Am J Cancer Res 2014;4(4):325-336
www.ajcr.us /ISSN:2156-6976/ajcr0000617

Original Article

Role of SCOX in determination of *Drosophila melanogaster* lifespan

Thanh Binh Nguyen[1,2], Hiroyuki Ida[1], Mai Shimamura[1], Daishi Kitazawa[1], Shinichi Akao[1], Hideki Yoshida[2,3], Yoshihiro H Inoue[3], Masamitsu Yamaguchi[2,3]

[1]Kankyo Eisei Yakuhin Co. Ltd, Kansai Gakken Laboratory, Hikaridai, Seika-cho, Souraku-gun, Kyoto 619-0237, Japan; [2]Department of Applied Biology, [3]Insect Biomedical Research Center, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606-8585, Japan

Received April 25, 2014; Accepted June 1, 2014; Epub July 16, 2014; Published July 30, 2014

Abstract: In man, COX (cytochrome c oxidase) deficiency is reported to be related to mutation of the *SCO2* (synthesis of cytochrome c oxidase 2) gene, which encodes one of the copper-donor chaperones involved in the assembly of mitochondrial cytochrome c oxidase. Such COX deficiency due to the genetic condition leads to heart disease and the Leigh syndrome and is frequently fatal in childhood. Synthesis of cytochrome c oxidase X (SCOX) is a *Drosophila* orthologue of human SCO2. Here, we generated *SCOX*-knockdown flies and the full length SCOX transgenic flies to investigate the *in vivo* roles of SCOX. Our results demonstrated knockdown of *SCOX* gene in all cells and tissues to be associated with lethality at larval or pupal stages and this correlated with a decrease in ATP level. In contrast, the full length SCOX transgenic flies showed a longer lifespan than wild type flies and control flies carrying *Act5C-GAL4* alone and this correlated with an increase in ATP level. Finally, when cultured on paraquat-added medium, full length SCOX transgenic flies also exhibited an elongated lifespan. Therefore, we hypothesized that SCOX plays an important role in ATP production and consumption, which helps to prevent production of mitochondrial reactive oxygen species and/or impairment of mitochondrial activity under oxidative stress.

Keywords: SCOX, SCO2, COX, ATP, ROS, lifespan

Introduction

Over the last few decades, a variety of disorders including cancers and numerous neurodegenerative diseases (e.g. Alzheimer's and Parkinson's), have been proved to be caused by defects in mitochondrial functions [1-4]. Mitochondria are well known to play a central role in the regulation of cellular function, metabolism, and cell death in cancer cells. Several functional changes in cancer-cell mitochondria have been observed, such as increased production of mitochondrial reactive oxygen species (mtROS), decreased oxidative phosphorylation, and a corresponding increase in glycolysis [5]. In addition, a high level of mtROS inhibits and damages mitochondrial proteins and lipids in several ways, which causes lower mitochondrial functions contributing to aging and disease.

In normal cells, it is recognized that the presence of oxygen results in the activation of an oxygen-dependent pathway of oxidative phosphorylation (OXPHOS) and inhibition of glycolysis, but most cancer cells show a higher rate of glycolysis even with only adequate oxygen levels. A high rate of aerobic glycolysis, or the Walburg effect, connects the high rate of glucose fermentation to cancer, and maintains relations with the p53-SCO2-respiration axis. Mutation of mitochondrial inner membrane gene- *synthesis of cytochrome c oxidase 2* (*SCO2*), one of two human SCO-encoding genes, is reported to result in severe cytochrome c oxidase (COX) deficiency [6]. In man, COX is a multimeric protein complex which is the last enzyme in the respiratory electron transport chain of mitochondria. COX deficiency encompasses a wide and heterogeneous spectrum of mostly severe multisystemic disorders, which primarily affect organs with high energy demand, such as the brain, skeletal muscle, heart and kidney [7].

Mutations in *SCO2* are reported to be associated with hypertrophic cardiomyopathy and encephalopathy that presents soon after birth

[8]. Affected infants have respiratory difficulties and metabolic acidosis, and die within the first year of life. In patients with *SCO2* mutations, neuropathological defects are various, including heterotopia, gliosis, early capillary proliferation, and atrophy [2, 7, 9, 10]. However, no child with *SCO2* mutations has neuropathological defects consistent with the Leigh syndrome, possibly because of their death before manifesting such features [10-12]. Moreover, on survival analysis, high SCO2-expressing breast-cancer patients show a significantly better prognosis than low SCO2-expressing counterparts [13].

To gain further insight into the *in vivo* roles and genetic functions of *SCO*, we used *Drosophila melanogaster* as a model system. In *Drosophila melanogaster*, a single SCOX-encoding gene (*CG8885*, *scox*) is present. Null mutations of the *scox* gene are reported to be associated with larval lethality while mutations in its 5'UTR are reported to be associated with motor dysfunction and female sterile phenotypes [14]. Here, *SCOX*-knockdown flies carrying *UAS-SCOXIR* and the full length SCOX transgenic flies carrying *UAS-SCOX* were established. Lifespan, morphology and mobility were examined in each transgenic fly strain, for comparison with wild type and control flies. Lethality at pharate adult and larval stages was observed with *SCOX*-knockdown flies, and appeared to correlate with decrease in ATP level. In contrast, longer lifespan and better mobility of full length SCOX transgenic flies could be demonstrated to be associated with increase in ATP.

Materials and methods

Fly stocks

Fly stocks were maintained at 25°C on standard food containing 0.7% agar, 5% glucose and 7% dry yeast. Canton S was used as the wild type strain. In this study, the full length SCOX transgenic fly strain (*Act5C*-GAL4>UAS-*SCOX*) carrying genotype of *w; UAS-SCOX/+; Act5C-GAL4/+* and the *SCOX*-knockdown fly strain (*Act5C*-GAL4>UAS-*SCOXIR*^{20}) carrying the genotype of *w; UAS-SCOXIR*^{20}*/+; Act5C-GAL4/+* were designed in our laboratory. The RNAi transgene on the second chromosome of *Act5C*-GAL4>UAS-*SCOXIR*^{20} fly strain was targeted to the region corresponding to residues 600-964 of *Drosophila* SCOX. The *SCOX*-knockdown fly strain (*Act5C*-GAL4>UAS-*SCOXIR*^{7861}) carrying *w; +; Act5C-GAL4/UAS-SCOXIR*^{7861} (CG8885) was obtained from the Vienna *Drosophila* RNAi center (VDRC). The RNAi transgene on the third chromosome of *Act5C*-GAL4>UAS-*SCOXIR*^{7861} fly strain was targeted to the region corresponding to amino acid residues 584-854 of *Drosophila* SCOX. The Act5C-GAL4 strain was obtained from the Bloomington *Drosophila* stock center.

Oligonucleotides

CG8885XbaI→5'-GCCTCTAGAGTACAACGAAACAACATTTG-3'

CG8885BamHI→5'-GGTCAACGGTGATCCCGATGGATCCTG-3'

CG8885BglII→5'-TTCAGATCTTCCCGCTCCCTGCAACGCTT-3'

CG8885KpnI→5'-TTCGGTCCCTAGCTGAACCATCCCTTTT-3'

Establishment of transgenic flies

PCR was carried out to construct the plasmid pUAS-SCOXIR$_{600-964}$ and pUAS-SCOX with *Drosophila* cDNA as a template.

