Supplementary Data

Animals

SAMP1/Sku Slc mice were provided from Japan SLC and raised in the Division of Laboratory Animal Research, Research Center for Human and Environmental Sciences, Shinshu University, under specific pathogen-free conditions at 24°C ± 2°C with a light-controlled regimen (12-h light/dark cycle). Tap water and food were available *ad libitum*. Ubiquinol-10 at a final concentration of 0.3% (w/w) was mixed into a standard laboratory mouse diet (CE-2, 25% protein, 4.6% fat, and 50% carbohydrate providing 343 kcal/100 g; CLEA Japan, Tokyo, Japan), using corn oil as a vehicle to 1% (v/w) of the diet. The control diet was prepared using corn oil only. CE-2 *ad libitum* has been widely used as the standard diet in many aging studies using SAM strains (4–7, 9). We used HPLC to measure the content of ubiquinol-10 in the pellets immediately after their preparation and after storage in the animal room for 3 days. The ratio of ubiquinol-10 was determined to be more than 80% of total CoQ10 (data not shown). Therefore, the food was preserved at −20°C, and the pellets were replaced every 3 days in this study. With regard to gender, in previous studies, no gender differences were observed in the aging process of SAMP1 mice (1, 2, 8, 9). Female mice were used in this study to avoid complications associated with aggressiveness in male mice. At the start of the longitudinal study, 4-week-old SAMP1 female mice were selected and housed in cages (20 cm wide × 30 cm long × 10 cm deep). At the start of the work, there were no differences in body weight as determined by statistical analysis. The housing groups remained the same, with no re-grouping throughout the animal life span. Every month, all mice were weighed, food intake was calculated, and the degree of senescence was evaluated. We tested the effect of starting ubiquinol-10 supplementation at different time points, 1 month (young), 7 months (middle), and 13 months (old age). Mice were inspected daily, and those that died spontaneously were necropsied immediately. The tissues of the whole body were fixed in 10% neutral buffered formalin, embedded in paraffin, and cut into 4 μm sections that were stained with hematoxylin and eosin or used for histopathologic evaluation. Female SAMP1 mice aged 2, 7, 13, and 19 months with ubiquinol-10 supplementation or fed a control diet were euthanized by cardiac puncture under diethyl ether anesthesia, and tissues were collected.

In our studies, liver samples from SAMP1 mice at 2, 7, 13, and 19 months of age were harvested and weighed, and then flash frozen in liquid nitrogen for storage at −70°C. We used subcellular samples from frozen tissues stored for different time periods. To check for differences between freshly dissected and stored frozen samples, we tested several indicators, including gene and protein expression levels by real-time PCR and western blot, respectively, and oxidative stress makers, mitochondria activity, and ATP levels. We observed no significant changes in these three indicators.

All experiments were performed with the consent of the Committee for Animal Experiments of Shinshu University.

Body Weight and Food Intake

The body weight and food intake of the mice was measured every month from 2 months of age until 19 months of age.

