LYMPHOID NEOPLASIA

Cereblon enhancer methylation and IMiD resistance in multiple myeloma

Larissa Haertle,[1,2,]* Santiago Barrio,[1,3,4,]* Umair Munawar,[1] Seungbin Han,[1] Xiang Zhou,[1] Cornelia Vogt,[1] Rafael Alonso Fernández,[4] Max Bittrich,[1] Yanira Ruiz-Heredia,[3,4] Matteo Da Viá,[1,5,6] Josip Zovko,[1] Andoni Garitano-Trojaola,[1] Niccolò Bolli,[5,6] Anna Ruckdeschel,[1] Thorsten Stühmer,[1] Manik Chatterjee,[1] Miriam Kull,[7] Jan Krönke,[7] Xabier Agirre,[8] Jose I. Martin-Subero,[9,10] Peter Raab,[11] Hermann Einsele,[1] Leo Rasche,[1] Joaquin Martinez-Lopez,[3,4] Thomas Haaf,[2] and K. Martin Kortüm[1]

[1]Department of Internal Medicine II, University Hospital Würzburg, Würzburg, Germany; [2]Institute of Human Genetics, Julius Maximilian University of Würzburg, Würzburg, Germany; [3]Altum Sequencing Co., Madrid, Spain; [4]Department of Hematology, Hospital Universitario 12 de Octubre, Spanish National Cancer Research Center, Complutense University Madrid, Madrid, Spain; [5]Department of Oncology and Hemato-Oncology, University of Milan, Milan, Italy; [6]Hematology Unit, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy; [7]Department of Internal Medicine III, University Hospital Ulm, Ulm, Germany; [8]Área de Hemato-Oncología, Centro de Investigación Médica Aplicada, Centro de Investigación Biomédica en Red-Cáncer (CIBERONC), Instituto de Investigación Sanitaria de Navarra, Universidad de Navarra, Pamplona, Spain; [9]Biomedical Epigenomics Group, Institut d'Investigacions Biomèdiques August Pi I Sunyer, University of Barcelona, Barcelona, Spain; [10]Institució Catalana de Recerca i Estudis Avançats, Barcelona, Spain; and [11]Department of Orthopaedic Surgery, König Ludwig Haus, University of Würzburg, Würzburg, Germany

---

KEY POINTS

- DNA hypermethylation of an active intronic *CRBN* enhancer region downregulates CRBN expression and mediates IMiD resistance in advanced MM.

Cereblon is the direct binding target of the immunomodulatory drugs (IMiDs) that are commonly used to treat multiple myeloma (MM), the second most frequent hematologic malignancy. Patients respond well to initial treatment with IMiDs, but virtually all patients develop drug resistance over time, and the underlying mechanisms are poorly understood. We identified an as yet undescribed DNA hypermethylation in an active intronic *CRBN* enhancer. Differential hypermethylation in this region was found to be increased in healthy plasma cells, but was more pronounced in IMiD-refractory MM. Methylation significantly correlated with decreased CRBN expression levels. DNA methyltransferase inhibitor (DNTMi) in vitro experiments induced *CRBN* enhancer demethylation, and sensitizing effects on lenalidomide treatment were observed in 2 MM cell lines. Thus, we provide first evidence that aberrant *CRBN* DNA methylation is a novel mechanism of IMiD resistance in MM and may predict IMiD response prior to treatment.

Introduction

Immunomodulatory drugs (IMiDs) are backbone agents of various multiple myeloma (MM) treatment regimens, with cereblon (CRBN) as the central orchestrator of their antitumor action.[1,2] CRBN is a substrate receptor of the CRL4^CRBN-E3–ubiquitin ligase complex.[1,3] Binding of IMiDs leads to the ubiquitination and degradation of the transcription factors Ikaros (IKZF1) and Aiolos (IKZF3) and the subsequent downregulation of c-Myc and IRF4, which causes inhibition of MM growth and apoptosis.[4,5] Reduced levels of or complete loss of CRBN expression, at both the messenger RNA and protein levels, have been associated with IMiD resistance.[2,3,6–9] Furthermore, in previous work, we identified underlying genomic mutations in the coding sequence of *CRBN*.[10,11]

