

Dual inhibition of EZH2 and EZH1 sensitizes PRC2-dependent tumors to proteasome inhibition

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Supplementary Materials and Methods

Human cell lines

Human MM cells MM.1S, NCI-H929 (H929) and RPMI8226; human prostate cancer cells LNCaP and DU145; and human embryonic HEK293T cells were obtained from American Type Culture Collection. Human KMS11 and bortezomib-resistant KMS11/BTZ (Ri et al., 2010) cells were obtained from Japanese Collection of Research Bioresources Cell Bank. Human OPM1 and OPM2 plasma cell leukemia cell lines and doxorubicin-resistant RPMI-DOX40 (DOX40) cells were kindly provided by Dr. Edward Thompson (University of Texas Medical Branch, Galveston, TX) and Dr. William Dalton (Lee Moffitt Cancer Center, Tampa, FL), respectively. MM and prostate cancer cells were cultured in RPMI-1640 containing 10% fetal bovine serum (FBS), 2 μ M L-glutamine, 100 U/mL penicillin and 100 μ g/mL streptomycin (Thermo Fisher). HEK293T cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 2 μ M L-glutamine, 100 U/mL penicillin and 100 μ g/mL streptomycin (Thermo Fisher).

Supplementary Reagents

HLM006474 (Ma et al., 2007) was purchased from Sigma-Aldrich and was diluted in DMSO to a stock of 10 mM.

Immunoblot analysis

Whole-cell lysates were prepared by lysis in RIPA (50 mM Tris, pH 8.0, 150 mM NaCl, 1mM EDTA, pH 8.0, 1% TritonX-100, 0.1% sodium deoxycholate and 0.1% SDS), PML (20 mM sodium phosphate, pH 7.0, 300 mM NaCl, 5 mM EDTA and 0.1% NP40) buffers supplemented with protease inhibitor cocktail (Roche) or SDS-sample buffer (25 mM Tris, pH 6.8, 1% SDS, 5% glycerol, 0.05% bromophenol blue and 1% β -mercaptoethanol). Lysates

were then sonicated (Bioruptor, COSMO BIO CO.) prior to SDS-PAGE. Immunoblotting was performed according to standard procedures. Membranes were probed with the indicated antibodies to: H3 (Abcam, ab1791), H3K27me3 (Millipore, 07449), EZH2 (clone DC9, Cell Signaling, 5246), EZH1 (Abcam, ab86128), caspase-3 (Cell Signaling, 9662), caspase-8 (clone 1C12, Cell Signaling, 9746), caspase-9 (Cell Signaling, 9502), PARP (Cell Signaling, 9542), GAPDH (clone 14C10, Cell Signaling, 2118), E2F1 (clone C20, Santa Cruz, sc-193), phospho-RB (clone Ser 780, Santa Cruz, sc-12901), p21 (clone 12D1, Cell Signaling, 2947), p27 (clone C-19, Santa Cruz, sc-528), α -tubulin (Calbiochem, CP06), Nur77 (D63C5, Cell Signaling, 3960), and MYC (clone N-262, Santa Cruz, sc-764). HRP-conjugated secondary antibodies were purchased from Amersham ECL. Immobilon Western Chemiluminescent substrates (EMD Millipore) were used for immunoblot detection. Sequential reprobing of the membranes was performed after stripping of primary and secondary antibodies using 62.5mM Tris, pH 6.8, 2% SDS and 0.7% β -mercaptoethanol. Protein expression was quantified using Image Lab software (BioRad).

Assay of apoptosis

MM cell lines were plated at 1×10^6 cells per well of a six-well plate and treated with or without UNC1999 (5 μ M) for 48 hours with bortezomib (5 nM) in the last 24 hours. Cells were harvested, washed with phosphate-buffered saline (PBS) and then stained with FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen, 556547). Flow cytometry was performed on a BD FACS Canto II (BD Biosciences, San Jose, CA, USA) and analyzed using FlowJo software (Tree Star). Annexin V-positive and PI-negative cells were considered as early apoptotic, whereas positivity for both Annexin V and PI was regarded as indicative of late apoptosis.

Assays of cytotoxicity

Cell lines were dissociated, counted and plated in flat-bottom tissue culture 96-well plates (TPP). MM cell lines were plated at 8,000-20,000 cells per well and cultured for 72 hours with the indicated doses of UNC1999 or GSK126, and then the indicated doses of bortezomib were added for the last 48 hours. For co-culture with BMSC medium experiments, MM cells were cultured in normal medium or conditioned medium derived from culture supernatant of BMSCs (BMSC medium) and then the cells were treated with UNC1999 and bortezomib as described above. Prostate cancer cell lines were plated at 4,000 cells per well and treated simultaneously with the indicated doses of UNC1999 or GSK126 and bortezomib for 72 hours. For MTS assay, CellTiter 96 AQueous One Solution (Promega) was added to the cells in the last four hours of the incubation period and absorbance was read on a plate reader (Synergy2, BioTeK, Winooski, VT, USA) to determine relative cell number in each well. Data were averaged for triplicates or quadruplicates and normalized to the untreated wells. Results are expressed as the percentage of untreated control. To calculate combination index, triplicate or quadruplicate data were averaged and input into CompuSyn software (ComboSyn) (Chou et al., 1984). CI values of less than 1.0, equal to 1.0, and greater than 1.0 indicate synergism, additive effect, and antagonism, respectively.

Quantitative RT-PCR

Total RNA was isolated using TRI Reagent (Clinical Research Center) and cDNA was made using the ThermoScript RT-PCR system (Invitrogen) with an oligo-dT primer. Real-time quantitative PCR was performed in triplicate using FastStart Universal Probe Master (Roche Applied Science) and the indicated combinations of the Universal Probe Library (Roche Applied Science) on a StepOnePlus Real-Time PCR System (Applied Biosystems). The real-

time PCR signals were examined in triplicates and normalized to those of *GAPDH* gene. The primers sequences and probes used are shown in supplementary table 6.

Chromatin immunoprecipitation assays (ChIP)

MM.1S cells incubated with bortezomib (5 nM) or DMSO for 24 hours were crosslinked with 1% para-formaldehyde (PFA) and then sonicated (ultrasonic homogenizer, MICROTEC.CO). After centrifugation, the soluble chromatin fraction was recovered, pre-cleared with anti-rabbit IgG conjugated Dynabeads (Thermo Fisher), and then incubated with an anti-E2F1 (clone C20, Santa Cruz, sc-193) for 6 hours at 4°C. On the following day, immunoprecipitates were thoroughly washed. A standard purification method was used to separate DNA from protein fragments. For ChIP assay, quantitative PCR was performed with Step One Plus real time PCR system using SYBR® Premix ExTaq II (Tli RNase Plus) from Takara. Primers for OCT4 (SIGMA-ALDRICH) were used as negative controls. Details of the primers used are shown in Supplementary Table 7.

Gene set enrichment analysis (GSEA)

Gene set enrichment analysis was conducted with the software GSEA (<http://www.broadinstitute.org/gsea>) (Subramanian et al., 2005). A pseudocount of 1 was added to the RPKM measure prior to GSEA.

Vectors

Lentiviral vectors (CS-H1-shRNA-EF-1 α -EGFP) expressing short hairpin RNA (shRNA) that targets human *EZH2* (target sequence: sh-*EZH2*-1, 50-GGAAAGAACGGAAATCTTA - 30; sh-*EZH2*-2, 50-GGATAGAGAATGTGGGTTT-30) and *luciferase* (*Luc*) were constructed (Chiba et al., 2012). Lentiviral vectors (CS-H1-shRNA-EF-1 α -RFP) expressing

short hairpin RNA (shRNA) that targets human *EZH1* (target sequence: sh-*EZH1*, 5'-GCGACTTCGACAACCTTAAACG-3') were constructed. The *EZH2* overexpression construct was generated by subcloning murine *Ezh2* cDNA into CSII-EF-MCS-IRES2-Venus. *E2F1* overexpression retroviral vector (pMCs-E2F1-IG) was a kind gift from Dr. Yuji Furukawa at Jichi Medical School. *NR4A1* overexpression retroviral vector (MIG-R1-NR4A1) was a kind gift from Dr. Akihiko Yoshimura at Keio University. Matched empty vectors were constructed. Recombinant lentiviruses and retroviruses were produced using established protocols (Iwama et al., 2004).

Luciferase assay

HEK293T cells (8×10^4 cells) were seeded in a 24 well plate and cultured for 24 hours. Cells were then transfected with 400 ng of the indicated expression vector (empty or *E2F1*) along with 100 ng of a reporter gene (pGL basic human *EZH2* promoter -1095bp, -442bp and -151bp to +48, respectively) and 20 pg of pRL-CMV, an expression vector of Renilla luciferase, using FuGENE HD Transfection Reagent (Promega). 24 hours after transfection, the cells were subjected to luciferase assay using the Dual-luciferase Reporter System (Promega). Relative firefly Luciferase activities were calculated by normalizing transfection efficiency to Renilla Luciferase activities.

Supplementary References

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Supplementary Tables

Supplementary Table S1	Combination index (CI) values.
Supplementary Table S2	Significantly upregulated PRC2 gene sets in UNC1999-treated MM.1S cells (FDR q-value < 0.05)
Supplementary Table S3	Significantly upregulated PRC2 gene sets in combination-treated MM.1S cells (FDR q-value < 0.05)
Supplementary Table S4	Top 74 PRC2 genes that are significantly altered in UNC1999-treated MM.1S cells
Supplementary Table S5	Significantly upregulated gene sets in RPMI8226 cells transduced with EZH2-overexpressing versus empty vectors.
Supplementary Table S6	List of primers used for Quantitative RT-PCR
Supplementary Table S7	List of primers used for CHIP-PCR

Supplementary Table S1. Combination index (CI) values.

The combined effect of two agents was analyzed by isobologram analysis using the Compu-Syn software program (ComboSyn, Inc).

- (A) Combination index (CI) values in H929 cells treated with the combination of UNC1999 and bortezomib.
- (B) Combination index (CI) values in RPMI8226 cells treated with the combination of UNC1999 and bortezomib.
- (C) Combination index (CI) values in DOX40 cells treated with the combination of UNC1999 and bortezomib.
- (D) Combination index (CI) values in MM.1S cells treated with the combination of UNC1999 and bortezomib in the presence of normal media or conditioned media derived from bone marrow stromal cells (BMSCs).
- (E) Combination index (CI) values in MM.1S cells treated with the combination of UNC1999 and carfilzomib.
- (F) Combination index (CI) values in H929 cells treated with bortezomib in combination with UNC1999 or GSK126.
- (G) Combination index (CI) values in RPMI8226 cells treated with bortezomib in combination with UNC1999 or GSK126.
- (H) Combination index (CI) values in LNCaP cells treated with bortezomib in combination with UNC1999 or GSK126.
- (I) Combination index (CI) values in DU145 cells treated with bortezomib in combination with UNC1999.

