The BET bromodomain inhibitor CPI203 improves lenalidomide and dexamethasone activity in *in vitro* and *in vivo* models of multiple myeloma by blockade of Ikaros and MYC signaling

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Received: January 20, 2017.

Accepted: July 19, 2017.

Pre-published: July 27, 2017.

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SUPPLEMENTARY METHODS

Cell lines and patient samples

Seven MM cell lines (ARP-1, JJN-3, U266, MM.1S, MM.1R, RMPI-8226 and KMM.1) were cultured in RPMI 1640 containing 10-15% fetal bovine serum, 2mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Thermo Fisher, Waltham, MA, USA). These cell lines were obtained from Division of Hematology and Oncology-IDIBAPS (Dolors Colomer, PhD, and Beatriz Martin, PhD).

Primary MM cells obtained from bone marrow aspiration of nine patients (4M/5F, median age: 63, range: 51-89) with symptomatic MM were used. None patient had received previous treatment with IMiDs. Four had previously undergone ASCT and bortezomib-induction regimens were administrated in 4 patients, one without ASCT. Mononuclear cells were isolated from bone marrow by Ficoll/Hypaque sedimentation (GE Healthcare, Chalfont St Giles, UK), cryopreserved and stored within the Hematopathology collection of our institution (Hospital Clínic-IDIBAPS Biobank R121001-094). The ethical approvals for this project, including the informed consent of the patients, were granted following the guidelines of the Hospital Clínic Ethics Committee (IRB).

Cell proliferation assays

Myeloma cell lines ($5x10^4$ per well) were incubated with CPI203 (kindly provided by Constellation Pharmaceuticals, Cambridge, MA, USA) and/or lenalidomide (Selleck Chemicals LLC, Houston, TX, USA) plus dexamethasone (Merck, S.L., Darmstadt Germany) at indicated doses in triplicates. MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) reagent (Sigma-Aldrich, St Louis, MO, USA) was added after 48h of treatment for 1-2 additional hours before spectrophotometric measurement (Synergy, Biotek. Winooski, VT, USA). The GI_{50} was calculated as the concentration that produced 50% growth inhibition. Combination indexes (CIs) were calculated by using the Calcusyn software version 2.0 (Biosoft, Ferguson, MO, USA), based on Chou-Talalay method. The interaction between two drugs was considered synergistic when $CI<0.8$.

Apoptosis was measured using the PE Annexin V Apoptosis Detection Kit I (Becton-Dickinson, San Jose, CA, USA) following manufacturer’s protocol.

Primary cells were labeled with CellTracker™ Red CMPTX dye (Thermo Fisher) following the manufacturer’s protocol and co-cultured with the mesenchymal stromal

cell line stromaNKtert in the presence of 10ng/ml IL-6 (RnD Systems, Minneapolis, MN, USA). Cell proliferation analysis was restricted to the MM population by co-staining with anti CD38 FITC antibody (Beckman Coulter, Marseille, France). Ten thousand stained cells per sample were acquired and analyzed in an Attune acoustic focusing cytometer using Attune software (Thermo Fisher).

Cell cycle analysis

ARP-1, JJN-3 and RMPI-8226 cells ($5x10^5$ cells) were incubated with 0.1μM CPI203 and/or 5μM lenalidomide with 0.1μM dexamethasone. After 24h, cells were washed in phosphate-buffered saline (PBS, Thermo Fisher), pelleted and fixed overnight at -20ºC in 70% ethanol. Fixed cells were then pelleted and resuspended in PBS containing 100 μg/ml RNase A (Roche Molecular Biochemicals, Pleasanton, CA, USA) and 20μg/ml propidium iodide (PI, Sigma Aldrich) at 37ºC for 30 minutes. Cells (10.000 events per condition) were acquired on a FACScalibur (Becton-Dickinson) and data were analyzed with the ModFit LT 1.01 software (Becton-Dickinson).

