S-adenosylmethionine biosynthesis is a targetable metabolic vulnerability in multiple myeloma

Yanmeng Wang,[1] Catharina Muylaert,[1] Arne Wyns,[1] Philip Vlummens,[1,2] Kim De Veirman,[1] Karin Vanderkerken,[1] Esther Zaal,[3] Celia Berkers,[3] Jérome Moreaux,[4,5,6] Elke De Bruyne[1] and Eline Menu[1]

[1]Department of Hematology and Immunology-Myeloma Center Brussels, Vrije Universiteit Brussel, Jette, Belgium; [2]Department of Clinical Hematology, Ghent University Hospital, Ghent, Belgium; [3]Utrecht Metabolism Expertise Center, Nieuw Gildestein, Utrecht, the Netherlands; [4]Laboratory for Monitoring Innovative Therapies, Department of Biological Hematology, CHU Montpellier, Montpellier, France; [5]Institute of Human Genetics, University of Montpellier, Montpellier, France and [6]Institut Universitaire de France, Paris, France

Correspondence: E.D. Bruyne
Elke.De.Bruyne@vub.be

E. Menu
Eline.Menu@vub.be

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Supplementary methods

Gene expression and survival analysis

Expression and survival analysis of (publicly available) gene-expression microarray data were done using Genomicscape (http://genomicscape.com). The Heidelberg-Montpellier (HM) cohort (Database E-MTAB-372) and the University of Arkansas for Medical Sciences (UAMS, Little Rock, AR) TT2 and TT3 cohorts (datasets GSE4581 and GSE2658) contain expression data of malignant plasma cells (PC) of newly diagnosed, untreated MM patients. We also used Affymetrix data of relapsed MM patients subsequently treated with bortezomib or high dose dexamethasone (GSE9782) from the study of Mulligan and colleagues and microarray data from normal BMPC, premalignant PC of monoclonal gammopathy of undetermined significance (MGUS; GSE5900) and HMCL (E-TABM-1088 and E-TABM-937). The MMRF CoMMpass Trial (NCT01454297), a longitudinal study in MM, comprises genomic data obtained at diagnosis and subsequent relapse(s). All data were obtained through the MMRF Researcher Gateway (https://research.themmrf.org). Survival curves were made using data from 653 patients.

Preparation of conditioned medium (CM)

For preparation of the HS-5-CM, upon reaching 80% confluence, the cells were washed three times using PBS and replaced by 10 ml of serum-free DMEM medium. After 24h, the culture medium was collected. The supernatant was centrifuged at 2000g for 10 min to remove any cell material. The CM were pooled together and used for further experiment or stored in -80 ºC.

Quantitative real-time PCR

RNA was extracted from cell pellets using the NucleoSpin@ RNA Plus Kit (Macherey-Nagel, Düren, Germany) and RNA concentration was measured using the NanoDrop (Thermo Fisher Scientific). RNA was converted to cDNA by the Verso cDNA Synthesis Kit (Thermo Fisher Scientific). Real-time PCR was performed in triplicate using SYBR-Green master mix (Thermo Fisher Scientific). Primers were purchased from Integrated DNA Technologies (Leuven, Belgium). Relative mRNA expression normalized to ABL1 was determined using the 2-ΔΔCt method. Gene-specific primer sequences were as follows, human MAT2A: forward (5'-TATCACCCAACGCTCCAAAG-3'), reverse (5'-CATTGCCAGACAGAGGCTATAA-3'), mouse Mat2a: forward (5'-GGGATGCTCTGAAGGAGAAAG-3'), reverse (5'-CACTTGGCTGTAGGTGGTAAA-3'), Human ABL1: forward (5'-GAGGGCGTGTGGAAGAAATA-3'), reverse (5'-CACCAGGTTAGGGTGTTTGA-3'), mouse Abl: forward (5'- CCGTGGGTGCCACTATATTT-3'), reverse (5'-GGGCACAGTGGTGAACTATT-3').

Viability and apoptosis assay

Cell viability was measured by using the CellTiter Glo® Luminescent Cell Viability Assay (Promega, Madison, WI, USA). Luminescence was determined using a Glomax luminometer (Promega). Apoptotic cells were quantified by performing a allophycocyanin (APC) coupled Annexin V (BD Biosciences, Belgium) and 7-AAD staining (BD Biosciences), followed by flow cytometric analysis on a FACSCanto flow cytometer (BD Biosciences), and the data were analyzed by FlowJo software V10.2 (FlowJo).

Western Blotting

Cell pellets were lysed in lysis buffer containing 50 mM Tris (pH 7.6), 150 mM NaCl, 1% Nonidet P40, and 0.25% sodium deoxycholate for 15 min on ice. The following protease and phosphatase inhibitors were added to the lysis buffer: 4 mM Na_3VO_4, 1 mM $Na_4P_2O_7$, 2 µg/mL aprotinin, 50 µg/mL leupeptin, 500 µg/mL trypsin inhibitor, 10 µM benzamidine, 2.5 mM pnp benzoate (all from Sigma-Aldrich), 50 mM NaF, 5 mM ethylenediaminetetraacetic acid (both from VWR International, Leuven, Belgium), 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, and 50µg/mL pepstatin A (both from ICN, Costa Mesa, CA, USA). Next, cell lysates were centrifuged (20,000 rpm) for 5 min at 4°C to pellet cell the debris. Supernatant was collected and quantified using the PierceTM Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Fisher scientific). Proteins (30 µg) were denaturated by adding an equal volume of 2x loading buffer (Bio-Rad Laboratories, Hercules, CA, USA) and boiling the samples for 5 min. Then, samples were loaded on 10%–15% SDS–PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad). Subsequently, membranes were blocked with PBS containing 5% skim milk and 0.1% Tween 20 for 1h at room temperature. After washing with phosphate buffered saline with 0.1% Tween-20 (PBST) three times at room temperature, the membranes were incubated with the primary antibodies at 4°C overnight. Antibodies used for analysis were: PARP (#9542), caspase-3 (#9662), caspase-9 (#9502), MCL-1 (#94296), $P21^{Waf1/Cip1}$ (#2947), $P27^{Kip1}$ (#3686), MYC (#18583), p-mTOR (#5536), mTOR (#2983), p-p70 (#9234), p70 (#2708), p-PRAS40 (#13,175), PRAS40 (#2691), p-S6 (#4858), S6 (#2217), p-4EBP1 (#2855), 4EBP1 (#9644), p-eIF4E (#9741), eIF4E (#2067), Tubulin (#2144), purchased from Cell Signaling Technology (Boston, MA), MAT2A (ab177484) from abcam

(Cambridge, MA) and anti-puromycin antibody from Sigma-Aldrich (#MABE343). Membranes were then incubated with an appropriate HRP-conjugated secondary antibody (ImTec Diagnostics, Antwerpen, Belgium) for 1 h at room temperature, and signals were developed using an enhanced chemiluminescence system (Thermo Fisher Scientific). For western blot analysis of the murine experiment, the 5TGM1 cells were purified by negative selection (CD11b-) using CD11b MACS beads (Miltenyi Biotec) according to the manufacturer's instructions and the further steps are as described above. Chemiluminescence was analyzed using an Odyssey Fc Imager (LI-COR Biosciences, Bad Homburg, Germany). Densitometric quantification was performed using Image Studio Lite v5.2.

Protein synthesis assay

For measurements of relative rates of protein synthesis, cells seeded in 6-well plates were treated as indicated and then incubated with 1 μM of puromycin (Cat nr S7417, Selleckchem) for 30 min prior to pellet isolation. Cells were lysed in lysis buffer and the protein concentration was normalized as described above. Puromycin uptake was then measured using western blot analysis as described above.

BrdU incorporation assay and cell cycle analysis

To assess proliferation, HMCLs were labeled with 10 μM 5-bromo-2-deoxyuridine (BrdU, Roche Diagnostics, Germany) for 4h at 37°C. Next, cells were harvested and washed with FACS flow before fixation in 4% paraformaldehyde for 10 min at room temperature and then further incubated with PBST overnight. Fixed cells were denatured with 2M HCl for 30 min

and rinsed free of acid. Following the denaturation step, cells were stained with 3 μL/sample of FITC-conjugated BrdU antibody (Sigma-Aldrich, St Louis, MO, USA). After 30 min, cells were rinsed and stained with propidium iodide solution. Samples were then analyzed by using a FACSCanto (Beckton Dickinson, San Jose, CA) and FlowJo was used to quantify the cell-cycle distribution.