Supplementary Table S1A

Bortezomib (nM)	UNC1999 (μ M)	Fraction of cells affected (Fa)	CI
1.5	2.5	0.3487	0.8905
1.5	5.0	0.4938	0.4291
2.0	2.5	0.3585	0.9820
2.0	5.0	0.6245	0.4247
2.5	2.5	0.4815	0.7245
2.5	5.0	0.9176	0.1978

Supplementary Table S1B

Bortezomib (nM)	UNC1999 (μ M)	Fraction of cells affected (Fa)	CI
4.0	2.5	0.2878	0.9819
4.0	5.0	0.5691	0.7992
5.0	2.5	0.5631	0.9220
5.0	5.0	0.8108	0.7432
6.0	2.5	0.7791	0.8921
6.0	5.0	0.8938	0.7673

Supplementary Table S1C

Bortezomib (nM)	UNC1999 (μ M)	Fraction of cells affected (Fa)	CI
10.0	1.25	0.4036	0.9349
12.5	1.25	0.6916	0.8849
15.0	1.25	0.8100	0.9226
10.0	2.5	0.6414	0.7518
12.5	2.5	0.8332	0.7446
15.0	2.5	0.8982	0.7894
10.0	5.0	0.8422	0.5904
12.5	5.0	0.8390	0.7406
15.0	5.0	0.8595	0.8567

Supplementary Table S1D

Bortezomib (nM)	UNC1999 (μ M)	Normal medium		BMSC medium	
		Fraction of cells affected (Fa)	CI	Fraction of cells affected (Fa)	CI
1.5	2.5	0.5132	1.2122	0.4711	1.0066
1.5	5	0.8326	0.8513	0.5918	0.8837
2	2.5	0.8336	0.9733	0.5250	1.0838
2	5	0.9288	0.8949	0.8160	0.7230
2.5	2.5	0.9043	1.1016	0.7168	0.9897
2.5	5	0.9505	1.0516	0.8860	0.8032

Supplementary Table S1E

Carfilzomib (nM)	UNC1999 (μ M)	Fraction of cells affected (Fa)	CI
3.0	1.25	0.4866	0.9434
3.0	2.5	0.8314	0.5910
3.0	5.0	0.9269	0.4738
4.0	1.25	0.7334	0.8531
4.0	2.5	0.8296	0.7625
4.0	5.0	0.9150	0.6399
5.0	1.25	0.8806	0.7891
5.0	2.5	0.8842	0.8113
5.0	5.0	0.9512	0.6444

Supplementary Table S1F

Bortezomib (nM)	UNC1999 or GSK126 (μ M)	UNC1999		GSK126	
		Fraction of cells affected (Fa)	CI	Fraction of cells affected (Fa)	CI
1.5	2.5	0.3933	0.7361	0.1168	1.7449
1.5	5.0	0.5720	0.5909	0.2767	1.6843
2.0	2.5	0.5815	0.7783	0.3345	1.4023
2.0	5.0	0.8981	0.5689	0.5177	1.4984
2.5	2.5	0.8233	0.7916	0.6617	1.0746
2.5	5.0	0.9823	0.5217	0.8597	1.0958

Supplementary Table S1G

Bortezomib (nM)	UNC1999 or GSK126 (μ M)	UNC1999		GSK126	
		Fraction of cells affected (Fa)	CI	Fraction of cells affected (Fa)	CI
4.0	1.25	0.2359	0.9560	0.1237	1.2524
4.0	2.5	0.3942	0.8674	0.2145	1.3443
4.0	5.0	0.8752	0.6364	0.5949	1.0580
5.0	1.25	0.7090	0.9129	0.4114	1.0679
5.0	2.5	0.8481	0.8194	0.6393	1.0383
5.0	5.0	0.9562	0.6861	0.8971	0.9307
6.0	1.25	0.8976	0.9272	0.8372	1.0595
6.0	2.5	0.9351	0.8691	0.9145	1.0373
6.0	5.0	0.9619	0.8079	0.9678	0.9967

Supplementary Table S1H

Bortezomib (nM)	UNC1999 or GSK126 (μ M)	UNC1999		GSK126	
		Fraction of cells affected (Fa)	CI	Fraction of cells affected (Fa)	CI
10.0	2.5	0.4179	0.5805	0.0696	1.8518
10.0	5.0	0.6637	0.3916	0.1637	1.3510
10.0	10.0	0.8513	0.3629	0.4715	0.7031
15.0	2.5	0.6991	0.3536	0.2634	1.0669
15.0	5.0	0.8198	0.3018	0.2615	1.2375
15.0	10.0	0.8943	0.3398	0.6492	0.6647
20.0	2.5	0.8234	0.2735	0.3447	1.1812
20.0	5.0	0.8788	0.2693	0.3525	1.2707
20.0	10.0	0.9058	0.3491	0.7609	0.6902

Supplementary Table S1I

Bortezomib (nM)	UNC1999 (μ M)	Fraction of cells affected (Fa)	CI
25	1.25	0.4186	0.9347
25	2.5	0.5510	0.8718
25	5.0	0.8185	0.7163
50	1.25	0.6036	0.9484
50	2.5	0.7099	0.8802
50	5.0	0.8801	0.7360
100	1.25	0.7067	0.9934
100	2.5	0.7937	0.9061
100	5.0	0.9106	0.7647

Supplementary Table S2. Significantly upregulated PRC2 gene sets in UNC1999-treated MM.1S cells.

Gene set enrichment analysis (GSEA) using our RNA-seq data identified PRC2 target gene sets that are significantly enriched in UNC1999-treated MM.1S cells (FDR q-value <0.05).

NAME	SIZE	NES	NOM p-val	FDR q-val
AGM_VE-CAD+CD45+ VS ABM_150+34-LSK UPON TOP500 2012	408	1.8839391	0	0.004089887
EZH2KO_SASHIDA_ABM_BMT_E2KO VS WT_UPON	543	1.844744	0	0.004125951
PLACENTA_45+KIT+CD34MED VS ABM_150+34-LSK UPON TOP500 2012	428	1.8432679	0	0.003615148
EZH1_2_OLA_UNC1999_MM.1S_K27ME3_GR_3FOLD	2742	1.7841836	0	0.008655357
UNC1999_CHIP_K27_TARGET_IN_PRC2_TARGET_OLA_MM.1S	2050	1.7660209	0	0.011018296
H2AK119UB1_WT-GMP > 5FOLD 766GENES	699	1.7608407	0	0.010083644
EZH1_2_OLA_UNC1999_MM.1S_K27ME3_GR_4FOLD	2156	1.7555077	0	0.009016174
EZH1_2_OLA_UNC1999_MM.1S_K27ME3_GR_2FOLD	3601	1.7482537	0	0.00856214
EZH2KOTET2KD_MUTO_FL-BMT_LSK_DKO VS WT UPON	237	1.7156032	0	0.013547997
EZH2KO_MUTO_FL-BMT_LSK_E2KO VS WT UPON	367	1.711354	0	0.012982707
H2AK119UB1_WT-GMP > 4FOLD 1859GENES	1684	1.696451	0	0.015298391
EZH2KOTET2KD-MDS_MUTO_FL-BMT_GMP_ET77 VS WT UPON	796	1.6943446	0	0.014246338
EZH2KO_SASHIDA_AHSCVECTOR_BMT_1000_E2KO VS WT_UPON	674	1.6850624	0	0.014819628
CD34-LSK_SPECIFIC GEROGGE-ARRAY UPON	546	1.6674124	0	0.018670889
TET2KD_MUTO_FL-BMT_GMP_T2KD VS WT UPON	116	1.660364	0.003194888	0.019254837
MPP_SPECIFIC GEROGGE-RNA-SEQ	108	1.6446334	0.001675042	0.022012768
MONOCYTES_GOODDEL	64	1.6385078	0.013445378	0.022561245
PRC2-TARGET IN ABM-GMP K27ME3 > 4FOLD_3859GENES	1979	1.6372403	0	0.021678675
LSKCD34- VS MPPFLK2+,- UPON 2014_ROSSI	204	1.618182	0.001718213	0.025311075
EZH2KO-MDS_CASCIO_DNOFF_B10-F14	948	1.6119399	0.001321004	0.02553239
TET2KD_MUTO_FL-BMT_LSK_T2KD VS WT UPON	284	1.6050408	0.001644737	0.02650931
PRC2-TARGET IN ABM-LK K27ME3 > 2FOLD_3773GENES	1983	1.600118	0	0.027115148
EZH2KOTET2KD-MDS_MUTO_FL-BMT_GMP_ET70 VS WT UPON	761	1.5943123	0	0.02759404
SOX17 OE CD48LSK-DEV-WT UP 376GENES 2011_MORRISON	292	1.593092	0	0.026844835
EZH1_2_OLA_UNC1999_MM.1S_K27ME3_GR_TOP1000	960	1.5877097	0	0.027534094
H2AK119UB1_WT-LSK > 2FOLD 3946GENES	1916	1.5817297	0	0.028337164
NK CELLS_GOODDEL	45	1.5793766	0.021812081	0.027997788
PRC2-TARGET IN ABM-BMT-LSK K27ME3_TOP500GENES	472	1.5725046	0	0.0288503
PRC2-TARGET IN BMT-LSK_HASEGAWA_K27 > 2FOLD_LSK-WT_4107GENES	1854	1.5693363	0	0.02906221
2008-ORKIN_ESC_EZH1_K27-EZH2KO_EZH1-EZH2KO	189	1.5691547	0.003284072	0.028240241
EZH2KO_MUTO_FL-BMT_GMP_EZH2KO VS WT UPON	356	1.5592595	0	0.03015549
PRC2-TARGET IN ABM-BMT-LSK K27ME3 > 2FOLD_2917GENES	1933	1.5576934	0	0.029507585
EEDKO VS WT BM_LT-HSC UP > 3FOLD 339GENES S2N 2014_ORKIN	302	1.5497996	0.001584786	0.031300623
EZH2KO-MDS_CASCIO_DNOFF_B11-F2	1019	1.5446994	0	0.031929474
BIVALENT IN FL-BMT-LSK_HASEGAWA_LSK-WT_K27ME3 > 2FOLD_K4ME3 > 1.5FOLD_1883GENES	1671	1.5433587	0	0.031772
EZH2KO-1M_K27_WT > 2_EZH2KO-VS-WT > 0.8_AOYAMA	526	1.5422975	0	0.03124281
EZH2KO-MDS-PLTHIGH_CASCIO_DNOFF_B10-F7	1539	1.542072	0	0.030435225
EZH2KO_MUTO_FL-BMT_GMP_E2KO VS WT UPON	345	1.5357662	0	0.031231508
EZH2KOTET2KD-MDS_MUTO_FL-BMT_LSK_E19 VS WT DNOFF	640	1.5337346	0	0.030878479
EZH2KO-MDSMPN_CASCIO_DNOFF_B11-F5	1434	1.5283102	0	0.03211698
EZH2KO-MDSMPN-2NDBMT_CASCIO_DNOFF_B10F52-F2	2580	1.5259757	0	0.032148264
H2AK119UB1_WT-LSK > 3.5FOLD 729GENES	686	1.5228395	0	0.032327462
EZH2KO-1M_K27_WT > 2_EZH2KO-VS-WT < 0.5_AOYAMA	1529	1.5196855	0	0.032762982
EZH2TARGET IN FL-BMT-LSK_HASEGAWA_LSK_CHIP_K27_WT > 2_EZH2KO VS WT < 1_MUTO-LSK_EXP_EZH2KO VS WT-UP,ON_31GENES	772	1.5169932	0	0.032860067
2008-ORKIN_ESC_EZH2_K27-WT_NOK27-EZH2KO	589	1.5149614	0.002890173	0.0330029
SOX17 OE HSCS-DEV-WT UP TOP500 2011_MORRISON	414	1.5130467	0.00309119	0.033010162
BIVALENT_SP_LSKCD150_K27ME3 > 3FOLD_K4ME3 > 10FOLD_04MO_CHIP-SEQ_2014_GOODSELL_GSE47819_1157GENES	2229	1.5080268	0	0.03377041
BMI1-KO_LSK DOWN OGURO	238	1.5073092	0.007849294	0.03332933
PRC2_EZH2KO-1M_K27_WT > 3_AOYAMA	2521	1.5071855	0	0.032679573

PRC2-TARGET IN ABM-LSK K27ME3_TOP1000GENES	2210	1.5023953	0	0.033841066
TET2KD_MUTO_FL-BMT_GMP_T2KD VS WT DNOFF	229	1.4862456	0.01821192	0.039234105
EZH2KOTET2KD_MUTO_FL-BMT_GMP_DKO VS WT UPON	356	1.482786	0.007496252	0.039773364
PRC2-TARGET IN ABM-LSK K27ME3 > 2FOLD_3192GENES	1933	1.4823841	0	0.039180823
BIVALENT IN FL-BMT-LSK_HASEGAWA_LSK-WT_K27ME3 > 2FOLD_K4ME3 > 2FOLD_759GENES	666	1.4818178	0.001410437	0.03864862
LTHSC_SPECIFIC GEROGGE-RNA-SEQ	258	1.4690437	0.012820513	0.04274336
PRC2-TARGET IN ABM-GMP K27ME3_TOP500GENES	1021	1.4656227	0	0.043372106
FL_13-5_150+48-LSK VS ABM_150+34-LSK DOWNOFF TOP500 2012	387	1.459586	0.006097561	0.045752805
SOX17 OE HSCS-DEV-WT UP TOP100 2011_MORRISON	86	1.4572768	0.0352349	0.045787722
EZH2KO-MDSMPNQ_CASCIO_DNOFF_F2	1584	1.4562854	0.001298701	0.04541264
EZH1_2_OLA_UNC1999_MM.1S_K27ME3_GR_TOP500	483	1.4529027	0.012102874	0.046120077
EZH2KO_MUTO_FL-BMT_GMP_E2KO VS WT DNOFF	154	1.4492344	0.026533997	0.046714045
MUTO_GMP-K27_KO VS WT-0.5_WT-3 UP ON	118	1.4412801	0.03642384	0.04965807
HSC_GODEL	207	1.4410642	0.014128729	0.048914943

Supplementary Table S3. Significantly upregulated PRC2 gene sets in combination-treated MM.1S cells.

