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

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Supplemental Methods

Meta-analysis

The meta-analysis was restricted to cohorts that include progression samples. Informed consent was obtained according to the declaration of Helsinki in all patients. We pooled 1,373 newly diagnosed MM (NDMM) cases including: Group_1D: 890 cases with WES data available at baseline (CoMMpass IA12 release).[23] Group_2D: own 331 M3P newly diagnosed cases. Group_3D: 152 untreated cases from previously published datasets (WES).[21,22] We compared the incidence in these NDMM groups with progression samples after therapy (465 cases): Group_1R: 148 WES data from confirmed last relapses in CoMMpass IA12. Group_2R: 164 M3P pretreated cases.[11,18–20], and Group_3R: 115 pretreated cases from previously published datasets.[21,22] Group_4R: 38 WGS advanced, multi-refractory MM cases obtained from a collaboration with the University of Heidelberg (unpublished data).

Targeted deep sequencing

Targeted deep sequencing was performed on paired tumor-germline DNA-samples using the Ion Torrent platform (PGM, Thermo Fisher) and Ion AmpliSeq Library kit. The M3P panel includes in its last version 1,327 amplicons covering the coding regions of 88 myeloma-relevant genes.[24] An average of 759× depth sequencing coverage was generated per nucleotide. Mutation analysis annotation and filtering was performed using Ion Reporter Software v4.4 and screened with the Integrative Genomics Viewer (IGV).[25]

Whole genome sequencing

WGS was performed on MACS sorted CD138+ tumor cells and germline controls. Preparation of DNA libraries was done using the TruSeq DNA Nano kit, sequencing was performed on the HiSeq X using the HiSeq X Ten Reagent kit v2.5 (all Illumina, Hayward, CA) obtaining a median coverage of 77x. Alignment workflow consisted of mapping of raw reads to the human reference genome build 37, version hs37d5, using BWA mem v0.7.8, sorting using SAMtools v0.1.19, and marking of duplicate reads using Sambamba v0.5.9. Somatic SNVs and small indel calling was performed using Mpileup v0.1.19 and Platypus v0.8.1, respectively, and high confidence variants were defined.

Structural analysis

The structural analysis was performed with Chimera 1.11 software[26] and data from public databases (Protein Data Bank). The complete structure of CRL4-CRBN E3 ubiquitin ligase was obtained by merging the structures of LEN-CRBN-DDB1 (PDB_4TZ4) and DDB1-CUL4B-ROC1 (PDB_4A0L), using DDB1 as common polypeptide domain.[7,27] *In silico* prediction of protein stability induced by mutations was performed with the Site Directed Mutator tool.[28]

Site-directed mutagenesis for functional CRBN mutation analysis

To establish mutant CRBN-expressing OCI-MY5 cell lines, we created lentivector pCDHPuroCRBN by subcloning wild-type (WT) CRBN from pCDHGFPCRBN and used this pCDHPuroCRBN as a template to introduce *CRBN* mutations by PCR as we previously described.[11] The ligated plasmid was amplified and isolated by using a Miniprep Plasmid Isolating Kit (QIAGEN) and then cloned into lentivector pCDH-CMV-MCS-EF1-Puro (SBI, Mountain View, CA) and packaged into a lentivirus. The lentivirus expressing WT-CRBN and mutant-CRBN were then used to infect OCI-MY5 myeloma cells to overexpress both WT and mutant CRBN protein. The infected cells were selected with Puromycin for two weeks. The cell viability was assessed in triplicate by using 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide (MTT) after a 5-day incubation time with different concentrations of LEN.

