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Generation, Analyzing and *in-vivo* Drug Treatment of *Drosophila* Models with IBMPFD

Ting Zhang[1], Bruce A. Hay[2] and Ming Guo[1, 3, 4, *]

[1]Department of Neurology, UCLA David Geffen School of Medicine, University of California, Los Angeles, CA 90095, USA; [2]Division of Biology and Biological Engineering, California Institute of Technology, Pasadena, CA 91125, USA; [3]Department of Molecular and Medical Pharmacology, UCLA David Geffen School of Medicine, University of California, Los Angeles, CA 90095, USA; [4]California Nanosystems Institute at UCLA, Los Angeles, CA 90095, USA

*For correspondence: mingfly@g.ucla.edu

[Abstract] Missense mutations of *p97/cdc48/Valosin-containing protein* (VCP) cause inclusion body myopathy, Paget disease with frontotemporal dementia (IBMPFD) and other neurodegenerative diseases. The pathological mechanism of IBMPFD is not clear and there is no treatment. We generated *Drosophila* models of IBMPFD in adult flight muscle *in vivo*. Here we describe a variety of assays to characterize disease pathology and dissect disease mechanism, and the consequences of *in vivo* feeding of VCP inhibitors.

Keywords: *Drosophila*, Inclusion body myopathy, Paget disease and frontotemporal dementia (IBMPFD), VCP/p97, Mitochondria, Muscle, Disease models, Inhibitors, Drug treatment

[Background] Mutations of *VCP/p97* cause inclusion body myopathy, Paget disease of the bone and frontotemporal dementia (IBMPFD), a degenerative disease in multiple systems including the brain, muscles and bones in an autosomal dominant fashion (Watts *et al.,* 2004). Mutations in VCP are also associated with 1-2% of cases of sporadic amyotrophic lateral sclerosis (ALS), as well as hereditary spastic paraplegia and Charcot-Marie-Tooth 2 neuropathy (Abramzon *et al.*, 2012; de Bot *et al.*, 2012; Gonzalez *et al.*, 2014). The R155H mutation is the most frequently identified in patients, while individuals with the A232E mutation have the most severe clinical manifestation (Kimonis *et al.*, 2008a; Ritson *et al.*, 2010). 90% of IBMPFD patients display myopathy, frequently the earliest symptom (Weihl *et al.,* 2009). 50% of patients will develop Paget's disease of bone, affecting skull, spine, hips and long bones. One-third of the patients develop frontotemporal dementia (Kimonis *et al.*, 2008b; Weihl *et al.*, 2009). Patients ultimately develop cardiopulmonary failure (Kimonis *et al.*, 2008b; Weihl *et al.*, 2009). *VCP* encodes a highly conserved and abundant AAA+ ATPase which participates in multiple cellular processes (Meyer *et al.*, 2012). Since VCP assembles as a hexamer, it has been controversial whether disease mutants with increased ATPase activity cause disease through a dominant-active (Chang *et al.*, 2011) or dominant-negative mechanism (Ju *et al.*, 2009; Ritz *et al.*, 2011; Bartolome *et al.*, 2013; Kim *et al.*, 2013; Kimura *et al.*, 2013). We built *in vivo* IBMPFD models to understand the pathogenesis of the disease and find potential treatments.

The specific assays for IBMPFD disease in flies include *in situ* cell death detection, muscle protein Western blot assays, immunofluorescence staining of disease markers, muscle integrity assay/Toluidine

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Blue staining, and muscle mitochondrial ultrastructural studies/Electron Microscopy (EM) imaging, and *in vivo* VCP inhibitors treatment (Yun *et al.*, 2008; Zhang *et al.*, 2017; Ma *et al.*, 2018).

