

1 **Supplementary Material and Methods**

2 **Proteomics A (4h treatments)**

3 *Sample preparation for LC-MS/MS*

4 For whole cell proteome analysis of OPM2, cells were lysed, reduced and alkylated in SDS-
5 lysis Buffer (2% SDS, 50mM Tris pH 8.5, 10mM TCEP, 40mM CAA) complemented with
6 protease inhibitor tablet. Cellular lysates were subsequently boiled, sonicated and subjected
7 to methanol-chloroform precipitation. The resulting dried pellet was resuspended in urea
8 digestion buffer (8M urea, 50mM Tris pH 8.2) and protein concentration was measured by BCA
9 assay (23225, Thermo Fischer Scientific, Pierce™ BCA Protein Assay Kit). 50 µg protein was
10 digested by Trypsin (enzymes to protein ratio 1:100) and Lys-C (enzymes to protein ratio 1:50)
11 overnight at 37°C in 1M urea, 50mM Tris pH 8.5. Proteolytic cleavage was stopped by TFA
12 (final concentration 1%) and peptides were subsequently desalted using tC18 Sep-Pak
13 cartridges (WAT054960, Waters, Milford, USA). Subsequently, digested peptides were
14 dissolved in 200 mM EPPS pH 8.2, 10% ACN buffer and peptide concentration was measured
15 by micro BCA assay (Micro BCATM Protein Assay Kit, 23235, Thermo Fischer Scientific). 10µg
16 of digested peptides were finally labelled (peptides to TMT ratio 1:2) with TMTpro reagents
17 (ThermoFisher Scientific) for 1 h at room temperature. Quenching of the labelling reaction was
18 performed by hydroxylamine at a final concentration of 0.5% and equal amounts of TMT-
19 labelled samples were pooled followed by cleaning up using tC18 Sep-Pak cartridges.

20 *High pH micro-flow fractionation*

21 Peptides were fractionated using high-pH liquid-chromatography on a micro-flow HPLC
22 (Dionex U3000 RSLC, ThermoFisher Scientific). 45µg of pooled and purified TMT labelled
23 peptides resuspended in Solvent A (5mM ammonium-bicarbonate, 5%ACN) were separated
24 on a C18 column (XSelect CSH, 1mm x 150mm, 3.5 µm particle size, Waters) using a multistep
25 gradient from 3-60% Solvent B (100% ACN) over 65 minutes at a flow rate of 30 µl/min. Eluting
26 peptides were collected every 43 seconds from minute 2 for 69 minutes into a total of 96

27 fractions, which were cross-concatenated into 24 fractions. Pooled fractions were evaporated
28 to dryness and stored at -20°C until mass spectrometry analysis.

29 *Mass spectrometry*

30 Fractions were resuspended in LC-MS grade water containing 2% ACN and 0.1% TFA.
31 Peptides were separated on an easy nLC 1200 (ThermoFisher Scientific) and a 35 cm long,
32 75µm ID fused-silica column, which has been packed in house with 1.9 µm C18 particles
33 (ReproSil-Pur, Dr. Maisch, Ammerbuch, Germany) and kept at 50°C using an integrated
34 column oven (Sonation). Peptides were eluted by a non-linear gradient optimised for each
35 fraction over 210 or 150 minutes for human and mouse samples, respectively, and directly
36 sprayed into a Fusion Lumos mass spectrometer equipped with a nanoFlex ion source
37 (ThermoFisher Scientific) at a spray voltage of 2.3 kV. MS analysis was performed using a
38 Top-Speed method (1.5s cycle time) with the RF lens at 30 %. Full scan MS spectra (350-1400
39 m/z) were acquired at a resolution of 120,000 at m/z 200, a maximum injection time of 100 ms
40 and an AGC target value of 4 x 10⁵. MS² scans were performed in the Ion trap (Rapid) using
41 an isolation window of 0.7 Th, a maximum injection time of 86ms and fragmented using CID
42 with a collision energy of 35%. SPS-MS³ was performed on the 10 most intense MS² fragment
43 ions with an isolation window of 0.7 Th (MS¹) and 2 m/z (MS²). Ions were fragmented using
44 HCD with a normalized collision energy of 65 and analyzed in the Orbitrap with a resolution
45 setting of 50,000 at m/z 200, scan range of 110-500 m/z, AGC target value of 1.5 x10⁵ and a
46 maximum injection time of 86ms. Repeated sequencing of already acquired precursors was
47 limited by setting a dynamic exclusion time of 45 seconds and 7 ppm.

48 *Raw data analysis and statistical significance evaluation*

49 Raw data analysis was done with Proteome Discoverer (v 2.4). SequenceHT node was
50 selected for database searches. Human trypsin digested proteome (Homo sapiens SwissProt
51 database [20531]) was used for protein identifications. Contaminants (MaxQuant
52 "contamination.fasta") were determined for quality control. TMTpro (K, +304.207 Da) for TMT
53 16 plex at the N terminus and carbamidomethyl (+57.021 Da) at cysteine residues were set as

54 fixed modifications. TMTpro (K, +304.207 Da) for TMT 16 plex, and methionine oxidation (M,
55 +15.995 Da) as well as Acetyl (+42.011 Da) at the protein N terminus were set for dynamic
56 modifications. Precursor mass tolerance was set to 7 ppm and fragment mass tolerance was
57 set to 0.02 Da. Default Percolator settings in PD were used to filter perfect spectrum matches
58 (PSMs). Reporter ion quantification was achieved with default settings in consensus workflow.
59 Peptide groups file was exported into .txt file and subsequent statistical analysis was done with
60 the Perseus software (version 1.6.15.0). Log₂ values of all the normalized abundances were
61 calculated. Using the histogram analysis function of the software, the normal distribution of the
62 abundance values was visually checked. Good correlation of the experimental replicates was
63 assured by multi-scatterplot analysis. Samples were then grouped into triplicates and a
64 Student's t-test was performed with randomization of 250 and permutation based FDR 0.05.
65 Then the datasets were exported and used for further analysis in Microsoft Excel. Significant
66 enrichment was defined in Excel based on the P-value and the Student's t-test difference
67 applying the following criteria: $-\log_{10} P\text{-value} > 1.34$ and $\log_2 \text{ratio} \geq 0.585$ (≥ 0.3 for comparing
68 CFZ+Subasumstat over CFZ) or ≤ -0.585 . Visual representation of data in volcano plots was
69 done using the online portal <https://huygens.science.uva.nl/VolcanoR/>. Enrichment of gene
70 ontology signatures was performed on an online portal ShinyGO v0.75: Gene Ontology
71 Enrichment Analysis.

