MIF as a biomarker and therapeutic target for overcoming resistance to proteasome inhibitors in human myeloma

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Supplemental Methods

Human MM cell lines

The MM cell line ARP-1 was kindly provided by the Arkansas Cancer Research Center, Little Rock, AR. KMS-12-BM, KMS-12-PE, JJN3, HRMM.09, and MWD MM.13 were kind gifts from Dr. Frederic J. Reu of the Cleveland Clinic[63]. Cfz-resistant KMS-11/Cfz and its WT KMS-11 were kindly provided by Dr. Robert G. Hawley[64]. Other cell lines were purchased from the American Type Culture Collection (Rockville, MD). All MM cells were cultured in RPMI-1640 medium supplemented with 10% FBS (Invitrogen, Carlsbad, CA).

Generation of knockdown, knockout, and overexpression MM cell lines

MIF-specific shRNA (target sequence: TCAACTATTACGACATGAA) was designed and synthesized by Sigma (St. Louis, MO) and inserted into a lentivirus system vector pLVTHM, which was a gift from Didier Trono (Addgene plasmid # 12247). CRISPR guide RNAs (1: GAGGAACCCGTCCGGCACGG; 2: ACAGCATCGGCAAGATCGG) for MIF were synthesized by Sigma (St. Louis, MO) and inserted into lentiCRISPRv2 GFP plasmid, which was constructed by replacing *puro* gene in lentiCRISPRv2 puro plasmid (a gift from Brett Stringer, Addgene plasmid # 98290) with *GFP*. After lentiviral infection, transduced cells were sorted for GFP^+ cells followed by western blot for examining MIF expression as described previously.

MIF overexpression was achieved using the lentiviral expression pUltra (carrying GFP selective marker; Addgene plasmid # 24129) or pUltra-hot (carrying mCherry selective marker; Addgene plasmid # 24130) vector, a gift from Malcolm Moore, according to the manufacturer's instruction. Mutant MIF-expressing pUltra-hot plasmids were generated using QuikChange II XL Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA) with primers listed in **Supplemental Table 1**. MIF-overexpressing MIF-KO MM cells were selected by sorting for $mCherry^+$ cells. MIF-overexpressing MIF-KI KMS-12-PE cells were selected by sorting for GFP^+ cells.

Gene-expression profiling and analysis of clinical datasets

MM microarray datasets, including array platform information and related clinic factors, were extracted and downloaded from the Oncomine (www.oncomine.org) and Gene Expression Omnibus (GEO) datasets (264 patients from GSE9782 and 414 patients from GSE2658). Normalized expression values of individual genes and clinical data from 1143 patients were downloaded from MMRF coMMpass study IA13 (https://research.themmrf.org). The significance of differential MIF or SOD1 expression between two patient groups was determined by the Wilcoxon test. Correlations between MIF and SOD1 expressions were analyzed by linear regression with R calculated. For OS analysis, samples with survival information were sorted according to their MIF and SOD1 expression levels and categorized into 2 groups (MIF^{high} vs. MIF^{low}; $SOD1^{high}$ vs. $SOD1^{low}$). The number of patients in each group is indicated in the figures. Patient survival curves were plotted using the Kaplan-Meier analysis and significance was measured using the log-rank test. The same method was used for the PFS and TTP analysis.

Microarray and RNAseq for MM samples

Microarray analysis of CTR-KO and MIF-KO ARP-1 and MM.1S MM cells (GSE141170) was performed using the Affymetrix WT Plus expression platform at the Gene Expression and Genotyping Facility at Case Western Reserve University, Cleveland, OH. RNAseq of DMSO- and Cfz-treated ARP-1 MM cells (GSE143406) was performed by BGI Americas Corporation. The DEseq2 method was used for differential expression analysis. IPA-Tox analysis of CTR-KO and MIF-KO MM cell array data was performed for assessment of dysregulated signaling and metabolic pathways in MIF-deficient MM cells. GSEA was run for each cell subset in pre-ranked list mode with 1000 permutations (nominal p value cutoff <0.01).

Antibodies and reagents

MIF antagonists ISO-1 and 4-IPP, and trimer-disrupting agent ebselen were purchased from EMD Millipore, the SOD1 inhibitor ATN-224 was from Cayman Chemical Company, Ann Arbor, MI, and DSF was from Sigma. NAC was purchased from Sigma. Human MIF-neutralizing mAb was a kind gift from Dr. Richard Bucala[65]. Western blot antibodies against XBP1s, ATF-4, ATF-6, Caspase 8, Caspase 9, Caspase 3, PARP, Mcl-1, β-actin, COX IV, and SOD1 were purchased from Cell Signaling Technology. Anti-MIF antibodies were purchased from Santa Cruz Biotechnology. Goat anti-mouse IgG secondary antibody labeled with Alexa

Fluor 555 was purchased from Invitrogen. Mouse anti-misfolded SOD1 conformational B8H10 mAb was from Medimabs. Protein G-conjugated sepharose beads were from Cell Signaling Technology. Flow cytometry antibodies for APC-Annexin V, human PE-CD138, and isotype controls were purchased from Biolegend.

PI treatment

To mimic *in vivo* patient pharmacokinetics of PIs, human myeloma cells were pulsed with high dose of PIs for 1 h and recovered in PI-free medium.

Cell viability assay

MTS assay (Promega, Madison, WI) was used to determine the relative number of viable cells after drug treatment as described previously[50]. Cells (2.5×10^5/ml) were treated with drugs and seeded in a 96 well-plate for 48-72 h. The viability assay was conducted based on manufacturer's protocol and the plates were read on a VICTOR Multilabel Plate Reader (PerkinElmer, Akron, Ohio). Values were normalized to DMSO-treated cells.

Apoptosis assay

MM (2.5×10^5/ml) cells were treated with 1 h pulse of 100 nM Btz or 80 nM (ARP-1, MM.1S, MM.1R, U266) or 200 nM (KMS-11, KMS-11/Cfz) Cfz, and allowed to recover in PI-free medium for 24 h. The fraction of apoptotic cells was determined by staining with APC-conjugated Annexin-V and analyzed by flow cytometry (BD LSRFortessa, BD Biosciences) as we described previously[66-68].

Untargeted metabolomic analysis

Twelve Cfz-treated ARP-1 cell samples (6 for CTR-KO and 6 for MIF-KO) were prepared and used for untargeted metabolomics analysis in Mass Spectrometry Laboratory at Lerner Research Institute, Cleveland, OH. The list of all known ions with statistical significance (FDR-adjusted p value <0.05) was analyzed by MetaboAnalyst 3.0 (www.metaboanalyst.ca/MetaboAnalyst/)[69] for determining the top impacted metabolic pathways.

Mitochondrial membrane potential assay

Mitochondrial Δψm was measured using a TMRE kit (Invitrogen, T669) according to manufacturer's instruction. Briefly, 5×10^5 MM cells were pulsed with 80nM Cfz for 1 h, and 14 h later incubated with 250 nM TMRE at 37 °C for 30 min before harvest. Carbonyl cyanide p-trifluoro-methoxyphenyl hydrazone (FCCP), an ionophore that depolarizes mitochondria and prevents binding of the dye to the mitochondria, was used as a negative control.

