SUPPLEMENTAL METHODS AND FIGURES

Cell lines and culture

The following human MM cell lines were used: MM.1S, MM.1R, H929, RPMI8226/S, U266 (obtained from ATCC), Melphalan-resistant RPMI-LR5 and doxorubicin (Dox)-resistant RPMI-Dox40 (kindly provided by Dr William Dalton, Lee Moffitt Cancer Center, Tampa, FL), KMS-20, KMS-18 and KMS-12BM (Japanese Collection of Research Bioresources), OPM1 and OPM2 (provided by Dr Edward Thompson, University of Texas Medical Branch, Galveston). IL-6–dependent human MM cell lines INA-6 and XG-1 were provided by Dr Renate Burger (University of Kiel, Kiel, Germany). UTMC2 and EJM were furnished by Dr Leif Bergsagel (Mayo Clinic, Scottsdale, AZ). ANBL-6-bortezomib–sensitive (ANBL-6.WT) and -resistant (ANBL-6.BR) cell lines were kindly provided by Dr. Robert Orlowski (M.D. Anderson Cancer Centre, Houston, TX). All MM cell lines were cultured in complete RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L L-glutamine. BMSCs were cultured in Dulbecco Modified Eagle medium (Sigma-Aldrich) containing 15% FBS, 2mM L-glutamine (Invitrogen), 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen). Blood samples collected from healthy volunteers were processed by Ficoll-Paque gradient to obtain peripheral blood mononuclear cells (PBMCs). MM Patient tumor cells and BMSCs were obtained from BM samples after informed consent was obtained, in accordance with the Declaration of Helsinki and approval by the Institutional Review Board of the Dana-Farber Cancer Institute. BM mononuclear cells were separated using Ficoll Paque density sedimentation, and plasma cells were purified by positive selection with anti-CD138 magnetic activated cell separation microbeads (Miltenyi Biotec). Residual CD138-negative BM mononuclear cells were cultured in RPMI 1640 at 20% FCS for 3 to 6 weeks to generate BMSCs, as previously described.[1]

Electron microscopy

RPMI8226/S, U266 and MM1S cells were treated with or without drug for 48h, collected, and fixed with 2.0% paraformaldehyde/2.5% electron microscope grade glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.4) at 37°C. After fixation, samples were placed in 2% osmium tetroxide in 0.1M sodium cacodylate buffer (pH 7.4), dehydrated in a graded series of ethyl alcohol, and embedded in resin. Ultrathin sections were cut and placed on formvar-coated slot copper grids. Sections were then counterstained with uranyl acetate and lead citrate, and viewed with a TecnaiG^2 Spirit BioTWIN electron microscope. Digital images were acquired with an AMT 2k CCD camera.

Flow cytometry assay for autophagy

RPMI8226/S and U266 -EGFP-LC3$^+$ cells were treated with vehicle, FK866 (10nM) or Rapamycin (10nM). After 48 hours, cells were harvested and washed with PBS containing 0.05% saponin or PBS alone. More than 30,000 events were captured on a FACS (BD Bioscences) and analyzed.

Mitochondrial transmembrane potential ($\Delta\psi$m) determination

Mitochondrial membrane integrity was measured with TMRE In brief, treated cells were harvested, washed, and incubated in the presence of 50 nM TMRE in regular RPMI-based medium for 15' at 37°C. Thereafter, cells were analyzed by flow cytometry.

Small interfering RNA (siRNA) Transfection

The ERK1/2 siRNA knockdown was performed using signal silence p44/42 MAPK (ErK1/2) siRNA kit (Cell Signaling, Beverly, MA). U266 cell lines were transiently transfected using Cell Line Nucleofector Kit C (Amaxa Biosystems), as per the manufacturer's instructions. 1.5 x 10^6 MM cells were transfected with 200 nM of Erk 1/2 or control (Cell Signaling) siRNA, and then separated into two groups. The first groups of cells were cultured for 72h; protein lysates were then analyzed for the expression of ERK1/2 by immunoblotting using anti-p44/42 MAPK Ab. In

the second group, U266 cells were transfected with Erk1/2 siRNA; after 24h incubation, FK866 (1-10nM) was added for an additional 72h, followed by analysis of viability using PI staining as described above.

