

Research Article

Overexpression of Fatty-Acid- β -Oxidation-Related Genes Extends the Lifespan of *Drosophila melanogaster*

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A better understanding of the aging process is necessary to ensure that the healthcare needs of an aging population are met. With the trend toward increased human life expectancies, identification of candidate genes affecting the regulation of lifespan and its relationship to environmental factors is essential. Through misexpression screening of EP mutant lines, we previously isolated several genes extending lifespan when ubiquitously overexpressed, including the two genes encoding the fatty-acid-binding protein and dodecenoyl-CoA delta-isomerase involved in fatty-acid β -oxidation, which is the main energy resource pathway in eukaryotic cells. In this study, we analyzed flies overexpressing the two main components of fatty-acid β -oxidation, and found that overexpression of fatty-acid- β -oxidation-related genes extended the *Drosophila* lifespan. Furthermore, we found that the ability of dietary restriction to extend lifespan was reduced by the overexpression of fatty-acid- β -oxidation-related genes. Moreover, the overexpression of fatty-acid- β -oxidation-related genes enhanced stress tolerance to oxidative and starvation stresses and activated the dFOXO signal, indicating translocation to the nucleus and transcriptional activation of the dFOXO target genes. Overall, the results of this study suggest that overexpression of fatty-acid- β -oxidation-related genes extends lifespan in a dietary-restriction-related manner, and that the mechanism of this process may be related to FOXO activation.

1. Introduction

The trend towards increased life expectancy demands a greater understanding of the aging process to ensure that healthcare needs of an aging population are met. This goal requires identification of the so-called “longevity candidate genes,” which are potential genes important to the regulation of lifespan, as well as appropriate understanding of how the effects of these genes are modulated by environmental factors such as diet. Numerous longevity candidate genes have been identified in model systems using extended longevity mutant phenotypes, offering important insights into the mechanisms of aging and lifespan determination [1–5]. Insulin/insulin-like growth factor (IGF) signaling (IIS), a major nutrient-sensing pathway, is a well-characterized age-related pathway. The loss of IIS function by mutations affecting insulin/IGF receptor, phosphatidylinositol-3 kinase (PI3K), Akt, and forkhead box (FOXO) has been found to

extend the lifespan of *C. elegans*, *Drosophila*, and mammals [3, 6–11]. In addition, energy-sensing pathways such as those associated with sirtuins, target of rapamycin (TOR) and AMP-activated protein kinase (AMPK) signaling are well known to be linked to the aging process [3–5, 12]. As nutrient-sensing pathways are linked to aging, the reduction of dietary intake, namely dietary restriction, also extends the lifespan of various model systems [13–16]. Furthermore, the ecdysteroid hormone pathway is known to modulate organismal lifespan [17, 18].

While investigating longevity candidate genes, we previously conducted misexpression screening of EP lines containing 14 copies of upstream activator sequence (UAS) to which Gal4 binds, allowing conditional overexpression or knockdown of genes of flanking genomic DNA located downstream of the basal promoter dependent on its insertion orientation [19]. In that study, we preliminarily selected 40 EP lines to demonstrate the lifespan extension, including

the two EP lines (EP^{CG6783}, EP^{CG13890}) targeting fatty-acid- β -oxidation-related genes (*CG6783*, *CG13890*), but they were excluded from further investigation since they were induced in the absence of Gal4 driver [19]. It has long been suggested that lipid metabolism plays a central role in regulation of the metazoan lifespan. One of the well-known longevity-candidate genes, AMPK, was reported to regulate fatty-acid synthesis and oxidation through the phosphorylation of acetyl-CoA-carboxylase [20]. In addition, calorie restriction and IIS mutation has been reported to promote fatty-acid β -oxidation [21, 22]. However, there has been no direct evidence of lifespan extension through the modulation of fatty-acid β -oxidation to date, except for our previous study [19], in which we did not investigate the relationship with dietary restriction and its underlying mechanisms.

In the current study, we analyzed EP lines that overexpressed two main components of fatty-acid β -oxidation and found that the overexpression of fatty-acid β -oxidation related genes extended their lifespan in a dietary-restriction-related manner, increased their stress resistance, and activated the FOXO transcription factor.

