Apoptosis reprogramming triggered by splicing inhibitors sensitizes multiple myeloma cells to Venetoclax treatment

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Supplementary Data

Reagents

Meayamicin B and Sudemycin D6 (SD6) were kindly gifts from Prof. Kazunori Koide (University of Pittsburgh) and Prof. Thomas Webb (SRI Biosciences, Dept. of Chemistry and Biochemistry UCSC), respectively. E7107 was kindly provided by H3 Biomedicine, Inc.

Cell lines and culture

The HMCL were purchased from ATCC or DSMZ. All cell lines were Mycoplasma-free and routinely tested for it. Cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS; GIBCO), 4mM glutamine, 100 U ml−1 penicillin, and 100 μg ml−1 streptomycin (GIBCO). 293T were cultured in DMEM high glucose containing 10% FBS (GIBCO), 4 mM glutamine, 100 U ml−1 penicillin, and 100 μg ml−1 streptomycin (GIBCO).

Primary cells

Peripheral blood samples collected from healthy volunteers were processed by Ficoll-Paque (GE Healthcare) gradient to obtain PBMCs. Malignant cells from individuals affected by MM were purified from bone marrow samples after informed consent was obtained in accordance with the Declaration of Helsinki and approval by the Ethical Committee at San Martino Policlinico in Genoa. Mononuclear cells were separated using Ficoll–Paque density sedimentation, and plasma cells were purified by positive selection (>95% CD138+) with anti–CD138 magnetic activated cell separation microbeads system (Miltenyi). Clinical and cytogenetic features of MM patients samples used for the study are shown in **Table S2**.

Lentiviral mediated gene transfer

pLV[shRNA]-EGFP:T2A:Puro-U6>Scramble_shRNA (shSCR) and pLV[shRNA]-EGFP:T2A:Puro-U6>hSF3B1[shRNA#1-4] (SF3B1 silencing plasmids), pLV[Exp]-CMV>EGFP:T2A:Puro (pLV empty vector) and pLV[Exp]-EGFP:T2A:Puro-SV40> hMYC (cMyc overexpressing plasmid) were used to create stable isogenic MM cell lines. pLV-EGFP/Neo-

SV40>Nluc was used to generate a stably expressing-NanoLucifease MM cell line. All lentiviral plasmids were purchased from Vector Builder.

Tumor cell-specific bioluminescence imaging in co-cultures with stromal cells

7×10^3 Luciferase$^+$ MM cells (H929 pLV-SV40-EGFP/Luc+) were plated in 96-well optical white plates in the presence or absence of pre-plated luciferase-negative primary stromal cells (20×10^3 cells seeded 24hours before) and treated with drugs or vehicle (DMSO), as indicated. After 48h of treatment, MM cell specific viability was assessed with Nano-Glo® Live Cell Assay System (Promega, #N2011) according to manufacturer's instructions.

Western blotting

Whole-cell lysates, SDS-page electrophoretic separation and blotting were performed as previously described[1]. Primary antibodies used are detailed in Supplementary material (**Table S3**). Signals were acquired by I bright™ 1500 Imaging System using standard ECL (Thermo Fisher).

Immunofluorescence staining

DNA damage and nuclear speckles were detected by confocal microscopy, as described previously[2]. The anti-γH2AX antibody and the anti-SC35 antibody [SC-35]-Nuclear Speckle Marker were purchased from Millipore (Ser139, #05-636) and Abcam (#ab11826), respectively. The secondary Alexa Fluor 488–conjugated antibody was from Jackson ImmunoResearch (#115-546-006). γH2AX foci and SC35 staining intensity were calculated by ImageJ-Find Maxima.

Cell Viability and Apoptosis Assay

MM cell lines viability and apoptosis were assessed as previously described[2]. For MM primary cells, 3.5×10^3 CD138$^+$ cells primary tumor cells were plated in white opaque 96-well plate in DMEM high glucose complete medium supplemented with IL-6 4ng/ml. Then, cells were treated with drugs and 48h later, cell viability was measured with CTG luminescent assay (Promega, #G7570) following manufacturer's instructions. For FACS analysis, 1×10^6 Bone Marrow-derived mononuclear cells from MM patients were treated with vehicle or drugs for 24 or 48 hours. After that, cells were stained with anti CD45-PC.5 (Beckman Coulter #A62835), anti CD38-PE (Becton

Dickinson #345806) and Annexin V-FITC (Miltenyi #130-093-060) and specific viability of $CD38^+$ $CD45^-$ or $CD38^-$/ $CD45^-$ gated MM cells was evaluated.

Transient transfection of MM cells

RPMI 8226 and AMO-1 SF3B1-silenced cells were generated by transient transfection of siRNAS (ON-TARGET plus SMART pool siRNA # L-020061-01-0005) using nucleofection. For each nucleofection, 2 x 10^6 cells were pulsed with the DN-100 and DA-100 program respectively, using Amaxa SF Cell line 4-D Nucleofector X KitL (Lonza). siRNAs were used at the final concentration of 500 nM. After 24h from nucleofection, cells were treated and used for further experiments.

RT-PCR and Quantitative Real-time PCR (qPCR)

Total RNA was extracted from cells using the RNeasy mini kit (Qiagen, #74134d) according to the manufacturer's instructions. 1 μg of RNA was reverse-transcribed in a final volume of 50 μl using High Capacity cDNA Reverse Transcription kit (Applied Biosystem, #4368814). For validation of splicing inhibitors targets, a 35-cycle PCR was performed (1 minute of annealing at 60°C and a 2-minute extension at 72°C). All PCR products were separated and visualized on 2% agarose gels. For qPCR, 5 μl of the resulting cDNA was used for QPCR with a QuantStudio™ 5 Real-Time PCR System (Applied Biosystem, Thermo Scientific). mRNA levels were detected using SYBR Select Master Mix (Applied Biosystem, #4472918) according to manufacturer's protocol. Gene expression was normalized to housekeeping gene expression (β-actin). Comparisons in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method. PCR and quantitative PCR (qPCR) primers are listed in **Table S4.**

TaqMan Array Plates

Total RNA from previously isolated Peripheral Blood Mononuclear Cells (PBMCs) or $CD138^+$ plasma cells was extracted using the RNeasy plus mini kit (Qiagen, #74134) according to the manufacturer's instructions and verified for quality with Nanodrop™ OneC (Thermo Scientific). 1μg of RNA was reverse-transcribed in a final volume of 20μl using SuperScript IV VILO Master Mix with ezDNase Enzyme (Applied Biosystem, #11766050) and diluted in 480μl of water to

obtain a final cDNA concentration of 10 ng per well. After that, 500µl of TaqMan Fast Advanced Master Mix (Applied Biosystem, # 4444963) were added to each sample and the mix was spotted on a 96-well 0.1µl Custom TaqMan Array plate (array ID: RPH49YH). Each plate contains 32 different TaqMan dried-down gene assays (including 3 housekeeping genes), in triplicate. QuantStudio™ 5 Real-Time PCR System (Applied Biosystem, Thermo Scientific) was employed. Results were analyzed with the ΔΔct method.[3] Log2 transformed Δct were used to draw the heatmap in R with heatmap.2 function in the gplots package and the RColorBrewer package. Z-scores of the expression levels among rows were calculated and genes divided in high expression (Z-score>1) and low expression (Z-score<-1). A list of genes included in the TaqMan Array plate is in **Table S5**.

