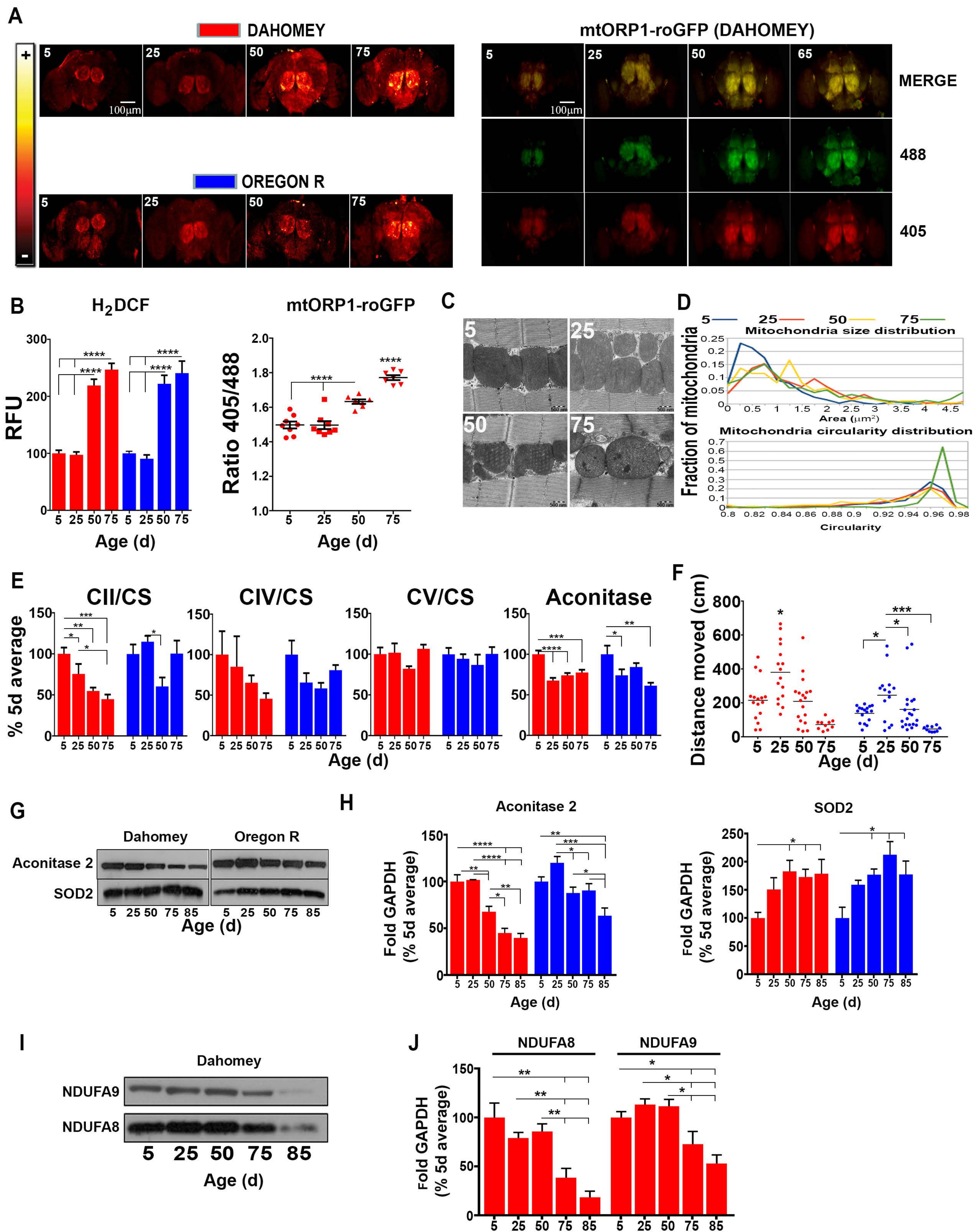
Cell Metabolism, Volume *23*

Supplemental Information

Mitochondrial ROS Produced via Reverse Electron Transport Extend Animal Lifespan

Filippo Scialò, Ashwin Sriram, Daniel Fernández-Ayala, Nina Gubina, Madis Lõhmus, Glyn Nelson, Angela Logan, Helen M. Cooper, Plácido Navas, Jose Antonio Enríquez, Michael P. Murphy, and Alberto Sanz


A
DAHOMEY
OREGON R
mtORP1-roGFP (DAHOMEY)
MERGE
488
405
100μm
B
H2DCF
RFU
Age (d)
mtORP1-roGFP
Ratio 405/488
C
D
Mitochondria size distribution
Mitochondria circularity distribution
Fraction of mitochondria
Area (μm2)
Circularity
E
CII/CS
CIV/CS
CV/CS
Aconitase
% 5d average
F
Distance moved (cm)
G
Dahomey
Oregon R
Aconitase 2
SOD2
H
Aconitase 2
SOD2
Fold GAPDH
(% 5d average)
I
Dahomey
NDUFA9
NDUFA8
J
NDUFA8
NDUFA9
Scialò et al. Supplemental Figure 1

Supplemental Figure 1. Related to Figure 1. Increased ROS production in aging flies correlates with mitochondrial dysfunction.

(A) Representative images of dissected fly brains stained with H_2DCF (left) from Dahomey and Oregon R of indicated ages and *in vivo* ROS measurements in brains dissected from Dahomey flies expressing a mitochondrially-localised redox-active GFP-based reporter (mtORP1-roGFP) of indicated ages.

(B) Quantification of A (n=7-8).

(C) Representative EM images of dissected female Dahomey dorsal flight muscle mitochondria at the indicated ages at 12000x magnification.

