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

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Abstract

Multiple myeloma (MM) is the second most prevalent hematologic malignancy and is incurable because of the inevitable development of drug resistance. Methionine adenosyltransferase 2α (MAT2A) is the primary producer of the methyl donor S-adenosylmethionine (SAM) and several studies have documented MAT2A deregulation in different solid cancers. As the role of MAT2A in MM has not been investigated yet, the aim of this study was to clarify the potential role and underlying molecular mechanisms of MAT2A in MM, exploring new therapeutic options to overcome drug resistance. By analyzing publicly available gene expression profiling data, MAT2A was found to be more highly expressed in patient-derived myeloma cells than in normal bone marrow plasma cells. The expression of MAT2A correlated with an unfavorable prognosis in relapsed patients. MAT2A inhibition in MM cells led to a reduction in intracellular SAM levels, which resulted in impaired cell viability and proliferation, and induction of apoptosis. Further mechanistic investigation demonstrated that MAT2A inhibition inactivated the mTOR-4EBP1 pathway, accompanied by a decrease in protein synthesis. MAT2A targeting *in vivo* with the small molecule compound FIDAS-5 was able to significantly reduce tumor burden in the 5TGM1 model. Finally, we found that MAT2A inhibition can synergistically enhance the anti-MM effect of the standard-of-care agent bortezomib on both MM cell lines and primary human CD138$^+$MM cells. In summary, we demonstrate that MAT2A inhibition reduces MM cell proliferation and survival by inhibiting mTOR-mediated protein synthesis. Moreover, our findings suggest that the MAT2A inhibitor FIDAS-5 could be a novel compound to improve bortezomib-based treatment of MM.

Introduction

Multiple myeloma (MM) is a hematologic malignancy characterized by the accumulation of malignant plasma cells in the bone marrow. MM is the second most frequent hematologic disorder and accounts for 10% of all hematologic malignancies.[1,2] Several drugs have already been approved to treat MM, including proteasome inhibitors, immunomodulatory drugs, steroids, histone deacetylase inhibitors and monoclonal antibodies.[3] Bortezomib was the first proteasome inhibitor approved by the US Food and Drug Administration for the treatment of MM and is currently one of the standard-of-care agents for first-line treatment.[4] However, despite the significant progress in treating MM patients, one of the major issues is the recurrence of the disease due to an incomplete eradication of myeloma cells.[5,6] Thus, therapies capable of inducing durable elimination of MM cells are still needed.

Recent studies provide evidence that dysregulation of cellular metabolism plays a central role in the pathogenesis of MM, including MM cell survival, growth, and drug resistance.[7-9] Methionine adenosyltransferase (MAT) is a key regulator of cellular metabolism and catalyzes the reaction of L-methionine and adenosine triphosphate (ATP) to S-adenosylmethionine (SAM), which is an essential methyl donor.[10] MAT includes MAT1A and MAT2A in mammals, which code for two different enzymes, MATI/III and MATII respectively. MAT1A is liver-specific, whereas MAT2A shows a wide distribution and is responsible for SAM synthesis in extrahepatic tissues.[11] MAT2A is dysregulated in

several cancer types, such as hepatic cancer, breast cancer, and colon cancer and it has been shown that silencing of MAT2A results in reduced cancer cell proliferation and cell death.[12-14] However, so far, the role of MAT2A in MM has not been explored.

A high rate of synthesis of immunoglobulins is a key feature of MM cells. Accordingly, MM cells are characterized by an expanded endoplasmic reticulum and partial activation of unfolded protein response pathways that contribute to the cells' excessive protein production.[15,16] The kinase mammalian target of rapamycin (mTOR) is the core component of two protein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2, and is a member of the family of phosphoinositide 3-kinase (PI3K)-related kinases.[17] The mTOR signaling pathway, which is regularly activated in tumors, not only plays an important role in tumor metabolism, but also regulates gene transcription and protein synthesis to regulate cell proliferation.[18-20] The inhibitory eukaryotic translation initiation factor 4E binding proteins 1 and 2 (4E-BP1 and 2) are phosphorylated by mTORC1, resulting in the release of the eukaryotic translation initiation factor 4E (eIF4E). By assisting in the formation of the translation initiation complex at the 5' ends of mRNA, the released eIF4E then enhances global translation.[21,22] Furthermore, mTORC1 phosphorylates the ribosomal proteins p70 S6 kinase (p70S6K) and S6, which are necessary for the positive regulation of the eukaryotic initiation factors 4A and 4B to augment translation.[23] MAT2A has been shown to impact different (patho)physiological processes (including protein synthesis) depending on the cell type.[24] Whether this is the case for MM cells remains to be determined.

In this study, we aimed to uncover the role of MAT2A in MM survival and response to drugs. We investigated the relationship between MAT2A expression and survival using publicly available gene expression profiling data. By using MAT2A-specific short interfering (si)RNA, we then studied the impact of MAT2A targeting on MM cell viability, proliferation, apoptosis, mTOR signaling and protein synthesis. To validate the therapeutic potential of targeting MAT2A further, we used the small molecule inhibitor FIDAS-5 to target MAT2A both *in vitro* and *in vivo*. Finally, we investigated whether the MAT2A inhibitor could increase the efficacy of bortezomib.

Methods

Cells and cell culture

Seven human myeloma cell lines (HMCL), ANBL6, AMO-1, JJN3, LP-1, OPM2, RPMI8226, and U266, and the human bone marrow stromal cell line HS-5 were obtained from the American Type Culture Collection. Two other HMCL, XG-2 and XG-7, were kindly provided by J. Moreaux (University of Montpellier, Montpellier, France). The HMCL and murine MM cell line 5TGM1 were cultured in RPMI1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) and HS-5 was cultured in DMEM (Thermo Fisher Scientific). All the cell cultures were supplemented with 100 U/mL penicillin/streptomycin, 2 mmol/L glutamine (Thermo Fisher Scientific), and 10% fetal calf serum (Hycone, Logan, UT, USA). Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2. All cell lines were authenticated by short-tandem repeat profiling and regularly tested to rule out contamination by mycoplasma.

Compounds

Bortezomib was purchased from Selleckchem (Munich, Germany), FIDAS-5 was purchased from Millipore Sigma (St. Louis, MO, USA) and MAT2A inhibitor 1 (Compound 196) was purchased from MedChemExpress (Monmouth Junction, NJ, USA). For *in vitro* studies, all compounds were dissolved in dimethylsulfoxide according to the manufacturer's instructions. For the murine experiments, FIDAS-5 was dissolved in polyethylene glycol 400 (Sigma-Aldrich, St Louis, MO, USA).

Murine experiments

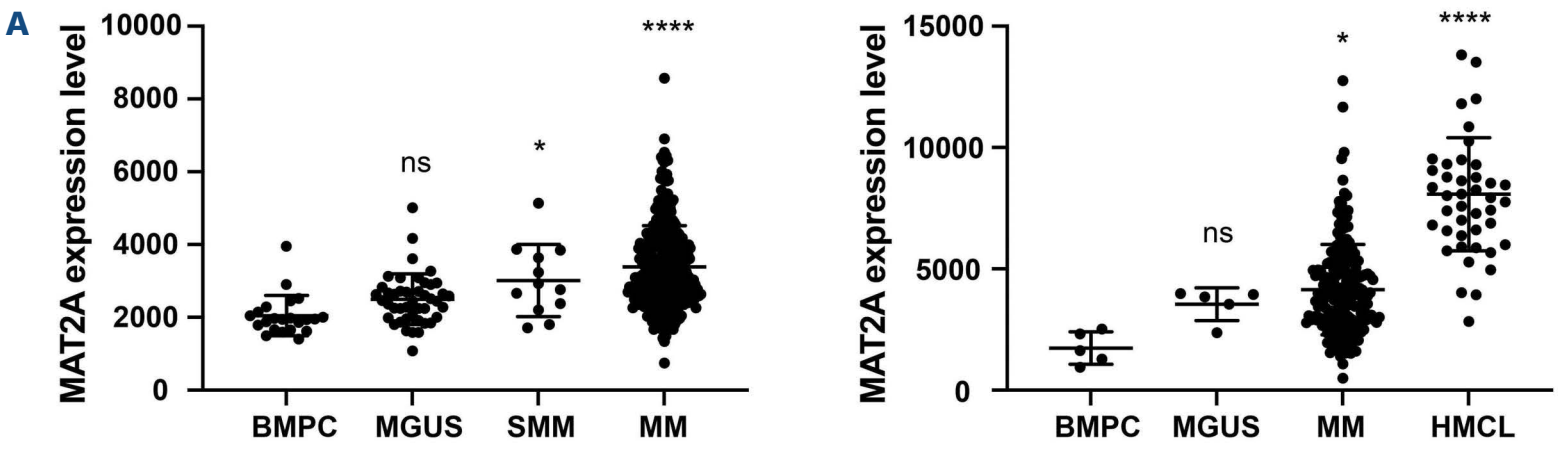
C57BL/KalwRij mice were purchased from Envigo Laboratories (Horst, the Netherlands). They were housed and treated following conditions approved by the Ethical Committee for Animal Experiments of the Vrije Universiteit Brussel (CEP N 20-281-6). On day 0, mice were injected intravenously with 1×10^6 5TGM1-eGFP-positive cells dissolved in 200 μL of RPMI-1640 medium. On day 3, mice were treated intraperitoneally with either vehicle or 20 mg/kg FIDAS-5 three times a week. After 28 days, all mice were sacrificed when the vehicle-treated mice had reached end-stage. We isolated bone marrow from the spine and legs, and then lysed the red blood cells. Tumor burden in the bone marrow was assessed by determining the percentage of eGFP-positive cells by flow cytometry and the percentage plasmacytosis on cytospins using May-Grünwald-Giemsa staining. Additionally, serum protein electrophoresis was performed to assess the serum M spike.

