FOXO1 suppresses PGC-1β gene expression in skeletal muscles

Shiho Nakai1, Mamoru Oyabu1, Yukino Hatazawa1, Shiori Akashi2, Tadahiro Kitamura3, Shinji Miura2 and Yasutomi Kamei1

1 Graduate School of Life and Environmental Sciences, Kyoto Prefectural University, Kyoto, Japan
2 Graduate School of Nutritional and Environmental Sciences, University of Shizuoka, Shizuoka, Japan
3 Metabolic Signal Research Center, Institute for Molecular and Cellular Regulation, Gunma University, Gunma, Japan

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Correspondence
Y. Kamei, Laboratory of Molecular Nutrition, Graduate School of Life and Environmental Sciences, Kyoto Prefectural University, Kyoto 606-8522, Japan
E-mail: kamei@kpu.ac.jp

Shiho Nakai and Mamoru Oyabu are equal contributors

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Peroxisome proliferator-activated receptor-gamma coactivator-1β (PGC-1β) is a transcriptional regulator whose increased expression activates energy expenditure-related genes in skeletal muscles. However, how PGC-1β is regulated remains largely unclear. Here, we show that PGC-1β gene expression is negatively correlated with the expression of a transcription factor, forkhead box protein O1 (FOXO1), whose expression is increased during muscle atrophy. In the skeletal muscles of FOXO1-overexpressing transgenic mice, PGC-1β gene expression is decreased. Denervation or plaster cast-based unloading, as well as fasting, increases endogenous FOXO1 expression in skeletal muscles, with decreased PGC-1β expression. In the skeletal muscles of FOXO1-knockout mice, the decrease in PGC-1β expression caused by fasting was attenuated. Tamoxifen-inducible FOXO1 activation in C2C12 myoblasts causes a marked decrease of PGC-1β expression. These findings together reveal that FOXO1 activation suppresses PGC-1β expression. During atrophy with FOXO1 activation, decreased PGC-1β may decrease energy expenditure and avoid wasting energy.

FOXO1 (Gene symbol; Foxo1) is a forkhead-type transcription factor, whose expression is markedly upregulated in skeletal muscles during atrophy, that is, under conditions such as starvation, unloading (plaster cast), and denervation [1,2]. Transgenic (Tg) overexpression of FOXO1 in skeletal muscles causes muscle atrophy [3], with increased expression of atrophy-related genes, including cathepsin L (Ctsl) and lysosomal proteinase [3,4]. In the skeletal muscles of FOXO1-knockout (FOXO1-KO) mice, the increase in cathepsin L gene expression caused by fasting was attenuated [4,5]. FOXO1 activation mostly increases the expression of its target genes [6,7]; however, the expression of some genes such as IGFBP5 (Igfbp5) and musclin (or osteocrin, Osn) is decreased by FOXO1 [3,8].

Peroxisome proliferator-activated receptor-gamma coactivator-1β (PGC-1β; Ppargc1b) is a transcriptional coactivator of nuclear receptors, which is a homolog of PGC-1α (Ppargc1a) [9,10]. Both PGC-1β and PGC-1α are known to increase the mitochondrial content in cells [11,12]. PGC-1β and PGC-1α activate nuclear receptors, such as the estrogen-related receptor [10], and activate target genes (i.e., medium-chain acyl CoA dehydrogenase, MCAD, Acadm) in skeletal muscles [10,13,14]. Indeed, the overexpression of PGC-1β in skeletal muscles in mice led to increased energy expenditure and an anti-obesity phenotype [10]. Regulation

Abbreviations
ER, estrogen receptor; FOXO1, forkhead box protein O1; MCAD, medium-chain acyl CoA dehydrogenase; PGC-1β, peroxisome proliferator-activated receptor-gamma coactivator-1β; Tg, transgenic.
of PGC-1α in skeletal muscles has been well studied. PGC-1α expression is markedly upregulated during exercise [15,16] and is considered to contribute to the expression of exercise-related genes, such as those involved in branched-chain amino acid metabolism [17]. In contrast, little is known about the regulation of the PGC-1β gene in skeletal muscles.

In this study, we attempted to analyze the possible FOXO1-mediated PGC-1β gene expression, as the level of PGC-1β mRNA was decreased in the skeletal muscles of FOXO1-overexpressing Tg mice. Thus, we examined the level of PGC-1β gene expression in various conditions with altered FOXO1 levels in skeletal muscles and cells.

**Materials and methods**

**Animals**

Tg mice overexpressing FOXO1 in skeletal muscles (FOXO1-Tg) have been previously described [3]. Skeletal muscle-specific FOXO1-KO mice were described previously [5]. C57BL/6J mice were purchased from Shimizu Laboratory Supplies Co., Ltd. (Kyoto, Japan) and maintained at a constant temperature (24 °C) with fixed artificial light (12-h light/12-h dark cycle). All animal experiments were performed in accordance with the guidelines of the Kyoto Prefectural University Committee on Animal Research. The protocol was approved by this committee (no. KPU260407, review board: Y. Tsukamoto).

**cDNA microarray analysis**

RNA was isolated from skeletal muscle (gastrocnemius) of FOXO1-Tg mice (age, 25 weeks) and age-matched wild-type control mice. Samples from wild-type (N = 6) and FOXO1-Tg mice (N = 5) were pooled and used. RNA was isolated using TRIzol reagent (Thermo Fisher Scientific Inc., Tokyo, Japan) and purified using an RNeasy Mini kit (Qiagen, Hilden, Germany). Each sample was labeled with cyanine 3-CTP using a Low Input Quick Amp Labeling Kit (Agilent technologies, Danvers, MA, USA). Cyanine 3-CTP-labeled cRNA (1.65 μg) was fragmented and hybridized to the Agilent whole mouse genome (8 × 60 K) microarray. Signal detection and data analysis were performed as described previously [17]. The microarray data were submitted to the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/). The records have been assigned GEO accession numbers as GSE146919.