Gene expression analysis of spliceosome signature in external MM datasets

Gene expression profiling data were derived from publicly available Gene Expression Omnibus (GEO) datasets profiled on Affymetrix Human Gene 1.0 ST array, available at accession numbers GSE66293 and GSE47552. Enrichment scores based on the expression levels of KEGG Spliceosome gene set (124 genes) from the Molecular Signatures Database (MSigDB) were calculated for microarray (GSE66293, GSE47552) and RNA-seq data (CoMMpass 774 MM dataset), respectively. As previously described [1], the gsva function in the Gene Set VariationAnalysis package for microarray and RNA-sequencing data (GSVA,version 3.8) was applied in R Bioconductor (version 4.0.2), by choosing the z-score method and Gaussian distribution. Samples were then stratified into low (z-score ≤-1), intermediate (-1<z-score>1), or high (z-score≥1) groups. The heatmap of KEGG Spliceosome signature was generated using DNA-Chip Analyzer software.[4]

Multi-Omics Data in CoMMpass Study

Multi-omics data about newly diagnosed bone marrow MM samples (BM_1) were freely available from Multiple Myeloma Research Foundation (MMRF) CoMMpass Study [5] and obtained from the Interim Analysis 15a (MMRF_CoMMpass_IA15a). Transcript per Million (TPM) reads values

were obtained by Salmon gene expression quantification (Salmon_V7.2) for 774 BM_1 MM patients. Non-synonymous (NS) somatic mutation variants data were derived from whole exome sequencing (WES) analyses; copy number alteration (CNA) data were obtained by means of Next generation Sequencing (NGS)-based fluorescence in situ hybridization (FISH) analyses in the 414 MM cases classified in the two extreme quartiles and for which molecular data were available. The presence of a specific CNA was determined at a 20 percent cut-off, when occurring in at least one of the investigated cytoband (1q21, 13q14, 13q34 or RB1 locus).

Survival analyses in CoMMpass study

Kaplan-Meier analysis was applied on Overall Survival (OS) and Progression Free Survival (PFS) data in 299 and 343 MM patients, respectively stratified in low and high z-score groups. Log-Rank test was applied to measure the difference between survival curves.

Cox proportional hazards model was applied as univariate analysis on single molecular variables and International Staging System (ISS) groups in relation to OS and PFS data, in 414 MM cases, classified in low or high spliceosome group and for which all information were accessible.

Global analysis of alternative splicing patterns in CoMMpass study

The analysis of genome-wide patterns in alternative splicing was performed by means of IsoformSwitchAnalyzeR package in R Bioconductor (v4.0.2)[6], by stratifying 774 MM samples of CoMMpass studyaccording to SF3B1 expression level and comparing the extreme quartiles, each including 194MM cases. Filtered Transcripts Per Million (TPM) and transcript counts obtained by RNA-sequencing (Salmon v7.2) and filtered GRCh37.74.gtf file from CoMMpass study were used to create isoform data matrices (194622 transcripts). After filtering out the isoforms not/low expressed in any samples, under stringent conditions (gene expression cut-off = 10, isoform expression cut-off = 3), isoforms switch analysis and prediction of alternative splicing was performed for 6388 gene comparisons by means of DRIMSeq.[7] Significant splicing enrichment results were finally obtained in top compared to bottom SF3B1quartile.

MM cell lines gene set enrichment analysis

For pathway enrichment analysis of Venetoclax resistant versus sensitive cell lines with published transcriptome data, activity areas were Pearson-correlated to the level of individual gene transcripts across cell lines. Gene-centric RMA-normalized mRNA expression data was acquired from CCLE.[8,9] The resulting ranked Pearson-correlation-score / gene transcript matrix was then used as an input for the pre-ranked list tool of gene set enrichment analysis (GSEA).[10] Enrichment scores were calculated for the following Molecular Signatures Database (MSigDB) collection CP:REACTOME: Reactome gene sets.[11] Significant pathways were selected based on FDR q-value <25%.

Highlighted REACTOME splicing / mRNA processing data sets: SNRNP_ASSEMBLY, PROCESSING_OF_CAPPED_INTRON_CONTAINING_PRE_MRNA, REACTOME_MRNA_SPLICING, REACTOME_MRNA_SPLICING_MINOR_PATHWAY. Enrichment plots were generated with GSEA software for REACTOME_MRNA_SPLICING and KEGG_SPLICEOSOME gene sets.

In situ detection of apoptosis and proliferation

Mice tumor sections were subjected to immunohistochemical staining for caspase-3 activation to detect apoptotic cell death. Visualization of apoptotic cells was done under a light microscope at ×40 magnication and was identified based on morphological features as described.[12] Tumor sections from treated and untreated mice stained with H&E and evaluated under light microscopy. Apoptotic cells were counted in 10 random fields. In each section, 1000 cells were evaluated for the presence of apoptotic cells and calculated as the number of apoptotic cells expressed as a percentage of the total number of non-apoptotic cells counted in each case.

Statistical analyses

All in vitro experiments were repeated at least three times and performed in triplicate; representative experiments are shown in figures. All data are shown as mean ± standard deviation (SD). The Student's t test was used to compare two experimental groups using Graph-Pad Prism software 8.4.3 (http://www.graphpad.com). The minimal level of significance was specified as

$p<0.05$. Tumor volume was analyzed by two-way Student's t-test and data are shown as mean ± s.e.m. Survival in *in vivo* experiments was evaluated by Log-rank (Mantel-Cox) Test. Drug interaction were assessed by CalcuSyn 2.0 software (Biosoft), which is based on the Chou-Talalay method. Combination Index (CI) = 1, indicates additive effect; $CI<1$ indicates synergism; $CI>1$ indicates antagonism.

TABLEs for supplementary data

Table S1. IC50 values for indicated spliceosome modulators

HMCLs	MeaymicinB IC50 (nM)	Sudamicin D6 IC50 (nM)	E7107 (nM)
KMS34	0.53	Not tested	Not tested
KMS20	1.03	Not tested	Not tested
LP1	2.66	Not tested	Not tested
MM1S	0.80	472.8	1.1
KMS11	1.16	Not tested	Not tested
U266	0.50	588.6	Not tested
MOLP8	0.51	Not tested	Not tested
AMO1	1.71	32.25	Not tested
KMS12PE	0.49	Not tested	Not tested
H929	1.01	165.8	0.57
MR20	3.5	Not tested	Not tested
OPM2	0.96	Not tested	Not tested
LR5	2.24	Not tested	Not tested
RPMI8226	2.01	2789	Not tested

