Alterations in organismal physiology, impaired stress resistance, and accelerated aging in *Drosophila* flies adapted to multigenerational proteome instability

Maria S. Manola, Eleni N. Tsakiri and Ioannis P. Trougakos

**Supplementary Information**

**Supplementary Figures Legends**

**Figure S1.** Drawing showing the different culture conditions and corresponding nomenclature of non-treated (NT) Oregon*^®* (A) and of G80-BTZ (exposed for >80 generations to 0.5 μM BTZ) (B) flies. NT or G80-BTZ flies were transiently exposed to standard medium (SM) or to SM containing either 0.5 or 1 μM BTZ as indicated.

**Figure S2.** Multigenerational developmentally non-lethal proteasome inhibition in *Drosophila* flies increased proteome instability and redox imbalance. (A1) Relative (%) CT-L and C-L proteasome activities in young G80-BTZ female and male flies’ somatic tissues lysates; comparisons were vs. proteasome activities of NT flies. (A2) Analysis of isolated 26S proteasomes from somatic tissues of young NT and G80-BTZ flies under ultraviolet light (UV) and in Native-PAGE following probing with a Prosβ5 antibody. (B) Immunoblot analysis of proteome ubiquitination (Ub) (B1) and carbonylation (dinitrophenol/DNP) (B2) in somatic tissues of young NT vs. G80-BTZ flies. GAPDH probing was used in (A2, B1, B2) as input reference. (C) (%) ROS levels in shown populations of middle-aged flies. Bars, ± SD (n=2); * P < 0.05; ** P < 0.01.

**Figure S3.** Analyses of proteasome activities after adding BTZ in lysates containing intact proteasomes or in shown flies’ tissues; and tissue ROS levels. (A) Relative (%) CT-L and C-L peptidase activities following addition of the shown BTZ concentrations in flies’ somatic tissue lysates containing intact proteasomes. (B) Relative (%) CT-L and C-L proteasome activities in somatic tissues of NT, G80 and G80-BTZ flies at the age of 4, 10, 20 or 30 days. (C) (%) ROS levels in somatic tissues of the indicated middle-aged flies. Bars, ± SD (n=2). * P < 0.05; ** P < 0.01.

**Figure S4.** Autophagic genes expression in NT and G80-BTZ aged flies and LysoTracker™ staining in control and G80-BTZ larvae fat bodies. (A) Relative expression of ref(2)P, Atg6, Atg8a and cathD
genes in somatic tissues of aged NT and G80-BTZ flies; gene expression was plotted vs. the respective control (NT flies). (B1) Staining with LysoTracker™ of isolated fat bodies from NT or G80-BTZ larvae and (B2) relative (%) lysosome quantification per µm², (n) nucleus. The Rp49 gene expression was used in (A) as input reference. Bars, ± SD (n=2); * P < 0.05.

**Figure S5. Proteome carbonylation and ubiquitination in mitochondria isolated from NT and G80-BTZ adult flies.** Blots were probed with antibodies against DNP (carbonylation) and Ub; ATP5a and GAPDH were used a loading references in mitochondria and cytosol samples respectively.

**Figure S6. The effect of high protein or low calories diet on G80-BTZ flies.** Longevity curves of G80-BTZ flies cultured in high protein (A) (HPM) or low calories (B) (CRM) medium. Equal numbers of female/male flies were used in each experiment. Statistics of the longevity assays are reported in Table S1; the shown G80-BTZ longevity curve is identical in (A), (B).

**Figure S7. The effect of low protein diet on NT flies treated (or not) with 0.5 μM BTZ.** Longevity curves (vs. controls) of NT flies exposed (B) [or not (A)] to 0.5 μM BTZ and co-cultured in low protein medium (LPM). Equal numbers of female/male flies were used in each experiment; comparative statistics of the longevity assays are reported in Table S1.

**Graphical Abstract.** Our summarized findings indicate that multigenerational proteotoxic stress and redox imbalance causes metabolic reprogramming and impaired stress resistance; it also promotes fecundity and neuromuscular defects, and accelerates aging.

**Supplementary Tables**

Table S1. Summary of lifespan experiments.

