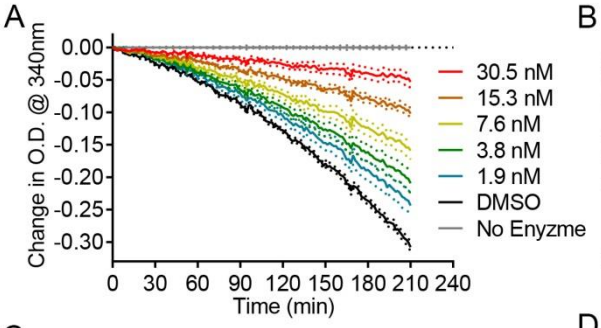
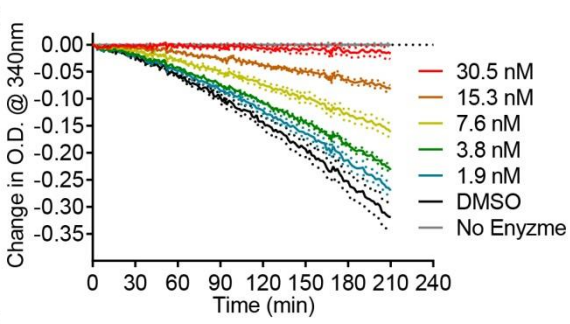
Supplemental Data

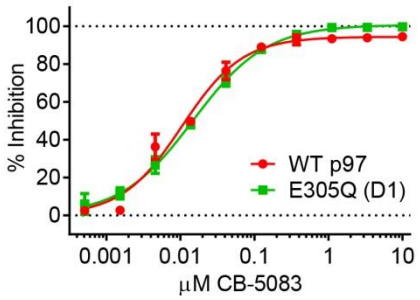
A



B



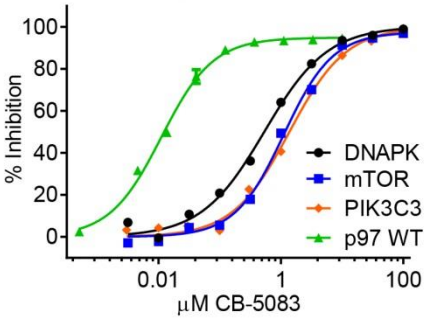
C



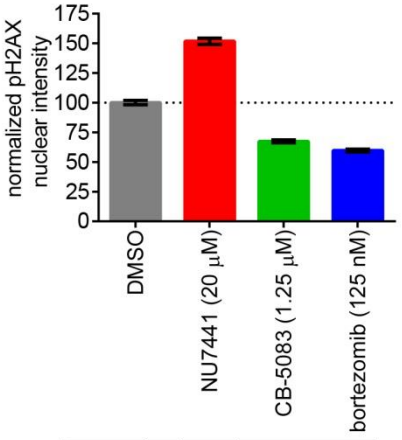
D

Target	Enzyme Class	Mean Percent Displacement (10 µM CB-5083)	K_i (nM)
p97	AAA ATPase	97	8.5
NSF	AAA ATPase	5	>50000
RPT1/PSMC2	AAA ATPase (19S)	-11	nd
RPT2/PSMC1	AAA ATPase (19S)	-1	nd
RPT4/PSMC6	AAA ATPase (19S)	-7	nd
RPT5/PSMC3	AAA ATPase (19S)	-30	nd
RPT6/PSMC5	AAA ATPase (19S)	-6	nd
DNA-PK	Kinase	87	252
mTOR	Kinase	58	1091
PIK3C3	Kinase	45.5	1345
nd = Not determined			

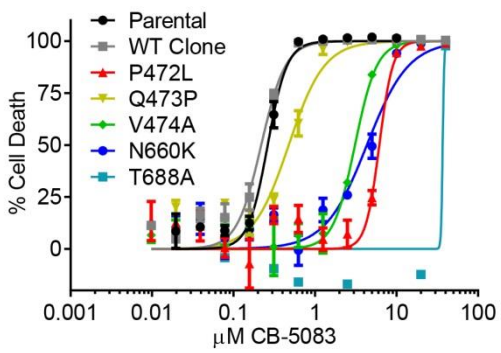
E



F



G



H

	Avg. IC_{50}	n	stdev	Avg Fold Change
Parental	0.35	23	0.11	-
WTClone	0.36	4	0.18	1.0
P472L	4.16	3	0.94	11.8
Q473P	1.18	2	0.03	3.4
V474A	3.94	3	1.51	11.2
N660K	7.31	4	3.26	20.7
T688A	22.37	4	11.86	63.4

I

	Avg. IC_{50}	n	stdev	Avg Fold Change
WT	0.010	3	0.002	-
Q473P	0.041	3	0.006	3.9
V474A	0.102	3	0.034	9.7
N660K	0.313	3	0.065	29.8
T688A	0.638	3	0.157	60.8

J

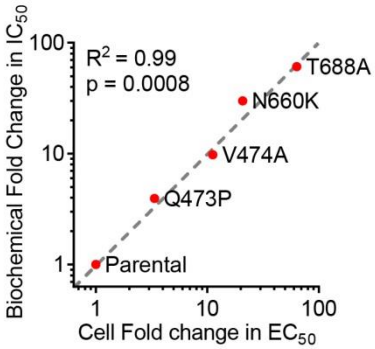


Figure S1. Selectivity of CB-5083. Related to Figure 1.

(A+B) Change in O.D at 340 nm measured over time in NADH biochemical assay reporting on the ATPase activity of WT p97 (A) or p87 E305Q (B) with a dose titration of CB-5083.

(C) Dose titration of CB-5083 in the ADP-glo ATPase assay for WT p97 and p97 E305Q. Dose responses were fit to a 4-parameter sigmoid curve with R^2 values of 0.98 and 1.00 for WT and E305Q respectively.

(D) Table summarizing off target activity of CB-5083 on select AAA-ATPases and kinases.

(E) Dose titration of CB-5083 in DNAPK, mTOR and PIK3C3 kinase assays compared to p97. Dose responses were fit to a 4-parameter sigmoid curve with R^2 values of 1.00, 1.00, 1.00 and 0.99 for DNAPK, mTOR, PIK3C3 and p97 WT respectively.

(F) Cellular activity of DNA-PK was measured by monitoring levels of nuclear phospho-H2AX by immunofluorescence in A549 cells treated with NU7441, CB-5083 or bortezomib for 6 hr.

(G) Dose titration of CB-5083 in 72 hr viability assay in cell lines with induced resistance to CB-5083 and identified point mutations in p97. Dose responses were fit to a 4-parameter sigmoid curve with R2 values of 0.98, 0.97, 0.84, 0.91, 0.90, 0.86 and 0.92 for parental, WT clone, P472L, Q473P, V474A, N660K and T688A respectively.

(H) Table summarizing the fold-change in viability EC_{50} for cell lines with identified p97 point mutations.

(I) Table summarizing the fold-change in biochemical IC_{50} for given p97 mutations.

(J) Scatterplot comparing fold-change in EC_{50} of CB-5083 in cell lines with given mutation to fold change in IC_{50} of CB-5083 for ATPase activity in recombinant p97 with given mutations.

Error bars are +/- SEM.

