SUPPLMENTAL METHODS

Vectors

The IKZF3 reporter vector ("Aiolos aa130-189 in Artichoke", Addgene #74451) contains SFFV.IKZF3(aa130-189)-17aaRigidLinker-EGFP.IRES.mCherry.cppt.EF1a.PuroR in a lentiviral backbone. The EF1a.PuroR and lentiviral backbone were derived from the Zhang lab LentiGuide-Puro plasmid (Addgene #52963). A derivative version of the IKZF3 reporter was generated which lacks EF1a.Puro ("Aiolos aa130-189 in Pea", Addgene #74459). The gRNA-EGFP vector used for flow cytometry-based competition assays ("Banana", Addgene) contains U6.gRNAclonesite.Scaffold.cppt.EF1a.PuroR.IRES.EGFP in a lentiviral backbone also derived from LentiGuide-Puro (Feng Zhang, Addgene #52963). gRNAs were cloned into the vector using BsmBI restriction enzyme sites. LentiGuide-Puro (Addgene #52963) and pLentiCas9-Blast (Addgene #52962) were both generated by the Feng Zhang lab. The following gRNAs sequencing were used for MM1S, NCIH929 and HEK293T CRISPR knockout experiments:

*CRBN*_gRNA_1	GCACCATACTGACTTCTTGA
*CRBN*_gRNA _2	TTACATACTGTATGTGATGT
*COPS5*_gRNA _1	ACAGCAGCAAGAAATCCTGG
*COPS5*_gRNA _2	GGAGGCAACTTGGAAGTGAT
*UBE2M*_gRNA _1	GCGCAGCTGCGGATCCAGAA
*UBE2M*_gRNA _2	TCCTGGAGCCAGGTGGGCCA
*UBE2D3*_gRNA _1	AATGACAGCCCATATCAAGG
*UBE2D3*_gRNA _2	CTTGCCTTAGGTGGTTTGAA
*UBE2G1*_gRNA _1	TGACAATGATCTCTACCGAT
*UBE2G1*_gRNA _2	CTACTGCGAAGACAGCTGGC
Control_gRNA _1	GACGGAGGCTAAGCGTCGCAA
Control_gRNA _2	GCGCTTCCGCGGCCCGTTCAA.

All novel vectors derived for use in this paper have been deposited in Addgene under "Ebert Lab".

Genome-Scale CRISPR-Cas9 Screen

The optimal lenalidomide dose and endpoints for the screen were identified using *CRBN* as a positive control. MM1.S cells with stable expression of Cas9 (pLentiCas9-Blast, Addgene #52962) were infected with a non-targeting control gRNA or *CRBN*-targeting gRNA + EGFP. The infected cells were then selected with 1µg puromycin for three days, then day 8 post-infection the control and *CRBN* populations were mixed at a 99:1 ratio respectively and cultured in lenalidomide (Selleck Chemicals) for 20 days **(Figure S1)**. Cells were dosed every two days and passaged every four. With each passage, the percentage of EGFP+ cells was measured using flow cytometry. The minimal lenalidomide dose required to elicit >60 fold enrichment by day 12 was 1µM lenalidomide, and we added a day 20 endpoint to identify hits with smaller effect sizes than *CRBN*.

For the genome-scale screen, we used the same MM1.S-Cas9 cells as those used in the above optimization assays. On day -8 of the screen the MM1.S-Cas9 cells were infected with the Human GeCKOv2 LentiGuide-Puro library (Addgene #1000000048, Broad Genetic Perturbations Platform),

and on day -7 the cells were selected with 1ug/mL puromycin for three days. The infection efficiency on day -4 was calculated to be 46% and the cells were passaged into puromycin-free media. Given a library size of ~120,000, we calculated that 120 and 60 million cells would result in a representation of each gRNA 1000 times and 500 times respectively. On day 0, 120 million cells were collected as a baseline control, 60 million cells were plated for DMSO treatment, and 6 replicates of 120 million cells were plated for 1μM lenalidomide treatment (3 replicates for the day 12 endpoint, and 3 replicates for the day 20 endpoint). The cells were dosed every two days with either DMSO or 1μM lenalidomide and passaged every four, maintaining a cell density of ~1 million/mL. On day 12, 60 million cells from the DMSO arm, and all remaining cells in the day 12 lenalidomide replicates were pelleted. On day 20, the screen concluded by pelleting 60 million cells from the DMSO arm and all remaining cells in the day 20 lenalidomide replicates.

