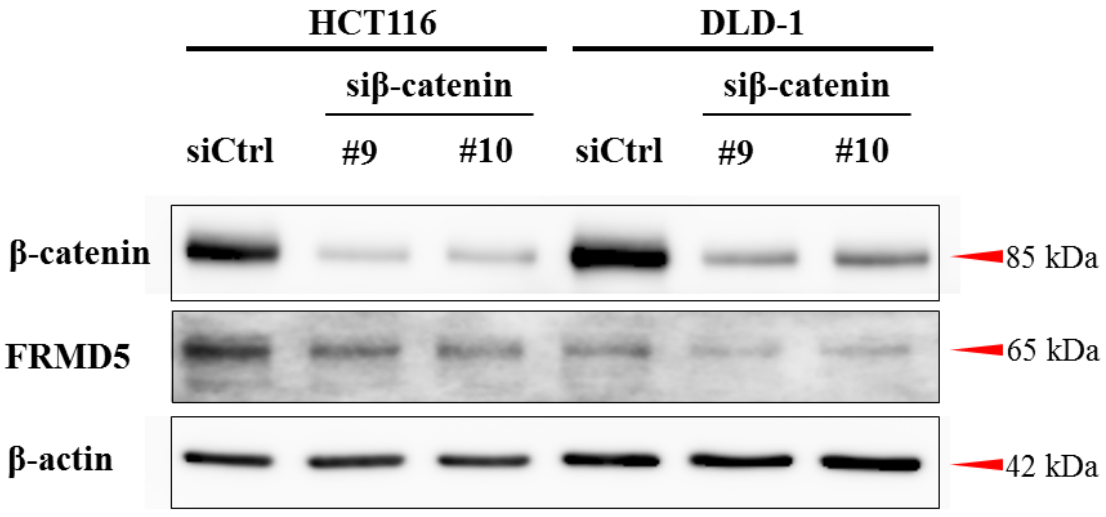

HCT116
DLD-1
siβ-catenin
siβ-catenin
siCtrl
#9
#10
siCtrl
#9
#10
β-catenin
85 kDa
FRMD5
65 kDa
β-actin
42 kDa

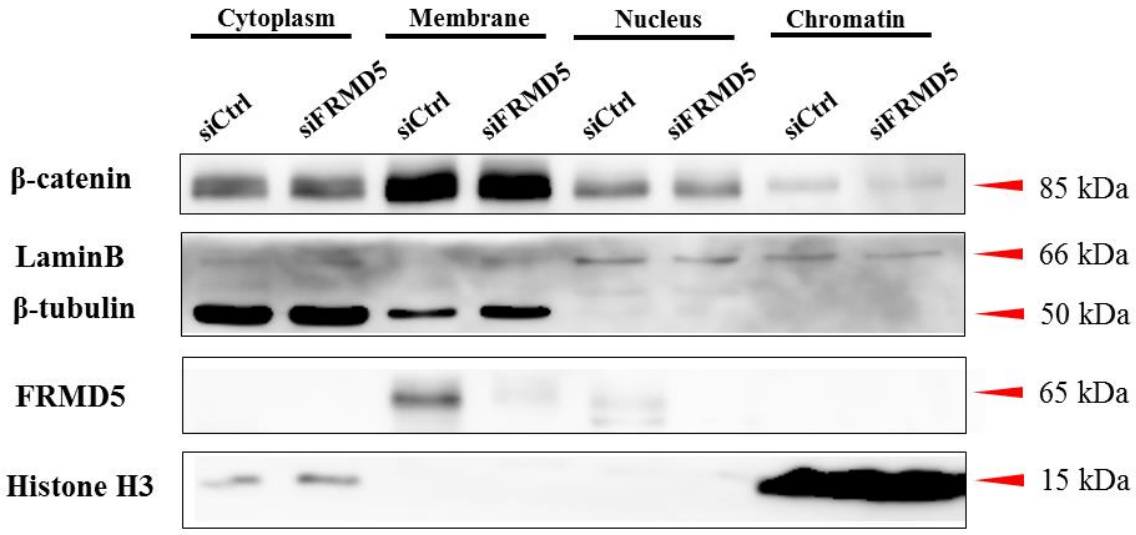
Figure 1 Screening of novel downstream genes of Wnt/β-catenin signaling. (A) Strategy for the identification of candidate target genes of β-catenin/TCF7L2 complex. Reported Wnt target genes are marked with an asterisk, "#". (B) Relative expression of the eleven candidate genes in HCT116 (upper panel) and SW480 (lower panel) cells treated with β-catenin siRNA (red and green, respectively) compared to the cells treated with control siRNA (blue). (C) HCT116 and DLD-1 were treated with siCtrl and two different siRNA of beta-catenin for 48 hours. An asterisk indicates statistical significance (Dunnett's test, p value < 0.05) between β-catenin siRNA and control siRNA.

5.2 Elevated FRMD5 expression in CRC

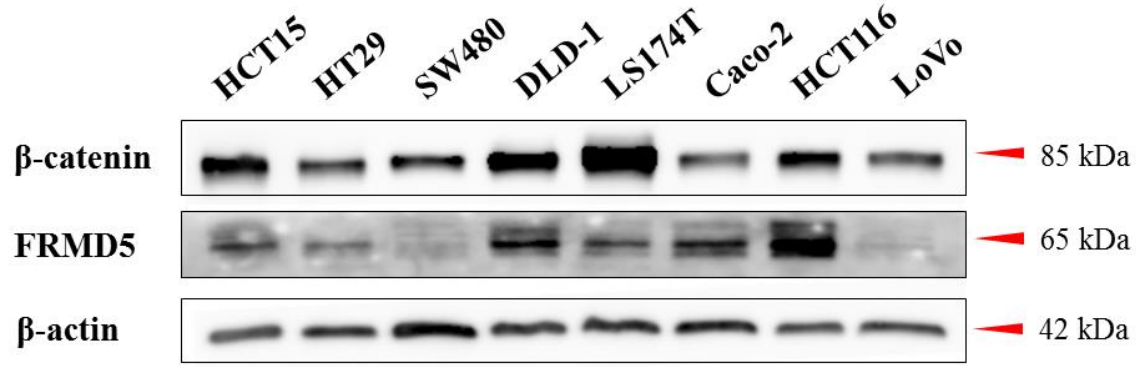
I examined subcellular localization of FRMD5 in HCT116 cells. Western blotting using subcellular fractions of HCT116 showed that FRMD5 mainly localizes at the membrane (Figure 2A). I next investigated the expression of FRMD5 in eight CRC cell lines with activated Wnt/β-catenin signaling. Western blot analysis showed that FRMD5 was expressed in all eight cell lines examined (Figure 2B). Among the eight, it was expressed abundantly in HCT116, HCT15, DLD-1 and Caco-2 cells, and slightly in HT29 and LS174T cells. Its expression was limited in SW480 and LoVo cells. Further analysis of the expression using ONCOMINE database (www.oncomine.org/resource/login.html) disclosed that four of eight microarray datasets (TCGA Colorectal, Sabates-Bellver Colon, Gaedcke Colorectal and Hong Colorectal) showed significant increase of *FRMD5* expression (≥ 2.0-fold) in CRC tissues compared with normal colonic tissues (p value <0.01). In addition, three of the

eight datasets showed a slight increase of *FRMD5* expression (<1.5-fold) in CRC tissues, indicating that FRMD5 is frequently up-regulated in CRC. Figure 2C showed top three datasets with minimal p value (TCGA Colorectal, Sabates-Bellver Colon, Gaedcke Colorectal).