TF-EB transfection of U266 cell line

U266 cells were transiently transfected with plasmids containing DKK-tagged ORF clone of Homo sapiens transcription factor EB (TFEB) (Origene Systems, Rockville, MD) using Cell Line Nucleofector Kit C (Amaxa Biosystems). DKK tagged TFEB cells were treated with vehicle, or FK866 (10nM) for 24-48 hrs. The cells were then subjected to nuclear and cytoplasmic fractionation with the Nuclear/Cytosol Fractionation Kit (BioVision) and blotted with antibody against DDK. GAPDH and Nucleolin were used as cytosolic and nuclear markers, respectively.

Gene expression profile by microarray analysis

Total RNA extraction and purification, cDNA synthesis and cRNA labeling, Affymetrix chip (human HG-U133 plus 2.0 array) hybridization and data analysis were performed as previously described. [2] Briefly, MM.1S cells were treated with 10 nM FK866 or with DMSO as a control for the indicated time points; total RNA was then extracted and purified, cDNA synthesized and cRNA labeled prior to hybridization to the HG-U133 plus 2.0 arrays. Gene expression data have been deposited in Gene Expression Omnibus (GEO, GSE35414). GeneChip arrays were scanned on a GeneChip Scanner 3000 (Affymetrix). The normalization of arrays and calculation of expression values was done using the DNAchip analyzer (dChip) program. Arrays were normalized based on relative signal produced for an invariant subset of genes. For each time-point of the analysis, gene expression profile of FK866-treated MM.1S cells was compared to profile of MM.1S cells cultured in absence of drug; filtering of upregulated or downregulated transcripts was based on conventional criteria for statistical significance incorporated in the software.[3,4] Probes were then filtered to identify those which have ≥2-fold change between vehicle vs FK866 treatment. Supervised hierarchical clustering of the dataset according to the list of transcripts significantly modulated was then performed using dChip. A Pearson product-moment correlation coefficient (PMCC) was obtained by comparing the gene expression profiles (vehicle- vs FK866-treatment).

To determine gene expression of Nampt in MM cells cultured in vitro in the presence versus absence of HS-5 bone marrow stromal cells, we used dataset from Gene Expression Omnibus (GSE 20540). Expression levels of Nampt (probe ID 217738_s_at) were compared in MM1R, MM1S and INA6 cells upon interaction with HS5 stromal cells.

