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

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Running title

DOT1L is a therapeutic target in myeloma

Conflict of interest

All authors declare no conflict of interest.

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Abstract

Epigenetic alterations play an important role in the pathogenesis in multiple myeloma (MM), but its biological and clinical relevance is not fully understood. Here, we show that DOT1L, which catalyzes methylation of histone H3 lysine 79, is required for the survival of MM cells. The levels of DOT1L expression were elevated in monoclonal gammopathy of undetermined significance (MGUS) and smoldering multiple myeloma (SmMM) than in normal plasma cells (NPCs). Treatment with DOT1L inhibitors induced cell cycle arrest and apoptosis in MM cells, and strongly suppressed cell proliferation in vitro. The anti-myeloma effect of DOT1L inhibition was also confirmed in a xenograft model in mice. Chromatin immunoprecipitation-sequencing (ChIP-seq) and microarray analysis revealed that DOT1L inhibition downregulated H3K79 dimethylation (H3K79me2) and expression levels of IRF4-MYC signaling genes in MM cells. Moreover, DOT1L inhibition upregulated genes associated with immune response and interferon signaling. MM cells with histone modifier mutations or lower expression of *IRF4*/*MYC* were less sensitive to DOT1L inhibition, but prolonged treatment exerted anti-proliferative effects in these cells. Our data suggest that DOT1L may play an essential role in the development of MM, and DOT1L inhibition may provide a new therapy for MM treatment.

Key words

multiple myeloma, histone methylation, DOT1L, H3K79, MYC, IRF4

Introduction

Multiple myeloma (MM) is a genetically complex disorder caused by monoclonal proliferation of abnormal plasma cells. MM accounts for 1 % of all cancers and 10 % of hematologic malignancies in the United States, and there are 101,000 deaths per year caused by MM in the world.[1] Although new therapies including proteasome inhibitors, immunomodulatory drugs, monoclonal antibodies and histone deacetylase inhibitors have been developed, MM is still an incurable disorder.[2]

Epigenetic alterations including aberrant DNA methylation and histone modifications play important roles in the pathogenesis of MM, and they are thought as potential therapeutic targets.[3, 4] For instance, a histone deacetylase inhibitor panobinostat reportedly exert synergistic anti-myeloma effect when combined with bortezomib and dexamethasone, and a complete or near complete response was observed in 27.6% of patients with relapsed or relapsed and refractory MM.[5]

Methylation of histone lysine residues is one of the major factors of epigenetic mechanism which regulates chromatin organization and gene expression [6]. Methylation of histone H3 lysine 4 (H3K4), H3K36 and H3K79 are associated with active transcription, while methylation of H3K9 and H3K27 are well characterized as repressive marks. Cumulative evidences suggest that dysregulation of histone methylation is involved in the pathogenesis of MM. Mutations in genes encoding histone modifiers including H3K27 demethylase UTX, H3K4 methyltransferases MLL, MLL2, MLL3, H3K9 methyltransferase G9a and H3K36 methyltransferase MMSET are reported in MM.[7, 8] MMSET is overexpressed in MM with t(4;14), which leads to global accumulation of H3K36 dimethylation (H3K36me2) and reduction of H3K27 trimethylation (H3K27me3).[9] EZH2 is also reportedly overexpressed in MM with poor prognosis, and is considered as a potential therapeutic target.[8, 10] In the current study, we aimed to clarify the pathological and therapeutic implication of histone methylation in MM.

Materials and methods

Cell lines and clinical specimens

MM cell lines (RPMI-8226, MM.1S, KMS-11, KMS-12BM, KMS-12PE and U-266) were obtained and cultured as described previously.[11] Total RNA and genomic DNA were extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany) and QIAamp DNA Mini kit (Qiagen) according to the manufacturer's instructions. Specimens of bone marrow or peripheral blood were collected from MM or plasma cell leukemia (PCL) patients, respectively, after which CD138-positive cells were isolated using the MACS manual cell separator (Miltenyi Biotec, Bergisch Gladbach, Germany). CD138-positive cells were cultured in RPMI-1640 medium supplemented with 20% fetal bovine serum (FBS) and 1% penicillin/streptomycin/amphotericin B for 24 h, after which drug treatment and cell viability assays were performed. This study was performed in accordance with the Declaration of Helsinki. Informed consent was obtained from all patients before collection of the specimens. Approval of this study was obtained from Institutional Review Board of Sapporo Medical University.

Reagents

A histone H3 lysine 4 (H3K4) methyltransferase LSD1 inhibitor S2101 was purchased from Merck Millipore (Burlington, MA, USA). A LSD1 inhibitor GSK2879552, a H3K27 methyltransferase EZH2 inhibitor GSK126 and a H3K79 methyltransferase DOT1L inhibitor EPZ-5676 were purchased from Chemietek (Indianapolis, IN, USA). A H3K9 methyltransferase G9a inhibitor UNC0638, a H3K27 demethylase JMJD3/UTX inhibitor GSKJ1, a DOT1L inhibitor SGC0946 and a MYC inhibitor 10058-F4 were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Drug treatment and cell viability assay

To screen the anti-proliferative effects of histone methyltransferases or demethylase inhibitors, MM cell lines (3×10^4 to 1×10^5 cells in 6-well plate) were treated with 1 μM of respective drugs or DMSO for up to 14 days, supplying the medium and drug every 3 to 4 days. Cell viabilities were assessed on days 3-4 and 11-14 using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) and a

microplate reader (Model 680; Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. To further analyze the effect of DOT1L inhibitors, MM cell lines (2×10^4 to 8×10^4 cells in 6-well plate) or patient derived CD138-positive cells (1.3×10^5 to 3×10^5 cells in 6-well plate) were treated with 0.25-1 μM of the respective inhibitors or DMSO for up to 18 days supplying the medium and drug every 3 days.

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 ACTB (β-actin). 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 1.

Xenograft studies

We used the ex vivo drug pre-treatment method.[12, 13] RPMI-8226 cells were pre-treated with 1 μM SGC0946 or DMSO for 3 days, after which 1×10^7 cells were resuspended in 200 μl of RPMI-1640 medium. Cells were injected subcutaneously in the area of the bilateral thighs of 6-week-old C.B-17 SCID mice. Tumor size was measured every 3 days using digital calipers, and tumor volume was calculated using the formula length × width^2/2. Animal experiments were conducted in compliance with the protocol which was approved by the Institutional Animal Care and Use Committee of Sapporo Medical University.

Flow cytometry analysis

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

Results

DOT1L is a potential therapeutic target in MM

To evaluate whether histone methylation modifiers could be therapeutic targets in MM, we first tested the effects of following compounds on the proliferation of MM cell lines: LSD1 inhibitors S2101 and GSK2879552, a G9a inhibitor UNC0638, an EZH2 inhibitor GSK126, a JMJD3 inhibitor GSKJ1 and a DOT1L inhibitor SGC0946 (Figure 1a). A series of 5 MM cell lines were treated with the drugs (1 μM) for up to 14 days, and cell viabilities were assessed at early (day 3-4) and late time points (day 11-14). We found that inhibitors against G9a, EZH2, JMJD3 and DOT1L moderately suppressed proliferation of more than 2 MM cell lines at the early time point (Figure 1a). Longer treatment with these drugs exerted stronger growth suppressive effects in many of the MM cell lines, except for KMS-12PE (Figure 1a). In contrast, LSD1 inhibitors were less effective in MM. We tested 2 LSD1 inhibitors (S2101 for KMS-12BM and MM.1S, GSK2879552 for RPMI-8226, KMS-12PE and U-266), but they didn't suppress or even promote MM cell proliferation (Figure 1a). Among them, we selected DOT1L for further analysis, because its inhibition exerted the strongest anti-proliferative effect.

Analysis using published data sets revealed that expression of DOT1L is increased during the progression from normal plasma cells (NPCs) to monoclonal gammopathy of undetermined significance (MGUS) and smoldering multiple myeloma (SmMM) (Figure 1b). In contrast, we didn't find significant difference in the levels of DOT1L expression between SmMM and symptomatic MM (SyMM) (Figure 1b). qRT-PCR showed that DOT1L is expressed at similar levels in all MM cell lines tested, irrespective of the sensitivities to the DOT1L inhibitor treatment (Supplementary Figure 1).

DOT1L inhibitors induce growth suppression , cell cycle arrest and apoptosis in MM cells

To evaluate the therapeutic potential of DOT1L inhibition in MM, we treated MM cell lines with two DOT1L inhibitors, SGC0946 and EPZ-5676. Western blot analysis showed that treatment with both drugs (1 μM, 3 days) significantly decreased the levels of H3K79me2 in RPMI-8226 cells (Supplementary Figure 2). We found that both drugs strongly suppressed proliferation of 4 MM cell lines, RPMI-8226, MM.1S, KMS-11 and KMS-12BM, while they were less effective or ineffective

in KMS-12PE and U-266 cells (Figure 2a). shRNA-mediated knockdown of DOT1L also suppressed MM cell proliferation (Supplementary Figure 3). Moreover, treatment with DOT1L inhibitors could completely suppress xenograft formation by MM cells in SCID mice (Figure 2b, Supplementary Figure 4). To evaluate the effects of DOT1L inhibitors in primary tumors, we isolated CD138-positive cells from MM and plasma cell leukemia (PCL) patients. We found that both drugs suppressed the proliferation of primary tumor cells (Supplementary Figure 5).

Cell cycle analysis using flow cytometry revealed that treatment with SGC0946 (1 μM, 6 days) or EPZ-5676 (1 μM, 6 days) leaded to increase in sub-G1 and G0-G1 phase population and decrease in S phase population in RPMI-8226 and MM.1S cells, suggesting that DOT1L inhibition induced G1-S arrest and apoptosis (Figure 3a). Induction of apoptosis by DOT1L inhibitors was also confirmed by Annexin V staining assays (Figure 3b).