HMCLs: human multiple myeloma cell lines

Table S2. Clinical and cytogenetic features of MM patients

PATIENTS	AGE	SEX	ISOTYPE	R-ISS	DISEASE STATUS	FISH	PREVIOUS THERAPIES
MM#1	82	M	IgG_k	II	RRMM	SD	Bz
MM#2	66	F	IgAk	III	NDMM	del17p	None
MM#3	74	F	IgGʎ	II	NDMM	SD	None
MM#4	74	F	IgGk	II	NDMM	1q amp	None
MM#5	78	F	micromolecular	III	RRMM	t(4;14)	Bz/Len
MM#6	75	F	IgGʎ	III	NDMM	t(14;16)	None
MM#7	74	M	IgG_K	II	RRMM	SD	Bz
MM#8	70	M	IgG_K	III	NDMM	del17p	None
MM#9	72	F	micromolecular	II	RRMM	SD	Bz/Len
MM#10	80	M	IgGʎ	III	RRMM	t(4;14)	Len
MM#11	78	M	micromolecular	II	NDMM	SD	None
MM#12	80	M	IgG_K	III	RRMM	del17p	Bz/Len
MM#13	75	M	IgG_K	I	RRMM	SD	Bz
MM#14	71	M	IgG_K	II	RRMM	t(11,14)	Bz
MM#15	80	F	IgG_K	II	RRMM	SD	Bz
MM#16	77	M	IgG_K	II	RRMM	SD	Bz/Len/Dara
MM#17	67	M	IgGk	II	NDMM	SD	None
MM#18	65	M	IgG_K	III	NDMM	del17p	None
MM#19	60	F	IgG_K	III	NDMM	del17p	None

MM#20	62	F	IgGʎ	II	RRMM	SD	None
MM#21	72	F	IgAʎ	III	RRMM	1q amp	Len
MM#22	67	M	IgAʎ	III	RRMM	del 17p	Bz/Len
MM#23	75	F	IgGʎ	II	NDMM	t(11;14)	None
MM#24	63	M	IgA_K	I	NDMM	SD	None
MM#25	82	M	IgGʎ	II	RRMM	SD	Bz/Len
MM#26	73	M	IgGk	III	RRMM	del17p	Bz
MM#27	79	M	IgGk	I	NDMM	SD	None
MM#28	79	M	IgAʎ	II	NDMM	SD	None

NDMM Newly diagnosed; RRMM resistant/refractory; SD standard risk, Bz: bortezomib; Len: lenalidomide; Dara: daratumumab

Table S3. Antibodies

mAb	Source	Product Number
Bcl-2	Cell Signalling Technology	4223
Bcl-xL	Cell Signalling Technology	2764
Mcl-1	Cell Signalling Technology	5453
SF3B1	Cell Signalling Technology	14434
phospho-SF3B1	Cell Signalling Technology	25009
PARP-1	Cell Signalling Technology	9542
Casapse3	Cell Signalling Technology	9662
cleaved-Caspase3	Cell Signalling Technology	9664
RAD51	SantaCruz Biotechnology	sc-8349
cMyc	Cell Signalling Technology	9402
γH2AX	Cell Signalling Technology	05-636
gamma-Tubulin	Thermo Scientific	MA1-850
GAPDH	Cell Signalling Technology	5174

Table S4. PCR and quantitative PCR (qPCR) primers

Gene name	primer FORWARD 5'-3'	primer REVERSE 5'-3'
MCL1	GAGGAGGAGGAGGACGAGTT	ACCAGCTCCTACTCCAGCAA
BCL2	ATGTGTGTGGAGAGCGTCAA	CACTTGTGGCCCAGATAGG
BCLXL	GGGCATTCAGTGACCTGACA	GGGAGGGTAGAGTGGATGGT
GAPDH	TCTCCTCTGACTTCAACAGCGAC	CCCTGTTGCTGTAGCCAAATTC
Delta2 MCL1	GCCAAGGACACAAAGCCAAT	AGTTTCCGAAGCATGCCTTG
Full length MCL1	GCCAAGGACACAAAGCCAAT	ACTCCACAAACCCATCCTTG

Table S5. Genes included in TaqMan Array plates

Genes included in Custom TaqMan Array plates			
18s rRNA	HNRNPA2B1	SF1	SNRPE
CLK1	HNRNPC	SF3A1	SNRPF
COIL	HNRNPD	SF3B1	SRSF1
GAPDH	HNRNPK	SF3B2	SRSF2
GEMIN2	HPRT1	SF3B6	STRAP
GEMIN6	PHF5A	SNRPD1	U2AF2
GEMIN7	PRMT5	SNRPD2	WDR77
HNRNPA1	PRPF8	SNRPD3	ZRSR2

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Supplementary Figures and legends

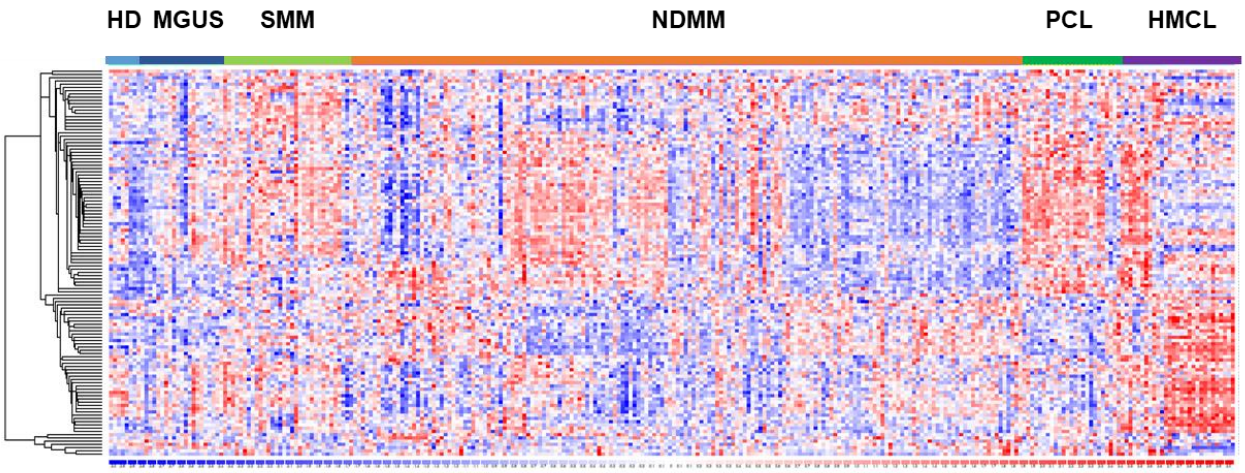

Figure S1. Heatmap showing expression levels for the genes corresponding to KEGG spliceosome gene set in plasma cells from healthy donors (normal, 9 N) or patients with monoclonal gammopathy of undetermined significance (20 MGUS), smoldering MM (33 SMM), active disease (170 MM), and secondary plasma cell leukemia (12 PCL) and in HMCLs (GSE66293 and GSE47552, profiled on Human Gene 1.0 ST Array). The color scale spans the relative gene expression changes standardized on the variance.