2. Materials and Methods

2.1. Fly Stocks and Food Preparation. *Drosophila melanogaster* were cultured and reared at 25°C. Cantonized *white* (*CS10* [23]) was used as wild-type control. The EP^{CG6783} (GX62810) and EP^{CG13890} (GX4385) lines, which carry the P-element mediated upstream activator sequence (UAS) on the 5' untranslated region of the *CG6783* or *CG13890* genes, respectively, were obtained from GenExel Inc. (KAIST Bio Medical Research Center, Korea). To generate *UAS-CG6783* flies, the full open reading frame of *fabp*-RA from RH46282 (*Drosophila* Genomics Resource Center, Bloomington, USA) was cloned into pUAST using *EcoRI/BglII* sites. Standard germline transformation into a *w*¹¹¹⁸ background was then performed for transgenic lines. Corn meal-sugar-yeast (CSY) media (5.2% cornmeal, 11% sucrose, 2.4% yeast, 0.8% agar, and 0.2% methyl-4-hydroxybenzoate (Sigma-Aldrich, St. Louis, MO, USA)) was used for larval development and routine culture. In the dietary restriction (DR) experiment, the concentration of yeast in the media fed to separate groups of flies was 2, 4, 8, 12, and 16%.

2.2. Lifespan Assays. Newly eclosed F1 generations were collected over 48 hours and the males were randomly assigned to 500 mL demography cages to achieve a final density of 100 male flies per cage. Food vials containing SY diet (10% sucrose, the indicated concentration of yeast, 0.2% methyl-4-hydroxybenzoate, and 0.8% agar) were affixed to separate cages and changed every two days, at which time the dead flies were removed and recorded. Three replicate cages were established for this experiment.

2.3. Stress-Resistance Assay

Oxidative Stress. Ten-day-old flies (20 males per vial) were fed SY medium supplemented with 18 mM paraquat (methyl

viologen dichloride hydrate, Sigma-Aldrich). The flies were transferred into fresh vials containing paraquat solution every six hours, and the dead flies were scored after each transfer. Fifteen replicates were established.

Starvation Stress. Newly eclosed flies were kept in vials (20 males per vial) containing 1% agar and transferred into fresh vials containing agar every six hours. Dead flies were scored after each transfer. Fifteen replicates were established.

2.4. Immunostaining of the Larval Fat Bodies. Dissected third instar larvae were fixed with 4% paraformaldehyde (USB Corp., Cleveland, OH, USA) for 30 min, washed with phosphate-buffered saline/0.1% Triton X-100/2% bovine serum albumin (PBST-BSA), and then incubated overnight with primary antibodies in PBST-BSA at 4°C. Samples were then washed in PBST-BSA, incubated with Alexa488 (Molecular Probes, Eugene, OR, USA) for 1 hour at 25°C, and washed and mounted with Vectashield (Vector Labs, CA, USA). The resulting images were analyzed using a Confocal Laser Scanning Microscope (LSM510 META, Carl Zeiss Inc., Germany). Anti-dFOXO antibody (a gift from O. Puig) was diluted to 1:300 in 2% BSA solution. DAPI was used to counterstain the nuclei.

2.5. Real-Time qPCR. Five-day-old adults were frozen in liquid nitrogen and stored at -80°C until analysis. Total RNA from homogenized whole-body lysates was prepared with RNAiso reagent (TAKARA, Japan). Next, total RNA (5 μ g) was reverse-transcribed using PrimeScript RT Reagent Kit (TAKARA) and real-time qPCR was performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, USA) using SYBR Premix Ex-Taq II (TAKARA). Mean induction folds were calculated from the values of 3–6 independent experiments and statistically evaluated by a Student *t*-test.

2.6. Statistical Analysis. Data are presented as the mean \pm SEM. Statistical analyses for the demographic data were carried out using standard survival models in the JMP statistical package (SAS, Cary, NC, USA).

