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1. Supplemental Methods

Cell Line and Culture

Human embryonic kidney cell lines 293FT (Invitrogen) and lenti-x 293 (Clontech) were maintained in Dulbecco's Modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 1x sodium pyruvate (Invitrogen), 1x non-essential amino acids (Invitrogen), 100 U/mL penicillin (Invitrogen), and 100 µg/mL streptomycin (Invitrogen). Acute myeloid leukemia cell lines KG-1, Kasumi-1, U937, MOLM-13, HL-60, and MV-4-11 were purchased from American Tissue Culture Collection (ATCC). NB-4, HNT-34, OCI-AML2, and OCI-AML3 cell lines were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. 293FT *CRBN*^{-/-}, MOLM-13 *CRBN*^{-/-} and OCI-AML2 *CRBN*^{-/-} cell lines were described previously.¹ KG-1, Kasumi-1, U937, MOLM-13, NB-4, and HNT-34 cell lines were maintained in Roswell Park Memorial Institute (RPMI) 1640 tissue culture medium (Invitrogen) supplemented with 10% FBS, 1X sodium pyruvate, 1X non-essential amino acids, 100 U/mL penicillin, and 100 µg/mL streptomycin. HL-60 and MV-4-11 cell lines were maintained in Iscove's Modified Dulbecco's medium (IMDM; Invitrogen) supplemented with 10% FBS, 1X sodium pyruvate, 1X non-essential amino acids, 100

U/mL penicillin, and 100 µg/mL streptomycin. OCI-AML2 and OCI-AML3 cell lines were maintained in minimal essential medium (MEM; Invitrogen) supplemented with 10% FBS, 1X sodium pyruvate, 1X non-essential amino acids, 100 U/mL penicillin, and 100 µg/mL streptomycin. U937 cells grown in flasks with agitation during the CRISPR screen were supplemented with 1X Pluronic F-68 Non-ionic surfactant (ThermoFisher). When applicable, Puromycin (Thermo Fisher), Blastidicin (Thermo Fisher) or Hygromycin (Thermo Fisher) was added to the cell culture medium at 1-2 µg/ml, 10-50 µg/ml, or 250-500 µg/ml, respectively. All cell lines were cultured at 37°C under an atmosphere of 5% CO₂ in a humidified cell incubator.

Cell Proliferation and Apoptosis Assays

Cell viability and proliferation of primary AML cells in the presences of indicated CC-90009 concentrations were assessed after 24 hours in culture by the trypan blue exclusion method using a Vi-Cell cell counter (Beckman-Coulter, Brea, CA). For human cancer cell lines, 5,000 to 10,000 cells per well were seeded in the optimized density of each cell line for cell growth in 96-well plates containing DMSO or test compounds with the recommended growth medium recommended by the vendor. After 72 hours, cell proliferation was assessed using CTG according to the manufacturer's instructions. The growth inhibitory IC₅₀ values of CC-90009 were determined using ActivityBase (IDBS). To test the effect of GSPT1 depletion on cell proliferation, U937 cells were infected with lentiviral shRNA vectors for 4 days, and cell proliferation was quantified using CTG every other day thereafter. Relative cell proliferation was normalized against Day 4 cell growth values.

The ability of CC-90009 to induce apoptosis was assessed in selected AML cell lines at the time points and compound concentrations indicated. AML primary cells and cell lines were collected after 24-48 hours in culture with DMSO or CC-90009. Collected cells were stained with Annexin V – PE (BD Biosciences) and 7AAD (BD Biosciences), or Annexin V – AF647 (Biolegend) and 7AAD (Biolegend) following the instructions of the manufactures. Stained cells were analyzed using the LSR II (BD) or Attune Flow Cytometer (Invitrogen).

AML Patient Sample Collection, Cell Culture and Colony Forming Assay

All patient samples used in these studies were collected with informed consent by the Princess Margaret Leukemia Bank and other respective hospitals. Samples were collected from patient's peripheral blood and were subjected to Ficoll gradient centrifugation to obtain mononuclear cells for viable cryopreservation. Bone marrow samples from adult AML patients were extracted into Vacutainer™ tubes containing heparin as an anticoagulant. A portion of frozen viable sample was sent to the laboratories of Vivia Biotech under currently ongoing research study protocols at the originating centers approved by the Center's Ethical Committees, after obtaining the donor patient's informed consent for the delivery of the sample to Vivia and for the research to be conducted with such a sample. All AML samples used for xenograft assays and in vivo CC-90009 treatment were tested for engraftment ability in NOD/SCID mice prior to use in these studies.

For in vitro cell culture of AML samples, primary cells were plated in IMDM supplemented with 15%BIT with addition of the following growth factors: SCF, IL-3,

FLT-3L, GM, G, IL-6, TPO, in the presence of CC-90009 at concentrations of 0, 3, 30 and 100nM.

Colony forming assays were performed with primary AML cells in methylcellulose semisolid culture medium supplemented with multiple growth factors and CC-90009 at concentrations of 0, 3, 30 and 100nM. Colonies were counted at day14. Only colonies with more than 50 cells were considered as AML-CFCs.

Xenotransplantation and Serial Limiting Dilution Assays

Animal experiments were performed in accordance with guidelines approved by the University Health Network Animal Care Committee. One day prior to transplantation, 8- to 12-week-old NOD.SCID mice were sublethally irradiated (225cGy) and pretreated with anti-CD122 antibody (200ug/mouse) to deplete residual host NK cells. Vially frozen mononuclear cells from patients diagnosed with AML were thawed and transplanted by intrafemoral injection into the preconditioned mice at a cell dose of 5×10^6 per mouse.³⁰ Following in vivo treatment, mice were euthanized and cells were flushed and collected from long bones using Iscove's modified Dulbecco's medium (IMDM). Human engraftment in the injected right femur (RF) and non-injected bone marrow (BM, left femur and 2 tibias) was evaluated by flow cytometry following using human-specific antibodies. For in vivo treatment, CC-90009 and vehicle treatment was initiated at day21 post transplantation of AML cells. CC-90009 was administered at a dose of 2.5 mg/kg IP twice a day at 3 hours apart for 4 weeks. CC-90009 was freshly prepared for each treatment following the instructions provided by Bristol-Myers Squibb (5%NMP, 45%PEG400 and 50%Saline). Vehicle solution without CC-90009 compound was administered to control

mice at the same volume (50 μ L/mouse) and on the same schedule as CC-90009 treatment. For the mice transplanted with normal hematopoietic cells, lineage negative cord blood cells were intrafemorally transplanted at 1x10⁵ per mouse. Mice were then treated with CC-90009 or vehicle using the same protocol as described above for the mice transplanted with AML.

For secondary transplantation limiting dilution assays, human leukemia cells were purified from vehicle- and CC-90009-treated mice through depletion of mouse cells and transplanted into preconditioned secondary NOD.SCID mice at doses ranging from 2x10⁶ to 200 cells per mouse. 12 weeks post transplantation, secondary mice were killed and the proportion of engrafted mice was determined by flow cytometry for each transplanted cell dose. The threshold for detection of AML engraftment was 1% human CD45⁺CD33⁺ cells. LSC frequency was calculated using ELDA software (bioinf.wehi.edu.au/software/elda).²

To evaluate leukemia and normal cord blood cell engraftment after CC-90009 treatment, immunophenotyping for cell surface markers was carried out using human-specific antibodies: anti-CD45-APC, anti-CD19-V450, anti-CD15-FITC, anti-CD34-APC-Cy7, anti-CD38-PE-Cy7 (BD), anti-CD14-PE, anti-CD19-PE, anti-CD14-ECD, and anti-33-PE-Cy5 (Beckman Coulter). Cells were stained with antibodies for 30 min at 4^oC, washed and analyzed using LSR II, and FlowJo software was used for data analysis. Xenografts were classified as leukemic if they were CD45⁺CD33⁺CD19⁻.

NanoString Assay Design, GE Profiling, Data Processing and Analysis

NanoString assays were performed as previously described³ using the RNA extracted from viably banked AML cells collected from patients at diagnosis. Briefly, a custom probeset was designed to hybridize to the 17 core genes making up the LSC17 score along with several reference genes chosen to cover a wide range of expression levels in AML. This NanoString assay has been implemented as a clinical diagnostic test at the Princess Margaret Hospital (Toronto, Canada) to determine the LSC17 scores of newly diagnosed AML patients. After hybridization on the nCounter Prep Station, transcript counts were determined using the nCounter Digital Analyzer (version 2.1.2.3) at the high-resolution setting. Reporter code count files containing raw transcript counts from each cartridge were analyzed for quality controls (QC) and normalization purposes by using default settings for gene expression (GE) analysis with the nSolver analysis software (version 2.0.72). The captured counts were normalized to the geometric mean of the reference genes and the codeset's internal positive control in our assay. The output files from the nSolver software were read into R for further QC, normalization and data processing to ensure that all of the signature and reference probe counts were well above 3 standard deviations over background noise levels. The fully normalized GE counts in each cartridge were then log₂-transformed after incrementing by 1. LSC17 scores were computed for each patient using the scaled data. Patients were classified as having a high (above median) or low (below median) LSC17 score based on the median LSC17 score of a reference cohort.

Gene Editing Vectors

Lentiviral vectors expressing non-targeting control sgRNA (pRSG17-U6-sgNT-1-UbiC-TagGFP2-2A-Puro and pRSG16-U6-sgNT-1-UbiC-TagRFP-2A-Puro) were purchased from Collecta. Complementary oligonucleotides containing sgRNAs targeting the non-coding region of the human genome (sgNC-1 and sgNC-8), and gene-specific sgRNAs targeting ILF2, ILF3, TSC1, TSC2, GCN1, GCN2, ATF4 or DDIT4 were annealed and cloned into pRSG16-U6-sgEV-UbiC-TagRFP-2A-Puro or plenti-H1TO-sgEV-EF1a-HTLV-TetRep-2A-TagRFP-2A-Puro, which were modified from pRSG16-U6-sg-HTS6C-UbiC-TagRFP-2A-Puro (Collecta) and pRSGT16-U6Tet-sg-CMV-TetRep-2A-TagRFP-Puro (Collecta), respectively. pRSG16-U6-sgCRBN-8-UbiC-TagRFP-2A-Puro was described previously.⁴ Complementary oligonucleotides containing single guide (sg)RNA targeting GCN2 were annealed and cloned into pLenti-CRISPR-Bla as previously described¹ to generate plenti-CRISPR-Bla-GCN2-T1.