We purified genomic DNA from the cell pellets using the Qiagen DNA Blood Maxi kit (Qiagen #51192). The gRNA sequences were the PCR amplified from the gDNA using primers tailed with barcodes and Illumina sequencing adaptors. The resulting amplicons were then pooled and sequenced across four lanes of the Illumina HiSeq. Reads counts were normalized and log2 transformed, and the triplicate read counts for a given gRNA were averaged. To identify gRNAs which increased in frequency in the lenalidomide treatment condition we subtracted the DMSO read count for a given gRNA from the corresponding lenalidomide day 12 or day 20 read count. The STARS algorithm was employed to collapse the gRNA enrichment results by gene and generate statistics (Mudra Henge and John Doench, Broad Genetic Perturbations Platform, http://www.broadinstitute.org/rnai/public/software/stars).

IKZF3 reporter counter screen

We designed gRNAs for the top 30 genes identified in the genome-scale screen (FDR<0.05) using the Broad Genetic Perturbations Platform gRNA prediction algorithm (https://iwww.broadinstitute.org/rnai/db/analysis-tools/sgrna-design). Three gRNAs per gene were chosen with priority given to gRNAs low predicted off-target effects, location in the first 30% of the protein, and target sequences located in different exons. 12 gRNAs targeting no known sequences were included as negative controls. gRNAs were ordered from IDT as paired, single-stranded oligos, after which they were annealed and cloned using BsmBI restriction enzyme sites into the LentiGuide-Puro vector (Addgene #52963). The resulting plasmids were then pooled and used to generate a lentiviral library

Gene_gRNA	gRNA Sequence (5'-3')
***CRBN*_1**	GCACCATACTGACTTCTTGA
***CRBN*_2**	GGGCGGTTACCAGGCAGGAG
***CRBN*_3**	TTACATACTGTATGTGATGT
***GPS1*_1**	CCAGGATCTGGAACAGTACG
***GPS1*_2**	TTAAACACCTGAACCGGCAG
***GPS1*_3**	ACCTGACGTCAACTACGTGG
***COPS2*_1**	GGAATTCTATGAAACAACAC
***COPS2*_2**	TTATATCTCTACTTCTAAAC
***COPS2*_3**	GGCATTAAGCAGTTTCCAAA
***COPS3*_1**	ACTGTTCACGAACTGCTCCA
***COPS3*_2**	AGGATGCCAATTCCTCGCAG

***COPS3*_3**	GATGGGATAAGTTCTTCGCA
***COPS4*_1**	GCCCAGCTCATGAATTCGAG
***COPS4*_2**	AGAAATCTATCACTTCACCT
***COPS4*_3**	AATGCAGCCCAAGTGTTGGT
***COPS5*_1**	ACAGCAGCAAGAAATCCTGG
***COPS5*_2**	GGAGGCAACTTGGAAGTGAT
***COPS5*_3**	GCCTTGAAAATGCAATCGGG
***COPS6*_1**	GAGATGTTGAGAATGACAAG
***COPS6*_2**	GCTCTGATTGGCAAGCAGGA
***COPS6*_3**	GCAGCTACGAACGGGACCGG
***COPS7A*_1**	AAACACTGTGAGCAGCCGGA
***COPS7A*_2**	CAGCTGCCGCACATTACGCA
***COPS7A*_3**	GAATCTTCCTCCACTAACAG
***COPS7B*_1**	TCAAGGCCAGAGAATGGCAG
***COPS7B*_2**	TCTTGATGCCAAGCTCACGA
***COPS7B*_3**	GGGACATACCCAGATTACAT
***COPS8*_1**	GCTGGACCGTCTCAGACCAC
***COPS8*_2**	AGAAGCTGACCATACACTGG
***COPS8*_3**	GGCCGCGAAGATGCCAGTGG
***CAND1*_1**	CCAGCCCTAGACTTGCAGTG
***CAND1*_2**	GCAGAAAGATTCCATCAAGT
***CAND1*_3**	AGATGAAGATGAAAATGCAA
***DDB1*_1**	CGACATGTCGTACAACTACG
***DDB1*_2**	CTACCAACCTGCGATCACCA
***DDB1*_3**	TGACATGCAGCTCCTCCAGG
***GLMN*_1**	AAGAGATAGCTGATTCCAAA
***GLMN*_2**	AGAATTGTGCTGTCAGCAAA
***GLMN*_3**	AATATGGGCTGGAATCTCGT
***OTUB1*_1**	CCAGCCGCTCTGACACCAGA
***OTUB1*_2**	GCGCTGTTTAAAGATGGCGG
***OTUB1*_3**	GCAGGACCGAATTCAGCAAG
***PLAA*_1**	GTAAAGATCTTACCTGAACA
***PLAA*_2**	AGACTTTGTGACAACAGCAG
***PLAA*_3**	TGAGGGTAGATGTCACTTGA
***UBE2M*_1**	GCGCAGCTGCGGATCCAGAA
***UBE2M*_2**	TCCTGGAGCCAGGTGGGCCA
***UBE2M*_3**	TGACCAGCTTGAAGTTGAGG
***UBE2D3*_1**	AATGACAGCCCATATCAAGG
***UBE2D3*_2**	TACTTACTATCATCCCCAAC
***UBE2D3*_3**	CTTGCCTTAGGTGGTTTGAA
***UBE2G1*_1**	TGACAATGATCTCTACCGAT
***UBE2G1*_2**	ACAGAAATCTGGCACCCAAA
***UBE2G1*_3**	CTACTGCGAAGACAGCTGGC
***TRAF2*_1**	TGTGCTGCCTGTGTTCACGA
***TRAF2*_2**	GGGGACCCTGAAAGAATACG