3. Results

3.1. Overexpression of Fatty-Acid β -Oxidation Components Extended Lifespan in a Dietary-Restriction-Dependent Manner. In the previous study, we selected long-lived EP lines, which extend lifespan when crossed to *da-Gal4* driver [19]. Among them, the two EP lines (GX62810, GX4385) targeting fatty-acid- β -oxidation-related genes (*CG6783*, *CG13890*) were of interest, however, they were excluded to further investigation in the previous study because they were induced in the absence of Gal4 driver [19]. *CG6783* encodes the fatty-acid-binding protein (FABP), which facilitates the intracellular movement of fatty acids, thus permitting the initiation of fatty-acid oxidation [24], while *CG13890* encodes the dodecenoyl-CoA delta-isomerase (DCI) localized in the inner mitochondria where it catalyzes the

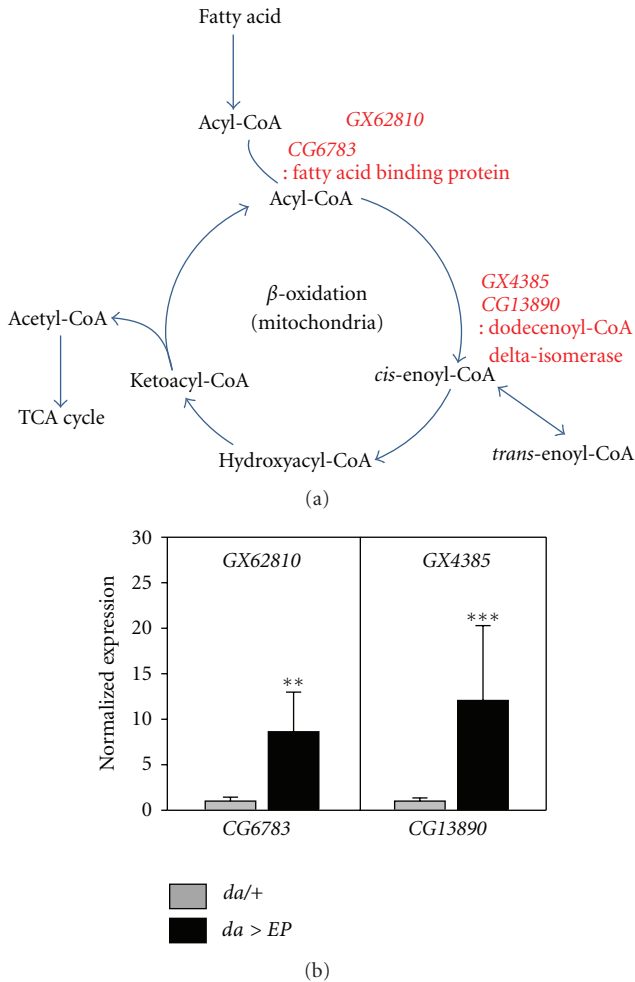


FIGURE 1: Overexpression of fatty-acid β -oxidation-related genes using EP lines. (a) Schematic representation of fatty-acid β -oxidation. *CG6783* encodes the fatty-acid-binding protein, which mediates the transportation of acyl-CoA to cellular organelles. *CG13890* encodes dodecenoyl-CoA delta-isomerase, which modifies *cis*-enoyl-CoA to *trans*-enoyl-CoA, a common substrate for enoyl-CoA hydratase in the β -oxidation cycle of saturated fatty acyl-CoA esters. (b) Overexpression of *CG6783* or *CG13890* using EP lines (*GX62810*, *GX4385*) and *da-Gal4*. The mRNA of *CG6783* or *CG13890* was analyzed in the whole body RNA extract from *da > EP* (black bars) and *da/+* (gray bars) flies. Significance was determined via a *t*-test (** $P < 0.001$, *** $P < 0.0001$).

degradation of long-chain fatty acids during fatty-acid β -oxidation [25, 26] (Figure 1(a)). We confirmed that the EP lines overexpressed *CG6783* or *CG13890* when crossed with the *da-Gal4* driver compared to *da/+* flies via real-time qPCR (Figure 1(b)).

