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(54) **Title**: METHODS AND MATERIALS FOR ASSESSING RESPONSIVENESS TO LENALIDOMIDE, THALIDOMIDE, AND/OR OTHER THALIDOMIDE ANALOGS

(57) **Abstract**: This document provides methods and materials related to assessing responsiveness to lenalidomide, thalidomide, and/or other IMiDs (structural and functional analogues of thalidomide that represent a promising new class of immunomodulators). For example, methods and materials for using CRBN levels to determine whether or not cancer cells (e.g., multiple myeloma cells) are susceptible to lenalidomide, thalidomide, and/or other IMiDs are provided.

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METHODS AND MATERIALS FOR ASSESSING RESPONSIVENESS TO LENALIDOMIDE, THALIDOMIDE, AND/OR OTHER THALIDOMIDE ANALOGS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application Serial No. 61/452,027, filed March 11, 2011. The disclosure of the prior application is considered part of (and are incorporated by reference in) the disclosure of this application.

BACKGROUND

1. Technical Field

This document relates to methods and materials involved in assessing responsiveness to lenalidomide, thalidomide, and/or other IMiDs (structural and functional analogues of thalidomide that represent a promising new class of immunomodulators). For example, this document relates to methods and materials for using cereblon (CRBN) levels to determine whether or not cancer cells are susceptible to lenalidomide, thalidomide, and/or other IMiDs.

2. Background Information

Thalidomide was used a sedative in the late 1950s and later withdrawn from use due to its teratogenicity. Because of its antiangiogenic activity, thalidomide was recently used to treat multiple myeloma, but its overall mechanism of action is unknown.

SUMMARY

This document provides methods and materials related to assessing responsiveness to lenalidomide, thalidomide, and/or other IMiDs (structural and functional analogues of thalidomide that represent a promising new class of immunomodulators). Examples of IMiDs include, without limitation, lenalidomide and pomalidomide. For example, this document provides methods and materials for using CRBN levels to determine whether or not cancer cells (e.g., multiple myeloma cells) are susceptible to lenalidomide, thalidomide, and/or other IMiDs. As described herein, cancer cells (e.g., multiple myeloma cells) expressing CRBN are susceptible

to treatment with lenalidomide, thalidomide, and/or other IMiDs, while cancer cells not expressing CRBN are not susceptible to treatment with lenalidomide, thalidomide, and/or other IMiDs.

Determining if a mammal (e.g., a human patient) has cancer cells expressing CRBN can allow physicians and the patient, in the case of humans, to determine a course of thalidomide and/or IMiDs treatment appropriate for that patient. For example, a patient found to have multiple myeloma cells expressing CRBN can be treated with lenalidomide, thalidomide, and/or other IMiDs. Likewise, determining if a mammal (e.g., a human patient) has cancer cells expressing little or no CRBN can allow physicians and the patient, in the case of humans, to determine a course of cancer treatment other than lenalidomide, thalidomide, and/or other IMiDs. For example, a patient found to have multiple myeloma cells not expressing CRBN can be treated with Bortezomib, alkylating agents, or corticosteroids.

In some cases, the methods and materials provided herein can be used to monitor a patient's cancer progression and/or treatment course over time. For example, a patient's cancer cells (e.g., multiple myeloma cells) can be assessed for CRBN levels before treatment starts and at various time points following the initiation of treatment. If CRBN expression levels drop in the patient's cancer cells, then the patient can be switched from a lenalidomide, thalidomide, and/or other IMiDs treatment to a treatment other than thalidomide and IMiDs. If the patient's cancer cells remain positive for CRBN expression, then the patient can continue to be treated with lenalidomide, thalidomide, and/or other IMiDs.

In general, one aspect of this document features a method for assessing responsiveness to a thalidomide or thalidomide analog treatment. The method comprises, or consists essentially of, (a) determining whether or not cancer cells from a mammal express CRBN, (b) classifying the cancer cells as being susceptible to treatment with thalidomide or a thalidomide analog if the cancer cells express CRBN, and (c) classifying the mammal as not being susceptible to treatment with thalidomide or a thalidomide analog if the cancer cells do not express CRBN. The cancer cells can be multiple myeloma cells. The mammal can be a human. The determining step can comprise determining whether or not the cancer cells express CRBN mRNA. The determining step can comprise determining whether or not the cancer cells express CRBN polypeptides. The method can comprise assessing responsiveness to the thalidomide analog treatment, wherein the thalidomide analog treatment can be a

lenalidomide treatment, and wherein the thalidomide analog can be lenalidomide.

In another aspect, this document features a method for assessing responsiveness to a thalidomide or thalidomide analog treatment. The method comprises, or consists essentially of, (a) determining whether or not cancer cells from a mammal comprise an elevated level of CRBN expression, (b) classifying the cancer cells as being susceptible to treatment with thalidomide or a thalidomide analog if the cancer cells comprise the elevated level, and (c) classifying the mammal as not being susceptible to treatment with thalidomide or a thalidomide analog if the cancer cells do not comprise the elevated level. The cancer cells can be multiple myeloma cells. The mammal can be a human. The CRBN expression can be CRBN mRNA expression. The CRBN expression can be CRBN polypeptide expression. The method can comprise assessing responsiveness to the thalidomide analog treatment, wherein the thalidomide analog treatment can be a lenalidomide treatment, and wherein the thalidomide analog can be lenalidomide.

In another aspect, this document features a method for identifying cancer cells susceptible to treatment with thalidomide or a thalidomide analog, wherein the method comprises, or consists essentially of, (a) detecting the presence of CRBN expression by cancer cells, and (b) classifying the cancer cells as being susceptible to treatment with thalidomide or a thalidomide analog based at least in part on the presence. The cancer cells can be multiple myeloma cells. The CRBN expression can be CRBN mRNA expression. The CRBN expression can be CRBN polypeptide expression. The method can comprise identifying cancer cells susceptible to treatment with the thalidomide analog, and wherein the thalidomide analog can be lenalidomide.

In another aspect, this document features a method for identifying cancer cells susceptible to treatment with thalidomide or a thalidomide analog, wherein the method comprises, or consists essentially of, (a) detecting the presence of an elevated level of CRBN expression by cancer cells, and (b) classifying the cancer cells as being susceptible to treatment with thalidomide or a thalidomide analog based at least in part on the presence. The cancer cells can be multiple myeloma cells. The CRBN expression can be CRBN mRNA expression. The CRBN expression can be CRBN polypeptide expression. The method can comprise identifying cancer cells susceptible to treatment with the thalidomide analog, and wherein the thalidomide analog can be lenalidomide.

In another aspect, this document features a method for monitoring treatment with a thalidomide or thalidomide analog, wherein the method comprises, or consists essentially of, (a) determining whether or not cancer cells obtained from a mammal treated with the thalidomide or thalidomide analog express CRBN, (b) classifying the cancer cells as being susceptible to treatment with thalidomide or a thalidomide analog if the cancer cells express CRBN, and (c) classifying the mammal as not being susceptible to treatment with thalidomide or a thalidomide analog if the cancer cells do not express CRBN. The method can comprise monitoring treatment with the thalidomide analog, and wherein the thalidomide analog can be lenalidomide.

In another aspect, this document features a method for monitoring treatment with a thalidomide or thalidomide analog, wherein the method comprises, or consists essentially of, (a) administering thalidomide or a thalidomide analog to a mammal having cancer cells that express CRBN, (b) determining whether or not cancer cells obtained from the mammal continue to express CRBN, (c) administering thalidomide or a thalidomide analog to the mammal if the cancer cells continue to express CRBN, and (d) administering a treatment other than thalidomide or a thalidomide analog to the mammal if the cancer cells do not continue to express CRBN. The method can comprise monitoring treatment with the thalidomide analog, and wherein the thalidomide analog can be lenalidomide.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

DESCRIPTION OF DRAWINGS

Figure 1 is a graph plotting the viability of the indicated cell lines treated with 0, 1.6, 3.1, 6.25, 12.5, 25, and 50 μM of lenalidomide for 72 hours.