Table S1 related to Figure 1. CB-5083 has little ATP-binding pocket competition in panel of kinases and ATPases.

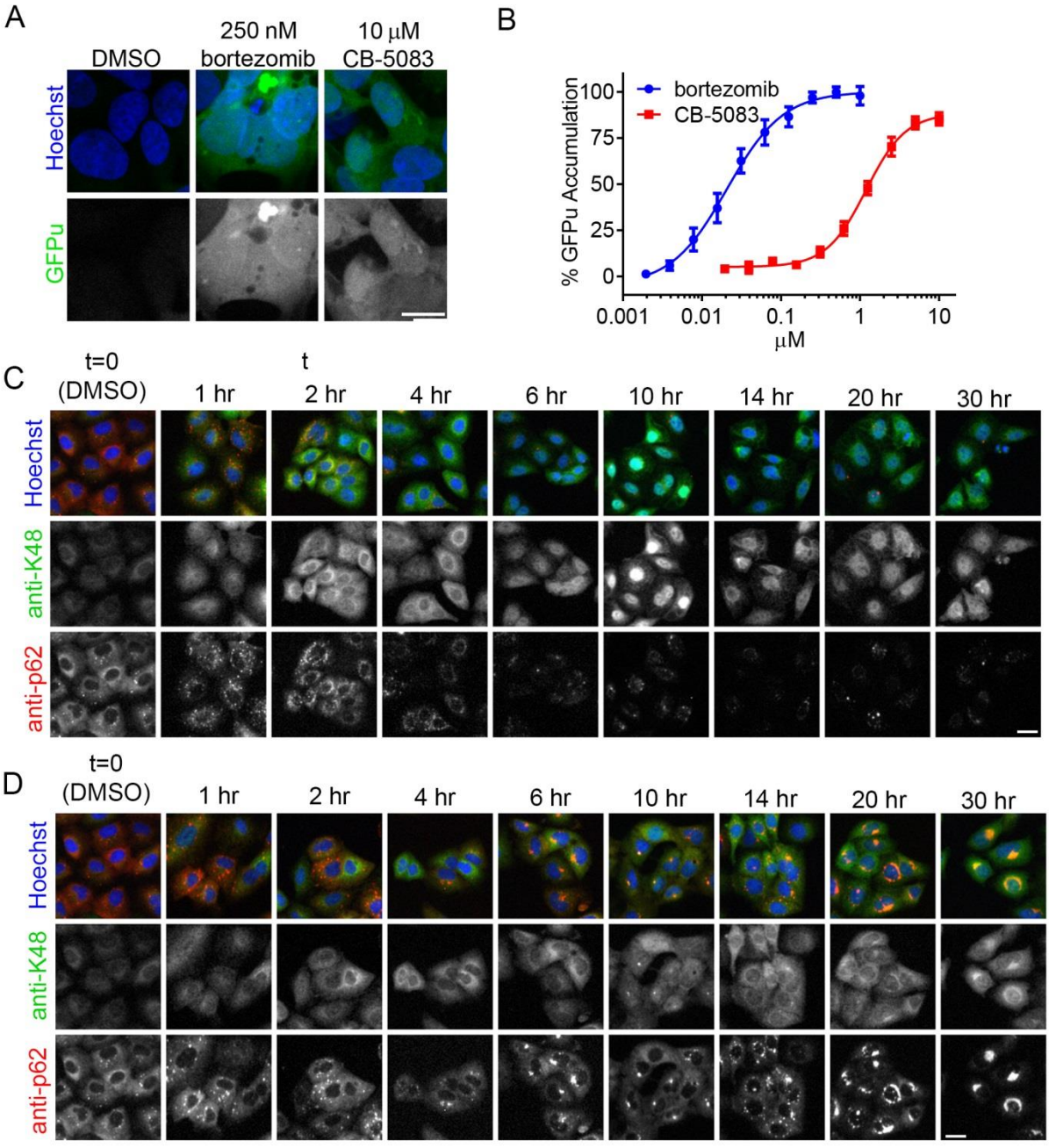
The percent displacement of the Desthiobiotin-ATP probe was measured by mass-spec for a panel of kinases and ATPases from HeLa cell lysates treated with 10 μM CB-5083.

Table S1 is attached separately.

Table S2 related to Figure 1. CB-5083 has minimal kinase inhibitor activity.

The percent activity in a panel of kinase assay after treatment with 10 μM CB-5083.

Table S2 is attached separately


A
DMSO
250 nM bortezomib
10 μM CB-5083
Hoechst
GFPu
B
% GFPu Accumulation
bortezomib
CB-5083
μM
C
t=0 (DMSO)
t
1 hr
2 hr
4 hr
6 hr
10 hr
14 hr
20 hr
30 hr
Hoechst
anti-K48
anti-p62
D
t=0 (DMSO)
1 hr
2 hr
4 hr
6 hr
10 hr
14 hr
20 hr
30 hr
Hoechst
anti-K48
anti-p62