To further assess the EP lines, they were crossed with *da-Gal4* driver or wild-type control stock to produce the *da/EP* and two controls (*EP/+* and *da/+*) after backcrossing eight times to rule out heterosis. Consistent with previous reports [19], the overexpression of these two fatty-acid β -oxidation-related genes using the *da-Gal4* driver increased lifespan. In media containing 16% yeast, the median lifespan

of *CG6783*- or *CG13890*-overexpressing flies was extended to nearly 58 or 42 days, respectively, from the 32 days observed for the *da/+* control flies (Figure 2(a)). In addition, the EP line overexpressing *CG6783* also showed extended maximum lifespan (closed-square line in Figure 2(a)), and the two EP lines consistently reduced mortality rate across adult ages (Figure 2(b)). However, it should be noted that the *EP/+* control cohorts without the *Gal4* driver showed longer lifespans than the parental cohorts (open-square line and open-triangle line, Figure 2(a)), which could be considered to be the side effect of EP insertion to express the target genes under control of the basal promoter possessed in the EP.

To further confirm the extension of lifespan by the β -oxidation-related gene, we used the *UAS-CG6783* transgenic line, which was generated by standard germ line transformation using a *pUAS-CG6783* construct, and analyzed the lifespan when the transgene was driven in adults with the conditional Gene Switch (GS) driver system [27] to produce cohorts of identical genetic background. Female offspring of the *Act-GS-Gal4 > UAS-CG6783* genotype showed increased median lifespan compared to uninduced control (Figure 2(c)), indicating that the extension of lifespan by the β -oxidation-related gene occurs independently from the insertion site and the genetic background.

To investigate whether or not the mechanistic basis of dietary restriction has an effect on fatty-acid β -oxidation in association with lifespan extension, flies overexpressing fatty-acid oxidation components were fed an SY diet ranging from 2-to-16% yeast. The median lifespan of the control cohorts increased with decreasing yeast concentration [13, 28]. While control flies showed a 31.4% increase in lifespan upon 2% SY compared to 16% SY conditions (circular lines, Figures 2(d) and 2(e)), the flies expressing *CG6783* or *CG13890* showed a reduction in the lifespan extension with dietary restriction (12% or 15%, respectively, Figures 2(d) and 2(e)). These results indicated that the promotion of fatty-acid β -oxidation extends lifespan via a mechanism similar to dietary restriction.

3.2. Overexpression of Fatty-Acid β -Oxidation Components Increased Resistance to Oxidative and/or Starvation Stress. A positive relationship between stress tolerance and longevity has been well defined [29, 30], and long-lived organisms tend to be resistant to various forms of environmental stress [31]. Thus, we investigated the effects of overexpressed fatty-acid β -oxidation-related genes on stress resistance. To induce oxidative stress, flies overexpressing fatty-acid- β -oxidation components were subjected to feed dosed with 18 mM paraquat. We found that both of the flies overexpressing each fatty-acid- β -oxidation component showed substantial resistance to oxidative stress (Figures 3(a) and 3(b)). In addition, when subjected to nutrient deprivation, flies overexpressing *CG13890* showed more resistance to starvation than the control (Figure 3(d)), whereas the survival rate of the flies overexpressing *CG6783* was not significantly altered by starvation (Figure 3(c)). These results indicated that overexpression of fatty-acid β -oxidation-related genes increases lifespan and stress tolerance.