***TRAF2*_3**	GGACACTCACCTTCTCCCGG
***EDC4*_1**	TTACTCACCCCGAATGGCAT
***EDC4*_2**	AGTGGAAACCTCATGATGGG
***EDC4*_3**	GTCCATATTCGGCAGCCAGA
***NCOR1*_1**	TTGCTTTCAGAATTTCACCC
***NCOR1*_2**	CCATGAGCTGATCATAACGC
***NCOR1*_3**	TCATGTTTAGAGTTGGGCAG
***DEPDC5*_1**	TTAGTCGCCACATATCCCCA
***DEPDC5*_2**	TCATGATCCTGACCAAGCAG
***DEPDC5*_3**	GATCCTGTCGAATTGAGGCT
***SRP14*_1**	GCTGATCTTCTTCTTCCCAT
***SRP14*_2**	TATAGACGCTGCCCGACGTC
***SRP14*_3**	GGTGTTGTTGGAGAGCGAGC
***XRN1*_1**	GCAAAAATGAACCAGCAGCG
***XRN1*_2**	TTTACAGATGGATCTCAGAG
***XRN1*_3**	TCTCAAATCGAGGTAAGTTG
***EIF4A1*_1**	CTCTGCTCAGATACAGAAGG
***EIF4A1*_2**	GAGGTCTCACCCTTGATACA
***EIF4A1*_3**	TCTCACCTCGATGACGCCTT
***SNRNP25*_1**	GGCACTCTGCACTACAACCA
***SNRNP25*_2**	ATCGGCAGATCGCAGAGCAG
***SNRNP25*_3**	AATAGCCCTAGAATACGGCC
***RARA*_1**	AAGCAAGGCTTGTAGATGCG
***RARA*_2**	AGAGTGGTCAGAGCGCCTGG
***RARA*_3**	TGTTCTTCTGGATGCTGCGG
***PPP6C*_1**	ACAAGTATGTGGAAATAGCG
***PPP6C*_2**	CAGTTCTGAACAGTTCACAA
***PPP6C*_3**	TGAGAGTAGACAGATAACAC
***SPOP*_1**	CCTCCGGCAGAAATGTCGAG
***SPOP*_2**	GTTTGCGAGTAAACCCCAAA
***SPOP*_3**	GCAAAAGCTAAAGTTATTGA
***SYCP2L*_1**	AGTCCCTGCCGAATTAGCAG
***SYCP2L*_2**	GATTCCTTCCTACTTAGCTT
***SYCP2L*_3**	AAGAAGCCTCGTTATGCAAG
***DCP2*_1**	CATCATGGAGACCAAACGGG
***DCP2*_2**	GTTCAATCTGAAAACACACT
***DCP2*_3**	TCACCTTGAGGCAGCAAAAA
***NFKBIA*_1**	GAGCCGCAGGAGGTGCCGCG
***NFKBIA*_2**	GGTTGGTGATCACAGCCAAG
***NFKBIA*_3**	GACCATGGAAGTGATCCGCC
***RBX1*_1**	ATGGATGTGGATACCCCGAG
***RBX1*_2**	ACCGTGTGTTTCCAAAATGG
***RBX1*_3**	CACAATATCCCAGGCCCAGA
***CUL4A*_1**	GCAGGACCACTGCAGACAAA
***CUL4A*_2**	GAGGACACTCACCAGATGGA

***CUL4A*_3**	CGAGCGCGAGAGGAGCGGCG
***CUL4B*_1**	GCAAAAACTGAAAGAAGCAG
***CUL4B*_2**	GTTTGATGCGAAGATGGCTG
***CUL4B*_3**	GCTACTACCTCTAAAGACGG
Control_1	GACGGAGGCTAAGCGTCGCAA
Control_2	GCGCTTCCGCGGCCCGTTCAA
Control_3	GATCGTTTCCGCTTAACGGCG
Control_4	GGTAGGCGCGCCGCTCTCTAC
Control_5	GCCATATCGGGGCGAGACATG
Control_6	GTACTAACGCCGCTCCTACAG
Control_7	GTGAGGATCATGTCGAGCGCC
Control_8	GGGGCCCGCATAGGATATCGC
Control_9	GTAGACAACCGCGGAGAATGC
Control_10	GACGGGCGGCTATCGCTGACT
Control_11	GCGCGGAAATTTTACCGACGA
Control_12	GCTTACAATCGTCGGTCCAAT

