Development of Antibody-based PROTACs (AbTACs) for the degradation of the cell-surface immune checkpoint protein PD-L1

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METHODS

Cell lines

Cells were grown and maintained in T75 flasks (Thermo Fisher) at 37°C and 5% CO_2. MDA-MB-231 and HeLa cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. T24 cells were grown in in McCoy's 5a supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. HCC827 cells were grown in in RPMI supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. HeLa, MDA-MB-231 and T24 cells were obtained from the UCSF Cell Culture Facility. HCC827 cells were obtained from American Type Culture Collection (ATCC). Cell lines were authenticated by the supplier.

Bio-layer interferometry (BLI) Experiments

Bio-layer interferometry data (BLI) was measured using an Octet RED384 (ForteBio) instrument. RNF43-Fc-Fusion was immobilized on a streptavidin biosensor and loaded until 1.0 nm signal was achieved. After blocking with 10 μM biotin, purified antibodies in solution was used as the analyte. PBSTB was used for all buffers. Data were analyzed using the ForteBio Octet analysis software and kinetic parameters were determined using a 1:1 monovalent binding model.

Whole cell proteomics

MDA-MB-231 cells in a T-75 flask (Corning) were treated with either 10 nM AC-1 or PBS. Following 24 hour, cells were lifted using Versene (Gibco) and pelleted by centrifugation. Cell pellets (~7 million cells) were then lysed in 6 M guanidine hydrochloride (Chem-Impex), 5 mM tris(2-carboxyethyl)phosphine (Fisher Scientific), 10 mM chloroacetamide (Sigma Aldrich), with 50 mM Tris (Thermo Fisher) for 10 minutes at 95°C with periodic vortexing. DNA in the lysate was sheared using a probe sonicator. Lysates were then diluted three-fold with 50 mM Tris, pH 8.5 and digested at room temperature overnight with 1:100 (enzyme mass:protein mass) proteomics-grade trypsin (Promega). Following digestion, peptides were desalted using SOLA HRP columns (Thermo Fisher), dried using a GeneVac system, and resuspended in 2% acetonitrile (ACN, Fisher Scientific) + 0.1% formic acid (FA, Sigma) for subsequent liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

For LC-MS/MS, 1 μg of peptides were separated using an UltiMate 3000 UHPLC system (Thermo) with a pre-packed 0.75mm x 150mm Acclaim Pepmap C18 reversed phase column (2μm pore size, Thermo) and analyzed with a Q Exactive Plus (Thermo Fisher Scientific) mass spectrometer. Separation was performed using a linear gradient of 3-35% solvent B (Solvent A: 0.1% FA, solvent B: 80% ACN, 0.1% FA) over 230 mins at 300 μL/min. A top 20 method for data-dependent acquisition was performed during analysis (dynamic exclusion 35 seconds; selection of peptides with a charge of 2, 3, or 4). A resolution of 140,000 (at 200 m/z) was used to gather full spectra in MS1 using an AGC target of 3e6, maximum injection time of 120 ms, and scan range of 400 - 1800 m/z. Centroided data from MS2 scans were collected at a resolution of 17,500 (at 200 m/z) and an AGC target of 5e4. The maximum injection time was set at 60 milliseconds. For MS2, a collision energy of 27 was used, with an isolation window of 1.5 m/z and an isolation offset of 0.5 m/z.

Raw mass spectrometry data from two independent experiments were searched and quantified using Label-free Quantitation (LFQ) in MaxQuant (Version 1.6.7). The Uniprot Human Reference Proteome was used to generate the search database (downloaded July 2019). Cysteine carbamidomethyl was set as the only fixed modification with methionine oxidation and N-terminal glutamate to pyroglutamate as variable modifications. The MaxQuant output was then filtered and data visualized using Perseus (Version 1.6.7). Briefly, contaminants and decoys were removed and only proteins identified with more than two unique peptides were carried forward. LFQ data were log_2 transformed and missing data were imputed using a normal distribution. A volcano plot was then generated using default Perseus statistical settings to visualize proteome changes following AbTAC treatment.