Western blot assays

Total protein from cell lines was isolated by resuspending cells in 400 μl of lysis buffer (Tris hydrochloride pH 7.5, Sodium Chloride and Triton X-100) complemented with proteases and phosphatases inhibitors and incubating them for 1 hour on ice. Samples were centrifuged at 15000x*g* for 15 minutes; equal amounts of protein (50 μg) were separated by sodium dodecyl sulfate–polyacrylamide electrophoresis in 12% Tris-HCl polyacrylamide gels and transferred to pure polyvinylidenedifluoride membranes (PVDF, Immobilon-P, Millipore, Billerica, MA, USA). Membranes were then incubated with antibodies against MYC (Cell Signaling Technology, Beverly, MA, USA), Ikaros (Cell Signaling Technology), IRF4 and GADD45β (Abcam, Cambridge, UK) and the endogenous β-actin (Sigma-Aldrich) followed by incubation with anti-rabbit (Cell Signaling Technology), anti-goat (Santa Cruz Biotechonology) or anti-mouse (Sigma-Aldrich) secondary antibodies. After incubation with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher), chemiluminescence was detected in a mini-LAS4000 device and analyzed with Image Gauge software (Fujifilm, Tokyo, Japan).

RNA isolation and real-time qPCR

Total RNA was isolated using Trizol reagent (Thermo Fisher) as per the manufacturer's protocol. cDNA was obtained from 500 ng of RNA using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher). mRNA expression was analyzed in

duplicate by quantitative real-time PCR on the StepOne system using TaqMan Gene Expression Assays (Thermo Fisher) for *IKZF1* (Hs00232635_m1), *MYC* (Hs00153408_m1) and *IRF4* (Hs01056533_m1). The relative expression of each gene was quantified by the comparative cycle threshold method ($\Delta\Delta C_t$). *GUSB* (Hs00939627_m1) was used as an endogenous control.

Gene expression profiling and gene set enrichment analysis

RNA integrity was examined with the Agilent 2100 Bioanalyzer (Agilent Technologies) and only high-quality RNA was used. Equal amounts of total RNA (2.5 µg) were reverse transcribed to cDNA and hybridized on Human Genome U219 microarray. All samples were simultaneously run in a GeneTITAN platform (Affymetrix, Santa Clara, CA, USA). Gene signatures overrepresented in each treatment group were identified using gene set enrichment analysis (GSEA) v2.0 (Broad Institute at MIT; http://www.broadinstitute.org/gsea/) interrogating C2CP and C3TFT gene sets from the Molecular Signature Database v2.5 and experimentally derived custom gene sets related to Ikaros(21). A two-class analysis (Control vs. CPI203) and an increasing profile analysis (Control vs. CPI203 vs. Combo) with 1,000 permutations of gene sets and a weighted metric was used. Bonferroni correction for multiple testing was applied. Gene sets were considered significantly enriched in a certain phenotype when false discovery rate (FDR) was lower than 0.1, and the normalized enrichment score (NES) was higher than 1.5 (for positively enriched gene sets) or lower than -1.5 for negatively enriched gene sets The leading edge genes of these enriched gene sets were displayed using Cluster (v2.11) and TreeView (v1.6) softwares (Eisen Laboratory, Stanford University, CA). The microarray data have been deposited in the NCBI's Gene Expression Omnibus and are accessible through GEO series accession number GSE (GSE87403).

Xenograft mouse model

SCID mice (Charles River Laboratories, L'Arbresle, France) were inoculated subcutaneously with $1.2x10^7$ cells of RPMI-8226 cell line. Mice were randomly assigned into cohorts of 5 mice each and received during 2 weeks (5 days on/2 days off), by intraperitoneal injection, a twice-daily dose of CPI203 (2.5 mg/kg), a daily dose of lenalidomide (25 mg/kg) plus twice weekly dexamethasone (1 mg/kg), the combination of both, or an equal volume of vehicle. Tumor growth was evaluated twice a week by measuring the shortest and longest diameters of the tumor with external calipers. Tumor volume (in mm^3) was calculated with the use of the following standard formula: $(\text{the shortest diameter})^2 \times (\text{the longest diameter}) \times 0.5$. Twenty-one days post

