Mitochondrial fission factor drives an actionable metabolic vulnerability in multiple myeloma

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Abstract

Proliferating multiple myeloma (MM) cells in the bone marrow fluctuate across various metabolic states to resist cancer treatments. Herein, we investigate how mitochondrial dynamics, which control mitochondrial fitness via coordinated fission and fusion events, shape MM cell metabolism impacting growth, survival and drug sensitivity. We identify mitochondrial fission factor (MFF), a pivotal driver of mitochondrial fragmentation, as being highly expressed in MM plasma cells bearing cytogenetic abnormalities predicting poor clinical outcome. In preclinical models, selective inhibition of MFF via multiple RNA-based strategies (short-hairpin RNA, short-interfering RNA or LNA gapmeR antisense oligonucleotides) reduces MM cell growth both *in vitro* and *in vivo*, enabling adaptive metabolic responses consistent with the induction of glycolysis and the inhibition of lactate-mediated oxidative phosphorylation. We also demonstrate that lactate supplementation, as well as clinically relevant drugs promoting lactate accumulation, such as AZD3965 and syrosingopine, trigger MFF-dependent metabolic changes, enhancing the sensitivity of MM cells to strategies targeting mitochondrial fission. Finally, we highlight a novel lactate-MFF axis involved in resistance to proteasome inhibitors, and show that combining AZD3965 or syrosingopine with bortezomib results in synergistic anti-MM activity along with MFF downregulation. Collectively, these data point to MFF-dependent mitochondrial fragmentation as a key metabolic hallmark of MM, providing a framework for the development of novel therapeutic strategies targeting mitochondrial dynamics and harnessing the metabolic plasticity of malignant plasma cells.

Introduction

Frequently recognized as the powerhouse of the cell, mitochondria are vital organelles that regulate critical biological processes, including energy production, cellular metabolism and cell death.[1] Proper mitochondrial function has been shown to depend on mechanical changes in the dynamic structure and architecture of these organelles.[2] In fact, to enable cells to adapt to various stressful conditions, mitochondria form a highly dynamic network through the opposing processes of fission and fusion, which divide or merge the two mitochondrial lipid bilayers, thereby modulating the

energetic machinery according to the cell's needs.[3,4] Due to their crucial role in maintaining mitochondrial homeostasis, fusion and fission events are tightly regulated, and their dysregulation leads to metabolic dysfunctions, which are associated with the onset and progression of various malignancies.[5]

In cancer, the elevated nutrient demands of proliferating cells are mainly fulfilled through drastic changes in energetic metabolism, in turn supported by variation in mitochondrial function and shape.[6] Elevated mitochondrial fission has been shown to drive metabolic reprogramming, cell cycle progression, and evasion of cell death, thereby enhancing migration and chemoresistance in a wide variety of solid[7-11] and hematologic malignancies.[12-14] On this basis, harnessing aberrant mitochondrial fission represents a novel potential approach for cancer treatment.

Mitochondrial fission factor (MFF) is an integral membrane protein of the outer mitochondrial membrane, which serves as the main molecular mediator regulating mitochondrial fragmentation.[15] During fission, the cytosolic protein Drp1 (dynamin-related protein 1) is recruited to the mitochondrial surface primarily via MFF, but also through other proteins such as Fis1 (mitochondrial fission protein 1), MiD49 and MiD51 (mitochondrial dynamics proteins of 49 and 51 kDa);[16,17] Drp1 then assembles into oligomeric complexes that form specific structures, named puncta, around mitochondrial tubules, thus driving the fission process.[18] Accumulating evidence has demonstrated MFF dysregulation in various types of cancer, in which it affects critical bioenergetic pathways and promotes rapid growth and survival of tumor cells.[19-21]

Multiple myeloma (MM), the second most common hematologic malignancy worldwide, is characterized by several mitochondrial defects that shape the metabolism of cancer cells and potently antagonize their responsiveness to therapeutic treatments.[4] Despite growing evidence supporting the oncogenic activity of MFF, its role in regulating mitochondrial dynamics has yet to be explored in the context of plasma cell disorders. In this study, we reveal a novel pro-survival function of MFF in MM, which may enhance our understanding of mitochondrial vulnerabilities and open new avenues for therapeutic intervention in this disease.

Methods

Cell culture and drugs

Detailed information regarding the cell cultures and drugs used in this study is provided in the *Online Supplementary Methods*.

Transmission electron microscopy

Samples were processed for ultrastructural transmission electron microscopy analysis according to standard protocols.[22,23] Detailed information is provided in the *Online Supplementary Methods*.

RNA-sequencing of multiple myeloma patients' samples

Multi-omics data about bone marrow MM samples at baseline (BM_1) were publicly accessible from the MMRF CoMMpass study (*https://research.themmrf.org/*). Detailed information is provided in the *Online Supplementary Methods*.

RNA-sequencing, differential gene expression and pathway analyses

Detailed information about RNA-sequencing, differential gene expression and pathway analyses is provided in the *Online Supplementary Methods*.

Virus generation and transduction of multiple myeloma cells

The plasmids used and the related protocols are described in the *Online Supplementary Methods*.

Transient transfection of multiple myeloma cells

MM cells were transfected by electroporation using the Neon Transfection System (Invitrogen, CA, USA), according to previously established protocols.[24] Detailed information can be found in the *Online Supplementary Methods*.

Cell viability, clonogenicity assays and cell cycle analysis

Details of the cell viability studies, clonogenicity assays and cell cycle analysis are presented in the *Online Supplementary Methods*.

Measurements of mitochondrial reactive oxygen species and mitochondrial membrane potential

Detailed information regarding the measurements of reactive oxygen species and mitochondrial membrane potential is supplied in the *Online Supplementary Methods*.

Measurement of oxygen consumption rate and extracellular acidification rate

The oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were quantified using a Seahorse Extracellular Flux Analyzer (XFe96, Agilent Technologies). Detailed information is provided in the *Online Supplementary Methods*.

Oroboros O2k-high resolution respirometry

Oxygen consumption was analyzed using an Oroboros Oxygraph-2k high-resolution respirometer (Oroboros Instrument, Innsbruck, Austria). Detailed information is provided in the *Online Supplementary Methods*.

Intracellular lactate measurement

The intracellular concentration of L-lactate was determined using the Lactate-Glo Assay (Promega, Madison, WI, USA), as described in detail in the *Online Supplementary Methods*.

Apoptosis detection

Detailed information regarding the detection of apoptosis is provided in the *Online Supplementary Methods*.

Western blotting

Total protein extracts were prepared using NP40 Cell Lysis Buffer supplemented with Halt Protease Inhibitor Single-Use Cocktail (Thermo Fisher Scientific, Waltham, MA, USA). Detailed information is provided in the *Online Supplementary Methods*.

Reverse transcription and quantitative real-time amplification

RNA extraction, reverse transcription, and quantitative real-time amplification (qRT-PCR) were carried out following previously described methods.[24] Detailed information is provided in the *Online Supplementary Methods*.

In vivo studies

Male NOD.SCID mice (6 to 8 weeks old; Envigo, Indianapolis, IN, USA) were housed and monitored in our Animal Research Facility. All the experimental procedures and protocols were previously approved by the Institutional Ethical Committee (Magna Graecia University of Catanzaro) and performed according to protocols approved by the National Directorate of Veterinary Services (Italy). Detailed information is provided in the *Online Supplementary Methods*.

Immunohistochemistry

Detailed information on the immunohistochemical studies is supplied in the *Online Supplementary Methods*.

Statistical analysis

Each *in vitro* experiment was performed at least three times and values are reported as mean ± standard deviation. Comparisons between groups were performed using a Student *t* test, while statistical significance of differences among multiple groups was determined by GraphPad software (*www.graphpad.com*). A *P* value <0.05 was accepted as statistically significant. Graphs were obtained using Graphpad Prism version 8.0 (GraphPad Software, La Jolla, CA, USA).

