SUPPLEMENTARY APPENDIX

Maternal embryonic leucine zipper kinase is a novel target for proliferation-associated high-risk myeloma

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Supplementary Materials and Methods

Methods

Reagents

OTSSP167, bendamustine, carfilzomib, bortezomib, lenalidomide, and pomalidomide were obtained from SelleckChem, dissolved in DMSO and stored at -20°C. Dexamethasone and melphalan were obtained from Sigma-Aldrich, dissolved in DMSO and stored at -20°C.

Cells and culture conditions

Human multiple myeloma cell lines (HMCLs) U266, KMS-12-BM, OPM-2, NCI-H929, SK-MM-1 and RPMI8226 were obtained from the German Collection of Microorganisms and Cell Cultures. MM.1S cells were kindly provided by Dr. Steven Rosen (Northwestern University, Chicago, IL). MM.1R and HEK293T cells were obtained from the American Type Culture Collection. Human bone marrow mesenchymal stromal cells immortalized by enforced expression of telomerase were kindly provided by Dr. Dario Campana (St. Jude Children's Research Hospital, Memphis, TN).

Primary MM and peripheral blood mononuclear cells (PBMCs) were purified from MM patients undergoing routine BM aspiration at the Department of Medicine I (Wihelminenspital, Vienna). Written informed consent for the use of material for scientific studies was obtained from these patients according to institutional guidelines. Mononuclear cells were isolated by Ficoll-Hypaque density sedimentation and MM cells were purified using the EasySep^{TM} Human CD138 Positive Selection Kit (Stem Cell Technologies).

Lentiviral transduction

MELK specific shRNA cloned into the pLKO.1 vector was obtained from Sigma-Aldrich (#TRCN0000196420). Lentiviral particles were produced using a lipofectamine 3000 (ThermoFisher Scientific) based protocol. Briefly, HEK293T cells were transfected with pLKO.1 vector in conjunction with pMD2.G envelope plasmid (kind gift from Didier Trono, Addgene plasmid #12259) and pCMVR8.74 packaging plasmid (kind gift from Didier Trono, Addgene plasmid #22036). Viral supernatant was collected 24h and 52h post transfection. For lentiviral transduction, HMCLs (5x 10^5) were spin transfected (1h, 800g) with 1 ml of viral supernatant and incubated overnight. Transduced MM cells were selected with puromycin (1µg/ml) and used for further experiments. Cells transduced with pLKO.1 carrying scrambled shRNA (kind gift from David Sabatini, Addgene plasmid #1864) served as control.

Flow cytometry

Induction of apoptosis was analysed by AnnexinV/7-AAD staining (BD Biosciences) 72h post treatment. Cell cycle analysis was performed 48h post treatment using FxCycle™ PI/RNase Staining solution (ThermoFisher Scientific). Depolarization of the mitochondrial membrane potential was analysed 24h post treatment using JC-1 assay (ThermoFisher Scientific). Cells positive for cleaved-PARP were determined 20h post treatment using anti-cleavedPARP antibody (BD Biosciences) and the Cytofix/Cytoperm staining procedure (BD Biosciences). All assays were performed according to the manufacturer's instructions. Analyses were performed on a FACScan and FACS Canto II (BD Biosciences).

Quantitative RT-PCR

Total RNA was isolated using RNeasy kit (Qiagen), and cDNA synthesis was performed with M-MuLV reverse transcriptase (New England Biolabs). *AURKA*, *IRF4*, *MYC*, *CCND1*, *CCNB1*, *PLK1*, *EZH2, FOXM1, DEPDC1* and *MCL-1* expression levels were analysed by quantitative PCR (qPCR) using TaqMan Universal PCR Master Mix and pre-designed TaqMan gene expression assays (ThermoFisher Scientific). RPLPO served as endogenous control. Reactions were carried out in 25 µl volumes and run on the ABI Prism7300 platform (ThermoFisher Scientific).

