Identification Of Cereblon Binding Proteins And Relationship With Response And Survival Following Pomalidomide And Dexamethasone In Multiple Myeloma

Short Title: IKZF1 predicts iMiDs response and survival

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Key points

We identified CRBN binding proteins and confirmed recent work implicating the Ikaros transcription factors as important members.

Low *IZKF1* levels predict lack of IMiD responsiveness and shorter OS. *IZKF1* expression is a potential predictive biomarker for clinic application.
Abstract

Cereblon (CRBN) mediates immunomodulatory drug (IMiD) action in multiple myeloma (MM). Using two different methodologies we identified 244 CRBN binding proteins and established relevance to MM biology by changes in their abundance after exposure to lenalidomide. Proteins most reproducibly binding CRBN (>4 fold versus controls) included DDB1, CUL4A, IKZF1, KPNA2, LTF, PFKL, PRKAR2A, RANGAP1 and SHMT2. After lenalidomide treatment abundance of 46 CRBN binding proteins decreased. We focused attention on two of these - IKZF1 and IKZF3. IZKF expression is similar across all MM stages or subtypes however IKZF1 is substantially lower in three of five IMiD resistant MM cell lines. The cell line (FR4) with the lowest IKZF1 levels also harbors a damaging mutation as well as a translocation which upregulates IRF4, an IKZF target. Clinical relevance of CRBN binding proteins was demonstrated in 44 refractory MM patients treated with pomalidomide and dexamethasone therapy in whom low IKZF1 gene expression predicted lack of response (0 of 11 responses in the lowest expression quartile). CRBN, IKZF1 and KPNA2 levels also correlate with significant differences in overall survival. Our study identifies CRBN binding proteins and demonstrates that in addition to CRBN, IKZF1 and KPNA2, expression can predict survival outcomes.

Keywords: Multiple Myeloma, Cereblon, Ikaros proteins, Mass Spectometry, Gene Expression Profiling, Immunomodulatory drugs, Biological Markers
Introduction

Immunomodulatory drugs (IMiDs) are key components of treatment for a number of hematologic malignancies\(^1\). The first drug in this class, thalidomide, became infamous for its teratogenic effects. Early mechanism of action studies implicated multiple potential, but no definitive drug activities, that explained this teratogenicity\(^2,3\). Recently however, Ito et al. described that cereblon (CRBN) is the primary target of thalidomide teratogenicity\(^4\). Thalidomide binds CRBN to alter the function of the E3 ubiquitin ligase complex (composed of CRBN, DDB1, and CUL4). Drug-induced downstream effects of altered CRBN activity include cell cycle arrest with up regulation of the cyclin-dependent kinase inhibitor p21\(^{\text{WAF-1}}\)\(^5\) and down regulation of interferon regulatory factor 4 (IRF4), a MM cell survival factor that targets critical genes including MYC, CDK6 and CASP\(^6-8\). Collateral effects on immune function including inhibition of tumor necrosis factor and upregulation of Interleukin 2, T lymphocytes and natural killer cells are also documented.

We and others previously reported that CRBN is also required for the anti-MM action of the thalidomide derivatives lenalidomide and pomalidomide, thus more accurately referred to collectively as “cereblon binding small molecules”\(^9\). Furthermore, we observed that CRBN expression decreases in MM patients that developed resistance to lenalidomide therapy\(^9,10\). Conversely, loss of CRBN expression did not affect response to other agents, such as bortezomib, dexamethasone, and melphalan\(^9\). Recent clinical correlative studies have observed a positive association between CRBN and response to thalidomide maintenance and upfront lenalidomide and dexamethasone therapy\(^11,12\). Furthermore, we have observed CRBN and IRF4 mutations in drug refractory patients,
supporting the key role of CRBN in the response to IMiDs\textsuperscript{13,14}. Finally we have reported that CRBN expression is highly predictive of response and survival outcomes following pomalidomide therapy\textsuperscript{15}. However, a majority of patients with low CRBN levels have no genomic mutation evident, thus transcriptional or post-transcriptional factors may influence CRBN gene expression and responsiveness to IMiD therapy\textsuperscript{16}.

