EZH2 inhibition overcomes immunomodulatory drug resistance in multiple myeloma via a cereblon-dependent pathway

Yigen Li,[1*] Amy Wilson,[1*] Yakinthi Chrisochoidou,[1] Shannon Martin,[1] Sarah Bird,[1,2] Salomon Morales,[1] Marc Leiro,[1] Zuza Kozik,[3] Nicholas T. Crump,[4] Theodoros I. Roumeliotis,[3] Jyoti Choudhary[3] and Charlotte Pawlyn[1,2]

[1]Division of Cancer Therapeutics, The Institute of Cancer Research; [2]The Royal Marsden Hospital NHS Foundation Trust; [3]Division of Cell and Molecular Biology, The Institute of Cancer Research and [4]Hugh and Josseline Langmuir Centre for Myeloma Research, Centre for Haematology, Department of Immunology and Inflammation, Imperial College London; London, UK

**YL and AW contributed equally as first authors.*

Correspondence: C. Pawlyn
charlotte.pawlyn@icr.ac.uk

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SUPPLEMENTARY MATERIAL

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Yigen Li[1]*, Amy Wilson[1]*, Yakinthi Chrisochoidou[1], Shannon Martin[1], Sarah Bird[1,2], Salomon Morales[1], Marc Leiro[1], Zuza Kozik[1], Nicholas T. Crump[3], Theodoros I. Roumeliotis[1], Jyoti Choudhary[1], Charlotte Pawlyn[1,2]

1. The Institute of Cancer Research, London
2. The Royal Marsden Hospital NHS Foundation Trust, London
3. Hugh and Josseline Langmuir Centre for Myeloma Research, Centre for Haematology, Department of Immunology and Inflammation, Imperial College London, London
*Joint first authors

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Supplementary Methods

Cell lines and compounds

MM.1S-Iberdomide-Resistant cell line was generated by long-term low-dose exposure of MM.1S to Iberdomide (0.01uM). Resistance was acquired gradually, with full resistance by 12 weeks of incubation associated with decreased CRBN protein expression. Cell lines underwent whole exome sequencing with no mutations in CRBN identified. Cell identity was confirmed using STR typing (Eurofins) and confirmed mycoplasma negative (MycoStrip, InvivoGen).

Lenalidomide and Pomalidomide were purchased from Fluorochem Ltd. Tazemetostat (EPZ-6438), Iberdomide and Mezigdomide were synthesized in-house using literature procedures.

Small interfering RNA (siRNA)

SMARTPool of siRNAs targeting Human IRF4 (E-019668-01-0005) and Non-targeting Pool (NT, D-001910-10-05) were purchased from Horizon (UK). Transfection was performed using DharmaFECT 1 (T-2001-01), following manufacturer's instructions. The final concentration of siRNAs was 100nM.

Western blot analysis

Cells were lysed in 2% SDS buffer. Protein concentration was measured by BCA protein assay (23225, Thermo Fisher Scientific). Equal amounts of protein were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto PVDF membrane (88518, Thermo Scientific), followed by immunoblot analysis. Antibodies used in this study were as follows: CRBN (71810, CST), IKZF1 (14859, CST), IKZF3 (15103, CST), β-Actin (A5441, SIGMA-ALDRICH), IRF4 (15106, CST). Western blots were visualized by Odyssey Fc Imager (LI-COR) using IRDye Infrared Dyes as secondary antibody. Densitometry analysis was performed using ImageJ (NIH, USA).

RNA extraction and quantitative RT-PCR analysis

Total RNA was extracted using RNeasy Plus Mini Kit (74134, QIAGEN) according to the manufacturer's instructions. First-strand cDNA was synthesized using cDNA Reverse Transcription Kit (10400745, Thermo Fisher Scientific). Expression of mRNA targets was measured using TaqMan Gene Expression Assays and GAPDH was served as housekeeping. The reaction was performed on ABI 7500 Real-Time PCR system using TaqMan Fast Advanced Master Mix (4444963, ABI) and primers for GAPDH (Hs99999905_m1) and IRF4 (Hs01056533_m1). The relative changes of gene expression were calculated according to the $2^{-\Delta\Delta CT}$ quantification method.

Apoptosis analysis

Apoptosis was determined using APC-Annexin V (550475, BD Biosciences) according to the manufacturer's instructions. Cells were primed in EZH2i for 5 days and then treated with IMiDs. After 72h and 120h, cells were harvested and stained with APC-Annexin V for 15min at room

temperature and PI for 5min at room temperature. Annexin/PI-positive cells were analyzed using flow cytometry (LSR II, BD Biosciences) within 1h.