Western Blot

Protein lysates were obtained using complete lysis solution (Sigma-Aldrich), quantified with Coomassie Protein-Assay Reagent (ThermoFisher Scientific) and analysed by immunoblotting using anti-MELK, anti-PLK1, anti-EZH2, anti-GAPDH, anti-βactin, anti-MCL1, anti-IRF4, anti-cleaved caspase3 (all from CellSignalingTechnology), anti-DEPDC1 (Abcam) and anti-FOXM1 antibodies (SantaCruz Biotechnology). After incubation with HRP-conjugated secondary antibodies immunoblots were analysed using ECL Prime Western Blotting Detection Reagent (GE Healthcare LifeSciences) and the FusionSolo 6S imaging-system (Vilber-Lamour).

***In vivo* study**

For studies in the murine 5TGM.1 myeloma model, $5x10^5$ 5TGM.1GFP+ cells were injected i.v. in C57/KaLwRij mice (Envigo). When mice presented signs of myeloma, such as paraplegia, all mice were sacrificed, blood was collected through an intracardiac puncture and spleen, femur and tibia were isolated. Bone marrow infiltration of MM cells was determined by flow cytometry. Blood cell counts were performed using Cell-Dyn 3700 (Abbott). Serum paraprotein levels were determined with the mouse IgG2b ELISA read-set-go kit (eBioscience) according to the supplier's protocol. A paraplegia score was determined throughout the study as required by our ethics protocol. This score takes the activity, posture and occurrence of hind-limb paraplegia into account.

Analysis of GEP data

The following datasets from the Gene Expression Omnibus (GEO) were selected for this study: GSE24080 (551 newly-diagnosed MM patients), GSE6477 (15 healthy controls, 22 MGUS patients, 24 SMM patients, 73 newly-diagnosed and 28 relapsed MM patients), GSE31161 (346 TT2-baseline and 127 TT2-relapse samples, 433 TT3-baseline and 29 TT3-relapse samples), GSE2113 (7 MGUS patients, 39 MM patients and 6 patients suffering from plasma cell leukemia (PCL)), GSE13591 (5 healthy controls, 11 MGUS, 133 MM, and 9 PCL patients) and GSE9782 (264 relapsed and/or refractory MM patients treated with bortezomib or dexamethasone). Raw CEL files were downloaded from GEO and analyses were performed on gcrma-normalized samples in R using the 'affy' package from Bioconductor. For GSE9782, mas5 expression sets were retrieved for analysis using the GEOquery package, since raw CEL files are not provided for this study.

Categorization of newly-diagnosed patients into distinct GEP-defined subgroups (GSE24080) was performed by Zhan *et al*. (Blood, 2006). This study identified seven distinct GEP-subgroups of myeloma. Four of these subgroups represent major genetic lesions in MM that arise from translocations on the immunoglobulin heavy chain locus. These groups are identified by the spiked expression of the translocated genes on the partner chromosome (*CCND1*, *CCND3*, c-MAF or MAFB, and MMSET). In contrast, hyperdiploidy had a distinct gene expression profile (associated with hyperdiploid karyotypes). The low bone disease group distinguished itself by low level expression of *Wnt* signalling inhibitory genes (*DKK1* and *FRZB*), interferon-induced genes and high expression of endothelin 1 (*EDN1*). Clinically, these patients were characterized by a lower number of MRI-defined focal lesions. The proliferation (PR) subgroup is defined by overexpression of various cell cycle/proliferation associated genes and cancer testis antigens as well as higher GEP-defined proliferation index. Finally, the authors defined a myeloid signature which is characterized by contamination of MM cell samples with myeloid and/or normal plasma cells but also superior clinical performance.

Metacore enrichment analysis

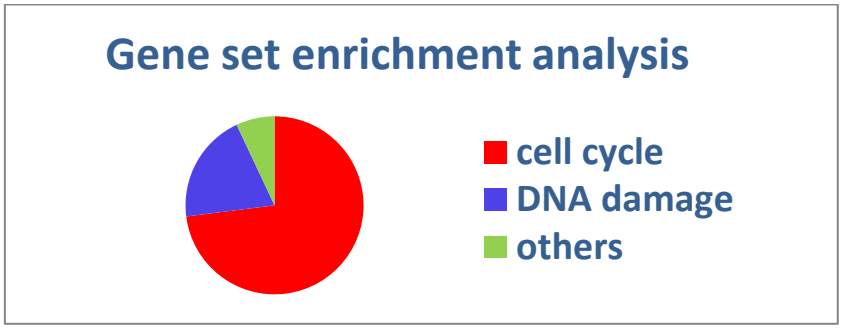
Pathway enrichment analysis was performed using MetaCore^{TM} (version 6.28) analytical software. Genes differentially expressed (min 2-fold up- or downregulation in samples with high *MELK* expression versus samples with low *MELK* expression, adjusted *P*-value <0.05) were imported into MetaCore. Enrichment analyses (hypergeometric test) were run on 3 ontologies: Pathway maps, GO processes, and process networks. These ontologies were ordered according to their *P*-value, based on hypergeometric distribution.