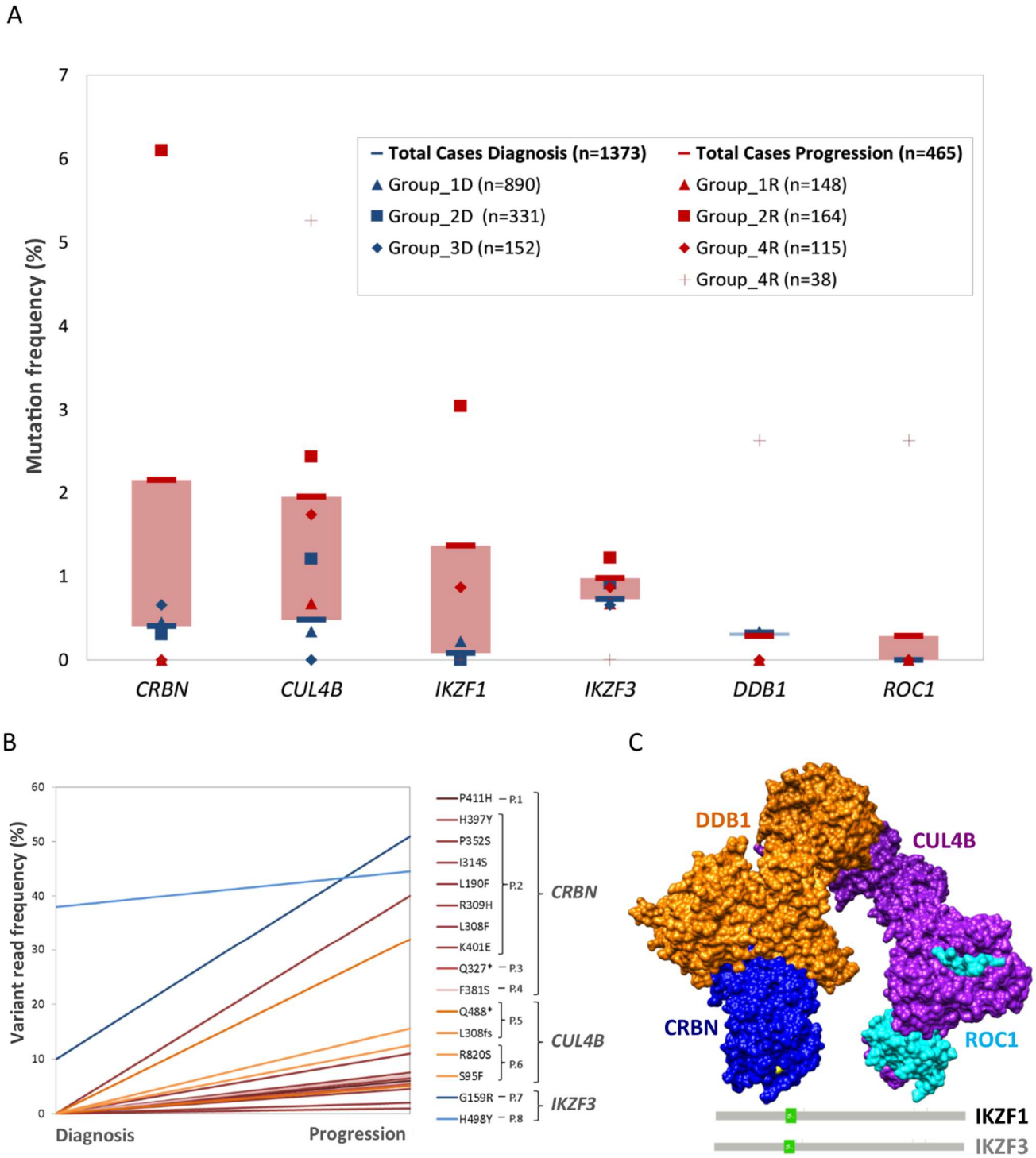
CUL4B knock out and mutated CUL4B reintroduction

Oligonucleotides for targeting Exon 3 of *CUL4B* gene with the help of CRISPR-Cas9 were designed and cloned into a GeneArt CRISPR nuclease vector (A21175, Thermo Fisher Scientific). L363 cells were co-electroporated with this vector and an expression vector for EGFP, and purified via CD4-microbead selection. Subsequently, undamaged bright green cells were manually picked and seeded into a 96-well plate for clonal upgrowth. Clones were screened for CUL4B status by Western blotting. The exact type of genomic alteration introduced by CRISPR-Cas9 was determined by Sanger sequencing and the complete knockout (KO) of CUL4B protein was verified by Western blotting as described before.[29] CUL4B-WT and CUL4B-mutant (D311H and R820S) protein was then expressed in these KO cells by transposition with Sleeping Beauty (SB) vectors coding for the respective genes. Polyclonal cultures of stable transformants were established by puromycin selection for about 2 weeks and then used in functional experiments such as viability (alamarBlue) and apoptosis assays (annexin V/propidiumiodide)[30], and for Western blotting. For functional experiments in 96-well format 1500 cells per well of either L363 CUL4B-KO, CUL4B-WT or CUL4B-mutant cells were seeded and treated with 10 µM LEN for 6 days.

