Supporting Information

Altered cofactor regulation with disease associated p97/VCP mutations

Xiaoyi Zhang\textsuperscript{a,b,1}, Lin Gui\textsuperscript{a,b,1}, Xiaoyan Zhang\textsuperscript{c}, Stacie L. Bulfer\textsuperscript{d}, Valentina Sanghez\textsuperscript{a}, Daniel E. Wong\textsuperscript{a}, YouJin Lee\textsuperscript{e}, Lynn Lehmann\textsuperscript{f}, James Siho Lee\textsuperscript{g}, Pei-Yin Shih\textsuperscript{g}, Henry J. Lin\textsuperscript{g}, Michelina Iacovino\textsuperscript{a}, Daniel E. Wong\textsuperscript{a}, YouJin Lee\textsuperscript{e}, Lynn Lehmann\textsuperscript{f}, James Siho Lee\textsuperscript{g}, Pei-Yin Shih\textsuperscript{g}, Henry J. Lin\textsuperscript{g}, Michelina Iacovino\textsuperscript{a}, Conrad C. Weihl\textsuperscript{f}, Michelle R. Arkin\textsuperscript{d}, Yanzhuang Wang\textsuperscript{c}, and Tsui-Fen Chou\textsuperscript{a,2}

\textsuperscript{a}Division of Medical Genetics, Department of Pediatrics, Harbor–UCLA Medical Center and Los Angeles Biomedical Research Institute, Torrance, CA 90502; \textsuperscript{b}College of Pharmaceutical Sciences, Capital Medical University, Beijing 100069, People’s Republic of China; \textsuperscript{c}Department of Molecular, Cellular and Developmental Biology, The University of Michigan, Ann Arbor, MI 48109-1048; \textsuperscript{d}Small Molecule Discovery Center, Department of Pharmaceutical Chemistry, University of California, San Francisco, CA 94158; \textsuperscript{e}Department of Neurology, Washington University School of Medicine, St. Louis, MO 63110; \textsuperscript{f}NanoTemper Technologies, Inc., South San Francisco, CA 94080; \textsuperscript{g}Division of Biology and Biological Engineering, California Institute of Technology, Pasadena, CA 91125.

\textsuperscript{1}These authors contributed equally to this work.
\textsuperscript{2}Corresponding author: Tsui-Fen Chou, Harbor-UCLA Medical Center and Los Angeles Biomedical Research Institute, Torrance, California 90502, USA. Tel: 1-424-201-3006; Email: tsuifen.chou@ucla.edu

Running Title: Cofactor regulation of p97/VCP disease mutants

Keywords: AAA ATPase; p97; VCP; IBMPFD; ALS; MSP1; steady-state kinetics; p37; p47

Classification: Biological Science

Biochemistry
SI Methods

BIOMOL Green ATPase assay — Purified p97 (25 µL of 50 µM; final concentration in the reaction was 25 nM monomer concentration) was diluted in 30 mL of assay buffer [10 mL of 5 x assay buffer A (1 x = 50 mM Tris pH 7.4, 20 mM MgCl₂, 1 mM EDTA,) mixed with 20 mL water and 50 µL 0.5 M TCEP, 50 µL 10% Triton] to make the enzyme solution. 30 µL of the enzyme solution was dispensed into each well of a 96-well plate and 10 µL of p97 cofactor (0 – 4000 nM) was added into each well. The ATPase assay was carried out by adding 10 µL of 1000 µM or 4000 µM ATP (pH 7.5) to each well and incubating the reaction at room temperature for 35 min. Reactions were stopped by adding 50 µL of BIOMOL Green reagent (Enzo Life Sciences). Absorbance at 635 nm was measured after 4 min. Eight final ATP concentrations were used to determine steady-state kinetic constants. For mutants, reaction times were adjusted according to specific activities in order to obtain acceptable absorbance readings. Michaelis-Menten constants were calculated from 8 replicates by fitting data to Equation 1 using GraphPad Prism 6.0.

\[ \nu = \frac{V_{\text{max}} \cdot [\text{ATP}]}{(K_m + [\text{ATP}])} \]  

In vitro His pull-down assays — 12 µM His-tagged p97 was mixed with 30 µM p47, p37, or p47 variants in 100 µL buffer [20 mM HEPES, pH 7.4, 250 mM KCl, 1mM MgCl₂, 200 µM ATP, 0.01% Triton, 10 µL MagneHis™ Ni-Particles (Promega, V8560)] at room temperature for 30 min. Unbound supernatant was removed, and Ni-Particles were washed with 500 µL washing buffer (50 mM HEPES, pH 7.4, 150 mM KCl, 5 mM MgCl₂, 20 mM imidazole) 3 times. Bound proteins were eluted by adding 100 µL 1x Laemmli Sample Buffer (Bio-Rad) and heated to 90°C for 5 min. Samples were separated on 4-20% Tris-Glycine Gel (Bio-Rad) and stained with Coomassie blue (Thermo Scientific Imperial Protein Stain).