cell inoculation, intratumoral glucose uptake was evaluated with an Odyssey infra-red scanner (Li-Cor, Lincoln, NE, USA) in representative mice previously injected with an IRDye 800CW 2- deoxyglucose probe (Li-Cor). Animals were then sacrificed according to institutional guidelines and tumor xenografts were isolated. Tumor samples were snap-frozen in OCT medium (Sakura Tissue Tek) or formalin-fixed before paraffin embedding on silane-coated slides in a fully automated immunostainer (Bond Max; Vision Biosystems). Paraffin-embedded tumor samples were subjected to immunohistochemical staining using primary antibodies against Ikaros, MYC, IRF4, GADD45β and pH3. Preparations were evaluated with an Olympus DP70 microscope by means of a 20×/0.75 NA objective and DPManager software v2.1.1 (Olympus).

Statistical analysis

All statistical analyses were performed using GraphPad software 5.0 (GraphPad Software Inc., San Diego, CA, USA). The comparisons between all analyzed groups were evaluated with a Kruskal-Wallis test and the comparisons between two groups were analyzed with Student's t-test or nonparametric Mann-Whitney test. Results were considered statistically significant if $p<0.05$ and data are represented as mean ± SEM of three independent experiments (*$p<0.05$; **$p<0.01$; ***$p<0.001$).

SUPPLEMENTARY TABLES

Table S1. Gene sets regulated by CPI203 treatment in MM cells

GENE SET NAME	NES	FDR q-val
Custom gene sets[1]		
BLIMP1 B-CELL REPRESSED *	1.98	0.0027
BLIMP1 TARGETS*	1.91	0.0038
GENES UPREGULATED BY MYC*	1.89	0.0037
IKAROS DEL DOWNREGULATED†	1.80	0.0114
IKAROS DEL UPREGULATED†	-1.62	0.0889
Motif gene sets (C3)[2]		
V$MYCMAX_01	1.74	0.0318
V$MYC_Q2	1.66	0.0436
V$MYCMAX_02	1.52	0.0545

GSEA was used to test for significant enrichment of defined gene signatures. NES indicates Normalized Enriched Score; FDR, False Discovery Rate. Threshold FDR<0.10 and NES>1.5.

[1] Custom genes set were experimentally derived:

(*) Downloaded from http://lymphochip.nih.gov/signaturedb/index.html[39]

(†) Gene signatures related to IKAROS deletion were extracted from Lacobucci L et al.[21]

[2] C3 motif gene transcription factor signatures were obtained from the Molecular Signature Database (v2.5).

Table S2. Gene sets regulated by CPI203, len/dex or combo treatment in MM cells

GENE SET NAME	NES	FDR q-val
Custom gene sets[1]		
GENES UPREGULATED BY MYC*	-2.39	<0.0001
CMYC TARGETS*	-2.16	0.0008
IKAROS DEL DOWNREGULATED†	-1.65	0.0871
MYELOMA IRF4*	-1.60	0.0835
BLIMP B-CELL REPRESSED*	-1.56	0.1096
BLIMP1 TARGETS*	-1.53	0.1073
Motif gene sets (C3)[2]		
V$MYCMAX_01	-2.12	0.0008
V$MYC_Q2	-1.65	0.0272
V$MYCMAX_B	-1.57	0.0427
V$MAX_01	-1.56	0.0413
V$MYCMAX_02	-1.50	0.0478