Generation of CRISPR knockdown cell line

RNF43 targeting sgRNAs, identified in a previously published genome-wide CRISPRi dataset[34], were cloned into a pLV hU6-sgRNA hUbC-dCas9-KRAB-T2a-Puro lentiviral vector (Addgene 71236). Lentivirus were produced by transfecting HEK293T cells with standard packaging vectors. The RNF43-knockdown MDA-MB-231 cell line was generated by transducing cells with the all-in-one CRISPRi lentivirus[35]. The stable cell line expressing dCas9-KRAB and sgRNA was selected with puromycin (1 μg/mL) and validated by quantitative reverse transcription PCR (RT-qPCR).

Degradation experiments

Cells at 70% confluency were treated with bispecific antibody or control antibody in complete growth medium. At the experiment end point, cells were washed with cold phosphate-buffered saline (PBS), lifted with Versene solution and pelleted by centrifugation (500g, 5 min, 4°C) and samples tested by either 'western blotting' or 'degradation flow cytometry'.

Western blotting

Cells pellets were lysed with RIPA buffer containing cOmplete mini protease inhibitor cocktail (Sigma) on ice for 45 min. The lysates were spun at 21,000g for 10 min at 4°C and protein concentrations were normalized using BCA assay (Pierce). 4x NuPAGE LDS sample buffer (Invitrogen) and 2-Mercaptoethanol (BME) was added to the lysates and boiled for 10 min. Equal amounts of lysates were loaded onto a 4-12% Bis-Tris gel and ran at 200V for 37 min. The gel was incubated in 20% ethanol for 10 min and then transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was then blocked in Odyssey Blocking Buffer (TBS) (LI-COR) for 45 min at room temperature with gentle shaking. Membranes were incubated overnight with rabbit-anti-PDL1 (CST: E1L3N, 1:1,000) and mouse-anti-tubulin (CST: DM1A, 1:2,000) at 4°C with gentle shaking in Odyssey Blocking Buffer (TBS) + 0.2% Tween-20, then washed five times with TBS + 0.1% Tween-20. The membrane was then incubated with HRP-anti-rabbit IgG (1:2000) for 1 h at room temperature in Odyssey Blocking Buffer (TBS) + 0.2% Tween-20. Membranes were washed five times with TBS + 0.1% Tween-20 and SuperSignal West Pico PLUS Chemiluminescent Substrate was added before imaging using a LICOR C-DiGit blot scanner. The membrane was then washed three times in TBS + 0.1% Tween-20, and 800CW goat anti-mouse IgG (1:10,000) in Odyssey Blocking Buffer (TBS) + 0.2% Tween-20 + 0.01% SDS for 1 h at room temperature. Membranes were washed five times with TBS + 0.1% Tween-20, then imaged using an OdysseyCLxImager (LI-COR). Band intensities were quantified using Image Studio Software (LI-COR)

Surface panning flow cytometry

Cells were washed with cold phosphate-buffered saline (PBS), lifted with Versene solution and pelleted by centrifugation (500g, 5 min, 4°C). Cell pellets were washed with cold PBS, before pelleting (500g, 5 min, 4°C). Cells were blocked with cold PBS + 3% BSA at 4°C for 10 min. Cells were washed three times with cold PBS + 3% BSA and 20 µg/mL biotinylated anti-RNF43 clone R3 IgG was added and incubated at 4°C for 30 min. Cells were washed three times with cold PBS + 3% BSA and neutravidin-647 (1:1,000) was added and incubated at 4°C for 30 min. Cells were washed three times with cold PBS + 3% BSA and finally resuspended in cold PBS. Flow cytometry was performed on a CytoFLEX cytometer (Beckman Coulter), and gating was performed on single cells and live cells, before acquisition of 10,000 cells. Analysis was performed using the FlowJo software package.

IgG Expression

IgGs were expressed and purified from Expi293 BirA cells according to established protocol from the manufacturer. Briefly, 30 µg of pFUSE (InvivoGen) vector was transiently transfected into 75 million Expi293 BirA cells using the Expifectamine kit. Enhancer was added 20 h after transfection. Cells were incubated for a total of 6 d at 37°C in a 8% CO_2 environment before the supernatants were harvested by centrifugation.

