

Rpd3 interacts with insulin signaling in *Drosophila* longevity extension

Jared K. Woods¹, Tahereh Ziafazeli^{1,3}, Blanka Rogina^{1,2}

¹Department of Genetics & Genome Sciences, School of Medicine, University of Connecticut Health, Farmington, CT 06030, USA

²Institute for Systems Genomics, School of Medicine, University of Connecticut Health, Farmington, CT 06030, USA

³current address: Division of Pediatric Endocrinology, Department of Pediatrics, Faculty of Health Sciences, McMaster University, Ontario, Canada

Correspondence to: Blanka Rogina; email: Rogina@uchc.edu

Keywords: Rpd3, Insulin/Insulin-like signaling, aging, *Drosophila melanogaster*

Received: September 10, 2016 Accepted: September 26, 2016 Published: November 14, 2016

ABSTRACT

Histone deacetylase (HDAC) 1 regulates chromatin compaction and gene expression by removing acetyl groups from lysine residues within histones. HDAC1 affects a variety of processes including proliferation, development, metabolism, and cancer. Reduction or inhibition of Rpd3, yeast and fly HDAC1 orthologue, extends longevity. However, the mechanism of *rpd3*'s effects on longevity remains unclear. Here we report an overlap between *rpd3* and the Insulin/Insulin-like growth factor signaling (IIS) longevity pathways. We demonstrated that *rpd3* reduction downregulates expression of members of the IIS pathway, which is associated with altered metabolism, increased energy storage, and higher resistance to starvation and oxidative stress. Genetic studies support the role of IIS in *rpd3* longevity pathway, as illustrated with reduced stress resistance and longevity of flies double mutant for *rpd3* and *dfoxo*, a downstream target of IIS pathway, compared to *rpd3* single mutant flies. Our data suggest that increased *dfoxo* is a mediator of *rpd3*'s effects on fly longevity and intermediary metabolism, and confer a new link between *rpd3* and IIS longevity pathways.

INTRODUCTION

Histone deacetylase (HDAC) proteins are highly conserved enzymes whose major role is regulation of chromatin structure. Histone deacetylation increases chromatin compaction with subsequent inhibition of gene transcription. Class I HDACs are zinc-dependent histone deacetylases and include HDACs 1, 2, 3, and 8. HDAC1 affects a variety of processes such as proliferation, differentiation, and development [1, 2]. Disruption of HDAC1 function has been associated with many disorders including cancer and neurodegenerative diseases including Alzheimer's disease [3, 4]. HDAC inhibitors are now used clinically for the treatment of several disorders including malignancies [5]. Rpd3, an orthologue of mammalian HDAC1, can also deacetylate lysine residues of various other proteins, such as P53, or bind to promoter region of other genes (*p21* and *p57*) and affect their transcription [6]. Reduced expression of Rpd3 extends lifespan in yeast, worms and fruit flies [7-12]. Similar effects on

Drosophila lifespan were achieved by feeding fruit flies 4-phenylbutyrate (PBA), a histone deacetylase inhibitor [3]. Despite all of these studies, the mechanism of the beneficial effects of decreased *rpd3* on longevity remains poorly understood.

The Insulin/insulin-like growth factor signaling (IIS) pathway is a nutrient-sensing pathway that regulates growth and development, energy homeostasis, stress response, and reproduction. Notably, mutations that reduce IIS activity are associated with longer lifespan in yeast, worms, flies, and mice [14-17]. *Drosophila* has eight insulin-like peptides (Dilps) that activate downstream events by binding to the insulin receptor [18]. dFOXO is the downstream target of IIS in flies [17]. When IIS is active, dFOXO is phosphorylated by dAkt, which leads to its binding to 14-3-3 proteins and its degradation. Reduced IIS results in decreased phosphorylation of dFOXO that promotes dFOXO nuclear translocation. dFOXO is a transcription factor and its nuclear localization is key to its influences on

growth, stress resistance, and metabolism [19]. The direct targets of dFOXO are conserved across several different mammalian tissues and species. Overexpression of nuclear localized dFOXO in fat body/gut extends longevity in flies and worms [20, 21]. In addition, overexpressing dFOXO in fly muscle extends lifespan [22].

Here, we investigate the effects and the mechanism of *rpd3* reduction on fly metabolism, stress resistance, and longevity. We found that flies with reduced *rpd3* levels have increased energy storage illustrated by increased levels of glucose, glycogen, trehalose, and triglycerides, which is consistent with their increased resistance to starvation. *rpd3* mutant flies have reduced IIS supported by decreased levels of *InR*, *chico*, and increased levels of *dfoxo* mRNA compared to controls. Genetic studies show an overlap between *rpd3* and IIS longevity pathways supported by a shorter life and reduced stress resistance of male flies with mutations in both *rpd3* and *dfoxo* compared to *rpd3* single mutant flies. Our data confer a novel link between *rpd3* and IIS and suggest IIS as a potential downstream mediator of the effects of *rpd3* mutation on fly health and metabolism.

RESULTS

rpd3 reduction affects energy storage in flies

To examine the mechanism of the longevity extension observed in *rpd3* mutant flies we examined how *Rpd3* reduction affects fly physiology by examining resistance to starvation, oxidative stress, and fly metabolism. We used two different heterozygous *rpd3* mutant alleles and their genetic controls, since homozygous *rpd3* mutation is embryonic lethal [23]. We used *rpd3* deficient (*rpd3^{def/+}*) and their genetic controls, F1 progeny of *rpd3^{def/+}* littermates. We also used *rpd3^{P-UTR/+}* flies, an *rpd3* hypomorph, and *rpd3^{P-1.8/+}*, which are genetic controls for *rpd3^{P-UTR/+}* flies and have *rpd3* reduction only in the eyes [24]. Here we show that *rpd3^{def/+}* mutant flies have higher starvation resistance at 10 and 40 days of age compared to control flies (Fig. 1 A,B; Supplemental Table 1A). Male *rpd3^{def/+}* flies are 38% and 44% more resistant to starvation at ages 10 and 40 days, respectively. Female *rpd3^{def/+}* flies are 28% and 108% more resistant at 10 and 40 days, respectively. To examine the potential mechanism of increased starvation resistance in *rpd3* alleles we examined the effects of *rpd3* reduction on fly metabolism. We quantified various forms of energy storage for the two *rpd3* mutant alleles. At 10 days of age *rpd3^{def/+}* females have increased triglyceride levels, but reduced glucose and glycogen levels (Fig. 1C-G). At 40 days, *rpd3^{def/+}* female flies have increased levels of glucose, glycogen, trehalose, and triglycerides.

rpd3^{def/+} males have increased levels of glucose and trehalose at 40 days, while no changes were observed at 10 days (Fig. 1C-F). Consistent with increased energy storage *rpd3^{def/+}* flies weighed more than control flies (Fig. 1H).

rpd3^{P-UTR/+} females live longer during starvation due to metabolic adaptation

At age 10, male and female *rpd3^{P-UTR/+}* flies have the same levels of energy stores as controls (Fig. 2A-F). At age 40, male *rpd3^{P-UTR/+}* flies have increased levels of triglycerides but reduced trehalose, glucose, and glycogen. When kept on standard lab food, female *rpd3^{P-UTR/+}* have the same triglyceride levels as controls at 40 days. To examine how *rpd3* reduction affects fly adaptation to starvation we kept flies at high calorie food (1.5N) for 10 or 40 days and then starved them for 24 hours. Triglyceride levels were much higher in *rpd3^{P-UTR/+}* female, compared to levels found in *rpd3^{P-1.8/+}* control female flies (Fig. 2G). Consistently, an increase in starvation resistance was observed in *rpd3^{P-UTR/+}* flies when compared to *rpd3^{P-1.8/+}* flies (Fig. 2H, Supplemental Table 1B). These data suggest that reduction in *rpd3* levels facilitates female adaptation to starvation, most likely by increasing efficiency in using metabolic reserves [25]. This is supported by our findings that *rpd3^{P-UTR/+}* flies have increased starvation resistance and consistent with our recent report that *rpd3^{P-UTR/+}* flies live longer compared to controls in conditions similar to starvation [11].

rpd3-mutant flies have altered gene expression of components of the IIS pathway

The IIS pathway plays an important role in metabolism, stress resistance, and aging. Decreasing IIS by inhibiting *InR*, *chico*, or ablating the IPCs of the brain has been shown to extend the lifespan of fruit flies [26]. Overexpressing dFOXO, a downstream target of the IIS pathway, increases fly stress resistance. Since both reduction of *rpd3* and of different members of IIS pathway extend fly longevity and increase stress resistance, we examined if these two longevity pathways overlap. We first examined how aging affects transcriptional levels of *rpd3* and different members of IIS in a control strain of *Drosophila*, *Canton-S* (CS) at age 10, 20, 40, and 60 days. We found age-related increase in the levels of *rpd3* and *dInR* mRNA in the heads and thoraces of CS flies and no changes in mRNA levels of *dfoxo* (Fig. 3A-C). Next we examined the status of IIS in *rpd3* mutant flies and determined the levels of genes of members of the IIS pathway in the heads and thoraces (H + T) of *rpd3^{def/+}* flies at 20 and 40 days of age. The mRNA levels of *InR* and *chico* were significantly decreased in heads and thoraces of