Construction of pUAS-SCOXIR$_{600-964}$: To establish the transgenic fly line carrying *UAS-SCOXIR*, a 365-bp fragment of *SCOX* was amplified by PCR using primers CG8885BamHI and CG8885XbaI, followed by subcloning into the pT7Blue-2vector (Novagen). The obtained DNA fragments were digested with *Xba*I *a*nd *Bam*HI and then inserted into the *Bgl*II and *Avr*II sites of pWIZ to create pWIZ-5'-CG8885. Then the 365-bp DNA fragment amplified from pT7-SCOX$_{600-964}$CG8885 was also digested by *Xba*II and inserted into the *Nhe*I and *Xba*I sites of pWIZ-5'-CG8885 to create pUAS-SCOXIR$_{600-964}$.

Construction of pUAS-SCOX containing full length *SCOX* cDNA: A 700-bp DNA fragment was amplified by PCR using the CG8885 cDNA as a template and primers CG8885BglII and CG8885KpnI. PCR products were digested with *Bgl*II and *Kpn*I and inserted between the *Bgl*II and *Kpn*I sites of the pUAS-Flag vector.

These plasmids were verified by sequencing and then injected into embryos to obtain stable transformant lines carring *UAS-SCOXIR* and

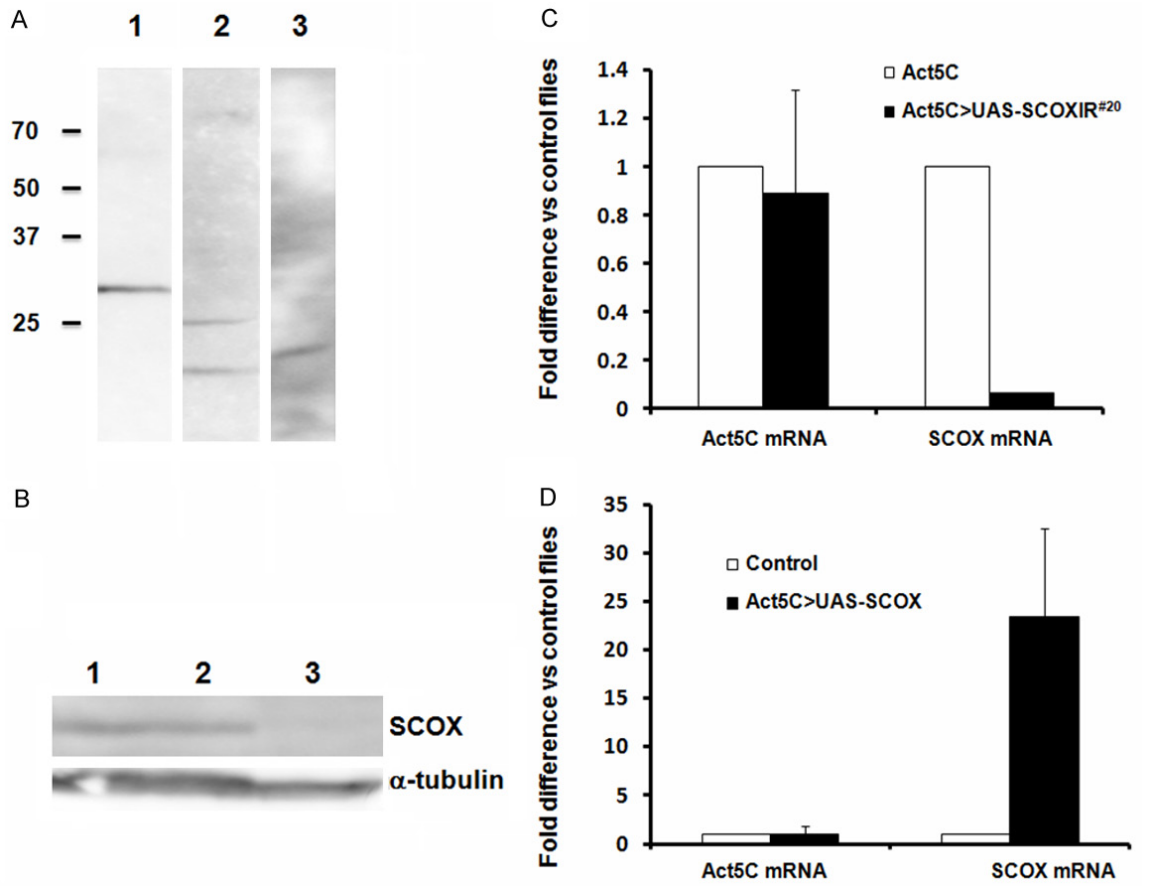

Figure 1. A. Western immunoblotting analysis with extracts from Canton S adult-fly and *E. coli* producing His-SCOX (full lenghth) fushion protein. Lane 1: *E. coli* extracts; lane 2: Canton S extracts and lane 3: Canton S extracts with only secondary antibody. Positions of size markers are shown in left side (kDa). B. Western immunoblotting analysis of the protein extract from whole bodies of adult flies. Lane 1: Canton S, lane 2: *w; UAS-SCOX/+; Act5C-GAL4/+*, and lane 3: *w; +; Act5C-GAL4/UAS-SCOXIR*^{7861}. In the *w; +; Act5C-GAL4/UAS-SCOXIR*^{7861} fly extracts no SCOX band was detectable. C. Quantitative RT-PCR for *SCOX* mRNA levels in *w; UAS-SCOXIR*^{20}*/+; Act5C-GAL4/+* and and *w; +; Act5C-GAL4/+* flies. A significant decrease of *SCOX* mRNA expression level in *w; UAS-SCOXIR*^{20}*/+; Act5C-GAL4/+* flies was observed, compared to *w*, +; *Act5C-GAL4/+* flies (n=3). D. Quantitative RT-PCR for *SCOX* mRNA levels in *w; UAS-SCOX/+; Act5C-GAL4/+* flies and *w; +; Act5C-GAL4/+* flies. An increase of *SCOX* mRNA expression level in *w; UAS-SCOX/+; Act5C-GAL4/+* flies was observed, compared to *w*, +; *Act5C-GAL4/+* flies (n=3). qPCR-SCOX-forward primer; 5'-GAGAAGGATGAGGCGAGAATG-3' and qPCR-SCOX-reverse primer; 5'-GCTCCCTGCGAATCAACTAA-3' were used in this assay.

UAS-SCOX. P element-mediated germline transformation was accomplished as described [15] and F1 transformants were selected on the basis of white eye color rescue [15]. Twenty-five lines were established for plasmid pUAS-SCOIR$_{600\text{-}964}$ but only line number 20 (UAS-*SCOXIR*^{20}) was determined to have an RNAi transgene on the second chromosome while others were determined to have examples on both the second and third chromosomes. Therefore, this line (UAS-*SCOXIR*^{20}) was mainly used in this study. Although twenty lines were established for the plasmid pUAS-SCOX, line number 8 (UAS-*SCOX*) showed the severest rough eye phenotype and therefore was primarily used in this study.