Seahorse Assay

The measurement of OCR and extracellular acidification rate (ECAR) in MM cells was performed using a Seahorse XF24 Extracellular Flux Analyzer[70,71]. Briefly, CTR-KO or MIF-KO MM (4×10^5) cells were pretreated with or without indicated chemotherapy drugs overnight in triplicates and then transferred into a pretreated Cell-Tak-coated XF 24-well. Prior to starting the assay, cells were washed and incubated in Seahorse Assay Medium supplemented with 5 mM glucose and 2 mM sodium pyruvate in a 37°C incubator without CO_2 for 45 min. Oligomycin (1 μM), FCCP (1.5 μM) and rotenone (0.5 μM) were injected as indicated and the OCR (pmol O_2/min) was measured in real time. Data normalization was achieved using protein concentration assay.

Measurement of mitochondrial Ca^{2+} levels

To measure mitochondrial Ca^{2+} levels in MM cells, a cold/warm incubation protocol was used to exclusively load mitochondria with Ca^{2+}-sensitive fluorescent dye, Rhod-2AM (Abcam, ab142780)[28,72]. Treated cells were incubated with 5 μM Rhod-2-AM in Tyrode's solution at 4°C for 90 min, followed by incubation at 37°C for 30 min, and then analyzed by flow cytometry. The steady state Ca^{2+} concentration was calculated as $[Ca^{2+}]_M$ = Ca^{2+} fluorescence intensities of MM cells in normal Tyrode's solution - fluorescence intensities of MM under calcium-free condition by ionomycin treatment.

Mitochondrial ATP assay

A luciferase assay was used for determination of mitochondrial ATP concentration of treated MM cells. The assay was done following the protocol of the ATP bioluminescence assay kit HS II (Sigma).

Immunofluorescent assay

Mitochondrial morphological changes of MM cells were examined by confocal microscope by staining the cells with MitoTracker^{TM} Red CMXRos (Invitrogen, M7513) for mitochondria and DAPI for nucleus according to manufacturer's instruction.

Measurement of mitochondrial superoxide levels

To measure the relative levels of mitochondrial superoxide, treated MM cells were stained with 5 μM Mitosox Red (Invitrogen, M36008) for 10 min at 37°C. Cells were then washed three times with media. MitoSOX Red excited at 488 nm and fluorescence emission at 610/20 nm were measured by a flow cytometer (FACSymphony, BD). Relative fluorescence intensity from biological triplicate of MM cells was used as an indicator of mitochondrial superoxide levels.

Real-time PCR

Total RNA from MM cells was extracted using TRIzol RNA isolation reagents (Invitrogen) or an RNeasy Mini Kit (QIAGEN), followed by cDNA synthesis with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems)[73]. Real-time quantitative PCR was conducted with SYBR Select Master Mix (Applied Biosystems). Expression was normalized to the expression of human housekeeping gene *GAPDH*. The primers used are listed in **Supplemental Table 1**.

Western blotting analysis

Cell lysates and immunoblotting were performed as previously described[74]. Cells were harvested and lysed with Cell Lysis Buffer (Cell Signaling Technology) supplemented with protease/phosphatase inhibitor cocktail (Cell Signaling Technology). Protein concentration was determined by the Bradford Protein Assay (Bio-Rad). Protein samples were subjected to NuPAGE 4-12% Bis-Tris Protein Gels (Invitrogen), transferred to a nitrocellulose membrane (Bio-Rad), and immunoblotted with primary antibodies overnight. Secondary antibodies conjugated to horseradish peroxidase were used for detection, followed by enhanced chemiluminescence (Pierce Biotechnology).

Subcellular fractionation

Mitochondria of 5×10^7 Cfz-pulsed CTR-KO or MIF-KO MM cells were purified using Mitochondria Isolation Kit for Cultured Cells as described previously[21].

Analysis of soluble misfolded human SOD1

Whole-cell extracts (100 μg), or cytosol or mitochondrial fractions (100 μg each) were solubilized in immunoprecipitation (IP) buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 0.5% NP-40 plus 1× protease inhibitors) and submitted to a pre-clearing step with 30 μl of protein G-conjugated sepharose beads for 2 h under rotation at 4°C. The conformation-specific B8H10 mAb was used since it is more efficient and specific in immunoprecipitation assays[40,41]. The antibody-protein G-conjugated sepharose beads (6 μl antibody/40 μl beads) were then incubated with 0.5 ml of the pre-cleared soluble extract overnight under rotation at 4°C. Finally, the beads were washed five times with IP buffer. Samples were eluted by boiling in a 2× sample loading buffer and analyzed by western blot. For primary MM cells, 5 Cfz-treated PI-sensitive or PI-resistant primary MM cells were used to obtain cell lysate for immunoprecipitation and western blot with anti-misfolded SOD1 B8H10 mAb.

Superoxide Dismutase activity assays

Treated MM cells were washed with PBS for 3 times and lysed using 0.1 M Tris/HCl, pH 7.4, 0.05% TritonX-100, 5 mM β-ME, and 0.1 mg/ml PMSF, followed by centrifuging for 5 min at 4°C at 14,000 g. The total SOD activity of supernatants was subjected to the Superoxide Dismutase Activity Assay Kit (Abcam, ab65354) according to manufacturer's recommendation. SOD2 activity was assayed in the presence of 5 μM potassium cyanide to inhibit SOD1 activity. SOD1 activity was determined by subtracting MnSOD activity from the total SOD activity.

Mouse study

To determine the role of MIF in MM response to Cfz *in vivo*, 6 to 8 week-old mice were injected s.c. with (2×10^6 cells per mouse) CTR-KO and MIF-KO ARP-1 MM cells. At day 7 after tumor inoculation, vehicle or 3 mg/kg Cfz were i.v. injected into MM-bearing mice (two consecutive days weekly for 3 weeks). At day 8, MM-bearing mice were i.p. injected with L 012 sodium salt followed by bioluminescent imaging for *in vivo* ROS signal. Tumor volume was calculated from