(D) Quantification of mitochondrial size (top) and circularity (bottom) in Figure 1C (5d, n=1069; 25d, n=753; 50d, n=901; 75d, n=744).

(E) Enzymatic activities in Dahomey and Oregon R flies (n=6).

(F) Locomotion (cms moved in 1 hour) for wild-type flies at different ages (n=10-20).

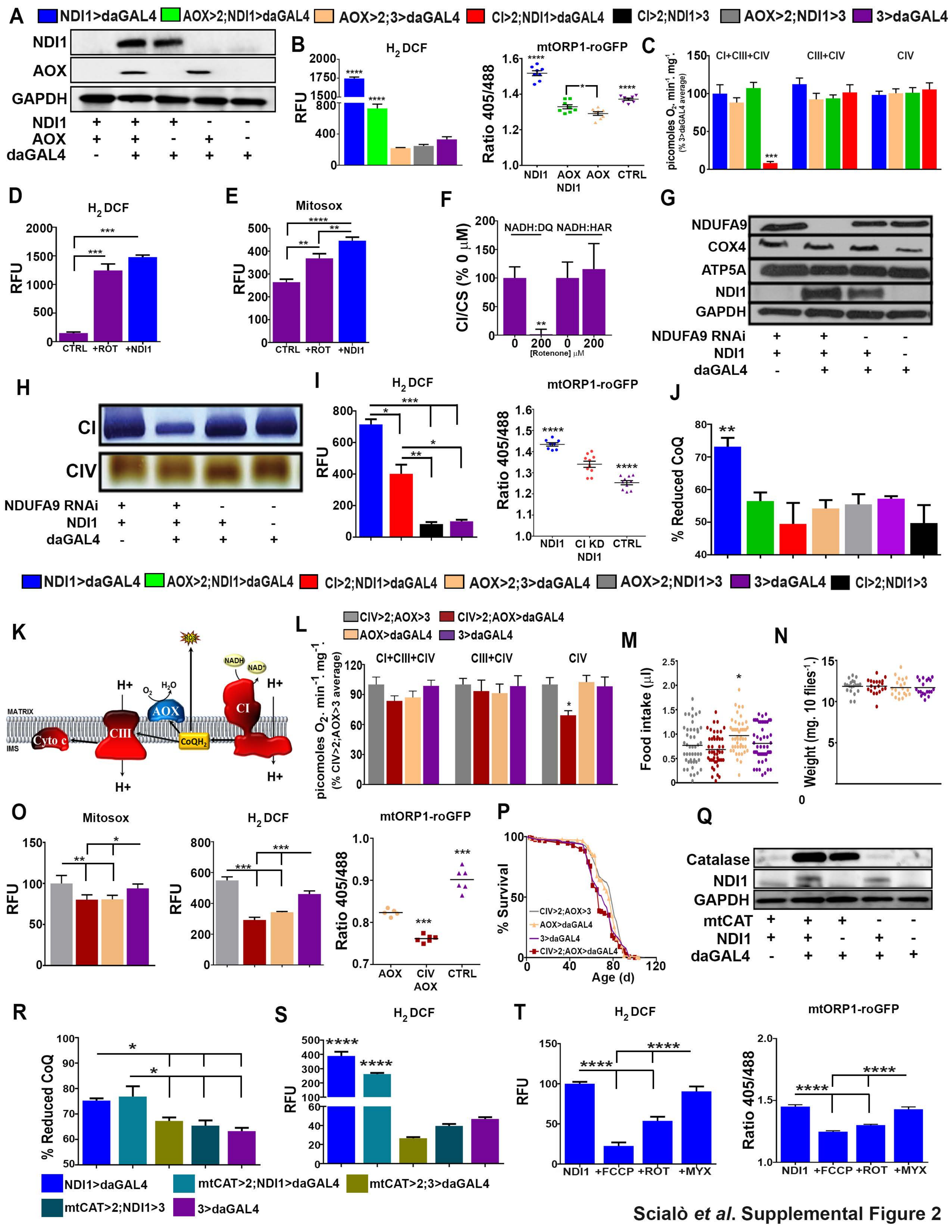
(G) Representative western blots.

(H) Quantification of **G**.

(I) Representative western blots for other CI subunits in Dahomey flies at different ages.

(J) Quantification of **I**.

Values shown represent means ± SEM of at least 3 biological replicates, unless otherwise stated.



Scialò *et al*. Supplemental Figure 2

Supplemental Figure 2. Related to Figure 2. Interrogating how NDI1 produces ROS and extends lifespan.

(A) Representative western blots showing expression of NDI1 and AOX.

(B) Quantification of fly brains dissected from the indicated genotypes stained with H_2DCF (n=5) or expressing mtORP1-roGFP (n=7-8).

(C) Mitochondrial respiration from flies of the indicated genotypes (n=3-18).

(D) Quantification of fluorescence from dissected fly brains stained with H_2DCF with (NDI1>daGAL4) and without (CTRL) NDI1 or rotenone (200 μM) treatment (+ROT) (n=5).

(E) Quantification of fluorescence from dissected fly brains stained with MitoSOX with (NDI1>daGAL4) and without (CTRL) NDI1 or rotenone (200 μM) treatment (+ROT) (n=5).

(F) CI activities in rotenone-fed wild-type flies.

(G) Representative western blots for ND-39/NDUFA9 (CI subunit), COXIV (CIV subunit), ATP5A (CV subunit) and NDI1 (GAPDH serves as loading control) in the indicated genotypes.

(H) Representative activity-stained blue-native gel for CI and CIV from fly mitochondria of the indicated phenotypes.

(I) Quantification of fluorescence in dissected fly brains from CI>2;NDI1>daGAL4 flies and controls stained with H_2DCF (n=5) or expressing mtORP1-roGFP (n=8-10).