**Supplementary list of Abbreviations**

19S regulatory particle, RP; 20S core particle, CP; 6-bromo-indirubin-3′-oxime, 6BIO; Adenosine triphosphate, ATP; Autophagy-lysosome pathway, ALP; Bortezomib, BTZ; Caspase-like activity, C-L; Chymotrypsin-like activity, CT-L; Forkhead box O, Foxo; Glucose, GLU; Glycogen synthase kinase, Gsk3; Glycogen, GLY; Heat shock protein, Hsp; Insulin/IGF-like signaling, IIS; Multiple myeloma, MM; Nuclear factor erythroid 2-related factor, Nrf2; Non-treated, NT; Proteostasis network, PN; Reactive oxygen species, ROS; Standard medium, SM; Trehalose, TREH; Ubiquitin, Ub; Ubiquitin-proteasome pathway, UPP.
### REAGENT OR RESOURCE | SOURCE | IDENTIFIER
--- | --- | ---
**Antibodies**
Prosβ5 (β5) | M. Figueiredo-Pereira | Vernace et al. 2007
26S proteasome α (IIG7) (20So) | Santa Cruz Biotechnology | sc-65755
Complex V subunit-ATP5a | Abcam | ab14748
Ilp2 | Prof. Ernst Hafen | N/A
Foxo | Cosmo Bio Co | CAC-THU-A-FOXO
ref(2)P (p62) | Prof. Gábor Juhász University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, University, Univers
| Chemical                  | Supplier          | Code   |
|---------------------------|-------------------|--------|
| Chloroquine               | Sigma-Aldrich     | C6628  |
| ClCH₂COONa                | Fluka             | 24610  |
| Coomassie Blue            | Fluka             | 27816  |
| Developer D-19            | Kodak             | 146 4593 |
| Diethyl malate            | Sigma-Aldrich     | W237418 |
| DMSO                      | Sigma             | 270431 |
| DTT                       | Applichem         | A2948  |
| EDTA                      | Applichem         | A2937  |
| EGTA                      | Applichem         | A0878  |
| ELIOH                     | Sigma-Aldrich     | 46139  |
| FCCP                      | CAYMAN CHEMICAL   | 370-86-5 |
| Fixer                     | Agfa              | G382B  |
| Glutamic acid             | Sigma-Aldrich     | G1251  |
| Glycerol                  | Applichem         | A2926  |
| Glycine                   | Serva             | 23390  |
| Hepes                     | Biosera           | PM-B2093 |
| KCl                       | Applichem         | 131494 |
| KH₂PO₄                    | Applichem         | A1043  |
| MeOH                      | Scharlau          | ME0316005l |
| MG-132                    | Enzo Life Sciences| 260-092-M05 |
| MgCl₂                     | Sigma-Aldrich     | 13152  |
| Mowiol®                   | Sigma-Aldrich     | 4-88   |
| Glutamic acid             | Sigma-Aldrich     | G1251  |
| Na₂HPO₄                   | Sigma-Aldrich     | S3264  |
| NaCl                      | Merck             | 1.06405.1000 |
| Native-PAGE sample additive 5% | Invitrogen    | BN2004 |
| Native-PAGE sample buffer | Invitrogen        | BN20032 |
| NP-40                     | Sigma             | NP40S  |
| Oligomycin                | Sigma-Aldrich     | 75351  |
| PBS                       | Sigma             | P4417  |
| PIC (proteases inhibitor cocktail) | Sigma | I5763 |
| Propionic acid            | Sigma-Aldrich     | 402907 |
| SDS                       | Serva             | 20765  |
| Product Code            | Company                        | Component Name               | Cat. No.  |
|------------------------|--------------------------------|------------------------------|----------|
| Suc-Leu-Leu-Val-Tyr-AMC-LLV | Enzo Life Sciences             | A2211                        | BML-P802-0005 |
| Sucrose                | Applichem                      | A3452                        |          |
| TEMED                  | Applichem                      | A1148                        |          |
| Trehalase              | Sigma-Aldrich                  | T8778                        |          |
| Tricine                | Sigma-Aldrich                  | 5704-04-1                    |          |
| Tris-HCl               | Applichem                      | A4975                        |          |
| Triton X-100           | Applichem                      | P-1379                       |          |
| Tween 20               | Sigma                          | BML-P139-0050                 |          |
| z-FR-AMC               | Enzo Life Sciences             | BML-ZW9345-0005              |          |
| Z-Leu-Leu-Glu-AMC-LLE  | Enzo Life Sciences             |                             |          |
| Critical Commercial Assays/Kits |                      |                              |          |
| Bradford assay         | Bio-Rad                        | 5000006                      |          |
| GLU Reagent            | Sigma-Aldrich                  | GAGO-20                      |          |
| Onescript cDNA synthesis kit | ABMGOOD                  | G233                         |          |
| HOT FIREPol Eva green qPCR Mix Plus | SolisBioDyne                   | 08-24-00001                  |          |
| Western ECL Blotting substrates | Bio-Rad                     | 1705060                      |          |
| Native-PAGE 3-12% gradient pre-cast Bis-Tris gels | Novex-Life Technologies | BN1001BOX                     |          |
| Nitrocellulose membrane | Macherey-Nagel GmbH           | 741280                       |          |
| PVDF membrane          | Macherey-Nagel GmbH           | 741290                       |          |
| OxyBlot                | Millipore                      | #s7150                       |          |
| Fluorescent dyes       |                                |                              |          |
| BODIPY 493/503         | Molecular Probes™ - TFS        | D3922                        |          |
| LysoTracker™ Deep Red  | Invitrogen                     | L12492                       |          |
| DAPI                   | Molecular Probes™ - TFS        | D1306                        |          |
| Oligonucleotides       | See Method Details for primer sequences | This study | N/A |
| Software and Algorithms |                                |                              |          |
| MS Excel               | Microsoft                      | N/A                          |          |
| IBM SPSS; version 24.0 | IBM                            | N/A                          |          |
| ImageJ                 | Wayne Rasband (NIH)           | N/A                          |          |
| Digital Eclipse Nikon C1 software | Nikon Inc.                     | N/A                          |          |
**Supplementary Materials and Methods**