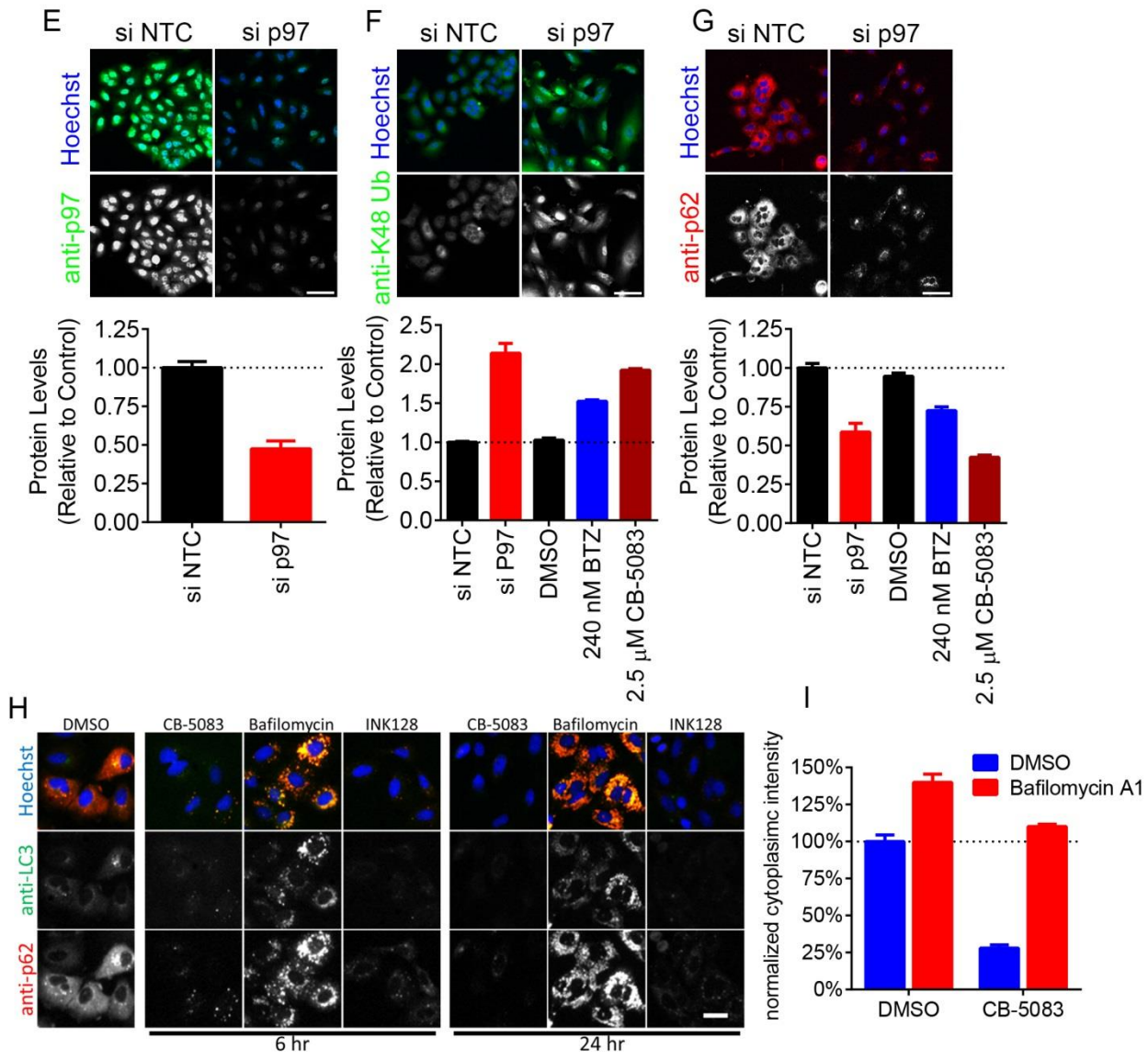


Figure S2. CB-5083 and p97 siRNA modulate pathway markers. Related to Figure 2.

(A) Example images of HEK293 cells expressing GFPu treated with CB-5083 or bortezomib for 8 hr. Scale bar is 20 μm and applies to all panels.

(B) Analysis of GFPu fluorescence in cells treated with dose titration of CB-5083 or bortezomib. Dose responses were fit to a 4-parameter sigmoid curve with R^2 values of 0.88 and 0.93 for bortezomib and CB-5083 respectively.

(C and D) Example images of A549 cells stained by immunofluorescence for p62 and K48-ub after a time-course of CB-5083 (5 μM, C) or bortezomib (100 nM, D) treatment. Scale bar is 20 μm and applies to all panels.

(E) Analysis of p97 protein levels by immunofluorescence in A549 cells 48 hr after transfection with siRNA oligos targeting p97. Scale bar is 50 μm and applies to all panels.

(F) Analysis of K48-ub levels by immunofluorescence in A549 cells 48 hr after transfection with siRNA oligos targeting p97 or 6 hr after CB-5083 (2.5 μM) or bortezomib (240 nM) treatment. Scale bar is 50 μm and applies to all panels.

(G) Analysis of p62 protein levels by immunofluorescence in A549 cells 48hr after transfection with siRNA oligos targeting p97 or 6 hr after CB-5083 (2.5 μM) or bortezomib (240 nM) treatment. Scale bar is 50 μm and applies to all panels.

(H) Example images of A549 cells stained by immunofluorescence for p62 and LC3 after treatment with CB-5083, Bafilomycin A1 or INK-128. Scale bar is 20 μm and applies to all panels.

(I) Quantification p62 as measured by immunofluorescence in A549 cells after treatment with 10 μM CB-5083 +/- 100 nM Bafilomycin A1.

Error bars are +/- SEM.

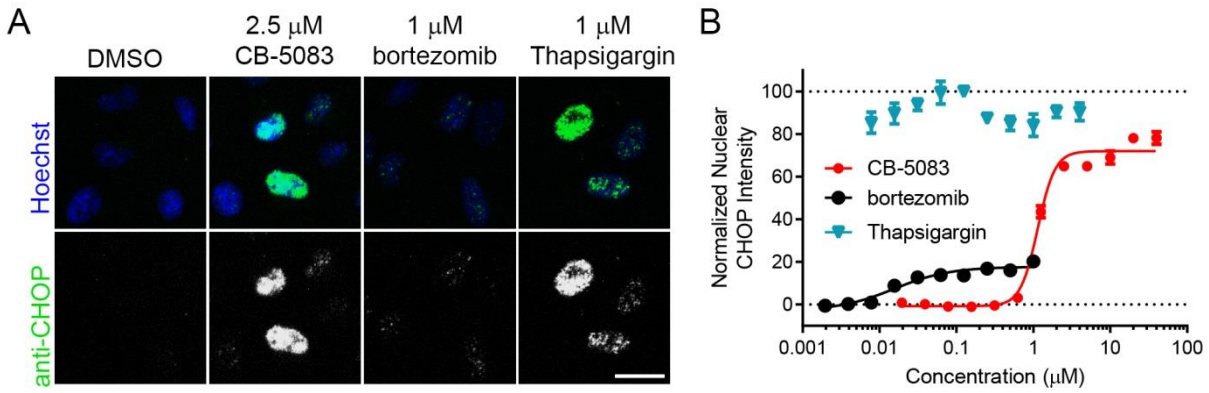


Figure S3. CB-5083 causes accumulation of CHOP protein. Related to Figure 3.

(A) Example images of NSCLC lung cancer cell line A549 labeled with anti-CHOP antibodies treated with CB-5083, bortezomib or Thapsigargin for 6 hr. Scale bar is 20 μm and applies to all panels.

(B) Analysis of nuclear levels of CHOP protein measured by immunofluorescence in A549 cells 6 hr after a dose titration of CB-5083, bortezomib or thapsigargin. Dose responses were fit to a 4-parameter sigmoid curve with R^2 values of 0.97 and 0.88 for CB-5083 and bortezomib respectively. Error bars are +/- SEM.

Table S3 related to Figure 3. CB-5083 induces the expression of UPR-related genes