Gene set enrichment analysis (GSEA) using our RNA-seq data identified PRC2 target gene sets that are significantly enriched in MM.1S cells treated with the combination of UNC1999 and bortezomib (FDR q-value <0.05).

NAME	SIZE	NES	NOM p-val	FDR q-val
EZH1_2_OLA UNC1999_MM.1S_K27ME3_GR_TOP1000	960	2.038131	0	0
TET2KD_MUTO_FL-BMT_GMP_T2KD VS WT UPON	116	1.993437	0	0
BMI1-KO_LSK DOWN OGURO	238	1.915446	0	0
AGM_VE-CAD+CD45+ VS ABM_150+34-LSK UPON TOP500 2012	408	1.756663	0	0.004732331
TET2KD_MUTO_FL-BMT_LSK_T2KD VS WT UPON	284	1.785852	0	0.005046993
EZH2KO_SASHIDA_ABM_BMT_E2KO VS WT_UPON	543	1.733326	0	0.005595749
EZH2KO-MDS_CASCIO_DNOFF_B10-F14	948	1.703561	0	0.006477696
EZH1_2_OLA UNC1999_MM.1S_K27ME3_GR_TOP500	483	1.739494	0	0.006528374
PLACENTA_45+KIT+CD34MED VS ABM_150+34-LSK UPON TOP500 2012	428	1.716587	0	0.006957409
EZH2KOTET2KD-MDS_MUTO_FL-BMT_LSK_E19 VS WT DNOFF	640	1.611375	0	0.024427976
EZH2KO-MDS_CASCIO_DNOFF_B11-F2	1019	1.557241	0	0.02966724
EZH2KOTET2KD-MDS_MUTO_FL-BMT_GMP_ET77 VS WT UPON	796	1.562724	0	0.030288149
EZH2KOTET2KD_MUTO_FL-BMT_LSK_DKO VS WT UPON	237	1.576857	0	0.031208133
EZH1_2_OLA UNC1999_MM.1S_K27ME3_GR_10FOLD	252	1.541668	0	0.03150973
EZH2KO_SASHIDA_AHSCVECTOR_BMT_1000_E2KO VS WT_UPON	674	1.531724	0	0.032200407
EEDKO VS WT BM_LT-HSC UP > 3FOLD 339GENES S2N 2014_ORKIN	302	1.565611	0	0.03236416
EZH2KO_MUTO_FL-BMT_LSK_E2KO VS WT UPON	367	1.577626	0	0.03377251
MPP_SPECIFIC GEROGES-RNA-SEQ	108	1.509353	0	0.03621907
EZH2KO-MDSMPN_CASCIO_DNOFF_B11-F5	1434	1.373092	0	0.09662716
BRUECKNER_TARGETS_OF_MIRLET7A3_UP	105	1.366569	0.021978023	0.09736389
LSKCD34- VS MPPFLK2+,- UPON 2014_ROSSI	204	1.336189	0.013888889	0.10865448
MONOCYTES_GOODDEL	64	1.337015	0.052173913	0.11252609
EZH2KO-MDS-PLTHIGH_CASCIO_DNOFF_B10-F7	1539	1.341122	0	0.11508455
CD34-LSK_SPECIFIC GEROGES-ARRAY UPON	546	1.282573	0	0.14296083
MUTO_GMP-K27_KO VS WT-0.5_WT-3 UP ON	118	1.282721	0.03508772	0.14867927
BRUECKNER_TARGETS_OF_MIRLET7A3_DN	77	1.288889	0.08050848	0.14956464
EZH2KOTET2KD-MDS_MUTO_FL-BMT_GMP_ET70 VS WT UPON	761	1.232986	0	0.19677146
H2AK119UB1_WT-LSK > 3.5FOLD 729GENES	686	1.223329	0	0.20442982
LTHSC_SPECIFIC GEROGES-RNA-SEQ	258	1.216009	0.046511628	0.20876718
BCORKO VS WT UPON BMT_LSK TANAKA_RNA-SEQ > 2FOLD_1INFLATE	769	1.201469	0	0.21420543
EZH2KO_MUTO_FL-BMT_GMP_E2KO VS WT DNOFF	154	1.204074	0.10191083	0.21776149
EZH2KOTET2KD-MDS_MUTO_FL-BMT_LSK_E18 VS WT DNOFF	703	1.187042	0	0.23063451
H2AK119UB1_WT-GMP > 5FOLD 766GENES	699	1.182274	0	0.23166198
EZH2KO-MDSMPNQ_CASCIO_DNOFF_F2	1584	1.162218	0	0.26081526
21MO VS 12MO UPON SPARKLS ARRAY 2007_GOODDELL	128	1.135571	0.16477273	0.2724012
CEBPA-KD_HSCS UP TOP100 TENEN 2013_D7	67	1.150959	0.19026549	0.27343008
HUMANHSCS OLD VS YOUNG UP S2N TOP100 GSE32719	93	1.131766	0.2292683	0.2738723
FL_14-5_150+48-LSK VS ABM_150+34-LSK DOWNOFF TOP500 2012	398	1.139702	0.06	0.27811852
24MO VS 04MO UP TOP100 S2N SPLSKCD150- RNA-SEQ 2014_GOODDELL	91	1.143477	0.17821783	0.2786928
EZH2KO_MUTO_FL-BMT_GMP_E2KO VS WT UPON	345	1.135812	0.060606062	0.2788061
SOX17 OE CD48LSK-DEV-WT UP 376GENES 2011_MORRISON	292	1.119467	0.11494253	0.28373903
FL_13-5_150+48-LSK VS ABM_150+34-LSK DOWNOFF TOP500 2012	387	1.113986	0.06153846	0.28644598
PRC2-TARGET IN ABM-BMT-LSK K27ME3_TOP500GENES	472	1.120644	0.04	0.28878835
EZH2KO_MUTO_FL-BMT_GMP_EZH2KO VS WT UPON	356	1.107892	0.12820514	0.29353318
EZH2KO-1M_K27_WT > 2_EZH2KO-VS-WT > 0.8_AOYAMA	526	1.103432	0.121212125	0.2957095
FL_12-5_VE-CAD+MAC1LOWLSK VS ABM_150+34-LSK DOWNOFF TOP500 2012	371	1.054261	0.12698413	0.41266543
CEBPA-KD_HSCS DOWN TOP500 TENEN 2013_D7	385	1.050906	0.25	0.41538653
EEDKO VS WT BM_LT-HSC UP < 0.33FOLD 109GENES S2N 2014_ORKIN	82	1.037325	0.3517588	0.45100906
EEDKO VS WT BM_LT-HSC UP TOP500 S2N 2014_ORKIN	452	1.033632	0.23076923	0.4516356

Supplementary Table S4. Top 74 PRC2 genes that are significantly altered in UNC1999-treated MM.1S cells.

Within PRC2 target genes, we defined genes that showed more than 2-fold reduction in H3K27me3 levels in UNC1999-treated MM.1S cells compared with DMSO-treated cells as "UNC1999 target genes". Among these genes, we selected 74 genes with significantly enhanced expression (>1.5-fold UNC1999/Control) and remarkable reduction of H3K27me3 (≥ 2 -fold) as major UNC1999 target genes in MM.1S cells.

Symbol	IP/Input		H3K27me3 (UNC1999/Control)	Expression (UNC1999/Control)
	Control	UNC1999		
B4GALNT1	2.77419355	1.08413095	0.390791389	1.653341911
PHLDA2	9.4789916	3.74200468	0.394768225	1.79486717
NOG	2.34104046	1.13709042	0.485720106	1.983382414
FURIN	6.16517857	2.24752007	0.364550684	1.782095703
PDLIM2	7.53278689	3.0464	0.404418716	2.076965762
FBXO2	3.80882353	1.82589142	0.47938462	2.666123933
TNFRSF12A	4.35344828	1.38966513	0.319210208	2.272379776
KCTD17	3.6875	1.80624361	0.489828775	1.8285526
LAPTM4B	4.06451613	1.59746918	0.393028132	1.524046495
PTP4A3	5.96052632	2.38434138	0.400021953	2.910717623
ARHGEF40	2.32222222	0.80438435	0.346385605	1.584748503
TP53I11	6.46666667	2.75738223	0.426399314	1.994753911
SLC2A6	7.45762712	2.22221121	0.297978322	2.023707513
MAP4K4	2.15929204	0.7315565	0.338794607	1.519566467
SERPINE1	5.5359116	2.57872779	0.465818094	4.916338636
C4orf48	2.55882353	1.18506892	0.463130383	1.628285412
ATF3	5.91946309	2.95269278	0.498810911	2.943320625
IFITM2	8.2556391	3.07271442	0.372195826	3.136479332
RRAD	6.78231293	3.36666443	0.496388837	2.480640192
CCND1	8.59482759	4.14082734	0.481781316	1.852545516
FAM19A5	4.35294118	1.70203725	0.391008557	1.552949065
TMEM54	5.29032258	2.33012558	0.440450568	1.99659116
LTB	9.81818182	3.97113341	0.404467292	1.82669
BCL9L	3.97849462	1.55465649	0.390765008	1.868135369
NEK6	2.71428571	1.24846346	0.459960223	1.958824163
BLVRB	2.97794118	0.86134443	0.289241587	2.059473255
EGR1	2.92907801	1.24672815	0.425638423	4.127642637
TYROBP	5.14173228	1.92188571	0.373781754	1.725858833
NR2F2	3.92934783	1.50006169	0.381758438	1.534643348
RAB31	3.45588235	1.21075153	0.350345123	1.524107525
PTMS	2.89449541	1.22665029	0.423787262	2.686701624
SLC2A3	2.2556391	1.10033708	0.487816106	2.25861547
RIMS3	2.27891156	0.80212289	0.351976312	1.509666845
MX1	2.97356828	1.38537915	0.465897878	1.596811224
UNC119	6.30882353	2.78851212	0.442001921	2.018590781
MXRA7	3.68589744	1.56674862	0.425065713	2.480453833
IFITM1	5.85714286	2.52040681	0.430313357	2.28369906
PCDH1	4.10638298	1.3018166	0.317022696	2.534735604
IGFBP6	2.25609756	1.01757544	0.451033439	2.016412101
C9orf7	3.65322581	1.61282946	0.441480913	1.521127762
WNT10B	2.08383234	0.75947141	0.364458983	1.528781495
TMEM158	5.20588235	1.39310252	0.267601614	1.733548817
SARDH	11.2	4.80102959	0.428663356	1.512089825
SORL1	2.85555556	1.10830332	0.388121786	2.098696811
ADAMTSL2	11.9512195	5.3077631	0.444118953	1.56281874
ATP13A2	5.75268817	1.93821141	0.336922731	1.560819085
IL4R	2.09625668	1.01129246	0.482427782	1.534315468
EGR3	10.3417722	3.63193428	0.351190708	1.503005664
SMOX	2.03797468	0.90615995	0.444637491	2.464241943

NR4A1	6.02083333	2.62618651	0.436183227	2.077228118
HLA-DMB	3.48421053	1.36821921	0.392691315	1.640276897
MT1X	4.75163399	1.73031029	0.364150584	5.592061664
HOXB7	3.42941176	1.23496656	0.360110318	1.704239346
RTN4RL2	2.67088608	0.68223808	0.255435111	3.593613969
ZNF467	4.63970588	1.92240815	0.414338365	1.912767935
PRKCB	3.07333333	1.41000119	0.458785637	1.975205174
LIPG	3.79432624	1.82798081	0.481766904	1.665232801
GSTP1	5.25862069	1.61917838	0.307909331	2.023897416
SLC43A2	2.40186916	1.16254568	0.484017074	1.609368534
SERINC2	8.60479042	3.76557723	0.437614055	1.646779373
CDKN1C	2.53278689	0.70657291	0.278970533	2.26714243
SYNGR1	4.43103448	1.64330228	0.370861993	1.576066531
RAB37	7.60769231	3.7459213	0.492386015	1.575599822
RTN2	2.57608696	1.19716146	0.464720905	2.295885849
C18orf1	5.29310345	2.41847618	0.456910809	3.045665033
AHNAK	3.23913043	1.56608078	0.483488027	1.881983502
HLA-DPA1	4.85840708	2.20413979	0.453675403	1.799515205
SERPINB6	3.79213483	1.87704561	0.494983879	2.883343692
KRT17	6.92622951	3.38479742	0.488692645	3.663495099
TSKU	2.08666667	0.97088566	0.465280668	1.533224646
LAMC1	2.06862745	0.75324202	0.364126472	1.880016543
IL32	6.00884956	2.88513739	0.480148049	4.787336672
CYR61	3.92	1.35632166	0.346000422	1.621716708
STK32C	6.76923077	2.843673	0.420088057	2.449710862

Supplementary Table S5. Significantly upregulated gene sets in RPMI8226 cells transduced with *EZH2* overexpressing versus empty vectors.