Figure 2A is a photograph of a Western Blot of proteins obtained from 293 cells expressing a FLAG-CRBN construct with or without an shRNA designed to reduce CRBN expression probed with either an anti-FLAG antibody or anti-tublin antibody. A negative control included cells not expressing the FLAG-CRBN cDNA and not containing an shRNA. Figure 2B is a graph plotting RT-PCR determined CRBN expression levels for cells containing non-targeting control shRNA (NT) or an shRNA designed to reduce CRBN expression (#1, #10, or #13). Each shRNA construct included a sequence encoding GFP. Figure 2C contains graphs plotting the percentage of GFP from the indicated cell lines containing non-targeting control shRNA (NT) or an shRNA designed to reduce CRBN expression (#1, #10, or #13). Figure 2D is a graph plotting viability of the indicated cell lines six days post infection with viruses containing non-targeting control shRNA (NT) or an shRNA designed to reduce CRBN expression (#1, #10, or #13).

Figure 3A contains graphs plotting the percentage of GFP from the indicated cell lines containing non-targeting control shRNA (NT) or an shRNA designed to reduce CRBN expression (CRBN shRNA). Each shRNA construct included a sequence encoding GFP. Figure 3B is a graph plotting RT-PCR for CRBN expression in the indicated cell lines containing non-targeting control shRNA (NT) or shRNA #13. Figure 3C contains graphs plotting cell viability for the indicated cells lines containing either a non-targeting control shRNA (NT) or shRNA #13 and treated with the indicated amount of lenalidomide.

Figure 4A is a graph plotting cell viability (O.D.) for OPM1 (myeloma) cells containing either a non-targeting control shRNA (NT) or shRNA #13 and treated with the indicated amount of revlimid. Figure 4B is a graph plotting cell viability (O.D.) for OPM1 cells containing either a non-targeting control shRNA (NT) or shRNA #13 and treated with the indicated amount of bortezomib. Figure 4C is a graph plotting cell viability (O.D.) for OPM1 cells containing either a non-targeting control shRNA (NT) or shRNA #13 and treated with the indicated amount of dexamethasone. Figure 4D is a graph plotting cell viability (O.D.) for OPM1 cells containing either a non-targeting control shRNA (NT) or shRNA #13 and treated with the indicated amount of melphalan.

Figure 5 is a diagram showing the overlap between up- and down-regulated genes for cells treated with shRNAs to reduce CRBN expression and cells treated with lenalidomide.

Figure 6A is a graph plotting RT-PCR expression of CRBN for MM1S cells and MM1s res cells. MM1s res cells were generated to be lenalidomide resistant in the laboratory. Figure 6B is a graph plotting cell viability of MM1S cells and MM1s res cells treated with the indicated amounts of lenalidomide. Figure 6C shows 3 regions on chromosome arm 3p that have an additional deletion in MM1S lenalidomide resistant cell line (right plot) compared with MM1S lenalidomide sensitive cell line (left plot). Figure 6D is a gene plot that confirms that one of the chromosomal regions in MMIs lenalidomide resistant cells shown in Figure 6C includes the 3' portion of CRBN.

Figure 7 is a graph plotting RT-PCR for CRBN in matched patient samples before and after exposure to lenalidomide.

Figure 8 is a list of up-regulated and down-regulated genes that are shared in OPM1 cells after CRBN knockdown and OPM1 cells after lenalidomide treatment.

DETAILED DESCRIPTION

This document provides methods and materials related to assessing responsiveness to lenalidomide, thalidomide, and/or other IMiDs. For example, this document provides methods and materials for using CRBN levels to determine whether or not cancer cells (e.g., multiple myeloma cells) are susceptible to lenalidomide, thalidomide, and/or other IMiDs. As described herein, cancer cells (e.g., multiple myeloma cells) expressing CRBN are susceptible to treatment with lenalidomide, thalidomide, and/or other IMiDs, while cancer cells not expressing CRBN are not susceptible to treatment with lenalidomide, thalidomide, and/or other IMiDs.

Any appropriate cancer cell can be assessed for CRBN expression to determine if it is susceptible to treatment with lenalidomide, thalidomide, and/or other IMiDs. For example, multiple myeloma cells, lymphoma cells, chronic lymphocytic leukemia cells, myelodysplasia cells, and other blood cancer cells can be assessed for CRBN expression to determine if such cells are susceptible to treatment with lenalidomide, thalidomide, and/or other IMiDs. In addition, the methods and materials provided herein can be used to assess cancer cells from any appropriate mammal. For example, cancer cells from a human, monkey, horse, dog, cat, cow, pig, mouse, or rat can be assessed for CRBN expression to determine if the cancer cells are susceptible to treatment with lenalidomide, thalidomide, and/or other IMiDs.

The amino acid sequence of a human CRBN polypeptide is set forth in GenBank® GI No. 16924279 (GenBank® Accession No. AAH17419.1), and the nucleic acid sequence encoding a human CRBN polypeptide is set forth in GenBank® GI No. 292658851 (GenBank® Accession No. NG_01684.1). The amino acid sequence of a monkey CRBN polypeptide is set forth in GenBank® GI No. 307548871 (GenBank® Accession No. NP_001182576.1), and the nucleic acid sequence encoding a monkey CRBN polypeptide is set forth in GenBank® GI No. 307548870 (GenBank® Accession No. NM_001195647.1). The amino acid sequence of a horse CRBN polypeptide is set forth in GenBank® GI No. 149728337 (GenBank® Accession No. XP_001496748.1), and the nucleic acid sequence encoding a horse CRBN polypeptide is set forth in GenBank® GI No. 194246364 (GenBank® Accession No. NC_009159.2). The amino acid sequence of a mouse CRBN polypeptide is set forth in GenBank® GI No. 47682727 (GenBank® Accession No. AAH69905.1), and the nucleic acid sequence encoding a mouse CRBN polypeptide is set forth in GenBank® GI No. 90403611 (GenBank® Accession No. NM_021449.2). Additional amino acid and nucleic acid sequences for CRBN polypeptides from other species can be obtained from GenBank® by performing standard sequence searches (e.g., BLAST searches) using one or more of the above listed sequences (e.g., a human CRBN amino acid or nucleic acid sequence).

Any appropriate method can be used to determine the level of CRBN mRNA or CRBN polypeptide present within cancer cells. For example, RT-PCR, quantitative PCR, Northern blotting and gene expression profiling techniques can be used to assess CRBN mRNA levels. In some cases, ELISAs, immunocytochemistry, flow cytometry, Western blotting, proteomic, and mass spectrometry techniques can be used to assess CRBN polypeptide levels. Any appropriate sample containing cancer cells can be obtained and assessed for CRBN expression. For example, fine-needle aspiration biopsies, surgical tissue biopsies, or blood samples can be obtained, and the level of CRBN expression within the cancer cells of such samples can be determined as described herein.

The term "elevated level" as used herein with respect to the level of CRBN expression can be in comparison with the median CRBN expression level present in normal non-cancer cells of the same cell type of the cancer to be assessed (e.g., the median CRBN expression level determined from a random sampling of 5, 10, 15, 20, 30, 40, 50, 100, 500, or more non-cancer cell samples from humans known not to

have cancer). In such cases, the presence of an elevated level can indicate that the patient's cancer cells are susceptible to treatment with lenalidomide, thalidomide, and/or other IMiDs, while the absence of such an elevated level can indicate that the patient's cancer cells are not susceptible to treatment with lenalidomide, thalidomide, or other IMiDs.

This document also provides methods and materials to assist medical or research professionals in determining if cancer cells (e.g., multiple myeloma cells) are susceptible or resistant to treatment with lenalidomide, thalidomide, and/or other IMiDs. Medical professionals can be, for example, doctors, nurses, medical laboratory technologists, and pharmacists. Research professionals can be, for example, principal investigators, research technicians, postdoctoral trainees, and graduate students. A professional can be assisted by (1) determining the level of CRBN expression in cancer cells as described herein, and (2) communicating information about the CRBN expression level to that professional.

