Protein Arginine Methyltransferase 5 (PRMT5) Promotes Survival of Lymphoma Cells via Activation of WNT/β-CATENIN and AKT/GSK3β Proliferative Signaling

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Supporting Information Table of Content:

Supplementary experimental procedures

Enzyme-linked immunosorbent assay (ELISA)

Human WIF1 DuoSet ELISA (Cat # DY134) was performed at room temperature as specified by the manufacturer (R&D systems, Minneapolis, MN, USA). A ninety six-well microplate was coated with 0.2 μg of anti-WIF1 antibody per well overnight, and washed three times with 400 μl of Wash Buffer before blocking with 300 μl of Reagent Diluent for 1-2 h. Next, the wells were washed three times with Wash buffer, and incubated 1-2 h with 100 μl of culture medium taken 72 h post-infection from uninfected lymphoma cells or lymphoma cells infected with lentivirus for expression of either control shGFP or shPRMT5 RNA. Similarly, when lymphoma cells were treated with either DMSO or CMP-5, 100 μl of culture medium was added to each well 48 h post-treatment and incubated for 1-2 h. All wells were washed, and incubated for an additional 2 h with 100 μl of alkaline-phosphatase-labeled secondary antibody, which was diluted in Reagent Diluent. After washing, a 100 μl of substrate solution was added to each well for 20 min, and reactions were stopped with 50 μl of Stop Solution before the concentration of WIF1 was determined by measuring the optical density using a microplate reader set to 450 nm.

Antibody neutralization assay

To assess the contribution of WIF1 to WNT/β-CATENIN target gene repression, we infected lymphoma cells with lentivirus that expresses sh-PRMT5 RNA or we treated them with CMP-5 before harvesting the medium. Approximately 1 or 10 μg of anti-WIF1 neutralizing antibody was incubated with 1 ml of medium harvested from either CMP5-treated or shPRMT5-infected JeKo, Pfeiffer, or SUDHL-2 cells for 1 h at 37°C. After incubation, treated or non-treated medium was added back to an equal number (0.5×10^6) of JeKo, Pfeiffer, or SUDHL-2 cells and

incubated at 37°C for 48 h. Whole cell extracts were prepared from either control lymphoma cells or lymphoma cells treated with or without anti-WIF1 antibody, and analyzed by Western blotting.