Fab Expression

Fabs were expressed and purified by an optimized autoinduction protocol previously described[14]. In brief, C43 (DE3) Pro + *E. coli* containing expression plasmids were grown in TB autoinduction media at 37°C for 6 h, then cooled to 30°C for 18 h. Fabs were purified by Protein A affinity chromatography. Purity was assessed by SDS/PAGE and intact protein mass spectrometry.

Bispecific IgG Expression and Assembly

Half IgG's with either knob or hole Fc domains were expressed using described IgG expression protocol. Hole half IgG's were purified using Protein A affinity chromatography. Knob half IgG's were purified using Nickel affinity chromatography, appropriating a C-terminal his tag. Half IgG's were mixed together in a 1:1 ratio in 10 mM Tris pH 7.5, 100 mM NaCl. The pH was adjusted to 8.5 with addition of 20% 800 mM L-Arg pH 10. 200-fold excess reduced glutathione in 800 mM L-Arg pH 10 was added and the mixture incubated overnight at 35°C with 150 rpm shaking. Reaction mixture was buffer exchanged into PBS pH 7.0 and purified by Nickel affinity chromatography, appropriating a C-terminal his tag on the knob half IgG. Purity was assessed by SDS/PAGE and BLI.

RT-qPCR

CRISPR knockdown cell lines were verified using RNAseq. Total RNA was extracted using the Qiagen RNAeasy kit. Reverse transcription was performed on 1µg of RNA using a Quantitect Reverse Transcription Kit. qPCR was performed on a BioRad CFX Connect, using primers: RNF43 - Hs.PT.58.19324722 – 52°C annealing temperature. And Actin - Hs.PT.39a.22214847

- 60°C annealing temperature. Experiments were performed with three biological and three technical replicates.

Phage display selections

Phage display was performed as previously described[34]. In brief selections with antibody phage Library E or Library UCSF were performed using biotinylated RNF43 Fc-fusion as the positive antigen or Biotinylated Fc-fusion for the negative selection. A 'catch and release' strategy was used with streptavidin-coated magnetic beads (Promega) and TEV protease. Four rounds of selections were performed with decreasing RNF43 Fc-fusion (100 nM, 50 nM, 10 nM, 10 nM). Individual clones from the fourth round of selection were carried onto fab-phage ELISA.

Fab-phage ELISA

384 Maxisorp plates were coated with Neutravidin (10 μg/mL) overnight at 4°C with gentle shaking and subsequently blocked with PBS + 0.05% Tween20 + 0.2% BSA (Blocking buffer) for 1 h at RT. Blocking buffer was removed and 20 nM biotinylated antigen or BSA was added and incubated at RT for 20 min. Antigen solution was removed and plates blocked for 10 min with 1 μM biotin in blocking buffer. The plate was washed three times with PBS + 0.05% Tween20. Phage diluted 1:5 in blocking buffer or blocking buffer with 20 nM soluble competitor was added and incubated for 20 min at RT. Plates were washed three times with PBS + 0.05% Tween20. Horseradish peroxidase (HRP)-conjugated anti-phage monoclonal antibody (GE LiveScience's 27-9421-01) diluted 1:5000 in blocking buffer was added and incubated for 30 min at RT. Plates were washed three times with PBS + 0.05% Tween20. HRP TMB substrate was added and incubated at RT until signal appeared. Reaction was quenched with 1 M phosphoric acid and plates analyzed at OD_{450nM} on a SpectraMax plate reader.

Confocal Microscopy

HeLa cells were transiently transfected with each described construct using FuGENE transfection reagent. After 24 h in complete growth medium cells were transferred to Mat-Tek 35 mm glass bottom petri dishes pre-treated with poly-d-lysine. After a further 12 h in complete growth medium cells were stained using standard protocols using DAPI (Cell Signaling Technologies) and LysoTracker (Invitrogen). Samples were imaged using a Nikon Ti Microscope with Yokogawa CSU-22 spinning disk confocal and a 100x objective lens. 405, 488 and 640 nM lasers were used to image DAPI, GFP, LysoTracker respectively with a step size of 0.1 μM. Images were deconvoluted and processed using NIS-Element and FIJI software packages.