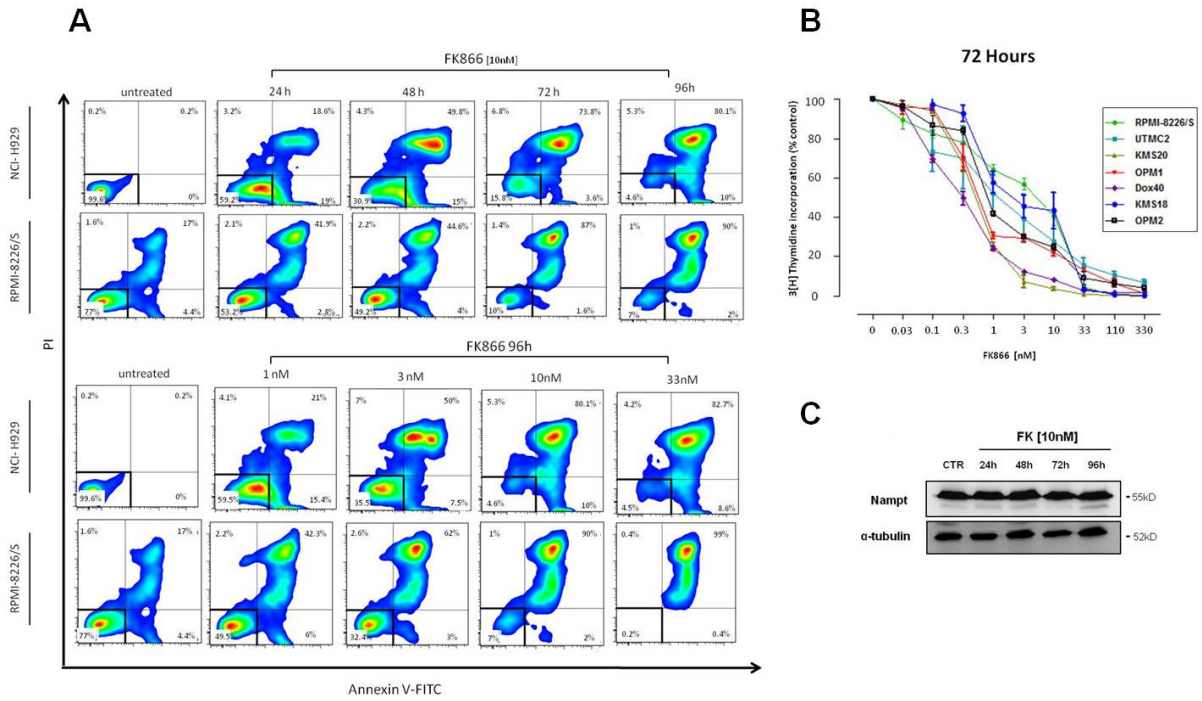

Figure S1. FK 866 induces time and dose dependent cell death and inhibits cell proliferation in human MM cell lines, without effect on Nampt protein level. (A) RPMI-8226/S and NCI-H929 cell lines were exposed to IC_{50} concentration of FK866 at indicated time (upper panel) or increasing dose (1-33nM) of FK866 for 96 hours (bottom panel). After incubation, cells were collected, washed, stained for Annexin V and PI, and analyzed using flow cytometry. FK866 treatment resulted in a dose- and time-dependent increase of Annexin V-positive and PI-positive cells. Representative graph from 3 independent experiments is shown. (B) A panel of 7 MM cell lines was treated with DMSO or increasing doses of FK866 (0.03-330nM) for 72 hours. Inhibition of cell proliferation was determined by 3[H]thymidine incorporation assay. Data represent mean ±SD of 3 independent experiments carried out in triplicate. (C) RPMI-8226/S cells were treated with 10nM FK866 for indicated times. Whole-cell lysates were subjected to immunoblotting using ant-Nampt antibody. α-tubulin was used as a loading control.