Epigenetic plasticity is an adaptive way for tumor cells to evade environmental factors such as anticancer therapy. But not much is known about epigenetic mechanisms or posttranscriptional factors that may regulate CRBN expression. Notably, acquired resistance to IMiDs in MM can be reversed in vitro by using a combination of a DNA methyltransferase and an EZH2 inhibitor.[12] The addition of oral 5 -azacytidine to lenalidomide-

dexamethasone induced meaningful clinical remissions in a lenalidomide-refractory MM cohort.[13] Previous studies suggested an epigenetic mechanism of IMiD resistance; however, it was shown that silencing of *CRBN* by promoter hypermethylation is not the underlying cause of CRBN downregulation.[12,14]

In this study, we investigated an alternative intragenic *CRBN* regulatory region (GRCH38: Chr.3 3172800-3173801 ENSR00000675670), which was identified as an active enhancer by its H3K4me1 and H3K27ac histone pattern.[15] This novel, yet unexplored enhancer is located downstream of the described 5' *CRBN* promoter region (GRCH38: Chr.3 3178000-3180601 ENSR00000147822). We analyzed primary samples from patients with newly diagnosed MM (NDMM) and IMiD relapsed or refractory MM (rrMM) as well as plasma cells from healthy volunteers, and we assessed the DNA methylation state of the known *CRBN* promoter and the novel *CRBN* enhancer.

Study design

We collected primary MM tumor samples from 131 patients at the Hospital Universitario 12 de Octubre (Madrid, Spain), the