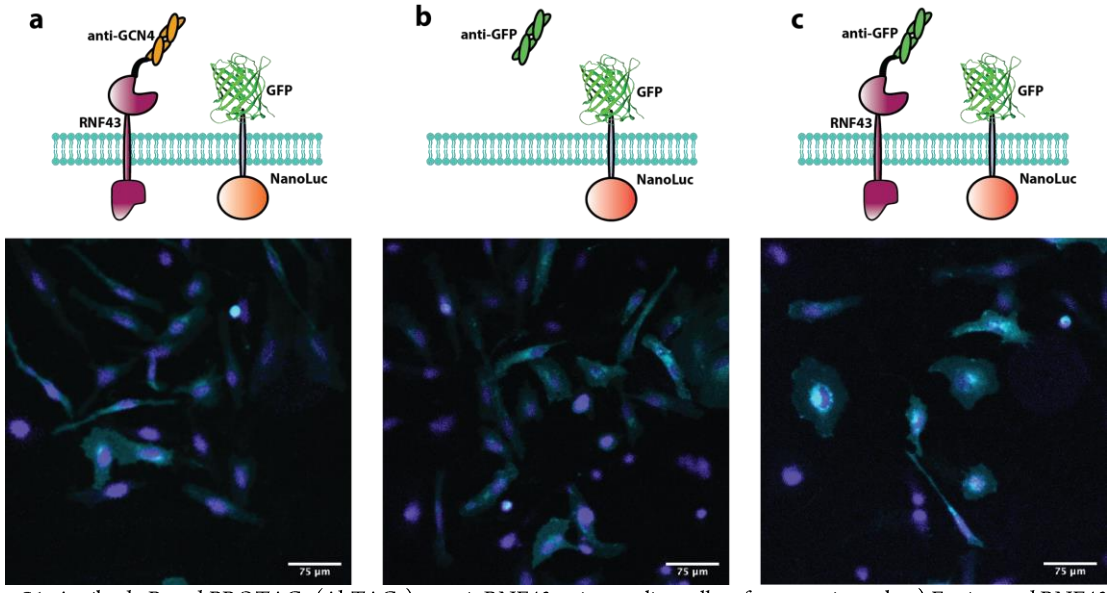

Figure S1: Antibody-Based-PROTACs (AbTACs) recruit RNF43 to internalize cell surface proteins. a, b, c) Engineered RNF43 constructs and the GFP-Nanoluciferase reporter. With their corresponding wide-field confocal microscopy images showing GFP localization for each experimental condition. a) Anti-GCN4-RNF43 fusion as an isotype control. b) Soluble Anti-GFP Fab to control for Fab binding effects. c) Anti-GFP-RNF43. (Green - GFP reporter protein, Blue – DAPI)

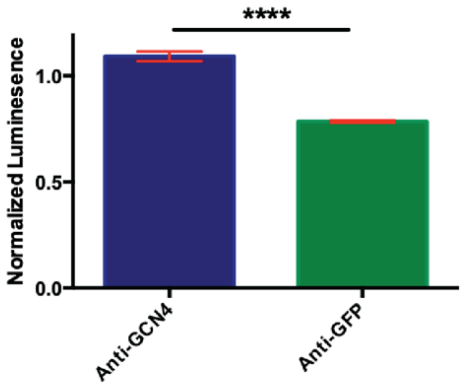

Figure S2: NanoLuciferase assay showing decrease in reporter signal after co-expression of either Anti-GFP or Anti-GCN4 scFab-RNF43 fusions. Error bars represents SD of 3 biological replicates (P value = 0.00002).