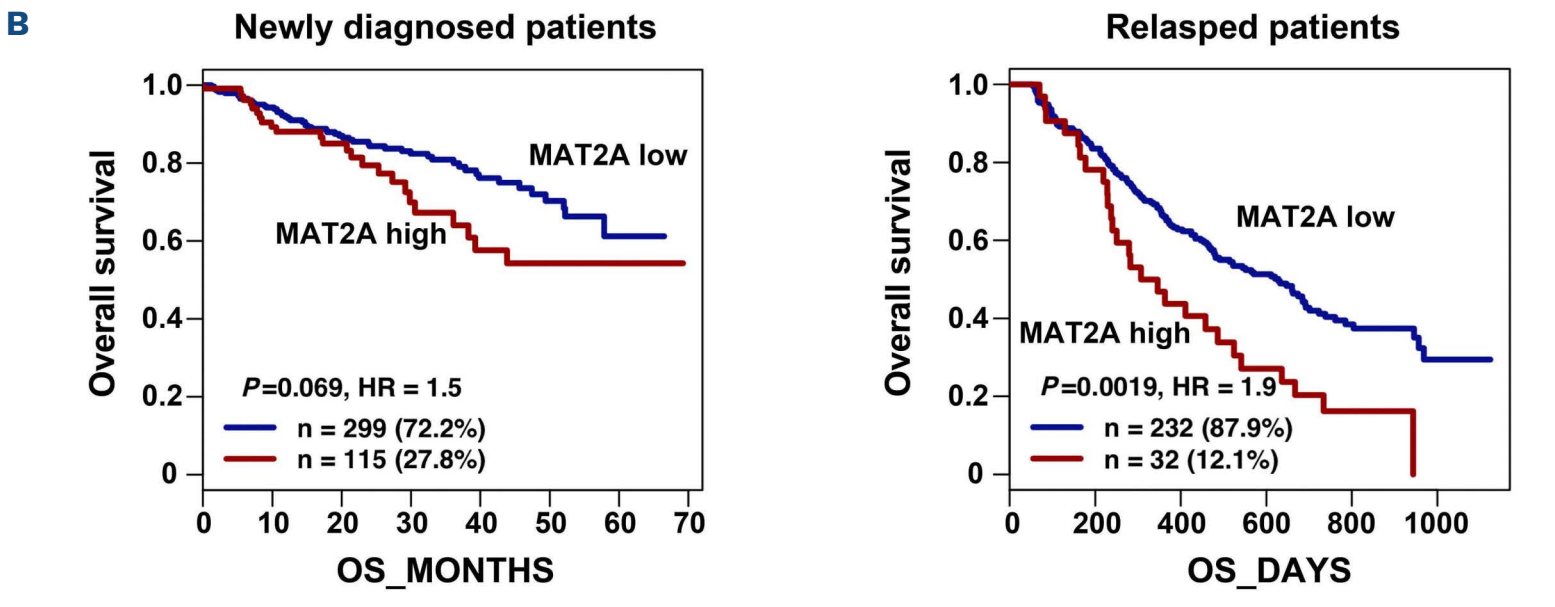
Statistical analysis

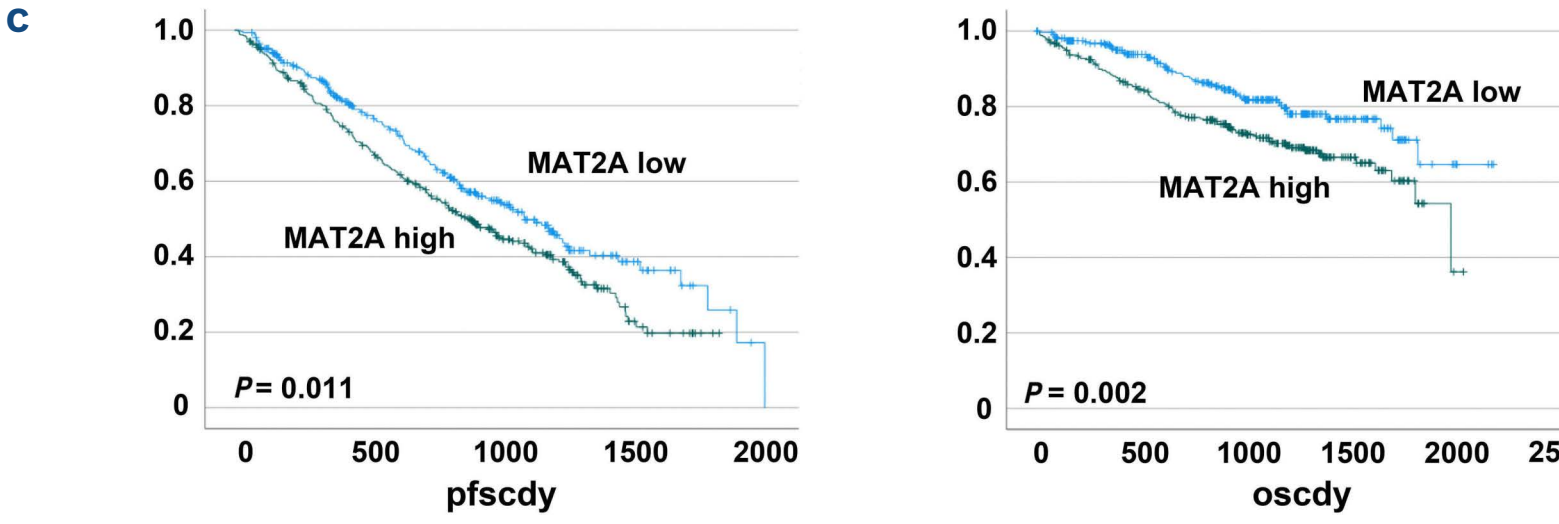
All results presented in this study were acquired from at least three independent experiments and analyzed with GraphPad Prism 9.0 software (GraphPad Software Inc, La Jolla, CA, USA). The data represent the mean ± standard deviation, and results were analyzed using the Mann–Whitney U non-parametric test or one-way analysis of variance for multiple testing. Values of $*P<0.05$, $**P<0.01$, $***P<0.001$ and $****P<0.0001$ were considered statistically significant.

Other methods

The *Online Supplementary Information* contains details on the other methods.







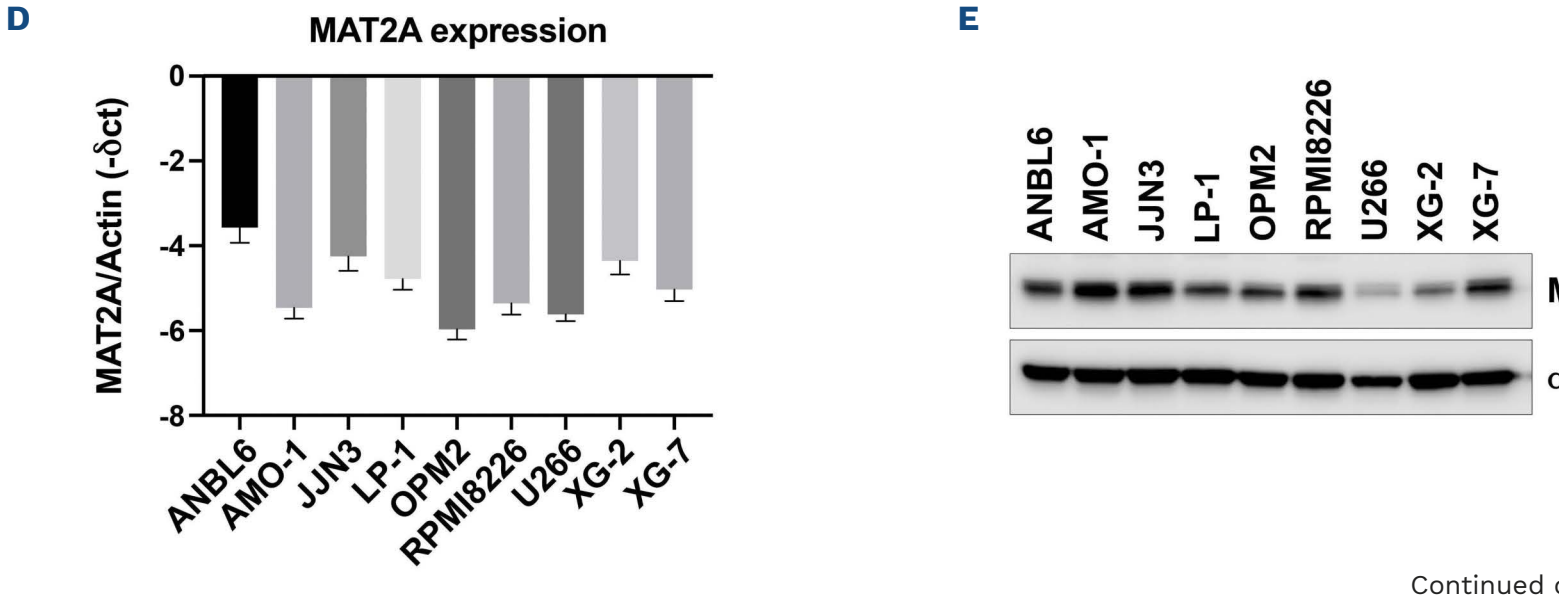


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Figure 1. Expression and prognostic value of MAT2A in multiple myeloma. (A) *Left side*. MAT2A mRNA expression levels in normal bone marrow plasma cells (BMPC; N=22), cells from patients with monoclonal gammopathy of undetermined significance (MGUS; (N=44), or smoldering multiple myeloma (SMM; N=12), and multiple myeloma (MM) cells from newly diagnosed patients (N=345) from the UAMS TT2 cohort. *Right side*. MAT2A mRNA expression levels in normal bone marrow plasma cells (N=5), MGUS (N=5), primary MM cells from newly diagnosed patients (N=206), and human MM cell lines (N=42) from the Heidelberg-Montpellier cohort. ns, non-significant; *P<0.05; ****P<0.0001. (B) Prognostic value of MAT2A mRNA levels in terms of overall survival in newly diagnosed patients from the UAMS TT2 (N=256), and UAMS TT3 (N=158) cohort and in relapsed MM patients from the Mulligan cohort (N=264). Maxstat analysis was used to calculate the optimal separation of patients based on a cutoff value. (C) Overall survival and progression-free survival curves for newly diagnosed MM patients with high or low MAT2A RNA expression (MMRF CoMMpass trial, N=653). The Kaplan-Meier method was used to plot survival curves and group comparisons were made using the log-rank test. P<0.05 was considered as statistically different. (D, E) Basal mRNA (D) and protein (E) expression of MAT2A in nine human MM cell lines (ANBL6, AMO-1, JJN3, LP-1, OPM2, RPMI8226, U266, XG-2 and XG-7). HR: hazard ratio; OS: overall survival; PFS: progression-free survival.