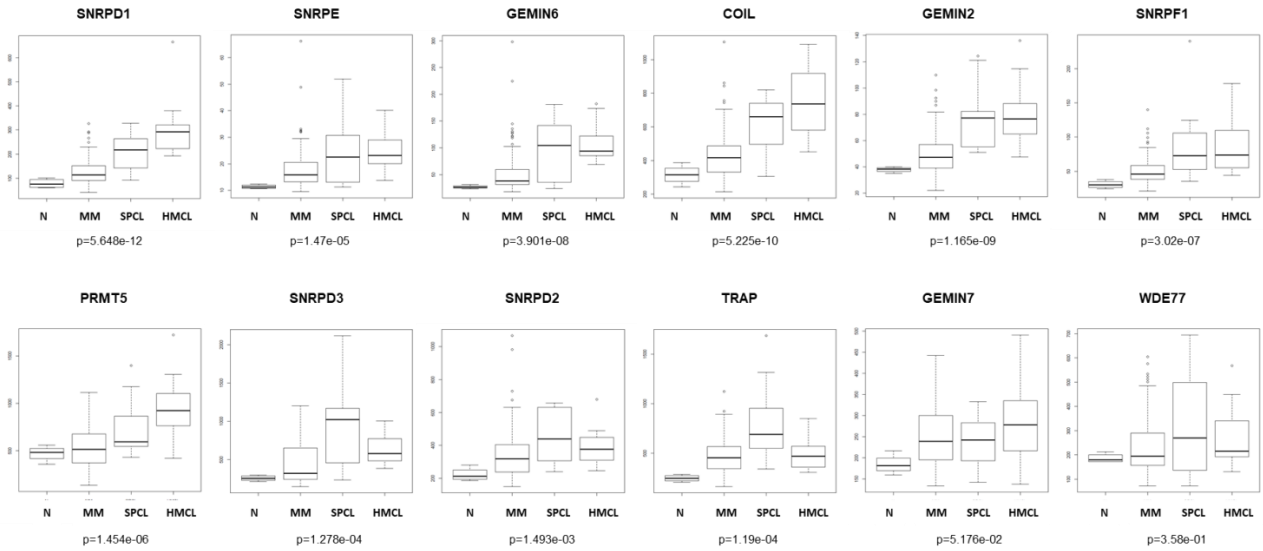

Figure S2. Box plot indicating the expression levels of SNRPD1, SNRPE, GEMIN6, COIL, GEMIN2, SNRPF1, PRMT5, SNRPD2, TRAP, GEMIN7 and WDE77 genes in a 323-sample dataset, including 4 healthy donors (N), 129 MM, 12SPCL patients, together with 18 HMCLs. This 323-sample dataset was generated using GSE66293. Global p-value (Kruskal-Wallis test) is indicated below.

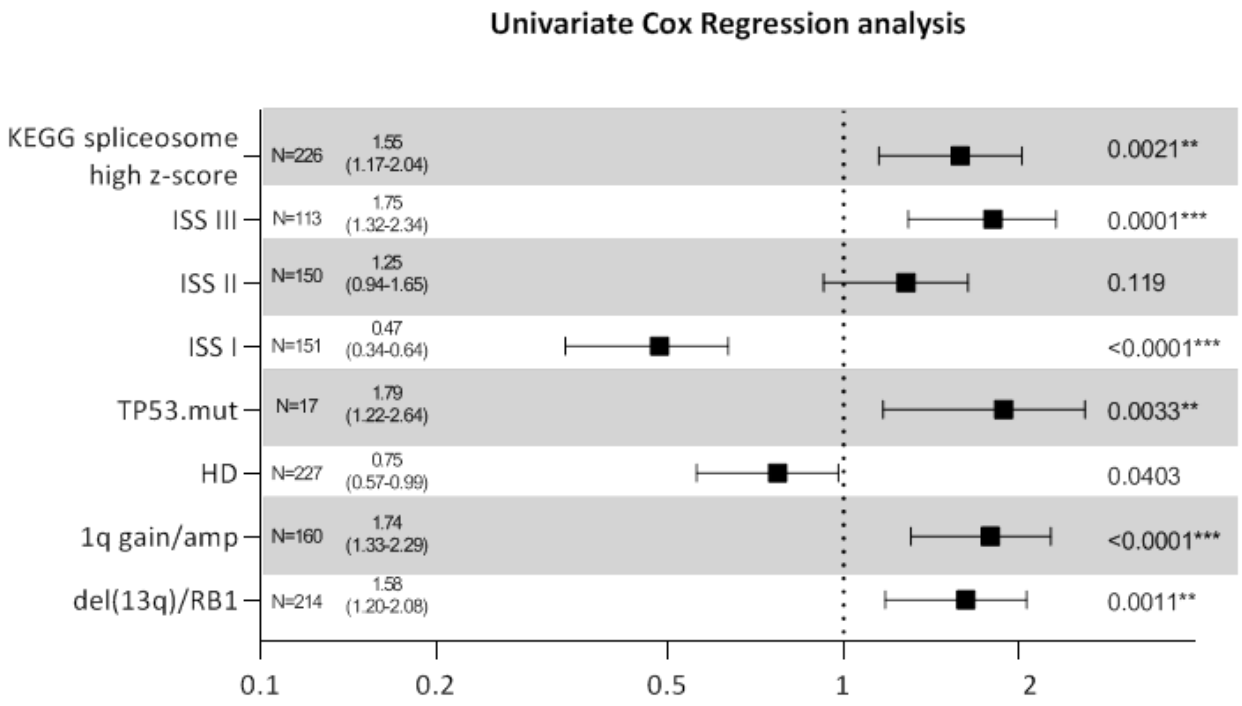

Figure S3. Forest plot based on Cox univariate analysis for progression free survival. Squares represent hazard ratios; bars represent 95% confidence intervals. p-values are reported on the right for each analysis.

