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Supplementary Information for

PRDM1 silences stem cell related genes and inhibits proliferation of human colon tumor organoids

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SI Materials and Methods

Genomic Deletion

Pairs of sgRNA gBlock PCR product with Cas9 plasmids were co-transfected into RKO cells via Lipofectamine 3000. Eight hours after transfection, GFP positive cells were collected by FACS and genomic DNA was extracted. To detect the effective genome deletion cased by a pair of sgRNAs, genomic PCR was carried out with a pair of primers flanking the deletion.

Lentiviral Vector Construction, Lentivirus Production, Concentration and Titration

The coding regions of PRDM1α and PRDM1β (kindly provided by Dr.Kenneth L. Wright, Moffitt Cancer Center) were amplified by PCR with primers PRDM1α_ATG_F: CCGAATTCATGTTG GATATTTGCTTGGAAAAACGTGT; PRDM1β_ATG_F: CCGAATTCATGGAA AAGATCTATTCCAGAGGGGAGCTT; PRDM1_R: AGGATCCATTGGTTCAAC TGTTTCTTGTTTG and then cloned into lentiviral vector (pLVX-EF1alpha-AcGFP21-C1, Clontech) with EcoRI site. The sequences of PRDM1α and PRDM1β were confirmed by sequencing.

The day before transfection, 293T cells were seeded at 9×10^6 cells per 150-cm^2 dish. On the next day, sixty micrograms plasmid DNA was used for transfection of one 150-cm2 dish. The DNA cocktail contained 10.5 μg envelope-coding plasmid pMD.G, 19.5 μg of the packaging plasmid pSPAX2 and 30 μg of transgene vector plasmid by CaCl2 method according to procedure published(1). 48 hours after transfection, virus-containing supernatants derived from these 293T cultures were filtered through a 0.45 μm cellulose acetate filter (Millipore, Cat.No: SCHVU01RE). Then the virus supernatants concentrated using PEG6000 and concentrated virus were stored in -80 °C freezer. For virus titration, RKO cells were seeded at 1×10^5 per well of six-well plate. 8 hours after seeding, the concentrated viruses were diluted with RKO grow medium and add the different volume to well of six-well plate with 6 μg/ml of polybrene. Twenty-four hours after transduction, change the medium with fresh RKO cell grow medium. 48 hours after transduction, GFP positive cells number was measured by flow cytometery and virus titer was calculated.

Virus Transduction and Cell line Establishment

RKO cells were seeded at 2×10^5 per well of six-well plate. Eight hours after seeding, 3 MOI of PRDM1α or PRDM1β viruses were used to infect RKO cell with 6 μg/ml polybrene. Twelve hours after transduction, change the medium with fresh RKO cell grow medium. 48 hours after transduction, GFP positive cells were collected by flow cytometery and cultured.

Western blotting analyses

Cell lysates and organoids lysates were prepared using RIPA buffer (150mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS and 50mMTris-

HCl, pH 8.0), supplemented with protease inhibitor cocktail (cat#: P8340, Sigma). The concentration of protein was determined using Pierce BCA Protein Assay Kit (cat#: 23227, Thermo Scientific). 20 ug of denatured cell lysates were separated by electrophoresis on 10% or/and 7% SDS-PAGE, and then were transferred to hydrophobic PVDF. The blot was blocked with TBST (10mMTris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween-20) containing 5% non-fat dry milkand then incubated with primary antibody in TBST at 4 °C overnight. Afterwashing with TBST, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 hour at room temperature with constant agitation. Signals were raised with SuperSignal™ West Pico Chemiluminescent substrate (cat#: 34077, Thermo Scientific) and detected using a ChemiDoc™ MP imaging system (Bio-RAD). Primary antibodies for PRDM1 (recognize both PRDM1α and PRDM1β) (1:1000, cat#: 9115, CST), c-myc (1:1,000, cat#: 9402, CST), p53 (1:400, cat#: sc-126, Santa Cruz), β-actin (1:1,000, cat#: 4970, CST) and horseradish peroxidase linked secondary antibodies for mouse IgG (1:2,000, cat#: 7076, CST) and rabbit IgG (1:2,000, cat#: 7074, CST) were used.

Cell proliferation assays

RKO cells were plated at 2,000 cells per well of a 96-well plate. Eight replicates were performed for each condition. On days 0, 1, 2, 3, 4, 5 and 6, 700 uM rezasurin (Sigma) in PBS was added to 10% by volume to each treatment well. Cells were incubated for four hours in 37 °C, 5% CO2 and then fluorescence was measured on Thermo Fluorskan Ascent plate reader with 544 nm emission/590 nm excitation filters.