Results

Mitochondrial fission factor is highly expressed in multiple myeloma cells and triggers mitochondrial fragmentation

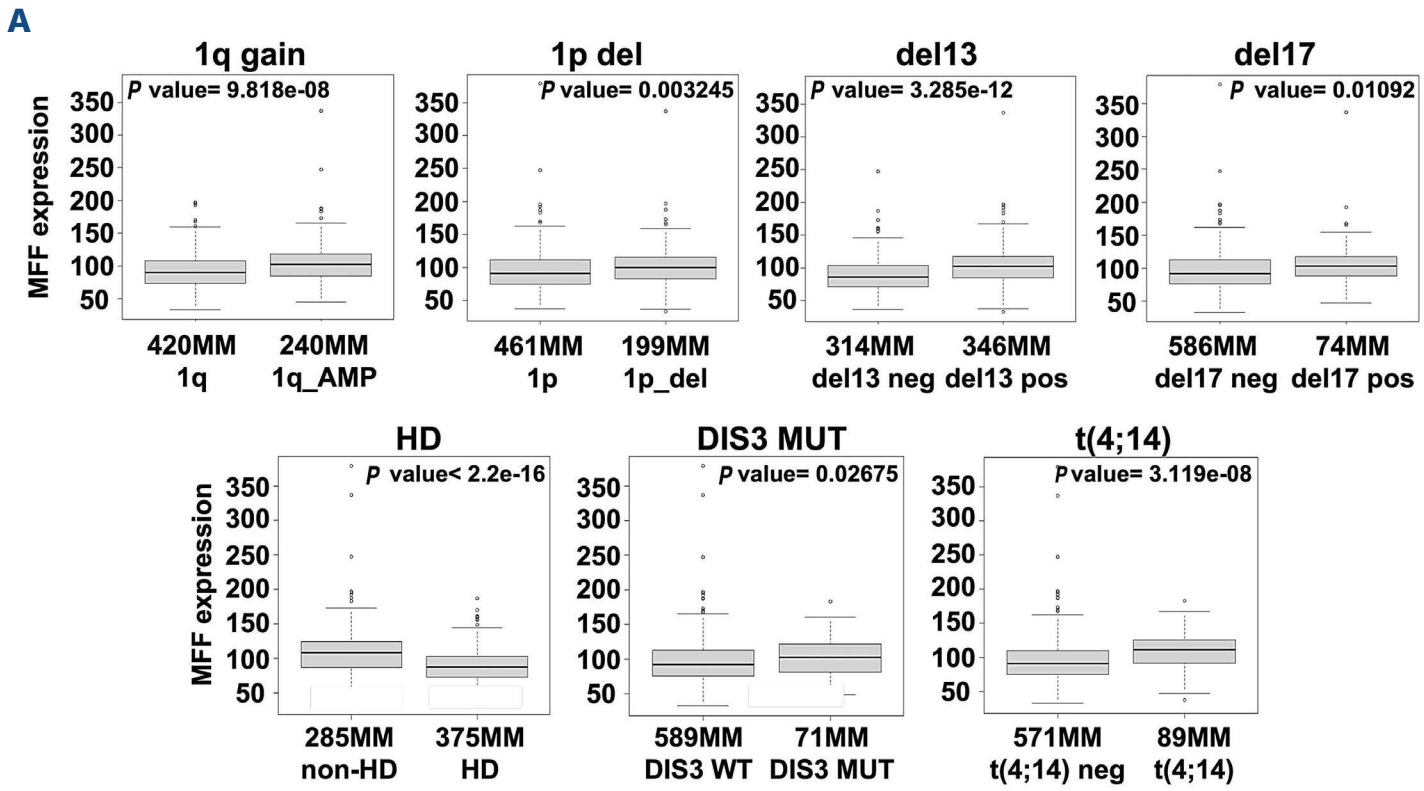
By exploiting the CoMMpass dataset, we first analyzed the expression pattern of MFF in MM patients stratified according to the presence of major molecular lesions. Interestingly, higher MFF gene expression was observed in patients harboring genomic alterations associated with poor prognosis, such as 1q amplification, 1p deletion, del(13), del(17), DIS3 mutation, t(4;14) translocation, and non-hyperdiploid status (Figure 1A). When patients were stratified according to major IgH translocations, MFF expression was significantly higher in the t(4;14) subgroup than in the other groups (*Online Supplementary Figure S1A*). Although microarray gene expression datasets did not show any significant increase in MFF transcript levels in MM plasma cells with respect to those in healthy controls (*data not shown*), immunohistochemistry staining of a tissue microarray demonstrated elevated MFF protein expression in primary MM samples compared to the expression in normal bone tissues (Figure 1B).

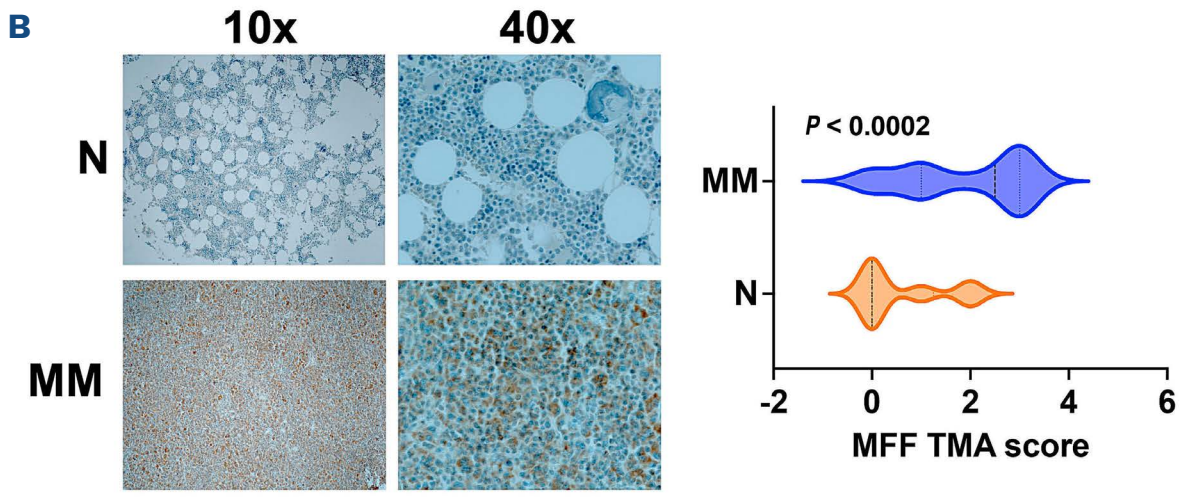
Consistent with the role of MFF as a Drp1 partner in mitochondrial fragmentation,[16,17,25] a physical interaction between the two proteins was demonstrated in MM cell lines, as MFF co-immunoprecipitated with Drp1 using an anti-Drp1 antibody (Figure 1C); conversely, Drp1 was also detected upon MFF immunoprecipitation (*Online Supplementary Figure S1B*).

Given that MFF is a key regulator of mitochondrial fragmentation in various cellular contexts, we next investigated whether MFF-targeted inhibition could affect mitochondrial morphology in MM cells. Using transmission electron microscopy, we observed a significant accumulation of elongated mitochondria following MFF knockdown (KD) (Figure 1D). These findings suggest that MFF is abundantly expressed in MM cells and plays a critical role in regulating mitochondrial dynamics by promoting mitochondrial fragmentation.

Mitochondrial fission factor targeting halts *in vitro* and *in vivo* multiple myeloma cell growth

Mitochondrial dynamics regulate biological processes relevant to the maintenance of metabolic homeostasis, affecting cell growth and proliferation.[26] We employed different knockdown strategies to evaluate the impact of MFF inhibition on MM cell growth and survival. As an initial approach, we attempted to induce degradation of MFF mRNA using LNA-gapmeR antisense oligonucleotides (ASO). Among six candidate molecules screened, we selected two gapmeRs, g_05 and g_06, as the most effective ASO in reducing MFF expression at both the mRNA and protein levels (*Online Supplementary Figure S2A, B*). Following transient transfection, g_05 and g_06 reduced MM cell growth (Figure 2A), and impaired colony-forming ability in semisolid cultures (*Online Supplementary Figure S2C*); similar inhibitory effects on cell viability were also observed using two different MFF-targeting short-interfering RNA (siRNA) (Figure 2B). To further validate our findings, we performed lentiviral-mediated MFF KD using short-hairpin RNA (shRNA), which also led to reduced cell proliferation and colony formation (Figure 2C, D). Additionally, MFF targeting increased levels of mitochondrial reactive oxygen species, suggesting mitochondrial dysfunction in MM cells (*Online Supplementary Figure S2D*). Consistent with these findings, MFF KD induced cell cycle defects, with an increase in both sub-G0 and G1 phases (Figure 2E). Moreover, MFF KD increased annexin-V positivity indicative of apoptosis (Figure 2F), further confirmed by PARP and