Supplementary Table 1. **Genes differentially expressed in patients with high compared to low *MELK* expression.** Genes listed in the supplemental Excel file (Suppl Table 1) are either > 2 fold (FC, fold change) under- or overexpressed in patients with high compared to low *MELK* expressing myeloma cells. Listed genes (symbol) with significant (adjusted *P*-value <0.05) deregulation compared to patients with low *MELK* expression are included in the list together with the corresponding microarray probeset IDs (Probe ID). AveExpr indicates gcrma-normalized average expression values of the corresponding genes in patients with high *MELK* expression. Analysis was performed using the GSE24080 expression dataset.

Supplementary Table 2. **Gene set enrichment analysis of genes upregulated in patients with high *MELK* expression.** This analysis confirmed the strong association between genes enriched in MELK^{high} patients and proliferation. As indicated by the graph at the bottom of the page 73% of top 10 GO processes, process networks and pathway maps are linked to cell cycle regulation.

Enrichment by GO processes	***P*-value**	**FDR**	**Nb of network genes enriched in MELK^{high} patients**
Mitotic cell cycle	8.45E-79	2.74E-75	103
Cell cycle	4.70E-74	7.62E-71	118
Cell cycle process	3.08E-72	3.33E-69	109
Mitotic cell cycle process	1.62E-66	1.31E-63	91
Nuclear division	2.99E-46	1.94E-43	63
Organelle fission	4.44E-45	2.40E-42	63
Cell division	6.71E-44	3.10E-41	60
Mitotic nuclear division	5.51E-41	2.23E-38	52
Organelle organization	2.67E-34	9.60E-32	119
Cell cycle phase transition	1.37E-33	4.45E-31	45
Enrichment by process networks			
Cell cycle core	8.75E-41	7.87E-39	37
Cell cycle mitosis	2.12E-22	9.54E-21	29
Cell cycle S phase	3.36E-22	1.01E-20	27
Cell cycle G2-M	2.27E-18	5.10E-17	27
Cytoskeleton spindle microtubules	1.14E-16	2.05E-15	20
Cell cycle G1-S	3.68E-12	5.06E-11	19
DNA damage checkpoint	3.93E-12	5.06E-11	17
DNA damage MMR repair	2.33E-07	2.62E-06	9
DNA damage BER-NER repair	7.64E-07	7.64E-06	11
DNA damage DBS repair	1.43E-06	1.29E-05	11
Enrichment by pathway maps			
Cell cycle The metaphase checkpoint	9.09E-18	2.01E-15	13
Cell cycle Role of APC in cell cycle regulation	1.03E-16	1.14E-14	12
Cell cycle Start of DNA replication in early S phase	1.69E-11	1.25E-09	9
Cell cycle Spindle assembly and chromosome separation	2.31E-11	1.27E-09	9
DNA damage Role of Brca1 and Brca2 in DNA repair	1.42E-08	6.29E-07	7
Cell cycle Role of Nek in cell cycle regulation	2.31E-08	8.52E-07	7
Cell cycle (generic schema)	4.29E-08	1.35E-06	6
Cell cycle Initiation of mitosis	1.36E-07	3.74E-06	6
DNA damage ATM/ATR regulation of G2/M checkpoint	1.75E-07	4.29E-06	6
Cell Cycle Transition and termination of DNA replication	2.81E-07	6.22E-06	6

FDR, False discovery rate; Nb, number.



