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Neuronal expression of a single-subunit yeast NADH–ubiquinone oxidoreductase (*Ndi1*) extends *Drosophila* lifespan

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Abstract

The ‘rate of living’ theory predicts that longevity should be inversely correlated with the rate of mitochondrial respiration. However, recent studies in a number of model organisms, including mice, have reported that interventions that retard the aging process are, in fact, associated with an increase in mitochondrial activity. To better understand the relationship between energy metabolism and longevity, we supplemented the endogenous respiratory chain machinery of the fruit fly *Drosophila melanogaster* with the alternative single-subunit NADH–ubiquinone oxidoreductase (*Ndi1*) of the baker's yeast *Saccharomyces cerevisiae*. Here, we report that expression of *Ndi1* in fly mitochondria leads to an increase in NADH–ubiquinone oxidoreductase activity, oxygen consumption and ATP levels. In addition, exogenous *Ndi1* expression results in increased CO_2 production in living flies. Using an inducible gene expression system, we expressed *Ndi1* in different cells and tissues and examined the impact on longevity. In doing so, we discovered that targeted expression of *Ndi1* in fly neurons significantly increases lifespan without compromising fertility or physical activity. These findings are consistent with the idea that enhanced respiratory chain activity in neuronal tissue can prolong fly lifespan.

Keywords

Aging; fly; longevity; mitochondria; respiration

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Introduction

Defects in mitochondrial energy metabolism and their effects on aging and age-related disease are widely reported (Wallace 2005; Lin & Beal 2006). Aged mammalian tissues show a decreased capacity to produce ATP and the impairment of mitochondrial function is due to decreased rates of electron transfer by the selectively diminished activities of complexes I and IV (Navarro & Boveris 2007). Dysfunctional mitochondria in aged rodents are characterized, besides decreased electron transfer and oxygen uptake, by an increased content of oxidation products of phospholipids, proteins and DNA, a decreased membrane potential, and altered morphology (Shigenaga *et al.* 1994). These features of the aging process appear to be conserved across the animal kingdom. For example, in *Drosophila* aging is also associated with changes in mitochondrial structure (Burch *et al.* 1970; Walker & Benzer 2004) and defects in mitochondrial respiratory chain function (Ferguson *et al.* 2005).

Although it is widely accepted that aging is associated with a decline in mitochondrial activity, an understanding of the causal relationship between the rate of respiratory chain activity and animal aging remains elusive. The 'rate of living' theory (Pearl 1928) predicts an inverse relationship between the rate of mitochondrial activity and longevity. However, inactivation of genes important for respiratory chain function has been associated with both increased (Feng *et al.* 2001; Dillin *et al.* 2002; Lee *et al.* 2003; Liu *et al.* 2005; Dell'agnello *et al.* 2007; Copeland *et al.* 2009) and decreased longevity (Ishii *et al.* 1998; Walker *et al.* 2006) and it is not yet known whether reduced mitochondrial *activity* is important for changes in longevity mediated by reduced expression of ETC genes. For example, long-lived flies with reduced expression of ETC genes do not consistently show energetic or physiological trade-offs (Copeland *et al.* 2009).

In recent years, a number of studies have reported that interventions that retard the aging process may, in fact, lead to an increase in mitochondrial respiratory chain activity (Guarente 2008). Moderate dietary restriction (DR) slows aging and delays the onset of age-related pathologies in a wide range of species (Mair & Dillin 2008). Recent studies have reported that DR is associated with an increase in mitochondrial activity in yeast (Lin *et al.* 2002), *C. elegans* (Bishop & Guarente 2007), *Drosophila* (Zid *et al.* 2009) and mice (Nisoli *et al.* 2005). In addition, long-lived mice with a fat-specific insulin receptor knockout (FIRKO) (Bluher *et al.* 2003) display an increase in basal metabolic rate and respiratory exchange ratio (Katic *et al.* 2007). The TOR (target of rapamycin) pathway modulates longevity in yeast, worms, flies and rodents (Vellai *et al.* 2003; Kapahi *et al.* 2004; Kaeberlein *et al.* 2005; Harrison *et al.* 2009). In a recent mechanistic study, it was shown that reduced TOR signaling extends chronological lifespan in yeast via increased respiration (Bonawitz *et al.* 2007). In both yeast (Barros *et al.* 2004) and mice (Caldeira da Silva *et al.* 2008), uncoupling agents such as 2,4-dinitrophenol (DNP) have been reported to increase both respiratory activity and longevity. In *Drosophila*, neuronal expression of human uncoupling protein 2 (hUCP2) was reported to increase longevity (Fridell *et al.* 2005). However, in an independent study, expression of human UCP3 (hUCP3) at moderate levels in adult neurons resulted in a marginal lifespan-extension in male flies and high expression of hUCP3 in neuronal tissue shortened lifespan (Humphrey *et al.* 2009). Interpretation of these findings is hampered by a lack of detailed understanding of the physiological function of individual UCPs. Interestingly, overexpression of neurofibromatosis-1 (NF1) was reported to increase both respiration and lifespan in the fly (Tong *et al.* 2007). It has been suggested that increased respiration is sufficient to prolong lifespan in yeast: overexpression of Hap4, a transcription factor expected to activate many genes involved in respiration, extends lifespan in yeast (Lin *et al.* 2002). Taken together, these studies raise an intriguing

question: Is increased mitochondrial respiratory chain activity sufficient to delay animal aging? And, if so, which tissues are important in mediating longevity?

Progress towards developing strategies to directly increase the activity of mitochondrial respiratory chain enzymes in animals has been confounded by technical difficulties associated with their coordinated assembly. For example, mitochondrial complex I (NADH–ubiquinone oxidoreductase), the major entry point for electrons into the respiratory chain, is comprised of at least 45 subunits encoded by both the nuclear and mitochondrial genomes (Carroll *et al.* 2006). In contrast, the alternative NADH-ubiquinone oxidoreductase (*Ndi1;* GeneID: 854919 Entrez Gene) of *Saccharomyces cerevisiae* mitochondria is composed of a single nuclear-encoded polypeptide (Luttik *et al.* 1998). Previously, *Ndi1* expression has been shown to restore mitochondrial activity in animal and cell-based models of respiratory chain disorders (DeCorby *et al.* 2007; Perales-Clemente *et al.* 2008) (Marella *et al.* 2008). *Ndi1* expression is also sufficient to restore mitochondrial activity in a fly mutant with impaired endogenous complex I activity (JC & DWW, manuscript in preparation). We reasoned that expression of *Ndi1* in wild-type *Drosophila* may be an effective approach to directly increase respiratory chain activity in an animal model. Here, we demonstrate that supplementation of the endogenous fly respiratory chain with the NDI1 enzyme leads to an increase in NADH–ubiquinone oxidoreductase activity, oxygen consumption and ATP levels. In addition, *Ndi1*-expressing flies display an increased metabolic rate. Using our transgenic fly model, we report the consequences of tissue-specific expression of *Ndi1* on longevity: expression in adipose tissue shortens lifespan, while neuronal expression leads to increased longevity. Long-lived flies display increased NADH–ubiquinone oxidoreductase activity and ATP levels in tissue isolated from heads.