Materials and Reagents

1. Razor blade (VWR North American, catalog number: 55411-050)
2. Microcentrifudge tube (Denville Scientific Inc., catalog number: C2171)
3. PCR tube (USA Scientific)
4. FORMVAR FILM on 100 Square Mesh Copper Grid (Electronic Microscopy Sciences, catalog number: FF100-Cu)
5. FORMVAR CARBON FILM on 2 x 1 mm oval slot Copper Grid (Electronic Microscopy Sciences, catalog number: FCF2010-Cu)
6. Disposable pellet pestle (Kimble, catalog number: 749521-0500)
7. PCR tube
8. Dissection dish
9. Needles
10. Pipette tips
11. Toothpick (Fisher Scientific Education, S04180)
12. 0.22 µm filter (Millipore, MillexGP Filter Unit)
13. Aluminum foil (Fisher Scientific, catalog number: 01-213-101)
14. Immobilon-P PVDF transfer membrane (Millipore, catalog number: IPVH00010)
15. *Drosophila* strains: UASt-VCP WT, VCP RH and AE lines were gifts from Dr. Tzu Kang Sang (Chang *et al*., 2011)
16. *Drosophila* Strain

    IFM-Gal4, with Gal4 under the control of the indirect flight muscle promoter, derived from the *flightin* gene (Yun *et al*., 2014). The IFM promoter provides a strong pulse of expression in late pupal stages and the first few days of adulthood, and is thereafter silent (Kandul *et al*., 2016). *Drosophila* strains were maintained in a 25 °C humidified incubator.
17. 20% paraformaldehyde, EM Grade (Electronic Microscopy Sciences, catalog number: 15713S)
18. 8% glutaraldehyde solution, EM Grade (Electronic Microscopy Sciences, catalog number: 16020)
19. 16% paraformaldehyde aqueous solution, EM Grade (Electronic Microscopy Sciences, catalog number: 15710)
20. Schneider's Buffer (GIBCO, catalog number: 21720-024)
21. *In Situ* Cell Death Detection Kit (Roche, catalog number: 11684795910)
22. Rhodamine Phalloidin (Life Technology, catalog number: R415)
23. Bovine Serum Albumin (Fisher Bio Reagent, catalog number: BP1600-100)
24. Fluoromount-G (Southern Biotech, catalog number: 0100-01)
25. Pierce RIPA lysate Buffer (Thermo Scientific, catalog number: 89900)

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26. Pierce Protease Inhibitor Tablets EDTA free (Thermo Scientific, catalog number: 88266)
27. Laemmli SDS Sample Buffer, 6x (Bioland Scientific LLC, catalog number: SAB02-02)
28. Anti-mouse IgG horseradish peroxidase linked whole antibody (from sheep) (GE Healthcare, catalog number: NXA931)
29. Anti-rabbit IgG horseradish peroxidase linked F(ab')2 fragments (from donkey) (GE Healthcare catalog number: NA9340V)
30. Goat anti-rabbit/mouse Alexa Fluor 546/488 secondary antibody (Life Technologies, catalog numbers: A11034/A11029/A11035/A10036)
31. Tween-20 500 ml (Hoefer, catalog number: 56-40-6)
32. Immobilon Western Chemiluminescent HRP Substrate (Millipore, catalog number: WBKLS0500).
33. Embed812 (Electronic Microscopy Sciences, catalog number: 14900)
34. Osmium Teroxide (OsO_4) 4% aqueous solution (Electronic Microscopy Sciences, catalog number: 19150)
35. DDSA (dodecenyl succinic anhydride) (Electronic Microscopy Sciences, catalog number: 13710)
36. NMA (nadic methyl anhydrate) (Electronic Microscopy Sciences, catalog number: 19000)
37. BDMA (benzyldimethylamine) (Electronic Microscopy Sciences, catalog number: 11400)
38. Propylene Oxide (Electronic Microscopy Sciences, catalog number: 20401)
39. Toluidine Blue O Powder (Electronic Microscopy Sciences, catalog number: 22050)
40. Sodium Borate (Fisher Scientific, catalog number: S249-500)
41. Uranyl Acetate Dihydrate (Ted Pella, catalog number: 19481)
42. Lead Nitrate (Sigma-Aldrich, catalog number: 22862-100G)
43. Sodium Citrate, dihydrate (EMD, catalog number: SX0442-1)
44. Sodium Hydroxide Certified ACS Pellets NaOH (Fisher Scientific, catalog number: S318-500)
45. NMS-873 (3-[3-(cyclopentylthio)-5-[[[2-methyl-4'-(methylsulfonyl) [1,1'-biphenyl]-4-yl] oxy] methyl]-4H-1,2,4-triazol-4-yl]-pyridine (Selleckchem, catalog number: S7285)
46. ML240,2-(2-Amino-1H-benzimidazol-1-yl)-8-methoxy-N-(phenylmethyl)-4-quinazolinamine (Sigma-Aldrich, catalog number: 1346527-98-7)
47. Permount mounting medium (Fisher Scientific, catalog number: SP15-100)
48. 0.1% Triton X-100
49. PBS
50. TUNEL Enzyme
51. β-mercaptoethanol
52. Ethanol
53. Muscle Dissection Fixative Buffer (see Recipes)
54. Dissection Buffer (see Recipes)
55. TUNEL Blocking Buffer (see Recipes)
56. 0.2 M Phosphate Buffer (pH = 7.4) (see Recipes)
57. 10 ml EM Fixative Buffer (see Recipes)