Any appropriate method can be used to communicate information to another person (e.g., a professional). For example, information can be given directly or indirectly to a professional. In addition, any type of communication can be used to communicate the information. For example, mail, e-mail, telephone, and face-to-face interactions can be used. The information also can be communicated to a professional by making that information electronically available to the professional. For example, the information can be communicated to a professional by placing the information on a computer database such that the professional can access the information. In addition, the information can be communicated to a hospital, clinic, or research facility serving as an agent for the professional.

The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

EXAMPLES

Example 1 – CRBN is required for the anti-myeloma activity of thalidomide, lenalidomide, and pomalidomide

Cell lines, compounds, siRNA, plasmids, and reagents

Myeloma cell lines were maintained in RPMI 1640, supplemented with 10% FCS and antibiotics. Lentiviral shRNA clones targeting human CRBN and non-targeting (NT) control lentiviral constructs were obtained from Sigma-Aldrich (St.

Louis, MO). Anti-Flag and anti-DDB1 antibodies were obtained from Cell Signaling Technology (Danvers, MA). Lipofectamin™ 2000 was obtained from Invitrogen (Carlsbad, CA). Annexin V apoptosis detection kit was obtained from BD Biosciences (San Jose, CA). Lenalidomide was obtained commercially.

CRBN shRNA lentiviral experiments

The lentiviruses expressing NT or CRBN shRNAs were subcloned into a GFP containing construct and used to infect various myeloma cell lines as described elsewhere (Zhu *et al.*, *Blood*, 117(14):3847-3857 (2011); Epublished Feb. 2, 2011). After a 48-hour infection period, efficiency was measured at 72 hours and 6 days post infection by FACScan analysis of GFP expression. Cell viability was measured by 3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium (MTT) dye absorbance according to the manufacturer's instructions (Boehringer Mannheim, Mannheim, Germany). Cells were also harvested at 72 hours post infection for real-time PCR analysis of CRBN levels in control and CRBN shRNA expressing cells. At three weeks post infection, GFP-positive cells were sorted and expanded. Real-time PCR was performed to measure CRBN levels in sorted cells.

In some experiments, cells were incubated with various doses of lenalidomide, dexamethasone, melphalan, or bortezomib. Plates were incubated for 72 hours to six days. Cell viability was determined using an MTT assay. Each experimental condition was performed in triplicate and repeated at least once.

Real-time PCR

Total RNA was isolated using RNeasy Plus Mini kit (Qiagen) and reverse transcribed using QuantiTect Reverse Transcription kit (Qiagen). The Quantitative PCR were performed using TaqMan Universal PCR Master Mix with pre-designed probes (Applied Biosystems, Foster City, CA), and the comparative C_T method was used for relative quantification on an ABI 7900HT Fast Real-Time PCR system (Applied Biosystems, Foster City, CA).

Gene expression profile analysis

Myeloma cells infected with NT or CRBN shRNA expressing lentiviruses were harvested, and total RNA was prepared using RNeasy Plus Mini Kit (Qiagen). The gene expression profiles (GEP) were generated from total RNA labeled using the

Affymetrix OneStep IVT labeling procedure and hybridized to the Affymetrix U133Plus2.0 genechip. All labeling, hybridization, washing, and scanning steps were performed following the manufacturer's recommended protocol by a MicroArray facility. The CEL files were processed and normalized by MAPP application with the following default settings: BG Correction: gcrma; Normalization: fastlo; PM Correction: affinities_only; Summarization: medianpolish; and computeCalls: TRUE. Differential expression between the treatment samples and the controls were selected using |FC|>2 to filter the genes for each comparison.

Immunoblotting

Western Blotting was performed. Briefly, equal amounts of protein were subjected to SDS-PAGE gel electrophoreses followed by transfer to PVDF membranes. Membranes were probed with primary antibodies overnight and then washed and incubated with HRP-conjugated-secondary antibodies. Detection was performed by the Enhanced Chemical Luminescence (ECL) method. The membranes were stripped and re-probed with anti- β-actin antibodies to confirm protein loading.

Array Comparative Genomic Hybridization

Genomic DNA was obtained using Puregene blood kit (Qiagen; Valencia, CA) according to the manufacturer's recommendations. High-resolution array-based comparative genomic hybridization (aCGH) was performed with the Human Genome 244A microarray (Agilent Technologies; Palo Alto, CA). DNA samples from a pool of nine female human lymphoblastoid cell lines (obtained from the Coriell repository) were used as the normal reference in the hybridization experiments. The digesting, labeling, and hybridizing steps were performed as described elsewhere with minor modifications (Braggio *et al.*, *Cancer Res.*, 69(8):3579-88 (2009)). Briefly, 1.2 μg of tumor and reference DNAs were separately digested with Bovine DNaseI (Ambion; Austin, TX) for 12 minutes at room temperature. Next, random primers and exo-Klenow fragment (Invitrogen; Carlsbad, CA) were used to differentially label tumor (Cy5) and reference (Cy3) genomic DNA samples (GE Healthcare; Piscataway, NJ). Labeled genomic reactions were cleaned-up with purification columns (Invitrogen) and hybridized at 65° C for 40 hours. Microarrays were scanned in a DNA Microarray Scanner (Agilent Technologies). Feature extraction was performed with Feature extraction Software, version 9.5 (Agilent Technologies). Log2 ratio data

were imported and analyzed using DNA Analytics software version 4.0.85 (Agilent Technologies).

Copy-number abnormalities (CNA) were calculated using aberration detection module (ADM)-1 algorithm (Lipson *et al., J. Comput. Biol.*,13:215–28 (2006)) with a threshold of 7.5. 2 probe, 0.25_log2 filters were used in the aberration detection, obtaining an average genomic resolution of 17 Kb.

Results

Myeloma cell responsiveness to IMiDs in vitro

The baseline cytotoxicity of thalidomide and lenalidomide was established. A MTT assay was performed on a panel of genetically heterogeneous human MM cells lines *in vitro* (Figure 1). Lenalidomide inhibited growth of 8 of 11 tested MM cell lines at the concentration between 1.6 μM to 50 μM *in vitro* (Figure 1). The same dose of thalidomide, however, had no effect on MM cell growth.

Suppression of Cereblon is cytotoxic

Lentiviral CRBN shRNAs were introduced into myeloma cell lines, and the effects of silencing CRBN on myeloma cell viability were measured. Five pre-designed CRBN shRNA lentiviral expression constructs were obtained. Three of the five exhibited a knockdown of CRBN expression using a Flag-tagged CRBN in 293 cells as the target (Figure 2A). The lentiviruses expressing these three CRBN shRNAs and lentiviruses expressing non-targeting (NT) control shRNAs were used to infect myeloma cell lines. At 48 hours post infection, the infection efficiency of each virus on different cell lines was measured by FACScan analysis of GFP positive cells (Figure 2B). Knock-down CRBN expression and cell viability were further measured at day 3 and day 6 post infection by RT-PCR and MTT, respectively. As shown in Figures 2C and 2D, MM cell lines infected with CRBN shRNAs exhibited reduced CRBN expression and cell viability as compared with control virus infected cells.

Suppression of Cereblon confers lenalidomide resistance

Although a mean of 70% myeloma cells stopped growing or died after knock-down of CRBN by day 6 post infection, a percentage of infected cells was found to survive. After sorting these cells by GFP expression, CRBN knock-down in those cells was still observed by real-time PCR (Figures 3A and 3B), suggesting that a non

CRBN-dependent survival pathway(s) may exist. Proliferation of those CRBN depleted but surviving MM cells were then tested in the absence or presence of different anti-myeloma drugs. As shown in Figure 3C, three different myeloma cell lines, which had more than 98% GFP positive cells and substantial CRBN knock-down, demonstrated acquired resistant to lenalidomide when compared with their NT control cells. Most of those cell lines exhibited resistance to both lenalidomide and pomalidomide, but exhibited similar sensitivity to melphalan and dexmethason. In some cases, some cell lines became more sensitive to Bortezomib after CRBN silencing (Figures 4A-D). These results demonstrate a requirement of CRBN for the activity of these IMiDs.