NCIH929 and HEK293T cells were transduced with pLentiCas9-Blast (Addgene #52962) and selected with 2μg/mL blasticidin to generate stable Cas9-expressing lines. The MM1.S-Cas9, NCIH929-Cas9, and HEK293T-Cas9 cells were then transduced with the IKZF3 reporter ("Aiolos aa130-189 in Pea", Addgene #74459) and infected cells were isolated using Fluorescence Activated Cell Sorting (BD FACS Aria). On day 0 of the screen three technical replicates of each reporter cell type were infected with the lentiviral library at an infection efficiency below 50%, and on day 1 the cells were selected with 1 or 2μg/mL puromycin. On day 4 cells were passaged into puromycin-free media, and on day 11 the cells were dosed for 20 hours with DMSO or 1μM lenalidomide. On day 12, unsorted baseline controls were harvested from both the DMSO and 1μM lenalidomide treatment groups, and 1μM lenalidomide-treated cells were then sorted for cells remaining EGFP+.

Genomic DNA was isolated from the resulting cell pellets using the QiaAmp DNA Blood Midi and Micro Kits (Qiagen #51183, #56304). We then PCR-amplified the gRNA sequences using primers tailed with barcodes and Illumina sequencing adaptors. The resulting amplicons were pooled and then sequenced across a single lane of the Illumina NextSeq. Reads counts were normalized and log2 transformed. To identify gRNAs which increased in frequency in the lenalidomide-treated EGFP+ population we subtracted the DMSO gRNA read count from the corresponding, EGFP+ 1μM lenalidomide read count, and then averaged the triplicate values. The averaged, log2 fold enrichment was then normalized to the average enrichment of the 12 non-targeting control gRNAs.

Cell Culture

MM1.S (MM.1s, multiple myeloma, ATCC #2974) and NCIH929 (multiple myeloma, Broad Institute Cell Line Repository) were grown in RPMI supplemented with 10% fetal bovine serum, penicillin, and streptomycin.

HEK293T cells were single cell cloned via limiting dilution and subsequently grown for ~21 days prior to harvesting. Genomic DNA purification was performed using the Zymo Research ZR-96 Quick–gDNA

kit (#D3012). PCR amplification of the genomic DNA target sites was performed with site-specific primers and sequenced via next generation sequencing at the MGH CCIB DNA Core.

Viability Assays

NCIH929-Cas9 cells infected with control or *UBE2G1* gRNAs were treated with DMSO, pomalidomide (Selleck), or CC-122 (Medkoo, Morrisville, NC, USA) for 10 days, with dosing at Day 0 and Day 5. At day 10, cell viability was measured using Cell Titer-Glo Luminescent Cell Viability Assays (Promega, Madison, WI, USA) and analyzed on an Envision Plate Reader (Perkin Elmer, Waltham, MA, USA).

Competition Assays

gRNAs targeting genes of interest were cloned using BsmBI restriction enzyme sites into the gRNA-EGFP reporter vector ("Banana", Addgene) and HEK293T cells were used to generate lentivirus. MM1.S cells stably expressing Cas9 (pLentiCas9-Blast Addgene #52962) were infected with the either the gRNA-EGFP vector or control gRNAs in LentiGuide-Puro vector (Addgene #52963), selected in 1ug/mL puromycin for three days, and then expanded for 20 days until sufficient cell numbers were achieved for the experiment.

Two biological replicates (different gRNAs targeting same gene) and three technical replicates were performed.

Reporter Assays in HEK293T Single Cell Clones

Single cell HEK293T cell clones were transduced with the IKZF3 aa130-189 reporter vector ("Aiolos aa130-189 in Artichoke", Addgene #74451) and selected for three days with 2ug/mL puromycin. On day 0, 15,000 cells were plated per well in a 96-well plate. On day 1 the cells were dosed with a titration of doses of lenalidomide (HP D300 digital dispenser) and 20 hours later on day 2 the cells were harvested for flow cytometry to read the EGFP and mCherry fluorescence. Analysis consisted of calculating the EGFP:mCherry fluorescence ratio on a per-cell basis and then taking the geometric mean of the values for ~1000 cells. Three experimental replicates were performed.

***In Vitro* Ubiquitination Assays**

In vitro ubiquitination assay was run on Tris-Glycine SDS-PAGE gel and immunoblotted as indicated. Secondary antibodies IRDye 800CW Goat anti-rabbit 926-32211 and IRDye680LT Goat anti-mouse 926-68020 were purchased from Licor and imaged on Licor Odyssey.