(A)



(B)



(C)

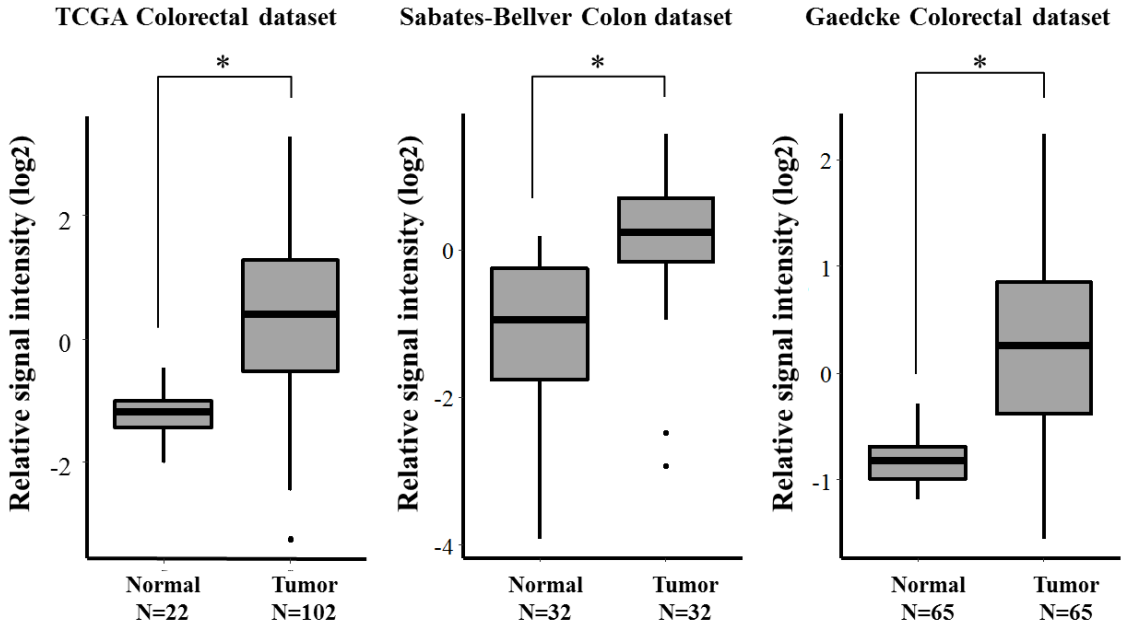


Figure 2 Expression of FRMD5 in colorectal carcinoma. (A) HCT116 was treated with siCtrl or siFRMD5 for 48 hours and cytoplasmic, membrane, nuclear and chromatic fractions were extracted based on protocol from extraction kit. (B) Eight CRC cell lines were cultured in proper medium for 48 hours and harvested. The lysates were subjected to western blot analysis. (C) Expression of *FRMD5* in colon cancer tissues. Significant increase of *FRMD5* expression in CRC tissues (Tumor) compared with normal colonic tissues (Normal) was found in three datasets (Student's t-test, p-value <0.01). Logarithmic values of relative *FRMD5* signal intensities in microarray data are shown in the boxplot.

5.3 Identification of the regulatory region of *FRMD5* as a downstream of Wnt/β-catenin signaling

5.3.1 Identification of a TCF7L2-binding site in *FRMD5*

I further investigated the TCF7L2-binding region(s) of FRMD5. The ChIP-seq data of TCF7L2 suggested a candidate region for TCF7L2-binding (UCSC hg19; chr15:44,449,680-44,450,487) in intron 1 of FRMD5, which was termed as FRMD5-TBE (Figure 3A). Consistent with our results, data of HCT116 in Encyclopedia of DNA Elements (wgENCODEEH000629, ENCODE: www.encodeproject.org) also demonstrated a TCF7L2-interacting peak (hg19; chr15:44,449,944-44,450,042) in intron 1. To confirm the interaction between the region and TCF7L2, Additional ChIP-quantitative PCR analysis corroborated that the DNA immunoprecipitated with anti-TCF7L2 antibody contained 89.4-fold higher concentration of the intronic region compared to that with control IgG. I used a region in *RNF43* that had been reported to interact with TCF7L2 as a positive control (Figure 3B). This analysis corroborated that TCF7L2 binds to the region in intron 1. A search of histone protein modifications (H3K4me1, H4K27Ac and H3K4me3) and DNase hypersensitive sites in ENCODE data of UCSC Genome Browser revealed that this region contains high levels of H3K4 mono-methylation and H3K27 acetylation and low levels of H3K4 tri-methylation, and

that the same region was overlapped by DNaseI hypersensitivity peak clusters (Figure 3A). These data supported that this region may play a crucial role as an enhancer.

5.3.2 Regulation of *FRMD5* enhancer and promoter activity

The ENCODE data additionally depicted a peak in the 5'-flanking region (hg19; chr15:44487635-44487877) of *FRMD5* which our ChIP-seq data failed to identify. To analyze the transcriptional activity of the 5'-flanking region and the TCF7L2-interacting region in the intron 1, I prepared two reporter plasmids, one containing a region between -1255 and +112 (pGL4.14-FRMD5-promoter), and the other containing the interacting region (pGL4.23-FRMD5-TBE-WT) (Figure 3A). The results showed that the activity of pGL4.14-FRMD5-promoter was 12.9-fold higher than that of the control plasmid (pGL4.14-Empty). However the reporter activity was unchanged by the knockdown of β-catenin (Figure 3C), suggesting that the transcriptional activity of the 5'-flanking region is not associated with the β-catenin/TCF7L2 complex. On the other hand, the reporter activity of pGL4.23-FRMD5-TBE-WT was 77.5-fold higher compared with the control plasmid (pGL4.23-Empty), suggesting an enhancer activity of the region. Importantly, the activity of pGL4.23-FRMD5-TBE-WT was reduced to 0.5-fold and 0.4-fold by β-catenin siRNA #9 and #10, respectively (Figure 3D). These

data tempted me to speculate that the enhancer activity is regulated by the β-catenin/TCF7L2 complex.

I therefore searched further for TCF7L2-binding elements (TBEs) in this region using a motif search for transcription factor-binding motifs, JASPAR (http://jaspar.genereg.net/). Motifs of putative TBEs with JASPAR score ≥ 10 were picked as candidates. As a result, I identified one putative TCF7L2 binding element (TBE), 5'-TTTCTTTCATCTCC-3', with a JASPAR score of 14.5 in the region (Figure 3A). I additionally prepared a mutant reporter plasmid (pGL4.23-FRMD5-TBE-MUT) by substituting TTTCTTTCATCTCC to TTTCGCTCATCTCC in pGL4.23-FRMD5-TBE-WT, and reporter assay was performed. As expected, the reporter activity of pGL4.23-FRMD5-TBE-MUT was significantly repressed to 0.6-fold compared with the activity of pGL4.23-FRMD5-TBE-WT. In addition, the activity of the mutant was unchanged by the treatment with β-catenin siRNA (Figure 3E).