Gene set enrichment analysis (GSEA) using our RNA-seq data identified gene sets that are significantly enriched in RPMI8226 cells transduced with *EZH2* overexpressing versus empty vectors. (FDR q-value <0.01).

NAME	SIZE	NES	NOM p-val	FDR q-val
REACTOME_PEPTIDE_CHAIN_ELONGATION	86	2.42	0	0
KEGG_RIBOSOME	87	2.41	0	0
REACTOME_TRANSLATION	146	2.41	0	0
REACTOME_INFLUENZA_VIRAL_RNA_TRANSCRIPTION_AND_REPLICATION	102	2.4	0	0
REACTOME_3_UTR_MEDIATED_TRANSLATIONAL_REGULATION	106	2.4	0	0
REACTOME_SRP_DEPENDENT_COTRANSLATIONAL_PROTEIN_TARGETING_TO_M EMBRANE	109	2.39	0	0
HSIAO_HOUSEKEEPING_GENES	387	2.34	0	0
REACTOME_NONSENSE_MEDIATED_DECAY_ENHANCED_BY_THE_EXON_JUNCTIO N_COMPLEX	107	2.31	0	0
RHEIN_ALL_GLUCOCORTICOID_THERAPY_DN	350	2.3	0	0
REACTOME_INFLUENZA_LIFE_CYCLE	136	2.27	0	0
YAO_TEMPORAL_RESPONSE_TO_PROGESTERONE_CLUSTER_13	159	2.27	0	0
REACTOME_METABOLISM_OF_MRNA	210	2.22	0	0
WONG_EMBRYONIC_STEM_CELL_CORE	331	2.22	0	0
REACTOME_METABOLISM_OF_RNA	255	2.2	0	0
MOOTHA_VOXPPOS	85	2.17	0	0
PECE_MAMMARY_STEM_CELL_UP	136	2.17	0	0
REACTOME_FORMATION_OF_THE_TERNARY_COMPLEX_AND_SUBSEQUENTLY_T HE_43S_COMPLEX	49	2.17	0	0
BILANGES_SERUM_AND_RAPAMYCIN_SENSITIVE_GENES	68	2.15	0	0
TARTE_PLASMA_CELL_VS_PLASMABLAST_DN	306	2.12	0	0
REACTOME_ACTIVATION_OF_THE_MRNA_UPON_BINDING_OF_THE_CAP_BINDING _COMPLEX_AND_EIFS_AND_SUBSEQUENT_BINDING_TO_43S	57	2.12	0	0
KEGG_PARKINSONS_DISEASE	112	2.12	0	0
YAMASHITA_LIVER_CANCER_WITH_EPCAM_UP	52	2.11	0	0
WONG_MITOCHONDRIA_GENE_MODULE	216	2.11	0	0
DANG_MYC_TARGETS_UP	139	2.11	0	0
YAO_TEMPORAL_RESPONSE_TO_PROGESTERONE_CLUSTER_17	173	2.11	0	0
KEGG_OXIDATIVE_PHOSPHORYLATION	116	2.11	0	0
MALONEY_RESPONSE_TO_17AAG_DN	78	2.11	0	0
REACTOME_RESPIRATORY_ELECTRON_TRANSPORT_ATP_SYNTHESIS_BY_CHEMI OSMOTIC_COUPLING_AND_HEAT_PRODUCTION_BY_UNCOUPLING_PROTEINS_	81	2.09	0	0
CHIANG_LIVER_CANCER_SUBCLASS_UNANNOTATED_DN	185	2.09	0	0
REACTOME_METABOLISM_OF_PROTEINS	422	2.08	0	0
PAL_PRMT5_TARGETS_UP	198	2.07	0	0
PROVENZANI_METASTASIS_DN	135	2.06	0	0
TAKAO_RESPONSE_TO_UVB_RADIATION_UP	86	2.06	0	0
ENK_UV_RESPONSE_KERATINOCYTE_UP	528	2.05	0	0
CAIRO_HEPATOBLASTOMA_CLASSES_UP	580	2.05	0	0
TIEN_INTESTINE_PROBIOTICS_6HR_UP	54	2.05	0	0
STARK_PREFRONTAL_CORTEX_22Q11_DELETION_DN	469	2.04	0	0
WANG_TUMOR_INVASIVENESS_UP	361	2.03	0	0
LEE_LIVER_CANCER_SURVIVAL_DN	166	2.03	0	0
TIEN_INTESTINE_PROBIOTICS_24HR_UP	537	2.03	0	0
REACTOME_RESPIRATORY_ELECTRON_TRANSPORT	65	2.01	0	0
PENG_Glutamine_DEPRIVATION_DN	331	2	0	0
PENG_LEUCINE_DEPRIVATION_DN	183	2	0	0
BOYALT_LIVER_CANCER_SUBCLASS_G3_UP	183	2	0	0
LI_CISPLATIN_RESISTANCE_UP	24	2	0	0
BLUM_RESPONSE_TO_SALIRASIB_DN	339	1.99	0	0
BERENJENO_TRANSFORMED_BY_RHOA_UP	519	1.99	0	0
PUJANA_CHEK2_PCC_NETWORK	762	1.99	0	0

REACTOME_TCA_CYCLE_AND_RESPIRATORY_ELECTRON_TRANSPORT	117	1.98	0	0
SWEET_KRAS_ONCOGENIC_SIGNATURE	89	1.98	0	0
ALONSO_METASTASIS_UP	191	1.98	0	0
KIM_BIPOLAR_DISORDER_OLIGODENDROCYTE_DENSITY_CORR_UP	657	1.98	0	0
YAO_TEMPORAL_RESPONSE_TO_PROGESTERONE_CLUSTER_14	135	1.96	0	0.001
IRITANI_MAD1_TARGETS_DN	46	1.96	0	0.001
CHNG_MULTIPLE_MYELOMA_HYPERPLOID_UP	52	1.95	0	0.001
LI_DCP2_BOUND_MRNA	87	1.95	0	0.001
RHODES_UNDIFFERENTIATED_CANCER	68	1.95	0	0.001
ZAMORA_NOS2_TARGETS_UP	66	1.95	0	0.001
REACTOME_AUTODEGRADATION_OF_CDH1_BY_CDH1_APC_C	56	1.94	0	0.001
JIANG_AGING_HYPOTHALAMUS_UP	46	1.94	0	0.001
KEGG_HUNTINGTONS_DISEASE	172	1.94	0	0.001
HILLION_HMGA1B_TARGETS	90	1.94	0	0.001
LI_AMPLIFIED_IN_LUNG_CANCER	175	1.94	0	0.001
MOOTHA_HUMAN_MITODB_6_2002	421	1.93	0	0.001
REACTOME_CYCLIN_E_ASSOCIATED_EVENTS_DURING_G1_S_TRANSITION_	62	1.93	0	0.001
GRAHAM_CML_DIVIDING_VS_NORMAL QUIESCENT_UP	178	1.93	0	0.001
BENPORATH_PROLIFERATION	136	1.93	0	0.001
BHATTACHARYA_EMBRYONIC_STEM_CELL	88	1.93	0	0.001
SHIPP_DLBCL_VS_FOLLICULAR_LYMPHOMA_UP	44	1.92	0	0.001
REACTOME_MITOTIC_G1_G1_S_PHASES	130	1.92	0	0.001
MENSSEN_MYC_TARGETS	51	1.92	0	0.001
MOREAUX_B_LYMPHOCYTE_MATURATION_BY_TACI_DN	67	1.92	0	0.001
KIM_ALL_DISORDERS_OLIGODENDROCYTE_NUMBER_CORR_UP	731	1.92	0	0.001
REACTOME_APC_C_CDH1_MEDIATED_DEGRADATION_OF_CDC20_AND_OTHER_A PC_C_CDH1_TARGETED_PROTEINS_IN_LATE_MITOSIS_EARLY_G1	64	1.92	0	0.001
REACTOME_CDK_MEDIATED_PHOSPHORYLATION_AND_REMOVAL_OF_CDC6	46	1.92	0	0.001
PROVENZANI_METASTASIS_UP	186	1.92	0	0.001
SESTO_RESPONSE_TO_UV_C0	107	1.91	0	0.001
REACTOME_REGULATION_OF_ORNITHINE_DECARBOXYLASE_ODC	48	1.91	0	0.002
REACTOME_APC_C_CDC20_MEDIATED_DEGRADATION_OF_MITOTIC_PROTEINS	65	1.91	0	0.002
REACTOME_ASSEMBLY_OF_THE_PRE_REPLICATIVE_COMPLEX	63	1.91	0	0.002
DAZARD_RESPONSE_TO_UV_SCC_UP	114	1.91	0	0.002
REACTOME_SCFSKP2_MEDIATED_DEGRADATION_OF_P27_P21	53	1.91	0	0.002
ZHOU_TNF_SIGNALING_30MIN	52	1.91	0	0.002
MANALO_HYPOXIA_DN	273	1.9	0	0.002
SPIELMAN_LYMPHOBLAST_EUROPEAN_VS_ASIAN_UP	469	1.9	0	0.002
WEI_MYCN_TARGETS_WITH_E_BOX	737	1.9	0	0.002
REACTOME_SCF_BETA_TRCP_MEDIATED_DEGRADATION_OF_EMI1	49	1.9	0	0.002
REACTOME_ER_PHAGOSOME_PATHWAY	58	1.9	0	0.002
SOTIRIOU_BREAST_CANCER_GRADE_1_VS_3_UP	145	1.9	0	0.002
REACTOME_VIF_MEDIATED_DEGRADATION_OF_APOBEC3G	49	1.9	0	0.002
REACTOME_REGULATION_OF_MITOTIC_CELL_CYCLE	77	1.89	0	0.002
REACTOME_G1_S_TRANSITION	106	1.89	0	0.002
GRADE_METASTASIS_DN	43	1.89	0	0.002
BILANGES_RAPAMYCIN_SENSITIVE_VIA_TSC1_AND_TSC2	71	1.89	0	0.002
GRADE_COLON_AND_RECTAL_CANCER_UP	275	1.89	0	0.002
REACTOME_P53_INDEPENDENT_G1_S_DNA_DAMAGE_CHECKPOINT	48	1.89	0	0.002
REACTOME_ANTIGEN_PROCESSING_CROSS_PRESENTATION	72	1.89	0	0.002
KEGG_ALZHEIMERS_DISEASE	156	1.89	0	0.002
PENG_RAPAMYCIN_RESPONSE_DN	238	1.89	0	0.002
OUELLET_OVARIAN_CANCER_INVASIVE_VS_LMP_UP	117	1.88	0	0.002
CASORELLI_ACUTE_PROMYELOCYTIC_LEUKEMIA_DN	643	1.88	0	0.003
REACTOME_CDT1_ASSOCIATION_WITH_THE_CDC6_ORC_ORIGIN_COMPLEX	54	1.88	0	0.003
REACTOME_DESTABILIZATION_OF_MRNA_BY_AUF1_HNRNP_D0	50	1.88	0	0.003