(J) Percentage of reduced CoQ in flies of the indicated genotypes.

(K) Diagram depicting the strategy (i.e. reduction of CIV levels) to specifically over-reduce ETC downstream of the CoQ pool to localize the ROS signal.

(L) Mitochondrial respiration in flies of the indicated phenotypes (n=6).

(M, N) Food intake (n=50) and body weight (n=20) in CIV>2;AOX>daGAL4 flies and controls.

(O) Quantification of brains dissected from flies of the indicated genotypes stained with MitoSOX, H_2DCF or expressing mtORP1-roGFP (n=5).

(P) Survival curves of the indicated genotypes (n=200). See Table S1 for statistical analysis.

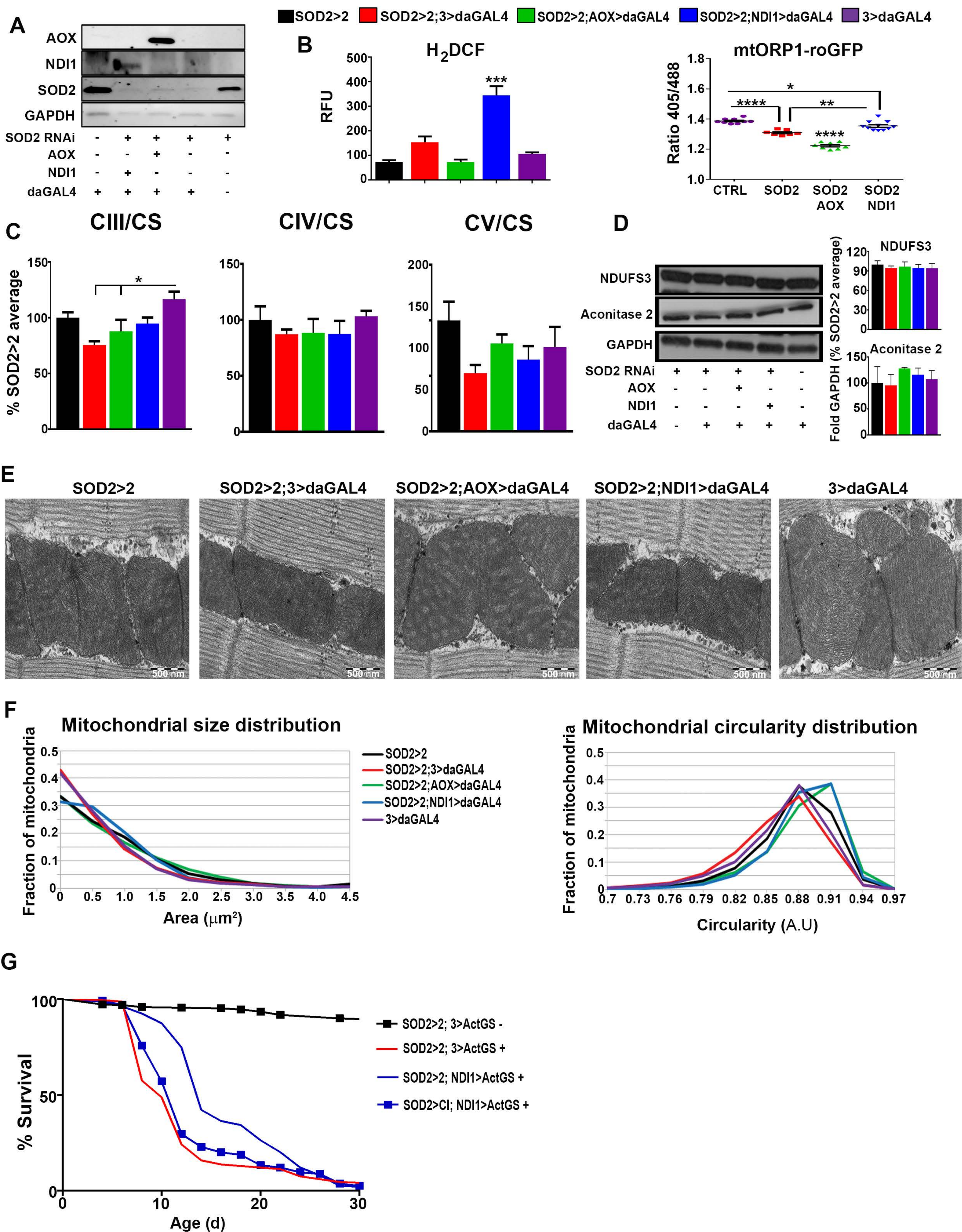
(Q) Representative western blots showing expression of Catalase, NDI1 and AOX.

(R) Percentage of reduced CoQ in flies of the indicated genotypes (n=4-5).

(S) Quantification of brains dissected from flies of the indicated genotypes stained with H_2DCF (n=4-5).

(T) Quantification of brains dissected from NDI1>daGAL4 flies fed with the indicated metabolic poisons, stained with H_2DCF (n=5) or expressing mtORP1-roGFP (n=7-10).

Values shown represent means ± SEM of at least 3 biological replicates, unless otherwise stated.



Scialò *et al*. Supplemental Figure 3

Supplemental Figure 3. Related to Figure 3. Effects of SOD2 knockdown together with co-expression of NDI1 or AOX on mitochondrial function.

(A) Representative western blot showing SOD2, AOX and NDI1 levels in the indicated genotypes.

(B) Quantification of brains dissected from flies of the indicated genotypes stained with H_2DCF (n=4) or expressing mtORP1-roGFP (n=7).

(C) Enzymatic assays showing activity of CIII, CIV (n=4) and CV in the indicated genotypes.