To drive expression of *SCOX* double stranded RNA (*SCOX* dsRNA) or SCOX in the whole body of the flies, we crossed the transgenic flies with the *Act5C-GAL4* driver line.

Expression of his-tag fusion proteins

The SCOX full length fragment was transferred to the pCold I expression vector to create pCold-scox which was inserted into *Escherichia coli* BL21 to express the His-SCOX full length fusion

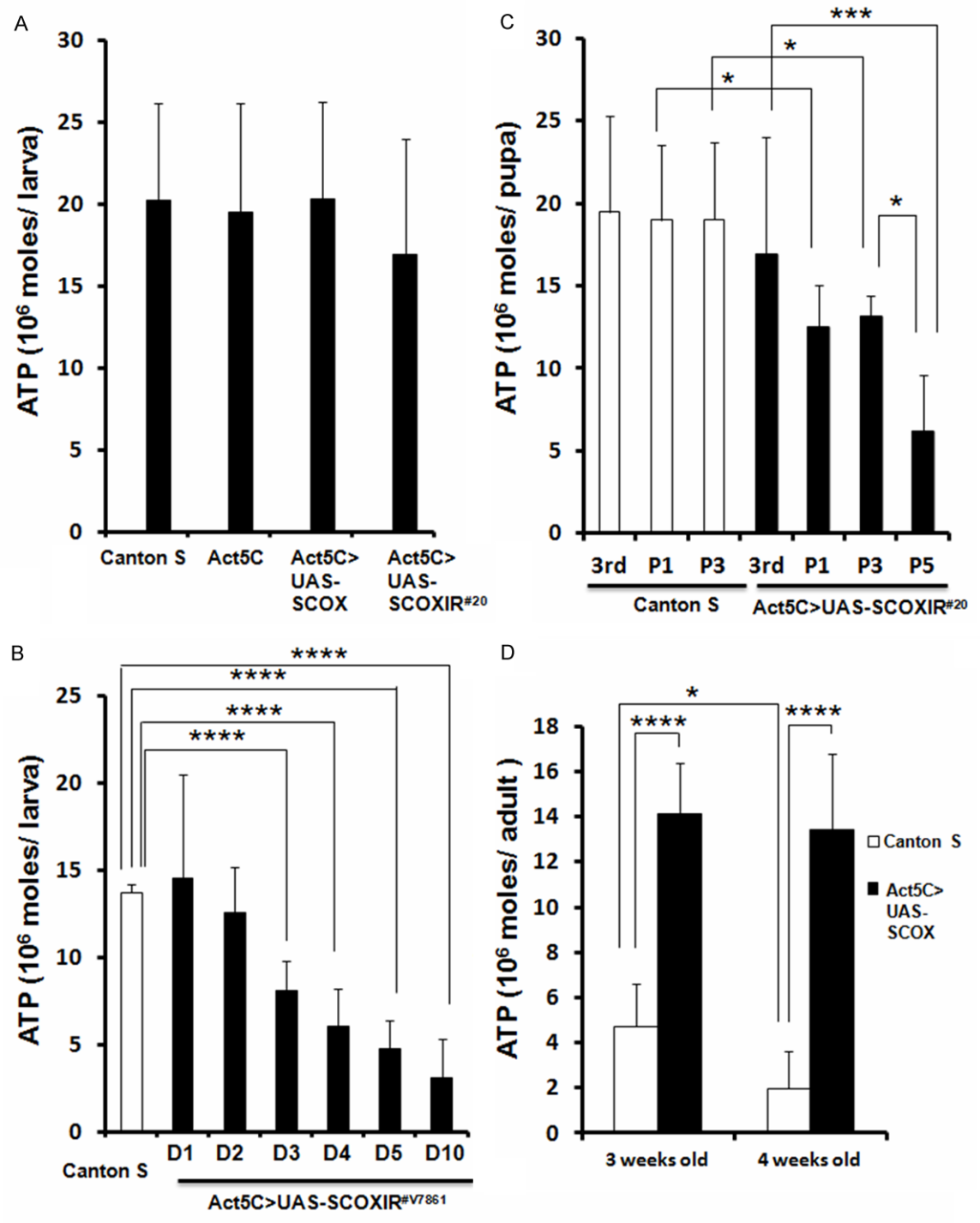

Figure 2. ATP Assay. A. ATP levels in third instar larvae. There was no significant difference in ATP levels among the indicated genotypes. B. ATP level in the larvae of *w; +; Act5C-GAL4/UAS-SCOXIR*^{7861} flies. The results showed that there was no significant difference in ATP level in the first two days of the second larval stage, but decrease was noted from the third day. C. ATP level in third instar larvae and pupae with *w; UAS-SCOXIR*^{20}*/+; Act5C-GAL4/+* flies or Canton S flies. A significant decrease was observed in the fifth day of the pupal stage (P5), compared to the third larvae stage (3rd) and the third day of the pupal stage (P3). Canton S flies showed constant levels of ATP. D. ATP level of adult flies with *w; UAS-SCOX/+; Act5C-GAL4/+* and Canton S at 3-weeks and 4 weeks of age. An increase in ATP level was observed in *w; UAS-SCOX/+; Act5C-GAL4/+* flies, compared to wild type flies. The horizontal bars indicate the standard errors of the mean values. ****p<0.001, ***p<0.005, **p<0.01, *p<0.05.