**List of Genes**

*Rpn11* (Regulatory particle non-ATPase 11, FBgn0028694, CG18174); *Rpn10* (Regulatory particle non-ATPase 10, FBgn0015283, CG7619); *Rpn6* (Regulatory particle non-ATPase 6, FBgn0028689, CG10149); *Prosa7* (Proteasome α7 subunit, FBgn0023175, CG1519); *Prosf5* (Proteasome β5 subunit, FBgn0029134, CG12323); *Prosf2* (Proteasome β2 subunit, FBgn0023174, CG3329); *Prosf1* (Proteasome β1 subunit, FBgn0010590, CG8392); *ref(2)P* (refractory to sigma P-RNA and export factor binding protein 2, FBgn0003231, CG10360); *Atg6* (Autophagy-related 6, FBgn0264325, CG5429); *Atg8a* (Autophagy-related 8a, FBgn0052672, CG32672); *cathD* (cathepsin D, FBgn0029093, CG1548); *PGC-1 (srl, spargel, FBgn0037248, CG9809); *TFAM* (mitochondrial transcription factor A, FBgn0038805, CG4217); *CG11267* (also known as *Hsp10*, 10kDa heat shock factor, mitochondria-like, FBgn0036334, CG11267); *Hsp60A* (Heat shock protein 60A, FBgn0015245, CG12101); *Hsc70-5* (Heat shock 70 kDa protein cognate 5, FBgn0001220, CG8542); *Marf* (Mitochondrial assembly regulatory factor, FBgn0029870, CG3869); *Opa1* (Optic atrophy 1, FBgn0261276, CG8479); *Drp1* (Dynamin related protein 1, FBgn0026479, CG3210); *ATPsynB* (ATP synthase, subunit B, FBgn0019644, CG8189); *SdhA* (Succinate dehydrogenase, subunit A, flavoprotein, FBgn0261439, CG17246); *Lon* (Lon protease, FBgn0036892, CG8798); *park* (parkin, FBgn0041100, CG10523); *Pink1* (PTEN-induced putative kinase 1, FBgn0029891, CG4523); *Ilp2* (Insulin-like peptide 2, FBgn0036046, CG8167); *Ilp6* (Insulin-like peptide 6, FBgn0044047, CG14049); *InR* (Insulin-like receptor, FBgn0283499, CG18402); *Pdk1* (Pyruvate dehydrogenase kinase, FBgn0017558, CG8808); *Akt1* (Akt1, FBgn0010379, CG4006); *foxo* (forkhead box, subgroup O, FBgn0038197, CG3143); *G6P* (Glucose-6-Phosphatase, FBgn0031463, CG15400); *Pepek* (Phosphoenolpyruvate carboxykinase, FBgn0003067, CG17725); *GlyP* (Glycogen Phosphorylase, FBgn0004507, CG7254); *GlyS* (Glycogen Synthase, FBgn0266064, CG6904); *Ide* (Insulin degrading metalloproteinase, FBgn0001247, CG5517); *PyK* (Pyruvate kinase, FBgn0267385, CG7070); *PEK* (pancreatic elf-2α kinase, FBgn0037327, CG2087); *Akh* (Adipokinetic hormone, FBgn0004552, CG1171); *ATGL* (Bmm, bummer- adipose triglyceride lipase, FBgn0036449, CG5295); *tgl* (tangled_Phasmatid Acid Phospholipase A1- triglyceride lipase, FBgn0084120); *Keap1* (Kelch-like ECH-associated protein 1, FBgn0038475, CG3962); *Trxr-1* (Thioredoxin reductase-1, FBgn0020653, CG2151); *Rp49* (*RpL32*, Ribosomal protein L32, FBgn0002626, CG7939).