Results

High expression of MAT2A is associated with progression of multiple myeloma and a worse outcome

To determine the role of MAT2A in MM, we first analyzed the mRNA expression levels of MAT2A in healthy donors and at different stages of MM disease using both publicly available and our own gene expression profiling data. We found that MAT2A expression was significantly upregulated in malignant plasma cells compared to normal bone marrow plasma cells and that MAT2A was even more highly expressed in HMCL (Figure 1A). By comparing MAT2A expression in the different molecular subgroups of MM, we observed a significantly elevated MAT2A mRNA level in the hyperdiploidy and the proliferation groups (*Online Supplementary Figure S1A*). Next, we investigated the relation between MAT2A mRNA expression and overall survival of MM patients using a cohort of newly diagnosed MM patients (TT2/TT3 cohort) and a cohort of relapsed MM patients (Mulligan cohort). In the cohort of relapsed patients, high MAT2A expression was found to be associated with a significantly worse overall survival, whereas no significant relation was observed in the newly diagnosed patients (Figure 1B). Nevertheless, after assessing the more recent RNA sequencing data from the CoMMpass trial, we did find a significant correlation between high mRNA expression of MAT2A and shorter progression-free survival and overall survival (Figure 1C). The prognostic value of high MAT2A gene expression was also validated in our own cohort of newly diagnosed MM patients in whom RNA sequencing was performed (*Online Supplementary Figure S1B*). We next determined MAT2A expression in a selected panel of HMCL using quantitative real-time polymerase chain reaction (qRT-PCR) and western blotting and confirmed that MAT2A is highly expressed in HMCL both on the mRNA and protein levels (Figure 1D, E). Together, these data show that MAT2A is highly expressed in MM patients and HMCL, correlating with an unfavorable prognosis. This indicates that MAT2A could be a novel promising target in MM.

Silencing of MAT2A impairs multiple myeloma cell viability and proliferation and induces apoptosis

To investigate the role of MAT2A in MM *in vitro*, we selected the JJN3 and OPM2 cell lines for further *in vitro* experiments, as these cell lines showed differential MAT2A expression. MAT2A-specific siRNA transfection was performed to inhibit MAT2A gene expression in MM cells. The silencing efficiency was confirmed by detecting MAT2A expression using qRT-PCR and western blotting (*Online Supplementary Figure S1C-E*). MAT2A expression was inhibited by 40% in JJN3 cells and by 70% in OPM2 cells. Next, we evaluated the long-term effects of MAT2A inhibition, as we assumed that it would take some time for downstream effects to come into play.[24] We found that silencing of MAT2A significantly reduced cell viability after 5 days, compared to scrambled siRNA, by 21% in JJN3 and 30% in OPM2 cells (Figure 2A). Furthermore, bromodeoxyuridine (BrdU) incorporation revealed that targeting MAT2A by siMAT2A was able to decrease proliferation in JJN3 cells from 55% to 45% and in OPM2 cells from 55% to 41% (Figure 2B; *Online Supplementary Figure S2A*). To better understand the effect on proliferation, we then analyzed cell cycle progression using propidium iodide staining. We found that the percentages of MM cells in the G0/G1 and G2 phases were modestly but significantly higher after MAT2A inhibition in the JJN3 and OPM2 cells, thereby confirming our previous results (Figure 2C; *Online Supplementary Figure S2B*). Finally, MAT2A interference also induced apoptosis in both cell lines, as evidenced by a significant increase in the number of annexin V and/or 7-amino-actinomycin D-positive cells (in JJN3 cells from 23% to 38% and in OPM2 cells from 11% to 18%) (Figure 2D; *Online Supplementary Figure S2C*) and increased cleavage of the apoptosis markers poly ADP ribose protein (PARP), caspase 9, and caspase 3 (Figure 2E; *Online Supplementary Figure S3A*). We further confirmed the anti-MM effect of MAT2A depletion by using OPM2 cells transduced with either MAT2A-short hairpin (sh)RNA or scrambled shRNA lentiviruses (*Online Supplementary Figure S4A-F*). Together, these data prove that MAT2A plays a crucial role in MM cell proliferation, cell cycle progression and survival.

MAT2A depletion impairs mTOR-regulated protein synthesis

Next, we investigated the underlying mechanisms contributing to the inhibition of MM cell proliferation and sur-

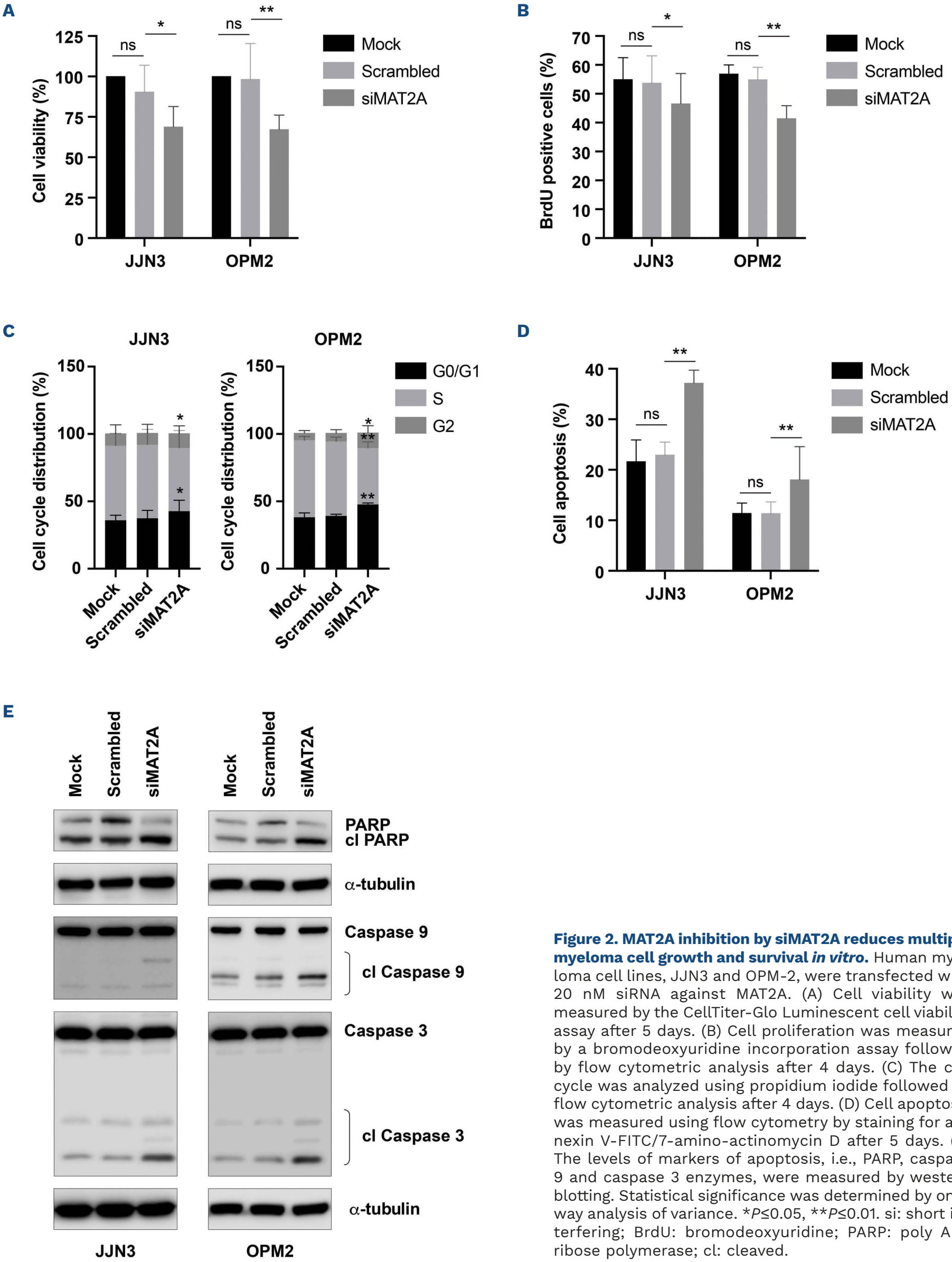


Figure 2. MAT2A inhibition by siMAT2A reduces multiple myeloma cell growth and survival *in vitro*. Human myeloma cell lines, JJN3 and OPM-2, were transfected with 20 nM siRNA against MAT2A. (A) Cell viability was measured by the CellTiter-Glo Luminescent cell viability assay after 5 days. (B) Cell proliferation was measured by a bromodeoxyuridine incorporation assay followed by flow cytometric analysis after 4 days. (C) The cell cycle was analyzed using propidium iodide followed by flow cytometric analysis after 4 days. (D) Cell apoptosis was measured using flow cytometry by staining for annexin V-FITC/7-amino-actinomycin D after 5 days. (E) The levels of markers of apoptosis, i.e., PARP, caspase 9 and caspase 3 enzymes, were measured by western blotting. Statistical significance was determined by one-way analysis of variance. **P*≤0.05, ***P*≤0.01. si: short interfering; BrdU: bromodeoxyuridine; PARP: poly ADP ribose polymerase; cl: cleaved.

vival upon MAT2A blockade. Since the main biological function of MAT2A is the synthesis of the methyl donor SAM, we first determined SAM levels using liquid chromatography-mass spectrometry (LC-MS). We found that silencing MAT2A did indeed lower SAM levels, suggesting inhibited SAM synthesis in OPM2 cells (Figure 3A). Importantly, recent research showed that SAM synthesis is required to support protein synthesis and tumor growth,