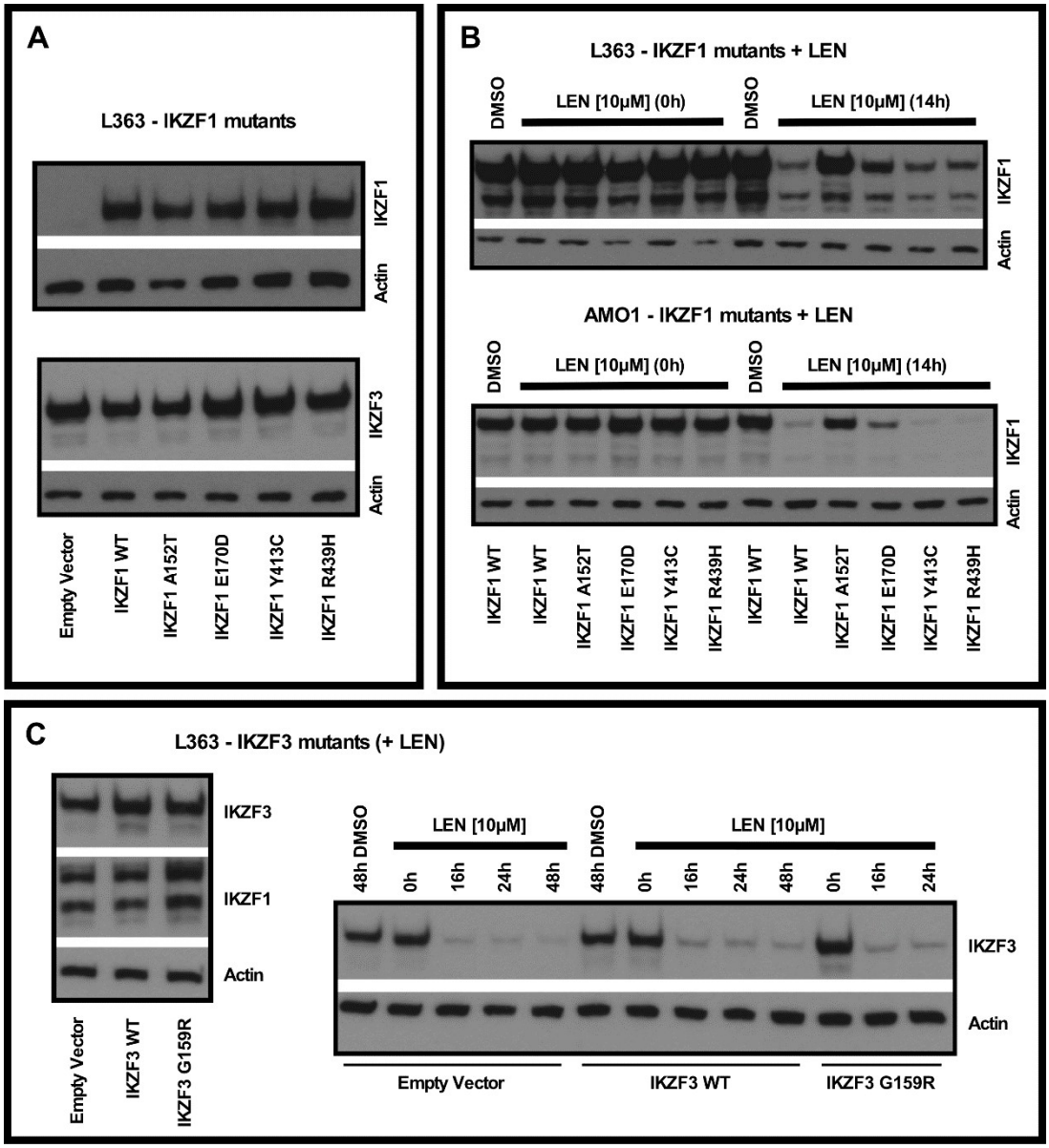
Clonal competition assays

To characterize the induction of cell resistance over time by the selected mutations in isogenic cells, we established a clonal competition assay system (CCA) using again a SB approach but with G418 selection to stably transfect L363 cells with EGFP and/or LSS mKate RFP. Briefly, after establishment of stably transfected polyclonal cultures of CUL4B mutant / WT marked with different fluorescence proteins, we mixed 90% of “reference-EGFP” cells with 10% of the “mutant-RFP” sub-line and co-cultured them for 40-60 days in the presence or absence of LEN. All experiments were conducted in triplicate to rule out that the observed effects were the result of coincidental alterations acquired over the long culture period. In addition, reference/mutant EGFP/RFP fluorescence was switched in one of the replicates to confirm that the effects are not related to the presence of any specific fluorescent protein. The ratio of EGFP and RFP cells was determined every 4-10 days by flow cytometry, and Sanger sequencing confirmed the genetic integrity of the mutants at the end of the experiment.