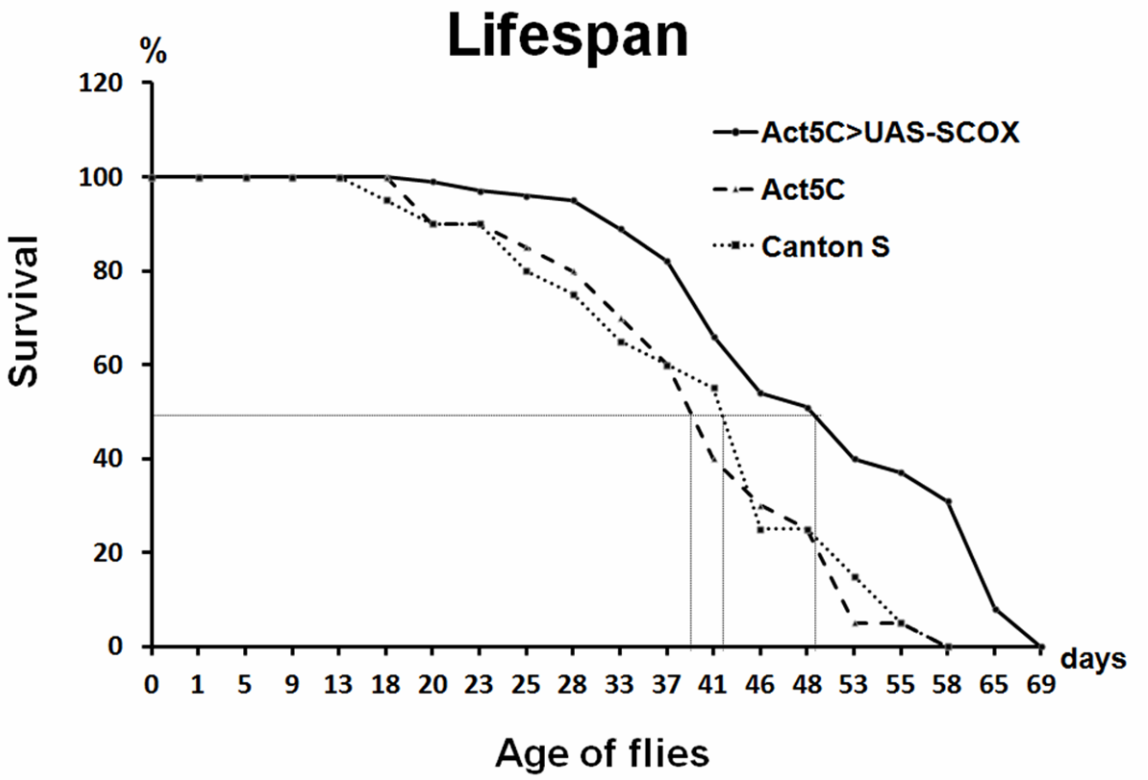

Figure 3. Lifespan analysis of *w; UAS-SCOX/+; Act5C-GAL4/+* flies, *w; +; Act5C-GAL4/+* flies and Canton S flies. Percentage survival of adult-male flies of the indicated genotypes is shown. Flies were collected and divided into 5 vials. The total number of counted flies was n=100 for each fly strain. There was no significant difference in the lifespan of wild type and *w*; +; *Act5C-GAL4/+*flies, but an extension of lifespan was observed in *w; UAS-SCOX/+; Act5C-GAL4/+*flies. The lifespan differences between *w; UAS-SCOX/+; Act5C-GAL4/+* flies and wild type flies or *w, +; Act5C-GAL4/+* flies were statistically significant ($p<0.05$ and $p<0.005$, respectively). Results were analyzed using OASIS software (http://sbi.postech.ac.kr/oasis).

protein. In brief, lysates of cells were prepared by sonication in PBS containing 1 mM phenylmethanesulfonyl fluoride (PMSF) and separated into supernatant and pellets by centrifugation at 12,000 g for 20 min at 4°C. Pellets were dissolved in 6 M urea buffer (6 M urea, 50 mMTris-HCl, pH 8.0, 1 mM EDTA), and purified by electrophoresis through Ni-NTA agarose (QIAGEN). After purification, the urea buffer was replaced with PBS by dialysis.

Preparation of anti-SCOX antibody

The purified GST-SCOX full length fusion protein was used to elicit polyclonal antibody production in a Guinea pig. The resulting antiserum was used at 1:5000 dilution for Western Blotting Assays.

Longevity assay

Wild-type flies, control flies carrying only *Act5C-GAL4* and *Act5C*-GAL4>UAS-*SCOX* flies were maintained at 28 ± 0.5°C in a tube with standard food. To estimate longevity, newly eclosed adult flies were collected during 24 hours from the onset of explosion for each experimental variant. Young males were separated from females and put into new conical tubes. Flies were transferred to new medium three times per week (each 2-3 days). Dead flies were counted daily. For each experimental variant, three biological replicators were pooled. Survival functions were estimated using the Kaplan-Meier procedure [16] and plotted as survival curves.

Immunoblotting analysis

Protein extracts from the whole bodies of wild type flies, control flies, *Act5C*-GAL4>UAS-*SCOX* flies, and *Act5C*-GAL4>UAS-*SCOXIR*^{7861} flies and from *E. coli* producing His-SCOX (full length) fusion protein were prepared in lysis buffer with proteinase inhibitors and then homogenized in