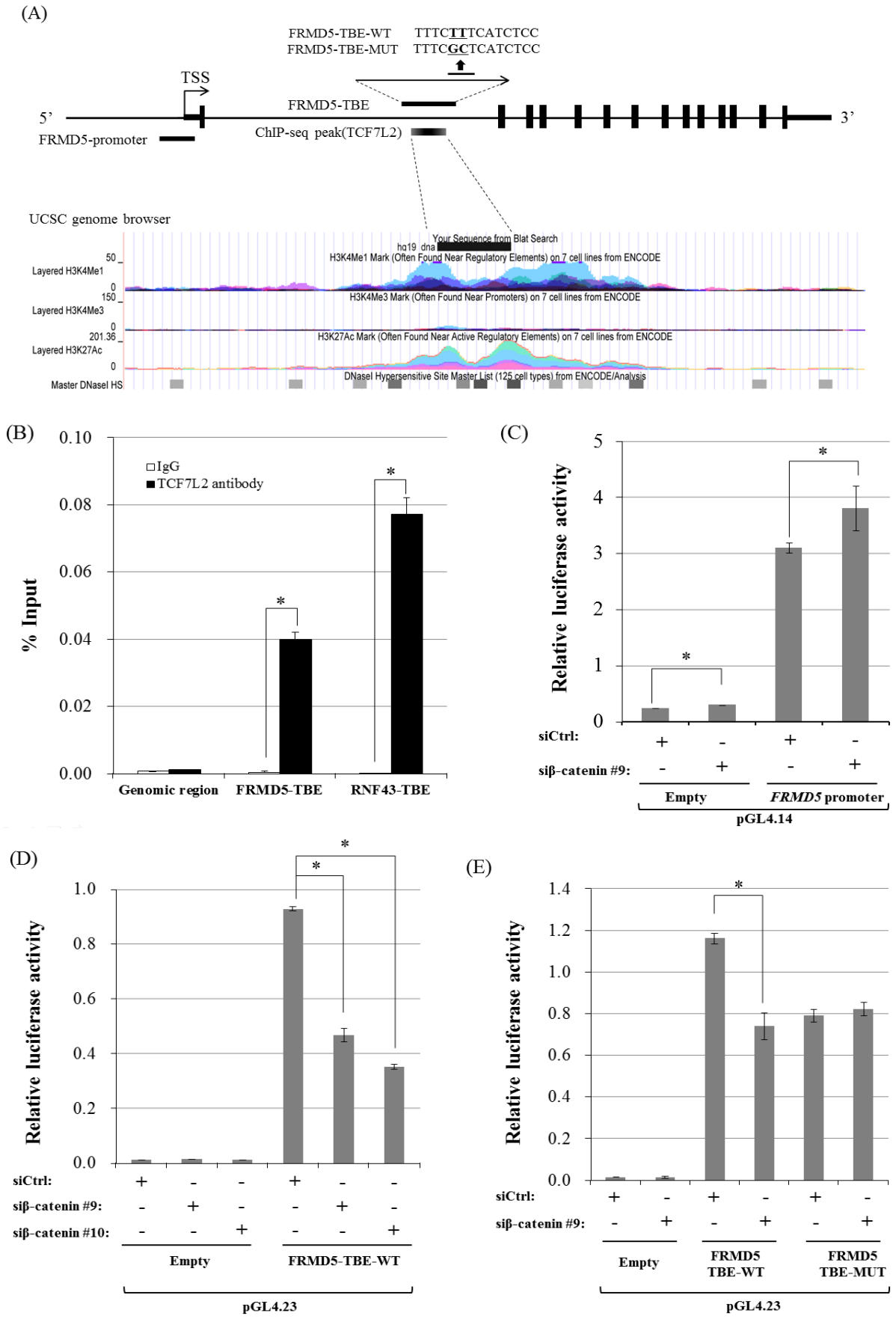

(A)
FRMD5-TBE-WT TTTCTTTCATCTCC
FRMD5-TBE-MUT TTTCGCTCATCTCC
TSS
FRMD5-TBE
5'
3'
FRMD5-promoter
ChIP-seq peak(TCF7L2)
UCSC genome browser
Your Sequence from Blat Search
hg19 dna
H3K4Me1 Mark (Often Found Near Regulatory Elements) on 7 cell lines from ENCODE
Layered H3K4Me1
H3K4Me3 Mark (Often Found Near Promoters) on 7 cell lines from ENCODE
Layered H3K4Me3
H3K27Ac Mark (Often Found Near Active Regulatory Elements) on 7 cell lines from ENCODE
Layered H3K27Ac
DNaseI Hypersensitive Site Master List (125 cell types) from ENCODE/Analysis
Master DNaseI HS
(B)
% Input
IgG
TCF7L2 antibody
Genomic region
FRMD5-TBE
RNF43-TBE
(C)
Relative luciferase activity
siCtrl:
siβ-catenin #9:
Empty
FRMD5 promoter
pGL4.14
(D)
Relative luciferase activity
siCtrl:
siβ-catenin #9:
siβ-catenin #10:
Empty
FRMD5-TBE-WT
pGL4.23
(E)
Relative luciferase activity
siCtrl:
siβ-catenin #9:
Empty
FRMD5 TBE-WT
FRMD5 TBE-MUT
pGL4.23

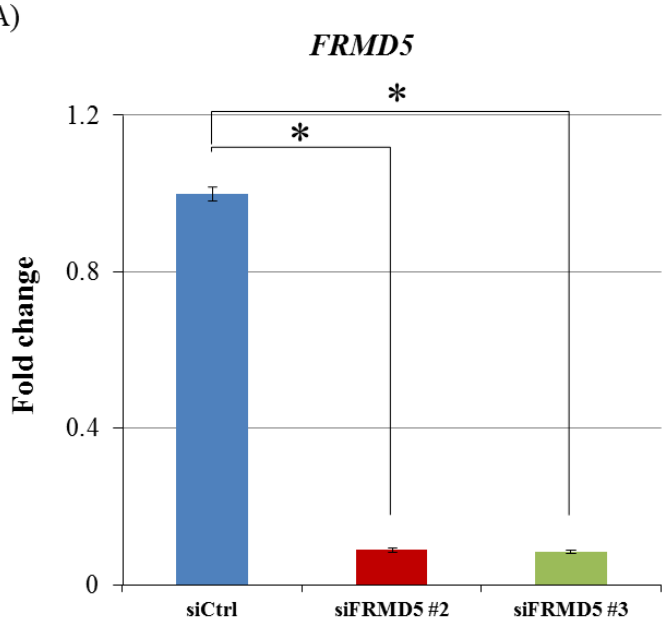
Figure 3 Regulation of promoter activity and enhancer activity of *FRMD5* .(A) Motif research was carried out by using JASPAR database and putative TBEs with jaspar score ≥ 10.0 were picked as candidates. (B) Quantification of precipitated regions by a ChIP assay with anti-TCF7L2 antibody was performed in HCT116 cells using real-time PCR (mean ± standard deviation). (C), (D), (E) Reporter plasmids were transfected in HCT116 cells and luciferase activities were measured in triplicate (mean ± standard deviation). An asterisk indicates statistical significance (Student's t-test and Dunnett's test, p value < 0.05).

