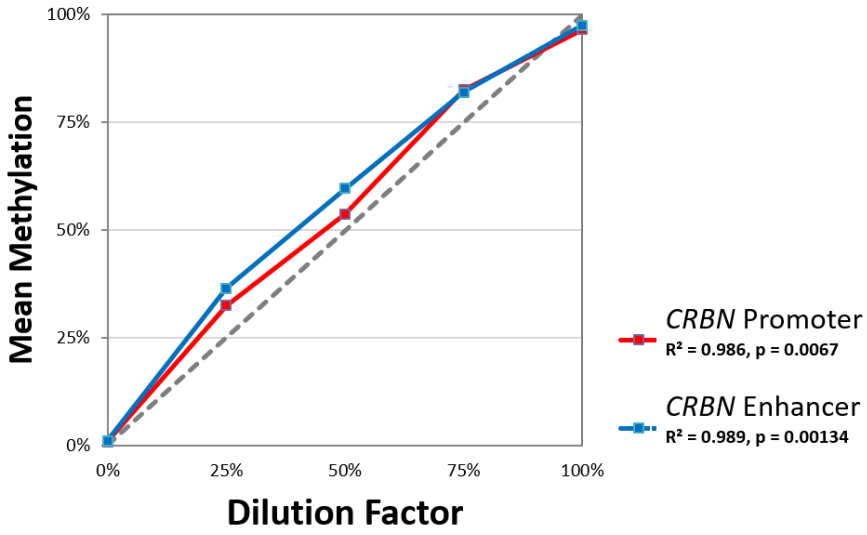
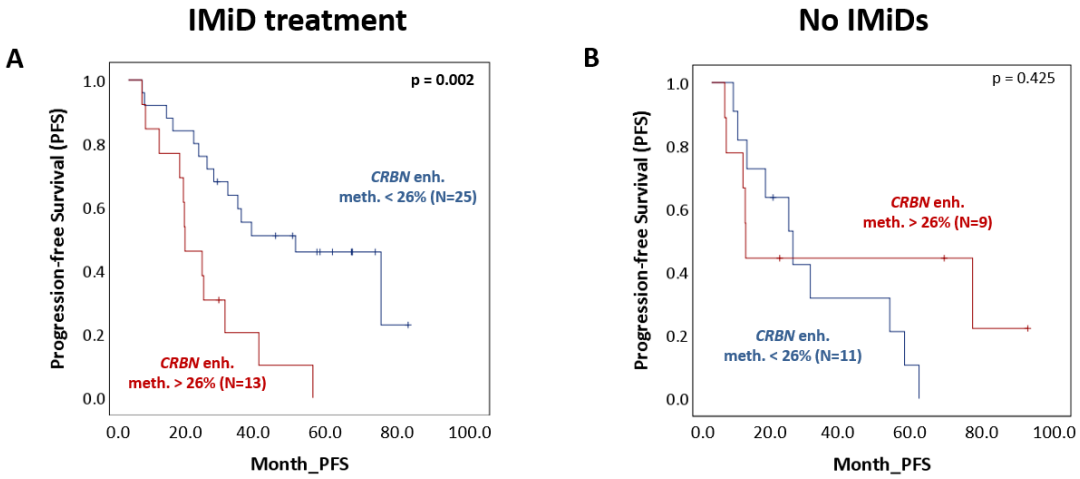
Supplementary Information



Supplementary figure 1: Artificially methylated standard DNAs for DBS assay confirmation.
DNA standards of 0%, 25%, 50%, 75% and 100% methylation were sequenced with the DBS assays for the two regulatory *CRBN* regions and correlated with the expected curves with R^2 = 0.986 for the promoter and R^2 = 0.989 for the enhancer region.