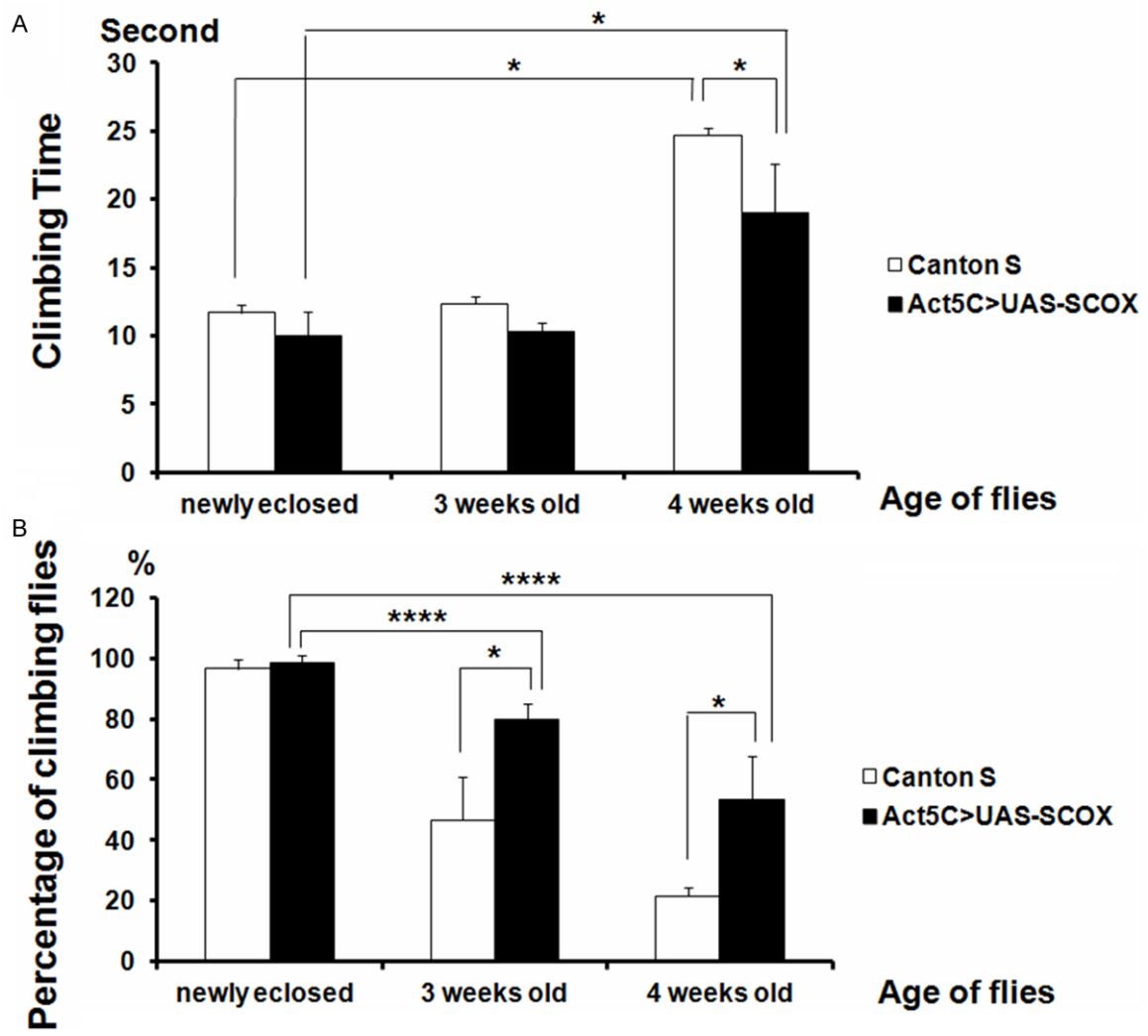

Figure 4. Climbing assay results. Two independent tests were performed for *w; UAS-SCOX/+; Act5C-GAL4/+* flies and Canton S as wild type flies. The total number of counted male-flies was n=60 for each fly strain. A. Time needed for the first fly climbing to the line (at height of 17.5 cm). There was no significant difference between *w; UAS-SCOX/+; Act5C-GAL4/+* flies and wild type flies at 3-weeks of age. However, with 4-week-old flies, while a longer climbing time was observed in each fly strain, *w; UAS-SCOX/+; Act5C-GAL4/+* flies took less time. B. Percentage of flies above the line 5 minutes after being put into tube. A decrease was observed at ages of 3-weeks and 4 weeks *w; UAS-SCOX/+; Act5C-GAL4/+* flies. Furthermore, at these latter ages *w; UAS-SCOX/+; Act5C-GAL4/+* flies were observed to keep being above the line longer than wild type flies. The horizontal bars indicate the standard errors of the mean values. ****p<0.001, ***p<0.005, *p<0.05.

a sample buffer containing 50 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 0.1% bromophenol blue and 1.2% β-mercaptoethanol. The homogenates were boiled at 100°C for 5 min, and then centrifuged. The supernatants of extracts were electrophoretically separated on SDS polyacrylamide gels containing 12% acrylamide and then transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Osaka, Japan). The blotted membranes were blocked with TBS/0.05% Tween containing 5% skim milk for 1 hour at 25°C, followed by incubation with Guinea pig polyclonal anti-SCOX at a 1:2,000 dilution for 16 hours at 4°C. After washing, the membranes were incubated with HRP-conjugated anti-Guinea pig IgG (GE Healthcare Bioscience, Tokyo, Japan) at 1:10,000 dilution for 2 hours at 25°C. Antibody binding was detected by using ECL Western blotting detection reagents (GE Healthcare Bioscience) and images were analyzed using a Lumivision Pro HSII image analyzer (Aisin Seiki,

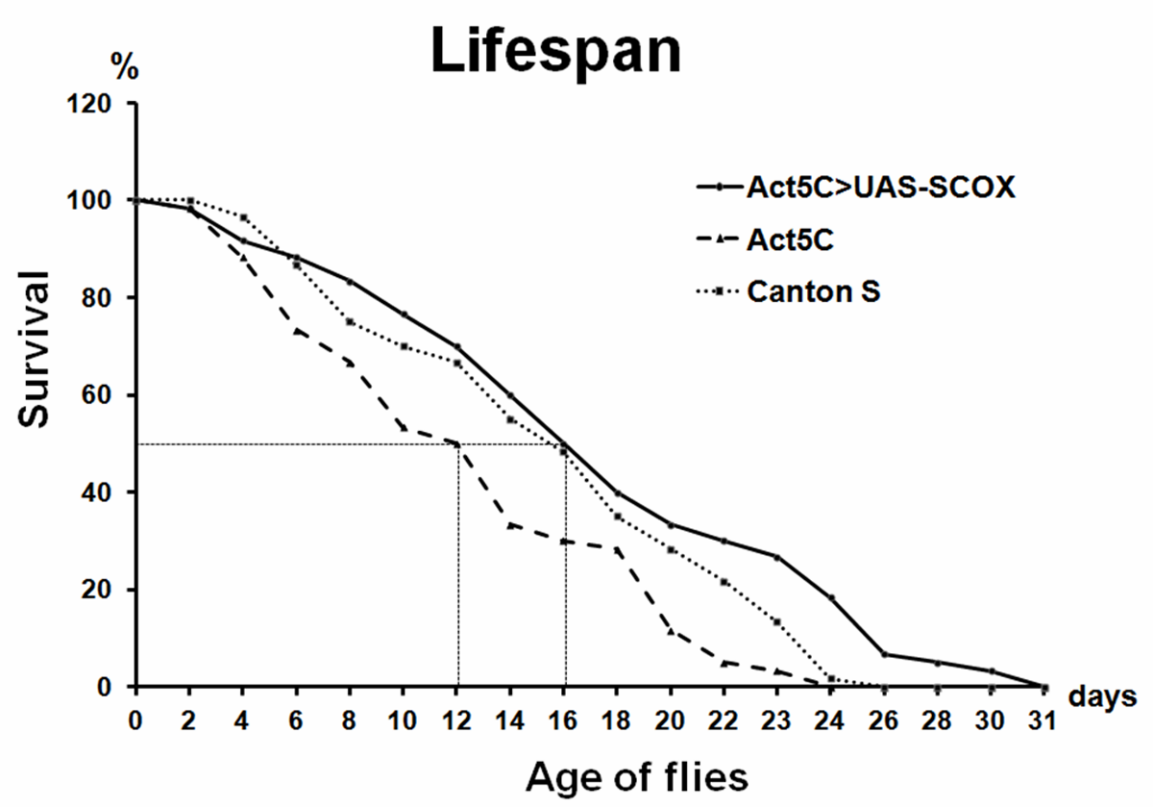

Figure 5. Lifespan analysis of each fly strain in the paraquat assay. Flies were collected and divided into 20 flies per vial (3 vials in total). Adult flies were cultured by food containing 10 mM paraquat. The total number of counted flies was n=60 for each fly strain. Percentage survival of adult male-flies of the indicated genotypes is shown. There was no significant differences in the lifespans of wild type and control flies, but an extension was observed in the lifespan of *w; UAS-SCOX/+; Act5C-GAL4/+* flies. The lifespan differences between *w; UAS-SCOX/+; Act5C-GAL4/+*, wild type and w; +; *Act5C-GAL4/+* flies were statistically significant ($p<0.005$, in both cases).

Kariya, Japan). To confirm equal amounts of protein loading in each lane, the membranes were also probed with an anti-α-tubulin antibody after stripping the complex of anti-SCOX antibody and HRP-conjugated anti-Guinea pig IgG. For the detection of α-tubulin, mouse anti-α-tubulin monoclonal antibody (1:5,000 dilution, Sigma, Tokyo, Japan) and an HRP-conjugated anti-mouse IgG (1:10,000 dilution, GE Healthcare Bioscience) were used as the primary and secondary antibody, respectively.

Quantitative RT-PCR

Total RNAs from whole bodies of *Drosophila* were purified with Trizol (Invitrogen) and 1 μg aliquots were reverse transcribed with oligo dT primers using a PrimeScript High Fidelity RT-PCR Kit (Takara). Then, quantitative RT-PCR was performed with a SYBR Premix Ex Taq (Tli RNaseH Plus) (Takara) kit and the Applied Biosystems 7500 quantitative RT-PCR system using 1 μg of reverse transcribed sample per reaction. Levels of mRNA in each fly strain were investigated by the C_T comparative method [17]. Rp49 was used as an endogenous reference gene. Experiments were performed in triplicate for each of three RNA batches isolated separately.

