# mTOR inhibitors sensitize multiple myeloma cells to venetoclax via IKZF3- and Blimp-1-mediated BCL-2 upregulation

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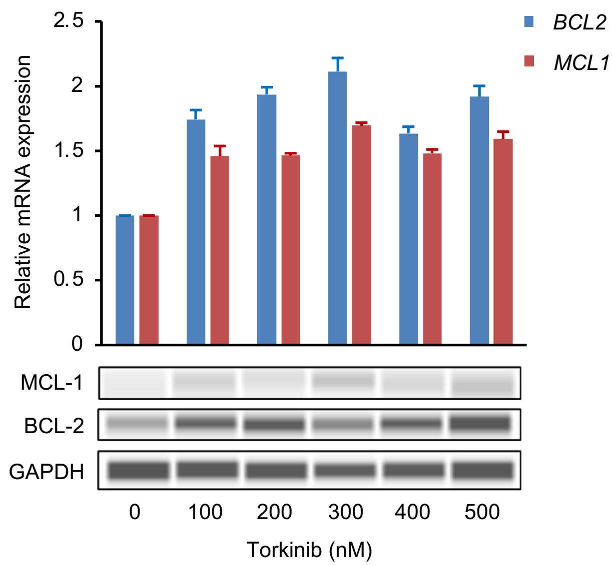
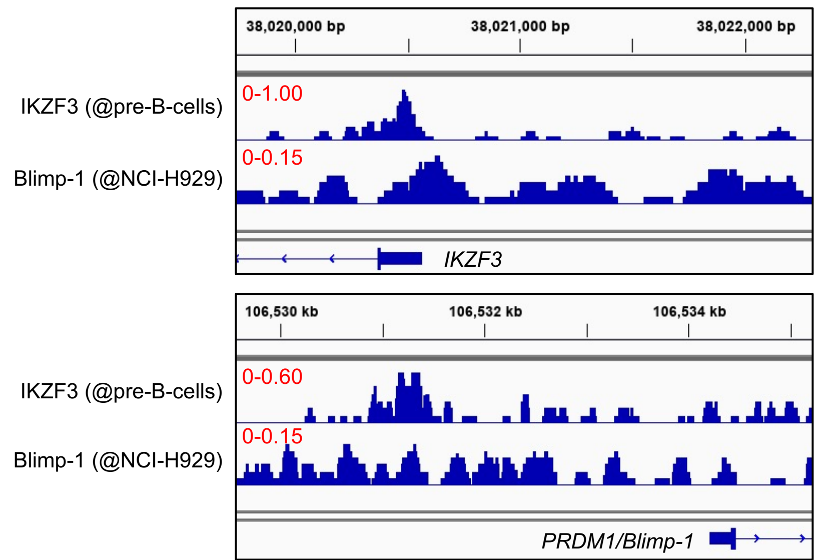
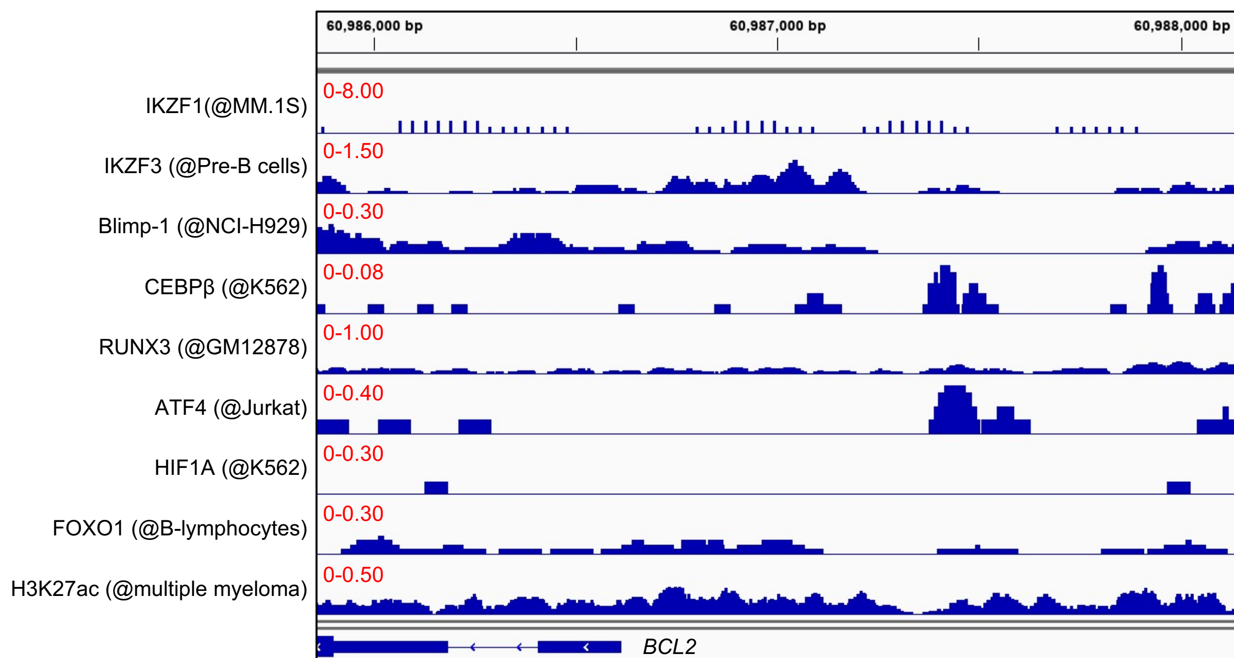
**Supplementary Table S1. Small molecular inhibitors used for the screening**