Supplementary figure 2: Survival of patients that underwent IMiD (THAL or LEN containing) therapies.
Kaplan-Meier analysis of NDMM patients that underwent IMiD (THAL or LEN) containing therapies. Patients with *CRBN* enhancer methylation over 26% (red) showed significantly inferior progression-free survival (PFS) (**A**) compared to patients with methylation levels lower than 26% (blue). NDMM patients not pretreated with IMiDs did not show differences in survival. The analyses were performed with IBM SPSS Statistics 26. A univariate log-ranked test (Mantel-Cox) and Chi-Square were used to compare the survival curves in different groups. A p-value of <0.05 was considered statistically significant.

Supplementary table 1: Primers used for DBS.

Gene	Primer	Template-specific sequence (5'-3')	Amplicon length	Chromosomal assay location[c]	Number of CpGs	Annealing Temp. [°C]
CRBN	F	[a]ATGTTAAGGGTTGGAATAAAGTGA	426 bp	Chr3:	39	56
Promoter	R	[b]TCCCAAACCCAACTACACC		3179543-3179968		
CRBN	F	[a]GTTTGGTTTGGGAAATGAGTTTAAAT	348 bp	Chr3:	7	57
Enhancer	R	[b]CAAATAATTCTCCTACCCCAACT		3173156-3173503		

[a] upstream universal adapter 1: ACACTCTTTCCCTACACGACGCTCTTCCGATCT

[b] upstream universal adapter 2: GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

[c] Ensembl release GRCh38p12

Abbreviations: F, forward primer; R, reverse primer

Supplementary table 2: Primers used for luciferase reporter assay.

Gene	Primer	Template-specific sequence (5'-3')	Amplicon length	Chromosomal assay location*	Number of CpGs	Annealing Temp. [°C]
CRBN	F	CCC**ACTAGT**TTGTACTTCCTGAGTCCTTC	412 bp	Chr3:	10	58
Enhancer	R	GGG**AGATCT**ACAGGTAAACAATTTATAG AATC		3:3173004-3173415		

Forward primer contains a SpeI recognition site (ACTAGT), reverse primer a BglII restriction site (AGATCT), both highlighted in bold

* Ensembl release GRCh38p12

Supplementary table 3. Raw data measurements of the dual-luciferase reporter assay in L363 cell line. All measurements were performed in triplicates.

Vector	Firefly			Renilla			Ratio		
CMV/EF1	2195890	2385475	2608292	436	504	576	5036.445	4733.085	4528.285
MP vector w/o insert	38	50	48	4036	6326	6428	0.009415	0.007904	0.007467
CRBN Enhancer **unmethylated**	2803	3268	2536	16523	16156	14802	0.169642	0.202278	0.171328
CRBN Enhancer **methylated**	89	73	65	14076	14569	14771	0.006323	0.005011	0.004401

Abbreviations: MP, minimal promoter; CMV, cytomegalovirus

Supplementary table 4: Characteristics of patients with relapsed/refractory multiple myeloma (rrMM) at sampling.

Parameter		
Patients, n		52
Gender, n (%)	Male	19 (37)
	Female	33 (63)
Age at diagnosis of MM, years (range)		57 (33-77)
Time between initial diagnosis and sampling, months (range)		50 (2-215)
Subtype, n (%)	IgG	29 (56)
	Non-IgG	11 (21)
	LC	12 (23)
ISS Stage, n (%)	I	22 (42)
	II	8 (15)
	III	4 (8)
	NA	18 (35)
Cytogenetics, n (%)	High risk*	15 (29)
	Standard-risk	28 (54)
	NA	9 (17)
Prior lines of therapies at the time point of sampling, n (%)	1-3	32 (61)