ATP assays

A CellTiter-Glo® Luminescent Cell Viability Assay Kit (Promega) was used to quantify the level of ATP present. Each adult fliy was ground in 100 μl Assay Kit buffer and then centrifuged at 12,000 g for 10 min. The supernatant was transferred into a new microtube and 10 μl of the supernatant was mixed with 100 μl of measure buffer, followed by incubation for 10 min at 25°C to stabilize luminescent signals before being read on a Lumat LB 9507 luminometer (Berthold).

Climbing assays

Climbing assays were performed at 25°C and 60% humidity. *Act5C*-GAL4>UAS-*SCOX* flies and wild type flies were placed at 28°C, and newly eclosed adult flies were separated and placed in vials at a density of 20 adult male flies per vial (3 vials was done in each genotype). Flies were transferred, without anesthesia, to a hinge tube with a line at the height of 17.5 cm. The tube was tapped to collect the flies to the bottom, and they were then given 5 min to climb the wall. The time when the first fly crossed the line was noted. After 5 min, the number of flies above the line was counted. These climbing assays were carried out at newly eclosed time and 3 weeks, and 4 weeks after eclosing.

Paraquat assays

Paraquat assays were carried out in a humidified, temperature controlled incubator at 28°C and 60% humidity under 12-hour light and 12-hour dark cycle culture condition. Instant food with added 10 mM paraquat was used. Newly eclosed male adult flies of control flies carrying *Act5C-GAL4* alone, and *Act5C*-GAL4>UAS-*SCOX* transgenic flies were separated and placed in vials at low density (20 flies per vial and in this experiment 3 vials for each genotype were used.). Every 2 days, they were transferred to new tubes containing fresh food and deaths were scored. Survival functions were estimated using the Kaplan-Meier procedure [16] and plotted as survival curves.

Data analysis

All statistical analyses were performed using Microsoft Excel. The Kaplan-Meier procedure was used for assessment of the statistical significance of comparisons between groups of data concerning the median lifespan. For other assays, two-way ANOVA was applied to assess the statistical significance of differences between groups of data. When two-way ANOVA showed significant variation, a Dunnet's test was subsequently used for pairwise comparisons. All data are shown as means ± SEM.

Results

Specificity of the anti-SCOX antibody and evaluation of SCOX level in transgenic flies carrying UAS-SCOXIR and UAS-SCOX

We raised a polyclonal antibody against SCOX as a tool for studying *in vivo* roles of *SCOX*. To investigate its specificity, Western immunoblotting was carried out with the extracts of adult-male flies of wild type Canton S. A band corresponding to approximately 25 kDa was detected in extracts from Canton S using the anti-SCOX antibody (**Figure 1A**, lane 2). The size of the detected band is nearly identical to the size (28,175.2 kDa) of SCOX protein which is predicted based on its amino acid composition. The faster migrating band was also detected with the Western blot without the first antibody, suggesting the band to be non-specifically detected with the secondary antibody. Moreover, a single band with an apparent molecular weight of 29 kDa was detected in extracts from *E. coli* producing His-SCOX (full length) fusion protein (**Figure 1A**, lane 1). The data indicate that the prepared antibody is highly specific to the SCOX protein.

To confirm the knockdown or expression of SCOX in the transgenic flies *Act5C*-GAL4>UAS-*SCOXIR*^{7861}, Western immunoblotting was performed again with anti-SCOX antibody. In extracts from the *SCOX*-knockdown *Act5C*-GAL4>UAS-*SCOXIR*^{7861} flies, no 25 kDa SCOX was detected, confirming that SCOX was effectively knocked down in the *Act5C*-GAL4>UAS-*SCOXIR*^{7861} flies (**Figure 1B**, lane 3).

To further examine the knockdown or expression of SCOX in the transgenic flies- *Act5C*-GAL4>UAS-*SCOXIR*^{20}, quantitative RT-PCR analysis was performed. In extracts from the *SCOX*-knockdown *Act5C*-GAL4>UAS-*SCOXIR*^{20} flies, a little of SCOX mRNA was detected as compared to control flies carrying *Act5c-GAL4* alone, confirming that SCOX was efficiently knocked down in the *Act5C*-GAL4>UAS-*SCOXIR*^{20} flies (**Figure 1C**). However, there was no significant increase in SCOX protein level in extracts from the transgenic *Act5C*-GAL4>UAS-*SCOX* flies in compared with the wild type flies (**Figure 1B**, lane 1 and 2). In contrast, the *SCOX* mRNA level was increased 23.8 fold in the transgenic *Act5C*-GAL4>UAS-*SCOX* flies as compared to control flies carrying *Act5C-GAL4* alone (**Figure 1D**).

Decreased ATP level correlates with lethality in SCOX-knockdown flies

Using the *Act5C-GAL4* driver that expresses GAL4 in the whole body of *Drosophila*, we examined the morphology, the life cycle, and the lifespan of the two independent *SCOX*-

knockdown flies, the *Act5C*-GAL4>UAS-*SCOXIR*^{7861} flies and the *Act5C*-GAL4>UAS-*SCOXIR*^{20} flies, in comparison with wild type flies and control flies carrying *Act5C-GAL4* alone.

No apparent difference in morphology was observed with larvae and adults from these flies. However, *Act5C*-GAL4>UAS-*SCOXIR*^{7861} was associated with lethality at the larval stage. Larvae of this knockdown strain survived for 12 days after hatching but demonstrated reduced motility day by day until death. The larvae stayed within the medium with low mobility or no climbing of the walls of culture tubes, in clear contrast to wild type and control flies. The other *SCOX*-knockdown *Act5C*-GAL4>UAS-*SCOXIR*^{20} flies died at the pupal stage. Dissection of the pupae revealed that the knockdown flies grew to the pharate adult stage, but could not hatch.

Previous studies provided evidence that SCOX is required for assembly of the cytochrome c oxidase (COX) complex [18, 19]. In eukaryotic cells, this is crucially important for aerobic respiration in mitochondria, where the majority of molecular oxygen is consumed and high amounts of energy (36-38 ATP) are released. Therefore, decrease of SCOX in the *SCOX*-knockdown flies may lead to decrease in activity of mitochondria, and lowered ATP. To examine this hypothesis, ATP levels were measured in *SCOX*-knockdown flies. That of *Act5C*-GAL4>UAS-*SCOXIR*^{7861} larvae was equal to that of wild type flies for the first two days but then significantly decreased from the third day (**Figure 2B**) ($p<0.001$) and continued decreasing until death. In contrast, the *Act5C*-GAL4>UAS-*SCOXIR*^{20} flies show no significant difference in ATP level as compared with wild type flies, and the control flies carrying *Act5C-GAL4* alone at the third larval stage (**Figure 2A**). However, at the pupal stage, a significant decrease in ATP level was observed at days 1 and 3 in compared to the wild type flies (**Figure 2C**). At the fifth day of the pupal stage (pharate adult stage), a further decrease in ATP level was observed, as comparing to the third day pupal stage (**Figure 2C**), while wild type flies showed a constant of ATP level. These results showed SCOX to be essential for the viability of *Drosophila* and the lethal phase coincides with the development stage of the knockdown flies showing the lowest ATP levels.