Cell cycle analysis

Cells were collected by trypsin and washed twice with DPBS. Cells were fixed in cold 70% ethanol at 4 °C overnight. The fixed cells were collected and incubated with 100 μg/ml RNase A (cat#: EN0531, Thermo Scientific) in DPBS with 0.1% Triton X-100 at 37 °C for 30 minutes, and then re-suspended in DPBS with propidium iodide at the concentration of 10 μg/ml for 30 minutes at room temperature. Cells were analyzed on a BD LSRII flow cytometer, and data was analyzed by FlowJo software.

Quantitative Reverse Transcription Polymerase Chain Reaction

The mRNA was purified from cell using Dynabeads® mRNA DIRECT™ Purification Kit (cat#: 61011, Invitrogen). The reverse transcription reaction was carried out with 20 ng of mRNA using SuperScript II Reverse Transcriptase (cat#: 18064014, Invitrogen). qPCR was performed using KAPA SYBR Fast Universal qPCR Master Mix (cat#: KK4602, Kapa Biosystems) and a Bio-Rad iCycler PCR System instrument. The relative quantities of genes were calculated using EEF1A1 as a reference. The primer sequences for each gene are provided in Table S1.

Edu Imaging

The proliferations of PRDM1 knockout RKO cells were evaluated by Click-iT Plus EdU Imaging Kit (cat#: C10638, Inivtrogen). In brief, cells were incubated with EdU for 16 hrs and fixed in 4% paraformaldehyde in DPBS and incubating for 15 minutes at room temperature. After washing 3 times with DPBS, the cells were permeabilized and blocked with blocking buffer (0.1% Triton-X 100 and 3% BSA in DPBS) for 20 min at room temperature. Click-iT Plus reaction cocktail were added and incubated with cells for 30 min. The cells were counterstained with Hoechst for 10 min. Images were taken by Olympus IX81 fluorescence microscope.