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58. Epon Mix (Medium) (see Recipes)
59. 1% Toluidine Blue Staining Solution (see Recipes)
60. Uranium Acetate Staining Solution (see Recipes)
61. Lead citrate Staining Solution (see Recipes)

Equipment

1. Pipette (Denville Scientific Inc., models: P10, P20, P200, P1000)
2. Diamond knife Ultra 45° (Diatome, catalog number: MX5341)
3. PELCO Reverse self-closing Tweezers (Ted Pella, catalog numbers: 5373/5375-NM)
4. DUMONT Biology Grade Tweezers (Ted Pella, catalog numbers: 505/505-U)
5. Pellet pestle motor (Kimble/Kontes, catalog number: 749540-0000)
6. JEOL 100CX transmission electron microscope (UCLA Brain Research Institute, Electronic Microscopic Core Facility)
7. Ultracut ultramicrotome (Leica EM UC6, Dr. Frank Laski, UCLA Molecular Cellular and Development Biology)
8. PELCO Pro Reverse (self-closing) tweezers (Ted Pella, catalog number: 5375-NM)
9. Dissection tweezers (Dumont Biology Switzerland, Electronic Microscopic Science)
10. Diamond knife (DiATOME, Ultra 45° MX5341)
11. Formvar mesh/slot grid (Electronic Microscopic Science, catalog numbers: FCF2010-Cu, FF100-Cu-50)
12. Water bath (Fisher Scientific, model: Isotemp)
13. The Mini-PROTEAN® tetra handcast systems and tetra blotting bodule (Bio-Rad)
14. Safety fume hood (UCLA facility 4315-3309-2)
15. Heat block (Fisher Scientific, model: Isotemp)

Procedure

A. Indirect flight muscle dissection
   1. Anesthetize flies on a CO_2 plate. Cut the head and abdomen off using a razor blade.
   2. Fix the thoraces in 4% paraformaldehyde/Schneider's Buffer for 45 min at room temperature in either a microcentrifuge tube or PCR tube.
   3. Transfer the post-fix thoraces into a dissection dish containing 0.1% Triton X-100/PBS. Triton X-100 helps to break the surface tension over the cuticles and facilitates muscle piece dissection in the following steps.
   4. Indirect flight muscles (IFM, indicated by red arrows in Figures 1A and 1B) are located in the middle of the thoraces. IFMs can be dissected out using either 30-gauge needles or tweezers. Use needles or tweezer to gently open up the thorax from either the dorsal or ventral midline and expose the IFM. The IFMs are two large groups of muscles that run anterior-posterior in the

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middle of the thorax (Figure 1C). Each group of IFMs contains 5 large muscles that lie parallel to each other and are located at the dorsal surface of the half-dissected thorax. Carefully isolate each muscle by cutting at the ends that attach the muscle to the cuticle. One well-fixed wildtype thorax can generate 5-10 fragments of intact muscle (Figure 1C). Flies that carry an IFM targeted GFP marker can be used to further confirm that the correct muscle has been dissected (Figure 1C).