Gene	HCT116	A549
CHOP	37.41	24.83
INHBE	23.56	6.69
HERPUD1	12.99	12.22
DNAJB9	11.01	7.57
TRIB3	8.63	11.58
ADM2	5.88	13.43
DNAJC3	5.77	4.52
EIF2AK3	5.59	3.19
PPP1R15A	5.34	4.83
ASNS	5.05	3.27
SEL1L	4.90	3.32
SYVN1	4.89	4.71
EDEM1	3.80	2.53
ERO1LB	3.75	3.35
HSPA5/BIP	3.57	5.62
XBP1	2.76	2.60
ERN1	2.60	3.86
CALR	2.47	2.04
ATF4	2.29	1.96
CEBPB	2.28	1.77
SERP1	2.25	2.53
BEX2	2.23	10.86
SIL1	2.17	1.24
PDIA3	2.07	2.27
OS9	1.93	1.13
MAPK8	1.90	1.53
KCNMB3	1.89	1.18
MANF	1.88	5.14
UGGT1	1.88	1.69
VIMP	1.82	3.18
SEC63	1.80	2.04
DNAJC10	1.73	1.62
VCP	1.70	1.33
ERP44	1.69	2.06
INSIG2	1.58	1.29
UBXN4	1.55	1.56
CREB3	1.52	1.85
ATF6	1.50	1.68
SREBF1	1.44	1.04
DERL1	1.39	1.78
ERN2	1.37	0.67
CANX	1.34	1.57
GANAB	1.32	1.24
RRM2	1.31	0.86
EIF2a	1.30	1.69
RPN1	1.29	1.56
NUCB1	1.25	1.29
SEC62	1.20	1.29
ERO1L	1.13	1.09
USP14	1.11	1.19
AMFR	1.11	1.08
ATF6B	1.11	0.83
MBTPS1	1.10	3.72
UFD1L	1.01	1.20
RNF5	1.01	1.13
GINS2	0.98	0.70
TCP1	0.96	0.77
MBTPS2	0.95	0.74
MAPK9	0.95	1.04
CCT4	0.94	1.03
TOR1A	0.93	1.05
NPLOC4	0.91	1.31
creB3L3	0.90	0.43
HSPA4	0.90	0.85
UBE2G2	0.88	0.85
HSPH1	0.88	0.83
BAX	0.88	1.08
MCM4	0.86	0.81
PFDN5	0.85	0.97
UHRF1	0.83	0.70
HPRT1	0.83	0.87
PPIA	0.81	0.76
HSPA4L	0.79	1.09
PRKCSH	0.78	0.80
SCAP	0.76	0.94
TYMS	0.74	1.00
GANC	0.68	0.82
PCNA	0.65	0.69
ATXN3	0.61	0.76
HTRA2	0.60	1.03
HTRA4	0.59	0.58
FBXO6	0.43	1.52
HGDC	0.34	0.05
SLC17A2	0.30	0.45
HSPA1B	0.22	0.25
HSPA2	0.08	0.62

Fold change in gene expression of HCT116 and A549 treated with 1 μM CB-5083 for 8 hr compared to DMSO treated cells. Genes were ordered by fold change.

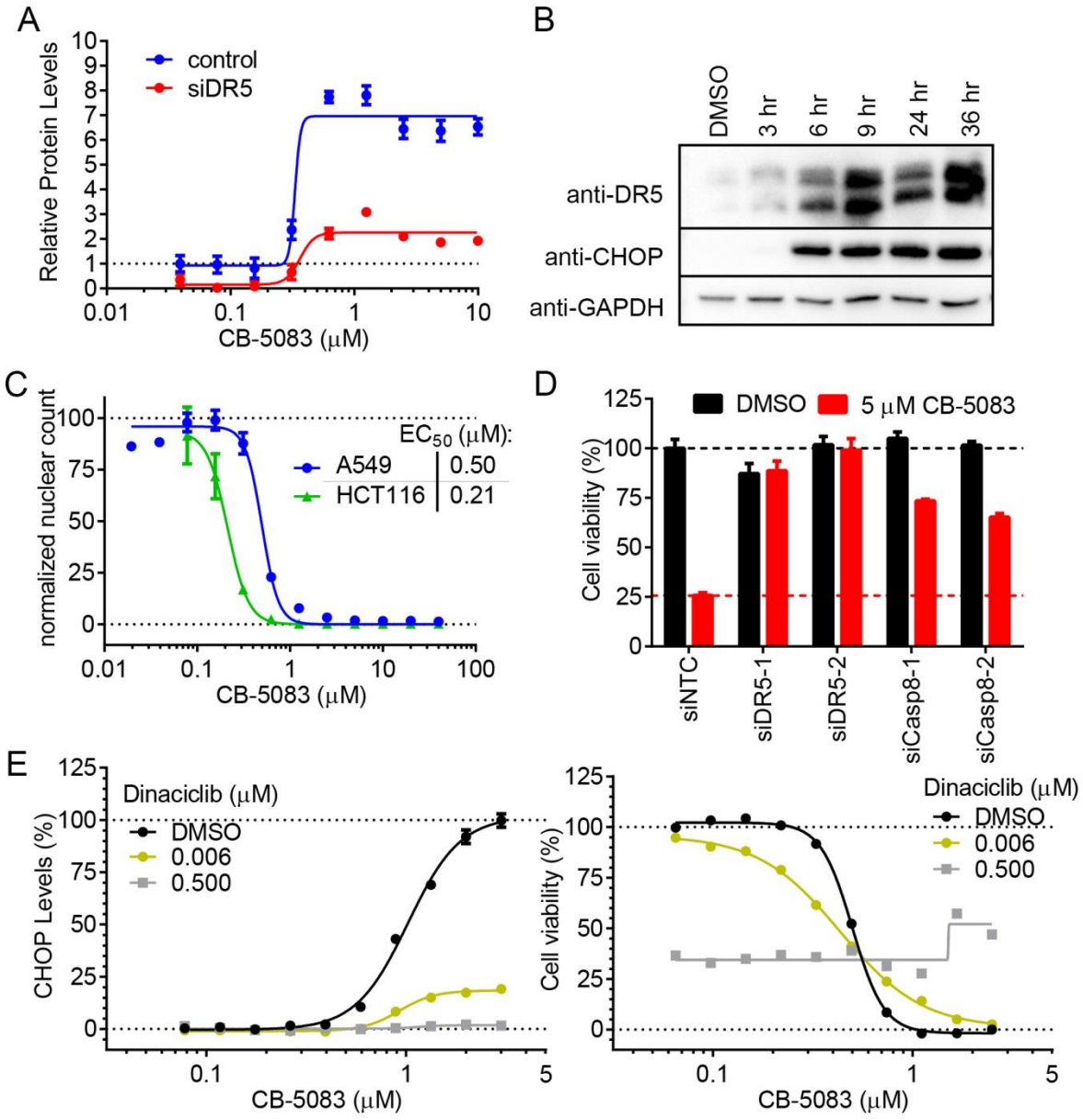


Figure S4. CB-5083 causes a strong accumulation of CHOP protein which is blocked by the CDK inhibitor Dinaciclib. Related to Figure 4.

(A) DR5 protein levels were measured by immunofluorescence and cellular intensities were quantified for a dose titration of CB-5083 with or without knockdown of DR5 by siRNA. Dose responses were fit to a 4-parameter sigmoid curve with R^2 values of 0.79 and 0.59 for control and siDR5 respectively.

(B) Western blot analysis of DR5 and CHOP in HCT116 cells treated with 1 μM CB-5083.

(C) Nuclear counts were measured by microscope in A549 and HCT116 cell lines following a 72 hr treatment of CB-5083 in dose titration. Dose responses were fit to a 4-parameter sigmoid curve with R^2 values of 0.97 and 0.87 for A549 and HCT116 respectively.

(D) DR5 and Caspase 8 were knocked down in A549 cells for 24 hr. Cells were then treated with CB-5083 (5 μM) for 48 hr and cell viability was measured by cell titer glo.

(E) Viability (left) and CHOP protein level (right) were measured in A549 cells following a 72 hr treatment of CB-5083 in dose titration in combination with constant doses of Dinaciclib. Dose responses were fit to a 4-parameter sigmoid curve with R^2 values of 0.98, 0.24 and 0.96 in DMSO, 0.006 μM and 0.5 μM CB-5083 respectively for CHOP levels and 1.00, 0.9991 and 0.78 for DMSO, 0.006 μM and 0.5 μM CB-5083 respectively for cell viability.