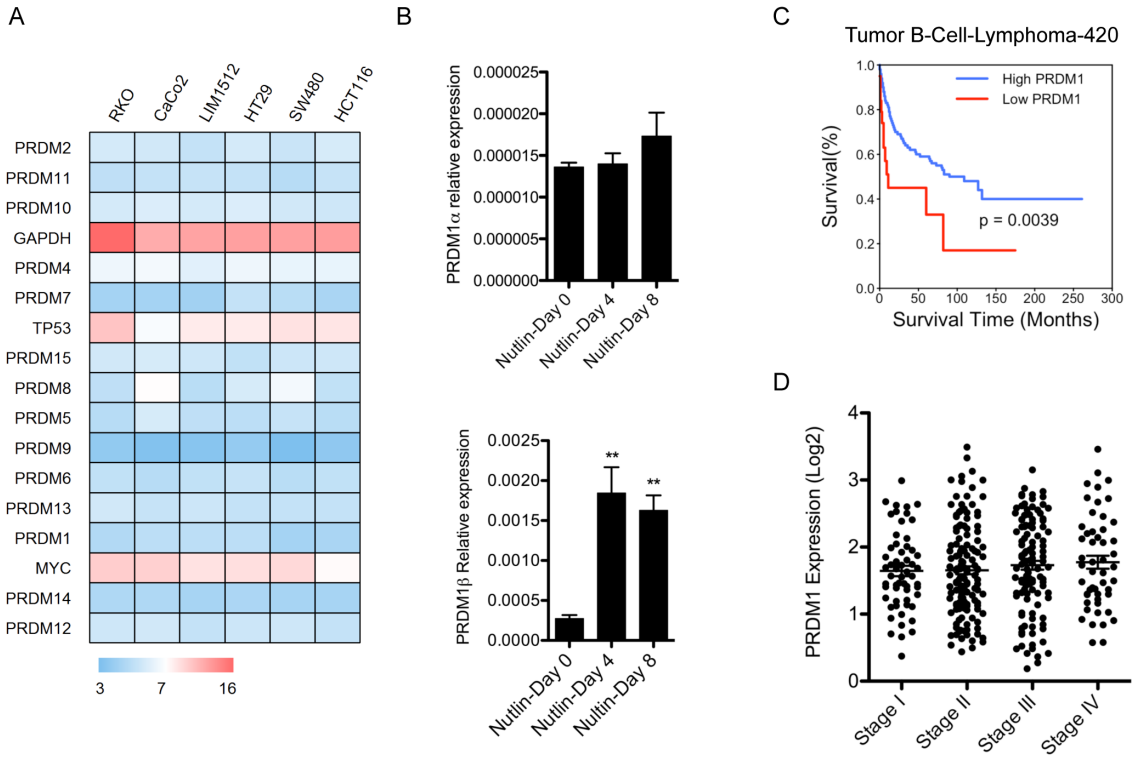


Fig. S1. PRDM1β is a p53 responsive gene and is correlated with disease free survival in colon cancers. (A) Datasets (GSE46549) were downloaded from NCBI GEO DataSets and normalized with RMA (Robust Multiarray Average) method. The gene expression level was showed as normalized value. The genes expression levels from PRDM family, TP53, MYC and GAPDH were showed. (B) Human colon normal organoids were treated with Nutlin3A and mRNA levels of PRDM1α and PRDM1β were evaluated by qPCR. Bar: Mean ± SD, Technical repeat. **, *P*< 0.01. (C) The Kaplan-Meier survival plots were obtained using R2 Genomics Analysis and Visualization Platform (https://hgserver1.amc.nl/cgi-bin/r2/main.cgi) and display the probability of disease-free survival of 414 B-cell lymphoma patients (Left panel, High PRDM1: n = 395; Low PRDM1: n = 19). *P* values on the plots are log-rank test for the comparisons of the low and high PRDM1 expression groups. (D) The expression levels of PRDM1 in colon cancer patients with different stage are showed. There is no significant different among different stage on PRDM1 expression.