Small interfering RNA transfection

HMCLs cells were transfected with small interfering RNA (siRNA) against MAT2A or negative Control siRNA (QIAGEN, Hilden, Germany) dissolved in sterile RNAse free water using Lipofectamine™ RNAiMAX Transfection Reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. The sequences for human MAT2A siRNA were as follow: sense, 5′-CAG UUG UGC CUG CGA AAU ATT-3′, and antisense, 5′-UAU UUC GCA GGC ACA ACU GCT-3′. siRNA for MAT2A was used at 20 nM as final concentration. To sustain efficient knockdown, we refreshed cells and re-added the same amount of siRNA on the third day of transduction.

Lentiviral Transduction

Three doxycycline-inducible lentiviral constructs containing a short hairpin RNA cassette against MAT2A (shMAT2A) and the control lentiviral construct containing a Scrambled shRNA were purchased from Horizon Discovery. The sequences of the shMAT2As were as follows: shRNA1, 5′-AGGTTTGTCTTGATGAAAT-3′, shRNA2, 5′-GGGTGATGCTGGTTTGACT-3′, shRNA3, 5′-AGATATTGCTCAAGGTGTT-3′. For the

lentiviral transductions, cells were first transduced at an MOI of 10 TU/cell, and subsequently selected using 2 μg/ml of puromycin (Bio-Connect, Huissen, Netherlands).

LC-MS based metabolomics

The concentration of SAM in OPM2 cells transfected for 72h with siMAT2A or negative control siRNA was determined using a liquid chromatography-tandem mass spectrometry (LC-MS) method as follows: 1×10^6 cells were washed with cold PBS and extracted with 100uL cold MS lysis buffer containing 40% Acetonitrile, 40% Methanol and 20% Milli-Q water. Samples were then centrifuged at 16.000g for 15 minutes at 4 °C to remove cell debris and proteins and supernatants were collected for LC-MS analysis. LC-MS analysis was performed on a Q-Exactive HF mass spectrometer (Thermo Scientific) coupled to a Vanquish autosampler and pump (Thermo Scientific). The MS operated in polarity-switching mode with spray voltages of 4.5 kV and -3.5 kV. Metabolites were separated using a Sequant ZIC-pHILIC column (2.1 x 150 mm, 5 μm, guard column 2.1 x 20 mm, 5 μm; Merck) with elution buffers acetonitrile (A) and eluent B (20 mM (NH4)2CO3, 0.1% NH4OH in ULC/MS grade water (Biosolve)). Gradient ran from 20% eluent B to 60% eluent B in 20 minutes, followed by a wash step at 80% and equilibration at 20%. Flow rate was set at 100 μl/min. Analysis was performed using TraceFinder software (Thermo Scientific). Metabolites were identified and quantified on the basis of exact mass within 5 ppm and further validated by concordance with retention times of standards. Peak intensities were normalized based on total ion count.

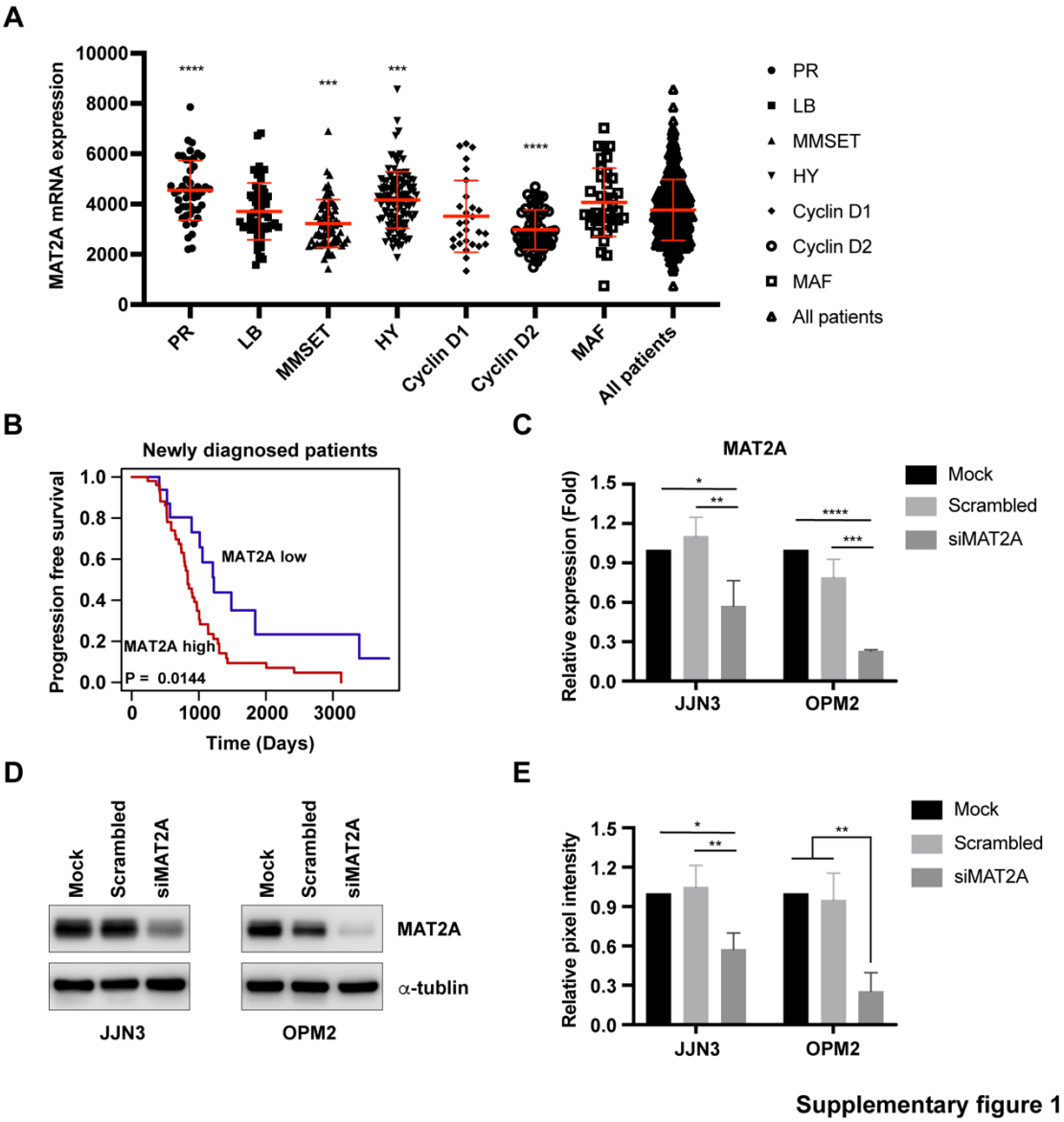
Analysis of Drug Interactions

To quantify drug interaction between FIDAS-5 and BZ, we employed the Bliss independence model. Cells were seeded in 96 well plates and treated with increasing doses of each single drug and all possible drug combinations for four days, after which cells were analyzed for cell viability using the CellTiter-Glo luminescent assay. Next, Bliss summary synergy scores were calculated as described previously (De Smedt E et al, Blood Advances 2021). Summary scores less than −10 indicate an antagonistic relationship, while scores ranging from −10 to 10 indicate additive effects and scores greater than 10 indicate synergistic effects.

Isolation of CD138+ cells from bone marrow samples

BM samples were collected in sterile EDTA tubes during routine diagnostics or evaluation purposes after patients' written informed consent and in accordance with the Declaration of Helsinki and institutional research board approval from Brussels University Hospital (B.U.N. 143201838414). Mononuclear cells were isolated by density gradient centrifugation with Lymphoprep™ (STEMCELL™ technologies, Grenoble, France). Next, $CD138^+$ cells were purified by magnetic sorting using the MACS system (Miltenyi Biotec, Gladbach, Germany), according to manufacturer's instructions.