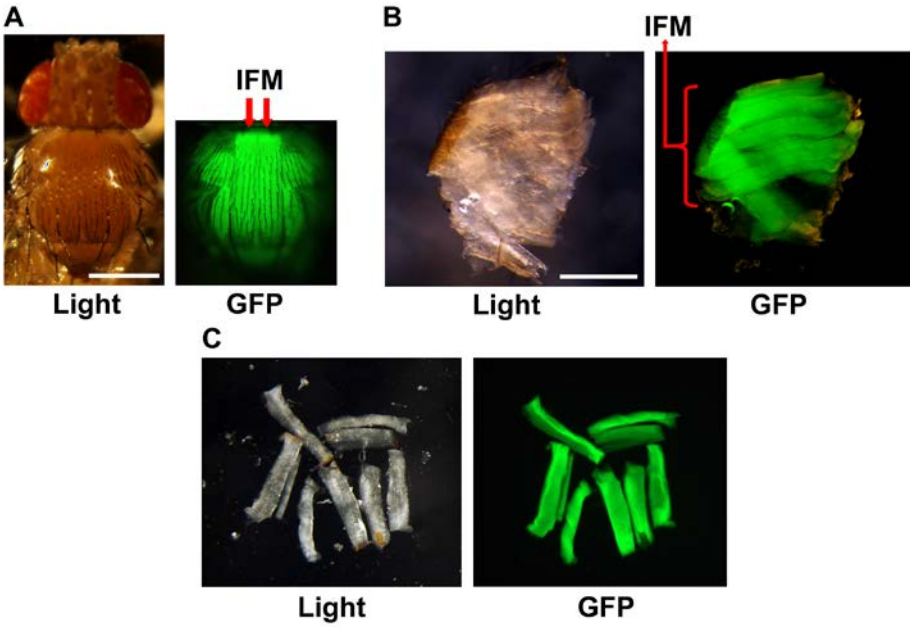

Figure 1. Indirect Flight Muscle (IFM) Dissection. A. The location of IFM in intact thorax (red arrows). B. The sagittal plane of a fixed thorax, red arrow indicating where the IFM are located. C. Dissected muscle pieces in the dissection dish. The genotype of the flies used is IFM-Gal4>UASt-mitoGFP. Scale bar: 0.5 mm.

B. *In situ* cell death detection/TUNEL Assay for IBMPFD flies

1. Fix the thoraces and dissect the IFMs from WT, VCP WT, RH and AE flies as described above and collect into PCR tubes.
2. Introduce TUNEL Blocking buffer into the tubes containing muscle for 30 min. The TUNEL Blocking buffer contains 0.2% Triton X-100 that permeabilizes and blocks the muscle sample at the same time.
3. Add TUNEL Enzyme (4 µl) and 10x Reaction Buffer (36 µl) from the *in-situ* Cell Death Detection Kit and incubate for 2-3 h at 37 °C in a water bath. Use a pipette to gently mix the sample every 30 min.
4. Wash the samples with PBS twice.
5. Replace PBS with Fluoromount-G mounting solution. Mount IFMs on a microscopic slide using either a glass pipette or 200 µl pipette tip and visualize the muscle with a confocal microscope

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using an excitation wavelength of 546 nm. In 6 days old flies, VCP RH and AE expression leads to significant cell death (Zhang *et al*., 2017). This is observed as extensive red nuclear staining.

C. Immunofluorescence staining and confocal microscopy imaging
   1. Dissect the fixed muscle as described above. Then wash with PBS and permeabilize muscles with 0.2% Triton X-100/PBS for 3-4 h on the rocker at room temperature followed by 5 min wash with 1x PBS.
   2. For myofibril staining, add Rhodamine Phalloidin (1:500 dilution) and stain for 2 h at room temperature or 4 °C overnight.
   3. For immunofluorescence staining, fixed muscle fragments are incubated with primary antibody diluted to the desired concentration (1:100 or 1:200) in 0.2% Triton X-100/PBS at 4 °C overnight. We used anti-TARTAR Binding Protein or anti-TDP43 at a concentration of 1:100.
   4. Wash 3x each for 10 min with 0.2% Triton X-100/PBS.
   5. Incubate muscle fragments with a goat anti-rabbit/mouse Alexa Fluor 488 or 546 secondary antibody (1:200 in 0.2% Triton X-100/PBS) for 2 h at room temperature or 4 °C overnight.
   6. Wash three times (10 min each time) with 0.2% Triton X-100/PBS and mount samples on a microscope slide with Fluoromount-G. Samples are visualized under a confocal microscope with an excitation wavelength at 488 nm or 546 nm corresponding to the secondary antibodies. In 6-day old flies, TDP43 localizes both in the nucleus and sarcoplasmic/cytosolic area of the IFM in wildtype flies; the nucleus localization pattern is lost and more aggregates are observed in the sarcoplasmic/cytosolic region in VCP RH and AE mutants (Zhang *et al*., 2017).