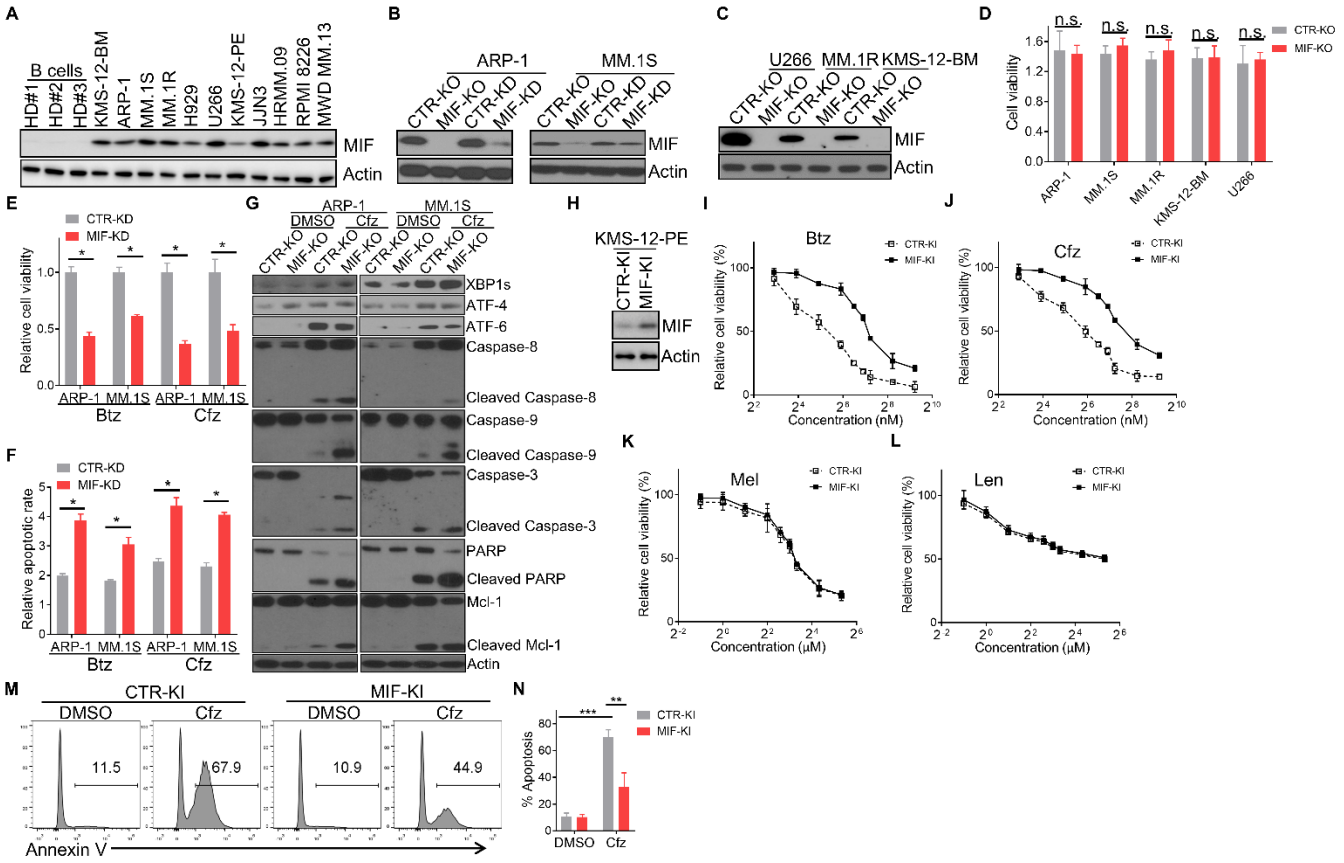
caliper measurements every 3 to 4 days. Tumor size was calculated as: 0.5 × L (length) × W (width)^2. To determine whether inhibiting MIF overcomes MM cell resistance to Cfz *in vivo*, NSG mice bearing large, established ARP-1 MM cells were treated, starting at day 8, with a low dose of Cfz (1 mg/kg, two consecutive days weekly till tumor relapsed). Tumor volumes were monitored and when the tumor size ≥ 10 mm, all mice received treatment of a high dose of Cfz (3 mg/kg, two consecutive days weekly), 4-IPP (0.5 mg per mouse, every 3 days), or a combination of both till the end of the study.

To determine whether MIF inhibitors may enhance the therapeutic efficacy of Cfz on MM *in vivo*, NSG mice were injected i.v. with 2×10^6 human MM cell lines ARP-1 or MM.1S. At day 14 after tumor inoculation, MM-bearing mice were injected with vehicle, 4-IPP, Cfz, or 4-IPP plus Cfz. Blood samples were collected once a week starting at day 16. Tumor burden was measured by ELISA, determining the level of human immunoglobulin light chain secreted by human MM cells in mouse plasma, which was normalized to control.

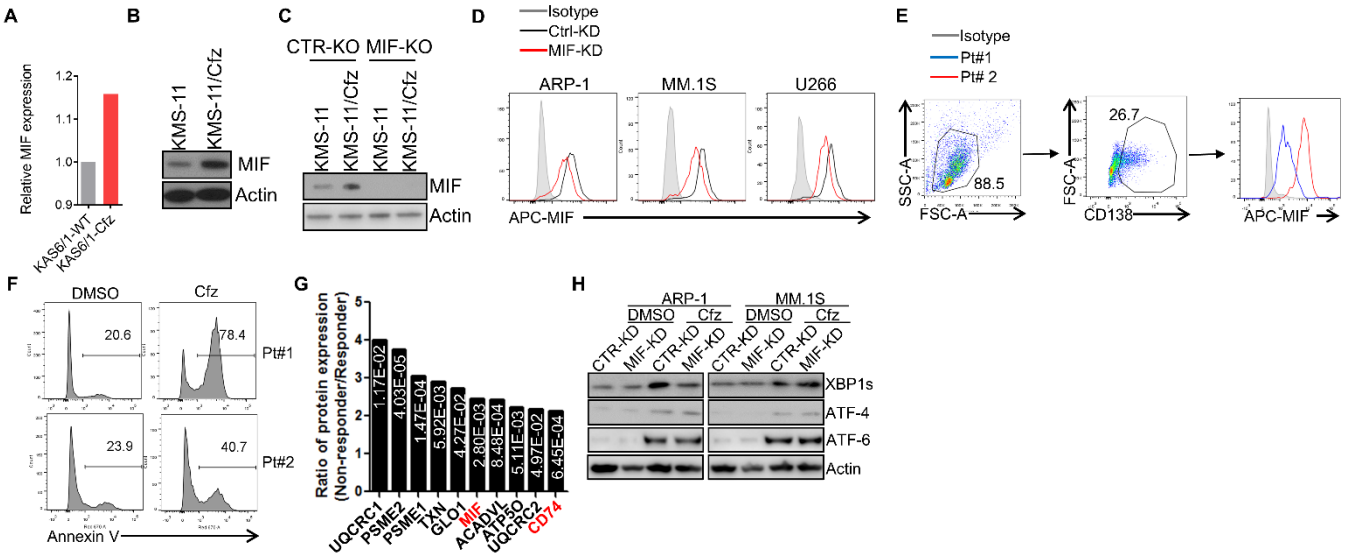
Statistical analysis

All data are shown as mean ± standard deviation. The Student two-tailed t test was used to compare 2 experimental groups, when more than 2 groups were included in an analysis, a Bonferroni corrected significance level was used. A p value less than 0.05 was considered significant. In some experiments, linear regression analyses were performed using GraphPad Prism 8, and slope and R values were calculated. Patient survival in MIF and SOD1 expression groups was analyzed by log-rank (Mantel-Cox) test using GraphPad Prism 8 software.