Supplementary results



Supplementary figure 1

Figure S1: A MAT2A mRNA levels in the different molecular MM subgroups from the UAMS TT2 patient cohort. Significance was determined by a Mann-Whitney test. ***P ≤ 0.001 and **** P ≤ 0.0001 compared to all patients. **B** Prognostic value of MAT2A mRNA levels in terms of progression free survival in newly diagnosed patients from the Montpellier cohort (n = 76). Maxstat analysis was used to calculate the optimal separation of patients based on a cut-off value. The Kaplan–Meier method was used to plot survival curves; group comparisons were made using the logrank test. $P < 0.05$ was considered as statistically different. **C-E** SiRNA-mediated knockdown reduced MAT2A expression both on mRNA and protein level. JJN3 and OPM2 cells were transfected with 20nM siMAT2A. **C** After 48h, RNA was extracted and the expression of MAT2A on mRNA level was measured by qRT-PCR. **D-E** The protein expression of MAT2A was measured by Western blot and the pixel intensity determined by Image Studio Lite v5.2. Significance was analyzed using one-way ANOVA. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

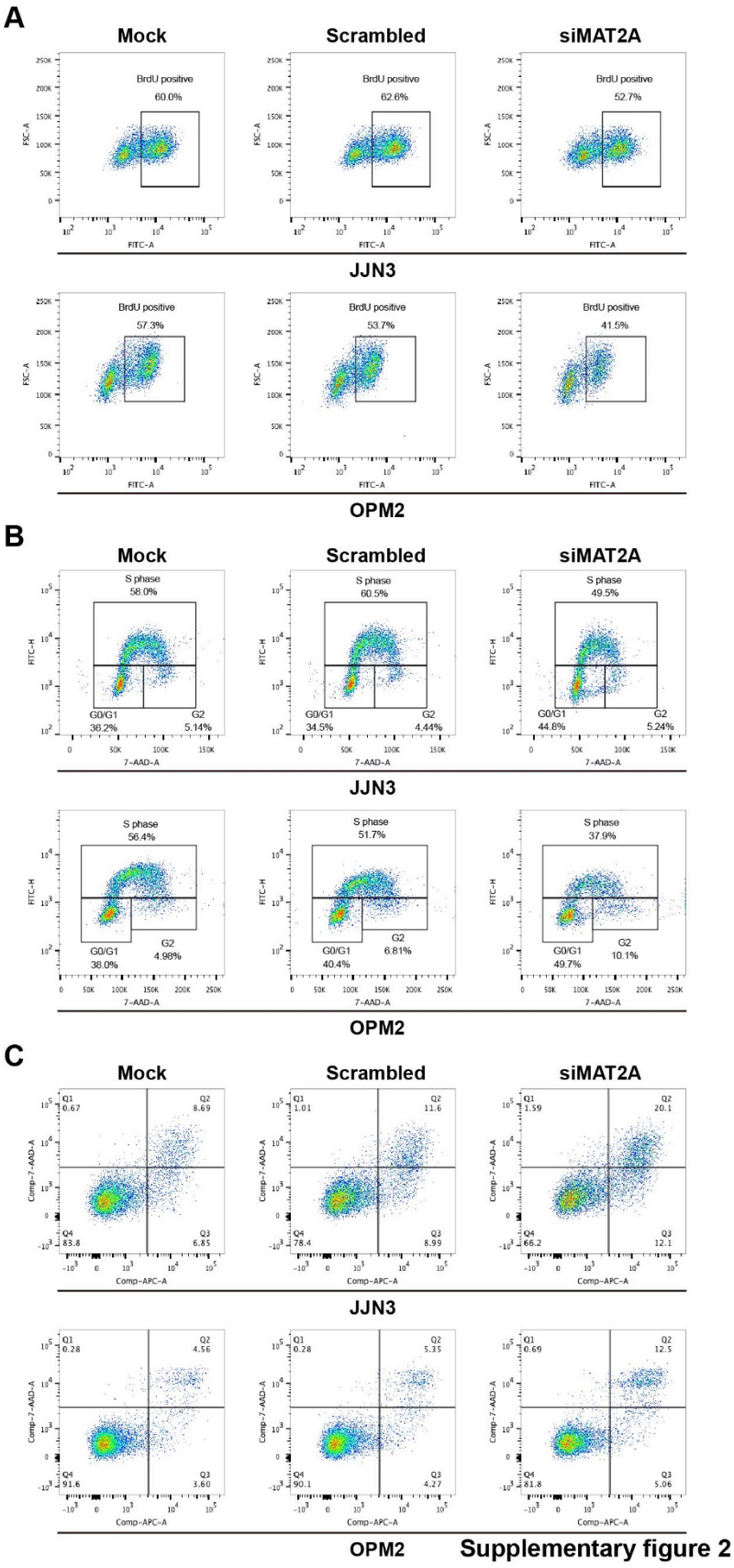
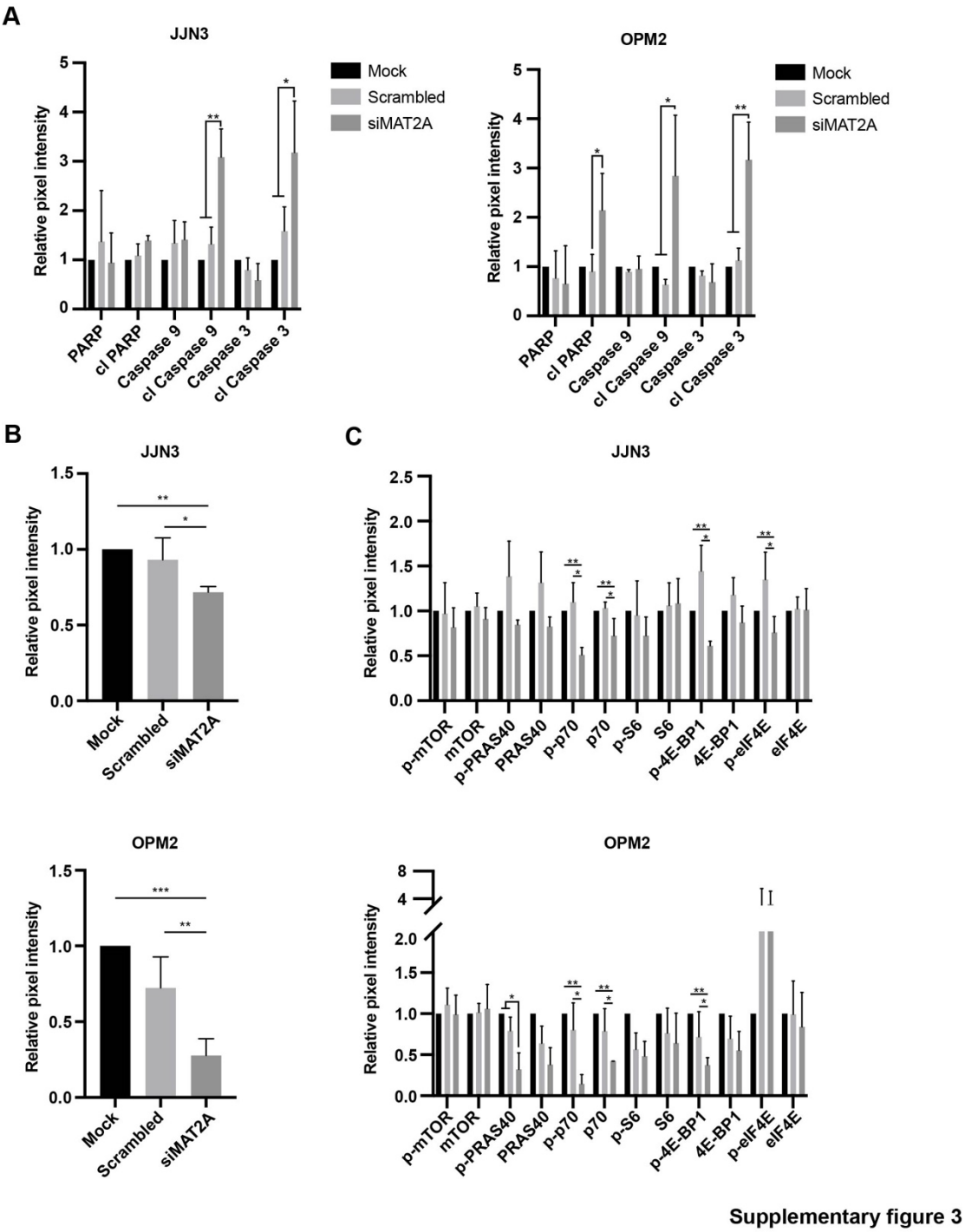


Figure S2: A-C Representative images of the flow cytometric analysis for proliferation, cell cycle progression and apoptosis (for Figure 2B-E).