Figure S1

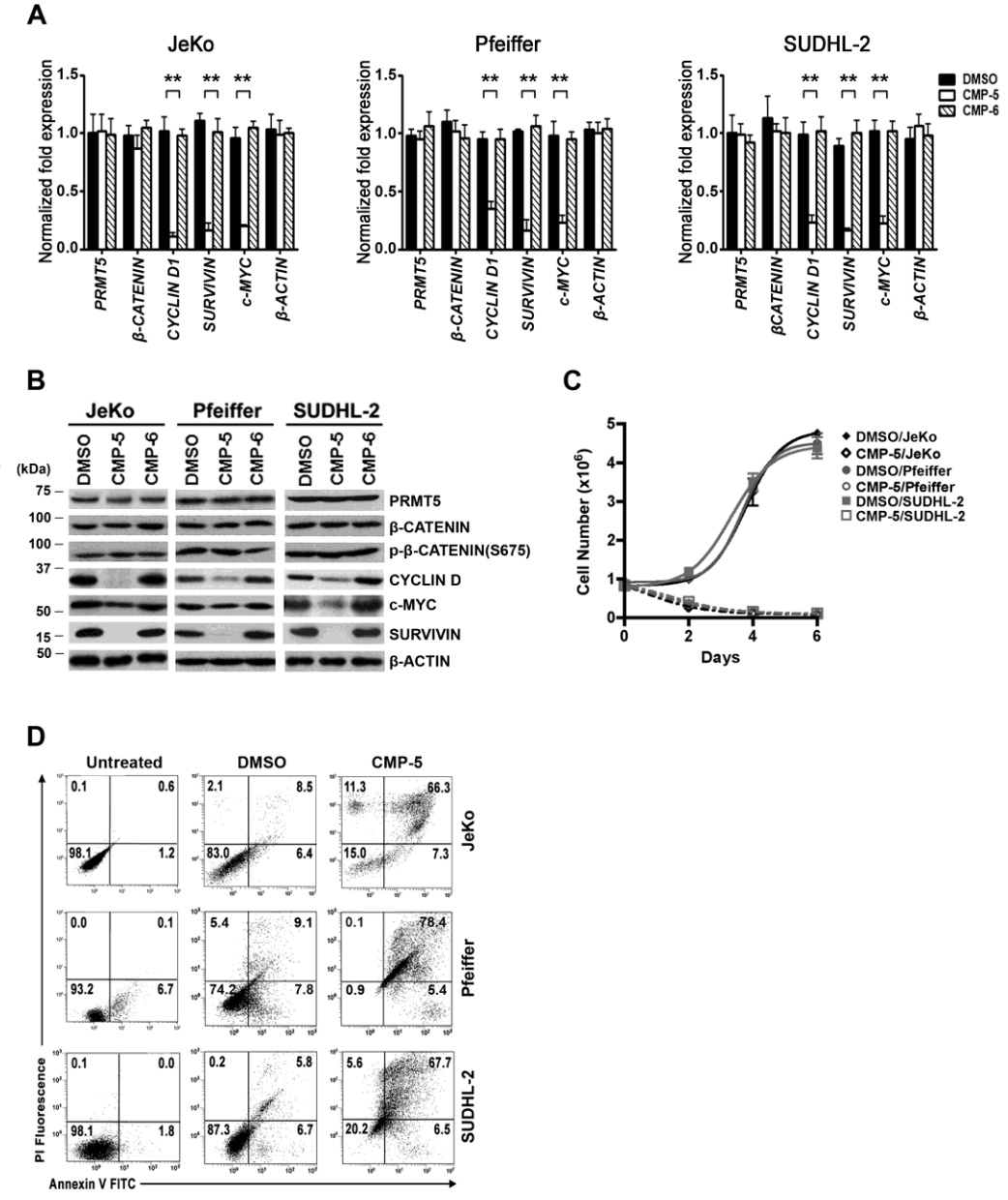
A
JeKo
Pfeiffer
SUDHL-2
Normalized fold expression
DMSO
CMP-5
CMP-6
PRMT5
β-CATENIN
CYCLIN D1
SURVIVIN
c-MYC
β-ACTIN
B
JeKo
Pfeiffer
SUDHL-2
(kDa)
PRMT5
β-CATENIN
p-β-CATENIN(S675)
CYCLIN D
c-MYC
SURVIVIN
β-ACTIN
C
Cell Number (x10^6)
Days
DMSO/JeKo
CMP-5/JeKo
DMSO/Pfeiffer
CMP-5/Pfeiffer
DMSO/SUDHL-2
CMP-5/SUDHL-2
D
Untreated
DMSO
CMP-5
JeKo
Pfeiffer
SUDHL-2
PI Fluorescence
Annexin V FITC

Figure S1. PRMT5 inhibition represses WNT/β-CATENIN target gene expression and induces lymphoma cell death. A) Levels of *PRMT5*, *β-CATENIN*, *CYCLIN D1*, *c-MYC*, and *SURVIVIN* mRNAs were measured by real time RT-PCR using total RNA from JeKo, Pfeiffer, and SUDHL-2 cells treated for 48 h with either DMSO, CMP-5, or CMP-6. Specific primers and probe sets were used to detect each of the indicated genes. This experiment was conducted using three biological replicates with three technical replicates, and the values represent mean ± SD. **B)** RIPA extracts (20 μg) were prepared from the indicated lymphoma cells treated with either DMSO, CMP-5, or CMP-6, and analyzed by immunoblotting. This experiment was conducted two times, and β-ACTIN is included to show equal loading. β-ACTIN loading control and PRMT5 in Supplemental Figures 1B and 3B are the same since this data resulted from the same experiment. **C)** Proliferation of lymphoma cells treated with either DMSO or CMP-5 was determined by seeding 8×10^5 cells in each well and counting the number of viable cells every 2 days for 6 days. This experiment was conducted using two biological replicates with three technical replicates, and the values are plotted as mean ± SD. **D)** Lymphoma cells were treated with either DMSO or CMP-5, and cell death was determined by FACS analysis using propidium iodide (PI)/Annexin V staining as described in experimental procedures. This experiment was performed using two biological replicates with three technical replicates.