**Quantitative real-time RT-PCR analysis**

Total RNA was isolated from skeletal muscles or cells using TRIzol reagent (Thermo Fisher Scientific Inc.). cDNA was synthesized using 500 ng of each RNA sample with ReverTraAce (Toyobo, Tokyo, Japan). Gene expression was measured as described previously [18]. Fold change for each target gene was calculated as follows: \( \Delta C_t = C_t \) (target gene) – \( C_t \) (reference gene), \( \Delta \Delta C_t = \Delta C_t \) (target gene) – \( \Delta C_t \) (reference gene). Due to the exponential nature of PCR, ‘fold change’ was calculated as \( 2^{-\Delta \Delta C_t}. \) [19]. The primer sequences used were as follows: FOXO1, forward 5'-GCACGGCTGAGAAATTCAAT-3' and reverse 5'-TCAGTTCTTCTATTTCTGCA-3'; cathepsin L, forward 5'-TCTACGCTCAAGGCAATCA-3' and reverse 5'-AA GC AAAATCCATCCGGCTTC-3'; PGC-1β, forward 5'- AGCTTGCAATGTCTCAGC GCAA-3' and reverse 5'- TTGTGCAATGTCTCAGCGCAA-3'; PGC-1α, forward 5'- CGAACCCAGACGGAAG-3' and reverse 5'-TTAGACCGCTCAGG-3'; MyoD, forward 5'- CGG GACATAGACTTGACAGGC-3' and reverse 5'- TGAAAA CACGGGCTCATCATAGA-3'; myogenin, forward 5'- CAT GGGCCCAGTG AATGCAACTC-3' and reverse 5'- TAT CCTCCACCCGT GATCCTGCAAC-3'; COX2, forward 5'- GGCACCCAGACGGAAG-3' and reverse 5'- TTGTGCAATGTCTCAGCGCAA-3' and reverse 5'- TTGTGCAATGTCTCAGCGCAA-3'.

**Western blotting analysis**

Western blotting analysis was performed as described previously [5]. The primary antibody used was anti-FOXO1 [FoxO1 (C29H4) Rabbit mAb #2880; Cell Signaling Technology, Danvers, MA, USA].

**Measurement of mitochondrial DNA content**

Mitochondrial DNA (mtDNA) content was measured as mtDNA copy number normalized to the copy number of a gene contained in the nuclear genome. The mitochondrial gene used for mtDNA copy estimation was cytochrome c oxidase subunit 2 (COX2), and the copy number of COX2 was normalized to the copy number of the 36B4 gene, contained in the nuclear genome, as described previously [20].

**Measurement of citrate synthase activity**

Citrate synthase (CS) activity was measured as described previously [21].

**Denervation, plaster cast, and fasting**

For the denervation model, a 4- to 5-mm section of the sciatic nerve in the hindlimb of the mice was removed [18]. After 12 days, skeletal muscles were collected.
A plaster cast for the mice was created as described previously [18]. The hindlimb skeletal muscles of the mice were immobilized (unloaded) by the plaster cast. After 11 days, skeletal muscles were collected.

For the fasting experiment, C57BL/6J mice (9 weeks old, male) were fasted for 8 or 24 h. For refeeding, the mice were fasted for 24 h and refed for 4 h. Then, skeletal muscles were collected [22].

**Cells**

C2C12 mouse myoblasts (Riken Cell Bank, Tsukuba, Japan) stably expressing the FOXO1-estrogen receptor (ER) fusion protein were prepared as previously described [4,23,24]. In brief, C2C12 cells were stably transfected with the pBABE retroviral vector expressing fusion proteins containing a constitutively active form of human FOXO1, in which the AKT phosphorylation sites Thr-24, Ser-256, and Ser-319 are replaced with alanine [FOXO1(3A)] in-frame with a modified tamoxifen-specific version of the ligand-binding domain murine ER [4,23]. Fusion proteins were restricted to the cytoplasmic compartment until activation with tamoxifen, which caused FOXO1-ER to relocate to the nucleus, where the FOXO1 moiety then functioned as a transcription factor [4,23]. The cells were then cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS. The medium was replaced every 2 days until the cells reached confluence. Two days after confluence, the cells (undifferentiated myoblasts) were treated with tamoxifen for 24 h and used for the RNA analysis.

**Statistical analyses**

Statistical analyses were performed using Student’s two-tailed unpaired t-test for comparisons between two groups, and one-way analysis of variance followed by Tukey’s post hoc test for comparisons between three or more groups. Two-way analysis of variance for FOXO1-KO mice analysis, P < 0.05 was considered significant.

**Results and Discussion**

**Decreased PGC-1β expression in the skeletal muscles of FOXO1-Tg mice**

First, we used a skeletal muscle sample of FOXO1-overexpressing Tg (FOXO1-Tg) mice [3]. The skeletal muscle weight of the wild-type control was 190 ± 9 mg (N = 5) and that of the FOXO1-Tg mice was 117 ± 7 mg (N = 5; P < 0.001), reflecting muscle atrophy in the latter group. We performed microarray analysis to understand the gene expression changes caused by FOXO1 overexpression. One-hundred-and-fifty-three genes were upregulated more than twofold, and 145 genes were downregulated more than 0.5-fold (Tables 1 and 2). Microarray data showed decreased PGC-1β expression in the skeletal muscles of FOXO1-Tg mice, compared with that in wild-type control mice (0.44-fold; Table 2). In order to confirm the microarray data, we examined the gene expression using real-time qPCR. As expected, FOXO1 transgene overexpression was observed in the skeletal muscles of the FOXO1-Tg mice (Fig. 1A). We observed increased FOXO1 protein levels in the skeletal muscle of FOXO1-Tg mice (Fig. 1B). Authentic FOXO1 target gene cathepsin L expression was markedly increased in the FOXO1-Tg mice (Fig. 1A), indicating the functional expression of the FOXO1 transgene. At the same time, PGC-1β gene expression was significantly decreased in the FOXO1-Tg mice (Fig. 1A), confirming the microarray data. In addition, the expression of the known PGC-1β target MCAD was significantly decreased. Thus, FOXO1 overexpression appears to decrease PGC-1β expression in skeletal muscles.