Increased ATP level correlates with prolonged lifespan in the transgenic flies carrying the full length SCOX

We also examined morphology and lifespan of full length SCOX transgenic flies (*Act5C*-GAL4>UAS-*SCOX*). While the *Act5C*-GAL4>UAS-*SCOXIR*^{20} and *Act5C*-GAL4>UAS-*SCOXIR*^{7861} knockdown flies showed pupal and larval lethality, respectively, the *Act5C*-GAL4>UAS-*SCOX* flies developed normally and eclosed a little faster than the control flies carrying *Act5C-GAL4* alone. In addition, there was no apparent difference in morphology throughout the developmental stages. However, a longer lifespan of adult flies was observed with the *Act5C*-GAL4>UAS-*SCOX* flies as compared with the control and wild type flies (**Figure 3**). The median lifespan of wild type flies was 60 days; that of the control flies was 58 days, whereas that of the *Act5C*-GAL4>UAS-*SCOX* flies was an average of 69 days. The differences in lifespan in comparing of the *Act5C*-GAL4>UAS-*SCOX* flies to wild type flies and the control flies were statistically significant ($p<0.05$ and $p<0.005$, respectively).

In order to evaluate the functional differences between *Act5C*-GAL4>UAS-*SCOX* and wild type flies, we performed climbing assays with newly eclosed flies and flies at 3-weeks and 4-weeks after eclosion. The *Act5C*-GAL4>UAS-*SCOX* flies showed better mobility than wild type flies at the same age (**Figure 4**), while there was no significant difference in mobility between wild type flies and the control flies (data not shown). We performed two different climbing assays and in both the *Act5C*-GAL4>UAS-*SCOX* flies showed better mobility than the wild type flies with a faster climbing time and a longer time remaining at elevation (**Figure 4**). All of these increases in mobility were statistically significant ($p<0.05$ and $p<0.01$, respectively).

To examine the activity of mitochondria at the age in which significant differences in climbing assays were observed in *Act5C*-GAL4>UAS-*SCOX* flies, ATP assays were performed with newly eclosed, 3-week-old and 4-week-old *Act5C*-GAL4>UAS-*SCOX* and wild type flies. The results showed increased ATP levels in the *Act5C*-GAL4>UAS-*SCOX* flies at both time points ($p<0.001$) (**Figure 2D**). Moreover, while decrease of ATP level was observed in 4-week as compared with 3-week-old wild type flies ($p<$

0.05), this was not observed with *Act5C*-GAL4>UAS-*SCOX* flies (**Figure 2D**).

In summary, on clear contrary to the *SCOX*-knockdown flies, the *Act5C*-GAL4>UAS-*SCOX* flies showed extension of lifespan. These results taken together suggest that decrease of ATP in *SCOX*-knockdown flies leads to the lethality at young age, while increase in the *Act5C*-GAL4>UAS-*SCOX* flies helps to extend their lifespan.

Role of SCOX in alleviating mitochondrial oxidative stress

Decrease of ATP observed in *SCOX*-knockdown larvae apparently correlated with death at young age, suggesting that the defect in SCOX affects activity of mitochondria. In previous studies, mitochondrial ROS (mtROS) production was reported to correlate with the lifespan in *Drosophila*; a decrease in mtROS production increased and increase in decreased the lifespan [17]. In the present study, when newly eclosed male adults of wild type flies and the *Act5C*-GAL4>UAS-*SCOX* flies were collected and fed food containing 10 mM paraquat, known to impair the mitochondrial functions by increasing the level of ROS [20-22], the lifespan of *Act5C*-GAL4>UAS-*SCOX* flies was observed to be longer than those of wild type and control flies under the same conditions (**Figure 5**). The median lifespan of both wild type and control flies was 25 days whereas that of *Act5C*-GAL4>UAS-*SCOX* flies was an average of 30 days. These data suggest that SCOX might reduce the level of mtROS and consequently prolong the lifespan.

Discussion

In a previous study, SCO2 mutation was found to lead to reduced-ATP levels [23]. Here we showed equivalent decrease in ATP in *SCOX*-knockdown flies, pointing to similarity between *Drosophila* SCOX and human SCO2 in functions. Recently, mutations in *SCO2* have been reported in patients with fatal infantile cardioencephalomyopathy and cause cytochrome c oxidase deficiency [11]. In man, cytochrome c oxidase deficiency is a genetic condition that can affect several parts of the body, including the muscles, the heart, the brain and the kidney [7]. Common signs or symptoms of cytochrome c oxidase defects are frequently found in childhood. Cytochrome c oxidase receives an electron from each of four cytochrome c molecules, transfers them to a single oxygen molecule and then converts molecular oxygen to two molecules of H_2O. In addition, it translocates four protons across the membrane, establishing a transmembrane difference of proton-electrochemical potential that allows ATP synthase to synthesize ATP. This means that the lack of functional cytochrome c oxidase disrupts the mechanism, called oxidative phosphorylation, and causes a decrease in energy production which may result in death. In the body of each individual, the brain, the muscle and the heart require the largest amount of energy for them to function. Decrease in energy may lead to decrease in their activity and induce death. In this study, the *SCOX*-knockdown flies demonstrated pupal or larval lethality which was accompanied by a decrease in ATP level, observed prior to the stage before death. Larval lethality is consistent with the previous report with scox null mutants [14]. Furthermore, full length SCOX transgenic adult flies showed longer lifespan and higher mobility than the wild type flies, accompanied by an increase in ATP level. Our data therefore suggest that SCOX plays an important role in controlling ATP production and/or consumption to keep cells and organs in normal activity.

In the full length SCOX transgenic flies, the SCOX mRNA level was observed to be increased as analyzed by quantitative RT-PCR, but no apparent increase was observed in SCOX protein level as analyzed by Western immunoblotting analysis. However, the full length SCOX transgenic flies show a longer lifespan than the wild type flies. These results suggest a possibility that overexpression of SCOX in the full length SCOX transgenic flies may be transient and some unknown negative feedback mechanism may operate to keep SCOX protein levels constant. Even under these conditions, some genes that act against aging may be affected.

Paraquat, a nonselective herbicide, induces oxidative stress by increasing the production of ROS, which is a toxic by-product of mitochondrial energy production and oxidative phosphorylation in cancer cells [24]. An increase of ROS may lead to signs associated with death and also linked to aging [21]. In our study, the *Act5C*-GAL4>UAS-*SCOX* flies exhibited a longer

lifespan than the wild type flies under conditions of 10 mM paraquat feeding. In a previous study, Ca^{2+} was shown to play a role in ATP synthesis and in ROS generation, and most of the mitochondrial effect of Ca^{2+} requires its entry across double-membrane into the matrix. Under physiological conditions, Ca^{2+} is beneficial for mitochondrial functions, but in the presence of an overriding pathological stimulus, Ca^{2+} may be detrimental which leads to an increase of ROS-associated cell death [25]. The available observations thus suggest that SCOX may help to prevent the formation of Ca^{2+} with pathological stimuli.