REACTOME_M_G1_TRANSITION	78	1.88	0	0.003
JIANG_AGING_CEREBRAL_CORTEX_UP	36	1.88	0	0.003
BORCZUK_MALIGNANT_MESOTHELIOMA_UP	296	1.87	0	0.003
MOOHA_MITOCHONDRIA	434	1.87	0	0.003
MORI_IMMATURE_B_LYMPHOCYTE_DN	90	1.87	0	0.003
KRIGE_RESPONSE_TO_TOSEDOSTAT_24HR_DN	936	1.87	0	0.003
HOLLMANN_APOPTOSIS_VIA_CD40_UP	191	1.87	0	0.003
NAKAMURA_TUMOR_ZONE_PERIPHERAL_VS_CENTRAL_UP	270	1.86	0	0.003
REACTOME_SIGNALING_BY_WNT	62	1.86	0	0.003
GRADE_COLON_CANCER_UP	818	1.86	0	0.004
BRIDEAU_IMPRINTED_GENES	63	1.86	0	0.004
REACTOME_CROSS_PRESENTATION_OF_SOLUBLE_EXOGENOUS_ANTIGENS_ENDOSOMES	47	1.85	0	0.004
REACTOME_AUTODEGRADATION_OF_THE_E3_UBIQUITIN_LIGASE_COP1	47	1.85	0	0.004
DITTMER_PTHLH_TARGETS_UP	111	1.85	0	0.004
REACTOME_S_PHASE	106	1.85	0	0.004
ZHANG_RESPONSE_TO_CANTHARIDIN_DN	67	1.85	0	0.005
KEGG_METABOLISM_OF_XENOBIOTICS_BY_CYTOCHROME_P450	70	1.84	0	0.005
NATSUME_RESPONSE_TO_INTERFERON_BETA_DN	52	1.84	0	0.005
TOOKER_GEMCITABINE_RESISTANCE_UP	77	1.84	0	0.005
ACEVEDO_LIVER_TUMOR_VS_NORMAL_ADJACENT_TISSUE_UP	796	1.84	0	0.005
CHAUHAN_RESPONSE_TO_METHOXYESTRADIOL_DN	100	1.84	0	0.006
KEGG_PROTEASOME	44	1.83	0	0.006
SESTO_RESPONSE_TO_UV_C7	68	1.83	0	0.006
BIOCARTA_PROTEASOME_PATHWAY	28	1.83	0	0.006
REACTOME_SYNTHESIS_OF_DNA	90	1.83	0	0.006
REACTOME_REGULATION_OF_MRNA_STABILITY_BY_PROTEINS_THAT_BIND_AURICHEL_ELEMENTS	81	1.83	0	0.006
KIM_ALL_DISORDERS_DURATION_CORR_DN	140	1.83	0	0.006
CHANG_CORE_SERUM_RESPONSE_UP	203	1.83	0	0.006
SMID_BREAST_CANCER_RELAPSE_IN_PLEURA_DN	25	1.83	0	0.006
SANA_RESPONSE_TO_IFNG_DN	83	1.83	0	0.006
SCHLOSSER_MYC_TARGETS_REPRESSED_BY_SERUM	154	1.82	0	0.006
HONMA_DOCETAXEL_RESISTANCE	32	1.81	0	0.007
MUELLER_PLURINET	299	1.81	0	0.007
REACTOME_HOST_INTERACTIONS_OF_HIV_FACTORS	120	1.81	0	0.007
TONKS_TARGETS_OF_RUNX1_RUNX1T1_FUSION_MONOCYTE_UP	198	1.81	0	0.007
HU_ANGIOGENESIS_DN	37	1.81	0	0.008
WANG_SMARCE1_TARGETS_DN	352	1.81	0	0.008
DAZARD_RESPONSE_TO_UV_NHEK_UP	237	1.81	0	0.008
MISSIAGLIA_REGULATED_BY_METHYLATION_DN	117	1.81	0	0.008
REACTOME_PROTEIN_FOLDING	51	1.81	0	0.008
PRAMOONJAGO_SOX4_TARGETS_DN	50	1.81	0	0.008
PETROVA_PROX1_TARGETS_UP	27	1.8	0	0.008
SWEET_LUNG_CANCER_KRAS_UP	475	1.8	0	0.008
ROSTY_CERVICAL_CANCER_PROLIFERATION_CLUSTER	137	1.8	0	0.008
WANG_TNF_TARGETS	23	1.8	0	0.008
KORKOLA_EMBRYONIC_CARCINOMA_VS_SEMINOMA_UP	21	1.8	0	0.008
REACTOME_REGULATION_OF_APOPTOSIS	56	1.8	0	0.008
HUANG_DASATINIB_RESISTANCE_UP	79	1.8	0	0.008
HOLLEMAN_ASPARAGINASE_RESISTANCE_B_ALL_UP	26	1.8	0	0.008
HORIUCHI_WTAP_TARGETS_DN	294	1.8	0	0.008
CROONQUIST_NRAS_SIGNALING_DN	72	1.8	0	0.009
REACTOME_MITOTIC_M_M_G1_PHASES	165	1.8	0	0.009
WELCSH_BRCA1_TARGETS_UP	194	1.79	0	0.009
GARY_CD5_TARGETS_DN	422	1.79	0	0.009
ZHU_CMV_24_HR_UP	93	1.79	0	0.009

FERRANDO_T_ALL_WITH_MLL_ENL_FUSION_DN	87	1.79	0	0.009
LUI_TARGETS_OF_PAX8_PPARG_FUSION	34	1.79	0	0.009
REACTOME_ORC1_REMOVAL_FROM_CHROMATIN	65	1.79	0	0.009

Supplementary Table S6. List of primers used for Quantitative RT-PCR.

Sequences of primers used for quantitative real time PCR.

Target Gene	Primers	Sequences (5' to 3')	Probe
<i>EZH2</i>	Forward Reverse	AGCTCCCGCTGAGGATGT CAGTGTGCAGCCCACAAC	25
<i>EZH1</i>	Forward Reverse	CATCCAGCGTGGACTTAAGAA CGTTCTTCTGCACAGACTCCT	62
<i>E2F1</i>	Forward Reverse	TACCTGGCCGAGAGCAGT GGTGGTCAGATTCAGTGAGGT	7
<i>E2F2</i>	Forward Reverse	AGGGGAAGTGCATCAGAGTG CCAGCGAAGTGTCATACCG	7
<i>MYC</i>	Forward Reverse	GCTGCTTAGACGCTGGATTT TAACGTTGAGGGGCATCG	66
<i>NR4A1</i>	Forward Reverse	ACAGCTTGCTTGTTCGATGTC GGTTCTGCAGCTCCTCCAC	34
<i>NR4A2</i>	Forward Reverse	ATGAAGAGAGACGCGGAGAA AAAAGCAATGGGGAGTCCA	63
<i>NR4A3</i>	Forward Reverse	TCTCAGTGTTGGAATGGTAAAAGA GGTTTGAAGGCAGACGAC	52
<i>GAPDH</i>	Forward Reverse	CTGACTTCAACAGCGACACC TAGCCAAATTCGTTGTCATACC	25

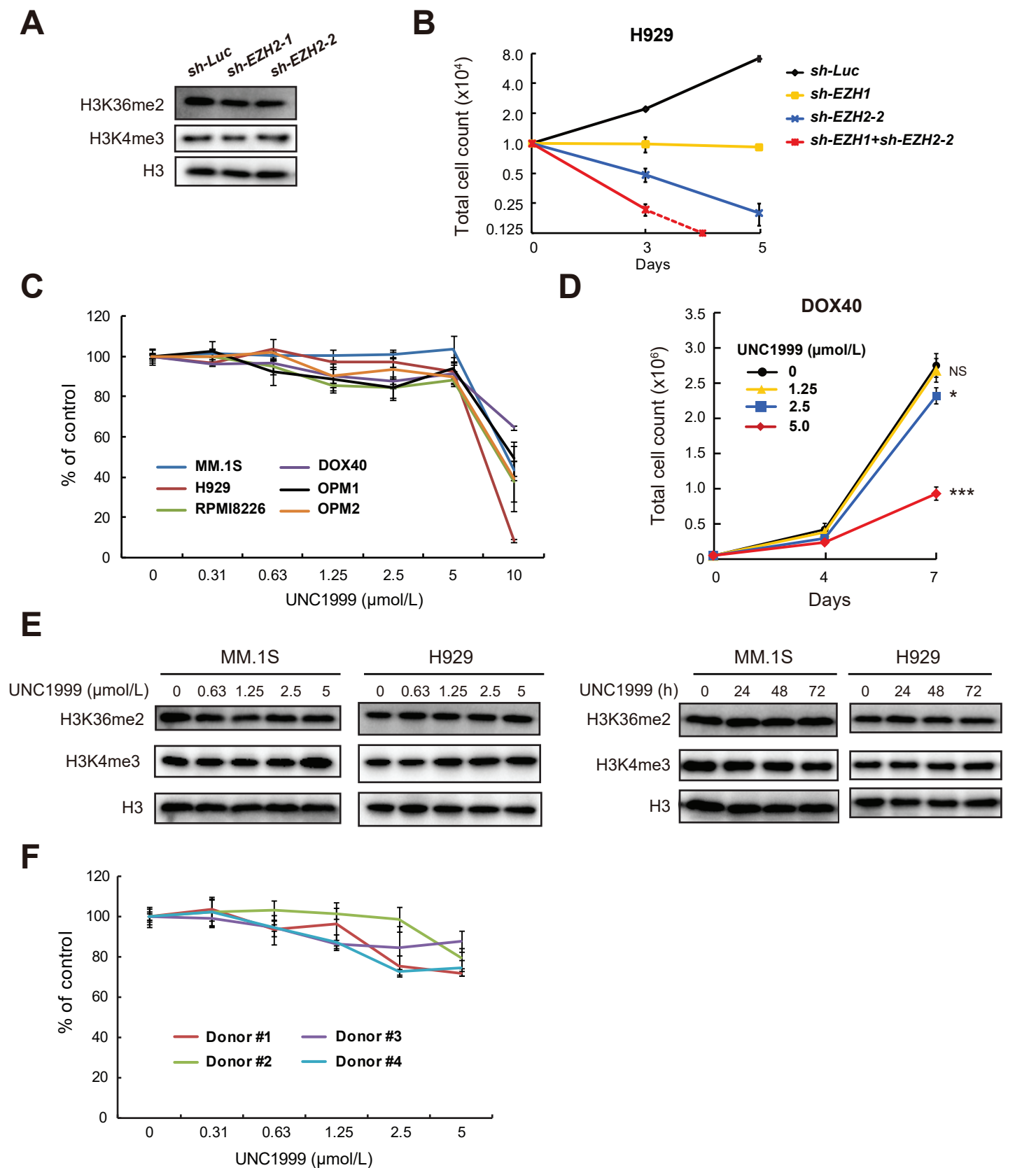
Supplementary Table S7. List of primers used for ChIP-PCR.

Sequences of primers used for ChIP-PCR.

Primer name	Primer sequence
EZH2-1 F ¹	GGAAGCCAAGTTTGAACCAG
EZH2-1 R ¹	GCGGTTAAAACCGTTACCAC
EZH2-2 F ²	AACTCTGCGGCGCCGGTCCCGCCAAGA
EZH2-2 R ²	TTCGCTGTAAGGGACGCCACTGGCCGTGT
NR4A1-TSS+2-2 Fw	CCCTGAGGCTGTGTCTTCTT
NR4A1-TSS+2-2 Rv	TCCCAGTCTGTAGGGAGACG

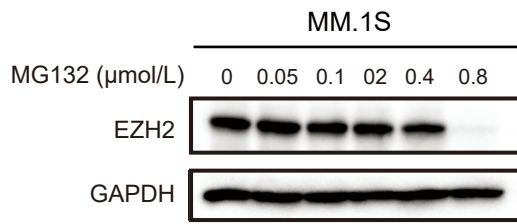
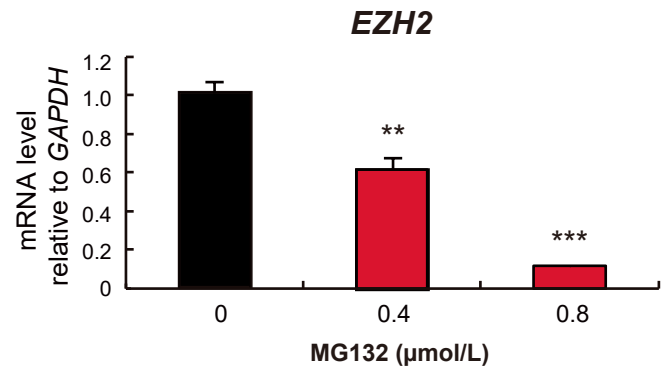
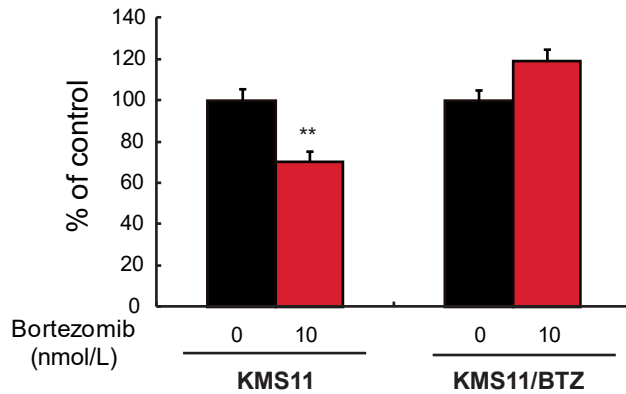
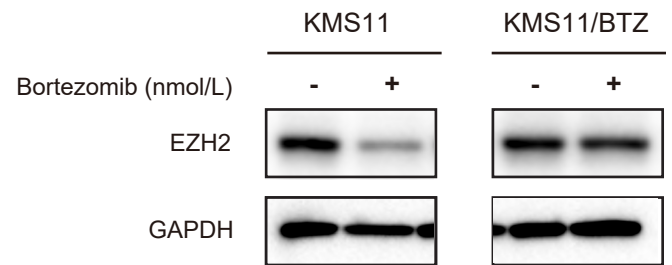
#1 Yu J, Yu J, Mani R-S, et al. An Integrated Network of Androgen Receptor, Polycomb, and TMPRSS2-ERG Gene Fusions in Prostate Cancer Progression. *Cancer cell*. 2010;17(5):443-454.