**Expression of PGC-1β gene in skeletal muscles with changed endogenous FOXO1 expression**

We analyzed the expression of the PGC-1β gene under other conditions with increased endogenous FOXO1 expression. For one of these conditions, we subjected the skeletal muscles of mice to denervation. After 12 days, we dissected the mice. The skeletal muscle (gastrocnemius) weights were 141 ± 6 mg (control, N = 3) and 82 ± 2 mg (denervation, N = 4; P < 0.001), showing muscle atrophy associated with the denervation. Increased FOXO1 as well as cathepsin L mRNA expression was also observed in the skeletal muscles with denervation (Fig. 2). We also observed increased FOXO1 protein levels in skeletal muscles with denervation (data not shown). A marked decrease of PGC-1β expression, as well as MCAD expression, was observed in the skeletal muscles with denervation (Fig. 2). Denervation increased 36B4 (reference gene) expression. We used another reference gene (18S), whose expression was not increased by denervation, and observed significant decrease of PGC-1β expression, as well as MCAD expression (Fig. 2).

Next, we used skeletal muscles subjected to unloading with a plaster cast. Unloading using a plaster cast for 11 days caused muscle atrophy. The skeletal muscle (gastrocnemius) weight was 150 ± 3 mg for the control group (N = 5) and 103 ± 5 mg for the group with a plaster cast (N = 4; P < 0.001). The plaster cast increased the mRNA expression of FOXO1 and its
Table 1. List of genes in skeletal muscle with increased expression levels in FOXO1-Tg mice compared with wild-type control mice. Top 100 genes are shown.