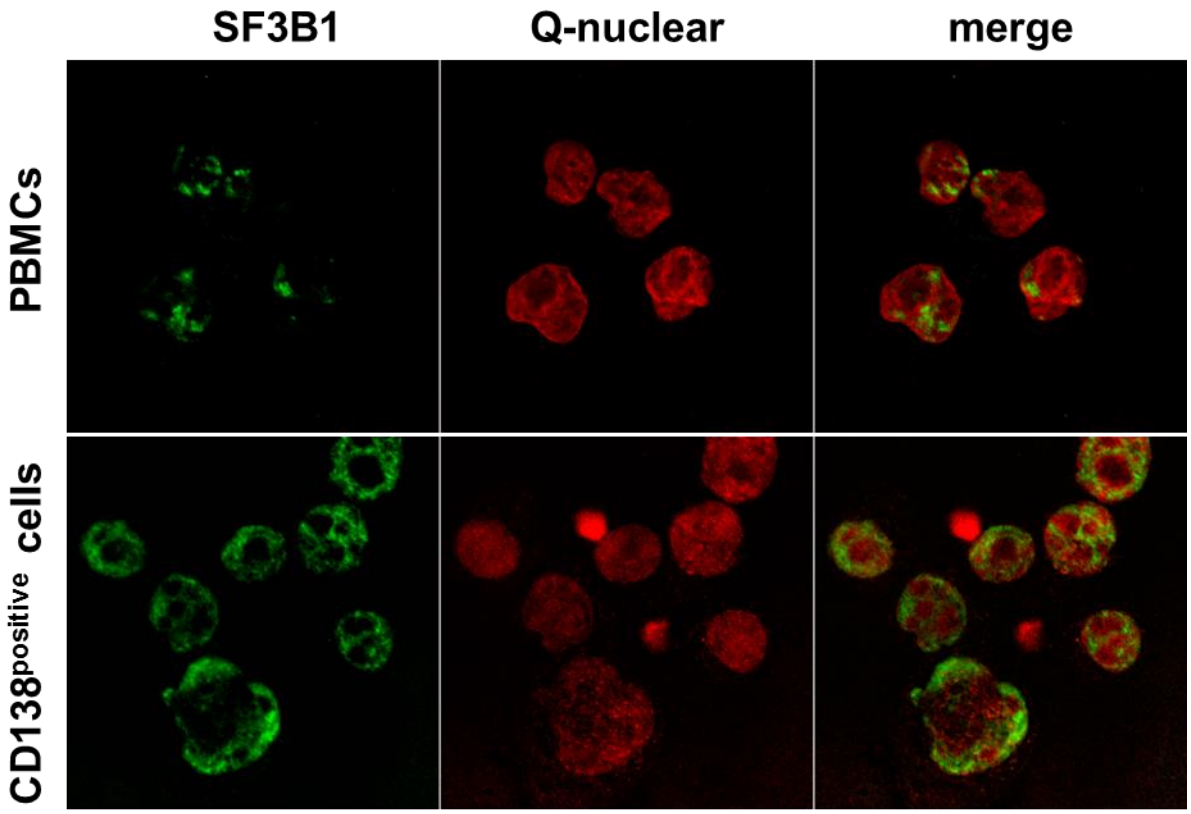

Figure S4. Detection of SF3B1 and Q-nuclear was measured by confocal microscopy in PBMCs (upper part) or MM tumor cells (bottom part) collected from a representative HD (healthy donor) and a MM patient, respectively.

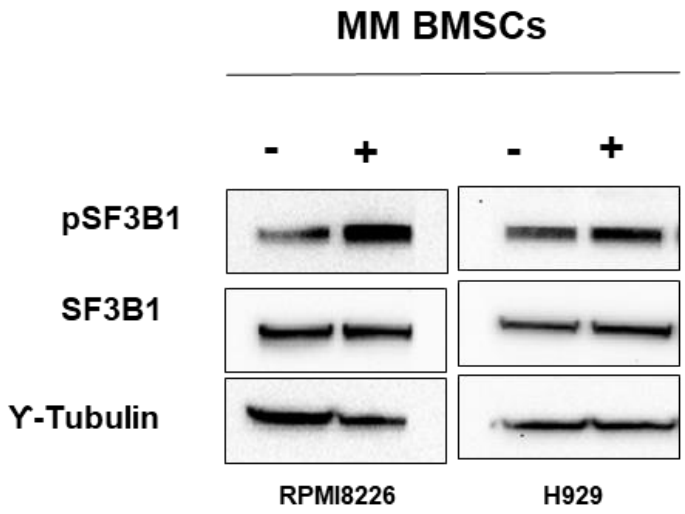

Figure S5. Western blot analysis of phopho-SF3B1 and SF3B1 protein expression in indicated MM cells alone and after 48 hours of co-incubation with MM-patient derived BMSCs. Gamma tubulin was used as a loading control.

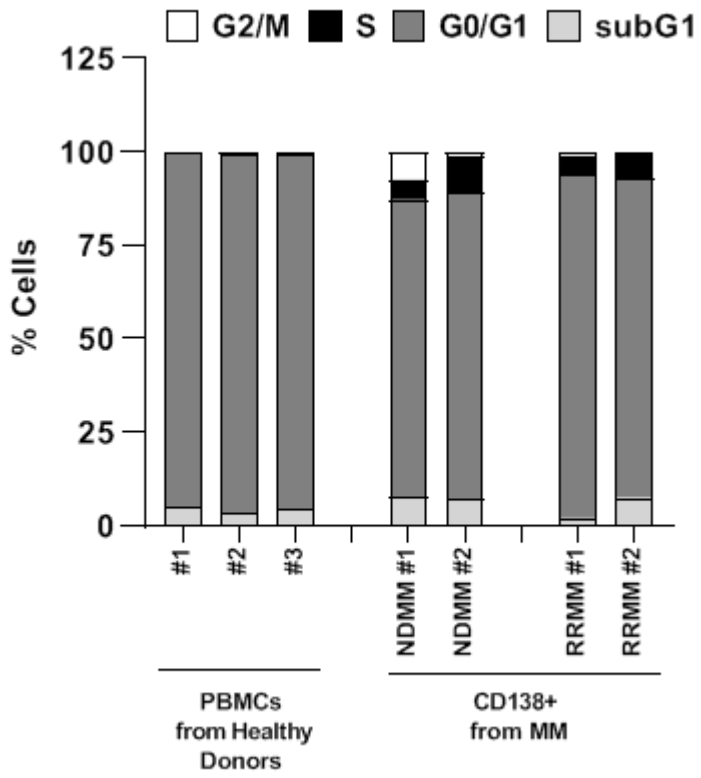

Figure S6. Cytofluorimetric analysis of cell cycle distributions of normal PBMCs cells and MM cells collected at indicated stage disease.

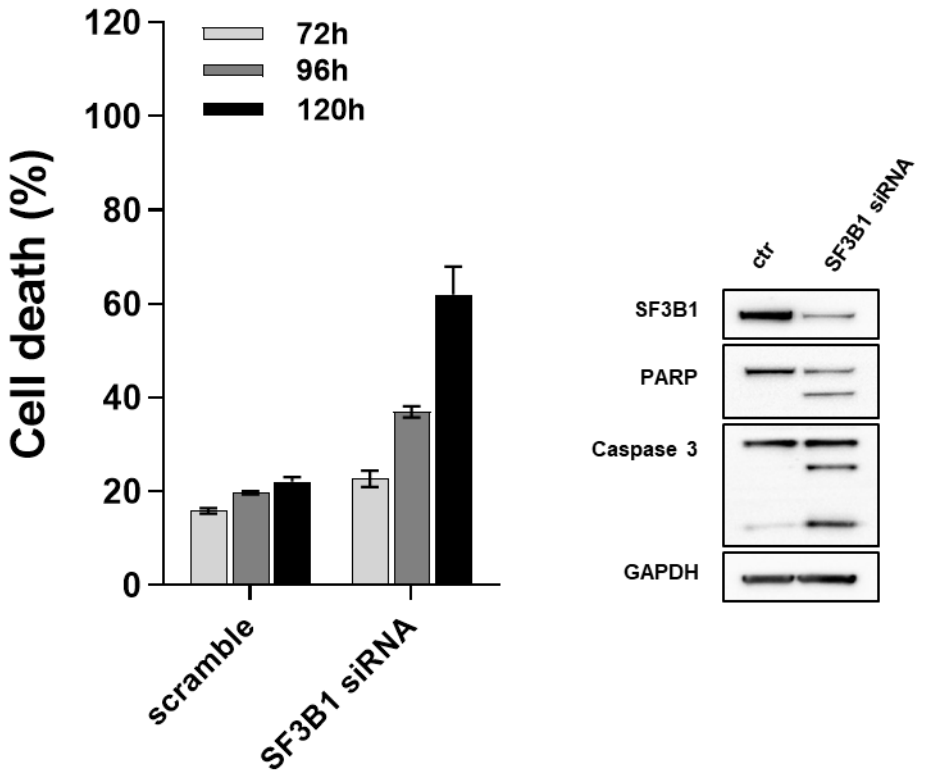

Figure S7. Cell death (measured with MTS assay) of SF3B1-silenced (nucleofected with specific siRNAs) or control (scramble siRNA) RPMI 8226 cells at indicated time points. On the right, western blot analysis of apoptotic markers (PARP1 and Caspase 3 cleavage) after 72hours from nucleofection. Data are representative of at least three independent experiments.