Evaluation of the Degree of Senescence

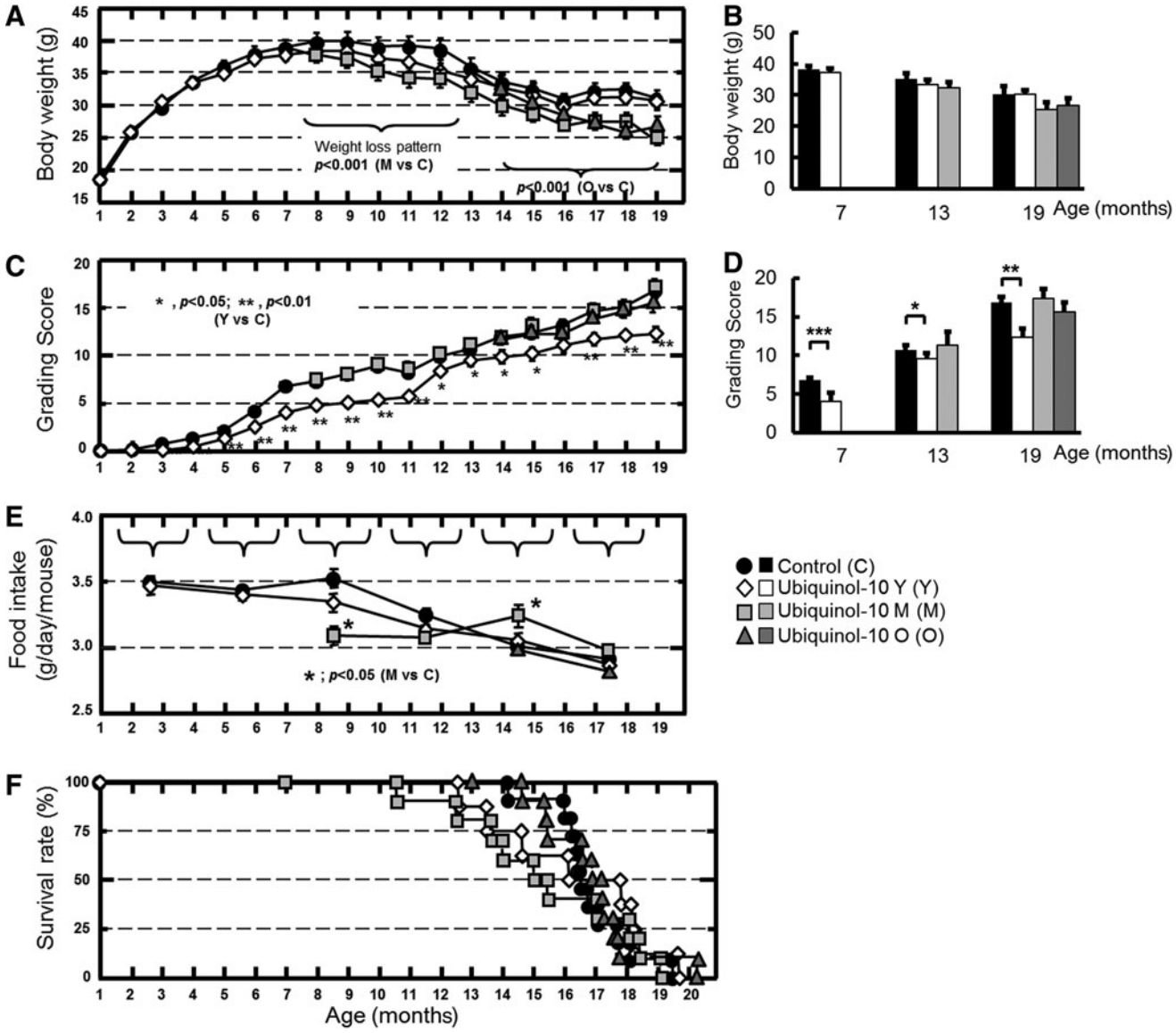
The degree of senescence was evaluated by a grading system (3). Eleven categories of behavioral activity and gross appearance of the skin, eyes, and spine were considered associated with the aging process: passivity, reactivity (general); glossiness, coarseness, hair loss, skin ulcers (skin); periophthalmic lesions, corneal opacity, corneal ulcer, cataracts (eye), and lordokyphosis. This grading score appears to be valid for evaluating the general degree of senescence, and grading was done at a fixed time (from 2 pm to 4 pm) by an observer who was blinded to the treatment group identities.

Assessment of Hearing

At 2, 7, 13, and 19 months, the auditory brainstem response (ABR) was measured with a tone burst stimulus (8, 16, and 32 kHz) using an ABR recording system (Intelligent Hearing System, Miami, FL) as previously described. Mice were anesthetized with a mixture of xylazine (10 mg/kg) and ketamine (100 mg/kg) administered by an intraperitoneal injection, and the ABR tests were performed in a soundproof room with a heating pad to maintain the body temperature. Click stimuli were performed at synthesized durations and specified amplitudes using a digital signal processing platform (Tucker-Davis Technologies, Alachua, FL), and analyzed with PowerLab systems (AD Instruments, Colorado Springs, CO) as described elsewhere. Click stimuli were 0.1 ms clicks, composed of a 0.1 ms square pulse. Stainless steel needle electrodes were placed at the vertex and ventrolateral to the left and right ears for the recording, and a tweeter modified with a coupler was inserted into the external canal to deliver acoustic stimuli. ABR waveforms were recorded in 5- to 10-decibel (dB) intervals stepped down from a maximum amplitude of 85 dB until no waveform could be visualized.

