SUPPLEMENTARY APPENDIX

DOT1L inhibition blocks multiple myeloma cell proliferation by suppressing IRF4-MYC signaling

Kazuya Ishiguro,[1,2] Hiroshi Kitajima,[2] Takeshi Niinuma,[2] Tadao Ishida,[3] Reo Maruyama,[4] Hiroshi Ikeda,[1] Toshiaki Hayashi,[1] Hajime Sasaki,[1] Hideki Wakasugi,[1] Koyo Nishiyama,[2] Tetsuya Shindo,[2] Eiichiro Yamamoto,[1,2] Masahiro Kai,[2] Yasushi Sasaki,[5] Takashi Tokino,[5] Hiroshi Nakase[1] and Hiromu Suzuki[2]

[1]Department of Gastroenterology and Hepatology, Sapporo Medical University School of Medicine; [2]Department of Molecular Biology, Sapporo Medical University School of Medicine; [3]Department of Hematology, Japanese Red Cross Medical Center, Tokyo; [4]Project for Cancer Epigenomics, Cancer Institute, Japanese Foundation for Cancer Research, Tokyo; [5]Department of Medical Genome Sciences, Research Institute for Frontier Medicine and Sapporo Medical University School of Medicine, Japan

 doi:10.3324/haematol.2018.191262

Received: February 15, 2018.

Accepted: August 29, 2018.

Pre-published: August 31, 2018.

Correspondence: hsuzuki@sapmed.ac.jp

Supplementary methods

Quantitative reverse transcription-PCR

Single-strand cDNA was prepared using PrimeScript RT Master Mix (Takara, Kusatsu, Japan), after which the integrity of the cDNA was confirmed by amplifying β-actin (*ACTB*). Quantitative reverse transcription-PCR (qRT-PCR) was carried out using SYBR Select Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) and a 7500 Fast Real-Time PCR System (Thermo Fisher Scientific). Primer sequences and PCR product sizes are listed in Supplementary Table 10.

Western blot analysis

Total proteins were extracted using M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Histones were then extracted using Triton Extraction Buffer (TEB) according to the protocol from Abcam (Cambridge, UK). Samples were separated using SDS-PAGE (12% acrylamide) and transferred to PVDF membranes (Bio-Rad, Hercules, California, USA). The membranes were then blocked using TBST with 5% BSA and incubated overnight with rabbit anti-Myc polyclonal Ab (1:1000 dilution, #9402; Cell Signaling Technology, Danvers, MA, USA), mouse anti-β-actin Ab (1:2000 dilution, #A5441; Sigma-Aldrich, St. Louis, MO, USA), rabbit anti-monomethyl histone H3K79 mAb (1:1000 dilution, #12522; Cell Signaling Technology), rabbit anti-dimethyl histone H3K79 mAb (1:1000 dilution, #5427; Cell Signaling Technology), rabbit anti-trimethyl histone H3K79 mAb (1:1000 dilution, #4260; Cell Signaling Technology), or rabbit anti-H3 mAb (1:2000 dilution, #4499; Cell Signaling Technology). Signals were detected using HRP-conjugated secondary antibodies (Cell Signaling Technology). Luminescent signals were detected using an ImageQuant LAS-4000 mini image reader (GE Healthcare Japan, Hino, Japan).

Lentiviral small hairpin RNA-mediated knockdown of DOT1L

Lentiviral small hairpin RNA (shRNA) vectors were produced using a Lenti-PacTM HIV Expression Packaging Kit (GeneCopoeia, Rockville, MD, USA) according to the manufacturer's instructions. Sequences of shRNA targeting DOT1L were designed by GeneCopoeia. For viral production, 293Ta cells were transfected with 2.5 μg of shRNA expression plasmid and 2.5 μg of Lenti-pac HIV mix using EndoFectin Lenti transfection reagent (GeneCopoeia). Media containing lentivirus was collected and passed through 0.45 μm Millex-HV membrane filters (Merck Millipore, Burlington, MA, USA). MM cells (2.5×10^5 cells) were mixed with 100 μl of lentiviral supernatant and 400 μl of medium in the presence of 8 μg/ml polybrene, after which the cells were centrifuged at 800 rpm for 2 h at room temperature. Four days after viral infection, GFP-positive cells were selected using a BD FACSAria II (BD Biosciences, Franklin Lakes, NJ,

USA) and subjected to qRT-PCR and cell viability assays.

Flow cytometry analysis

MM cells were treated for 3 or 6 days with 1 μM DOT1L inhibitor or with DMSO as described above, after which cells were stained with propidium iodide (Dojindo, Kumamoto, Japan) and a ApoScreen Annexin V Apoptosis Kit (Southern Biotech, Birmingham, AL, USA) according to the manufacturer's instructions. Flow cytometric analysis was then performed using a BD FACSCant II (BD Biosciences) with BD FACSDiva software (BD Biosciences). Data were analyzed using FlowJo software version 10 (FlowJo LLC, Ashland, OR, USA).