5.4 The role of FRMD5 as a downstream target of Wnt signaling.

To uncover the role of FRMD5 in CRC, I carried out expression profile analysis of HCT116 cells treated with/without FRMD5 siRNA. As expected, expression of FRMD5 was decreased to 10.3-fold and 12.4-fold by the treatment with siFRMD5 #2 and siFRMD5 #3, respectively, compared with control siRNA (Figure 4A). I selected genes whose expression levels were commonly decreased or increased more than two-fold by the FRMD5 siRNA. As a result, I identified a total of 189 commonly down-regulated genes and 226 commonly up-regulated genes by the two siRNAs. Among the genes, I further selected genes whose expression was consistently altered by β-catenin siRNA in HCT116 cells to elucidate the function of FRMD5 as a downstream target of canonical Wnt signaling. Consequently, I found a total of 36 and 53 genes that were either down-regulated or up-regulated, respectively, by FRMD5 siRNA as well as β-catenin

siRNA (Table S2 and Table S3). Additional qPCR analysis validated altered expression in four (*MYB*, *PIGC*, *RPRML*, and *DTL*) of the five top down-regulated genes and three (*GRM8*, *CAMP* and *KRT80*) of the five top up-regulated genes (Figure 4B). Additionally the 36 and 53 genes were applied for gene set enrichment analysis, which identified ten enriched gene sets such as DNA replication, cell cycle, and extracellular matrix (ECM) (Table 1). The gene set associated with DNA replication and cell cycle included *CDC45*, *CDT1*, *POLA2*, *MCM10* and *SMC1A*, which are all in the 36 down-regulated genes. Meanwhile the gene set associated with ECM included *WNT7A*, *WNT7B*, *ANGPTL4*, *LEFTY1*, *TCHH*, *EGLN3*, *PLAU* and *PLXDC1*, which are all in the 53 up-regulated genes. These results indicated that FRMD5 may play an important role in the regulation of DNA replication and cell cycle, and/or the interaction with extracellular matrix through these genes.

(A)



(B)

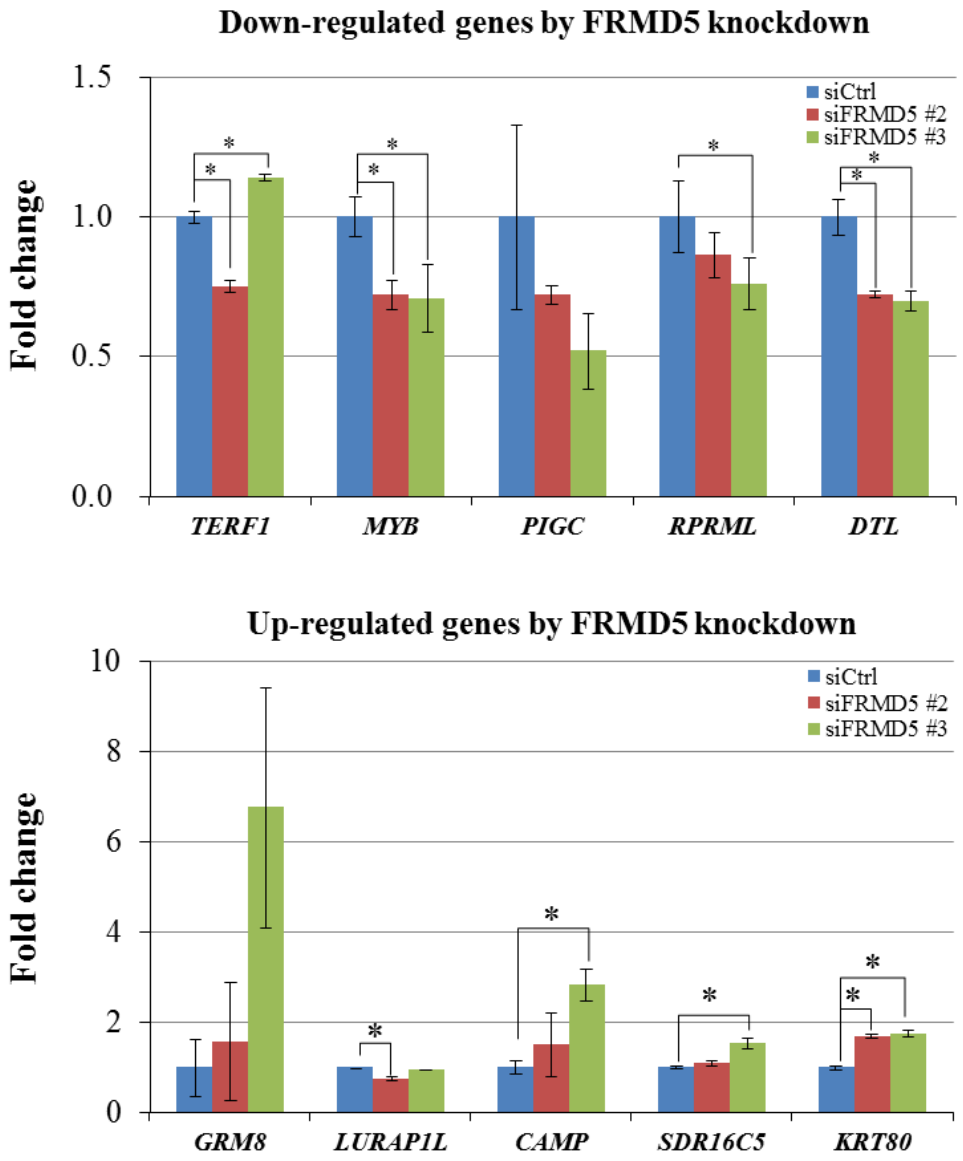


Figure 4. Genes with altered expression by the knockdown of FRMD5. (A) Decreased expression of FRMD5 in response to FRMD5 siRNA #2 and #3. (B) Top five down-regulated genes and top five up-regulated genes by FRMD5 siRNA in HCT116. Quantitative PCR was performed in triplicate using RNA from the cells treated with siRNA #2 (red) or #3 (green) or control siRNA (blue). Relative expression levels of the ten genes are shown in the histogram (mean ± standard deviation). An asterisk indicates

statistical significance (Dunnett's test, p value < 0.05) between β-catenin siRNA and control siRNA.

