IKZF1/3 and CRL4^{CRBN} E3 ubiquitin ligase mutations and resistance to immunomodulatory drugs in multiple myeloma

Cereblon (CRBN), a target of immunomodulatory drugs (IMiD), forms the CRL4^{CRBN} E3 ubiquitin ligase (CRL4) complex with DDB1, CUL4B and ROC1.[1,2] Under the influence of IMiD, CRL4 polyubiquitinates and thus depletes the transcription factors IKZF1 and IKZF3, resulting in cytotoxicity to multiple myeloma (MM) cells. *In vitro*, CRBN and IKZF1/3 mutations affecting the CRBN-lenalidomide binding site (degron) cause drug resistance to IMiD.[3-5] We hypothesized that mutations in the other components of the CRL4 complex and its targets, Ikaros and Aiolos, likewise interfere with ubiquitin ligase activity, thus contributing to resistance to IMiD. In order to select the most promising patient-derived candidate mutations for functional validation, we first generated a comprehensive overview of point mutations affecting *IKZF1*, *IKZF3* or *CRL4* genes in patients with advanced MM. Next, we contextualized all described mutations at the protein level, to investigate their structural impact on complex formation and stability. Based on these analyses, we then selected a subset for functional validation by expressing mutant IKZF1, CRBN or CUL4B in MM cell lines and analyzed their effects on resistance to IMiD, thus probing the relevance of such alterations for complex integrity and the transmission of IMiD activity.

To select relevant candidate mutations, we analyzed data from different Multiple Myeloma Mutation panel (M3P) cohorts[3,6-8] and from other published and unpublished datasets[9,10] for a total of 1,838 MM cases (*Online Supplementary Methods*). In this meta-analysis we observed that the mutation frequency increased significantly after treatment (Z-score: 4.5; P<0.00001), from 2.0% (28/1373) in untreated cases to 6.2% (29/465) in pretreated cases. Notably, this increase occurred predominantly in three genes, *IKZF1* (0.15% to 1.3%, Z-