DOT1L inhibitors suppress IRF4 and MYC signaling in MM cells

To clarify the mechanism underlying the antitumor effects of DOT1L inhibition, we analyzed H3K79me2 and gene expression status in MM cells. Earlier studies have shown that 2 to 3 days of DOT1L inhibitors treatment resulted in evident depletion of H3K79me2 in tumor cells, while mRNA expression of target genes were significantly reduced after 6 to 7 days treatment.[14, 15] We thus performed ChIP-seq analysis of H3K79me2 in RPMI-8226 and MM.1S cell lines treated with 1 μM SGC0946 or DMSO for 3 days. Gene expression microarray analysis was performed in MM cells treated with the drug for 6 days. ChIP-seq analysis identified 1753 and 1701 genes of which H3K79me2 levels were significantly reduced by DOT1L inhibition in RPMI-8226 and MM.1S cells, respectively (Figure 4a, Supplementary Figure 6a, Supplementary Tables 2,3). Microarray analysis revealed that expression levels of 912 and 390 genes were downregulated (> 1.5-fold) by SGC0946 in these cells (Supplementary Tables 4,5). Collectively, we identified 77 genes (including 49 protein coding genes) of which H3K79me2 and expression levels were significantly decreased by the treatment in RPMI-8226 cells (Figure 4a). A set of 51 genes (including 45 protein coding genes) were similarly identified in MM.1S cells (Figure 4a). Among them, we noted that genes associated with IRF4 and MYC signaling (*MYC*, *IRF4*, *PRDM1* and *KLF2*) were affected in both cell lines (Figure 4a). Reduced H3K79me2 levels of these genes by SGC0946 are shown in Figure 4b.

Although microarray analysis failed to detect MYC in MM.1S cells, qRT-PCR and western blot analysis confirmed that both SGC0946 and EPZ-5676 suppressed the expression of the IRF4-MYC signaling genes in MM cells (Figure 4b, Supplementary Figure 6b).

DOT1L inhibitors affect immune response and interferon signaling in MM cells

In addition to the suppressive effects on H3K79me2 and gene expression, DOT1L inhibitors increased expression of a number of genes in MM cells. Microarray analysis showed that 1255 and 492 probe sets were upregulated (>1.5-fold) by SGC0946 in RPMI-8226 and MM.1S cells, respectively (Supplementary Tables 6,7). Among them, 143 probe sets (including 125 protein coding genes) were commonly upregulated in the both cell lines (Supplementary Figure 7a). GO analysis suggested that genes involved in "immune system" were significantly enriched in the upregulated genes in both cell lines (Figure 5a, Supplementary Figure 7b). Pathway analysis also revealed that genes associated with "interferon (IFN) signaling" were significantly enriched in the upregulated genes in both cell lines (Figure 5a, Supplementary Figure 7b). qRT-PCR analysis confirmed that a series of IFN-stimulated genes were upregulated by SGC0946 and EPZ-5676 in MM cells, although H3K79me2 levels were not affected (Figure 5b, Supplementary Figure 7c). These results suggest that DOT1L inhibitors may affect immune response and IFN signaling in a H3K79me2-independent mechanism in MM.

Gene mutations are potentially associated with the DOT1L sensitivity in MM cells

Although KMS-12BM and KMS-12PE were established from the same patient [16], KMS-12PE was less sensitive to DOT1L inhibitors than KMS-12BM (Figures 1a,2a). To clarify the mechanism underlying the drug sensitivity, we performed targeted sequencing of cancer-related genes. Of the 409 genes analyzed, KMS-12BM exhibited mutations in 12 genes, while KMS-12PE showed 14 mutations in 13 genes (Supplementary Table 8). Among them, 8 mutations were found in both cell lines (Supplementary Figure 8). Interestingly, we noted that KMS-12PE exhibited mutations in multiple histone modifier genes (*EP300*, *KMT2C* and *KMT2D*), while they were not detected in KMS-12 BE (Supplementary Figure 8).

Prolonged treatment enhances the effect of DOT1L inhibitors in MM cells

We next tried to clarify whether the IRF4-MYC signaling is associated with the different sensitivities to DOT1L inhibitors among KMS-12BM and KMS-12PE cells. qRT-PCR revealed that *MYC* and *IRF4* were expressed at lower levels in KMS-12PE than in KMS-12BM, suggesting that lower IRF4-MYC signaling may be associated with the impaired effect of DOT1L inhibitors (Figure 6a). Consistent with this hypothesis, KMS-12PE was less sensitive to a MYC inhibitor 10058-F4 than KMS-12BM (Figure 6b).

However, we also noted that DOT1L inhibitors suppressed *MYC* and *IRF4* expression in KMS-12PE (Figure 7a). Moreover, prolonged treatment with DOT1L inhibitors for up to 18 days exerted growth suppressive effects in less sensitive cell lines, KMS-12PE and U-266 cells (Figure 7b). Gene expression microarray analysis in KMS-12PE treated with SGC0946 or DMSO for 12 days identified 509 (401 unique genes) and 865 probe sets (739 unique genes) which were down- and upregulated (> 1.5-fold) by SGC0946, respectively (Supplementary Tables 9,10). Among them, we found that multiple IRF4-MYC signaling genes were downregulated (Supplementary Figure 9a). Moreover, GO and pathway analyses showed that genes associated with immune response and IFN signaling were significantly enriched in the upregulated genes (Supplementary Figure 9b).

Discussion

In the current study, we show that DOT1L inhibitors exerted strong anti-proliferative effects in MM cells. Expression of DOT1L is significantly elevated in SmMM compared to NPC, suggesting that DOT1L may be causally associated with the myelomagenesis. DOT1L is the only known histone methyltransferase which catalyzes mono-, di- and trimethylation at H3K79.[17, 18] In mammals, most of H3K79 is unmethylated, while H3K79 methylation is linked to active transcription.[17, 19] DOT1L composes large transcription protein complexes with transcription factors including AF4, AF9, AF10, ENL and P-TEFb.[17, 20-23] DOT1L is also characterized as a drug target in mixed lineage leukemia (MLL) gene rearranged leukemia. DOT1L forms a complex with the MLL fusion proteins, and DOT1L-mediated H3K79 methylation leads to enhanced expression of target oncogenes including *HOXA9* and *MEIS1*.[23, 24] Recent studies demonstrated selective and strong antitumor effects of DOT1L inhibitors against MLL-rearranged leukemia.[14, 15, 25] DOT1L is also reported as a potential therapeutic target in lung and breast cancer with high DOT1L expression and neuroblastoma with *MYCN* amplification.[26-28]

Notably, we found that DOT1L inhibition targets IRF4-MYC axis in MM cells (Supplementary Figure 10). Aberrant activation of a number of transcription factors including MYC, MAF, NF-κB and IRF4 is involved in the development of MM.[4] Survival of MM cell is strongly addicted to IRF4 and MYC, and *MYC* is a direct target gene of IRF4 while *IRF4* is also a direct target of MYC transactivation.[29, 30] IRF4-MYC axis is thus considered as an important therapeutic target in MM, and a recent study showed that CBP/EP300 bromodomain inhibitors directly suppress IRF4 expression and inhibited MM cell viability.[31] Moreover, dependence on KDM3A-KLF2-IRF4 axis was recently reported in MM.[32] KDM3A maintains *KLF2* and *IRF4* expression via H3K9 demethylation, and KLF2 directly targets *IRF4* while IRF4 reciprocally activates *KLF2*, forming a positive autoregulatory circuit.[32] We found that DOT1L inhibition leaded to decreased levels of H3K79me2 and expression of *IRF4* and its target genes, including *MYC*, *PRDM1* (also known as BLIMP1) and *KLF2* in MM cells.[29] As previously shown, H3K79me2 peaks just behind the transcriptional start site of the active genes and gradually declines throughout the gene bodies[17, 33], while it was significantly depleted in MM cells with DOT1L inhibition.

We found that genes associated with immune response and IFN signaling were significantly

upregulated by DOT1L inhibition in MM cells. IFN is strongly implicated in the clinical treatment of MM. Interferon-alpha (IFN-α) reportedly induces apoptosis and inhibits growth in MM cell lines.[34] In 1980s, IFN-α was used as monotherapy in MM with an overall response rate of 15-20%.[35] In this study, we found that a series of INF-stimulated genes were upregulated by DOT1L inhibitors in MM cells, which may contribute to the anti-myeloma effects. The mechanism underlying the activation of immune response genes and INF signaling remain unclear. Recent studies showed that DNA methyltransferase inhibitors stimulate an interferon response via inducing endogenous double strand RNAs, which contributes to the antitumor effect.[36, 37] Although it is unclear whether DOT1L inhibition exerts similar effects in MM cells, further study is warranted to reveal its anti-myeloma mechanism.

Finally, we tried to investigate the mechanism underlying the sensitivity to DOT1L. We focused on 2 MM cell lines with different sensitivities to DOT1L inhibitors (KMS-12BM and KMS-12PE). These cells were established from a 64 years-old female MM patient, and KMS-12BE was obtained from bone marrow while the less sensitive KMS-12PE was obtained from pleural effusion.[16] Targeted sequencing of a panel of cancer-related genes revealed that, although many of the mutations were commonly found in both cell lines, the less sensitive KMS-12PE cells harbored mutations in multiple histone modifier genes. A recent study reported that mutations in epigenetic modifier genes were more frequently found in previously treated MM patients than in newly diagnosed patients, suggesting that these mutations may be associated with disease progression and chemoresistance.[8] Thus, the mutations of epigenetic modifier genes in KMS-12PE cells may be associated with less sensitivity to DOT1L inhibitors. In addition, we also noted that KMS-12PE cells express MYC and IRF4 at lower levels than KMS-12BM, suggesting that decreased addiction to the IRF4-MYC axis may lead to lower sensitivity to DOT1L inhibitors in KMS-12PE cells. However, it is of noteworthy that prolonged treatment with DOT1L inhibitors exerted suppressive effects on *IRF4*/*MYC* expression and cell viabilities in the less sensitive MM cell lines. Our results suggest that DOT1L is a promising therapeutic target in MM, and further exploration of DOT1L inhibitors for MM treatment is warranted.