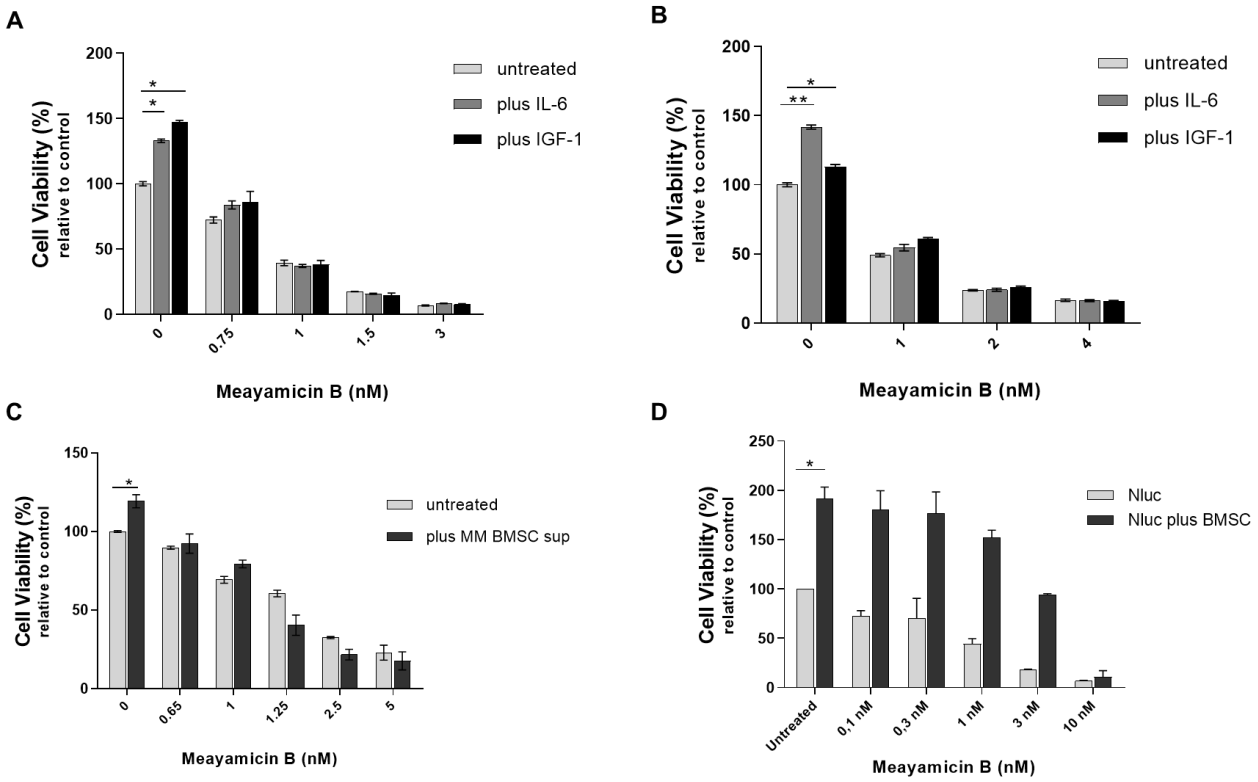

Figure S8. H929 (**A**) or MM1S (**B**) cells were treated with different doses of Meaymicin B for 48hours, (0.75-3nM) in presence or absence of rhIL-6 (10 ng/mL) or rhIGF-1 (100 ng/mL) for 48 hours, and viability was then measured by MTS assay. **C**) Viability of H929 cells treated with increasing doses of Meaymicin B for 48 hours, in presence or absence of BMSC-conditioned medium. Viability was then measured by MTS assay. **D)** Viability of H929 Nano Luc+ cells treated with increasing doses of Meaymicin B for 48 hours, alone and in the presence of MM patient–derived BMSCs (grey), measured by luciferase-based luminescence assay. The results presented in all panels are a mean ± SD of triplicate samples (*P≤ 0.05; **P ≤ 0.01; unpaired t test).

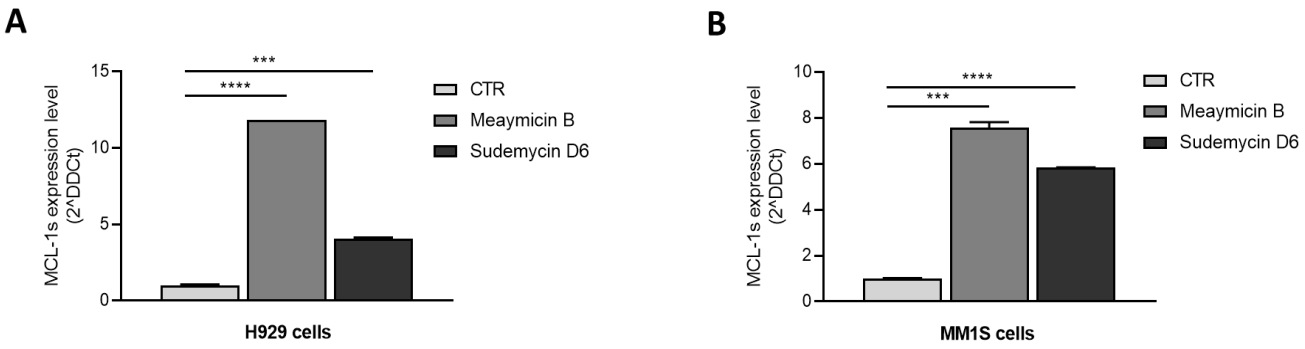

Figure S9. A-B) qPCR analysis for the pro-apoptotic short-form of MCL-1 (exon 2-skipped, MCL-1s) in H929 (**A**) or MM1S (**B**) cells treated for 6 hours with Meaymicin B (3 nM) or Sudemycin D6 (500 nM for H929 and 1.5μM for MM1S). GAPDH was used as housekeeping gene and 2^-DDCt method was used for the analysis. Results are a mean ± SD of triplicate samples (***P≤ 0.001; ****P ≤ 0.0001).