Table 1. Enriched genesets in expression profile of HCT116 with FRMD5-knockdown

#	Gene Set	Description	Genes in Overlap	p value	FDR q value
1	REACTOME_ACTIVATION_OF_THE_PRE_REPLICAICATIVE_COMPLEX [31]	Genes involved in Activation of the pre-replicative complex	4	2.1 e-7	2.79 e-4
2	NABA_MATRISOME_ASSOCIATED [753]	Ensemble of genes encoding ECM-associated proteins including ECM-affilaited proteins, ECM regulators and secreted factors	9	4.35 e-6	2.77 e-3
3	NABA_MATRISOME [1028]	Ensemble of genes encoding extracellular matrix and extracellular matrix-associated proteins	10	7.54 e-6	2.77 e-3
4	REACTOME_MITOTIC_M_M_G1_PHASES [172]	Genes involved in Mitotic M-M/G1 phases	5	1.03 e-5	2.77 e-3
5	REACTOME_M_G1_TRANSITION [81]	Genes involved in M/G1 Transition	4	1.04 e-5	2.77 e-3
6	REACTOME_DNA_REPLICATION [192]	Genes involved in DNA Replication	5	1.75 e-5	3.89 e-3
7	NABA_SECRETED_FACTORS [344]	Genes encoding secreted soluble factors	6	2.37 e-5	4.5 e-3
8	REACTOME_E2F_MEDIATED_REGULATION_OF_DNA_REPLICATION [35]	Genes involved in E2F mediated regulation of DNA replication	3	2.74 e-5	4.55 e-3
9	REACTOME_G1_S_TRANSITION [112]	Genes involved in G1/S Transition	4	3.75 e-5	5.53 e-3
10	REACTOME_CELL_CYCLE [421]	Genes involved in Cell Cycle	6	7.26 e-5	9.65 e-3

Gene list were generated from genes whose expression levels were commonly decreased or increased by both FRMD5 siRNA and β-catenin siRNA. Gene set enrichment analysis was performed using MSigDB with gene sets derived from Canonical pathways, BioCarta, KEGG, and Reactome. Genesets in the table are with FDR q-value < 0.05.

5.5 FRMD5 regulates cell cycle in a cell-context dependent manner.

Because expression profile showed that FRMD5 is associated with cell cycle regulation and DNA replication, I carried out cell cycle profile analysis and investigated S phase population in HCT116, DLD-1, HCT-15 and LS174T (Figure 5). Consistent with the microarray data of HCT116 cells, treatment of the cells with siFRMD5 decreased S phase compared to that with siControl (33.3% and 36.1% with siFRMD5 #2, and #3, respectively, and 47.9% with siControl). Meanwhile, DLD-1 showed marginal decrease of S phase by FRMD5 #2, and #3 (30.3% and 27.4%, respectively) compared with siControl (32.7%). However, knockdown of FRMD5 did not induce significant changes in S phase in LS174T and HCT-15 cells, indicating that FRMD5 may regulate cell cycle in a cell-context dependent manner.

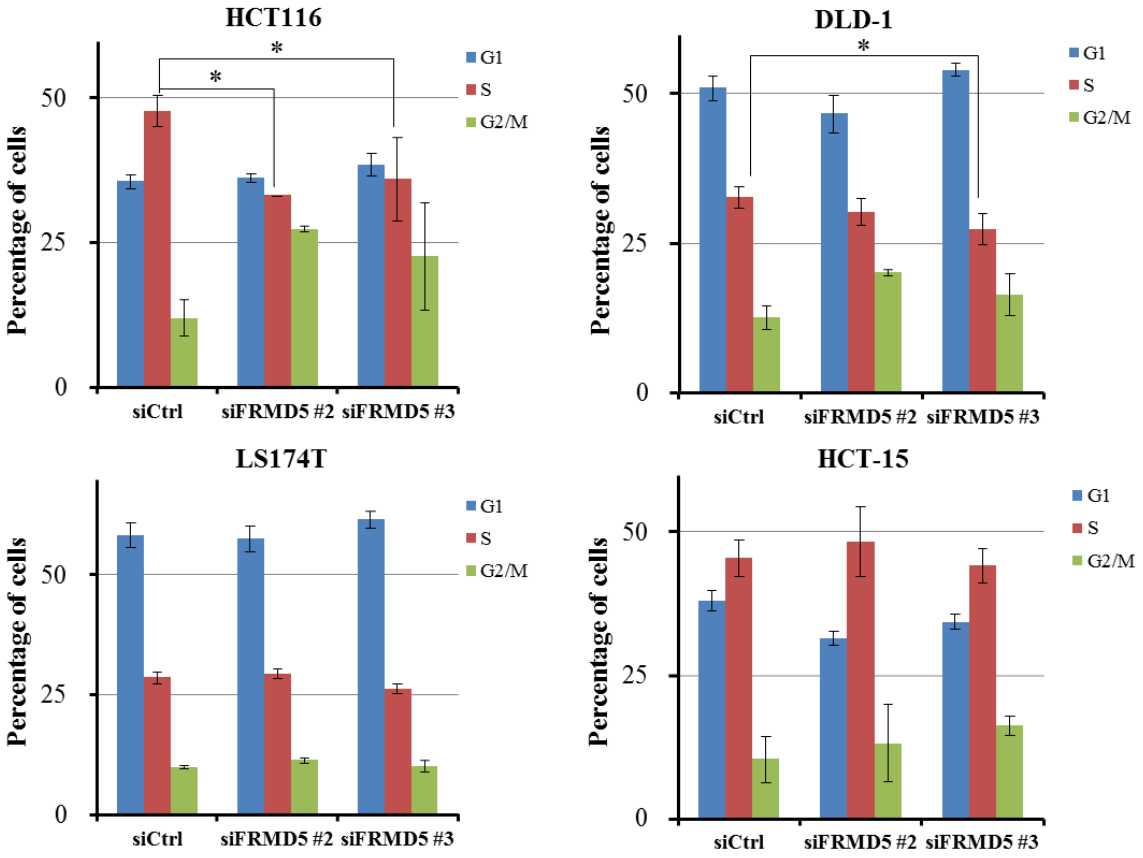


Figure 5. FRMD5 regulates cell cycle in a cell context-dependent manner. HCT116, DLD-1, LS174T and HCT-15 were treated with FRMD5 siRNA #2, #3 or control siRNA. G1 (blue), S (red) and G2/M (green) phases are measured by FACS. Percentage (%) of each phase was showed in the histogram (mean ± SD). An asterisk indicates statistical significance (Dunnett's test, p-value<0.05) between FRMD5 siRNA and control siRNA.