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Author contributions

KI, RM and HS designed the study. KI, HK and TN performed experiments and data analysis. YS and TT performed sequencing analysis. HI, TH, HS, HW, KN, TS, EY and MK contributed to administrative, technical and material support. HN supervised the study. KI, TI and HS wrote the manuscript.

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37. Roulois D, Loo Yau H, Singhania R, Wang Y, Danesh A, Shen SY*, et al.* DNA-Demethylating Agents Target Colorectal Cancer Cells by Inducing Viral Mimicry by Endogenous Transcripts. *Cell* 2015; **162:** 961-973.

Figure legends

Figure 1

Identification of DOT1L as a potential therapeutic target in MM. (a) Effects of inhibitors against histone methylation modifiers on MM cell proliferation. Summarized results of cell viability assays in MM cell lines treated with indicated drugs at early and late time points are shown. Results are normalized to cells treated with DMSO, and shown are means of two replicates. (b) Expression of DOT1L mRNA in normal plasma cells (NPC, n = 22), monoclonal gammopathy of undetermined significance (MGUS, n = 44) and smoldering multiple myeloma (SmMM, n = 12) (left) and those in SmMM (n = 24) and symptomatic MM (SyMM, n = 69) (right) in datasets.

Figure 2

Antiproliferative effects of DOT1L inhibitors in MM. (a) Cell viability assays in MM cell lines with indicated concentrations of DOT1L inhibitors. Results are normalized to non-treated cells. Shown are means of 3 replications; error bars represent SEMs. (b) Tumor growth in mice injected with RPMI-8226 cells pretreated with SGC0946 (left thigh) or DMSO (right thigh). Growth curves are means of 5 replicates; error bars represent SEMs.

Figure 3

Effects of DOT1L inhibitors on cell cycle and apoptosis in MM. (a) Results of cell cycle analysis in MM cells treated with indicated DOT1L inhibitors (1μM, 6 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 indicated DOT1L inhibitors.

Figure 4

Effects of DOT1L inhibitors on H3K79me2 and gene expression in MM. (a) Integrated analysis of H3K79me2 and gene expression in RPMI-8226 and MM.1S cells treated with SGC0946. Scatter plots showing the changes in H3K79me2 and expression levels induced by SGC0946 (left). Middle; Venn diagrams of genes whose H3K79me2 or expression levels were suppressed by SGC0946 (middle). Protein coding genes with suppressed H3K79me2 and expression levels (right). Large

letters indicate genes shared by both cell lines. Red letters indicate IRF4-MYC signaling genes. (b) Representative results of ChIP-seq analysis showing decreased H3K79me3 levels of IRF4-MYC signaling genes by SGC0946 (upper). qRT-PCR analysis of IRF4-MYC signaling genes in MM cells treated with indicated DOT1L inhibitors (lower). Results are normalized to ACTB expression.

Figure 5

DOT1L inhibition affects immune response and interferon signaling in MM. (a) GO analysis of genes of upregulated by SGC0946 in RPMI-8226 cells (left). Pathway analysis of genes upregulated by SGC0946 in RPMI-8226 cells (right). (c) qRT-PCR analysis of IFN-stimulated genes in MM cell lines treated with indicated DOT1L inhibitors. Results are normalized to ACTB expression. Shown are means of 3 replications; error bars represent SEMs.

Figure 6

Expression levels of IRF4 and MYC may be associated with the sensitivity of MM cells to DOT1L inhibitors. (a) qRT-PCR of MYC and IRF4. Results are normalized to ACTB expression. Shown are means of 3 replications; error bars represent SEMs. (c) Results of cell viability assays in KMS-12BM and KMS-12PE treated with a MYC inhibitor 10058-F4 for 2 days. Results are normalized to cells treated with DMSO. Shown are means of 3 replications; error bars represent SEMs.

Figure 7

Prolonged treatment enhances the effect of DOT1L inhibitors in MM. (a) qRT-PCR of MYC and IRF4 in MM cells treated with indicated DOT1L inhibitors (1 μM, 6 days). (b) Results of cell viability assays in KMS-12PE and U-266 with prolonged DOT1L inhibitor treatment. Results are normalized to cells treated with DMSO. Shown are means of 3 replications; error bars represent SEMs.

Figure 1

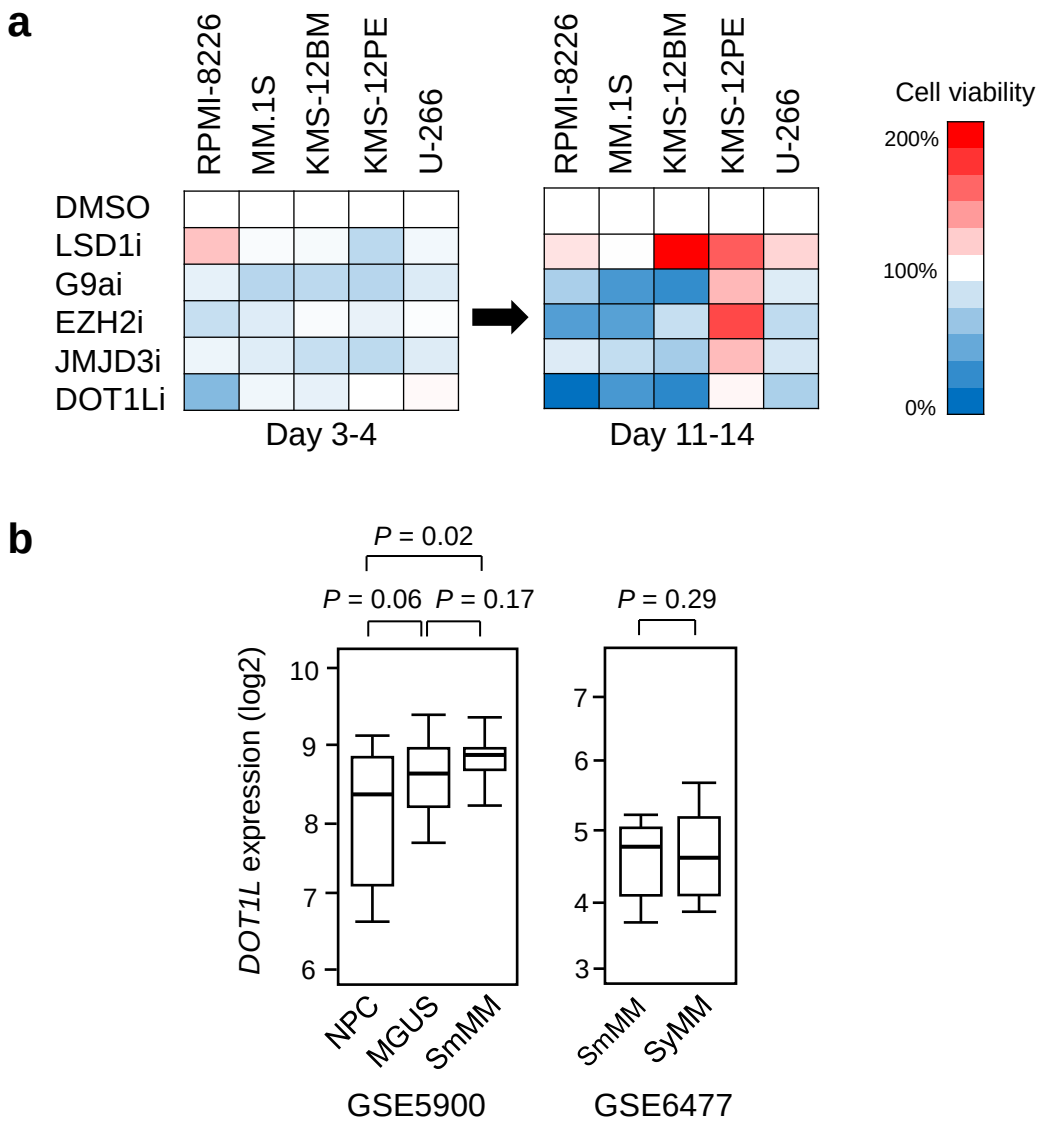
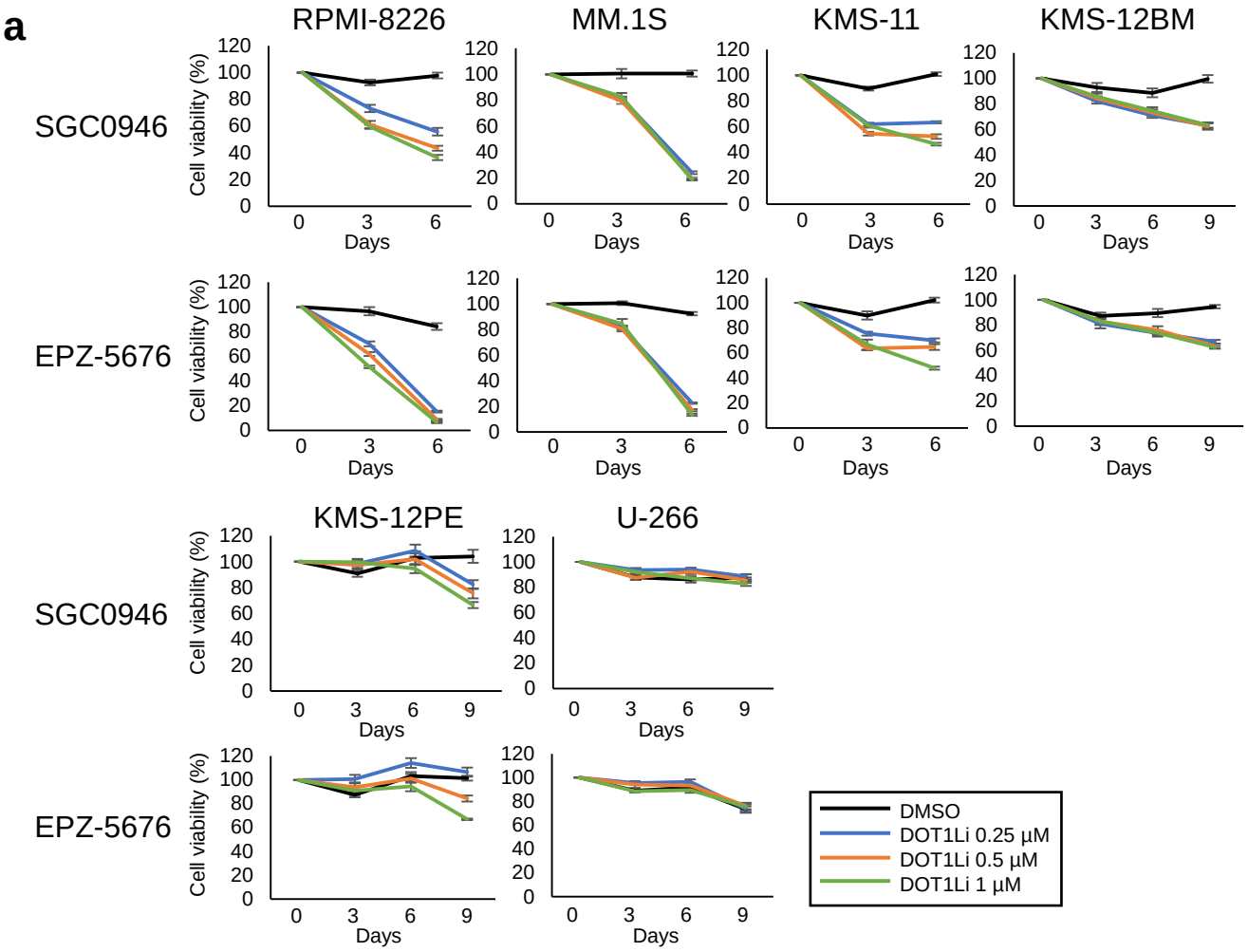