Results

Expression of the yeast *Ndi1* gene in *Drosophila* mitochondria leads to an increase in respiratory chain activity

To investigate whether expression of the alternative, rotenone-insensitive NADH-ubiquinone oxidoreductase (*Ndi1*) of *Saccharomyces cerevisiae* was sufficient to enhance mitochondrial activity in *Drosophila*, we expressed *Ndi1* in flies using the GAL4/UAS system (Brand & Perrimon 1993). We transformed flies with UAS-constructs containing the *Ndi1* gene and performed seven rounds of backcrossing into a w^{1118} background. In all subsequent experiments, w^{1118} was used as a control strain. Except for the insertion of the *UAS-Ndi1* transgene, these control flies were genetically very similar to the experimental flies. Using a ubiquitous expression GAL4 driver line, *daughterless (da)*-GAL4, we confirmed the mitochondrial-specific expression of the NDI1 protein by Western blot (Figure 1A). To determine whether expression of an exogenous inner membrane protein (*Ndi1*) may affect the structure of the endogenous respiratory chain enzymes, we examined their assembly by Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE). Expression of *Ndi1* in fly mitochondria did not affect the expression or assembly of the endogenous respiratory chain machinery (Figure 1B). To determine whether NDI1 could function to promote respiration in fly mitochondria, we examined oxygen consumption in the presence of various substrate/inhibitor combinations. To distinguish the NADH–ubiquinone oxidoreductase activites of the endogenous *Drosophila* complex I and of the yeast NDI1 enzyme, we monitored oxygen consumption in the presence of the endogenous complex I inhibitor rotenone. In contrast to control flies, mitochondria from *Ndi1*-expressing flies supported a substantial rotenone-insensitive substrate oxidation (Figure 1C). We confirmed that this rotenone-insensitive respiration was due to the yeast enzyme, using the *Ndi1*-specific inhibitor flavone. Moreover, we observed that expression of *Ndi1* in fly mitochondria leads to a 30% increase in glutamate/malate-stimulated (complex I-specific) respiration, while complex II (succinate-stimulated)-specific respiration was unaffected by

Ndi1 expression (Figure 1C). Our results indicate that NDI1 can function in fly mitochondria to promote respiration without affecting the structure or function of the endogenous respiratory chain.

We observe that expression of NDI1 in fly mitochondria leads to an increase in complex I-specific oxygen consumption. Next, we assayed NADH-ubiquinone oxidoreductase (complex I) enzymatic activity and confirmed that mitochondria from *Ndi1*-expressing flies display a 28% increase in this activity (Figure 2A). In addition, *Ndi1* conferred a 2.6-fold increase in rotenone-insensitive NADH-ubiquinone oxidoreductase activity. To determine whether this increase in NADH-ubiquinone oxidoreductase activity affects overall energy production, we took two independent approaches. Firstly, we examined ATP levels in *Ndi1*-expressing flies and controls. Expression of *Ndi1* conferred a 24% increase in ATP levels (Figure 2B). Next, we set out to determine whether expression of *Ndi1* affects respiratory chain activity in living flies. To do so, we assayed CO_2 production in *Ndi1*-expressing flies and controls. Flies expressing *Ndi1* display a 16% increase in metabolic rate compared to controls (Figure 2C). This result indicates that *Ndi1* can promote an increase in respiratory rate in the intracellular environment in which mitochondria respire *in vivo*. Unlike the endogenous fly complex I enzyme, NDI1 cannot promote proton pumping. Therefore, the NDI1-mediated increase in CO_2 production and ATP levels likely results from an increase in electron flow through the ETC. In Figure S1, we depict a schematic drawing of the putative mode of *Ndi1* function in fly mitochondria.

The relationship between the rate of respiratory chain activity and the production of reactive oxygen species (ROS) is complicated and controversial (Balaban *et al.* 2005). We examined whether *Ndi1*-mediated increased respiratory chain activity affects ROS production in fly mitochondria using the MitoSOX fluorescent indicator to monitor superoxide anion production. We observe that increased NADH-ubiquinone oxidoreductase activity, resulting from *Ndi1* expression, did not affect mitochondrial ROS production (Figure 2D). Our data indicate that *Ndi1* expression in fly mitochondria can increase respiratory chain activity without affecting ROS production.

Targeted expression of *Ndi1* in adipose tissue shortens lifespan, while expression in neurons increases lifespan

Having established that expression of *Ndi1* in fly mitochondria can increase respiratory chain activity, we next set out to examine the impact on longevity of targeted expression of *Ndi1* in different cells and tissues. To do so, we used the mifepristone (RU486) inducible-GAL4 system (annotated P[Switch] or Gene-Switch (Osterwalder *et al.* 2001; Roman *et al.* 2001)). This system eliminates genetic background effects since all flies share the same genetic background and only differ with respect to the presence of the inducing agent (RU486) in the food. To eliminate the possible contribution of induction of endogenous genes at the site of insertion of the *Ndi1* transgene, we tested two independent insertions of the *UAS-Ndi1* transgene in all longevity experiments. Firstly, we set out to investigate the impact of expression of *Ndi1* in all cells and tissues. Ubiquitous induction of *Ndi1* expression during both development and adulthood using tubulin *(tub)-Gene-Switch* (GS) had no robust positive effects on longevity in male (Figures S2A & S2B; Table S1) or female flies (Figures 3A & 3B; Table S1). Next, we set out to investigate the effects of targeted expression of *Ndi1* in specific tissues. Adipose tissue has been reported to play an important role in insulin/IGF-1-mediated longevity across the animal kingdom (Russell & Kahn 2007). To examine the impact of *Ndi1* expression in adipose tissue, we used the Gene-Switch driver S_1106, which is predominately expressed in the fat-body (Poirier *et al.* 2008). Expression of *Ndi1* in adipose tissue resulted in shortened lifespan in both male (Figures S2C & S2D; Table S2) and female flies (Figure 3C & 3D; Table S2).

Previous studies have reported that the nervous system is an important target tissue in mediating longevity in flies (Bauer *et al.* 2005; Fridell *et al.* 2005; Copeland *et al.* 2009). Therefore, we used the pan-neuronal *Elav-Gene-Switch* (*Elav*-GS) driver line to induce *Ndi1* expression specifically in neurons during both development and adulthood. In contrast to the effects we observed using both the ubiquitous and the fat-body driver lines, we observed life extension when *Ndi1* was expressed in neurons in both male (Figures S2E, S2F; Table S3) and female flies (Figures 3E & 3F; S3A & S3B; Table S3). Specifically, neuronal expression of *Ndi1* increased lifespan by up to 21% in females and by up to 7% in males. We investigated the possibility that RU486 itself may increase longevity in our fly strains by simultaneously feeding RU486 or diluent alone to the progeny of *Elav-GS* crossed to w^{1118}, the genetic background of the two *Ndi1* transgenic lines. RU486 had an overall negative impact on longevity in control flies (Table S3). Therefore, it is possible that the negative effects of RU486 on lifespan may partially 'mask' the beneficial effects of NDI1.

To examine the impact of *Ndi1* expression in muscle cells on longevity, we used the constitutive muscle specific driver *MHC-GAL4*. We used this constitutive driver line because *MHC-GeneSwitch* displays very high-level inducer-independent ('leaky') expression in muscle, but does not induce in a broad set of muscles, but instead induces in digestive tissue (Poirier *et al.* 2008). Expression of two independent *UAS-Ndi1* transgenes mediated by *MHC-GAL4* had no major impact on longevity in male or female flies (Figure S4; Table S4).