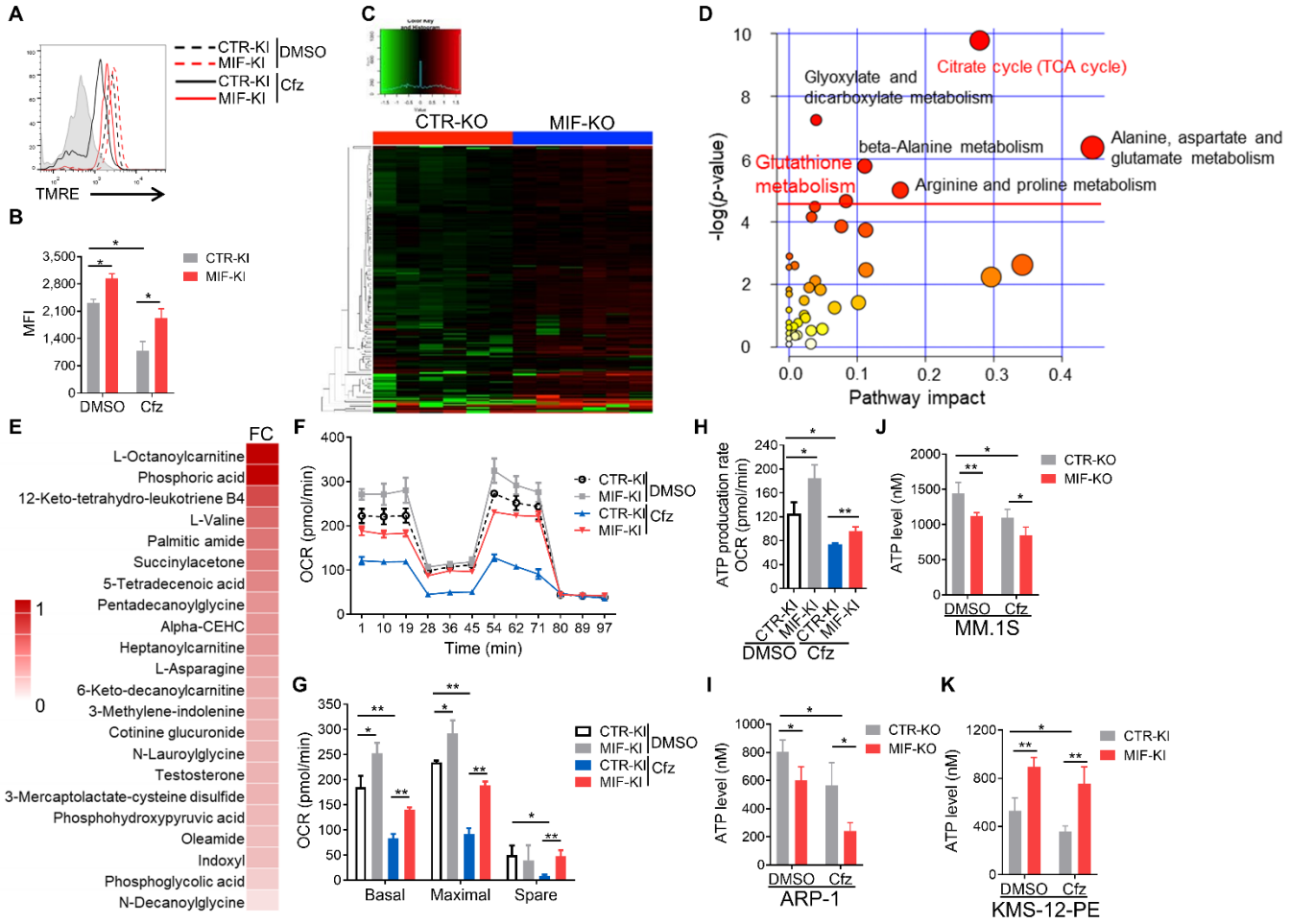
Supplemental Figures



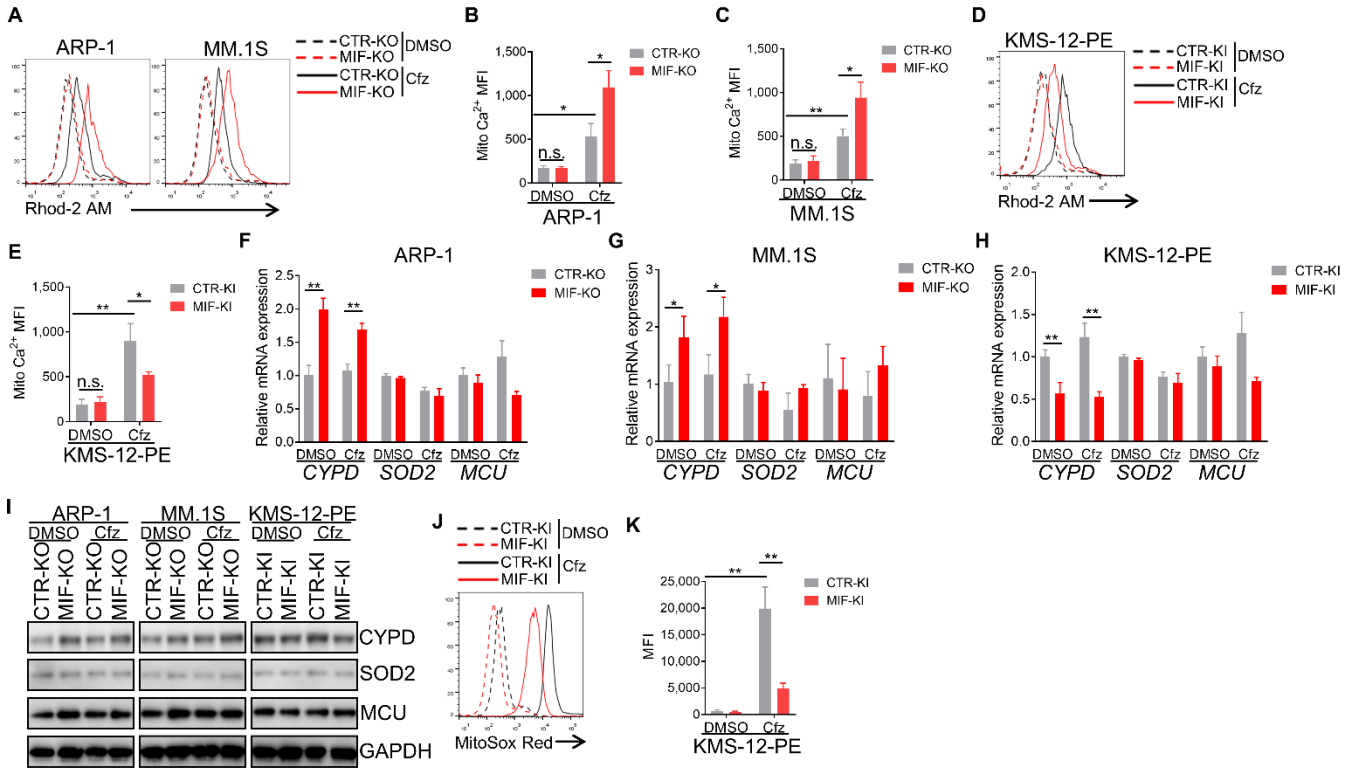
Supplemental Figure 1. MIF expression and drug response in human MM cell lines and primary MM cells. (**A**) Western blot showing MIF expression in B cells from 3 healthy donors (HD#1-3) and human myeloma cell lines. The efficiency of MIF depletion in control-knockdown (CTR-KD) or MIF-KD and control-knockout (CTR-KO) or MIF-KO ARP-1 and MM.1S (**B**), and in CTR-KO or MIF-KO U266, MM.1R, and KMS-12-BM (**C**). (**D**) Cell viability of CTR-KD or MIF-KD MM cells. Cell viability (**E**) and apoptosis (**F**) of CTR-KD or MIF-KD ARP-1 and MM.1S MM cells treated with DMSO, Btz, or Cfz; values were normalized to DMSO-treated MM cells. (**G**) Western blot showing the expression of indicated proteins involved in the ER stress and apoptotic pathways in CTR-KO and MIF-KO ARP-1 and MM.1S MM cells treated with DMSO or Cfz for 18 h. (**H**) Western blot showing MIF expression in CTR-knockin (CTR-KI) and MIF-KI KMS-12-PE cells. Cell viability of CTR-KI or MIF-KI KMS-12-PE MM cell lines with pulse treatment with Btz (**I**) or Cfz (**J**) for 1 h followed by 48 h culture in drug-free medium, or with continuous treatment with melphalan (Mel) (**K**), or lenalidomide (Len) (**L**) by MTS assay; significance was analyzed by one-way ANOVA with Tukey's post hoc test at each concentration point. Histogram (**M**) and summarized results (**N**) of apoptotic rate of CTR-KI or MIF-KI KMS-12-PE cells pulsed with 80 nM Cfz for 1 h and allowed to recover for 24 h by Annexin V assay. Student t test was used to compare 2 samples, n.s., no significance, *p<0.05, **p<0.01, ***p<0.001.