| SystematicName | GeneName | Description | Fold (FOXO1-Tg/ Wild-type) |
|----------------|----------|-------------|---------------------------|
| 1              | NM_025540| Sln         | Sarcolipin                | 154.62                     |
| 2              | NM_001081187| Htra4     | HtrA serine peptidase 4    | 66.04                      |
| 3              | NM_019739| Foxo1       | Forkhead box O1            | 56.54                      |
| 4              | NM_010868| Myl4        | Myosin, light polypeptide 4 | 14.98                      |
| 5              | NM_001134697| Ctxn3     | Cortexin 3                 | 11.80                      |
| 6              | NM_013803| Casr        | Calcium-sensing receptor   | 9.63                       |
| 7              | NM_007836| Gadd45a     | Growth arrest and DNA-damage-inducible 45 alpha | 7.61 |
| 8              | NM_013492| Clu         | Clusterin                  | 7.22                       |
| 9              | NM_025359| Tspan13     | Tetraspanin 13             | 7.18                       |
| 10             | NM_030695| Lrba        | LFS-responsive beige-like anchor | 6.99 |
| 11             | NM_010597| Kcnab1      | Potassium voltage-gated channel, shaker-related subfamily, beta member 1 | 5.78 |
| 12             | NM_146085| Apbb3       | Amyloid beta (A4) precursor protein-binding, family B, member 3 | 5.16 |
| 13             | NM_008362| Il1r1       | Interleukin 1 receptor, type I | 5.07 |
| 14             | NM_007913| Egr1        | Early growth response 1    | 4.71                       |
| 15             | NM_153578| Nipa1       | Nonimprinted in Prader-Willi/Angelman syndrome 1 homolog (human) | 4.62 |
| 16             | NM_011044| Pck1        | Phosphoenolpyruvate carboxykinase 1, cytosolic | 4.46 |
| 17             | NM_008258| Hn1         | Hematological and neurological expressed sequence 1 | 4.36 |
| 18             | NM_201256| Eif4ebp3    | Eukaryotic translation initiation factor 4E binding protein 3 | 4.28 |
| 19             | NM_001102405| Acp5    | Acid phosphatase 5, tartrate resistant | 4.15 |
| 20             | NM_021282| Cyp2e1      | Cytochrome P450, family 2, subfamily e, polypeptide 1 | 4.15 |
| 21             | NM_009876| Cdkn1c      | Cyclin-dependent kinase inhibitor 1C (P57) | 4.15 |
| 22             | NM_021282| Cyp2e1      | Cytochrome P450, family 2, subfamily e, polypeptide 1 | 4.14 |
| 23             | NM_025439| Tmem9       | Transmembrane protein 9    | 4.12                       |
| 24             | NM_144936| Tmem45b     | Transmembrane protein 45b  | 4.01                       |
| 25             | NM_008086| Gas1        | Growth arrest-specific 1   | 4.00                       |
| 26             | NM_013614| Odc1        | Ornithine decarboxylase, structural 1 | 3.90 |
| 27             | NM_011858| Tenm4       | Teneurin transmembrane protein 4 | 3.87 |
| 28             | NM_001204959| Retn     | Resistin                   | 3.84                       |
| 29             | NM_178373| Cidec       | Cell death-inducing DFFA-like effector c | 3.76 |
| 30             | NM_009605| Adipoq      | Adiponectin, C1Q, and collagen domain containing | 3.69 |
| 31             | NM_008161| Gpx3        | Glutathione peroxidase 3   | 3.66                       |
| 32             | NM_007389| Chma1       | Cholinergic receptor, nicotinic, alpha polypeptide 1 (muscle) | 3.55 |
| 33             | NM_025869| Dusp26      | Dual specificity phosphatase 26 (putative) | 3.50 |
| 34             | NM_011158| Prkar2b     | Protein kinase, cAMP-dependent regulatory, type II beta | 3.44 |
| 35             | NM_175640| Plin1       | Perilipin 1                | 3.42                       |
| 36             | NM_001159487| Rbp4     | Retinol binding protein 4, plasma | 3.37 |
| 37             | NM_033037| Cdo1        | Cysteine dioxygenase 1, cytosolic | 3.37 |
| 38             | NM_026929| Chac1       | ChaC, cation transport regulator 1 | 3.37 |
| 39             | NM_181072| Myo1e       | Myosin IE                  | 3.35                       |
| 40             | NM_013459| Cfd         | Complement factor D (adipsin) | 3.34 |
| 41             | NM_029385| Nudt16      | Nudix (nucleoside diphosphate linked moiety X)-type motif 16 | 3.34 |
| 42             | NM_009675| Aoc3        | Amine oxidase, copper containing 3 | 3.32 |
| 43             | NM_009127| Scd1        | Stearoyl-Coenzyme A desaturase 1 | 3.30 |
| 44             | NM_007469| Apoc1       | Apolipoprotein C-I         | 3.27                       |
