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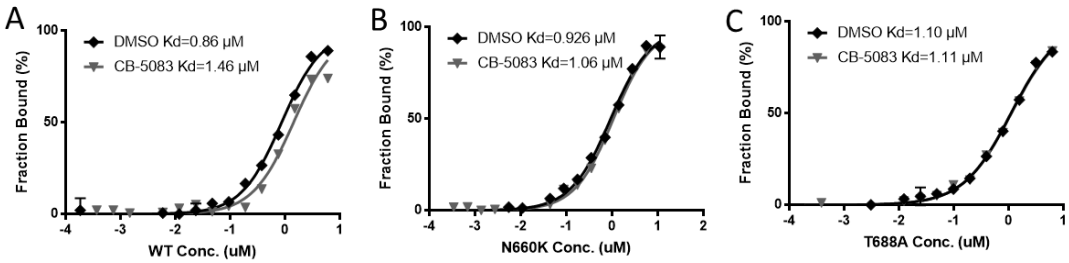

Figure S1. Binding of BODIPY™ FL ATP-γ-S to WT p97 (A) and CB-5083 resistant mutants (B, C), in the presence or absence of CB-5083.

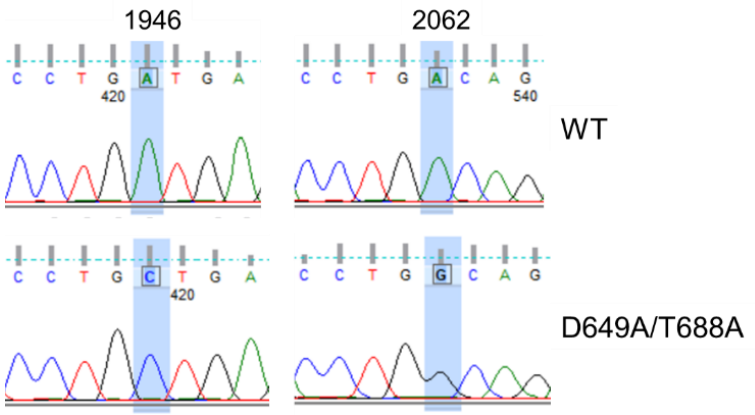

Figure S2. cDNA sequencing results of WT and D649A/T688A p97.

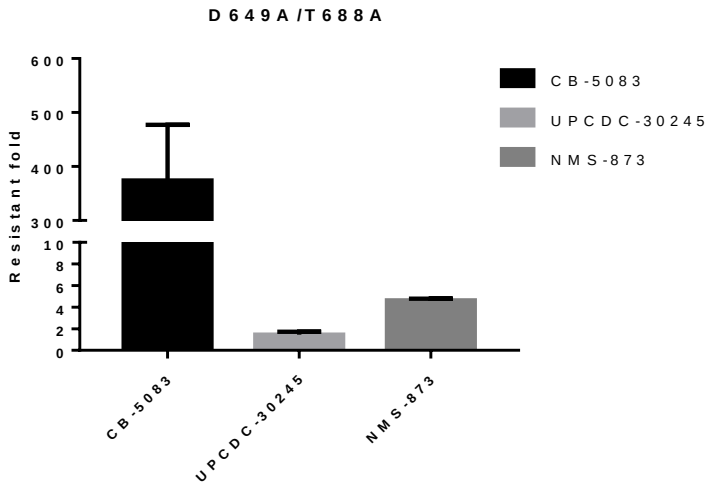

Figure S3. Fold resistance of mutant p97 D649A/T688A toward p97 inhibitors, as compared to WT p97.