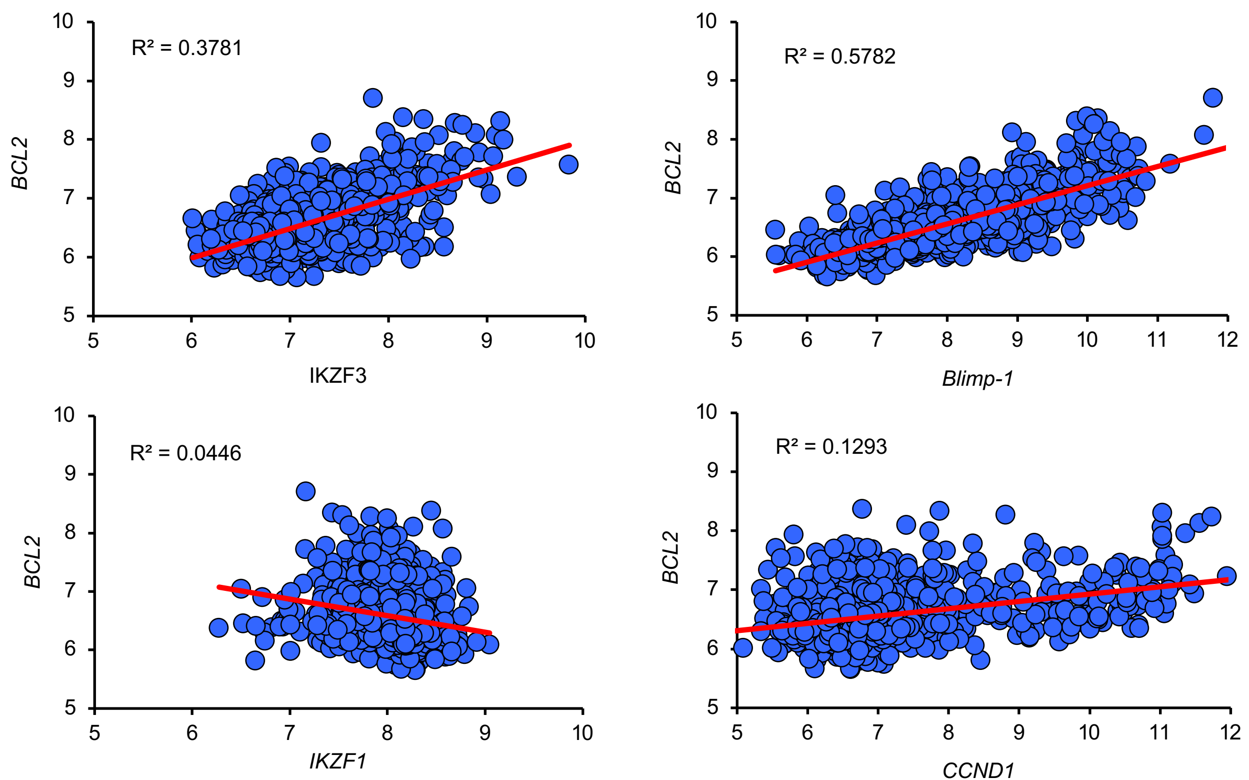
| Target category | Compound | $IC_{50}$ | Target | BCL2/MCL1 ratio |
|---|---|---|---|---|
| Epigenetics | UNC0638 | 1.5 µM | G9a | 1.09 |
| | BIX01294 | 1.5 µM | G9a | 0.96 |
| | Chaetocin | 50 nM | G9a | 0.7 |
| | GSKJ4 | 1.0 µM | JMJD3/UTX | 2.05 |
| | S2101 | 1.5 µM | LSD1 | 0.99 |
| | C646 | 2.5 µM | P300 | 0.66 |
| | JQ1 | 0.5 µM | BRD4 | 0.39 |
| | GSK343 | 10 µM | EZH2 | 1.33 |
| | DZNep | 0.5 µM | EZH2 | 1.08 |
| | Decitabine | 1.0 µM | DNMT | 0.33 |
| HDAC | Panobinostat | 1.0 µM | Pan HDAC | 0.08 |
| | Romidepsin | 0.5 nM | Class I HDAC | 1.29 |
| | Tubastatin A | 1.5 µM | Class II HDAC | 1.62 |
| | Vorinostat | 1.0 µM | Pan HDAC | 13.81 |
| | PCI-34051 | 2.5 µM | HDAC8 | 1.17 |
| | Tenovin-6 | 1.5 µM | SIRT1/2 | 1.76 |
| | SIRT1 inhibitor III | 1.5 µM | SIRT1 | 0.84 |
| Receptor tyrosine kinase (RTK) | OSI-906 | 0.1 µM | IGF-IR | 1.73 |
| | PD173074 | 1.5 µM | FGFR | 0.96 |
| | LY2157299 | 1.5 µM | TGFβR | 0.64 |
| | Lapatinib | 2.5 µM | EGFR/Her2 | 1 |
| | PF04217903 | 1.5 µM | MET | 0.32 |
| | Gefitinib | 1.5 µM | EGFR | 0.77 |
| | Erlotinib | 2.5 µM | EGFR | 12.53 |
| Multi-kinase | Sorafenib | 2.5 µM | Multiple kinases | 1.08 |
| | Sunitinib | 1.5 µM | Multiple kinases | 1.09 |
| | Pazopanib | 2.5 µM | Multiple kinases | 12.45 |
| | Vandetanib | 1.5 µM | Multiple kinases | 0.27 |
| PARP | XAV939 | 1.5 µM | PARP | 0.87 |
| | PJ-34 | 1.5 µM | PARP | 0.76 |
| | BSI-201 | 1.5 µM | PARP | 1.44 |
| PI3K/Akt/CDK | PF04691502 | 0.25 µM | PI3K | 5.62 |