a
RPMI-8226
MM.1S
KMS-12BM
KMS-12PE
U-266
DMSO
LSD1i
G9ai
EZH2i
JMJD3i
DOT1Li
Day 3-4
Day 11-14
Cell viability
200%
100%
0%
b
P = 0.02
P = 0.06
P = 0.17
P = 0.29
DOT1L expression (log2)
NPC
MGUS
SmMM
SmMM
SyMM
GSE5900
GSE6477

Figure 2


a
RPMI-8226
MM.1S
KMS-11
KMS-12BM
SGC0946
EPZ-5676
KMS-12PE
U-266
Cell viability (%)
Days
DMSO
DOT1Li 0.25 μM
DOT1Li 0.5 μM
DOT1Li 1 μM

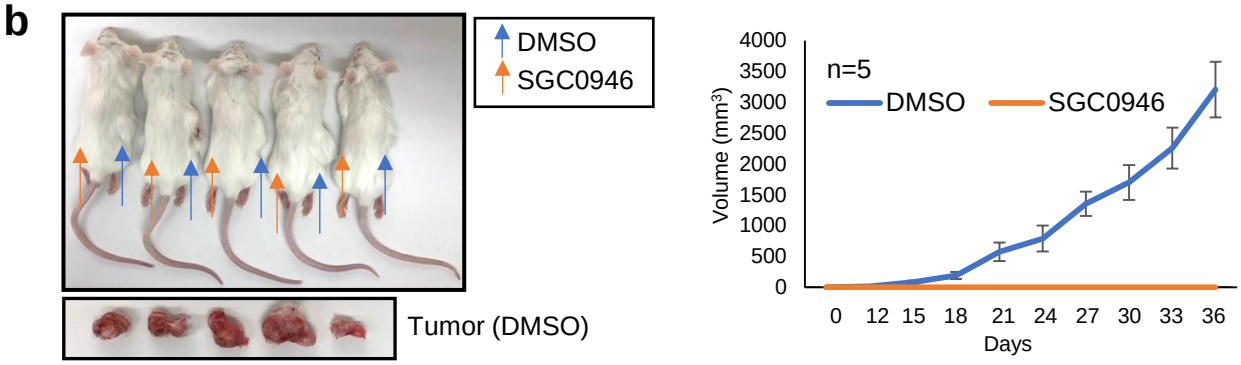

b
DMSO
SGC0946
Tumor (DMSO)
n=5
Volume (mm³)
Days

Figure 3

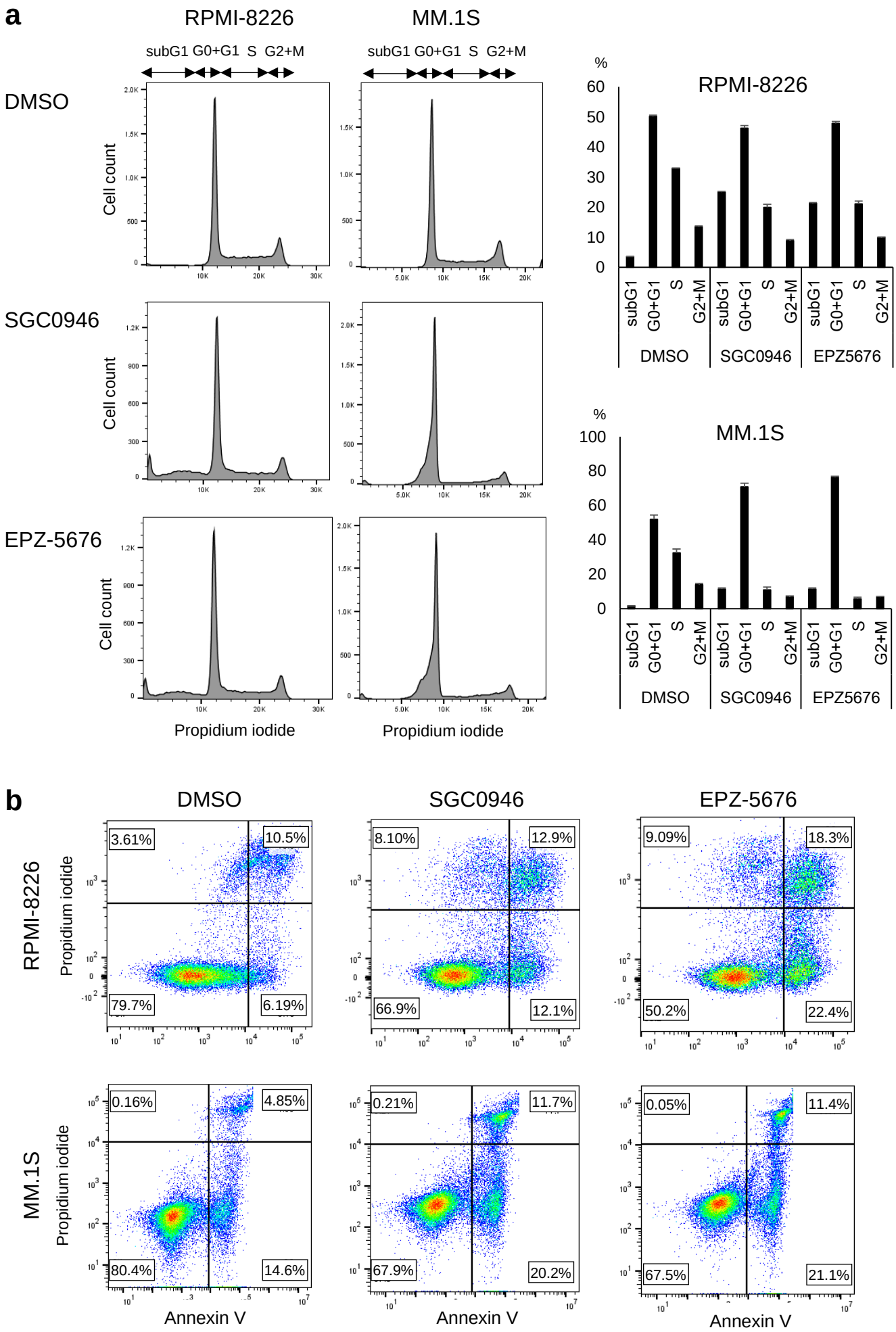
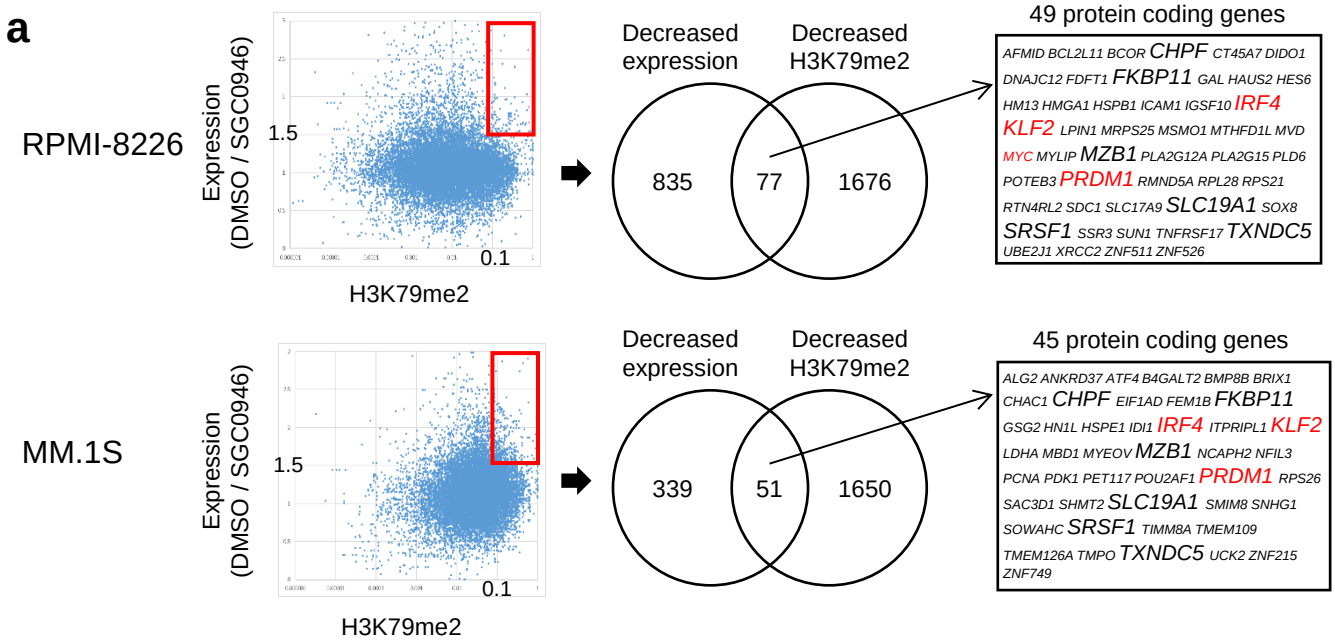

a
RPMI-8226
MM.1S
subG1 G0+G1 S G2+M
DMSO
SGC0946
EPZ-5676
Cell count
Propidium iodide
%
subG1
G0+G1
S
G2+M
DMSO
SGC0946
EPZ5676
b
DMSO
SGC0946
EPZ-5676
RPMI-8226
MM.1S
Propidium iodide
Annexin V
3.61%
10.5%
79.7%
6.19%
8.10%
12.9%
66.9%
12.1%
9.09%
18.3%
50.2%
22.4%
0.16%
4.85%
80.4%
14.6%
0.21%
11.7%
67.9%
20.2%
0.05%
11.4%
67.5%
21.1%