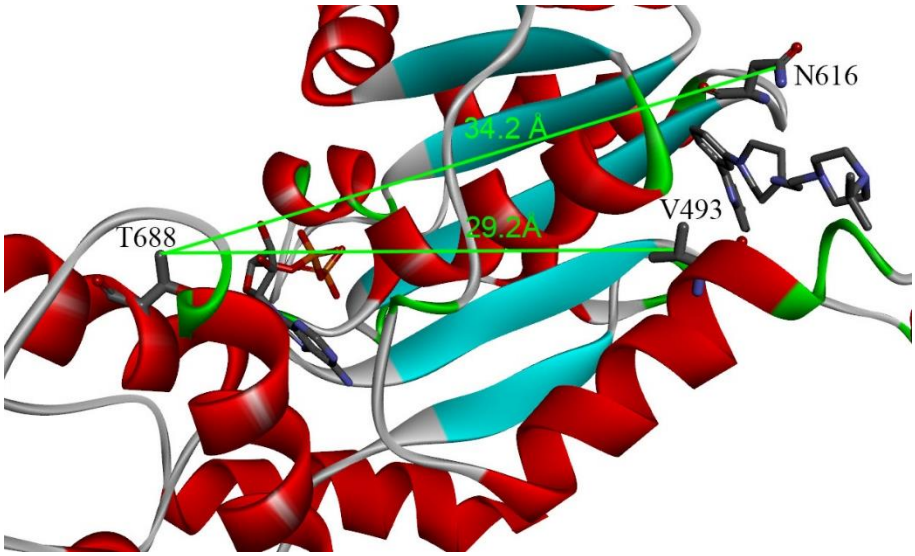

Figure S4. Distance between NMS-873 or UPCDC-30245 binding site and T688.

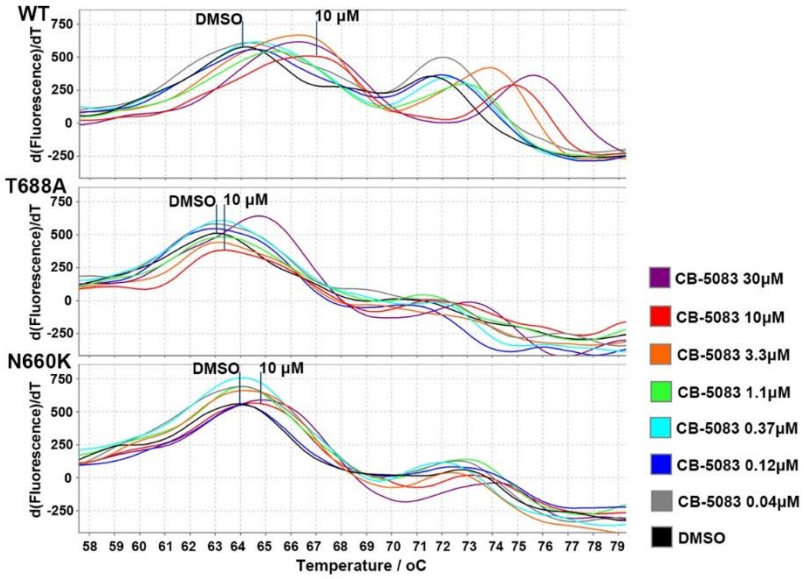

Figure S5. Thermal shift assay (TSA) of WT p97 and p97 mutants in the presence of different concentrations of CB-5083.

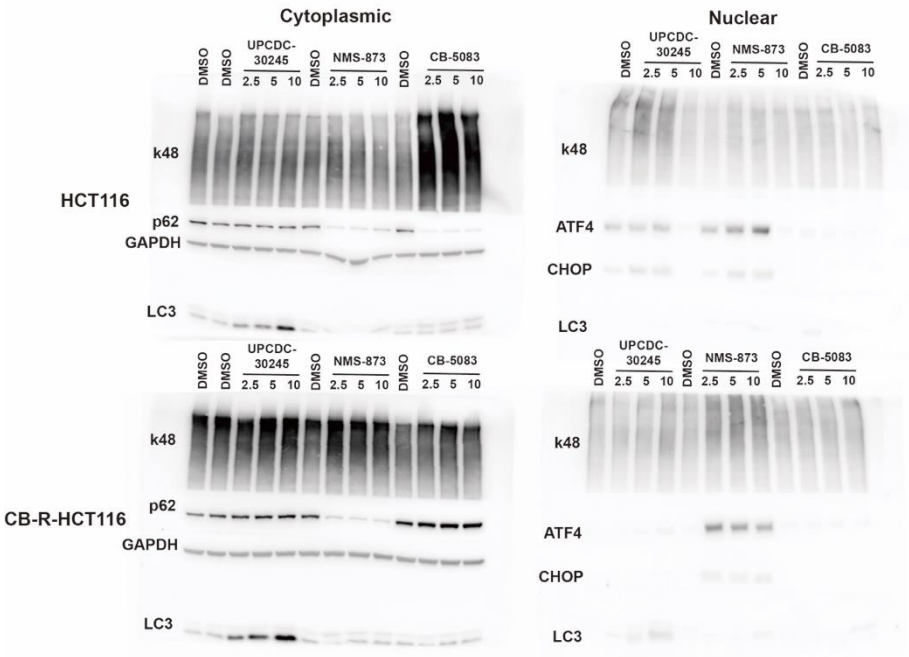

Figure S6. Western blot assay carried out to determine the change in biomarkers caused by p97 inhibition.