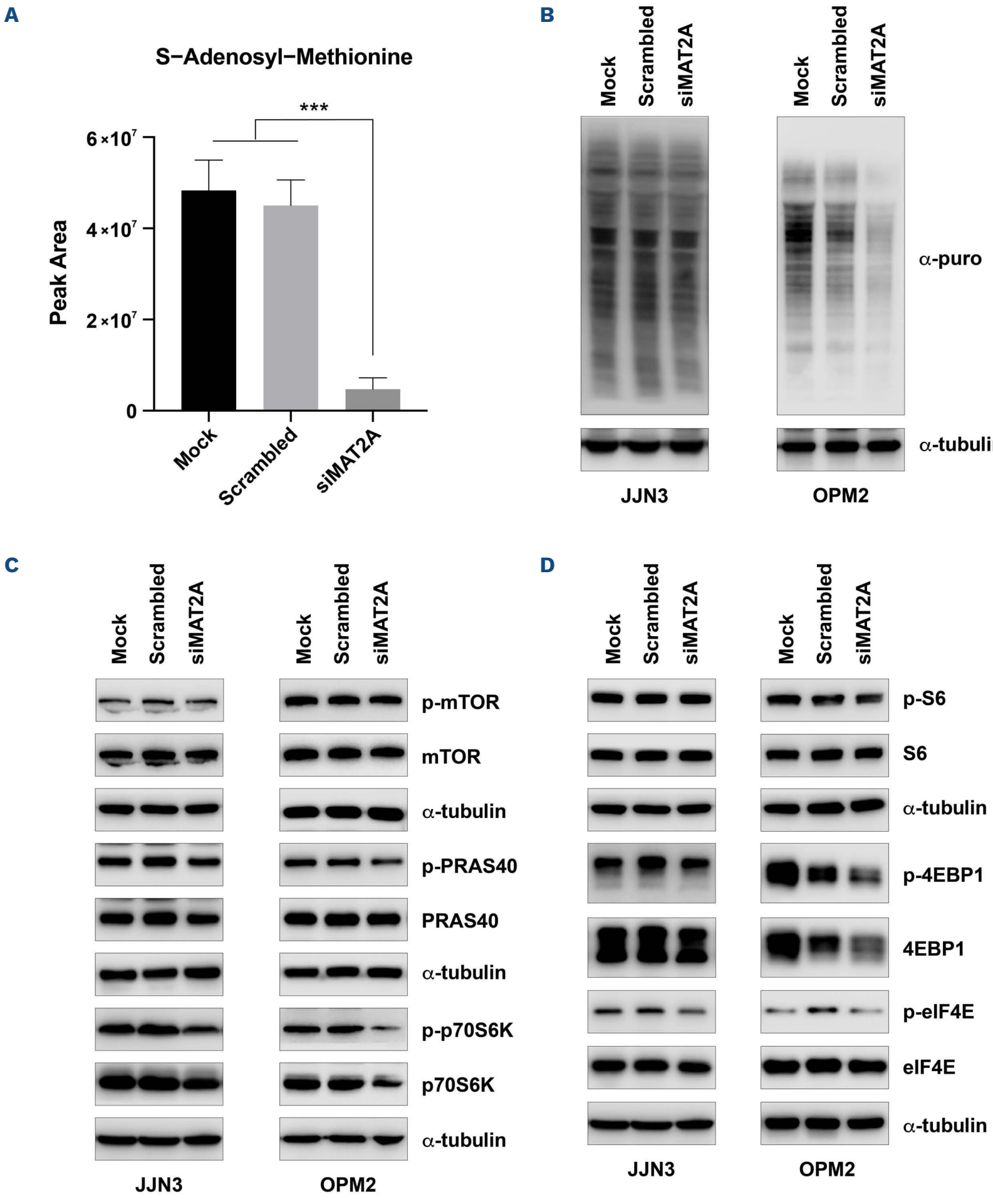
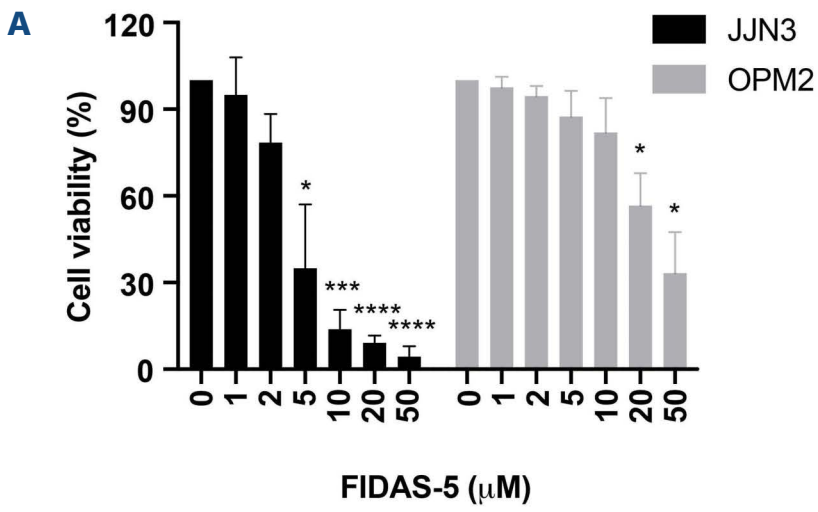
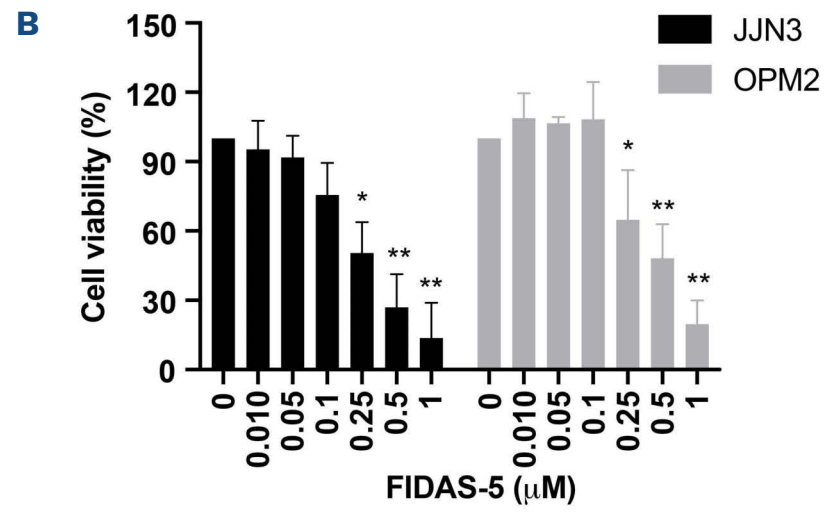
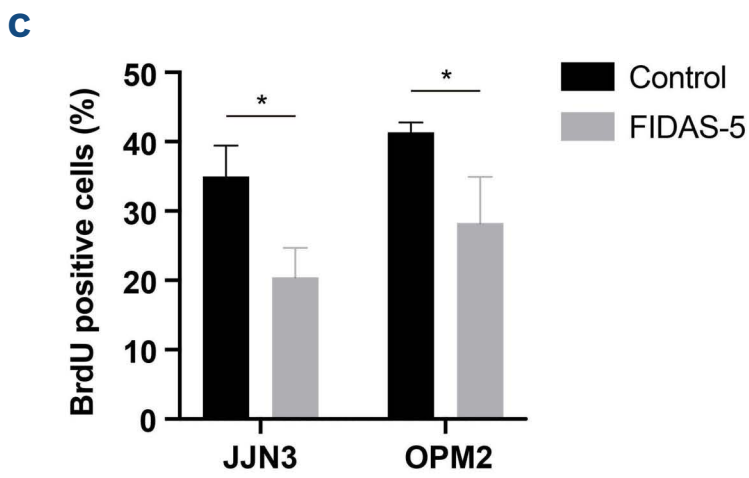
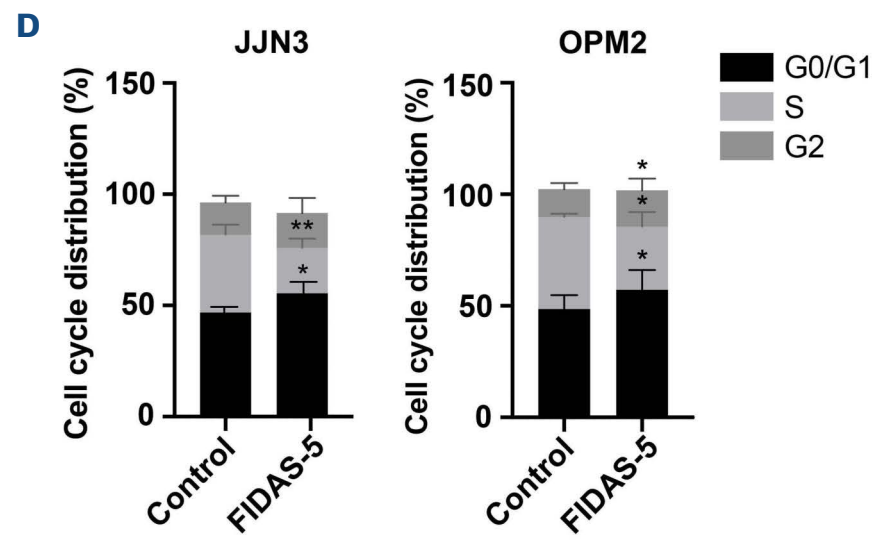


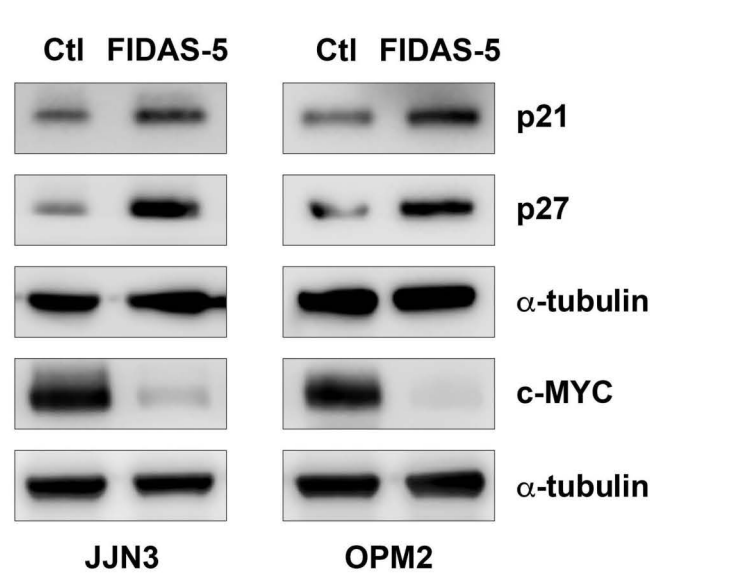
Figure 3. MAT2A interference inhibits protein synthesis by downregulating the mTOR pathway. (A) OPM2 cells were treated with 20 nM siMAT2A for 3 days and s-adenosyl-methionine concentration levels were measured by liquid chromatography-mass spectrometry. Significance was determined by one-way analysis of variance. (B) Western blot analysis of puromycin uptake after MAT2A knockdown for 4 days in JJN3 and OPM2 cells. (C) Western blot analysis of protein synthesis-related proteins, isolated after 4 days of MAT2A knockdown. ***$P \leq 0.001$. si: short interfering; α-puro: α-puromycin.