Microscale Thermophoresis (MST) — MST was carried out at 25 °C on a Monolith NT.115pico instrument (Nano-Temper Technologies). Full-length p37 and p47 were exchanged to 1x assay Buffer A containing TCEP and Pluronic acid (50 mM Tris pH 7.4, 20 mM MgCl₂, 1 mM EDTA, 0.5 mM TCEP, 0.05% Pluronic acid) and labeled with NanoTemper 647 cysteine-reactive red dye per the manufacturer’s instructions. In this assay, unlabeled p37 or p47 was titrated against 400 pM of NT-647 labeled p37 or p47 in two fold steps from 250 nM to 61 pM. Assays were performed in hydrophilic capillaries in two separate experiments.

Surface plasmon resonance (SPR) — Binding affinities for p37 and p47 cofactors to p97 proteins were measured on a Biacore 4000 instrument. NeutrAvidin-coated sensor chips were prepared as described (1) with the following exceptions: 60 mM N-hydroxysuccinimide and 240 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide was injected for 4 min, followed by a 2 min injection of 0.25 µg/mL NeutrAvidin. p97 proteins were immobilized in 10 mM HEPES, pH 7.5, 150 mM NaCl, 0.5 mM TCEP, 0.05% Tween 20 to 500-600 RU by injecting 4-6 µg/mL protein for 2 min.

Binding of cofactor proteins was measured in 25 mM Tris, pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 0.5 mM TCEP, 0.05% Tween 20 and 0.1% Prionex (Calbiochem) at 20 °C. Sensorgrams were reduced, double-referenced, and fit to a 1:1 kinetic interaction model (p37) or to an equilibrium binding model for a 1:1 interaction (F253S p47) or a 2:1 interaction (WT p47, Δ69-92 p47, Δ83-92 p47 and 83-88 (DE₅ to NQ₅) p47) in Scrubber 2 (BioLogic Software).
**p97-mediated post-mitotic Golgi reassembly assay** — The assay was carried out in vitro with purified Golgi membranes, p97, and its cofactors (2,3). Briefly, purified rat liver Golgi stacks were treated with mitotic cytosol, and the resulting membrane fragments were incubated with indicated amounts of purified p97/p47, p97/Δ69-92 p47, or p97/p37 proteins for reassembly. Membranes were processed for EM, and the results were quantified to estimate the activity of membrane fusion to form cisternal membranes. Mitotic Golgi fragments were normalized to 0%. Reassembly with WT p97 (167 nM hexamer) and p47 (167 nM trimer) was normalized to 100%. The results represent the mean of at least ten EM images ± SEM.

**Western blot analysis** — Human osteosarcoma U2OS cells were maintained in DMEM supplemented with penicillin/streptomycin and 10% fetal bovine serum. Cells were 80% confluent at the time of transfection with Flag-p47 plasmid. Transfections were performed with BioT reagent (Bioland Scientific LLC). Cells were lysed with Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific). The sample (25 µg) was loaded on a 4-20% gel for SDS-PAGE (Bio-Rad). Proteins were transferred to nitrocellulose membranes using the Trans-Blot Turbo system (Bio-Rad). Antibodies used include p97 (MA3-004, Thermo Scientific), Flag (F3165, Sigma-Aldrich), p62 (M162-3, MBL International Corporation), p47 (15620-1-AP, Proteintech Group Inc.), LC3 (PM036, MBL International Corporation), and GAPDH (2118, Cell Signaling Technology).

**Determining IC\textsubscript{50} values of p97 inhibitors in ATPase assays** — The detailed method was described previously (1,4). Inhibition of human p97 (25 nM monomer) was carried out in assay buffer (50 mM Tris pH 7.4, 20 mM MgCl\textsubscript{2}, 1 mM EDTA, 0.5 mM TCEP) containing 0.01% Triton X-100 and 200 µM ATP. The 8-dose titration was performed at 40, 13.3, 4.4, 1.48, 0.49, 0.16, 0.05, and 0 µM. ATPase activity was determined through the addition of Biomol Green Reagent (Enzo Life Sciences). p47 was added to a final concentration of 400 nM. NMS-873 was purchased from Xcess Biosciences Inc.
Supplemental Tables.