Chromatin immunoprecipitation (ChIP)-qPCR assay

KMS-11 cells were incubated in 2μM Tazemetostat or DMSO for 10 days. Then cells were fixed with 1% formaldehyde solution for 15 min and incubated with 125 nM glycine for 5 min. DNA fragments ranging from 200 to 500 bp were generated using sonication. Antibodies including H3K27me3 (61017, ACTIVE MOTIF), IKZF1 (39356, ACTIVE MOTIF) and IgG (2729, CST) were employed for specific immunoprecipitation. qPCR was used to analyze the precipitated DNA. Primers used for detecting indicated enrichment were as follows, H3K27me3 (F, ACTCTCAGTTTCACCGCTCG; R, CTCCGGGTCCTCTCTGGTAT); MYT1 (F, ACAAAGGCAGATACCCAACG; R, GCAGTTTCAAAAAGCCATCC); ACTB (71023, Active Motif); IKZF1 (#1F, AGTTGCAGGTTGACCTACGG; #1R, AGCTTTCACCCGTTGAGCTT; #2F, GCCCCCATCTCTTTCATGCT; #2R, CCTCTCCGCGGTGTTTAGAG; #3F, GGACCATTCCTCCGTCTTCC; #3R, AATGCGAATCTCGCCTTTGC; SE1 F, CAGGTGACCTTCAGAGTTTGT; SE1 R, CCAGGTCTCTCCCATCCTATTA; SE2 F, GCCTCTCCTTACAACTGAAGAC; SE2 R, CAGGTGACTGCTCAAGTACAG; SE3 F, GCAGAAAGCCTATTCCAGAGAG; SE3 R, ATCCATGATCTCTCCCTCCTAC); Neg (F, CGTGGCTATGTTTGCTTGGG; R, AGCAGGCCTCTTGGTTGTTT).

Proteomics

Briefly, LC-MS analysis was performed with an UltiMate 3000 RSLCnano system (Thermo Fisher Scientific) coupled to the Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific) using a 25cm capillary column (Waters, nanoE MZ PST BEH130 C18, 1.7μm, 75μm × 250mm) over a 100min gradient 5%-35% of mobile phase B composed of 80% acetonitrile, 0.1% formic acid. Peptides were preconcentrated onto an Acclaim PepMap 100, 100μm × 2cm C18, 5μm trapping column at 10μL/min of 0.1% TFA and the analytical column was connected to an EASY-Spray emitter (ES991, Thermo Fisher Scientific). MS spectra were collected at Orbitrap mass resolution of 120,000 and precursors were targeted for HCD fragmentation in the top speed mode (3 sec) with collision energy 36% and Orbitrap detection with a resolution of 45,000. Targeted precursors were dynamically excluded from further activation for 45 seconds with 10ppm mass tolerance.

The Sequest HT node in Proteome Discoverer 3.0 (Thermo) was used to search the raw mass spectra against a FASTA file containing reviewed UniProt Homo sapiens entries. The precursor mass tolerance was set at 20ppm and the fragment ion mass tolerance at 0.02Da with up to 2 trypsin missed-cleavages allowed. TMTpro at N-terminus/K and Carbamidomethyl at C were defined as static modifications. Dynamic modifications were oxidation of M and deamidation of N/Q. Peptide confidence was estimated with the Percolator node and peptide FDR was set at 0.01 based on target-decoy search. Only unique peptides were used for quantification, considering protein groups for peptide uniqueness. Peptides with average reporter signal-to-noise greater than 3 were used for protein quantification.