The sgRNA sequences are shown below:

sgNT-1, 5'- GTAGCGAACGTGTCCGGCGT -3'

sgNC-1, 5'- GACCTTGTGATCTTCTCATG -3'

sgNC-8, 5'- GGCCAGTGACTAGTGCTTGA -3'

sgILF2-1, 5'- AAGGAAGTTTCATCAGGTGC -3'

sgILF2-6, 5'- CTCACCAAATAGAAGTCAAA -3'

sgILF3-2, 5'- TCCCCCTCTGACGGGTACTG -3'

sgILF3-4, 5'- CGGCATTCATGTAGCCTCCA -3'

sgILF3-8, 5'- CCTGCGGGGAGTGATGCGGG -3'

sgTSC1-4, 5'- TTTATCCATCCTCTCGTTAC -3'

sgTSC1-6, 5'- ATTCGTTAATCCTGTCCAAG -3'

sgTSC1-8, 5'- AGAGTGCGTACACACTGGCA -3'

sgTSC2-4, 5'- GTGGCCTCAACAATCGCATC -3'

sgTSC2-5, 5'- CCAACGAAGACCTTCACGAA -3'

sgTSC2-6, 5'- AGCACGCAGTGGAAGCACTC -3'

sgTSC2-8, 5'- CATGGCATGTCCGAACGAGG -3'

sgGCN1-3, 5'- GCAGGCAGCCATCCAGCAGT -3'

sgGCN1-5, 5'- GGTGACAACAGCCAGTGTA -3'

sgGCN2-1, 5'- CGCTGAGAAATGACTGCACG -3'

sgGCN2-2, 5'- CATATACTTCTTCACCAGTT -3'

sgGCN2-3, 5'- ATGTA CT CACACATCTGGAT -3'

sgGCN2-T1, 5'-ACTTTCATTTGATAGACCTT -3'

sgATF4-4, 5'- AGGATCGTAAGGTTTGGGAC -3'

sgATF4-7, 5'- TCTCTTAGATGATTACCTGG -3'

sgATF4-8, 5'- GCAACGTAAGCAGTG TAGTC -3'

sgDDIT4-1, 5'- GTTTGACCGCTCCACGAGCC -3'

sgDDIT4-3, 5'- CGCACGGCTCGCTGTAGGCC -3'

sgDDIT4-6, 5'- GGCGCTGCTGGACGTCTGCG -3'

ORF Expression Vectors

pDONR223-FLAG-HA-CRBN-STOP, pcDNA3.2-GSPT1-V5, pcDNA3.2-IKZF1-V5, and pLKO.5pur vectors expressing control shRNA or shRNA targeting GSPT1 were described previously.¹ Y384 and W386A double mutations were introduced into pDONR223-FLAG-HA-CRBN-STOP via site-directed mutagenesis and overlapping PCR to generate pDONR223-FLAG-HA-YWAA-STOP. pDONR223-FLAG-HA-CRBN-STOP and pDONR223-FLAG-HA-YWAA-STOP were shuttled into pcDNA3.2-gateway-V5 (Invitrogen) to generate pcDNA3.2-FLAG-HA-CRBN and pcDNA3.2-FLAG-HA-YWAA. The coding region of human GCN2 was PCR amplified from a GCN2 cDNA clone (GE Dharmacon), and cloned into pDONR223 via BP recombination (Invitrogen). GCN2 cDNA resistant to plenti-CRISPR-Bla GCN2-T1 (a single CRISPR vector encoding both Cas9 and GCN2-specific sgRNA) was generated by overlapping PCR and cloned into pDONR223 to generate pDONR223-GCN2-CR (CRISPR resistant). Additional mutations including T899A/T904A, F1143L/R1144L, and K519R were introduced into pDONR223-GCN2-CR via site-directed mutagenesis and overlapping PCR. The CMV promoter in pLenti-CMV-3xHA-gateway-mPGK-Pur as previously described¹ was replaced by the human pGK promoter, which was in vitro synthesized by Invitrogen, to generate plenti-hPGK-3xHA-gateway-mPGK-Pur. Then, pDONR223-GCN2-CR wild-type and mutants were cloned into plenti-hPGK-3xHA-gateway-mPGK-Pur to generate plenti-hPGK-3xHA-GCN2 wild-type or mutants-mPGK-Pur.

Gene Editing

U937 parental cells were transduced with plenti-CRISPR-Bla-GCN2-T1. Twenty-four hours after lentiviral transduction, cells were treated with 10 µg/mL Blasticidin for 3 days, followed by limiting dilution for clonal selection in 96-well plates. After 1 week in culture, multiple single clones were picked and expanded into T25 flasks. The knockout of GCN2 in each individual clone was confirmed by immunoblot analysis.

PharmaFlow Assay

The thawed AML patient samples were diluted with StemSpan SFEM II, supplemented with 20% (v/v) FBS and 1% antibiotic to a final volume of 60µL per well. A small part of the sample was stained with specific monoclonal antibodies (MAb) in order to determine the best combination of markers to identify leukemic cells in each sample. Additionally, Annexin V was included in this combination to evaluate the initial viability of the sample. Combinations of Annexin-V and monoclonal antibodies (Mab) used for initial evaluation, depending on the type of sample, are shown below:

MAb panel 1: Annexin-V, CD34, CD45, HLADR, CD117, CD19

MAb panel 2: Annexin-V, CD34, CD45, CD64, CD14, CD33

The mixture was dispensed into 96-well plates containing DMSO or CC-90009, previously prepared using an Echo 550 Liquid Handler. The plates containing the sample were incubated at different timepoints as indicated, at 37°C in humidified air containing 5% CO₂. To prepare the sample for analysis, the red blood cell population was lysed at the end of the incubation, and then the sample was stained with the MAb

that best identified pathological cells plus Annexin V. Finally, plates were analyzed in Vivia's PharmaFlow™ platform.

Intracellular Flow Cytometry Assay

For intracellular flow cytometry to detect the expression of GSPT1, cells harvested after 24 hours in culture with CC-90009 were fixed and permeabilized in 200µl of BD Cytotfix/Cytoperm Buffer per tube for 20 minutes on ice. AML cells were then washed in 1ml 1xPerm Wash Buffer per sample and collected for intracellular antibody staining using Alexa Fluor 647-anti-GSPT1 (BMS). Stained AML cells were washed and data was acquired on a BD LSR II and analysed using FlowJo.

To evaluate GSPT1 expression in AML xenografts, NOD.SCID mice were randomly separated into vehicle and CC-90009 treated groups at 4 to 6 weeks post transplantation of AML cells (3 to 4 mice per treatment group). CC-90009 or vehicle was administered by intraperitoneal (IP) injection at a dose of 2.5mg/kg twice a day at 3 hours apart. At 2 hours after the third dose of treatment, mice were sacrificed and cells collected from RF and BM were stained with anti-human CD45 and CD33 antibodies. After washing, intracellular flow cytometry was performed on stained cells described as above.

Proteomics

KG1 cells were treated with DMSO or 100 nM CC-90009 for 4 hours. All treatments were performed with four replicates and protein lysates were digested with trypsin, labeled by isobaric tandem-mass-tags (TMT), fractionated, and analyzed using an Orbitrap Fusion Lumos mass spectrometer at IQ Proteomics (Cambridge, MA). Peptide to protein mapping, protein identification, and quantification of relative protein

abundance with statistical analyses were performed at Celgene using custom software. 7957 human proteins were unambiguously quantified by at least one peptide in at least one of the MS runs. Proteins were defined by the Uniprot identifier, with some represented by more than one isoform.

Lentiviral Production and Infection

Lentiviral plasmid was cotransfected with the 2nd Generation packaging system (ABM) into lenti-X 293 cells using Lipofectamine® 2000. After 12 hours of incubation, media was changed to fresh DMEM media supplemented with 20% FBS. At 36 hours post transfection, viral supernatant was collected and fresh media was replenished. At 56 hours, viral supernatant was collected again, combined with the first viral harvest, cleared via centrifugation at 2000 rpm for 5 minutes, and then filtered through a 0.45 micron cellulose acetate or nylon filter unit. All lentiviral supernatants except those of lentiviral small hairpin ribonucleic acid (shRNA) and small guide ribonucleic acid (sgRNA) vectors were concentrated using a lenti-X concentrator according to the manufacturer's instructions. Acute myeloid leukemia cell lines were spin-inoculated with lentivirus at 2000 rpm for 90 minutes. After twelve hours, viral supernatant was removed and complete culture media was added to the cells. Forty-eight hours later, cells were incubated with Puromycin or Blasticidin for an additional 3 days to select cells stably integrated with lentiviral vectors.

Genome-Wide CRISPR Screen, Genomic DNA Isolation and Sequencing Library Preparation

The pooled human sgRNA library (Cellecta) used expressed 4-8 sgRNAs targeting each of over 19,000 protein-coding genes for a total of 150,076 unique sgRNAs. Vectors also expressed a RFP reporter and a puromycin resistance gene. Twenty-four hours after transduction with the lentiviral sgRNA library, U937-Cas9 cells were treated with 1 µg/mL puromycin. After 48 hours of puromycin selection, transduced cells were split into two arms and treated with either DMSO or 10 µM CC-90009 for an additional 9 days. The samples collected on day 3 represented T0 controls for comparison to treatment day 12. To generate sgRNA libraries for Next Generation Sequencing (NGS), 9×10^7 cells per sample were collected and lysed with Qiagen Buffer P1 with added RNase A. Lysates were adjusted to 0.5% SDS and chromatin was sheared into 10-100 kb fragments with a probe sonicator. After treatment with Proteinase K, genomic DNA was extracted with Phenol:Chloroform:Isoamyl Alcohol and precipitated overnight with isopropanol and 10% sodium acetate. The DNA pellet was washed with ethanol and dissolved in water. Total genomic DNA was quantified on the Nanodrop.

To amplify the sgRNA portions of the transduced sgRNA construct in each cell, PCR was performed on all of the genomic DNA isolated per sample with 25 µg DNA amplified per reaction. Twenty-four cycles of PCR were performed with an annealing temperature of 65°C. After visualizing the 477 base-pair PCR products on an agarose gel, PCR reactions from each sample were combined, mixed, and 100 µl per sample was cleaned with 1X volume of SPRIselect beads in a 2-step cleanup protocol to eliminate both primers and genomic DNA carryover. Products were eluted in Qiagen Elution Buffer and

measured on the Nanodrop. A second PCR reaction was performed on the first PCR product to incorporate dual-indexed Illumina primers into the final sgRNA libraries. Six cycles of PCR were run with an annealing temperature of 65°C and four cycles were run with an annealing temperature of 71°C to reduce non-specific products. After mixing the four reactions per sample and confirming the 339 base-pair libraries on an agarose gel, 100 µl of each library was cleaned with 1X volume of SPRIselect beads in a 2-step cleanup protocol to eliminate both primers and carryover of the first PCR reaction.

Final sgRNA libraries were visualized on the Agilent Bioanalyzer and quantified with the KAPA library quantitation kit. Samples were diluted to 3 nM and 2-3 samples were run per lane on the Illumina HiSeq 4000. Each lane also contained 5% molar ratio spike-in of PhiX to enhance sequence diversity. Data were analyzed by comparing sgRNA counts between samples. Enriched sgRNAs in samples treated with lethal doses relative to the DMSO condition at the same timepoint indicated genes whose knockout conferred resistance.