#2 Fujii S, Tokita K, Wada N, et al. MEK-ERK pathway regulates EZH2 overexpression in association with aggressive breast cancer subtypes. *Oncogene*. 2011;30(39):4118-4128.



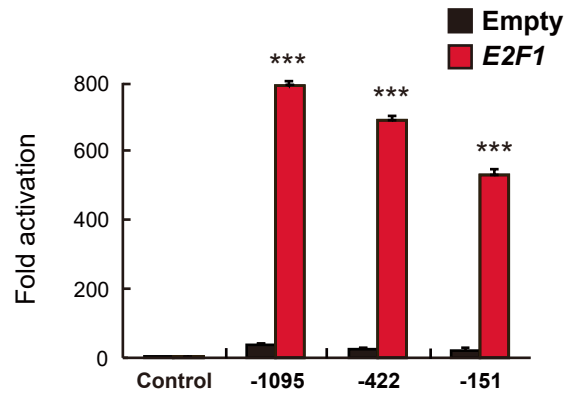
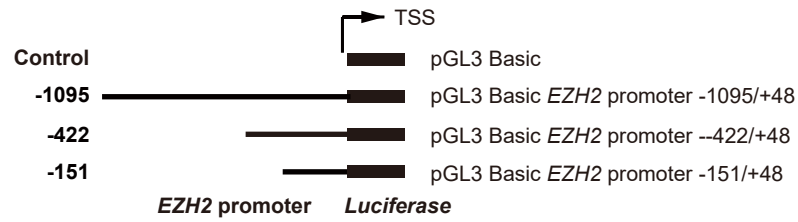
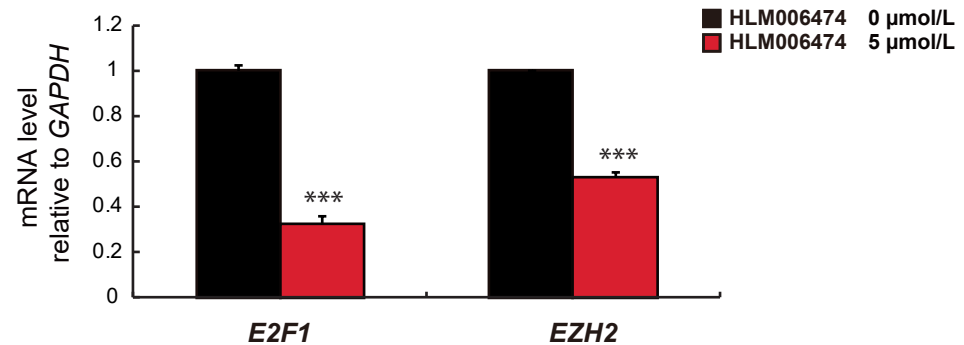
Supplementary Figure S1. Pharmacological and lentiviral suppression of EZH2 and EZH1 in MM cell lines.

(A) H929 cells transduced with the indicated lentiviruses were selected by cell sorting for GFP expression, and subjected to immunoblot analysis for the indicated proteins. H3 served as a loading control. (B) H929 cells transduced with the indicated lentiviruses were selected by cell sorting for GFP or RFP expression, and subjected to cell proliferation assay. Cell counting was performed using trypan blue on the indicated days of cultures. Y-axis represents log₂. Red dashed line indicates that no live sh-EZH1+sh-EZH2-2 cells were detected on day 5. Data represent mean \pm SD of triplicate cultures. (C) MTS assay showing viability of MM cell lines upon treatment with the indicated doses of UNC1999 (72 hours) relative to untreated control. Data represent mean \pm SD of triplicates. (D) Cell proliferation assay of DOX40 cells treated with a range of UNC1999 concentrations. Cell counting was performed using trypan blue on the indicated days of cultures. Data represent mean \pm SD of triplicate cultures. (E) Immunoblot analyses for H3K36me2 and H3K4me3 in MM cells described in Fig. 1E. H3 served as a loading control. (F) MTS assay showing viability of peripheral blood mononuclear cells (PBMNCs) isolated from healthy donors upon treatment with the indicated doses of UNC1999 (48 hours) relative to untreated control. Data represent mean \pm SD (n = 3~4). *P < 0.05; ***P < 0.001; NS, not significant.

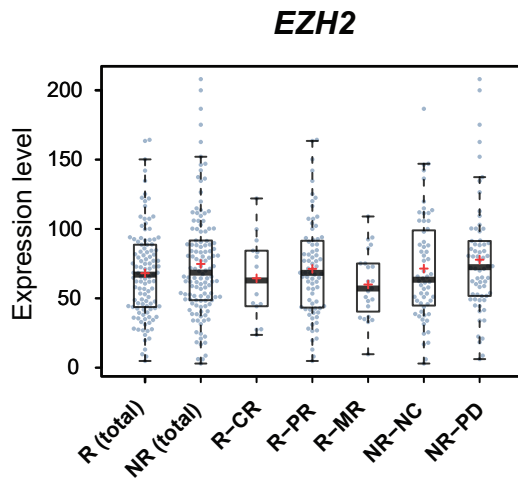
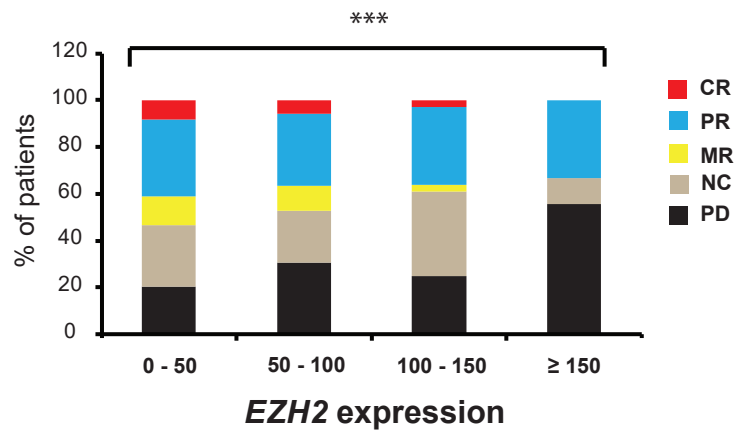
A**B****C****D**

Supplementary Figure S2. Proteasome inhibitors downregulate EZH2 in MM cell lines.

(A) Immunoblot analysis of EZH2 in MM.1S cells treated with the indicated doses of MG132 for 24 hours. GAPDH served as a loading control. (B) Quantitative RT-PCR analysis of *EZH2* mRNA expression in MM.1S cells treated with the indicated doses of MG132 for 12 hours. Y-axis represents fold-change after normalization to *GAPDH*, and error bars represent SD of triplicates. (C) MTS assay showing viability of KMS11 or KMS11/BTZ cells upon treatment with 10 nmol/L of bortezomib for 24 hours relative to untreated control. Data represent mean \pm SD of triplicate cultures. (D) Immunoblot analysis of EZH2 in KMS11 or KMS11/BTZ cells treated with 10 nmol/L of bortezomib for 24 hours. GAPDH served as a loading control. **P < 0.01; ***P < 0.001.

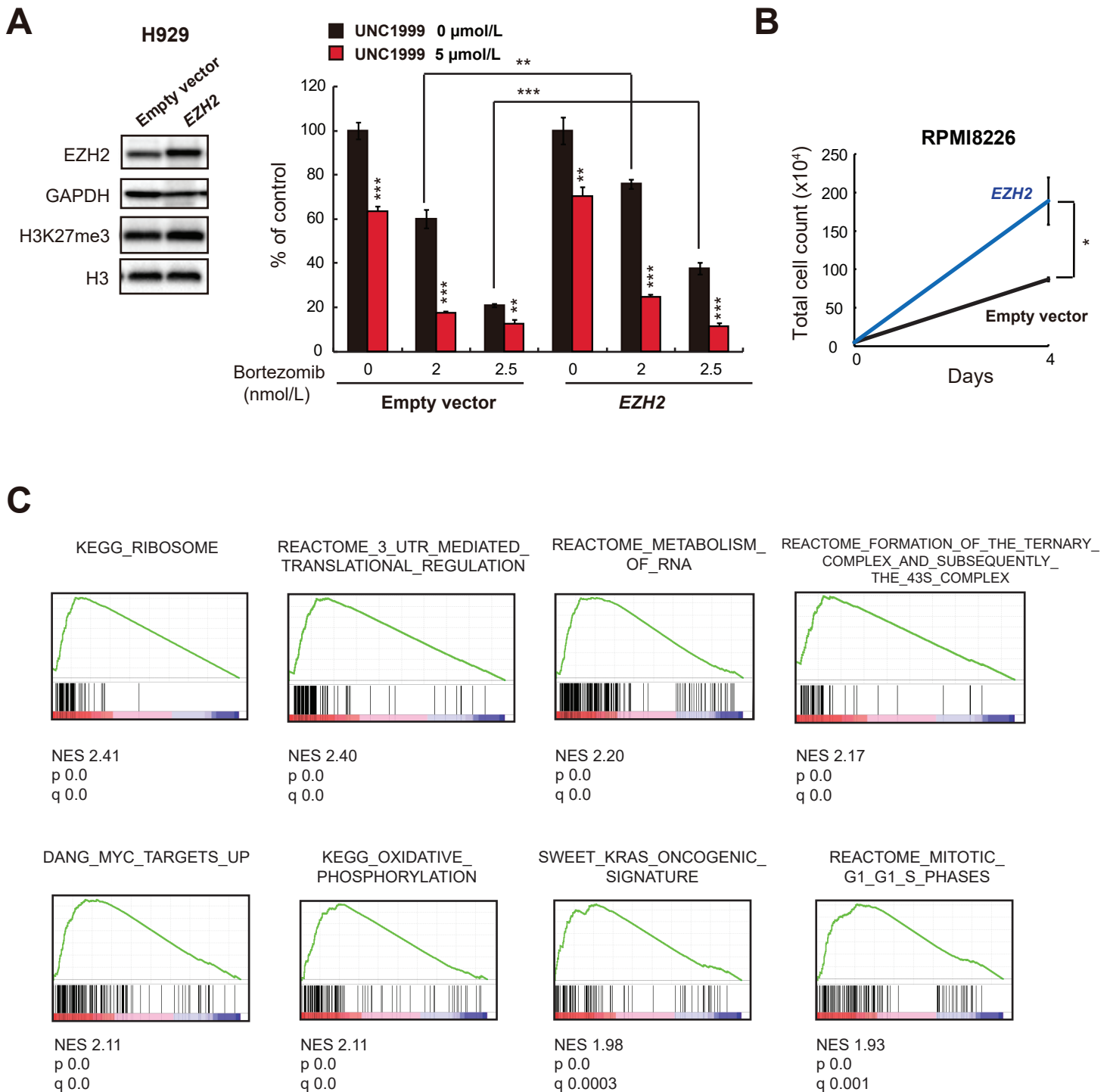
A**B****Supplementary Figure S3. EZH2 suppression by bortezomib is mediated through E2F1.**

(A) Luciferase reporter assays were performed in HEK293T cells with the indicated *EZH2* promoter constructs. Reporters were co-transfected with either empty or *E2F1* expression plasmids. Transfections were normalized by using a simultaneously delivered Renilla luciferase expression plasmid. Schematic representation of the reporters constructs are depicted in upper panels. Data represent mean \pm SD of triplicates. (B) Quantitative RT-PCR analysis of *E2F1* and *EZH2* mRNA expression in MM.1S cells treated with 5 μ mol/L of HLM006474 for 12 hours. Y-axis represents fold-change after normalization to *GAPDH*, and error bars represent SD of triplicates. *** $P < 0.001$.