In conclusion, our study identified characteristics of the whole bodies of *SCOX*-knockdown and full length SCOX transgenic flies. We propose that SCOX plays an important role in mitochondrial anti-oxidative stress mechanisms or in a mitochondrial death pathway that is important for *Drosophila* lifespan.

Acknowledgements

We thank Dr M. Moore for advice in English usage. This study was supported by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan, JST and JSPS Core-to-Core Program, B. Asia-Africa Science Platforms.

Disclosure of conflict of interest

None.

Address correspondence to: Masamitsu Yamaguchi, Department of Applied Biology, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606-8585, Japan. Tel: +81-75-724-7781; Fax: +81-75-724-7799; E-mail: myamaguc@kit.ac.jp

References

[1] Jake GH, Kathleen SM, Jing Z and Thomas JM. Mitochondrial therapeutics in Alzheimer's disease and Parkinson's disease. Alzheimers Res Ther 2011; 3: 21.

[2] Chial H and Craig J. mtDNA and mitochondrial diseases. Nat Edu 2008; 1: 217.

[3] Aleksandra M, Katarzyna GW, Ireneusz S and Cerazy Z. Acta Neurobiol 2006; 66: 153-176.

[4] Timothy H and Gino C. A mitochondria DNA clone is associated with increased risk for Alzheimer disease. Proc Natl Acad Sci U S A 1995; 92: 6892-6895.

[5] Chen Y, Cairns R, Papandreou I, Koong A, Denko NC. Oxygen consumption can regulate the growth of tumors, a new perspective on the Warburg effect. PLoS One 2009; 4: e7033.

[6] Jaksch M, Paret C, Stucka R, Horn N, Muller-Hocker J, Horvarth R, Trepesch N, Stecker G, Freisinger P, Thirion C, Muller J, Lunkwitz R, Rodel G, Shoubridge AE, Lockmuller. Cytochrome coxidase deficiency due to mutations in SCO2, encoding a mitochondrial copper-binding protein, is rescued by copper in human myoblasts. Hum Mol Genet 2001; 10: 3025-35.

[7] Zeviani M, Tiranti V and Piantadosi C. Mitochondrial disorders. Medicine (Baltimore) 1998; 77: 59-72.

[8] Sanz A, Fernández-Ayala DJ, Stefanatos RK, Jacobs HT. Mitochondrial ROS production correlates with, but does not directly regulate lifespan in *Drosophila*. Aging 2010; 2: 200-223.

[9] Papadopoulou LC, Sue CM, Davidson MM, Tanji K, Nishino I, Sadlock JE, Krishna S, Walker W, Selby J, Glerum DM, Van Coster R, Lyon G, Scalais E, Lebel R, Kaplan P, Shanske S, De Vivo DC, Bonilla E, Hirano M, DiMauro S, Schon EA. Fatal infantile cardioencephalomyopathy with COX deficiency and mutations in SCO2, a COX assembly gene. Nat Genet 1999; 23: 333-337.

[10] Sue CM, Karadimas C, Checcarelli N, Tanji K, Papadopoulou LC, Pallotti F, Guo FL, Shanske S, Hirano M, De Vivo DC, van Coster R, Kaplan P, Bonilla E, DiMauro S. Differential features of patients with mutations in two COX assembly genes, *SURF-1* and *SCO2*. Ann Neurol 2000; 47: 589-595.

[11] Vesela K, Hansikova H, Tesarova M, Martasek P, Elleder M, Houstek J, Zeman J. Clinical, biochemical and molecular analyses of six patients with isolated cytochrome c oxidase deficiency due to mutations in the *SCO2* gene. Acta Paediatr 2004; 93: 1312-1317.

[12] Bohm M, Pronicka E, Karczmarewicz E, Pronicki M, Piekutowska-Abramczuk D, Sykut-Cegielska J, Mierzewska H, Hansikova H, Vesela K, Tesarova M, Houstkova H, Houstek J and Zeman J. Retrospective, multicentric study of 180 children with cytochrome c oxidase deficiency. Pediatrian Res 2006; 59: 21-26.

[13] Won KY, Lim SJ, Kim GY, Kim YW, Han SA, Song JY, Lee DK. Regulatory role of p53 in cancer metabolism via SCO2 and TIGAR in human breast cancer. Hum Pathol 2011; 43: 221-228.

[14] Porcelli D, Oliva M, Duchi S, Latorre D, Cavaliere V, Barsanti P, Villani G, Gargiulo G, Caggesse C. Genetic, functional and evolutionary characterization of scox, the *Drosophila mela-*

nogaster ortholog of human SCO1 gene. Mitochondrion 2010; 10: 433-448.
[15] Spradling AC. P-element-mediated transformation. In: Robertsd B, editor. *Drosophila*: A Practical Approach. Oxford: IRL Press; 1986. pp. 175-197.
[16] Yang JS, Nam HJ, Seo M, Han SK, Choi Y, Nam HG, Lee SJ, Kim S. OASIS: Online application for the survival analysis of lifespan assays performef in aging research. PLoS One 2011; 6, e23525.
[17] Morrison TB, Weiss JJ, Wittwer CT. Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. Biotechniques 1998; 24: 954-958, 960, 962.
[18] Diaz F. Cytochrome c oxidase deficiency: patients and animal models. Biochim Biophys Acta 2009; 1802: 100-110.
[19] Leary SC, Kaufman BA, Pellecchia G, Guercin GH, Mattman A, Jaksh M, Shoubridge EA. Human SCO1 and SCO2 have independent, cooperative functions in copper delivery to cytochrome c oxidase. Hum Mol Genet 2004; 13: 1839-1848.
[20] Ramiro L, Nacira M, German R, Marianela R, Mariana M, Victorio T and Gaston Q. In: Mohammed Nagib Hasaneen, editor. Paraquat: an oxidative stress inducer, Herbicides - Properties, Synthesis and Control of Weeds. ISBN 2012: 978-953-307-803-8, InTech: pp. 135-148.
[21] McCarthy S, Somayajulu M, Sikorska M, Borowy-Borowski H, Pandey S. Paraquat induces oxidative stress and neuron cell death; neuroprotection by water-solube coenzyme Q10. Toxicol Appl Pharmacol 2004; 201: 21-31.
[22] Bus JS, Aust SD, Gibson JE. Paraquat toxicity: Proposed mechanism of action involving lipid peroxidation. Environ Health Perspect 1976; 16: 139-146.
[23] Matoba S, Kang JG, Patino WD, Wragg A, Boehm M, Gavrilova O, Hurey PJ, Bunz F, Hwang PM. p53 regulates mitochondrial respiration. Science 2006; 312: 1650-1653.
[24] Vijay P, Stewart F, Alan GL and Blanka R. The effect of age on radiation resistance and oxidative stress in adult Drosophila melanogaster. Radiat Res 2008; 169: 707-711.
[25] Brookes PS, Yisang Y, James LR, Anders MW, Shey-Shing S. Calcium, ATP, and ROS: a mitochondrial love-rate triangle. Am J Physiol Cell Physiol 2004; 287: 817-833.