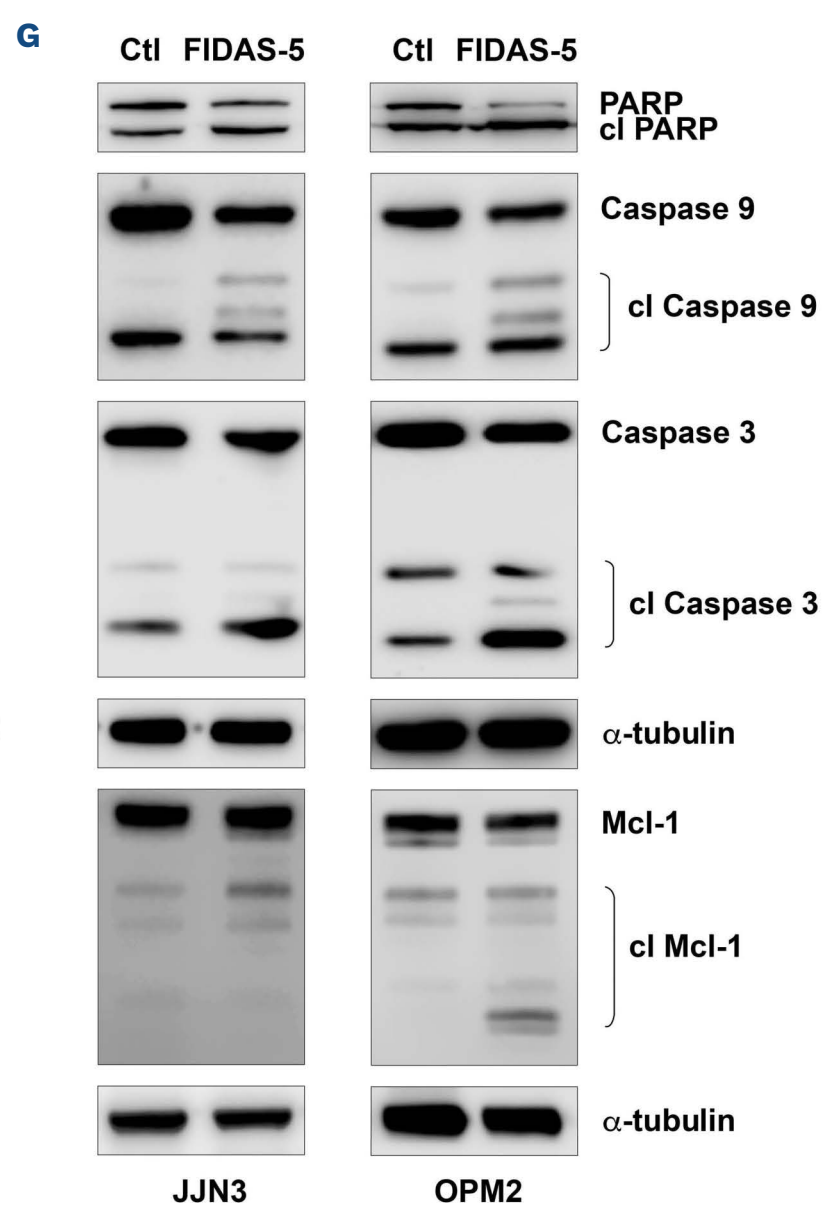












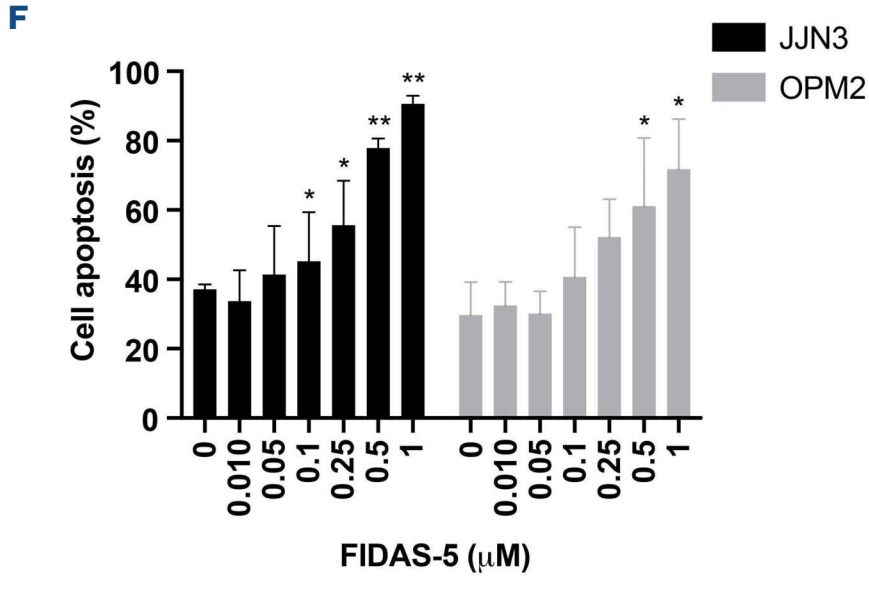


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Figure 4. MAT2A inhibition by FIDAS-5 reduces multiple myeloma cell survival *in vitro*. (A, B) JJN3 and OPM2 cells were treated with increasing doses of FIDAS-5 for 48 h (A) or 5 days (B) and cell viability was measured by a CellTiter-Glo Luminescent cell viability assay. (C) Cell proliferation was measured using a bromodeoxyuridine incorporation assay followed by flow cytometric analysis after treatment with FIDAS-5 (250 nM) for 4 days. (D) Cell cycle was analyzed using propidium iodide staining followed by flow cytometry after treatment with FIDAS-5 (250 nM) for 4 days. (E) Western blot analysis of cell cycle proteins, isolated after 4 days of FIDAS-5 (250 nM) treatment. (F) Cell apoptosis was measured using flow cytometry by staining for annexin V-FITC/7-amino-actinomycin D after 5 days of treatment with increasing doses of FIDAS-5. (G) The protein levels of the apoptosis markers PARP, caspase 9 and caspase 3 and MCL-1 were measured by western blotting after treatment with FIDAS-5 (250 nM) for 5 days. All experiments were performed in conditioned medium. Statistical significance was determined by one-way analysis of variance or the Mann-Whitney U test. **P*≤0.05, ***P*≤0.01, ****P*≤0.001, *****P*≤0.0001. BrdU: bromodeoxyuridine; PARP: poly ADP ribose polymerase; cl: cleaved.

mediated by the mTOR pathway.[24] Therefore, we next evaluated whether MAT2A is also involved in controlling protein synthesis in MM cells by using the non-radioactive surface sensing of translation (SUnSET) method. After 4 days, we observed that silencing of MAT2A did indeed significantly impair protein synthesis in JJN3 and even more markedly so in OPM2 cells (Figure 3B; *Online SupplementaryFigure S3B*). Next, we investigated the mTOR pathways and found a significant decrease in p-p70 and p-4EBP1 levels in OPM2 and JJN3 cells after 4 days of MAT2A inhibition. We also observed a significant decrease of p-eIF4E in JJN3 cells and p-PRAS40 in OPM2 cells (Figure 3C; *Online Supplementary Figure S3C*). Similar results were obtained with OPM2-shMAT2A cells, in which we observed clear reductions of p-mTOR, p-p70, and p-4E-BP1 (*Online Supplementary Figure S3G, H*). These findings indicate that the inhibition of protein synthesis by targeting MAT2A is mainly mediated through mTOR pathways.

FIDAS-5 restrains cell cycle progression and induces apoptosis of multiple myeloma cells

Recently, several new inhibitors of MAT2A have been developed, allowing us to further validate the impact of MAT2A inhibition on MM cell survival. FIDAS-5 and MAT2A inhibitor 1 (or Compound 196) are two potent MAT2A inhibitors, inducing strong anti-cancer effects.[25,26] Compared to Compound 196, the anti-tumor effect of FIDAS-5 is supported by more literature, and its *in vivo* pharmacokinetic properties and applicability have also been described. All tests with MAT2A inhibitors were performed with MM cells cultured in conditioned medium of HS-5 cells, which was used to mimic the bone marrow environment *in vitro*. We confirmed that this medium did not alter the expression of MAT2A in MM cells (*Online Supplementary Figure S5*). We treated MM cells (JJN3 and OPM2) for 2 days with either FIDAS-5 or Compound 196 and observed a dose-dependent decrease in cell viability and increase in apoptosis (Figure 4A; *Online Supplementary Figure S6A-C*). In line with genetic MAT2A inhibition, FIDAS-5 was also able to increase the levels of cleaved caspase-3, caspase-9 and PARP in MM cells (*Online Supplementary Figure S6D, E*). When the treatment period was increased to 5 days (long-term treatment), we found that the dose of FIDAS-5 could be reduced to a range of 0.1-1 μM in both cell lines (Figure 4B). This dose was also found to be effective in four other MM cell lines, while the bone marrow stromal cell line HS-5 was only affected at about 4-fold higher doses (*Online Supplementary Figure S7*). Furthermore, targeting MAT2A with FIDAS-5 for 4 days significantly suppressed cell proliferation in both cell lines, as evidenced by a decrease in BrdU incorporation and a G0/G1 and G2 phase arrest (Figure 4C, D; *Online Supplementary Figure S8A, B*). In addition, we confirmed the effect of FIDAS-5 on cell cycle-related proteins by western blot analysis. The results showed that MAT2A inhibition with FIDAS-5 potently increased the expression of the cyclin-dependent kinase inhibitor 1 ($p21^{Waf1/Cip1}$) and 1B ($p27^{kip1}$) (Figure 4E; *Online Supplementary Figure S8C*), while strongly reducing the protein levels of the master cell cycle regulator MYC.[27] Moreover, we found that long-term inhibition of MAT2A using FIDAS-5 also led to increased, dose-dependent apoptosis of MM cells (Figure 4F; *Online Supplementary Figure S9A*). On a protein level, again significant increases in cleavage of PARP, caspase 9, and caspase 3 were detected (Figure 4G; *Online Supplementary Figure S9B*). In addition, western blotting analysis revealed that FIDAS-5 induced cleavage of MCL-1 after 4 days in JJN3 and OPM2 cells (Figure 4G; *Online Supplementary Figure S9B*). These data confirm that MAT2A targeting leads to a reduction in proliferation and survival of MM cells.