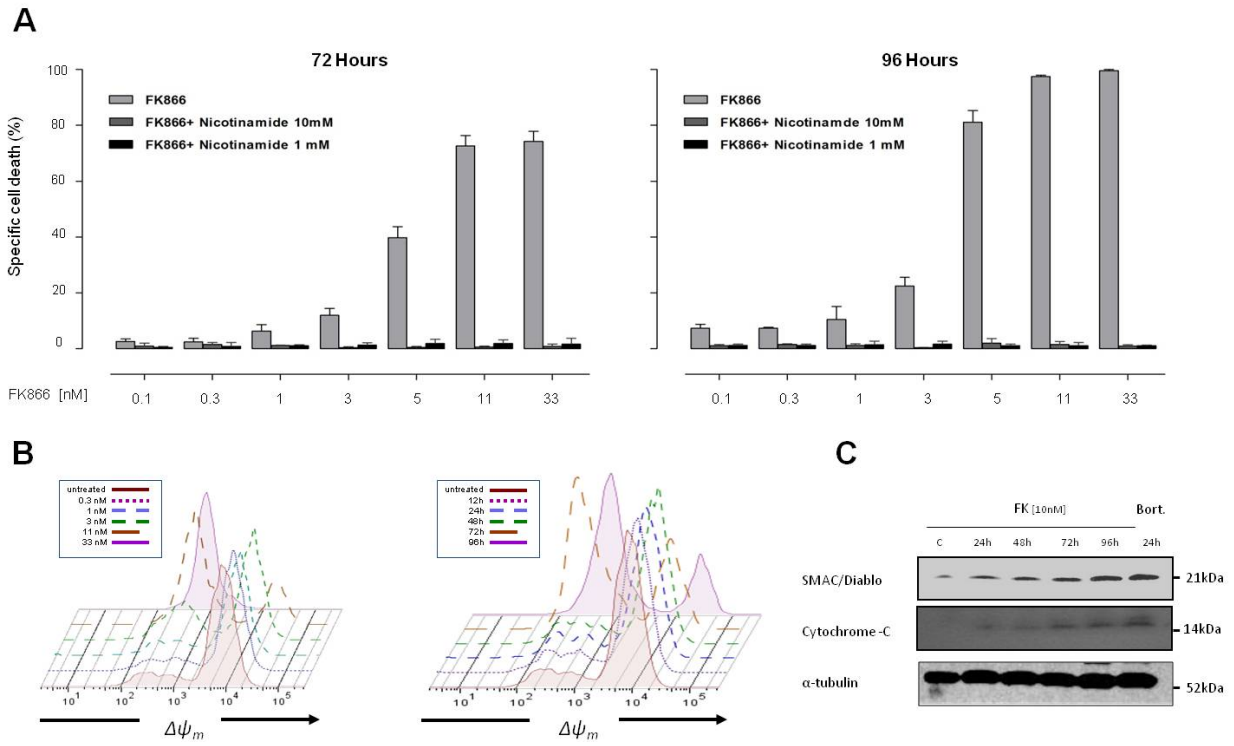

Figure S2. Anti-MM activity of FK866 is associated with mitochondria depolarization and is rescued by Nicotinamide. (A) RPMI-8226/S cells were treated with increasing doses of FK866 (0.1-33nM), in the presence or absence of 1-10 mM Nam. After 72-96h, specific cell death was determined by PI staining and flow cytometry. Each treatment was tested in triplicate wells, and results are means ± SD of three independent experiments. (B) RPMI-8226/S cells were incubated with increasing doses (0.3-33nM) of FK866 for 96 hours (left panel) or IC50 concentration of FK866 for indicated times (right panel). $\Delta_{\psi m}$ was assessed by TMRE staining and flow cytometric analysis. (C) RPMI-8226/S cells were treated with 10 nM FK866 at indicated times, and cytosolic fractions were subjected to immunoblot analysis using the indicated antibodies. Bortezomib was used as a positive control (25nM, 24h).