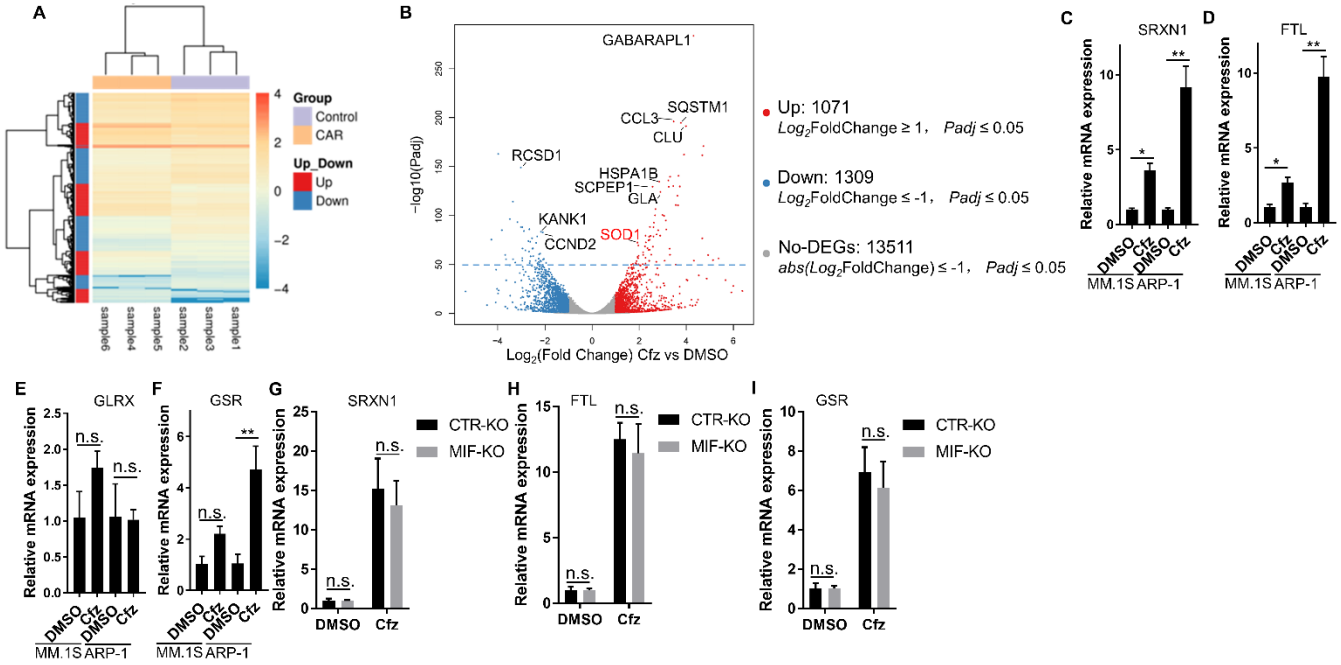
Supplemental Figure 2. MIF expression and drug response in Cfz-resistant cell line and primary MM cells. (**A**) MIF expression in WT KAS-6/1 and KAS-6/1-Cfz identified by gene expression profiling data from Wang et al. (GSE62237). Western blot showing MIF expression in normal (**B**), CTR-KO, and MIF-KO (**C**) KMS-11 and KMS-11/Cfz MM cells. (**D**) Intracellular staining for MIF expression by flow cytometry was validated in CTR-KD or MIF-KD MM cells. Fresh primary MM cells from patient BMs were divided into two parts for: 1) intracellular staining of MIF (**E**), and 2) determining apoptotic rate induced by Cfz treatment for 24 h (**F**). Two representative results of 16 patient samples are shown. (**G**) Histogram showing the top 10 upregulated proteins in the proteomic profiling data of MM plasma cells from patients with poor response compared to patients with good response according to the fold change; MIF and its receptor CD74 are marked as red. (**H**) Western blot showing the expression of indicated proteins involved in ER stress pathway in CTR-KD and MIF-KD ARP-1 and MM.1S MM cells treated with DMSO or Cfz for 18 h.