Gene expression microarray analysis

Gene expression was analyzed as described previously.[1] Briefly, 100 ng of total RNA were amplified and labeled using a Low-input Quick Amp Labeling kit One-color (Agilent Technologies, Santa Clara, CA, USA). The synthesized cRNA was hybridized to a SurePrint G3 Human GE microarray v2 (G4858A #39494; Agilent Technologies). The microarray data were imported to Gene Spring GX version 13 (Agilent Technologies). Gene ontology and pathway analyses were also performed using Gene Spring GX. The Gene Expression Omnibus accession number for the microarray data is GSE108661.

Chromatin immunoprecipitation sequencing and quantitative PCR

Chromatin immunoprecipitation (ChIP) was performed as described previously.[2] Briefly, 1×10^6 cells were treated for 10 min with 0.5% formaldehyde. After washing, the cells were resuspended in 110 μL of lysis buffer and sonicated. Chromatin was immunoprecipitated for 12 h at 4°C using 1 μL of rabbit anti-dimethyl histone H3K79 mAb (#5427; Cell Signaling Technology) or rabbit (DA1E) mAb IgG XP isotype control (#3900; Cell Signaling Technology) as a control. Before adding the antibody, 10 μL of each cell lysate was saved as an internal control for the input DNA. After washing, elution, reversal of the cross-links and DNA purification, the immunoprecipitated DNA was end-repaired and ligated to Ion-compatible barcode adapters (Thermo Fisher Scientific). Samples were then nick repaired and PCR amplified. After size selection and a quality check, samples were sequenced using an Ion Proton System (Thermo Fisher Scientific). The numbers of sequence reads in the ChIP-seq analysis are summarized in Supplementary Table 11. Sequencing data were mapped to the human genome (UCSC hg19), and peaks were called using MACS2.0 software (GitHub, San Francisco, CA, USA) with the default broad peak setting. To identify genes whose H3K79me2 status was altered by drug treatment, we used the bdgdiff tool in MACS2.0 software (GitHub). Differential peaks with a log 10 likelihood ratio greater than 0.8 were called and were annotated using HOMER (GitHub). Peaks within intergenic regions were excluded,

while those within protein coding genes were selected for further analysis. ChIP-quantitative PCR (ChIP-qPCR) was carried out using Power Up SYBR Green Master Mix (Thermo Fisher Scientific) and a 7500 Fast Real-Time PCR System (Thermo Fisher Scientific). For ChIP of DOT1L, a rabbit anti-DOT1L polyclonal antibody (A300-953A; Bethyl Laboratories, Montgomery, TX, USA) and a ChIP-IT High Sensitivity Kit (Active Motif Japan, Tokyo, Japan) were used according to manufacturer's instructions. DOT1L ChIP products were then analyzed by ChIP-qPCR or sequenced using a MiSeq system (Illumina, San Diego, CA, USA). Primer sequences and PCR product sizes are listed in Supplementary Table 10.

Mutation analysis

Targeted sequencing of 409 cancer-related genes was performed using an Ion Ampliseq Comprehensive Cancer Panel (Thermo Fisher Scientific) and an Ion Proton System (Thermo Fisher Scientific) as described.[3] Nucleotide variants were detected using Ion Reporter (Thermo Fisher Scientific).

Statistical analysis

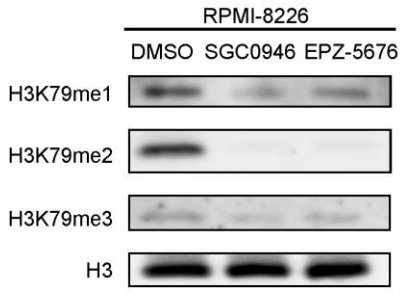
To analyze DOT1L expression in clinical samples, published datasets (GSE5900 and GSE6477) were obtained from the Gene Expression Omnibus.[4,5] Expression levels were analyzed using Student's t-test. Results of cell viability assays, qRT-PCR and ChIP-qPCR were analyzed using Student's t-test or one-way ANOVA. Values of $P < 0.05$ (two-sided) were considered significant. Data were analyzed using EZR version 1.32 (Saitama Medical Center, Jichi Medical University, Saitama, Japan).[6]

References

1. Kai M, Yamamoto E, Sato A, et al. Epigenetic silencing of diacylglycerol kinase gamma in colorectal cancer. Mol Carcinog. 2017;56(7):1743-1752.
2. Kamimae S, Yamamoto E, Kai M, et al. Epigenetic silencing of NTSR1 is associated with lateral and noninvasive growth of colorectal tumors. Oncotarget. 2015;6(30):29975-29990.
3. Ikeda H, Ishiguro K, Igarashi T, et al. Molecular diagnostics of a single drug-resistant multiple myeloma case using targeted next-generation sequencing. Onco Targets Ther. 2015;8:2805-2815.
4. Zhan F, Barlogie B, Arzoumanian V, et al. Gene-expression signature of benign monoclonal gammopathy evident in multiple myeloma is linked to good prognosis. Blood. 2007;109(4):1692-1700.
5. Chng WJ, Kumar S, Vanwier S, et al. Molecular dissection of hyperdiploid multiple myeloma by gene expression profiling. Cancer Res. 2007;67(7):2982-2989.