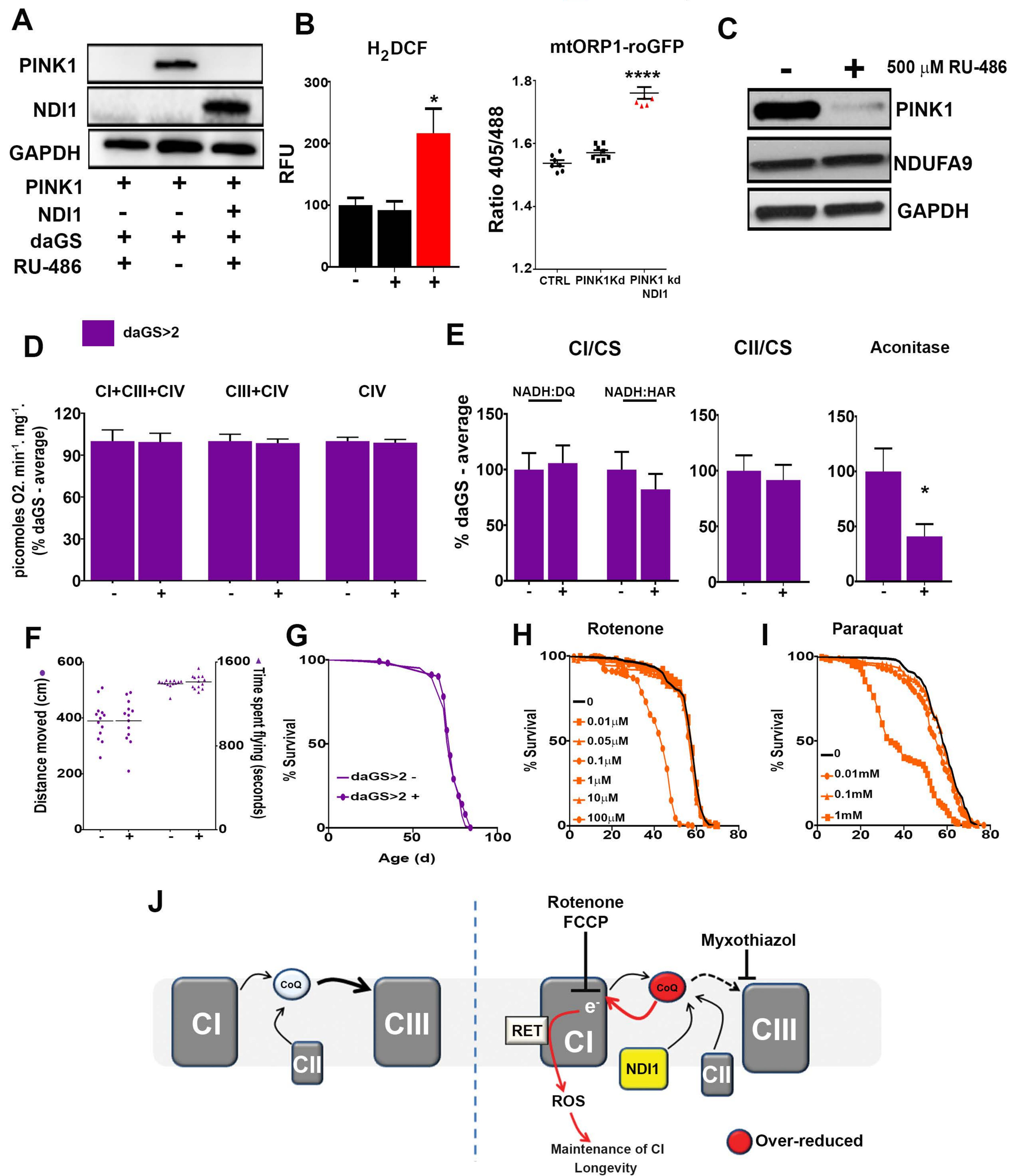
(D) Representative western blots and quantification of levels of NDUFS3 (CI subunit) and Aconitase 2 in the indicated genotypes (GAPDH is used as a loading control)

(E) Representative electron micrographs of dissected dorsal flight muscle mitochondria (20,000x magnification) from the indicated genotypes.

(F) Quantification of mitochondrial size (left) and circularity (right) in **E**.

(G) Survival curves of indicated genotypes (n=120-180). -/+ indicates presence/absence of 300 μM RU-486. See Table S1 for statistical analysis.

Values shown represent means ± SEM of at least 3 biological replicates, unless otherwise stated.



Scialò *et al.* Supplemental Figure 4

Supplemental Figure 4. Related to Figure 4. Preventing reduction of CoQ mediates age-related pathology.

(A) Representative western blots of PINK1 and NDI1 levels in the indicated genotypes.

(B) Quantification of fluorescence in brains dissected from flies from the indicated genotypes stained with H_2DCF (n=4) or expressing mtORP1-roGFP (n=4-8).

(C) Representative western blots of PINK1 and NDUFA9 levels in the indicated genotypes.

(D) Mitochondrial respiration in daGS>2 flies fed with RU-486 (+) and controls (-) (n=4).

(E) CI, CII and aconitase activities in daGS>2 flies fed with RU-486 (+) and controls (-) (n=4).

(F) Locomotive activity and flight time in daGS>2 flies fed with RU-486 (+) and controls (-) (n=13).

(G) Survival curves in daGS>2 flies fed with RU-486 (+) and controls (-) (n=200). See Table S1 for statistical analysis

(H) Survival curves of Dahomey flies fed with the indicated doses of rotenone (n=156-200). See Table S1 for statistical analysis.

(I) Survival curves of Dahomey flies fed with the indicated doses of paraquat (n=160). See Table S1 for statistical analysis.