---

University Hospital of Würzburg (Würzburg, Germany), and the University Hospital of Ulm (Ulm, Germany). Of these patients, 79 were newly diagnosed[16] and 52 had relapsed MM (rMM), including 27 patients who were refractory to IMiDs (rrMM) according to the International Myeloma Working Group criteria (supplemental Table 4, available on the *Blood* Web site). We analyzed 45 peripheral blood mononuclear cells (PBMCs) and 52 CD138+ plasma cells from bone marrow aspirates (femoral head) from healthy donors; 26 samples were collected at the University Hospital of Würzburg, 18 at the Hospital Universitario 12 de Octubre, and 8 at the Department of Oncology and Hemato-Oncology, University of Milan (Milan, Italy) and used those samples as controls. An independent control group of 32 white blood cell samples from healthy donors was provided by the Institute of Human Genetics,
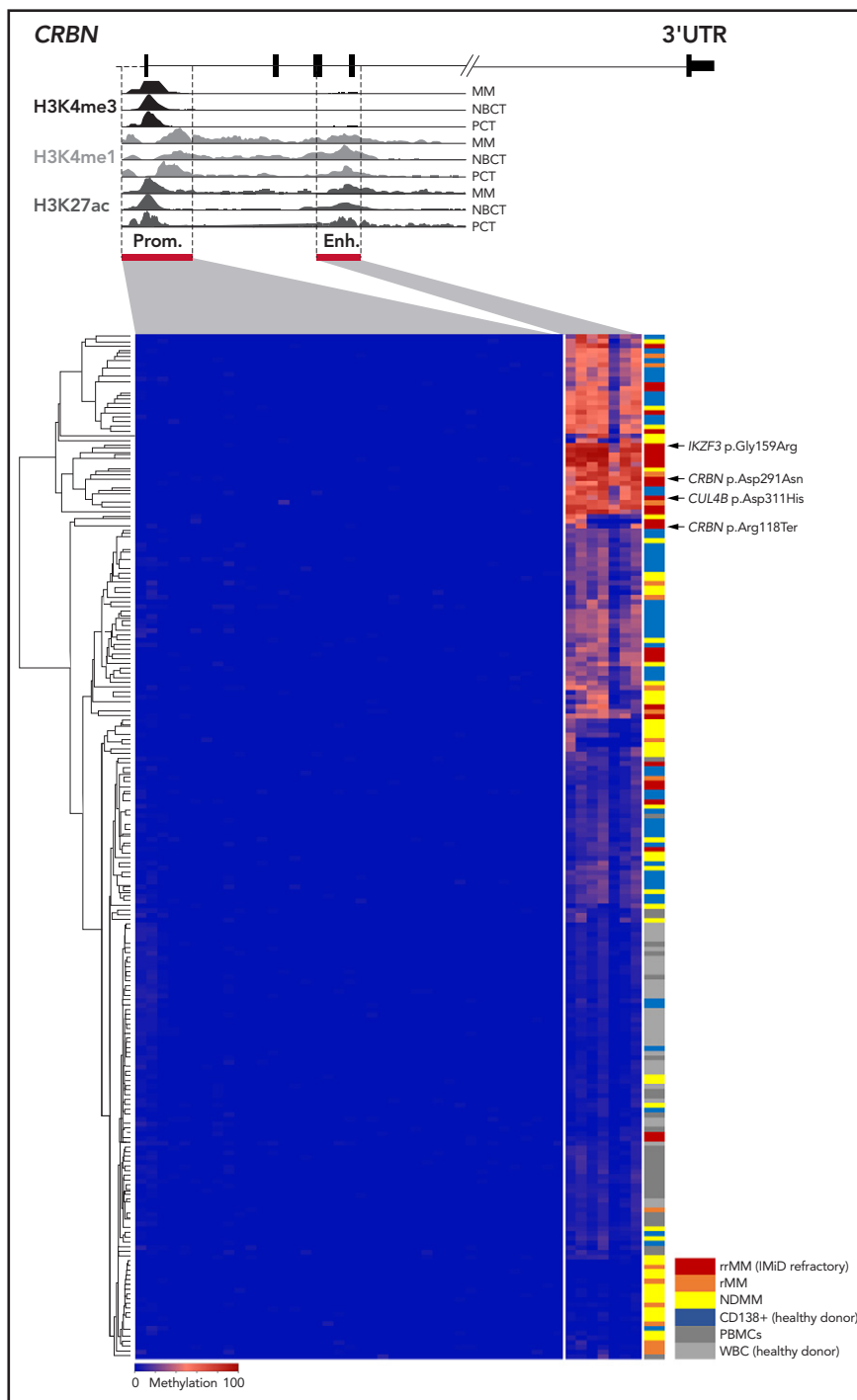
Figure 1. DNA methylation of the *CRBN* promoter and enhancer in MM patients and CD138+ plasma cells from healthy donors. *CRBN* has 2 regulatory regions, a 5' promoter (Prom.) and a 6039 bp downstream located active intronic enhancer (Enh.) region as shown in a UCSC plot of representative primary MM as well as naïve B cells from tonsil (NBCT) and plasma cells from tonsil (PCT) from healthy donors. DNA methylation was detected only in the enhancer region and accumulated in IMiD-resistant MM patients. Each line represents an individual sample, and each column represents a single CpG; blue encodes for an unmethylated state, red for a methylated state. The different study cohorts are marked by a second color code. UTR, untranslated region.