Error bars are +/- SEM.

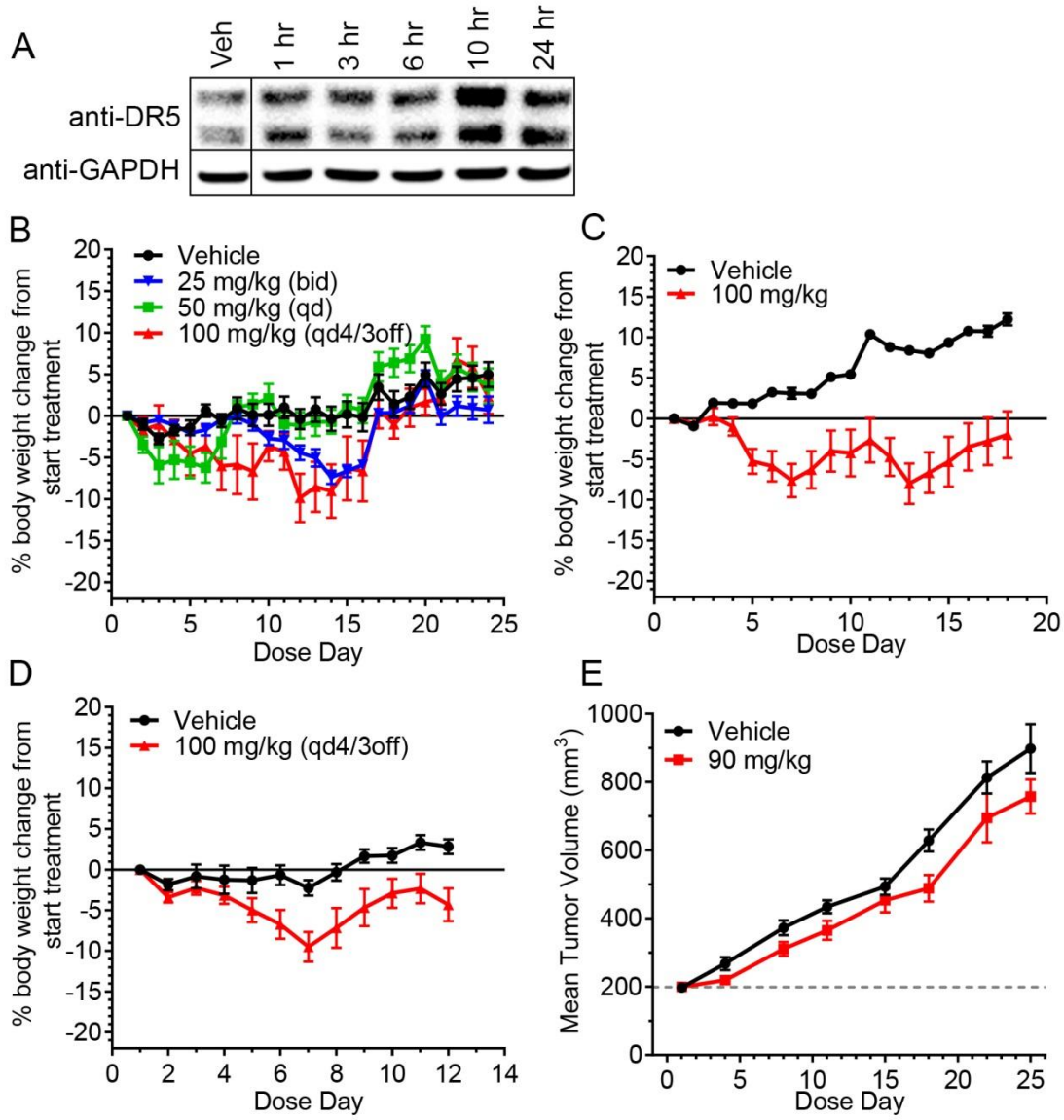


Figure S5 In vivo effects of CB-5083. Related to Figure 5.

(A) Western blot analysis of DR5 in HCT116 xenograft treated with 100 mg/kg CB-5083.

(B-D) Change in body weights were measured daily in HCT116 (B), NCI-H1838 (C) and AMO1 implanted xenograft for efficacy studies described in Figures 5D-F and 6D-E

(E) Tumor growth was measured for CB-5083 dosing in HCT116 xenograft model harboring the p97 T688A mutation. Female nude mice with established HCT 116 T688A xenografts were

treated for 25 days (n=10-12). CB-5083, 90 mg/kg PO, qd4/3off (4 days treatment, 3 days holidays per cycle), TGI = 20 %, p = 0.0521.

Error bars are +/- SEM.

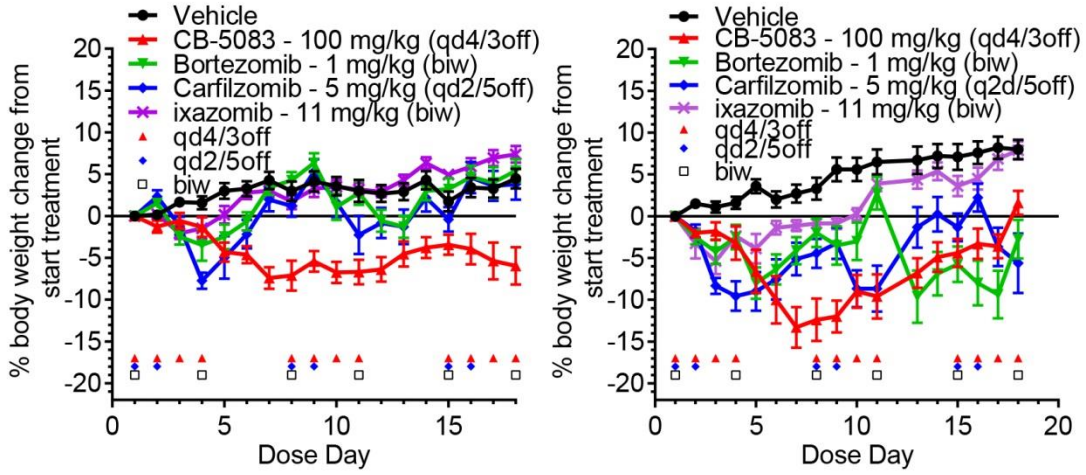


Figure S6 In vivo effects of CB-5083 and proteasome inhibitors. Related to Figure 6. Change in body weights were measured daily in HCT116 (left) and A549 (right) implanted xenograft for efficacy studies described in Figures 6D-E.

Error bars are +/- SEM.