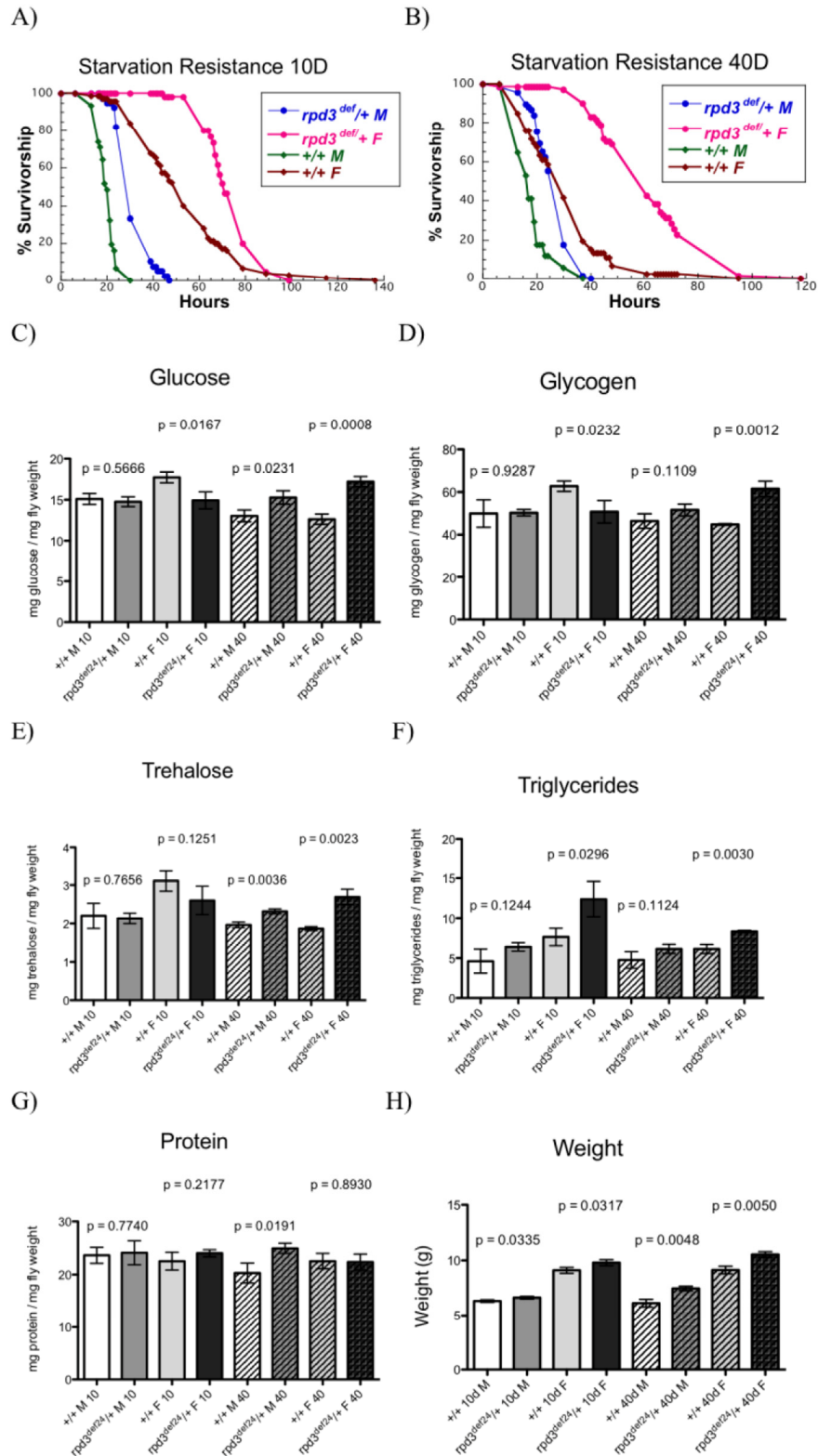


Figure 1. *rpd3* reduction affects stress resistance and metabolism. (A,B) Reduced *rpd3* levels increase stress resistance. Survival curves for male and female *rpd3^{def/+}* and control flies during starvation at age 10 (A) and 40 (B). **(C-G) *rpd3* reduction affects intermediary metabolism:** Total levels of glucose (C), glycogen (D), trehalose (E), triglyceride (F) and protein (G) in *rpd3^{def/+}* and control male and female flies at age 10 and 40 days. (H) Weight of *rpd3^{def}* and control flies used in C-G. Data are presented as means + SD (n=3, 30 flies per replicate. t test).

rpd3^{def} mutants compared to their genetic controls at age 40 (Fig. 3D-I). The mRNA levels of *PI3K*, another

member of IIS pathway, were the same as in controls (Fig. 3G). *dFOXO* is a downstream effector of the IIS

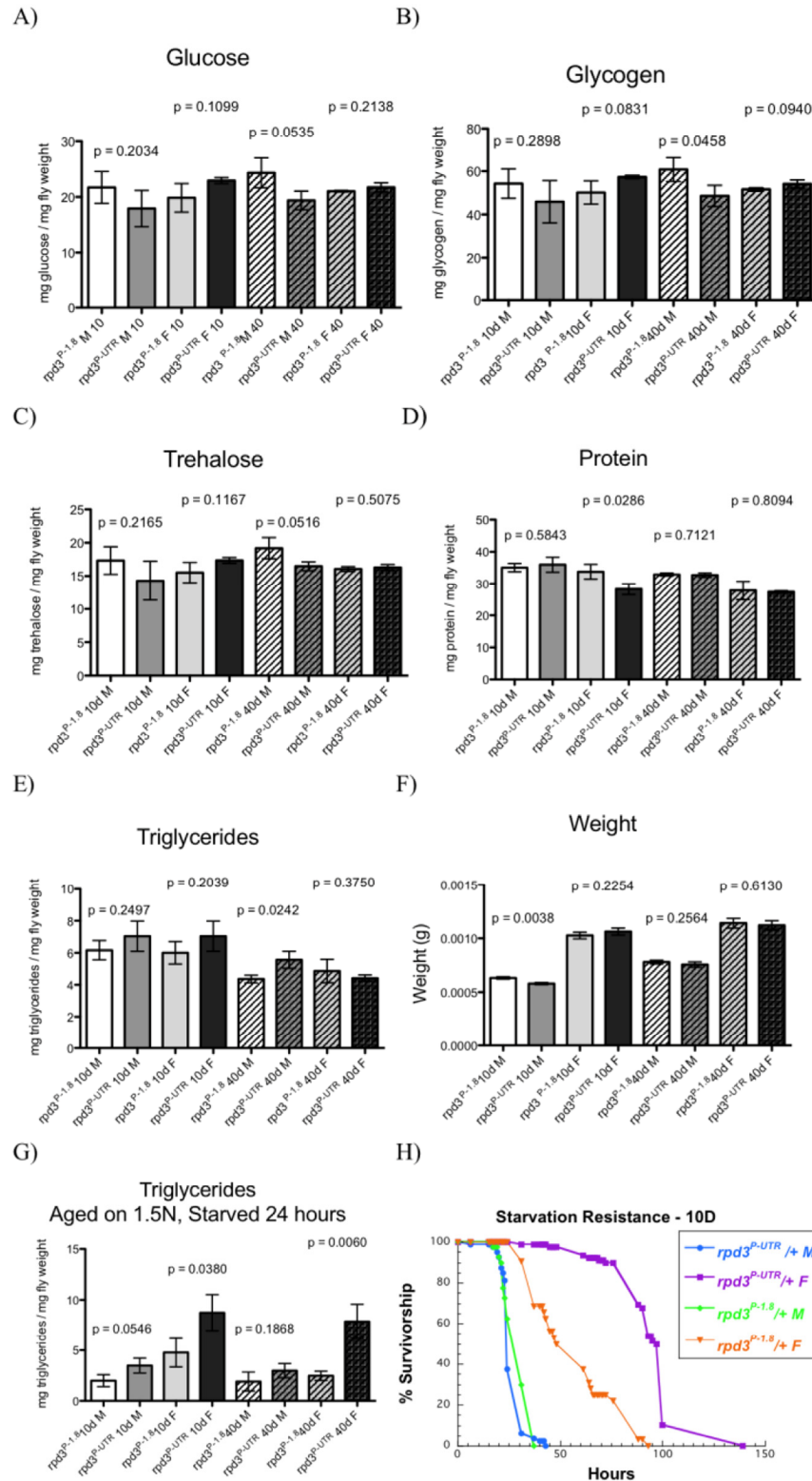


Figure 2. *rpd3* reduction affects fly intermediary metabolism. (A-E) Total glucose (A), glycogen (B), trehalose (C), protein (D) and triglyceride (E) levels in *rpd3^{P-UTR}/+* (experimental) and *rpd3^{P-1.8}/+* (control) male and female flies at age 10 and 40 days. (F) Weight of male and female flies used in (C-E). (G) Total triglyceride levels in *rpd3^{P-UTR}/+* and *rpd3^{P-1.8}/+* flies aged on 1.5N for 10 or 40 days and then starved for 24 hours. Data are presented as means + SD (n=3, 30 flies per replicate. t test) (H) *rpd3* reduction increases stress resistance in flies. Survival curves of *rpd3^{P-UTR}/+* and *rpd3^{P-1.8}/+* male and female flies during starvation at 10 of age.

pathway, whose activity is inversely related to IIS. Consistently with reduced IIS in *rpd3* mutant flies, we found increased *dfoxo* mRNA expression in both *rpd3^{def/+}* flies and *rpd3^{P-UTR/+}* flies at 40 of age (Fig. 3H,I). We used only thoraces of *rpd3^{P-UTR}* because their genetic controls *rpd3^{P-1.8}* flies have reduced levels of *rpd3* mRNA in the eyes. Similarly, increased expression of *dfoxo* mRNA was found in whole body RNA isolated from heart-specific *rpd3* downregulation [12].