HAERTLE et al
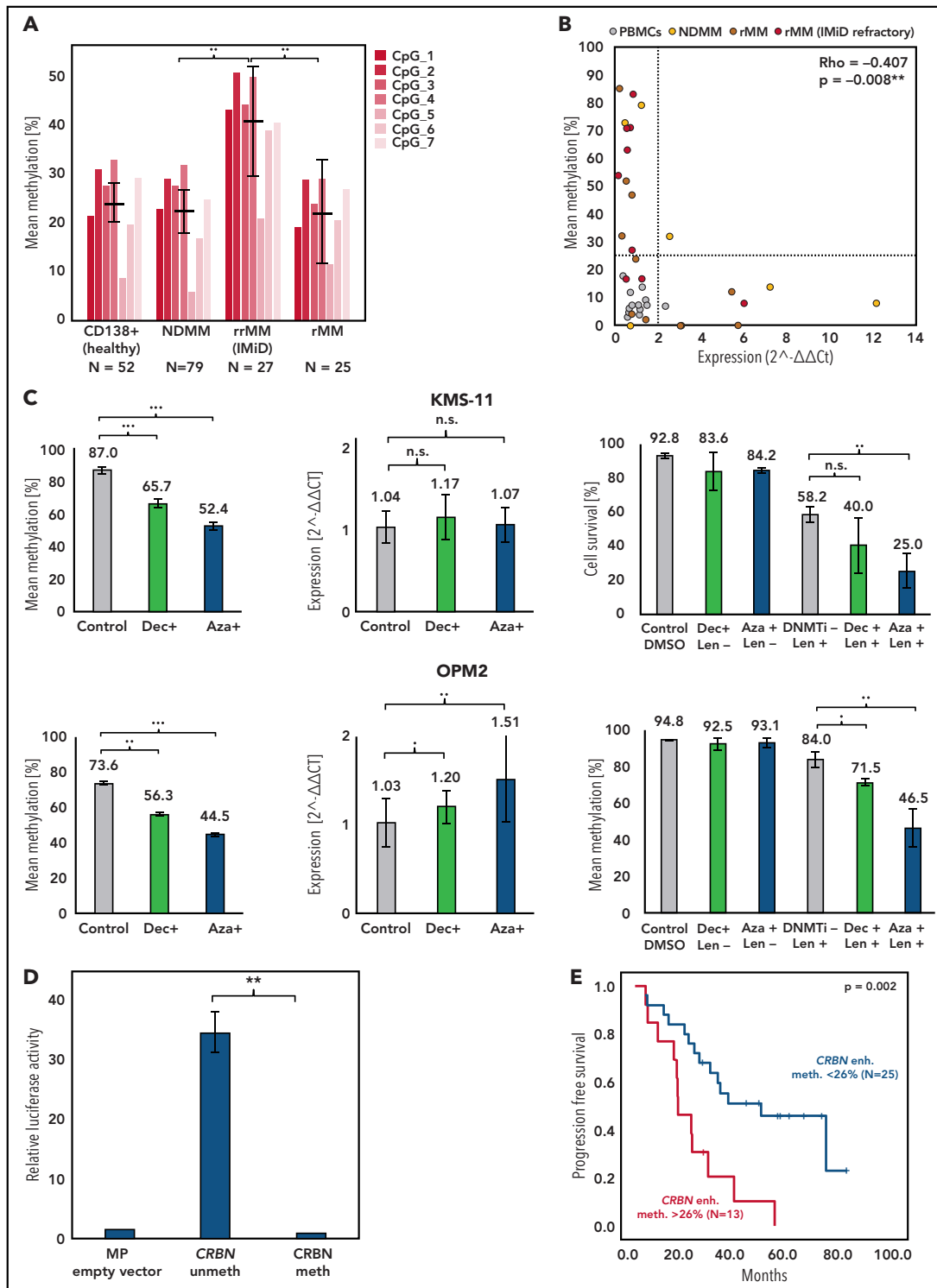
Figure 2. *CRBN* enhancer DNA methylation correlates with gene expression, affects IMiD sensitivity in vitro, and impacts clinical outcome of MM patients exposed to IMiDs. (A) Methylation significantly increased from NDMM to IMiD rrMM (*P* < .002). (B) *CRBN* enhancer methylation and gene expression showed a significant negative correlation (Spearman's rho = −0.407; *P* = .008). For patients with high methylation, gene expression was suppressed. (C) Significantly decreased *CRBN* enhancer methylation, CRBN expression and lenalidomide (Len) sensitization of KMS-11 (upper panel) and OPM2 cells (lower panel) after exposure to DNMTi's (500 nM 5-azacytidine [Aza] for 48 hours or 100 nM 5-aza-2′-deoxycytidine [Dec] for 72 hours). Left: *CRBN* enhancer methylation, measured by pyrosequencing. Center: CRBN expression 5 days after incubation with DNA methyltranferase inhibitors (DNMTi) analyzed by using RT-PCR TaqMan assays, ΔΔCt method, and GUSB as housekeeping control. Right: after pretreatment with DNMTi for 48 hours with 500 nM 5-azacytidine or 72 hours with 100 nM 5-aza-2′-deoxycytidine, cells were treated for 5 days with 10 μM lenalidomide. Cell survival was measured by annexin V-propidium iodide (PI) fluorescence-activated cell sorting. Significantly restored IMiD sensitivity was observed in both cell lines. (D) Functional luciferase activity of CpG-free pCpGL vector containing in vitro methylated or unmethylated *CRBN* enhancer (Firefly), normalized to internal *Renilla* control activity in the L363 cell line. Empty MP -pCpGL vector served as a negative control. (E) Kaplan-Meier analysis of NDMM patients who underwent IMiD containing therapies (thalidomide or lenalidomide). Patients with *CRBN* enhancer (enh.) methylation (meth.) >26% (red) showed significantly inferior PFS compared with patients with methylation levels <26% (blue). Significance level: **P* < .05; ***P* < .01; ****P* < .001. DMSO, dimethylsulfoxide; n.s., not significant.