Neuronal expression of *Ndi1* can increase respiratory chain activity

To investigate the mechanism by which neuronal *Ndi1* expression extends lifespan, we examined the physiology of mitochondria from heads of *Ndi1*-expressing flies. First, using quantitative real-time PCR (qRT-PCR), we confirmed that the *Ndi1* transcript was induced in heads, which are greatly enriched for neuronal tissue (Figure 4A). Next, we examined whether neuron-specific expression of *Ndi1* leads to an increase in NADH-ubiquinone oxidoreductase activity. We observe that induced neuronal expression of *Ndi1* leads to a 16% increase in complex I enzymatic activity in mitochondria isolated from heads (Figure 4B). In addition, *Ndi1* conferred a 65% increase in rotenone-insensitive NADH-ubiquinone oxidoreductase activity in head tissue. RU486 had no effect on in NADH-ubiquinone oxidoreductase activity in control flies (w^{1118}*/Elav-GS*; Figure S5A).

To determine whether this increase in NADH-ubiquinone oxidoreductase activity affects overall energy production in neuronal tissue, we examined ATP levels in heads of *Ndi1*-expressing flies and controls. Expression of *Ndi1* in fly neurons resulted in a 28% increase in ATP levels in the heads of long-lived flies (Figure 4C). RU486 had no effect on ATP levels in control flies (w^{1118}*/Elav-GS*; Figure S5B). To investigate whether this increase in energy production was due to an increase in the activity of the endogenous *Drosophila* respiratory chain machinery, we assayed the activities of each of the individual fly respiratory chain enzymes using BN-PAGE followed by in-gel enzyme activity staining. As Figure 4D illustrates, the activities of the endogenous respiratory enzymes were unaffected in the heads of long-lived flies. To determine whether neuron-specific expression of *Ndi1* could affect metabolism at the whole-animal level, we examined the metabolic rate of *Ndi1* flies and controls. Neuron-specific expression of *Ndi1* did not lead to an increase in CO_2 production in living flies (Figure 4E). RU486 had no effect on CO_2 production in control flies either (w^{1118}*/Elav-GS*; Figure S5C).

Neuronal expression of *Ndi1* protects against oxidative stress and rotenone toxicity

To investigate whether neuronal expression of *Ndi1* leads to a reduction in oxidative stress, we assayed ROS levels in dissected brains of long-lived flies. Dihydroethidium staining

revealed that targeted expression of *Ndi1* conferred a 51% decrease in ROS levels in the brains of 45-day old flies compared to uninduced controls (Figures 5A & 5B). Inducer has no impact on ROS levels in control flies (w^{1118}*/Elav-GS*; S.B & D.W.W, unpublished observations). We observe that expression of *Ndi1* in fly mitochondria supports rotenone-insensitive respiration (Figure 1C). To determine whether *Ndi1* can function *in vivo* to support respiration in the presence of an endogenous complex I inhibitor, we tested flies for resistance to rotenone. Induced expression of *Ndi1* in fly neurons results in an improved tolerance to rotenone poisoning (Figure 6A), with RU486 having no effect on rotenone resistance in control flies (w^{1118}*/Elav-GS*; Figure S6A). We observe that targeted neuronal expression of *NdiI* leads to reduced ROS production in fly brains (Figure 5A & 5B). Next, we investigated whether neuronal *Ndi1* expression can protect against acute oxidative stress at the whole-animal level. To do so, we examined whether long-lived flies, with targeted neuronal expression of *Ndi1*, were more resistant to dietary paraquat which has been shown to generate ROS *in vivo* (Hassan & Fridovich 1979). Expression of *Ndi1* in fly neurons resulted in improved tolerance to paraquat poisoning relative to uninduced cohorts (Figure 6B), while RU486 had no effect on paraquat tolerance in control flies (w^{1118}*/Elav-GS*; Figure S6B). To determine whether *Ndi1*-expressing flies display a general resistance to extrinsic stressors, we examined their resistance to starvation. As opposed to the increase in resistance to both dietary paraquat and rotenone, expression of *Ndi1* in fly neurons did not improve their ability to withstand starvation (Figure 6C). RU486 had no effect on starvation resistance in control flies (w^{1118}*/Elav-GS*; Figure S6C).

Long-lived *Ndi1*-expressing flies display normal physical activity and fertility

In many cases, interventions that extend longevity result in reproductive trade-offs (Partridge *et al.* 2005). Therefore, we examined female fertility over the first 30 days of adulthood in *Ndi1*-expressing flies and uninduced controls. As has been previously reported (Fridell *et al.* 2005), we observed that RU486 had a negative impact on fertility in control flies (w^{1118}*/Elav-GS*; Figure S7A). In contrast, RU486-induced expression of *Ndi1* in fly neurons did not decrease fertility relative to uninduced cohorts (Figure 7A). Decreased physical activity has also been associated with extended lifespan in flies (Helfand & Rogina 2003). To determine whether a decline in physical activity contributes to the lifespan extension of the *Ndi1*-expressing flies, we monitored the physical activity of flies with a 24 hr *Drosophila* activity monitor and found that long-lived *Ndi1*-expressing flies exhibited spontaneous physical activity equal to uninduced controls (Figure 7B). RU486 had no effect on physical activity in control flies (w^{1118}*/Elav-GS*; Figure S7B). Thus, *Ndi1*-mediated life extension is not a result of a decrease in reproduction or physical activity.

Discussion

The mechanisms that cause the deterioration of cellular functions in aging animals remain poorly understood. One attractive hypothesis, supported by a growing body of correlative data (Shigenaga *et al.* 1994; Wallace 2005), is that impaired mitochondrial respiratory chain function may play an important role in the aging process. However, the causal relationship between the rate of mitochondrial activity and animal aging remains poorly understood. The ‘rate of living – free-radical damage’ theory (Pearl 1928; Harman 1956; Sohal 2002) suggests that higher rates of metabolism should be linked to increased ROS and, as a result, decreased longevity. In recent years, however, this idea has been challenged by a number of studies reporting a positive correlation between respiratory activity and longevity (Speakman *et al.* 2004; Guarente 2008).

In this study, we report that targeted expression of the single subunit alternative NADH-ubiquinone oxidoreductase (*Ndi1*) of *Saccharomyces cerevisiae* can increase respiratory chain activity in fly mitochondria and in living flies. While expression of *Ndi1* in fat tissue

leads to detrimental effects on longevity, expression in neurons can promote life extension. These contradictory effects in different tissues may explain our observation that ubiquitous expression of *Ndi1* had no major effect on longevity. Flies expressing *Ndi1* in neurons displayed increased NADH-ubiquinone oxidoreductase activity and ATP levels in tissue prepared from heads. However, expression of *Ndi1* in neurons did not result in an increase in CO_2 production in living flies. This result implies that increased respiration in neurons did not lead to an increase in energy production in other tissues of the animal.

Our study suggests that reduced oxidative stress in neurons may play a role in *Ndi1*-mediated longevity. In cell culture, *Ndi1* has been reported to reduce ROS production under conditions of endogenous complex I deficiency (Seo *et al.* 2006; Park *et al.* 2007). Here, we report that expression of *Ndi1* in wild-type neurons leads to reduced ROS levels in the brains of long-lived flies. In addition, neuronal expression of *Ndi1* results in improved tolerance to the free radical generator paraquat. However, at present, the underlying mechanism by which neuronal expression of *Ndi1* results in reduced oxidative stress remains unknown. We observed that *Ndi1* expression does not affect ROS production in mitochondria isolated from whole flies. It is possible that expression of *Ndi1* may lead to different effects on ROS production in different cells and tissues. Another possibility is that *Ndi1* may reduce oxidative stress indirectly via altered cell signaling, resulting in an up-regulation of genes important for ROS detoxification.