When the IIS is active, dFOXO is phosphorylated by Akt and localized in cytoplasm. Reduction in IIS decreases dFOXO phosphorylation, which allows nuclear dFOXO localization and activation of trans-

cription of many genes known to mediate beneficial effects of reduced IIS. Our Western blots did not reveal any difference in the levels of phosphorylated dFOXO or in the ratio of nuclear and cytoplasmic fraction (Supplemental Fig. 1A-C). Nevertheless, this result could be due to low stability of the FOXO protein or possibly due to the quality of our anti-dFOXO antibody. Our data show that *rpd3* reduction prevents age-related increase in *InR* and reduction in *dfoxo* mRNA and suggest that decreased IIS could mediate the effects of *rpd3* mutation on *Drosophila* longevity.

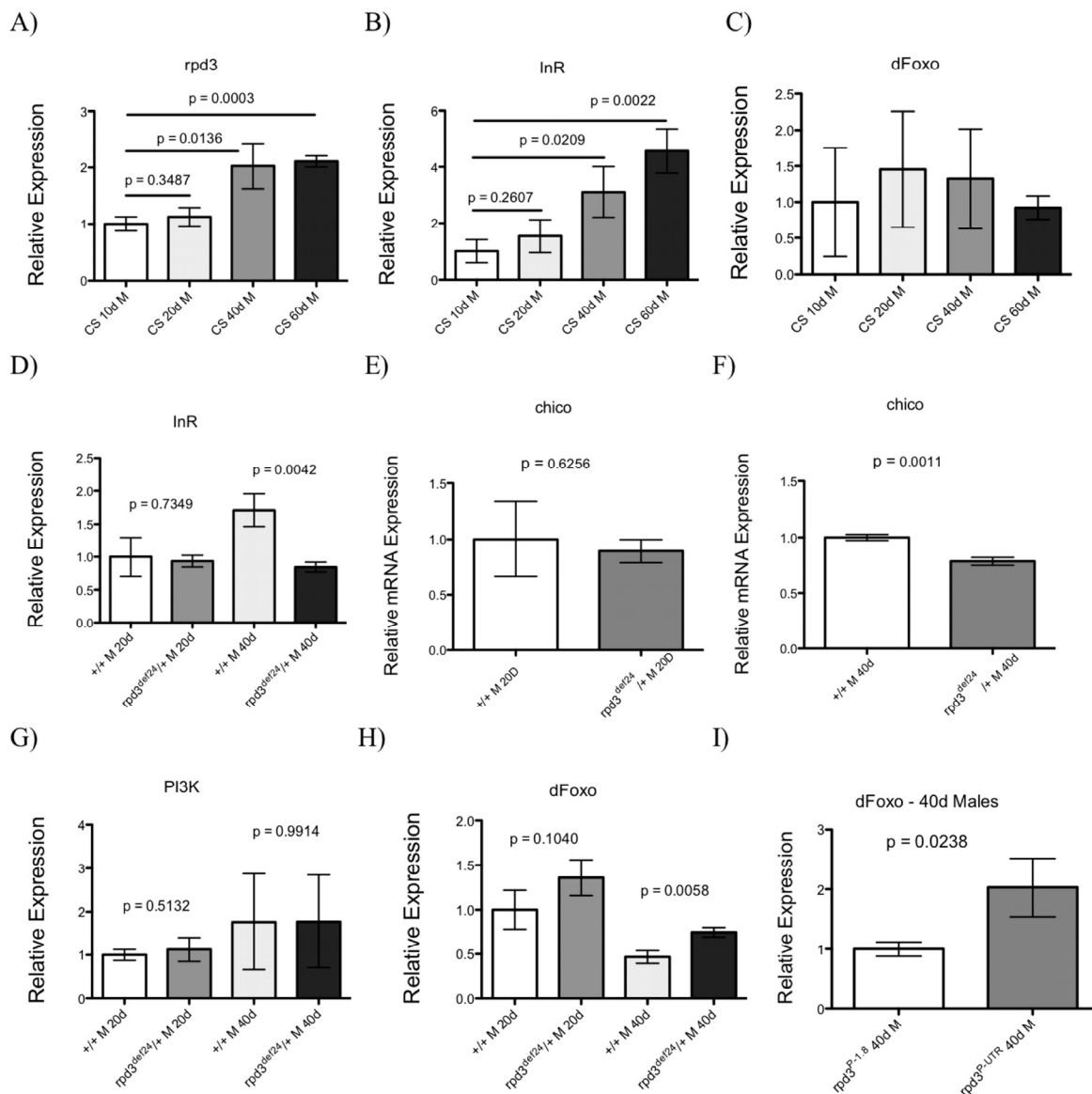


Figure 3. *rpd3* reduction decreases IIS. (A-C) Aging affects *rpd3* and *InR* mRNA levels. The levels of *rpd3* (A), *InR* (B) or *dfoxo* (C) mRNA in the heads and thoraces of Canton S (CS) wild type male flies at 10, 20, 40 or 60 days determined by qPCR. Controls show an age-related increase in *rpd3* and *InR* mRNA levels. (D-I) The levels of *InR* (D), *chico* (E,F), PI3K (G), and *dfoxo* (H) mRNA in heads and thoraces of *rpd3^{def/+}* male flies at ages 20 and 40 and their genetic controls determined by qPCR. (I) The levels of *dfoxo* mRNA are increased in thoraces of *rpd3^{P-UTR}*/CS male flies compared to *rpd3^{P-1.8}*/CS controls at 40 days (n=3, A-H: 30 heads and thoraces per replicate. I: 30 thoraces per replicate. p as noted, t test).