Supplementary Figures

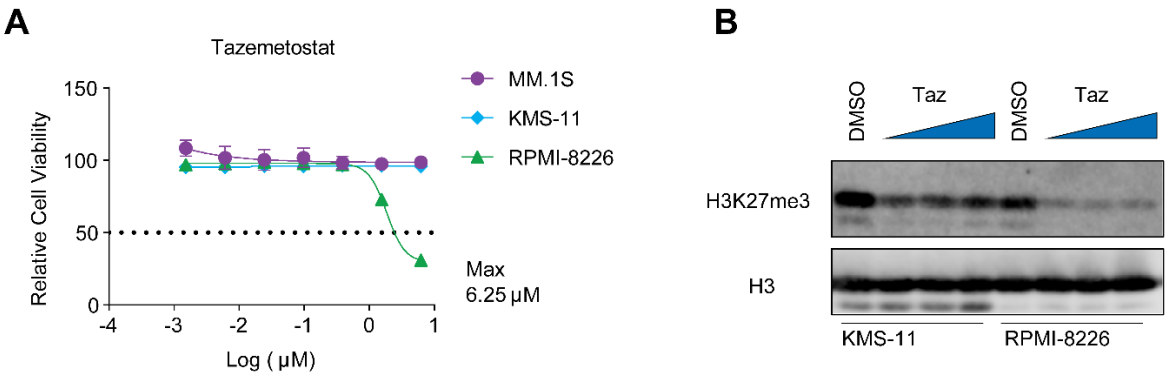

Figure S1 Optimization of Tazemetostat concentration used for priming
A) Dose-response curve in MM cell lines treated with Tazemetostat (highest 6.25µM) for 5 days.
B) WB analysis of H3K27me3 with titration of Tazemetostat concentration (0.5, 1, 2µM) for 5 days.

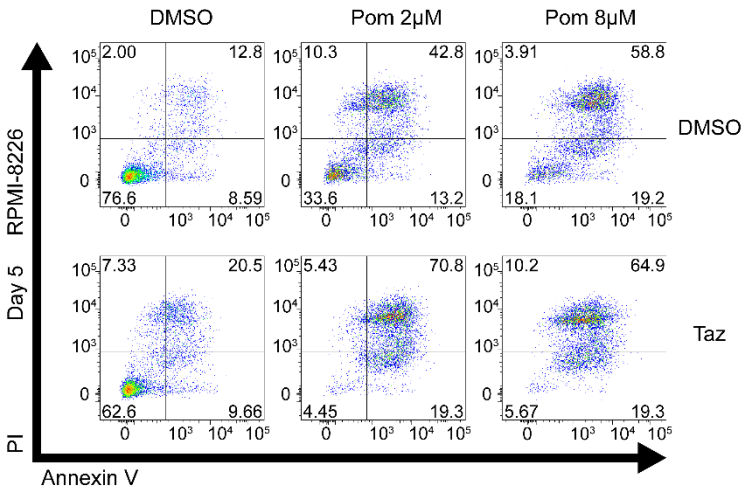

Figure S2 Tazemetostat-Pomalidomide induces apoptosis in RPMI-8226
Representative plot of Annexin V/PI staining for detecting apoptosis in RPMI-8226. Cells were primed in 0.125μM Tazemetostat or DMSO for 5 days and then treated with indicated Pomalidomide combination (alongside Tazemetostat/DMSO) for 5 days. Equivalent plot for KMS-11 is shown in Figure 2C.