6. Discussion

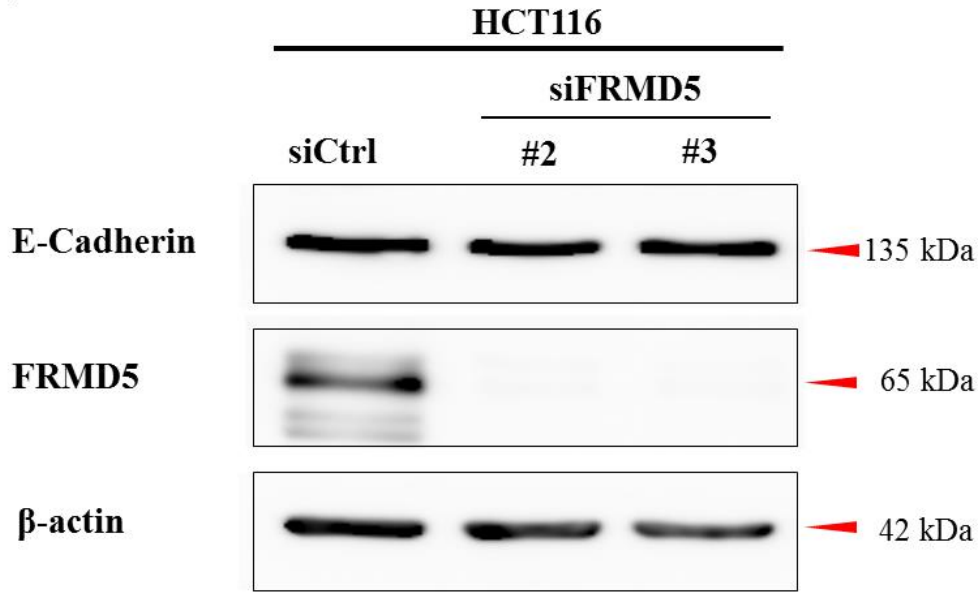
FRMD5, a member of the FERM protein family, encodes a 570 amino acid protein containing an N-terminal FERM domain. It was first identified as a gene down-regulated by mutant $p53^{R273H}$ protein[15]. In this study, I showed that FRMD5 is a novel direct target gene of β-catenin/TCF7L2 in CRC cells. Its abundant expression was detected in four of six CRC cell lines carrying a mutation in *APC* or *CTNNB1*. Consistent with the previous report, limited expression of FRMD5 was observed in SW480 cells that carries a mutated p53 ($p53^{R273H}$) allele[16]. These data indicates that several factors including β-catenin/TCF7L2 and mutant $p53^{R273H}$ play a role in FRMD5 expression.

I identified a region that is responsible for the expression of *FRMD5*. The additional search of histone protein modifications in the ENCODE database indicated that high levels of H3K4 mono-methylation, H3K27 acetylation and DNaseI hypersensitivity peak clusters enriched in this region. Reportedly, high levels of H3K4 mono-methylation with low levels H3K4 tri-methylation indicate enhancer regions[10, 11] and augmented acetylation of H3K27 is a marker for active enhancer rather than poised enhancer[19]. In addition, DNaseI hypersensitivity sites is linked to the regions with open chromatin, and thus may serve as binding regions for transcription factors[20]. These data

are consistent with the notion that this region interacts with β-catenin/TCF7L2 complex, and functions as an enhancer.

The biological function of FRMD5 remains to be fully elucidated. It was reported that FRMD5 interacts with p120-catenin and E-cadherin in lung H1299 cancer cells[12], and that FRMD5 represses epithelial-mesenchymal transition (EMT) and cell motility in H1299 cancer cells through interacting with integrin β5[13]. However, knockdown of FRMD5 in HCT116 did not change the expression of E-cadherin (Figure 6A). Additionally, FRMD5-knockout cells did not show consistent alteration of cell migration in HCT116 cells (Figure 6B and C). Therefore, FRMD5 may play a role in cell migration in a cell-type or tissue-type manner. Further investigation is needed to clarify the role of FRMD5 in carcinogenesis.

(A)



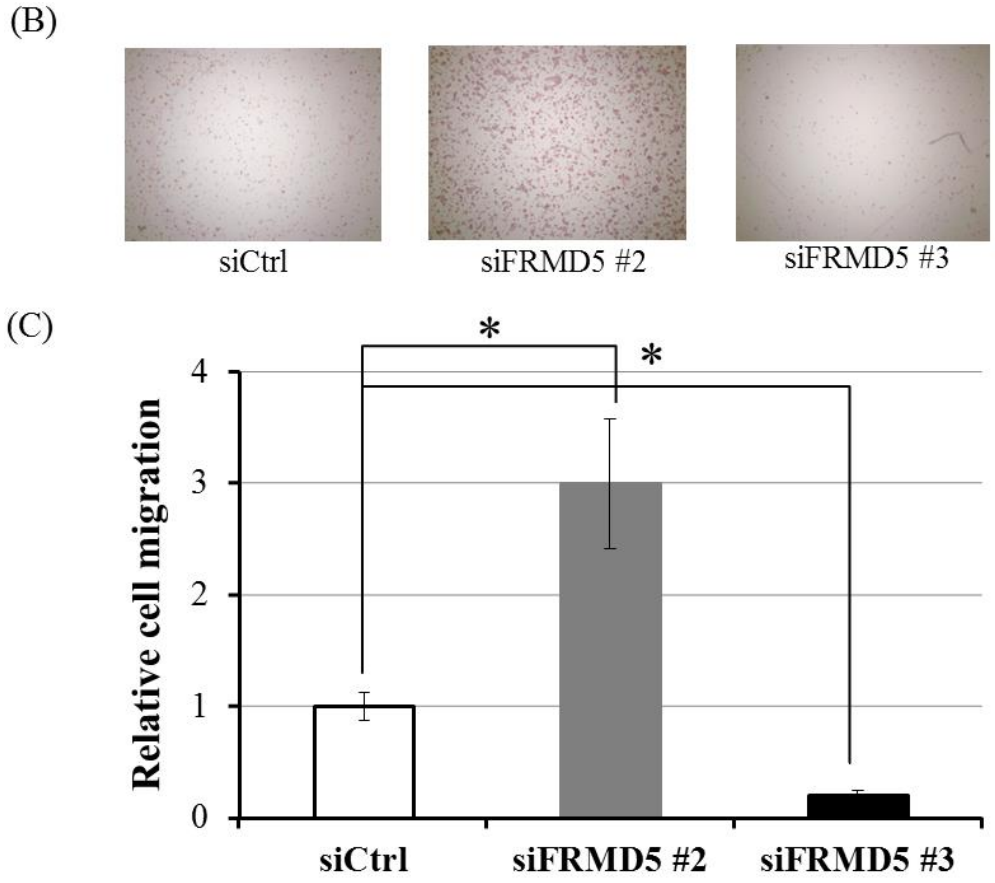


Figure 6. (A) Expression of E-cadherin in HCT116 cells treated with FRMD5 siRNA (siFRMD5 #2 and #3) or control siRNA (siCtrl). (B) Images of migrated HCT116 cells in the transwell assay. Cells treated with control siRNA (siCtrl), or FRMD5 siRNA (siFRMD5 #2, or #3). (C) Relative number of migrated cells. The number of migrated cells (mean ± SD) in the cells treated with siFRMD5 #2 or #3 was normalized by that in the cells treated with siCtrl. An asterisk indicates statistical significance (Dunnett's test, p-value < 0.05) between FRMD5 siRNA and control siRNA.