	4-6	15 (29)
	>6	4 (8)
	NA	1 (2)
Pretreatment at the time point of sampling, n (%)		
IMiD, n (%)	Lenalidomide	41 (79)
	Pomalidomide	17 (33)
	Thalidomide	11 (21)
	None	11 (21)
PI, n (%)	Bortezomib	45 (87)
	Carfilzomib	15 (29)
	None	6 (12)
Monoclonal antibody, n (%)	Elotuzumab	2 (4)
	Daratumumab	4 (8)
	None	46 (88)
Autologous stem cell transplant, n (%)	Yes	45 (87)
	No	7 (13)
IMiD-refractory patients, n (%)		27 (52)
	Lenalidomide-refractory	19 (37)
	Pomalidomide-refractory	14 (27)
	Both	6 (12)

Abbreviations: IMiD, immunomodulatory drugs; PI, proteasome inhibitors; * defined as presence of at least one of the following: t(4;14), t(14;16), t(14;20), del17p

Supplementary table 5: Characteristics of patients with newly diagnosed MM (NDMM) for survival analysis.

Parameter		
Patients, n		58
Gender, n (%)	Male	32 (55)
	Female	26 (45)
Age at diagnosis of MM, years (range)		65 (42-92)
Increased LDH level at diagnosis, n (%)	Yes	6 (10)
	No	52 (90)
Subtype, n (%)	IgG	37 (64)
	Non-IgG	16 (28)
	LC	5 (8)
ISS Stage, n (%)	I	16 (28)
	II	26 (44)
	III	16 (28)
Cytogenetics, n (%)	High risk*	10 (17)
	Standard-risk	35 (60)
	NA	13 (23)
Bone lesions at diagnosis, n (%)	Yes	46 (79)
	No	12 (21)
Extramedullary disease at diagnosis, n (%)	Yes	2 (3)
	No	56 (97)
Renal impairment at diagnosis, n (%)	Yes**	0 (0)
	No	49 (84)
	NA	9 (16)
Treatment received, n (%)	Yes	38 (77)
Frontline therapy including IMiD, n (%)	No	20 (23)
Autologous stem cell transplant, n (%)	Yes	35 (60)
	No	23 (40)

Abbreviations: IMiD, immunomodulatory drugs; PI, proteasome inhibitors; * defined as presence of at least one of the following: t(4;14), t(14;16), t(14;20), del17p; **defined as serum creatinine levels ≥2 mg/dL

Supplementary table 6: Patients with mutations in IMiD resistance associated genes (M3P panel sequencing) and *CRBN* enhancer methylation status (DBS) at sampling time point.

Case	M3P						DBS	
	Associated Gene	Mutation location*	Base Change	Putative Consequence	Mutation Type	Het/Hom	*CRBN* Methylation	Coverage
1	*CRBN*	chr3:3195724	C/T	p.Asp291Asn	missense	het	71%	15,603
2	*CUL4B*	chrX:119679342	C/G	p.Asp311His	missense	het	56%	22,264
3	*CRBN*	chr3:3215768	T/A	p.Arg118Ter	nonsense	het	23%	15,205
4	*IKZF3*	chr17:37947786	C/G	p.Gly159Arg	missense	het	72%	13,972

Abbreviations: DBS, Deep Bisulfite Sequencing; M3P, Mutation Panel Version 3, het, heterozygous, *GRCH37

Supplementary table 7: IMiD refractory patients subdivided by POM and LEN. Patients with methylation degrees over 26% are highlighted.