Würzburg University. Sample collection was performed in accordance with the Declaration of Helsinki, and all participants gave written informed consent.

Plasma cells were enriched from bone marrow aspirates using anti-CD138$^+$ immunomagnetic beads, and PBMCs were separated using gradient centrifugation. DNA was isolated and 100 to 200 ng was used for bisulfite conversion. We analyzed the promoter and enhancer DNA methylation status by using deep bisulfite sequencing on the Illumina MiSeq next-generation sequencer as described elsewhere.[17] Real-time quantitative polymerase chain reaction (RT-qPCR) to correlate CRBN methylation and gene expression was performed in a subset of 26 MM samples and in 15 PBMCs with available RNA. Functional validation of the novel CRBN enhancer was confirmed through luciferase reporter assay.[18,19] MM cell lines KMS-11 and OPM2 were treated with the demethylating drugs 5-aza-2'-deoxycytidine and 5-azacytidine. Demethylation of the CRBN enhancer region was monitored by pyrosequencing. Lenalidomide sensitivity was measured by annexin V-propidium iodide fluorescence-activated cell sorting. Detailed experimental workup settings are described in the supplemental Methods.

Results and discussion

In epigenome-wide screens, B-cell malignancies usually present global DNA hypomethylation and loci-specific hypermethylation within tumor suppressor promoter regions.[20,21] In MM, changes in DNA methylation have been demonstrated to have an impact on pathogenesis, prognosis, and disease progression.[21-23] Hypomethylating agents synergize with IMiDs and may even revert resistance; however, the underlying mechanisms have not been fully explained.[12,13] Other researchers have demonstrated that the CRBN promoter region (ENSR00000147822) is unmethylated in IMiD-resistant disease in vitro and in vivo.[12,14] This was confirmed in 92 of our MM samples and 52 CD138$^+$ plasma cells from healthy donors by using in-house targeted deep bisulfite sequencing methodology,[17] in which no promoter hypermethylation was detectable in 39 investigated CpGs (mean methylation, 0.22% ± 0.13% standard deviation; Figure 1). Strikingly, we found remarkable variation in DNA methylation of the novel CRBN enhancer region (ENSR00000675670) located 6039 bp downstream of the promoter in our 131-patient cohort (range, 0.00% to 84.43%). It is noteworthy that this area is not represented on the commonly used Illumina Infinium MethylationEPIC array, which may explain why this hypermethylation remained undetected in previous large-scale epigenome-wide studies. DNA methylation was largely absent in PBMCs (mean, 6.46% ± 3.04%; n = 45) and white blood cells from healthy donors (mean, 4.08% ± 1.11%; n = 32), but it was present in CD138$^+$ plasma cells from healthy donors (24.27% ± 14.95%; n = 52) and CD138$^+$ plasma cells from MM patients (mean, 26.21% ± 23.56%; n = 131; Figure 1). Interestingly, CRBN enhancer methylation significantly increased from NDMM patients (mean, 22.45% ± 19.93%; n = 79) to IMiD-refractory patients (rrMM mean, 40.59% ± 27.65%; n = 27; P = .002). Patients with rMM not refractory to IMiDs, unexposed or with unknown response status (rMM mean, 22.54% ± 23.04%; n = 25), showed significantly lower methylation compared with patients who had rrMM (P = .004) (Figure 2A). The mean methylation of all 131 investigated MM patients (26.21%) was chosen to define the threshold of a hypermethylated state. Accordingly, 39.24% (31 of 79) of NDMM and 66.67% (18 of 27) of IMiD rrMM patients were identified to be hypermethylated.