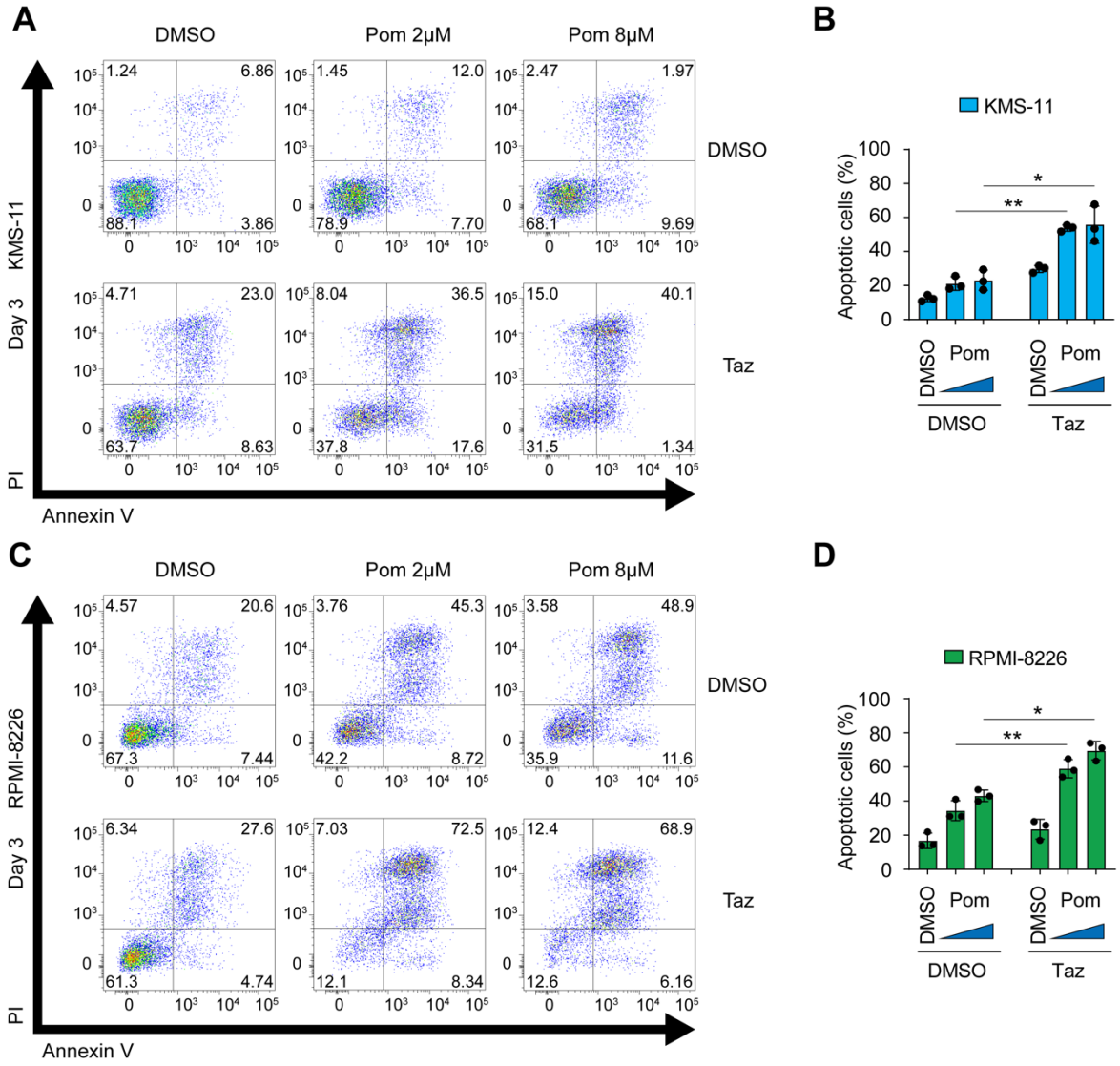

Figure S3 Tazemetostat-Pomalidomide induces apoptosis in KMS-11 and RPMI-8226

Representative plot and quantification of Annexin V/PI staining for detecting apoptosis in KMS-11 (A&B) and RPMI-8226 (C&D), which were primed in 0.25 or 0.125μM Tazemetostat respectively for 5 days and then treated with indicated Pomalidomide concentration (alongside Tazemetostat/DMSO) for 3 days. For statistical comparison the drug combination was compared to pomalidomide monotherapy. Data shown are mean ± SD, n=3 biological replicates. *p<0.05; **p<0.001.