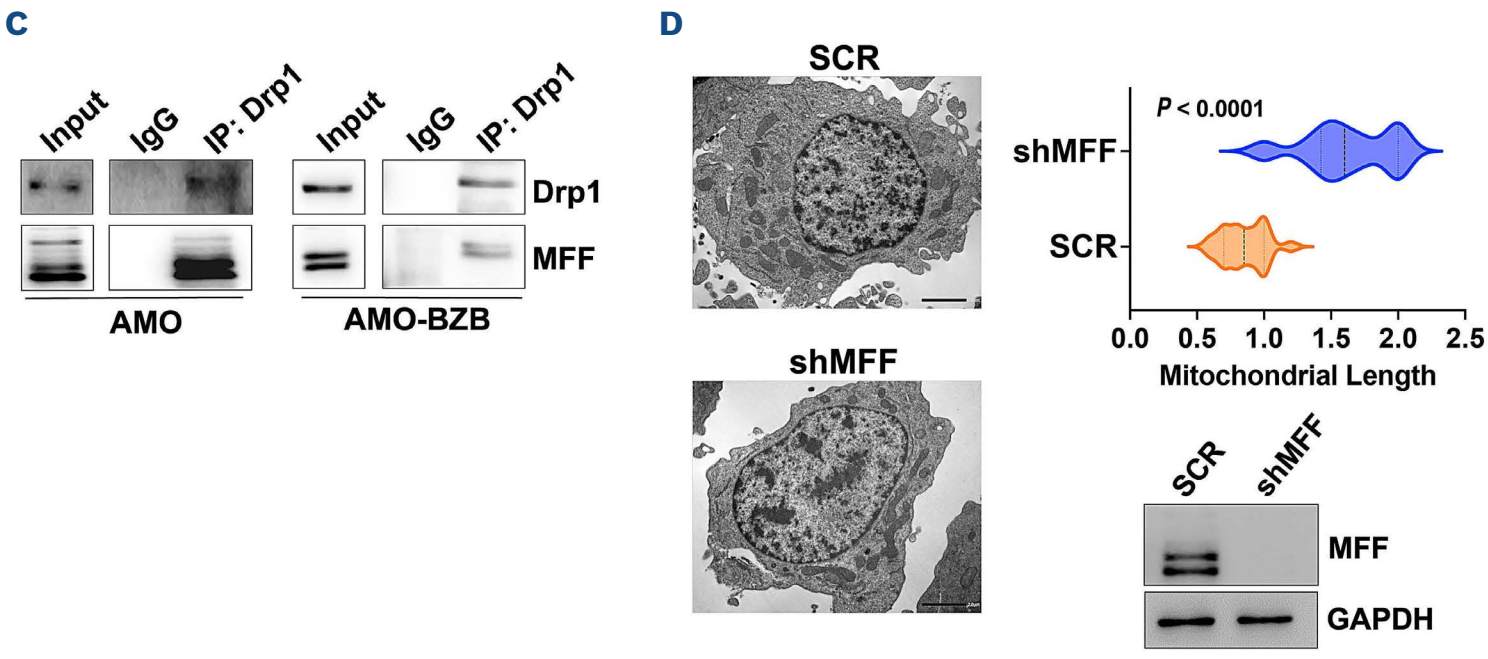


Figure 1. Mitochondrial fission factor is highly expressed and drives mitochondrial fragmentation in multiple myeloma. (A) Box plots of mitochondrial fission factor (MFF) expression level in 660 cases of multiple myeloma (MM) in the CoMMpass dataset stratified according to the presence of molecular lesions. Differential expression was tested by the Wilcoxon rank-sum test with continuity correction. (B) Immunohistochemical analysis of MFF expression in MM and normal bone tissue cores from a tissue microarray (TMA) slide; representative images (10x and 40x magnification) are shown. The TMA score was calculated as the average of MFF intensity values across all tissue samples on the TMA slide. (C) Western blot analysis of Drp1 and MFF proteins in

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whole-cell lysates (Input) and anti-Drp1 immunoprecipitation products derived from AMO or AMO-BZB cells. (D) Transmission electron microscopy (TEM) analysis of mitochondrial structure and morphology in AMO-BZB cells stably silenced for MFF (shMFF); a scrambled control vector was used as a control (SCR). Representative TEM images are shown (12,000x magnification). Mitochondrial length is reported as the average of acquisitions from different samples across various tissue specimens. The western blot shows MFF protein in shMFF and SCR AMO-BZB cells; GAPDH was used as a loading control. HD: hyperdiploid; WT: wildtype; MUT: mutated; N: normal bone tissue; IP: immunoprecipitation; AMO: parental cell line; AMO-BZB: bortezomib-resistant cell line.