72

73 **Proteomics B (16h treatment in OPM2)**

74 *Sample preparation for LC -MS/MS*

75 OPM2 cells treated for 16h with subasumstat were analyzed with isobaric tandem mass tags
76 (TMT) as described previously (1). In brief, cells were lysed with 8M urea buffer (8 M urea, 50
77 mM Tris (pH 8), 150 mM NaCl) containing protease inhibitors (2 $\mu\text{g/ml}$ aprotinin, 10 $\mu\text{g/ml}$
78 leupeptin 1 mM phenylmethylsulfonylflourid). Samples were reduced with 5 mM dithiothreitol
79 for 1 h and alkylated with 10 mM iodoacetamide for 45 min in the dark. Proteins were digested
80 with sequencing grade LysC (Wako) at a ratio of 1:50 for 2 h and subsequently diluted 1:4 with

81 50 mM Tris-HCl pH8. Sequencing grade trypsin (Promega) was added at a ratio of 1:50 and
82 digestion was completed overnight. Samples were acidified with formic acid and desalted with
83 Sep-Pak C18 cc Cartridges (Waters). Dried samples were resuspended in 10mM HEPES (pH
84 8.5) and peptide concentration was determined. 50 µg peptides of each sample were labeled
85 with TMTpro reagents according to the manufacturer's instructions and combined into one
86 TMT plex. An internal reference sample composed of equal amounts of peptide material from
87 all samples was included to provide a standard for relative quantification.

88 *High pH micro-flow fractionation*

89 Labeled samples were combined, dried and resuspended in high pH buffer A (5mM ammonium
90 formate, 2% ACN) prior to offline high pH reverse phase fractionation on an Agilent 1290
91 Infinity II HPLC system. The separation was performed on a XBridge Peptide BEH C18 (130Å,
92 3.5µm; 2.1mm x 250mm) column (Waters) with a 96 minute multi-step gradient from 0 to 60%
93 high pH buffer B (5mM ammonium formate, 90% ACN). Samples were collected into 96
94 fractions (1min/fraction) that were pooled into 28 fractions. Pooled fractions were evaporated
95 to dryness.

96 *Liquid chromatography mass spectrometry*

97 Mass spectrometry raw data was acquired on an Orbitrap Exploris™ 480 mass spectrometer
98 connected to an EASY-nLC 1200 system (both Thermo Fisher Scientific). HpH fraction were
99 resuspended in LC buffer A (0.1% formic acid and 3% acetonitrile in water) and separated
100 online on a 25cm column packed in-house with C18-AQ 1.9 µm beads (Dr. Maisch Repronil-
101 Pur 120). A gradient of LC buffer A and LC buffer B (0.1% formic acid, 90% acetonitrile in
102 water) was used to separate the samples online at a flow rate of 250 µl/min. LC buffer B was
103 ramped from 4% to 30% in the first 88 min, followed by an increase to 60% B in 10 min and a
104 plateau of 90% B for 5 min. Temperature of the column was kept constant at 45 °C and spray
105 voltage static at 2kV. MS data was acquired with a Top-Speed method (1s cycle time) in data-
106 dependent acquisition. Full scan MS spectra (375-1500 m/z) were acquired in profile mode at
107 a resolution of 60,000, RF lens at 55%, a maximum injection time of 50 ms and AGC target

108 set to 300%. Monoisotopic peak determination was set to peptide and intensity threshold filter
109 to 5.0e4. Isolation window for MS2 scans was set to 0.4 m/z and normalized HCD collision
110 energy to 31 %. MS2 scans were acquired in centroid mode at a resolution of 45.000. First
111 mass set to 110 m/z, AGC Target was at 100% and maximum injection time was 86 ms.
112 Unknown charge states and charge states of 1 or > 6 were excluded from fragmentation.
113 Dynamic exclusion was set to 20 s and 10 ppm.

114 *Raw data analysis and statistical significance evaluation*

115 Raw data was analyzed with MaxQuant (Version 1.6.10.43) (2) with default parameters unless
116 otherwise stated. Data was searched against the human reference proteome downloaded from
117 UniProt in 07/2018 and default protein contaminants included in MaxQuant. Quantitation type
118 was set to reporter type MS2 and reporter ion correction factors were entered as supplied by
119 the manufacturer. PIF filter was set to 0.5. Fixed modifications were set to
120 carbamidomethylation of C. Variable modifications were set to M-oxidation and acetylation of
121 protein N-termini including neo protein N-terms after cleavage of first methionine. A maximum
122 of 5 modification per peptide were allowed. N-terminal acetylation and M-oxidation were used
123 in protein quantification (unmodified counterpart discarded). Unique and razor peptides were
124 used for quantification. MaxQuant output was further analyzed with the R statistical software
125 environment. Protein groups were filtered for ≥ 1 unique peptides and ≥ 2 detected peptides
126 and contaminants and reverse hits were removed. Corrected reporter ion intensities were log2
127 transformed and the internal standard channel was subtracted, followed by median-MAD
128 normalization. Significance was assessed with a 2-sided moderated - sample t-test (treatment
129 vs DMSO). Resulting p-values were corrected for multiple testing with the Benjamini-Hochberg
130 method. Fold changes were subjected to FGSEA analysis.

131

132 Cell viability assay

133 On day 1, cells were seeded in 96-well plates (10 000/well). On day 2, treatments were done
134 to the cell as indicated. After 72 hours of incubation, viability was assessed by adding
135 CellTiterGlo (G7572, Promega, Madison, USA) and measuring luminescence. Luminescence
136 reads were then normalized to DMSO control and converted to the percentage of living cells.
137 All cell viability assays were performed as biological triplicates. GI50 values define at which
138 concentration the growth of the cells were inhibited by half.