Cohort	Patient ID	Methylation	Prior lines of therapy
LEN refractory	1882_M_2_48	82.00%	9
	1809_M_2_45	70.86%	2
	1745_M_1_2	71.00%	4
	1758_M_1_6	56.29%	2
	1752_M_2_43	53.57%	4
	1829_M_1_28	46.29%	3
	1772_M_1_11	44.86%	1
	1802_M_1_20	27.57%	3
	1867_M_1_31	27.29%	2
	1912_M_1_35	12.86%	2
	1908_M_1_34	11.71%	2
	1833_M_1_29	10.29%	6
	1754_M_1_5	7.71%	3
Percentage of cases hypermethylated		**69% (9/13)**	**3.31 ± 2.14**
POM refractory	1976_M_1_48	83.29%	6
	1868_M_1_32	71.71%	2
	1944_M__42	70.71%	5
	1774_M_1_12	65.43%	3
	1926_M_1_39	62.71%	5
	1960_M_1_46	34.29%	2
	1744_M_1_1	8.71%	7
	1957_M_1_45	5.00%	4
Percentage of cases hypermethylated		**75% (6/8)**	**4.25 ± 2.14**
LEN and POM refractory	1914_M_1_37	81.57%	10
	1787_M_1_16	29.29%	6
	1800_M_1_19	27.14%	4
	1806_M_1_22	22.57%	4
	1644_M_2_41	7.57%	7
	1797_M_1_18	3.57%	3
Percentage of cases hypermethylated		**50% (3/6)**	**5.67 ± 2.58**

Supplementary table 8: Annexin V Propidium Iodide (PI) cell survival data after DNMTi/Len treatment.

Cell Line	Drug Exposure	Mean Survival	Standard Deviation (SD)	Relative to control [%]	p-Value
OPM2	**Control DMSO**	94.8	0.53	100.0	
	Dec + / Len -	92.5	3.03	97.5	n.s. 0.265
	Aza + / Len -	93.1	2.68	98.2	n.s. 0.333
	DNMTi - / Len +	84.0	4.54	100.0	
	Dec + / Len +	71.5	2.08	85.0	*0.011
	Aza + / Len +	46.5	10.65	55.3	**0.005
KMS-11	**Control DMSO**	92.8	1.56	100.0	
	Dec + / Len -	83.6	11.26	90.1	n.s. 0.234
	Aza + / Len -	84.2	1.75	90.1	n.s. 0.135
	DNMTi - / Len +	58.2	4.49	100.0	
	Dec + / Len +	40.0	16.54	68.8	n.s. (0.141)
	Aza + / Len +	25.0	10.22	43.0	** 0.007

Abbreviations: Aza +, 48 hours exposure to 500 nM 5-azacytidine; Dec +, 72 hours exposure to 100 nM 5-aza-2'-deoxycytidine; Len +, 5 days exposure to 10µM lenalidomide after DNMTi pretreatment; all experiments were performed at least in triplicates. (Significance level: *p < 0.05, **p < 0.01, and ***p < 0.001).

Supplementary Methods

Deep Bisulfite Sequencing (DBS)

DNA isolation was performed with the AllPrep DNA/RNA Micro Kit (Qiagen). For bisulfite conversion, 100-200ng genomic DNA per sample was processed using the EpiTect® Fast 96 Bisulfite Conversion Kit (Qiagen). Deep Bisulfite Sequencing (DBS) libraries were paired-end sequenced with the Illumina MiSeq platform and prepared by enrichment PCRs with target-specific primers (**SI table 1**). Assays were established with artificially (un)methylated DNA standards with R^2=0.986 for *CRBN* promoter and R^2=0.989 for *CRBN* enhancer (**SI figure 1**). FASTQ files were aligned using the Amplikyzer2 pipeline (https://doi.org/10.7287/peerj.preprints.122v2).

Expression analysis

Real-time qPCR of CRBN was performed on 26 MM samples and 15 PBMCs. RNA was isolated from cell pellets using the AllPrep DNA/RNA kit (Qiagen) and reverse transcribed by SuperScript IV VILO Mastermix (Thermo Fisher Scientific). The StepOnePlus was used with TaqMan reagent kits and molecular probes (Applied Biosystems): Hs00372266_m1 for CRBN and Hs00939627_m1 for the control GUSB. All samples were analyzed in triplicates.