In this study, expression profile analysis detected enriched gene sets of DNA replication, cell cycle, and ECM in HCT116 cells treated with siFRMD5. The gene set associated with DNA replication and cell cycle included *CDC45*, *CDT1*, *POLA2*, *MCM10* and *SMC1A*. *CDT1* encodes chromatin licensing factor 1 that plays an essential role in the initiation of DNA replication[21] together with origin recognition complex-associated (ORCA)[22]. Minichromosome maintenance complex component 10 (MCM10) and DNA-polymerase alpha 2 (*POLA2*) are components of DNA polymerase α-primase complex[18, 19], which plays a crucial role in eukaryotic DNA replication. Previous studies showed that MCM10 is recruited to the replication sites[25] and regulates the stability and association of DNA polymerase-alpha[26]. POLA2 enhances the protein level of p180, a catalytic subunit of DNA polymerase-alpha 1 (POLA1)[24], and increases DNA polymerase activity. Expression of *CDT1*, *MCM10*, and *POLA2* was reduced by the knockdown of FRMD5. Therefore, enhanced expression of *CDT1*, *MCM10*, and *POLA2* by *FRMD5* may facilitate DNA replication in the proliferating cancer cells. Regarding the gene set of ECM that included *ANGPTL4*and *LEFTY1*, left-right determination factor 1 (*LEFTY1)* encodes a secreted ligand of TGF-β family member, and is involved in the determination of leftness during development. It is reported that expression of LEFTY1 is reduced in metastasized colorectal tumors in the liver

compared with their primary tumors[27]. In addition, angiopoietin-like 4 (ANGPTL4) prevented metastasis of mouse cancer cells through the inhibition of their intravasation and/or extravasation[28]. These data suggest that FRMD5 may render metastasizing potential to colon cancer cells through the reduced expression of *LEFTY1* and/or *ANGPTL4*.

In addition, I found that knockdown of FRMD5 decreased the expression of *MYB*, a well-known oncogene. It was reported that Myb blocks differentiation of colon epithelial cells[29] and regulates the renewal of intestinal stem cells[30], and that Myb is often overexpressed in colon cancer with poor prognosis[31]. Consistently, I found that colorectal cancer patients with a high expression level of FRMD5 showed poor prognosis compared to patients with a low expression level (PrognoScan http://www.abren.net/PrognoScan/, dataset GSE17536 and GSE17537) (Figure 7). Interestingly, a recent study revealed that intestinal-specific activation of Myb in pre-carcinogen-induced mouse model accelerated the tumorigenesis in colon by expanding the intestinal stem cell pool[32]. Therefore, elevated expression of FRMD5 may render malignant properties to colorectal cancer cells through the induction of *MYB* and subsequent induction of stemness in the tumor cells.

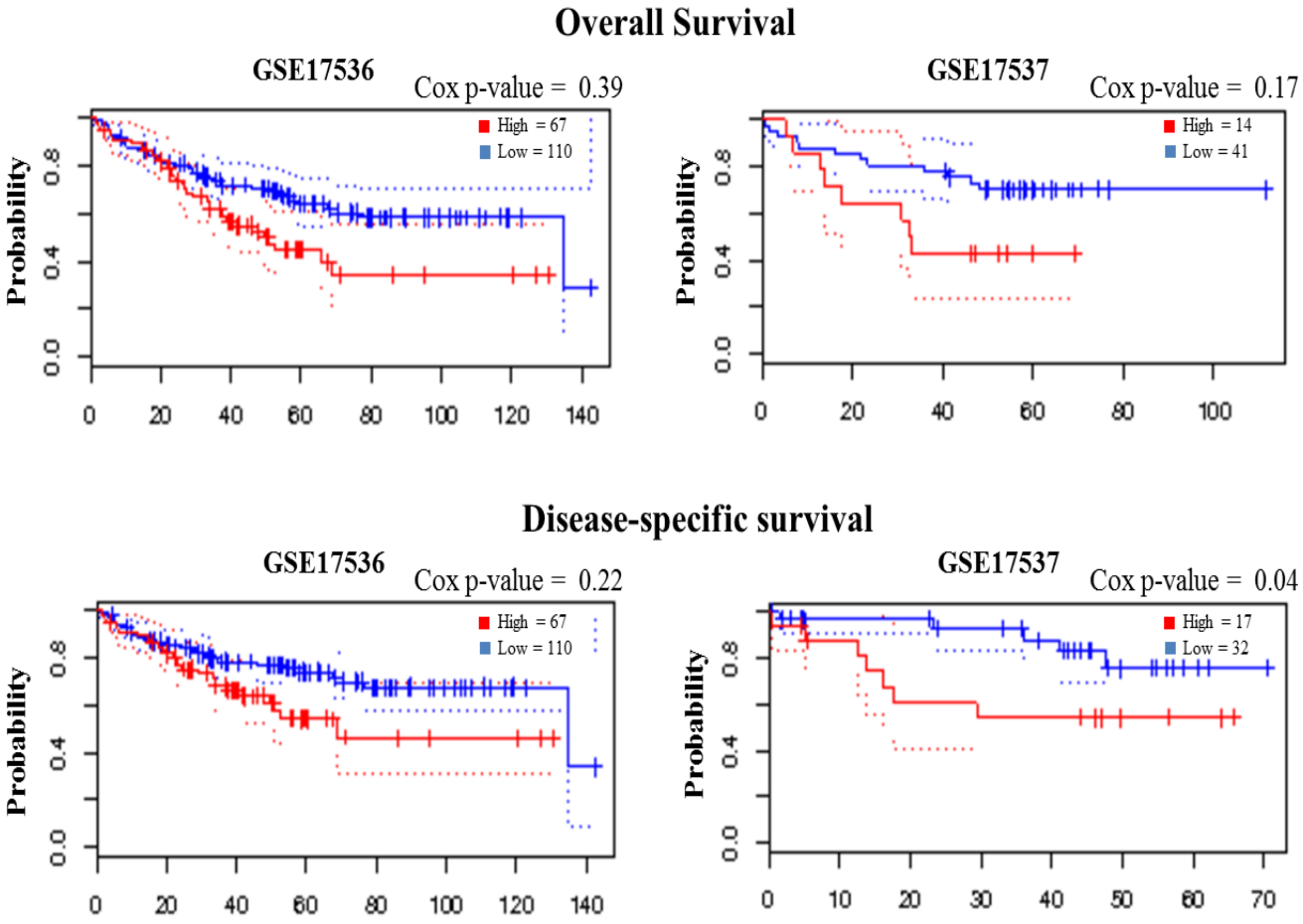


Figure 7. Kaplan-Meier plot of overall survival and disease-specific survival. Survival of patients with high (red) and low (blue) expression of FRMD5 was analyzed. Data were cited from PrognoScan; dataset GSE17536 (left) and GSE17537 (right). The COX p-value and 95% confidence intervals for each group indicated by dotted lines are provided by PrognoScan.

In summary, my results demonstrated that FRMD5 was a novel target of the Wnt signaling pathway, and that a region in intron 1 was responsible for the transcriptional regulation of *FRMD5*. Expression profile analysis and subsequent gene enrichment analysis revealed that FRMD5 may be involved in regulating cell cycle and ECM-regulation in CRC. Further studies are essential to clarify the role played by FRMD5 in colorectal tumorigenesis.