| | LY-294002 | 1.0 µM | PI3K | 0.07 |
| | Perifosine | 10 µM | AKT | 1.85 |
| | AT7519 | 0.25 µM | CDK | 0.98 |
| mTOR | Temsirolimus | 0.5 µM | mTOR | 2.91 |
| | Everolimus | 0.5 µM | mTOR | 37.66 |
| | Torkinib | 0.5 µM | mTOR | 3.29 |
| Bcr-Abl | Nilotinib | 2.5 µM | BCR-ABL | 1.43 |
| | Dasatinib | 1.0 µM | BCR-ABL/SRC family | 14.95 |
| | Imatinib | 2.5 µM | BCR-ABL/c-KIT | 0.84 |
| ALK | Crizotinib | 1.5 µM | EML4-ALK | 1.17 |
| | A83-01 | 1.5 µM | ALK | 0.85 |
| | LDN193189 | 1.5 µM | ALK | 0.72 |
| GSK-3 | BIO | 1.5 µM | GSK-3 | 0.93 |
| | CT99021 | 1.5 µM | GSK-3 | 0.88 |
| IMiDs | Thalidomide | 1.5 µM | Cereblon | 0.7 |
| | Lenalidomide | 0.5 µM | Cereblon | 0.87 |
| JAK/STAT | Ruxolitinib | 1.5 µM | JAK | 0.23 |
| | WP1066 | 1.0 µM | STAT3 | 1.01 |
| Proteasome | Bortezomib | 5.0 nM | Proteasome | 2.88 |
| Others | CCG-1423 | 1.5 µM | RhoA | 11.51 |
| | AY 9944 | 1.5 µM | Hedgehog | 0.96 |
| | IWP-2 | 1.5 µM | Wnt | 0.77 |
| | DAPT | 1.5 µM | Notch | 0.98 |
| | Chlorpromazine | 1.5 µM | Potassium channel | 0.86 |
| | Desipramine | 1.5 µM | Norepinephrine reuptake | 0.79 |
| | Brefeldin A | 0.5 µM | Protein transport | 1.04 |
| | Anisomycin | 30 nM | Protein synthesis | 0.81 |
| | Tretinoin | 1.5 µM | Retinoic acid receptor | 0.65 |
| | Temozolomide | 1.5 µM | DNA synthesis | 0.71 |
| | Orlistat | 1.5 µM | Lipase | 0.96 |
| | MDV3100 | 1.5 µM | AR | 0.9 |
| | PAC-1 | 1.0 µM | Procaspase-3 | 0.73 |
| | ABT-737 | 1.0 µM | BCL-2 | 1.28 |
| | AMI-1 | 1.5 µM | PRMT1 | 0.88 |
| | PIM1/2 kinase inhibitor V | 1.5 µM | PIM | 12.43 |