CRISPR Screen Data Quality Control and Analysis

The MAGeCK pipeline (version 0.5.6)^{5,6} was used to process and analyze the genome-wide CRISPR/Cas9 screen data. After using the command 'mageck count' to identify raw sequencing reads with a perfect match to the sgRNA sequences in the CRISPR library (Cellecta), we assessed the data quality based on total reads generated per sample, percentage of mapped reads, zero sgRNA count, and the Gini index (which is a measure of statistical dispersion in sgRNA count distribution). All screen data passed the quality control guideline provided by MAGeCK (supplemental Table 4). Raw sgRNA counts were then normalized to the median count of the 100 non-targeting sgRNAs

included in the library. Next, the command 'mageck test' was used to rank all sgRNAs based on the log₂FC of their normalized counts between 10 μM CC-90009 treated samples and DMSO control samples at T12. MAGeCK uses the Robust Rank Aggregation method to aggregate the sgRNA level result at gene level which produces a p-value as a statistical assessment of how concordant the sgRNAs mapping to the same gene behave. These gene level p-values were further converted to False Discovery Rate (FDR) after correcting for multiple hypothesis using the Benjamini & Hochberg method. Genes with an average log₂FC (across all its sgRNAs) > 1 and FDR < 0.05 were identified as resistant hits and listed in supplementary Table 1. We tested for molecular pathways over-represented among the top ranked genes enriched by CC-90009 using a Fisher's Exact Test (FET) against the REACTOME database⁷, as implemented in the clusterProfiler package⁸ in R. The FET p-values were corrected for multiple hypothesis testing again using the Benjamini & Hochberg method to generate FDRs. Forty-seven statistically significant pathways were identified at FDR<0.05 and were used to interpret the resistant mechanism of CC-90009 in U937 cells. An enrichment map was also generated using Cytoscape(3.7)⁹ to display the relationship among the enriched pathways and the resistant gene hits (supplementary Figure 2B).

Flow Cytometry Competition Assay

U937 and OCI-AML2 cell lines were transduced with plenti-EF1α-Cas9-P2A-Blast, followed by Blasticidin selection to establish Cas9-expressing cell lines. U937-Cas9 and OCI-AML2-Cas9 cells were then transduced with pRSG17-U6-sgNT-1-UbiC-TagGFP2-2A-Puro, or pRSG16-U6-UbiC-TagRFP-2A-Puro vectors expressing sgNT-1, sgNC-1, sgNC-8 or gene-specific sgRNAs. Transduced cells were washed with 1X PBS 72 hours

post-transduction. Cells were assessed for transduction via RFP or GFP fluorescence reporter expression. Once fluorescence was confirmed, RFP- and GFP-transduced cells were mixed at a 1:1 ratio, seeded in 2 ml culture media per well in 12-well tissue culture plates at a cell density of 200,000 cells per mL, and treated with DMSO or an appropriate dose of CC-90009. Leftover cells underwent puromycin selection for 3-7 days and were harvested for immunoblot analysis to confirm gene knock-out. After seeding cells for culture in 12-well plates, 100 μ L per well was removed for flow cytometric analysis of the baseline “Day 0” RFP- and GFP-positive percentages in each well. This was repeated every 2-4 days for flow cytometric analysis of the RFP- and GFP-positive percentages over the indicated timecourse for each experiment. The RFP⁺/GFP⁺ ratios at each timepoint were normalized to their respective RFP⁺/GFP⁺ ratio on “Day 0.”

To evaluate the effects of ILF3 knockout by the flow cytometry competition assay, U937-Cas9 cells were transduced with plenti-H1TO-EF1a-HTLV-TetR-P2A-RFP-P2A-Puro vectors expressing sgNT-1, sgNC-1, sgILF3-2, or sgILF3-4 to generate stable, doxycycline-inducible sgRNA cell lines. U937-Cas9 cells inducibly expressing sgNT-1, sgNC-1, sgILF3-2 or sgILF3-4 were treated with 1 μ g/ml of doxycycline, and on the same day, U937-Cas9 cells were transduced with pRSG17-U6-sgNT-1-UbiC-TagGFP2-2A-Puro. After 3 days, RFP- and GFP-expressing cells were mixed at a 1:1 ratio and treated with DMSO or CC-90009, followed by cell viability assessment by flow cytometry as described above.

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Analysis

Following incubation with DMSO or CC-90009, cells were collected via centrifugation at 2000 rpm for 2 minutes. Cell pellets were then washed once in ice-cold PBS and snap-frozen in liquid nitrogen. Total RNA was extracted using the RNeasy Mini Kit according to the manufacturer's instructions, and reverse-transcribed into first-strand cDNA using the AffinityScript QPCR cDNA Synthesis Kit with random primers. The total cDNA transcripts of CRBN, ILF3, Actin, GADPH, DDIT3 (CHOP), DDIT4 (REDD), and ATF3 were quantified by the ViiA™ 7 Real-Time PCR System using TaqMan Gene Expression Assay probes (Invitrogen). The cDNA level of various CRBN transcripts was quantified using RT² SYBR Green qPCR Mastermixes (Qiagen). Each reaction was performed in triplicates or quadruplicates, and values were averaged to calculate the relative expression level. Primer sequences for detected alternatively transcribed CRBN transcripts are listed below:

Probe 1: CRBN.201/202-1 For, GCAGTGCTATCCAGCGACTT

CRBN.201/202-1 Rev, GCCGGCCTATCAGATTCAA

Probe 2: CRBN.201/202-2 For, AGCGACTTCGCTGTGAATTA

CRBN.201/202-2 Rev, AGGCATACCCAGGAAACCAG

Probe 3: CRBN.213-1 For, AAAGGGAAGCACAGTTTGGGA

CRBN.213-1 Rev, GATGGGAGATGAGGTTGGAA

Probe 4: CRBN.213-2 For, AGAACCTTTGCTGTTCTTGCA

CRBN.213-2 Rev, AGCTTCCCAGGTGATTCTGA

Control: GADPH For, gaaggtgaaggtcggagtca

GADPH Rev, gacaagcttcccgttctcag

RNAseq and Data Analysis

U937-Cas9 cells expressing inducible sgNT-1 or sgILF3-2 were treated with or without 1 µg/ml of doxycycline in triplicate for 5 days. Poly-A selected mRNA libraries were prepared using the TruSeq mRNA kit (Illumina) according to the manufacturer's protocol. In brief, total RNA was extracted from cell pellets using the RNeasy Mini Kit, followed by mRNA isolation via poly-A selection. Purified mRNAs were fragmented and converted to 1st strand cDNAs with reverse transcriptase. The resulting 1st strand cDNAs were converted to double stranded cDNAs, and subjected to end-repair, A-tailing, and adapter ligation. The libraries were amplified and sequenced using Illumina's HiSeq 4000 (2x150bp configuration, single index, per lane). Fastq converted sequence files were adapter and quality trimmed using Cutadapt v1.15⁸ and aligned to genome version hg38 using STAR v2.5^{10,11}. A flattened exon file based on Gencode v24 annotation was prepared using the python script, "dexseq_prepare_annotation.py" provided with R package DEXSeq.¹² This script generates unique exon bins by collapsing overlapping exons or exon regions as well as representing regions of unique exon boundaries from different transcripts. The "featureCounts" function from Subread v1.6.2 package was used to generate exon bin counts for each STAR aligned bam file¹³. Processed read counts were assessed for differential gene expression and alternative splicing between the two experimental conditions, ILF3 KO versus non-targeting (NT) controls, using R package edgeR¹⁴. The "diffSpliceDGE" function applied

to normalized exon bin counts fitted by a generalized linear model, tests for differential exon usage (DEU). DEU is a measure comparing the log₂ fold change (logFC) of an exon bin relative to the overall gene's logFC. Significant DEU thus signifies significant differences in relative abundance of an exon or exon region compared to abundance across the entire gene between the two experimental conditions. A false discovery rate (FDR) <0.05 was applied to define statistically significant gene and exon abundance.

Pathway enrichment analysis was performed using a hypergeometric model to identify Reactome pathways associated with a greater than expected number of differentially expressed genes between sgILF3-2 and sgNT-1 transfected cells. Genes with evidence of differential exon usage were similarly interrogated for Reactome pathway enrichment, each using the R package ReactomePA¹⁵.

Antibodies

Rabbit anti-human CRBN65 monoclonal antibody (mAb) (Celgene, San Diego, CA);
Mouse anti-human GSPT1 monoclonal antibody (mAb) (Celgene, San Diego, CA);
rabbit anti-human GSPT1 polyclonal antibody (pAb; Abcam), rabbit anti-IKZF1 mAb (Cell Signaling), rabbit anti-eIF2 α pAb (Cell Signaling), rabbit anti-phospho-eIF2 α Ser51 pAb (Cell Signaling), rabbit anti-GCN2 pAb (Cell Signaling), mouse anti-Caspase-8 mAb (Cell Signaling), rabbit anti-human Caspase-3 pAb (Cell Signaling), rabbit anti-human Cleaved Caspase-3 pAb (Cell Signaling), rabbit anti-human TSC1 pAb (Cell Signaling), rabbit anti-human TSC2 pAb (Cell Signaling), rabbit anti-human S6K1 pAb (Cell Signaling), rabbit anti-human phospho-S6K1 T389 pAb (Cell Signaling), mouse anti-human ILF2 pAb (Santa Cruz), rabbit anti-human ILF3 pAb (Abcam), rabbit anti-ATF4 mAb (Cell Signaling), rabbit anti-DDIT3/CHOP mAb (Cell Signaling), rabbit anti-ATF3

pAb (Santa Cruz), rabbit-anti-DDIT4/REDD1 pAb (Cell Signaling), mouse anti-HA mAb (Covance), rabbit anti-FLAG mAb (Cell Signaling), and mouse anti-human Actin and Tubulin (Sigma) were used as primary antibodies. Goat anti-mouse 800 antibody (LI-COR Biosciences), goat anti-rabbit 680 antibody (LI-COR Biosciences), goat anti-mouse 800 antibody (LI-COR Biosciences) and goat anti-rabbit 680 antibody (LI-COR Biosciences) were used as secondary antibodies.

Immunoblot Analysis

Cells were washed in ice-cold 1X PBS twice before harvest in Buffer A [50 mM Tris.Cl (pH 7.6), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM β -glycerophosphate, 2.5 mM sodium pyrophosphate, 1 mM Na₃VO₄, 1 μ g/mL leupeptin, one tablet of Complete ULTRA protease inhibitor cocktail (Roche), and one tablet of PhosSTOP phosphatase inhibitor cocktail (Roche)]. Whole cell extracts were collected after centrifugation at top speed for 10 minutes and total protein was quantified using Bradford Reagent (Bio-Rad) according to the manufacturer's instructions. 10-20 μ g of total protein per sample were resolved by SDS-PAGE gel electrophoresis, transferred onto a nitrocellulose membrane using the Turboblot system (Bio-Rad), and probed with the indicated primary antibodies. Bound antibodies were detected with IRDye-680 or -800 conjugated secondary antibodies using a LI-COR scanner.