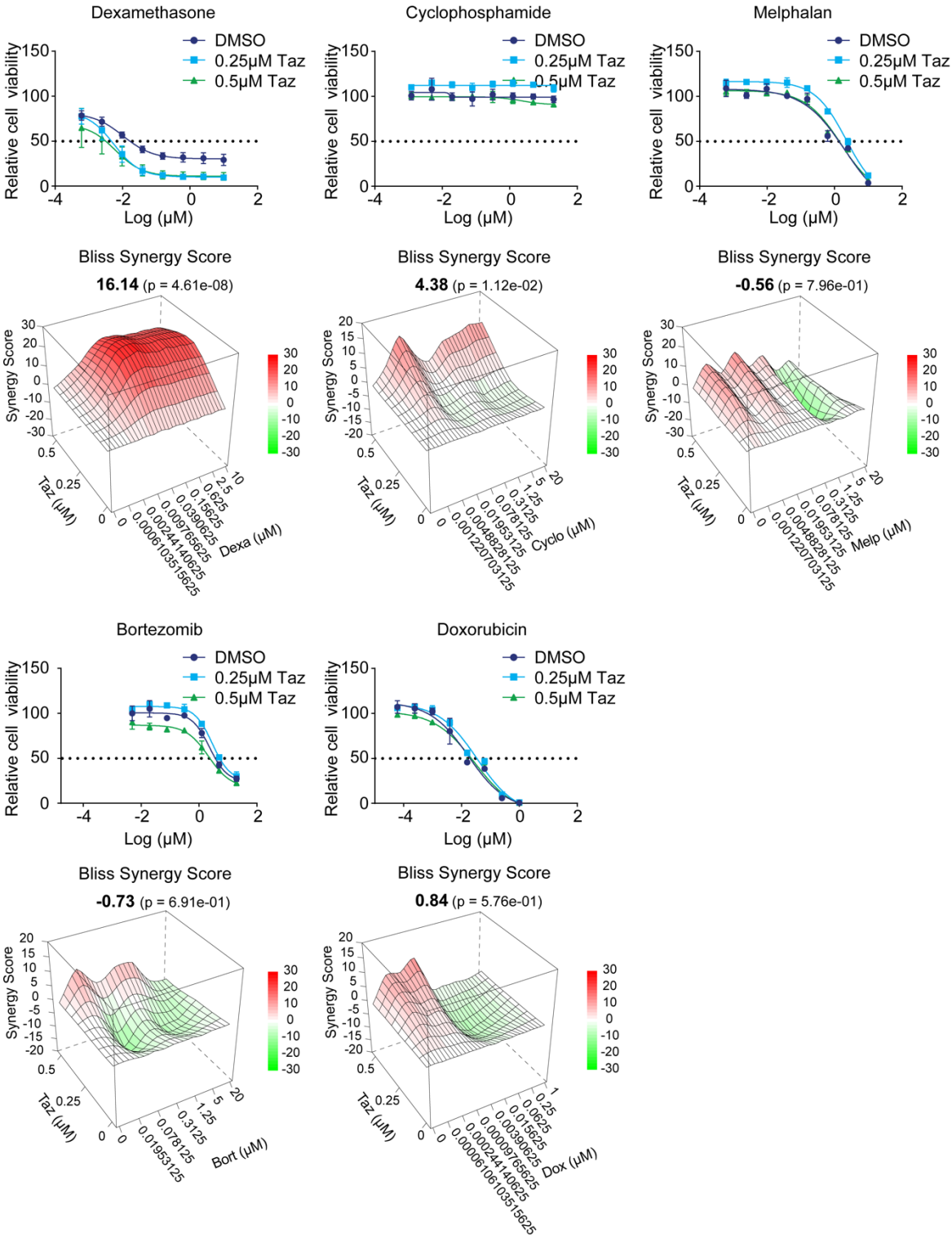

Figure S4 Tazemetostat is synergistic with Dexamethasone

Dose-response curve in KMS-11 treated with multi-dose drug combinations. Data shown are mean ± SD, n=3 biological replicates. The mean cell viability was used to calculate the synergy score.

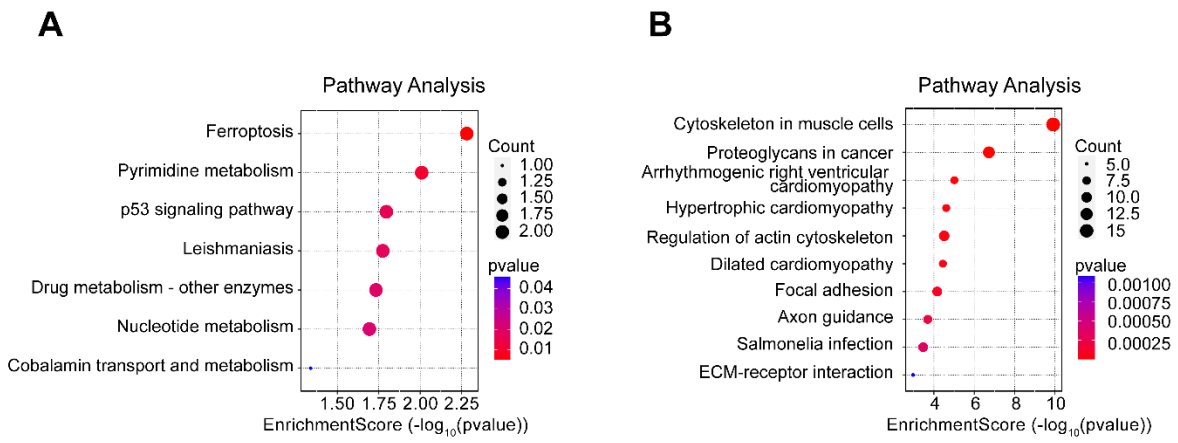

Figure S5 KEGG pathway analysis

A) KEGG pathway enrichment analysis for downregulated proteins with the drug combination (Tazemetostat and Pomalidomide).

B) KEGG pathway enrichment analysis for upregulated proteins with the drug combination (Tazemetostat and Pomalidomide).