Previous studies in mammalian model systems have reported a positive correlation between increased respiratory chain activity and longevity (Speakman *et al.* 2004; Nisoli *et al.* 2005; Katic *et al.* 2007; Caldeira da Silva *et al.* 2008). Our *Ndi1* fly model builds upon these studies. However, as yet, we have not demonstrated that the observed changes in mitochondrial function in response to *Ndi1* expression are a *cause* of increased longevity. Future work will focus on examining the molecular and cellular correlates of NDI1-mediated longevity and establishing causal relationships between rates of energy metabolism and rates of aging in different tissues.

Experimental Procedures

Drosophila strains

Tubulin-Gene-Switch was provided by S. Pletcher. *Elav-Gene-Switch* was provided by H. Keshishian. S_1106 was provided by L. Seroude. We transformed flies with pUAST plasmids containing *Ndi1* (amplified by PCR from yeast genomic DNA) and performed seven rounds of backcrossing into a w^{1118} background.

Survivorship

Flies were collected under light nitrogen-induced anesthesia and housed at a density of 25-30 flies per vial. At least 6 vials were assayed per treatment. All flies were kept in a humidified, temperature-controlled incubator with 12 h on/off light cycle at 25 °C in vials containing standard cornmeal medium (Lewis 1960). RU486 was administered by adding 200 μl of 0, 2, 10, or 50 μg/ml RU486 dissolved in ethanol to the top of the fly food, as previously described (Poirier *et al.* 2008; Copeland *et al.* 2009). Flies were flipped to fresh vials twice per week and scored for death.

Purification of Mitochondria

Mitochondria were isolated from adult flies as previously described (Walker *et al.* 2006). Fifty flies were gently crushed in 1 ml chilled mitochondrial isolation medium (MIM: 250 mM sucrose, 10 mM Tris (pH 7.4), 0.15 mM $MgCl_2$) by using a glass-on-glass homogenizer, then spun twice at 1,000 × g for 5 min at 4°C to remove debris. The

supernatant was then spun at 13,000 × g, for 5 min at 4°C. The pellet, containing the mitochondria, was washed with 1 ml MIM and resuspended in 50 μl MIM.

NADH-ubiquinone oxidoreductase Activity assay

Mitochondrial complex I (NADH-ubiquinone oxidoreductase) activity was measured spectrophotometrically at 600 nm in an incubation volume of 1 ml containing 25 mM potassium phosphate, 3.5 g/l BSA, 60 μM DCIP, 70 μM decylubiquinone, 1 μM antimycin A, and 0.2 mM NADH, pH 7.8. We preincubated an aliquot of 10 μl mitochondrial suspension, prepared as described above, at 25°C in 960 μl incubation mixture without NADH. After 3 minutes, 20 μl of 10 mM NADH was added, and the absorbance was measured at 30 sec intervals for 4 minutes at 25°C. After 4 minutes, we added 1 μl rotenone (1 mM) and measured the absorbance again at 30 sec intervals for 4 minutes. Complex I activity was expressed as a reduction rate of DCIP.

Analysis of CO_2 release

The rate of CO_2 emission was used to determine the metabolic rate of 8 groups of flies (10 individuals per group) from each treatment. We used flow through respirometry to measure CO_2 release with Sable Systems (Las Vegas, NV, USA) data acquisition software logging data from an infrared CO_2 analyzer (Li-Cor model 6251; Lincoln, NE, USA). During an experimental run, room air was pumped through two silica and one ascarite/drierite column to be scrubbed of water and CO_2. Air then flowed through one of five 30 ml chambers attached to the multiplexor and subsequently passed into the CO_2 analyzer. The chambers connected to the multiplexor included an empty control chamber used to collect baseline values, and four chambers containing flies from a particular treatment. The order in which each chamber was measured was controlled with SABLE system data acquisition software (Las Vegas, NV, USA). When a chamber was not being measured, it was still perfused with H_20- and CO_2 -free air at a rate equal to the regulated flow entering the measured chamber. Flow rate was adjusted using a mass flow controller (Sierra Instruments, Monterey, CA, USA) and was held at 100 ml/min.

An experimental run lasted a total of 75 minutes. Three five-minute baseline readings were recorded at the beginning, middle and end of the run and used to provide accurate zero values. The rate of CO_2 emission of four fly groups was measured for 15 minutes within each run. The room temperature was maintained at 25 ± 1°C. The CO2 levels (ppm) were averaged and recorded once/second using Sable Systems data acquisition software. Sable Systems (Las Vegas, NV, USA) Expedata analysis software was used to process VCO_2 measurements. CO_2 levels were recorded in parts per million and, after data were zeroed using baseline values, converted to milliliters/minute.

Dihydroethidium (DHE) staining

Fly brains were dissected in PBS buffer (8 mM Na_2HPO_4, 150 mM NaCl, 2 mM KH_2PO_4, 3 mM KCl) and stained with 30 μM DHE solution for 10-15 minutes. Brains were washed 3 times in PBS buffer and visualized by confocal microscopy. The intensity of the brains was measured using the Scion Image Software (Scion Corp., Frederick, MD). To minimize variations in signal intensity, brains were mounted on the same slide and fluorescence intensities of five different brains of each treatment were averaged to give one data point.

Immunoblot assay

Polyclonal rabbit antisera against NDI1 (1:2000), polyclonal rabbit antisera against dPorin1 (1:1000), and monoclonal mouse antibody against α-tubulin (1:5000 dilution, Cell Signalling). The primary antibodies were detected by HRP-conjugated anti-mouse or anti-

rabbit IgG antibodies (1:10000 dilution, Sigma) and SuperSignal West Pico Chemiluminescent reagents (Thermo Scientific).

Mitochondrial respiration assay

Rate of oxygen consumption was measured polarographically using a Clark-type oxygen electrode connected to a computer-operated Oxygraph control unit (Hansatech Instruments) at 25°C. Freshly isolated mitochondria was prepared from 50 flies and then suspended in a respiration medium containing 20 mM Hepes-KOH (pH 7.1), 110 mM sucrose, 10 mM KH_2PO_4, 40mM KCl, 3 mM $MgCl_2$ and 0.5 mM EGTA. The rate was measured continuously, with the following sequential additions: 5 mM glutamate plus 5 mM malate, 200 μM ADP, 0.5 μM rotenone, 5 mM succinate, 2.5 μM antimycin A, 1 mM ascorbate plus 0.25 mM N′-tetramethyl-1,4-phenylenediamine (TMPD).

MitoSox assay

Superoxide production was assayed, in isolated mitochondria, in respiration medium with 5 mM glutamate, 5 mM malate and 50 μM MitoSox (Invitrogen) at 510 nm excitation/580 nm emission.

ATP assay

Five flies were homogenized in 100 μl of 6 M guanidine-HCl in extraction buffer (100 mM Tris and 4 mM EDTA, pH 7.8) and boiled for 3 minutes. The samples were then centrifuged to collect the supernatant, which was then diluted (1/750) with extraction buffer. The luminescence was measured by a luminometer using autoinjection of the luminescent solution (Invitrogen), and the results were compared to the standards. The ATP level was then calculated by dividing the luminescence by the total protein concentration, which was determined by the Bradford method.