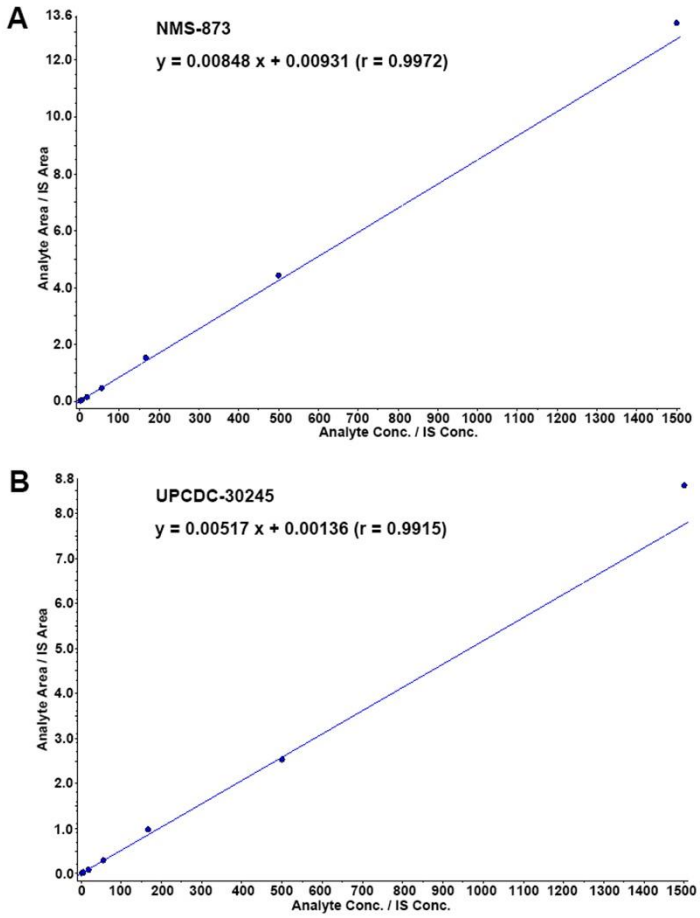

Figure S7. LC-MS/MS standard curves of (A) NMS-873 and (B) UPCDC-30245.

Supplemental Tables

Table S1. Plasmids used in this study.

Plasmid Number	Plasmid name	Vector	Source and Reference
TCB-197	Human p97 pET15_T	pET15b_TEV linker	Chou, 2014 [1]
TCB-494	Human E470D p97 pET15_T	pET15b_TEV linker	Bastola, 2017 [2]
TCB-484	Human E470K p97 pET15_T	pET15b_TEV linker	Bastola, 2017 [2]
TCB-530	Human V474A p97 pET15_T	pET15b_TEV linker	This study
TCB-487	Human N660K p97 pET15_T	pET15b_TEV linker	This study
TCB-496	Human T688A p97 pET15_T	pET15b_TEV linker	This study
TCB-592	Human D649A/T688A p97 pET15_T	pET15b_TEV linker	This study

Table S2. Primers used in this study.

Name of primer	Target of primer	Sequence of primer
PCR Primer 1	VCP-Cloning F	5'- CAG CGT TGT TCG CCC -3'
PCR Primer 2	VCP-Cloning R	5'- ACC CCC AGG GAA CAA G -3'
Sequencing Primer 1	VCP-Cloning F	5'- CAG CGT TGT TCG CCC -3'
Sequencing Primer 2	p97seq301F	5'- GCA TCC AGC CAT GCC CTG ATG TG -3'
Sequencing Primer 3	p97bp803F	5'- GAC CCT GAT TGC TCG AGC TG -3'
Sequencing Primer 4	p97 b1189F	5'- GAA CAG GTA GCC AAT GAG ACT -3'
Sequencing Primer 5	Hp97 1501-F	5'- GAC AAA TTC CTG AAG TTT GGC -3'
Sequencing Primer 6	p97seq1976F	5'- CTA ACC TGC GCA AGT CCC CAG TTG -3'

Table S3. HPLC conditions for NMS-873 intracellular concentration measurement.