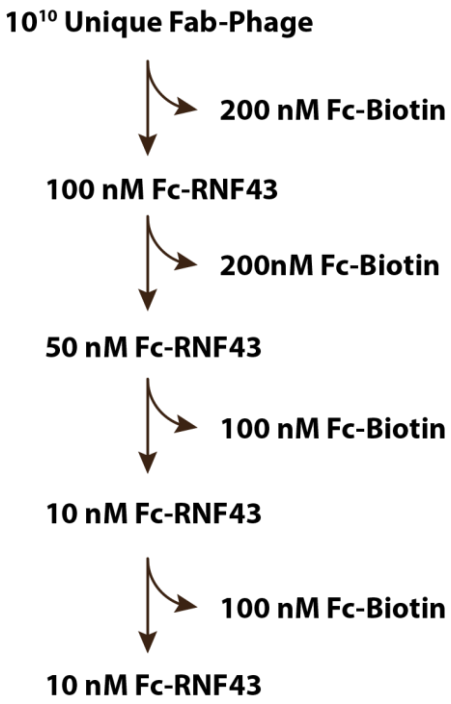

Figure S3: Phage display selection strategy utilized against RNF43 Fc-fusion.

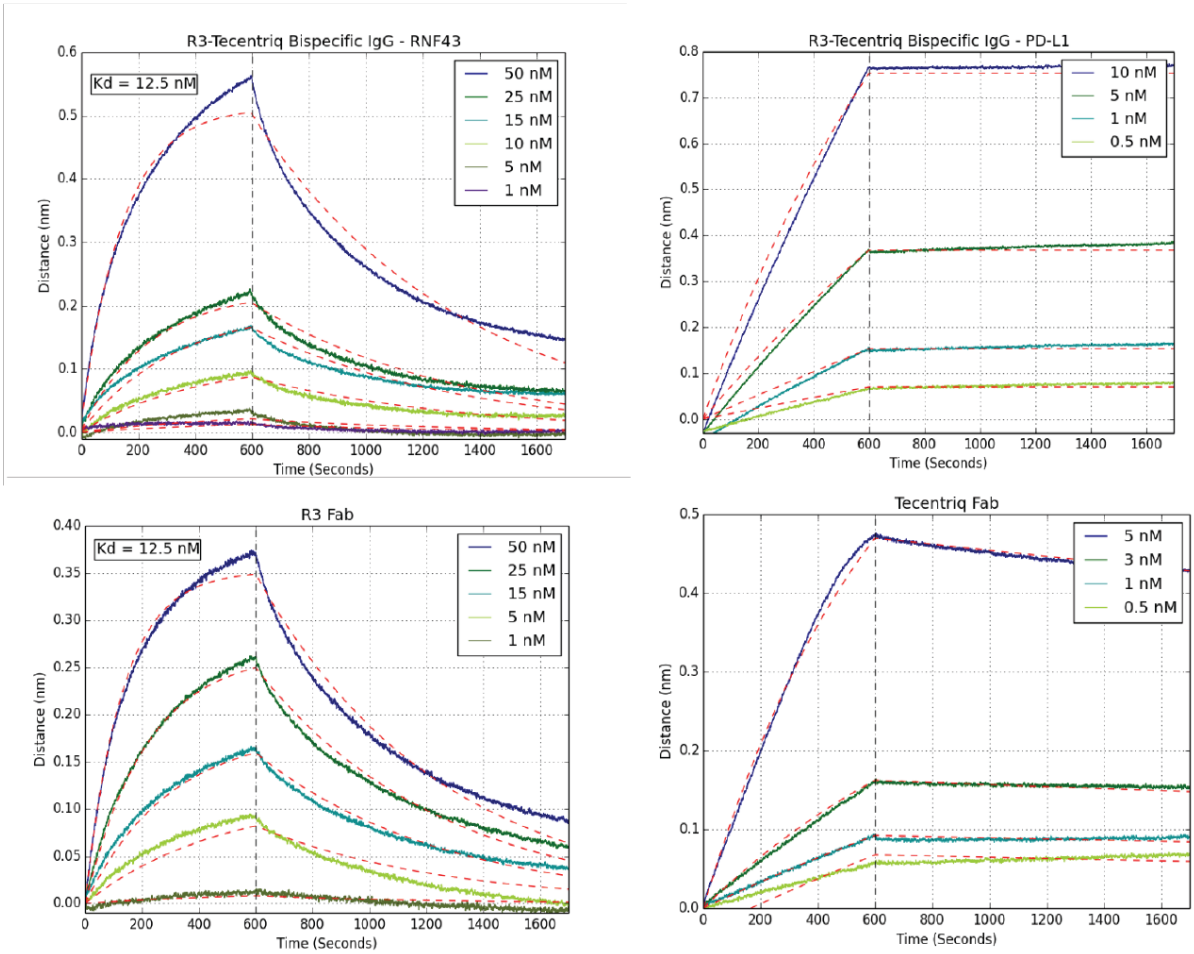

Figure S4: Dose escalation BLI experiment showing binding of AC-1 either in Fab or BsIgG format to either RNF43 (left) or PD-L1 (right).

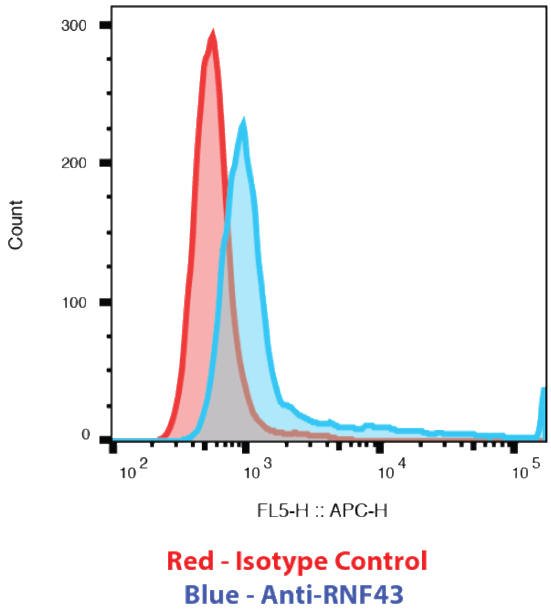