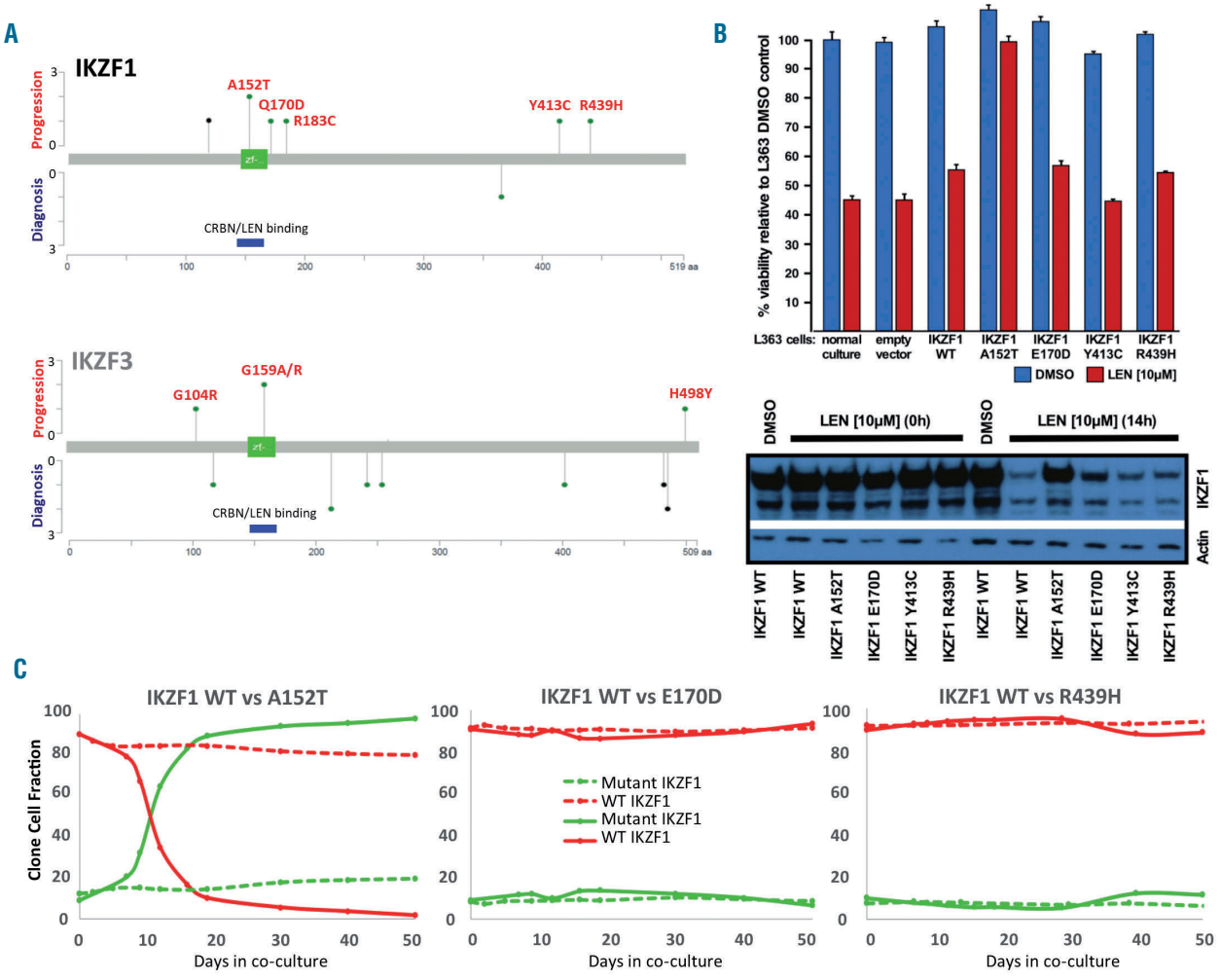

Figure 1. Only IKZF1/3 mutations affecting the lenalidomide/cereblon binding area induce resistance to lenalidomide. (A) Location of the mutations within the *IKZF3* and *IKZF1* genes. Lollipop plots of all mutations described in IKZF1 and IKZF3. Blue bar; degron sequence of the lenalidomide binding site. Bottom of the lollipop plot: mutations detected at diagnosis. Top: alterations detected after exposure to therapy. Green dots indicate missense mutations and black dots nonsense mutations. (B) Viability and molecular effects of expression of mutated IKZF1 in L363 cells. (C) Clonal competition assay (CCA) results for IKZF1-WT (red) *vs.* IKZF-A152T, IKZF-E170D or IKZF1-R439H (green). Dashed line: CAA without drug (10% mutant/90% WT). Solid line: CCA with 2.5 μM lenalidomide. DMSO: dimethylsulfoxide; LEN: lenalidomide; WT: wildtype.

score: 2.9; *P*=0.001), *CRBN* (0.44% to 2.15%, Z-score: 3.4; *P*=0.006) and *CUL4B* (0.44% to 1.93%, Z-score: 3.4; *P*=0.004) (*Online Supplementary Figure S1*). Of the *IKZF1/3*, *CRBN*, *DDB1*, *CUL4B* and *ROC1* mutations found in treated patients, 71% (24/34) were nonsense mutations or mutations located within previously described binding areas, whereas at diagnosis the distribution was spread out all along the gene loci[2,4,11] suggesting passenger mutations. We identified three potential new hotspots (*IKZF3* G159R/A, *IKZF1* A152T and *CUL4B* R820T/S) each with mutations in two different patients. All the detected mutations and variant read frequencies are summarized in *Online Supplementary Table S1*. Of interest, in eight patients with samples available from diagnosis and relapse, *CRBN* and *CUL4B* mutations were acquired after IMiD treatment, whereas *IKZF3* mutations were also identified in *de novo* disease (*Online Supplementary Figure S1*). Together, these results suggest that CRL4 mutations play an important role in disease progression and clonal evolution.