Supplementary figure 3

Figure S3: A-C The pixel intensity of Figure 2E (**A**) and Figure 3B-C (**B** and **C**) were measured by Image Studio Lite v5.2. Significance was determined by one-way ANOVA. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

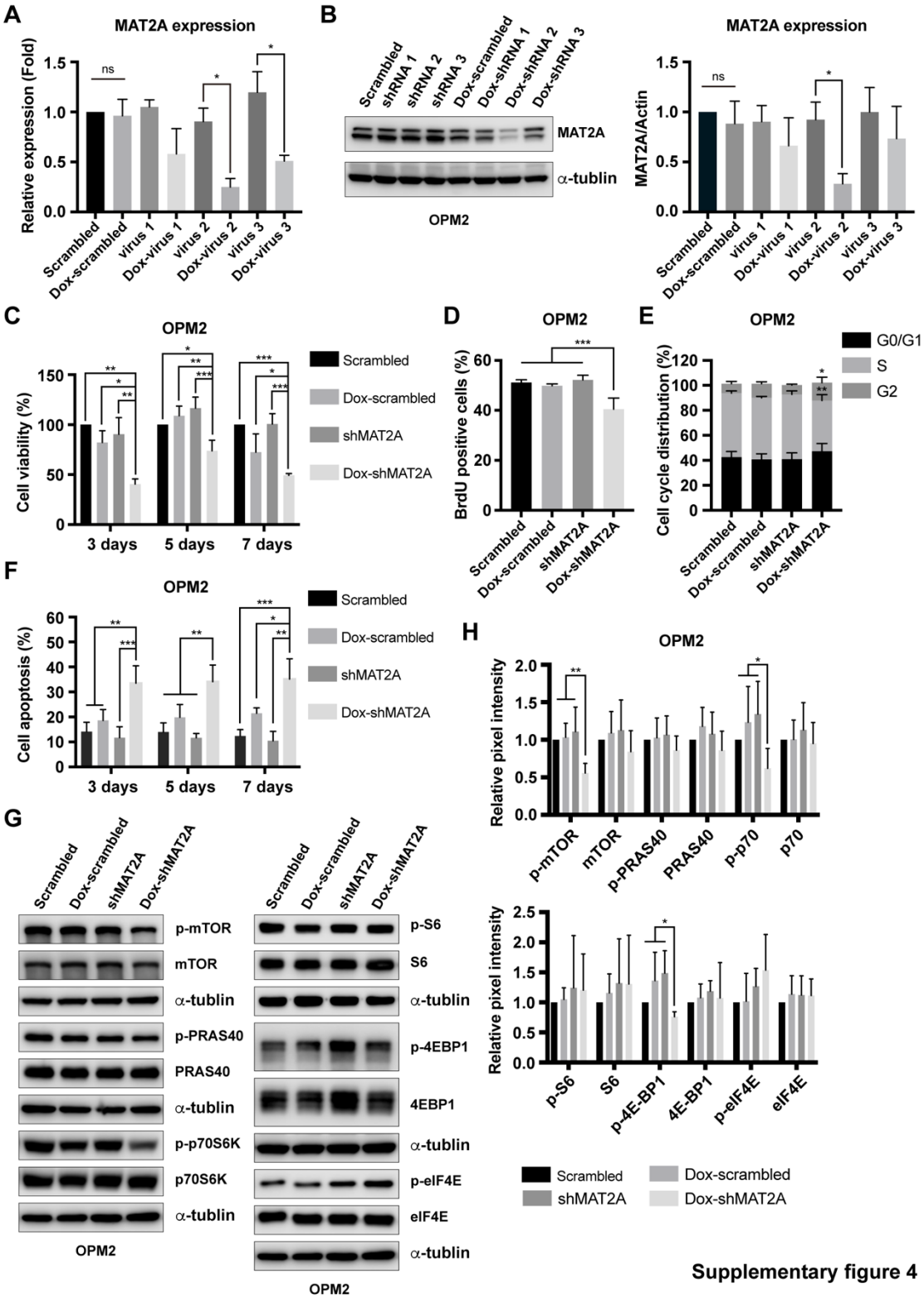


Figure S4: Conditional MAT2A KD using inducible shRNA against MAT2A impairs MM cell survival and m-TOR pathway activation in OPM2 cells. **A-B** MAT2A conditional KD OPM2 cells were established by using doxycycline inducible lentiviral vectors containing a shRNA-cassette against MAT2A, followed by puromycin selection and doxycycline administration. **A** After three days of doxycycline (0.5µg/mL) treatment, RNA was extracted and the expression

of MAT2A on mRNA level was measured by qRT-PCR. **B** Protein expression of MAT2A was measured by Western blot after five days of doxycycline treatment (Left) and the pixel intensity was determined by Image Studio Lite v5.2 (Right). **C** Cell viability was measured by the CellTiter-Glo Luminescent cell viability assay 3, 5 and 7 days post-doxycycline treatment respectively. **D-E** After four days of doxycycline treatment, cell proliferation and cell cycle analysis were measured using BrdU incorporation assay and PI staining respectively, followed by flow cytometric analysis. **F** Cell apoptosis was measured using flow cytometry by staining for Annexin V-FITC/7AAD after 3, 5 and 7days respectively. Significance was analyzed using an one-way ANOVA test. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. **G-H** Following doxycycline treatment for four days, key proteins related to the mTOR pathway were analyzed by WB (**G**) and the pixel intensity determined by Image Studio Lite v5.2 (**H**). Significance was analyzed using an one-way ANOVA test. * $P \leq 0.05$, ** $P \leq 0.01$.

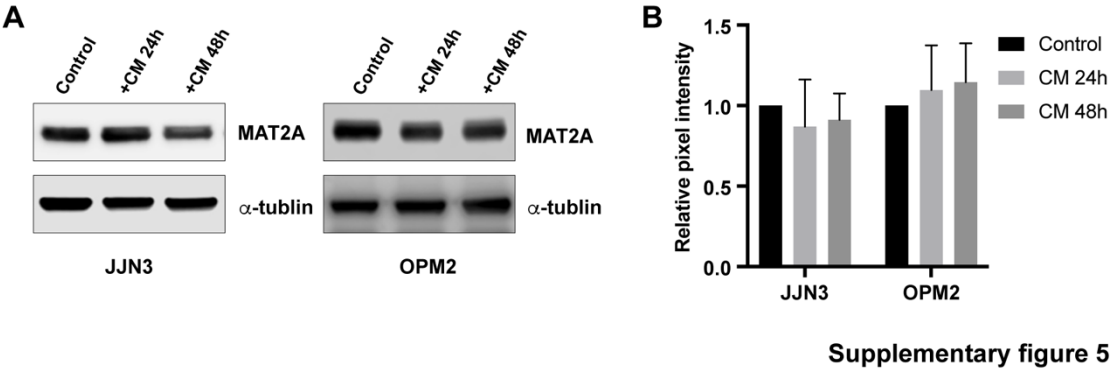


Figure S5: Conditioned medium form HS-5 bone marrow stromal cells does not alter the expression of MAT2A in MM cells. JJN3 and OPM2 cells were treated with the HS-5 conditioned medium (CM) for 24h or 48h after which the protein level of MAT2A was measured by WB (**A**) and the pixel intensity determined by Image Studio Lite v5.2 (**B**).