Figure 4


a
RPMI-8226
Expression (DMSO / SGC0946)
1.5
0.1
H3K79me2
Decreased expression
Decreased H3K79me2
835
77
1676
49 protein coding genes
AFMID BCL2L11 BCOR CHPF CT45A7 DIDO1 DNAJC12 FDFT1 FKBP11 GAL HAUS2 HES6 HM13 HMGA1 HSPB1 ICAM1 IGSF10 IRF4 KLF2 LPIN1 MRPS25 MSMO1 MTHFD1L MVD MYC MYLIP MZB1 PLA2G12A PLA2G15 PLD6 POTEB3 PRDM1 RMND5A RPL28 RPS21 RTN4RL2 SDC1 SLC17A9 SLC19A1 SOX8 SRSF1 SSR3 SUN1 TNFRSF17 TXNDC5 UBE2J1 XRCC2 ZNF511 ZNF526
MM.1S
Expression (DMSO / SGC0946)
1.5
0.1
H3K79me2
Decreased expression
Decreased H3K79me2
339
51
1650
45 protein coding genes
ALG2 ANKRD37 ATF4 B4GALT2 BMP8B BRIX1 CHAC1 CHPF EIF1AD FEM1B FKBP11 GSG2 HN1L HSPE1 IDI1 IRF4 ITPRIPL1 KLF2 LDHA MBD1 MYEOV MZB1 NCAPH2 NFIL3 PCNA PDK1 PET117 POU2AF1 PRDM1 RPS26 SAC3D1 SHMT2 SLC19A1 SMIM8 SNHG1 SOWAHC SRSF1 TIMM8A TMEM109 TMEM126A TMPO TXNDC5 UCK2 ZNF215 ZNF749

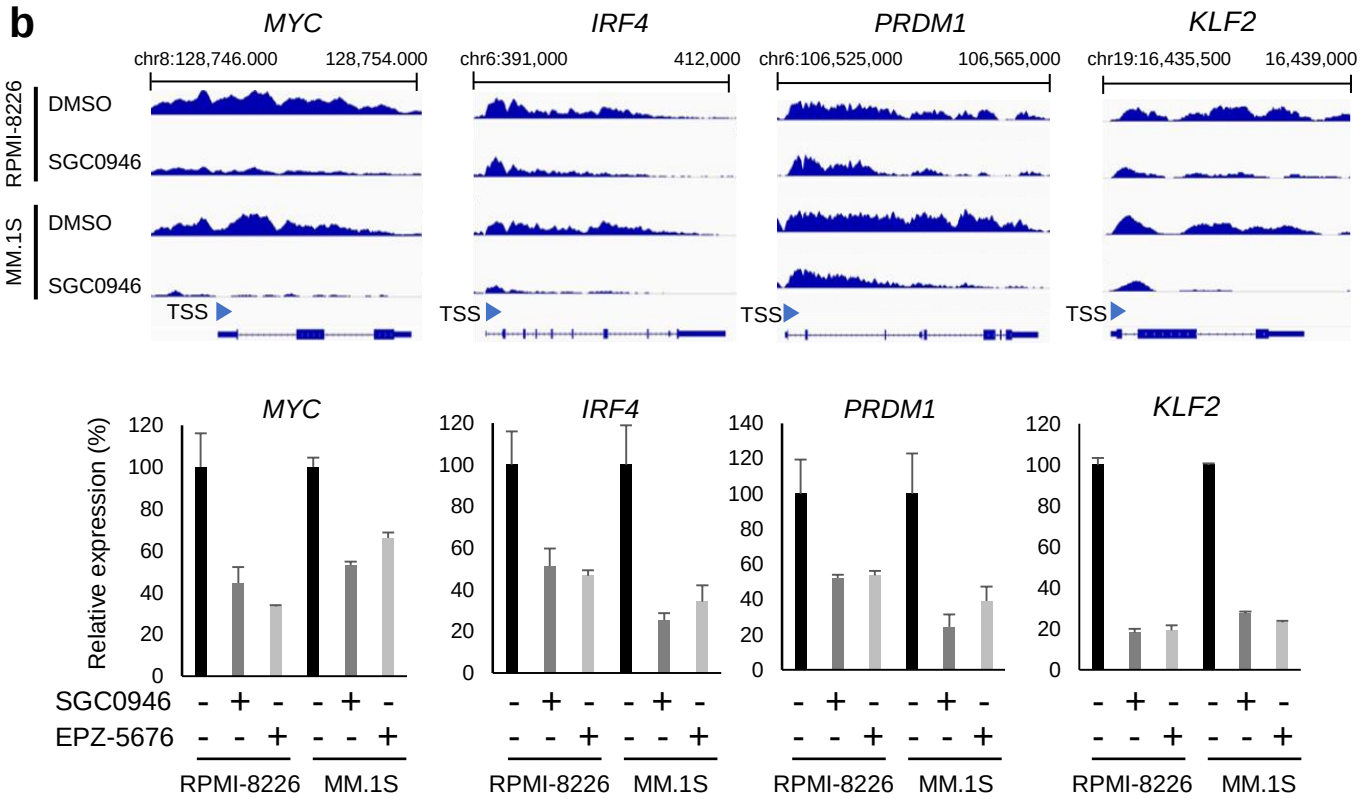

b
MYC
IRF4
PRDM1
KLF2
chr8:128,746.000
128,754.000
chr6:391,000
412,000
chr6:106,525,000
106,565,000
chr19:16,435,500
16,439,000
RPMI-8226
MM.1S
DMSO
SGC0946
TSS
Relative expression (%)
SGC0946
EPZ-5676
RPMI-8226
MM.1S

Figure 5

a

GO ID	GO Term	*P* value
1679	Immune system process	1.27E-20
5032	Immune response	1.27E-20
5029	Defense response	1.35E-19
1985	Regulation of immune system process	3.57E-17
5031	Inflammatory response	1.38E-16
25101	Regulation of response to stimulus	5.02E-16
11151	Cytokine-mediated signaling pathway	1.14E-15
31362	Cellular response to cytokine stimulus	1.14E-15
16311	Response to cytokine	1.82E-15
20235	Response to external biotic stimulus	7.47E-14
27078	Response to other organism	7.47E-14
1987	Positive regulation of immune system process	1.10E-13
6861	Response to biotic stimulus	3.09E-13
6859	Response to external stimulus	4.00E-13
5028	Response to stress	8.42E-13
1555	Immune effector process	9.01E-12
5198	Cell surface receptor signaling pathway	2.30E-11
22108	Leukocyte activation	4.30E-11
1997	Regulation of leukocyte activation	8.55E-11
16548	Response to interferon-gamma	9.61E-11

Pathway	*P* value
Type II interferon signaling (IFNG)	0.00E-10
Allograft rejection	3.27E-10
Interferon alpha-beta signaling	4.08E-10
Regulation of toll-like receptor signaling pathway	1.81E-09
Toll-like receptor signaling pathway	1.17E-08
Benzo(a)pyrene metabolism	9.18E-07
Cytokines and inflammatory response	2.28E-06
NOD pathway	1.34E-05
Spinal cord injury	1.70E-05
IL-3 signaling pathway	6.27E-05
Myometrial relaxation and contraction pathways	1.34E-04
IL-2 signaling pathway	1.58E-04
Sulindac metabolic pathway	2.14E-04
Calcium regulation in the cardiac cell	3.23E-04
Adipogenesis	3.23E-04
TSLP signaling pathway	3.25E-04
Oncostatin M signaling pathway	4.72E-04
Complement and coagulation cascades	5.43E-04
IL-4 signaling pathway	6.12E-04
Parkinsons disease pathway	6.30E-04