Next, we assessed the functional effects of patient-derived mutations in Ikaros and Aiolos. We constructed Sleeping Beauty vectors for expression of four IKZF1 mutations (A152T, E170D, Y413C and R439H), and one mutation in IKZF3 (G159R) (Figure 1A), and stably introduced these into MM cell lines (MM1.S and L363: IMiD sensitive, AMO1: less sensitive). Whereas transfection led to strong expression for all IKZF1 constructs, the IKZF3 expression vectors (wildtype and G159R), unfortunately, did not lead to noticeable expression even though two different versions using either cytomegalovirus- or CAAG-driven expression cassettes, were tested (*Online Supplementary Figure S2*). Viability assessments for all L363 IKZF1 sub-lines after 6 days of treatment with 10 μM lenalidomide showed that only the mutation at the lenalidomide binding site (A152T) conferred resistance to the drug (Figure 1B, top). This observation was supported by western blotting studies, which showed that only in the A152T subline did overnight treatment with lenalidomide have no effect on the level of Ikaros (Figure 1B, bottom). Furthermore, a minor inhibitory effect on IKZF1 degradation was also observed for the mutation E170D, which is located in close proximity to the degron sequence. This result was

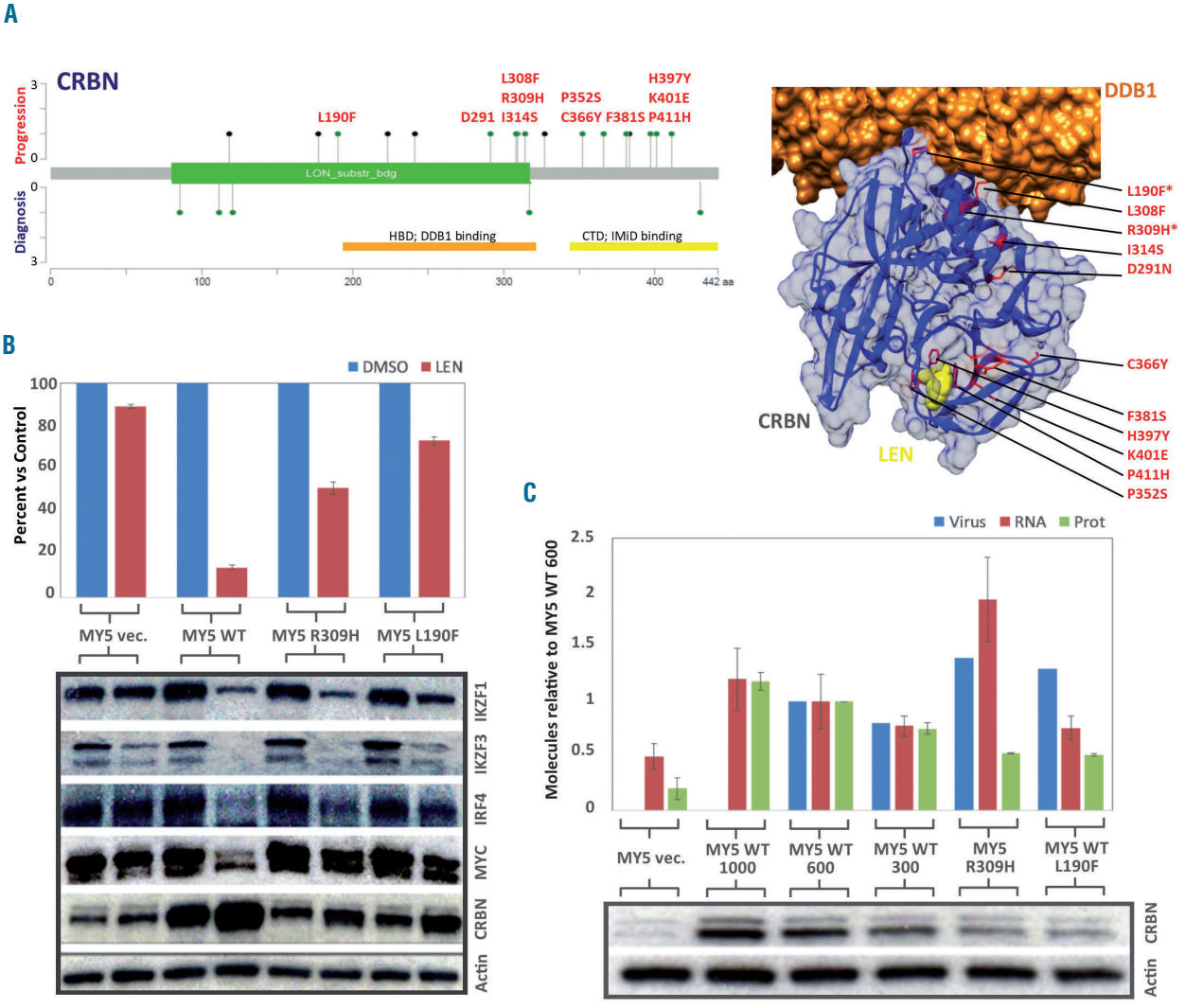