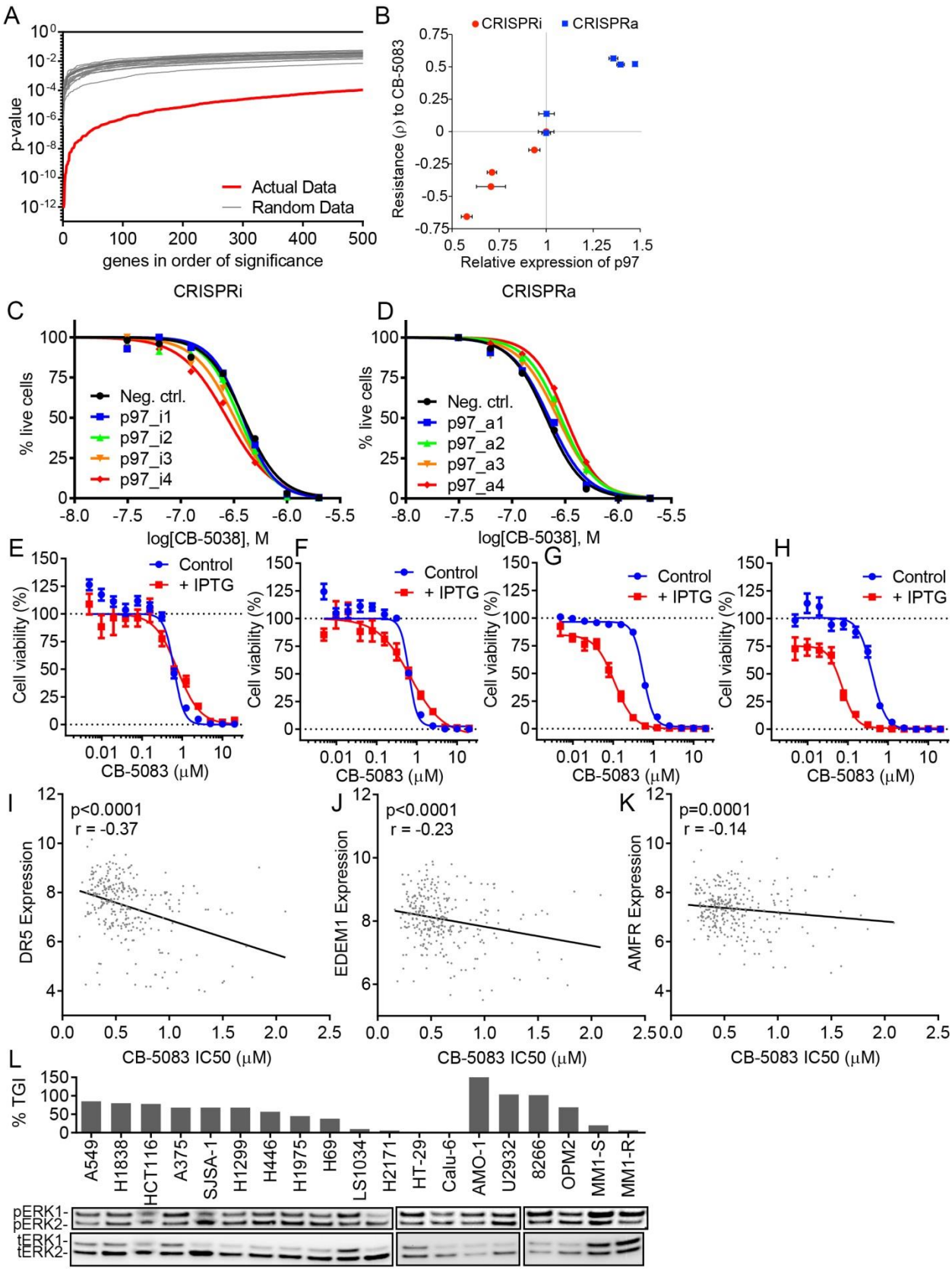

A
p-value
Actual Data
Random Data
genes in order of significance
B
CRISPRi
CRISPRa
Resistance (ρ) to CB-5083
Relative expression of p97
CRISPRi
CRISPRa
C
D
% live cells
Neg. ctrl.
p97_i1
p97_i2
p97_i3
p97_i4
p97_a1
p97_a2
p97_a3
p97_a4
log[CB-5038], M
E
F
G
H
Control
+ IPTG
Cell viability (%)
CB-5083 (μM)
I
J
K
p<0.0001
r = -0.37
p<0.0001
r = -0.23
p=0.0001
r = -0.14
DR5 Expression
EDEM1 Expression
AMFR Expression
CB-5083 IC50 (μM)
L
% TGI
A549
H1838
HCT116
A375
SJSA-1
H1299
H446
H1975
H69
LS1034
H2171
HT-29
Calu-6
AMO-1
U2932
8266
OPM2
MM1-S
MM1-R
pERK1-
pERK2-
tERK1-
tERK2-

Figure S7. Cell line sensitivity to CB-5083 correlates with gene expression of p97 or select UPR genes. Related to Figure 7.

(A) Top gene expression correlates are plotted in order of significance for cell line EC_{50} of viability (red) as well as 20 iterations of randomized EC_{50} data (grey).

(B) Relative resistance to CB-5083 (ρ) was plotted against p97 expression across a set of K562 cell lines expressing CRISPRi and CRISPRa sgRNAs.

(C-D) CRISPRi or CRISPRa lines of human K562 leukemia cells expressing different sgRNAs targeting p97 or a negative control sgRNA were treated with different concentrations of CB-5083 and numbers of live cells were determined by cell titer glo after 72 hr of treatment. Dose responses were fit to a 4-parameter sigmoid curve with R^2 values of 0.98, 0.97, 0.97, 0.99 and 0.99 for Neg. ctrl, p97_i1, p97_i2, p97_i3 and p97_i4 respectively in (B) and 0.99, 0.99, 0.99, 0.98 and 0.99 in Neg. ctrl, p97_a1, p97_a2, p97_a3 and p97_a4 respectively in (C).

(E-H) Viability in A549 cells stably expressing inducible control shRNA (clone 1 is (E), clone 2 in (F)) or inducible shRNA targeting p97 (clone 1 in (G),m clone 2 in (H)) was measured by cell titer glo after 3 days of 500 μM IPTG treatment followed by 3 days of CB-5083 treatment. Dose responses were fit to a 4-parameter sigmoid curve with R^2 values of 0.92, 0.70 for control and +IPTG respectively in (E), 0.93, 0.75 for control and +IPTG respectively in (F), 0.98, 0.89 for control and +IPTG respectively in (G) and 0.91, 0.87 for control and +IPTG respectively in (H).

(I-K) DR5, EDEM1 and AMFR gene expression as measured by microarray was plotted against CB-5083 EC_{50} of viability in a panel of cancer cell lines.

(L) Western blot analysis of phosphorylated ERK1/2 and total ERK1/2 across a panel of xenograft models along with their corresponding % TGI.

Error bars are +/- SEM.

Supplemental Experimental Procedures

ATPase and enzymatic assays

CB-5083 IC_{50} analyses for p97 and mutants were conducted utilizing a standard NADH-based coupled kinetic ATPase assay. Three-fold eleven-point serial dilution of CB-5083 was conducted in DMSO to achieve assay concentrations ranging from 50 μM to 0.8 nM. The assay was conducted in 384 well plates in 50 μl volume with 60 nM p97 enzyme, 500 μM ATP, 3 units/ml each of pyruvate kinase and lactate-dehydrogenase, 250 μM NADH and 3.75 mM phosphoenolpyruvate. ATP hydrolysis dependent NADH reduction was measured at 340 nM wavelength after 2 hours incubation at 37 °C. The IC_{50} for each enzyme was derived by fitting the slope of the reaction velocity values to a 4 point sigmoidal curve. ATP competition with CB-5083 was measured in the NADH assay with increasing concentrations of ATP.