Quantitative Real-Time PCR

Total RNA was extracted from heads of female flies using Trizol reagent. RNA concentration was measured with a Nanodrop spectrophotometer, and 1 μg RNA was used for qRT-PCR measurements. Actin5C was the reference gene used to normalize amplicon amounts. The following primer pairs were used: *Act57B*: GTGCTATGTTGCCCTGGACT, GCTGGAAGGTGGACAGAGAG; *Ndi1*: GGTGGGCCTACTGGTGTAGA, CAATGGCGAAAATGTTGTTG. cDNA synthesis and qRT-PCR were combined into one step using Power SYBR Green RNA-to-CT 1-Step kit (Applied Biosystems) and DNA amount was monitored during the 40-cycle PCR by using a 7300 thermal cycler (Applied Biosystems).

Blue native-polyacrylamide gel electrophoresis (BN-PAGE) and In-gel enzyme activity staining

BN-PAGE was performed using Novex Native PAGE Bis-Tris Gel System followed the manufacturer's (Invitrogen) procedure. In brief, 80 μg of the purified mitochondria were resuspended in 25 μl of 1× Native PAGE Sample buffer (Invitrogen) with 1% digitonin and protease inhibitors (Roche) and incubated on ice for 15 min. After centrifugation at 20,000 × g for 30 min, the 25 μl of supernatant was resuspended with 1.25 μl of 5% G-250 sample additive and 10 μl of 4× Native PAGE Sample Buffer (Invitrogen). The final volume was adjusted to 40 μl by addition of double-distilled H_2O. Samples were loaded on 3-12% Bis-Tris Native PAGE gels and electrophoresed using 1× Native PAGE Running buffer system (Invitrogen). The upper buffer included 1× Cathode Buffer Additive (Invitrogen). NativeMark Protein standard (Invitrogen) was used as the molecular weight marker.

For in-gel activity assay, the gels were rinsed briefly and equilibrated in the appropriate reaction buffer without chromogenic reagents for 10 minutes. The gels were then incubated in fresh reaction buffer plus chromogenic reagents for varying lengths of time. For complex I, the reaction buffer contains 5 mM Tris-HCl (pH 7.4), 2.5 mg/ml NTB (nitrotetrazolium blue) and 0.1 mg/ml NADH. For Complex II, the reaction buffer contains 5 mM Tris-HCl (pH 7.4), 20 mM sodium succinate, 0.2 mM phenazine methosulfate, and 2.5 mg/ml NTB. For complex III and IV, the reaction buffer contains 50mM sodium phosphate (pH 7.2), 0.05% 3,3′-diaminobenzidine tetrahydrochloride, 50 mM horse heart cytochrome c. All activity staining was carried out at room temperature. The reactions were stopped by fixing the gels in 45% (v/v) methanol and 10% (v/v) acetic acid after color development. The band intensities of both protein contents and activity staining were estimated by optical densitometry.

Stress resistance

Paraquat resistance—Female flies were aged on ethanol (diluent) or RU486-supplemented media for 10 days, starved for 6 h and then transferred to vials containing two 2.4cm glass fiber filter circles (Whatman) wetted with 150μl of 30 mM paraquat (Sigma) in 5% sucrose solution. Flies were kept in the dark at all times, except for scoring death and the daily addition of 150μl 30mM paraquat solution.

Rotenone resistance—female flies were aged on ethanol (diluent) or RU486-supplemented media for 10 days and then transferred to 250 μM rotenone-supplemented medium with diluent or RU486. Survival rate was measured every 2 days, with survivors transferred into fresh vials every 4 days.

Starvation resistance—Female flies were aged on ethanol (diluent) or RU486-supplemented medium for 10 days and then transferred to 1% agar solution with 200 μl diluent or RU486. Percentage survival was measured every day, with survivors transferred to fresh vials every 3-4 days.

Fertility

Four individual crosses were established each with five virgin females that developed on either 0 μg/ml or 2 μg/ml RU486 and five wild-type (Canton-S) males. Crosses were transferred twice per week to fresh food containing either 0 μg/ml or 10 μg/ml RU486. Adult progeny from each vial were recorded and averaged to generate the fertility over time.

Physical Activity

30 adult female flies were placed in a *Drosophila* activity meter (TriKinetics Inc., Massachusetts). Movements were recorded continuously under normal culturing conditions for 24 hours on a 12:12-hour dark:light cycle. Triplicate samples were used for each activity measurements.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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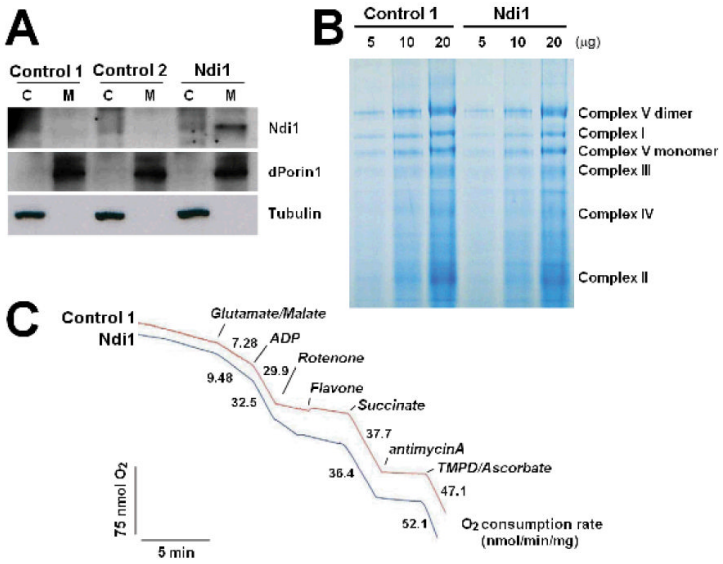

Figure 1. NDI1 can function in *Drosophila* mitochondria

All assays were performed on female flies between 6-10 days of age.

A Targeted expression of NDI1 in fly mitochondria. The purity of cytoplasmic (C) and mitochondrial (M) fractions was confirmed using antibodies against *Drosophila* Porin 1 (dPorin1) and α-tubulin as mitochondrial and cytoplasmic markers, respectively. The expression of NDI1 protein in each fraction was examined by immunoblotting with polyclonal rabbit antisera against NDI1. Genotypes: Control 1: +/*da-GAL4*, Control 2: *UAS-Ndi1*/+; Ndi1: *UAS-Ndi1*/*da-GAL4*. **B** Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE) was performed on mitochondrial proteins isolated from *Ndi1*-expressing flies and controls. Each lane represents increasing amount (5, 10, 20μg) of protein. Expression of NDI1 does not affect the assembly of the endogenous respiratory chain enzymes. Genotypes: Control: +/*da-GAL4*, Ndi1: *UAS-Ndi1*/*da-GAL4*. **C**. Representative polarographic trace showing rates of oxygen consumption in mitochondria isolated from *Ndi1*-expressing flies and controls. *Ndi1* confers a 30% increase in glutamate/malate-stimulated (complex I-specific) respiration and promotes rotenone-insensitive respiration, without affecting succinate-stimulated (complex II-specific) respiration. Genotypes: Control: +/*da-GAL4*, Ndi1: *UAS-Ndi1*/*da-GAL4*.