Figure 2. *CRBN* point mutations outside the lenalidomide binding pocket induce protein destabilization. (A) *CRBN* mutations at diagnosis and progression. (B) Viability test analyses (MTT) after 5 days of incubation with 5 μM lenalidomide and western blotting for the different CRBN constructs. (C) Virus titration of the CRBN constructs, compared to RNA expression level (TaqMan assays) and protein quantity (Image).

confirmed in cell lines AMO-1 (*Online Supplementary Figure S2B*) and MM1.S (*data not shown*). However, the slightly enhanced levels of IKZF-E170D after lenalidomide treatment did not lead to measurable changes in cell viability and apoptosis assays. In order to investigate the biological effects of IKZF1-E170D on proliferation fitness under lenalidomide treatment, we employed a clonal competition assay and tested L363 co-cultures of fluorescently identifiable sublines (IKZF1-WT *vs.* IKZF-A152T, IKZF-E170D or IKZF-R439H) growing under the selective pressure of a modest concentration of lenalidomide (2.5 μM, *Online Supplementary Methods*). Of note, IKZF1-A152T cells had a significant survival advantage compared to IKZF1-WT cells. Complete dominance in the culture (inversion from an initial ratio of 10% mutated/90% wildtype to 90% mutated/10 % wildtype) was observed after 21 days in co-culture. No such effect was observed for IKZF-E170D and IKZF-R439H cells (Figure 1C), which is in line with published reports that only mutations affecting the IKZF1 degron sequence[4] induce IMiD resistance.