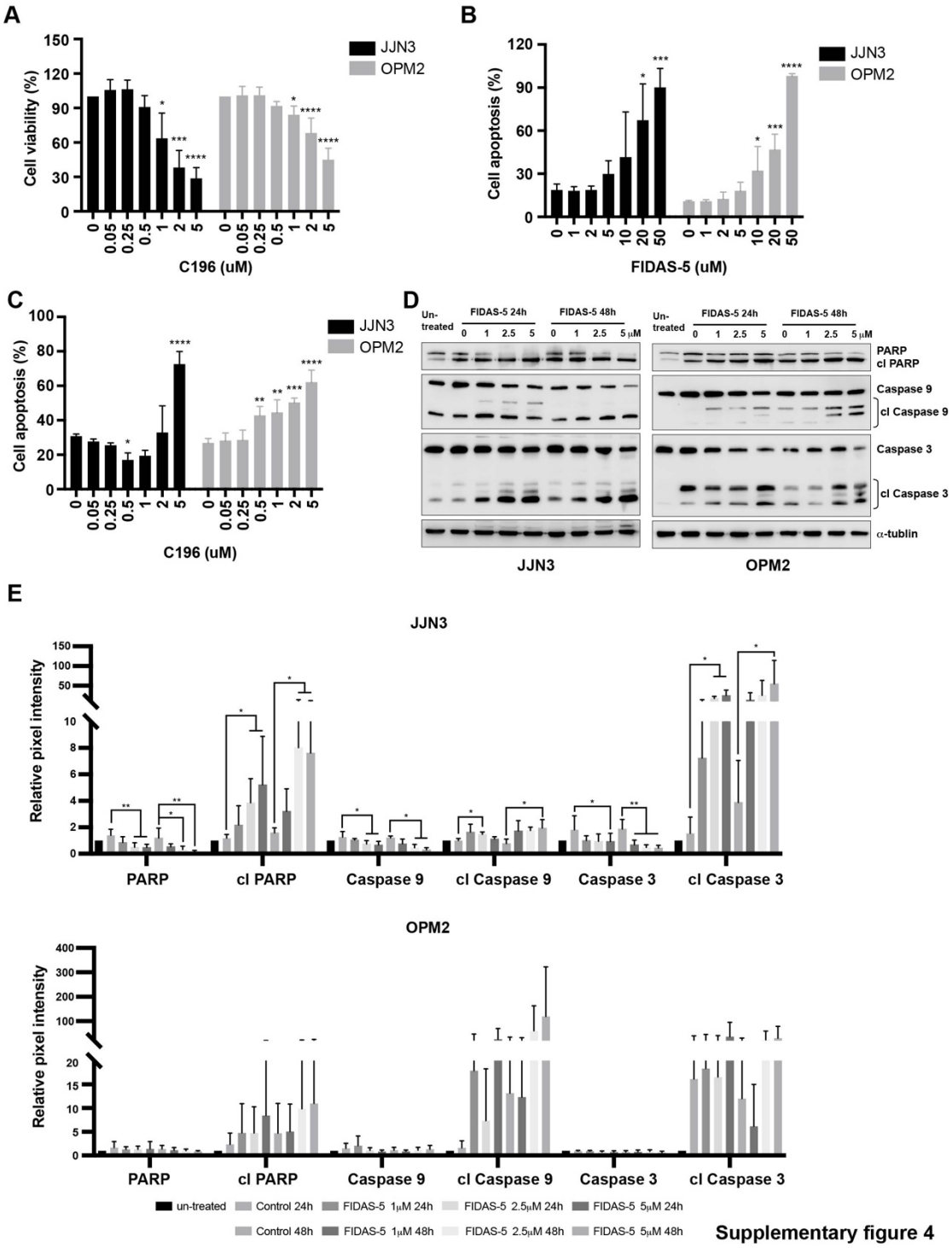
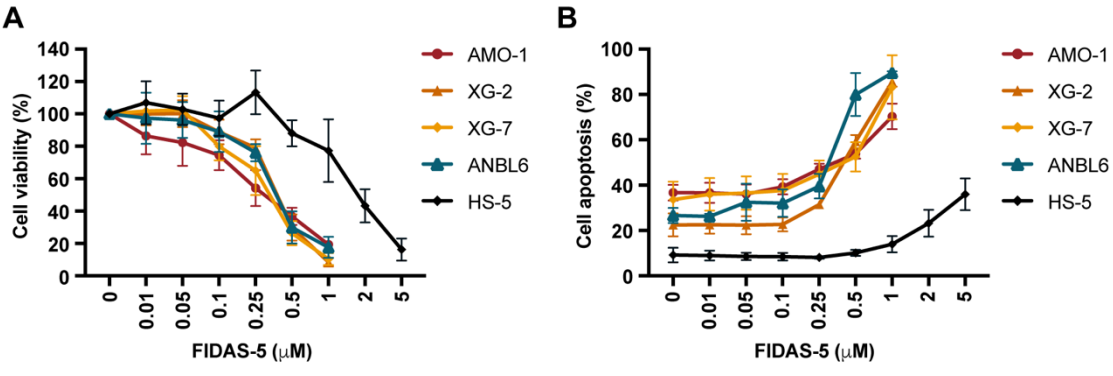


Figure S6: Short-term MAT2A inhibition by C196 or FIDAS-5 reduces MM cell survival *in vitro*. HMCL JJN3 and OPM2 were treated with increasing doses of C196 or FIDAS-5 for 48h. **A** Cell viability was measured by a CellTiter-Glo Luminescent cell viability assay after treatment with C196. **B-C** Cell apoptosis was measured using flow cytometry by staining for Annexin V-FITC/7AAD after treatment with FIDAS-5 (**B**) or C196 (**C**). **D-E** HMCL JJN3 and OPM2 were treated with 1, 2.5 and 5 µM of FIDAS-5 for 24h or 48h. Apoptosis markers PARP, Caspase 9 and Caspase 3 were measured on protein level by western blotting (**D**) and the pixel

intensity was measured by Image Studio Lite v5.2 (**E**). All experiments were performed in conditioned medium. Significance was determined by one-way ANOVA. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.



C

	AMO-1	XG-2	XG-7	ANBL6	JJN3	OPM2	HS-5
IC50 (μM)	0.367	0.375	0.334	0.337	0.243	0.320	1.314

Supplementary figure 7

Figure S7: MAT2A inhibition by FIDAS-5 reduces MM cell survival. Four HMCLs and the human bone marrow stromal cell line HS-5 were treated with increasing doses of FIDAS-5 for five days. **A** Cell viability was measured by a CellTiter-Glo Luminescent cell viability assay. **B** Cell apoptosis was measured using Annexin V-FITC/7AAD staining followed by FACS. **C** The half maximal inhibitory concentration (IC50) values of FIDAS-5 for the MM cell lines and HS-5 cells were calculated with GraphPad Prism 9.0 software.

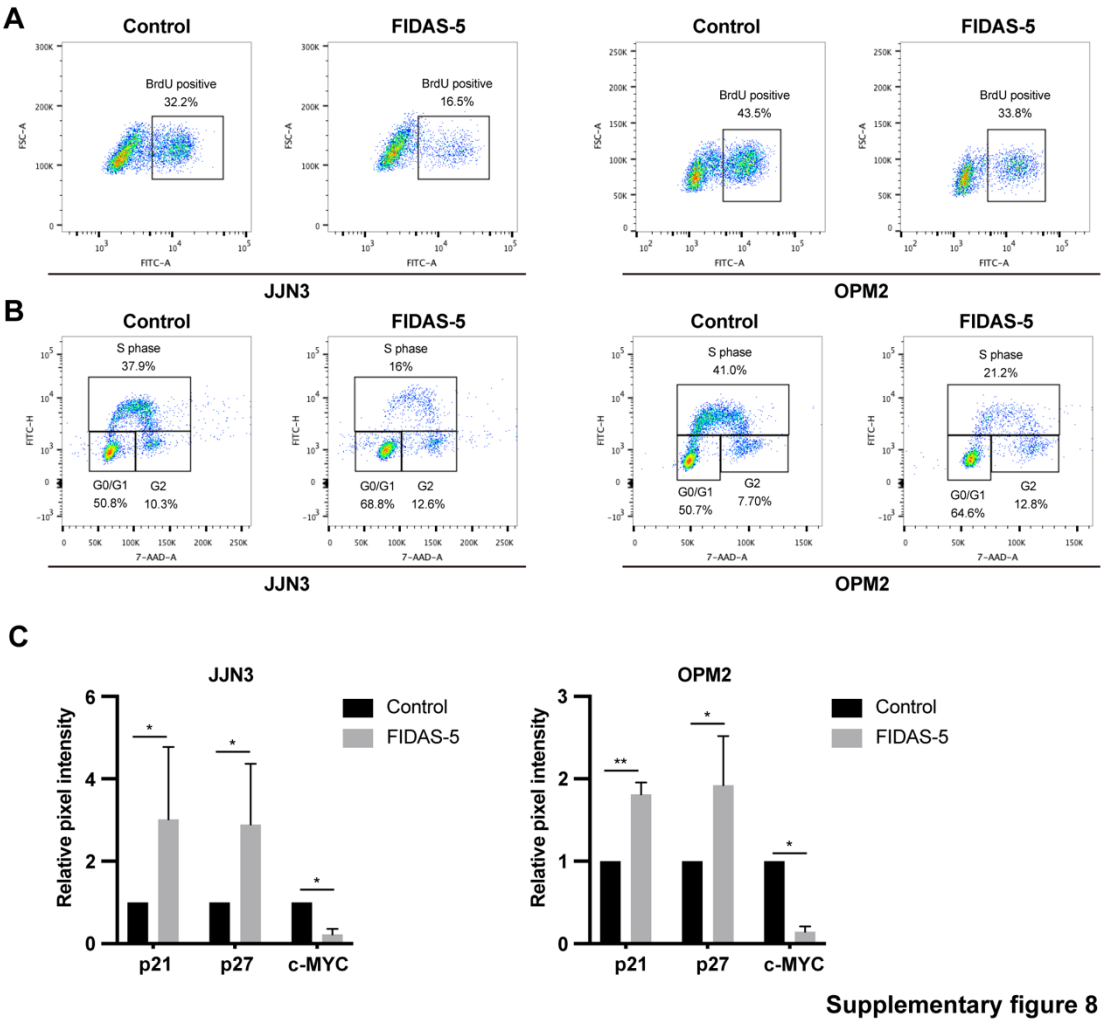


Figure S8: A-B Representative images of the flow cytometric analysis for proliferation and cell cycle (For Figure 4C-D). **C** The pixel intensity of Figure 4E measured by Image Studio Lite v5.2. Significance was determined by Mann–Whitney U test. * $P \leq 0.05$, ** $P \leq 0.01$.