Cell Culture and Reagents

The human hepatoma HepG2 cell line (JCRB, Osaka, Japan) was cultured in Dulbecco's-modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Cells were incubated in a humidified atmosphere of 5% CO_2 at 37°C. The HepG2 cells were cultured for 2 days to achieve 75% confluence before treatment with ubiquinol-10. Ubiquinol-10, donated by Kaneka (Kaneka Corporation, Osaka, Japan), was solubilized in water using a mixture of glycerol and the emulsifying agent PEG 60-hydrogenated castor oil (HCO60; Nikko Chemicals Co., Ltd., Tokyo, Japan) (CoQ10:glycerol:HCO60 0.4:0.6:1). A 1 m*M* stock solution was kept at −80°C until use. Oxidation under these conditions was minimal after several months of storage. Cells were treated with ubiquinol-10 at 0.5, 1, 3, or 10 μM and incubated for 48 h as described earlier.



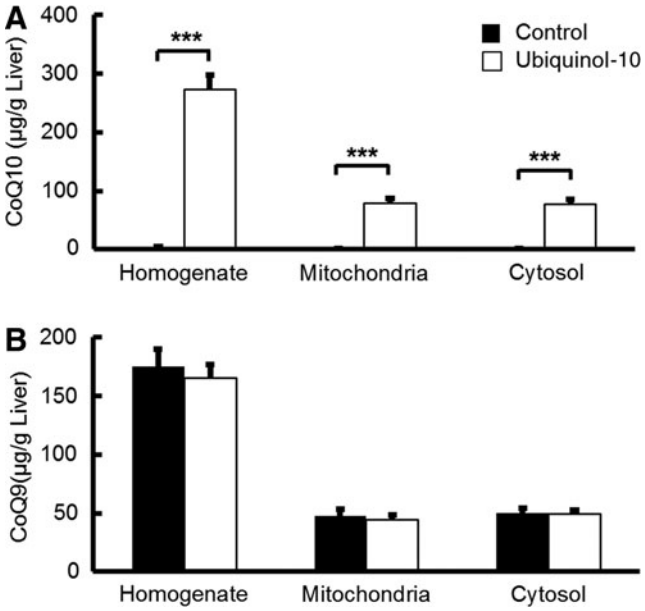
SUPPLEMENTARY FIG. S1. Body weight, degree of senescence, food intake, and survival rate related to ubiquinol-10 supplementation. (A, B) Body weight was measured monthly for SAMP1 mice fed a control or ubiquinol-10-supplemented diet from 1 month of age until 19 months of age **(A)** and at 7, 13, and 19 months of age **(B)**. Each point represents the mean ± SE of mice in each age group ($n = 11–20$). **(C, D)** Age-related changes in senescence grading scores for 2-, 7-, 13-, and 19-month-old SAMP1 mice fed a control or ubiquinol-10-supplemented diet were measured every month from 2 months of age until 19 months of age **(C)** and at 7, 13, and 19 months of age **(D)**. Each point represents the mean ± SE of mice in each age group ($n = 11–20$).**(E)** Control diet and ubiquinol-10 diet intake for SAMP1 mice were measured every month from 1 month of age until 19 months of age. Each point is the mean ± SE of mice in each age group ($n = 11–20$). **(F)** Survival curves of 2-, 7-, 13-, and 19-month-old SAMP1 mice fed a control or ubiquinol-10-supplemented diet.

Cell Fractionation and HPLC Analysis

The Mitochondria Isolation Kit for Tissue (Pierce, Rockford, IL) was used on cell fractions from SAMP1 mouse livers. Each cell fraction (homogenate, mitochondria, and cytosol) was obtained by extraction with 2-propanol that was more than five times that of each cell fraction volume. Extracts (10 μl) were subjected to HPLC analysis, as described by Kubo *et al.*, to determine the concentration of CoQ9 and CoQ10 (sum of the concentrations of oxidized and reduced forms of CoQ9 or CoQ10).