| 45             | NM_177733| E2f2        | E2F transcription factor 2  | 3.25                       |
| 46             | NM_013869| Tnfrsf19    | Tumor necrosis factor receptor superfamily, member 19 | 3.23 |
| 47             | NM_010864| Myo5a       | Myosin VA                  | 3.21                       |
| 48             | NM_029803| Ifi272a     | Interferon, alpha-inducible protein 27 like 2A | 3.19 |
| 49             | NM_010828| Cited2      | Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2 | 3.14 |
| 50             | NM_017370| Hp          | Haptoglobin                | 3.13                       |
| SystematicName | GeneName | Description | Fold (FOXO1-Tg/ Wild-type) |
|----------------|----------|-------------|--------------------------|
| NM_145400      | Ube4a    | Ubiquitination factor E4A, UFD2 homolog (S. cerevisiae) | 3.06 |
| NM_133838      | Ehd4     | EH-domain containing 4 | 3.05 |
| NM_007639      | Cd1d1    | CD1d1 antigen | 3.05 |
| NM_001013826   | Dupd1    | Dual specificity phosphatase and pro isomerase domain containing 1 | 2.95 |
| NM_023625      | Plbd2    | Phospholipase B domain containing 2 | 2.95 |
| NM_013822      | Jag1     | Jagged 1 | 2.93 |
| NM_177409      | Tram2    | Translocating chain-associating membrane protein 2 | 2.90 |
| NM_020581      | Angprd4  | Angiopoietin-like 4 | 2.89 |
| NM_009822      | Runxd1   | Runx-related transcription factor 1; translocated to, 1 (cyclin D-related) | 2.89 |
| NM_146001      | Hip1     | Huntington-interacting protein 1 | 2.89 |
| NM_011430      | Snog     | Synuclein, gamma | 2.89 |
| NM_007679      | Cebpd    | CCAAT/enhancer binding protein (C/EBP), delta | 2.88 |
| NM_011580      | Thbs1    | Thrombospondin 1 | 2.85 |
| NM_008630      | Mi2      | Metallothionein 2 | 2.84 |
| NM_133955      | Rhou     | Ras homolog gene family, member U | 2.83 |
| NM_025888      | Kctd20   | Potassium channel tetramerization domain containing 20 | 2.82 |
| NM_008198      | C1b      | Complement factor B | 2.81 |
| NM_019432      | Tmem37   | Transmembrane protein 37 | 2.71 |
| NM_013468      | Ankrd1   | Ankyrin repeat domain 1 (cardiac muscle) | 2.71 |
| NM_025593      | Polr2l   | Polymerase (RNA) II (DNA directed) polypeptide L | 2.70 |
| NM_001198823   | App      | Amyloid beta (A4) precursor protein (App) | 2.69 |
| NM_178087      | Pml      | Promyelocytic leukemia | 2.68 |
| NM_138673      | Stab2    | Stabilin 2 | 2.66 |
| NM_007569      | Btg1     | B-cell translocation gene 1, antiproliferative | 2.66 |
| NM_009984      | Cts1     | Cathepsin L | 2.63 |
| NM_009508      | Car2     | Carbonic anhydrase 2 | 2.63 |
| NM_008055      | Fzd4     | Frizzled homolog 4 (Drosophila) | 2.60 |
| ENSMUST00000030257 | Cachd1 | Cache domain containing 1 | 2.58 |
| NM_146251      | Prpla7   | Patatin-like phospholipase domain containing 7 | 2.58 |
| NM_197986      | Tmem140  | Transmembrane protein 140 | 2.58 |
| NM_001198884   | Tcof1    | Treacher Collins Fraschetti syndrome 1, homolog | 2.58 |
| NM_009201      | Slc1a5   | Solute carrier family 1 (neutral amino acid transporter), member 5 | 2.54 |
| NM_001145963   | Lgals3   | Lectin, galactose binding, soluble 3 | 2.52 |
| NM_133977      | Trf      | Transferrin | 2.50 |
| NM_001081349   | Slc43a1  | Solute carrier family 43, member 1 | 2.50 |
| NM_029083      | Ddit4    | DNA damage-inducible transcript 4 | 2.50 |
| NM_009780      | C4b      | Complement component 4B | 2.50 |
| NM_010097      | Sparcl1  | SPARC-like 1 | 2.49 |
| NM_001101433   | Zcchc24  | Zinc finger, CCHC domain containing 24 | 2.48 |
| NM_133198      | Pygl     | Liver glycogen phosphorylase | 2.44 |
| NM_026439      | Ccdc80   | Coiled-coil domain containing 80 | 2.44 |
| NM_019412      | Prx      | Periakin | 2.41 |
| NM_148927      | Plekh4a  | Pleckstrin homology domain containing, family A | 2.41 |
| NM_181390      | Mus1n1   | Musculoskeletal, embryonic nuclear protein 1 | 2.40 |
| NM_001097644   | Ccnly1   | Cyclin Y-like 1 | 2.39 |
| NM_026330      | Nsmce1   | Non-SMC element 1 homolog (S. cerevisiae) | 2.38 |
| NM_008037      | Fosl2    | Fos-like antigen 2 | 2.36 |
| NM_001039386   | Nsmf     | NMDA receptor synaptonuclear signaling and neuronal migration factor | 2.35 |
| NM_023587      | Ptptb    | Protein tyrosine phosphatase-like (proline instead of catalytic arginine), member b | 2.32 |
| NM_011785      | Akt3     | Thymoma viral proto-oncogene 3 | 2.32 |
### Table 2. List of genes in skeletal muscle with decreased expression levels in FOXO1-Tg mice compared with wild-type control mice. Top 100 genes are shown. PGC-1β is highlighted.