Supplemental Figures and tables



Supplemental Figure 1: Mutation frequency in CRL4^{CRBN} E3 ubiquitin ligase and *IKZF1/3* increase after therapy exposition. A: Incidence of somatic mutations in the CRL4^{CRBN} E3 ubiquitin ligase. Color bars represent the increase from diagnosis to progression. **B:** Clonal dynamics for eight patients mutated under therapy (p1-p8) that represent paired samples, including one at diagnosis. * indicates nonsense mutations. **C**: CRL4 protein structure obtained after merging PDB_IDs 4TZ4, and 4A0L. LEN is shown in yellow, CRBN in blue, DDB1 in orange, CUL4B in purple and ROC1 in cyan. IKZF1 and IKZF3 amino acid sequences with the degron sequence highlighted in green are also included.



Supplemental Figure 2: Western Blot of the different mutants prepared. A: IKZF1 and IKZF3 expression level after sleeping beauty stable transfection. IKZF1 shows a clear overexpression compared to Empty Vector, but the expression level of IKZF3 are not increased after IKZF3 transfection. **B:** Effect of Lenalidomide (LEN) in L363 and AMO1 IKZF1 mutants. IKZF1 A152T is not degraded after LEN addiction and IKZF1 E170D suffers less degradation than IKZF1 WT. **C:** Effect of LEN in IKZF3 mutants.