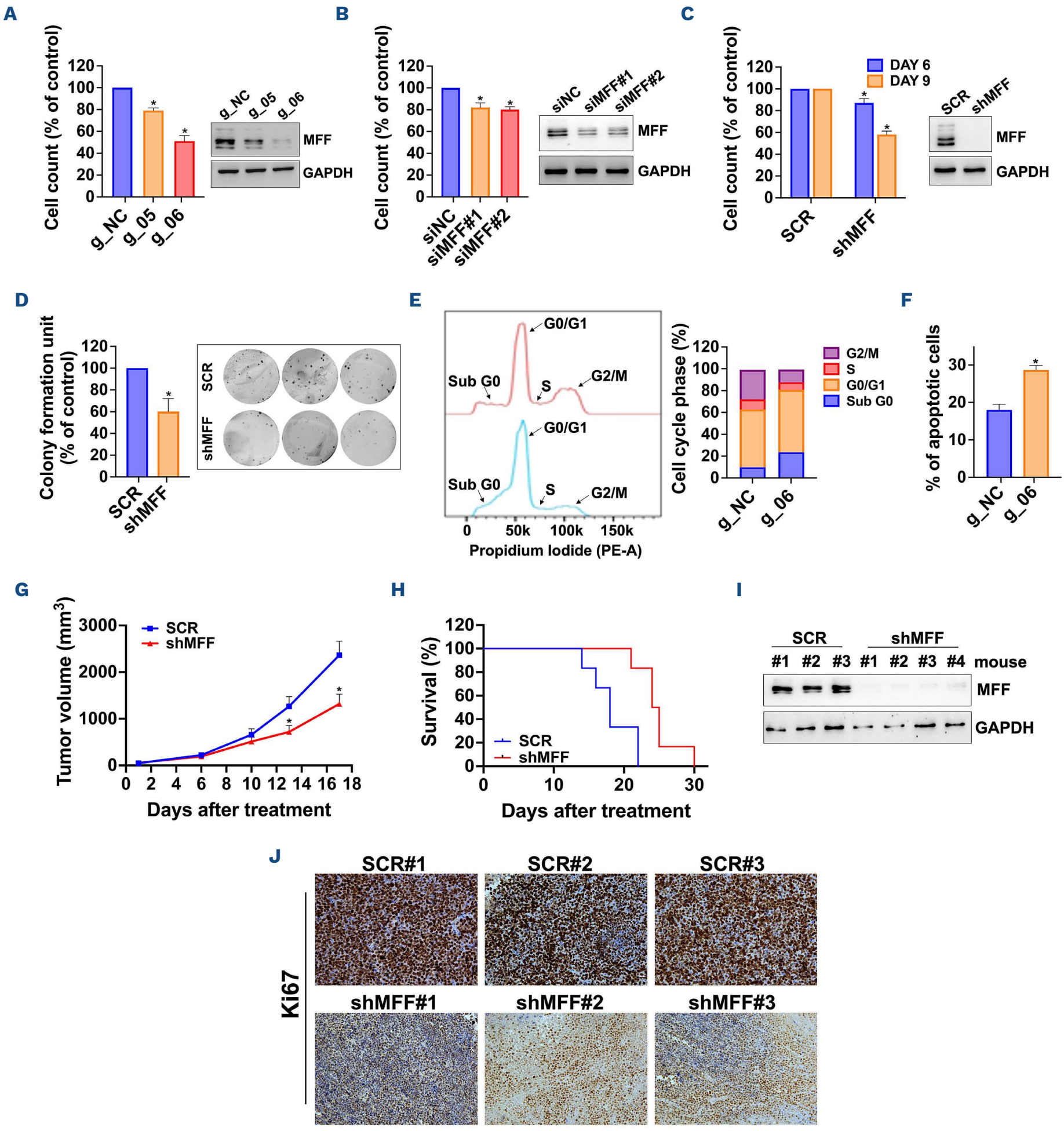

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Figure 2. Mitochondrial fission factor targeting induces anti-multiple myeloma effects *in vitro* and *in vivo*. (A-C) Automated cell count performed in AMO-BZB cells, 48 h after electroporation with (A) g_05, g_06 or g_NC negative control gapmeR, (B) short-interfering RNA (siRNA) targeting mitochondrial fission factor (MFF), or (C) after lentiviral transduction with an MFF-targeting short hairpin RNA (shRNA). Western blot analysis of MFF in AMO-BZB cells, 48 h after electroporation with gapmeR, siRNA, or lentiviral transduction, is reported; GAPDH was used as a loading control. **P*<0.05. (D) Colony formation assay performed on AMO-BZB cells stably silenced for MFF (shMFF), 10 days after seeding; a scrambled (SCR) vector was used as control. Histogram bars reported the mean ± standard deviation (SD) of three independent experiments. Representative images of colonies at day 10 are reported. **P*<0.05. (E) Cell-cycle analysis of AMO-BZB cells, 48 h after electroporation with g_06 or g_NC; histogram bars represent the percentage of cells in each cell cycle phase. (F) Flow cytometry analysis of AMO-BZB cells stained with annexin V/7-aminoactinomycin D, 48 h after electroporation with g_06 or g_NC; histogram bars represent the mean from three independent biological replicates. **P*<0.05. (G) *In vivo* tumor growth evaluation of shMFF AMO-BZB xenografts in NOD.SCID mice; SCR was used as the control. Average ± SD of the tumor volume for each group is shown; **P*<0.05. (H) Kaplan-Meier curves relative to shMFF and SCR AMO-BZB xenografts (log-rank test, ***P*=0.0045). Survival was evaluated from the first day of palpable tumor until death or sacrifice; the percentage of mice alive is shown. (I) Western blot analysis of MFF expression in SCR and shMFF AMO-BZB xenografts retrieved from mice; GAPDH was used as the loading control. (J) Immunohistochemical analysis (20x magnification) of Ki-67 expression in shMFF and SCR AMO-BZB xenografts retrieved from mice; representative images are shown.

caspase-3 cleavage (*Online Supplementary Figure S2E*) and a decrease in TMRM staining (*Online Supplementary Figure S2F*). Notably, MFF targeting also reduced the viability of MM cells co-cultured with HS5 stromal cells, highlighting its potential to overcome the protective bone marrow microenvironment (*Online Supplementary Figure S2G*).

Finally, using NOD.SCID mice xenografted with AMO-BZB cells stably transduced with shMFF or a scrambled control (SCR), we validated the inhibitory effect of MFF KD on MM growth *in vivo*. MFF targeting reduced the growth of MM xenografts (Figure 2G) and prolonged animal survival (Figure 2H). The analysis of retrieved xenografts confirmed MFF downregulation (Figure 2I), along with reduced Ki-67 expression following MFF silencing (Figure 2J). Collectively, these data indicate that impairing mitochondrial fission via MFF targeting triggers detrimental effects on MM cell growth and survival, both *in vitro* and *in vivo*.

Mitochondrial fission factor inhibition triggers a metabolic switch in multiple myeloma cells

To gain deeper insights into the biological pathways regulated by MFF in MM, we generated an isogenic MM cell line overexpressing MFF (H929 OE MFF), alongside a corresponding control carrying the empty vector (H929 EV). After confirming MFF protein overexpression (*Online Supplementary Figure S3A*), the newly established cell line underwent transcriptomic analyses to identify differentially expressed genes, which were compared to those derived from MFF KD cells. Among the differentially expressed genes, we identified 1,446 genes that were deregulated in both shMFF and OE MFF transcript profiles; of these, 1,103 genes were upregulated in OE MFF and, *vice versa*, downregulated in shMFF, while 292 differentially expressed genes exhibited the opposite expression pattern (Figure 3A).

We hypothesized that the differential modulation of the pathways regulated by such genes, which displayed opposing trends between the two experimental conditions (OE and shMFF), could unveil MFF function in MM. To address this point, we performed gene set enrichment analysis (GSEA) comparing each condition to its corresponding control. Upon analyzing the GSEA results from shMFF or OE MFF cells, we observed five top deregulated processes exhibiting opposite patterns (Figure 3B). Notably, glycolysis was among the top upregulated metabolic pathways in shMFF cells, while it was downregulated in MFF-overexpressing cells. Volcano plot analysis showed upregulation of key glycolytic genes in shMFF cells, which were, conversely, downregulated in MFF-overexpressing cells (*Online Supplementary Figure S3B*). Additionally, among the glycolysis and oxidative phosphorylation (OXPHOS)-related genes from the GSEA database, 34 glycolysis-related genes and 15 OXPHOS-related genes showed opposite expression patterns in our experimental setting (*Online Supplementary Figure S3C*). The expression of a set of these glycolytic genes, including HK2, ALDOC, ENO2, PDK4, and SLC2A1, was validated by quantitative real-time amplification both *in vitro* in shMFF or OE MFF cells (Figure 3C), and *in vivo* in tumors retrieved from treated mice (*Online Supplementary Figure S3D*).

Mitochondrial fission factor-dependent metabolic reprogramming of multiple myeloma cells is mediated by intracellular lactate accumulation

To directly correlate the MFF-associated transcriptomic changes reported above to the metabolic phenotype, we performed metabolic analyses using high-resolution respirometry (Oroboros) and/or Seahorse analysis on MM cells after knockdown or overexpression of MFF. High-resolution respirometry showed that MFF overexpression caused a significant increase in OXPHOS, as evidenced by enhanced basal and maximal respiration rates compared to those of control cells. Specifically, the increased maximal respiration following injection of FCCP, a mitochondrial uncoupler, indicated an elevated mitochondrial capacity, improved electron transport chain efficiency, and greater oxygen consumption during cellular respiration, reflecting the enhanced ability of mitochondria to produce ATP (Figure 4A). Moreover, MFF-overexpressing cells showed significantly higher spare respiratory capacity, suggesting an enhanced ability to adapt to energetic stress. Collectively, this response was abrogated by treatment with IACS-010759, a