Despite these findings the intermediaries and facilitators of cell death following CRBN and IMiD interaction that ultimately degrade IRF4 and MYC are as yet not fully elucidated. In the current study, we set out to identify CRBN binding proteins and then to determine which of these proteins were relevant to MM cytotoxicity by assessing alterations in abundance of CRBN binding partners in the presence of lenalidomide. In the interim two recent papers described that the transcription factors Ikaros (IKZF1) and Aiolos (IKZF3) were important intermediaries that are rapidly ubiquitinated and degraded after IMiDs bind to CRBN\textsuperscript{17,18} We describe our identification of these but also other binding proteins of relevance and the correlation of these proteins with response and survival outcomes in MM-patients.
Methods

Cells and reagents

Human myeloma cell lines (HMCLs) were maintained in RPMI-1640 medium, supplemented with 5% of sterile fetal calf serum and antibiotics. Anti-CRBN antibodies were from Sigma-Aldrich (St. Louis, MO). Anti-IRF4, anti-PARP, anti-KPNA2 and anti-DDB1 were from Cell Signaling Technology (Danvers, MA). Anti–IKZF1 antibodies were from Cell Signaling Technology and Santa Cruz Biotechnology (Dallas, TX). Anti-IKZF3 was from Imgenex (San Diego, CA) and anti-MYC antibody was from Epitomics (Burlingame, CA). Lenalidomide was from LC Laboratories (Woburn, MA). Ni-NTA agarose was from Qiagen (Valencia, CA).

All experiments were conducted under approval of IRB (IRB2207-02). Patient samples are collected and stored under IRB919-04. This study was conducted in accordance with the Declaration of Helsinki.

Preparation of lentiviral expressing virus and infection of myeloma cells

Human CRBN, IKZF1 and IKZF3 cDNAs were purchased from Thermo Scientific (Rockford, IL) and were sub-cloned into a lentiviral expression vector, pCDH-CMV-MCS-EF1-copGFP (System Bioscience, Mountain View, CA). CRBN tagged with His at its C-terminal was generated by PCR method and confirmed by sequencing. IKZF1 and IKZF3 lentiviral shRNAs constructs were purchased from Sigma-Aldrich (St. Louis, MO). Lentivirus harboring control vector and all expression constructs were used to infect human myeloma cell lines (HMCLs)\textsuperscript{19}. The infection efficiency was measured by FACScan analysis of GFP expression at day 3 after infection. OCI-MY5 cells infected by
control virus (vector alone) and CRBN expressing virus were sorted for GFP expression at 14 days after infection. Overexpression of CRBN was confirmed by immunoblotting assay.

**Identification of CRBN binding proteins using Co-IP and Ni Charged (Ni⁺) beads**

OCI-MY5 cells stably expressing vector alone (OCI-MY5/vec) or His-tagged CRBN (OCI-MY5/CRBN-His) were harvested and processed using two different pull-down methodologies:

1. **Co-immunoprecipitation (Co-IP)** with an anti-CRBN antibody. OCI-MY5/CRBN-His cells were lysed using lysis buffer (50mM Tris pH8.0, 150mM NaCl, 1%NP40, 0.05% Tween20 and protease inhibitors) and protein quantified using a BCA assay. 1mg of protein was pre-cleaned by incubation with protein A/G slurry at 4°C with rocking and resultant supernatant was transferred to a fresh tube. Normal mouse IgG (NlgG, as control) or CRBN antibody were added and incubated at 4°C overnight (with rocking). 30 μl of A/G slurry were added and incubated for 1 hour. After washing three times with lysis buffer, loading buffer (Cell Signaling Technology) was added and samples were boiled and loaded to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels.

2. **Pull-down using Ni⁺ beads.** OCI-MY5/vec or OCI-MY5/CRBN-His (untreated or pre-treated with Lenalidomide for 48 hours) were harvested and stored at – 80°C. The cell pellets were re-suspended in 1 X binding buffer (20 mM Tris/HCl, pH7.9; 500 mM NaCl; and 10% glycerol) with protease inhibitors and then equilibrated on ice for 30 minutes at 800 p.s.i.in a Parr nitrogen cavitation bomb. After releasing the pressure, the cell lysates were centrifuged at 33,000 x g for 30 minutes at 4°C to collect supernatant.
Protein amount was quantified and the same amount of proteins from each samples were incubated with Ni-NTA agarose on a rocker for 2 hours at 4˚C. The loaded Ni-NTA agarose beads were packed into a column and washed with 1 X binding buffer containing 10 mM imidazole (1st wash), 20 mM imidazole (2nd wash) and 40 mM imidazole (3rd wash) ²¹. The bound proteins were eluted with 1 X binding buffer containing 400 mM imidazole and were used for mass spectrometry analysis.