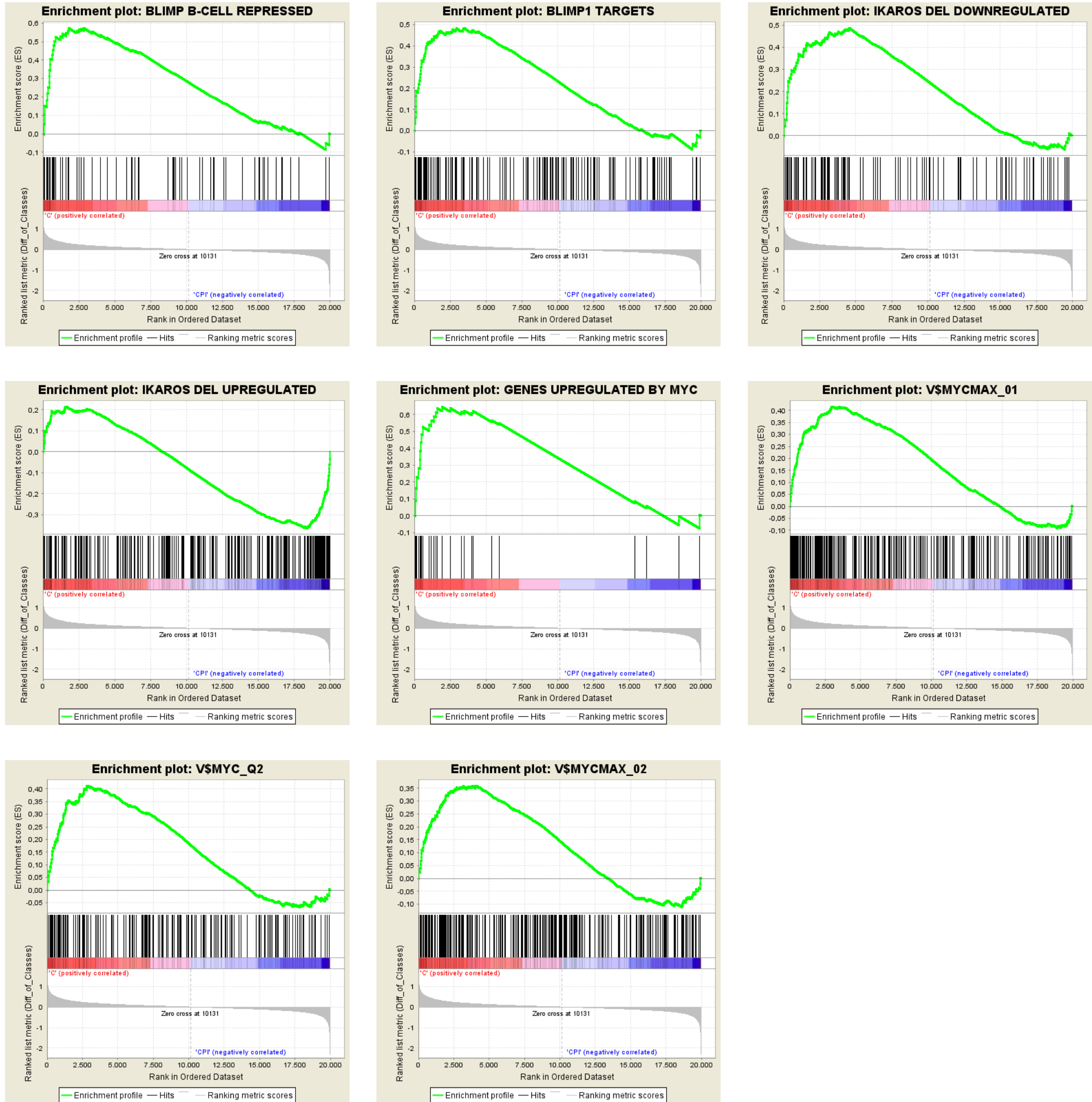
GSEA was used to test for significant enrichment of defined gene signatures. NES indicates Normalized Enriched Score; FDR, False Discovery Rate. Threshold FDR<0.10 and NES>1.5.

[1] Custom genes set were experimentally derived:

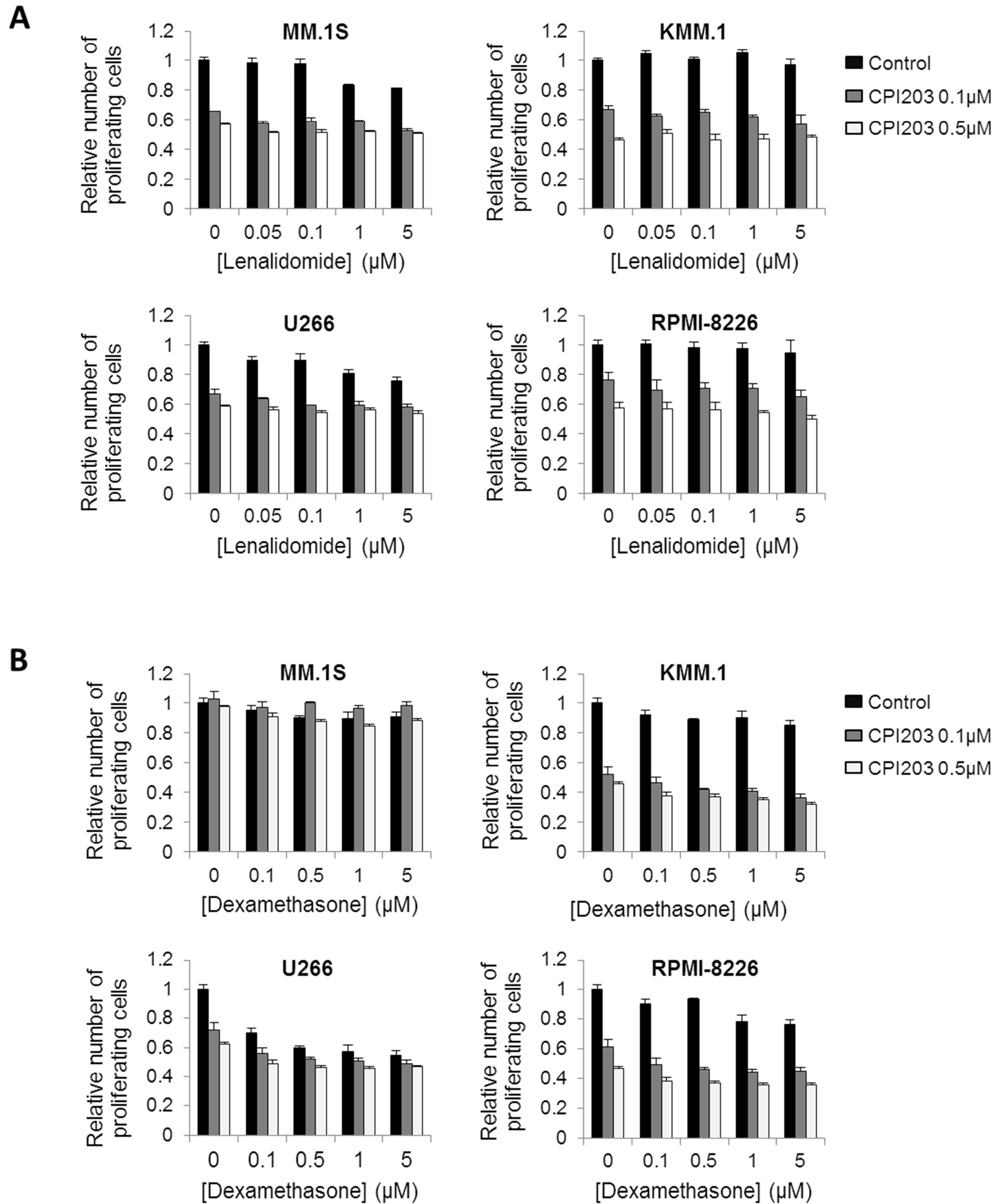
*downloaded from http://lymphochip.nih.gov/signaturedb/index.html[39];

†Gene signatures related to IKAROS deletion were extracted from Lacobucci L et al.[21]