*All compounds were supplied from the Screening Committee of Anticancer Drugs (Tokyo, Japan), dissolved in dimethyl sulfoxide, and used at a dilution that made a final concentration of the solvent <0.1% so as not to affect drug effects and cell growth.

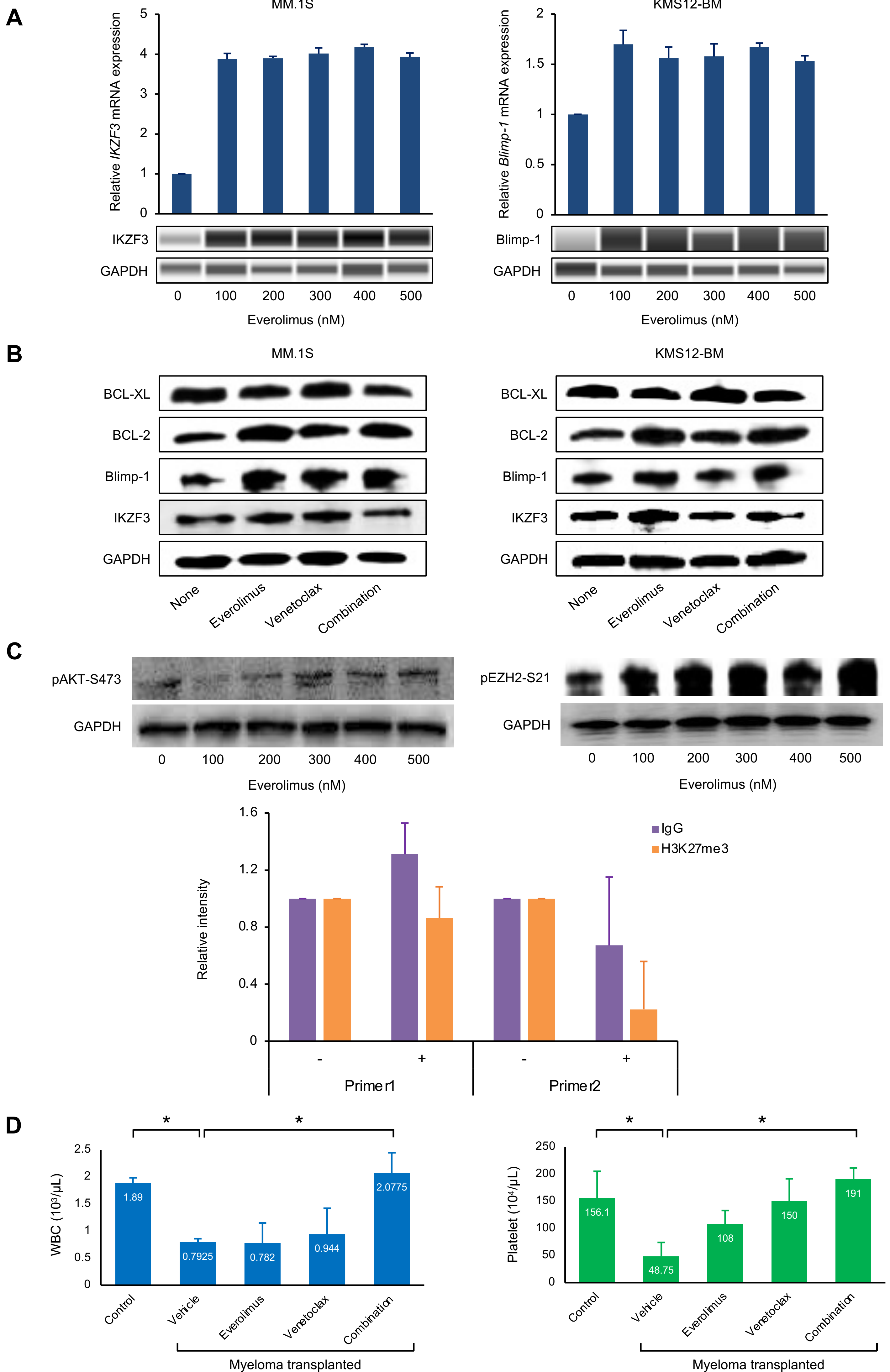

A
BCL2
MCL1
Relative mRNA expression
2.5
2
1.5
1
0.5
0
MCL-1
BCL-2
GAPDH
0
100
200
300
400
500
Torkinib (nM)


D
38,020,000 bp
38,021,000 bp
38,022,000 bp
IKZF3 (@pre-B-cells)
0-1.00
Blimp-1 (@NCI-H929)
0-0.15
IKZF3
106,530 kb
106,532 kb
106,534 kb
IKZF3 (@pre-B-cells)
0-0.60
Blimp-1 (@NCI-H929)
0-0.15
PRDM1/Blimp-1


B
60,986,000 bp
60,987,000 bp
60,988,000 bp
IKZF1(@MM.1S)
0-8.00
IKZF3 (@Pre-B cells)
0-1.50
Blimp-1 (@NCI-H929)
0-0.30
CEBPβ (@K562)
0-0.08
RUNX3 (@GM12878)
0-1.00
ATF4 (@Jurkat)
0-0.40
HIF1A (@K562)
0-0.30
FOXO1 (@B-lymphocytes)
0-0.30
H3K27ac (@multiple myeloma)
0-0.50
BCL2


C
R² = 0.3781
BCL2
IKZF3
R² = 0.5782
BCL2
Blimp-1
R² = 0.0446
BCL2
IKZF1
R² = 0.1293
BCL2
CCND1

**Supplementary Figure S1.** (A) KMS12-BM cells were treated with various concentrations of torkinib for 24 hours. The expression level of *BCL2* and *MCL1* mRNAs was determined by real-time quantitative RT-PCR (Q-PCR), normalized to that of GAPDH, quantified