6. Kanda Y. Investigation of the freely available easy-to-use software 'EZR' for medical statistics. Bone Marrow Transplant. 2013;48(3):452-458.

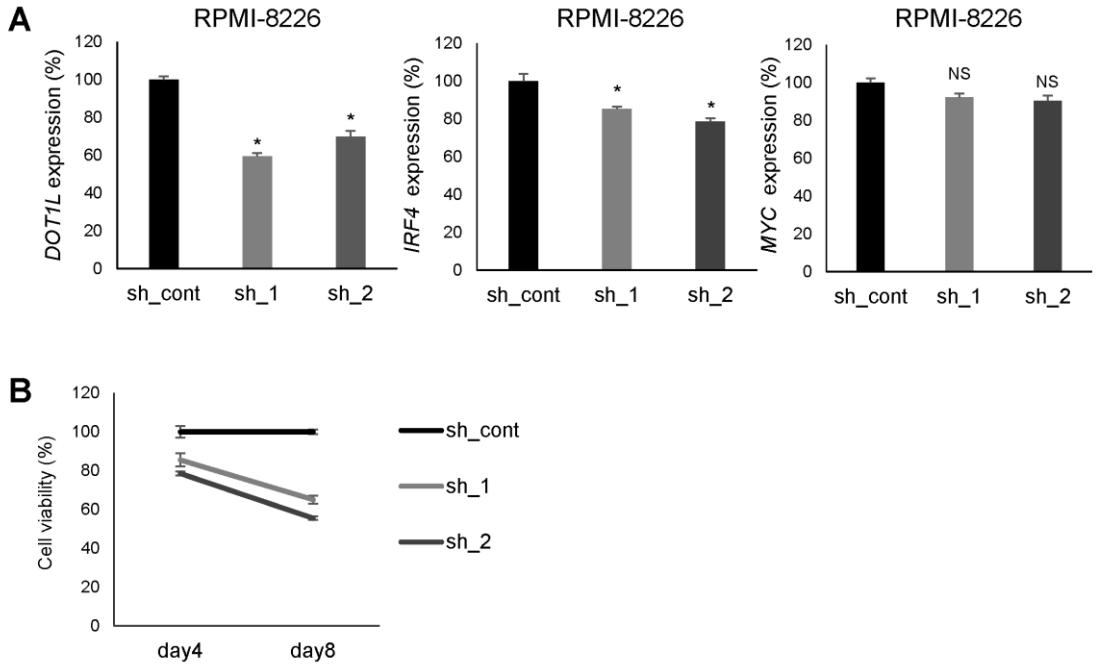
Supplementary Figure 1



Supplementary Figure 1

Western blot analysis of H3K79me1, H3K79me2 and H3K79me3 in RPMI-8226 cells treated with the indicated DOT1L inhibitors (1 μM, 3 days). Histone H3 is shown as a loading control. The exposure times for H3K79me1, H3K79me2, H3K79me3 and H3 were 10 s, 80 s, 110 s and 2 s, respectively. The results were confirmed in 2 independent experiments, and representative results are shown.