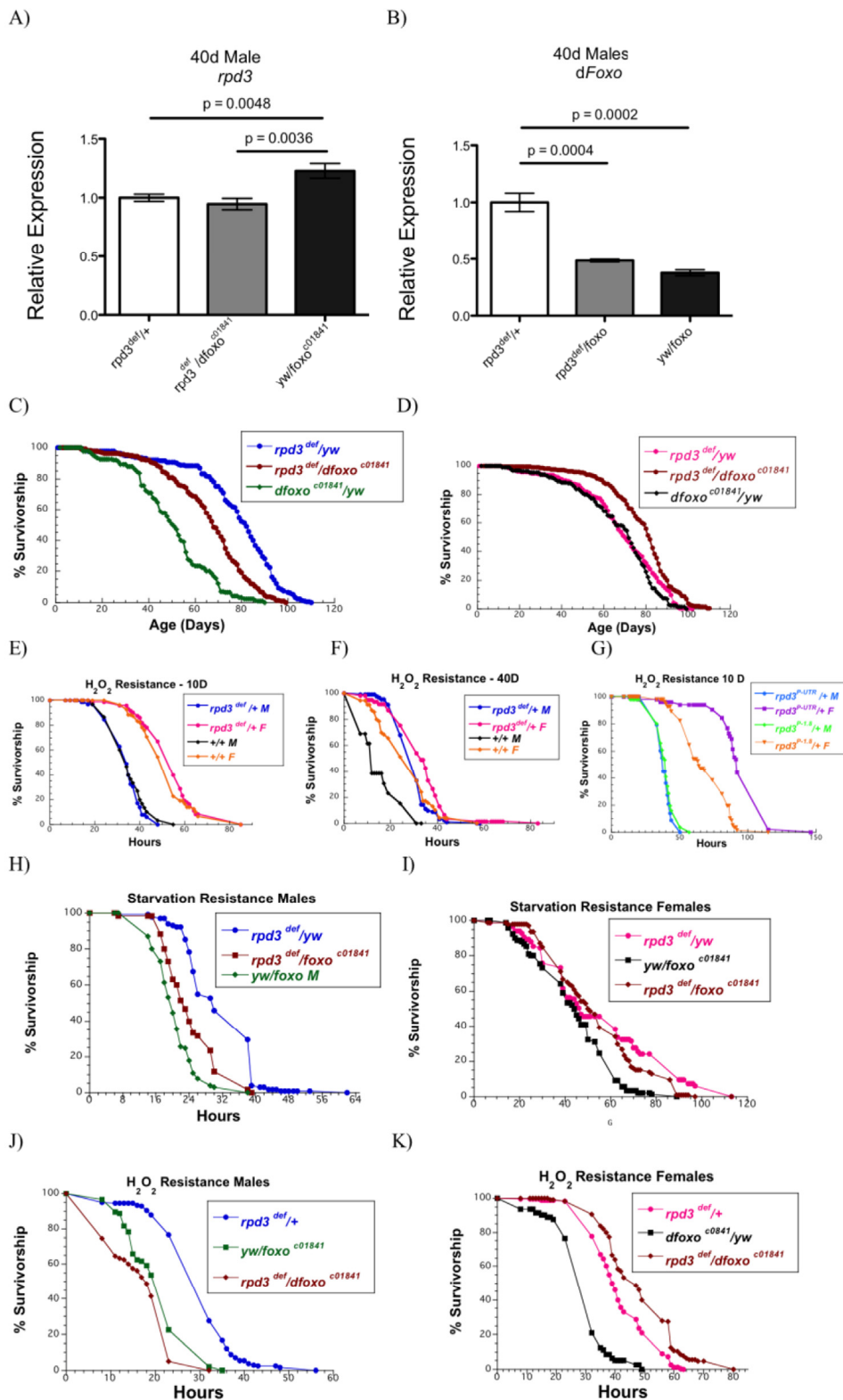


Figure 4. *dfoxo* partially mediates effects on longevity and stress resistance observed in male *rpd3* mutant flies. (A, B) *rpd3* (A) and *dfoxo* (B) mRNA levels in the heads and thoraces of *rpd3^{def}/yw*, *rpd3^{def}/dfoxo^{c01841}* and *dfoxo^{c01841}/yw* male flies at 40 days determined by qPCR (n=3, 30 Heads and thoraces per replicate. p as noted, t test, error bars represent SEM). (C,D) Survival curves of male (C) and female (D) *rpd3^{def}/yw*, *rpd3^{def}/dfoxo^{c01841}* and *dfoxo^{c01841}/yw* flies. (E-F) Survival curves of *rpd3^{def}/+* and control flies male and female flies on 5% H₂O₂ at age 10 (E) and 40 (F) days. (G) Survival curves of *rpd3^{P-1.8}/+* and *rpd3^{P-1.8}/+* male and female flies exposed to 5% H₂O₂ at age 10 days of age. (H, K) Survival curves for male (H, J) and female (I, K) *rpd3^{def}/yw*, *rpd3^{def}/dfoxo^{c01841}* and *dfoxo^{c01841}/yw* flies during starvation (H, I) or on 5% H₂O₂ (J, K) at age 40.

***dfoxo* partially mediates lifespan extension and increased stress resistance in *rpd3*-mutant flies**

To strengthen the link between *rpd3* mutation and reduced IIS, we generated flies that were double mutants for both *rpd3* and *dfoxo* and examined their longevity and stress resistance. *dfoxo* is the downstream IIS target that mediates beneficial effects of reduced IIS signaling. We confirmed that the levels of *rpd3* and *dfoxo* expression were reduced based on the presence of the mutations in male flies (Fig. 4A,B). *rpd3^{def}/yw* mutants and *rpd3^{def}/dfoxo^{c01841}* double mutants had lower levels of *rpd3* mRNA expression compared to *dfoxo^{c01841}/yw* mutant flies (Fig. 4A). *dfoxo^{c01841}/yw* and *rpd3^{def}/dfoxo^{c01841}* males have decreased levels of *dfoxo* mRNA expression compared to *rpd3^{def}/yw* flies (Fig. 4B). Notably, male *rpd3^{def}/dfoxo^{c01841}* double mutant flies have higher levels of *dfoxo* mRNA compared to single *dfoxo^{c01841}/yw* flies, consistent with our data that *rpd3* mutation increases *dfoxo* mRNA levels (Fig. 4B). Lifespan experiments were performed on these flies. Heterozygous *rpd3^{def}/+* males lived the longest (Fig. 4C, Supplemental Table 2) *dfoxo^{c01841}/yw* males had the shortest lifespan, whereas *rpd3^{def}/dfoxo^{c01841}* double mutants had a longevity in the middle of these two. These data suggest that the full beneficial effects of *rpd3* mutation on fly longevity require both copies of *dfoxo*, and that small increases in *dfoxo* levels in *rpd3^{def}/dfoxo^{c01841}* may increase longevity of double mutant flies compared to *dfoxo^{c01841}/yw* males. In contrast, female *rpd3^{def}/dfoxo^{c01841}* flies live longer compared to single mutant flies, which have similar lifespans. The differences between male and female in longevity effects of double mutations may be due to sexual dimorphism previously described to be associated with IIS, FOXO, and p53 [27]. Similar differences were observed in stress response, which is shown in next paragraph and discussed later.

Increased stress resistance in flies with reduced *rpd3* and IIS

Reduced IIS is associated with increased resistance to oxidative stress. Since *rpd3* reduction is associated with IIS reduction we examined if flies with reduced *rpd3* levels are more resistant to hydrogen peroxide (H₂O₂). Male and female *rpd3^{def}/+* are more resistant to H₂O₂ compared to control flies at 40 days of age, while no difference was observed at age 10 days (Fig. 4E,F; Supplemental Table 3). Female *rpd3^{P-UTR}/+* flies are more resistant to H₂O₂ at both ages but no difference was observed in male *rpd3^{P-UTR}/+* flies (Fig 4G, Supplemental Table 3 and data not shown). To examine the role of *dfoxo* in increased stress resistance of *rpd3* mutant flies, we determined starvation and H₂O₂ resistance of *rpd3* mutant flies with or without *dfoxo*

mutation. Similarly to longevity studies, *rpd3^{def}/+* male flies had the highest mean survival rate when exposed to starvation at 40d of age (Fig. 4H,I; Supplemental Table 4). *rpd3^{def}/dfoxo^{c01841}* double mutants had a lower mean survival, but they were more resistant to starvation than *dfoxo^{c01841}/yw* flies (Fig. 4H,J). Similarly, male *rpd3^{def}/+* mutants are more resistant to H₂O₂ compared to *rpd3^{def}/dfoxo^{c01841}* or *dfoxo^{c01841}/yw* flies. These results suggest that *dfoxo* mediates some of the effects of *rpd3* mutation on lifespan in *Drosophila* males and is required for full beneficial effects of *rpd3* mutation on longevity and stress resistance. Female flies double mutant for *rpd3^{def}* and *dfoxo^{c01841}* live longer and have similar starvation and H₂O₂ resistance compared to single *rpd3^{def}/+*, but are more resistance compared to *dfoxo^{c01841}/yw* mutant flies (Fig. 4D,I,K; Supplemental Table 5).

DISCUSSION

***rpd3* reduction affects fly metabolism and IIS**

Members of the class I HDAC family are vital regulators of chromatin structure and gene expression. They have multiple functions including a role in development, metabolism, and aging [1, 28]. Deletion of *rpd3* in yeast extends their replicative lifespan [7]. Flies heterozygous for a null or a hypomorphic *rpd3* mutant alleles had an extended lifespan compared to genetic controls [9, 11]. Moreover, heart-specific *rpd3* downregulation in flies increases heart function, stress resistance, and extends longevity [12]. In addition, HDAC inhibitors trichostatin A and butyrate also extend fly lifespan [13, 29]. Longevity extension of *rpd3* mutant flies was not further extended by dietary restriction (DR) and was absent in flies double mutant for *dSir2* and *rpd3* mutations [30]. Both DR flies and flies with reduced *rpd3* have increased *dSir2* levels [12, 30]. These findings suggested that the mechanism of longevity extension in *rpd3* mutant and DR flies overlap. However, the full understanding of the mechanism of longevity extension associated with *rpd3* reduction is missing. Therefore we examined intermediary metabolism and starvation resistance of two *rpd3* heterozygous mutant alleles. Increased starvation resistance in female *rpd3^{def}/+* flies is the result of increased energy storage in forms of triglyceride at young and old age, as well as increased carbohydrate levels at age 40. Metabolic adaptation to fasting is key to preserving homeostasis of an organism. This adaptation includes mobilization of lipids followed by their oxidation into ketone bodies, which are used as a source of energy in other tissues. Metabolic adaptation to starvation in *rpd3* mutant flies is illustrated by the findings that *rpd3* reduction prevents a fasting-induced decrease in triglycerides in female *rpd3^{P-UTR}/+* flies.