by the $2^{-\Delta\Delta Ct}$ method, and shown as fold increases of untreated cells. Whole cell lysates were simultaneously prepared and subjected to immunoblot analysis for the expression of MCL-1, BCL-2 and GAPDH (loading control). (B) The chromatin landscape of the promoter/enhancer region of the *BCL2* gene assembled from the data of the MM.1S cell line (IKZF1), pre-B cells (IKZF3), human MM cell line NCI-H929 (Blimp-1), human AML cell line K562 (CEBPβ, HIF1α), human peripheral blood mononuclear cells GM12878 (RUNX3), human T-cell line Jurkat (ATF4), peripheral blood B-lymphocytes (FOXO1), and human primary MM cells (H3K27ac) deposited in the ChIP-Atlas database. (C) The correlation between the expression level of *BCL2* mRNA and that of *IKZF3*, *PRDM1*/*Blimp-1*, *IKZF1* or *CCND1* mRNA in bone marrow mononuclear cells from MM patients (n=867). The data were extracted from the MIAME-compliant GEO data GSE136400. (D) Transcription of *IKZF3* and *PRDM1*/*Blimp-1* is mutually regulated in MM cells. The binding of IKZF3 and Blimp-1 to promoter/enhancer regions of the *IKZF3* and *PRDM1*/*Blimp-1* genes was analyzed using the data of pre-B cells (IKZF3) and human MM cell line NCI-H929 (Blimp-1) in the ChIP-Atlas database.