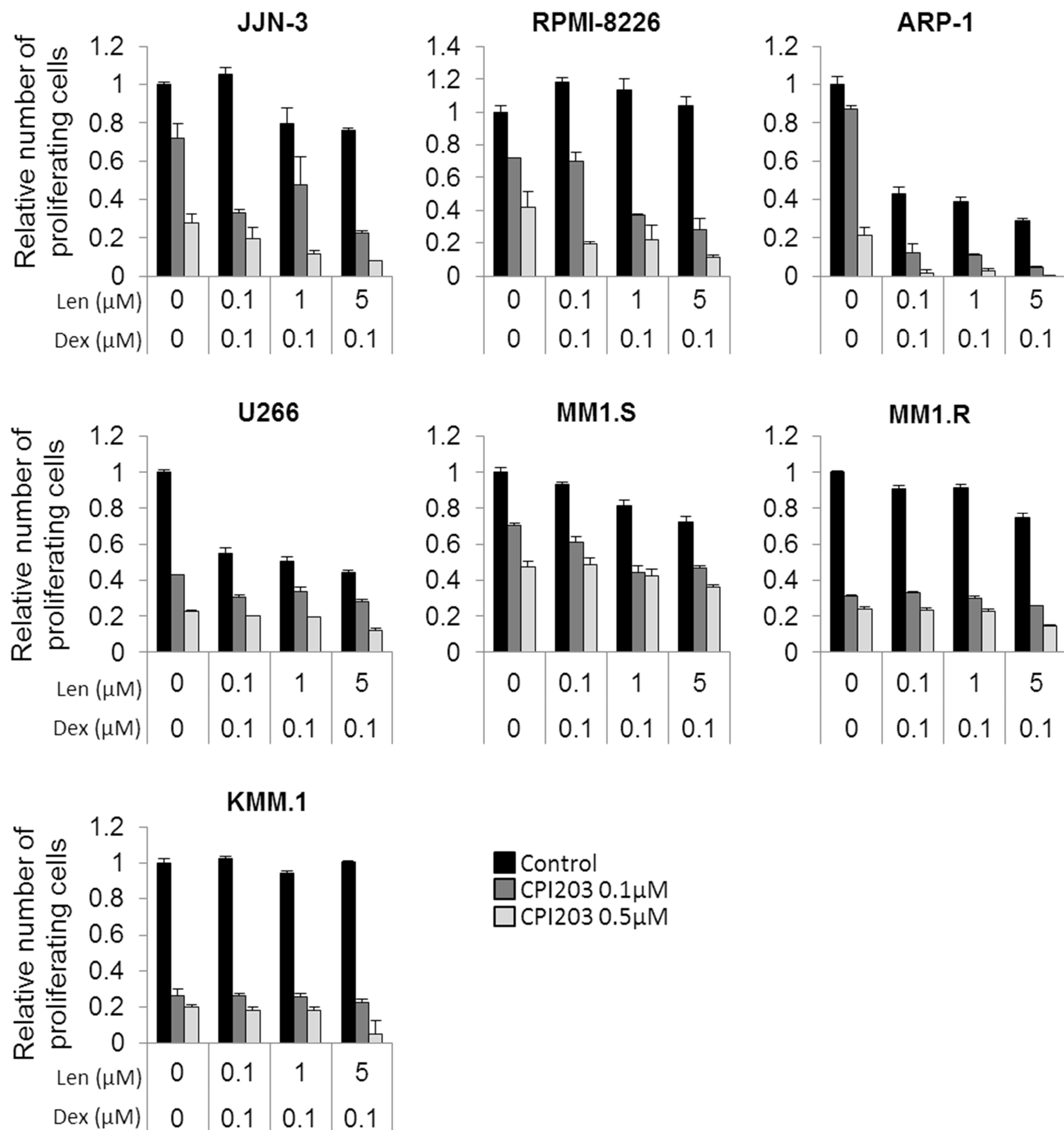
[2] C3 motif gene transcription factor signatures were obtained from the Molecular Signature Database (v2.5).