To confirm knockout of the target genes in U937 and OCI-AML2 cells used for the flow cytometry-based CRISPR competition assay, one million cells per sample were washed with ice-cold 1X PBS and lysed with 100 μ l 2x LDS buffer containing 2-mercaptoethanol. Lysates were boiled for 10 minutes at 95°C. Whole cell extracts were resolved by SDS-PAGE gel electrophoresis, transferred onto a nitrocellulose membrane

using the Turboblot system (Bio-Rad), and probed with the indicated primary antibodies. Bound antibodies were detected with IRDye-680 or -800 conjugated secondary antibodies using a LI-COR scanner.

Immunoprecipitation

To test the in vitro binding of CRBN to GSPT1 and IKZF1, 293FT CRBN^{-/-} cells were transiently transfected with pcDNA3.2- FLAG-HA-CRBN, pcDNA3.2- FLAG-HA-YWAA, or both pcDNA3.2-GSPT1-V5 and pcDNA3.2-IKZF1-V5. After 48 hours, cells were harvested and lysed in Buffer B [50 mM Tris (pH 7.4), 150 mM NaCl, 0.5% NP-40, one tablet of Complete ULTRA protease inhibitor cocktail (Roche), and one tablet of PhosSTOP phosphatase inhibitor cocktail (Roche)]. Whole cell extracts were clarified by centrifugation at top speed for 10 minutes. FLAG-HA-CRBN or FLAG-HA-YWAA cell extracts were mixed with the whole cell extract containing both GSPT1-V5 and IKZF1-V5 at a 1:1 ratio, and incubated with DMSO, CC-90009 (2 μ M), or LEN (2 μ M) at 4°C overnight. Next, the whole cell extracts were incubated with Anti-HA Affinity Beads (Roche) for an additional 4 hours. Beads were then washed with Buffer B 6 times, and bound proteins were eluted by boiling in LDS loading buffer and then detected by immunoblot analysis.

To test the CC-90009-induced interaction between CRBN and HA-tagged GSPT1, U937-Cas9 cells infected with lentiviral vectors expressing HA-GSPT1 and sgNT-1, sgTSC1-4 or sgTSC1-6. After treatment with MLN-4924 (1 μ M) and DMSO or CC-90009 (10 μ M) for 8 hours, cells were harvested and lysed in Buffer B. Whole cell extracts were clarified by centrifugation at top speed for 10 minutes. Whole cell extracts were then incubated with Anti-HA Affinity Beads (Roche) for an additional 6 hours.

Beads were washed with Buffer B for 6 times, and bound proteins were eluted by boiling in LDS loading buffer and detected by immunoblot analysis.

DDB1-cereblon and GSPT1 Protein Expression and Purification

ZZ-domain-6xHis-thrombin-tagged human cereblon (amino acids 40 – 442) and full length human DDB1 were co-expressed in SF9 insect cells in ESF921 medium (Expression Systems) with 50 μ M zinc acetate. Cells were lysed in 50 mM Tris-HCl pH 7.5, 500 mM NaCl, 10 mM imidazole, 10% glycerol, 5 mM BME, Salt Active Nuclease (Sigma-Aldrich), and Protease Inhibitor XL Capsules, EDTA-free (Pierce) using a handheld homogenizer. The lysate was centrifuged at 38,400 x g for 45 minutes, and the clarified lysate was incubated with Ni-NTA affinity resin (Qiagen) with rotation for 1 hr. The complex was eluted with lysis buffer with 250mM imidazole, and the ZZ-domain-6xHis tag was removed by thrombin cleavage (Enzyme Research) overnight, and combined with dialysis in lysis buffer. The cleaved protein was run over a HisTrap column (GE Healthcare), and the flow-through and wash was diluted to 150mM NaCl and run over an ANX HiTrap ion exchange column (GE Healthcare). The ANX column was washed with 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 3 mM TCEP, followed by 50 mM Bis-Tris pH 6.0, 150 mM NaCl, 3 mM TCEP. The CRBN-DDB1 peak was run over a Superdex S200 26/60 column (GE Healthcare) in 10 mM HEPES pH 7.0, 240 mM NaCl, and 3 mM TCEP. The cereblon-DDB1 complex was concentrated to 50 mg/mL.

GSPT1 domains 2 and 3 (amino acids 437–633) were expressed as an MBP-fusion in *Escherichia coli* BL21 (DE3) Star cells (Life Technologies) using 2XYT media (Teknova). Cells were induced at OD600 0.6 and grown overnight at 16 °C. Cells were pelleted, resuspended in 50 mM Tris pH 7.5, 200 mM NaCl, 1 mM TCEP, 10% glycerol,

lysozyme (Sigma), Benzonase (Novagen), and Protease Inhibitor XL Capsules, EDTA-free (Pierce). Cells were sonicated and the lysate was centrifuged at 384,000 x g for 45 min. The clarified lysate was incubated with amylose resin (NEB) at 4 °C with rotation for 1 hr. The protein was eluted in lysis buffer with 10 mM maltose, and the MBP tag was removed by overnight cleavage with thrombin (Enzyme Research). Cleaved GSPT1 was diluted to 90 mM NaCl and run over a Heparin HiTrap column (GE Healthcare). The GSPT1 peak was run over a Superdex 75 26/600 column (GE Healthcare) in 10 mM HEPES pH 7.0, 240 mM NaCl, and 3 mM TECP. The peak containing GSPT1 was concentrated to 10 mg/mL for crystallization trials.

DDB1-cereblon-GSPT1-CC90009 Crystallization and Structure Solution

Crystallization of the complex was achieved by sitting-drop vapour diffusion. Cereblon–DDB1 and GSPT1 were mixed together to equimolar stoichiometry at a final concentration of 150µM. The solution of cereblon–DDB1–GSPT1 in the presence of 500µM CC-90009 was mixed 1:1 with, and subsequently equilibrated against, a mother liquor solution of 300mM sodium citrate, 100mM Tris-HCl (pH 7.5), 20% PEG 3350 and incubated at 20 °C. Crystals were cryoprotected in the reservoir solution supplemented with 20% ethylene glycol and cooled under liquid nitrogen. Data were collected from a single crystal at the Advanced Light Source, beamline 5.0.2. The structure of human cereblon–DDB1–GSPT1–CC-90009 was solved by molecular replacement using Phaser¹⁶, with human cereblon–DDB1–GSPT1–CC-885 (PDB code 5HXB) as a search model. Subsequent manual model building using Coot and refinement were performed using Phenix with non-crystallographic symmetry and external structure restraints.¹⁷ Crystallographic statistics are summarized in supplemental Table 5.

Statistics analysis

All data were analyzed and graphed using GraphPad Prism software version 6.0 and 7.

The comparison of the data in drug- versus vehicle-treated mice was performed using multiple t tests, or a paired two-tailed Student's t test.

Data Sharing Statement

RNAseq data are available at GEO under access number GSE154257

2. Supplemental Tables

Excel files for supplemental Tables 1, 2 and 3 are uploaded separately

Supplemental Table 1. List of CC-90009 enriched genes with $\log_2FC >1$ and $FDR < 0.05$ relative to DMSO control. Sheet 1, related to Figure 3C-D, CC-90009 enrichment score and gene essentiality score of 705 top enriched genes; Sheet 2, related to Figure 3E-F, CC-90009 enrichment score of individual sgRNA for the 705 top enriched genes; Sheet 3, related to Figure 3D, CC-90009 enrichment score and gene essentiality score of 10 common essential genes; Sheet 4, related to Figure 3D, CC-90009 enrichment score and gene essentiality score of sgRNAs targeting the intronic regions of 9 genes.

Supplemental Table 2. Pathway enrichment analysis of top ranked genes enriched by CC-90009 in the U937 CRISPR screen. Related to Figure 3C. Fisher's Exact Test of CC-90009 enriched genes with $\log_2FC >2$ and $FDR < 0.05$ relative to DMSO control.

Supplemental Table 3. Gene and exons with evidence of statistical differential expression in *ILF3*^{-/-} U937 cells versus control U937 cells. Related to Figure 4E-F. Sheet 1, genes with evidence of statistical differential expression in U937-Cas9 cells expressing ILF3-specific sgRNA (sgILF3-2) versus non-targeting sgRNA control (sgNT-1); Sheet 2, genes with statistical differential exon usage in U937-Cas9 cells expressing

ILF3-specific sgRNA (sgILF3-2) versus non-targeting sgRNA control (sgNT-1); Sheet 3, combined list of genes shown in sheet 1 and sheet 2.

Supplement table 4. CRISPR screening data quality control

Label	Total sgRNAs	Total sgRNA Reads	Mapped sgRNA Reads	Percentage of sgRNAs with perfect match	sgRNAs with Zero counts	Gini Index	Average count per sgRNA
T3-DMSO-1	150076	165139568	146834928	88.92%	0	0.02595	978.4
T3-DMSO-2	150076	159072185	142723755	89.72%	0	0.02614	951
T12-DMSO-1	150076	171283985	151317107	88.34%	12	0.04416	1008.3
T12-DMSO-2	150076	164833679	145700462	88.39%	13	0.04447	970.8
T12-10uM-1	150076	70926567	62690261	88.39%	331	0.06669	417.7
T12-10uM-2	150076	91821268	81932541	89.23%	336	0.06394	545.9