For the ADP-glo assay, compounds were diluted in DMSO with a three-fold ten-point serial dilution to achieve assay concentrations ranging from 10 μM to 0.16 nM. The assay was performed by incubating 20 nM p97, 20 μM ATP and serial diluted compounds in 5 μl volume at 37 °C for 15 min. ADP glo reagents 1 and 2 were added according to the manufacturer's protocol (Promega). The IC_{50} of each compound was derived by reading luminescence values and fitting the values to a 4 point sigmoidal curve.

Kinase assays were also performed using the ADP glo technology and were conducted in the presence of substrate. ATP concentrations were 1 μM for DNA-PK and mTOR and 20 μM for PIK3C3. Substrates were S6-peptide, casein and PI for DNA-PK, mTOR and PIK3C3, respectively.

Reagents

ER-RFP was obtained from Life Technologies (CellLight). Antibodies utilized were anti-K48 ubiquitin (Millipore #05-1307), GAPDH (Cell Signaling #5174), Spliced XBP1 (Biolegend #647501), BiP (Cell Signaling #3177), ATF6 (Abcam #37149), CHOP (Western blot - Cell signaling #2895, Imaging – Santa Cruz #7351), DR5 (Cell Signaling #8074), LC3 (Cell Signaling #3868), p53 (Santa Cruz #126), p62 (Santa Cruz #28359), p97 (Abcam #109240), PERK (Cell Signaling #5683), phospho-EIF2a (Cell Signaling #3597) and phospho-ERK1/2 (Cell Signaling #4370). Secondary antibodies were from BioRad (for western blot) or Life Technologies (immunofluorescence). NMS-873 was synthesized as described (Magnaghi et al., 2013). Bortezomib, carlfizomib, ixazomib, dinaciclib and INK128 were obtained from Selleck Chemicals. Tunicamycin, thapsigargin and bafiloymcyin A1 were from Sigma. siRNA oligos were obtained from Sigma or Dharmacon and are listed in table S4. IPTG inducible vectors (pLKO-puro-IPTG-3xLacO) with shRNA sequences targeting p97 were generated by Sigma.

Oligos used in siRNA study

siRNA Name	Gene	Target Sequence
siBiP#1	*HSPA5*	GAAAGAAGGUUACCCAUGC
siBIP#2	*HSPA5*	GUUACUGUACCAGCCUAUU
siPERK#1	*EIF2AK3*	CCAAUGGGAUAGUGACGAA
siPERK#2	*EIF2AK3*	GGUAGGAUCUGAUGAAUUU
siCHOP#1	*DDIT3*	CACUCUUGACCCUGCUUCU
siCHOP#2	*DDIT3*	GGUAUGAGGACCUGCAAGA
siDR5#1	*TNFRSF10B*	CAAGGUCGGUGAUUGUACA
siDR5#2	*TNFRSF10B*	GAUGGAACAUCCUGUAACU
siCaspase8#1	*CASP8*	GAUAAUCAACGACUAUGAA
siCaspase8#2	*CASP8*	CAUCUCAGUUCACUGGUUU

Table S4

Cellular Assays

Cells were cultured in clear bottomed, tissue culture-treated 384-well plates and treated with 10-point dose titrations of compound in well duplicates. For immunofluorescence staining, paraformaldehyde (4% final) was added to plates after treatment. Cells were blocked in PBS with 1% BSA, 0.3% Triton-X100 and Hoechst (1:10,000) for 1 hour and then incubated in primary antibodies at 4 degrees Celsius for 16 hr. Cells were washed three times in PBS and secondary antibodies were added for 2 hours at 25 degrees Celsius. Cells were washed four

times in PBS and imaged with an automated wide field fluorescence microscope (Cell Insight, Thermo Fisher Scientific). Automated image analysis was written in Matlab (Mathworks) to count nuclei, mask cellular compartments and measure fluorescence intensities within cellular compartments. Fluorescence intensity values were fit to a 4 parameter sigmoidal curve to determine EC_{50} concentrations for each marker. Cell viability was assessed by nuclear count or Cell Titer Glo (Promega) after 72 hr of treatment. Values were fit to a 4 parameter sigmoidal curve to determine EC_{50} concentrations. Caspase activity was measured using the Caspase-Glo luminescent assay (Promega). HCT116 resistant cell lines were generated as described in Wacker et al(Wacker et al., 2012). cDNA was generated from resistant clones and p97 was sequenced from cDNA using Sanger sequencing.

Western Blotting

Cells were cultured in tissue culture-treated 6-well plates for 24 hours prior to treatment. After compound treatment cells were rinsed with PBS and then lysed with RIPA buffer supplemented with protease inhibitors (Roche Applied Science) and phosphatase inhibitors (Sigma). Lysates were cleared and protein was quantified by Pierce BCA protein assay kit. Western blotting was performed using the Novex® NuPAGE® SDS-PAGE Gel system. Briefly, 10 µg of protein was resolved on 4-12% Bis-Tris gradient gels and then transferred onto Nitrocellulose membranes. Membranes were blocked for one hour at room temperature in TBS + 0.1% Tween (TBST) with 5% non-fat dry milk. Membranes were probed overnight at 4 degrees Celsius with primary antibodies. Membranes were washed three times in TBST and then incubated with goat anti-rabbit or goat anti-mouse secondary antibodies for 1 hour at room temperature. Membranes were then washed three times with TBST and developed with SuperSignal West Dura

Chemiluminescent Substrate. Images were taken using a BioRad Gel Doc Imager system, exported as TIF files and cropped using NIH ImageJ.

qPCR

For qPCR assays RNA was extracted using the PureLink® RNA Mini Kit (Life Technologies). cDNA was prepared by amplifying 100 ng of RNA in the SuperScript® VILO™ cDNA Synthesis Kit (Life Technologies). Spliced or un-spliced specific XBP1 primers were synthesized as previously described (Maiuolo et al., 2011). Threshold cycle (Ct) values were automatically determined using QuantStudio 6 software (Life Technologies, Carlsbad, CA). Delta Ct values were calculated using the geometric mean of at least three housekeeping genes (GAPDH, PGK1, TBP, MRLP19). RNAseq was performed by Expression Analysis. Gene Ontology analysis was performed as in Ashburner et al. Gene ontology: tool for the unification of biology (2000) Nat Genet 25(1):25-9.

RNA Sequencing

RNA samples were converted into cDNA libraries using the Illumina TruSeq Stranded mRNA sample preparation kit (Illumina # RS-122-2103). The pipeline mRNAv8-RSEM (EA-Quintiles) was used to analyze RNA-seq data. To prepare the reads for alignment, the sequencing adapters and other low quality bases were clipped. To calculate a limit of detection of transcript copy number and correlation with RNA amounts, the reads were aligned to External RNA Controls Consortium (ERCC) sequences since these oligos were spiked into the RNA sample at known amounts. A subset of the reads (~1 million reads) were aligned to spiked-in control sequences (PhiX and other Illumina controls used during library preparation), residual sequences (globin and ribosomal RNA), and poly-A/T sequences that persisted after clipping. The reads were also

aligned to a sampling of intergenic regions to assess DNA contamination level and set the RPK (reads per thousand bases) threshold to call a transcript as present. RSEM was used to quantify genes and transcripts. The RSEM v1.2.0 program, rsem-calculate-expression, was run with parameters optimized for Illumina 50x50 paired-end sequencing. The UCSC knownGene transcriptome was used as a reference. For cross sample analysis, upper-quartile normalization of the read counts was performed. Gene ontology analysis was performed on the top 500 upregulated genes as previously described (Ashburner et al., 2000).