**Mass spectrometry (MS) analysis**

For proteomics experiments, proteins were separated by 10% SDS-PAGE and were stained with Coomassie Blue. Each lane of the SDS-PAGE gel was cut into 10 slices, followed by de-staining and digestion with trypsin. The peptides were extracted and then purified by solid-phase extraction (C18 ZipTip, Millipore, Billerica, MA).

HPLC-ESI-MS/MS was performed on a (ThermoScientific, San Jose, CA) Orbitrap Elite Velos PRO mass spectrometer fitted with a NanoFlex source (New Objective, Woburn, MA) coupled to a DIONEX (Sunnyvale, CA) Ultimate 3000 Series Nano/Cap System NCS-3500RS. NanoLC was performed using an Acclaim PepMap100 trap column an Acclaim PepMap RSLC analytical column (Thermo Scientific). A “top-10” data-dependent MS/MS analysis was performed. The fragment mass spectra were then searched against the human SwissProt_2013_02 database using Mascot (Matrix Science, London, UK; version 2.4). Cross-correlation of Mascot search results with X! Tandem was accomplished with Scaffold (version Scaffold_4.2.1; Proteome Software, Portland, OR, USA). Probability assessment of peptide assignments and protein identifications were made through the use of Scaffold. Only peptides with ≥ 95%
probability were considered.

**Analysis of IKZF1 expression by flow cytometry**

500,000 cells were re-suspended in 0.1 ml PBS in 5 ml U-bottom tubes. Cells were fixed by adding 40ul 10% Formaldehyde at room temperature for 10 minutes (min) and then cooled on ice for 1 min. 0.6 ml ice-cold methanol was added and left on ice for 20-30 min. After washing with PBS buffer, anti–IKZF1 at room temperature for 20 min. After washing with PBS buffer, 2nd Ab was added and incubated at room temperature, in dark for 15 min, washed once with PBS+3%FBS and samples were then analyzed on flow cytometer.

**In silico Network Analysis and Pathway Enrichment Analysis**

Cytoscape Reactome FI plugin was used to assess the molecular function, biological pathways and additional protein-protein interactions as described 22. Lists of proteins upregulated or downregulated in response to lenalidomide were evaluated against the latest versions of the following databases (Covering >50% of human proteins): CellMap, Reactome, KEGG, NCI-PID, and BioCartel. Linker proteins were included in the network modeling and comparative pathway enrichment predictions were generated using input lists including or excluding linker proteins. Results were filtered for p-value <0.05 and False Discovery Rate < 0.05.
Primary patient samples

We screened the gene expression profiling (GEP) levels from 44 MM patients from Mayo Clinic homogeneously treated in two prospective clinical trials with pomalidomide and dexamethasone\textsuperscript{23,24}. The first trial included 35 relapsed or refractory patients that received pomalidomide 2mg daily, continuously on a 28 day cycle, and dexamethasone 40mg weekly. The second trial included 35 relapsed or refractory patients that received pomalidomide 4mg daily, continuously on a 28 day cycle, as well as dexamethasone 40mg weekly. 44 of the 70 patients on these two trials were successfully analyzed for gene expression prior to therapy initiation.

Gene expression profiling

RNA was isolated from marrow CD138 positive plasma cells. GEP was performed from total RNA using the Affymetrix U133Plus2.0 array. Microarrays were scanned with an Affymetrix Scanner 3000 and data normalization was performed using Expression Console (Affymetrix) and the Robust Multi-array Average (RMA). RMA intensity values were log2 transformed for subsequent analyses. Public datasets were used for comparative expression studies on different stages of plasma cell neoplasm (GSE6477) and for analysing the association between copy-number and gene expression, (http://www.broadinstitute.org/mmgp/home).

Response criteria

Response was assessed according to the International Myeloma Working Group criteria\textsuperscript{25}. A positive response to therapy in this study required a partial response or
better.

Statistical Analysis

For the comparison of gene expression levels between groups, we used Fisher two-tailed test (comparison between two groups) and Kruskal-Wallis test (three or more groups).

In order to evaluate the association of CRBN binding protein level with PFS (Progression Free Survival, time from registration to the earlier of disease progression or death) and OS (Overall survival, time from registration to death), median level was used to separate patients into two groups, followed by analysis using Kaplan Meier methods. COX survival model was also used to analyze the effects of 11 proteins identified from CRBN complex altered by lenalidomide on survival outcomes. To evaluate connection between IKZF1 and response, we determined the quartile cut-off points for IKZF1 expression (for all MM patients). Optimal cutoff points for survival analyses were determined using the Contal and O'Quigley method.