A
MM.1S
Relative IKZF3 mRNA expression
IKZF3
GAPDH
0 100 200 300 400 500
Everolimus (nM)
KMS12-BM
Relative Blimp-1 mRNA expression
Blimp-1
GAPDH
0 100 200 300 400 500
Everolimus (nM)
B
MM.1S
BCL-XL
BCL-2
Blimp-1
IKZF3
GAPDH
None
Everolimus
Venetoclax
Combination
KMS12-BM
C
pAKT-S473
GAPDH
0 100 200 300 400 500
Everolimus (nM)
pEZH2-S21
GAPDH
0 100 200 300 400 500
Everolimus (nM)
Relative intensity
IgG
H3K27me3
Primer1
Primer2
D
WBC (10³/μL)
1.89
0.7925
0.782
0.944
2.0775
Control
Vehicle
Everolimus
Venetoclax
Combination
Myeloma transplanted
Platelet (10⁴/μL)
156.1
48.75
108
150
191
Control
Vehicle
Everolimus
Venetoclax
Combination
Myeloma transplanted

**Supplementary Figure S2.** (A) Left pane: MM.1S cells were treated with various concentrations of everolimus for 24 hours. The expression level of *IKZF3* mRNA was determined by Q-PCR, normalized to that of *GAPDH*, quantified by the $2^{-\Delta\Delta Ct}$ method, and shown as fold increases of untreated cells. Whole cell lysates were simultaneously prepared and subjected to immunoblot analysis for the expression of IKZF3 and GAPDH (loading control). Right panel: KMS12-BM cells were treated with various concentrations of everolimus for 24 hours. The expression level of *PRDM1/Blimp-1* mRNA was determined by Q-PCR, normalized to that of *GAPDH*, quantified by the $2^{-\Delta\Delta Ct}$ method, and shown as fold increases of untreated cells. Whole cell lysates were simultaneously prepared and subjected to immunoblot analysis for the expression of Blimp-1 and GAPDH (loading control). (B) MM.1S and KMS12-BM cells were cultured in the absence (None) or presence of everolimus (100 nM), venetoclax (500 nM for MM.1S and 10 nM for KMS12-BM) or the combination of everolimus and venetoclax (at corresponding doses). Whole cell lysates were prepared after 24 hours and subjected to immunoblot analysis for the expression of BCL-XL, BCL-2, Blimp-1, IKZF3 and GAPDH (loading control). (C) Upper panel: Whole cell lysates were simultaneously prepared from KMS12-BM cells during the experiments described in Figure 1C and subjected to immunoblot analysis for serine-473 and serine-21 phosphorylation of AKT and EZH2, respectively. Lower panel: Chromatin suspensions were prepared from KMS12-BM cells cultured in the absence (–) or presence (+) of 200 nM everolimus for 24 hours and immunoprecipitated with specific antibody against trimethylated histone H3 at lysine-27 (H3K27me3) or isotype-matched IgG. The resulting precipitates were subjected to Q-PCR to amplify the regions containing the upstream (primer 2) and downstream (primer 1) IKZF3-binding sites of *BCL2* promoter as shown in Figure 2A. The data were normalized to the values of input, quantified by the $2^{-\Delta\Delta Ct}$ method, and shown as fold increases against the values obtained with control IgG immunoprecipitants from untreated KMS12-BM cells. (D) We inoculated $5 \times 10^5$ luciferase-expressing KMS12-BM cells subcutaneously in the right thigh of male NOD/SCID mice (Charles River Laboratories, Wilmington, MA) and randomized them into four treatment groups when measurable tumors developed (day 0). Each group was treated with the vehicle alone (0.9% NaCl, orally, 5 times a week), everolimus alone (4 mg/kg, orally, twice a week), venetoclax alone (40 mg/kg, orally, 5 times a week), and the combination of everolimus and venetoclax for three weeks. We measured the counts of white blood cells (WBC) and platelets in peripheral blood of recipient mice on day 21 of treatment. The means ± S.D. (bars) are shown (n=4). $^*P$ <0.01 by Student's *t* test. The control values were adapted from the database of Charles River Laboratories (shown below).