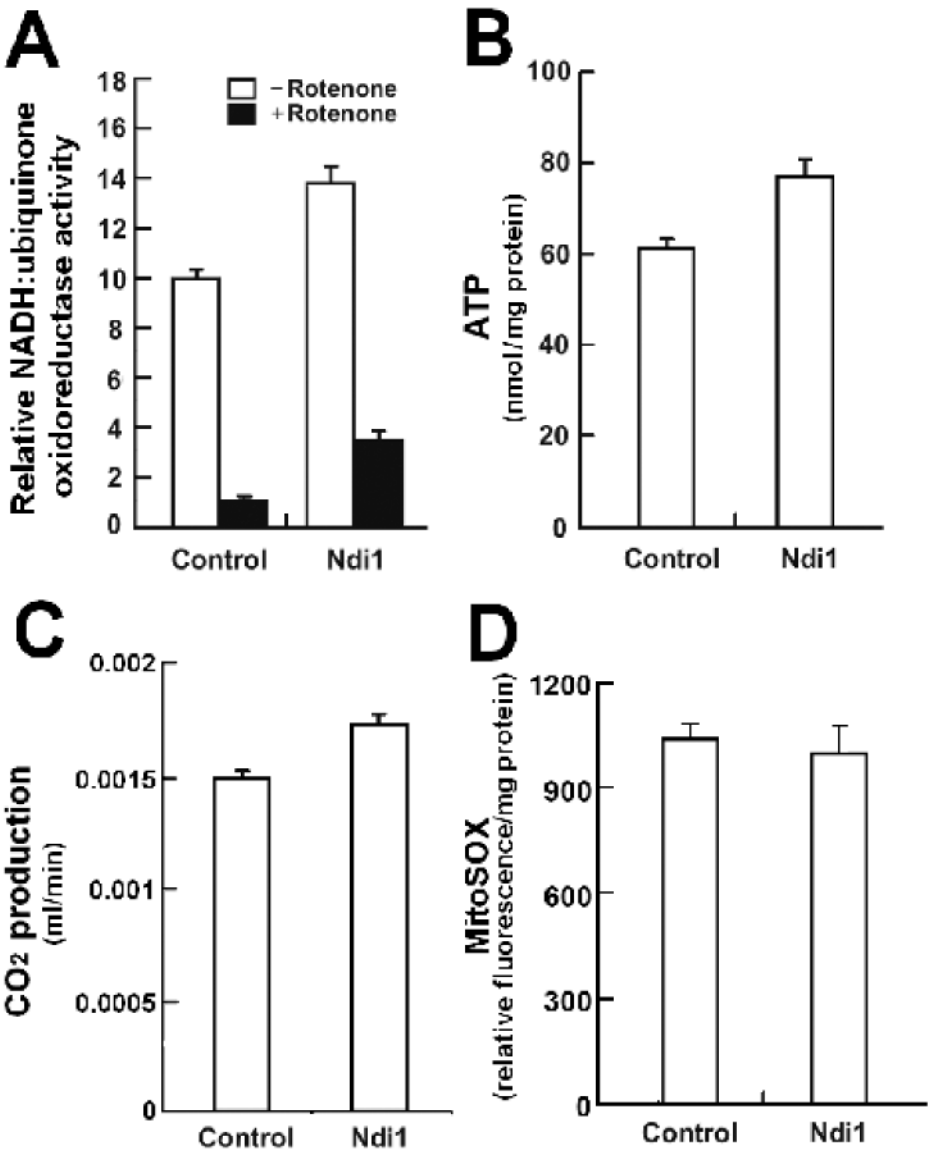

Figure 2. Expression of *Ndi1* can increase respiratory chain activity in *Drosophila*

Genotypes: Control: +/*da-GAL4*, Ndi1: *UAS-Ndi1*/*da-GAL4*. All assays were performed on female flies between 6-10 days of age.

A *Ndi1* confers a significant increase in NADH-ubiquinone oxidoreductase (complex I) enzymatic activity compared to controls (p=0.0007). Rotenone insensitive activity was measured after addition of 1 μM rotenone. **B** *Ndi1*-expressing flies display a significant increase in mean ATP levels compared to controls (p=0.004). **C**. *Ndi1*-expressing flies display a significant increase in CO_2 production compared to controls ($P < 0.001$). **D**. Expression of *Ndi1* in fly mitochondria does not affect ROS production (p>0.05). **A, B, D** n = 3, **C** n=8. Error bars indicate standard deviation. Student's *t*-test was used.

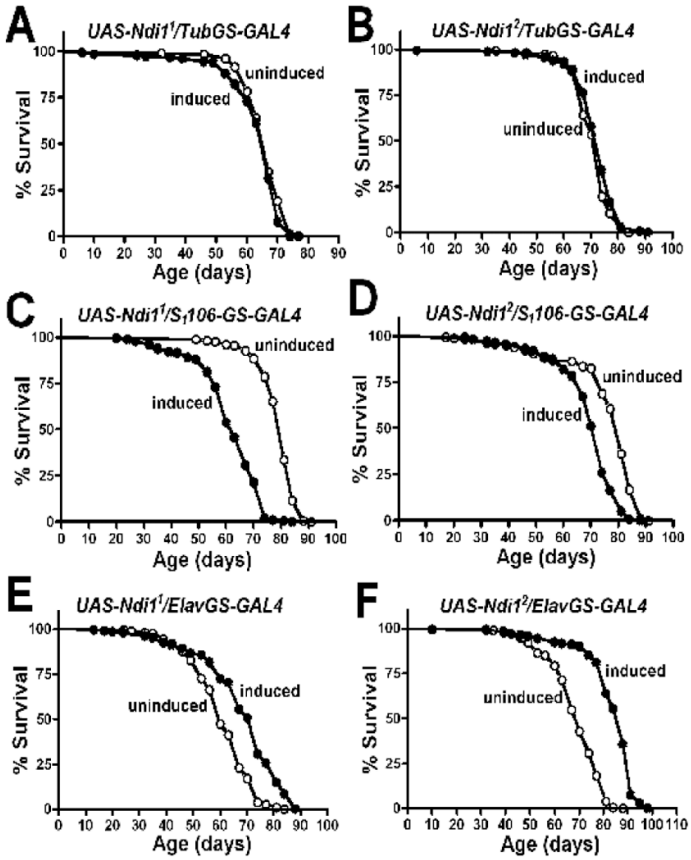

Figure 3. Effects of tissue-specific expression of *Ndi1* on fly longevity

Two independent *UAS-Ndi1* lines (*UAS-Ndi1*^{1} & *UAS-Ndi1*^{2}), were crossed to GeneSwitch driver lines (A-B the ubiquitous *Tubulin (tub)-GS* driver, C-D the fat-body driver *S*$_{1}$*106*, E-F the pan-neuronal driver *Elav-GS*) and lifespan curves are shown as induced (2 mg/ml RU486 during development and 10 mg/ml RU486 from the onset of adulthood (black circles) or uninduced (−RU486, open circles).

A. Lifespan curves of *UAS-Ndi1*^{1}/*tub-GS* females. A moderate decrease in survival was observed in response to RU486 (P=0.0441). **B**. Lifespan curves of *UAS-Ndi1*^{2}/*tub-GS* females. A moderate increase in survival was observed in response to RU486 (P=0.018). **C**. Lifespan curves of *UAS-Ndi1*^{1}/S$_{1}$106 females. A 22% decrease in survival was observed in response to RU486 ($p<0.0001$). **D**. Lifespan curves of *UAS-Ndi1*^{2}/S$_{1}$106 females. A 9% decrease in survival was observed in response to RU486 ($p<0.0001$). **E**. Lifespan curves of *UAS-Ndi1*^{1}/*Elav-GS* females. A 12% increase in survival was observed in response to RU486 ($p<0.0001$). **F**. Lifespan curves of *UAS-Ndi1*^{2}/*Elav-GS* females. A 21% increase in survival was observed in response to RU486 ($p<0.0001$). Complete survival data on different RU486 concentrations are given in Tables S1, S2, S3. The significance of the difference between survival curves was analyzed using log-rank statistical test.