FIDAS-5 suppresses multiple myeloma cell protein synthesis through SAM synthesis

To determine whether FIDAS-5 does indeed impair MM cell survival by inhibiting enzymatic activity of MAT2A, we next treated MAT2A-silenced and parental OPM2 cells with different doses of FIDAS-5. Briefly, OPM2 cells were transduced with siMAT2A or scrambled siRNA for 3 days. Next, the cells were refreshed and treated with FIDAS-5 for 2 additional days. The antitumor effect of FIDAS-5 on MAT2A-silenced cells was impaired compared to that of the cells transduced with scrambled siRNA (Figure 5A, B; *Online Supplementary Figure S10A*), indicating that FIDAS-5 does inhibit MM cell survival partly by targeting MAT2A. Next, the two cell lines were further treated with 250 nM

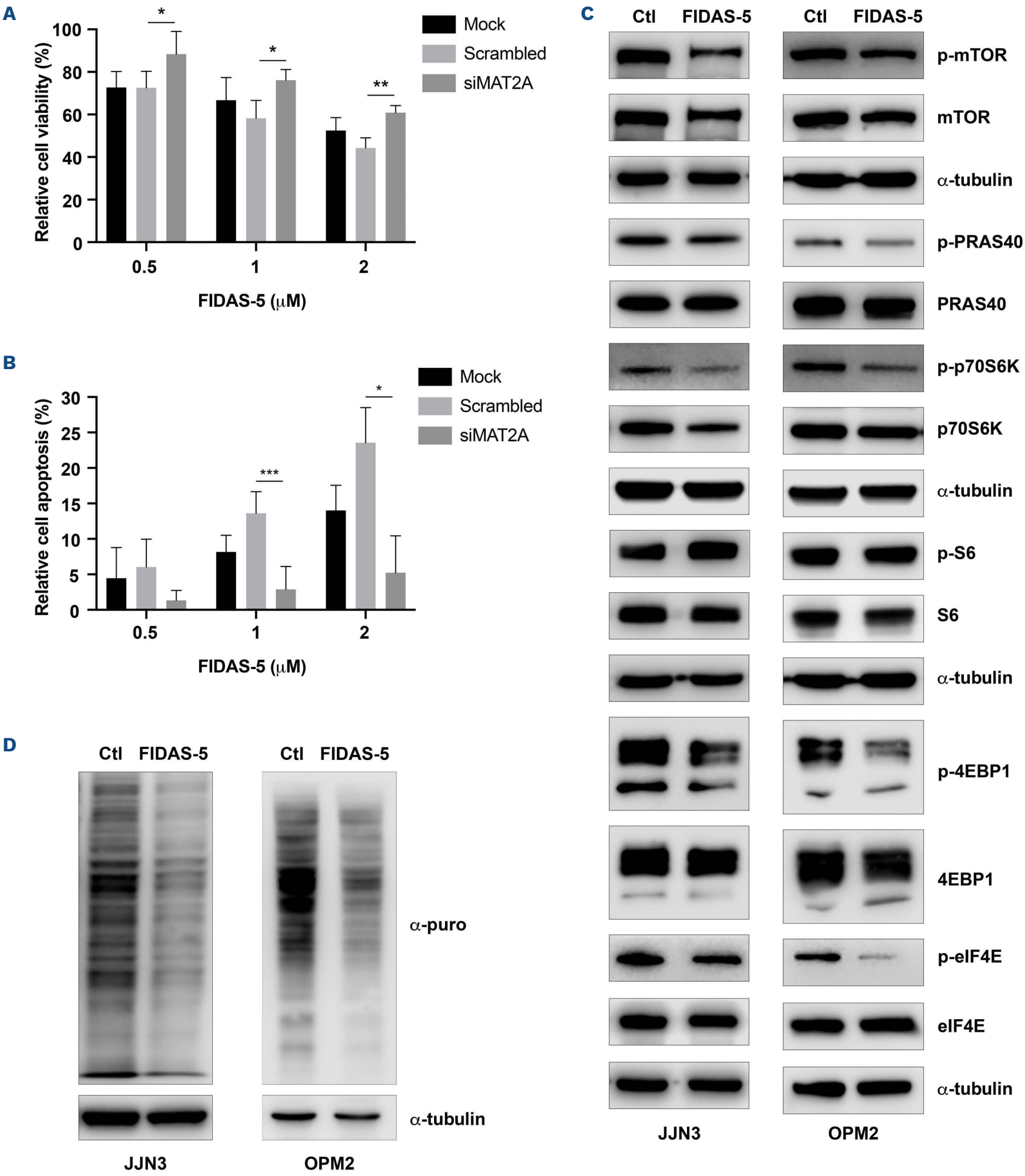

Figure 5. Inhibition of MAT2A with FIDAS-5 impairs protein synthesis by downregulating the mTOR pathway. (A, B) siRNA-mediated knockdown was established using 20 nM siMAT2A in OPM2 cells for 3 days, after which the cells were treated with 0.5, 1, or 2 μM FIDAS-5 for 48 h. Cell viability was evaluated using a CellTiter-Glo Luminescent Cell viability assay (A) and cell apoptosis was measured using flow cytometry by staining for annexin V-FITC/7-amino-actinomycin D (B). (C) Western blot analysis of protein synthesis-related proteins, isolated after 4 days of FIDAS-5 (250 nM) treatment. (D) Western blot analysis of puromycin uptake after FIDAS-5 (250 nM) treatment for 4 days. All experiments were performed in conditioned medium. Statistical significance was determined by one-way analysis of variance. *P≤0.05, **P≤0.01, ***P≤0.001. Ctl: control; si: short interfering; α-puro: α-puromycin.

FIDAS-5 for 4 days and the key kinases/proteins related to the mTOR pathway were investigated. Similarly to previous results obtained with silencing MAT2A, we observed a clear decrease in phosphorylation of mTOR, PRAS40, p70, 4EBP1 and eIF4E in JJN3 and OPM2 cells (Figure 5C; *Online Supplementary Figure S10B*). Moreover, we observed a decrease in puromycin uptake in the same two cell lines (Figure 5D; *Online Supplementary Figure S10C*). Glycolysis is an important pathway for energy generation and cell growth and is often associated with cancer, including MM.[28] LC-MS-based metabolomics showed that long-term FIDAS-5 treatment resulted in lower glucose uptake and less lactate secretion, indicative of lower glycolytic activity (Figure 6). More specifically, glycolytic intermediates of upper glycolysis were increased after FIDAS-5 treatment, while intermediates of lower glycolysis were decreased, suggesting a blockage of glycolysis. In addition, FIDAS-5 treatment lowered tricarboxylic acid cycle intermediates,

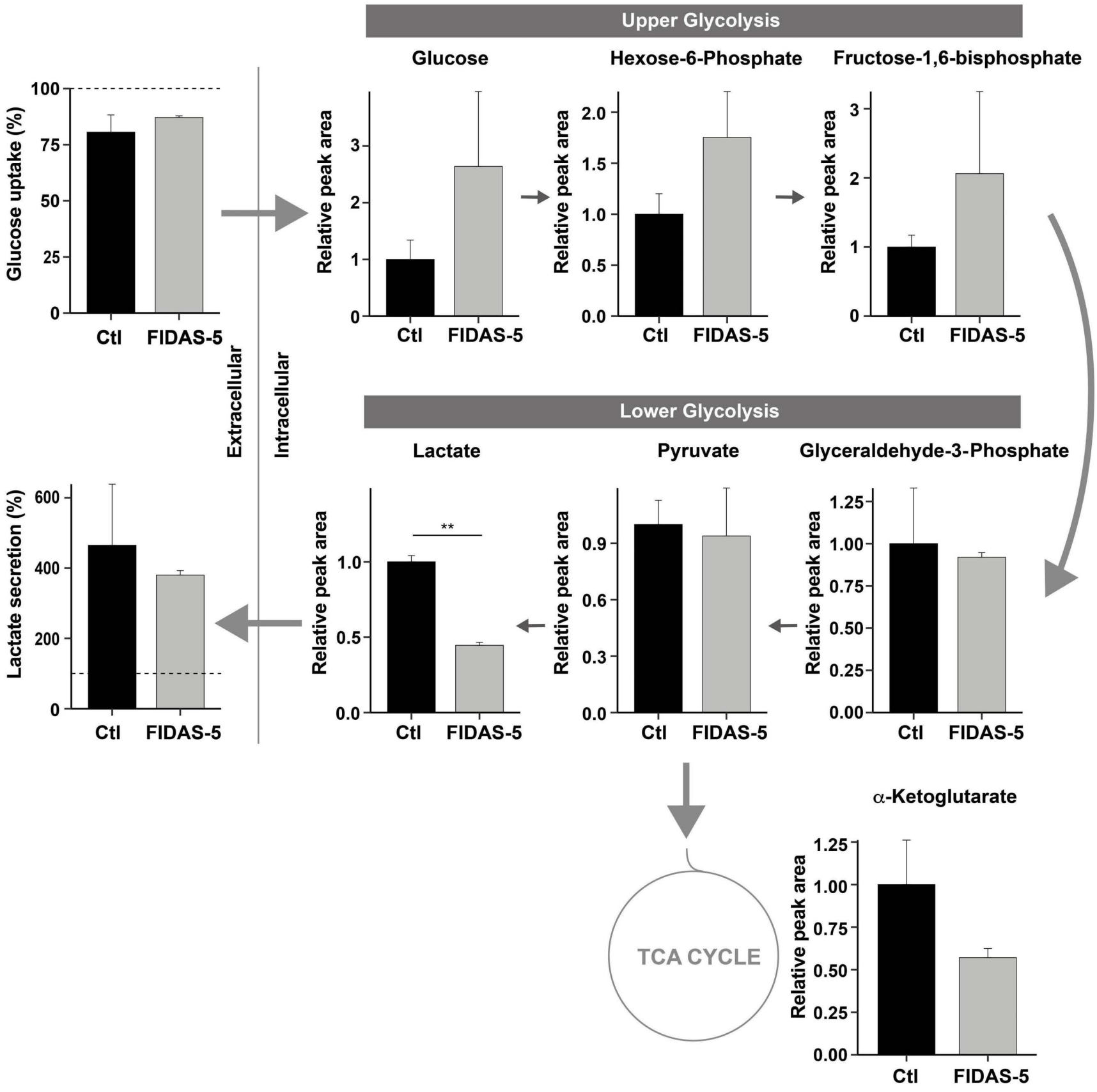

Figure 6. Inhibition of MAT2A with FIDAS-5 blocks glycolysis and the tricarboxylic acid cycle. Media and cell samples were collected after 5 days of FIDAS-5 (250 nM) treatment, followed by liquid chromatography-mass spectrometry analysis of extracellular glucose and lactate and intracellular metabolites. For the analysis of extracellular metabolite, results represent % peak area ± standard deviation (SD) compared to that of cell-free media. For analysis of intracellular metabolites, data represent relative peak area of metabolite ± SD compared to that of the non-treated controls. Unpaired *t* tests were performed. ***P*≤0.01. Ctl: control; TCA: tricarboxylic acid.

such as α-ketoglutarate. These results indicate that FIDAS-5 treatment impairs energy metabolism, which can influence cell growth.