Next, we addressed the question of whether CRBN enhancer hypermethylation has an impact on expression and thus may mediate drug resistance. In fact, we confirmed in 26 of our MM patients and in 15 PBMCs with available RNA that the CRBN enhancer methylation degree correlated significantly with decreased CRBN gene expression (Spearman's r = −0.406; P = .008; Figure 2B). To explore IMiD desensitizing effects by using demethylating agents, we selected 2 tMM cell lines with intrinsic CRBN enhancer hypermethylation (OPM2 and KMS-11). After exposure to DNMTi (48 hours with 500 nM 5-azacytidine or 72 hours with 100 nM 5-aza-2′-deoxycytidine), we observed a significant reduction of the CRBN enhancer methylation (P < .001). As we had hypothesized, cells demonstrated significantly increased sensitivity to lenalidomide (P < .01) (Figure 2C), and significantly increased expression of CRBN was assessed by qPCR in OPM2 (OPM2 5-aza-2'-deoxycytidine$^+$, P = .013; OPM2 5-azacytidine$^+$, P < .001). However, in KMS-11 cells, the positive trend did not reach statistical significance. Finally, we validated the regulatory properties of the novel CRBN enhancer at a functional level by using a dual-luciferase reporter assay, in which the unmethylated insert demonstrated a significant 34.5-fold increased luciferase activity compared with the methylated insert (P < .001; Figure 2D). Together these results strongly suggest yet undescribed epigenetic regulation of CRBN impacting IMiD response in MM.

Patients with MM are commonly treated with combinations of multiple drugs, which makes it challenging to retrospectively assess the clinical relevance of CRBN enhancer methylation, in particular the degree of IMiD impairment for individual patients. Nevertheless, among the top 5 methylated patients in our cohort for whom clinical information was available, we found confirming evidence of IMiD-resistant disease: 1 patient was IMiD naïve at the time of sampling and had 71% DNA methylation before second-line therapy with carfilzomib-lenalidomide-dexamethasone to which she progressed unexpectedly early after not more than 4 cycles. One patient was primary IMiD refractory with no response to first-line lenalidomide-doxorubicin-dexamethasone. She had 71% methylation when she had no response to pomalidomide-dexamethasone therapy. A patient with 72% methylation receiving fourth-line therapy did not respond to subsequent pomalidomide-bortezomib-doxorubicin-dexamethasone, and another patient with 82% methylation receiving eleventh-line therapy did not respond to either pomalidomide-bortezomib-doxorubicin-dexamethasone or later dosing with elotuzumab-thalidomide-dexamethasone. Still, one patient with 83% CRBN enhancer methylation was initially responsive to first-line lenalidomide-doxorubicin-dexamethasone induction and third-line bortezomib-doxorubicin-lenalidomide-dexamethasone but progressed under fourth-line lenalidomide-elotuzumab-dexamethasone and had no response to fifth-line pomalidomide and subsequent pomalidomide-bortezomib-doxorubicin-dexamethasone or daratumumab-carfilzomib-lenalidomide-dexamethasone.

In a progression-free survival (PFS) analysis, NDMM patients who underwent IMiD based therapy and that had a hypermethylated CRBN enhancer status faced an inferior PFS compared with those who had no CRBN enhancer hypermethylation (median PFS, CRBN methylation >26% at 17 months; 95% confidence interval [CI], 10-23 months vs CRBN methylation <26% at 74 months; 95% CI, 21-127 months; P = .004) (Figure 2E). Notably, this effect was absent in patients who had not received IMiD first-line therapy (supplemental Figure 2).