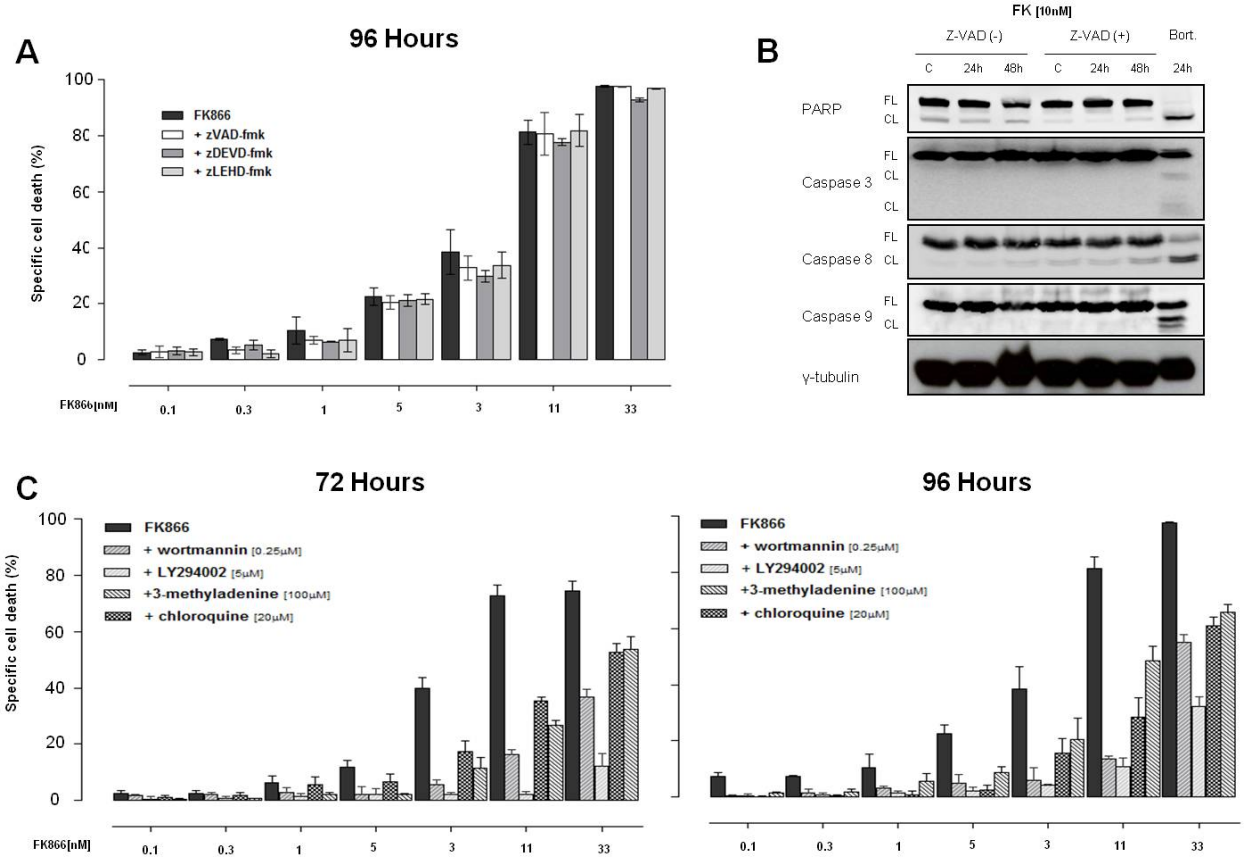

Figure S3. Anti-MM activity of FK866 is independent of caspase activation and involves autophagy. (A) RPMI-8226/S cells were pretreated with caspase inhibitors (zVAD-fmk, zDEVD-fmk or zLEHD-fmk), and then incubated with FK866 at indicated concentrations for 96 hours. Specific cell death was measured with PI staining and flow cytometry analysis. (B) RPMI-8226/S cells were cultured with or without FK866 10nM (for 24-48 hours) ± zVAD-fmk. Total cell lysates were subjected to immunoblotting using indicated antibodies. Bortezomib (50nM for 24h) was used as a positive control. (C) RPMI-8226/S cells were treated for 72-96h with increasing doses of FK866, in the presence or absence of the indicated concentrations of wortmannin, LY294002, 3-MA and chloroquine. Cell death was subsequently assessed by flow cytometric enumeration of PI+ cells. One representative experiment of three is presented.