gene	Patient id	Cohort	Timepoint	IMID treated	IMiD refractory/relapse	Chr	position	reference	genotype	function	VRF (%)	Baseline VRF (%)	previous timepoint VRF (%)	aa change	Predicted ΔΔG SDM	Outcome SDM
CRBN	152	M3P	pretreated	yes	yes	chr3	3192646	G	G/T	Missense	5	0	-	P411H	1.16	Increased stability
CUL4B	126	M3P	Pretreated	yes	yes	chrX	119668435	T	T/C	Missense	5	-	-	K741E	-	-
CUL4B	135	M3P	Pretreated	yes	yes	chrX	119666311	C	C/G	Missense	25	-	-	R820T	-	-
CUL4B	158	M3P	Pretreated	yes	yes	chrX	119677622	C	C/T	Missense	5	-	-	E424K	-	-
CUL4B	158	M3P	Pretreated	yes	yes	chrX	119677977	C	C/T	Missense	5	-	-	E407K	-	-
IKZF1	122	M3P	pretreated	yes	yes	chr7	50450270	G	G/A	Missense	31	-	-	A152T	-	-
IKZF3	176	M3P	Untreated	-	-	chr17	37933948	C	T	Missense	54	-	-	S118N	-	-
IKZF1	540	M3P	pretreated	uk	uk	chr7	50450270	G	A	Missense	4	-	-	A152T	-	-
IKZF3	486	M3P	pretreated	uk	uk	chr17	37947690	-	-	Missense	4	-	-	G104R	-	-
CUL4B	459	M3P	Untreated	-	-	chrX	119666359	-	T/G	Missense	50	-	-	D804A	-	-
CRBN	121	M3P	pretreated	yes	yes	chr3	3192689	G	G/A	Missense	6	0	0	H397Y	0.61	Increased stability
CRBN	121	M3P	pretreated	yes	yes	chr3	3194234	G	G/A	Missense	2	0	0	P352S	0.1	Increased stability
CRBN	121	M3P	pretreated	yes	yes	chr3	3195654	A	A/C	Missense	11	0	0	I314S	-2.08	Reduced stability
CRBN	121	M3P	pretreated	yes	yes	chr3	3209435	C	C/A	Missense	5	0	0	L190F	0.2	Increased stability
CRBN	121	M3P	pretreated	yes	yes	chr3	3209432	C	C/A	Missense	1	0	0	R309H	-0.48	Reduced stability
CRBN	121	M3P	pretreated	yes	yes	chr3	3195673	-	-	Missense	5	0	0	L308F	-1.48	Reduced stability
CRBN	121	M3P	pretreated	yes	yes	chr3	3195673	-	-	Missense	40	0	0	K401E	0.4	Increased stability
IKZF1	119	M3P	pretreated	yes	uk	chr7	50450326	G	G/C	Missense	28	-	37	Q170D	-	-
CRBN	MMRF_1796_1_BM	MMRF	Untreated	-	-	chr3	3215867	G	A	Missense	42	-	-	P85S	-0.63	Reduced stability
CRBN	MMRF_2305_1_BM	MMRF	Untreated	-	-	chr3	3215762	A	C	Missense	10.4	-	-	F120V	-1.54	Reduced stability
CRBN	MMRF_2402_1_BM	MMRF	Untreated	-	-	chr3	3192592	G	A	Missense	28.1	-	-	T429I	-	-
CRBN	MMRF_2754_1_BM	MMRF	untreated	-	-	chr3	3194191	C	T	Missense	29.5	-	-	C366Y	0.09	Increased stability
CUL4B	MMRF_2118_1_BM	MMRF	Untreated	-	-	chrX	119672540	T	A	Missense	5.5	-	-	K627N	-	-
CUL4B	MMRF_2336_1_BM	MMRF	Untreated	-	-	chrX	119669779	A	G	Missense	31.3	-	-	I707T	-	-
CUL4B	MMRF_2746_1_BM	MMRF	untreated	-	-	chrX	119694456	G	T	Missense	23.6	-	-	A31D	-	-
DDB1	MMRF_1327_1_BM	MMRF	Untreated	-	-	chr11	61070587	T	G	Missense	6.6	-	-	E958A	0.27	Increased stability
DDB1	MMRF_1602_1_BM	MMRF	Untreated	-	-	chr11	61096942	C	A	Missense	4.5	-	-	D148Y	-0.09	Reduced stability
DDB1	MMRF_2041_1_BM	MMRF	Untreated	-	-	chr11	61070086	G	A	Missense	53	-	-	S1027L	1.27	Increased stability
DDB1	MMRF_2734_1_BM	MMRF	untreated	-	-	chr11	61081591	A	T	Missense	53.7	-	-	W561R	-0.92	Reduced stability
DDB1	MMRF_2734_1_BM	MMRF	untreated	-	-	chr11	61081602	G	C	Missense	56.8	-	-	A557G	-1.57	Reduced stability
IKZF1	MMRF_1725_1_BM	MMRF	Untreated	-	-	chr7	50467856	C	T	Missense	35.8	-	-	S364L	-	-
IKZF3	MMRF_1210_2_BM	MMRF	pretreated	-	-	chr17	37947785	C	G	Missense	8	-	-	G159A	-	-
IKZF3	MMRF_1721_1_BM	MMRF	Untreated	-	-	chr17	37933970	G	C	Missense	50	-	-	L254V	-	-
IKZF3	MMRF_1796_1_BM	MMRF	Untreated	-	-	chr17	37944581	C	G	Missense	32.5	-	-	Q213H	-	-