Molecular basis of drug resistance after CRBN knock-down and effects of CRBN silencing

In order to determine the molecular basis of drug resistance after CRBN knock-down, a gene expression profile (GEP) analysis was performed on OPM1 cells which stably express NT or CRBN shRNA after lenalidomide treatment for 48 hours. Compared with NT controls (i.e., lenalidomide responsive cells, which exhibited about six hundred genes up or down regulated after 48 hours lenalidomide treatment), CRBN depleted and thus lenalidomide resistant cells only exhibited 30 genes down regulated (3% of control) and 150 genes (24% of control) up-regulated after treatment, further demonstrating that CRBN is involved in a full complement drug response.

In order to know whether knock-down of CRBN induces the same effects on gene expression as lenalidomide treatment, a GEP analysis was performed on OPM1 cells either treated with lenalidomide for 24, 48, or 72 hours or transfected with CRBN shRNA for 48 or 72 hours. A comparison of the profile induced by lenalidomide alone versus the profile induced by CRBN knockdown alone was performed. Only 123 genes were found to have shared GEP changes between the two groups (Figures 5 and 8). A pathway analysis indicated that those genes are enriched on cell survival and immune response cell signaling and regulated by several transcription factors such as c-myc, Sp1, and P53.

Genomic deletion of Cereblon confers IMiD resistance in cell lines

To determine whether down-regulation of CRBN also exists in a lenalidomide resistant MM1.1S cell line (MM1.S res; a gift from Dr. Orlwoski), which was generated by culturing MM1.S cells in gradually increasing concentrations of lenalidomide, RT-PCR was performed to quantitate CRBN levels in parental MM1.S cells and MM1.S res cells. Compared with parental MM1.S cells, lenalidomide resistant cells exhibited much lower levels of CRBN expression (Figure 6A). An array CGH analysis was performed using the isogenic cell lines MM1.S and MMI.S res to identify the genetic basis for why CRBN is down-regulated in the resistant cell line. A mono-allelic deletion of *CRBN* was noted in MM1.S, which becomes bi-allelic in MM1.S res. This confirms a requirement for CRBN in lenalidomide activity.

Lenalidomide refractory patients demonstrate low Cereblon levels post treatment

Since CRBN was determined to be required for lenalidomide and pomalidomide response, the following was performed to investigate whether lenalidomide resistance in MM is the result of lower levels of CRBN in patients. In three patients for which pre- and post-treatment samples were available, CRBN expression levels were assessed using RT-PCR. The expression of CRBN in MM cells from samples obtained from three patients who relapsed was lower than the levels measured in their samples collected at diagnosis stage. These RT-PCR results are consistent with mRNA sequencing analysis data.

The results provided herein demonstrate a requirement for IMiDs to bind CRBN in order to be effective and demonstrate that loss of CRBN function confers resistance to the IMiD class of drugs. Gene expression changes induced by lenalidomide exposure were to a large extent vanquished when CRBN was depleted. Interestingly, depleting CRBN is directly cytotoxic to MM cells, further implicating CRBN as a mediator of IMiD function. In patients exposed to and resistant to lenalidomide, CRBN levels appear to decline. Thus, the presence of CRBN can be a requirement for activity of this drug class and while its consequent suppression mediates cell death, its complete loss renders the drug class ineffective.

OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended

to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

WHAT IS CLAIMED IS:

1. A method for assessing responsiveness to a thalidomide or thalidomide analog treatment, wherein said method comprises:

(a) determining whether or not cancer cells from a mammal express CRBN,

(b) classifying said cancer cells as being susceptible to treatment with thalidomide or a thalidomide analog if said cancer cells express CRBN, and

(c) classifying said mammal as not being susceptible to treatment with thalidomide or a thalidomide analog if said cancer cells do not express CRBN.

2. The method of claim 1, wherein said cancer cells are multiple myeloma cells.

3. The method of claim 1, wherein said mammal is a human.

4. The method of claim 1, wherein determining step comprises determining whether or not said cancer cells express CRBN mRNA.

5. The method of claim 1, wherein determining step comprises determining whether or not said cancer cells express CRBN polypeptides.

6. The method of claim 1, wherein said method comprises assessing responsiveness to said thalidomide analog treatment, wherein said thalidomide analog treatment is a lenalidomide treatment, and wherein said thalidomide analog is lenalidomide.

7. A method for assessing responsiveness to a thalidomide or thalidomide analog treatment, wherein said method comprises:

(a) determining whether or not cancer cells from a mammal comprise an elevated level of CRBN expression,

(b) classifying said cancer cells as being susceptible to treatment with thalidomide or a thalidomide analog if said cancer cells comprise said elevated level, and

(c) classifying said mammal as not being susceptible to treatment with thalidomide or a thalidomide analog if said cancer cells do not comprise said elevated

level.

8. The method of claim 7, wherein said cancer cells are multiple myeloma cells.

9. The method of claim 7, wherein said mammal is a human.

10. The method of claim 7, wherein said CRBN expression is CRBN mRNA expression.

11. The method of claim 7, wherein said CRBN expression is CRBN polypeptide expression.

12. The method of claim 7, wherein said method comprises assessing responsiveness to said thalidomide analog treatment, wherein said thalidomide analog treatment is a lenalidomide treatment, and wherein said thalidomide analog is lenalidomide.

13. A method for identifying cancer cells susceptible to treatment with thalidomide or a thalidomide analog, wherein said method comprises:

(a) detecting the presence of CRBN expression by cancer cells, and

(b) classifying said cancer cells as being susceptible to treatment with thalidomide or a thalidomide analog based at least in part on said presence.

14. The method of claim 13, wherein said cancer cells are multiple myeloma cells.

15. The method of claim 13, wherein said CRBN expression is CRBN mRNA expression.

16. The method of claim 13, wherein said CRBN expression is CRBN polypeptide expression.

17. The method of claim 13, wherein said method comprises identifying cancer cells susceptible to treatment with said thalidomide analog, and wherein said thalidomide analog is lenalidomide.

18. A method for identifying cancer cells susceptible to treatment with thalidomide or a thalidomide analog, wherein said method comprises:

(a) detecting the presence of an elevated level of CRBN expression by cancer cells, and

(b) classifying said cancer cells as being susceptible to treatment with thalidomide or a thalidomide analog based at least in part on said presence.

19. The method of claim 18, wherein said cancer cells are multiple myeloma cells.

20. The method of claim 18, wherein said CRBN expression is CRBN mRNA expression.

21. The method of claim 18, wherein said CRBN expression is CRBN polypeptide expression.

22. The method of claim 18, wherein said method comprises identifying cancer cells susceptible to treatment with said thalidomide analog, and wherein said thalidomide analog is lenalidomide.

23. A method for monitoring treatment with a thalidomide or thalidomide analog, wherein said method comprises:

(a) determining whether or not cancer cells obtained from a mammal treated with said thalidomide or thalidomide analog express CRBN,

(b) classifying said cancer cells as being susceptible to treatment with thalidomide or a thalidomide analog if said cancer cells express CRBN, and

(c) classifying said mammal as not being susceptible to treatment with thalidomide or a thalidomide analog if said cancer cells do not express CRBN.

24. The method of claim 23, wherein said method comprises monitoring treatment with said thalidomide analog, and wherein said thalidomide analog is lenalidomide.

25. A method for monitoring treatment with a thalidomide or thalidomide analog, wherein said method comprises:

(a) administering thalidomide or a thalidomide analog to a mammal having cancer cells that express CRBN,

(b) determining whether or not cancer cells obtained from said mammal continue to express CRBN,

(c) administering thalidomide or a thalidomide analog to said mammal if said cancer cells continue to express CRBN, and

(d) administering a treatment other than thalidomide or a thalidomide analog to said mammal if said cancer cells do not continue to express CRBN.