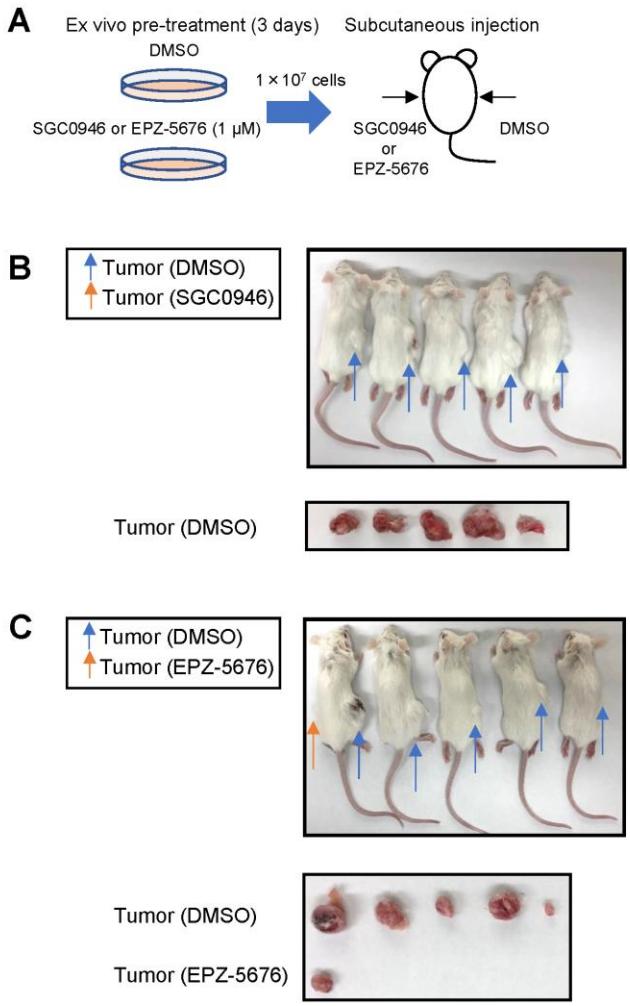
Supplementary Figure 2



Supplementary Figure 2

shRNA-mediated knockdown of DOT1L suppresses MM cell proliferation. (A) qRT-PCR analysis of *DOT1L*, *IRF4* and *MYC* in RPMI-8226 cells infected with lentiviral vectors containing control shRNA or 2 different shRNAs targeting DOT1L. Total RNA was extracted 4 days after infection. Shown are means of 3 replications; error bars represent SEMs. **P* <0.05. (B) Results of cell viability assays in RPMI-8226 cells with DOT1L knockdown. RPMI-8226 cells were infected with the indicated lentiviral shRNA vectors; cell viability assays were started 4 days after infection (9×10^4 cells/well in 6-well plate). Shown are means of 3 replications; error bars represent SEMs.

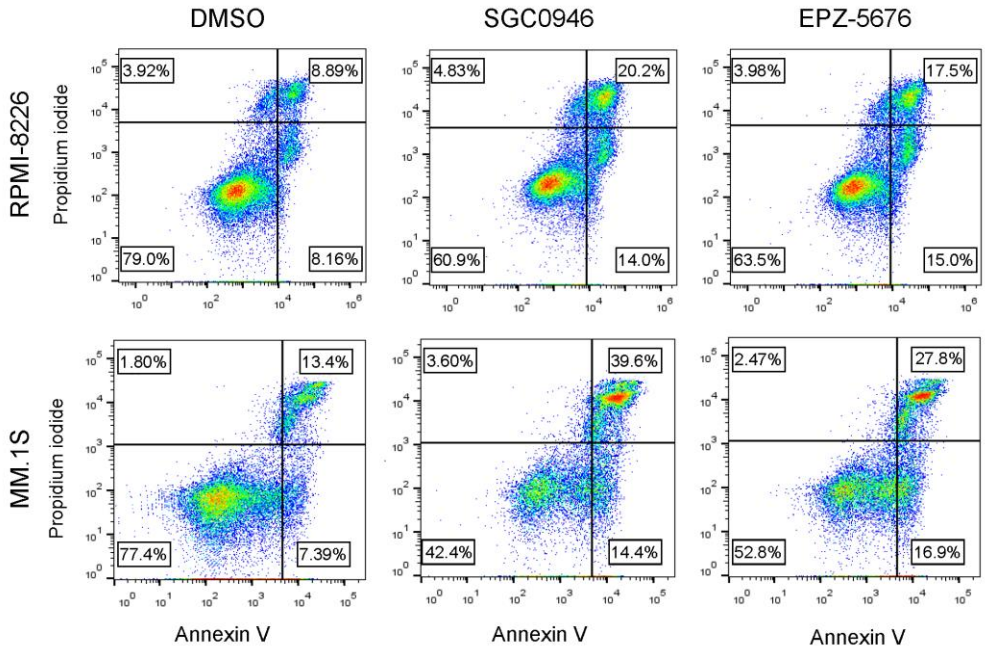
Supplementary Figure 3



Supplementary Figure 3

DOT1L inhibition suppresses tumor formation by MM cells in SCID mice. (A) Workflow of the ex vivo treatment study. RPMI-8226 cells were pretreated with 1 μM SGC0946 or EPZ-5676 or with DMSO for 3 days, after which 1×10^7 cells were subcutaneously injected into the left (SGC0946 or EPZ-5676) and right (DMSO) thighs of SCID mice. (B) Representative tumor xenografts derived from DMSO- and SGC0946-treated cells. Cells treated with SGC0946 did not form tumors. (C) Representative tumor xenografts derived from DMSO- and EPZ-5676-treated cells. Cells treated with EPZ-5676 formed a tumor in only one mouse.