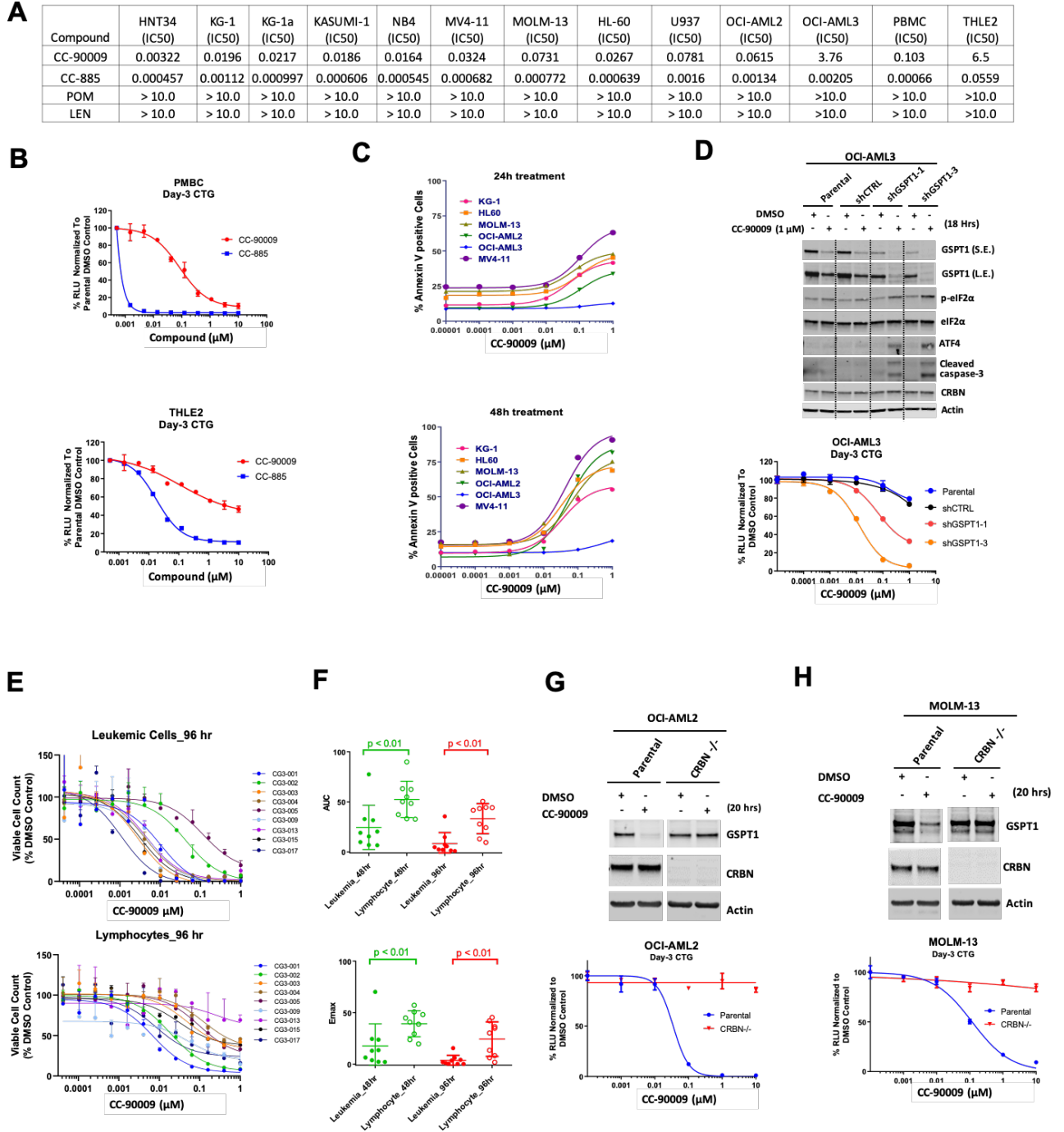
Supplemental table 5. Data collection and refinement statistics

	GSPT1-CC90009-cereblon-DDB1
Wavelength	0.977
Resolution range (Å)	74.45 - 3.64 (3.77 - 3.64)
Space group	<i>P</i> 1 2 1
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	157.616 112.041 176.582
α , β , γ (°)	90 95.7906 90
Total reflections	225387 (22547)
Unique reflections	68917 (6809)
Multiplicity	3.3 (3.3)
Completeness (%)	99.82 (99.66)
Mean <i>I</i> / σ (<i>I</i>)	4.49 (2.33)
Wilson <i>B</i> -factor	86.75
<i>R</i> -merge	0.1184 (0.4706)
<i>R</i> -meas	0.1419 (0.5644)
<i>R</i> -pim	0.07746 (0.3084)
CC1/2	0.992 (0.696)
CC*	0.998 (0.906)
Reflections used in refinement	68917 (6804)
Reflections used for <i>R</i> -free	3355 (303)
<i>R</i> -work	0.2079 (0.2972)
<i>R</i> -free	0.2455 (0.3016)
CC(work)	0.929 (0.736)
CC(free)	0.922 (0.753)
Number of non-hydrogen atoms	25483
macromolecules	25417
ligands	66
Protein residues	3347
RMS(bonds)	0.003
RMS(angles)	0.68
Ramachandran favored (%)	92.71
Ramachandran allowed (%)	6.96
Ramachandran outliers (%)	0.33
Rotamer outliers (%)	0.07
Clashscore	8.19
Average <i>B</i> -factor	93.1
macromolecules	93.14
ligands	77.48

Statistics for the highest-resolution shell are shown in parentheses

3. Supplemental Figures

Supplemental Figure 1



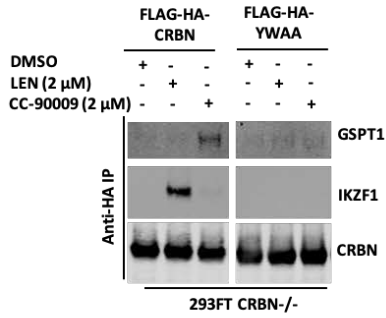
Supplemental Figure 1. The antiproliferative effect of CC-90009 in AML is cereblon-dependent

A) The growth inhibitory IC_{50} values of CC-90009, CC-885, pomalidomide (POM) and lenalidomide (LEN) in AML cell lines, PBMC and THLE. Cells were incubated with DMSO or increasing concentrations of test compounds ranging from 0.1 nM to 10 μ M for 3 days. The IC_{50} value was determined using ActivityBase (IDBS). Data are the mean of 4 or more biological replicates. **B)** The growth inhibitory effect of CC-90009 and CC-885 on PBMC (top) and THLE2 (bottom). Cells were incubated with DMSO, CC-90009 or CC-885 at the indicated concentrations. On Day 3 cell proliferation was assessed using CTG. **C)** Induction of apoptosis in AML cell lines by CC-90009. Cells were treated with DMSO or CC-90009 at the indicated concentrations for 24 (top) or 48 hours (bottom), followed by flow cytometry assessment of cell viability using 7-amino-actinomycin D (7-AAD) and Annexin V. **D)** Immunoblot analysis (top panel) and cell proliferation (bottom panel) of OCI-AML3 parental cells or cells transiently transduced with lentiviral vectors expressing control shRNA (shCNTL) or *GSPT1* specific shRNAs as indicated. Twenty-four hours after lentiviral transduction, cells were selected with 1 μ g/mL Puromycin for an additional 2 days. Then, cells were treated with DMSO or CC-90009 at indicated concentrations for 18 hours (top) or 3 days (bottom). S.E., short exposure; L.E., long exposure. **E)** The effect of CC-90009 on cell viability of leukemic cells (top) and normal lymphocytes (bottom) in bone marrow aspirates of AML patients. Cells were treated with DMSO or increasing concentrations of CC-90009 for 96 hours. Cells were then stained with fluorescently labeled antibodies and Annexin-V, followed by flow cytometry to determine the cell number of live leukemic cells and lymphocytes.

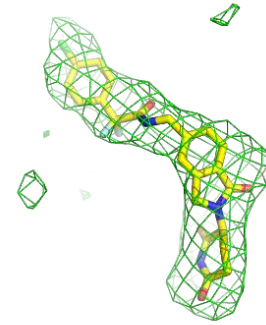
F) The activity under the curve (AUC; **top**) and maximum efficacy (E_{max}; **bottom**) of CC-90009 in leukemic cells (solid circle) and lymphocytes (open circle) as shown in Figure 1C and supplemental figure 1E. A paired two-tailed Student's t-test was used to determine the statistical significance of the difference between the AUC of leukemic cells compared to lymphocytes. **G)** and **H)** Immunoblot analysis (top) and cell proliferation (bottom) of OCI-AML2 (**G**) and MOLM-13 (**H**) parental and *CRBN*^{-/-} cells. Cells were treated with DMSO or CC-90009 at indicated concentrations. Data in (**B-H**) are shown as mean ± SD, n=3 technical replicates. Result shown in all figure panels is representative of at least three biological replicates.

Supplemental Figure 2

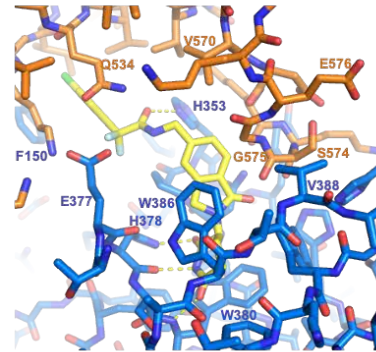
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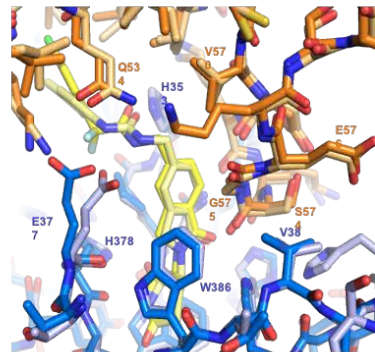
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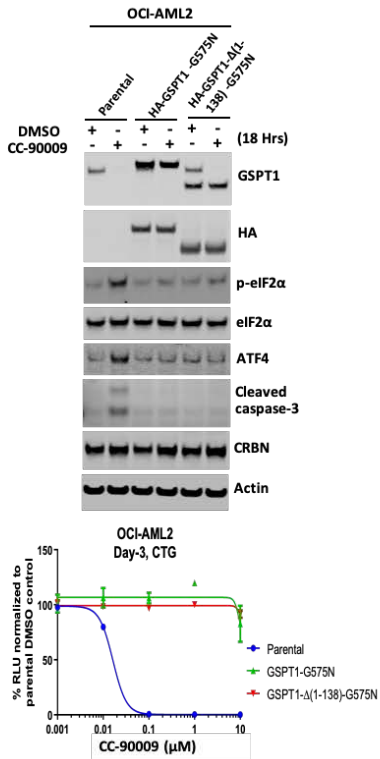
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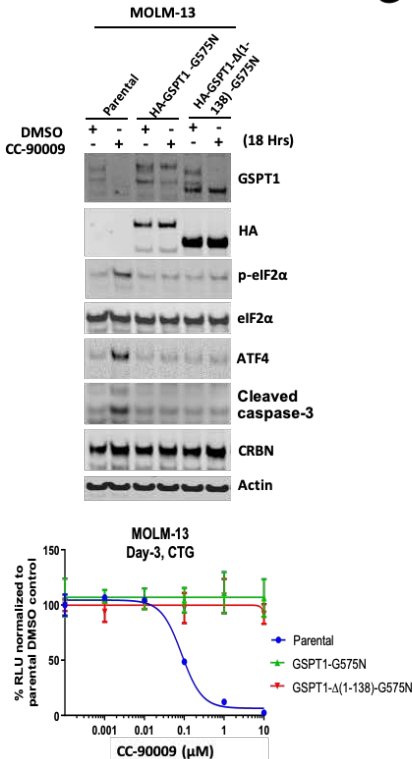
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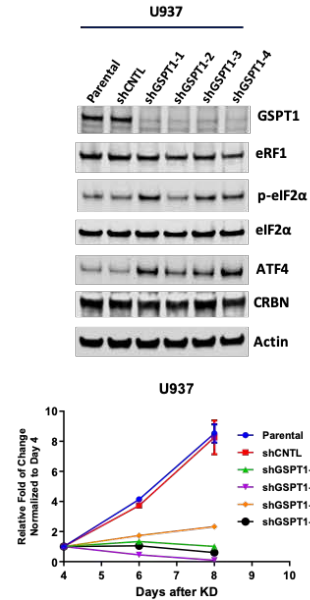
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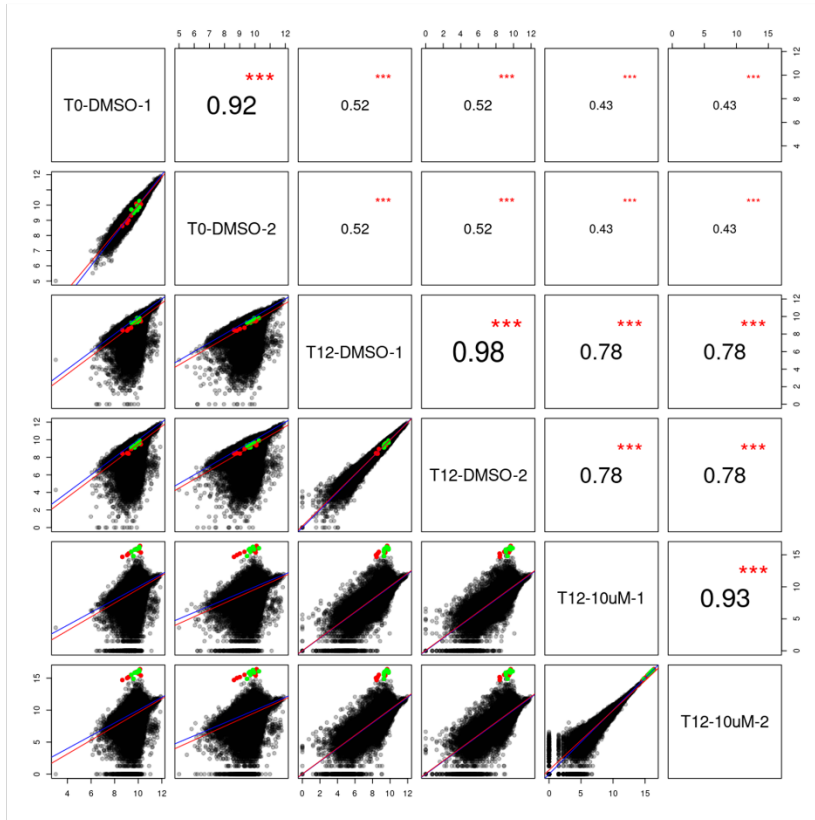
Supplemental Figure 2. Selective degradation of GSPT1 induced by CC-90009 results in growth inhibitory effects in AML