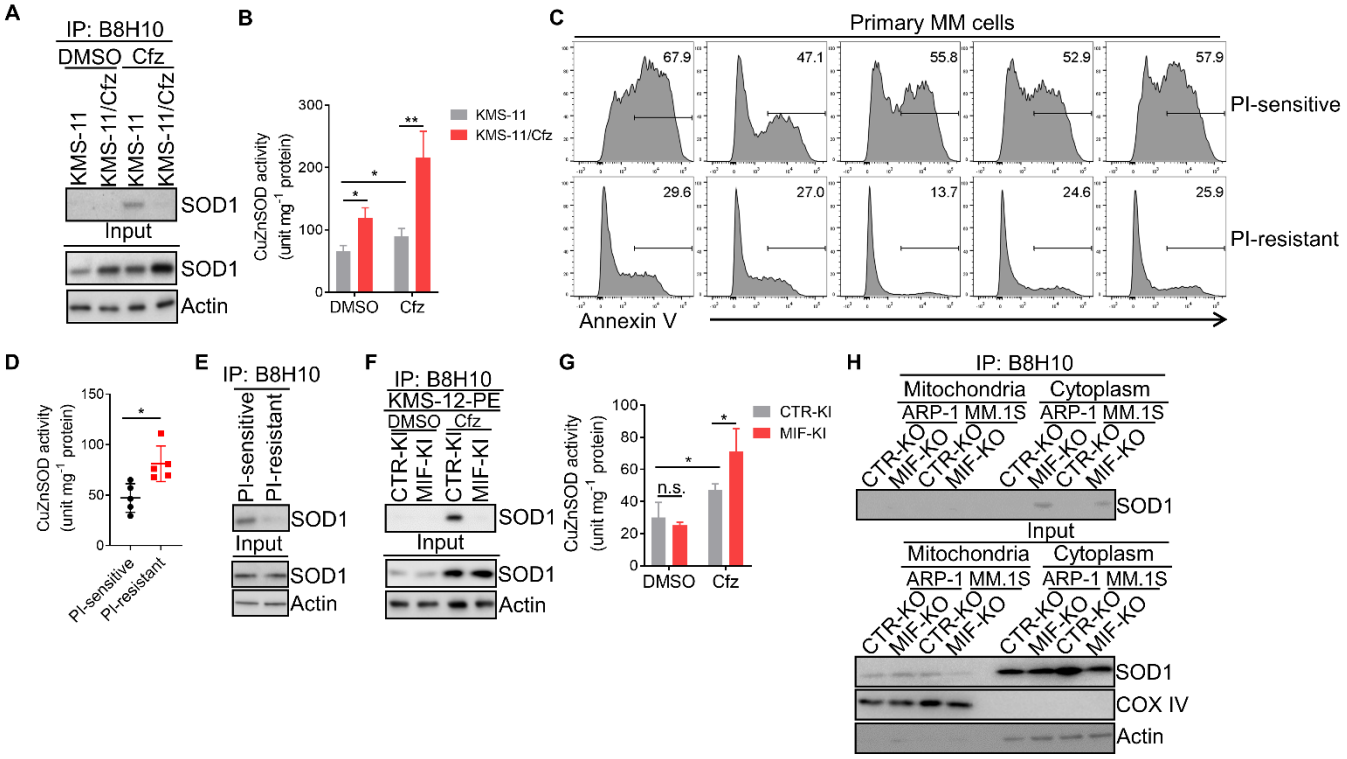
Supplemental Figure 3. Mitochondrial functions and untargeted metabolic profiling of MM cells. Flow cytometry histogram (**A**) and summarized results (**B**) of TMRE fluorescence of CTR-KI or MIF-KI KMS-12-PE MM cells after pulse treatment with DMSO or Cfz for 14 h. (**C**) Consensus hierarchical clustering from untargeted metabolic profiling of CTR-KO or MIF-KO ARP-1 MM cells treated with Cfz for 16 h. Heatmap showing ions that had the most significant (p-value<0.05, FDR=0.1) difference between the MIF-KO and CTR-KO MM cells. (**D**) MetaboAnalyst elaboration of the impact of pathways in MIF-KO versus CTR-KO MM cells. Circles that are larger, higher and closer to the Y-axis show higher impact. The red line indicates p-value <0.01. (**E**) Heatmap showing the fold change (FC) of 22 known ions that are upregulated in Cfz-treated MIF-KO ARP-1 compared with CTR-KO MM cells with the highest statistical significance. (**F**) Oxygen consumption rates (OCRs) for mitochondrial respiration function in CTR-KI or MIF-KI KMS-12-PE MM cells treated with DMSO or Cfz were measured by Seahorse XF Cell Mito Stress Test. Summarized results of basal OCR, maximum OCR, spare OCR (**G**), or ATP production OCR (**H**) are shown for 3 independent replicates as described in **F**. Mitochondrial ATP concentrations of 5×10^5 ARP-1 (**I**), MM.1S (**J**), and KMS-12-PE cells (**K**) treated with DMSO or Cfz were determined based on luciferase-catalyzed ATP-dependent oxidation of luciferin. For Panels **A**, one representative result of at least three independent experiments is shown. Student t test was used to compare 2 samples, *p<0.05, **p<0.01.