139

**140 Zero Interaction Potency (ZIP) synergy score and Chou-Talalay Combination Index (CI)
141 score**

142 MM cells were treated with different concentrations of indicated compounds and viability was
143 measured by CellTiterGlo. Viability data were used to calculate both ZIP synergy scores using
144 SynergyFinder (3) at synergyfinder.fimm.fi and Combination Index (CI) scores and generate
145 isobolograms using CompuSyn (4). ZIP-scores near 0 give limited confidence on synergy or
146 antagonism, but likely represent additive effects. Positive ZIP-scores indicate likely synergistic
147 effects, whereas negative ZIP-scores likely indicate antagonistic effects. CI-scores near 1
148 indicate additive effects. CI-scores <1 indicate synergistic effects, whereas CI-scores >1
149 indicate antagonistic effects.

150

151 Generation of CFZ-resistant cells

152 AMO1 and JJN3 cells were cultured in medium containing steadily increasing concentrations
153 of CFZ (up to 12 nM CFZ for AMO1 and 6 nM for JJN3), starting from a sublethal dose of 1nM.
154 Cells were cultured in CFZ-containing medium for at least 12 weeks and passaged every 3-4
155 days in fresh CFZ-containing medium. After becoming CFZ-resistant, AMO1-R and JJN3-R
156 cells were continuously cultured in 12 nM and 6 nM CFZ-containing medium, respectively.

157

158 Immunoblotting

159 Cell samples were lysed in RIPA buffer (50 mM Tris pH7.4, 150 mM NaCl, 1% NP40, 0.1%
160 SDS and 0.5% Sodium deoxycholate) supplemented with protease inhibitor
161 (#11836153001, Roche, Basel, Switzerland), phosphatase inhibitor cocktails 2 and 3
162 (#P57261 and #P0044, Sigma-Aldrich, Burlington, USA) and N-ethylmaleimide (E3876,
163 Sigma-Aldrich). Protein concentrations were determined by Pierce BCA protein assay kit
164 (#23225, Thermo Scientific). Proteins were separated by SDS-PAGE in Laemmli buffer
165 (0.25M Tris, 1.92M glycine, 1% SDS), transferred to PVDF membranes, pore size 0.45 µM
166 (Carl Roth, Karlsruhe, Germany) in transfer buffer (25 mM Tris, 192 mM glycine, 20%
167 methanol) and subsequently incubated overnight at 4°C with indicated antibodies in 5% BSA
168 in TBS-T. For dot blotting, 2µl of protein lysate with a concentration of 2µg, 4µg or 8µg was
169 spotted on a nitrocellulose membrane. After drying, membrane was blocked for 1h and
170 incubated with the indicated antibodies overnight. ECL (NEL104001EA , Perkin Elmer,
171 Waltham, USA) was used to detect antibodies on an Intas ECL Chemocam. Immunoblotting
172 experiments were performed in biological triplicates and representative results are shown.

173

174 Antibodies

175 Purchased from Cell Signalling (Danvers, USA): Phospho-CHEK1 S345 (#2348), cleaved
176 caspase-3 (#9664), SUMO 2/3 (#4971), SUMO 1 (#4930). From Abcam (Cambridge, UK): γ-
177 H2AX S139 (11174). From BD Biosciences (Franklin Lakes, USA): Cleaved PARP (51-
178 9000017). From Sigma-Aldrich: β-actin (A1978). From Santa Cruz Biotechnologies (Dallas,
179 USA): p53 (sc-126), HSP90 (sc-13119), p63a (sc-5301). Antibodies used for FACS analysis
180 were purchased from Biolegend (San Diego, USA): Annexin V-FITC (640906), CD38-APC
181 (356606), CD138-PE (356504). DAPI was purchased from Invitrogen (Waltham, USA, D3571).

182

183

184 Transcriptomics / RNA sequencing

185 JLN3, OPM2 and AMO1 cells were treated for four hours with DMSO, 5 nM CFZ, 250
186 subasumstat and the combination of CFZ and subasumstat. mRNA was isolated from cells
187 and samples using Qiagen RNeasy Mini Kit (74106, Qiagen, Venlo, The Netherlands). RNA
188 concentration was determined with a Nanodrop spectrophotometer. 1 µg of total RNA was
189 enriched in mRNA with NEBNext Poly(A) mRNA Magnetic Isolation (# E7490, NEB, Ipswich,
190 USA). Libraries were prepared with NEBNext Ultra II Directional RNA Library (#E7765L, NEB)
191 and indexes were added by PCR with NEBNext Multiplex Oligos for Illumina (#E7600, NEB)
192 according to manufacturer's protocols. Libraries were quantified and checked for fragment size
193 with Agilent High Sensitivity DNA Kit (Agilent Technologies, Santa Clare, USA). They were
194 pooled in equimolar ratios and sequenced on an Illumina NovaSeq 6000 for 150 bps in paired-
195 ended fashion. Raw reads were quality checked, adapters trimmed using Trimmomatic (5).
196 Reads were aligned to the human reference genome (GRCh38) using HISAT2 with default
197 parameters. Additionally, a study cohort of 16 multiple myeloma patients with paired diagnosed
198 and relapsed samples were included in the RNAseq study. Patient characteristics and
199 treatment are summarized in (Ng, Ramberger et al., Nat. Comm., accepted). All samples were
200 obtained from the iliac crest of patients and were CD138+ enriched by MACS (Miltenyi,
201 Cologne, Germany). All patients provided written informed consent according to the
202 Declaration of Helsinki and the study was approved by the institutional review board (IRB) of
203 Ulm University. Library preparation was performed from 100 ng of input total RNA using the
204 TruSeq Stranded Exome RNA Kit (Illumina, San Diego, CA, USA) according to the
205 manufacturer's instructions. The pooled RNA libraries were sequenced on an Illumina
206 HiSeq2000 with 50bp single-end reads with an average coverage of 36.6×10^6 reads per
207 sample. RNA-Seq data were aligned and quantified with STAR (6) and mRNA reads were
208 identified using an in-house analysis pipeline detecting exons in a shuffled order. Differential
209 gene expression analysis was carried out with DEseq2 (7) and gene set enrichment analysis
210 (GSEA) was performed using significant expressed genes (adj.p/FDR <0.05) and the FGSEA
211 package (8) and molecular signatures of the REACTOME knowledgebase (9). Data can be