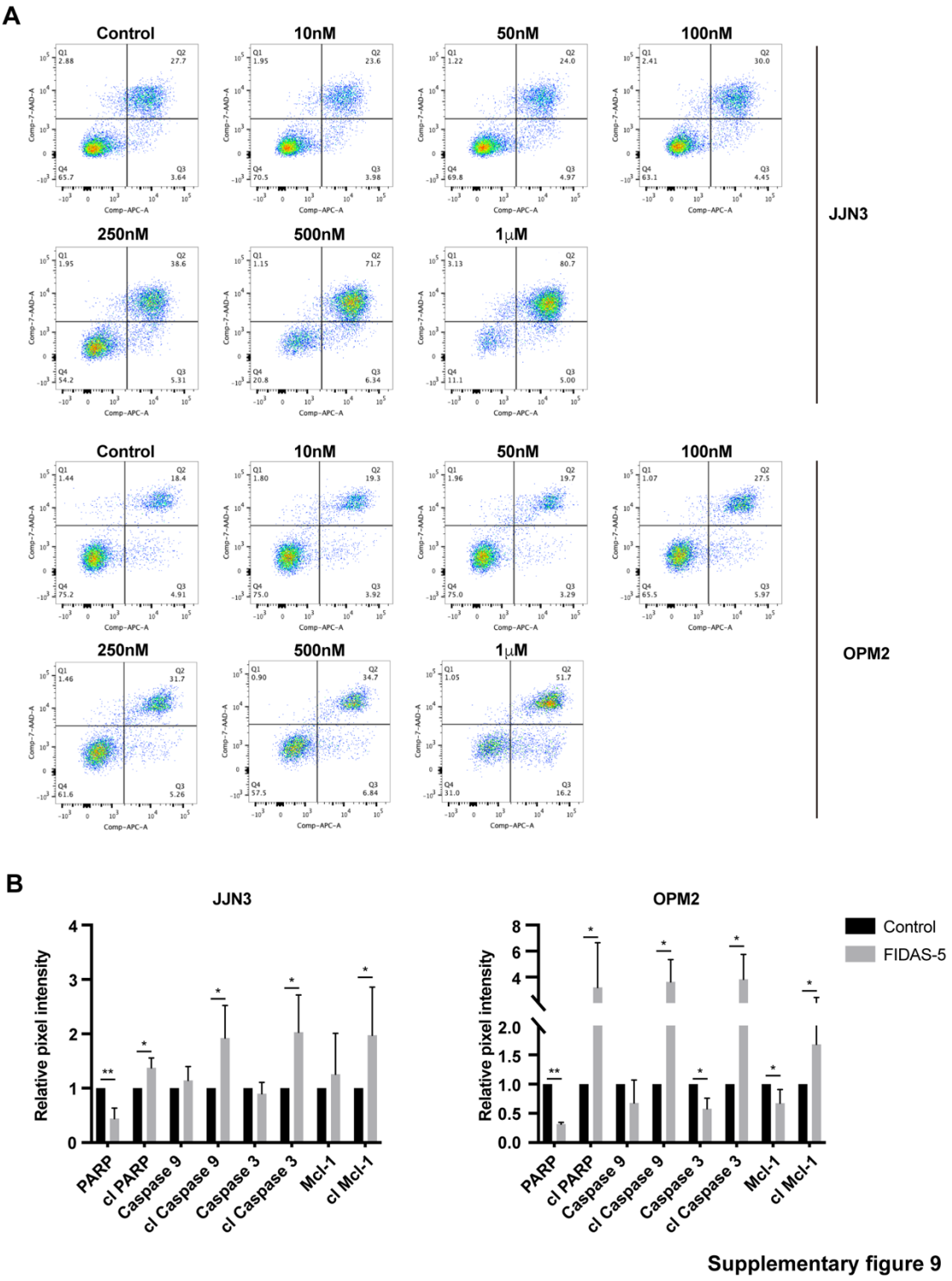


Figure S9: A Representative images of the flow cytometric analysis for cell apoptosis (For Figure 4F). **B** The pixel intensity of Figure 4G measured by Image Studio Lite v5.2. Significance was determined by Mann–Whitney U test. * $P \leq 0.05$, ** $P \leq 0.01$.

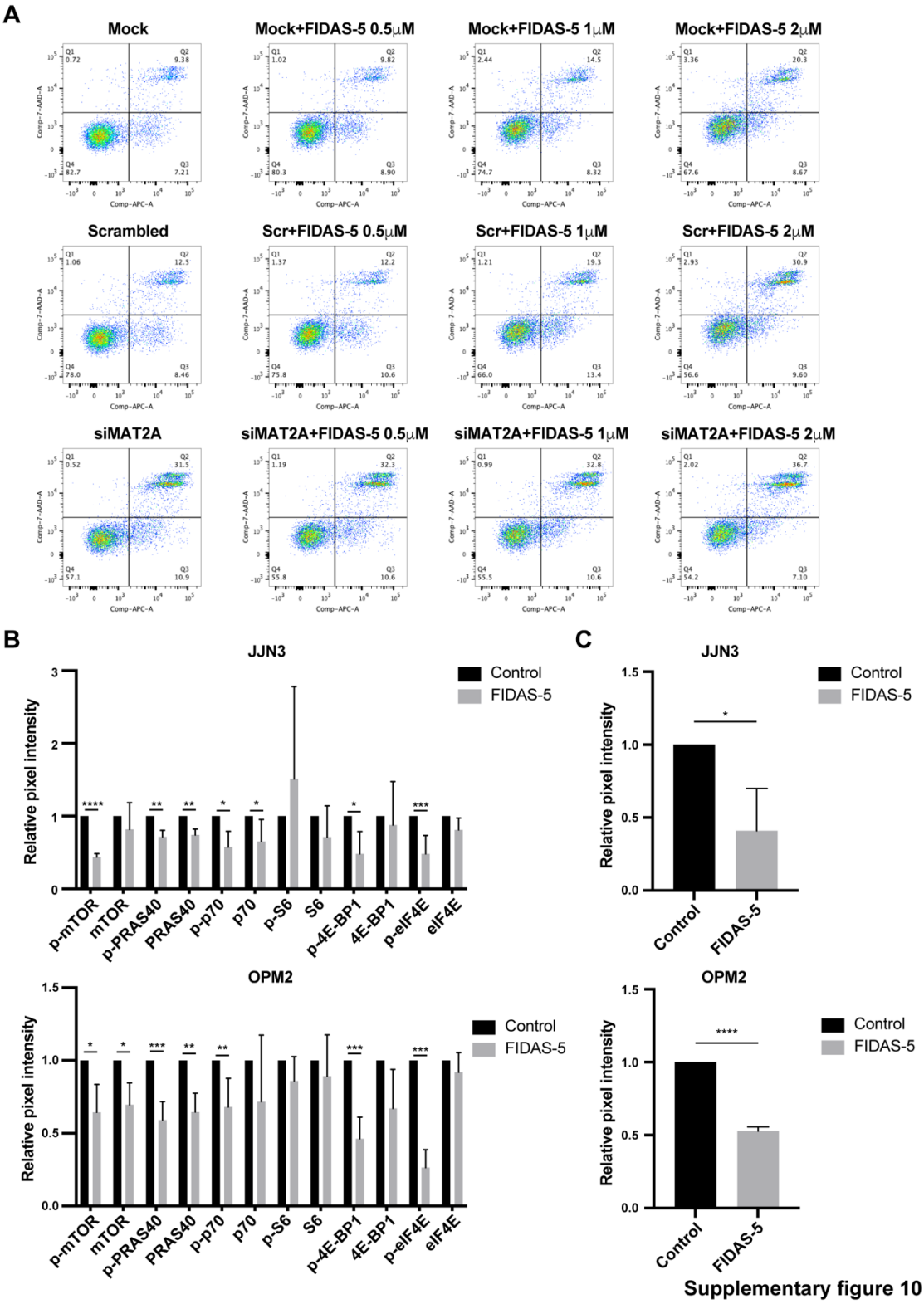


Figure S10: A Representative images of the flow cytometric analysis for cell apoptosis (For Figure 5B). **B-C** The pixel intensity of Figure 5C (**B**) and Figure 5D (**C**) was measured by Image Studio Lite v5.2. Significance was determined by one-way ANOVA. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