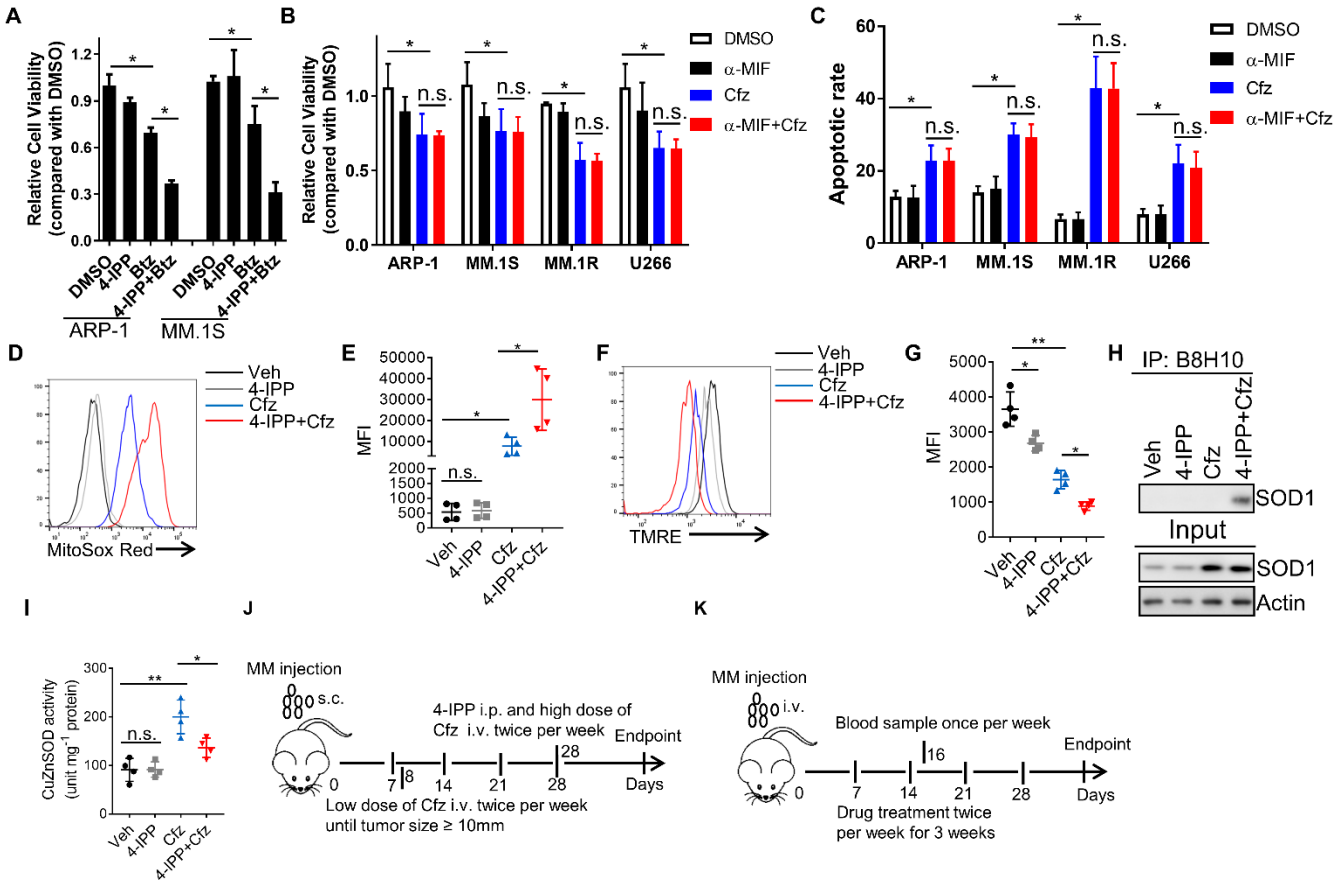
Supplemental Figure 4. Mitochondrial functions and function-related gene expressions in MM cells. Flow cytometry histogram (**A,D**) and summarized results (**B,C,E**) of mitochondrial Ca^{2+} concentrations (Mito Ca^{2+}) of CTR-KO or MIF-KO ARP-1 and MM.1S, and CTR-KI or MIF-KI KMS-12-PE MM cells after pulse treatment with DMSO or Cfz for 20 h. mRNA expression of *CYPD*, *SOD2*, and *MCU* in ARP-1 (**F**), MM.1S (**G**), and KMS-12-PE (**H**) MM cells treated with DMSO or Cfz; the housekeeping gene GAPDH was used for normalization of the qRT-PCR results. (**I**) Western blot showing the expression of indicated proteins involved in mitochondrial functions in ARP-1, MM.1S, and KMS-12-PE MM cells treated with DMSO or Cfz for 18 h. Flow cytometry histogram (**J**) and summarized data (**K**) showing MitoSOX Red fluorescence in CTR-KI or MIF-KI KMS-12-PE cells after pulse treatment with DMSO or Cfz for 16 h. For Panels **A**, **D**, and **J**, one representative result of at least three independent experiments is shown. Student t test was used to compare 2 samples, n.s., no significance, *p<0.05, **p<0.01.



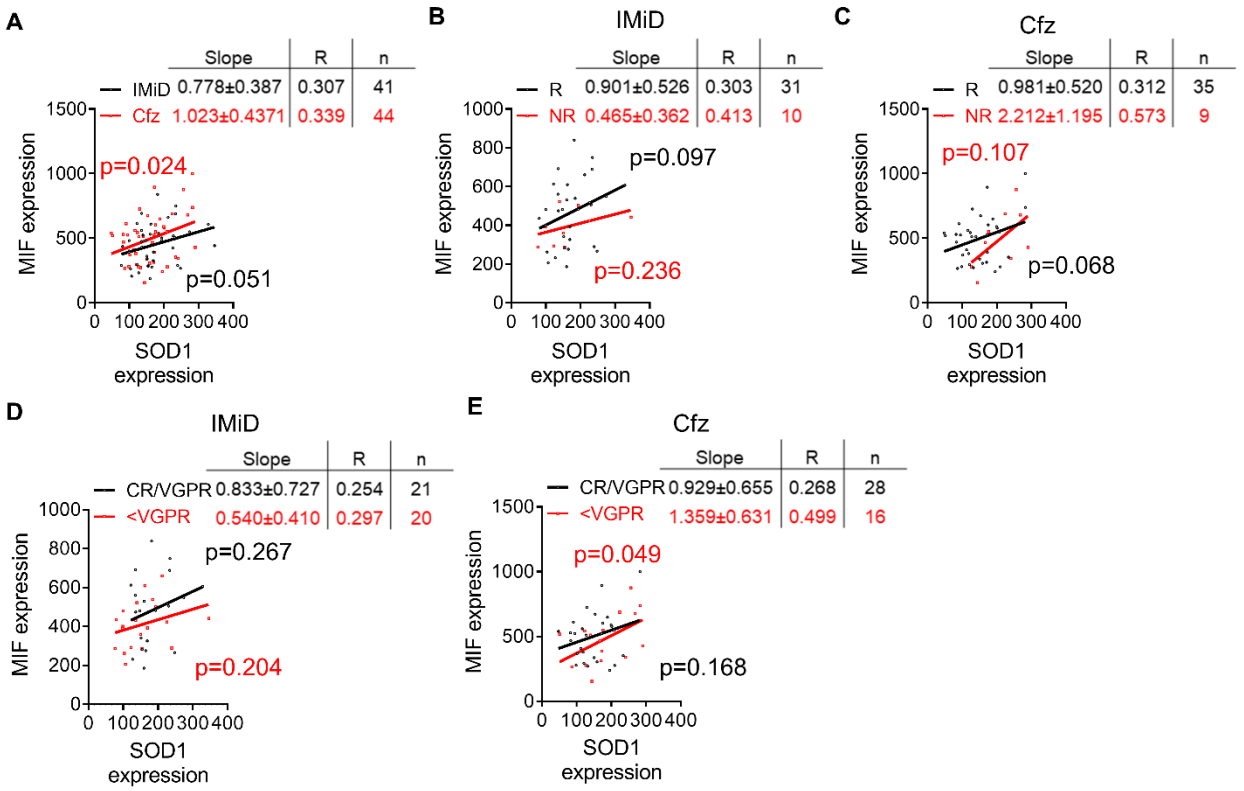
Supplemental Figure 5. Reactive oxygen species (ROS) pathway gene expressions in human MM cells. (**A**) Consensus hierarchical clustering from RNA-seq data of DMSO (Control)- or Cfz (CAR)-treated ARP-1 MM cells. (**B**) Volcano plot (mRNA fold difference versus P value) of gene profiling data of Cfz- versus DMSO- treated ARP-1 MM cells. mRNA expression of *SRXN1*, *FTL*, *GLRX*, and *GSR* in MM.1S and ARP-1 MM cells treated with DMSO or Cfz (**C-F**), and in CTR-KO or MIF-KO ARP-1 MM cells (**G-I**); the housekeeping gene GAPDH was used for normalization of the qRT-PCR results. Student t test was used to compare 2 samples, n.s., no significance, *p<0.05, **p<0.01, ***p<0.001.