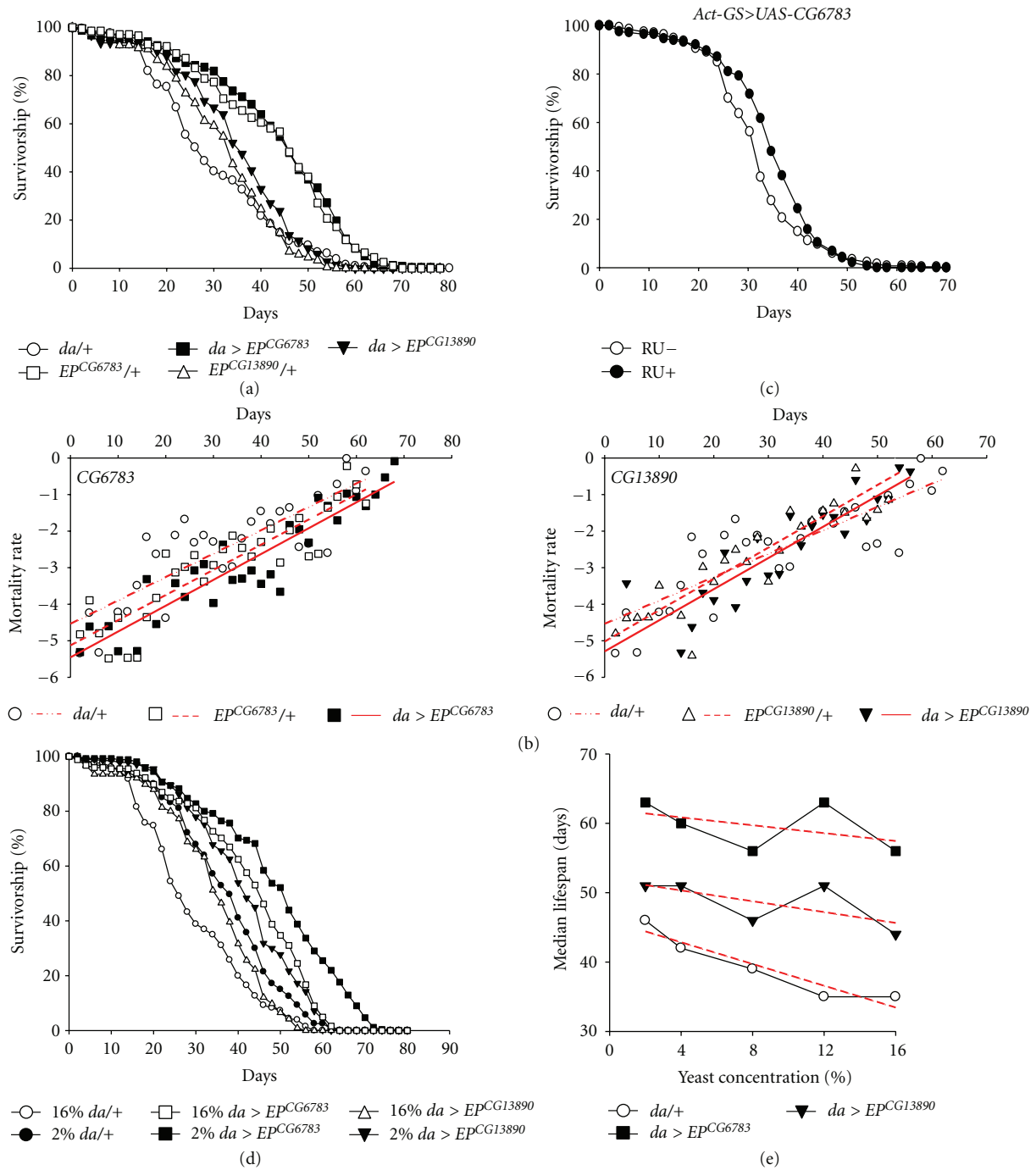


FIGURE 2: Overexpression of *CG6783* or *CG13890* throughout the whole body extends lifespan, which is associated with diet restriction. (a) Flies exhibiting overexpression of *EP^{CG6783}* (closed-square line) and *EP^{CG13890}* (closed-triangle line) fed 16% SY media display increased median and/or maximum lifespan when compared to the *da-Gal4* driver alone (open-circular line). Significance was determined via a log-rank test (*CG6783*, $P < 0.001$ to *da/+*, $P = 0.6$ to *EP^{CG6783}/+*; *CG13890*, $P < 0.1$ to *da/+*, $P < 0.05$ to *EP^{CG13890}/+*). (b) Flies overexpressing *CG6783* using *UAS-CG6783* and *Act-GS-Gal4* showed increased median lifespan in response to feeding with RU486-containing food from day 3 of adulthood (RU+, closed-symbol line) when compared to the uninduced control (RU-, open-symbol line). Significance was determined via a log-rank test ($P < 0.0001$). (c) Mortality curves of the flies that overexpressed *CG6783* or *CG13890*. The natural log of the mortality rate was plotted using the Gompertz mortality model. Red lines indicate linear regressions for each category. (d) Overexpression of *CG6783* or *CG13890* throughout the whole body reduced the lifespan extension with dietary restriction. Survival curves of the flies that overexpressed *CG6783* (square line) or *CG13890* (triangular line) fed 2% or 16% SY media. (e) Dietary restriction in adult *Drosophila* when *CG6783* or *CG13890* are overexpressed throughout the whole body. The median lifespan was calculated from Kaplan-Meier survival analysis of the EP lines fed a range of yeast concentrations. Red dashed lines indicate linear regressions for each line.