212 accessed via the European Nucleotide Archive accession ID: PRJEB51059. Analysis of public
213 available data using MMRF-CoMMpass data(10) and gene expression omnibus (GEO)
214 accession IDs: GSE2658 and GSE39754. Zscore of normalized read counts were visualized
215 in heatmaps using ClustVis (11). Hierarchical clustering by euclidean distance of SUMO core
216 components (SAE1, UBA2, UBE2I, SUMO1, SUMO2, SUMO3) are indicated in the heatmap
217 plots. Survival data of these data sets was assigned to the respective SUMO^{high/low} clusters and
218 plotted using Kaplan-Meier-curves. Log-rank test was used to determine significance.

219

220 **Quantification of western blot band intensity**

221 ImageJ was used to quantify intensity of whole SUMO1 and SUMO2/3 lane immunoblots of
222 figure S1E, and relative band intensities were normalized to Actin loading control for each
223 sample.

224

225 **References**

- 226 1. Mertins P, Tang LC, Krug K, Clark DJ, Gritsenko MA, Chen L, Clauser KR, Clauss TR,
 227 Shah P, Gillette MA, Petyuk VA, Thomas SN, Mani DR, Mundt F, Moore RJ, Hu Y, Zhao R,
 228 Schnaubelt M, Keshishian H, Monroe ME, Zhang Z, Udeshi ND, Mani D, Davies SR,
 229 Townsend RR, Chan DW, Smith RD, Zhang H, Liu T, Carr SA. Reproducible workflow for
 230 multiplexed deep-scale proteome and phosphoproteome analysis of tumor tissues by liquid
 231 chromatography-mass spectrometry. *Nat Protoc.* 2018 Jul;13(7):1632-1661. Epub
 232 2018/07/11. doi:10.1038/s41596-018-0006-9. Cited in: Pubmed; PMID 29988108.
- 233 2. Cox J, Mann M. MaxQuant enables high peptide identification rates, individualized p.p.b.-
 234 range mass accuracies and proteome-wide protein quantification. *Nat Biotechnol.* 2008
 235 Dec;26(12):1367-72. Epub 2008/11/26. doi:10.1038/nbt.1511. Cited in: Pubmed; PMID
 236 19029910.
- 238 3. Ianevski A, Giri AK, Aittokallio T. SynergyFinder 2.0: visual analytics of multi-drug
 239 combination synergies. *Nucleic Acids Res.* 2020 Jul 2;48(W1):W488-W493. Epub
 240 2020/04/05. doi:10.1093/nar/gkaa216. Cited in: Pubmed; PMID 32246720.
- 242 4. Chou TC. Drug combination studies and their synergy quantification using the Chou-
 243 Talalay method. *Cancer Res.* 2010 Jan 15;70(2):440-6. Epub 2010/01/14. doi:10.1158/0008-
 244 5472.CAN-09-1947. Cited in: Pubmed; PMID 20068163.
- 246 5. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence
 247 data. *Bioinformatics.* 2014 Aug 1;30(15):2114-20. Epub 2014/04/04.
 248 doi:10.1093/bioinformatics/btu170. Cited in: Pubmed; PMID 24695404.
- 250 6. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M,
 251 Gingeras TR. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics.* 2013 Jan
 252 1;29(1):15-21. Epub 2012/10/30. doi:10.1093/bioinformatics/bts635. Cited in: Pubmed; PMID
 253 23104886.
- 255 7. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for
 256 RNA-seq data with DESeq2. *Genome Biol.* 2014;15(12):550. Epub 2014/12/18.
 257 doi:10.1186/s13059-014-0550-8. Cited in: Pubmed; PMID 25516281.
- 259 8. Sergushichev AA. An algorithm for fast preranked gene set enrichment analysis using
 260 cumulative statistic calculation. *bioRxiv.* 2016:060012. doi:10.1101/060012.
- 262 9. Gillespie M, Jassal B, Stephan R, Milacic M, Rothfels K, Senff-Ribeiro A, Griss J, Sevilla
 263 C, Matthews L, Gong C, Deng C, Varusai T, Ragueneau E, Haider Y, May B, Shamovsky V,
 264 Weiser J, Brunson T, Sanati N, Beckman L, Shao X, Fabregat A, Sidiropoulos K, Murillo J,
 265 Viteri G, Cook J, Shorser S, Bader G, Demir E, Sander C, Haw R, Wu G, Stein L, Hermjakob
 266 H, D'Eustachio P. The reactome pathway knowledgebase 2022. *Nucleic Acids Res.* 2022
 267 Jan 7;50(D1):D687-D692. Epub 2021/11/18. doi:10.1093/nar/gkab1028. Cited in: Pubmed;
 268 PMID 34788843.