(J) Model of NDI1-mediated extension of lifespan and maintenance of CI function. Black arrows indicate electron (e^-) transport events in forward direction; red arrows indicated e- transport events in reverse direction; flat-headed arrows indicate inhibition. Left, without NDI1; right, with NDI1.

Values shown represent means ± SEM of at least 3 biological replicates, unless otherwise stated. -/+ indicates presence/absence of 500 µM RU-486.

Table S1 -Related to Figures 2, 3 and 4 and Supplemental Figures 2, 3 and 4. Lifespan summary.

Statistical analysis of all lifespan experiments presented in this study. In parentheses, reference to the appropriate Figure panel is indicated.

NS indicates not significant differences.

Table S2. ***Drosophila transgenic lines.*** **Related to Experimental Procedures.**

Fly Strain	Source	Details
w^{Dah} ; UAS-Ndi1	(Sanz et al., 2010)	Dahomey backcrossed UAS-Ndi1 on 3rd Chromosome
w^{Dah} ; UAS-AOX	(Fernandez-Ayala et al., 2009)	Dahomey backcrossed UAS-AOX on 2nd Chromosome
w^{Dah} ; UAS-AOX	(Fernandez-Ayala et al., 2009)	Dahomey backcrossed UAS-AOX on 3rd Chromosome
w^{Dah} ; UAS-SOD2	Vienna Drosophila Resource Center (VDRC)	Dahomey backcrossed UAS-SOD2 RNAi on 2nd Chromosome (VDRC ID: 42162)
w^{Dah} ; UAS-ND-39	VDRC	Dahomey backcrossed UAS-ND-39 (CI) RNAi on 2nd Chromosome (VDRC ID: 13131)
w^{Dah} ; UAS-levy	VDRC	Dahomey backcrossed UAS-levy RNAi (CIV) on 2nd Chromosome (VDRC ID: 101523)
w^{Dah} ; UAS-Pink1	VDRC	Dahomey backcrossed UAS-Pink1 RNAi on 2nd Chromosome (VDRC ID: 109614)
w^{Dah} ; daGAL4	Bloomington *Drosophila* Stock Center (BDSC)	Dahomey backcrossed daughterless GAL4 driver on 3rd Chromosome (BDSC N^o: 55849)
w^{Dah}; daGS	(Tricoire et al., 2009)	Dahomey backcrossed daughterless GeneSwitch GAL4 driver on 2nd Chromosome
w^{Dah}; actGS	BDSC	Dahomey backcrossed Actin5c GeneSwitch GAL4 driver on 3rd Chromosome (BDSC N^o: 9431)
w^{Dah}; UAS- mito-ORP1-roGFP2	(Albrecht et al., 2011)	Dahomey backcrossed mitochondrially targeted ROS reporter on 2nd Chromosome
w^{Dah}; UAS-mtCatalase	(Mockett et al., 2010)	Dahomey backcrossed mitochondrially targeted catalase on 2nd Chromosome

Description of all transgenic fly lines (including the final background) used in this study.

Supplemental Experimental Procedures

Fly husbandry. Following eclosion, flies were tipped to new food and allowed to mate for 24 hours before being sorted and collected using CO_2 anesthesia. Female flies were used in all experiments. Flies were maintained at a density of 20 flies/vial and transferred to new food every 2-3 days. For all lifespan experiments, at least 80 flies were used. Each experiment was repeated at least 2-4 times as indicated in Table S1. Wild-type *Dahomey* and *Oregor R* strains were obtained from the laboratory of Prof Howy Jacobs (University of Helsinki). UAS-NDI1 flies and UAS-AOX flies have been described previously by Sanz *et al*. (2010) and Fernandez-Ayala *et al.* (2009), respectively. Flies containing UAS-RNAi constructs against *CG8905* (SOD2), *CG6020* (ND-39/NDUFA9), *CG17280* (levy, complex IV subunit VIa) and PINK1 were obtained from the Vienna Drosophila RNAi stock center (VDRC). Daughterless-GAL4 (daGAL4) and Actin-GeneSwitch-GAL4 (ActGS) flies were obtained from Bloomington Drosophila Stock Center. Daughterless-GeneSwitch-GAL4 (daGS) flies were a kind gift from Dr. Veronique Monnier (University of Paris). mito-ORP1-roGFP2 (mtORP1-roGFP) flies were a gift from Dr. Tobias Dick (German Cancer Research Center, Heidelberg). Mitochondrially targeted catalase (mtCAT) flies were a gift from Prof Rajindar Sohal. See Table S2 for details of the different fly strains used in this study. SOD2 RNAi expressing flies, CI>2;NDI1>daGAL4 flies, AOX>2;NDI1>daGAL4 flies, mtCAT>2;NDI1>daGAL4 flies and CIV>2;AOX>daGAL4 flies with respective controls were allowed to developed at 18°C before being aged in 25°C post-eclosion to reduce lethality in the experimental group during development. All strains and balancers used were backcrossed for a minimum of 6 generations to our white-eyed Dahomey background, derived from backcrossing the w^{1118} mutation into Dahomey for 11 generations. Dahomey virgin females were first crossed with males of each strain to ensure that all lines carried the same mitochondrial DNA. Virgin females from the F_1 generation were mated with Dahomey males and this cross was repeated for a minimum of six generations. Experimental flies were obtained by crossing females homozygous for the appropriate Gal4 driver (e.g. daGAL4>daGAL4) with males homozygous for one (e.g. NDI1>NDI1) or more transgenes (e.g. AOX>AOX;NDI1>NDI1). The experimental progeny carried one copy of the appropriate Gal4 driver and one copy of the desired transgene (e.g. AOX>2;NDI1>daGAL4). The only exception to this, was SOD2>CI;NDI1>ActGS flies (and controls for this experiment) that were produced by crossing virgin females carrying two copies of a UAS-SOD2 RNAi construct and one copy of the ActGS driver (SOD2>SOD2;ActGS>CyO) with either males homozygous for NDI1 (NDI1>NDI1) or males homozygous for NDI1 and heterozygous for a UAS-RNAi construct against ND-39 (CI>CyO;NDI1>NDI1). Flies containing more than two transgenes (e.g. AOX>AOX;NDI1>NDI1) were generated from backcrossed strains. Following backcrossing to Dahomey , flies homozygous for single