b

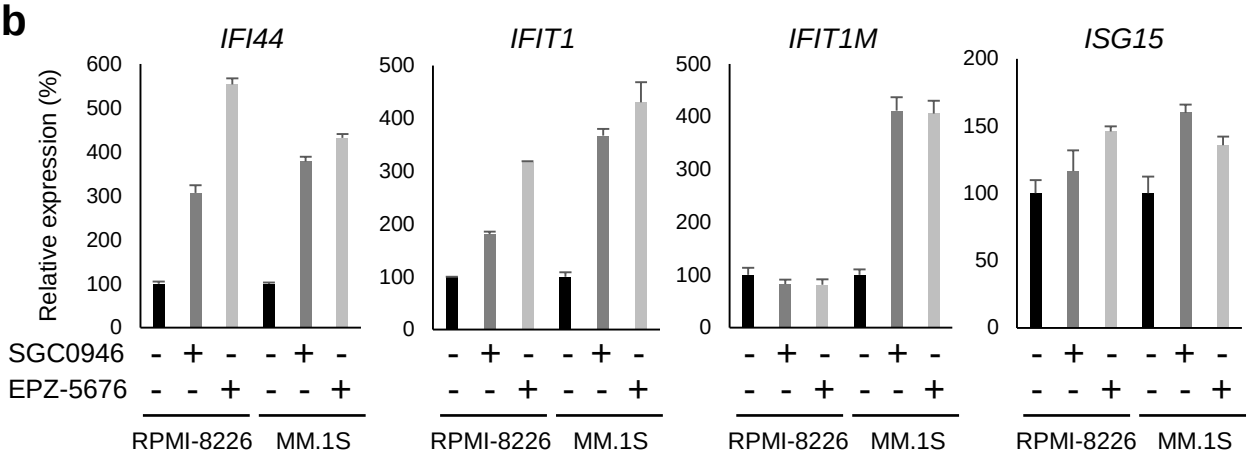


Figure 6

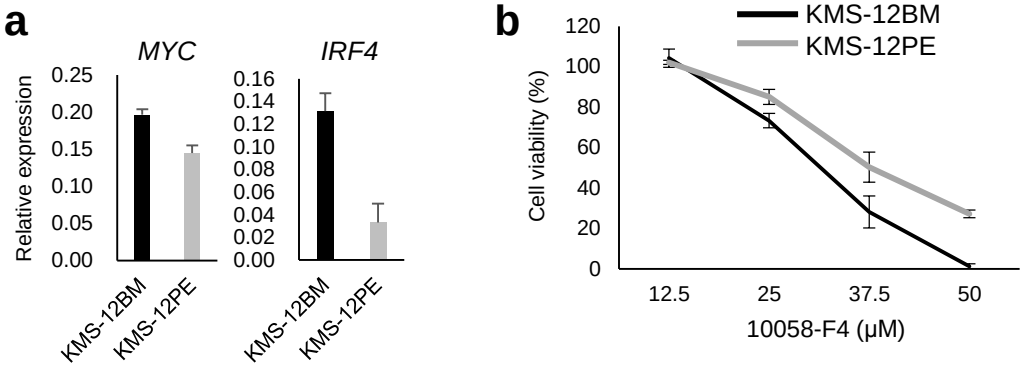
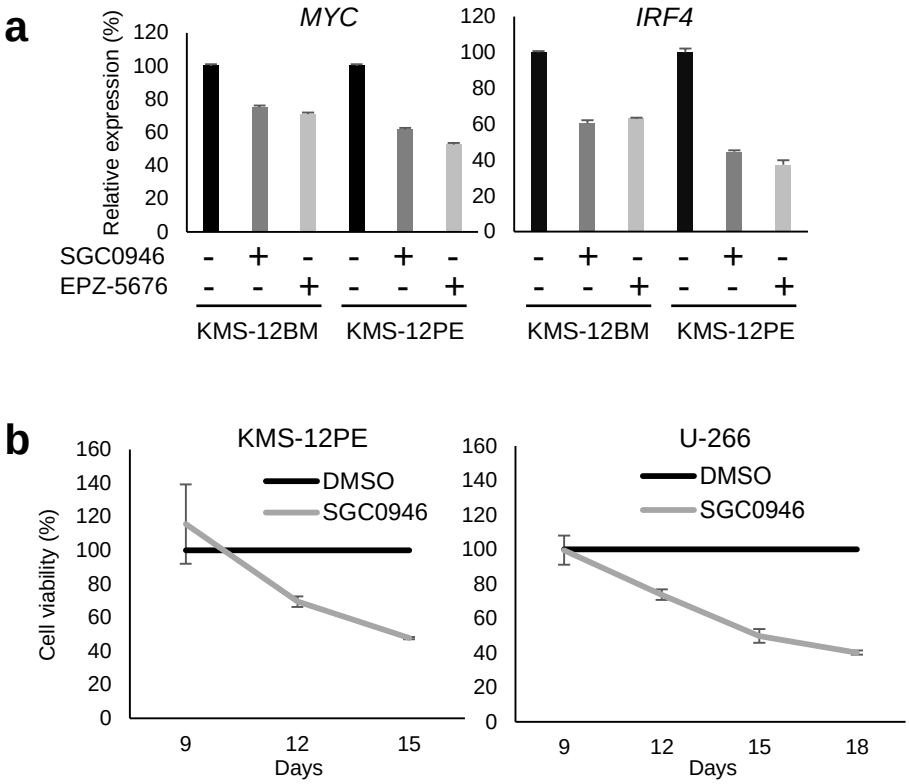
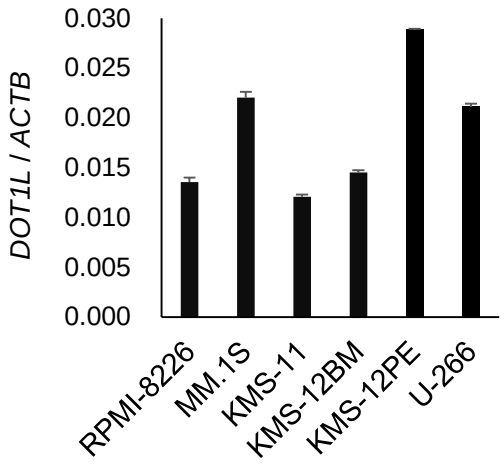

a
MYC
IRF4
Relative expression
0.25
0.20
0.15
0.10
0.05
0.00
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
KMS-12BM
KMS-12PE
KMS-12BM
KMS-12PE
b
KMS-12BM
KMS-12PE
Cell viability (%)
120
100
80
60
40
20
0
12.5
25
37.5
50
10058-F4 (μM)

Figure 7

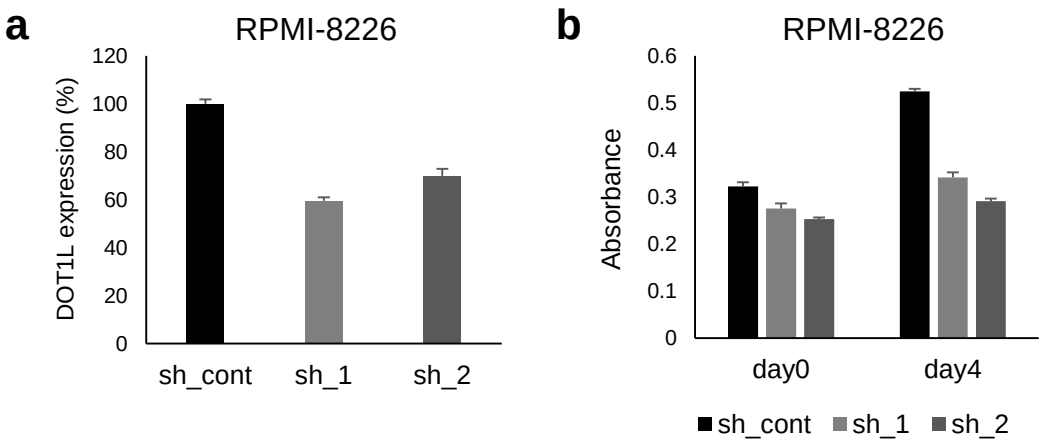

a
MYC
IRF4
Relative expression (%)
120
100
80
60
40
20
0
SGC0946
EPZ-5676
KMS-12BM
KMS-12PE
KMS-12BM
KMS-12PE
b
KMS-12PE
U-266
DMSO
SGC0946
Cell viability (%)
160
140
120
100
80
60
40
20
0
9
12
15
18
Days