A) FLAG-HA tagged cereblon wild-type (FLAG-HA-CRBN) or Y384A/W386A mutant (FLAG-HA-YWAA) produced in 293FT *CRBN*^{-/-} cells was used to capture V5 tagged GSPT1 (GSPT1-V5) and V5 tagged IKZF1 (IKZF1-V5) transiently expressed in 293FT *CRBN*^{-/-} cells. DMSO, lenalidomide (LEN) or CC-90009 was added into the binding assay. Left panel, immunoblot analysis of anti-HA immunoprecipitates; Right panel, total input for Figure 2A. Immunoblot analysis of 293FT *CRBN*^{-/-} cells transiently transfected to produce to FLAG-HA tagged cereblon wild-type or YWAA mutant, or V5-tagged GSPT1 or IKZF1. **B)** *F_o* - *F_c* omit electron density (green mesh) for CC-90009 (yellow sticks) contoured at 3.0 σ . **C)** and **D)** Crystal structure of GSPT1 in complex with cereblon, DDB1 and CC-90009. **C)** Details of the binding interface between cereblon and GSPT1. CC-90009 is represented as yellow sticks. Predicted polar interactions between CC-90009 and cereblon are shown as yellow dashes. **D)** Superimposition of the DDB1-cereblon-GSPT1-CC-90009 and DDB1-cereblon-GSPT1-CC-885 structures. For the CC-885 structure GSPT1 is shown in light orange, cereblon in light blue, and CC-885 in light yellow. **E)** and **F)** Immunoblot analysis (top panel) and cell proliferation (bottom panel) of OCI-AML2 (**E)** and MOLM-13 (**F)** parental cells and cells stably expressing HA tagged GSPT1-G575N. Cells were treated with DMSO or CC-90009 at indicated concentrations. **G)** Immunoblot analysis (top panel) and cell proliferation (bottom panel) of U937 parental cells or cells transiently transduced with lentiviral vectors expressing control shRNA (shCNTL) or *GSPT1* specific shRNAs (shGSPT1-1 to

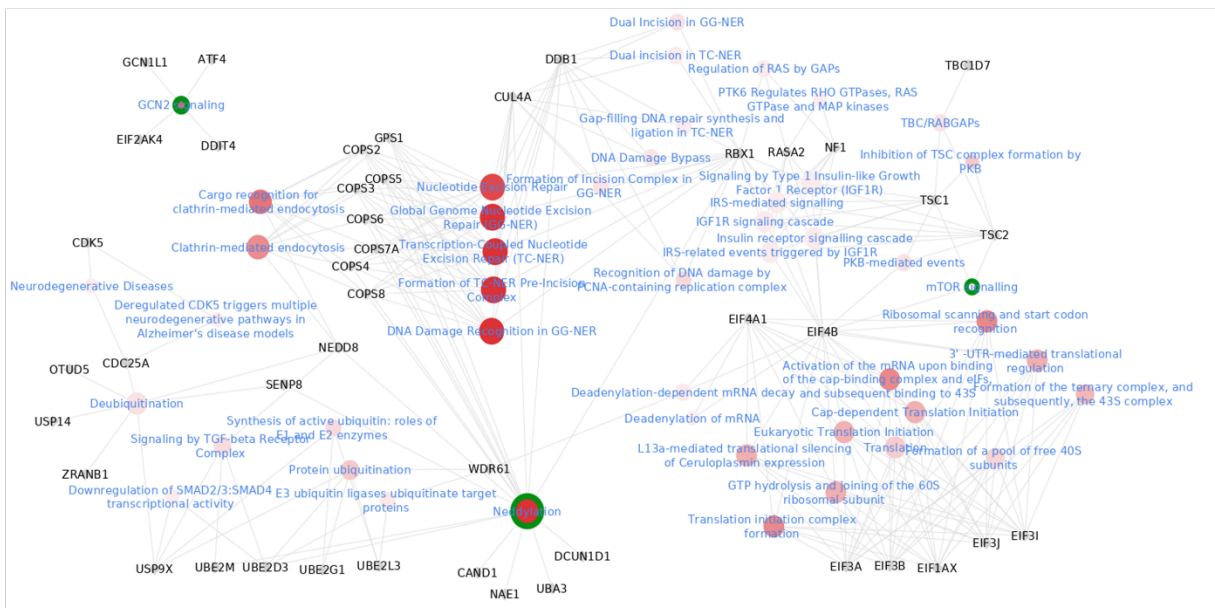
4). Twenty-four hours after lentiviral transduction, cells were selected with 1 $\mu\text{g}/\text{mL}$ Puromycin for an additional 2 days (top) or 3 days (bottom), followed by immunoblot analysis (top) or cell proliferation assessment (bottom). Data in (**E-G**, bottom panel) are shown as mean \pm SD, n=3 technical replicates. Result shown in all figure panels is representative of at least three biological replicates.

Supplemental Figure 3

A



B

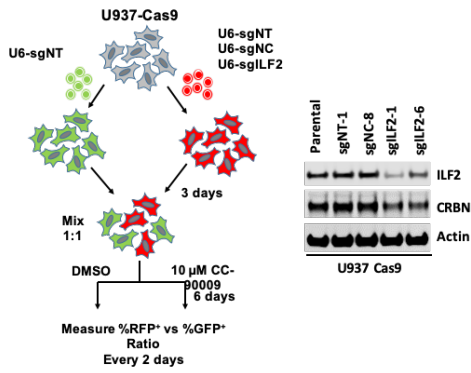


Supplemental Figure 3. CRISPR screen quality control and network analysis

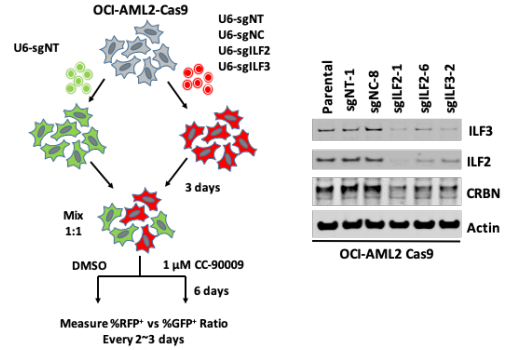
A) Normalized sgRNA read count comparison of different treatment conditions and technical replicates at day 3 and day 12 post transduction of the lentiviral sgRNA library. 150k sgRNAs are used in the scatter plots. Numbers in the upper right boxes indicate the Pearson correlation coefficient between samples. “****” indicates the correlation p-value is < 0.001 . Note a subset of sgRNAs including those targeting *CRBN* (green) and *UBE2G1* (red) clustered separately from the remaining sgRNAs. **B)** Network graph of enriched pathways among 78 top-ranked genes enriched by CC-90009 treatment in U937. Enriched pathways from the Reactome database were identified using Fisher’s exact test and were selected by adjusted p-value (FDR) < 0.05 . Pathway nodes are color-coded with different shades of red according to their statistical significance. The grey nodes in the graph depict pathway genes that were enriched by CC-90009 treatment. The core enriched pathways modulating the response to CC-90009 are highlighted with green circles.