transgenes of interest were crossed to flies carrying balancers for the second and third chromosomes (CyO>2;TM3, Sb[1]>3), as all the transgenes (Gal4/GS, UAS-cDNA or UAS-RNAi) we are working with are located on either 2nd or 3rd chromosomes. These balancer flies are regularly rederived to avoid accumulation of mutations. The progeny (e.g. F_1 UAS-X>CyO;TM3, Sb[1]>3 or CyO>2;TM3, UAS-Y>Sb[1]) were crossed to produce flies homozygous for a single transgene and heterozygous for a balancer (F_2 UAS-X>UAS-X; TM3, Sb[1]>3 or CyO>2;TM3, UAS-Y>UAS-Y). In order to combine two transgenes, flies homozygous for single transgenes and heterozygous for a balancer (F_2) were crossed to generate flies with one copy of each transgene and each balancer (e.g. UAS-X>CyO; UAS-Y>TM3, Sb[1]). These flies were then crossed together to generate flies homozygous for both constructs (eg UAS-X>UAS-X; UAS-Y>UAS-Y). Flies carrying single transgenes (e.g. daGal4, daGS, NDI1, AOX flies or UAS-CG2060 RNAi) were derived from the same crossing scheme in order to prevent background differences that can affect lifespan studies. Finally, transgene expression in experimental flies was confirmed by western blot or qPCR when antibodies were not available. To induce transgene expression in flies using GS drivers (i.e. daGS or i.e ActGS), food was supplemented with 300 or 500 μM RU-486. Food supplemented with rotenone (ROT; CI Q-site inhibitor), carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP; uncoupler) or myxothiazol (MYX; CIII Q_o-site inhibitor) was prepared by adding 5 mM stock solutions in absolute EtOH to 2 ml of fly food to produce the desired concentrations. Food supplemented with paraquat was prepared by adding 2.5 mM stock solution in H_2O to 2 ml of fly food to the desired concentration. Control food was supplemented with EtOH or H_2O (vehicle) alone.

Isolation of crude mitochondrial fractions. All steps were performed at 4°C. 20-40 flies were homogenized in mitochondrial isolation buffer (MIB; 250 mM sucrose, 2 mM EGTA, 0.1% (w/v) bovine serum albumin (BSA), 5 mM tris-HCl pH 7.4) and filtered through 200μM netting. Following centrifugation at 200 x g, the supernatant was collected and centrifuged a second time at 9,000 x g. The mitochondrial pellet was resuspended in MIB without BSA. Mitochondria were used immediately for blue-native gel electrophoresis.

Blue-native gel electrophoresis (BNGE). 100 μg isolated mitochondria prepared as described above were pelleted and resuspended in 25 μl sample buffer (50 mM BisTris, 6N HCl, 50 mM NaCl, 10% w/v Glycerol, 0.001% Ponceau S, 1% w/v digitonin, supplemented with protease inhibitor cocktail (Roche)). Following a 15 minute incubation on ice, samples were centrifuged at max speed and the supernatant containing solubilized inner membrane complexes was collected

and supplemented with 10 µl sample buffer without digitonin and 1.5 µl PageBlue protein staining solution (ThermoFisher Scientific). 15 µl sample was then run on Novex Bis-Tris pre-cast gels and stained according to (Sanz et al., 2010).

Behavioral and locomotive activity assays. Individual flies were placed into empty vials. Live tracking of the flies was performed using a high-sensitivity The Imaging Source USB camera (equipped with a Computar 1/3" Varifocal Lens (2.8-12mm)), for 60 minutes per individual experiment. The recorded video files were saved and used to calculate distance moved per hour for each fly using the EthoVision XT video tracking software (Noldus Information Technology). Each video file was quantified at least two times (two replicates per individual fly). The distance moved was measured in centimeters and tabulated as centimeters/hour. The flying time of the individual fruit flies was calculated by manual set scoring using an inbuilt software timer. A 60 minute video recording was also used for this purpose. During manual scoring two behaviors, namely flying time and non-flying time were measured. This was repeated twice for each video file. The flying time was calculated in seconds and tabulated.

ROS detection. MitoSOX and dichlorofluorescein (H_2DCF) were used to measure either mitochondrial matrix superoxide or total cellular ROS levels, respectively, in adult fly brains dissected in PBS. Following dissection, fly brains were incubated in either 30 µM MitoSOX or 30 µM H_2DCF for 10 minutes before being washed 3 times with PBS and imaged immediately. Images were acquired using an LSM510 confocal microscope (Zeiss) equipped with a 10x 0.3 NA objective as Z stacks throughout the sample, using multi-line Argon laser. For *in vivo* ROS imaging using mtORP1-roGFP reporter lines, whole flies containing the reporter and driver, along with any other indicated constructs were put to sleep on ice before being dissected and imaged under Ex. 488 (reduced) or 405 (oxidized) nm/Em. 510 nm. The total (average) intensity of each individual brain imaged was quantified using ImageJ.