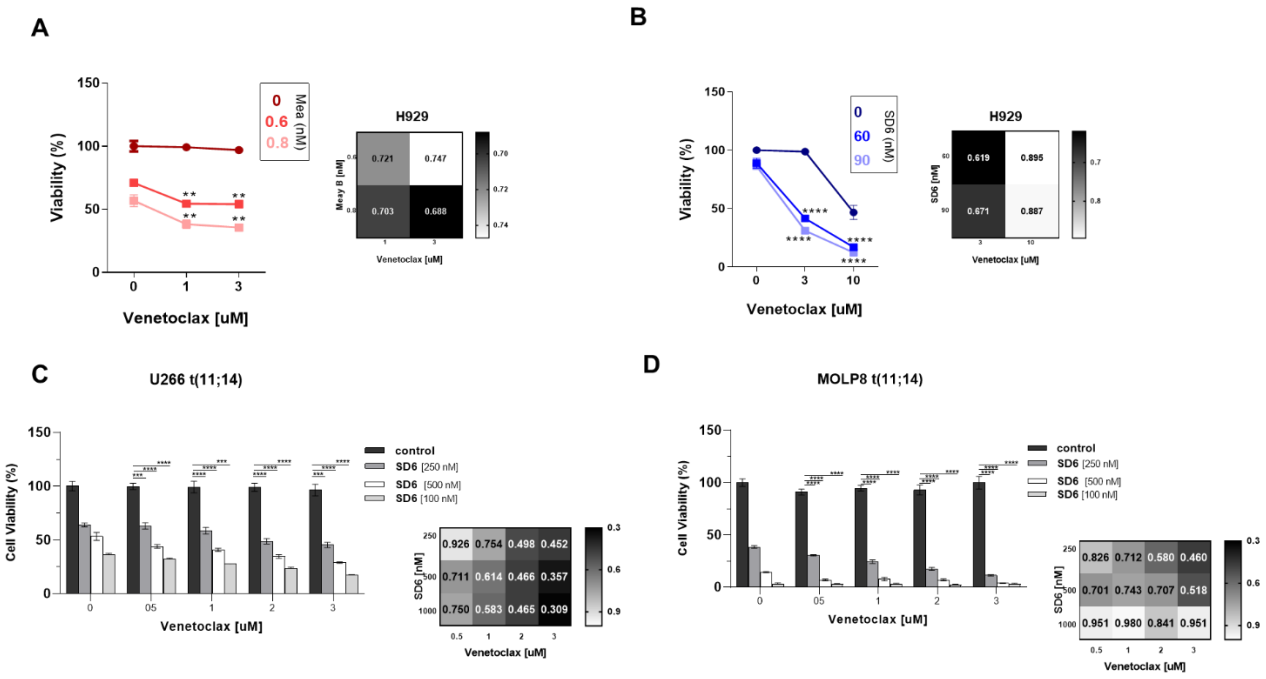

Figure S10. A-B) Cell viability curves of H929 combination therapy with MeaymcinB (left) or Sudemycin D6 (right) and Venetoclax. **C-D)** Cell viability curves of indicated t(11;14) MM cell lines following combination therapy with Sudemycin D6 and Venetoclax. CI synergy score (calculated with CalcuSyn software) for each set of drugs combination is indicated. Data are presented as mean ± S.D (n=3). (*** p≤0.01, **** p ≤ 0.001; unpaired t test).

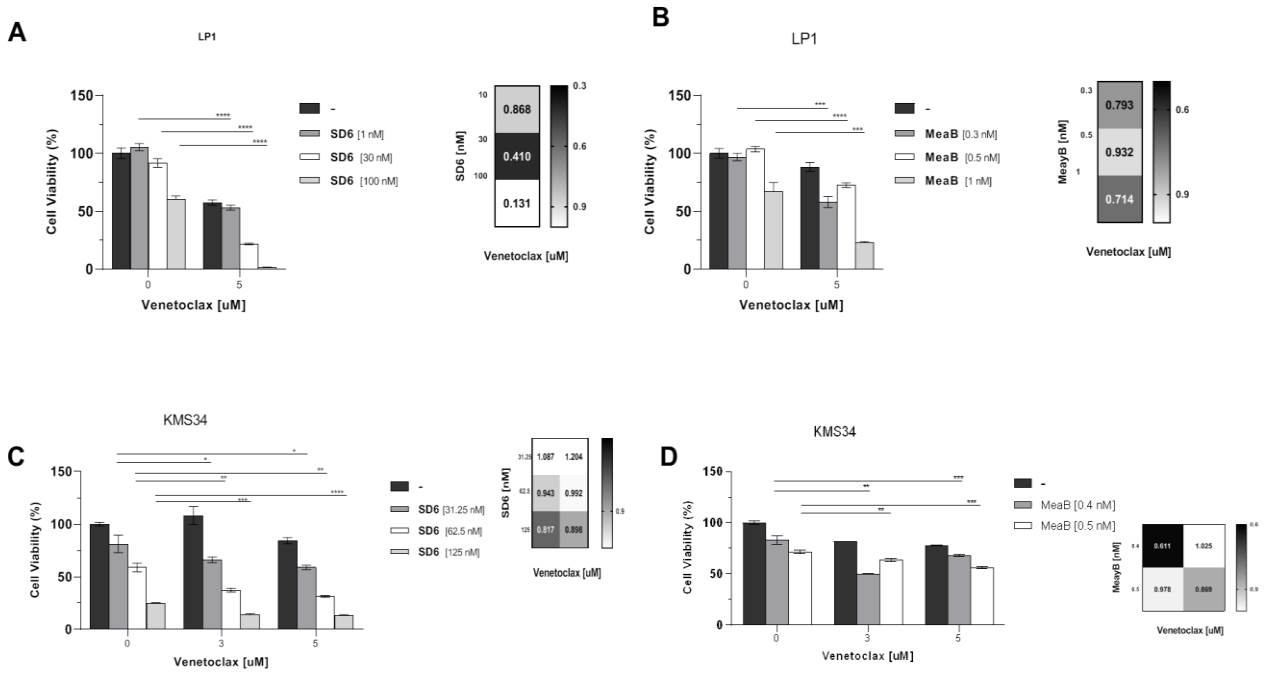

Figure S11. Cell viability curves of LP1 and KMS34 combination therapy with Sudemycin D6 (upper) or MeaymcinB (bottom) and Venetoclax. CI synergy score (calculated with CalcuSyn software) for each set of drugs combination is indicated. Data are presented as mean ± S.D (n=3). (*** $p \leq 0.01$, **** $p \leq 0.001$; unpaired t test).