Luciferase reporter assay

Functional analysis of the *CRBN* enhancer was performed with a dual-luciferase reporter assay. To properly initiate the transcription, a CpG free minimal promoter (MP, 5´-AGAGGGTATATAATGGAAGCTTAACTTCCAG-3´) was cloned into the pCpGL empty vector. The backbone of the pCpGL vector is completely CpG dinucleotide free to guarantee that the vector is only repressed due to the presence of functionally important methylated CpG dinucleotides within the insert. The forward primer contained a SpeI and the reverse primers a BglII recognition site (**SI table 2**). After double digestion, the insert was ligated into the multiple cloning site of the pCpGL vector upstream of the luciferase gene and transformed into one shot PIR1 competent cells (Thermo Fisher Scientific). Selection was conducted via Zeocin resistance and confirmed by Sanger sequencing. A colony PCR was done with CCTGTAAAGTCTTTATCACACTACC as forward primer and CCTCACAGACATCTCAAAGTATTC as reverse primer. Plasmid DNA was purified and *in vitro* methylated using SssI, HhaI and HpaII methyltransferases (New England Biolabs). A confirmation digestion experiment was conducted with the enzymes HpaII (methylation sensitive) and MspI (methylation insensitive) (New England Biolabs). Either the methylated or the unmethylated pCgGL vector was co-transfected with Renilla control vector (20:1 ratio) into L363 cells by electroporation. To overcome transfection efficiency bias, a CD4Δ-expression plasmid was cotransfected and the positive population was isolated by magnetic bead retention followed by OptiPrep purification. Luciferase activity of 300.000 cells was measured in triplicates with a Spark microplate reader (Tecan Life Sciences) and a normalization of the pCpGL reporter (firefly fluorescence) was done against the activity of the Renilla control vector (**SI table 3**). Empty pGpGL vector with a minimal promoter (MP) served as negative control, pGpGL with a cytomegalovirus (CMV) promoter inserted as positive control.

Cell culture and drug treatment

The human L363 cell line was purchased from DSMZ (Leibniz Institute DSMZ–German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). OPM2 and KMS-11 were kindly provided by Dr. Leif Bergsagel's laboratory. All myeloma cell lines were cultured under sterile conditions at 37°C with 5% CO_2 in a humidified atmosphere in RPMI-1640 media, supplemented with 10% fetal calf serum, 1mM sodium pyruvate, 2mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. OPM2 and KMS-11 cells were pretreated for 48 hours in 500 nM 5-azacytidine (Sigma Aldrich, St. Louis, MO, USA) or 72 hours in 100 nM 5-aza-2'-deoxycytidine (decitabine; Selleck Chemicals, Houston, TX, USA). The demethylation of the *CRBN* enhancer region after pretreatment with DNMTi was monitored via pyrosequencing of bisulfite converted DNA using the following primers: forward primer GTTTGGTTTGGGAAATGAGTTTAAAT, reverse primer Biotin-CAAATAATTCTCCTACCCCAACT, pyrosequencing primer 1 TGTGATTTAAAAATAAATAAAATTG and pyrosequencing primer 2 GGGTGGATTATTTGAGG. Four of the 7 CpGs within the target region were covered. Pyrosequencing was performed on a PyroMark Q96 MD system (Qiagen, Hilden, Germany) using the PyroMark Gold Q96 CDT reagent kit (Qiagen), 10 pmol of the sequencing primer, and Pyro Q-CpG software for data analysis (Qiagen). Then, 10.000 cells per well were seeded into 96 well plates and incubated with lenalidomide (Selleck Chemicals, Houston, TX, USA) for 5 days. Cell survival was measured with Annexin V Propidium Iodide (PI) viability/cell death measurements. All drug solutions were always freshly prepared from concentrated stocks. The solvent for the stock solution was DMSO. All experiments were performed in triplicates and repeated at least once. Flow cytometry was performed with a FACS Calibur (BD Biosciences, Heidelberg, Germany) and data was analysed with FlowJo version 8.8.7 (Tree Star, Inc., Ashland, USA). Statistical analyses were performed with IBM SPSS Statistics 26 using T-Test for normally distributed measurements and Mann-Whitney U for nonparametric data.