Results

Identification of Cereblon interacting proteins

OCI-MY5 MM cells have very low CRBN expression and are highly resistant to IMiDs. We were able to restore IMiD responsiveness by introducing wild-type CRBN with a His-tag at its C-terminal (OCI-MY5 CRBN-His) (supplementary figure 1).
We then used an anti-CRBN antibody to immunoprecipitate CRBN and its associated proteins, using a normal mouse IgG negative control (Fig.1A), and performed quantitative proteomics to define the CRBN interactome. 83 putative CRBN protein binding partners with an average of 2-fold higher assigned spectra as compared with controls were identified from duplicate experiments (supplementary data 1). The most differentially associated 10 proteins (>4 fold higher than the negative controls in both runs) include CRBN, and its known associated proteins, CUL4A and DDB1 (Table 1), confirming the specificity of the method. Six proteins (CRBN, ACAD9, FLOT1, FN1, HDDC3 and IKZF1) were detected in CRBN immunoprecipitates, and were completely absent in both negative control immunoprecipitates.

We independently also employed Ni charged (Ni+) beads to identify CRBN associated proteins (Fig.1). In duplicate experiments, 171 CRBN associated proteins with higher assigned spectra (average ≥2 fold higher) were identified in the CRBN overexpressing cells versus the negative control vector-expressing cells (supplementary data 1). The most differentially identified proteins again included CRBN, DDB1 and CUL4A. Over all, ten proteins were identified to be shared between the Co-IP and Ni+ bead methods, including CRBN, CUL4A, DDB1, IKZF1, KPNA2, LTF, PFKL, PRKAR2A, RANGAP1 and SHMT2 (Table 2). Among the 13 unique proteins (not part of the CRBN E3 ligase complex) shown in Tables 1 and 2, three are involved in metabolic pathways (Phosphofructokinase, ACAD9, a member of the acyl-CoA dehydrogenase family, PRKAR2A which engages in lipid and glucose metabolism) while two of these strong CRBN binding proteins are engaged in nuclear transport (RANGAP1, KPNA2).
CRBN interacting proteins relevant to IMiD treatment

We next examined the influence of lenalidomide treatment. Proteomic analysis identified 114 proteins that decreased in CRBN binding abundance (≥2 fold lower in the Ni⁺ beads pull-down samples from OCI-MY5/CRBN cells 48 hours after lenalidomide treatment versus no treatment, n=3, supplementary data 1). Of these 114 proteins 46 were also identified to significantly bind to CRBN complex in the absence of drug. Two of the most downregulated CRBN associated proteins after lenalidomide treatment included the Ikaros family members IKZF1 (Ikaros) and IKZF3 (Aiolos) (Table 3). On the other hand, 16 proteins underwent an increase in abundance following lenalidomide treatment (supplementary data 1), including CRBN, its associated proteins DDB1, CUL4B, CUL4A and SQSTM1 (Table 3).

Pathway analysis of all CRBN binding proteins altered by lenalidomide revealed that the molecular pathways most affected included; the regulation of cytoplasmic and nucleus SMAD2/3 signaling, ubiquitin mediated proteolysis, P53 tumor suppressor pathways, signaling by HDAC, NOTCH and Wnt (Fig. 2 and supplementary data 2). Prominent networks, implicated the transcriptional co-activator and histone deacteylase EP300, the proteasome and ubiquitin complex, MYC complex associated proteins, E2F1 and XPO1.

Validation of IKZF1 and IKZF3 as CRBN-binding proteins

Immunoblotting (Fig.3A) confirmed that CRBN, DDB1, and CUL4A were enriched in the CRBN binding-fraction from OCI-MY5/CRBN-His but not from control cells (vector).
After lenalidomide treatment, CRBN, DDB1 and CUL4A were also more abundant in the CRBN pull-down complex while IKZF1, IKZF3 and KPNA2 decreased (Fig. 3A). We found that both IKZF1 and IKZF3 were downregulated within 3-6 hours of lenalidomide treatment, and that this change occurred significantly before downregulation of IRF4, MYC and other binding proteins such as KPNA2 (Fig. 3B-C).

Binding of IKZF1 and IKZF3 to CRBN was actually initially increased after lenalidomide treatment (3 hours) whereas protein levels in lysates were decreased (Fig. 3D), suggesting a rapid binding and then later degradation of IKZF1 and IKZF3. The CRBN level in Co-IP samples increased after lenalidomide treatment, but unlike Ikaros proteins, CRBN protein level increased in lysates (Fig. 3D).