Immunoblots

Cells were washed with phosphate buffered saline and lysed in buffer (150 mM NaCl, 50 mM Tris pH 7.5, 1% NP-40, 1% glycerol, 1X Halt Cocktail protease and phosphatase inhibitors) for 20 minutes on

ice. Cullin probed lysates were lysed with additional 2mM 1,10-orthophenathroline (SIGMA). To preclear cell lysates, we centrifuged at 13,000 rpm at 4°C for 15 minutes. We measured protein concentration with Pierce BCA protein assay kit and resolved lysates on BioRad any kD TGX polyacrylamide gels by SDS-PAGE and transferred by electrophoresis to nitrocellulose membrane (Life Technologies) at 100V for 70 minutes. Membranes were blocked in 5% milk/ TBST for 1 hour. Blots were incubated, with indicated primary antibody, overnight at 4°C. The membrane was then washed in TBST, three times at 15 minute intervals, before 1 hour secondary horseradish peroxidase-conjugated antibody incubation at room temperature. We again washed nitrocellulose membranes in TBST, three times for 15 minutes, prior to enhanced chemiluminescent substrate detection (Pierce).

Antibodies

Anti-HA (#2367), anti-UBE2M (# 4913), anti-β Actin (#3700) primary antibodies were purchased from Cell Signaling Technologies. Anti-CK1α (sc-6477) and anti-IKZF1 (sc-13039) were obtained from Santa Cruz. Anti-CRBN (#NBP1-918010) and Anti-IKZF3 (NBP2-16938) antibodies were acquired from Novus. Antibodies detecting UBE2G1 (ab135508), UBE2D3 (ab127578), COPS5 (ab12323), and cullin 4A (ab72548) were purchased from Abcam. Horseradish peroxidase-conjugated anti-rabbit and anti-mouse immunoglobulin G (IgG) antibodies were purchased from Millipore

SUPPLEMENTAL FIGURES

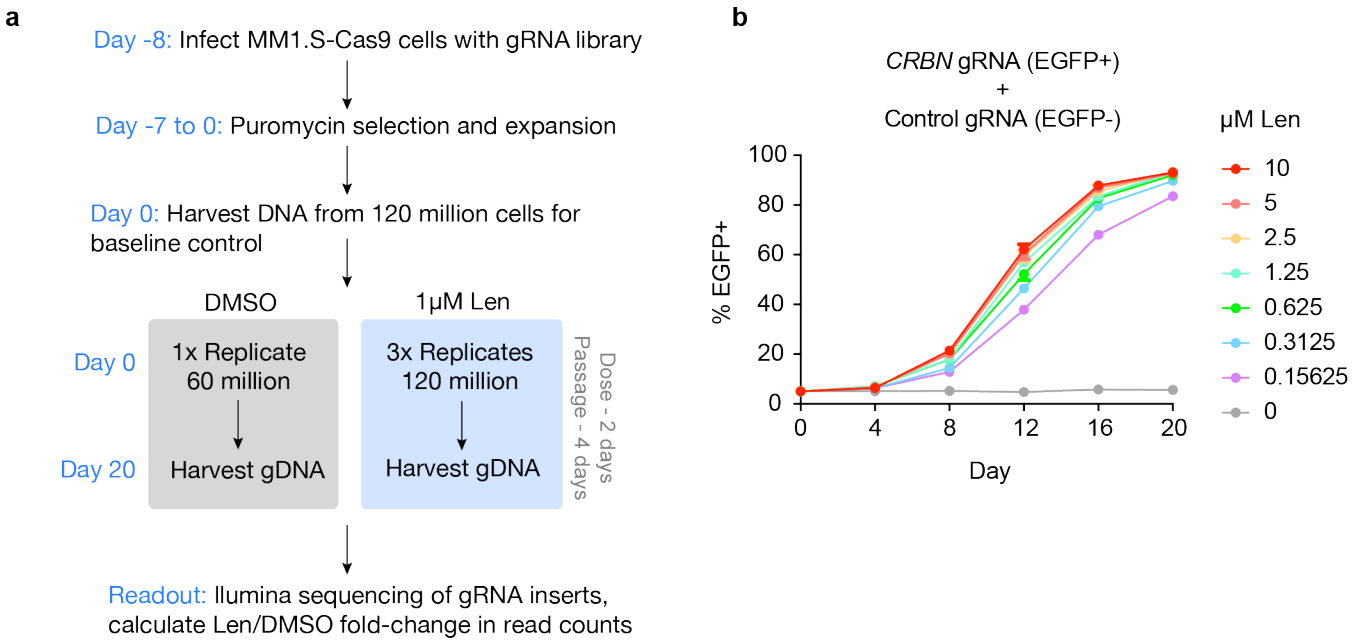

Figure S1 | *CRBN* is required for lenalidomide's anti-myeloma effects and is a non-essential gene
a, Detailed schematic of the genome-scale CRISPR-Cas9 screen.
b, MM1.S-Cas9 cells expressing EGFP were infected with gRNAs targeting *CRBN* and mixed at a 5:95 ratio with control gRNA-infected cells lacking EGFP. The cells were then grow for 20 days in the presence of lenalidomide and the percentage of EGFP+ cells was quantified every four days using flow cytometry.