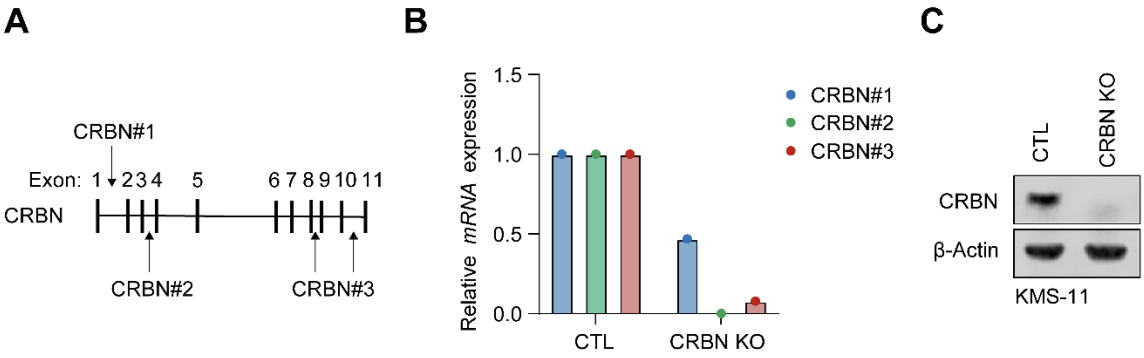

Figure S6 Validation of CRBN KO in KMS-11

A) Schematic of TaqMan probes targeting CRBN. CRBN#1 (HS01020593): Interrogated sequence in the boundaries of exons 1 & 2; CRBN#2 (HS00372266): Interrogated sequence in the boundaries of exons 3 & 4; CRBN#3 (HS00372271): Interrogated sequence in the boundaries of exons 8 & 9, and exons 10 & 11.

B&C) qPCR and WB analysis for validation of CRBN KO in KMS-11.

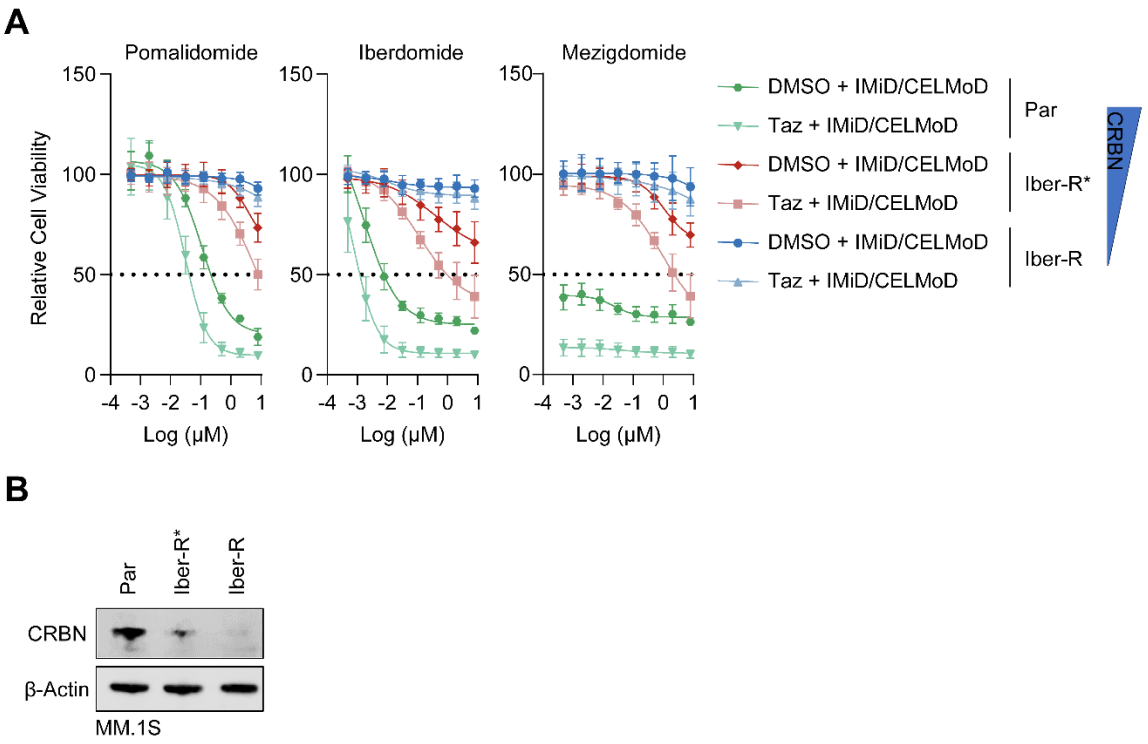

Figure S7 Tazemetostat overcoming IMiD/CELMoD resistance is CRBN-dependent

A) Dose-response (highest 8μM) curve assessed by cell viability assay for drug combinations in MM.1S (Iber-R) cell line, which developed acquired resistance after exposure to Iberdomide (0.01μM) for 6 months. Iber-R*, which retains residual CRBN expression as shown in (B), is not completely resistant to Iberdomide. Data shown are mean ± SD, n ⩾ 4 biological replicates.