Table S1. Plasmids used in this study

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<tr>
<th>Plasmid Number</th>
<th>Plasmid name</th>
<th>Vector</th>
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<td>pBirAcm</td>
<td>pACYC184</td>
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Table S2. Gel filtration to determine the oligomeric states of p37, p47, and p47 variants.

Gel filtration was carried out with Superdex 75 10/300 GL (GE Healthcare). The column was calibrated with molecular weight (MW) standards kit (Sigma). The standards are Blue Dextran [MW is 2000 kDa; used to determine the void volume (Vo) of the column], β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa). The linear molecular weight calibration plotted Ve/Vo versus Log MW (see standard curve), where Ve is the elution volume of the MW standard and Vo is the void volume. To determine the apparent molecular weight of p37, p47, or p47 variants, we injected 100 µL of 20 µM proteins, determined their elution volumes (Ve), and then calculated apparent molecular weights using the equation obtained from the standard curve. The calculated MW was divided by the monomer MW to calculate the oligomeric state.

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<th>Molecular Weight Standard</th>
<th>Molecular Weight (kDa)</th>
<th>Elution Volume (mL)</th>
<th>Ve/Vo</th>
<th>Log MW</th>
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<td>1.462</td>
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<td>Bovine Serum Albumin</td>
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<td>10.2</td>
<td>1.192</td>
<td>1.819</td>
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<td>Alcohol Dehydrogenase</td>
<td>150</td>
<td>9.3</td>
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<td>β-Amylase</td>
<td>200</td>
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<td>Blue Dextran</td>
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![Standard Curve Image]

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<tr>
<th>Proteins</th>
<th>Ve (mL)</th>
<th>Ve/Vo</th>
<th>Log (calculated MW)</th>
<th>Calculated MW (kDa)</th>
<th>Monomer MW (kDa)</th>
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<td>107.3</td>
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<td>101.0</td>
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<td>107.3</td>
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* The calculated MW was divided by the monomer MW to calculate the oligomer number.
Table S3. Binding affinities for p97 cofactors to WT p97 and disease mutants R155H and A232E, measured by SPR.

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<th>R155H p97</th>
<th>A232E p97</th>
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<td>$K_{D1}$ µM</td>
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<td>WT p37$^1$</td>
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<td>0.032</td>
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$^1$ $K_D$ values determined using a one-site kinetic fit.
$^2$ $K_D$ values determined using a two-site equilibrium fit.
$^3$ $K_D$ values determined using a one-site equilibrium fit.
Supplemental Figure
Figure S1

A

Walker B mutants:
- Can bind to ATP/ADP
- Can not hydrolyze ATP

p97 disease mutants:
- N-domain: R155H
- ND1 linker: L198W
- D1-domain: A232E

B

N-domain
- "Down" conformation
- "Up" conformation

D1 domain
- ATP
- ADP

D2 domain
- ATP
- ADP

WT p97
Disease Mutant

C

WT p97/p47

ATPase activity (%)

200 µM ATP
800 µM ATP

p47 (nM)

D

WT p97/p47-800uM ATP

ATPase activity (%)

4.17 nM p97
20.8 nM p97

p47 (nM)

E

p47-800uM ATP

ATPase activity (%)

Phase 1
Phase 2

WT
R155H
L198W
A232E

p47 (nM)

F

WT p97/p37

ATPase activity (%)

200 µM ATP
800 µM ATP

p37 (nM)

G

p37-800uM ATP

ATPase activity (%)