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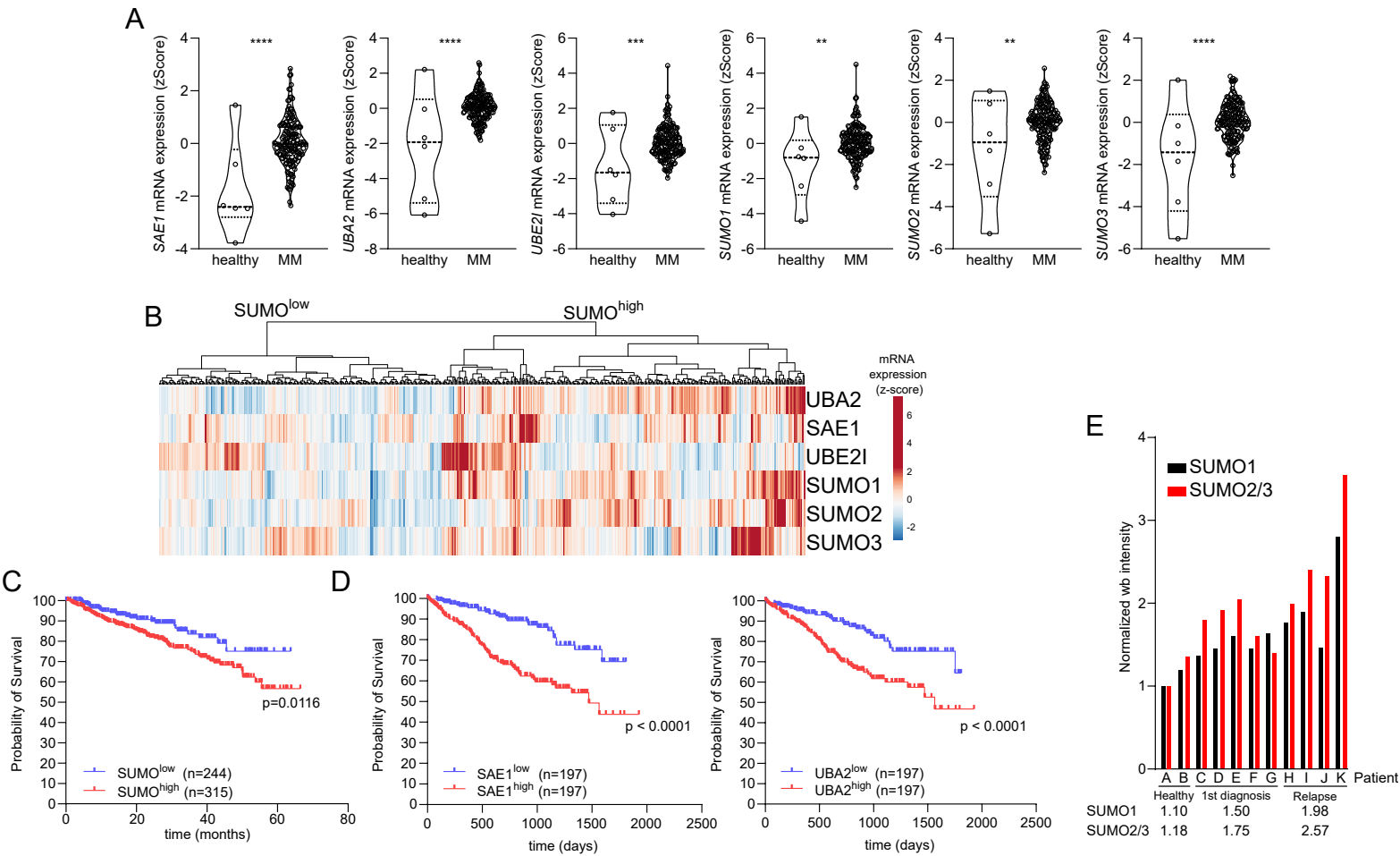
271 10. Study TMC. Multiple Myeloma Research Foundation (MMRF) CoMMpass study
272 [30.05.2022]. Available from: [https://themmrf.org/we-are-curing-multiple-myeloma/mmr-](https://themmrf.org/we-are-curing-multiple-myeloma/mmr-compass-study/)
273 [compass-study/](https://themmrf.org/we-are-curing-multiple-myeloma/mmr-compass-study/).

274
275 11. Metsalu T, Vilo J. ClustVis: a web tool for visualizing clustering of multivariate data using
276 Principal Component Analysis and heatmap. *Nucleic Acids Res.* 2015 Jul 1;43(W1):W566-
277 70. Epub 2015/05/15. doi:10.1093/nar/gkv468. Cited in: Pubmed; PMID 25969447.

278

279

Figure S1



Supplementary Figure S1.

(A) mRNA expression (zScore) of indicated SUMO core components in CD138⁺ healthy cells and CD138⁺ multiple myeloma cells. Data were derived from GSE39754. t-test: **p<0.01, ***p<0.001, ****p<0.0001

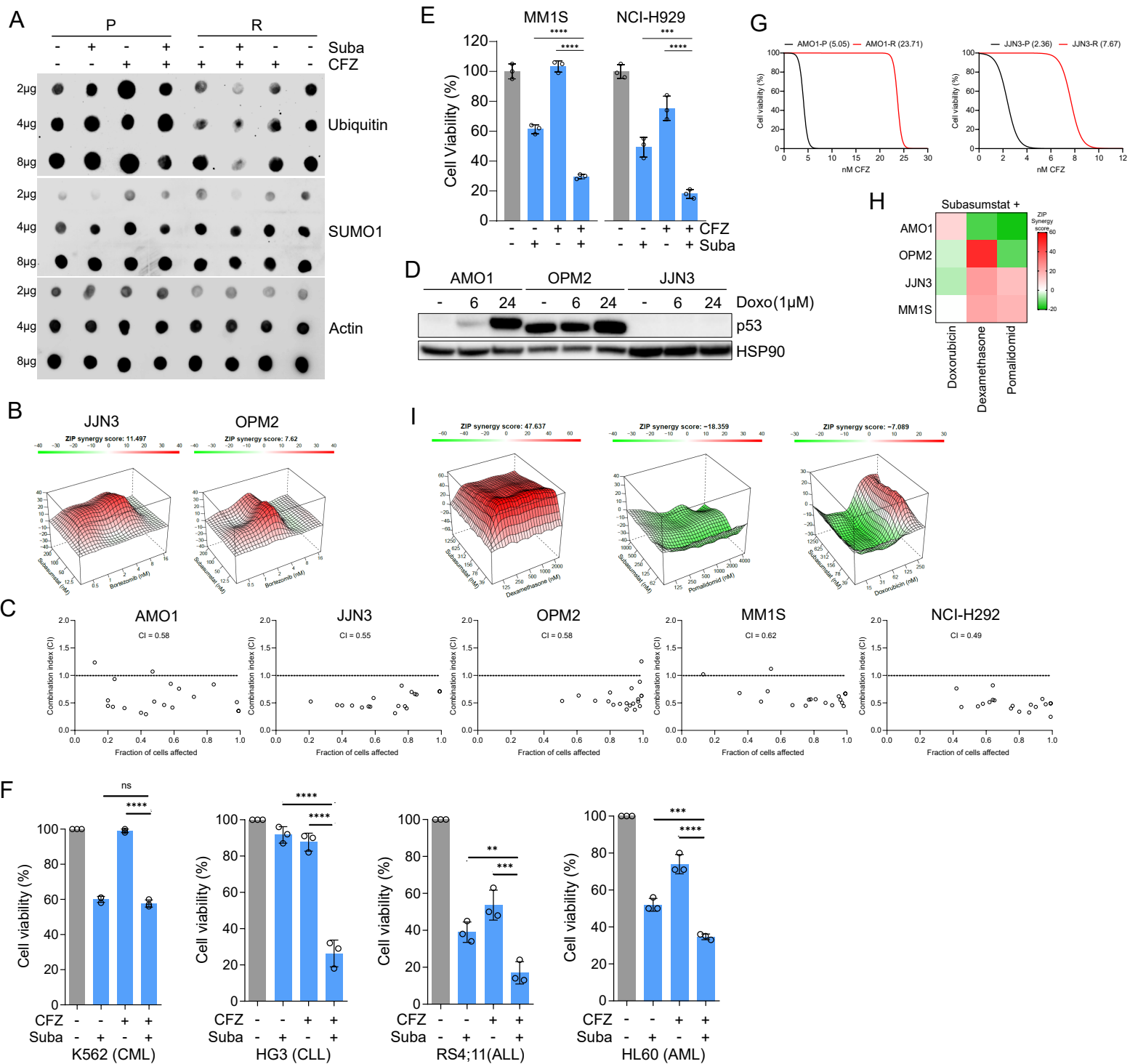
(B) Heatmap and hierarchical clustering of the SUMO core components *SAE1*, *UBA2*, *UBE21*, *SUMO1*, *SUMO2* and *SUMO3* derived from transcriptome data from n=559 multiple myeloma patients of the GSE2658 dataset with indicated clustering into SUMO^{high} and SUMO^{low} groups.