Mitochondrial Protein Preparation

Mitochondrial proteins were prepared using a glass Dounce homogenizer. Each homogenate was centrifuged at 1000*g* at 4°C for 10 min. The supernatant was transferred to a new tube, and centrifuged again at 12,000 *g* at 4°C for 15 min to separate the crude mitochondrial preparation. The pellet was washed and re-suspended in 30 μl isolation buffer as the mitochondria protein sample. The protein concentration was determined using the BCA protein assay kit (ThermoFisher, Waltham, MA) according to the manufacturer's suggested protocol.

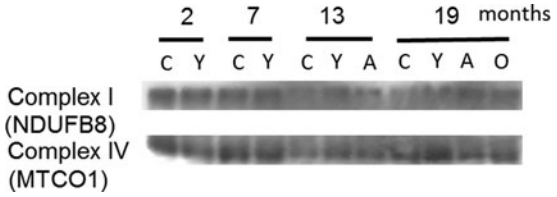


SUPPLEMENTARY FIG. S2. Ubiquinol-10 supplementation increased CoQ10 but not CoQ9 levels in SAMP1 mice livers. (A) Homogenate, mitochondrial, and cytosolic CoQ10 and **(B)** CoQ9 concentrations were measured in livers from 7 month SAMP1 mice fed a control or ubiquinol-10-supplemented diet. Each point is the mean ± SD of mice in each age group ($n = 5$).

Western Blotting and Immunoprecipitation Analysis

Liver and cochleae proteins of ubiquinol-10 supplemented and control SAMP1 mice and proteins from HepG2 cells treated with or without ubiquinol-10 were separated by electrophoresis at 20 mA for 4 h on tris-glycine/SDS-12% polyacrylamide gels (SDS-PAGE). After electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membranes using a semidry western blot apparatus at 150 mA for 1.5 h. The membranes were then probed with the given antibodies in 3% bovine serum albumin in TBS containing 0.1% Tween-20 (T-TBS) for 1 h at room temperature. Subsequently, the membranes were incubated for 1 h with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG. Target proteins were then detected with the enhanced chemiluminescence system and quantified using a densitometric image analyzer with NIH Image version 1.61 (Bethesda, MD). To calculate the relative values, we used pooled liver protein samples from 2-month-old SAMP1 mice fed a control diet as the internal standard.

PGC-1α, SOD2, and IDH2 acetylation was visualized by immunoprecipitation from tissue and cell extracts using PGC-1α, SOD2, and IDH2 antibodies followed by immunoblotting with antibodies that were specific for acetylated lysine (1:1000; Abcam, Cambridge, United Kingdom) or for PGC-1α, SOD2, and IDH2. Levels of PGC-1α, SOD2, and IDH2 acetylation were then quantified by scanning densitometry.



SUPPLEMENTARY FIG. S3. Ubiquinol-10 supplementation increased mitochondrial complex proteins in mitochondria fractions. Western blot analysis of mitochondrial complex proteins in 10 μg liver mitochondria fractions from 2-, 7-, 13-, and 19-month-old SAMP1 mice fed a control or ubiquinol-10-supplemented diet beginning during young, middle, or old age [mixed protein samples ($n = 3$)].

Antibody Information

The following primary antibodies and their corresponding ratios were used: protein carbonyls (protein carbonyl western blot detection kit; SHIMA, Tokyo, Japan), malondialdehyde antibody (1:1000; APB, Richmond, Canada), SIRT1 antibody (1:1000; Santa Cruz, Dallas, TX), SIRT3 antibody (1:1000; Abcam), PGC-1α antibody (1:500; Santa Cruz), ERRα antibody (1:1000; Abcam), SOD2 antibody (1:1000; AbFrontier, Seoul, Korea), IDH2 antibody (1:200; Santa Cruz), NRF2 antibody (1:1000; Abcam), TFAM antibody (1:1000; Abcam), acetylated lysine antibody (1:1000; Abcam), NDUF8 antibody (1:1000; Abcam), MTCO1 antibody (1:1000; Abcam), CREB antibody (1:1000; CST, Danvers, MA), p-CREB antibody (1:1000; CST), LKB1 antibody (1:1000; CST), p-LKB1 antibody (1:1000; CST), AMPK antibody (1:1000; CST), p-AMPK antibody (1:1000; CST), ACC antibody (1:1000; CST), p-ACC antibody (1:1000; CST), and β actin (1:5000; BWT, Louis Park, MN). The secondary antibody used was goat anti-rabbit IgG-HRP (1:3000; CST).

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