4.5-fold
1.8-fold

p37 (nM)
Figure S1. Regulation of p97 ATPase activity by p37 and p47. (A) Diagram showing the domain representation of human p97 and the mutations analyzed in this study. (B) Differences in N-domain conformations between WT and p97 disease mutants. (C) Normalized WT ATPase activities (4.17 nM hexamer) in the presence of increasing amounts of p47 (0 to 800 nM) for 200 µM versus 800 µM ATP. Error bars indicate ± SD (n=6). (D) Normalized WT ATPase activities (4.17 nM versus 20.8 nM hexamer) in the presence of increasing amounts of p47 (0 to 800 nM) for 800 µM ATP. Error bars indicate ± SD (n=6). (E) Normalized ATPase activities of WT, R155H, L198W, and A232E p97 (4.17 nM hexamer) in the presence of increasing amounts of p47 (0 to 800 nM) with 800 µM ATP. Error bars indicate ± SD (n=12, excluding L198W, where n=6). (F) Normalized WT ATPase activities (4.17 nM hexamer) in the presence of increasing amounts of p37 (0 to 800 nM) for 200 µM versus 800 µM ATP. Error bars indicate ± SD (n=6). (G) Normalized ATPase activities of WT, R155H, L198W, and A232E p97 (4.17 nM hexamer) in the presence of increasing amounts of p37 (0 to 800 nM) with 800 µM ATP. Error bars indicate ± SD (n=6). (H) ATPase activities of WT, D1-E305Q, R155H, and the R155H, D1-E305Q double mutant were measured in the presence of p47 (0 to 800 nM) with 200 µM ATP. Error bars indicate ± SD (n=12, excluding double mutant R155H,E305Q, where n=6). (I) Normalized ATPase activities of WT, D1-E305Q, A232E, and the A232E, D1-E305Q double mutant were measured in the presence of p47 (0 to 800 nM). Error bars indicate ± SD (n=12). (J) ATPase activities of D1-E305Q and D2-E578Q were measured in the presence of p37 with 200 µM ATP. Error bars indicate ± SD (n=6). (K) Normalized ATPase activities of WT (4.17 nM hexamer) in the presence of increasing amounts of WT p47 and ΔUBA p47 (0 to 800 nM) with 200 µM ATP. The activity of each p97 protein was normalized to its basal activity in the absence of cofactor. Blue lettering indicates the active ATPase domain in each protein, and green lettering indicates the Walker B mutant. Error bars indicate ± SD (n=12 for WT p47, n=6 for ΔUBA p47).
Figure S2. Gel filtration and microscale thermophoresis analysis. Gel filtration was used to determine the stability of the p97-p47 complex for both WT and R155H p97. 1.67 μM p97 hexamer was mixed with 80 μM p47 for 10 min and fractionated in HEPES buffer (20 mM HEPES, 150 mM KCl, 1 mM MgCl₂, 5% glycerol, pH 7.4) with a gel filtration column (Superdex 20010/300 GL, GE Healthcare). (A) Western blot for Fractions 17 to 23. (B) Western blot for Fractions 24 to 30. Microscale thermophoresis (MST) analysis was used to determine the equilibrium binding constant.
(K_D) for trimer formation in solution for both (C) p37 and (D) p47. Unlabeled p97 cofactor was titrated against 400 pM of NT-647 labeled cofactor in two fold steps from 500,000 pM to 15 pM. Assays were performed in hydrophilic capillaries using a Monolith NT.115pico instrument.
Figure S3

A

Figure S3. WT p97 and disease mutants display similar binding to p47, and removal of p47 amino acids 69-92 does not affect p47-p97 interactions. (A) p47 and p37 sequence alignment.
Amino acid sequences were aligned using CLC Sequence Viewer 6 (CLC Bio). The alignment showed that p47 amino acid residues 69-92 (in red) are completely absent in p37. (B) and (C) An in vitro His pull-down assay was carried out by mixing 12 µM His-tagged p97 with 30 µM p47, p37, F253S p47, or Δ69-92 p47 at room temperature for 30 min. Unbound supernatant was removed, and Ni-Particles were washed with 500 µL washing buffer (50 mM HEPES, pH 7.4, 150 mM KCl, 5 mM MgCl₂, 20 mM imidazole) 3 times. Bound proteins were eluted by adding 100 µL 1x Laemmli Sample Buffer (Bio-Rad) and heated at 90°C for 5 min. Samples were separated on 4-20% Tris-Glycine Gels (Bio-Rad) and stained with Coomassie blue (Thermo Scientific Imperial Protein Stain). (B) Comparison of WT and A232E p97. (C) Comparison of WT and R155H p97.
Figure S4. SPR sensorgrams for p37 and p47 binding to WT p97 and disease mutants R155H and A232E. (A) Binding of WT p37 (67 pM-102.4 nM; 2.5 fold dilutions) to immobilized p97 fit to a 1:1 kinetic model (orange line). (B) Binding of WT p47 (419 pM-4 µM; 2.5 fold dilutions) to p97 fit to a two-site equilibrium-binding model (inset). (C) Binding of F253S p47 (2.6 nM-25 µM; 2.5 fold dilutions) to p97 fit to a one-site equilibrium-binding model (inset). (D-F) Binding of Δ69-92 p47 (D) Δ83-92 p47 (E) and 83-88 DE5 to NQ5 (F) (419 pM-4 µM; 2.5 fold dilutions) to p97 fit to a two-site equilibrium-binding model (insets).
Figure S5