We previously demonstrated that loss of CRBN or the

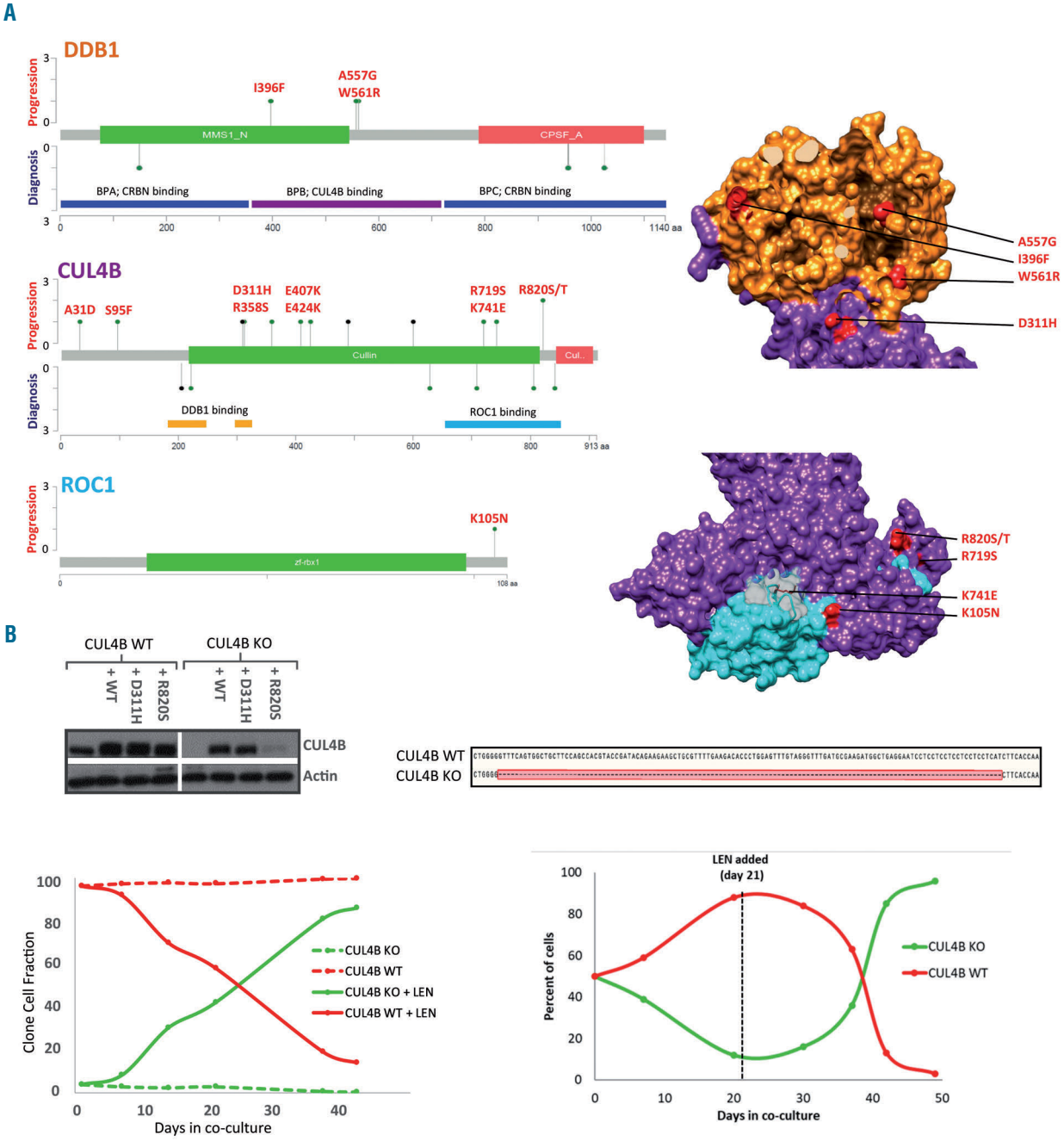

Figure 3. Loss of CUL4B induces resistance to lenalidomide *in vitro*. (A) DDB1, CUL4B and ROC1 mutations in myeloma patients. (B) Top left: western blotting of CUL4B wildtype (WT) and knockout (KO) before and after the introduction of the CUL4B WT or mutant by transfection. Top right: sequence of the CUL4B KO clone compared to WT. Bottom left: clonal competition assay (CCA) results for CUL4B KO transfected with CUL4B WT (red) vs. CUL4B KO (green). Dashed line: untreated cells. Solid lines: CCA with 2.5 μM lenalidomide (LEN). Bottom right: CCA of the same subline combinations without LEN (days 0-21) and after subsequent LEN addition (days 21-40) showing the clonal dynamics induced by the drug.

occurrence of mutations at the IMiD binding site of CRBN induces resistance to IMiD.[3] However, 56% of the missense *CRBN* mutations did not affect the IMiD binding pocket, but were present in the previously described CRBN Lon-like domain (LLD; residues 76-318), which contains a DDB1-binding motif (Figure 2A).[11] Furthermore, *in silico* analysis[12] suggests that seven of nine mutations affect this region (P85S, R111Q, F120V, D291N, L308F, R309H and I314S), which may thus lead to a reduction of CRBN stability (predicted stability change ΔΔG<0) (*Online Supplementary Table S1*). To understand whether these alterations also affect sensitivity to IMID, we selected two CRBN mutations detected in IMID-resistant patients (R309H and L190F) and lentivirally introduced them into OCIMY5 cells (cells that have very low levels of endogenous CRBN expression and are, therefore, resistant to IMiD). As shown in Figure 2B, both types of CRBN mutant-transduced cells showed a substantial reduction of sensitivity to lenalidomide compared with cells transduced with wildtype CRBN. However, further immunoblotting indicated that exogenous CRBN expression in the mutant-transfected cells was much lower than in cells transduced with wildtype CRBN. In order to analyze whether this effect was related to intrinsic features of the mutations, we performed a titration of wildtype CRBN virus (1000 virus equivalents to 75) and found that a decrease of virus equivalents correlated with decreases in RNA and protein levels of CRBN in the infected MM cells. Although both CRBN mutant virus preparations showed the highest virus equivalents, and although this corresponded to high levels of expressed CRBN RNA in OCIMY5 cells, these cells failed to express equivalent levels of CRBN protein (Figure 2C). These results suggest that in addition to CRBN mutations in the IMiD binding site, other point mutations may play a role in the development of resistance to IMiD either through impaired binding with other proteins of the complex or through destabilizing effects on the protein.