HPLC	Shimadzu LC-20AD	
Column Type	Hypersil Gold, 50x2.1 mm, 5 µm, Thermo Fisher	
Mobile Phases	A: Water with 0.1% formic acid	
	B: Acetonitrile with 0.1% formic acid	
Pump Program	Time (min)	B (%)
	0	30
	0.02	30
	0.8	95
	1.8	95
	1.9	30
	3	30
Flow Rate	400 µL/min	

Table S4. HPLC conditions for UPCDC-30245 intracellular concentration measurement.

HPLC	Shimadzu LC-20AD	
Column Type	Atlantis T3, 50x4.6 mm, 5 µm, Waters	
Mobile Phases	A: Water with 0.1% formic acid	
	B: Acetonitrile with 0.1% formic acid	
Pump Program	Time (min)	B (%)

	0	30
	0.02	30
	0.8	90
	1.8	90
	1.9	30
	3	30
Flow Rate	700 µL/min	

Table S5. Intracellular concentrations (µM per cell) of NMS-873 and UPCDC-30245 in A549 cells.

	NMS-873		UPCDC-30245	
Treated time / Treated Conc.	3 h	6 h	3 h	6 h
2.5 µM	423.2 ± 40.8	468.1 ± 13.5	4970.0 ± 353.0	5079.8 ± 284.6
5 µM	700.68 ± 101.4	825.4 ± 79.0	9481.0 ± 599.8	9900.2 ± 428.0
10 µM	994.0 ± 60.1	1246.5 ± 65.7	4620.8 ± 224.7	3333.3 ± 287.7

Supplemental Methods

Protein purification

Plasmids used for generating WT and mutant p97 proteins can be found in the Supplemental Table S1; proteins were purified as previously described [1, 3].

ATPase activity

To compare the ATPase activity of WT and mutant p97 proteins, each purified protein was diluted to a final monomer concentration of 25 nM in 50 µL ATPase assay buffer (50 mM Tris pH 7.4, 20 mM $MgCl_2$, 1mM EDTA, 0.5 mM TCEP, and 0.01% Triton X-100) containing 200 µM ATP. After 60 min incubation at room temperature, 50 µL Biomol Green reagent (Enzo Life Sciences) was added to stop the reaction and the absorbance at 635 nm was measured using a BioTek Synergy Neo plate reader. The eight-dose titrations were performed by adding 30, 10, 3.3, 1.1, 0.37, 0.12, 0.04, and 0 µM compound to the reaction to determine the IC_{50} values of p97 inhibitors. The steady-state kinetic experiments were performed at 2 or 3 different concentrations of compound and 10 different ATP concentrations. The results were calculated from 6 replicates using GraphPad Prism 7.0.

Cell viability

Cell viability was measured using CellTiter Glo® Luminescent Cell Viability Assay (Promega) according to the manufacturer's procedure. RMPI1640 or DMEM containing 5% FBS and 1% Penicillin-Streptomycin (Thermo Fisher) was used as cell viability assay medium. To find the linear relationship between the relative luminescence unit and the number of viable cells, a standard curve for each cell line was generated. Generally, 30 µL of cell suspension was plated in 384-well white plates (Greiner) with serial 2 fold-dilutions (from 30000 to 284 cells per well). Twenty-four hours after seeding, 8 uL of assay media containing 5% DMSO was added into each well and plates were incubated for an additional 48 h at 37 °C in a 5% CO_2 incubator. To test the anti-proliferative activity of p97 inhibitors,

cells were seeded at 750 to 3000 cells per well according to the linear range determined from the standard curve of each cell line. Twenty-four hours after seeding, cells were treated with the compounds (three-fold dilution, eight concentrations). After 48 hours of treatment, cell viability was measured using CellTiter Glo and IC_{50} values were calculated using the percentage of growth of treated cells versus the DMSO control.

Western blot

Parental or CB-5083 resistant human HCT116 cells were maintained in RPMI1640 medium containing 5% FBS and 1% penicillin/streptomycin in 10 cm cell dishes. Cells were replated into 6-well plates (10^6 cells/well) when they were 80% confluent. The next day inhibitors were added and cells were harvested after 6 hours of treatment. Cytoplasmic and nuclear fractions were isolated from the cell pellets with NE-PER Nuclear and Cytoplasmic Extraction Reagents (78835, Thermo Scientific). After determining protein concentration using Bradford reagent (Bio-Rad), 10 µg samples were loaded on a 4-20% Tris-Glycine Mini-PROTEAN Gels (Bio-Rad). Proteins were transferred to nitrocellulose membranes using the Trans-Blot Turbo system (Bio-Rad). Primary antibodies used were K48 Ubiquitin (A101, Boston Biochem), p97 (MA3-004, Thermo Scientific), p62 (M162-3, MBL International Corporation), ATF4 (SC-200, Santa Cruz Biotechnology), GAPDH (CST2118s, Cell Signaling Technology), CHOP (CST2895s, Cell Signaling Technology) and LC3 (PM036, MBL International Corporation). ECL reagent (WBKLS0500, MilliporeSigma) and ChemiDoc MP Imaging System (Bio-Rad) were used to image the blots.