D. Protein lysates and Western blot
   1. Anesthetize flies with CO_2, isolate thoraces as above, and put them in a microcentrifuge tube on ice.
      Note: The thorax is not fixed.
   2. Add RIPA lysis buffer with protease inhibitors, 10-15 µl for each thorax, 5-10 thoraces for each genotype.
   3. Homogenize the thoraces in the lysis buffer with prechilled the polypropylene pellet pestles 5-6 times, followed by spinning the pestle with the mortar at the highest speed for 10-15 s. Incubate lysate on ice for 30 min to fully lyse the tissue.
   4. Centrifuge protein lysates at 10,000 *x g* for 15 min and transfer the supernatant to a clean microcentrifuge tube.
   5. Boil the supernatant with 6x SDS sample buffer with β-mercaptoethanol at 95 °C for 5 min.
   6. Load proteins and let them separate in 8% SDS-PAGE Gels in Tris/SDS running buffer and run it at 80 volts.
   7. Transfer proteins to PVDF membrane for 2 h in Tris/Glycine Buffer with 15% methanol.
   8. Incubate transfer membrane with 3% BSA/PBS for 1 h.

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9. Incubate the membrane with primary antibody diluted in 1% BSA/0.01% Tween-PBS at 4 °C overnight.
10. Wash the membrane in 0.01% Tween-PBS for three times (10 min each) and incubate with anti-mouse/rabbit IgG HRP linked secondary antibodies diluted in 5% non-fat milk/0.01% Tween-PBS for 2 h at room temperature.
11. Develop the membrane with Immobilon Western Chemiluminescent HRP Substrate Kit. We expressed VCP WT, VCP RH and AE at comparable levels in the thoraces (Zhang *et al.*, 2017).

E. Toluidine blue staining and electronic microscopy for IBMPFD flies

Fixation and Embedding

Note: Perform Step E4 and subsequent steps in a safety fume hood.

1. Anesthetize flies and cut the thoraces, quickly dip in 95% ethanol (this helps to break the surface tension so as to facilitate subsequent immersion in the EM fixative buffer). Then transfer thoraces to 0.5 ml ice-old EM Fixative Buffer (below) in a microcentrifuge tube.
2. Fix for a minimum of 2 h on ice with rocking. If fixation is carried out for longer or the protocol needs to be paused, samples may be left in fixative after the 2 h time point and placed at 4 °C overnight or until ready to proceed. Thoraces will sink to the bottom of microcentrifuge tube.
3. Rinse each sample with 0.1 M phosphate buffer for three times (10 min each time) at room temperature.

   Note: All subsequent steps should be carried out in a Bio-safety hood.
4. Post-Fix samples in 1% OsO_4 in ddH_2O (freshly prepared) for 2 h at room temperature. Samples should not be fixed with OsO_4 for more than 2 h or the samples will become brittle.
5. Rinse sample with ddH_2O for three times (10 min each) at room temperature.
6. Dehydrate samples in 70% and 95% ethanol for 5 min each at room temperature.
7. Dehydrate samples in 100% ethanol twice (10 min each) at room temperature.
8. Dehydrate samples in 100% propylene oxide twice (7 min each) at room temperature.
9. Infiltrate samples in 1:1 mixture of 100% propylene oxide and Epon mix for 30 min at room temperature.
10. Incubate samples in 100% Epon Mix (1.5% BDMA added) overnight at room temperature.
11. Introduce samples in 100% Epon Mix (1.5% BDMA added) into an embedding mold. In the bottom right of each well, put a piece of paper with sample name/number (Figure 2A).
12. Polymerize Epon at 60-70 °C overnight.