**Tissues dissection, sorting of flies and haemolymph extraction**

Proteasome is regulated in a tissue, sex and age-dependent manner [26]; thus, experiments were performed in dissected female or male somatic (head and thorax) tissues collected from young, middle-aged or old flies. For sorting experiments, female and male flies were anesthetized using CO₂ 24 h prior to the experiment and the same number of individuals was used per sample. Haemolymph was isolated as described previously (Tsakiri et al., 2013).
**Total RNA extraction and quantitative Real-Time PCR (Q-RT-PCR) analyses**

Total RNA was extracted from flies’ somatic tissues and converted to cDNA with the OneScript® cDNA Synthesis Kit of ABMGOOD (G234). cDNA was then subjected to Q-RT-PCR analysis using the HOT FIREPol® EvaGreen® qPCR Mix Plus of SOLISBIODYNE (08-24-00001) as described previously [21,33]. Primer sets were as described before [8,33,46].

**Extraction of protein; immunoblot analyses and detection of protein carbonyl groups**

Pooled or sex-sorted flies’ somatic tissues or isolated mitochondria were homogenized on ice in Nonident P-40 (NP-40) lysis buffer [0.1 % Nonidet P-40, 150 mM NaCl, 50 mm Tris/HCl buffer (pH 8.0)] enriched with protease inhibitors and centrifuged for 10 min at 19,000 x g (4°C). The protein content of each sample was quantified by Bradford assay (Bio-Rad) and analyzed by SDS-PAGE/immunoblotting as described before [26,46]. Immunoblots were developed using an enhanced chemiluminescence Western blot detection kit (Bio-Rad Laboratories, Inc; 1705060S). For the detection of protein carbonyl groups, the OxyBlot protein oxidation detection kit (Millipore, Billerica, MA; #s7150) was used as per manufacturer’s instructions. ImageJ was used to quantify protein expression in blots.

**Native gel electrophoresis of proteasomes; measurement of ROS, cathepsin B, L and proteasome enzymatic activities**

Proteasomes were analyzed according to Elsasser et al. (2005) with minor modifications. Somatic tissues from 20 young flies were collected in lysis buffer [50 mM Tris·HCl (pH 7.6), 5mM MgCl₂, 10% Glycerol, 5mM ATP and 1mM DTT]; tissues were homogenized, and samples were centrifuged 10 min at 9,000x g (4°C). Supernatants were collected, and the protein content of each sample was quantified using Bradford assay. Native-PAGE 3-12% gradient pre-cast Bis-Tris gels (Novex-Life Technologies, Thermo-Fisher Scientific) were used to resolve 26S proteasomes. Prepared samples (50 μg of purified proteasomes) were mixed with 5x sample buffer [250 mM Tris-HCl (pH 7.4), 50% glycerol, 60 ng/ml xylene cyanol] just before loading. Electrophoresis was carried out at 4°C with an applied voltage of 100-110 Volts (~23-25 mA) for 3 h. Following completion of electrophoresis, gels were carefully transferred to a clear glass dish containing developing buffer [50 mM Tris-HCl (pH 7.4), 5 mM MgCl₂, and 1 mM ATP]. To assess proteasome activity, gels were incubated with 50 μM of suc-LLVY-AMC, a fluorogenic substrate for CT-L peptidase activity, in developing buffer for 30 min at 37°C. Next, gels were exposed to UV and fluorescent bands were visualized by standard gel-imaging systems. For immunoblotting, remaining proteins were transferred to polyvinylidene fluoride membranes and immunoblotting was performed as described above. ROS levels, cathepsin and proteasome activities were measured as described previously [26,30, Tsakiri et al., 2013] and
expressed as (%) values vs. respective controls. When both male/female samples were analyzed equal numbers of male/female flies were used.