We also hypothesized that mutations in *DDB1*, *CUL4B* and *ROC1* may affect the formation of the CRL4 complex (Figure 3A). To confirm this, we prepared a *CUL4B* knockout model (in L363 cells) using the CRISPR/Cas9 system. Of ten viable clones, we selected one with clear knockout of CUL4B (Figure 3B, top). Compared to the naïve L363 cells, the knockout clone was more resistant to lenalidomide treatment. This effect was confirmed by a clonal competition assay, in which it was observed that under the selective pressure of lenalidomide treatment, the CUL4B knockout clone had an advantage, in terms of survival fitness, over the CUL4B wildtype cells (Figure 3B, bottom left). The clonal dynamics became even clearer when both CUL4B knockout and wildtype cells were initially present in equal amounts and cultured without lenalidomide. The wildtype cells easily outcompeted the CUL4B knockout clone under these conditions, but once the drug was added (2,5 μM, at day 21 in co-culture) the resistant clone took over (Figure 3B, bottom right). Of note, the re-introduction of wildtype CUL4B into the CUL4B knockout cells re-sensitized the cells to lenalidomide, confirming our hypothesis. Likewise, reintroduction of two other CUL4B mutants from IMiD pretreated patients (D311H, and R820S, a hotspot mutation detected in two MM patients) also reverted the resistance, suggesting that these two mutations do not alter the response to IMiD (*Online Supplementary Figure S3*).

We confirmed an increase of mutations in *CRBN*[3] and detected similar results for *CUL4B* and *IKZF1* after therapy. For eight cases of our cohort with acquired mutations in the CRL4 complex, a prior tumor sample was available (diagnosis-progression/relapse). In these sequential samples *CRBN* and *CUL4B* mutations were acquired after exposure to IMiD; in contrast, alterations in *IKZF3* were already detectable at diagnosis. This suggests that *IKZF3* mutations might play a role in the pathogenesis of MM rather than in the development of therapy-induced resistance. Recently, CRBN protein loss and point mutations in the CRBN-lenalidomide binding area were associated with IMiD resistance.[3] Mutations in the CRBN-DDB1 binding motif[11] may also induce resistance to lenalidomide (Figure 2B). However, the underlying mechanisms of the mutations investigated were not related to binding, but may involve destabilization of the protein folding that induces CRBN degradation (Figure 2C). The knockout of CUL4B by CRISPR/Cas9 induced resistance to lenalidomide, highlighting the importance of this protein for the anti-tumor action of IMiD. The fact that this resistance was overcome by the reintroduction of CUL4B WT using Sleeping Beauty proved the specificity of our *in vitro* approach (Figure 3B).

This is the first comprehensive analysis of the impact of *CRL4* point mutations on responses to IMiD in MM. Mutations are predominantly selected for by therapy and affect the function of the ubiquitin ligase complex through loss of a subunit by a nonsense mutation, point mutations affecting protein stability, or by impairment of substrate binding. Some alterations, including *IKZF3* hotspot mutations, are detectable prior to treatment, at initial diagnosis. The possible implications of such alterations in the pathophysiology of MM deserve further investigation.