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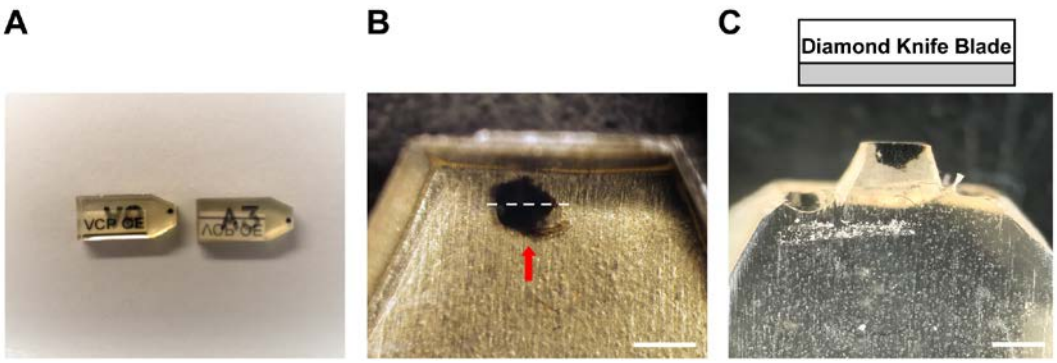

Figure 2. *Drosophila* thorax embedding and section position. A. Two individual samples embedded and blocked with labels. B. Properly embedded fly thorax (red arrow) in the Epon Resin. The sagittal plane of the thorax (white dotted lines) should be parallel to the edge of the resin block so as to facilitate the section. C. A well-trimmed resin block with a thorax sample after thick section. Glass knife or Diamond knife blades should run parallel to the sagittal plane of the thorax. Scale bar: 1 mm.

Sectioning and Staining

1. Toluidine Blue Staining
    a. Well-embedded thorax samples are positioned in the resin block as shown (Figure 2B). The sagittal midline of the thorax is parallel to the edge of the resin block.
    b. Use a razor blade to cut away the Epon around the sample as much as possible (Figure 2C). Decreasing the section surface will increase the section quality.
    c. Cut thick sections (1.5-2 µm) for Toluidine Blue staining and to determine the area within which thin sections will be taken for electron microscopy imaging.
        i. Use a microtome with glass knife to cut thick sections. Transfer sections to water drops on the glass slide using a toothpick.
        ii. Evaporate the water by putting the slide on a 70 °C heat block. This will flatten the section samples.
        iii. Apply 1% Toluidine Blue to fully cover the section sample area and put the slide on the 70 °C heat block until the edge of the Toluidine Blue liquid starts to dry (2-5 min). Do not over dry the Toluidine Blue solution or the section will become contaminated by the dry solute in the staining buffer, which results in degradation of image quality.
        iv. Rinse the slide in ddH_2O until non-bound stain is removed.
        v. Let the section dry completely before applying the Permount mounting solution.
    d. A well-aligned thick section sample of a wildtype thorax is shown in Figure 3A (Indirect flight muscle pieces can be easily observed). Higher magnification images of the tissue samples can be utilized (Figure 3B) to assay muscle tissue integrity. In 6-day old flies, VCP RH and AE expressing flies have disrupted muscle tissue integrity as compared to WT and VCP WT controls (Zhang *et al.*, 2017).