This is consistent with our recent report that *rpd3^{P-UTR/+}* flies live longer compared to controls in conditions similar to starvation and findings that heart-specific Rpd3 downregulation increases fly resistance to starvation at age 2 days [11, 12]. To get insights into the mechanism associated with *rpd3* reduction we examined if these changes are mediated by IIS. The IIS pathway is a nutrient sensing pathway, which also affects the activity of metabolic enzymes. When nutrients are abundant IIS is active, dFOXO is phosphorylated, and it is localized in the cytoplasm. Reduced IIS decreases phosphorylation of dFOXO, which promotes dFOXO nuclear translocation. In the nucleus, dFOXO regulates glucose, glycogen, and lipid metabolism by activating transcription of key enzymes involved in these metabolic pathways. For instance, dFOXO activates glycogenolysis and gluconeogenesis by activating transcription of glucose-6-phosphatase (G6P) and phosphoenolpyruvate carboxykinase (PEPCK) mRNA, respectively [31]. dFOXO regulates autophagy in response to starvation, which promotes recycling of the cellular components [32]. Intriguingly, adult dFOXO null mutant flies have no difference in starvation resistance or energy storage [33]. Under conditions when nutrients are limited, activated dFOXO upregulates *InR* transcription. This allows the cells to accumulate *InR* mRNA, and prime them to respond quickly when nutrients become available [34]. Once IIS

is activated, it will upregulate growth and inhibit dFOXO activity via its phosphorylation. Here we show that in a control strain of *Drosophila*, *CS*, the levels of *InR* mRNA expression gradually increase throughout the lifespan. We found that *rpd3* reduction affects the IIS pathway and prevents age-associated changes in the transcript levels of IIS genes. *rpd3^{def}* flies have reduced levels of *InR* and *chico* and increased *dfoxo* mRNA at 40 days. These changes in IIS are consistent with metabolic changes found in 40 day old *rpd3* mutant flies.

dFOXO mediates some longevity effects observed in *rpd3* mutant flies

Our genetic studies suggest that dFOXO mediates some longevity effects of *rpd3^{def}* mutant flies. This is illustrated by shorter lifespan of male flies double mutant for *rpd3* and *dfoxo* compared to *rpd3^{def/+}* flies. *rpd3^{def/dfoxo^{c0184}}* flies live longer compared to *dfoxo* single mutant flies, which is most likely mediated by higher *dfoxo* mRNA expression in double mutants compared to *dfoxo* single mutants. Similarly, *rpd3^{def/dfoxo^{c0184}}* flies have reduced resistance to H₂O₂ compared *rpd3^{def/+}* indicating that increased dFOXO mediates resistance to stress in *rpd3* mutant male flies. This is consistent with findings that overexpression of nuclear localized dFOXO mediates increased resistance to oxidative stress in flies and mammalian cells [33].

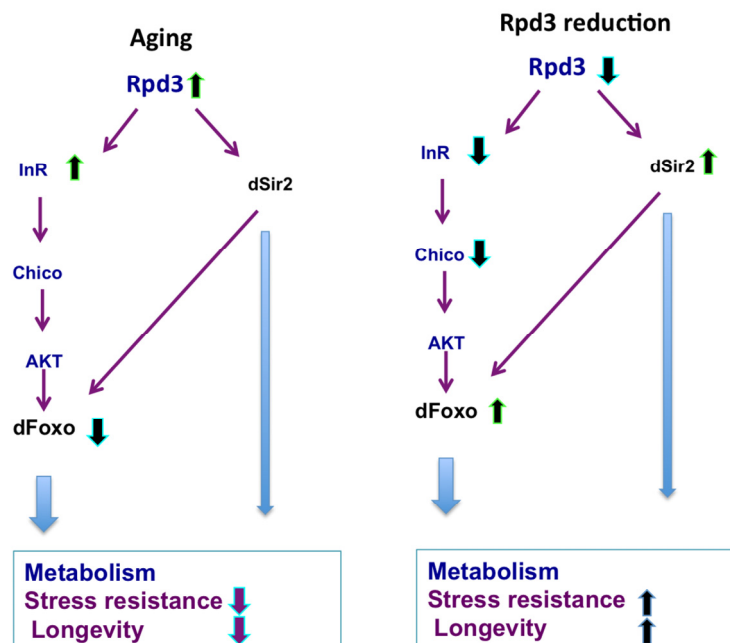


Figure 5. *rpd3* reduction prevents age-associated changes in IIS. Age-associated increases in the *rpd3*, *InR*, and a decrease in *dfoxo* mRNA are observed in wild type flies, Reduced *rpd3* activity decreases *InR* and *chico* mRNA, while increases *dfoxo* and *dSir2* mRNA levels. Reduced *rpd3* affects metabolism, increases stress resistance and longevity by reducing IIS and increasing *dSir2* levels. Purple and light blue arrows indicate downstream effects, green arrows represent increase and blue reduction in mRNA levels.

Moreover, treatment with PBA, a HDAC1 inhibitor, increases expression of genes that have been implicated in response to oxidative stress, such as SOD, glutathione S-transferase, and heat-shock protein. However, female *rp3^{def}/dfoxo^{c01841}* live longer and are similarly more resistant to H₂O₂ compared to both single *rp3^{def/+}* and *dfoxo^{c01841/+}* mutant flies. It is possible that reduced *rp3* levels in *rp3^{def/+}* female flies increase *dfoxo* levels of the remaining wild type copy of the gene, which contribute to longer lifespan. The differences may be also due to sexual dimorphism previously described to be associated with IIS, FOXO, and p53. It was found that nervous-system specific overexpression of *p53* increases female lifespan in a *foxo* null background. In contrast, in males *foxo* null mutation caused the tissue-specific effects of *p53* [27]. Several studies have examined the relationship between IIS and HDAC inhibition. β -hydroxybutyrate (β OHB) is an endogenous inhibitor of HDACs 1, 3, and 4 (Class I and Class II). β OHB is one of the ketone bodies released during fasting and exercise. β OHB inhibition of HDAC1 and HDAC2 activity results in increased histone acetylation and gene expression. Particularly important is induction of *Foxo3*, the mammalian orthologue of the dFOXO, expression by removing HDAC-mediated *Foxo3* repression via hypoacetylation of its promoter [35]. Ye reviewed the effects of an HDAC1 inhibitor on energy metabolism and insulin sensitivity [36]. It was also reported that use of butyrate, a class I and II HDAC inhibitor, improves glucose metabolism and prevents age-related atrophy [37]. However, less is known about the specific role of *rp3* reduction on metabolism in flies. The data presented here provide new information about the effects of *rp3* reduction on fly metabolism and link the changes in metabolism to a reduction in IIS. Taken together, our genetic data strengthen the link between *rp3* reduction and reduced IIS.

Longevity extension in *rp3* mutant flies has been linked to dSir2, 4E-BP, dFOXO, and CR longevity pathways [9, 11, 12, 30]. The data presented here add to our understanding of the mechanism of *rp3*'s effects on longevity by identifying a novel genetic link between *rp3* reduction and IIS (Fig. 5). However, considering that *rp3* has multiple targets, the reduction of *rp3* does not completely reproduce phenotypes of the flies with reduced IIS. In *Drosophila*, reduction in IIS results in female sterility and longevity extension independent of fertility [15,16]. *Chico* mutants are smaller and sterile, but they contain twice as much lipid content per weight as do genetic controls [38]. Overexpression of *dfoxo* in adult fat body and the gut has no effect on fecundity, total triglyceride, total trehalose, or total glycogen content, but it reduced fly weight and total protein content [39]. *rp3* mutant flies have reduced

levels of *InR* and *chico*, increased levels of *dfoxo* mRNA, and increased resistance to starvation and oxidative stress. However, *rp3* flies are as fertile as controls on a standard diet, and their weight is higher or the same compared to controls. These differences highlight the complex mechanism of the beneficial effects of *rp3* reduction on fly health and longevity.

Here we show that *rp3* reduction affects fly metabolism characterized by increased energy storages, higher resistance to starvation and oxidative stress, and increased longevity. These effects are mediated at least partially by reduced IIS, confirmed by genetic studies showing that longevity extension requires increased dFOXO (Fig. 5). Previous studies highlight the role of dSir2 in *rp3* effects on lifespan [12, 30]. Histone modifying enzymes link changes in nutrient availability to changes in intermediary metabolism by affecting the activity and stability of the enzymes involved in glycogenesis, glycolysis, gluconeogenesis, and β -oxidation through acetylation [40-42]. The acetylation pattern differs in tissues and cell types, suggesting a complex, highly orchestrated regulation of acetylation levels at the organismal level. Future studies on the acetylation status of enzymes involved in intermediary metabolism in different tissues of *rp3* mutant flies would further expand our knowledge of the effects of *rp3* reduction on metabolism, health, and longevity. Our data illustrates how these complex interactions result in phenotypic changes at the organismal level. Further studies are necessary to examine how tissue-specific alterations in *rp3* levels orchestrate these changes.

MATERIALS AND METHODS

Fly strains and maintenance

rp3-deficient (*rp3^{def24}*) and *rp3*-hypomorphic (*rp3^{P-UTR}*) flies and their genetic controls were used in the experiments. Genetic controls for *rp3^{def24}* were progeny generated by crossing F1 *rp3^{def24/+}* littermates. The hypomorphic *rp3^{P-UTR}* allele has a P-element inserted in the 5'UTR region of the *rp3* genes, which affects expression throughout the fly's bod. The control *rp3^{P-1.8}* allele has a P-element inserted 1.8 Kb upstream from the transcriptional start site, which only decreases expression in the eye [24]. *Canton S*, *yw*, and *dfoxo^{c01841}* were kindly provided by the Bloomington Stock center. *dfoxo^{c01841}* flies were backcrossed to *yw* strain for 10 generations to eliminate difference in genetic background. Flies were collected within 24 hours of eclosion and maintained using standard culture media in plastic vials. They were kept at 25°C in a humidified incubator. About 25 males and 25 females were kept together in each vial, and they were passed to a fresh vial every Monday, Wednesday, and Friday.

Standard corn and 1.5N (High calorie diet) diet was prepared as previously reported [43].

Lifespan studies

Flies were collected within 24 hours of eclosion and maintained as described above. 25 males or 25 females were placed in each vial. They were passed every 2 days up to age 30 days and every day after that and the number of dead flies were counted. The number of flies in each survivorship study is listed in Tables 1-7.