7. Acknowledgements

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9. Supplementary Tables

Table S1. Primers of real-time qPCR for candidate genes and *GAPDH*

Gene		Primer (5’-3’)
CDCA7L	Forward	CAATGTTAAGGAATATCTGGAGAGC
	Reverse	CATGTCTTGTTGGAGTACTCTATGG
DPYSL3	Forward	GATCGTCTCTGCCAAGAACC
	Reverse	CCTGGCAGATGACAACCAG
FRMD5	Forward	GATCTCTACCATGGCCGACT
	Reverse	CCCTGAGTCATAATCCCCAAT
JPH1	Forward	GACATCGCGAGAGCTGTG
	Reverse	TTCCTGAAATCTCTGTTTGACG
MOSPD1	Forward	TTTTGAGCAGTCGTTTCAACCAG
	Reverse	ACATCCCCCAGTGTAGGCAG
MSI2	Forward	GGTCATGAGAGATCCCACTACG
	Reverse	TCTACACTTGCTGGGTCTGC
MTBP	Forward	CCGAGACTCATGAATGTTTCACT
	Reverse	GACCCCTTGAAGTTTTAAGATCC
OXR1	Forward	ACATCTGCTGATGGACACATAGA

	Reverse	ACGTTTTCCTCATTGGTTTTCCA
PDE4D	Forward	TTGTCCAGTCTACTCATGTGCTATT
	Reverse	TTGCTGCAAGAATCTCCAAA
PHLDB2	Forward	GGAAAGACTCAGGGAGCAGG
	Reverse	AAGCGACTGTCAGGCTTTGT
SNTB1	Forward	TCCACAGAGACCGGAAAAGC
	Reverse	CCGTGTGCTTAGCATCTGGA
TERF1	Forward	TGCTAAGTGAAAAATCATCAACCTT
	Reverse	TTGTTCTTGTCCTTTTGCTTTCT
PIGC	Forward	GCCCCGAAGCGGGAAAAAG
	Reverse	AAGAGAGACTGTGAACCCGAGA
MYB	Forward	TGGGAAGGGGACAGTCTGAA
	Reverse	TCTTCTGATGCTGGTGCCAT
RPRML	Forward	TGGGGTGAGGGTTCTTACCA
	Reverse	CCGCAGTCCCAAATTGCATC
DTL	Forward	CCTCTTTCAAGACGAGAATACCTT
	Reverse	TCCCATACTTTGATTATCCCATC
GRM8	Forward	TTGGCTGCAAGTTAGGATCA

	Reverse	CATAAGATGAATCCCGAGCAA
LURAP1L	Forward	AGCTTCACCTCCTCAGGCAA
	Reverse	CACTTGATGGACTCGATGCTCT
CAMP	Forward	TCGGATGCTAACCTCTACCG
	Reverse	GTCTGGGTCCCCATCCAT
SDR16C5	Forward	AAGAGTGCACGCCTATACCTG
	Reverse	GAAACATCGCCGACTTCTTT
KRT80	Forward	CACCTACAGGAAGCTGGTGG
	Reverse	GTTTTGCACCTGGACTGCAC
CNTN1	Forward	TGGAGGAGATGGAGTGGTGT
	Reverse	GCAGCAGTAAGCCGAGAAGA
GAPDH	Forward	AGCCACATCGCTCAGACAC
	Reverse	GCCCAATACGACCAAATCC

Primer sets were designed by Universal Probe Library from Roche or Primer Design Tool from NCBI.

Table S2. Down-regualted genes after FRMD5 knockdown

Gene ID	Microarray Fold Change	
	siFRMD5-#02	siFRMD5-#03
*TERF1**	-24.0	-20.3
FRMD5	-10.3	-12.4
*PIGC**	-2.6	-5.7
*MYB**	-5.4	-2.2
*RPRML**	-4.4	-2.3
*DTL**	-3.3	-3.1
LRRC14B	-3.2	-3.1
MTHFD2P1	-3.0	-3.4
BEX4	-2.7	-3.3
CRY1	-3.0	-2.9
ZNF486	-3.3	-2.6
SLC2A13	-2.2	-3.6
CHAF1A	-3.4	-2.2
CLSPN	-3.2	-2.4
MCM10	-3.1	-2.3
FZD3	-2.8	-2.6
ZNF678	-2.9	-2.5
CDT1	-2.8	-2.4
SMC1A	-2.1	-3.1
BRIP1	-2.9	-2.2
EXO1	-2.8	-2.3
ARHGEF25	-2.3	-2.9
AATK	-2.6	-2.3
PPP1R3E	-2.2	-2.7
LOC728819	-2.5	-2.4
CDC45	-2.6	-2.2
SOX12	-2.1	-2.6
LOC102724747	-2.6	-2.1
XRCC2	-2.4	-2.2
FGFBP2	-2.3	-2.1
POLA2	-2.1	-2.3
GPSM1	-2.3	-2.1
FAM122B	-2.0	-2.3

ARHGEF10	-2.1	-2.1
LINC00706	-2.2	-2.0
AMIGO1	-2.1	-2.1

Genes whose expression levels were down-regulated more than two-fold by the FRMD5 siRNA compared with control siRNA were selected from the genes whose expression levels were consistently changed by β-catenin siRNA in HCT116 cells. Genes with "*" were validated by qPCR.

Table S3. Up-regualted genes after FRMD5 knockdown

Gene ID	Microarray Fold Change	
	siFRMD5-#02	siFRMD5-#03
*GRM8**	6.7	4.0
*LURAP1L**	4.8	3.8
*CAMP**	2.1	6.4
*SDR16C5**	2.0	6.1
*KRT80**	4.9	3.0
CNTN1	3.6	4.2
C2	4.3	3.5
TMSB4X	4.3	3.4
LRRC66	3.9	3.3
CCRL2	2.7	4.4
DAPP1	3.8	3.3
WNT7A	2.3	4.8
CCDC85A	2.0	4.9
WNT7B	2.3	4.3
LINC00707	2.1	4.5
LOC102723652	4.0	2.6
CCNI2	2.8	3.5
AQP3	2.8	3.5
BMF	3.3	2.6
POFUT1	3.8	2.1
PPP1R1B	2.3	3.6
KRT86	3.1	2.5
FTL	3.2	2.4
AKR1B10	2.6	3.0
LOC101928880	2.9	2.6
TSPAN8	2.1	3.3
MKNK2	3.3	2.0
TCHH	2.1	3.2
AKR1B10	2.3	3.0
LOC653712	2.9	2.4
CALHM3	2.9	2.4
MINOS1	3.1	2.1
LAMA3	2.8	2.5

LEFTY1	2.7	2.5
PLXDC1	2.9	2.2
AKR1B15	2.1	3.0
EGLN3	2.2	2.8
PNRC1	2.5	2.4
SCEL	2.6	2.2
NEK10	2.3	2.5
CFAP70	2.8	2.0
RASAL2	2.3	2.5
ANGPTL4	2.3	2.4
SLC46A3	2.0	2.6
CDRT1	2.4	2.2
ANK2	2.3	2.3
RAB19	2.5	2.0
MAP6	2.1	2.4
SLC35D1	2.1	2.3
VSTM2A-OT1	2.0	2.3
PLAU	2.1	2.2
FAM214B	2.2	2.0
GALNT9	2.1	2.0

Genes whose expression levels were up-regulated more than two-fold by the FRMD5 siRNA compared with control siRNA were selected from the genes whose expression levels were consistently changed by β-catenin siRNA in HCT116 cells. Genes with "*" were validated by qPCR.