**Mitochondria isolation, measurement of mitochondrial respiration, blue native-PAGE and measurement of GLU, TREH and GLY Levels**

Mitochondria isolation, respiration analyses and blue native PAGE were performed as described before (Nijtmans et al., 2002; Ferguson et al., 2005; Cogliati et al., 2013); in most cases dissected somatic tissues from 30 flies were analyzed. GLU, GLY and TREH levels from indicated tissues were measured as described previously [46] (Barrio et al., 2014). At least 3 replicates per genotype or experimental condition were performed.

**Adipose and muscle tissue preparation for immunohistochemistry and CLSM viewing**

Adipose tissue was attached to the dorsal abdominal area and was isolated after removing head, thorax and the internal organs. Muscle tissue was recovered from the thoracic area after removing head and abdomen. Dissections were performed in PBS and tissues were fixed in 4 % formaldehyde for 15 min, washed in PBS containing 0.3 % Triton X-100 and were then incubated with the primary antibody (1:100) for 1 h in RT. Secondary antibodies (1:500), BODIPY (Molecular Probes™, TFS) (1:100), or DAPI (Thermo Fischer Scientific) staining were applied for 30 min in RT. Samples were washed in PBS, mounted in Mowiol® 4-88 (Sigma) and viewed at a Nikon C1 Confocal Laser Scanning Microscope (CLSM) equipped with a 40×, 1.0 NA differential interference contrast (DIC), 60×, 1.4 NA DIC Plan Apochromat objectives using the EZC1 acquisition and analysis software (Nikon). In most experiments 5-7 animals per population were analyzed. Measurement of CLSM stained structures (e.g. lipid droplets) was performed by ImageJ.

**Larval fat bodies preparation for LysoTracker™ staining**

Following dissection of 3rd instar stage larvae and removing all internal organs, fat bodies were attached to the dorsal abdominal area. Isolated fat bodies were simultaneously stained with DAPI and 100 μM LysoTracker Red (L-7528, Molecular Probes) as per manufacturer’s instructions; samples were viewed using CLSM. The relative (%) quantification of the lysosomes per larval fat body area (μm²) was performed using Image J.

**Statistical Analysis**

Statistical analysis of the results was performed as previously described [26]. Presented experiments were analyzed at least in duplicates, unless otherwise indicated. Data points correspond to the means of the independent experiments; error bars denote standard deviation (SD). For statistical analysis, MS Excel (Microsoft, Redmond, WA, USA) and the Statistical Package for Social Sciences (IBM
SPSS 19.0 for Windows; IBM, Armonk, NY, USA) were used. Significance at $P < 0.05$ or $P < 0.01$ is indicated in graphs by one or two asterisks, respectively.

**Supplementary References**

L. Barrio, A. Dekanty and M. Milán, “MicroRNA-mediated regulation of Dp53 in the *Drosophila* fat body contributes to metabolic adaptation to nutrient deprivation,” *Cell Reports*, vol. 8, pp. 528-41, 2014.

S. Cogliati, C. Frezza, M.E. Soriano and et al., “Mitochondrial Cristae Shape Determines Respiratory Chain Supercomplexes Assembly and Respiratory Efficiency,” *Cell*, vol. 155, no. 1, pp. 160-171, 2013.

S. Elsasser, M. Schmidt and D.B. Finley, “Characterization of the Proteasome Using Native Gel Electrophoresis,” *Ubiquitin and Protein Degradation, Part A. Academic Press*, vol. 398, pp. 353–363, 2005.

M. Ferguson, M.J. Mockett, Y. Shen and et al., “Age-associated decline in mitochondrial respiration and electron transport in *Drosophila melanogaster*,” *Biochemical Journal*, vol. 390, pp. 501–511, 2005.