Starvation and oxidative stress resistance

Flies were collected as described above and aged until 10 or 40 days of age. They were separated into vials of 20 males or 20 females and transferred into new vials containing 2 filter papers with 300 μ l of DI H₂O for starvation studies. 300 μ l of 5% H₂O₂ with 5% sucrose was used instead of water for oxidative stress studies following initial 6 hours of starvation. The number of dead flies was counted at regular intervals, and the vials were changed ever 24 hours.

Resistance to paraquat: flies were collected as described above. 20 flies were transferred to a vial containing filter paper soaked with 300 μ L of 20 mM paraquat following initial starvation for 6 hours. The number of dead flies was counted hourly during the day and twice overnight until no flies remained alive. Stress resistance data were analyzed by log-rank tests using the JMP 10 program. Total number of flies per experiment is listed in Supplemental Tables 1, 2, and 3.

Biochemistry

Flies were collected and aged as described above. At ages 10 and 40 days, flies were weighed, homogenized, and 25 μ l of homogenate was aliquoted into 96-well plates. For glucose, PGO enzyme plus color reagent was added to each well, the plate was incubated at 37°C, and the optic density was read at 450 nm. For glycogen, the procedure was the same except amyloglucosidase was added to each well in addition to the other enzyme. For trehalose, the procedure was the same as glucose except the samples were incubated with trehalase before addition of PGO. For protein, BioRad protein solution was diluted 1:5, added to each well, and then the plate was read at 595 nm. For triglycerides, free glycerol and triglyceride reagents were added to each well, the plate was incubated at 37°C, and the optic density was read at 540 nm [45, 46].

Quantitative PCR (qPCR)

Flies were frozen at the appropriate age, and RNA was

isolated from the heads and thoraces, thoraces, or heads. cDNA was synthesized from RNA. Using TaqMan primers and the Applied Biosystems Thermal Cycler, levels of gene expression were determined. The data were normalized to the results of the control flies using *ankryn* as standard [47].

Western blots

40 day old flies were dissected on CO₂ and placed in tubes containing Kinase Lysis Buffer. The tissue was homogenized using Teflon pestles. Protein concentrations were quantified using BioRad Protein determination Kit. Lysates were cleared by centrifugation, run on a Lithium Dodecyl Sulfate (LDS) PAGE using NuPAGE NOVEX 4-12% gradient gels (Life Technologies), and transferred overnight onto nitrocellulose membranes in transfer buffer (20mM CAPS pH 11, 20% MeOH). Western blotting was performed using standard procedures with washes done in TBST. Membranes were blocked in 5% milk for an hour followed by overnight, 1:500 primary antibody incubation at 4°C. Secondary antibody incubation was done for an hour at room temperature at a concentration of 1:5,000. Secondary antibodies were labeled with Horseradish Peroxidase. Blots were imaged using the Kodak Image Station 4000 MM following application of Enhanced Chemiluminescence reagent (Perkin Elmer). The dFOXO antibody was a gift from Mark Tatar, and the two anti-phospho-Foxo3A antibodies (Cat #9466) were gifts from Cell Signaling Technology to test if they would work in *Drosophila* tissue. Nuclear and cytoplasmic fractions were isolated from 40-day old flies following instruction for The Active Motif Nuclear Extract Kit (Carlsbad, CA. USA, Cat #40010).

Statistical analysis

Significance was determined using a two-tailed, unpaired t-test from at least three independent experiments and expressed as P values. P < 0.05 is considered to be significant. P values are specifically indicated in each figure. Error bars represent standard deviation (SD). Longevity data were censored for early mortality (1-9 Days) and analyzed by log-rank tests using the JMP 12 program.

ACKNOWLEDGEMENTS

We would like to thank Suzanne Kowalski, Alexander Pokorski, Bhavin Gupta and Dr. Ryan Rogers for excellent technical assistance. We thank Dr. Stewart Frankel for critical reading of the manuscript.

CONFLICTS OF INTEREST

The authors have no conflict of interests to declare.

FUNDING

This work was supported by grant from the National Institute on Health RO1AG 023088 to B. Rogina. B. Rogina is a recipient of a Glenn Award for Research in Biological Mechanisms of Aging.

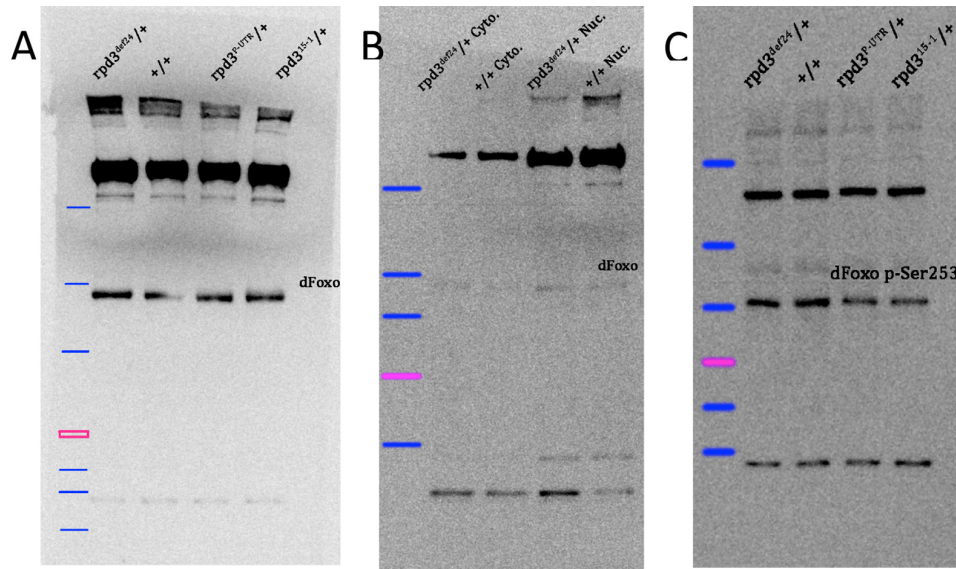
REFERENCES

1. Seto E, Yoshida M. Erasers of histone acetylation: the histone deacetylase enzymes. *Cold Spring Harb Perspect Biol.* 2014; 6:a018713. doi: 10.1101/cshperspect.a018713
2. Moser MA, Hagelkruys A, Seiser C. Transcription and beyond: the role of mammalian class I lysine deacetylases. *Chromosoma.* 2014; 123:67–78. doi: 10.1007/s00412-013-0441-x
3. Willis-Martinez D, Richards HW, Timchenko NA, Medrano EE. Role of HDAC1 in senescence, aging, and cancer. *Exp Gerontol.* 2010; 45:279–85. doi: 10.1016/j.exger.2009.10.001
4. Woods JK, Rogina B. The effects of Rpd3 on fly metabolism, health, and longevity. *Exp Gerontol.* 2016; doi: 10.1016/j.exger.2016.02.015. Epub ahead of print
5. Drummond DC, Noble CO, Kirpotin DB, Guo Z, Scott GK, Benz CC. Clinical development of histone deacetylase inhibitors as anticancer agents. *Annu Rev Pharmacol Toxicol.* 2005; 45:495–528. doi: 10.1146/annurev.pharmtox.45.120403.095825
6. Juan LJ, Shia WJ, Chen MH, Yang WM, Seto E, Lin YS, Wu CW. Histone deacetylases specifically down-regulate p53-dependent gene activation. *J Biol Chem.* 2000; 275:20436–43. doi: 10.1074/jbc.M000202200
7. Kim S, Benguria A, Lai C-Y, Jazwinski SM. Modulation of life-span by histone deacetylase genes in *Saccharomyces cerevisiae*. *Mol Biol Cell.* 1999; 10:3125–36. doi: 10.1091/mbc.10.10.3125
8. Edwards C, Canfield J, Copes N, Rehan M, Lipps D, Bradshaw PC. D-beta-hydroxybutyrate extends lifespan in *C. elegans*. *Aging (Albany NY).* 2014; 6:621–44. doi: 10.18632/aging.100683
9. Rogina B, Helfand SL, Frankel S. Longevity regulation by *Drosophila* Rpd3 deacetylase and caloric restriction. *Science.* 2002; 298:1745. doi: 10.1126/science.1078986
10. Pallos J, Bodai L, Lukacsovich T, Purcell JM, Steffan JS, Thompson LM, Marsh JL. Inhibition of specific HDACs and sirtuins suppresses pathogenesis in a *Drosophila* model of Huntington's disease. *Hum Mol Genet.* 2008; 17:3767–75. doi: 10.1093/hmg/ddn273
11. Frankel S, Woods J, Ziafazeli T, Rogina B. RPD3 histone deacetylase and nutrition have distinct but interacting effects on *Drosophila* longevity. *Aging (Albany NY).* 2015; 7:1112–29. doi: 10.18632/aging.100856
12. Kopp ZA, Hsieh JL, Li A, Wang W, Bhatt DT, Lee A, Kim SY, Fan D, Shah V, Siddiqui E, Ragam R, Park K, Ardeshtna D, et al. Heart-specific Rpd3 downregulation enhances cardiac function and longevity. *Aging (Albany NY).* 2015; 7:648–63. doi: 10.18632/aging.100806
13. Kang H-L, Benzer S, Min K-T. Life extension in *Drosophila* by feeding a drug. *Proc Natl Acad Sci USA.* 2002; 99:838–43. doi: 10.1073/pnas.022631999
14. Kenyon C, Chang J, Gensch E, Rudner A, Tabtiang R. A *C. elegans* mutant that lives twice as long as wild type. *Nature.* 1993; 366:461–64. doi: 10.1038/366461a0
15. Clancy DJ, Gems D, Harshman LG, Oldham S, Stocker H, Hafen E, Leivers SJ, Partridge L. Extension of life-span by loss of CHICO, a *Drosophila* insulin receptor substrate protein. *Science.* 2001; 292:104–06. doi: 10.1126/science.1057991
16. Tatar M, Kopelman A, Epstein D, Tu M-P, Yin C-M, Garofalo RS. A mutant *Drosophila* insulin receptor homolog that extends life-span and impairs neuroendocrine function. *Science.* 2001; 292:107–10. doi: 10.1126/science.1057987
17. Yamamoto R, Tatar M. Insulin receptor substrate chico acts with the transcription factor FOXO to extend *Drosophila* lifespan. *Aging Cell.* 2011; 10:729–32. doi: 10.1111/j.1474-9726.2011.00716.x
18. Kannan K, Fridell YW. Functional implications of *Drosophila* insulin-like peptides in metabolism, aging, and dietary restriction. *Front Physiol.* 2013; 4:288. doi: 10.3389/fphys.2013.00288
19. Webb AE, Kundaje A, Brunet A. Characterization of the direct targets of FOXO transcription factors throughout evolution. *Aging Cell.* 2016; 15:673–85. doi: 10.1111/accel.12479
20. Giannakou ME, Goss M, Jünger MA, Hafen E, Leivers SJ, Partridge L. Long-lived *Drosophila* with overexpressed dFOXO in adult fat body. *Science.* 2004; 305:361. doi: 10.1126/science.1098219
21. Hwangbo DS, Gershman B, Tu MP, Palmer M, Tatar M. *Drosophila* dFOXO controls lifespan and regulates insulin signalling in brain and fat body. *Nature.* 2004; 429:562–66. doi: 10.1038/nature02549
22. Demontis F, Perrimon N. FOXO/4E-BP signaling in *Drosophila* muscles regulates organism-wide