Figure S2

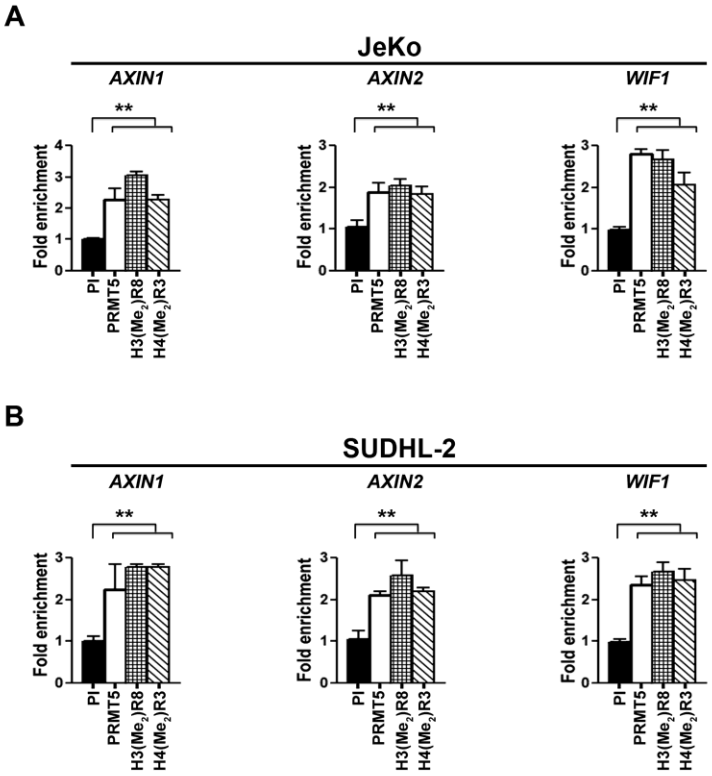
A
JeKo
AXIN1
AXIN2
WIF1
**
Fold enrichment
PI
PRMT5
H3(Me2)R8
H4(Me2)R3
B
SUDHL-2
AXIN1
AXIN2
WIF1
**
Fold enrichment
PI
PRMT5
H3(Me2)R8
H4(Me2)R3

Figure S2. PRMT5 recruitment to the *AXIN1*, *AXIN2* and *WIF1* promoters is increased in JeKo and SUDHL-2 lymphoma cells. Cross-linked chromatin from either JeKo (**A**) or SUDHL-2 (**B**) cells was immunoprecipitated using pre-immune (PI), anti-PRMT5, anti-H3(Me_2)R8, or anti-H4(Me_2)R3 antibody. *AXIN1*, *AXIN2*, and *WIF1* promoter sequences were detected by real time PCR. Fold enrichment with each antibody was calculated relative to the PI sample, and ChIP assays were conducted using two biological replicates with three technical replicates. Values in each graph represent the mean ± SD.