(C) Kaplan-Meier curves for probability of survival of SUMO^{high} and SUMO^{low} groups as described in (B). Curve comparison by log-rank test with indicated p-value.

(D) Kaplan-Meier curves for probability of survival of *SAE1*^{high} / *UBA2*^{high} (upper quartile) and *SAE1*^{low} / *UBA2*^{low} (lower quartile) groups from MMRF-CoMMpass data. Curve comparison by log-rank test with indicated p-value.

(E) Quantification of SUMO1 and SUMO2/3 western blots. Whole lanes were quantified using ImageJ and relative values normalized to beta-Actin expression are depicted. For each group mean intensity is indicated.

Figure S2



Supplementary Figure S2.

(A) AMO1 cells (parental (P) and resistant (R), as indicated in Fig. 1E) were treated with 12nM Carfilzomib (CFZ) and/or 1µM Subasumstat (Suba) for 4 hours and examined by dot blots for total Ubiquitin, SUMO1 and Actin concentrations with the amounts of loading proteins indicated.

(B) Landscape plots depicting the synergistic area of concentrations for subasumstat and BTZ combination treatment in JJN3 and OPM2. Cells were treated for 72 hours with the indicated concentrations of subasumstat and BTZ and cell viability was measured. Subsequently, cell viability data was used to generate landscape-plots using SynergyFinder.

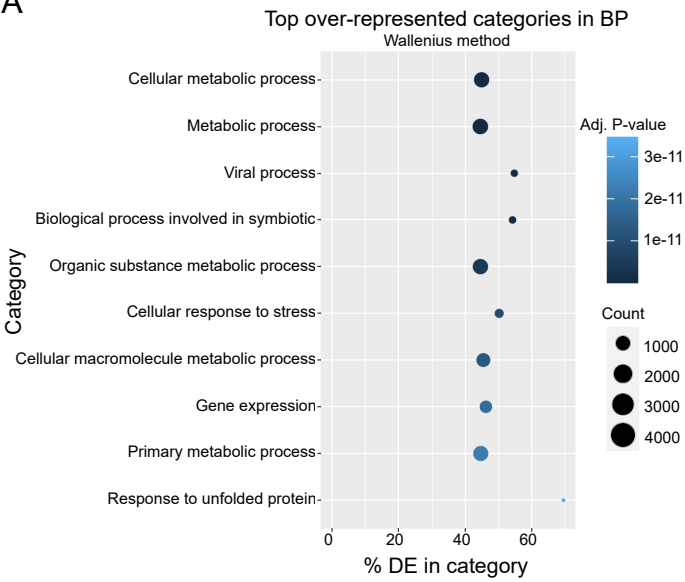
(C) Combination Index (CI) of indicated cell lines, treated with subasumstat and carfilzomib for 72h. Viability was determined using CellTiterGlo.

(D) TP53 status of AMO1, OPM2 and JJN3 cells. AMO1 cells, TP53wt, induce TP53 over time upon treatment with 1µM DNA damaging agent doxorubicin. OPM2 TP53mut cells constitutively express mutant TP53 and JJN3 cells are TP53null.