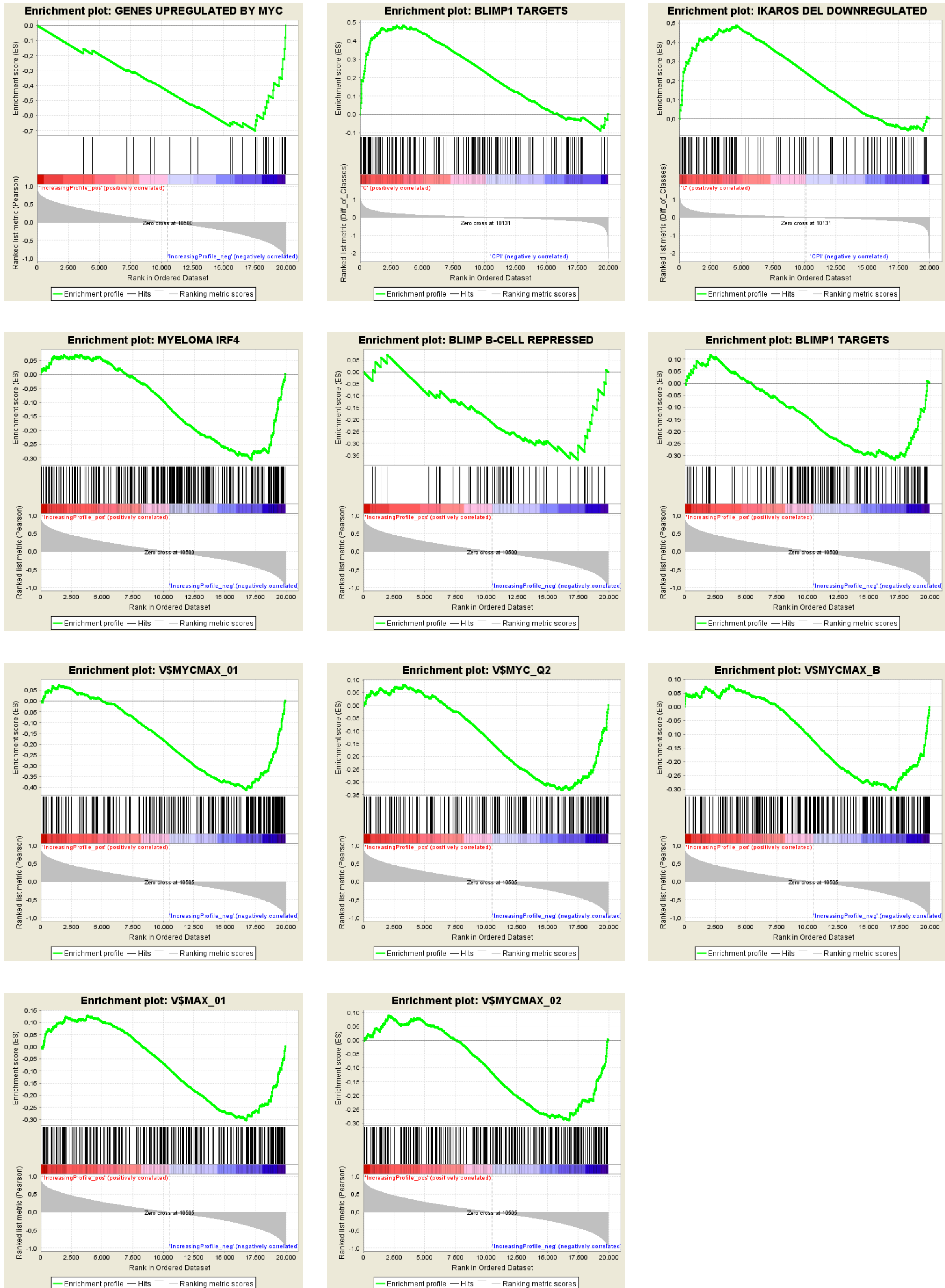
Supplementary Figure S1. Enrichment plots of gene sets regulated by CPI203 in MM cell lines



Supplementary Figure S2. Cell proliferation in MM cell lines after CPI203 treatment combined with lenalidomide (A) or dexamethasone (B). Relative proliferation of 4 representative MM cell lines after 48 hours of treatment with increasing doses of CPI203 and/or Lenalidomide and/or dexamethasone analyzed by MTT proliferation assay.



Supplementary Figure S3. Cell proliferation in MM cell lines after CPI203, Len/Dex or three-drug combination treatment. Relative proliferation of 7 cell lines after 48 hours of treatment with increasing doses of CPI203 and/or Len/Dex analyzed by MTT proliferation assay.



Supplementary Figure S4. Enrichment plots of gene sets regulated by CPI203, Len/Dex or three-drug combination in MM cell lines