Figure S3

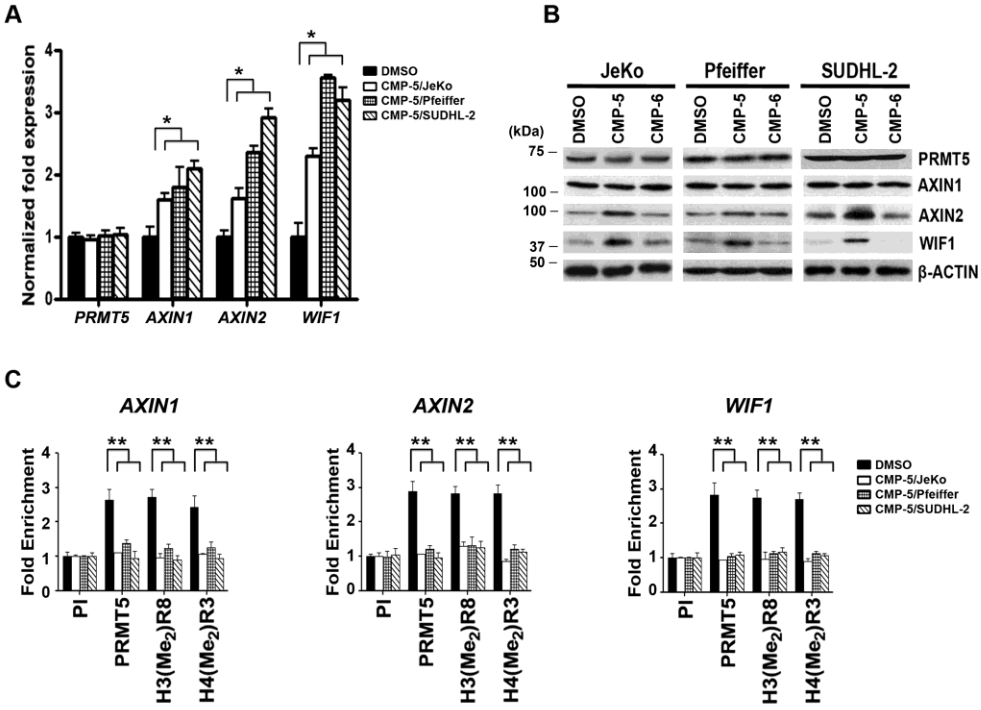
A
Normalized fold expression
DMSO
CMP-5/JeKo
CMP-5/Pfeiffer
CMP-5/SUDHL-2
PRMT5
AXIN1
AXIN2
WIF1
B
JeKo
Pfeiffer
SUDHL-2
DMSO
CMP-5
CMP-6
(kDa)
75
100
100
37
50
PRMT5
AXIN1
AXIN2
WIF1
β-ACTIN
C
AXIN1
AXIN2
WIF1
Fold Enrichment
PI
PRMT5
H3(Me2)R8
H4(Me2)R3
DMSO
CMP-5/JeKo
CMP-5/Pfeiffer
CMP-5/SUDHL-2

Figure S3. PRMT5 inhibition abolishes its recruitment and enrichment of its epigenetic marks, and reactivates *AXIN2* and *WIF1* expression. A) Levels of *PRMT5*, *AXIN1*, *AXIN2*, and *WIF1* mRNAs were measured by real time RT-PCR using total RNA from lymphoma cells treated with either DMSO or CMP-5 48 h post-treatment. Values in this graph represent the average from three biological replicates with three technical replicates, and are reported as mean ± SD. *18S* rRNA was used as an internal control. * indicates *P* values < 0.05. **B)** RIPA extracts (20 μg) prepared from the indicated lymphoma cells treated with either DMSO, CMP-5, or CMP-6, were analyzed by immunoblotting 48 h post-infection. β-ACTIN serves as a loading control. β-ACTIN loading control and PRMT5 in Supplemental Figures 1B and 3B are the same since this data resulted from the same experiment. **C)** Cross-linked chromatin from the indicated lymphoma cells treated with either DMSO or CMP-5 was immunoprecipitated using anti-PRMT5, anti-H3(Me_2)R8, or anti-H4(Me_2)R3 antibody, and promoter sequences were detected by real time PCR. The data points in each graph represent the mean ± SD from two biological replicates with three technical replicates each.