L.G. Nijtmans, N.S. Henderson and I.J. Holt, “Blue Native electrophoresis to study mitochondrial and other protein complexes,” *Methods*, vol. 26, pp.327–334, 2002.

E.N. Tsakiri, K.K. Iliaki, A. Höhn, S. Grimm, I.S. Papassideri, T. Grune and I.P. Trougakos, “Diet-derived advanced glycation end products or lipofuscin disrupts proteostasis and reduces life span in *Drosophila melanogaster*,” *Free Radical Biology and Medicine*, vol. 65, pp. 1155–1163, 2013.

V.A. Vernace, L. Arnaud, T. Schmidt-Glenewinkel, M.E. Figueiredo-Pereira, “Aging perturbs 26S proteasome assembly in *Drosophila melanogaster*.” *FASEB Journal*, vol. 21, pp 2672–2682, 2007.
Manola et al. Fig. S1

Parental Oregon\textsuperscript{R} flies (NT)

Nomenclature used for non-treated Oregon\textsuperscript{R} flies (NT) cultured in standard medium (SM) and \textit{transiently} exposed to 0.5 or 1 μM BTZ

G80-BTZ flies

Nomenclature used for Oregon\textsuperscript{R} flies cultured in SM containing 0.5 μM BTZ for >80 generations (G80-BTZ) and \textit{transiently} exposed to BTZ-free SM or to SM containing 1 μM BTZ
Manola et al. Fig. S2
Manola et al. Fig. S3

**A**

CT-L

C-L

**B**

CT-L

C-L

**C**

(% ROS levels)

NT

G80
A

Aged flies

Relative mRNA expression levels

NT   G80-BTZ   NT   G80-BTZ   NT   G80-BTZ   NT   G80-BTZ

ref(2)P
Atg6
Atg8a
*cathD

B1

Larvae fat bodies

50 μM

NT   G80-BTZ

DAPI
LysoTracker™

B2

(%) number of lysosomes/ larval fat body area (μm²)

NT   G80-BTZ

Manola et al. Fig. S4
Survival Time in days

A

G80-BTZ

G80-BTZ-HPM

B

G80-BTZ

G80-BTZ-CRM

Manola et al. Fig. S6
Manola et al. Fig. S7

(A) Survival Time in days

(B) Survival Time in days

Survival vs Time in days

- NT
- NT-LPM
- NT-BTZ
- LPM
Manola et al. Graphical Abstract

- Sustained proteotoxic/oxidative stress
- Fecundity defects
- Neuromuscular defects
- Impaired stress resistance
- Metabolic reprogramming
- Accelerated aging

Pharmacological developmentally non-lethal proteasome inhibition

>80 generations
## Supplementary Table S1. Summary of lifespan experiments.