| **NOD.CB17-*Prkdc*$^{scid}$/NcrCrl** | | **WBC** | **NEUT** | **LYMPH** | **MONO** | **EOS** | **BASO** | **NEUT** | **LYMPH** | **MONO** | **EOS** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | (K/µL) | (K/µL) | (K/µL) | (K/µL) | (K/µL) | (K/µL) | (%) | (%) | (%) | (%) |
| Male (♂) | Mean | 1.89 | 1.21 | 0.50 | 0.11 | 0.06 | 0.01 | 64.14 | 26.58 | 5.82 | 2.84 |
| 95% interval | Low | 0.94 | 0.54 | 0.26 | 0.03 | 0.01 | 0.00 | 44.21 | 16.83 | 1.74 | 0.30 |
| | High | 4.12 | 2.15 | 1.56 | 0.25 | 0.33 | 0.11 | 79.12 | 40.93 | 11.74 | 8.67 |
| | N | 120 | 120 | 120 | 120 | 120 | 120 | 120 | 120 | 120 | 120 |
| Female (♀) | Mean | 2.18 | 1.44 | 0.52 | 0.11 | 0.09 | 0.02 | 66.35 | 24.13 | 5.37 | 3.46 |
| 95% interval | Low | 0.96 | 0.60 | 0.23 | 0.03 | 0.00 | 0.00 | 47.00 | 13.51 | 1.71 | 0.29 |
| | High | 4.68 | 3.16 | 1.32 | 0.26 | 0.39 | 0.15 | 79.92 | 42.61 | 10.93 | 10.32 |
| | N | 120 | 120 | 120 | 120 | 120 | 120 | 120 | 120 | 120 | 120 |

| **NOD.CB17-*Prkdc*$^{scid}$/NcrCrl** | | **BASO** | **RBC** | **HGB** | **HCT** | **MCV** | **MCH** | **MCHC** | **RDW** | **PLT** | **MPV** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | (%) | (M/µL) | (g/dL) | (%) | (fL) | (pg) | (g/dL) | (%) | (K/µL) | (fL) |
| Male (♂) | Mean | 0.62 | 9.19 | 14.0 | 49.9 | 54.4 | 15.3 | 28.1 | 18.4 | 1561 | 5.2 |
| 95% interval | Low | 0.00 | 7.84 | 11.8 | 44.1 | 51.1 | 13.7 | 25.1 | 17.3 | 914 | 4.3 |
| | High | 2.76 | 10.84 | 16.6 | 57.2 | 58.6 | 17.2 | 31.2 | 20.3 | 2055 | 6.0 |
| | N | 120 | 120 | 120 | 120 | 120 | 120 | 120 | 120 | 120 | 120 |
| Female (♀) | Mean | 0.68 | 9.23 | 14.2 | 49.7 | 53.8 | 15.4 | 28.5 | 18.2 | 1284 | 5.2 |
| 95% interval | Low | 0.00 | 8.21 | 12.1 | 44.6 | 51.3 | 13.9 | 25.7 | 17.4 | 651 | 4.2 |
| | High | 3.55 | 10.48 | 17.6 | 58.3 | 56.3 | 17.1 | 31.3 | 19.5 | 1878 | 6.3 |
| | N | 120 | 120 | 120 | 120 | 120 | 120 | 120 | 120 | 120 | 120 |

*The data were collected from the actual measurement of North American colonies of NOD/SCID mice in Charles River Laboratories. Age: 8-10 weeks.