**Drug resistance and cereblon target proteins in MM**

We compared Ikaros and Aiolos (IKZF1 and IKZF3) gene expression levels across different human tissues and malignancies. *IKZF1* (Fig. 4A) and *IKZF3* (Fig. 4B) were expressed in most hematological malignancies, including MM, but not in solid tumors (source: Broad-Novartis Encyclopaedia and The Genotype Tissue Expression Portal). IKZF1 and IKZF3 gene expression levels did not change in different stages of disease progression from normal PC to MGUS, SMM and MM. Furthermore, no significant differences were found in the expression level across genetic subtypes (data not shown).

By western blot IKZF1 levels are adequate in all sensitive MM lines examined while in three of five tested lenalidomide resistant cell lines IKZF1 were low (Fig. 5A). As an alternate and potentially clinically useful strategy we were also able to detect IKZF1
expression and its changes after IMiD using flow cytometry analysis and the result was consistent with western blot results (Fig. 5B).

Using lentiviral shRNA expression, knockdown of IKZF1 and IKZF3 induced a reduction of MM cell viability and down-regulation of IRF4 (Fig. 5 C-D), indeed no stable MM line with low IKZF1 could be generated by this methodology although MM cell lines with low IKZF1 expression exist. Forced overexpression of IKZF1 and IKZF3 did decrease lenalidomide sensitivity, albeit not dramatically (Fig. 5 E-G).

To explain the low IKZF1 in cell lines we next screened public datasets and 69 MM cell lines for mutations in IKZF1 and IKZF3. As with CRBN, mutations were very uncommon in primary MM with less than 1% of MM patients harboring non-synonymous mutations in either IKZF1 or IKZF3. It is important to note however that this sequence data is generated from patients mostly early in their treatment course. Indeed in contrast, mutations of IKZF1/3 were observed in six of 69 MM cell lines (8.7%) (supplementary data 3) including an LR208R single amino acid deletion resulting from an in-frame L208R substitution resulting in a codon deletion in the FR4 cell line which has very low levels of IKZF1 and which is highly IMiD resistant likely also due to high IRF4 levels from a known translocation t(6;14) IgH-IRF4. Recurrent mutations at Q156K in both the XG1 and H1112 cell lines and at G158R in OCI-MY7 all occur within the critical amino acid binding domain of IKZF1 and IKZF3 to CRBN. The significance of the Q156K mutation is unknown as XG1 cells are very sensitive to IMiD therapy. Further evidence of pathway mutation is evident in the observation that IRF4 mutations are found in 4% of MM genomes sequenced and the t(6;14) IgH-IRF4 is present in 3 of 69 cell lines. Since mutations or deletions of cereblon have also been reported in drug
resistant patients it seems that mutation of the CRBN-IKZF-IRF4 pathway may end up explaining IMiD resistance in more patients than previously suspected.

**Correlation of cereblon binding proteins with IMiD sensitivity and survival outcomes**

Next, we used gene expression profiling to examine the association between drug response and survival for the 11 top lenalidomide-regulated CRBN-interacting proteins. Baseline gene expression levels were measured in a cohort of 44 refractory MM patients prior to initiation of pomalidomide and dexamethasone therapy as part of phase II, clinical trials. Responders were defined as patients achieving PR or higher after two cycles of treatment. No significant difference was observed in responder and non-responder between the high and low IKZF1 expression groups. However, when we set the quartile cut-off points for IKZF1 expression (for all MM patients), we found lack of clinical response for patients in the lowest quartile range of IKZF1 expression. Response rate was 0% in the lowest quartile of IKZF1 expression versus 30% in the highest three quartiles (P = 0.04) (Fig. 6A). On the other hand, IKZF3, Eos (IKZF4) and Pegasus (IKZF5) did not show differences between responders and non-responders to pomalidomide.

Expression of CRBN, IKZF1 and KPNA2 correlated with OS after this therapy (Fig. 6B). Patients with low IKZF1 expression had a median OS of 7.3 months, compared with 27.2 months in the patients with higher IKZF1 expression (Fig. 6C, p=0.004). The group with lower IKZF1 expression also exhibited shorter median PFS (4.9 months) compared with the patients with a higher IKZF1 expression (7.3 months), but the
differences were not statistically significant. Besides IKZF1 and CRBN (previously published), high KPNA2 expression level was also found to be associated with shorter OS (Fig. 6D).