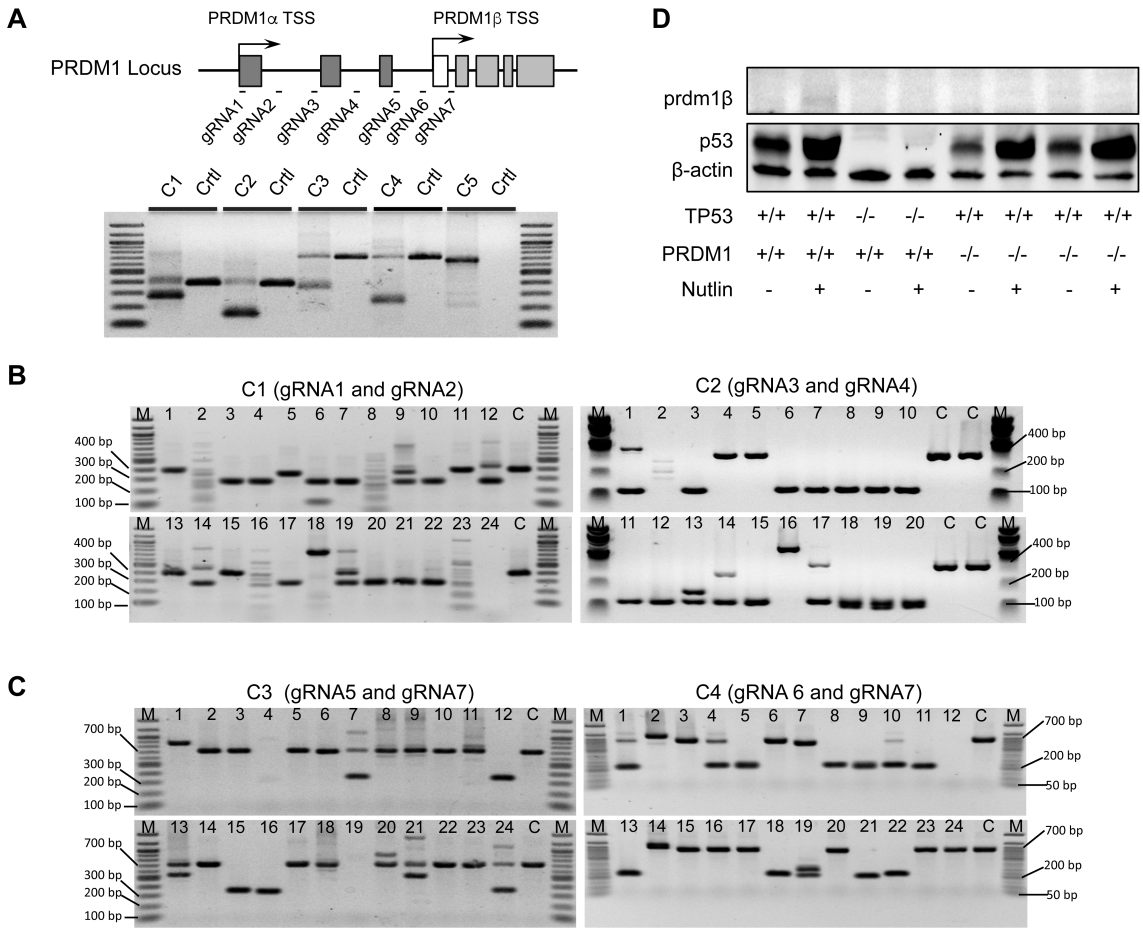


Fig. S2. Generating PRDM1 knockouts by using combination of different small gRNAs. (A) RKO cells were transfected with various combinations of gRNAs (as indicated). GFP positive cells were sorted with flow cytometry. Genomic DNA was extracted from GFP positive cells. PCR was carried out to detect genomic DNA deletion. C1: gRNA1 +gRNA2+Cas9; C2: gRNA3 +gRNA4 + Cas9; C, C3: gRNA5 + gRNA7 + Cas9; C4: gRNA6 + gRNA7 + Cas9; C5: gRNA1 + gRNA7+ Cas9. Crtl: Cas9 only. (C1--WT:382 bp, Predicted KO: 266 bp; C2--WT: 355 bp, Predicted KO: 133 bp; C3--WT: 692 bp, Predicted KO: 334 bp; C4--WT: 692 bp, Predicted KO: 242 bp; C5--WT: 13000bp (Can't be amplified by PCR), Predicted KO: 636 bp). (B) Agarose gel of PCR product of individual knockout clones from C1 and C2. (C) Agarose gel of PCR product of individual knockout clones from C3 and C4. (D) PRDM1β protein and p53 levels were determined by Western blot in TP53 wild-type RKO, TP53 knockout RKO, PRDM1 wild-type RKO and PRDM1 knockout RKO cells that were treated with DMSO or Nutlin3a for 96 hours. PRDM1β was activated only in TP53 wild-type RKO cells by Nutlin3A treatment.

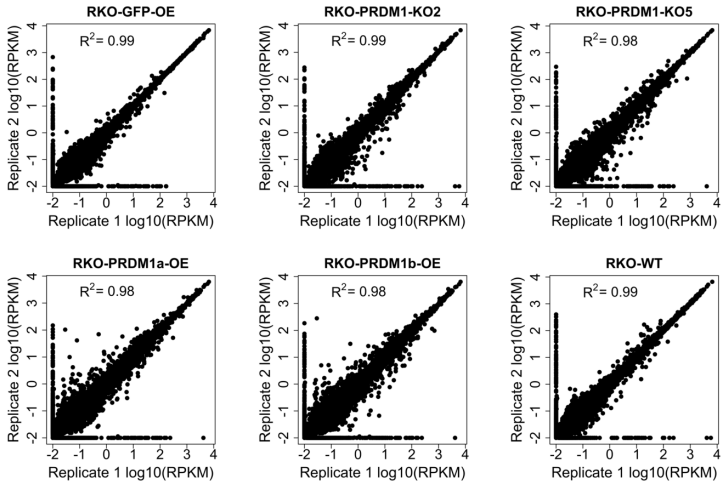
A

Samples	Description	PRDM1 Gene
RKO-WT	Wild type RKO cells	+/+
RKO-PRDM1-KO2	PRDM1 knock-out RKO cells	-/-
RKO-PRDM1-KO5	PRDM1 knock-out RKO cells	-/-
RKO-GFP-OE	GFP overexpressed RKO cells	+/+
RKO-PRDM1α-OE	PRDM1α over-expressed RKO cells	+/+
RKO-PRDM1β-OE	PRDM1β over-expressed RKO cells	+/+

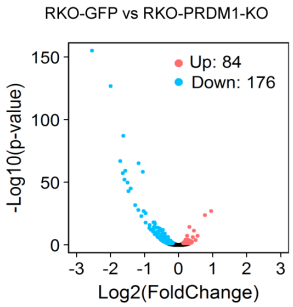
B

Samples	Replicates	Total Reads	Uniquely Aligned Reads	Uniquely Aligned Rate
RKO-WT	Replicate 1	6.74E+07	6.02E+07	89.30%
	Replicate 2	9.91E+07	8.97E+07	90.53%
RKO-PRDM1-KO2	Replicate 1	7.82E+07	7.08E+07	90.58%
	Replicate 2	6.35E+07	5.74E+07	90.34%
RKO-PRDM1-KO5	Replicate 1	5.35E+07	4.84E+07	90.56%
	Replicate 2	5.16E+07	4.66E+07	90.30%
RKO-GFP-OE	Replicate 1	4.60E+07	4.10E+07	89.19%
	Replicate 2	4.87E+07	4.37E+07	89.73%
RKO-PRDM1α-OE	Replicate 1	4.24E+07	3.79E+07	89.43%
	Replicate 2	3.62E+07	3.25E+07	89.64%
RKO-PRDM1β-OE	Replicate 1	3.42E+07	3.09E+07	90.27%
	Replicate 2	4.29E+07	3.87E+07	90.13%

C



D



E

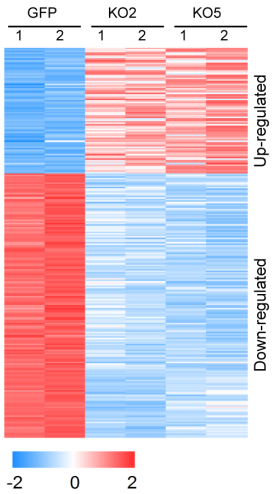


Fig. S3. Summary of RNA-seq information. (A) Description of samples used in this study. (B) Summary of total and uniquely aligned reads for each sample of PRDM1 RKO cell lines with two biological repeats used in this study. (C) Scatterplot evaluation of the reproducibility of two different biological repeats. (D)

Volcano plot comparing differential gene expression levels between PRDM1-KO and parental RKO-GFP cells. Red dots show up regulated genes and blue dots show repressed genes in PRDM1 overexpressed compared to PRDM1-KO cells (FDR $p < 0.05$). (E) Heat map showing common differentially expressed genes in each replicates between PRDM1-GFP and PRDM1-KO RKO cells.