26. The method of claim 25, wherein said method comprises monitoring treatment with said thalidomide analog, and wherein said thalidomide analog is lenalidomide.

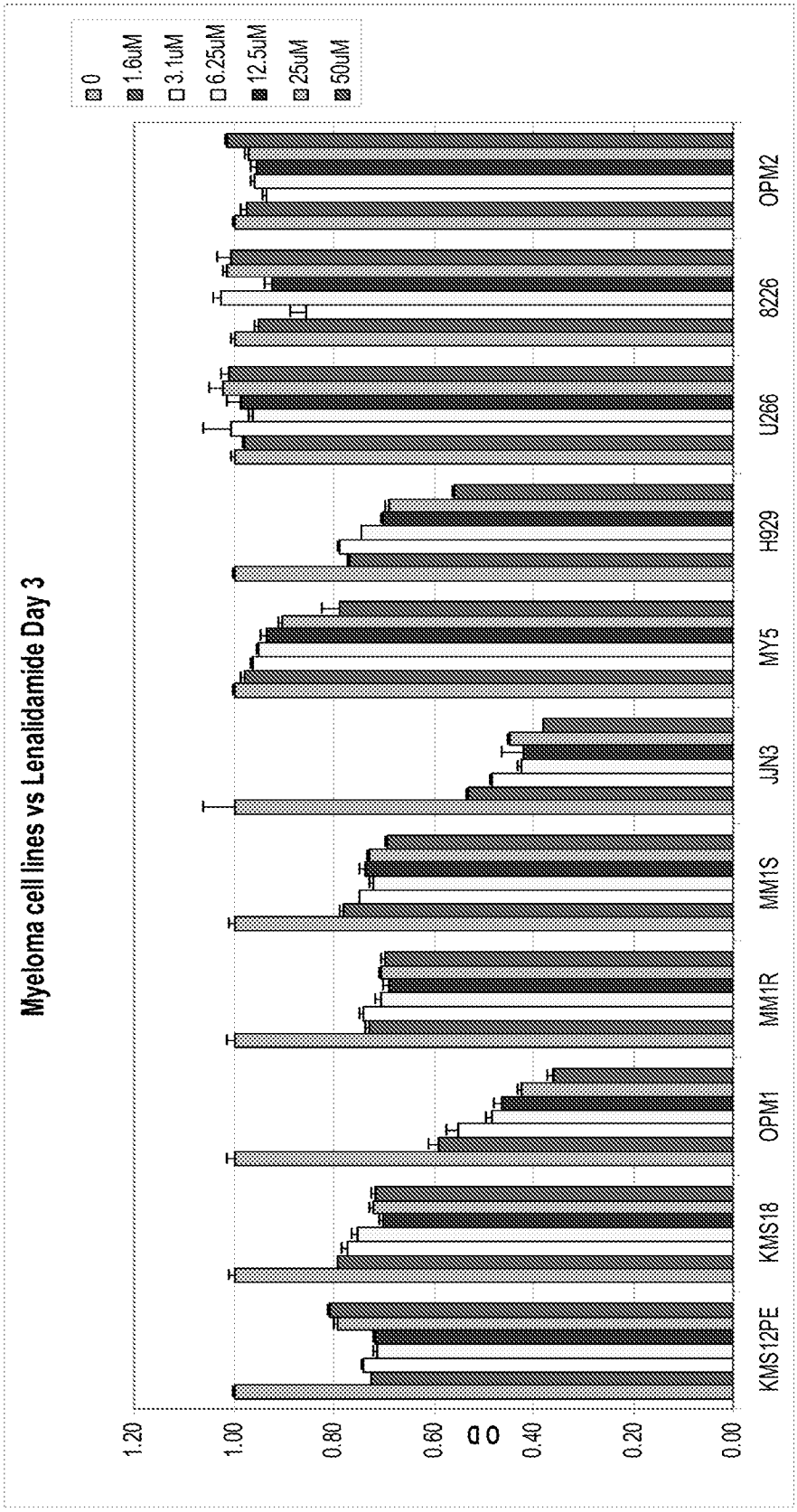

Myeloma cell lines vs Lenalidamide Day 3
0
1.6uM
3.1uM
6.25uM
12.5uM
25uM
50uM
OD
1.20
1.00
0.80
0.60
0.40
0.20
0.00
KMS12PE
KMS18
OPM1
MM1R
MM1S
JJN3
MY5
H929
U266
8226
OPM2