We then analyzed the Apex clinical trial data set a trial that compared MM patients treated with single agent dexamethasone or bortezomib. No significant difference in the response rate between patients with the lowest and the highest quartile of IKZF1 expression was found (data not shown). We actually noted that IKZF1 low expressors have a longer OS with Bortezomib (supplementary figure 2A), the opposite of the pomalidomide trial. We also demonstrated that lenalidomide resistant cell lines with low IKZF1 expression are just as responsive to bortezomib as lenalidomide sensitive MM cell lines with high IKZF1 expression (supplementary figure 2A).

Discussion
In this study, we sought to determine CRBN binding proteins and to establish their relevance to MM biology by exploring changes in protein abundance after exposure to the IMiD, Lenalidomide. Two independent pull-down methods were used to isolate proteins bound in the CRBN complex, followed by identification via MS analysis. Both methods identified CRBN and its known associated proteins DDB1 and CUL4A as among the highest associating proteins, demonstrating the validity of methodologies. Ten proteins were identified in the CRBN complex by both methods, including IKZF1, a protein that has recently been identified by others as a substrate of CRBN E3 ubiquitin ligase activity. We also identified the second implicated Ikaros member, IKZF3, as one of the CRBN binding proteins most downregulated after lenalidomide treatment for 48 hours. IKZF1 and IKZF3 are members of the Ikaros zinc-finger proteins and both are
chromatin remodeling transcription regulators involved in the regulation of lymphocyte development\textsuperscript{27-31}. IKZF1 and IKZF3 were previously demonstrated to regulate B cell activation and differentiation\textsuperscript{28}. Dysregulated IKZF1 and IKZF3 are associated with B-ALL and B-CLL\textsuperscript{32-35}. Recently, two landmark studies have independently shown that IKZF1 and IKZF3 are substrates of CRBN ubiquitin ligase\textsuperscript{17,18}. Consistent with these studies, we validated that binding of IKZF1 and IKZF3 with CRBN was greatly enhanced within a few hours of lenalidomide treatment but that IKZF1 and IKZF3 level are later downregulated in the same samples, suggesting they are substrates of CRBN ubiquitin ligase. However, our data also identified that CRBN and DDB1, in contrast to Ikaros proteins, are upregulated in the same pull-down implying their degradation is blocked after lenalidomide treatment. This result was consistent with previous studies that suggest that CRBN itself is a substrate of CRBN ubiquitin ligase. A possible explanation for this paradox is that after binding to IMiDs, CRBN may become resistant to proteasomal degradation. Theoretically, accumulated CRBN will enhance its function.

Consistent with recent reports, knockdown of \textit{IFZF1} and \textit{IKZF3} in our study induced MM cytotoxicity, confirming that this family is important for MM survival. Interestingly, although IKZF1 and IKZF3 were downregulated within hours after lenalidomide treatment in most MM cell lines, it usually takes another 24-72 hours to see lenalidomide induced downregulation of IRF4 and associated MM cytotoxicity, suggesting additional interactions or signaling cascades may be required to achieve a full drug response. We have observed but cannot yet explain why some lenalidomide resistant cell lines have downregulation of IKZF1 and IKZF3 after lenalidomide treatment but do not have lenalidomide–induced downregulation of IRF4 and
cytotoxicity (data not shown). Another unexplained observation we have made is that the doses required to downregulate Ikaros are also much lower than those required to mediate MM cytotoxicity.

In addition to Ikaros family members our MS data identified other interesting proteins which may bind the CRBN complex and are downregulated by IMiDs including for example, several binding partners involved in metabolism, nuclear transport and protein folding such as KPNA2, NUP153 and DNAJA2. Interestingly, several proteasome subunits were also detected, consistent with a recent report that CRBN was identified by two-hybrid screen to associate with a proteasome subunit\(^3\). The significance of this observation is not yet understood but is provocative since the scientific evidence would currently suggest that proteasome inhibitors and IMiDs work in opposite directions in either requiring proteasomal degradation or inhibiting this function, a scientific observation which is at odds with the clinical experience.

KPNA2 is of note as this nuclear transport protein has been also associated with B cell development via Pax5\(^3\). KPNA2 appears to be depleted later than the Ikaros family. Our data suggests that low levels of KPNA2 at baseline (perhaps as a marker for likelihood of drug activity) associates with a better prognosis.