Supplementary Table 3. **Patient characteristics of primary MM cell samples used for *in vitro* testing of OTSSP167.**

#	Gender	Age	Status	Isotype	ISS	FISH
MM1	Female	75	Newly-diagnosed MM	κ– light chains	III	amp1q21
MM2	Female	76	Relapsed MM	IgGκ	II	Trisomy 11, trisomy 13q14
MM3	Female	79	Relapsed MM	IgGκ	II	N/A
MM4	Female	68	Primary Plasma Cell Leukaemia	IgGλ	III	t(14;16), del13q14, del17p, amp1q21
MM5	Female	37	Secondary Plasma Cell Leukaemia	IgGκ	III	del13q14, translocation 14q32 (unknown partner)
MM6	Male	65	Refractory MM	IgGκ	N/A	amp1q21
MM7	Male	78	Relapsed MM	IgGκ	II	normal

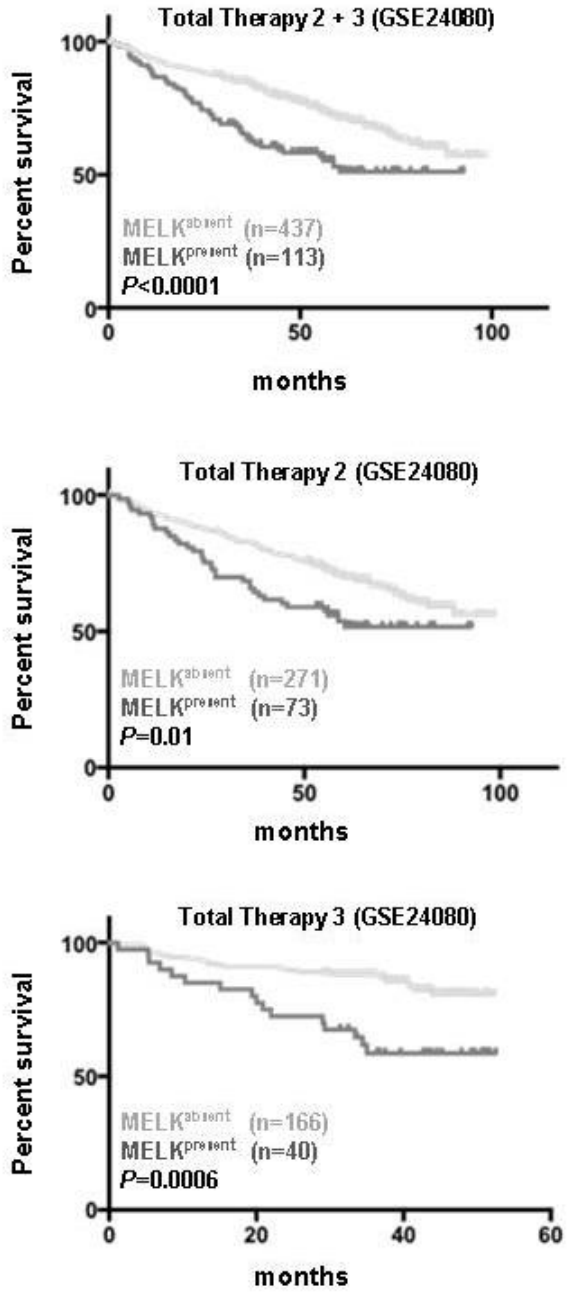
ISS, international staging system; N/A, not available.

Supplementary Table 4. **Synergistic activity of OTSSP167 in combination with immunomodulatory drugs (lenalidomide or pomalidomide) or dexamethasone at varying concentrations.** Combination index (CI) values were determined with Compusyn. CI values <0.85, 0.85-1.15, or >1.15 indicate synergistic, additive or antagonistic drug activity, respectively.