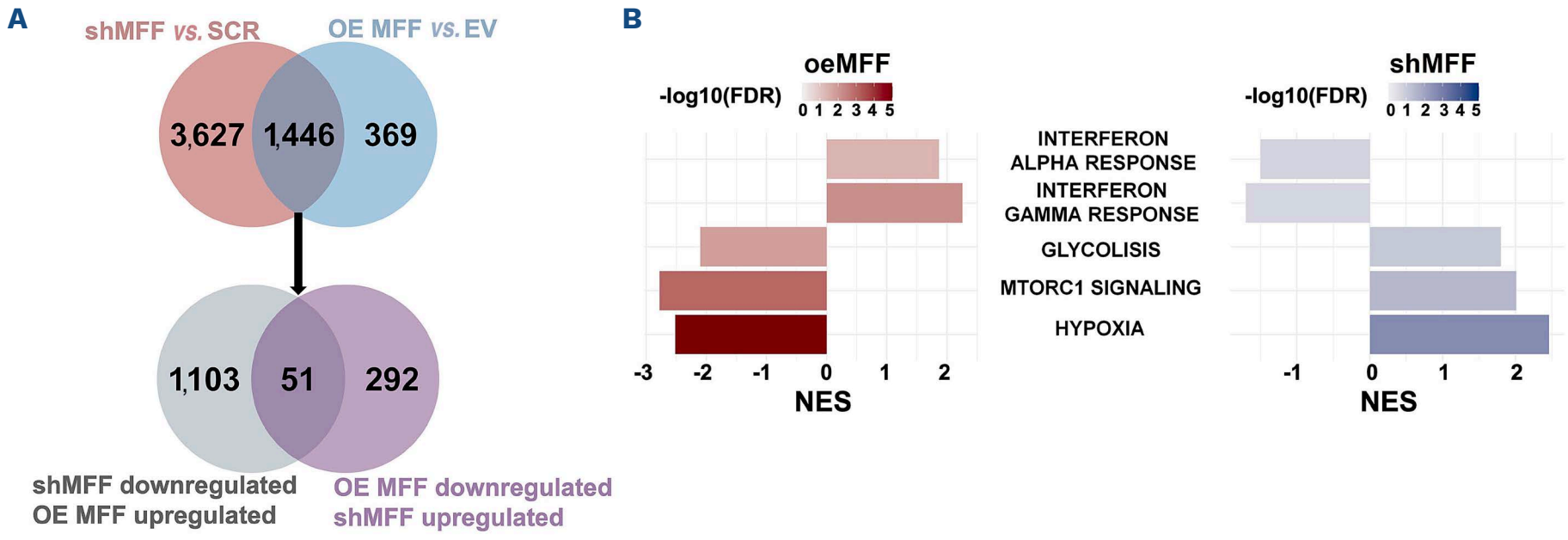

Figure 3. Mitochondrial fission factor targeting prompts a shift towards glycolytic metabolism. (A) Venn diagrams illustrating the number of differentially expressed genes that overlap (top), or have opposite expression (bottom) between AMO-BZB cells stably silenced for MFF (shMFF) and H929 cells overexpressing MFF (OE MFF). (B) Gene set enrichment analysis indicating the top five hallmark gene sets with opposite patterns enriched between H929 OE MFF (left side) and shMFF AMO-BZB cells (right side). The normalized enrichment score is indicated on the y-axis. (C) Quantitative reverse transcriptase polymerase chain reaction analysis of ALDOC, ENO2, HK2, PDK4, and SLC2A1 mRNA expression levels in shMFF AMO-BZB and H929 OE MFF cells. The results show the average mRNA expression levels after normalization with β-actin and ΔΔCt calculations and are expressed as percentage ± standard deviation normalized to each respective control. ***P*<0.01. FDR: false discovery rate; NES: normalized expression score; ALDOC: aldolase C; ENO2: enolase 2; HK2: hexokinase 2; PDK4: pyruvate dehydrogenase kinase 4; SLC2A1: solute carrier family 2A1.

selective complex I inhibitor (*Online Supplementary Figure S4A*), underscoring the OXPHOS dependence of MFF-overexpressing cells. This shift toward OXPHOS may thus provide an advantage to highly proliferating malignant MM cells, in which rapid ATP production is needed.

MFF targeting, on the other hand, led to a reduction in both basal and maximal respiration in MM cells (Figure 4B; *Online Supplementary Figure S4B*), which was further supported by the downregulation of proteins of the electron transport chain, such as NDUFS1, NDUFAB1, UQCRFS1, COX1, and COX4 (*Online Supplementary Figure S4C*). This reduction resulted in an increase in ECAR (Figure 4C), but was not associated with lactate accumulation. In fact, when intracellular lactate was measured in MFF-depleted cells, we observed a reduction in lactate levels (Figure 4D), which was associated with decreased LDHB expression (*Online Supplementary Figure S4D, E*). Consistent with these findings, MFF KD caused a decrease in protein lactylation, which conversely increased upon MFF overexpression (Figure 4E). These results were further supported by the downregulation of lactate transporters, MCT1 and MCT4, both *in vitro* and *in vivo* following MFF KD (Figure 4F; *Online Supplementary Figure S4F*). Additionally, a positive correlation between MFF and MCT4 mRNA expression was observed in the CoMMpass cohort (*Online Supplementary Figure S4G*), reinforcing the clinical relevance of this interaction.

Previous studies have indicated that excessive lactate can drive OXPHOS while suppressing glucose fermentation.[27,28] In agreement with these findings, lactate supplementation increased cellular OCR, enhancing both basal and maximal respiration in MM cells. Notably, this effect was abrogated by MFF KD (Figure 4G), suggesting that MFF acts as a critical determinant of the lactate-induced metabolic shift.