Supplementary Figure 1

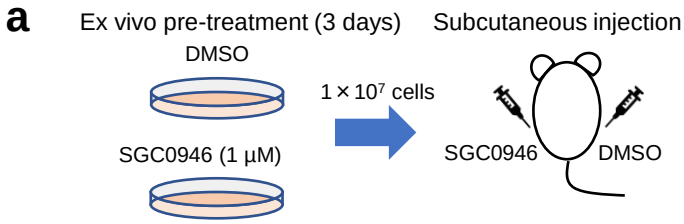


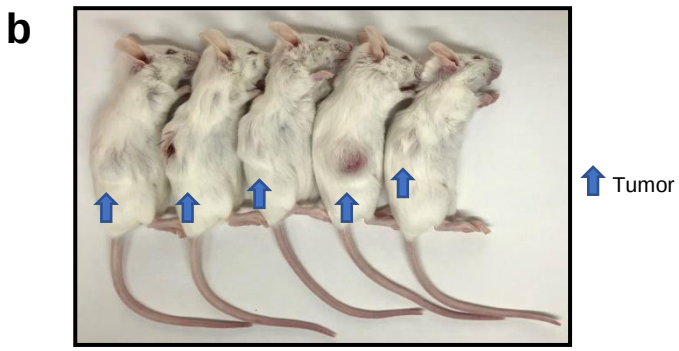
Supplementary Figure 2

Supplementary Figure 3



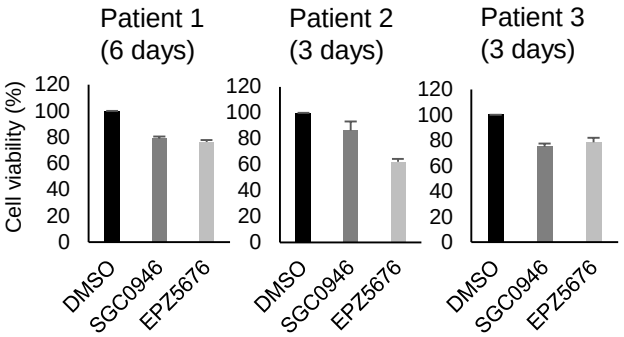
Supplementary Figure 4



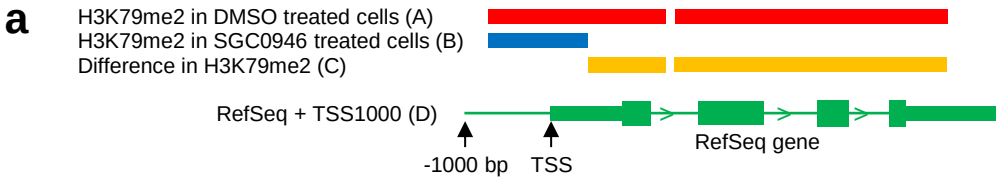


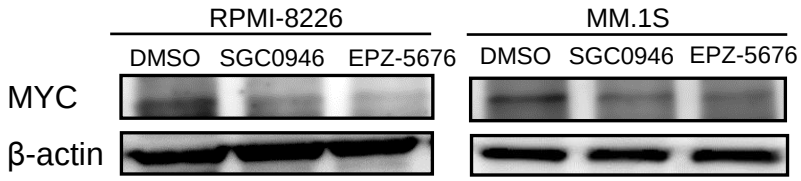
Supplementary Figure 5

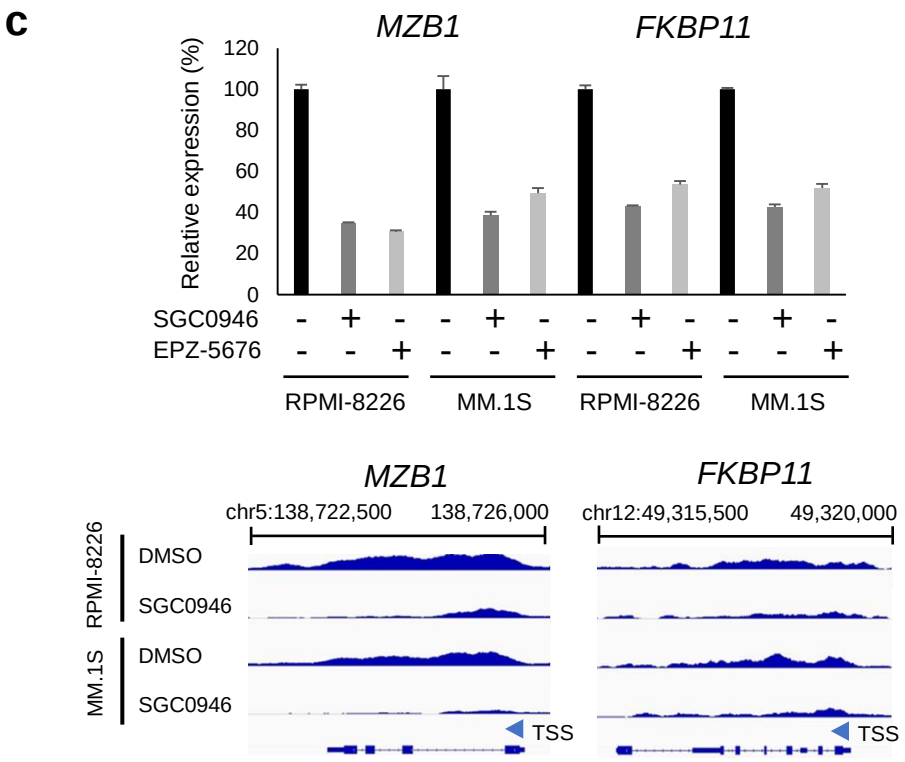
Patient	Age	Gender	Disease	Previous treatment
1	82	F	PCL, relapse	MPB, Bd, Ld
2	82	F	MM, relapse	Bd
3	86	M	MM, relapse	MP, Bd, Ld



Supplementary Figure 6

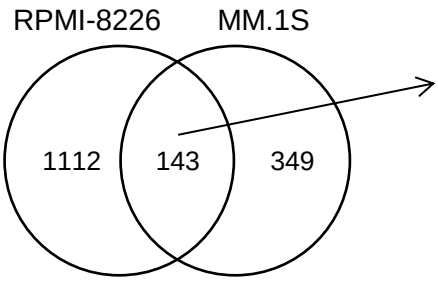






Supplementary Figure 7

a



125 protein coding genes

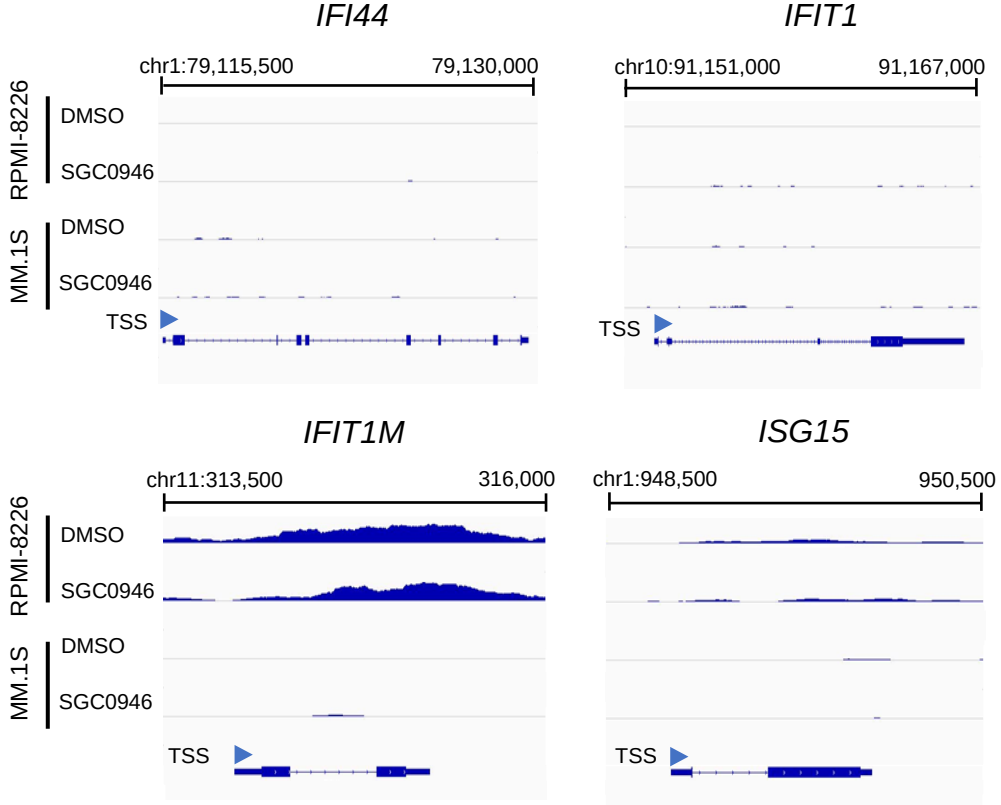
ACOT11 AGPAT9 AIF1 ALOX5AP ALPK1 AMY1C ANTXR2 ANXA2R APH1B APOL4 BATF3 BCL6 BHLHE22 BMF CACNB3 CAPG CAPN2 CARD6 CCDC74B CCDC96 CCL2 CCR2 CCR5 CFH CHRNA6 CKMT1A CLEC3B CLIP2 CMTM3 COL16A1 CPED1 CPQ CTSH CXCL10 CXCL12 CXCL9 DDIT4L EMID1 EMILIN2 ENC1 FAM200B FBXO2 FCGR2B FNBP1 FNBP1L FZD1 GLYATL1 GNAI1 GPD2 GRHL3 GTF2IRD2 HLA-DMA HLA-DMB HLA-DPB1 HLA-DQB1 HLA-DRA HLA-DRB1 HLA-DRB5 HOXB6 ID1 ID2 IFI44 IFIT1 IFITM1 IFITM3 IGFBP6 IL13RA1 IL1RAP IQCE ISG15 LAMB3 LRRC56 LY86 MAFB MILR1 MME MMP9 MS4A4A MYO1F MYOF NEURL1B NOG NREP NRIP1 NTNG1 NXF3 PARP8 POPDC2 PPP1R1C PRDM8 PRRT2 PTGER4 PTPN13 PTPRD PVRL2 PYCARD RAB37 RAPGEF2 RARRES3 RGS1 RIMBP3 RORC S100A10 SAP30L SERPINB1 SGK1 SLC9A9 SMIM14 SNTB1 SNX10 SOAT2 SORL1 SPIB TLE1 TMEM229B TP53I11 TP53I3 TP53TG3 TRAF3IP3 TTC39C XKR3 ZG16B ZNF75D ZNF862 ZSCAN31

b

GO ID	GO Term	*P* value
1679	Immune system process	2.31E-09
5032	Immune response	4.97E-07
3906	Protein binding	1.75E-06
5198	Cell surface receptor signaling pathway	2.00E-06
16311	Response to cytokine	6.11E-06
31362	Cellular response to cytokine stimulus	2.54E-05
11151	Cytokine-mediated signaling pathway	2.78E-05
1985	Regulation of immune system process	3.62E-05
26678	Regulation of cellular component movement	3.62E-05
5197	Signal transduction	4.99E-05
25101	Regulation of response to stimulus	4.99E-05
1555	Immune effector process	5.42E-05
12802	Regulation of cell migration	5.43E-05
19112	Regulation of locomotion	5.43E-05
19684	MHC class II protein complex	6.27E-05
12518	Signaling	7.92E-05
21659	Single organism signaling	7.92E-05
5186	Cell communication	1.23E-04
41363	Regulation of cell motility	1.23E-04
31328	Cellular response to organic substance	1.52E-04