A**B**

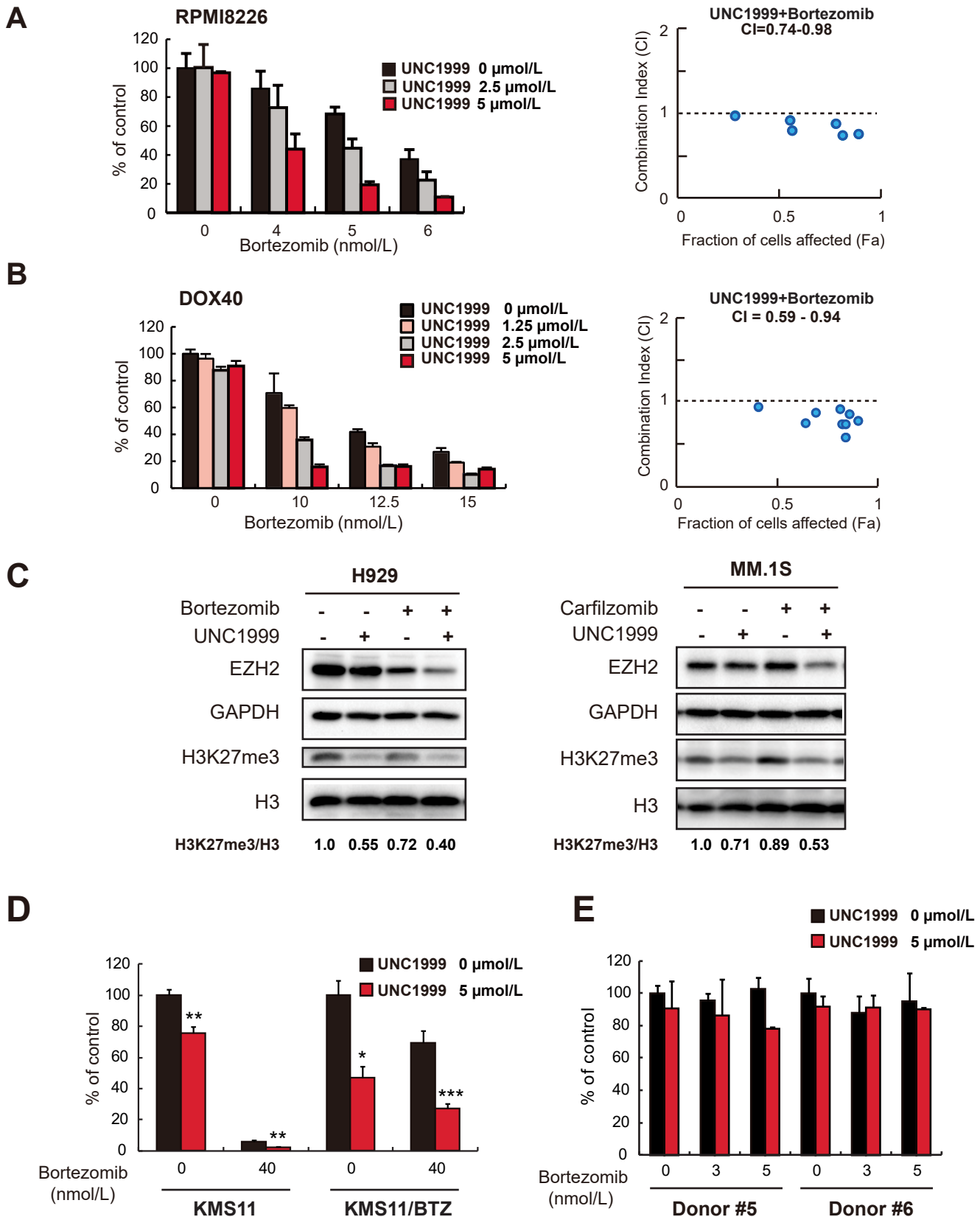
Supplementary Figure S4. MM patients with higher levels of *EZH2* expression have poorer response to bortezomib.

(A) Box-and-whisker plots showing the expression levels of *EZH2* in pre-treatment samples from MM patients enrolled on the APEX 039 clinical study who received bortezomib treatment. Boxes represent 25 to 75 percentile ranges. Whiskers represent the most extreme data point which is no more than 1.5 times the interquartile range from the box. Red + represents mean value. Horizontal bars represent median. (B) *EZH2* gene expression in MM patients in (A) is represented as a stacked bar chart. The range at the bottom of each bar represents the expression levels of *EZH2* in the total cohort in (A). Bars are subdivided by patients' response to treatment, with segments proportional to percentage of patients. R, response; NR, nonresponse; CR, complete response; PR, partial response; MR, minimal response; NC, no change; PD, progressive disease. *** $P < 0.001$. (Kruskal-Wallis test).



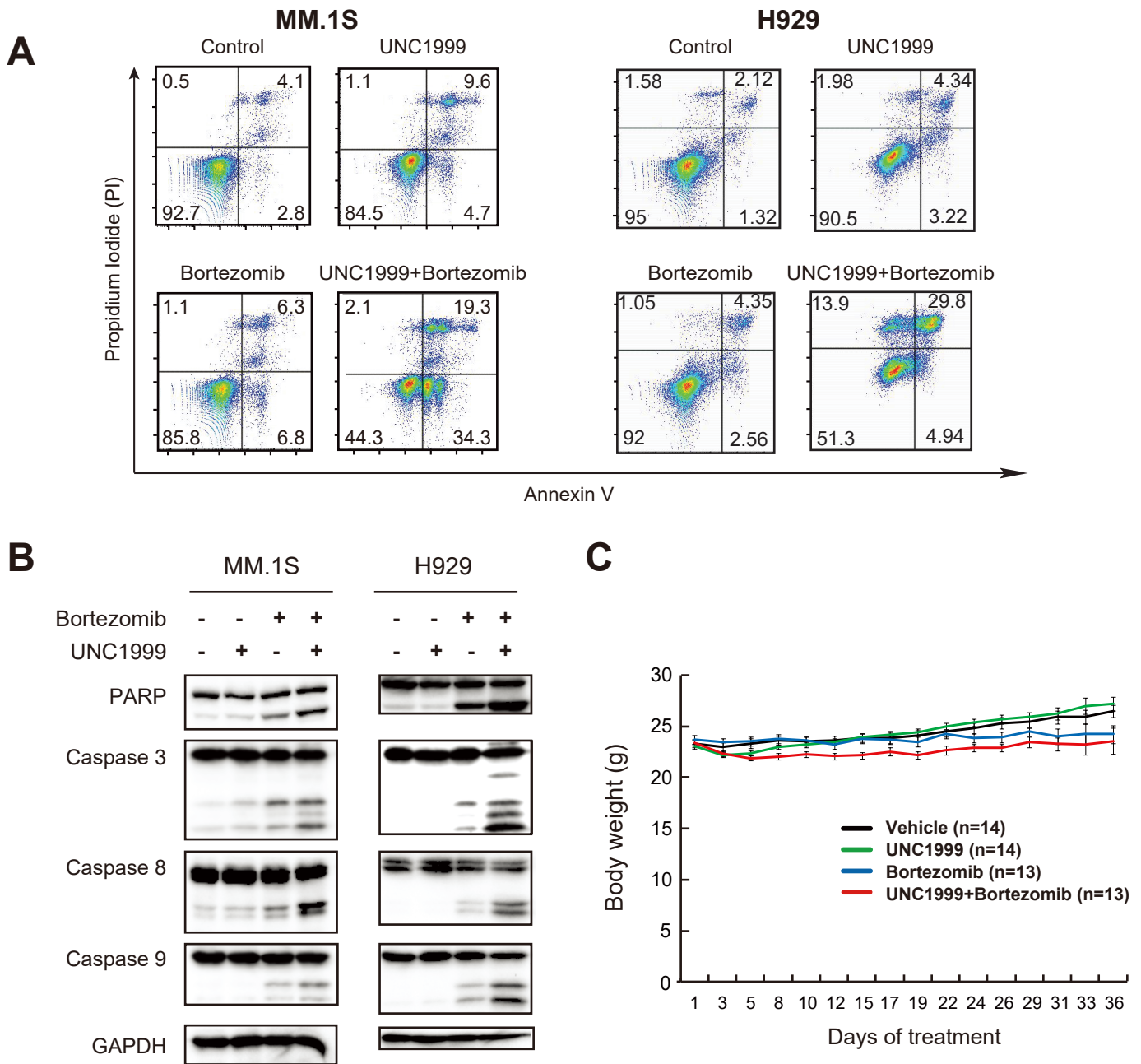
Supplementary Figure S5. EZH2 overexpression in MM cells confers resistance to bortezomib through upregulation of gene sets related to MYC, cell cycle and metabolism.

(A) MTS assay showing the viability of H929 cells transduced with *EZH2*-overexpressing or empty vectors upon treatment with 5 $\mu\text{mol/L}$ of UNC1999 (72 hours) and the indicated doses of bortezomib (last 48 hours) relative to untreated cells. Data represent mean \pm SD of triplicates. Immunoblot analyses of *EZH2* and H3K27me3 in *EZH2*-overexpressing or empty vector-transduced H929 cells are shown to the left of the graph. GAPDH and H3 served as loading controls. (B) Cell proliferation assay of RPMI8226 cells (5×10^4 cells per well in 6-well plate) transduced with the indicated lentiviruses. Cell counting was performed using trypan blue at the indicated times. Y-axis is presented as the mean cell number \pm SD of triplicates. (C) Representative gene sets significantly enriched in RPMI8226 cells transduced with *EZH2*-overexpressing versus empty vectors. NES, normalized enrichment score; q, FDR (false discovery rate) q value; p, p value. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.



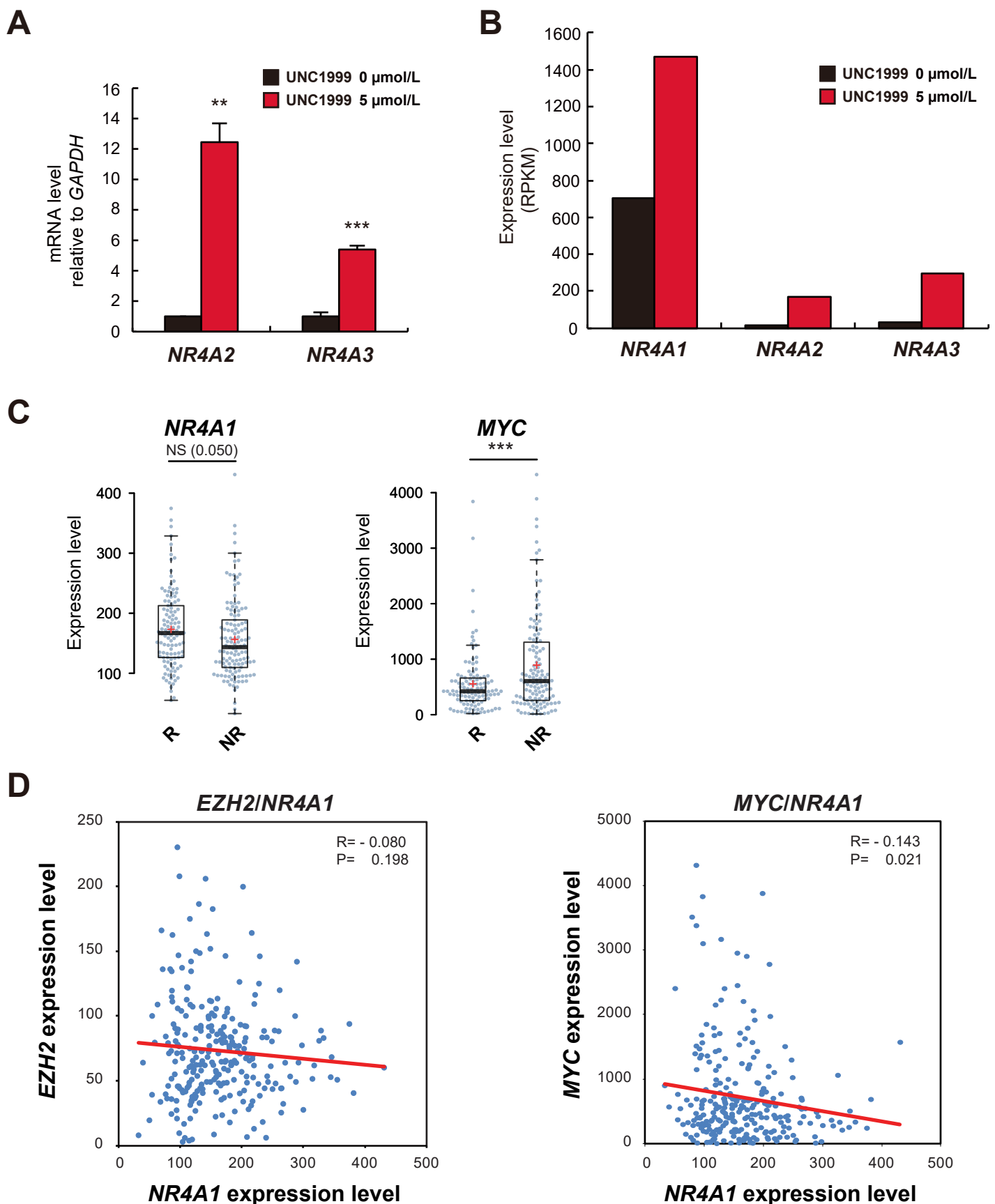
Supplementary Figure S6. UNC1999 enhances the cytotoxicity induced by bortezomib in MM cell lines including resistant ones but not in PBMCs from healthy donors.