- proteostasis during aging. *Cell*. 2010; 143:813–25. doi: 10.1016/j.cell.2010.10.007
23. Mannervik M, Levine M. The Rpd3 histone deacetylase is required for segmentation of the *Drosophila* embryo. *Proc Natl Acad Sci USA*. 1999; 96:6797–801. doi: 10.1073/pnas.96.12.6797
 24. Mottus R, Sobel RE, Grigliatti TA. Mutational analysis of a histone deacetylase in *Drosophila melanogaster*: missense mutations suppress gene silencing associated with position effect variegation. *Genetics*. 2000; 154:657–68.
 25. Rion S, Kawecki TJ. Evolutionary biology of starvation resistance: what we have learned from *Drosophila*. *J Evol Biol*. 2007; 20:1655–64. doi: 10.1111/j.1420-9101.2007.01405.x
 26. Kenyon CJ. The genetics of ageing. *Nature*. 2010; 464:504–12. doi: 10.1038/nature08980
 27. Shen J, Tower J. *Drosophila* foxo acts in males to cause sexual-dimorphism in tissue-specific p53 life span effects. *Exp Gerontol*. 2010; 45:97–105. doi: 10.1016/j.exger.2009.10.009
 28. Mihaylova MM, Shaw RJ. Metabolic reprogramming by class I and II histone deacetylases. *Trends Endocrinol Metab*. 2013; 24:48–57. doi: 10.1016/j.tem.2012.09.003
 29. Zhao Y, Sun H, Lu J, Li X, Chen X, Tao D, Huang W, Huang B. Lifespan extension and elevated hsp gene expression in *Drosophila* caused by histone deacetylase inhibitors. *J Exp Biol*. 2005; 208:697–705. doi: 10.1242/jeb.01439
 30. Rogina B, Helfand SL. Sir2 mediates longevity in the fly through a pathway related to calorie restriction. *Proc Natl Acad Sci USA*. 2004; 101:15998–6003. doi: 10.1073/pnas.0404184101
 31. Barthel A, Schmoll D, Unterman TG. FoxO proteins in insulin action and metabolism. *Trends Endocrinol Metab*. 2005; 16:183–89. doi: 10.1016/j.tem.2005.03.010
 32. Martins R, Lithgow GJ, Link W. Long live FOXO: unraveling the role of FOXO proteins in aging and longevity. *Aging Cell*. 2016; 15:196–207. doi: 10.1111/accel.12427
 33. Jünger MA, Rintelen F, Stocker H, Wasserman JD, Végh M, Radimerski T, Greenberg ME, Hafen E. The *Drosophila* forkhead transcription factor FOXO mediates the reduction in cell number associated with reduced insulin signaling. *J Biol*. 2003; 2:20. doi: 10.1186/1475-4924-2-20
 34. Puig O, Tjian R. Transcriptional feedback control of insulin receptor by dFOXO/FOXO1. *Genes Dev*. 2005; 19:2435–46. doi: 10.1101/gad.1340505
 35. Shimazu T, Hirschey MD, Newman J, He W, Shirakawa K, Le Moan N, Grueter CA, Lim H, Saunders LR, Stevens RD, Newgard CB, Farese RV Jr, de Cabo R, et al. Suppression of oxidative stress by β -hydroxybutyrate, an endogenous histone deacetylase inhibitor. *Science*. 2013; 339:211–14. doi: 10.1126/science.1227166
 36. Ye J. Improving insulin sensitivity with HDAC inhibitor. *Diabetes*. 2013; 62:685–87. doi: 10.2337/db12-1354
 37. Walsh ME, Bhattacharya A, Sataranatarajan K, Qaisar R, Sloane L, Rahman MM, Kinter M, Van Remmen H. The histone deacetylase inhibitor butyrate improves metabolism and reduces muscle atrophy during aging. *Aging Cell*. 2015; 14:957–70. doi: 10.1111/accel.12387
 38. Böhni R, Riesgo-Escovar J, Oldham S, Brogiolo W, Stocker H, Andrus BF, Beckingham K, Hafen E. Autonomous control of cell and organ size by CHICO, a *Drosophila* homolog of vertebrate IRS1-4. *Cell*. 1999; 97:865–75. doi: 10.1016/S0092-8674(00)80799-0
 39. Alic N, Tullet JM, Niccoli T, Broughton S, Hoddinott MP, Slack C, Gems D, Partridge L. Cell-nonautonomous effects of dFOXO/DAF-16 in aging. *Cell Reports*. 2014; 6:608–16. doi: 10.1016/j.celrep.2014.01.015
 40. Zhao S, Xu W, Jiang W, Yu W, Lin Y, Zhang T, Yao J, Zhou L, Zeng Y, Li H, Li Y, Shi J, An W, et al. Regulation of cellular metabolism by protein lysine acetylation. *Science*. 2010; 327:1000–04. doi: 10.1126/science.1179689
 41. Peleg S, Feller C, Forne I, Schiller E, Sévin DC, Schauer T, Regnard C, Straub T, Prestel M, Klima C, Schmitt Nogueira M, Becker L, Klopstock T, et al. Life span extension by targeting a link between metabolism and histone acetylation in *Drosophila*. *EMBO Rep*. 2016; 17:455–69. doi: 10.15252/embr.201541132
 42. Gut P, Verdin E. The nexus of chromatin regulation and intermediary metabolism. *Nature*. 2013; 502:489–98. doi: 10.1038/nature12752
 43. Woods JK, Kowalski S, Rogina B. Determination of the spontaneous locomotor activity in *Drosophila melanogaster*. *J Vis Exp*. 2014; 86. 10.3791/51449
 44. Bross TG, Rogina B, Helfand SL. Behavioral, physical, and demographic changes in *Drosophila* populations through dietary restriction. *Aging Cell*. 2005; 4:309–17. doi: 10.1111/j.1474-9726.2005.00181.x
 45. Wang PY, Neretti N, Whitaker R, Hosier S, Chang C, Lu D, Rogina B, Helfand SL. Long-lived Indy and calorie restriction interact to extend life span. *Proc Natl Acad Sci USA*. 2009; 106:9262–67. doi: 10.1073/pnas.0904115106

46. Rogers RP, Rogina B. Increased mitochondrial biogenesis preserves intestinal stem cell homeostasis and contributes to longevity in Indy mutant flies. *Aging (Albany NY)*. 2014; 6:335–50. doi: 10.18632/aging.100658

SUPPLEMENTAL MATERIAL



Supplemental Figure 1. (A) Western blot staining for dFOXO in 40 day old *rpd3^{def24}/+*, control (+/+), *rpd3^{P-UTR}/CS* and *rpd3^{P-1.8}/CS* male flies. (B) Western blot staining for dFOXO in the cytoplasmic and nuclear fractions of 40 day old *rpd3^{def24}/+* and control (+/+) male flies. (C) Western blot staining for phosphorylated Serine 253 dFOXO in 40 day old *rpd3^{def24}/+*, control (+/+), *rpd3^{P-UTR}/CS*, and *rpd3^{P-1.8}/CS* (control) male flies.