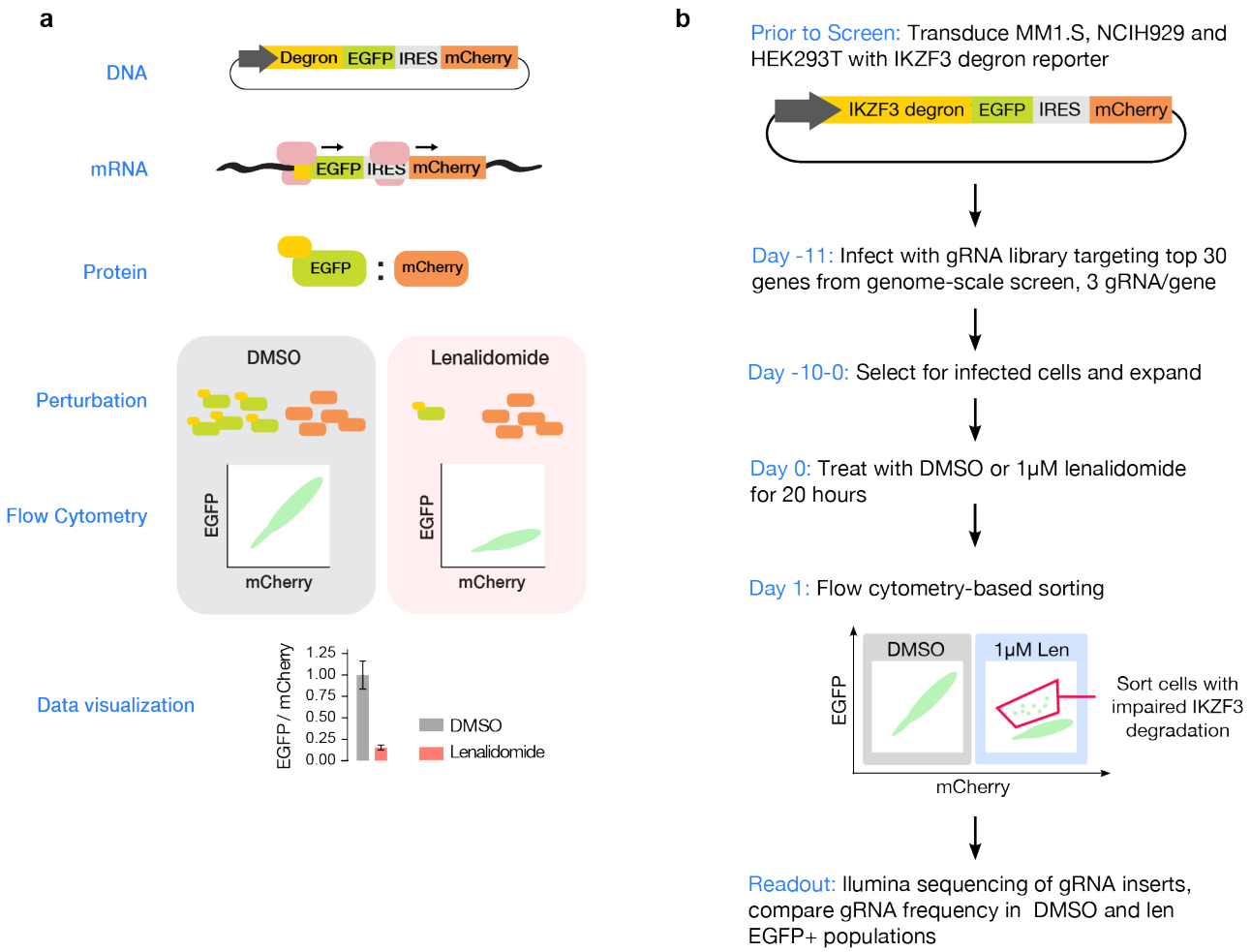

Figure S2 | IKZF3 degron reporter vector and detailed schematic of the counter screen
a, Schematic of the reporter vector and how it functions.
b, Detailed overview of the reporter-based flow cytometry counter screen.