Electron microscopy. Dorsal flight muscles were dissected from flies of the indicated genotypes and indicated ages suspended in 0.16 M sym-collidine buffer pH 7.4 (Electron Microscopy Sciences) supplemented with 5% glutaraldehyde (fixing solution). Dissected muscles were maintained at 4°C in fixing solution for at least 3 hours before undergoing osmium tetroxide (OsO_4) postfixation, followed by dehydration, embedding, sectioning and imaging according to (Ban-Ishihara et al., 2013). Muscle sections were imaged using a JEOL JEM-1400 Plus transmission electron microscope with attached 11 Mpx Olympus Quemesa digital camera. For quantification of size and circularity in SOD2 knockdown models, the mitochondria were

separated from the rest of the image by thresholding. The resulting thresholded binary image was then segmented using ImageJ watersheding algorithm (http://rsb.info.nih.gov/ij/plugins/watershed.html). The resulting segmented image was then used as an input for the particle analysis tool of ImageJ (http://rsbweb.nih.gov/ij/docs/guide/146-30.html). From the results table, the area and circularity (http://rsb.info.nih.gov/ij/plugins/circularity.html) distributions were plotted. For wild type Dahomey flies of different ages the images were manually segmented using GIMP (http://www.gimp.org/). The resulting segmented image was then again used as an input for the ImageJ particle analysis tool.

High-resolution respirometry. Whole-fly homogenates were used for respirometry measurements. Briefly, 20-40 flies were homogenized in MIB without BSA and filtered before being immediately measured using an OROBOROS O2k oxygraph. Homogenates were incubated in assay buffer (120 mM KCl, 5 mM KH_2PO_4, 3 mM Hepes, 1 mM EGTA, 1 mM $MgCl_2$, 0.2% bovine serum albumin, pH 7.2 at 25°C). State 4 respiration was initiated by addition of 5 mM pyruvate and 5 mM proline. State 3 was initiated with the addition of 1 mM ADP. CI-linked respiration (CI+CIII+CIV in figures) was inhibited adding 0.5 μM rotenone and 20 mM glycerol 3-phosphate was added to stimulate CIII-linked respiration (CIII+CIV in figures). CIII-linked respiration was inhibited with the addition of 2.5 μM antimycin A. CIV respiration (CIV in figures) was initiated by addition of 4 mM ascorbate and 2 mM TMPD. CIV respiration was inhibited by adding 0.5 mM KCN. Data presented corresponded to state 3 respiration (i.e.after ADP addition). Values were normalized to protein concentration as calculated by the Bradford method. Samples were flash frozen in liquid nitrogen and stored at -80°C prior to use in subsequent enzymatic assays.

Enzymatic assays. All assays were adapted for use in a 96-well plate with a final volume of 300 μl using either a PerkinElmer EnVision 2104 (PerkinElmer) or a Fluostar Omega (BMG Labtech) plate reader. For ETC and citrate synthase assays, homogenates used in respirometry were thawed on ice and protein concentration was calculated via the Bradford method. CI activity was measured as rotenone-sensitive NADH dehydrogenase activity. 30 μl homogenate was added to 264 μl assay buffer consisting of 50 mM KH_2PO_4 pH 7.6, 3.5 mg/ml BSA and 50 μM decylubiquinone (DQ). The reaction was started with the addition of 6 μl 10 mM NADH and the decrease in absorbance at 340 nm was measured for 3 minutes at 25°C. Rotenone-insensitive activity was followed for another 3 minutes following the addition of 1 μl 1 mM rotenone. Non-physiological activity through the matrix arm of CI was measured as

NADH:hexaammineruthenium chloride (HAR) oxidoreductase activity. Following addition of rotenone to inhibit CoQ/DQ-dependent activity, 6 µl 10 mM NADH and 7.5 µl 10 mM HAR were added and the decrease in absorbance at 340 nm was measured for 3 minutes at 25°C. CIII activity (antimycin-sensitive decylubiquinol:cytochrome c oxidoreductase) was measured according to the optimized method used by (Luo et al., 2008). Complex II (CII), IV and V activities were measured according to (Barrientos, 2002) with slight modifications. Briefly, CII activity (malonate-sensitive succinate:dichlorophenolindophenol (DCPIP) oxidoreductase) was measured by adding 40 µl of homogenate to 258 µl of assay buffer (10 mM KH_2PO_4 pH 7.6, 1 mg/ml BSA, 2 mM EDTA, 80 µM DCPIP, 4 µM rotenone, 10 mM succinate, 0.2 mM ATP). Following 10 minutes of pre-incubation at 25°C to allow succinate to overcome the inhibitory effect of oxaloacetate, 2 µl of 1.55 mM decylubiquinone was added to initiate the reaction and the decrease in absorbance at 600 nm was measured for 3 minutes. Non-CII activity was discarded by the addition of 2 µl 1 M malonate. CIV activity (potassium cyanide-sensitive cytochrome c oxidase) was measured by resuspending 30 µl fly homogenate in 210 µl assay buffer (10 mM KH_2PO_4 pH 6.5, 250 mM sucrose, 1 mg/ml BSA). The reaction was initiated by the addition of 60 µl 0.22 mM reduced cytochrome c (0.22 mM cytochrome c solution supplemented with 0.5 mM DTT and incubated at room temperature for 15 minutes) and the decrease in absorbance was measured at 550 nm for 3 minutes before being supplemented with 2 µl 0.1 M KCN and measured again. Complex V activity (oligomycin-sensitive NADH-coupled ATPase activity) by resuspending 20 µl homogenate in 243 µl assay buffer (50 mM Tris pH 8, 5 mg/ml BSA, 20 mM $MgCl_2$, 50 mM KCl). The reaction was initiated by the addition of 37 µl of initiation mix (24.3 µM FCCP, 8.1 µM Antimycin A, 4.05 mM ATP, 109 U/ml Lactate dehydrogenase, 108 U/ml pyruvate kinase, 2.43 mM NADH and 24.3 mM phosphoenolpyruvate) and the decrease in absorbance at 340 nm was measured for 3 minutes. 2 µl 0.5 mg/ml oligomycin was added to each well and the rate was measured again. All ETC activities were normalized to mitochondrial density calculated by citrate synthase (CS) activity measured according to (Magwere et al., 2006). Whole and mitochondrial fractions of fly homogenates were supplemented with 1 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride before use in CS activity assay. Mitochondrial aconitase activity was measured as in (Muller et al., 2004) with a few modifications. Mitochondria (0.01 mg/ml) were pre-incubated in assay buffer (50 mM KH_2PO_4 (pH 7.4), 0.6 mM $MgCl_2$, 5 mM sodium citrate, 0.2 mM $NADP^+$, 0.4 U/ml Isocitrate dehydrogenase ($NADP^+$)). The assay was initiated by permeabilisation of mitochondrial membranes with the addition of 0.01% Triton X-100. Activity was measured at 25°C using a PerkinElmer EnVision 2104 plate reader at 355 nm/460 nm Ex./Em. for 30 minutes. Non-specific activity (assay without isocitrate dehydrogenase) was subtracted from final values.