Supplemental Table 1A. Starvation resistance of *rpd3^{def}/+* heterozygous flies and genetic controls.

Gender	Genotype	N	Age	Mean LS (% change)	Median LS	χ^2	p	Maximal life span (% change)
M	<i>rpd3^{def}/+</i>	39	10	32 (38)	30	54.82	<0.0001*	44 (40)
M	<i>Control</i>	31	10	20	20			26
F	<i>rpd3^{def}/+</i>	60	10	74 (28)	53	24.880	<0.0001*	92 (2)
F	<i>Controls</i>	75	10	54	72			90
M	<i>rpd3^{def}/+</i>	69	40	27 (44)	30	20.9381	<0.0001*	37 (11)
M	<i>Control</i>	17	40	18	17			34
F	<i>rpd3^{def}/+</i>	71	40	64 (108)	61	70.1045	<0.0001*	96 (54)
F	<i>Controls</i>	46	40	31	30			63

The mean, median, and maximal lifespan of *rpd3^{def}/+* and genetic control (+/+) heterozygous male (M) and female (F) flies after exposure to starvation at 10 or 40 days of age. Control values are compared to either male or female *rpd3^{def}/+* groups to determine the percent change in mean, median, and maximal lifespan. Mean, median, and maximal lifespan are in hours. Log-rank analyses were performed using the JMP 12 program. M = Males, F = Females, N = number of flies in the experiment.

Supplemental Table 1B. Starvation resistance of *rpd3*^{P-UTR} heterozygous flies and genetic controls *rpd3*^{P-1.8}/+ flies.

Gender	Genotype	N	Age	Mean LS (% change)	Median LS	X ²	p	Maximal life span (% change)
M	<i>rpd3</i> ^{P-UTR} /+	80	10	32 (9)	31	3.6315	0.0567	38
M	<i>rpd3</i> ^{P-1.8} /+	40	10	29	31			37
F	<i>rpd3</i> ^{P-UTR} /+	78	10	96 (40)	99	81.6546	<0.0001*	139 (36)
F	<i>rpd3</i> ^{P-1.8} /+	32	10	57	55			89

The mean, median, and maximal lifespan of *rpd3*^{P-UTR}/+ and their genetic control (*rpd3*^{P-1.8}/+) heterozygous male (M) and female (F) flies after exposure to starvation at 10 days of age. Control values are compared to either male or female *rpd3*^{P-UTR}/+ to determine the percent change in mean and maximal lifespan. Mean and median lifespan are in hours. N = number of flies in the experiment. Log-rank analyses were performed using the JMP 12 program.

Supplemental Table 2. *dfoxo* is required for longevity extension in *rpd3*^{def} mutant flies

Gender	Genotype	N	Mean (% change)	Median LS	X ²	p	Maximal life span (% change)
M	<i>rpd3</i> ^{def} /+	180	77.7	82			101.1
M	<i>rpd3</i> ^{def} / <i>dfoxo</i> ^{c01841}	225	65.6 (-16)	68	65.99	<0.0001*	91.5 (-9.5)
M	<i>dfoxo</i> ^{c0184} /+	123	50 (36)	51	176.40	<0.0001*	76.7 (-24)
F	<i>rpd3</i> ^{def} /+	200	68.6	70.5			93.7
F	<i>rpd3</i> ^{def} / <i>dfoxo</i> ^{c0184}	238	78.8 (-15)	82	27.868	<0.0001*	101.2 (8)
F	<i>dfoxo</i> ^{c0184} /+	132	67 (2)	72	2.474	0.1158	91.4 (3)

The mean, median, and maximal lifespans of *rpd3*^{def}/+, *rpd3*^{def}/*dfoxo*^{c01841}, and *dfoxo*^{x01841}/+ heterozygous male (M) and female (F) flies. *dfoxo*^{x01841} flies are hypomorphic for *dfoxo*. All values are compared to either male or female *rpd3*^{def}/+ groups to determine the percent change in median and maximal lifespan. Data are censored for 0-10 days. N: number of flies used in the experiment. Median and maximal lifespan are in days. Log-rank analyses were performed using the JMP 12 program.

Supplemental Table 3. Heterozygous flies with reduced *rpd3* mRNA levels have increased resistance to H₂O₂ compared to the genetic control flies at 40 days.

Gender	Genotype	N	Age	Mean (% change)	Median LS	χ^2	p	Maximal life span (% change)
M	<i>rpd3^{def}/+</i>	65	10	34 (-2.5)	34	0.4934	0.4824	43 (-8.5)
M	<i>Control</i>	30	10	35	34			49
F	<i>rpd3^{def}/+</i>	73	10	55 (4.9)	55	1.3942	0.2377	80 (2.7)
F	<i>Controls</i>	79	10	52	55			78
M	<i>rpd3^{def}/+</i>	53	40	25 (155.8)	24	31.3716	0.0001*	31 (17.8)
M	<i>Control</i>	31	40	15	12			26
F	<i>rpd3^{def}/+</i>	55	40	39 (39.5)	36	5.7003	0.0170*	56 (-7.9)
F	<i>Controls</i>	39	40	28	31			60
M	<i>rpd3^{P-UTR}/+</i>	58	10	39 (-3)	38	1.4847	0.2230	48 (-60)
M	<i>rpd3^{P-1.8}/+</i>	56	10	40	40			51
F	<i>rpd3^{P-UTR}/+</i>	52	10	97 (30)	92	55.2690	<0.0001*	116 (20)
F	<i>rpd3^{P-1.8}/+</i>	57	10	68	64			94

The mean, median, and maximal lifespan of *rpd3^{def}/+* and genetic control (+/+), *rpd3^{P-UTR}/+*, and genetic control (*rpd3^{P-1.8}/+*) heterozygous male (M) and female (F) flies after exposure to H₂O₂ at 10 or 40 days of age. Control values are compared to either male or female *rpd3^{def}/+* groups to determine the percent change in mean and maximal lifespan. Mean and median lifespan are in hours. N = number of flies in the experiment. Log-rank analyses were performed using the JMP 12 program.

Supplemental Table 4. *dfoxo* is required for increased starvation resistance in *rpd3^{def}* heterozygous flies at 40 days.

Gender	Genotype	N	Mean (% change)	Median LS	χ^2	p
M	<i>rpd3^{def}/yw</i>	102	29	29		
M	<i>dfoxo^{c01841}/yw</i>	101	20 (-31)	20	82.3659	<0.0001
M	<i>rpd3^{def}/yw</i>	102	29	29		
M	<i>rpd3^{def}/dfoxo^{c01841}</i>	60	24 (-17)	23	16.1367	<0.0001
F	<i>rpd3^{def}/yw</i>	83	55	47		
F	<i>dfoxo^{c01841}/yw</i>	94	53 (-4)	51	1.7	0.18
F	<i>rpd3^{def}/yw</i>	83	55	47		
F	<i>rpd3^{def}/dfoxo^{c01841}</i>	90	44 (20)	45	16.3721	<0.0001*

The mean and median lifespans of *rpd3^{def}/+*, *rpd3^{def}/dfoxo^{c01841}*, and *dfoxo^{c01841}/+* heterozygous male (M) and female (F) flies. *dfoxo^{c01841}* flies are hypomorphic for *dfoxo*. All values are compared to either male or female *rpd3^{def}/+* groups to determine the percent change in mean and median lifespan. Mean and median lifespan are in hours. The similarity in stress resistance between *rpd3^{def}/dfoxo^{c01841}* and *dfoxo^{c01841}/+* male and female flies was also determined. N: number of flies used in the experiment. Three independent experiments with similar results were combined. Mean and Median lifespans are in hours. Log-rank analyses were performed using the JMP 12 program.

Supplemental Table 5. *dfoxo* is required for increased H₂O₂ resistance in *rpd3^{def}* heterozygous flies.

Gender	Genotype	N	Mean (% change)	Median LS	χ^2	p	Maximal life span (% change)
M	<i>rpd3^{def}/+</i>	166	30	32			42
M	<i>rpd3^{def}/dfoxo^{c01841}</i>	82	17 (43)	18	139.6062	<0.0001*	24 (42)
M	<i>dfoxo^{c01841}/+</i>	88	21 (30)	23	82.7128	<0.0001*	32 (22)
F	<i>rpd3^{def}/+</i>	166	41	40			57
F	<i>rpd3^{def}/dfoxo^{c01841}</i>	128	48 (-17)	47	26.7491	<0.0001*	71 (-20)
F	<i>dfoxo^{c01841}/+</i>	80	30 (27)	32	89.8302	<0.0001*	43 (25)

The mean and median lifespans of *rpd3^{def}/+*, *rpd3^{def}/dfoxo^{c01841}*, and *dfoxo^{c01841}/+* heterozygous male (M) and female (F) flies after exposure to H₂O₂ at 40 days of age. *Dfoxo^{c01841}* flies are hypomorphic for *dfoxo*. All values are compared to either male or female *rpd3^{def}/+* groups to determine the percent change in mean or median lifespan. N: number of flies used in the experiment. Three independent experiments with similar results were combined. Mean and median lifespan are in hours. Log-rank analyses were performed using the JMP 12 program.