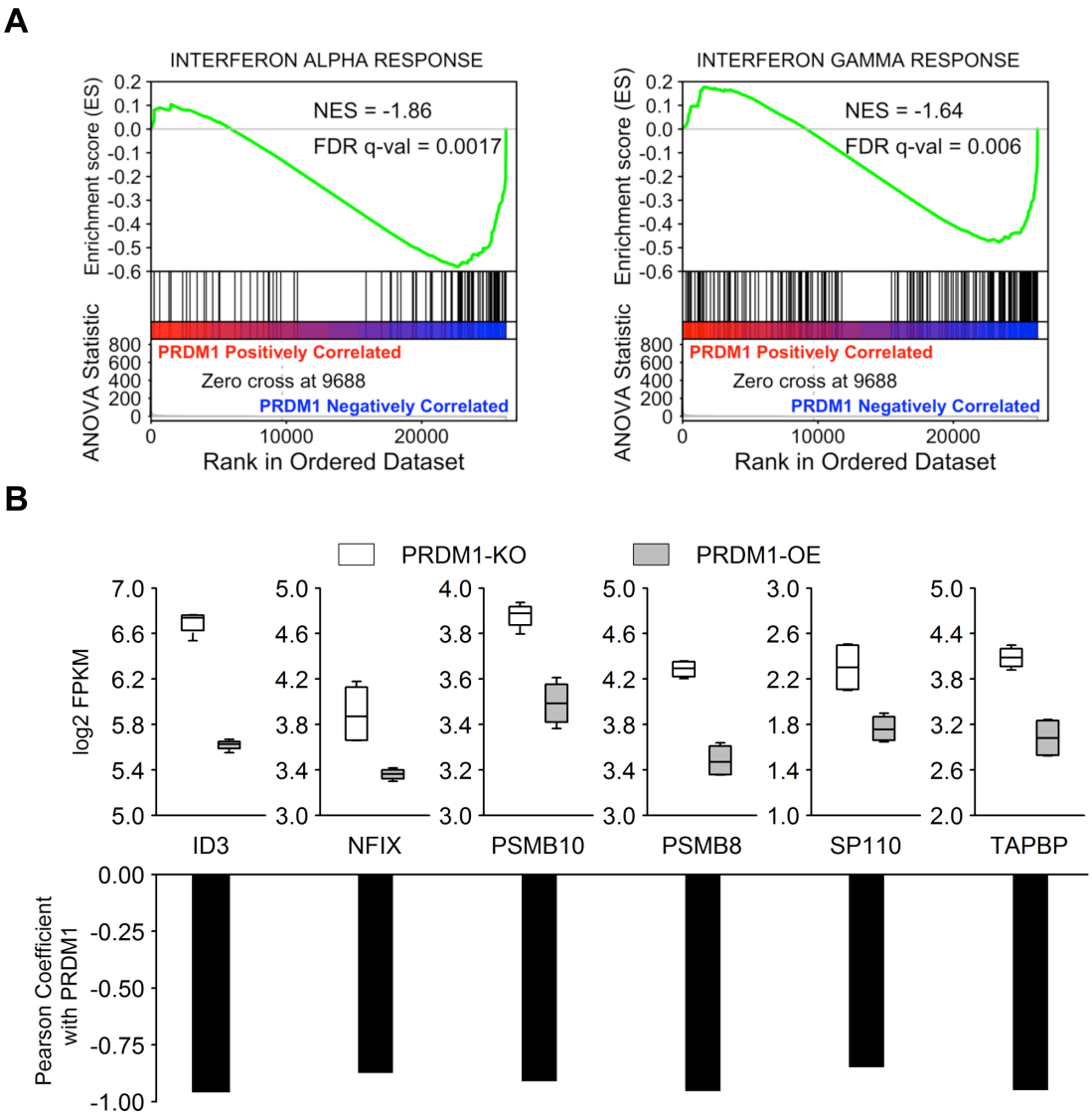


Fig. S4. **Known genes regulated by PRDM1**. (A) GSEA showing enrichment for interferon alpha and interferon gamma response gene sets in PRDM1 overexpressed versus knockout RKO cells. (B) PRDM1 regulates genes in RKO cells similar to what are regulated in B cells. Expression levels and Pearson correlation coefficients with PRDM1 of known PRDM1 target genes in B cells.