Figure S4

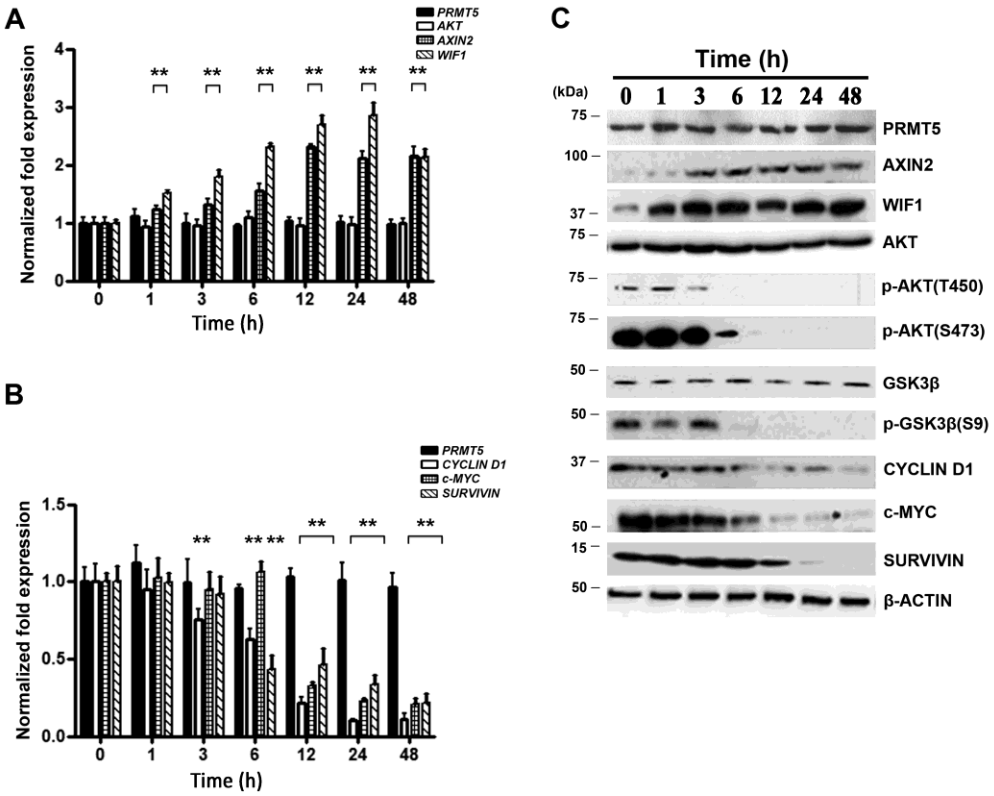
A
PRMT5
AKT
AXIN2
WIF1
Normalized fold expression
Time (h)
B
PRMT5
CYCLIN D1
c-MYC
SURVIVIN
Normalized fold expression
Time (h)
C
Time (h)
(kDa)
0 1 3 6 12 24 48
PRMT5
AXIN2
WIF1
AKT
p-AKT(T450)
p-AKT(S473)
GSK3β
p-GSK3β(S9)
CYCLIN D1
c-MYC
SURVIVIN
β-ACTIN

Figure S4. PRMT5 inhibition reactivates *AXIN2* and *WIF1* gene expression, and inactivates AKT/GSK3β and WNT/β-CATENIN signaling. A-B) Pfeiffer cells were treated with either DMSO or CMP-5, and mRNA levels of *PRMT5, AKT, AXIN2, WIF1, CYCLIN D1, c-MYC* and *SURVIVIN* were measured by real time RT-PCR at different time points using *18S* rRNA as internal control. Values represent the mean ± SD from three biological replicates with three technical replicates each. **C)** RIPA extracts (20 μg) were prepared at the indicated time points from control untreated (0 h) or CMP5-treated Pfeiffer cells, and analyzed by Western blotting. β-ACTIN was detected to show equal loading.