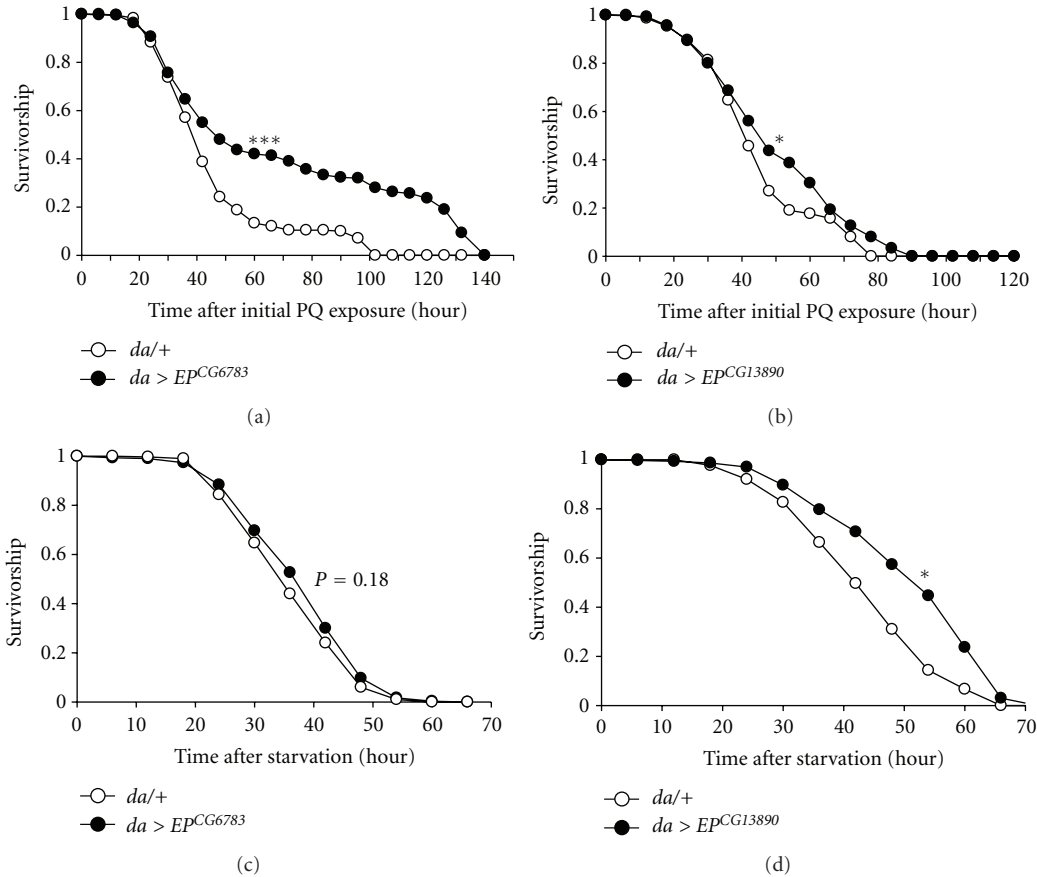


FIGURE 3: Overexpression of *CG6783* or *CG13890* increases resistance to stresses. Flies expressing *CG6783* or *CG13890* exhibited elevated resistance to oxidative stress (a, b) and starvation (c, d) when compared to the control (*da/+*, open-circle lines). Significance was determined via a log-rank test (* $P < 0.01$, *** $P < 0.0001$).