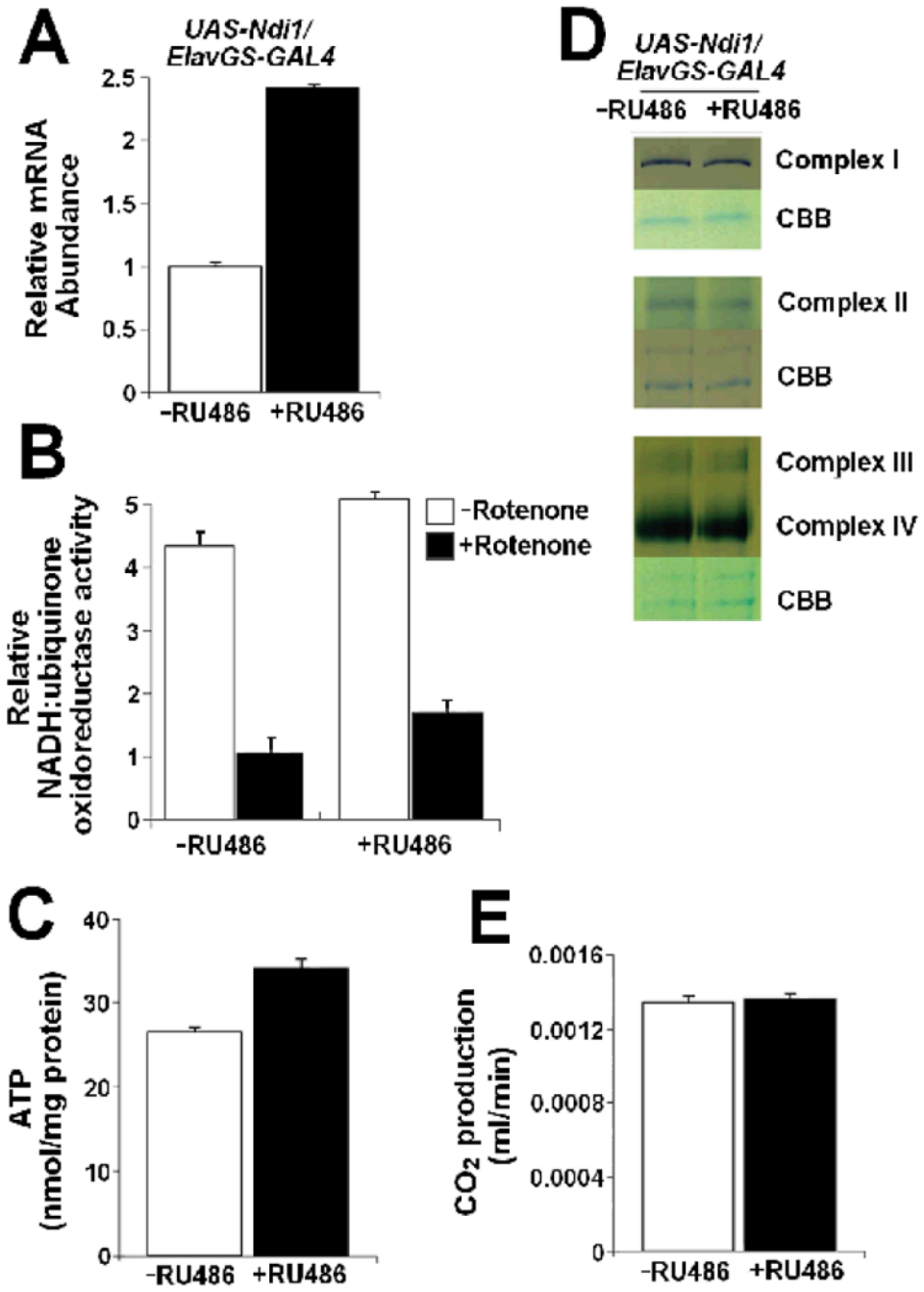

Figure 4. Neuronal expression of *Ndi1* increases complex I enzymatic activity and ATP levels in heads of long-lived flies

A-**E**. *UAS-Ndi1/Elav-GeneSwitch* flies were fed with 2 μg/ml RU486 during development and 10 μg/ml RU486 during the adulthood stage; uninduced flies were fed with diluent. All assays were performed on female flies between 6-10 days of age.

A. Increased *Ndi1* mRNA in heads of long-lived flies ($p<0.0001$). **B**. Increased NADH-ubiquinone oxidoreductase (complex I) enzymatic activity in heads of long-lived flies compared to uninduced controls ($p = 0.0029$). Rotenone insensitive activity was measured after addition of 1μM rotenone. **C**. Increased ATP levels in heads of long-lived flies ($p=0.0002$). **D**. The expression and activities of endogenous respiratory chain enzymes are

unchanged in heads of long-lived flies as assayed by Blue native-polyacrylamide gel electrophoresis (BN-PAGE) followed by in-gel activity staining. Coomasie Brilliant Blue (CBB) was used for estimating the amounts of the separated protein complexes. **E**. Neuronal expression of *Ndi1* does not affect CO_2 production in living flies ($p>0.05$). **A-D** n = 3, **E** n=8. Error bars indicate standard deviation. Student's *t*-test was used.

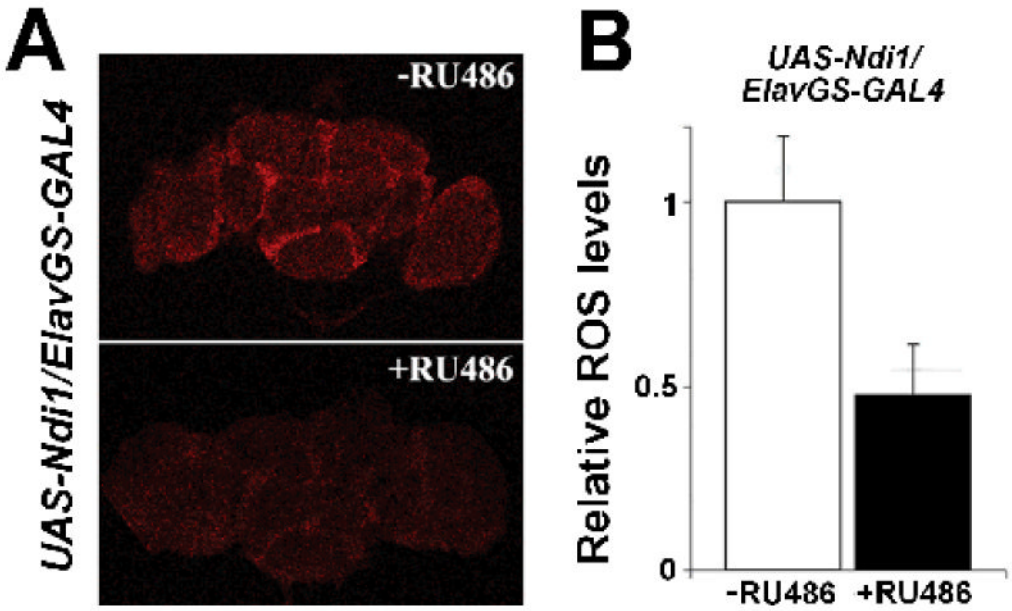

Figure 5. Reduced levels of reactive oxygen species (ROS) in brains of *Ndi1*-expressing flies

A-B. *UAS-Ndi1/Elav-GeneSwitch* flies were fed with 2 μg/ml RU486 during development and 10 μg/ml RU486 during the adulthood stage; uninduced flies were fed with diluent. Assays were performed on 45 day-old female flies.