Supplemental Figure 4

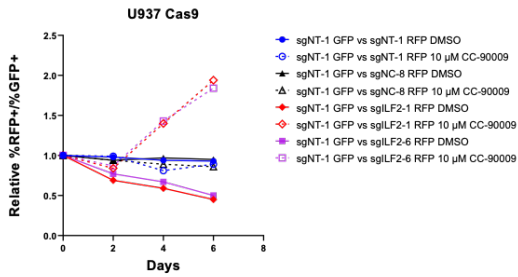
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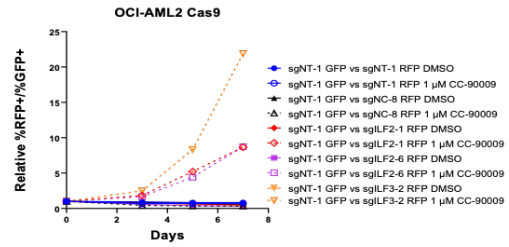
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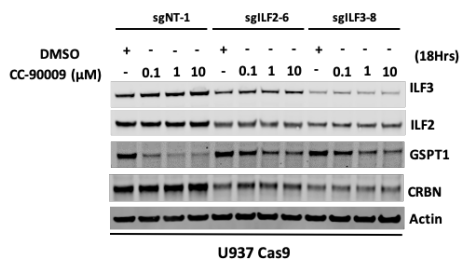
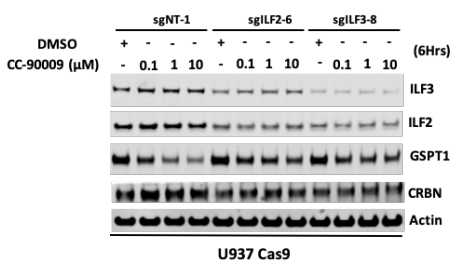
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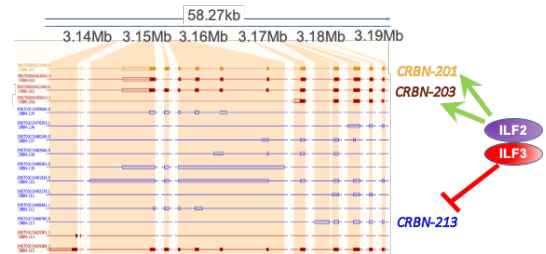
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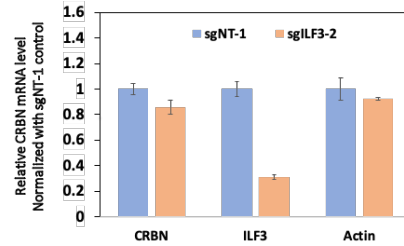
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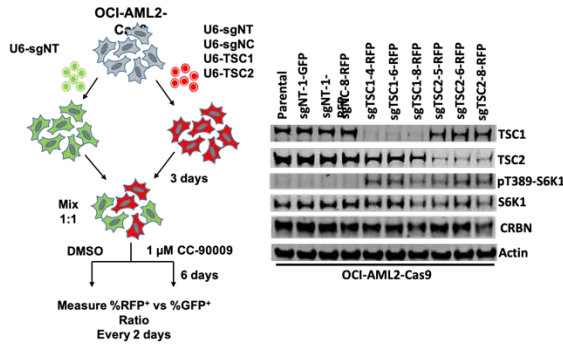
Supplemental Figure 4. The ILF2 and ILF3 complex modulates the response to CC-90009

A-B) Evaluation of the effect of *ILF2* knockout on CC-90009 response in U937 cells using a flow cytometry-based CRISPR competition assay. **A)** Left, schematic showing the design of the CRISPR competition assay; Right, immunoblot analysis of U937-Cas9 parental cells or cells expressing control sgRNAs (sgNT-1 or sgNC-8) or *ILF2*-specific sgRNAs (sgILF2-1 or sgILF2-6). **B)** The RFP+/GFP+ ratios of U937-Cas9 cells co-expressing RFP and sgNT-1, sgNC-8, sgILF2-1, or sgILF2-6 mixed with cells co-expressing GFP and sgNT-1 at each indicated timepoint were normalized to the RFP+/GFP+ ratio of the cell mixtures on “Day 0.” **C)** Immunoblot analysis of U937-Cas9 cells stably expressing the indicated sgRNAs. Cells were treated with DMSO or CC-90009 at the indicated concentrations for 6 hours (top panel) or 18 hours (bottom panel). **D-E)** Evaluation of the effect of *ILF2* or *ILF3* knockout on CC-90009 response in OCI-AML2 using a flow cytometry-based CRISPR competition assay. **D)** Left, schematic showing the design of the CRISPR competition assay; Right, immunoblot analysis of OCI-AML2-Cas9 parental cells or cells expressing sgRNAs as indicated. **E)** The RFP+/GFP+ ratios of OCI-AML2-Cas9 cells co-expressing RFP and sgNT-1, sgNC-8, sgILF2-1, sgILF2-6 or sgILF3-2 mixed with cells co-expressing GFP and sgNT-1 at each indicated timepoint were normalized to the RFP+/GFP+ ratio of the cell mixtures on “Day 0.” **F)** A schematic diagram adopted from Ensembl showing the genomic locus of *CRBN* and the gene structures of 15 *CRBN* mRNA transcripts. Box, exon; solid line, intron; shaded area in each box, protein coding region; unfilled area in each box,

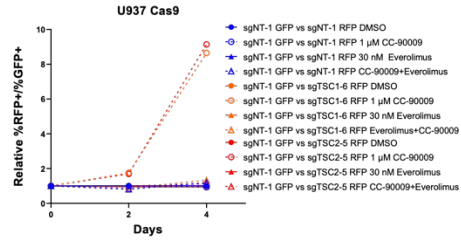
untranslated region. *CRBN* transcripts 201 and 203 encode two full-length cereblon proteins with one amino acid difference in the N-terminus. *CRBN* transcript 213 containing a cryptic exon 5 with a premature stop codon encodes a truncated cereblon protein lacking most of its functional domain. **G)** Quantitative PCR analysis of total *CRBN* mRNA level in U937-Cas9 cells stably expressing sgNT-1 or sgILF3-2. Result shown in all figure panels is representative of at least three biological replicates

Supplemental Figure 5

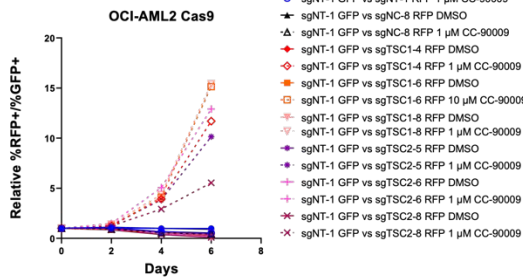
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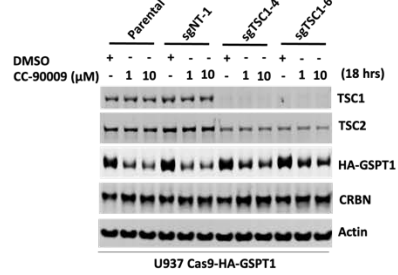
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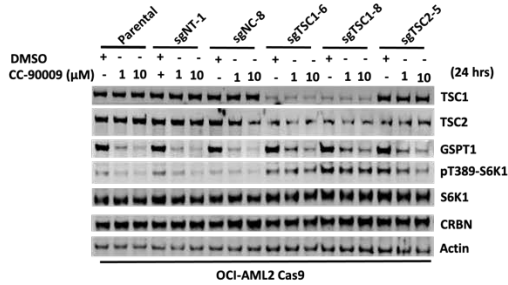
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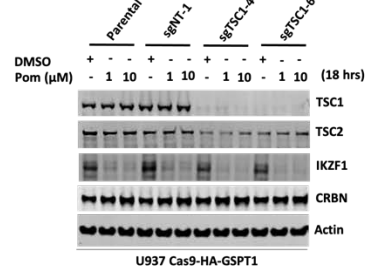
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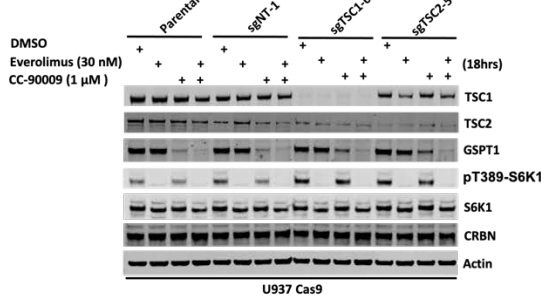
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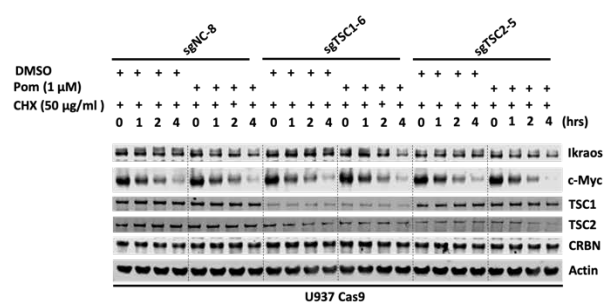
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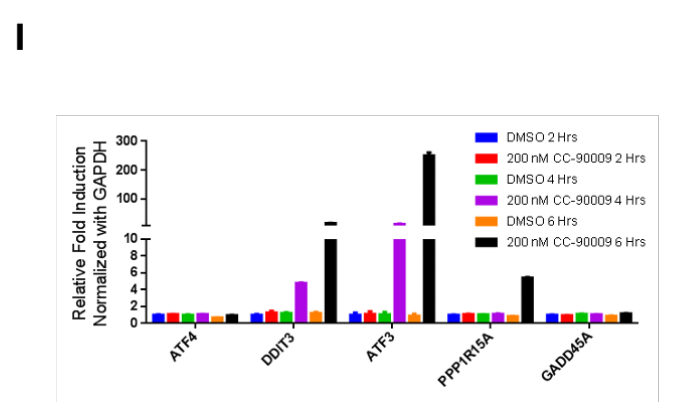
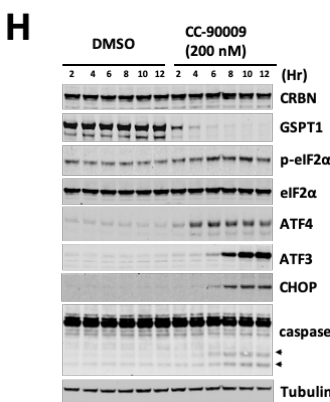
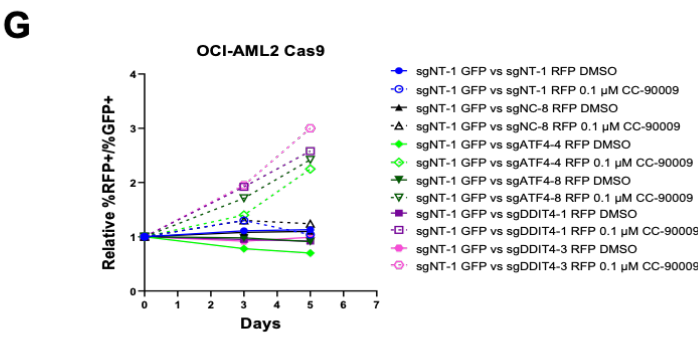
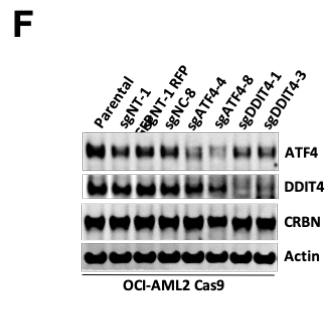
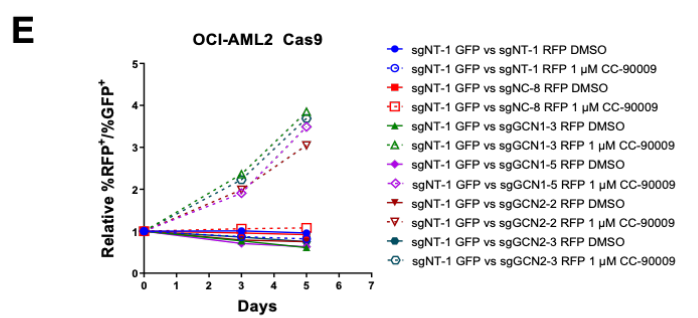
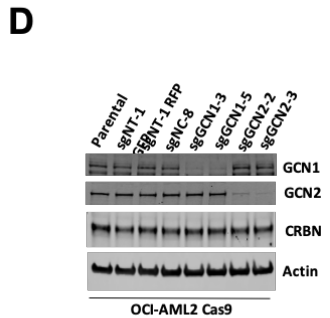
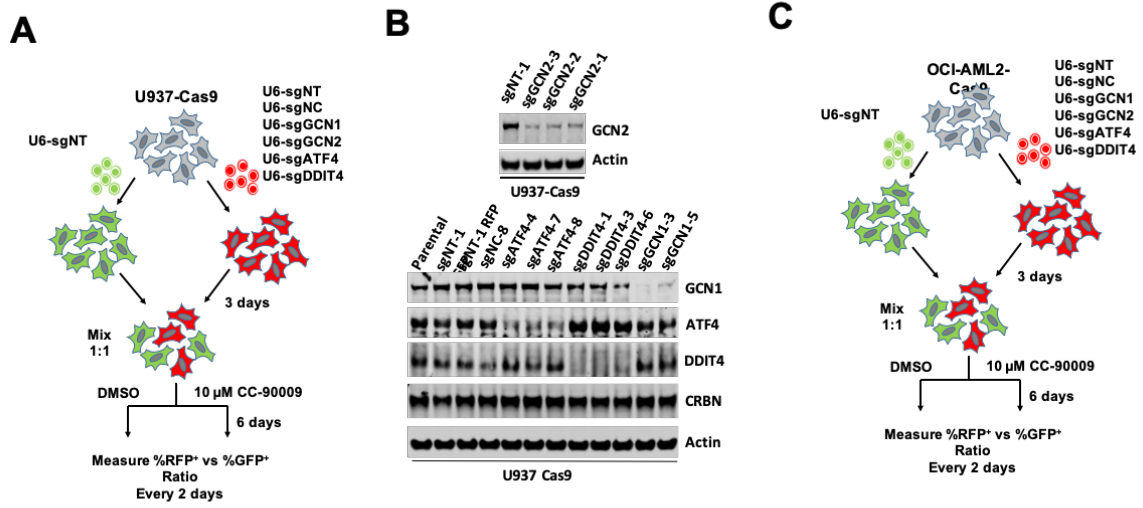