Further research is needed to determine the clinical effects of *CRBN* enhancer regulation on IMiD sensitivity. In addition, the druggable properties of the epigenetic modifications need to be further explored. Importantly, epimutations and genetic mutations that we described earlier to inhibit CRBN-IMiD interactions[10] were not mutually exclusive in 4 of our patients with available mutation status from M3P analysis[24,25] (supplemental Table 5). This provides evidence that both mechanisms in parallel contribute to IMiD resistance in MM.

Acknowledgments

Authorship

Contribution: S.B., L.H., T.H., K.M.K., and L.R. designed the research; S.B., A.G.-T., L.H., S.H., U.M., and C.V. performed the experiments; X.A. and J.I.M.-S. helped with data analysis and gave technical support; M.B., N.B., M.C., M.D.V., H.E., R.A.F., J.K., A.R., M.K., K.M.K., J.M.-L, L.R., P.R., T.S., Y.R.-H., J.Z., and X.Z. provided patient samples and clinical data; L.H., S.B., L.R., and K.M.K. wrote the manuscript which was approved by all authors; and all authors analyzed and interpreted the data.

Conflict-of-interest disclosure: S.B. and Y.R.-H. are employees of and S.B. and J.M.-L. are equity shareholders of Altum Sequencing Co. The remaining authors declare no competing financial interests.

ORCID profiles: L.H., 0000-0002-3927-9163; U.M., 0000-0001-9710-9923; S.H., 0000-0003-1117-3102; M.B., 0000-0003-0917-4173; M.D.V., 0000-0002-5396-6584; J.K., 0000-0002-4649-0506; L.R., 0000-0002-9536-9649; T.H., 0000-0002-0737-0763; K.M.K., 0000-0002-7011-0286.

Correspondence: K. Martin Kortüm, Department of Hematology and Oncology, University Hospital Würzburg, Oberdürrbacherstr. 6, 97080 Würzburg, Germany; e-mail: kortuem_m@ukw.de.

Footnotes

REFERENCES

1. Kortüm KM, Zhu YX, Shi CX, Jedlowski P, Stewart AK. Cereblon binding molecules in multiple myeloma. *Blood Rev.* 2015;29(5): 329-334.

2. Zhu YX, Braggio E, Shi CX, et al. Cereblon expression is required for the antimyeloma activity of lenalidomide and pomalidomide. *Blood.* 2011;118(18):4771-4779.

3. Lopez-Girona A, Mendy D, Ito T, et al. Cereblon is a direct protein target for immunomodulatory and antiproliferative activities of lenalidomide and pomalidomide. *Leukemia.* 2012;26(11):2326-2335.

4. Krönke J, Udeshi ND, Narla A, et al. Lenalidomide causes selective degradation of IKZF1 and IKZF3 in multiple myeloma cells. *Science.* 2014;343(6168):301-305.

5. Lu G, Middleton RE, Sun H, et al. The myeloma drug lenalidomide promotes the cereblon-dependent destruction of Ikaros proteins. *Science.* 2014;343(6168):305-309.

6. Franssen LE, Nijhof IS, Couto S, et al. Cereblon loss and up-regulation of c-Myc are associated with lenalidomide resistance in multiple myeloma patients. *Haematologica.* 2018;103(8):e368-e371.

7. Schuster SR, Kortuem KM, Zhu YX, et al. The clinical significance of cereblon expression in multiple myeloma. *Leuk Res.* 2014;38(1): 23-28.

8. Huang SY, Lin CW, Lin HH, et al. Expression of cereblon protein assessed by immunohistochemical staining in myeloma cells is associated with superior response of thalidomide- and lenalidomide-based treatment, but not bortezomib-based treatment, in patients with multiple myeloma. *Ann Hematol.* 2014;93(8):1371-1380.