We found in the current study that low \(IKZF1\) expression levels are associated with poor response to IMiDs and shorter overall survival. Response rate was 0% in the lowest quartile of \(IKZF1\) expression versus 30% in the highest 3 quartiles \((P = 0.04)\). This observation appears to contradict our data and that of recent publications that deliberate overexpression of \(IKZF1\) in MM cell lines induced resistance to lenalidomide\(^1\).\(^1\). A
possible explanation is that myeloma cells surviving with low IKZF1 are less dependent on IKZF1-associated signaling for proliferation and survival and are therefore resistant to IMiDs. In at least one lenalidomide resistant cell line, FR4, a genomic explanation of resistance emerges in that FR4 has a predicted damaging genomic mutation of IKZF1, very low levels of IKZF1 expression and a translocation upregulating IRF4 which together would seem to explain the ability to survive without IKZF1.

In summary, this study demonstrates numerous novel CRBN binding proteins altered in MM cells after IMiD treatment and confirms recent work implicating the Ikaros transcription factors as important members of this group. Unique to our studies is not only identifying novel CRBN binding proteins but also correlating IKZF1 and KPNA2 with clinical outcome of IMiD therapy.
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Authorship contribution

A.K.S., Y.X.Z., E.B., X.B., P.L.B and R.F. contributed to conception and design of the study; Y.X.Z., E.B., C.X.S., L.B., J. S., K. M. K., P. L., M.L., P. J., B. L., K.L., J.M., M.L., M.D. C. contributed to acquisition of data and analysis of the data; all authors contributed to interpretation of data; Y.X.Z., E.B. and A.K.S drafted the article; and all authors read, revised the article critically for important intellectual content, and gave final approval of the version to be published.

Conflict-of-interest disclosure

The authors declare no competing financial interests.
References


Table 1. Top differentially associated proteins identified in Co-IP

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*The number in each column represents the number of assigned spectra for that protein.
Table 2. The proteins in CRBN complex identified by both Co-IP and pull-down assays

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<tr>
<th>Proteins</th>
<th>NlgG</th>
<th>CRBN Ab</th>
<th>NlgG</th>
<th>CRBN Ab</th>
<th>Vector</th>
<th>CRBN-His</th>
<th>Vector</th>
<th>CRBN-His</th>
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*The number in each column represents the number of assigned spectra for that protein.
Table 3. Top lenalidomide-regulated putative CRBN-interacting proteins

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<tr>
<th>Proteins</th>
<th>Experiment 1*</th>
<th>Experiment 2*</th>
<th>Experiment 3*</th>
<th>Regulation</th>
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</table>

*The number in each column represents the number of assigned spectra for that protein.
Figure legends

Figure 1. Identification of proteins in CRBN protein-protein interaction. Schematic diagrams show the workflow designed for identifying CRBN binding partners via Co-IP or Ni\(^+\) beads pull-down and proteomic-based analysis. The first approach includes co-immunoprecipitation, separation by SDS-PAGE, in-gel digestion and peptide extraction and HPLC-ESI-MS/MS quantitative analysis. The second approach include using Ni\(^+\) beads to pull down the proteins from control cells (OCI-MY5 vector) and CRBN overexpressing cells (OCI-MY5 CRBN-His) untreated or pre-treated with lenalidomide for 48 hours, separation by SDS-PAGE, in-gel digestion and peptide extraction and HPLC-ESI-MS/MS quantitative analysis.

Figure 2. In silico network analysis and pathway enrichment analysis.

Lists of the proteins in CRBN complex that upregulated (A) or downregulated (B) in response to lenolidomide were evaluated against the latest versions of the following databases (Covering >50% of human proteins): CellMap, Reactome, KEGG, NCI-PID, and BioCartel. Linker genes were included in the network modeling and pathway enrichment predictions. Results were filtered for p-value <0.05 and False Discovery Rate < 0.05. The separate networks modeled from downregulation vs. upregulation lists are present as panel A. and B. and the association or connection between IKZF1 and CRBN was a custom annotation added to the figures.
Figure 3. Validation of IKZF1 and IKZF3 as CRBN-binding proteins.