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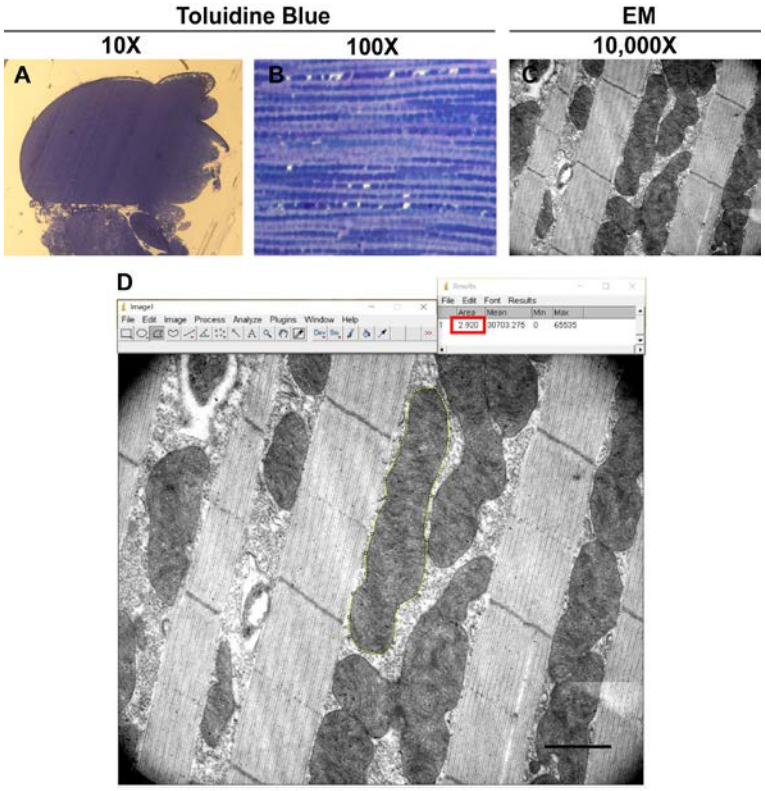

Figure 3. Toluidine Blue and EM images of IFM. A-B. Toluidine Blue staining of thick sections of thorax at different magnifications. C. EM image of a thin section of wildtype thorax. D. Mitochondrial size quantification using ImageJ. Scale bar: 1 μm (119 pixels). Cross-section area of selected mitochondrion is 2.92 μm^2 (Red square).

2. Electronic microscopy sample preparation
   a. Select well-aligned samples from the Toluidine Blue staining to be used in thin sectioning (80-90 nm).
      i. Use a microtome with a Diamond knife (with sterilized ddH_2O) to cut thin sections. The sections should appear silver to gold color.
      ii. Use a fine needle to position 1-4 sections in a line on the water surface for grid mounting.
      iii. Pick up an empty slot grid that is not coated with formvar with tweezers. Gently touch the grid to sections floating on the surface. The sections will attach to the middle of the slot within the water drop.
      iv. Use a pair of self-closing tweezers to hold a new formvar-sealed slot grid or mesh grid with the shiny-side up. Place the section-attached slot grid onto the shiny side of the new formvar grid.
      v. Remove the water drop between two grids by placing the sharp corner of a triangle-shaped filter paper at the edge between the grids. Sections will attach to the formvar-sealed new grids and flatten.
      vi. Remove the empty grid and reuse it to pick up additional sections. The section-attached grid can be stored in the grid box for further staining as described below.

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b. Prepare the uranium acetate (UA) solution and filter through a 0.22 µm filter system. Float the section side of the grid on the UA solution for 15 min at room temperature. Wash the grid five times (1 min each) in a ddH_2O water drop or three times (1 min each) in 3 separate beakers containing ddH_2O.
c. Put the grid section with shiny side down on a drop of the lead citrate solution and stain for 5-10 min at room temperature. Wear a mask to prevent breathing on to the sections as excess CO_2 will affect staining quality. Keep the staining dishes covered with the plastic cover that comes with the staining dish.
d. Wash the grid with ddH_2O as above.
e. Transfer the stained grid to the grid box for further imaging.
f. Figure 3C shows an image of wildtype muscle visualized using TEM at 10,000x. In 6-day old flies, VCP RH and AE expressing flies display disrupted actin and mitochondrial structure as compared with wildtype and VCP WT flies (Zhang *et al*., 2017).

3. Mitochondrial cross-section size quantification
a. Import an ultrastructural electron microscopy image (10,000x magnification) into ImageJ Software (National Institute of Health), Figure 3D.
b. After setting the scale (analyze>set scale: 119 pixels = 1 µm), using Polygon selection (Yellow line) trace out each mitochondrion on the image and measure its area (analyze > measure), Figure 3D. All the mitochondria on the image are individually measured.
c. At least three images are analyzed for each thorax and 3 thoraxes of each genotype were examined.
d. An independent Student's *t*-test is used to test for statistical significance between different genotypes.