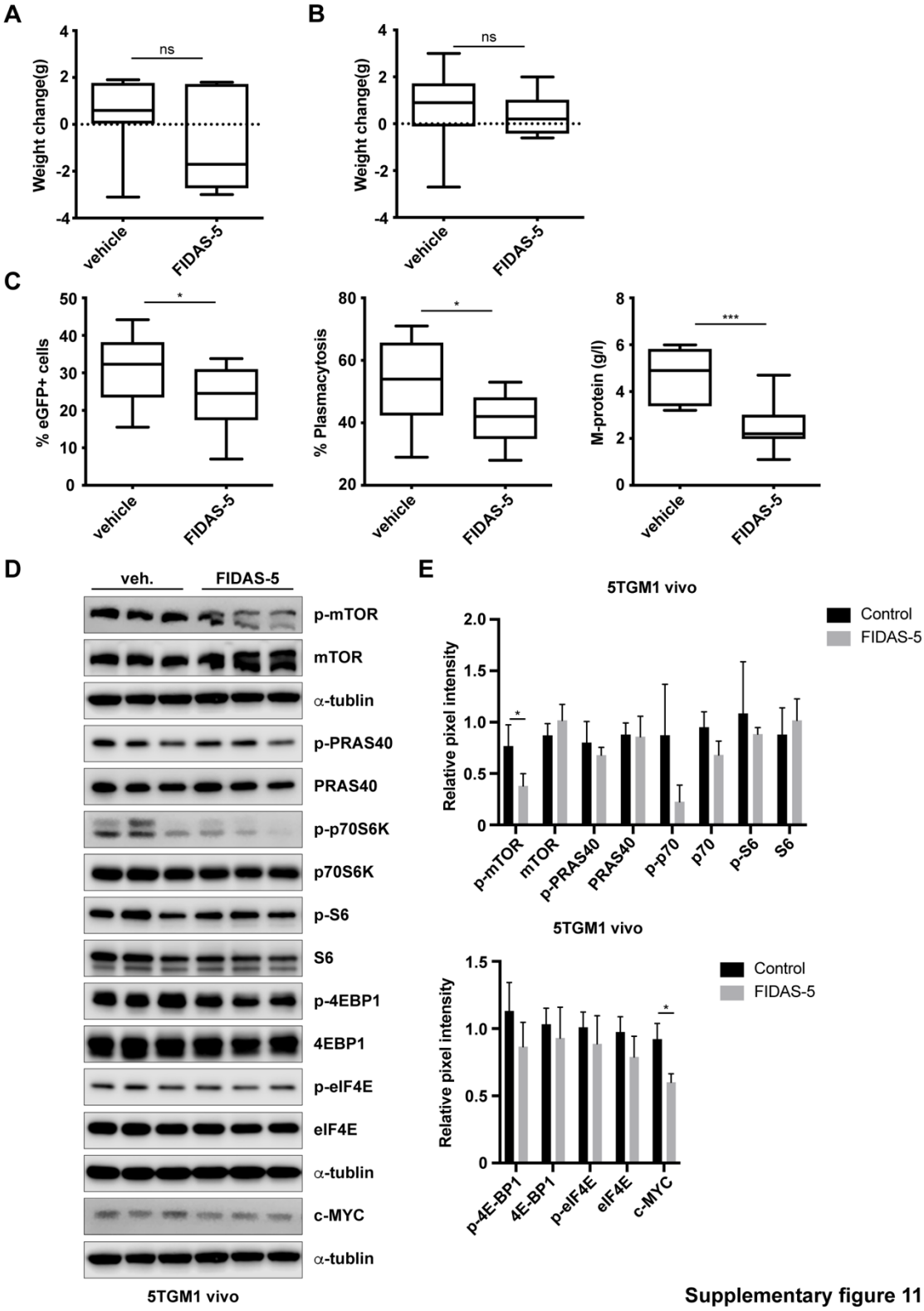


Figure S11: MAT2A inhibition by FIDAS-5 reduces tumor burden *in vivo.* In the repeat experiment, the mice received the same treatment as in the first *in vivo* experiment. **A-B** The weight changes of the mice at end-stage disease relative to day 0 (**A**, first experiment; **B**, repeated experiment). **C** Tumor burden was analyzed by assessing the percentage of eGFP positive cells using flow cytometry, BM plasmacytosis on cytosmears and the amount of M-

protein in murine serum. **D-E** Western blot analysis of protein synthesis-related proteins. Bar graphs represent the pixel intensity as measured by Image Studio Lite v5.2. Significance was determined by Mann–Whitney U test. ns, non-significant, * $P \leq 0.05$, *** $P \leq 0.001$.