Pathway	*P* value
Allograft rejection	3.97E-09
Spinal cord injury	5.32E-06
Interferon alpha-beta signaling	6.31E-06
Insulin signaling	1.59E-05
Type II interferon signaling (IFNG)	5.74E-05
Myometrial relaxation and contraction pathways	7.12E-05
Kit receptor signaling pathway	5.38E-04
TCR signaling pathway	5.54E-04
Oncostatin M signaling pathway	8.40E-04
Cell differentiation	1.20E-03
Cytokines and inflammatory response	2.12E-03
Apoptosis modulation and signaling	2.13E-03
EGF-EGFR signaling pathway	2.43E-03
Adipogenesis	3.65E-03
DNA damage response (only ATM dependent)	4.28E-03
Regulation of toll-like receptor signaling pathway	4.54E-03
Prostaglandin synthesis and regulation	4.60E-03
Toll-like receptor signaling pathway	4.68E-03
BDNF signaling pathway	5.22E-03
Integrin-mediated cell adhesion	5.33E-03

c



Supplementary Figure 8

KMS-12BM

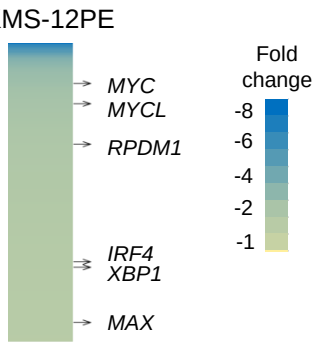
Locus number	Gene	Mutation
chr1:147092639	*BCL9*	missense
chr15:91326152	*BLM*	missense
chr9:136905311	*BRD3*	missense
chr17:59934523	*BRIP1*	nonsense
chr1:51436101	*CDKN2C*	fsDel
chr3:89456423	*EPHA3*	missense
chr4:1803120	*FGFR3*	missense
chr12:123214499	*HCAR1*	missense
chr6:152749427	*SYNE1*	missense
chr19:1621885	*TCF3*	missense
chr17:7574017	*TP53*	missense
chr14:92470910	*TRIP11*	missense

KMS-12PE

Locus number	Gene	Mutation
chr15:91326152	*BLM*	missense
chr9:136905311	*BRD3*	missense
chr2:208425880	*CREB1*	missense
chr22:41565521	*EP300*	missense
chr3:89456423	*EPHA3*	missense
chr4:1806582	*FGFR3*	missense
chr4:1803120	*FGFR3*	missense
chr12:123214499	*HCAR1*	missense
chr7:152007138	*KMT2C*	fsDel
chr12:49441845	*KMT2D*	missense
chr2:141092126	*LRP1B*	missense
chr19:1621885	*TCF3*	missense
chr17:7574017	*TP53*	missense
chr14:92470910	*TRIP11*	missense

Supplementary Figure 9

a

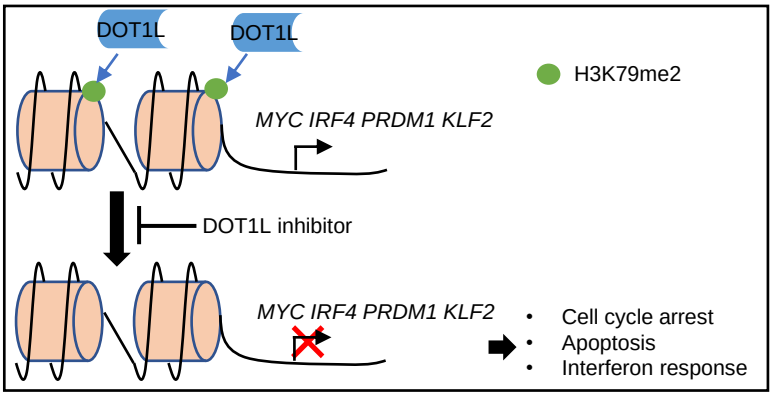


b

GO Term	*P* value
MHC class II protein complex	1.09E-12
MHC protein complex	5.91E-12
Response to interferon-gamma	7.44E-11
Immune response	8.02E-10
Defense response	3.75E-09
Cytokine-mediated signaling pathway	8.54E-09
Interferon-gamma-mediated signaling pathway	1.58E-08
Cellular response to interferon-gamma	2.08E-08
Immune system process	3.37E-08
Response to cytokine	3.55E-08

Pathway	*P* value
Allograft rejection	3.26E-10
Interferon alpha-beta signaling	1.12E-08
Type II interferon signaling	4.66E-07
Focal adhesion	7.46E-07
Interferon gamma signaling	4.21E-05
Myometrial relaxation and contraction pathways	5.60E-05
Cytokines and inflammatory response	9.39E-04
Apoptosis	1.28E-03
Integrin cell surface interactions	2.28E-03
Inflammatory response pathway	2.61E-03

Supplementary Figure 10



Supplementary methods

Western blot analysis

Total proteins were extracted using a M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific) according to the manufacturer's instruction. Histone proteins were extracted using a Triton Extraction Buffer (TEB) according to the protocol by Abcam (Cambridge, UK). Samples were separated using SDS-PAGE (12% acrylamide) and transferred to PVDF membranes (Bio-Rad). 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), rabbit anti-dimethyl histone H3K79 mAb (1:1000 dilution, #5427; 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 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 a 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). MM cells (2.5×10^5 cells) were mixed with 100 μl of lentiviral supernatant and 400 μl of media in the presence of 8 μg/ml of polybrene, after which cell were centrifuged at 800 rpm for 2h at room temperature. Twelve hours after viral infection, GFP-positive cells were selected using a BD FACSAria II (BD Biosciences, Franklin Lakes, NJ, USA).

Chromatin immunoprecipitation sequencing

Chromatin immunoprecipitation (ChIP) was performed as described previously.[1] Briefly, 1×10^6 cells were treated with 0.5 % formaldehyde for 10 minutes. 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 anti-dimethyl histone H3K79 antibody (#5427; Cell signaling technology). Before adding antibodies, 10 μL of the each cell lysate was saved as an internal control for the input DNA to use for ChIP-qPCR. 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 quality check, samples were sequenced using the Ion Proton system (Thermo Fisher Scientific). 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 with altered H3K79me2 status by the drug treatment, we subtracted the lengths of H3K79me2 peaks in respective gene regions (including 1000 bp upstream of transcription start sites) in cells treated with SGC0946 from those in cells treated with DMSO. The proportions of the subtracted peak lengths in the respective gene regions (including 1000 bp upstream of transcription start sites) were calculated, and genes with more than 0.1 were selected.

Gene expression microarray analysis

Gene expression analysis was carried out as described previously.[2] 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 (GO) and pathway analyses were also performed using Gene Spring GX. The Gene Expression Omnibus accession number for the microarray data is GSE108661.

Mutation analysis

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

Statistical analysis

To analyze the expression of DOT1L in clinical samples, published datasets (GSE5900 and GSE6477) were obtained from Gene Expression Omnibus [4, 5]. Expression levels were analyzed using standard t-test. Values of $P < 0.05$ (two-sided) were considered as statistically significant. Data were analyzed using EZR version 1.32 (Saitama Medical Center, Jichi Medical University, Saitama, Japan).[6]

References

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Supplementary figure legends

Supplementary Figure 1

qRT-PCR analysis of DOT1L in indicated MM cell lines. Results are normalized to ACTB expression. Shown are means of 3 replications; error bars represent standard error of means (SEMs).

Supplementary Figure 2

Western blot analysis of H3K79me2 in RPMI-8226 cells treated with indicated DOT1L inhibitors (1 μM, 3 days). Histone H3 is shown as a loading control.

Supplementary Figure 3

shRNA-mediated knockdown of DOT1L suppress MM cell proliferation. (a) qRT-PCR analysis of DOT1L in RPMI-8226 cells with a control shRNA or 2 different shRNAs targeting DOT1L. (b) Results of cell viability assays in RPMI-8226 cells with DOT1L knockdown. RPMI-8226 cells were infected with lentiviral shRNA vectors and cell viabilities were assessed at indicated time points. Shown are means of 3 replications; error bars represent SEMs.

Supplementary Figure 4

DOT1L inhibitor suppresses in vivo tumor formation by MM cells in SCID mice. (a) Workflow of the xenograft study. RPMI-8226 cells were pretreated with 1 μM SGC0946 or DMSO for 3 days, after which 1×10^7 cells were subcutaneously injected in the area of the left (SGC0946) and right (DMSO) thighs of SCID mice. (b) Representative tumor xenografts of DMSO treated cells. (c) Representative tumor xenografts of SGC0946 treated cells. Note that no tumor formation was found.

Supplementary Figure 5

Results of cell viability assays in primary tumor cells treated with DOT1L inhibitors. CD138-positive cells were isolated from MM or PCL patients and were treated with DOT1L inhibitors (1 μM) for indicated periods. Summary of the patients are shown on the top. Shown are means of 3-6 replications; error bars represent SEMs. MPB, Melphalan + Prednisolone + Bortezomib; Bd, Bortezomib + Dexamethasone; Ld, Lenalidomide + Dexamethasone; MP, Melphalan + Prednisolone.

Supplementary Figure 6

Analysis of H3K79me2 and gene expression in MM cells treated with DOT1L inhibitors. (a)

Schema showing the method to evaluate H3K79me2 changes induced by DOT1L inhibition. Reference sequence (RefSeq) genes satisfying C/D > 0.1 were defined as genes with reduced H3K79me2. TSS, transcription start site. (b) Western blot analysis of MYC in MM cells treated with indicated DOT1L inhibitors (1 μM, 6 days). (c) qRT-PCR analysis of MZB1 and FKBP11 in MM cell lines treated with indicated DOT1L inhibitors (upper). Results of ChIP-seq analysis showing decreased H3K79me3 levels of *MZB1* and *FKBP11* by SGC0946 (lower).

Supplementary Figure 7

DOT1L inhibition affects genes associated with 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) GO analysis of genes of upregulated (> 1.5-fold) by SGC0946 (1 μM, 6 days) in MM.1S cells (left). Pathway analysis of genes upregulated by SGC0946 in indicated MM.1S cells (right). (c) H3K79me2 status of representative interferon-stimulated genes in MM cell lines treated with DMSO or SGC0946.

Supplementary Figure 8

Mutations of cancer-related genes detected in KMS-12BM and KMS-12PE. Genes shared by both cell lines are indicated in gray letters. fsDel, frameshift deletion.

Supplementary Figure 9

Gene expression microarray analysis in KMS-12PE cells with prolonged DOT1L inhibitor treatment. (a) Heat map showing genes downregulated (> 1.5-fold) by SGC0946 (1 μM, 12 days) in KMS-12PE. (b) GO (left) and pathway (right) analyses of genes upregulated (> 1.5-fold) by SGC0946 (1 μM, 12 days) in KMS-12PE.

Supplementary Figure 10

Hypothesized mechanism underlying the antitumor effect of DOT1L inhibitors in MM cells.