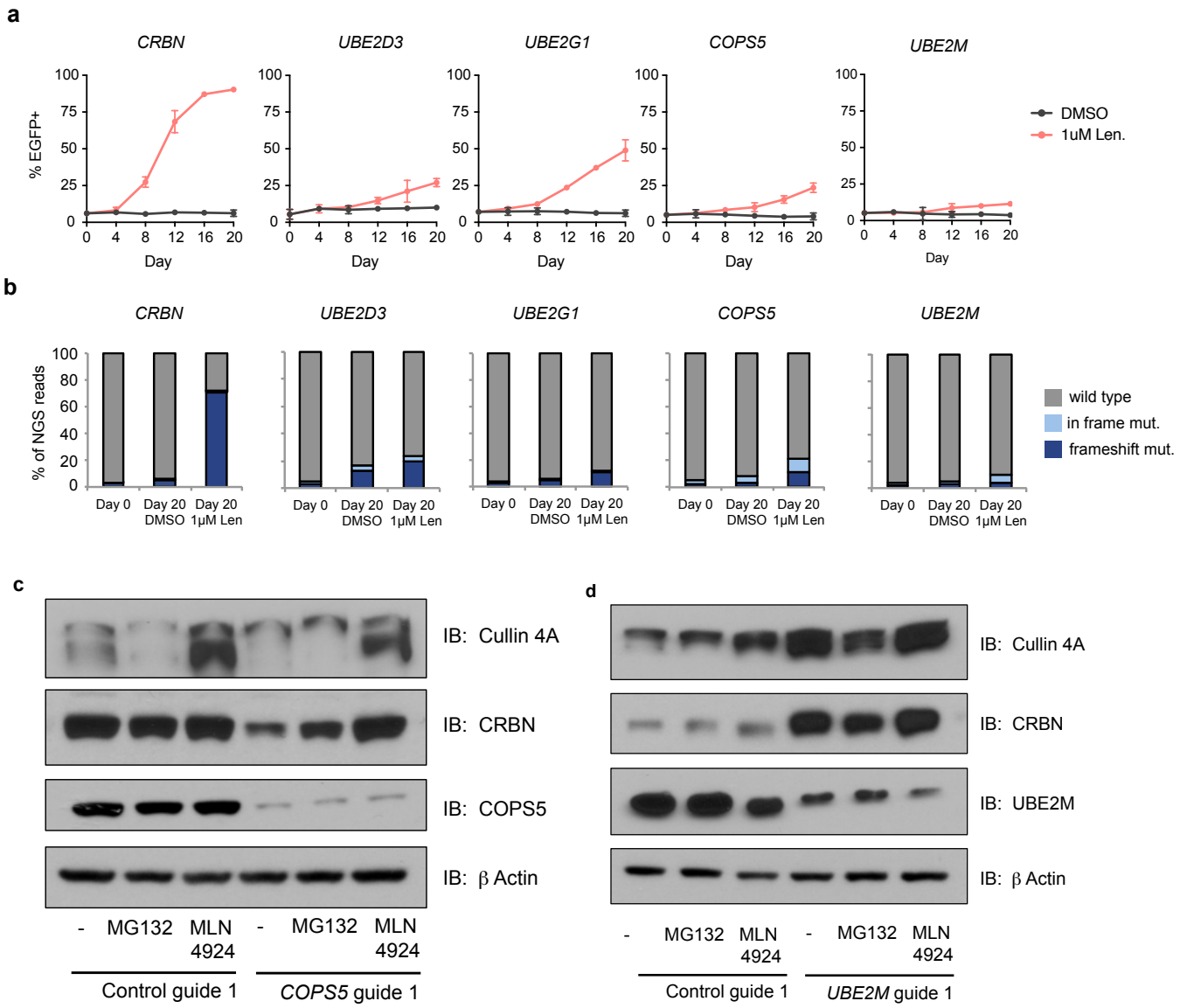

Figure S3 | Replicate competition assays and sequencing

a, Replicate experiment of Figure 3a with distinct gene-targeting and control gRNAs: MM1.S-Cas9 cells expressing EGFP were infected with gRNAs targeting indicated genes and mixed at a 5:95 ratio with control gRNA-infected cells. The cells were then grown for 20 days in the presence of DMSO or 1μM lenalidomide and the percentage of EGFP+ cells was quantified every 4 days using flow cytometry. Data points are average of three technical replicates and error bars represent 95% confidence interval.

b, gDNA was harvested from the day 0 and day 20 DMSO or 1μM Len samples, the gRNA target site was PCR amplified, and next generation sequencing was used to quantify the percentage of reads containing frameshifting indels. (indel frequency is likely underrepresented due to use of sequencing parameters for gRNA target site limiting product size to 300 base pairs, which may systematically omit detection of large indels).

c, HEK293T-Cas9 cells were infected with *COPS5* or control targeting gRNAs. The cells were then treated with 5μM MG132, 5μM MLN4924 or DMSO for 12 hours. Protein lysates were harvested and immunoblotted (IB) for the indicated proteins.

d, HEK293T-Cas9 cells were infected with *UBE2M* or control targeting gRNAs. The cells were then treated with 5μM MG132, 5μM MLN4924 or DMSO for 12 hours. Protein lysates were harvested and immunoblotted (IB) for the indicated proteins.