B) WB analysis of CRBN expression in MM.1S Iber-R cell line, compared to the parental MM.1S cell line. Iber-R*, cell lines with residual CRBN expression.

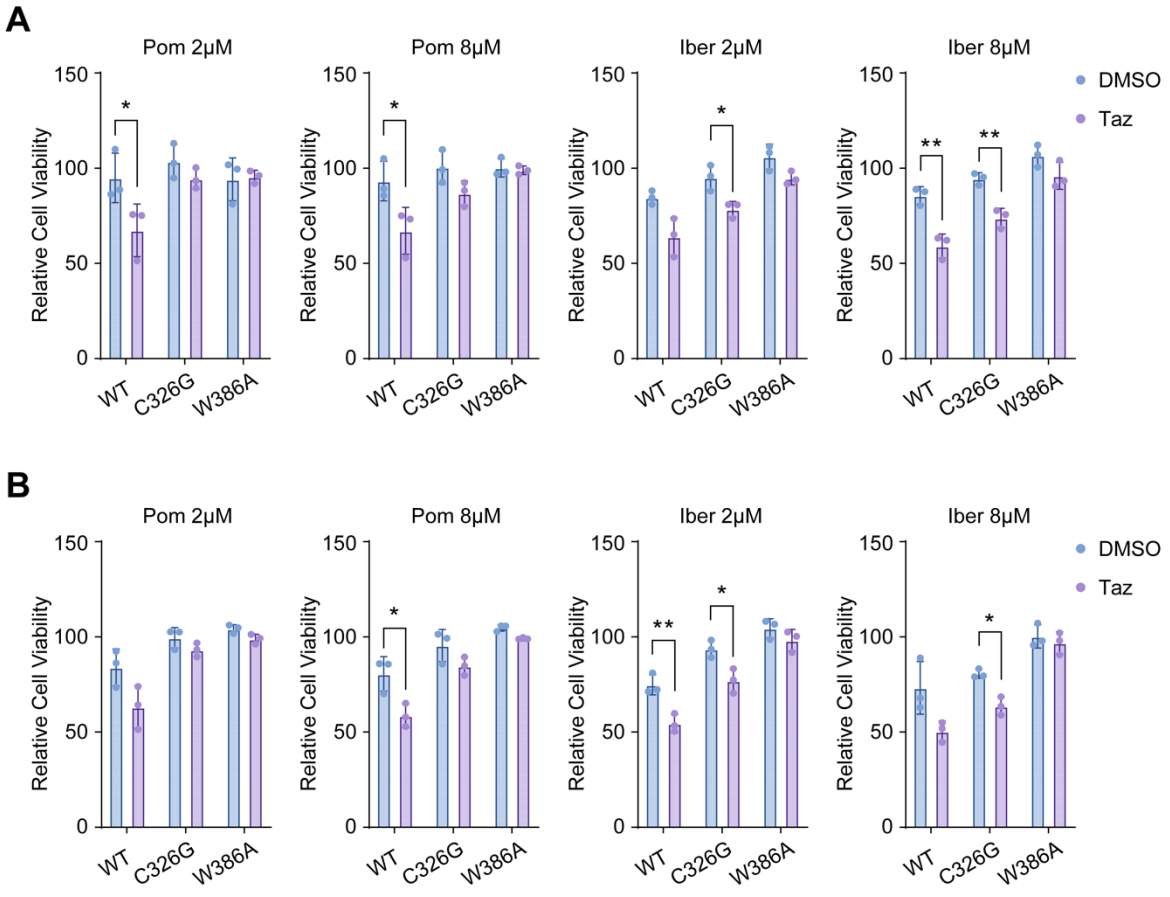

Figure S8 Tazemetostat and IMiD/CELMoD synergy depends on functional CRBN
Cell viability measured in CRBN mutants KMS-11 (A) or MM.1S (B) cell lines, which were primed in 0.25μM Tazemetostat for 5 days and treated with indicated concentrations of IMiD/CELMoD as per Figure 2A. Data shown are mean ± SD, n=3 biological replicates. *p<0.05; **p<0.001.