Collectively, this set of data suggests that MFF fine-tunes

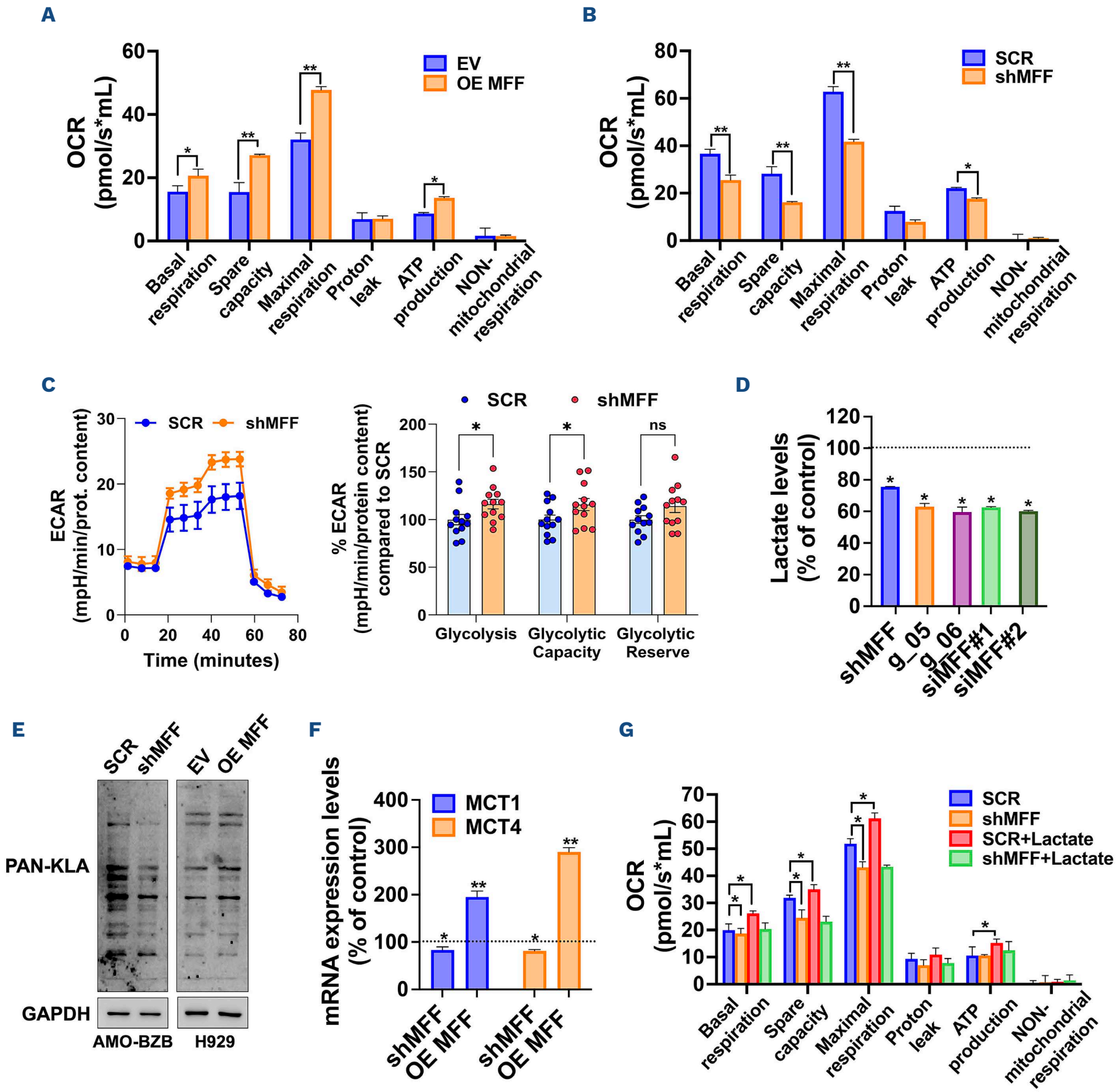

Figure 4. Lactate accumulation drives mitochondrial fission factor-dependent metabolic reprogramming in multiple myeloma cells. (A, B) Real-time oxygen consumption rate (OCR) measured via OROBOROS on (A) H929 cells overexpressing mitochondrial fission factor (OE MFF) or (B) AMO-BZB cells stably silenced for MFF (shMFF); histogram bars report the average of three independent experiments of multiple key parameters, including basal respiration, spare capacity, maximal respiration, leak state, ATP production, and non-mitochondrial respiration. *P<0.05; **P<0.01. (C) Seahorse analysis of extracellular acidification rate of shMFF AMO-BZB cells; at least six replicates were analyzed in each experiment. Results are the average of independent experiments and are expressed as percentages normalized to the control ± standard error of the mean. *P<0.05. (D) Relative intracellular lactate levels assessed by a Lactate-Glo assay in shMFF AMO-BZB cells. *P<0.05. (E) Western blot analysis of total lactylated proteins in shMFF AMO-BZB or H929 OE MFF cells using an anti-pan-KLA antibody; GAPDH was used as a loading control. (F) Quantitative reverse transcriptase polymerase chain reaction analysis of MCT1 and MCT4 mRNA levels in shMFF AMO-BZB and in H929 OE MFF cells. The results show the average of mRNA expression levels after normalization with GAPDH and ΔΔCt calculations, and are expressed as percentage ± standard deviation normalized to each control. *P<0.05; **P<0.01. (G) Real-time OCR measurement via OROBOROS, 24 h after lactate supplementation (20 mM) in SCR or shMFF AMO-BZB cells; histogram bars report the average of three independent experiments. *P<0.05. EV: empty vector; SCR: scrambled control; ECAR: extracellular acidification rate.

the lactate-dependent balance between OXPHOS and glycolysis, positioning MFF as a key determinant of metabolic reprogramming in MM cells.

Inhibitors of mitochondrial fission synergize with lactate transporter inhibitors to induce multiple myeloma cell death

Emerging evidence strengthens the link between lactate and mitochondrial fission, which subsequently affects cellular metabolic reprogramming.[29] Given that lactate supplementation can promote a shift towards OXPHOS,[27] we aimed to unravel the contribution of MFF to such metabolic effects. Notably, western blotting analysis showed that lactate exposure elicited an upregulation of MFF protein levels in MM cells (Figure 5A; *Online Supplementary Figure S5A*). Since lactate transporter inhibitors, such as AZD3965 (a selective MCT1 inhibitor) and syrosingopine (an inhibitor of both MCT1 and MCT4) have been shown to increase intracellular lactate levels,[30,31] we hypothesized that these drugs could mimic exogenous lactate. As expected, both AZD3965 and syrosingopine raised intracellular lactate levels in MM cells (Figure 5B), which in turn stimulated mitochondrial respiration by enhancing OCR (*Online Supplementary Figure S5B, C*), whereas this effect was abrogated after MFF silencing (*data not shown*). Moreover, both inhibitors induced the upregulation of MFF protein expression (Figure 5C, D). This metabolic effect mirrored that observed after MFF overexpression, and suggests that MM cells may upregulate MFF to counteract elevated lactate levels, thus promoting mitochondrial fission and boosting OXPHOS.

Using loss-of-function approaches, we next investigated the impact of MFF targeting on the antitumor activity of lactate transporter inhibitors. As shown in Figure 5E, MFF KD cells were more sensitive to lactate supplementation, with increased cell death likely due to the inability of MM cells to prompt OXPHOS; similar results were obtained using g_06 (Figure 5F). Moreover, increased anti-MM activity could be observed when g_06 was combined with syrosingopine or AZD3965 (Figure 5G); similarly, Mdivi-1, a well-known inhibitor of Drp1, the major partner of MFF in the execution of mitochondrial fission,[32] synergistically enhanced AZD3965 or syrosingopine antitumor activity in MM cells (Figure 5H, I), while sparing healthy peripheral blood mononuclear cells (Figure 5J), suggesting a favorable therapeutic window of these combinatorial strategies.

Overall, these data indicate that targeting mitochondrial fission can enhance the efficacy of lactate transporter inhibitors in MM, with a safe toxicity profile *in vitro*.

Mitochondrial fission factor promotes bortezomib resistance of multiple myeloma cells

Recent reports have highlighted the capability of lactate to ignite MM cell resistance to proteasome inhibitors (PI).[33] Since lactate induces MFF expression, we hypothesized that MFF might mediate lactate-dependent mechanisms of drug resistance. Western blotting analysis showed differential expression of MFF between PI-sensitive and PI-resistant isogenic MM cell lines, with MFF being notably upregulated in the PI-resistant counterpart (Figure 6A). Notably, upon lentiviral-enforced expression of MFF, the PI-sensitive cell line became resistant to bortezomib treatment, as evidenced by enhanced cell viability and reduced apoptosis compared to those of control cells (Figure 6B, C); conversely, targeting MFF with g_06 increased the *in vitro* anti-MM activity of bortezomib (Figure 6D).

As both AZD3965 and syrosingopine promote MFF expression, likely as an adaptive mechanism to cope with lactate overload, we tested whether these two drugs could affect bortezomib anti-MM activity *in vitro*. Bortezomib treatment did indeed synergistically enhance the inhibitory effects of both lactate transporter inhibitors on MM cell viability (Figure 6E), even in co-culture with HS5 cells (*Online Supplementary Figure S6*), and this was accompanied by MFF protein downregulation in the combination setting (Figure 6F). Taken together, these results suggest that bortezomib hampers MCT1/4 inhibitor-induced MFF elevation, leading to synergistic anti-MM activity *in vitro* when combined with AZD3965 or syrosingopine.

Discussion

MM is a plasma cell malignancy characterized by a complex array of clinical manifestations, including hypercalcemia, renal dysfunction, anemia, and bone lesions (collectively referred to as CRAB symptoms); the disease also presents a wide spectrum of clinical variants, ranging from benign monoclonal gammopathy of undetermined significance and smoldering/indolent MM to more aggressive forms, such as overt MM and plasma cell leukemia.[34]

Mitochondria are vital organelles often referred to as the powerhouse of the cell; as such, they regulate critical cellular processes, including energy production, cellular metabolism, and apoptosis.[1] Defects in mitochondrial functions prompt metabolic and phenotypic changes, which have been shown to contribute to the onset and progression of different types of neoplasias, including MM. The complex and heterogeneous genomic landscape of MM, including prognostically relevant cytogenetic abnormalities, gene mutations, and altered gene expression profiles, leads to highly diverse metabolic derangements during MM onset and progression.[4] Partly depending on their genomic alterations, MM cells not only utilize glucose uptake, aerobic glycolysis, and lactate production but also rely on mitochondria to activate the tricarboxylic acid cycle and OXPHOS, thereby sustaining elevated protein synthesis, folding, and secretion.[35] Additionally, bioenergetic plasticity, which is associated with increased mitochondrial biomass and function, can also emerge as an adaptive response to therapeutic treatments, potentially leading to drug resistance.[36,37]