RNA extraction, molecular cloning and sequencing

RNA extraction was performed using Direct-zol™ RNA MiniPrep Plus (Zymo Research Corporation, Cat. No. R2072) and TRIZOL LS REAGENT (Thermofisher, Cat. No. 10296028) according to the manufacturer's protocol. One microgram of total RNA was reverse transcribed using Oligo(dT)20 Primer (Thermofisher, Cat. No. 18418020) and ThermoScript Reverse Transcriptase (Thermofisher, Cat. No. 12236-014) according to the manufacturer's protocol. cDNA sequence of p97 was amplified using Platinum SuperFi PCR Master Mix (Thermofisher, Cat. No. 12358-010). PCR product was purified after electrophoresis through 0.8% Agarose and extracted using QIAquick Gel Extraction kit (QIAGEN, Cat. No. 28115). Molecular cloning was performed using NEB PCR Cloning Kit (Biolabs, NEB #E1202) according to the manufacturer's protocol. After overnight incubation, 5 clones were picked up and plasmids were purified using Plasmid miniprep kit (Bioland, Cat. No. PD01-01). Purified plasmids were sequenced by Sanger sequencing. PCR and sequencing primers refer to the Supplemental Table S2.

Microscale Thermophoresis (MST)

MST was carried out on a Monolith NT.115pico instrument (Nano-Temper Technologies) at 25 °C. Full-length wild type p97 or p97 mutants were exchanged to 1x assay Buffer (50 mM Tris pH 7.4, 20 mM $MgCl_2$, 1 mM EDTA, 0.5 mM TCEP, and 0.05% Tween 20). p97 was titrated against 25 nM of BODIPY™ FL ATP-γ-S (Thermofisher) in two-fold steps from 6.25 µM to 191 pM, with or without 1 µM of CB-5083. Assays were performed in hydrophilic capillaries in three independent experiments.

Protein structure modeling

Protein structure viewing and modeling were performed with Discovery Studio 4.0. The Build Mutants protocol was used to generate the mutant structures based on the wild type p97 structure (PDB 5FTJ).

LC-MS/MS standard curves preparation

20,000 A549 cells per well in 90 µL of DMEM with 2.5% FBS and 1% Penicillin/Streptomycin were plated in a 96 well plate. After 24 hours, medium was removed, and cells were washed twice with ice-cold DPBS. 90 µL of ACN was added to each well and shaken for 60 min at 800 rpm at room temperature. 10 µL of 10x compound (in 10% DMSO mixed with 90% ACN) was added to yield calibration curve samples with 1% DMSO in 100 µL ACN. The plate was centrifuged at 4000 rpm for 15 min at 23°C, and 75 µL of supernatant was transferred to a fresh plate; the plate can be sealed and stored at -80°C. The concentrations for the standard curve are 0, 2.1, 6.2, 18.5, 55.6, 166.7, 500, and 1500 nM. The standard curves are showed as Fig. S6.

References:

[1] T. F. Chou, S. L. Bulfer, C. C. Weihl, K. Li, L. G. Lis, M. A. Walters, F. J. Schoenen, H. J. Lin, R. J. Deshaies, M. R. Arkin, *J. Mol. Biol.* **2014**, *426*(15), 2886-2899.

[2] P. Bastola, F. Wang, M. A Schaich, T. Gan, B. D Freudenthal, T.-F. Chou, J. Chien, *Cell Death Discovery* **2017**, *3*.

[3] T.-F. Chou, S. J. Brown, D. Minond, B.E. Nordin, K. Li, A.C. Jones, P. Chase, P. R. Porubsky, B.M. Stoltz, F. J. Schoenen, M.P. Patricelli, P. Hodder, H. Rosen, R. J. Deshaies, *Proc. Natl. Acad. Sci. USA* **2011**, *108*(12), 4834-4839.