Inhibition of MAT2A delays disease progression *in vivo*

To evaluate whether the anti-tumor effects observed *in vitro* could be translated *in vivo*, we used the murine

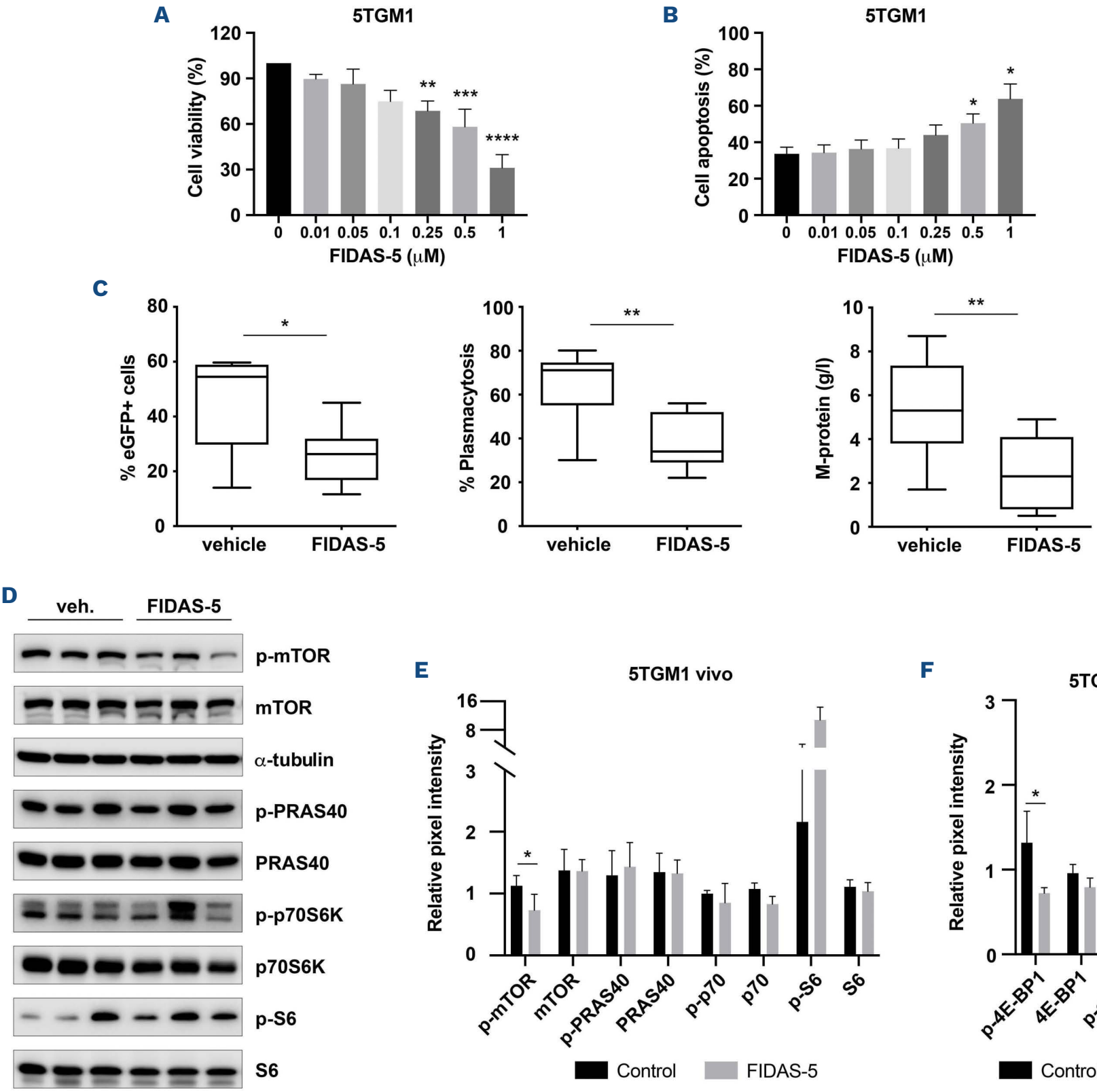

Figure 7. MAT2A inhibition by FIDAS-5 reduces tumor burden *in vivo*. (A, B) Murine multiple myeloma cells 5TGM1 were treated with increasing doses of FIDAS-5 for 5 days. Next, cell viability was measured by a CellTiter-Glo Luminescent cell viability assay (A), while cell apoptosis was measured using flow cytometry by staining for annexin V-FITC/7-amino-actinomycin D (B). (C) Tumor burden was analyzed by assessing the percentage of eGFP-positive cells using flow cytometry, bone marrow plasmacytosis on cytosmears and the amount of M-protein in 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. Statistical significance was determined by one-way analysis of variance or the Mann-Whitney U test. *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001.

5TGM1 MM model to investigate the effect of FIDAS-5 on tumor growth. We first confirmed that FIDAS-5 significantly inhibited cell viability and induced cell apoptosis of the murine MM cell line 5TGM1 (Figure 7A, B). Then, two groups of nine C57BL/KaLwRij mice were injected intravenously with 1×10^6 5TGM1-eGFP-positive cells. On day 3 after inoculation, mice were treated with either vehicle or 20 mg/kg FIDAS-5. When vehicle-treated mice reached end-stage disease, we evaluated effects on tumor burden by assessing the percentages of eGFP-positive cells and bone marrow plasmacytosis, and the amount of M-protein in the serum. In line with the *in vitro* data, the results showed that FIDAS-5 was able to significantly decrease tumor load as a single agent (Figure 7C). On a protein level, FIDAS-5 again significantly reduced p-mTOR, p-4EBP1 and MYC levels (Figure 7D, E). Importantly, following treatment, mice were also monitored for body weight loss and signs of toxicity. No significant body weight loss or overt signs of toxicity were observed (*Online Supplementary Figure S11A*). A repetition of the *in vivo* experiment had the same effect on mice, again confirming the therapeutic potential of FIDAS-5 in MM (*Online Supplementary Figure S11B-E*).

MAT2A inhibition sensitizes multiple myeloma cells to bortezomib

Finally, we investigated whether MAT2A inhibition influences MM cell sensitivity towards the standard-of-care agent bortezomib. Inhibition of MAT2A either by FIDAS-5 or siMAT2A significantly enhanced the effect of bortezomib on cell viability in both cell lines (Figure 8A, B). Furthermore, we found strong synergy for the FIDAS-5 and bortezomib long-term combination in both JJN3 and OPM2 cells, with the highest synergy scores being 30.46 (bortezomib: 2.5 nM, FIDAS-5: 500 nM) and 34.41 (bortezomib: 1.25 nM, FIDAS-5: 125 nM), respectively (*Online Supplementary Figure S12*). We also noticed a significant decrease in BrdU incorporation compared to that for both single agents alone, accompanied by a further reduction in MYC and increase in $p21^{Waf1/Cip1}$ protein levels. For the JJN3 cells, we also observed a further increase in $p27^{kip1}$ levels (Figure 8C, D; *Online Supplementary Figure S13*). In addition, we found that both FIDAS-5 and siMAT2A could significantly enhance the bortezomib-mediated effect on apoptosis (Figure 8E, F; *Online Supplementary Figure S4A, B*) together with inducing a significant increase in cleaved PARP, cleaved caspase 9 and cleaved caspase 3 levels in both cell lines (Figure 8G; *Online Supplementary Figure S14C*). Moreover, the combination therapy was beneficial on human primary MM samples ($CD138^+$ cells). The combination further reduced the viability of the MM cells, even though each patient's sample responded to the treatments differently (Figure 8H). Taken together, these findings provide the rationale to test the anti-MM activity of bortezomib in combination with FIDAS-5 in patients.

Discussion

Our study, for the first time, provides evidence supporting a key role for MAT2A in myeloma progression, thus constituting a promising novel target for the treatment of (relapsed) MM. Mechanistically, we demonstrated that MAT2A inhibition in MM cells triggers, at least in part, inhibition of the mTOR pathway leading to reduced protein synthesis, thereby inhibiting MM cell growth and survival. Using public gene expression profiling data, we showed that high MAT2A mRNA expression is associated with MM malignancy and adverse survival in MM patients. Previous studies showed that MAT2A is overexpressed in various tumors, such as hepatic cancer, breast cancer, glioma cells and colon cancer, and plays a significant role in the pathogenesis of those tumors.[29-32] In MM, research by Janker *et al*. showed that MAT2A is upregulated in patients with a high tumor burden.[33] Moreover, we recently demonstrated that MAT2A is upregulated in HMCL resistant to EZH2 inhibition.[34] MAT2A may therefore be an interesting therapeutic target in MM and an indicator of poor prognosis.

In order to study the function of MAT2A in MM cell biology, we conducted both genetic silencing experiments, in which we knocked down MAT2A using siRNA or shRNA, and chemical inhibition experiments using FIDAS-5 treatment. Inhibiting MAT2A strongly affected MM cell viability, proliferation and protein synthesis, resulting in apoptosis. To understand the mechanistic aspects, we first investigated the effect of MAT2A inhibition on SAM synthesis by mass spectrometry. We observed that silencing MAT2A is able to decrease SAM levels in OPM2 cells. As mentioned before, silencing the expression of MAT2A in solid tumors is also able to affect tumor growth. For example, in the HepG2 cell line, MAT2A silencing could lower intracellular SAM levels and restrict polyamine production, preventing leptin's pro-survival signal, which is required for cell growth.[35] In 2013, Zhang *et al.* showed that blocking MAT2A activity with FIDAS agents resulted in reduced SAM synthesis, which significantly inhibited the growth of colorectal cancer *in vitro*. Additionally, using cell-based assays, they found that FIDAS-5 is more potent than other family members and that FIDAS-5 was able to significantly inhibit the growth of tumors *in vivo*, with minimal differences in body weight of the animals treated.[25] The anticancer activity of FIDAS-5 was also validated in gastric cancer cells, in which an obvious downregulation of SAM sensitized the gastric cancer cells to ferroptosis.[36] We confirmed that MAT2A silencing attenuates the antitumor effect of FIDAS-5, indicating that MAT2A is indeed a target of FIDAS-5. Additionally, SAM inhibition in tumors causes cell cycle arrest in the G1 and G2 phases, which we were able to detect in MM using both siMAT2A and FIDAS-5. We also demonstrated that MYC expression is suppressed by