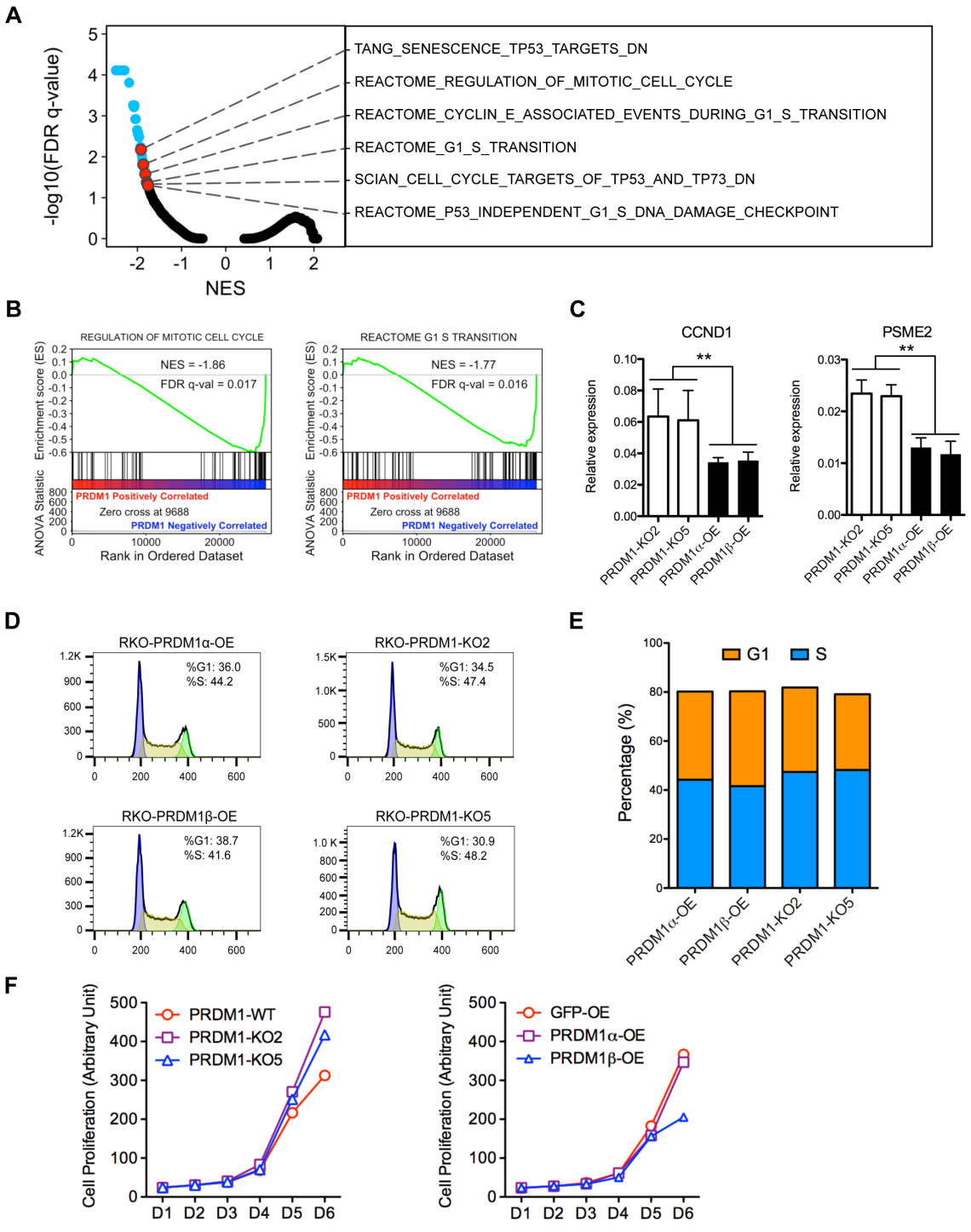


Fig. S5. Ectopic Expression of PRDM1 may lead to inhibition of G1/S transition. (A) Gene set enrichment analysis of the MSigDB C2ALL database. False discovery rate (FDR) versus normalized enrichment score (NES) for each evaluated gene set is shown. Colored dots indicate significantly enriched gene sets (FDR adjusted p value less than 0.05). (B) GSEA of mitotic cell cycle gene

sets and G1/S transition. (C) mRNA levels of CCND1 and PSME2 were determined by qPCR in PRDM1-KO and PRDM1-OE RKO cells. Bar: Mean ± SEM, technical triplicate, **, $p < 0.01$. (D) Representative flow cytometry histograms from RKO cell cycle analysis. (E) The stacked bar plot show the percentage of each stage of the cell cycle in D. (F). RKO cell proliferation levels were tested by resazurin assay at the indicated day.