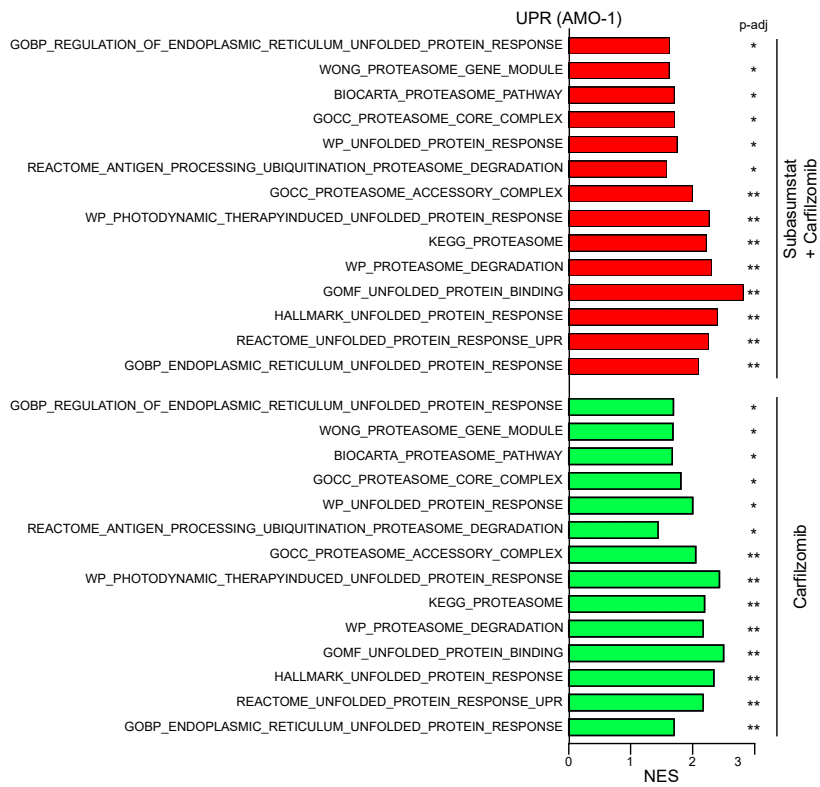
- (E)** Bar diagrams showing the effect on viability after 72 hours of treatment with CFZ, subasumstat and the combination thereof in MM1S (2nM CFZ, 100nM Suba) and NCI-H929 (4nM CFZ, 200nM Suba) cells. Treatment of five MM cell lines with 250 nM subasumstat (S) inhibits 2/3 SUMOylation and increases the pool of free SUMO 2/3 compared to DMSO treated control cells (D).
- (F)** Bar diagrams showing the effect on viability after 72 hours of treatment with CFZ, subasumstat and the combination thereof in K562 (2nM CFZ, 50nM Suba), HG3 (2nM CFZ, 12.5nM Suba), RS4;11 (2nM CFZ, 50nM Suba), and HL60 (2nM CFZ, 50nM Suba) cells. One-Way ANOVA; ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns: not significant. CML: chronic myeloid leukemia, CLL: chronic lymphocytic leukemia, ALL: acute lymphocytic leukemia, AML: acute myeloid leukemia.
- (G)** Viability of parental (P) and carfilzomib-resistant (R) AMO1 and JJN3 cells, treated for 72h with different concentrations of carfilzomib (CFZ). Viability was determined using CellTiterGlo. GI50 is indicated.
- (H)** ZIP synergy score heatmap in a panel of MM cell lines for the combination treatment of subasumstat with three clinically used drugs to treat MM. The synergistic/antagonistic effect is calculated by the ZIP synergy score using SynergyFinder. The presented ZIP synergy scores are the average of three independent experiments for each cell line.
- (I)** Landscape plots depicting the synergistic (red) and antagonistic (green) areas for combination treatment of subasumstat with doxorubicin (left), dexamethasone (middle) and pomalidomid (right) in OPM2 cells. Cells were treated for 72 hours with the indicated concentrations of drugs and cell viability was measured. Subsequently, cell viability data was used to generate landscape-plots using SynergyFinder.

Figure S3

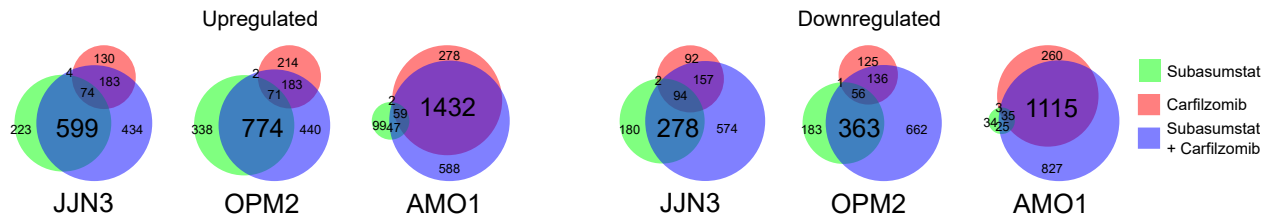
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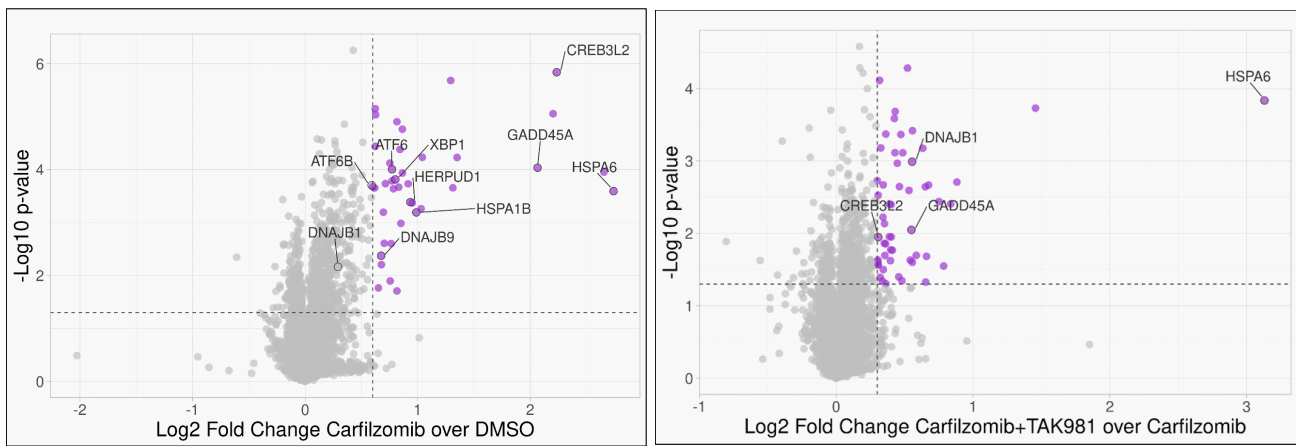
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C



D



Supplementary Figure S3.