(A) Immunoblotting of the Ni\textsuperscript+ beads pull-down proteins from OCI-MY5 vector (control) and OCI-MY5 CRBN-His with (+) without (-) lenalidomide treatment for 48 hours (two independent experiments). CRBN, DDB1, CUL4A, IKZF1, IKZF3 and KPNA2 showed similar changes detected by MS analysis. (B-C) OCI-MY5 CRBN-His and MM1.S cells were treated with lenalidomide (10\textmu M) in a time course and the changes of some top proteins identified from MS analysis or related with lenalidomide induced cytotoxicity are measured by western blot. IKZF1 and IKZF3 downregulation was identified as the earliest change after lenalidomide treatment. (D) OCI-MY5 CRBN-His cells were treated with DMSO or lenalidomide for three hours and co-IP were performed with anti-CRBN antibody to immunoprecipitate CRBN and CRBN–associated proteins, using a normal mouse IgG as a negative control. IKZF1 and IKZF3 were greatly increased in CRBN antibody immunoprecipitated samples after lenalidomide treatment (left panels), but they are decreased in cell lysate after lenalidomide treatment (right panels). CRBN increased in both immunoprecipitated and non-immunoprecipitated lysate.

Figure 4. Gene expression analysis of \textit{IKZF1} and \textit{IKZF3} across cell lines originated from multiple hematological and solid tumors. The number of cases of each tumor entity is indicated in parenthesis. Red boxes indicate the set of multiple myeloma cell lines. Results show expression of \textit{IKZF1} (A) and \textit{IKZF3} (B) expression in most cell lines established from hematological tumors. The findings in hematological neoplasms contrast with the lack of expression of both genes found in solid-tumor cell lines. Abbreviations: Acute myeloid leukemia (AML), B-cell acute lymphoblastic

**Figure 5. Expression of Ikaros proteins in HMCLs and induction of cytotoxicity after knockdown of IKZF1 and IKZF3.** (A) Expression of IKZF1 and IKZF3 in HMCLs. Nine HMLCs either sensitive or resistant to lenalidomide were analyzed for the expression of ikraros by western blot. (B) Expression of IKZF1 in HMCLs was detected by flow cytometry analysis. (C-D) Knockdown of IKZF1 and IKZF3 induced cytotoxicity. MM1.S cells were infected with lentivirus expressing non-targeting control or IKZF1 or IKZF3 shRNAs. MTT assays were performed at day 6 after infection and western blot were performed at day 3 after infection. (E-G) overexpression of IKZF1 and IKZF3 in lenalidomide sensitive cell line, MM1.S, did not substantially change lenalidomide sensitivity. MM1.S cells were infected with control virus (expressing vector alone) or lentivirus expressing IKZF1 and IKZF3, after 72 hours (E) or after sorting GFP positive cells in MM1.S cells infected by IKZF1 lentivirus at 14 days after infection (F), cells were seeded in a 96 well plate and were treated with lenalidomide. MTT assay was performed at day 6 after treatment and western blot was performed at day 3 to confirm overexpression of IKZF1 and IKZF3 (G).
Figure 6. Correlation of IMiD responsiveness and overall survival with *IKZF1* and *KPNA2* gene expression level in pomalidomide and dexamethasone therapy

A cohort of 44 MM patients treated with pomalidomide with gene expression profiling measured prior to initiation of chemotherapy was used in this study. (A) Responsiveness to pomalidomide in quartiles of *IKZF1* expression. No patient in the lowest *IKZF1* quartile expression responded to pomalidomide treatment. (B) The gene expression of the 11 top lenalidomide-regulated putative CRBN-interacting proteins was analyzed to examine the association between drug response and survival by using COX modeling. *IKZF1* was identified as the top gene which expression is associated with overall survival (p-value 0.002) (C-D) Low *IKZF1* and high *KPNA2* expression levels were correlated with shorter overall survival.
Fig 6

(A) Bar chart showing the percentage of responders and non-responders across quartiles.

(B) Table showing overall survival - univariate Cox models (25 events).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Hazard Ratio</th>
<th>95% CI</th>
<th>Chi Square p-value</th>
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<td>0.91</td>
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<tr>
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<td>0.200-0.816</td>
<td>0.01</td>
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<tr>
<td>CUL4A</td>
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<tr>
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<td>0.526-4.100</td>
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(C) Survival curve for IKZF1 with p-value 0.004.

(D) Survival curve for KPNA2 with p-value 0.0004.
Identification of cereblon binding proteins and relationship with response and survival following pomalidomide and dexamethasone in multiple myeloma

Yuan Xiao Zhu, Esteban Braggio, Chang-Xin Shi, K. Martin Kortuem, Laura A. Bruins, Jessica E. Schmidt, Xiu-Bao Chang, Paul Langlais, Moulun Luo, Patrick Jedlowski, Betsy LaPlant, Kristina Laumann, Rafael Fonseca, P. Leif Bergsagel, Joseph Mikhael, Martha Lacy, Mia D. Champion and A. Keith Stewart