A

**Golgi Cisternal Regrowth(%)**

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<td>+</td>
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<td>-</td>
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Figure S5. The R155H disease mutant displays elevated activity and reduced responses to the activating effect of p37, as shown by a p97-mediated post-mitotic Golgi reassembly assay. The assay was carried out in vitro with p97 cofactors. Purified rat liver Golgi stacks were treated with mitotic cytosol, and the resulting membrane fragments were re-isolated by centrifugation and further incubated with purified p97 and either p47, Δ69-92 p47, or p37. Membranes were processed for EM, and the results were quantified to estimate the percentage of cisternal membranes from the activity of cisternal membrane regrowth. Mitotic Golgi fragments were normalized to 0%. Reassembly with p97 (167 nM hexamer) and p47 (167 nM trimer) was normalized to 100%. The results represent the mean of at least ten EM images ± SEM. (A) p97-mediated post-mitotic Golgi reassembly assays. Column 1: Control (mitotic Golgi fragments), 2: WT p97 alone (167 nM hexamer), 3: R155H p97 alone (167 nM...
**Figure S6**

(A) Western blot assays to evaluate the effect of p47 in the autophagy pathway. U2OS cells were transiently transfected with Flag-p47 plasmid for 48 h to overexpress Flag-p47 (lanes 2 and 4). After 44 h of transfection, U2OS cells were treated with 200 nM Bafilomycin A for 4 h, to perform an “autophagic flux” assay (lane 3 and 4). GAPDH served as a loading control.

(B) Western blot assays to evaluate the effects of p47 on the disease mutant in the autophagy pathway. U2OS cells stably expressing WT, L198W, or A232E p97 proteins were used to determine the effect of p47 on the autophagy pathway. Cells were transiently transfected with Flag-p47 plasmid (2.5 or 5 µg) for 30 h. GAPDH served as a loading control.

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**Figure S6. p47 improves p97/VCP disease associated autophagy impairment.** (A) Western blot assays to evaluate the effect of p47 in the autophagy pathway. U2OS cells were transiently transfected with Flag-p47 plasmid for 48 h to overexpress Flag-p47 (lanes 2 and 4). After 44 h of transfection, U2OS cells were treated with 200 nM Bafilomycin A for 4 h, to perform an “autophagic flux” assay (lane 3 and 4). GAPDH served as a loading control. (B) Western blot assays to evaluate the effects of p47 on the disease mutant in the autophagy pathway. U2OS cells stably expressing WT, L198W, or A232E p97 proteins were used to determine the effect of p47 on the autophagy pathway. Cells were transiently transfected with Flag-p47 plasmid (2.5 or 5 µg) for 30 h. GAPDH served as a loading control.
Figure S7

A

[DBeQ](N,N)[HN]_{2}[N=N]\text{OCH}_3[N,N][O,N][S,N][S,O]

ML240

ML241

NMS-873

B

\( IC_{50} \) values of p97 inhibitors against WT p97, the p97-p47 complex, R155H p97, and the R155H p97-p47 complex. (A) Chemical structures of DBeQ, ML240, ML241, and NMS-873. (B) \( IC_{50} \) values of DBeQ. (C) \( IC_{50} \) values of ML240. (D) \( IC_{50} \) values of ML241. (E) \( IC_{50} \) values of NMS-873.
Figure S8

A

N-domain

“Down” conformation

“Up” conformation

D1 domain

D2 domain

p37 monomer

(major species at low concentration)

p37 trimer

(major species at high concentration)

Δ69-92 p47 monomer

(p37 mimic, major species at low concentration)

Δ69-92 p47 trimer

(p37 mimic, major species at high concentration)

Figure S8. Cofactor-induced changes in p97 ATPase activity. Diagram showing the domain representation of human p97 and p37, or Δ69-92 p47 cofactors. Illustrations depict changes in p97 ATPase activity of WT and disease mutants caused by the presence of (A) p37, or (B) Δ69-92 p47.
SI References