Western blots. All sample preparation steps were performed at 4°C as described in (Fernandez-Ayala et al., 2009). Protein concentration was calculated by the Bradford method before loading into pre-cast AnykD Criterion TGX Stain-Free (BIO-RAD) polyacrylamide gels for electrophoresis. Following electrophoresis, proteins were blotted onto Hybond nitrocellulose membranes and blocked with 5% milk in PBS-Tween 20 for 1 hour at room temperature before being probed overnight at 4°C with the indicated antibodies diluted in 5% milk in PBS-Tween 20. Antibody dilutions are as follows: NDUFS3 (Abcam), 1:10,000; Aconitase 2 (Abcam), 1: 2,000; PARKIN (Sigma-Aldrich), 1:2,000; PINK1 (Sigma-Aldrich), 1:500; GAPDH (Everest Biotech), 1:30,000; SDHB (Abcam), 1:250; COXIV (Abcam), 1:7,500; Cytochrome b (Abmart), 1:1,000; ATP5A (Abcam), 1:500,000; SOD2 (Abcam), 1:1,000; NDUFA8 and NDUFA9 (A gift from Prof Howy Jacobs (University of Helsinki)), 1:1,000 and 1:2,500 respectively; NDI1 (a gift from the lab of Prof Takao Yagi (Scripps)) 1:10,000; AOX (a gift from the lab of Prof Howy Jacobs (University of Helsinki)), 1:20,000; Catalase (GeneTex), 1:1,000.

CAFÉ assay to measure food intake. Glass capillaries containing CAFÉ assay food (5% sucrose and 5% yeast extract) were inserted into the tops of 1.5 ml tubes (1 per tube). Another hole was made in the tube to allow air circulation. Flies were anesthetized with CO_2 and transferred to the tube. Quantification of food intake was performed by adding a known amount of food into the capillaries, and then measuring changes in this volume every 24 hours. Food evaporation was controlled for by measuring capillaries in tubes without flies. The analysis was carried out for around 120 hours.

RNA quantification. The methods for the isolation of mRNA and cDNA synthesis and q-RT-PCR analysis have been described in detail in (Sanz et al., 2010). The data were extracted and analysed using Applied Biosystems StepOne software version 2.1. Primer sequences are available upon request.

Measurement of redox state of CoQ. 20 frozen flies were homogenized according to (Sanz et al., 2010). A single sample was processed each time to prevent artificial oxidation of ubiquinol. Lipids were extracted by adding 300 µl of 2-propanol and 1:1000 beta-mercaptoethanol to 100 µl of fly homogenate. Samples were centrifuged at 16,000 x g for 1 minute at 4 ºC and 100 µl of the supernatant was immediately injected into a 166-126 HPLC system (Beckman-Coulter) equipped with an UV/Vis detector (System Gold R 168, Beckman-Coulter) and an electrochemical

(Coulochem III ESA) detector. Separation was carried out in a 15 cm Kromasil C18 column (Scharlab, Spain) at 40ºC with a mobile phase of methanol/n-propanol (65:35) containing 1.42 mM lithium perclorate at a flow-rate of 1 ml/min. UV-spectrum was used to identify the different forms of ubiquinone (oxidized CoQ9 and CoQ10 with maximum absorption at 275 nm) and ubiquinol (reduced CoQ, CoQ9H2 and CoQ10H2, with maximum absorption at 290 nm) using specific standards, and the electrochemical readings were used for their quantification. Redox status of CoQ was calculated as follows:

$$\% \text{ reduced CoQ} = (CoQ9H2+CoQ10H2)/(CoQ9H2+CoQ10H2+CoQ9+CoQ10).$$

Statistical analysis. Values shown represent means ± SEM. $p<0.05$ was taken as statistically significant. Data were analyzed with Prism 6 (GraphPad) using either 1-way ANOVA with Newman-Keul's post-test or using the unpaired Student's t-test where appropriate. Lifespan data were analyzed using the log-rank Mantel Cox Test. Different symbols in graphs denote significantly different groups ($p<0.05$). * = $p<0.05$; ** = $p<0.01$; *** = $p<0.005$; **** = $p<0.001$ indicate significant difference from all other groups unless indicated otherwise by line art. NS = not significantly different. Individual and combined lifespan experiments with statistical analysis are summarized in Table S1.

Supplemental References

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