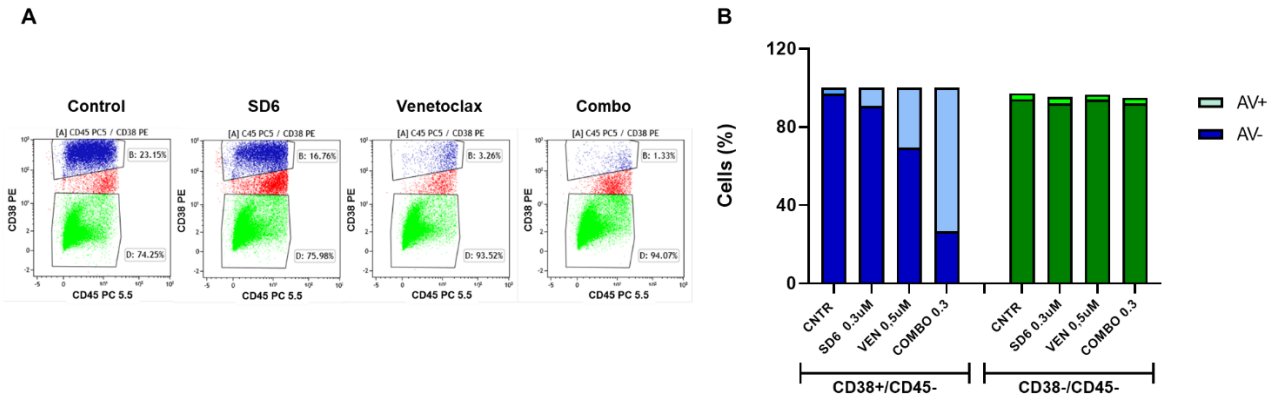

Figure S12. A) Flow plots of one representative MM patient sample (MM17). MM ($CD38^+$ $CD45^-$) and BMSCs-gated cells ($CD38^-$ $CD45^-$) were quantified. **B)** Histograms showing percentages of Annexin V positive and negative cells in each gated population after 48hours of drugs exposure.

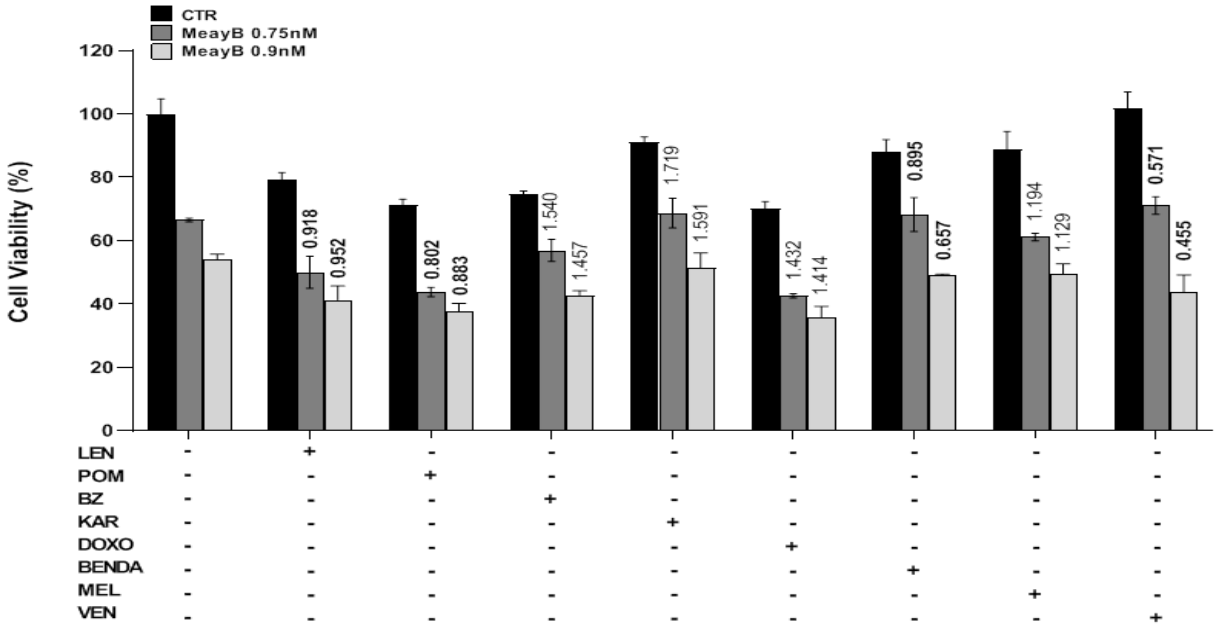

Figure S13. NCI-H929 cells were incubated in 96-well plates in the presence or absence of 0.75 or 0.9 nM MeaymicinB and different anti-MM drugs (2.5 µM Lenalidomide, 0.25 µM Pomalidomide, 3 nM Bortezomib, 2 nM Carfilzomib, 60 nM Doxorubicin, 30 µM Bendamustine, 1µM Melphalan, and 3 µM Venetoclax) or their combination. Cell death was assessed 48 hours later by MTS-viability assay. Results are means of triplicates ± SD; CI synergy score (calculated with CalcuSyn software) for each set of drugs combination is also indicated.

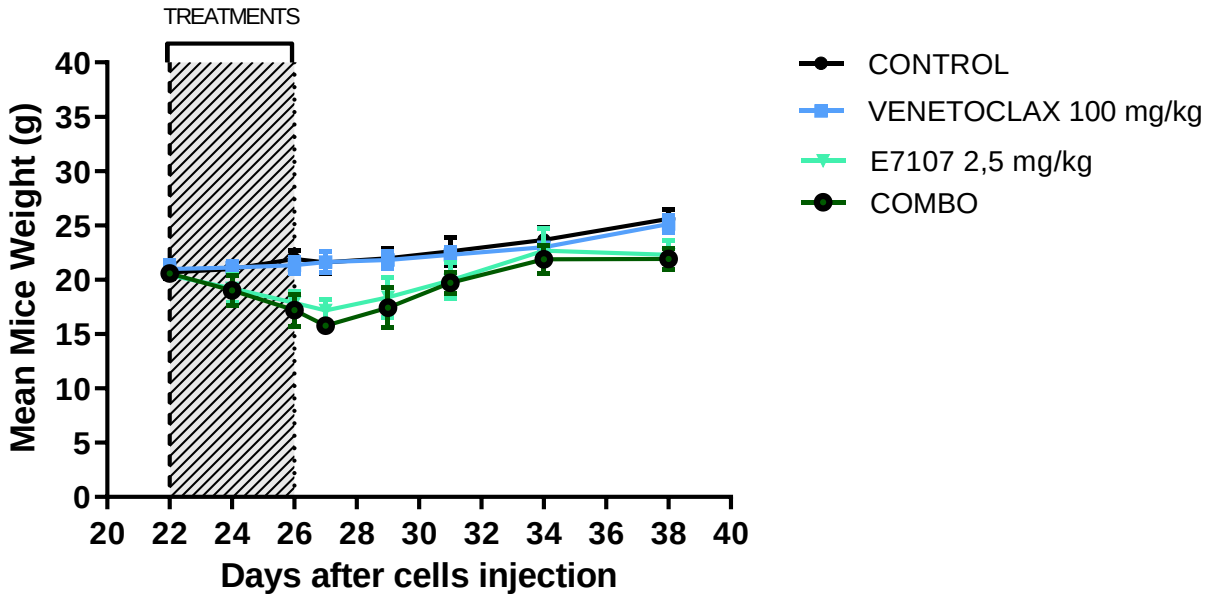

Figure S14. Mean mice weight ± SD for each treated group over time. Days after MM cell injection and treatment schedule are indicated as well.