Santiago Barrio,[1] Umair Munawar,[2] Yuan Xiao Zhu,[3] Nicola Giesen,[4] Chang-Xin Shi,[3] Matteo Da Viá,[1] Ricardo Sanchez,[5] Laura Bruins,[3] Theresa Demler,[2] Nicole Müller,[1] Larissa Haertle,[1] Andoni Garitano,[1] Torsten Steinbrunn,[1] Sophia Danhof,[1] Isabel Cuenca,[5] Clara Barrio-Garcia,[6] Esteban Braggio,[3] Andreas Rosenwald,[7] Joaquin Martinez-Lopez,[5] Leo Rasche,[1] Marc S. Raab,[4] A. Keith Stewart,[3,8] Hermann Einsele,[1] Thorsten Stühmer[2] and K. Martin Kortüm[1]

[1]Department of Internal Medicine II, University Hospital of Würzburg, Würzburg, Germany; [2]Comprehensive Cancer Center Mainfranken, University Hospital of Würzburg, Würzburg, Germany; [3]Department of Hematology, Mayo Clinic, Scottsdale, AZ, USA; [4]Department of Internal Medicine V, Heidelberg University Hospital, Heidelberg, Germany and CCU Molecular Hematology/Oncology, German Cancer Research Center (DKFZ), Germany; [5]Hematology Department Hospital 12 de Octubre, Complutense University, H12O-CNIO Clinical Research Unit, CIBERONC Madrid, Spain; [6]Gene Center, Ludwig-Maximilians University, Munich, Germany; [7]Institute of Pathology, University of Würzburg, Würzburg, Germany and [8]Center for Individualized Medicine of the Mayo Clinic, Rochester, MN, USA.

Correspondence: K. MARTIN KORTÜM
kortuem_m@ukw.de

doi:10.3324/haematol.2019.217943

Acknowledgments: this work was supported by the Deutsche Forschungsgemeinschaft (KFO216), the IZKF, the BTHA and the CDW Stiftung (KMK). UM was supported by a grant of the German Excellence Initiative to the Graduate School of Life Sciences, University of Würzburg.

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

References

1. Kortum KM, Zhu YX, Shi CX, Jedlowski P, Stewart AK. Cereblon binding molecules in multiple myeloma. Blood Rev. 2015;29(5):329-334.
2. Fischer ES, Scrima A, Bohm K, et al. The molecular basis of CRL4DDB2/CSA ubiquitin ligase architecture, targeting, and activation. Cell. 2011;147(5):1024-1039.
3. Kortum 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.
4. Kronke 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. Zhu YX, Braggio E, Shi C-X, et al. Identification of cereblon-binding proteins and relationship with response and survival after IMiDs in multiple myeloma. Blood. 2014;124(4):536-545.
6. Kortuem KM, Braggio E, Bruins L, et al. Panel sequencing for clinically oriented variant screening and copy number detection in 142 untreated multiple myeloma patients. Blood Cancer J. 2016;6(2):e397.
7. Kortum KM, Langer C, Monge J, et al. Longitudinal analysis of 25 sequential sample-pairs using a custom multiple myeloma mutation sequencing panel (M(3)P). Ann Hematol. 2015;94(7):1205-1211.
8. Kortum KM, Langer C, Monge J, et al. Targeted sequencing using a 47 gene multiple myeloma mutation panel (M(3) P) in -17p high risk disease. Br J Haematol. 2015;168(4):507-510.
9. Bolli N, Avet-Loiseau H, Wedge DC, et al. Heterogeneity of genomic evolution and mutational profiles in multiple myeloma. Nat Commun. 2014;5:2997.
10. Lohr JG, Stojanov P, Carter SL, et al. Widespread genetic heterogeneity in multiple myeloma: implications for targeted therapy. Cancer Cell. 2014;25(1):91-101.
11. Chamberlain PP, Lopez-Girona A, Miller K, et al. Structure of the human Cereblon-DDB1-lenalidomide complex reveals basis for responsiveness to thalidomide analogs. Nat Struct Mol Biol. 2014;21(9):803-809.
12. Pandurangan AP, Ochoa-Montaño B, Ascher DB, Blundell TL. SDM: a server for predicting effects of mutations on protein stability. Nucleic Acids Res. 2017;45(W1):W229-W235.