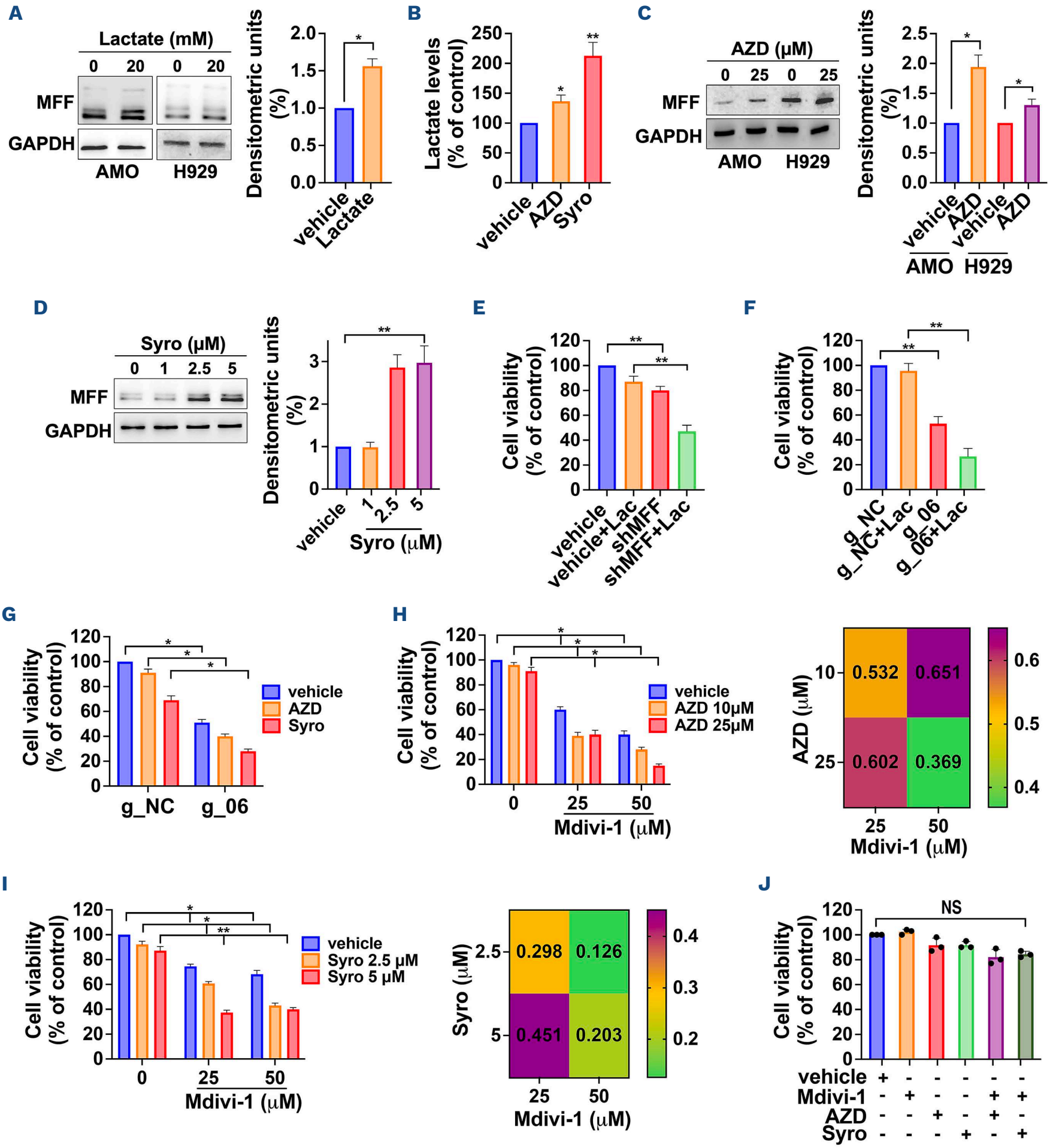

Figure 5. Targeting of mitochondrial fission enhances the *in vitro* anti-multiple myeloma activity of lactate transporter inhibitors. (A) Western blot analysis of mitochondrial fission factor (MFF) expression, 24 h after lactate supplementation. GAPDH was used as a loading control. Histogram bars represent the densitometric analysis, reported as fold increase relative to the control. **P*<0.05. (B) Relative intracellular lactate levels assessed by a Lactate-Glo assay, 24h after treatment with AZD3965 (25 μM) or syrosingopine (5 μM) in AMO cells. **P*<0.05; ***P*<0.01. (C, D) Western blot analysis of MFF expression, 24 h after treatment with AZD3965 (C) or syrosingopine (D); GAPDH was used as a loading control. Histogram bars represent the densitometric analysis, reported as fold change relative to the vehicle. **P*<0.05; ***P*<0.01. (E, F) Cell Titer Glo assay after lactate supplementation (20 mM) in AMO-BZB cells stably silenced for MFF (shMFF) (E) or in AMO-BZB cells electroporated with g_06 or g_NC (F); viable cells are reported as percentage of control. ***P*<0.01. (G) Cell Titer Glo assay performed in AMO-BZB cells after electroporation with g_06 or

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g_NC, alone or in combination with AZD3965 (25 μM) or syrosingopine (5 μM); viable cells are reported as percentage of control. **P*<0.05. (H, I) Cell Titer Glo assay performed in AMO-BZB cells after treatment with Mdivi-1 alone or in combination with AZD3965 (H) or syrosingopine (I). **P*<0.05; ***P*<0.01. Heat-maps show combination indexes, determined by Calcusyn software, of the combination treatments. (J) Cell Titer Glo assay performed on peripheral blood mononuclear cells from healthy donors (N=3), after treatment with Mdivi-1 (50 μM) alone or in combination with AZD3965 (50 μM) or syrosingopine (5 μM). Viable cells are represented as percentage of vehicle. AZD: AZD3965; Syro: syrosingopine; Lac: lactate; NS: not statistically significant.

Over the last decade, it has become evident that the tight regulation of mitochondrial morphology, carried out by the mitochondrial dynamics rheostat, is mandatory for regulating cellular homeostasis and survival, and its disruption or imbalance can lead to mitochondrial dysfunction which, in turn, promotes a wide range of diseases, including cancer.[38] The balance of mitochondrial dynamics is controlled by the presence of fusion-promoting or fission-promoting molecules with GTPase activity. Elevated mitochondrial fission, driven by increased expression and/or activity of Drp1, has been proven to promote metabolic reprogramming, cell cycle progression and evasion of cell death in a wide variety of solid tumors.[7-11] Alterations in the mitochondrial dynamics pathway have also been identified in hematologic malignancies, such as T-cell acute lymphoblastic leukemia[12] and MM,[13] leading to increased Drp1 expression, activity and tunneling nanotube-mediated mitochondrial trafficking, in the bone marrow microenvironment, of plasma cell-derived mitochondria in a post-fission state,[39] which triggered survival and drug resistance.

Although deregulated mitochondrial fission is emerging in cancer and is starting to be linked to MM pathobiology,[13,14] the precise role of the MFF protein remains to be elucidated in this malignancy. MFF is the predominant Drp1 receptor in mammalian cells,[16] and it is highly expressed in tissues with high energy demands, such as heart, brain, muscle, kidney, and liver.[15] Knockdown of MFF results in elongated mitochondria, whereas its overexpression increases mitochondrial fragmentation.[40]

Overexpression of MFF has been reported in non-small cell lung,[19] ovarian,[41] prostate,[42] platinum-resistant head and neck,[43] and hepatocellular[44] cancer tissues compared to their normal counterparts, as well as in liver[20] and prostate[45] cancer-initiating cells, where elevated mitochondrial fission plays a pro-survival role through the enhancement of mitochondrial functions that sustain the rapid metabolism of tumor cells. Here, we show that MFF is a feature of aggressive myelomas, as higher expression was observed in plasma cells from MM patients bearing genomic alterations predictive of poor outcome; moreover, elevated MFF protein expression was detected in primary MM samples spotted onto a tissue microarray.

Consistent with a general collapse of mitochondrial integrity, the induction of mitochondrial elongation via multiple MFF targeting strategies (siRNA, shRNA or ASO) delivered preclinical anticancer activity, as indicated by inhibition of MM proliferation, blockade of the cell cycle, suppression of colony formation and impaired growth in mice.

Overall, these data point to MFF as a novel potential therapeutic target in MM. Transcriptomic analyses were carried out to assess the molecular perturbations produced by MFF targeting and revealed that mitochondrial fission blockade is associated with substantial alterations in metabolic pathways, consistent with the inhibition of mitochondrial OXPHOS and a shift toward glycolysis. This was validated by high resolution respirometry and Seahorse measurement of metabolic fluxes. Intriguingly, this glycolytic signature was associated with a decrease in intracellular lactate, highlighting the broad and still not fully understood metabolic plasticity of MM. For a long time considered a metabolic waste product, it is now acknowledged that lactate can act as fuel for oxidative phosphorylation;[46] accordingly, we demonstrated that, in MM cells, lactate-induced oxidative metabolism led to higher basal respiration, mitochondrial ATP production and maximal respiratory capacity, all of which were significantly antagonized by MFF knockdown. These data suggest that MFF acts as a restriction point in the lactate-dependent fine-tuning between oxidative metabolism and glycolysis, strengthening the role of mitochondrial dynamics in shaping the metabolic plasticity of malignant plasma cells.