Figure S5: Flow cytometry showing presence of RNF43 on MDA-MB-231 cells. Cells were stained with biotinylated IgG clone R3 and Neutravidin-Alexa Fluor 647.

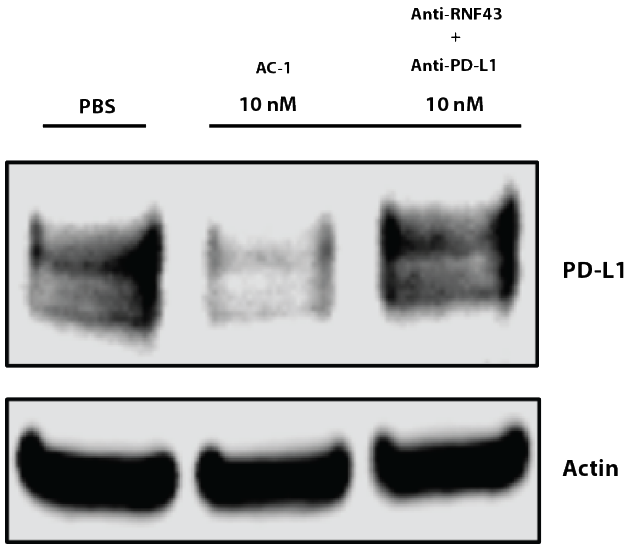

Figure S6: Degradation experiment showing PD-L1 degradation is dependent on the dimerization of RNF43 and PD-L1, where simultaneous targeting has no effect of PD-L1 levels.