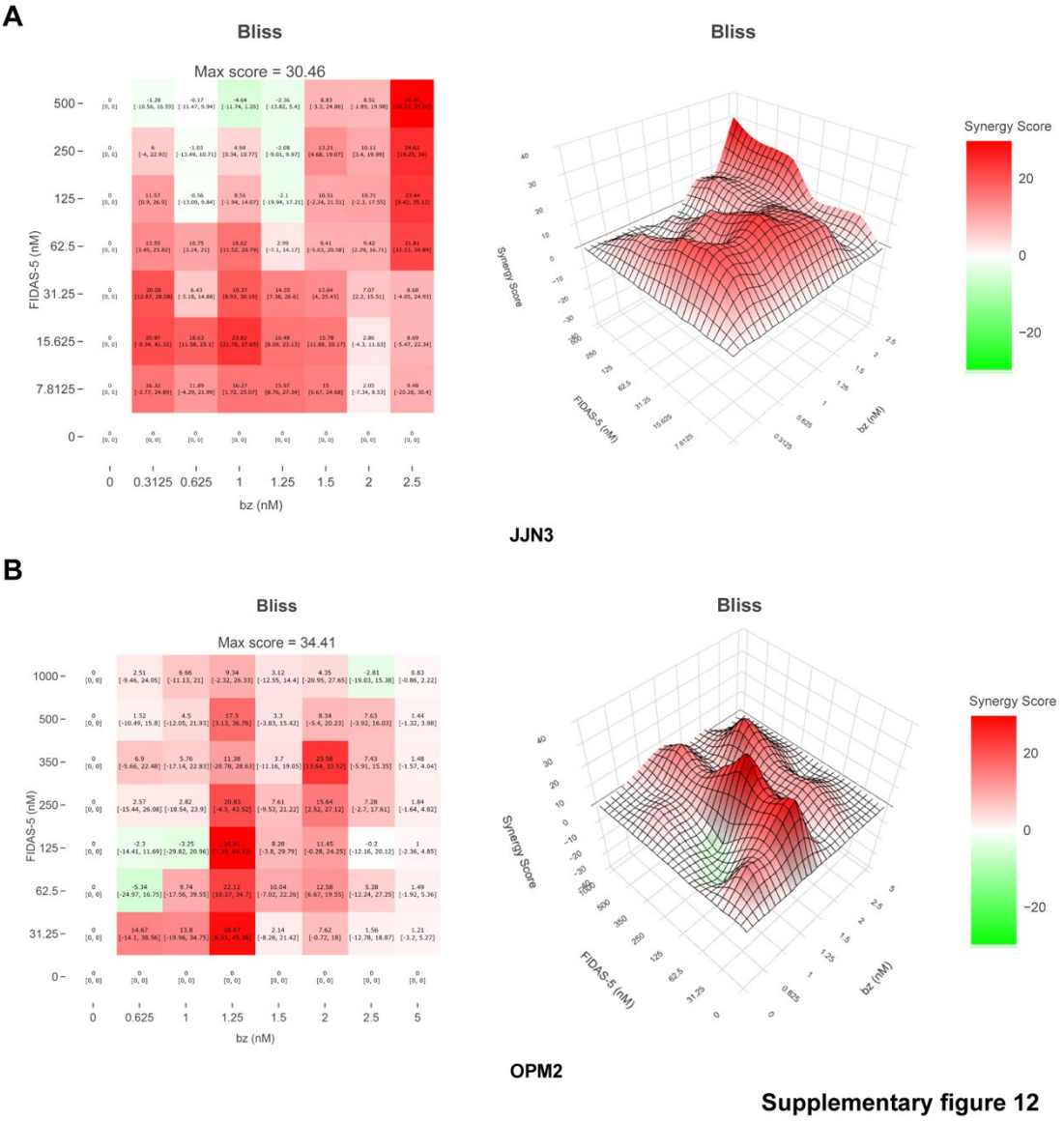
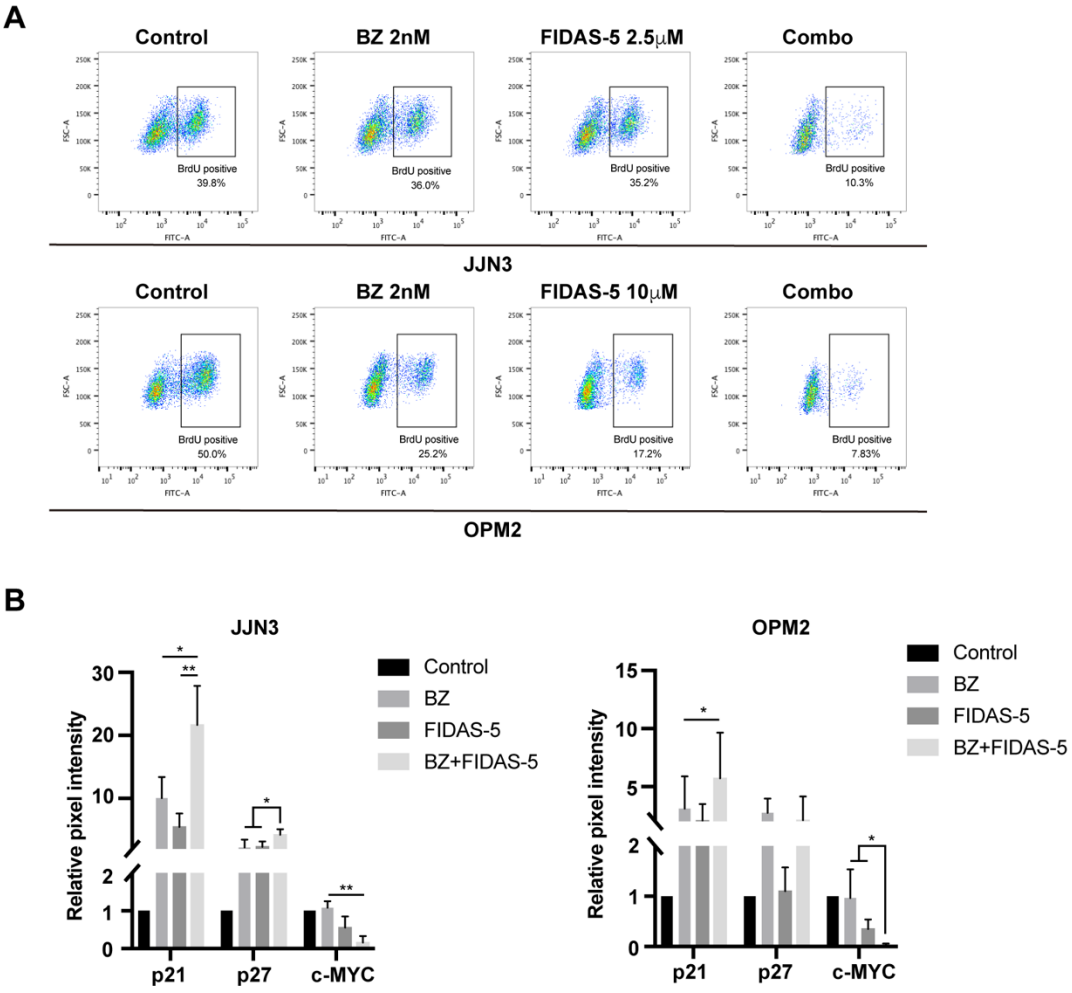
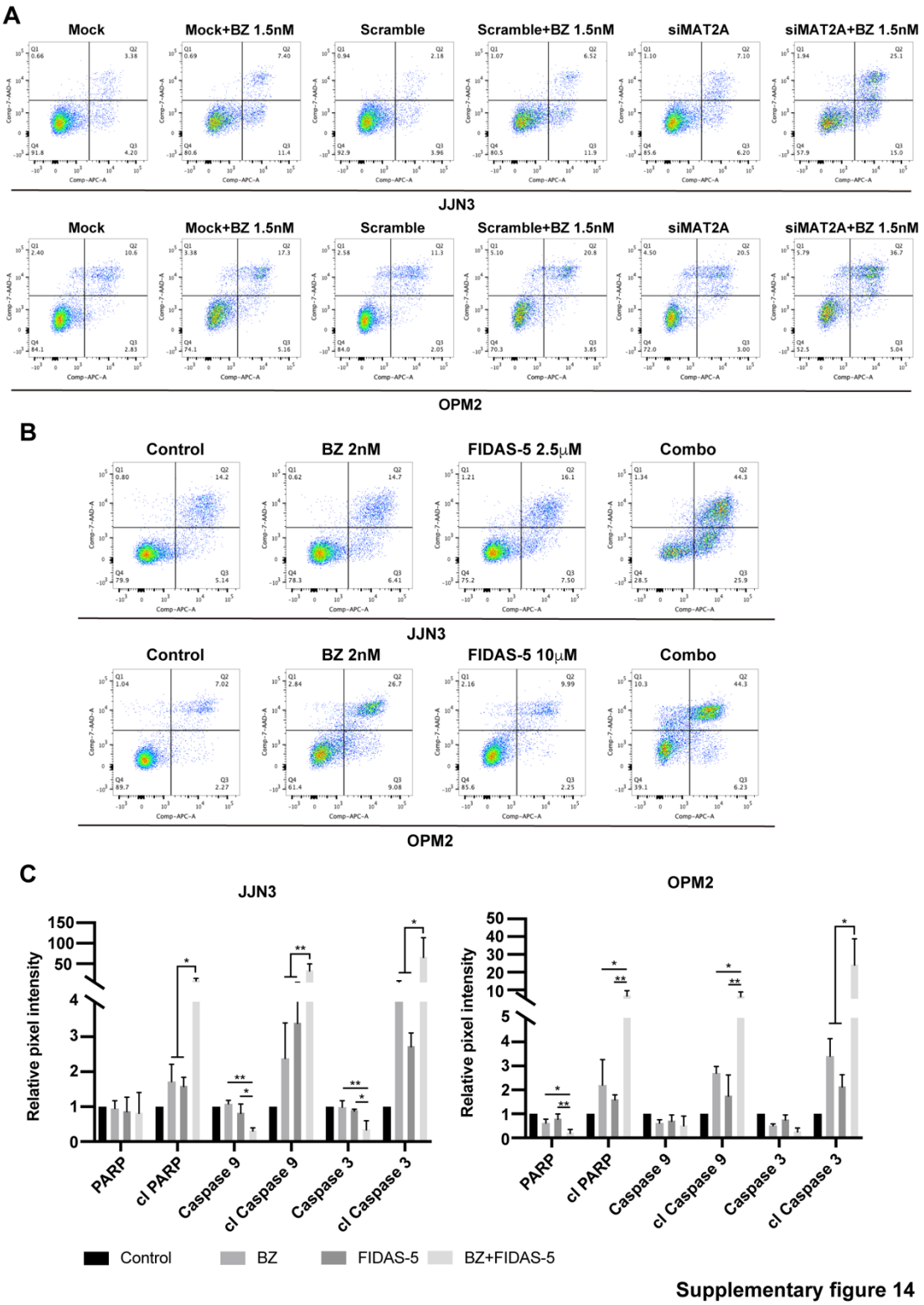


Figure S12: FIDAS-5 and BZ exert synergistic anti-tumor effects on MM cells. MM cells were treated with a concentration range of FIDAS-5 and/or BZ for four days. Next, viability of JJN3 (**A**) and OPM2 (**B**) cells was measured by a CellTiter-Glo luminescent assay and Bliss summary synergy scores were calculated. The 2D (Left) and 3D (Right) synergy maps highlight synergistic and antagonistic dose regions in red and green colors, respectively. Synergy scores above 10 are considered synergic interactions.



Supplementary figure 13

Figure S13: A Representative images of the flow cytometric analysis for cell proliferation (For Figure 8C). **B** The pixel intensity of Figure 8D was measured by Image Studio Lite v5.2. Significance was determined by one-way ANOVA. * $P \leq 0.05$, ** $P \leq 0.01$.



Supplementary figure 14

Figure S14: A-B Representative images of the flow cytometric analysis for cell apoptosis (For Figure 8E-F). **C** The pixel intensity of Figure 8G was measured by Image Studio Lite v5.2. Significance was determined by one-way ANOVA. * $P \leq 0.05$, ** $P \leq 0.01$.