| SystematicName | GeneName | Description | Fold (FOXO1-Tg/ Wild-type) |
|----------------|----------|-------------|---------------------------|
| NM_010292      | Gck      | Glucokinase | 0.06                      |
| NM_001081324   | Neto2    | Neuropilin (NRP) and tolloid (TLL)-like 2 | 0.06                      |
| NM_001033473   | Odf3l2   | Outer dense fiber of sperm tails 3-like 2 | 0.06                      |
| NM_198112      | Ostein   | Osteocrin   | 0.06                      |
| NM_011825      | Grem2    | Gremlin 2 homolog, cysteine knot superfamily (Xenopus laevis) | 0.06                      |
| NM_009867      | Cdh4     | Cadherin 4 | 0.08                      |
| NM_053250      | Crip3    | Cysteine-rich protein 3 (Crip3), transcript variant TLP-B | 0.09                      |
| NM_009700      | Aqp4     | Aquaporin 4 | 0.10                      |
| NM_177787      | Slc15a5  | Solute carrier family 15, member 5 | 0.12                      |
| NM_144547      | Amhr2    | Anti-Mullerian hormone type 2 receptor | 0.13                      |
| NM_013467      | Aldh1a1  | Aldehyde dehydrogenase family 1, subfamily A1 | 0.14                      |
| NM_030017      | Rdh12    | Retinol dehydrogenase 12 | 0.15                      |
| NM_011487      | Aurora   | Aurora kinase A | 0.16                      |
| NM_001081160   | Mga1     | MAM domain containing glycosylphosphatidylinositol anchor 1 | 0.16                      |
| NM_001012799   | Methyltransferase like 21C | 0.16                      |
| NM_001024539   | Shc2     | SHC (Src homology 2 domain containing) transforming protein 2 | 0.17                      |
| NM_144860      | Mb1      | Mindbomb homolog 1 (Drosophila) | 0.18                      |
| NM_176920      | Ltrn1    | Leucine-rich repeats and transmembrane domains 1 | 0.18                      |
| NM_016749      | Mybph    | Myosin binding protein H | 0.18                      |
| NM_010061      | Dnase1   | Deoxyribonuclease I | 0.19                      |
| NM_029104      | Mss51    | MSS51 mitochondrial translational activator (Mss51), nuclear gene encoding mitochondrial protein | 0.20                      |
| NM_001177841   | Otub2    | OTU domain, ubiquitin aldehyde binding 2 | 0.20                      |
| NM_010019      | Dapk2    | Death-associated protein kinase 2 | 0.20                      |
| NM_025998      | Nkain1   | Na+/K+-transporting ATPase interacting 1 | 0.20                      |
| NM_011943      | Map2k6   | Mitogen-activated protein kinase kinase 6 | 0.22                      |
| NM_194060      | Foxo6    | Forkhead box O6 | 0.22                      |
| NM_028638      | Gad1     | Glutamate decarboxylase-like 1 | 0.22                      |
| NM_009393      | Itpr1    | Inositol 1,4,5-trisphosphate receptor 1 | 0.25                      |
| NM_01253822    | Inx3     | Iroquois-related homeobox 3 (Drosophila) | 0.26                      |
| NM_013737      | Pla2g7   | Phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasmal) | 0.26                      |
| NM_019636      | Tbc1d1   | TBC1 domain family, member 1 | 0.26                      |
| NM_016719      | Grb14    | Growth factor receptor bound protein 14 | 0.27                      |
| NM_015814      | Dkk3     | dickkopf homolog 3 (Xenopus laevis) | 0.28                      |
| NM_010267      | Gdap1    | Ganglioside-induced differentiation-associated-protein 1 | 0.29                      |
| NM_022314      | Tpm3     | Troponin C, cardiac/slow skeletal | 0.29                      |
| NM_007642      | Cd28     | CD28 antigen | 0.29                      |
| NM_010246      | Fzd9     | frizzled homolog 9 (Drosophila) | 0.29                      |
| NM_026999      | Zfp688   | Zinc finger protein 688 | 0.30                      |
| NM_031997      | Tmem2    | Transmembrane Protein 2 | 0.30                      |
| NM_016854      | Ppp1r3c  | Protein phosphatase 1, regulatory (inhibitor) subunit 3C | 0.30                      |
| NM_001159344   | Casz1    | Castor zinc finger 1 | 0.30                      |
| NM_027402      | Fndc5    | Fibronectin type III domain containing 5 | 0.30                      |
| BC019757       | Hist1h4i | Histone cluster 1, H4i | 0.30                      |
| NM_010859      | Myl3     | Myosin, light polypeptide 3 | 0.30                      |
| NM_207161      | Dnph1    | 2’-deoxynucleoside 5’-phosphate N-hydrolase 1 | 0.31                      |
| NM_026884      | Fam57b   | Family with sequence similarity 57, member B | 0.32                      |
| NM_010861      | Myl2     | Myosin, light polypeptide 2, regulatory, cardiac, slow | 0.32                      |
| NM_010518      | Igfbp5   | Insulin-like growth factor binding protein 5 | 0.32                      |
| NM_027963      | Wdr16    | WD repeat domain 16 | 0.32                      |
| SystematicName | GeneName | Description | Fold (FOXO1-Tg/Wild-type) |
|----------------|----------|-------------|--------------------------|
| NM_001081063   | Prss55   | Protease, serine, 55 | 0.32                     |
| NR_037996      | Hmga2-ps1| HIGH-mobility group AT-hook 2, pseudogene 1 | 0.32                     |
| NM_027161      | Tmem52   | Transmembrane protein 52 | 0.32                     |
| NM_019563      | Cited4   | Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 4 | 0.33                     |
| NM_175511      | Fam78a   | Family with sequence similarity 78, member A | 0.33                     |
| NM_175276      | Phod3    | Formin homology 2 domain containing 3 | 0.34                     |
| NM_018760      | Sloc4a4  | Solute carrier family 4 (anion exchanger), member 4 | 0.34                     |
| ENSMUST00000108587 | Tnt1 | Troponin T1, skeletal, slow | 0.35                     |
| NM_008852      | Pitx3    | Paired-like homeodomain transcription factor 3 | 0.35                     |
| NM_080728      | Myh7     | Myosin, heavy polypeptide 7, cardiac muscle, beta | 0.36                     |
| NM_018832      | Magix    | MAGI family member, X-linked | 0.37                     |
| NM_001170488   | Tprkb    | Tp53r binding protein | 0.37                     |
| NM_030241      | Setdb1   | SET domain containing (lysine methyltransferase) 8 | 0.37                     |
| NM_007431      | Alpi     | Alkaline phosphatase, liver/bone/kidney | 0.37                     |
| NM_181577      | Ccd85a   | Coiled-coil domain containing 85A | 0.37                     |
| NM_001122683   | Bdh1     | 3-hydroxybutyrate dehydrogenase, type 1 | 0.37                     |
| NM_011983      | Homer2   | Homer homolog 2 (Drosophila) | 0.37                     |
| NM_011638      | Tfic     | Transferrin receptor | 0.37                     |
| NM_030179      | Clip4    | CAP-GLY domain containing linker protein family, member 4 | 0.37                     |
| NM_198190      | Tnni1    | Troponin T1, skeletal, slow | 0.40                     |
| NM_0010834     | Mstn     | myostatin | 0.38                     |
| NM_001085378   | Myh7b    | Myosin, heavy chain 7B, cardiac muscle, beta | 0.38                     |
| NM_177603      | Frat2    | Frequently rearranged in advanced T cell lymphomas 2 | 0.38                     |
| NM_000519      | Wnt11    | Wingless-related MMTV integration site 11 | 0.39                     |
| NM_133363      | Myoz3    | Myogenin 3 | 0.39                     |
| NM_027307      | Gom1     | Golgi membrane protein 1 | 0.39                     |
| NM_027678      | Znab3    | Zinc finger, RAN-binding domain containing 3 | 0.40                     |
| NM_001160262   | Fam78b   | Family with sequence similarity 78, member B | 0.40                     |
| NM_148958      | Obsp10   | Oxysterol binding protein-like 10 | 0.40                     |
| EU616813       | Mrg      | Clone E19 5E_C11 maternally expressed gene 9 | 0.40                     |
| NM_021467      | Tnn1     | Troponin I, skeletal, slow | 0.40                     |
| NR_003280      | Rs5-8s1  | 5.8S ribosomal RNA | 0.41                     |
| NM_080595      | Emid1    | EMI domain containing 1 | 0.41                     |
| NM_001109040   | Kif21a   | Kinesin family member 21A | 0.41                     |
| NM_0033478     | Ly6g6d   | Lymphocyte antigen 6 complex, locus G6D | 0.41                     |
| NM_173745      | Dusp18   | Dual specificity phosphatase 18 | 0.41                     |
| NM_018803      | Syt10    | Synaptotagmin X | 0.41                     |
| NM_001252310   | Fam19a   | Family with sequence similarity 19, member A5 | 0.41                     |
| NM_011160      | Prkg1    | Protein kinase, cGMP-dependent, type I | 0.42                     |
| NM_0020263     | Psd3     | Pleckstrin and Sec7 domain containing 3 | 0.43                     |
| NM_010866      | Myod1    | Myogenic differentiation 1 | 0.43                     |
| NM_008421      | Kcc1     | Potassium voltage-gated channel, Shaw-related subfamily, member 1 | 0.43                     |
| NM_009107      | Rrgx     | Retinoid X receptor gamma | 0.44                     |
| NM_133249      | Pparc1b  | Peroxisome proliferative activated receptor, gamma, coactivator 1 beta | 0.44                     |
| NM_008596      | Sypl2    | Sypaptophysin-like 2 | 0.44                     |
| NM_001272024   | Sema6c   | Sema domain, transmembrane domain (TM), and cytoplasmic domain (semaphorin) 6C | 0.44                     |
| NM_011103      | Prkcd    | Protein kinase C, delta | 0.44                     |
target cathepsin L, along with decreased PGC-1β and MCAD expression (Fig. 3). Plaster cast also increased 36B4 (reference gene) expression. We used another reference gene (18S), whose expression was not increased by plaster cast, and observed significant decrease in PGC-1β expression, as well as MCAD expression (Fig. 3).