3.3. Overexpression of Fatty-Acid- β -Oxidation Components Activated the dFOXO Signal. Forkhead box (FOXO) is a key mediator of the aging-related pathway that is regulated by signaling pathways including IIS/PI3K/Akt, JNK, AMPK, MST1, CBP, and Sirt1 [12, 32–36]. In addition, dFOXO activation in *Drosophila* fat body is reportedly associated with physiological traits such as aging, stress resistance, and lipid metabolism [15, 37, 38]. To determine whether lifespan extension and stress resistance produced by the overexpression of fatty-acid- β -oxidation components were associated with dFOXO activation, we immunostained larval fat bodies with anti-dFOXO antibody. In the control fat bodies, an endogenous dFOXO signal was detected in the cytoplasm of all cells and the nuclei of some of the cells (Figure 4(a)). However, the dFOXO signal increased in the nuclei of the fat body as a result of overexpression of the fatty-acid- β -oxidation components (Figure 4(a)).

To further assess whether the fatty-acid β -oxidation-related genes activate dFOXO in adults, we analyzed the expression level of the dFOXO transcriptional target gene *l(2)efl* and *4E-BP* in adult whole bodies. The mRNA level of *l(2)efl* and *4E-BP* in the adult whole bodies increased in response to overexpression of the fatty-acid β -oxidation component (Figure 4(b)). These results indicated

that increased fatty-acid β -oxidation leads to the activation of FOXO signaling, suggesting that fatty-acid- β -oxidation-induced lifespan extension is linked to FOXO activation.

4. Discussion

In this study, we demonstrated that the overexpression of fatty-acid- β -oxidation-related genes extended median and maximum lifespan and increased stress resistance, suggesting that the level of fatty-acid β -oxidation regulates lifespan. Consistent with our results, many investigations have suggested fatty-acid β -oxidation as a lifespan determinant. One of the well-known longevity-candidate genes, AMPK reportedly regulates fatty-acid synthesis and oxidation [20]. Moreover, calorie restriction and IIS have been reported to promote fatty-acid β -oxidation [21, 22]. In addition, *enigma* mutant, which exhibits oxidative stress resistance and a longevity phenotype, was found to encode a fatty-acid- β -oxidation related enzyme [39]. A mutant of *Withered*, which contains the carnitine palmitoyltransferase activity used to import long-chain fatty acids into the mitochondria, was found to be hypersensitive to oxidative and starvation stresses [40]. Furthermore, the mutant fly for *mitochondria trifunctional protein* containing three kinds of