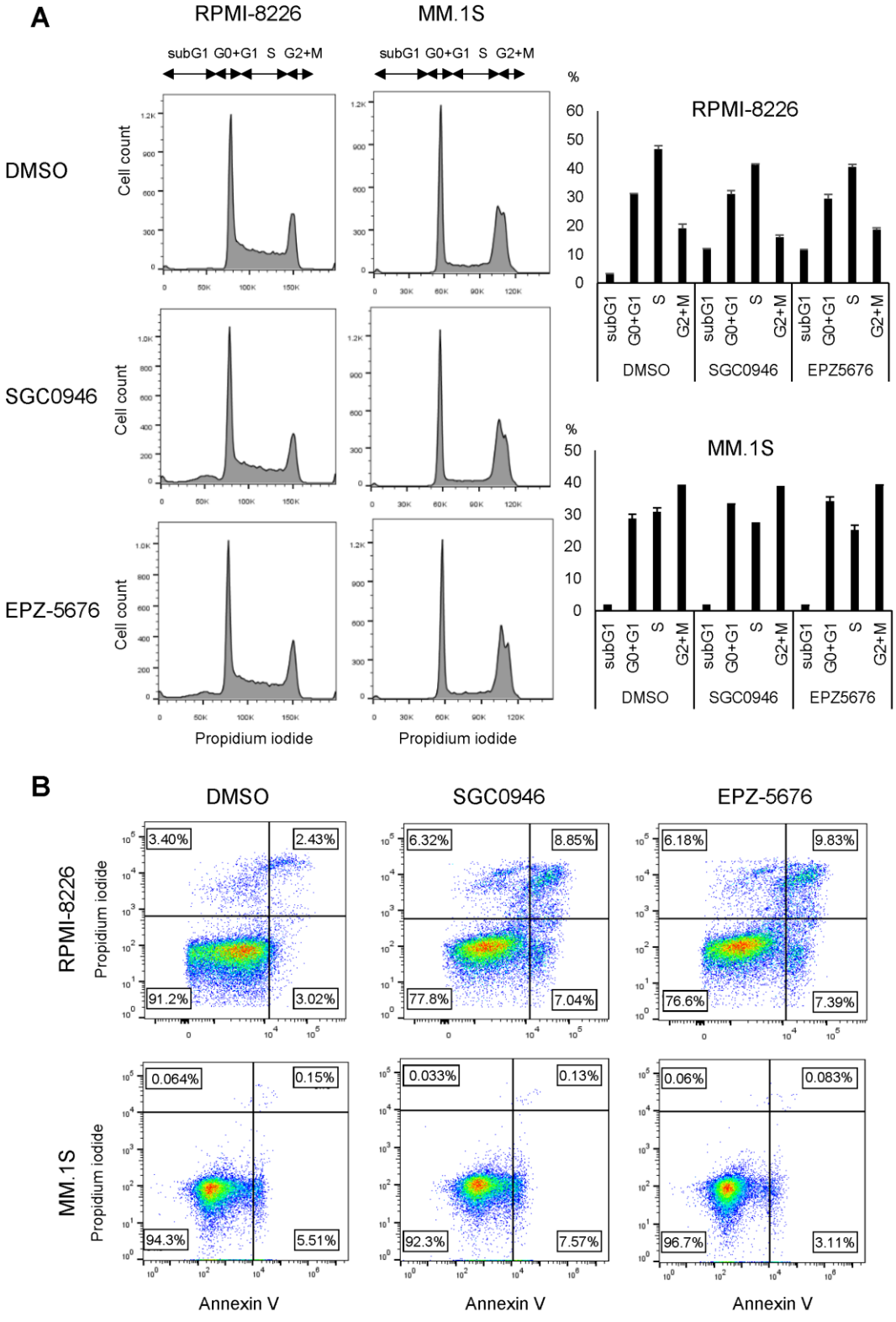
Supplementary Figure 4



Supplementary Figure 4

Effects of DOT1L inhibitors on apoptosis in MM cells. Results shown in Figure 3B were confirmed in independent experiments. Cells were treated with the indicated DOT1L inhibitors (1μM, 6 days), and apoptosis was analyzed.