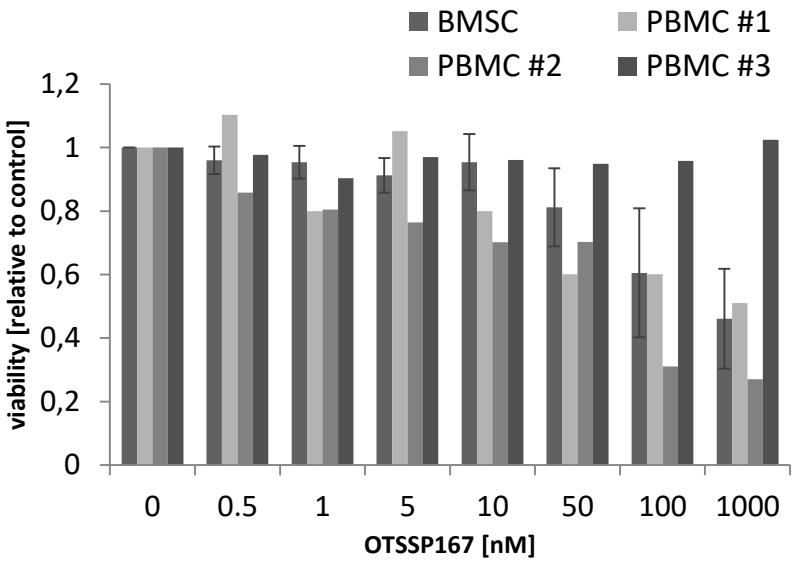
NCI-H929								
OTSSP167 [nM]	**Dex [nM]**	**CI**	**OTSSP167 [nM]**	**Len [µM]**	**CI**	**OTSSP167 [nM]**	**Pom [µM]**	**CI**
5	10	2.62	5	0.5	1	5	0.5	0.78
5	100	0.45	5	1.0	0.91	5	1.0	0.67
5	250	0.7	5	2.5	0.74	5	2.5	0.7
10	10	1.4	10	0.5	0.66	10	0.5	0.32
10	100	0.45	10	1.0	0.62	10	1.0	0.5
10	250	0.56	10	2.5	0.63	10	2.5	0.62
20	10	0.86	20	0.5	0.39	20	0.5	0.22
20	100	0.36	20	1.0	0.36	20	1.0	0.2
20	250	0.38	20	2.5	0.36	20	2.5	0.26
MEDIAN		**0.56**			**0.63**			**0.50**

OPM-2								
OTSSP167 [nM]	**Dex [nM]**	**CI**	**OTSSP167 [nM]**	**Len [µM]**	**CI**	**OTSSP167 [nM]**	**Pom [µM]**	**CI**
5	10	0.62	5	0.5	1.04	5	0.5	0.52
5	100	0.29	5	1.0	0.78	5	1.0	0.44
5	250	0.38	5	2.5	1.21	5	2.5	0.64
10	10	0.8	10	0.5	0.59	10	0.5	0.28
10	100	0.32	10	1.0	0.76	10	1.0	0.28
10	250	0.34	10	2.5	0.81	10	2.5	0.34
20	10	0.74	20	0.5	0.5	20	0.5	0.21
20	100	0.27	20	1.0	0.52	20	1.0	0.22
20	250	0.26	20	2.5	0.51	20	2.5	0.23
MEDIAN		**0.34**			**0.76**			**0.28**