A. Neuronal expression of *Ndi1* reduces ROS abundance in fly brains detected by dihydroethidium. **B**. Quantification of the relative ROS levels in brains of *Ndi1*-expressing flies normalized to uninduced control brains; *Ndi1* expression in neurons reduces ROS levels by 51% (p=0.001). n = 5 brains per treatment. Error bars indicate standard deviation. Student's *t*-test was used.

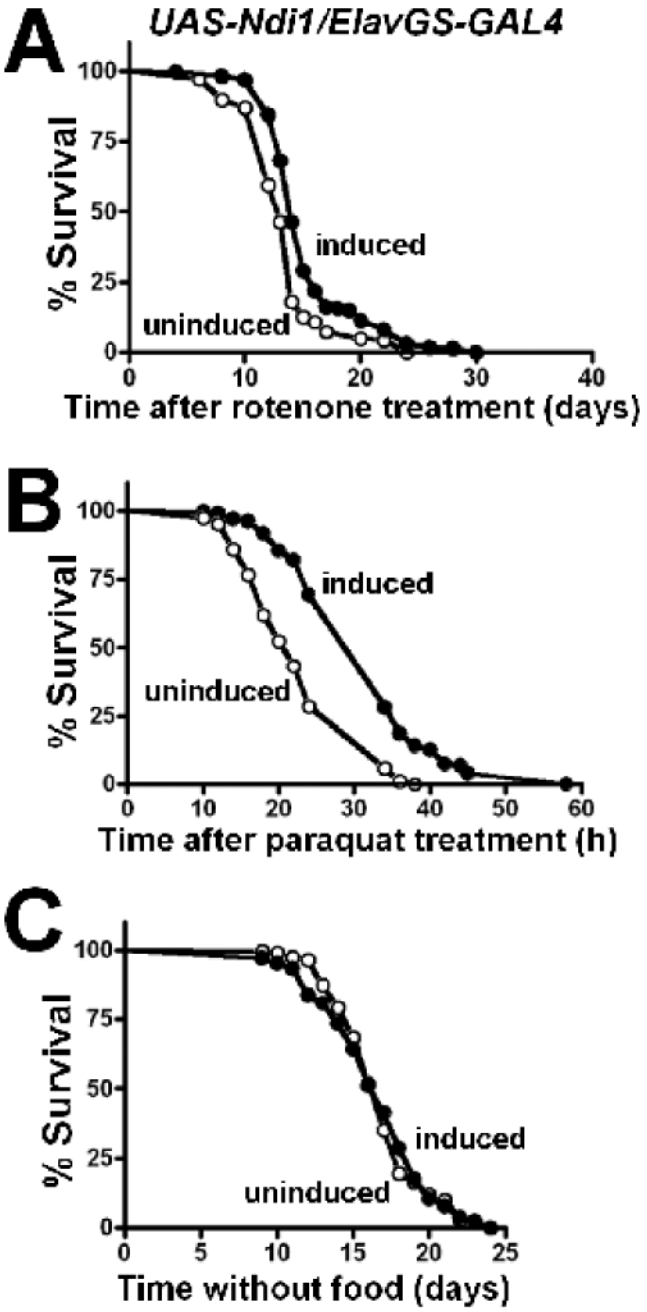

Figure 6. ***Ndi1*** **expressing flies are resistant to the endogenous complex I inhibitor rotenone and the free radical generator paraquat**

A-C, *UAS-Ndi1/Elav-GeneSwitch* flies were fed with 2 μg/ml RU486 during development and 10 μg/ml RU486 during the adulthood stage; uninduced flies were fed with diluent. Survival curves are shown as induced (+RU486, solid circles) or uninduced (-RU486, open circles). All assays were performed on female flies between 6-10 days of age. **A** *Ndi1* confers tolerance to 250 μM rotenone ($p < 0.0001$) and **B** 30 mM paraquat ($p < 0.0001$), but does not affect **C** starvation resistance ($p=0.9278$). The significance of the difference between survival curves was analyzed using the log-rank statistical test.

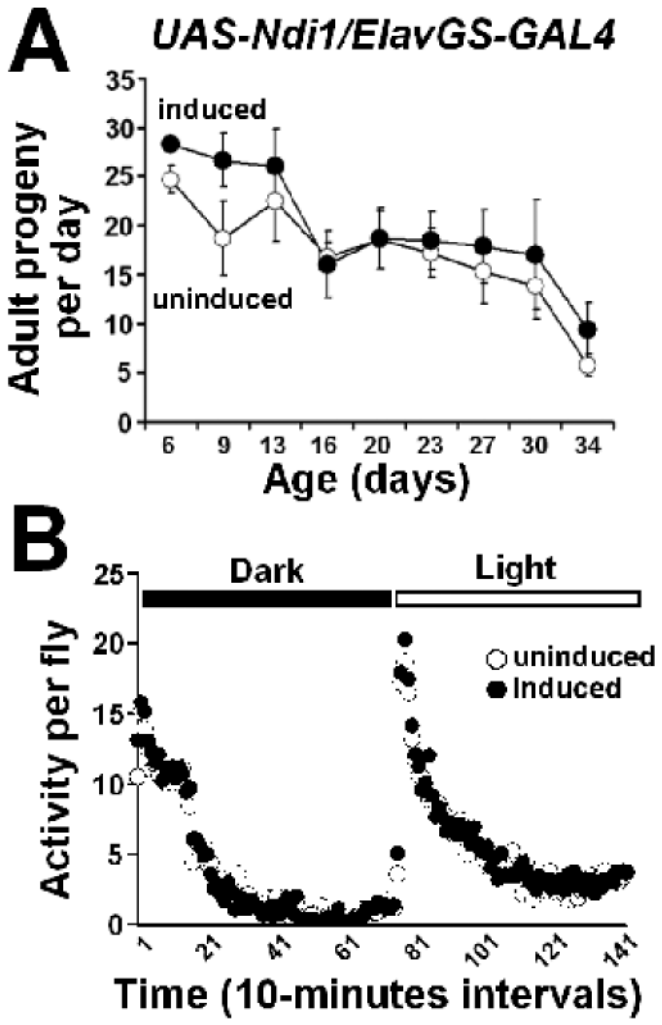

Figure 7. Long-lived *Ndi1*-expressing flies display normal fertility and physical activity

UAS-Ndi1/Elav-GeneSwitch flies were fed with 2 μg/ml RU486 during development and 10 μg/ml RU486 during the adulthood stage; uninduced flies were fed with diluent.

A. Average number of progeny produced from crosses between 5 wild-type males and 5 *UAS-Ndi1/Elav-GeneSwitch-GAL4* females fed RU486 or diluent. Long-lived *Ndi1* expressing flies display normal fertility compared to uninduced controls ($p>0.05$; two-way Analysis of Variance). **B**. Activity of 6- 10 day-old flies recorded with *Drosophila* Activity Monitor over a 24 hour period is shown. Long-lived *Ndi1* expressing flies display normal physical activity compared to uninduced controls ($p>0.05$; two-way Analysis of Variance).