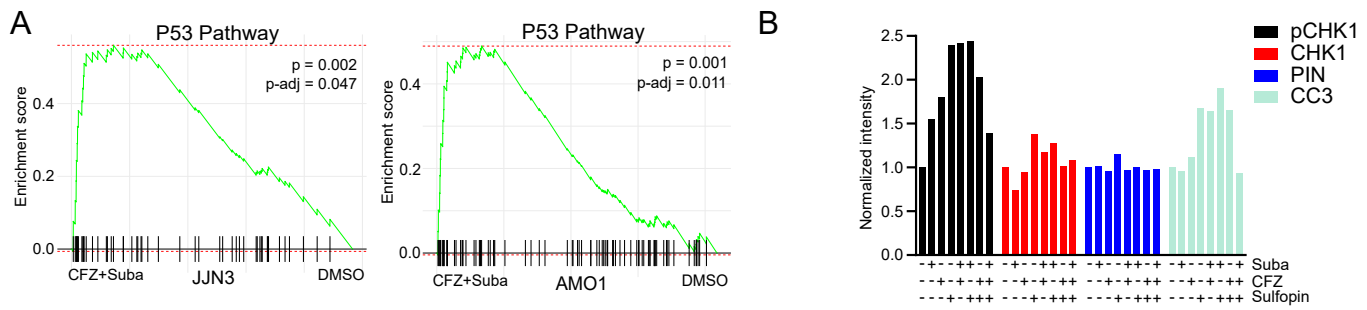
(A) Pathway analysis of combined RNA sequencing data of JJN3, OPM2 and AMO1 cells that were treated with 250 nM subasumstat and 5 nM CFZ for 4 hours. Gene expression, unfolded protein response and responses to cellular stress are significantly upregulated.

(B) Unfolded protein response signature gene sets are upregulated in AMO1 cells that are treated for 4 hours with 5 nM CFZ or 250 nM subasumstat + 5 nM CFZ compared to DMSO control.

(C) Venn diagrams illustrating mRNA expression of genes that are significantly up- or downregulated in JJN3, OPM2 and AMO1 cells that are treated for 4 hours with 250 nM subasumstat, 5 nM CFZ or the combination treatment. Significance has been determined by DESeq2 and an adj-p<0.05.

(D) Graphical representation of quantitative proteomics data of OPM2 cells that are treated for 4 hours with 5 nM CFZ over DMSO control cells or treated for 4 h with 250 nM subasumstat and 5 nM CFZ over 5 nM CFZ treated cells. Proteins are ranked in a volcano plot according to their statistical P-value (y-axis) and their relative abundance ratio (log₂ fold change, x-axis).

Figure S4

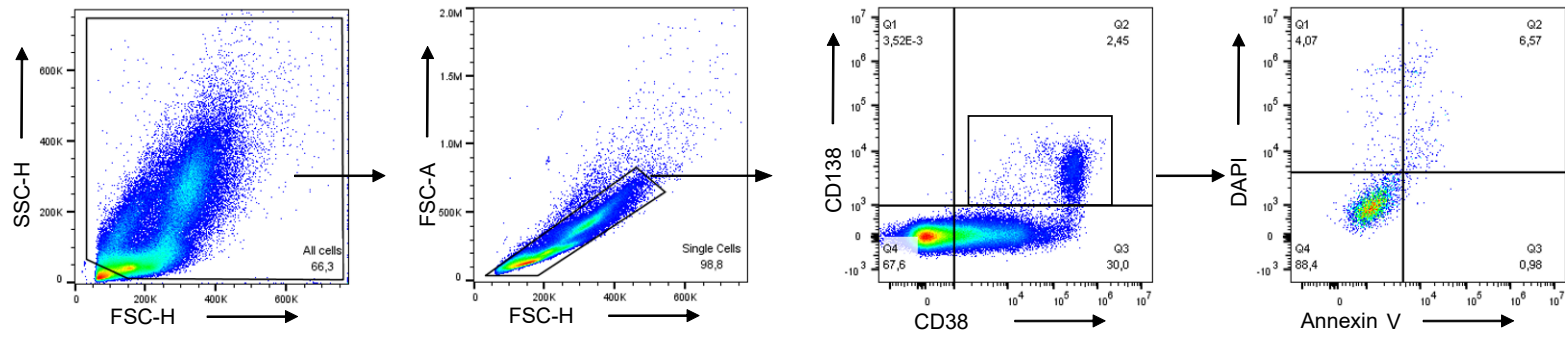


Supplementary Figure S4.

(A) JYN3 and AMO1 cells were treated for 4 hours with DMSO, 250 nM subasumstat, 5 nM CFZ or the combination thereof and subsequently analyzed by RNA sequencing. GSEA analysis by the FGSEA package of the combination treatment (4 h) versus DMSO control shows enriched p53 signatures of the Hallmark set from the molecular signature database. FGSEA p-values and adjusted p-values (false discovery rate) are indicated.

(B) Quantification of Immunoblots from Fig. 5E. Signal intensity was normalized to loading control (β -Actin). 4h Treatment with 250 nM Subasumstat (Suba), 5nM carfilzomib (CFZ), and 2 μ M Sulfopin in OPM2 cells.

Figure S5



Supplementary Figure S5

FACS sorting strategy for Figure 6C. After live cell population was gated, MM cells positive for CD38⁺ (PE) and CD138⁺ (APC) were gated for Annexin V positivity.

Table S2. Clinical characteristics of MM patients.

Patient #	Sample Time Point	ISS	Ig-Type	Treatment	Time until relapse
1	1st diagnosis	II	Lambda-LC	Not applicable	Not applicable
2	1st diagnosis	I	IgA Kappa	Not applicable	Not applicable
3	Relapse	I	IgA Kappa	BCD	17 months
4	Relapse	III	IgG Lambda	BCD + HD+auto-SCT CRD + HD+auto-SCT Len Pom / Dex	3 months
5	Relapse	II	IgA Lambda	BCD + HD+auto-SCT Dara / Len / Dex CFZ / Dex	12 months

ISS = International Staging System

LC = Light chain

BCD = Bortezomib / Cyclophosphamide / Dexamethasone

HD+auto-SCT = High dose chemotherapy+autologous stem cell transplantation

CRD = Carfilzomib / Lenalidomide / Dexamethasone

Len = Lenalidomide

Pom = Pomalidomide

Dex = Dexamethasone

Dara = Daratumumab

CFZ = Carfilzomib