Supplemental Figure 5. Hyperactivation of mTOR signaling results in diminished response to CC-90009

A-B) Evaluation of the effect of *TSC1* or *TSC2* knockout on CC-90009 response in OCI-AML2 cells using a flow cytometry-based CRISPR competition assay. **A)** Left, schematic showing the design of the CRISPR competition assay; Right, immunoblot analysis of OCI-AML2-Cas9 parental cells or cells expressing the indicated sgRNAs. **B)** The RFP+/GFP+ ratios of OCI-AML2-Cas9 cells co-expressing RFP and the indicated sgRNAs mixed with cells co-expressing GFP and sgNT-1 at each indicated timepoint were normalized to the RFP+/GFP+ ratio of the cell mixtures on “Day 0”. **C)** Immunoblot analysis of OCI-AML2-Cas9 cells stably expressing the indicated sgRNAs. Cells were treated with DMSO or CC-90009 at the indicated concentrations for 24 hours. **D)** Immunoblot analysis of U937-Cas9 cells stably expressing the indicated sgRNAs. Cells were treated with DMSO, CC-90009 (1 μ M), everolimus (30 nM), or both CC-90009 and everolimus for 18 hours. **E)** Evaluation of the effect of *TSC1* or *TSC2* knockout on CC-90009 response in U937 cells in the presence or absence of everolimus using a flow cytometry-based CRISPR competition assay. The RFP+/GFP+ ratios of U937-Cas9 cells co-expressing RFP and the indicated sgRNAs mixed with cells co-expressing GFP and sgNT-1 at each indicated timepoint were normalized to the RFP+/GFP+ ratio of the cell mixtures on “Day 0”. **F-G)** Immunoblot analysis of U937-Cas9 cells stably expressing HA-tagged GSPT1 and the indicated sgRNAs. Cells were treated with DMSO, CC-90009 (**F**) or pomalidomide (Pom; **G**) at the indicated concentrations for 18 hours. **H)** Immunoblot analysis of U937-Cas9 cells stably expressing sgRNAs as indicated. Following treatment with 50 μ g/L cycloheximide and DMSO or 1 μ M

Pomalidomide (Pom), cells were collected at the indicated time points and subjected to immunoblot analysis. Result shown in all figure panels is representative of at least three biological replicates.

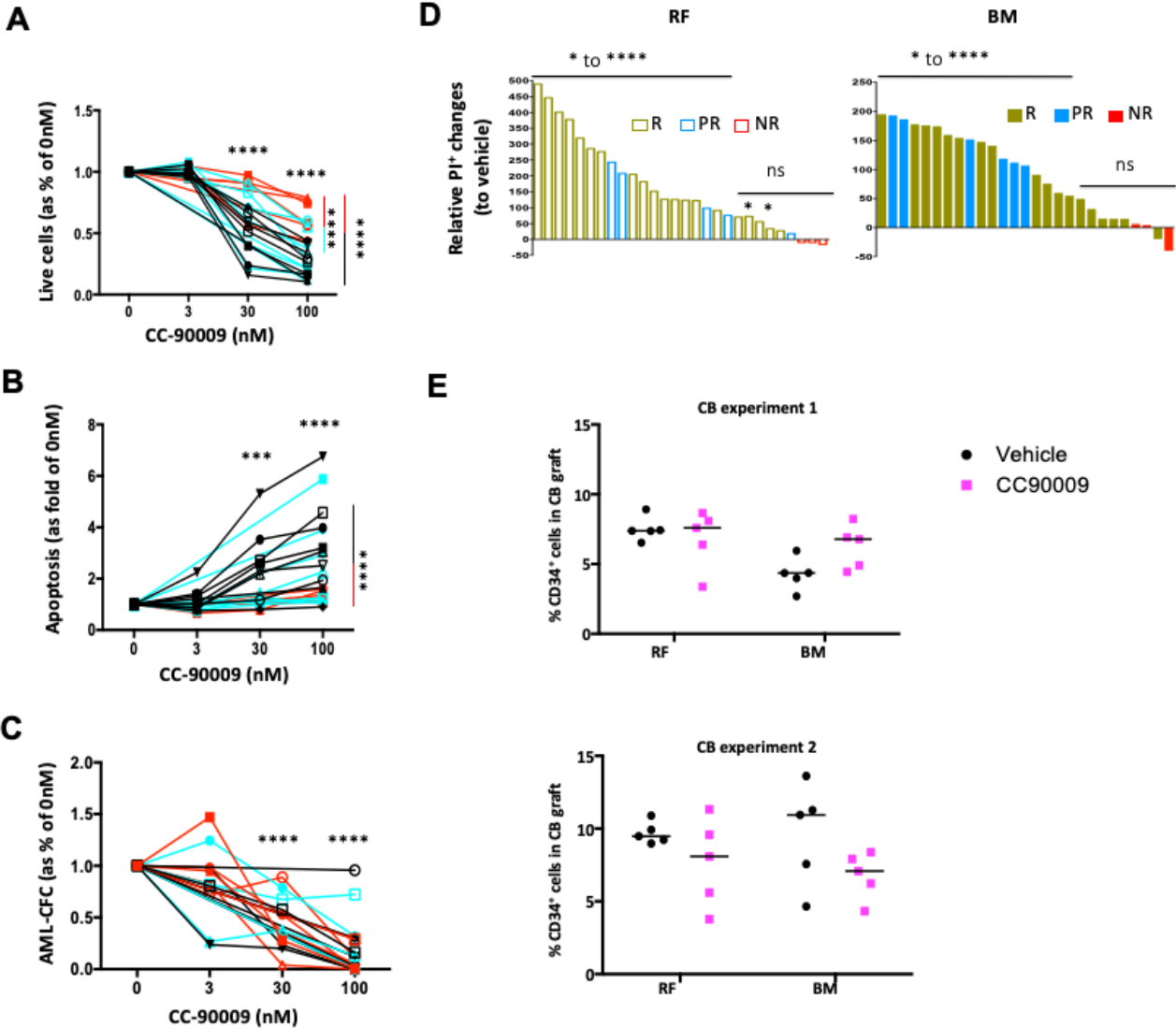
Supplemental Figure 6



Supplemental Figure 6. The AML activity of CC-90009 is mediated by the GCN1/GCN2/ATF4/DDIT4 signaling cascade

A) A schematic diagram showing the design of the flow cytometry-based CRISPR competition assay as shown in Figure 6A-D. **B)** Immunoblotting analysis of U937-Cas9 parental cell or cells expressing the indicated sgRNAs used in the CRISPR competition assay as shown in Figure 6A-D. **B-G)** Evaluation of the effect of *GCN1*, *GCN2*, *ATF4* or *DDIT4* knockout on CC-90009 response in OCI-AML2 cells using a flow cytometry-based CRISPR competition assay. **C)** A schematic diagram showing the design of the CRISPR competition assay. **D, F)** Immunoblot analysis of OCI-AML2-Cas9 parental cells or cells stably expressing the indicated sgRNAs. **E, G)** The RFP+/GFP+ ratios of OCI-AML2-Cas9 cells co-expressing RFP and the indicated sgRNAs mixed with cells co-expressing GFP and sgNT-1 at each indicated timepoint were normalized to the RFP+/GFP+ ratio of the cell mixtures on “Day 0”. **H)** Immunoblot analysis of whole cell extracts of KG-1 cells. Cells were incubated with DMSO or 200 nM CC-90009 and lysed at the indicated time points. Arrows pointing to bands in the blot on the right designate the cleaved forms of caspase-3. **I)** Quantitative RT-PCR analysis of indicated mRNA transcript in KG-1 cells incubated with DMSO or 200 nM CC-90009 for 2, 4 or 6 hours. Data in **(I)** are shown as mean \pm SD, n=3 technical replicates. Result shown in all figure panels is representative of at least three biological replicates.

Supplemental Figure 7



Supplemental Figure 7. Inhibitory effect of CC-90009 on primary AML cells in vitro and in vivo.

(A) Number of live AML cells at 24 hours in culture with CC90009 at indicated concentrations. (B) Apoptosis of AML cells after treatment with CC-90009 at indicated concentrations. (C) Numbers of colony-forming AML cells after treatment with CC-90009 at indicated concentrations. Each line indicates data relative to 0nM CC-90009

for an individual patient sample (n=23). Lines in black, cyan and red represent samples with greater than 70%, 50-70%, and less than 50% GSPT1 reduction, respectively, at the 100nM treatment dose. (D) Ratio of PI⁺ events in the mouse RF and BM of CC-90009-treated mice relative to controls. Relative change for each patient sample was calculated as (Median of CC90009 – median of Vehicle)/Median of vehicle x 100. R: responder; PR: partial responder; NR: non-responder. (E) Percentage of normal CD34⁺ primitive cells in the cord blood graft from the vehicle- and CC-90009-treated mice. Each dot indicates data from each individual mouse. ns: not-significant; * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

4. Supplemental References

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