We also attempted to apply another condition with changed FOXO1 expression: fasting and refeeding. Fasting for 8 or 24 h increased FOXO1 expression in skeletal muscles. Fasting for 24 h followed by refeeding for 4 h downregulated the FOXO1 mRNA expression. Previously, we confirmed increased endogenous FOXO1 protein levels after 24-h fasting [5]. Cathepsin

**Fig. 1.** Gene expression analysis of FOXO1, cathepsin L, PGC-1β, and MCAD in the skeletal muscles of FOXO1-overexpressing mice. (A) FOXO1 was remarkably expressed in FOXO1-Tg mice. Cathepsin L, the target gene of FOXO1, was also increased in FOXO1-Tg mice. In contrast, the expression of PGC-1β and MCAD decreased in FOXO1-Tg mice. Quantitative real-time RT-PCR data from wild-type (WT) control mice were set at 100 arbitrary units. Each value is presented as the mean ± standard error (SE; N = 5). Statistical analyses were performed using Student’s two-tailed unpaired t-test. ***P < 0.001, **P < 0.01 versus wild-type. (B) Western blotting analysis of skeletal muscle from FOXO1-Tg mice.

**Fig. 2.** Gene expression analysis of FOXO1, cathepsin L, PGC-1β, and MCAD in the skeletal muscles of denervated mice. The expression of FOXO1 increased in mice that had undergone denervation. Cathepsin L was also increased in denervated mice. Denervation significantly reduced the expression of PGC-1β and MCAD. Quantitative real-time RT-PCR data from control samples were set at 100 arbitrary units. Each value is presented as the mean ± SE (control: N = 3, denervation: N = 4). Statistical analyses were performed using Student’s two-tailed unpaired t-test. ***P < 0.001, **P < 0.01, *P < 0.05 versus control.
L expression was gradually increased by fasting for 8 and 24 h, but it was not decreased by refeeding for 4 h. Therefore, cathepsin L mRNA may be stable against degradation for this period. PGC-1β expression was gradually decreased by fasting for 8 and 24 h. Interestingly, refeeding for 4 h after fasting for 24 h recovered the PGC-1β expression, compared with that upon fasting for 24 h alone (Fig. 4). MCAD expression was slightly decreased by fasting (8 or 24 h) and not markedly changed by refeeding (Fig. 4). Taking these findings together, in the skeletal muscles of mice, an inverse correlation was observed between FOXO1 and PGC-1β (Figs 1–4), suggesting that PGC-1β expression is negatively regulated by FOXO1.

**Decreased PGC-1β expression and decreased markers of mitochondrial density**

PGC-1β is known to increase mitochondrial content [12]; therefore, we examined mtDNA levels and mitochondrial enzyme CS activity as markers of mitochondrial density. Mitochondrially encoded COX2 (Cox2)
DNA levels were slightly decreased, and CS activity was also significantly decreased in FOXO1-Tg mice (Fig. 5A). In addition, decreased mtDNA levels and decreased CS activity were observed after denervation (Fig. 5B). Moreover, fasting for 24 h caused decreased mtDNA level and decreased CS activity (Fig. 5C). Thus, decreased PGC-1β mRNA levels caused by FOXO1 appeared to lead to decreased functional PGC-1β protein expression, concomitant with decreased mitochondrial content.

Attenuation of decreased PGC-1β expression by fasting in the skeletal muscles of FOXO1-KO mice

For loss-of-function experiments, we used skeletal muscle-specific FOXO1-KO mice [5]. In wild-type control mice, fasting caused increased FOXO1 mRNA levels concomitant with increased cathepsin L mRNA (Fig. 6). In FOXO1-KO mice, FOXO1 mRNA levels were very low in both fed and fasting samples, as expected. In a previous study, we confirmed diminished endogenous FOXO1 protein levels in the skeletal muscle of FOXO1-KO mice in fed and fasting conditions [5]. In FOXO1-KO mice, fasting-induced cathepsin L mRNA expression was significantly attenuated (Fig. 6). In wild-type mice, PGC-1β mRNA levels were decreased by fasting. On the other hand, the fasting-induced PGC-1β mRNA decrease was significantly attenuated in FOXO1-KO mice. The data indicated that fasting-caused PGC-1β mRNA decrease was likely to be mediated by FOXO1.

PGC-1β gene expression change induced by FOXO1 activation in C2C12 cells

In order to understand the causal relationship between FOXO1 expression and PGC-1β expression, we used a
tamoxifen-inducible FOXO1 activation system in C2C12 myoblast cells. Namely, tamoxifen treatment induces the translocation of FOXO1-ER fusion protein (FOXO1-ER) from the cytoplasm to the nucleus and causes FOXO1-mediated target gene activation [4,23]. In the presence of tamoxifen (for 24 h), FOXO1 mRNA expression remained unchanged (Fig. 7A), which is consistent with the findings of a previous study [4]. Cathepsin L expression was increased by tamoxifen treatment, indicating FOXO1 activation. Interestingly, in the presence of tamoxifen (FOXO1 activation), there was a marked decrease of PGC-1β expression (Fig. 7A). Thus, PGC-1β gene expression was negatively regulated by FOXO1 in C2C12 myoblast cells. In this experiment, MCAD expression was slightly increased by tamoxifen treatment.

Forkhead box protein O1 was reported to suppress muscle cell differentiation [1,25,26]; therefore, we examined the change in muscle differentiation marker gene expression during this experimental period. We examined the time course of PGC-1β and differentiation marker gene expression. C2C12 cells expressing FOXO1-ER were treated with tamoxifen, and at 3, 6, 8, and 24 h after treatment, mRNA expression levels were examined. Three hours after treatment, PGC-1β and MyoD and myogenin (differentiation marker genes) mRNA levels were decreased (Fig. 7B). We also observed microscopic views of cells; there were no marked phenotypical changes between the vehicle (control) and tamoxifen treatment groups during this time period (Fig. 7B). We used cells without differentiation stimuli (not using differentiation medium) in confluent cells. Thus, we consider that the cells did not differentiate in these conditions. Thus, the decreased PGC-1β mRNA levels were not likely to be caused by decreased differentiation (not a result of the differentiation process), but by direct suppression by FOXO1.

**FOXO1 expression and PGC-1α expression changes**

For comparison, we also examined PGC-1α (PGC-1β homologue) expression in the samples used for Figs 1–4, 6, and 7 (Fig. 8). PGC-1α expression was decreased in FOXO1-Tg mice and in those subjected to denervation, but not unloading with a plaster cast, while PGC-1α expression was decreased in these groups. Meanwhile, upon fasting for 8 h, PGC-1α expression did not change; however, upon fasting for 24 h followed by refeeding, PGC-1α expression was decreased (Fig. 8). In FOXO1-KO mice, fasting (24 h) caused decrease of PGC-1α expression was attenuated (Fig. 8). In the case of the tamoxifen-activated FOXO1-ER experiment, PGC-1α expression was not decreased but rather significantly increased (Fig. 8). Thus, PGC-1α expression appears not to be simply downregulated by FOXO1 activation. PGC-1α is also known to increase MCAD expression [11]. Thus, the increased MCAD level observed in the FOXO1-ER cells (Fig. 7A) may be explained by the increased PGC-1α expression, but other possibilities should also be considered.
FOXO1 suppresses PGC-1β expression in muscles