Figure 1

Figure 2

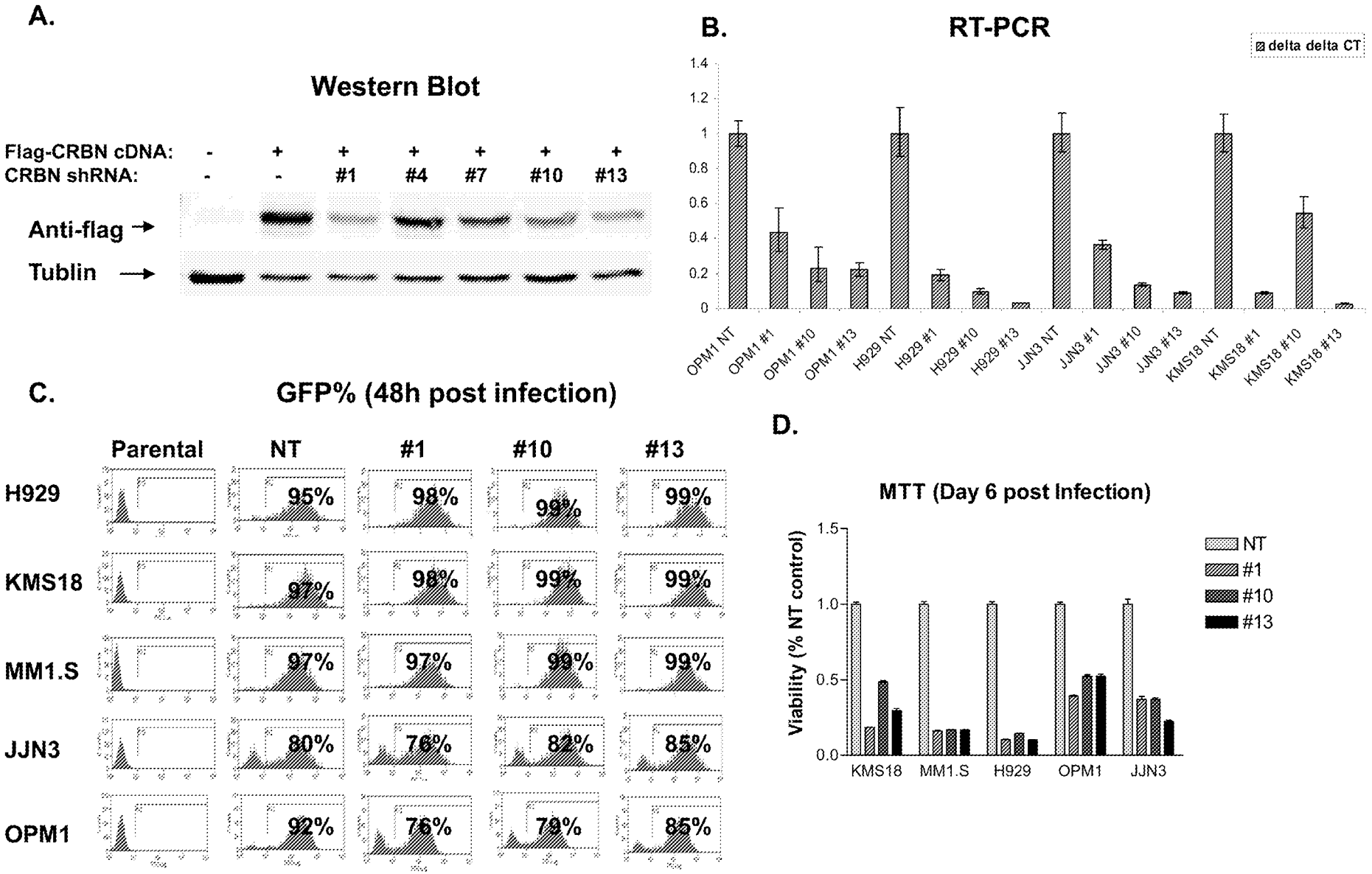
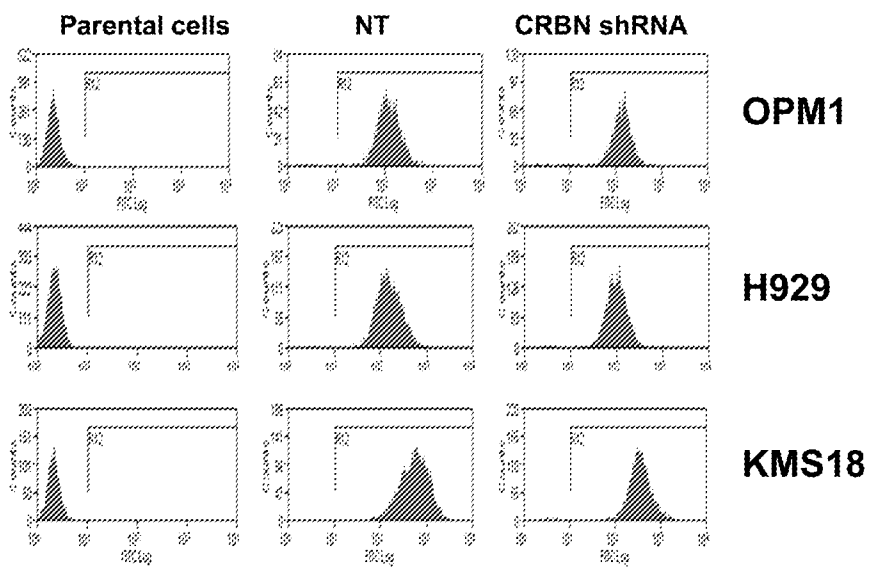
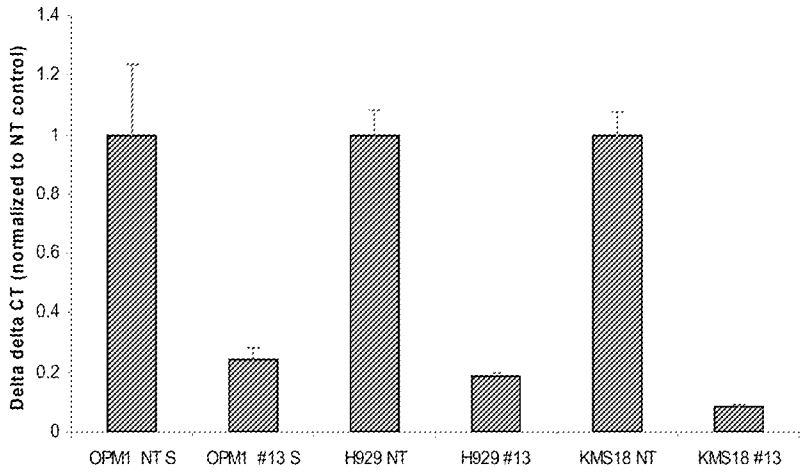

A.
Western Blot
Flag-CRBN cDNA: - + + + + + +
CRBN shRNA: - - #1 #4 #7 #10 #13
Anti-flag
Tublin
B.
RT-PCR
delta delta CT
1.4
1.2
1
0.8
0.6
0.4
0.2
0
OPM1 NT
OPM1 #1
OPM1 #10
OPM1 #13
H929 NT
H929 #1
H929 #10
H929 #13
JJN3 NT
JJN3 #1
JJN3 #10
JJN3 #13
KMS18 NT
KMS18 #1
KMS18 #10
KMS18 #13
C.
GFP% (48h post infection)
Parental
NT
#1
#10
#13
H929
95%
98%
99%
99%
KMS18
97%
98%
99%
99%
MM1.S
97%
97%
99%
99%
JJN3
80%
76%
82%
85%
OPM1
92%
76%
79%
85%
D.
MTT (Day 6 post Infection)
Viability (% NT control)
1.5
1.0
0.5
0.0
KMS18
MM1.S
H929
OPM1
JJN3
NT
#1
#10
#13

Figure 3

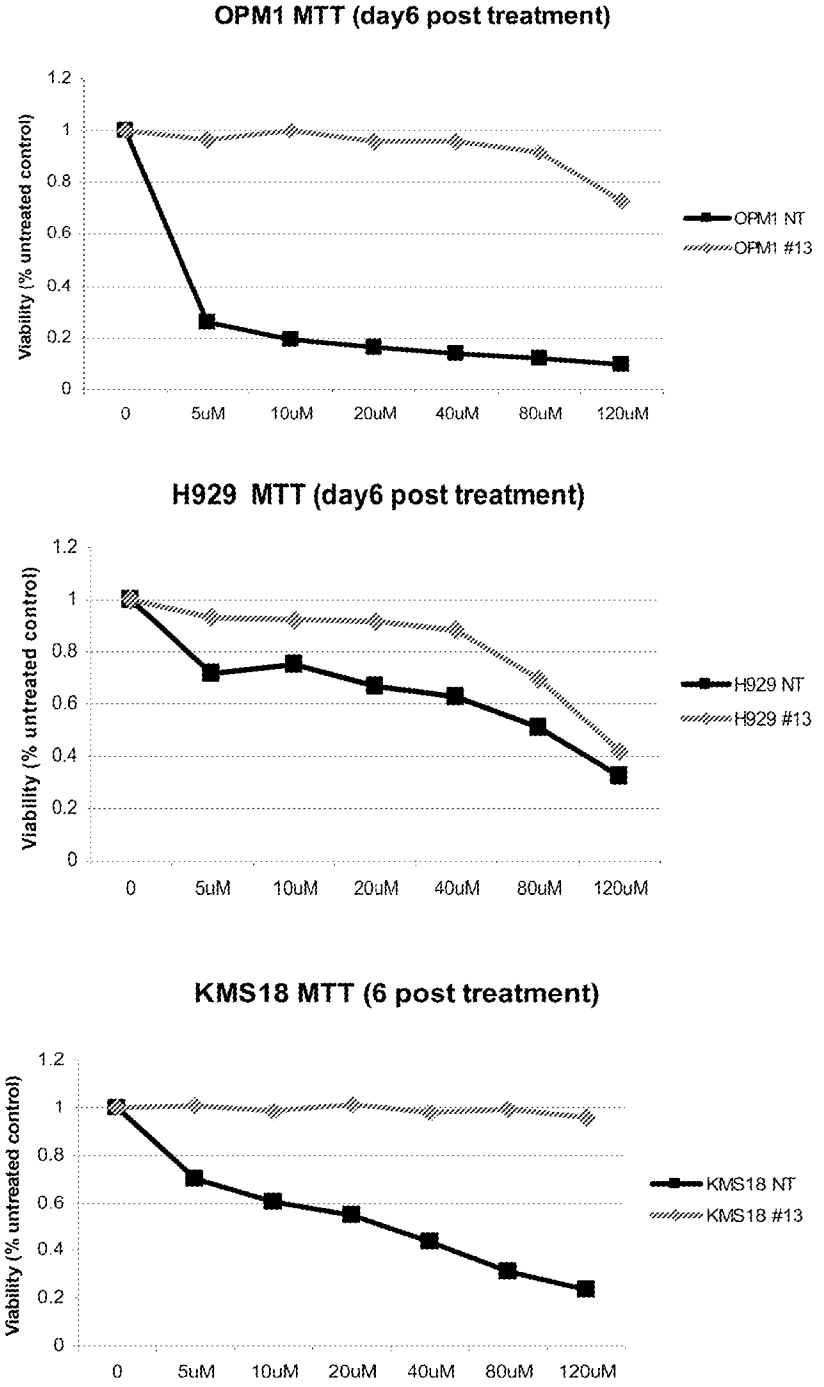
A.