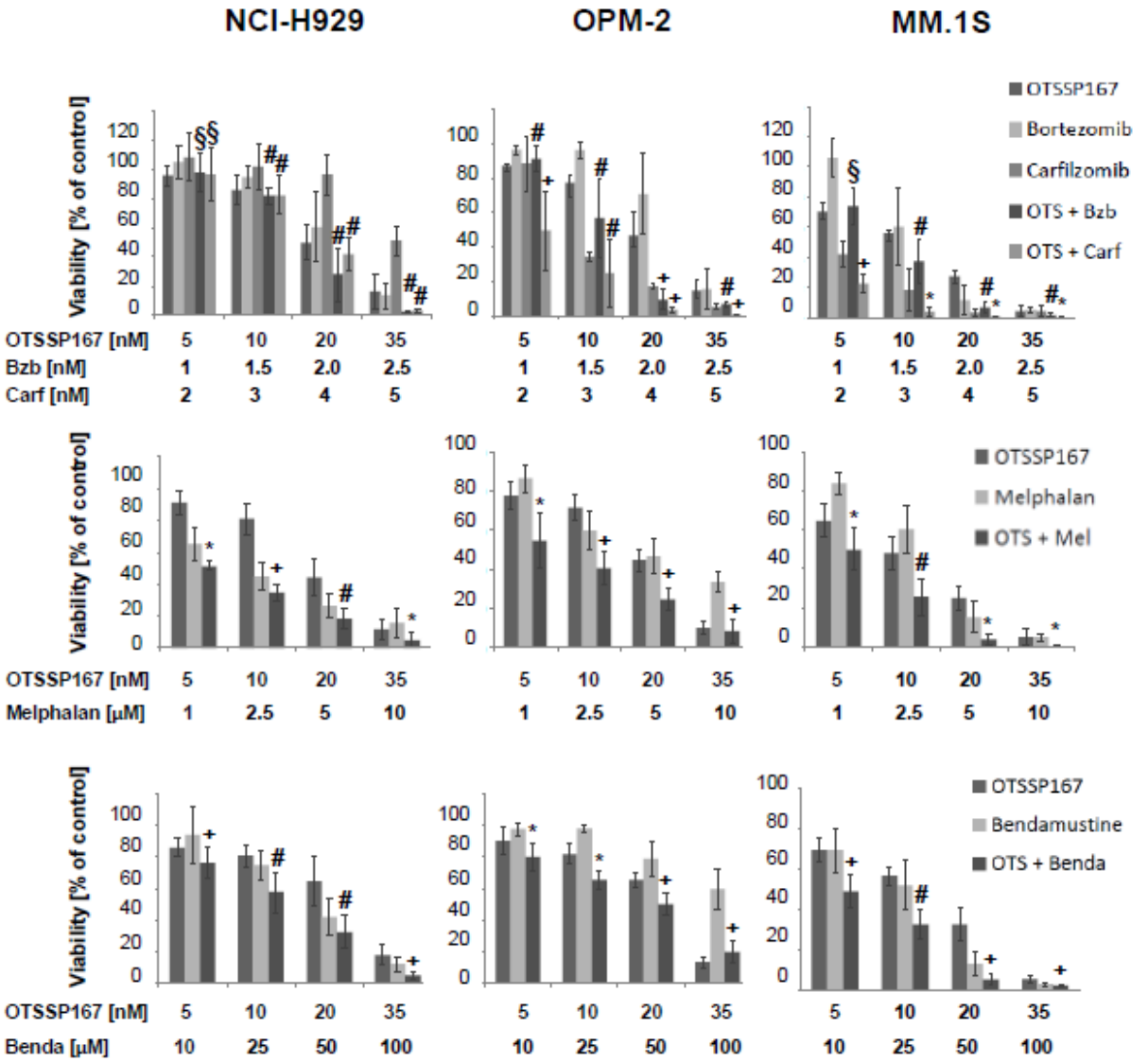
MM.1S								
OTSSP167 [nM]	**Dex [nM]**	**CI**	**OTSSP167 [nM]**	**Len [µM]**	**CI**	**OTSSP167 [nM]**	**Pom [µM]**	**CI**
5	10	0,45	5	0.5	0,36	5	0.5	0,25
5	100	0,24	5	1.0	0,26	5	1.0	0,21
5	250	0,29	5	2.5	0,33	5	2.5	0,21
10	10	0,49	10	0.5	0,46	10	0.5	0,34
10	100	0,21	10	1.0	0,36	10	1.0	0,29
10	250	0,23	10	2.5	0,35	10	2.5	0,28
20	10	0,34	20	0.5	0,4	20	0.5	0,39
20	100	0,17	20	1.0	0,36	20	1.0	0,34
20	250	0,15	20	2.5	0,36	20	2.5	0,37
MEDIAN		**0.24**			**0.36**			**0.29**