MTS assays showing viability of (A) RPMI8226 or (B) DOX40 cells upon treatment with the indicated doses of UNC1999 (72 hours) in combination with the indicated doses of bortezomib (last 48 hours) relative to untreated control. Data represent mean \pm SD of triplicate cultures. Combination index (CI) calculation is shown to the right of each graph. (C) Immunoblot analyses for EZH2 and H3K27me3 after treatment of H929 (left panels) or MM.1S (right panels) cells with 5 $\mu\text{mol/L}$ of UNC1999 for 72 hours and/or 5 nmol/L of bortezomib or carfilzomib for the last 12 or 24 hours, respectively. H3 and GAPDH served as loading controls. H3K27me3 amounts relative to total H3 are shown. (D) MTS assay showing viability of KMS11 or KMS11/BTZ cells upon treatment with 5 $\mu\text{mol/L}$ of UNC1999 (72 hours) in combination with 40 nmol/L of bortezomib (last 48 hours) relative to untreated control. Data represent mean \pm SD of triplicate cultures. (E) MTS assay showing the viability of PBMCs isolated from healthy donors treated simultaneously with 5 $\mu\text{mol/L}$ of UNC1999 and the indicated doses of bortezomib for 48 hours relative to untreated control. Data represent mean \pm SD of triplicate cultures. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.



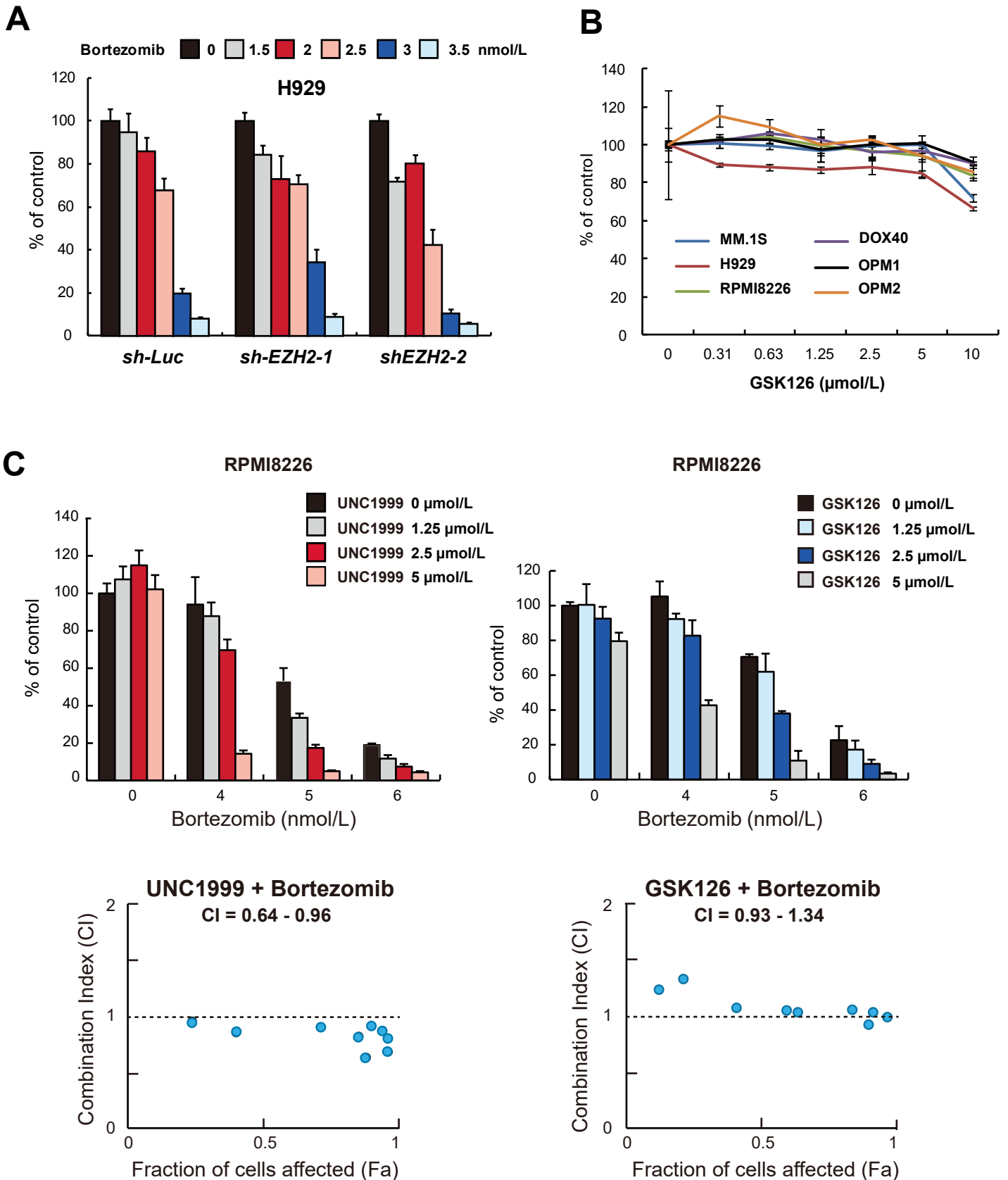
Supplementary Figure S7. The combination of UNC1999 and bortezomib induces significant apoptosis in vitro and is tolerated in vivo.

(A) Propidium iodide (PI) and annexin V staining of MM.1S cells (left panels) or H929 (right panels) after incubation with or without UNC1999 (5 $\mu\text{mol/L}$) for 48 hours, or bortezomib (2.5 nmol/L) for 24 hours, or the combination (UNC1999 for 48 hours with bortezomib in the last 24 hours). Apoptotic cells were analyzed using flow cytometry. (B) Immunoblot analysis for the indicated proteins in MM.1S (left panels) and H929 (right panels) cells after treatment with 5 $\mu\text{mol/L}$ of UNC1999 for 36 hours and/or 5 nmol/L of bortezomib for the last 12 hours. GAPDH served as a loading control. (C) Body weight of mice described in Figure 3F-G. Data represent mean \pm SE.



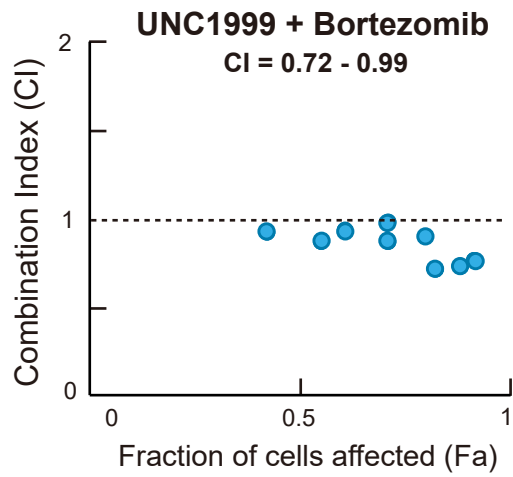
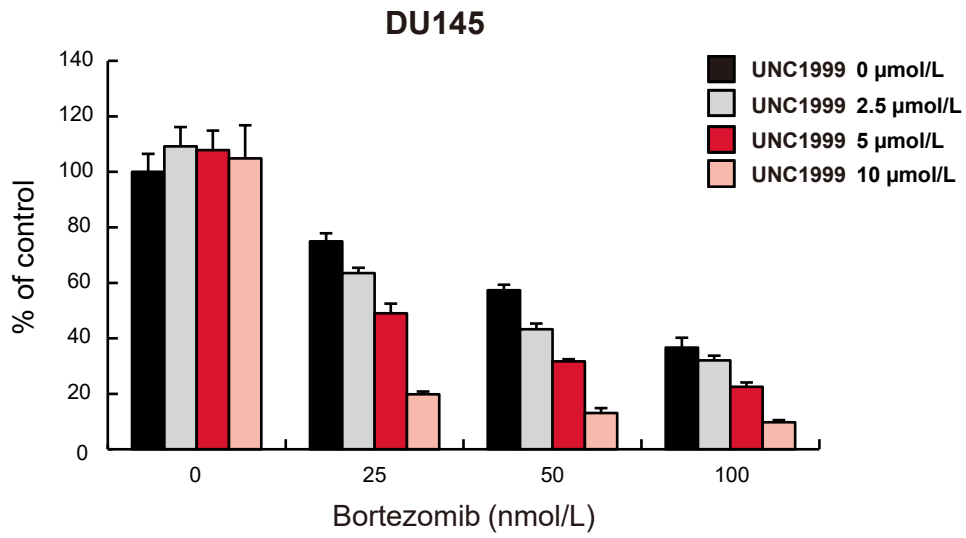
Supplementary Figure S8. The expression of NR4As in MM.

(A) Quantitative RT-PCR analysis of mRNA expression of *NR4A2* and *NR4A3* in MM.1S cells treated with 5 μmol/L of UNC1999 for 72 hours. Y-axis represents fold-change after normalization to *GAPDH*, and error bars represent SD of triplicates. (B) Expression levels of *NR4A1/2/3* in RPKM detected in RNA sequencing analysis of MM.1S cells treated with 5 μmol/L of UNC1999 for 72 hours versus DMSO-treated cells. (C) Box-and-whisker plots showing the expression levels of *NR4A1* (left) or *MYC* (right) in pre-treatment samples in responsive (R) vs. NR (nonresponsive) MM patients enrolled on the APEX 039 clinical study who received bortezomib treatment. Boxes represent 25 to 75 percentile ranges. Whiskers represent the most extreme data point which is no more than 1.5 times the interquartile range from the box. Red + represents mean value. Horizontal bars represent median. (D) Scatter plots showing the correlation between the expression of *EZH2* and *NR4A1* (left) or *MYC* and *NR4A1* (right) in patients described in (C). Pearson's product-moment correlation was used to determine correlation. **P<0.01; ***P<0.001; NS, not significant.



Supplementary Figure S9. Dual inhibition of EZH2 and EZH1, but not specific EZH2 inhibition, is critical for the synergy with bortezomib in MM cell lines.

(A) H929 cells transduced with the indicated lentiviruses were selected by cell sorting for GFP expression, and subjected to MTS assay. MTS assay showing viability of H929 cells upon treatment with the indicated doses of bortezomib (48 hours) relative to untreated control. Data represent mean \pm SD of triplicates. (B) MTS assays showing viability of MM cell lines upon treatment with the indicated doses of GSK126 (72 hours) relative to untreated control. Data represent mean \pm SD of triplicates. (C) MTS assays performed side by side showing viability of RPMI8226 cells upon treatment for 72 hours with the indicated doses of UNC1999 (left) or GSK126 (right) with the indicated doses of bortezomib in the last 48 hours relative to untreated control. Data represent mean \pm SD of triplicate cultures. Combination index (CI) calculation is shown below each graph.



Supplementary Figure S10. UNC1999 exhibits synergy with bortezomib in DU145 prostate cancer cells. MTS assay showing viability of DU145 cells upon simultaneous treatment for 72 hours with the indicated doses of UNC1999 and bortezomib relative to untreated control. Data represent mean \pm SD of triplicate cultures. Combination index (CI) calculation is shown below the graph.