GFP%
Parental cells
NT
CRBN shRNA
OPM1
H929
KMS18

B.


RT-PCR (CRBN)
Delta delta CT (normalized to NT control)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
OPM1 NT S
OPM1 #13 S
H929 NT
H929 #13
KMS18 NT
KMS18 #13

C.


OPM1 MTT (day6 post treatment)
Viability (% untreated control)
1.2
1
0.8
0.6
0.4
0.2
0
0
5uM
10uM
20uM
40uM
80uM
120uM
OPM1 NT
OPM1 #13
H929 MTT (day6 post treatment)
Viability (% untreated control)
H929 NT
H929 #13
KMS18 MTT (6 post treatment)
Viability (% untreated control)
KMS18 NT
KMS18 #13

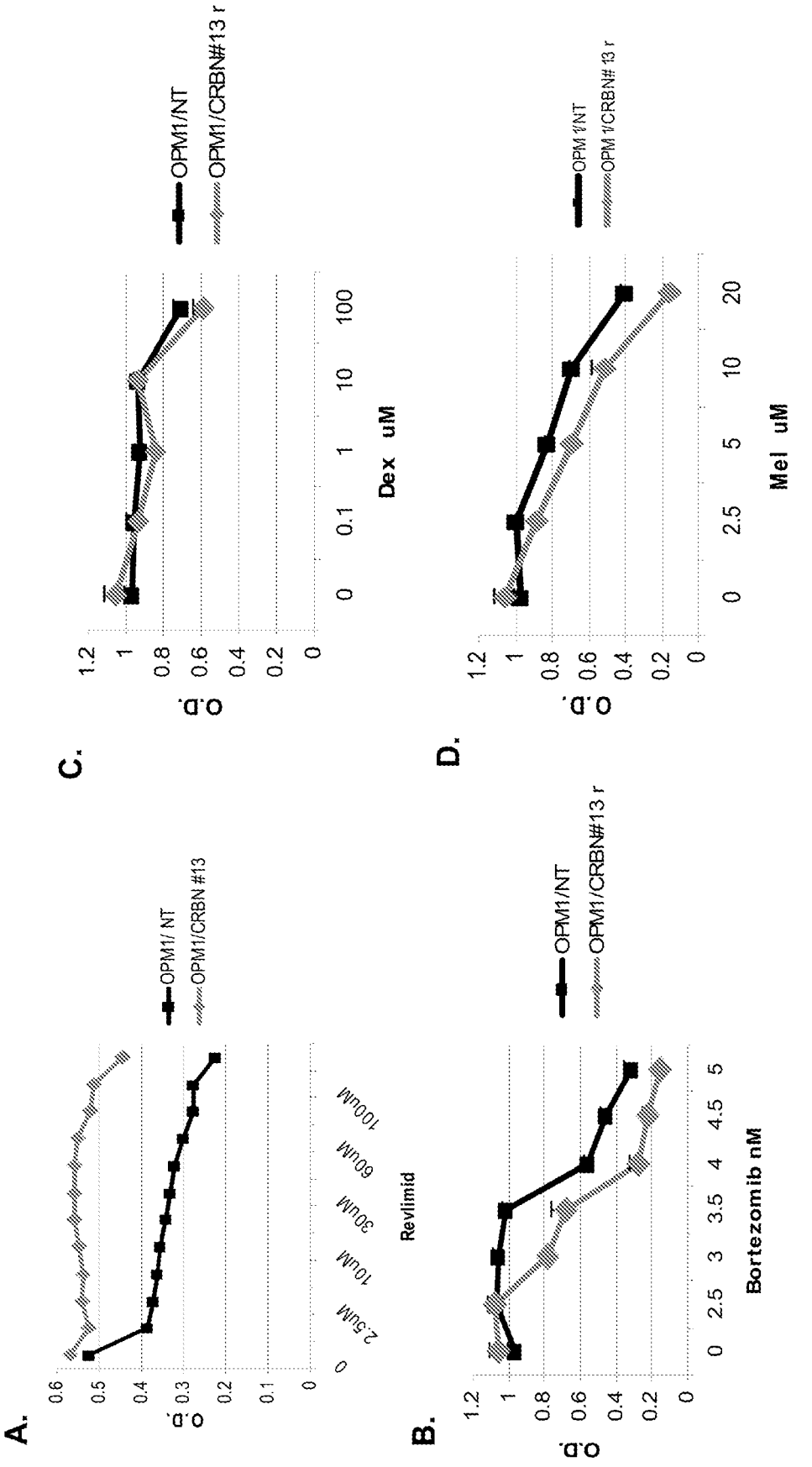

MTT (day 3 post treatment)
A.
O.D.
OPM1/ NT
OPM1/CRBN #13
Revlimid
0
2.5uM
10uM
30uM
60uM
100uM
B.
O.D.
OPM1/NT
OPM1/CRBN#13 r
Bortezomib nM
C.
O.D.
OPM1/NT
OPM1/CRBN#13 r
Dex uM
D.
O.D.
OPM 1/NT
OPM 1/CRBN# 13 r
Mel uM

Figure 4

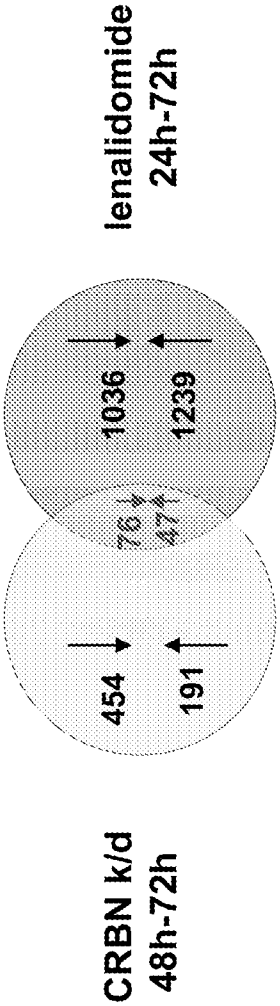
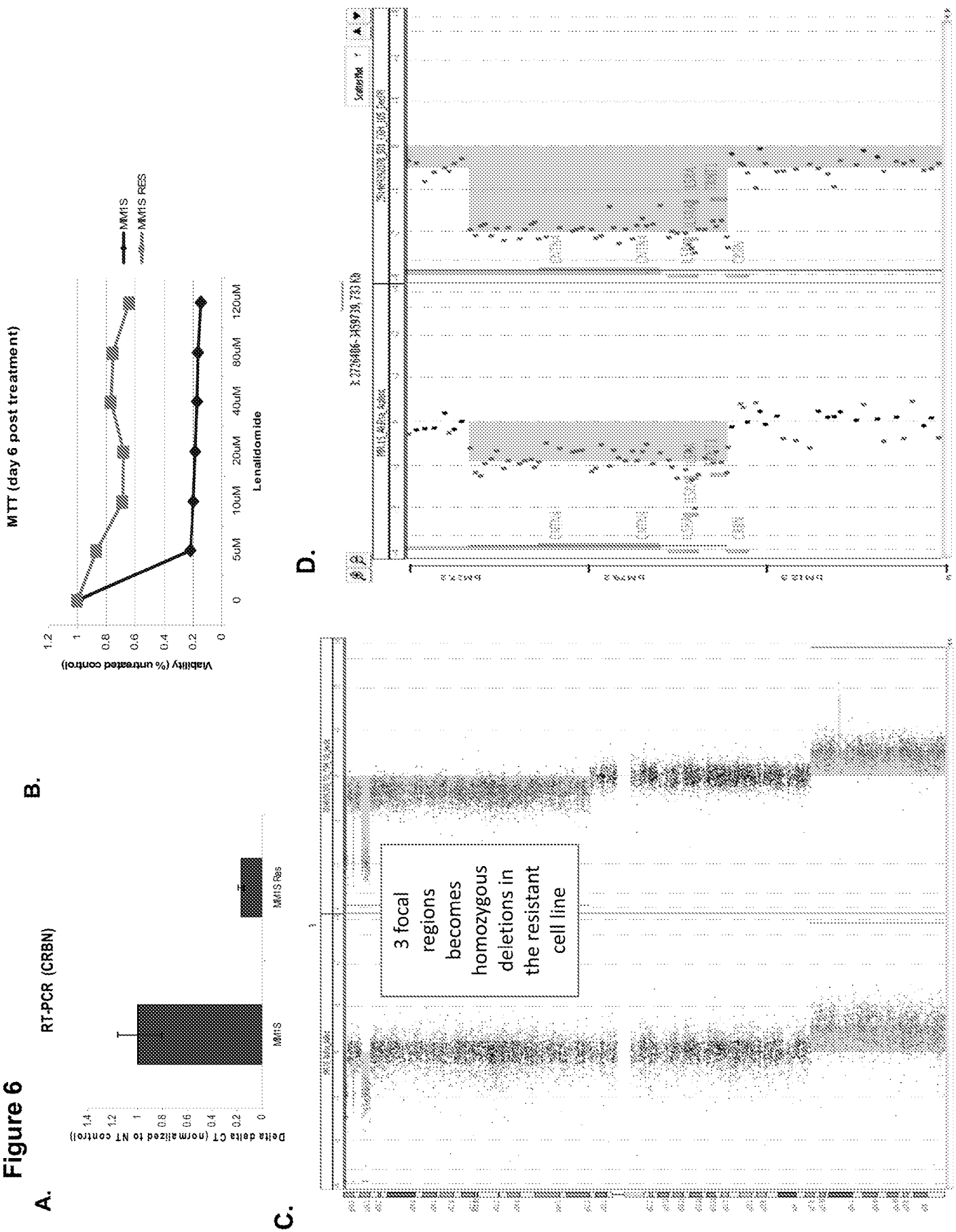