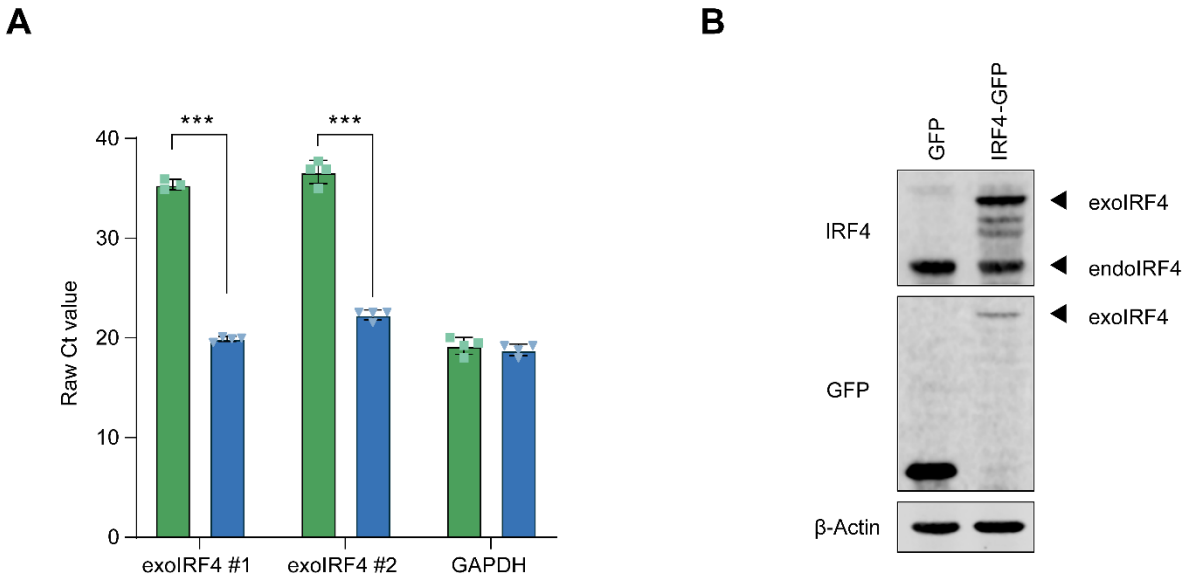

Figure S9 Validation of exogenous IRF4 overexpression

A) qPCR analysis for detecting exogenous *IRF4* in KMS-11. Primers were designed to specifically target exogenous *IRF4* (codon optimised) mRNA sequence. Raw Ct values were used to indicate IRF4 overexpression. Data shown are mean ± SD, n⩾3 biological replicates. ***p<0.001.

B) WB analysis for detecting exogenous and endogenous IRF4 expression in KMS-11.

Supplementary Tables

Table S1 IC50 of MM cell lines treated with indicated IMiD/CELMoD

	Len (μM)	Pom (μM)	Iber (μM)
MM.1S	3.3698 ± 3.4057	0.1642 ± 0.0550	0.0038 ± 0.0011
NCI-H929	3.4057 ± 0.3738	0.1387 ± 0.1192	0.0076 ± 0.0005
RPMI-8226	>20	>20	>20
KMS-11	>20	>20	>20
L363	>20	>20	>20
AMO-1	>20	>20	>20
U266	>20	>20	>20
LP1	>20	>20	>20
MOLP-8	>20	>20	>20
The maximum drug concentration is 20μM.			

Table S2 MM cell lines characteristics

Data from https://www.keatslab.org/myeloma-cell-lines/hmcl-characteristics

Public Name	Sex	Ancestry	Clinical Heavy Chain	Clinical Light Chain	Canonical Translocations	TP53
MM.1S	Female	Africa	IgA	Lambda	t(14:16) + t(8;14)	Wt
NCI-H929	Female	Europe	IgA	Kappa	t(4;14)	Wt
RPMI-8226	Male	Africa	IgG	Lambda	t(16;22) + t(8;22)	E285K - Homo (cc)
KMS-11	Female	East Asia	IgG	Kappa	t(4;14) + t(8;14) + t(14;16)	
L363	Female	Europe	IgG	Lambda	t(20;22)	S261T - Homo
AMO-1	Female	East Asia	IgA	Kappa	t(8;14)	Wt
U266	Male	Europe	IgE	Lambda	t(11;14)	A161T - Homo (cc)
LP-1	Female	Europe	IgG	Lambda	t(4;14) + t(8;14)	E286K - Homo (cc)
MOLP-8	Male	East Asia	IgD	Lambda	t(11;14)	

Supplementary Appendix

Data for Figure 4: Supplementary Appendix_DEP Data for Figure 4.xlsx