We also explored whether the adaptive metabolic responses induced by MFF targeting could be leveraged for therapeutic purposes. To this end, we employed two drugs, AZD3965 and syrosingopine, both known to promote lactate accumulation via MCT4 or MCT1/4 inhibition, respectively. Notably, the excessive intracellular lactate produced by both compounds resulted in MFF upregulation and stronger anti-MM activity when MFF, or its partner Drp1, was inhibited; conversely, no cytotoxic effects were observed in healthy cells. These data suggest that lactate overload, which can no longer support growth-promoting OXPHOS when mitochondrial fission is impaired, leads to MM cell death. This provides the first preclinical rationale for exploiting therapeutic strategies targeting mitochondrial fission to enhance the limited single-agent activity of drugs inhibiting MCT1 and/or MCT4 transporters, both of which are overexpressed and predict poor survival in MM.[33,47]

The proteasomal machinery, i.e., the most relevant therapeutic target in MM, is involved in the regulation of cellular metabolism.[36,48] To survive proteasome inhibition, MM cells do indeed modulate their metabolism, mitochondria and endoplasmic reticulum to redistribute cellular resources and decrease their metabolic fitness.[36] Notably, lactate-dependent OXPHOS stimulation takes part in the mechanisms by which plasma cells metabolically adapt and maintain bioenergetic plasticity,[48,49] and AZD3965 was shown to target the metabolic rewiring of malignant plasma cells, overcoming

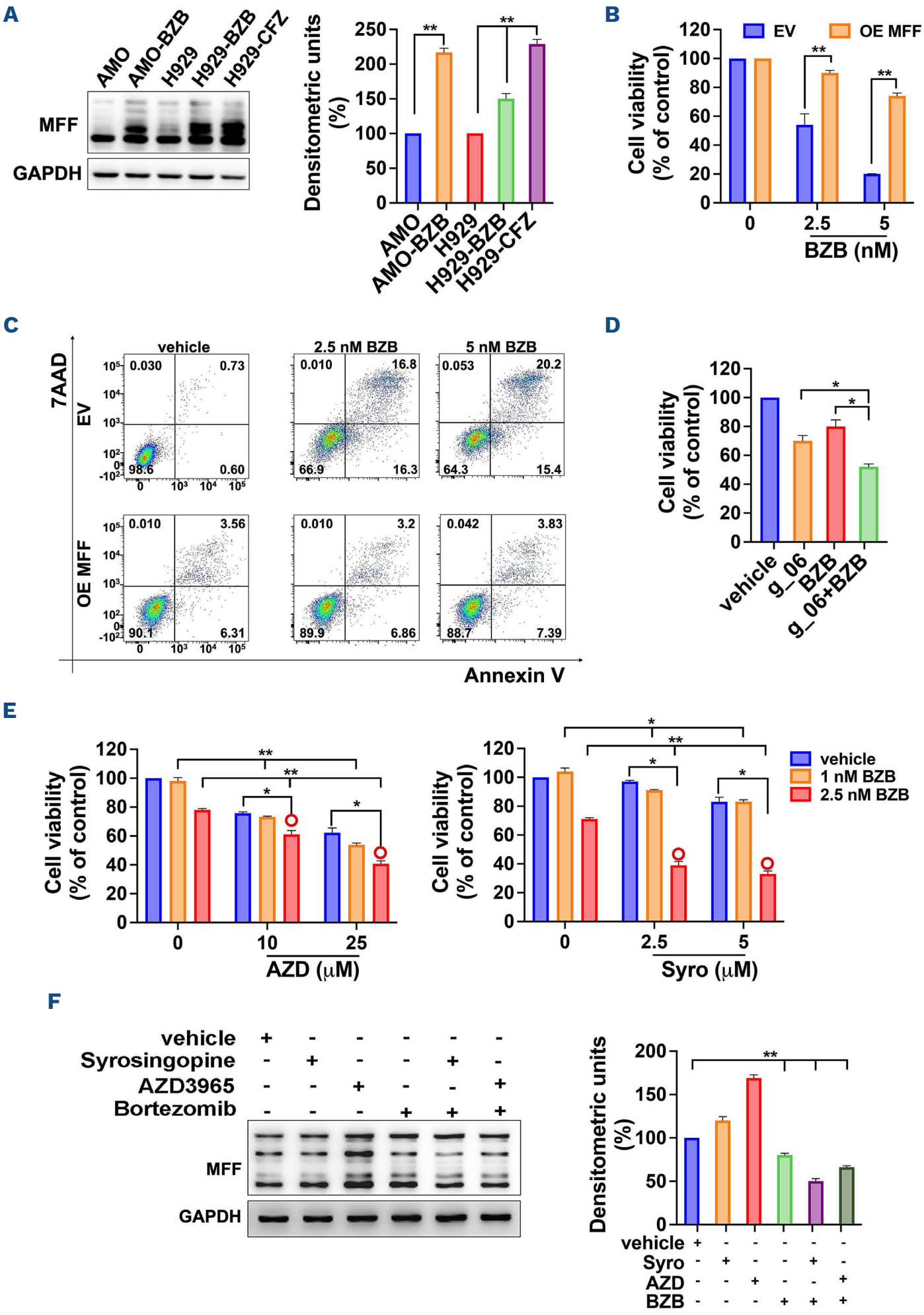

Figure 6. Mitochondrial fission factor triggers resistance to proteasome inhibitors via a lactate-dependent pathway. (A) Western blot analysis of mitochondrial fission factor (MFF) expression in isogenic multiple myeloma (MM) cell lines sensitive to proteasome inhibitors (AMO; H929) and resistant to bortezomib (AMO-BZB, H929-BZB) or carfilzomib (H929-CFZ); GAPDH was used as a loading control. Histogram bars represent the densitometric analysis of the blot, reported as fold increase respect to each parental cell line. ***P*<0.01. (B) Cell Titer Glo assay performed in H929 cells overexpressing MFF (OE MFF), after treatment with bortezomib; viable cells are reported as percentage of control. ***P*<0.01. (C) Flow cytometry analysis of H929 OE MFF cells stained

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with annexin V/7-aminoactinomycin D, 24 h after treatment with bortezomib. Dot plots represent the data from an independent biological replicate (N=3). (D) Cell Titer Glo assay performed in AMO cells electroporated with g_06 or g_NC, and treated with 2.5 nM bortezomib for 24 h. Viable cells are reported as percentage of control. *P<0.05. (E) Cell Titer Glo assay performed in AMO cells, 24 h after treatment with bortezomib alone or in combination with AZD3965 or syrosingopine. Viable cells are reported as percentage of control; red circles indicate synergistic effects of drug combinations (combination index <1). *P<0.05; **P<0.01. (F) Western blot analysis of MFF expression in AMO cells, 24 h after treatment with bortezomib (5 nM), alone or in combination with 25 μM AZD3965 or 5 μM syrosingopine; GAPDH was used as a loading control. Histogram bars represent the blot densitometric analysis, reported as fold increase respect to vehicle. **P<0.01. EV: empty vector; BZB: bortezomib; 7AAD: 7-aminoactinomycin D; AZD: AZD3965; Syro: syrosingopine.

bortezomib resistance.[33] In line with these observations, our findings demonstrate that mitochondrial fragmentation may drive lactate-induced PI resistance. Indeed, PI-resistant isogenic cell lines significantly upregulated MFF with respect to their parental counterparts, and MFF overexpression could dampen bortezomib sensitivity, while anti-MFF ASO enhanced its anti-tumor effects. Importantly, we observed that bortezomib reversed the induction of MFF promoted by MCT1/4 inhibitors, leading to deeper antitumor responses. This finding informs novel potential combination therapies aimed at halting lactate-induced adaptive phenomena to enhance PI activity. Future studies will focus on validating the antitumor effects of bortezomib-MCT1/4 inhibitor combination regimens in *in vivo* preclinical models of MM.

In conclusion, the data presented highlight novel aberrations in mitochondrial dynamics that underlie the metabolic adaptability of MM cells, providing a compelling framework for the development of innovative therapeutic approaches to treat MM and potentially other cancers characterized by elevated mitochondrial fission.

Disclosures

No conflicts of interest to disclose.

Contributions

NA and MEGC were responsible for the overall design of the study, experimental validation, and writing the manuscript. MEGC, IV, RT, LG, PM, MM and CG performed most of the in vitro and in vivo experiments. CV and CDM performed transcriptomic experiments. IP carried out the transmission electron microscopy studies. FT performed immunohistochemistry experiments. MF and ARC contributed to metabolic flux analysis. VA, TP, CG, MG, DT, GV and AN provided critical comments. All the authors read and approved the final manuscript.

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Data-sharing statement

Transcriptomic data are available through GEO accession number GSE286009. All the other data generated or analyzed during this study are included in this manuscript.

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