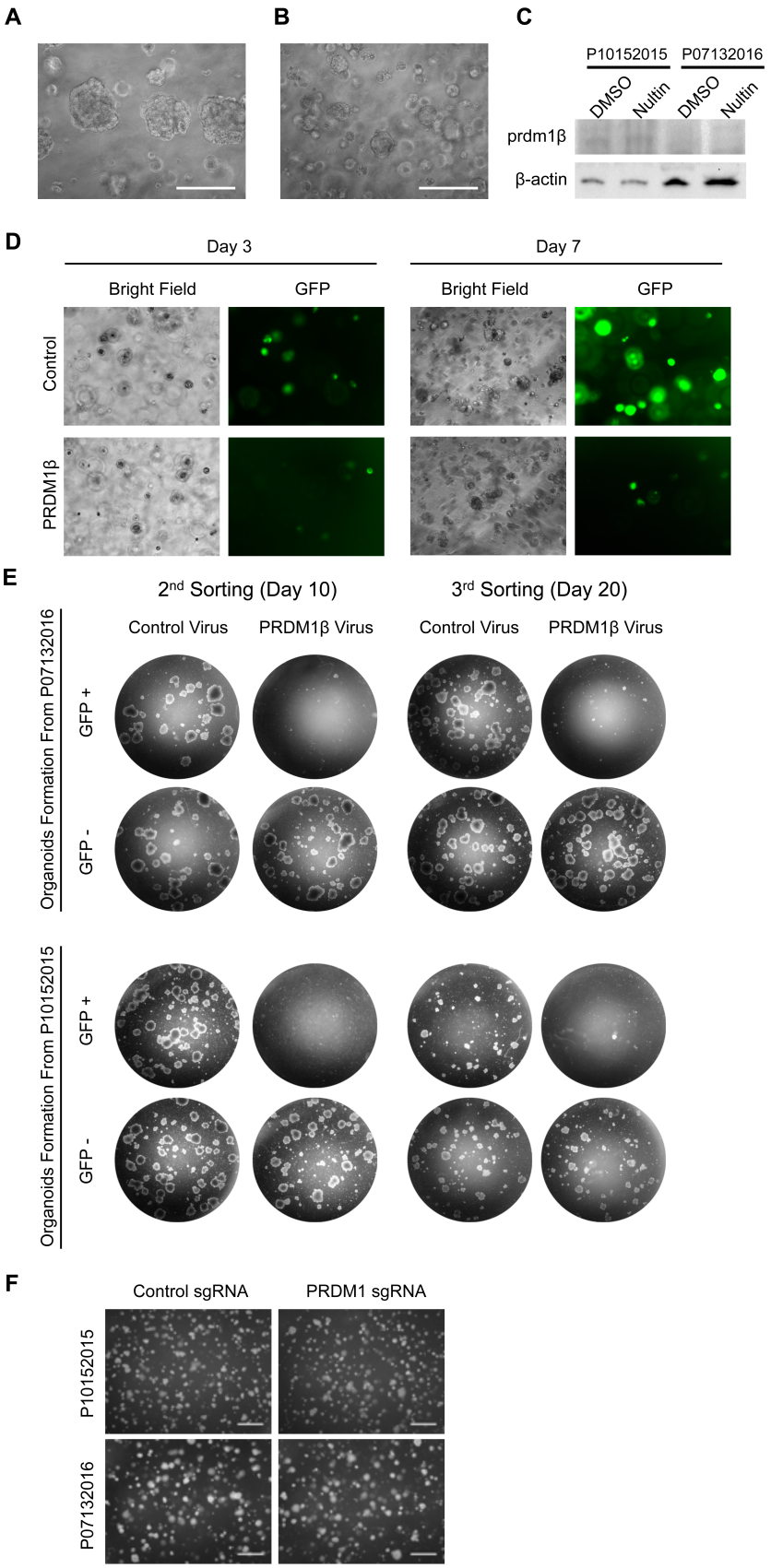

A
B
C
P10152015
P07132016
DMSO
Nultin
DMSO
Nultin
prdm1β
β-actin
D
Day 3
Day 7
Bright Field
GFP
Bright Field
GFP
Control
PRDM1β
E
2nd Sorting (Day 10)
3rd Sorting (Day 20)
Control Virus
PRDM1β Virus
Control Virus
PRDM1β Virus
Organoids Formation From P07132016
GFP +
GFP -
Organoids Formation From P10152015
GFP +
GFP -
F
Control sgRNA
PRDM1 sgRNA
P10152015
P07132016