Supplemental Figure 6. SOD1 misfolding and activity in Cfz-treated primary MM cells and human MM cell lines. KMS-11 or KMS-11/Cfz MM cells were pulsed with DMSO or 250 nM Cfz for 1 h. After 16 h, misfolded SOD1 (**A**) was detected by immunoblotting of immunoprecipitates with anti-misfolded SOD1 B8H10 mAb and SOD1 activity (**B**) was determined by ELISA. Histogram (**C**) showing apoptosis of Cfz-treated primary MM cells from MM patients, MM cells with apoptosis rate less than 40% were categorized as PI-resistant. (**D**) SOD1 activities of PI-sensitive and PI-resistant primary cells (n = 5 per group) treated with 100 nM Cfz were determined by ELISA. (**E**) Misfolded SOD1 was detected by immunoblotting of immunoprecipitates of PI-sensitive or PI-resistant primary MM cell lysate together with anti-misfolded SOD1 B8H10 mAb. CTR-KI or MIF-KI KMS-12-PE MM cells were pulsed with DMSO or Cfz for 1 h. After 16 h, misfolded SOD1 (**F**) was detected by immunoblotting of immunoprecipitates with anti-misfolded SOD1 B8H10 mAb and SOD1 activity (**G**) was determined by ELISA. (**H**) The expression of misfolded SOD1 and total SOD1 in cytosolic and mitochondrial fractions measured by western blot in CTR-KO and MIF-KO MM.1S and ARP-1 MM cells pretreated with Cfz for 16 h; β-actin and COX IV were used as loading controls. One representative result of two repeated experiments is shown.



Supplemental Figure 7. The role of MIF inhibition on human MM cell response to PIs. (**A**) Cell viability of ARP-1 and MM.1S MM cells treated with DMSO, 4-IPP, Btz, or 4-IPP+Btz. Cell viability (**B**) and apoptosis (**C**) of ARP-1, MM.1S, MM.1R, and U266 MM cell treated with DMSO, 20 μg/ml MIF-neutralizing mAb (α-MIF), Cfz, or α-MIF+Cfz. NSG mice were injected s.c. with 4 × 10^6 ARP-1 MM cells. At day 8 after tumor inoculation, MM-bearing mice received treatment of a high dose of Cfz (3 mg/kg for two consecutive days weekly; n=4) or 4-IPP (0.5 mg per mouse for every 3 days; n=4) alone, or their combinations (n=4). Ten days later after treatment, tumors were collected and sorted for $CD138^+$ MM cells followed by mitochondrial superoxide assay (**D,E**), mitochondrial membrane potential assay (**F,G**), SOD1 misfolding (**H**) and activity detection (**I**). **(J)** NSG mice were injected s.c. with 2 × 10^6 ARP-1 MM cells. At day 8 after tumor inoculation, vehicle or 1 mg/kg Cfz were i.v. injected, two consecutive days weekly, into MM-bearing mice. At day 28 when the size of some tumors enlarged to 10 mm, MM-bearing mice received treatment of a high dose of Cfz (3 mg/kg for two consecutive days weekly; n=6) or 4-IPP (0.5 mg per mouse for every 3 days; n=7) alone, or their combinations (n=6) till the end of the experiment. **(K)** NSG mice were injected i.v. with 2 × 10^6 MM.1S or ARP-1 MM cells. At day 14 after tumor inoculation, vehicle, 4-IPP, Cfz, or 4-IPP+Cfz were injected into MM-bearing mice (n=5/group). Blood samples were collected weekly starting at day 16. Student t test was used to compare 2 samples, n.s., no significance, *p<0.05, **p<0.01.



Supplemental Figure 8. Correlation of SOD1 and MIF in MMRF coMMpass datasets. (A) Dot plots showing co-expression patterns of SOD1 and MIF in patients with IMiD-based therapy alone or Cfz-based therapy alone. **(B)** Dot plots showing co-expression patterns of SOD1 and MIF in responder (R) or non-responder (NR) patients with IMiD-based therapy. **(C)** Dot plots showing co-expression patterns of SOD1 and MIF in R or NR patients with Cfz-based therapy. **(D)** Dot plots showing co-expression patterns of SOD1 and MIF in IMiD-treated patients with good response (CR/VGPR; including complete response, stringent complete response, and very good partial response) or poor response (< VGPR; including partial response, stable disease, and progressive disease). **(E)** Dot plots showing co-expression patterns of SOD1 and MIF in Cfz-treated patients with good response (CR/VGPR) or poor response (< VGPR). Correlations were analyzed by linear regression with R calculated. The linear slope and sample number were indicated. The significance of linear relationship between SOD1 and MIF expression were indicated as p value.

Cloning and mutagenesis primers	Sequence
MIF clone XbaI F	AGCGTCTAGAATGCCGATGTTCATCGTAAACACCAAC
MIF clone EcRI R	AGCGGAATTCTTAGGCGAAGGTGGAGTTGTTCCAGCC
MIF A48P F	GACCAGCTCATGCCCTTCGGCGGCT
MIF A48P R	AGCCGCCGAAGGGCATGAGCTGGTC
MIF L46A F	GCCGAAGGCCATGGCCTGGTCCGGGACC
MIF L46A R	GGTCCCGGACCAGGCCATGGCCTTCGGC
MIF P107 F	GCGGCCAATGTGCCGGGCTGGAAC
MIF P107 R	GTTGTTCCAGCCCGGCACATTGGC
MIF N110C F	CTTAGGCGAAGGTGGAGCAGTTCCAGCCCACATTGG
MIF N110C R	CCAATGTGGGCTGGAACTGCTCCACCTTCGCCTAAG
MIF P2A F	GTTTACGATGAACATCGCCATTCTAGAAGGCCCGG
MIF P2A R	CCGGGCCTTCTAGAATGGCGATGTTCATCGTAAAC
MIF C60S F	GTGCAGGCTGCTGAGCGCGCACG
MIF C60S R	CGTGCGCGCTCAGCAGCCTGCAC
RT qPCR primers	Sequence
SRXN1 F	CAGGGAGGTGACTACTTCTACTC
SRXN1 R	CAGGTACACCCTTAGGTCTGA
FTL F	CAGCCTGGTCAATTTGTACCT
FTL R	GCCAATTCGCGGAAGAAGTG
GLRX F	CCCATCAAACAAGGGCTTCTG
GLRX R	CTGCATCCGCCTATACAATCTT
SOD1 F	ACTGGTGGTCCATGAAAAAGC
SOD1 R	AACGACTTCCAGCGTTTCCT
GSR F	CACTTGCGTGAATGTTGGATG
GSR R	TGGGATCACTCGTGAAGGCT
SYT1 RT F	TCATTCAGCTTTGAAGTACCTTT
SYT1 RT R	GCATCGTTCTTGCCAATCTT
CYPD RT F	CGACTTCACCAACCACAATG
CYPD RT R	TCTTGGATGTCCTCCCACTC
SOD2 RT F	GGAAGCCATCAAACGTGACT
SOD2 RT R	ACACATCAATCCCCAGCAGT
MCU RT F	GAATTTGGGAGCTGTTTATTGC
MCU RT R	AGGTCTCTTTTTGGTGGTCGTA

Supplemental Table 1. Primer list for cloning and mutagenesis and real time qPCR.