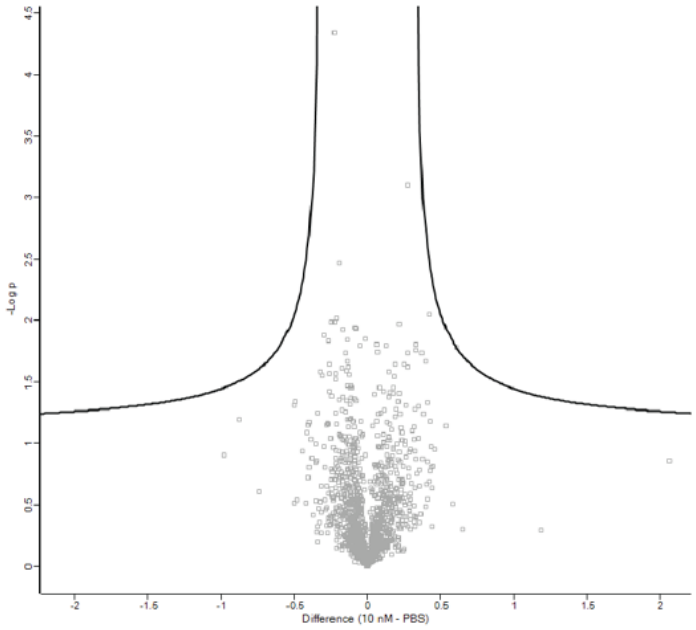

Figure S7: Whole cell proteomics showing no significant change in protein expression in MDA-MB-231 cells after 24 hours treatment with 10 nM AC-1.

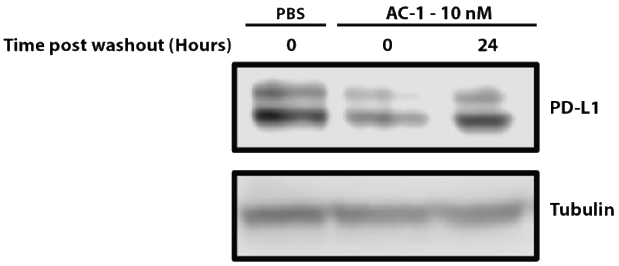

Figure S8: Wash-out experiment showing PD-L1 levels largely recover 24 hours post treatment with 10 nM AC-1.

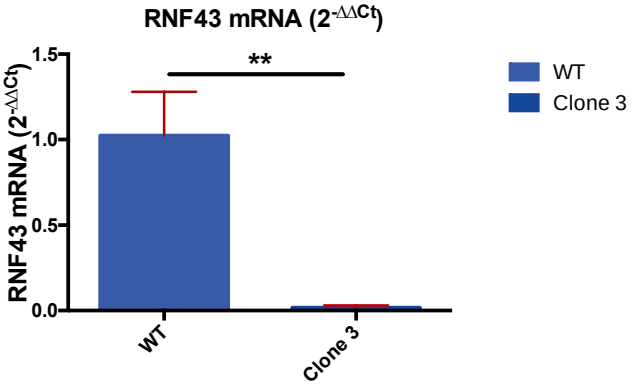

Figure S9: qPCR confirms RNF43 knockdown in the engineered MDA-MB-231 cells. Error bars represents SD of 3 biological replicates and 2 technical replicates (P value = 0.0009).

Table S1: Primers used for sgRNA cloning into the all-in-one CRISPR constructs.

sgRNA-RNF43-A-fwd	caccGGGCCCACTGGAATCCACGG
sgRNA-RNF43-A-rev	aaacCCGTGGATTCCAGTGGGCCC
sgRNA-RNF43-B-fwd	caccGTGGTTGCAGAGTAAGAAGG
sgRNA-RNF43-B-rev	aaacCCTTCTTACTCTGCAACCAC
sgRNA-RNF43-C-fwd	caccGTCGGGCCCACTGGAATCCA
sgRNA-RNF43-C-rev	aaacTGGATTCCAGTGGGCCCGAC
sgRNA-RNF43-D-fwd	caccGGGCACCTACCTGTAGTATG
sgRNA-RNF43-D-rev	aaacCATACTACAGGTAGGTGCCC
sgRNA-RNF43-E-fwd	caccGAGGCAGTATCTCTGAATCA
sgRNA-RNF43-E-rev	aaacTGATTCAGAGATACTGCCTC
sgRNA-RNF43-A-fwd	caccGGGCCCACTGGAATCCACGG