9. Heintel D, Rocci A, Ludwig H, et al. High expression of cereblon (CRBN) is associated with improved clinical response in patients with multiple myeloma treated with lenalidomide and dexamethasone. *Br J Haematol.* 2013;161(5):695-700.

10. Kortüm KM, Mai EK, Hanafiah NH, et al. Targeted sequencing of refractory myeloma reveals a high incidence of mutations in CRBN and Ras pathway genes. *Blood.* 2016;128(9): 1226-1233.

11. Egan JB, Kortuem KM, Kurdoglu A, et al. Extramedullary myeloma whole genome sequencing reveals novel mutations in Cereblon, proteasome subunit G2 and the glucocorticoid receptor in multi drug resistant disease. *Br J Haematol.* 2013; 161(5):748-751.

12. Dimopoulos K, Søgaard Helbo A, Fibiger Munch-Petersen H, et al. Dual inhibition of DNMTs and EZH2 can overcome both intrinsic and acquired resistance of myeloma cells to IMiDs in a cereblon-independent manner. *Mol Oncol.* 2018;12(2):180-195.

13. Kalff A, Khong T, Mithraprabhu S, et al. Oral azacitidine (CC-486) in combination with lenalidomide and dexamethasone in advanced, lenalidomide-refractory multiple myeloma (ROAR study). *Leuk Lymphoma.* 2019;60(9):2143-2151.

14. Tachita T, Kinoshita S, Ri M, et al. Expression, mutation, and methylation of cereblon-pathway genes at pre- and post-lenalidomide treatment in multiple myeloma. *Cancer Sci.* 2020;111(4):1333-1343.

15. Ordoñez R, Kulis M, Russiñol N, et al. Chromatin activation as a unifying principle underlying pathogenic mechanisms in multiple myeloma. *Genome Res.* 2020;30(9): 1217-1227.

16. Ruiz-Heredia Y, Sánchez-Vega B, Onecha E, et al. Mutational screening of newly diagnosed multiple myeloma patients by deep targeted sequencing. *Haematologica.* 2018;103(11):e544-e548.

17. Haertle L, Maierhofer A, Böck J, et al. Hypermethylation of the non-imprinted maternal MEG3 and paternal MEST alleles is highly variable among normal individuals. *PLoS One.* 2017;12(8):e0184030.

18. Klug M, Rehli M. Functional analysis of promoter CpG methylation using a CpG-free luciferase reporter vector. *Epigenetics.* 2006; 1(3):127-130.

19. Agirre X, Castellano G, Pascual M, et al. Whole-epigenome analysis in multiple myeloma reveals DNA hypermethylation of B cell-specific enhancers. *Genome Res.* 2015; 25(4):478-487.

20. Oakes CC, Martin-Subero JI. Insight into origins, mechanisms, and utility of DNA

methylation in B-cell malignancies. *Blood.* 2018;132(10):999-1006.

21. Walker BA, Wardell CP, Chiecchio L, et al. Aberrant global methylation patterns affect the molecular pathogenesis and prognosis of multiple myeloma. *Blood.* 2011; 117(2):553-562.

22. Alzrigat M, Párraga AA, Jernberg-Wiklund H. Epigenetics in multiple myeloma: From

mechanisms to therapy. *Semin Cancer Biol.* 2018;51:101-115.

23. Chim CS, Liang R, Leung MH, Yip SF, Kwong YL. Aberrant gene promoter methylation marking disease progression in multiple myeloma. *Leukemia.* 2006;20(6): 1190-1192.

24. Barrio S, Munawar U, Zhu YX, et al. IKZF1/3 and CRL4[CRBN] E3 ubiquitin

ligase mutations and resistance to immunomodulatory drugs in multiple myeloma. *Haematologica.* 2020;105(5): e237-e241.

25. Barrio S, DáVia M, Bruins L, et al. Protocol for M[3]P: A comprehensive and clinical oriented targeted sequencing panel for routine molecular analysis in multiple myeloma. *Methods Mol Biol.* 2018; 1792:117-128.