Figure S5

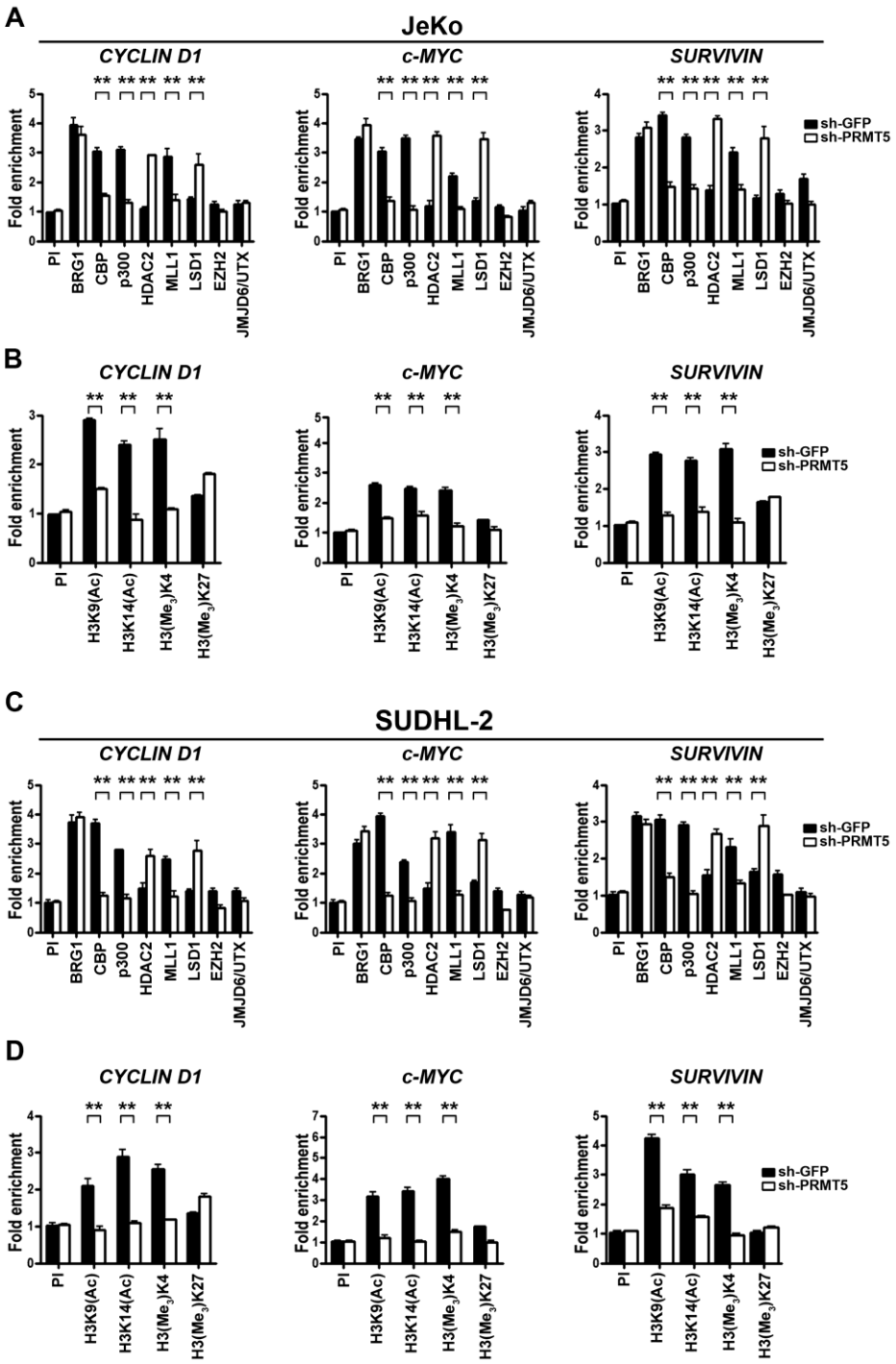
A
JeKo
CYCLIN D1
c-MYC
SURVIVIN
Fold enrichment
sh-GFP
sh-PRMT5
PI
BRG1
CBP
p300
HDAC2
MLL1
LSD1
EZH2
JMJD6/UTX
B
H3K9(Ac)
H3K14(Ac)
H3(Me3)K4
H3(Me3)K27
C
SUDHL-2
D
**

Figure S5. PRMT5 knock down results in enhanced recruitment of co-repressors and decreased binding of co-activators to the *CYCLIN D1*, *c-MYC* and *SURVIVIN* promoters. **A-B)** JeKo cells were infected with lentivirus that expresses either control sh-GFP or sh-PRMT5, and cross-linked chromatin was prepared and immunoprecipitated using the indicated antibodies. *CYCLIN D1*, *c-MYC* and *SURVIVIN* promoter sequences were detected by real time PCR as described in Supplementary Figure 2S. **C-D)** SUDHL-2 cells were infected as described in panels A and B, and cross-linked chromatin was immunoprecipitated using the indicated antibodies. Promoter sequences of WNT/β-CATENIN target genes were also detected as described in panels A and B.