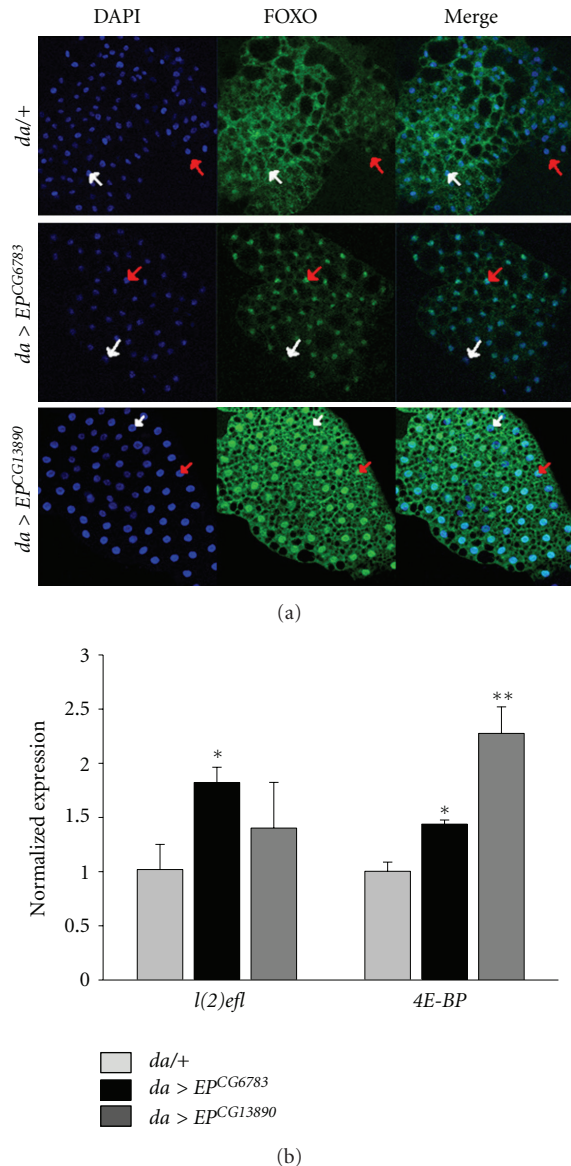


FIGURE 4: Overexpression of *CG6783* or *CG13890* activates dFOXO. (a) Overexpression of *CG6783* or *CG13890* induced the translocation of dFOXO to the nucleus. The fat bodies of the third instar larvae expressing *CG6783* or *CG13890* under *da-Gal4* were stained with anti-dFOXO (green) and DAPI (blue). White arrows indicate dFOXO-negative cells and red arrows indicate dFOXO-positive cells. Original magnification is 200x. (b) Overexpression of *CG6783* or *CG13890* increased the expression of dFOXO target genes. The mRNA of *l(2)efl* and *4E-BP* was analyzed in the whole body RNA extract from *da* > *EP* (black or dark gray bars) and *da*+/+ (gray bars) flies. Significance was determined via a *t*-test (* $P < 0.01$, ** $P < 0.001$).

enzyme activities associated with fatty-acid β -oxidation, was recently reported to have a shortened lifespan and decreased locomotion and fecundity [41]. However, the present study is the first to provide direct evidence that the modulation of fatty-acid- β -oxidation components extends lifespan.

Our data showed that lifespan extension by dietary restriction decreased with the overexpression of fatty-acid β -oxidation-related genes, indicating that lifespan extension by fatty-acid- β -oxidation components is associated with dietary restriction. It was previously reported that calorie restriction increased whole-body-fat oxidation [21]. Energy deprivation subsequent to calorie restriction activates AMPK, which subsequently enables the increase of fatty-acid oxidation necessary to utilize the energy resource. These findings suggested that fatty acid oxidation and dietary restriction are related by same underlying mechanisms. However, it should be noted that flies expressing fatty-acid- β -oxidation-related genes still responded to dietary restriction, especially in the lowest-yeast-feeding group. This result suggests that the flies have gained longevity through changes in the fatty-acid- β -oxidation-related genes and also other mechanism(s) unrelated to fatty-acid- β -oxidation in the dietary restriction condition.

Overexpression of the two fatty-acid- β -oxidation components showed similar effects, such as the extension of lifespan, mortality, stress resistance and dFOXO activation, throughout current study. However, flies overexpressing FABP (*CG6783*) were more resistant to oxidative stress, while DCI (*CG13890*) expressing flies were more resistant to starvation when compared to each other. FABP facilitates the intracellular movement of fatty acids, thus permitting the translocation of fatty acids to the mitochondria for fatty-acid oxidation and to the nucleus for activation of transcription of the FABP target gene via the fatty-acid nuclear receptors [42]. Therefore, the different effects of the two components on stress resistance may be caused by distinct functions of FABP and DCI.

In this study, we showed that the enhancement of fatty-acid oxidation components activates FOXO transcription factor, suggesting that fatty-acid- β -oxidation-induced lifespan extension is associated with FOXO activation. Fasting DCI homozygous mutants have been found to deposit large amounts of triglycerides in their hepatocytes and accumulated unsaturated fatty acyl groups in their ester lipids [25]. Surprisingly, our data showed that overexpression of FABP and DCI genes led to a mild increase in triglycerides levels (data not shown). This finding was likely the result of activation of FOXO by FABP or DCI overexpression, as it was recently reported that constitutively nuclear FOXO1 in mouse liver produces increased triglyceride accumulation [43]. As a mediator of aging-related signaling pathways, dFOXO is known to be regulated by several factors, including AMPK, JNK, MST1, Sir2, and IIS [12, 32–36]. Thus, further investigations are needed to determine whether the FOXO activation is required for longevity and stress resistance in flies overexpressing fatty acid oxidation-related genes and which signaling pathways are associated with fatty-acid- β -oxidation-related FOXO activation.

Authors' Contribution

S.-H. Lee and S.-K. Lee are contributed equally to the paper.

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