| Figure | Sample | Mean Lifespan (LF) +/- s.e.m. (Days) | Median Lifespan +/- s.e.m. (Days) | % Median LF vs. control | Max (Days) | Log Rank P Value | Total Animals Died/Total |
|--------|--------|--------------------------------------|------------------------------------|--------------------------|------------|------------------|--------------------------|
| Fig. 1E1 | NT ♀ | 53.3 ± 1.8 | 58 ± 1.3 | 100 | 84 | G80-BTZ ♀ | NT ♀ | G80-BTZ ♀ | Total Animals Died/Total |
|         | G80-BTZ ♀ | 30.0 ± 1.8 | 32 ± 1.6 | 55 | 51 | 0.000 | 0.000 | 0.182 | 54/55 |
| NT ♂    | 49.8 ± 1.9 | 50 ± 2.9 | 86 | 84 | 0.246 | 0.000 | 0.000 | 82/93 |
| G80-BTZ ♂ | 27.0 ± 1.4 | 27 ± 0.9 | 47 | 51 | 0.000 | 0.000 | 0.000 | 86/90 |
| Fig. 1E2 | NT ♀ | 46.4 ± 1.3 | 49 ± 2.1 | 100 | 69 | G80 ♀ | NT ♀ | G80 ♀ | Total Animals Died/Total |
|         | G80 ♀ | 44.7 ± 1.0 | 35 ± 0.7 | 76 | 71 | 0.000 | 0.000 | 0.000 | 240/292 |
| Fig. 2D | NT-BTZ / 1 ♀ | 19.7 ± 0.7 | 20 ± 0.7 | 100 | 29 | NT-BTZ / 1 ♀ | G80-BTZ / 1 ♀ | NT-BTZ / 1 ♀ | G80-BTZ / 1 ♀ | Total Animals Died/Total |
|         | G80-BTZ / 1 ♀ | 25.6 ± 0.9 | 27 ± 1.2 | 136 | 39 | 0.000 | 0.000 | 0.000 | 88/81 |
| NT-BTZ / 1 ♀ | 21.4 ± 0.9 | 23 ± 1.8 | 115 | 30 | 0.048 | 0.000 | 0.261 | 38/40 |
| G80-BTZ / 1 ♀ | 21.7 ± 0.8 | 20 ± 1.3 | 36 | 72 | 0.024 | 0.000 | 0.000 | 69/75 |
| Fig. 3E1 | NT ♀ | 54.9 ± 1.4 | 59 ± 0.9 | 100 | 64 | G80-BTZ ♀ | NT ♀ | G80-BTZ ♀ | Total Animals Died/Total |
|         | G80-BTZ ♀ | 45.1 ± 2.2 | 43 ± 1.5 | 73 | 69 | 0.000 | 0.000 | 0.000 | 110/129 |
| NT-ΒΤΖ / 1 ♀ | 32.0 ± 1.3 | 33 ± 1.0 | 56 | 53 | 0.000 | 0.000 | 0.000 | 109/106 |
| G80-ΒΤΖ / 1 ♀ | 16.9 ± 1.0 | 16 ± 0.8 | 27 | 27 | 0.000 | 0.000 | 0.000 | 34/38 |
| Fig. 3E2 | NT ♀ | 46.6 ± 1.5 | 50 ± 2.1 | 100 | 75 | NT-ΒΤΖ / - CQ | G80-ΒΤΖ / - CQ | NT-ΒΤΖ / - CQ | G80-ΒΤΖ / - CQ | Total Animals Died/Total |
|         | G80-ΒΤΖ / - CQ | 38.2 ± 2.0 | 37 ± 3.1 | 74 | 64 | 0.000 | 0.000 | 0.000 | 131/130 |
| NT-ΒΤΖ / - CQ | 25.1 ± 1.2 | 27 ± 0.8 | 54 | 51 | 0.000 | 0.000 | 0.000 | 72/72 |
| G80-ΒΤΖ / - CQ | 15.5 ± 0.9 | 17 ± 0.6 | 34 | 28 | 0.000 | 0.000 | 0.000 | 35/37 |
| Fig. 5A1 | G80-BTZ | 29.5 ± 2.6 | 30 ± 4.7 | 100 | 52 | G80-BTZ-LPM | G80-BTZ-LPM | Total Animals Died/Total |
|         | G80-BTZ-LPM | 34.7 ± 2.1 | 36 ± 3.7 | 120 | 59 | 0.304 | 0.000 | 35/36 |
| Fig. 5A2 | G80-BTZ | 32.1 ± 1.5 | 34 ± 2.2 | 100 | 52 | G80-BTZ-LPM | G80-BTZ-LPM | Total Animals Died/Total |
|         | G80-BTZ-LPM | 25.6 ± 1.2 | 26 ± 2.9 | 74 | 41 | 0.000 | 0.000 | 78/80 |
| Fig. 5B1 | G80-BTZ | 32.1 ± 1.5 | 34 ± 1.2 | 100 | 52 | G80-BTZ-CRM | G80-BTZ-CRM | Total Animals Died/Total |
|         | G80-BTZ-CRM | 18.4 ± 0.7 | 14 ± 0.7 | 41 | 33 | 0.000 | 0.000 | 139/148 |
| Fig. 5A1 | NT ♀ | 46.0 ± 1.3 | 46 ± 2.2 | 100 | 89 | NT-LPM | NT-LPM | Total Animals Died/Total |
|         | NT-LPM | 43.0 ± 1.8 | 42 ± 3.1 | 61 | 60 | 0.095 | 0.000 | 138/142 |
| Fig. 5B1 | NT-BTZ | 35.8 ± 1.2 | 36 ± 1.2 | 100 | 58 | NT-BTZ-LPM | NT-BTZ-LPM | Total Animals Died/Total |
|         | NT-BTZ-LPM | 31.9 ± 1.2 | 31 ± 1.7 | 46 | 56 | 0.06 | 0.000 | 93/104 |

* shown also in S6A