F. *In vivo* VCP inhibitor treatment
1. Dissolve powdered forms of NMS-873 (19.2 mM, stock concentration) and ML240 (25.2 mM, stock concentration) in DMSO as stocks.
2. Dilute Stock solution in ethanol/ddH_2O (100 µl Ethanol + 600 µl ddH_2O) to the desired concentration. Ethanol can help keep the compound in solution. Heat *Drosophila* food in a microwave for 20 s until it fully melts, and then let it cool down to < 50 °C. Add the chemical in DMSO/ethanol H_2O to the food along with 10 µl of food dye per 4 ml of fly food, and hand-mix it until the color appears homogenous. Vials with cotton plugs can be dried at room temperature if food seems wet. DMSO/ethanol in comparable amounts is used as a vehicle control.
3. Put *Drosophila* parents of desired genotypes in food containing DMSO/ethanol or inhibitors for 3 days to lay eggs and then remove them. Progeny growth then occurs in the presence of vehicle control or test compound.
4. Immediately after eclosion of adults (hatch from their pupal case), transfer these adults to freshly prepared food containing the same concentration of DMSO or inhibitor, and feed the flies for the desired number of days before assaying them.

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5. VCP inhibitors feeding significantly reversed the muscle disintegration, muscle cell death and ultrastructural mitochondrial defects in VCP RH and AE flies (Zhang *et al*., 2017).

Recipes

1. Muscle Dissection Fixative Buffer (500 µl)
   100 µl 20% paraformaldehyde
   400 µl Schneider's Buffer
   Store at 4 °C
   Note: Preferably prepare fresh each experiment.
2. Dissection Buffer
   0.1% Triton X-100
   1x PBS Buffer
   Store at room temperature
3. TUNEL Blocking Buffer
   50 mM Tris-Cl (pH = 7.4)
   188 mM NaCl
   0.2% Triton X-100
   1% BSA
   Store at 4 °C
4. 0.2 M Phosphate Buffer (pH = 7.4)
   Solution X: 3.516 g $Na_2HPO_4 \cdot 2H_2O$/100 ml ddH_2O
   Solution Y: 2.76 g $NaH_2PO_4 \cdot H_2O$/100 ml ddH_2O
   Mix 40.5 ml of Solution X with 9.5 ml of Solution Y, then get 50 ml of 0.2 M Phosphate Buffer. The pH should be 7.4
   Store at room temperature
5. 10 ml EM Fixative Buffer
   1.25 ml 8% glutaraldehyde
   0.625 ml 16% paraformaldehyde
   5.0 ml 0.2 M phosphate buffer
   3.125 ml ddH_2O Sterile
   Store at 4 °C
6. Epon Mix (Medium)

Embed812	20 ml
DDSA (dodecenyl succinic anhydride)	16 ml
NMA (nadic methyl anhydrate)	8 ml
BDMA (benzyldimethylamine)	0.9 ml (Add fresh for each embedding)

   Mix thoroughly at least 1 h before embedding
   Can be stored at room temperature in light-proof container. Preferably prepare fresh each time

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7. 1% Toluidine Blue Staining Solution
   1 g Toluidine blue powder
   1 g Sodium Borate
   100 ml of ddH_2O
   0.22 µm filtered before use.
   Store at room temperature in light-proof container
8. Uranium Acetate Staining Solution
   4 g Uranyl acetate
   100 ml ddH_2O
   Dissolve fully (heat up to 70 °C to facilitate dissolving)
   0.22 µm filtered before use
   Store at room temperature in light-proof acrylic storage container that protects from beta radiation
9. Lead Citrate Staining Solution
   Boil 100 ml ddH_2O to get rid of CO_2 in a small beaker covered with aluminum foil let it cool to room temperature
   1.33 g lead nitrate
   1.76 g sodium citrate
   30 ml CO_2-free ddH_2O
   8 ml 1 N NaOH (4 g NaOH in 100 ml ddH_2O)
   Add CO_2-free ddH_2O to a total volume of 50 ml
   Store at 4 °C in light-proof container

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Competing interests

We have no competing interests.

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