IKZF3	MMRF_1986_1_BM	MMRF	Untreated	-	-	chr17	37944581	C	G	Missense	52.4	-	-	Q213H	-	-
CRBN	299	M3P	Untreated	-	-	chr3	3195647	A	A/C	Missense	10	-	-	N316K	0.19	Increased stability
CUL4B	347	M3P	Untreated	-	-	chrX	119691847	G	G/C	Missense	45	-	-	L220V	-	-
IKZF3	278	M3P	Untreated	-	-	chr17	37934006	T	T/C	Missense	26	-	-	R242G	-	-
CRBN	553	M3P	Pretreated	yes	yes	chr3	3195724	-	C/T	Missense	17	-	-	D291N	-0.54	Reduced stability
CUL4B	-	M3P	Pretreated	yes	yes	chrX	119679342	C	C/G	Missense	47	-	-	D311H	-	-
IKZF1	526	M3P	pretreated	no	no	chr7	50468081	G	G/A	Missense	4	-	-	R439H	-	-
IKZF1	534	M3P	pretreated	uk	uk	chr7	50468003	A	A/G	Missense	4	-	-	Y413C	-	-
IKZF3	558	M3P	pretreated	yes	yes	chr17	37947786	-	C/G	Missense	51	10	-	G159R	-	-
CUL4B	445	M3P	Untreated	-	-	chrX	hrX:11966408	-	C/G	Missense	32	-	-	E840Q	-	-
CRBN	125	M3P	pretreated	yes	yes	chr3	3192699	CACTGGGCAACAGTCCAC	TGGGCAACAGTCCA	Splice_Site	38	-	-	Y383_splice	-	-
CRBN	132	M3P	pretreated	yes	yes	chr3	3195145	G	G/A	Nonsense_Mutation	7	0	-	Q327*	-	-
CRBN	154	M3P	pretreated	yes	yes	chr3	3209479	T	T/C	Splice_Site	21	-	-	I177_splice	-	-
CRBN	158	M3P	pretreated	yes	yes	chr3	3197931	G	G/GGCCC	Frame_Shift_Del	25	-	-	P241fs	-	-
CRBN	532	M3P	pretreated	yes	yes	3	chr3:3209336	C	C/T	Nonsense_Mutation	2.23	-	-	W223*	-	-
CRBN	537	M3P	pretreated	yes	yes	3	chr3:3215768	T	T/A	Nonsense_Mutation	63.2	-	-	R118*	-	-
CUL4B	126	M3P	pretreated	yes	yes	chrX	119673122	CC	CC/AT	Splice_Site	17.9	-	-	G599_splice	-	-
CUL4B	394	M3P	untreated	-	-	chrX	119691895	C	C/G	Splice_Site	5	-	-	G204_splice	-	-
IKZF3	355	M3P	untreated	-	-	chr17	37922121	CAT	CAT/C	Frame_Shift_Del	40	-	-	M484fs	-	-
CUL4B	MMRF_1153_4_BM	MMRF	pretreated	yes	yes	X	119675492	G	A	Nonsense_Mutation	5.1	0	-	Q488*	-	-
CUL4B	MMRF_1153_4_BM	MMRF	pretreated	yes	yes	X	119679349	C	CA	Frame_Shift_Del	32	0	-	L308fs	-	-
IKZF3	MMRF_1832_1_BM	MMRF	Untreated	-	-	17	37922133	G	GA	Frame_Shift_Del	46.8	-	-	G481fs	-	-
IKZF3	MMRF_1963_1_BM	MMRF	Untreated	-	-	17	37922121	CAT	C	Frame_Shift_Del	48.1	-	-	M484fs	-	-
CRBN	144	M3P	pretreated	yes	yes	chr3	3194146	A	A/C	missense	7	0	-	F381S	-2.32	Reduced stability
IKZF3	MMRF2168	MMRF	untreated	-	-	17	37933954	A	G	Missense	91	96.3	-	L259S	-	-
CUL4B	PD4291	Bolli et al.	pretreated	uk	uk	chrX	119666310	C	G	Missense	12.5	0	-	R820S	-	-
CUL4B	PD4291	Bolli et al.	pretreated	uk	uk	chrX	119694264	G	A	Missense	15.6	0	-	S95F	-	-
IKZF3	PD4286	Bolli et al.	pretreated	uk	uk	chr17	37922081	G	A	Missense	44.5	38	-	H498Y	-	-
CRBN	MM-0550-Tumor	Lohr et al.	Untreated	-	-	chr3	3190788	C	T	Missense	48.7	-	-	R111Q	-0.01	Reduced stability
CUL4B	MM-0423-Tumor	Lohr et al.	pretreated	uk	uk	chr23	119553770	C	A	Missense	9.8	-	-	R719S	-	-
IKZF1	IKZF1	Lohr et al.	pretreated	uk	uk	chr7	50417857	C	T	Missense	22.7	-	-	R183C	-	-
IKZF3	MM-0547-Tumor	Lohr et al.	Untreated	-	-	chr17	35175897	T	C	Missense	12.4	-	-	Y401C	-	-
DDB1	N1	Heidelberg	pretreated	yes	yes	-	-	-	-	Missense	56	-	-	I396F	-	-
ROC1	N2	Heidelberg	pretreated	yes	yes	-	-	-	-	Missense	16	-	-	K105N	-	-
CUL4B	N3	Heidelberg	pretreated	yes	yes	-	-	-	-	Missense	-	-	-	R358S	-	-
IKZF1	MMRF2787	MMRF	untreated	-	-	chr7	50444493	T	A	G141_splice	-	-	-	-	-	-

Supplemental Table 1. Summary of all studied mutations in the CRL4 complex.