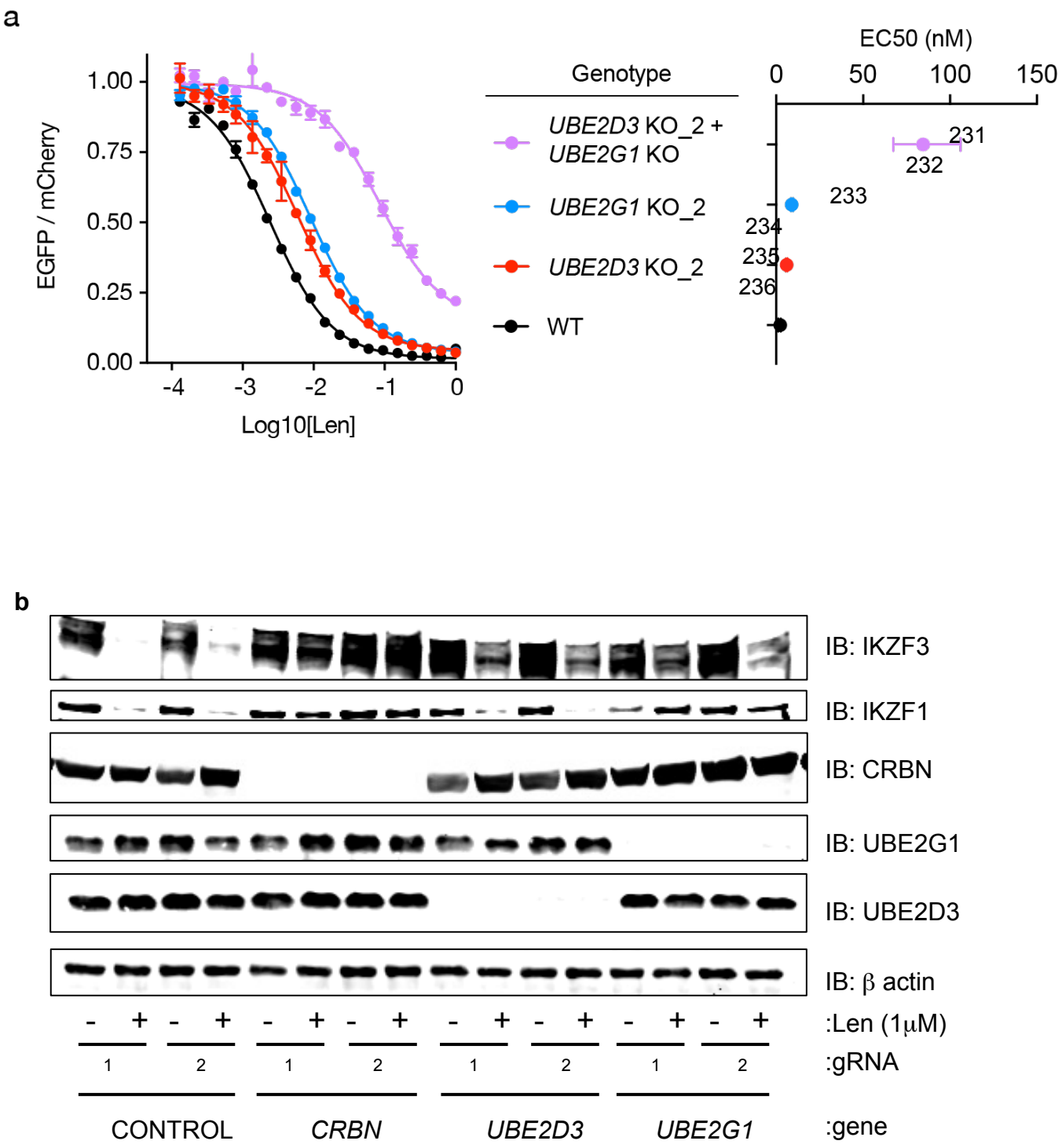

Figure S4 | Biological replicate of lenalidomide titration in HEK293T single cell clones

a, HEK293T single cell clones in which *UBE2G1* and/or *UBE2D3* were knocked out via CRISPR-Cas9 were transduced with the IKZF3 degron reporter and were then treated with a titration of lenalidomide. After 20 hours the EGFP/mCherry ratio was assayed via flow cytometry. Data points are an average of three experimental replicates and error bars represent standard error of the mean.

b, NCIH929-Cas9 cells were infected with gRNAs targeting the indicated genes, treated with DMSO or 1μM lenalidomide for 20 hours, then lysates were harvested and immunoblotted as indicated. Data are representative of three experimental replicates

256
257
258
259