Animals

Mice were kept in a barrier system with controlled temperature (70º ± 10°F), humidity (50% ± 20%) and a lighting cycle of 12 hours light/12 hours dark. Mice were housed in isolator cages (5 mice per cage) and had free access to standard pellet food and water during the experimental period.

Cancer cell line xenografts were established by implanting subcutaneously 5 X 10^6 HCT116 in nude mice, 10 X 10^6 A549 with 50% matrigel in SCID Beige, 5 million AMO-1 with 50% matrigel in SCID Beige mice and 10 X 10^6 NCI-H1838 with 50% matrigel in SCID Beige mice. The PDX model CTG-0360 was tested at Champions Oncology (Baltimore, MD). Animals were randomized and treatments were initiated when tumor size reached 200-300 mm^3 (established tumors) or 300-500 mm^3 (advanced tumors). Tumor growth inhibition (TGI) was calculated using the formula [1-[(Xf-X0)/(Yf-X0)]*100] where X0 equals the average tumor weight of all tumors on Day 0, Xf equals the average tumor volume of the treated group at the end of the study and Yf equals the average tumor weight of the vehicle control group the end of the study.

Statistical analysis was performed using two-way analysis of variance (ANOVA) on delta tumor values in GraphPad Prism.

In vivo protein analysis

Tumors were excised, flash frozen and stored at -60°C to -90°C until processing. Following thawing, tissues were resuspended in ice cold lysis buffer (50 mM Tris HCl, pH 7.5, 50 mM sodium chloride, 1.0% Triton X-100, 1.0% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, pH 8.0) supplemented with standard protease (Sigma) and phosphatase (Sigma) inhibitors and homogenized using Qiagen's TissueLyser II. Clarified supernatants were stored at -60°C to -90°C until analysis. Determination of polyubiquitin accumulation in tumor lysates was performed using a commercially available polyubiquitin ELISA kit (Cyclex, Nagano, Japan). Determination of cleaved PARP, cleaved caspase 3, CHOP and p62 accumulation in tumor lysates was performed using commercially available MesoScale kits (MSD; Rockville, MD). Briefly, total protein concentrations were determined for clarified supernatants from above (BCA Protein Assay #23225, ThermoFisher Scientific, Rockford, IL) and then normalized to 2 mg/mL, aliquots were added to the ELISA/MSD plate and the remaining assay procedures carried out according to the manufacturer's protocol.

CRISPRi/CRISPRa control of p97 expression

We cloned individual sgRNAs targeting p97 for inactivation (CRISPRi) or activation (CRISPRa) into the lentiviral expression vector pU6-sgRNA EF1Alpha-puro-T2A-BFP(Gilbert et al., 2014). sgRNA sequences are listed in Table S3. Using lentiviral infection, these constructs were introduced into human K562 leukemia cell lines we previously described(Gilbert et al., 2014):

for CRISPRi, cells expressing a doxycycline-inducible KRAB-dCas9 fusion protein and for CRISPRa, cells expressing the sunCas9 system(Tanenbaum et al., 2014). Initial rates of infection were between ~30% and ~70%, as quantified by flow cytometry measurements of the percentage of the cell population expressing BFP. For the competitive growth experiments, CRISPRi cell lines were grown for 48 hours in the presence of 25ng/ml doxycycline. Then, CRISPRi and CRISPRa cell lines were treated for 24 hours with 1 μM CB-5083, after which the cells were washed and continued to grow in CB-5083-free medium. Medium used for CRISPRi cell lines contained 25ng/ml doxycycline. Cells were diluted two-fold each day and the percentage of BFP-positive cells was quantified by flow cytometry before CB-5083 treatment and 24, 48 and 72 hours after CB-5083 removal. Cell growth was monitored over the time course of the experiment. Experiments were carried out in triplicates. To compare drug resistance phenotypes across cell lines with differing initial rates of infection, we calculated the metric ρ we previously defined(Kampmann et al., 2013). For quantification of p97 expression, cell lines described above were subjected to fluorescence-activated cell sorting (FACS) based on BFP expression to isolate pure populations expressing the sgRNA of interest. CRISPRi lines were grown for 48 hours in the presence of 25 ng/ml doxycycline. Fractions of cells expressing sgRNAs were quantified again based on BFP expression and found to be between 93% and 99%. RNA was isolated and p97 transcript levels were quantified by qPCR using the primer pair 5'-gaagcgtatcgacccatc-3', 5'-gcaacaatgcaataagggc-3'. Results were normalized by quantifying levels of β-actin (ACTB) mRNA using the primer pair 5'-gctacgagctgcctgacg-3', 5'-ggctggaagagtgcctca-3'. For dose-response curves, FACS-sorted cell populations described above were used to determine CB-5083 dose responses. CRISPRi cell lines were grown in the presence

of 25 ng/ml doxycycline for 48 hours. CRISPRi and CRISPRa cells were plated in 96-well plates in the absence or presence of CB-5083 at concentrations between 31.25 nM and 2 μM. For CRISPRi cell lines, 25 ng/ml doxycycline was included. After 72 hours of CB-5083 treatment, cell numbers were quantified using cell titer glo (Promega). Experiments were carried out in duplicates. Dose-response curves were fitted using Graphpad Prism.

sgRNA sequences targeting p97 for CRISPRi or CRISPRa

sgRNA	sgRNA protospacer sequences (5' to 3')
Negative control	GAACGACTAGTTAGGCGTGTA
p97_i1	GTCTATTGTGCCACGTTCTTCC
p97_i2	GAGAAGGAGCAAGAAGTGTC
p97_i3	GCTAGTCAGAAAGTTCAGAGT
p97_i4	GGCTCCGGAGTTTATCCTCC
p97_a1	GGTGGTCTAGATAGGCCTCTC
p97_a2	GTCCCTGTTAGTAGTCAAC
p97_a3	GCCCAGGACAGTGACAATGAG
p97_a4	GGTACCTGTTACTTAGAGAC

Supplemental References

Ashburner, M., Ball, C. A., Blake, J. A., Botstein, D., Butler, H., Cherry, J. M., Davis, A. P., Dolinski, K., Dwight, S. S., Eppig, J. T.*, et al.* (2000). Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat Genet *25*, 25-29.

Kampmann, M., Bassik, M. C., and Weissman, J. S. (2013). Integrated platform for genome-wide screening and construction of high-density genetic interaction maps in mammalian cells. Proceedings of the National Academy of Sciences of the United States of America *110*, E2317-2326.

Tanenbaum, M. E., Gilbert, L. A., Qi, L. S., Weissman, J. S., and Vale, R. D. (2014). A protein-tagging system for signal amplification in gene expression and fluorescence imaging. Cell *159*, 635-646.