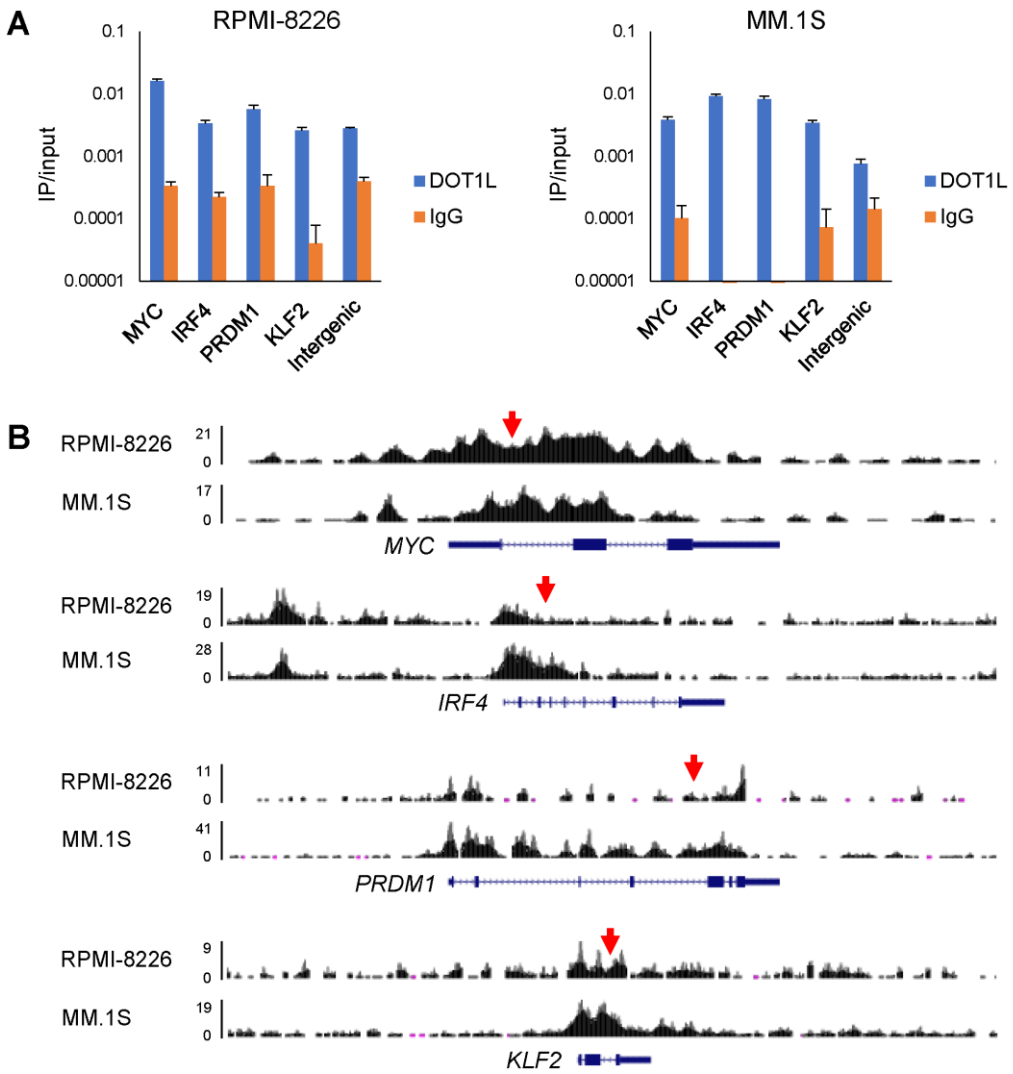
Supplementary Figure 5



Supplementary Figure 5

Effects of DOT1L inhibitors on cell cycling and apoptosis in MM cells. (A) Results of cell cycle analysis in MM cells treated with the indicated DOT1L inhibitors (1μM, 3 days). Representative results are shown on the left. Summarized results of 3 replications are shown on the right; error bars represent SEMs. (B) Results of apoptosis assays in MM cell lines treated with the indicated DOT1L inhibitors (1μM, 3 days). The results were confirmed in 3 independent experiments, and representative results are shown.

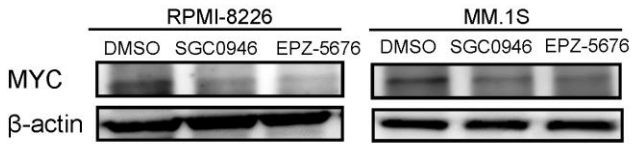
Supplementary Figure 6



Supplementary Figure 6

Enrichment of DOT1L in IRF4-MYC signaling genes in MM cells. (A) ChIP-qPCR analysis of DOT1L in IRF4-MYC signaling genes in indicated MM cell lines. Results are normalized to respective input DNAs. Shown are means of 3 replications; error bars represent SEMs. An intergenic region located 28 kb upstream of *KLF2* was used as a negative control. (B) ChIP-seq analysis of IRF4-MYC signaling genes in indicated MM cell lines. ChIP products used in (A) were analyzed by deep sequencing. The numbers on the vertical axis indicate the numbers of sequence reads. Regions analyzed by ChIP-PCR are indicated by red arrows.