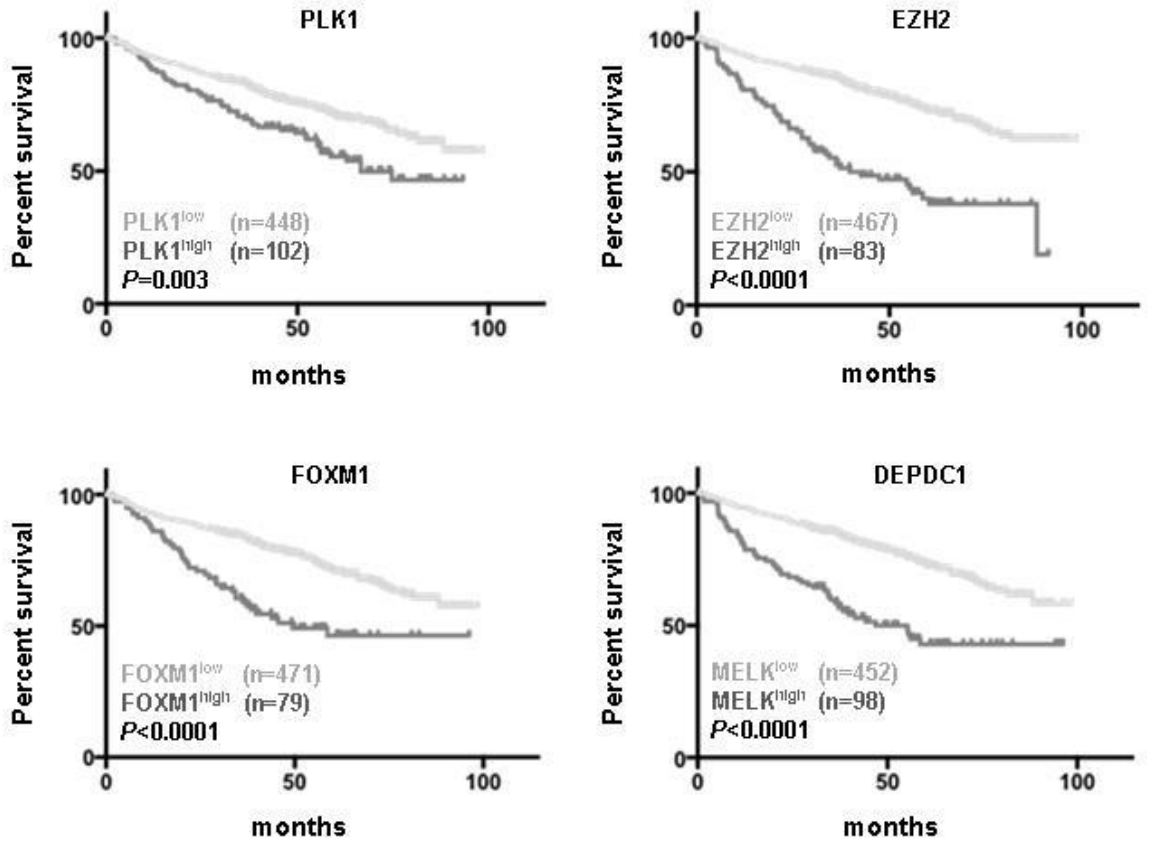
Supplementary Figure 1 | Overall survival of patients with MELK^{present} and MELK^{absent} myeloma cells. Presence or absence of MELK expression in MM cells was determined using the Presence-Absence calls with Negative Probesets (PANP) method. MELK^{present} expression was associated with poor outcome in newly-diagnosed patients treated within the total therapy 2 and 3 protocols (GSE24080).



Supplementary Figure 2 | Bone marrow stromal cell (BMSC) and peripheral blood mononuclear cell (PBMC) viability post OTSSP167 treatment. Viability of bone marrow stromal cells (BMSCs) or peripheral blood mononuclear cells (PBMCs) 96 h post treatment with OTSSP167 at the indicated concentrations.



Supplementary Figure 3 | Drug combination effects of OTSSP167 with proteasome inhibitors and alkylating agents. HMCLs were treated with OTSSP167 in combination with proteasome inhibitors (bortezomib, carfilzomib), melphalan or bendamustine for 96 h at the indicated concentrations. Combination index (CI) values were determined with Compusyn. CI values <0.85, 0.85-1.15, or >1.15 indicate synergistic (*), additive (+) or antagonistic (#) drug activity, respectively. §, not evaluable due to viability >100% with one or both drugs of the combination.



Supplementary Figure 4 | High expression of proliferation associated genes is associated with poor prognosis. Newly- diagnosed patients treated within the total therapy 2 and 3 protocols (GSE24080) were grouped according to high or low expression of proliferation associated genes. High expression levels of *PLK1, EZH2, FOXM1*, and *DEPDC1* were associated poor outcome.