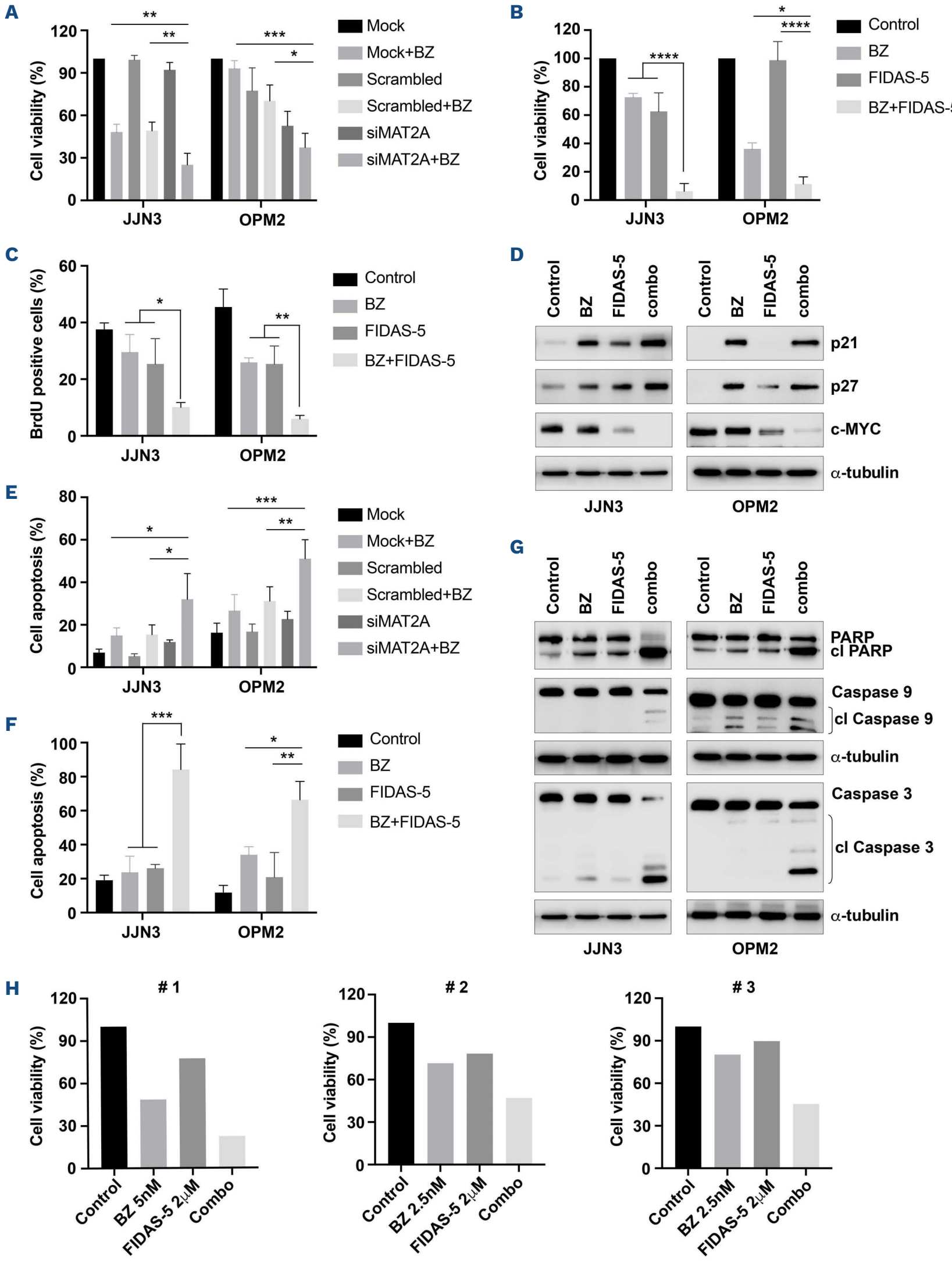

Figure 8. Inhibition of MAT2A sensitizes multiple myeloma cells to bortezomib. (A) Cells were transfected with 20 nM siRNA against MAT2A for 5 days. On day 3, cells were treated with bortezomib (JJN3: 1.5 nM, OPM2: 2.5 nM) for an additional 2 days. Cell viability was measured by a CellTiter-Glo Luminescent cell viability assay. (B) Cells were treated with FIDAS-5 (JJN3: 2.5 μM, OPM2: 10 μM) and bortezomib (JJN3: 1.5 nM, OPM2: 2.5 nM) for 48 h in conditioned medium and cell viability was measured by a CellTiter-Glo Luminescent cell viability assay. (C, D) Cells were treated with FIDAS-5 (JJN3: 2.5 μM, OPM2: 10 μM) and bortezomib

Continued on following page.

(JJN3: 1.5 nM, OPM2: 2.5 nM) for 24 h in conditioned medium. Next, cell proliferation was measured by a bromodeoxyuridine incorporation assay followed by flow cytometry (C) and cell cycle proteins were analyzed by western blot (D). (E, F) Cells were transfected with 20 nM siMAT2A (E) or treated with FIDAS-5 (JJN3: 2.5 μM, OPM2: 10 μM) in conditioned medium (F), combined with bortezomib (JJN3: 1.5 nM, OPM2: 2.5 nM). After 48 h, cell apoptosis was measured using flow cytometry by staining for annexin V-FITC/7-amino-actinomycin D. (G) The protein levels of the apoptosis markers PARP, caspase 9 and caspase 3 were measured by western blotting after treatment with FIDAS-5 (JJN3: 2.5 μM, OPM2: 10 μM) and bortezomib (JJN3: 1.5 nM, OPM2: 2.5 nM) for 48 h in conditioned medium. (H) $CD138^+$ MM cells isolated from three patients' samples were cultured for 24 h in conditioned medium and treated with bortezomib and FIDAS-5. Cell viability was measured by CellTiter-Glo assay. Statistical significance was determined by one-way analysis of variance. *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001. BZ: bortezomib; BrdU: bromodeoxyuridine; Combo: combination of bortezomib and FIDAS-5; PARP: poly ADP ribose polymerase; cl: cleaved.

MAT2A inhibition. It is well known that MYC contributes significantly to a number of biological functions, including proliferation and cell cycle progression.[37] Recently, it was discovered that MYC induces the expression of MAT2A, explaining why MAT2A is elevated in proliferative tissues, notably cancer.[24] In colorectal cancer cells, FIDAS-5 decreased the expression of the oncogenes MYC and Cyclin D1, while increasing the expression of the cell cycle inhibitor $p21^{Waf1/Cip125}$.

A copious amount of protein must be synthesized in order for cells to survive and this is especially true for malignant plasma cells. According to our data, MAT2A seems to contribute to cell proliferation by supporting protein synthesis, since both MAT2A depletion and pharmacological inhibition of MAT2A activity resulted in a decrease of protein synthesis. This is in line with the study of Villa and colleagues, who demonstrated that MAT2A targeting inhibits protein synthesis, and tumor development by diminishing intracellular SAM levels. Moreover, we observed that MAT2A regulates translation via mTOR signaling. According to Gu *et al.*, the SAM sensor SAMTOR forms a stable dimer with Gap Activity TOward Rags 1 (GATOR1) to limit mTORC1 activity in HEK293T cells when SAM levels are decreasing. In contrast, when sufficient SAM levels are present, SAM binds to SAMTOR thereby disassembling the SAMTOR-GATOR1 complex and thus activating mTORC1 signaling and triggering translation.[38] As a key player in drug resistance and the pathogenesis of MM, the PI3K/AKT/mTOR pathway has been well described in MM.[39] The progression of MM is decelerated and cell death is induced when p-p70, p-S6, p-4EBP1, and p-eIF4E are inhibited.[16] mTOR is also a regulator of glycolysis, an important pathway for energy and cell growth, and our metabolomics data showed that MAT2A inhibition results in lower glycolytic activity, which could also result in lower cell proliferation. As a major methyl donor, SAM plays a pivotal role in the methylation of DNA, RNA and proteins that regulate fundamental cellular activities. In MM, hypermethylation of tumor suppressor genes is involved in the regulation of cell cycle progression, DNA repair, apoptosis, drug response and key signaling pathways.[40] Therefore, investigating the involvement of MAT2A in the DNA methylation landscape of MM cells will be an interesting topic for future research.

We also performed *in vivo* experiments using FIDAS-5 to target MAT2A in order to analyze its therapeutic potential. In our study, we found that FIDAS-5, as a single treatment, was able to considerably reduce tumor burden in two separate experiments, without inducing obvious toxicity. Moreover, FIDAS-5 significantly decreased the levels of p-mTOR and p-4EBP1 *in vivo*, while p-PRAS40 and p-p70 also showed modest decreases in one experiment. Furthermore, there was a considerable decrease in the levels of MYC. Previous research revealed that FIDAS-5 dramatically delayed the development of HT29 tumor xenografts in nude mice, while only minimally altering the body weight of the animals.[25] It could also decrease tumor growth in the CAL-51 cancer cell line-derived xenograft model.[24]

The effectiveness of bortezomib in MM was the first evidence that disrupting protein homeostasis may be used as a cancer treatment strategy.[41] Currently, bortezomib is one of the main standard-of-care agents for the treatment of newly diagnosed MM patients.[42] However, although the majority of the MM patients initially respond well to bortezomib treatment, most of them will eventually relapse. Since we demonstrated in our study that inhibiting MAT2A reduced protein synthesis, we hypothesized that the combination of MAT2A inhibition and bortezomib might have enhanced antitumor efficacy. In addition, enhanced glycolysis and tricarboxylic acid cycle activity have been associated with resistance to bortezomib, and FIDAS-5 inhibits both these pathways as well.[43,44] Our data showed that the combination did indeed significantly decrease cell proliferation and synergistically inhibited cell viability compared to treatment with the two drugs as single agents. Furthermore, FIDAS-5 and bortezomib induced cell death in a caspase-dependent manner. Thus, combining FIDAS-5 with bortezomib may be a novel way to delay or even overcome bortezomib resistance in MM. The clinical phase I trial with AG-270, another MAT2A inhibitor with strong affinity, in patients with lymphoma or solid tumors (NCT03435250, clinical trials.gov) further supports the idea that targeting MAT2A could be clinically feasible.

Disclosures

No conflicts of interest to disclose.

Contributions

EM, EDB, and YW contributed to the conception, design and

revision of the manuscript. YM and AW performed experiments. EDB, JM, CM, and PV provided bioinformatic analyses. EZ and CB performed the liquid chromatography-mass spectometry. EM, EDB, YW, CM, AW, AM, PV, KDV, KV, JM, EZ, and CB were responsible for writing, reviewing and editing the manuscript. All authors read and approved the final version of the manuscript.

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Data-sharing statement

The data generated in this study are available upon request from the corresponding authors.

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