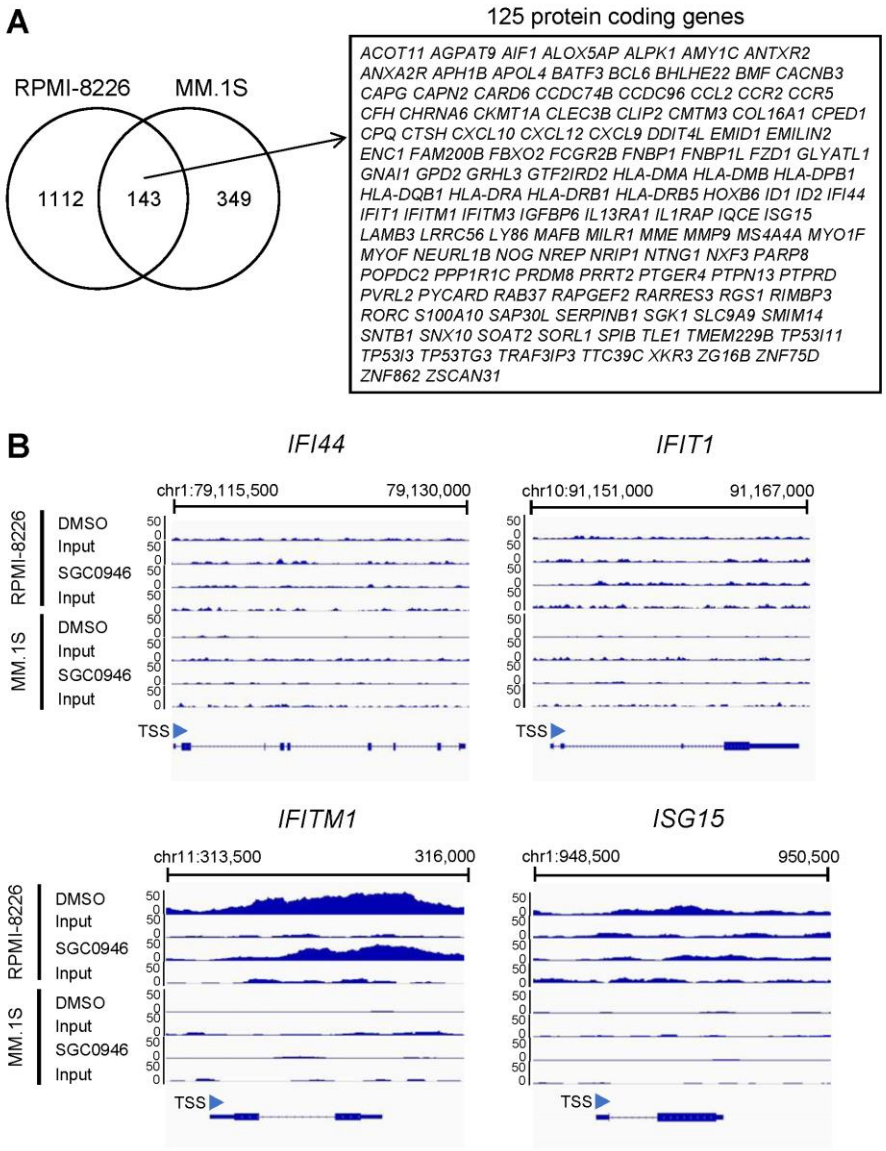
Supplementary Figure 7



Supplementary Figure 7

Western blot analysis of MYC in MM cells treated with the indicated DOT1L inhibitors (1 μM, 6 days). The results were confirmed in two independent experiments, and representative results are shown.

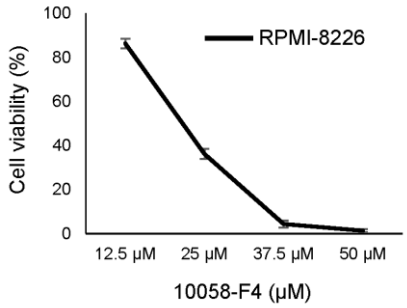
Supplementary Figure 8



Supplementary Figure 8

DOT1L inhibition affects genes associated with the immune system and interferon signaling in MM cells. (A) Venn diagram of the microarray probe sets upregulated (> 1.5-fold) by SGC0946 (1 μM, 6 days) in RPMI-8226 and MM.1S cells. (B) H3K79me2 status of representative interferon-stimulated genes in MM cell lines treated with DMSO or SGC0946. The numbers on the vertical axis indicate the numbers of sequence reads. Results of input DNA are shown below. TSS, transcription start site. Note that H3K79me2 levels in interferon-stimulated genes were not altered by DOT1L inhibition.

Supplementary Figure 9



Supplementary Figure 9

Results of cell viability assays using RPMI-8226 cells treated for 2 days with the indicated concentrations of the MYC inhibitor 10058-F4. Results are normalized to cells treated with DMSO. Shown are means of 3 replications; error bars represent SEMs.

Supplementary Table 1. Genes whose H3K79me2 levels were decreased by SGC0946 in RPMI-8226 cells

Supplementary Table 2. Genes whose H3K79me2 levels were decreased by SGC0946 in MM.1S cells

Supplementary Table 3. Genes whose expression was downregulated by SGC0946 in RPMI-8226 cells

Supplementary Table 4. Genes whose expression was downregulated by SGC0946 in MM.1S cells

Supplementary Table 5. Genes whose expression was upregulated by SGC0946 in RPMI-8226 cells

Supplementary Table 6. Genes whose expression was upregulated by SGC0946 in MM.1S cells

Supplementary Table 8. Genes whose expression was downregulated by SGC0946 in KMS-12PE cells

Supplementary Table 9. Genes whose expression was upregulated by SGC0946 in KMS-12PE cells

These Supplementary Tables are shown in separate files.

Supplementary Table 7. Mutations detected in KMS-12BM and KMS-12PE cells

KMS-12BM

Locus number	Gene	Function	Codon	Protein	Coding
chr1:51436101	CDKN2C	fsDel	CAC	p.Leu21fs	c.62_63delTT
chr17:7574017	TP53	missense	CTC	p.Arg337Leu	c.1010G>T
chr19:1621885	TCF3	missense	AGC	p.Gly303Ser	c.907G>A
chr14:92470910	TRIP11	missense	CGA	p.Gln1137Arg	c.3410A>G
chr12:123214499	HCAR1	missense	TGG	p.Arg130Trp	c.388C>T
chr9:136905311	BRD3	missense	GAC	p.Glu496Asp	c.1488G>C
chr3:89456423	EPHA3	missense	TTA	p.Phe533Leu	c.1599C>A
chr15:91326152	BLM	missense	AAC	p.His886Asn	c.2656C>A
chr17:59934523	BRIP1	nonsense	TGA	p.Ser92Ter	c.275C>G
chr4:1803120	FGFR3	missense	TGG	p.Arg158Trp	c.472C>T
chr6:152749427	SYNE1	missense	GGG	p.Ala1630Gly	c.4889C>G
chr1:147092639	BCL9	missense	GAA	p.Ala893Glu	c.2678C>A