Figure S6

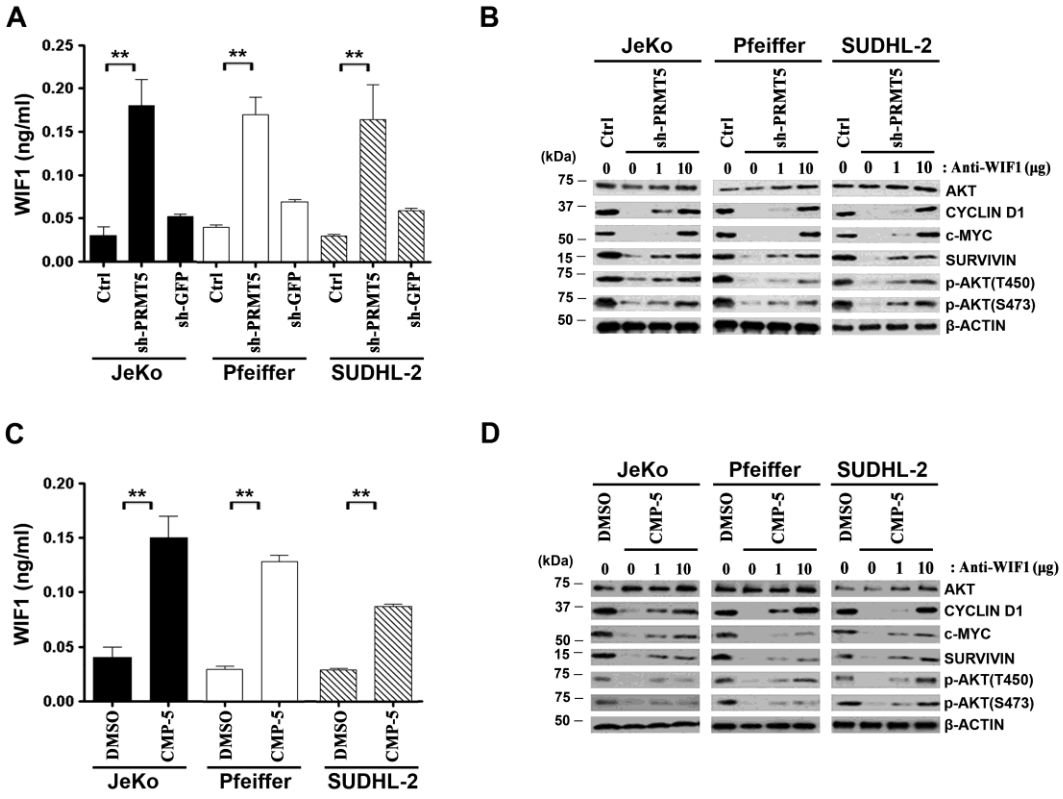
A
WIF1 (ng/ml)
0.25
0.20
0.15
0.10
0.05
0.00
**
Ctrl
sh-PRMT5
sh-GFP
JeKo
Pfeiffer
SUDHL-2
B
JeKo
Pfeiffer
SUDHL-2
Ctrl
sh-PRMT5
(kDa)
0 0 1 10 : Anti-WIF1 (μg)
75 – AKT
37 – CYCLIN D1
50 – c-MYC
15 – SURVIVIN
75 – p-AKT(T450)
75 – p-AKT(S473)
50 – β-ACTIN
C
WIF1 (ng/ml)
0.20
0.15
0.10
0.05
0.00
**
DMSO
CMP-5
JeKo
Pfeiffer
SUDHL-2
D
JeKo
Pfeiffer
SUDHL-2
DMSO
CMP-5
(kDa)
0 0 1 10 : Anti-WIF1 (μg)
75 – AKT
37 – CYCLIN D1
50 – c-MYC
15 – SURVIVIN
75 – p-AKT(T450)
75 – p-AKT(S473)
50 – β-ACTIN

Figure S6. PRMT5 knock down restores WIF1 expression, which can in turn inactivate AKT signaling and inhibit WNT/β-CATENIN target gene expression. Cell culture media collected from JeKo, Pfeiffer, and SUDHL-2 cells either infected with lentivirus that expresses control sh-GFP or sh-PRMT5 RNA (**A**), or treated with DMSO or CMP-5 (**C**) was analyzed for the presence of WIF1 protein by ELISA 72 h post-infection as described in Supplementary experimental procedures. This experiment was conducted with two biological replicates with three technical replicates each, and the data was plotted as mean ± SD. ** indicates *P* values < 10^{-3}. Increasing amounts of anti-WIF1 antibody was incubated with 1 ml of cell culture medium from lymphoma cells either infected with lentivirus that expresses sh-PRMT5 RNA (**B**), or treated with CMP-5 (**D**). After anti-WIF1 antibody neutralization, the medium was added back to an equal number of freshly seeded JeKo, Pfeiffer, or SUDHL-2 cells, and RIPA extracts (20 μg) were prepared from either control (Ctrl) uninfected or untreated, and either infected or treated lymphoma cells, and analyzed by immunoblotting using the indicated antibodies. β-ACTIN serves as a loading control.