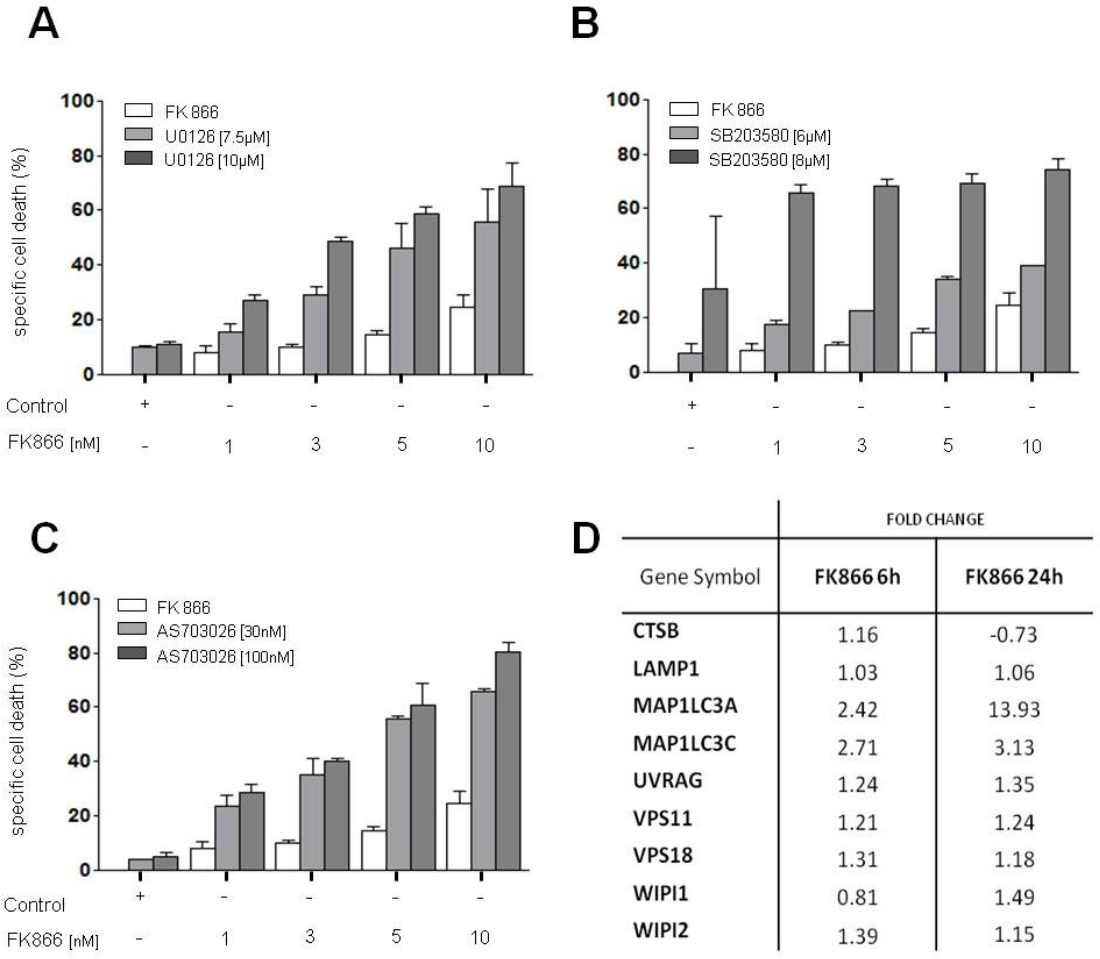

Figure S4. MAPK pathway deregulation contributes to FK866-induced autophagy via TFEB. (A-C) U266 cells were pretreated with MAPK inhibitors (U0126, SB203580, AS703026) for 30 min, followed by FK866 treatment (0-10nM). After 96 hours, specific cell death was measured by PI staining and cytometry Data represents mean ±SD of two independent experiments carried out in triplicate. (D) MM1S cells were cultured in the absence or presence of FK866 10nM for 6 and 24h. Purified cDNA was hybridized to human HG-U133Plus2.0 GeneChip (Affymetrix). The expression level of autophagic TFEB-target genes is represented.

SUPPLEMENTAL REFERENCES

1. Hideshima T, Chauhan D, Hayashi T, Akiyama M, Mitsiades N et al. Proteasome inhibitor PS-341 abrogates IL-6 triggered signaling cascades via caspase-dependent downregulation of gp130 in multiple myeloma. *Oncogene.* 2003 Nov 20;22(52):8386-93.
2. Mitsiades CS, Mitsiades NS, McMullan CJ, Poulaki V, Shringarpure R et al. Inhibition of the insulin-like growth factor receptor-1 tyrosine kinase activity as a therapeutic strategy for multiple myeloma, other hematologic malignancies, and solid tumors. *Cancer Cell.* 2004; 5(3):221-30.
3. Li, C., and Hung Wong, W. (2001). Model-based analysis of oligonucleotide arrays: model validation, design issues and standard error application. *Genome Biology 2, RESEARCH0032.*
4. Li, C., and Wong, W. H. (2001). Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection. *Proc Natl Acad Sci USA 98*, 31-36.