KMS-12PE

Locus number	Gene	Function	Codon	Protein	Coding
chr17:7574017	TP53	missense	CTC	p.Arg337Leu	c.1010G>T
chr19:1621885	TCF3	missense	AGC	p.Gly303Ser	c.907G>A
chr22:41565521	EP300	missense	TGT	p.Ser1396Cys	c.4187C>G
chr2:141092126	LRP1B	missense	ACG	p.Met4040Thr	c.12119T>C
chr4:1806582	FGFR3	missense	TGC	p.Ser435Cys	c.1304C>G
chr4:1803120	FGFR3	missense	TGG	p.Arg158Trp	c.472C>T
chr3:89456423	EPHA3	missense	TTA	p.Phe533Leu	c.1599C>A
chr12:49441845	KMT2D	missense	TTT	p.Cys1380Phe	c.4139G>T
chr12:123214499	HCAR1	missense	TGG	p.Arg130Trp	c.388C>T
chr9:136905311	BRD3	missense	GAC	p.Glu496Asp	c.1488G>C
chr15:91326152	BLM	missense	AAC	p.His886Asn	c.2656C>A
chr14:92470910	TRIP11	missense	CGA	p.Gln1137Arg	c.3410A>G
chr2:208425880	CREB1	missense	AAA	p.Thr100Lys	c.299C>A
chr7:152007138	KMT2C	fsDel	TTG	p.His253fs	c.757_761delCACCG

Supplementary Table 10. Sequences of the primers used in this study

	Forward	Reverse	Product size
qRT-PCR			
DOT1L	5'-ACGACCTGTTTGTGGACTTG-3'	5'-TTCTCGACGCCATAGTGATG-3'	94 bp
MYC	5'-AGGACCCGCTTCTCTGAAAG-3'	5'-CCTGTTGGTGAAGCTAACGTTG-3'	119 bp
IRF4	5'-GCAATGACTTTGAGGAACTGG-3'	5'-AAGGGTAAGGCGTTGTCATG-3'	168 bp
PRDM1	5'-TACATACCAAAGGGCACACG-3'	5'-GATTCACATAGCGCATCCAG-3'	175 bp
KLF2	5'-CCAAGAGTTCGCATCTGAAGG-3'	5'-CGTGTGCTTTCGGTAGTGG-3'	131 bp
MZB1	5'-CGAGAAGTGGACCAAGTGAAAC-3'	5'-CCCAAGTAGTGCAAACATGTCC-3'	131 bp
FKBP11	5'-GCCTATGGAAAACGGGGATTTC-3'	5'-GCTCGGATTAGTGCAATCAGC-3'	86 bp
IFI44	5'-ACTGGGGCTGAGTGAGAAAG-3'	5'-GCGATGGGGAATCAATGTAGTC-3'	143 bp
IFIT1	5'-ACGGCTGCCTAATTTACAGCA-3'	5'-GGATAACTCCCATGTAAAGTGA-3'	102 bp
IFITM1	5'-TCTTGAACTGGTGCTGTCTGG-3'	5'-CAGATGTTCAGGCACTTGGC-3'	127 bp
ISG15	5'-GCGAACTCATCTTTGCCAGTAC-3'	5'-TTCAGCTCTGACACCGACATG-3'	148 bp
ACTB	5'-GCCAACCGCGAGAAGATGA-3'	5'-AGCACAGCCTGGATAGCAAC-3'	80 bp
ChIP-qPCR			
MYC	5'-TGCTGAGATGAGTCGAATGC-3'	5'-ACAAGTCACTTTACCCCGATCC-3'	138 bp
IRF4	5'-TTCGCATGCCATCTGTCATG-3'	5'-TTTTCAGCAACTCCCTTGGG-3'	113 bp
PRDM1	5'-TGTCCTCATTGCTGCTGTTC -3'	5'-AGTCACCTCTGCCAAACCAC-3'	159 bp
KLF2	5'-AGTGTGATCCGAGAGGTTGG-3'	5'-GGGGATTCTGAGCCAAGTG-3'	174 bp
Intergenic region	5'-AGCGCGTGATTTGATTGGAC-3'	5'-TGGAGGCTGCATTTTCACAC-3'	79 bp

Supplementary Table 11. Summary of the ChIP-seq experiments in this study

Cell line	Treatment	Antibody	IP or input	Number of total reads
RPMI-8226	DMSO	anti-H3K79me2 mAb	IP	60,827,288
RPMI-8226	SGC0946	anti-H3K79me2 mAb	IP	80,072,177
RPMI-8226	DMSO	anti-H3K79me2 mAb	Input	76,887,129
RPMI-8226	SGC0946	anti-H3K79me2 mAb	Input	87,075,037
MM.1S	DMSO	anti-H3K79me2 mAb	IP	68,024,520
MM.1S	SGC0946	anti-H3K79me2 mAb	IP	67,227,216
MM.1S	DMSO	anti-H3K79me2 mAb	Input	39,059,759
MM.1S	SGC0946	anti-H3K79me2 mAb	Input	40,929,037
RPMI-8226	None	anti-DOT1L Ab	IP	5,508,743
MM.1S	None	anti-DOT1L Ab	IP	6,065,385