lenalidomide
24h-72h
1036
1239
764
471
454
191
CRBN k/d
48h-72h

Figure 5

Figure 6


A.
RT-PCR (CRBN)
Delta delta CT (normalized to NT control)
MM1S
MM1S Res
B.
MTT (day 6 post treatment)
Viability (% untreated control)
MM1S
MM1S RES
Lenalidomide
C.
3 focal regions becomes homozygous deletions in the resistant cell line
D.

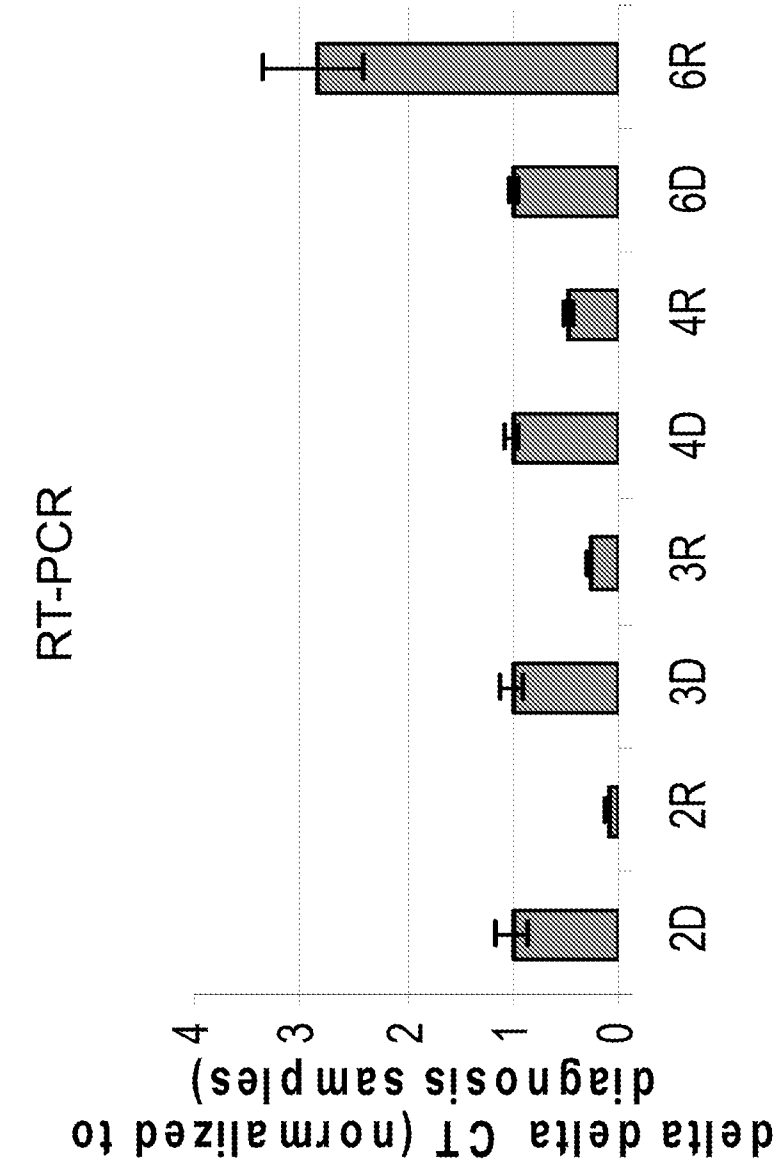

RT-PCR
delta delta CT (normalized to diagnosis samples)
0
1
2
3
4
2D
2R
3D
3R
4D
4R
6D
6R

Figure 7

Figure 8: List of genes shared in OPM1 cells after CRBN knockdown and Lenalidomide treatment

Down-regulated			
AGPS	GALNTL4	PGM2	TMEM33
AJAP1	GINS4	PPIF	TMEM64
APTX	GNPNAT1	PPP2R1B	TNFRSF21
BCL11B	HAUS7 /// TREX2	RAD18	TRIM4
BEST3	HINT3	RFC3	USP9X
BID	HS3ST3B1	RGS1	WDR4
C12orf66	ITPR1	RGS16	WDR72
C13orf1	LOC730631	ROR1	ZNF148
C18orf54	LOC84740	RRP1B	ZNF561
C7orf68	LPL	SEC24A	ZNF706
CEP72	LRRC8B	SEL1L	ZWILCH
CNKSR2	MMACHC	SETD7	
COX11	MRS2	SETMAR	
DFFA	MTAP	SLC7A2	
DNAJC25	MYB	SMCR7L	
DTNA	NEK6	SMEK2	
EIF3M	NETO2	SNHG3	
FAM76B	NUDCD1	SNRPN	
FANCI	NUP43	STAMBPL1	
FKBP11	ORC5L	SUV39H2	
FLJ25006	PDK1	TET1	
GABRB2			

Up-regulated		
ADAT3	HLA-DPB1	RTP4
ATXN1	HLA-DQA1	SUMF1
AUTS2	HLA-DQA1 ///	TMEM185A
BTBD7	HLA-DRB1 /// HLA-DRB3	UTRN
C10orf10	HLA-DRB1 /// HLA-DRB4	WDFY3
C2orf68	HLA-DRB1 /// HLA-DRB4	
CARD16	IFIH1	
CASP1	IGF2BP3	
CTTNBP2	LIMA1	
DCC	LRRC4C	
DHTKD1	MAML2	
DLEU2	MARCH2	
DNAJC5B	MB	
ELF3	NFIB	
EXTL2	NMI	
FCHO2	PCBP2	
GIMAP2	PLOD2	
HIPK2	PTEN	
HIST1H2AD /// HIST1H3D	PTPRM	
HIST1H2BG	RASGRP3	
HIST2H4A /// HIST2H4B	RCAN3	