Fig. S6. Organoids formation from PRDM1 overexpressing and knockout colon tumor organoids (A) Morphology of human colon tumor organoids from clinical surgery specimen P07132016. (B), Morphology of human colon tumor organoids from clinical surgery specimen P10152015. Scale Bar: 2 mm. (C) PRDM1β protein levels were determined by Western blot in organoids P10152015 and P07132016 that were treated with Nutlin3A or DMSO as control for 48 hours. (D) Tumor organoids P10152015 were infected with PRDM1β or control virus. Tumor organoids were digested with TrypLE to single cells, and then infected with PRDM1β or control virus. After transduction, cells were plated in matrigel. Pictures were taken at day 3 and day 7. Representative images are shown. (E) Representative images from organoid forming assay from P07132016 and P10152015 colon tumor organoids at different time points as indicated. GFP positive and negative cells from control or PRDM1 infected organoids were plated in Matrigel at density of 500 cells per well of 24-well plate and cultured for three to five weeks. Representative images are shown. (F) Representative images from organoid forming assay from PRDM1 knockout P07132016 and P10152015 organoids. Single cells from PRDM1 knockout or control organoids were plated in Matrigel at density of 1000 cells per well of 24-well plate and cultured for three weeks. Representative images are shown. Scale bar: 3 mm.

Table S1. Primer pairs used for qPCR and knockout

PCR primer Name	Sequence (5'-3')	Purpose
cDNA_PRDM1β_F	TTTGCCATTCACTGCAGTAGCA	qPCR
cDNA_PRDM1β_R	GTTGCTTTTCTCTTCATTAAAGCCG	qPCR
cDNA_MYC_F	CACCGAGTCGTAGTCGAGGT	qPCR
cDNA_MYC_R	TTTCGGGTAGTGGAAAACCA	qPCR
cDNA_PRDM1α_F	GGGAGAATGTGGACTGGGTA	qPCR
cDNA_PRDM1α_R	ATATCCGCATCCTCCATGTC	qPCR
GDF15_F2	AAGATTCGAACACCGACC	qPCR
GDF15_R2	GAGAGATACGCAGGTGCAG	qPCR
qPCR-CCND1-F	GGCGGATTGGAAATGAACTT	qPCR
qPCR-CCND1-R	TCCTCTCCAAAATGCCAGAG	qPCR
qPCR-PSME2-F	CAATCTTGGGGATCAGGTGT	qPCR
qPCR-PSME2-R	GAAAGTCCTGTCCCTGCTTG	qPCR
EEF1A1_F2	GGCATCGACAAAAGAACCAT	qPCR
EEF1A1_R2	CCCAGGCATACTTGAAGGAG	qPCR
PRDM1α_E1_F2	AAGTGCTGCCGTGACACTC	Knockout
PRDM1α _E1_R2	TGCACTAAAGCAGAAGCATGT	Knockout
PRDM1α_E2_F	GCATCTGCTTTTAAAGTCTTCAGAC	Knockout
PRDM1α_E2_R	TCAATAAACCAGAGGCTTACCTC	Knockout
PRDM1β_E1_F3	CTTCCCTCCTTTGCATTGAA	Knockout
PRDM1β_E1_R2	CCCCAGCACCTCTCAGAATA	Knockout
PRDM1α_E1_F3	AAGCCAGACGGTTAACACAGA	Knockout

Note: PRDM1α_E1_F2 and PRDM1α_E1_R2 for Combination 1 (WT:382 bp, Predicted KO: 266 bp); PRDM1α_E2_F and PRDM1α_E2_R for Combination 2 (WT: 355 bp, Predicted KO: 133 bp); PRDM1β_E1_F3 and PRDM1β_E1_R2 for Combination 3 (WT: 692 bp, Predicted KO: 334 bp) and Combination 4 (WT: 692 bp, Predicted KO: 242 bp); PRDM1α _ E1_F3 and PRDM1β_E1_R2 for Combination 5 (WT: 13000bp, Predicted KO: 636 bp)

Table S2. Reagents for Human Intestinal Stem Cell

Reagent Name	Catalog Number	Supplier
Advanced DMEM/F12	12634-010	Invitrogen
Penicillin/Streptomycin	15140-122	Invitrogen
GlutaMAX™ Supplement	35050-061	Invitrogen
HEPES (1 M)	15630-080	Invitrogen
N-2 Supplement (100X)	17502-048	Invitrogen
B-27® Supplement (50X), minus vitamin A	12587-010	Invitrogen
N-Acetyl-L-cysteine	A9165-5G	Sigma-Aldrich
[Leu15]Gastrin I	G9145	Sigma-Aldrich
EGF	PHG0311	Invitrogen
A-83-01	SML0788-5mg	Sigma
SB202190	S7067-5MG	Sigma-Aldrich
Noggin	120-10C	Peprotech
Nicotinamide	N0636-100G	Sigma-Aldrich
Matrigel	CB-40230C	Fisher
Y-27632	Y0503-1MG	Sigma-Aldrich
Primocin	ant-pm-1	Invivogen

References:

1. Kutner RH, Zhang XY, & Reiser J (2009) Production, concentration and titration of pseudotyped HIV-1-based lentiviral vectors. *Nat Protoc* 4(4):495-505.