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Fig. 7. Effect of FOXO1 activation on the expression of PGC-1α in C2C12 cells. (A) Tamoxifen (TAM) was added to FOXO1-ER cells, and 24 h later, mRNA expression was analyzed. Expression levels of FOXO1, cathepsin L, PGC-1α, and MCAD are shown. Quantitative real-time RT-PCR data from controls were set at 100 arbitrary units. Each value is presented as the mean ± SE (N = 4). Statistical analyses were performed using Student’s two-tailed unpaired t-test. ***P < 0.001, **P < 0.01, *P < 0.05 versus control. (B) Time course (3, 6, 8, and 24 h) after tamoxifen treatment; microscopic views were observed, and mRNA expression levels were analyzed. Scale bars, 100 μm. Quantitative real-time RT-PCR data from 0 h were set at 100 arbitrary units. Each value is presented as the mean ± SE (N = 4). Statistical analyses were performed using Student’s two-tailed unpaired t-test. ***P < 0.001, **P < 0.01, *P < 0.05 versus control (vehicle).

Possible physiological significance and mechanism of FOXO1-mediated suppressed PGC-1α expression

In this study, we observed that the activation of FOXO1 suppressed PGC-1α expression in skeletal muscles and myoblast cells. We obtained a clue regarding the mechanism of PGC-1α gene regulation, which was previously largely unknown. FOXO1 activation causes skeletal muscle atrophy [3], and PGC-1α activation causes increased energy expenditure [10]. During atrophy with FOXO1 activation, decreased PGC-1α with decreased energy expenditure appears to be physiologically reasonable, to avoid wasting energy in order to prevent a greater decrease of muscle mass.

Forkhead box protein O1 has been reported to increase the degradation of mitochondria, leading to a decrease in mitochondrial content [27]. As described in the Introduction, PGC-1α increases mitochondrial content [12]. Thus, FOXO1 caused downregulation of PGC-1α as described in this study, which is consistent with decreased mitochondrial content. Indeed, in FOXO1-Tg mice, the amount of red muscle fiber, which is rich in mitochondria, is decreased [3].

Fig. 8. Gene expression of PGC-1α in skeletal muscles and cells. The expression of PGC-1α was examined in the samples used in Figs 1–4, 6, and 7A. Each value is presented as the mean ± SE. For FOXO1-Tg experiment, statistical analyses were performed using Student’s two-tailed unpaired t-test (N = 5). **P < 0.01 versus wild-type. For denervation experiment, statistical analyses were performed using Student’s two-tailed unpaired t-test (control: N = 3, denervation: N = 4). ***P < 0.001 versus control. For casting experiment, statistical analyses were performed using Student’s two-tailed unpaired t-test (control: N = 5, casting: N = 4). For fasting experiment, statistical analyses were performed using one-way analysis of variance followed by Tukey’s post hoc test (N = 6). ***P < 0.001 versus fast for 8 h; †††P < 0.001 versus fast for 24 h. For FOXO1-KO experiment, statistical analyses were performed using two-way analysis of variance followed by Tukey’s post hoc test (wild-type fed, n = 3; wild-type fasted, n = 4; KO fed, n = 4; KO fasted, n = 4). ***P < 0.001, *P < 0.05. For FOXO1-ER experiment, statistical analyses were performed using Student’s two-tailed unpaired t-test (N = 6). ***P < 0.001 versus control.
Additionally, the skeletal muscle of mice with plaster cast or denervation shows a decreased red muscle fiber level, that is, decreased mitochondria concomitant with increased FOXO1 expression [3,18]. Thus, decreased mitochondrial content with increased FOXO1 expression may be mediated by FOXO1-induced PGC-1β suppression.

Meanwhile, how FOXO1 downregulates the PGC-1β gene is currently unclear. FOXO1 binds to the genomic DNA sequence, with the Daf16 binding element (DBE) (consensus: TT[G/A]TTTAC) [28] or insulin response element (IRE; consensus: TT[G/A]TTTTG) [29]. However, there were no consensus DBE or IRE up to 2 kb upstream from the transcription start site. Meanwhile, FOXO1 has been reported to physically interact with other transcription factors, such as nuclear receptors, and to positively and negatively regulate target gene expression [30–32]. However, there were no typical nuclear receptor binding sites, such as glucocorticoid (atrophic hormone) receptor response elements (GREs; consensus: AGAAACA), up to 2 kb upstream from the transcription site. Meanwhile, Yasui et al. [8] reported Sp1 binding sites are involved in FOXO1-mediated repression of musclin gene expression. Notably, there are two putative Sp1 binding sites (consensus: GGGGCGGGG) [33] in the mouse PGC-1β gene at 0.1 and 1.2 kb upstream from the transcription start site (Fig. 9). Moreover, Shintaku et al. [34] showed transcription factors MyoD and RelB bind within the first intron of the PGC-1β gene and activate transcription. FOXO1 may regulate the PGC-1β gene expression by directly binding to the PGC-1β promoter, or by interacting with other transcription regulators (such as Sp1, MyoD, and RelB) binding to the PGC-1β promoter. On the other hand, microarray data showed

Putative Sp1 binding sites in the mouse PGC-1β gene

| Position | Sequence |
|----------|----------|
| -1306    | ATGGAAACACCCCTCGTCCCGCCTCCACACGGGTAATTATCCAGAC -1257 |
| -1256    | GCGAAGGAGCAGGAAGGTAGACTCTCGGAGGTCATGTAGGGGCGGGG -1207 |
| -1206    | GCACTCTGTGTGATCTACGGACGAGAACCCTTGTGCTCTCTCTACTCCAAG -1157 |
| -1156    | GTCCAAACTTCTAGTCTTCTCCAAGAAGGAGCCGGCGCAGTGCAAGG -1107 |
| -1106    | ACCAGACGTGAGACTCGGATCGGAGGAGTAGACATCTCTACTACCAC -1057 |

| Position | Sequence |
|----------|----------|
| -546     | TTGTATACTGAGCTTGGGAGACTCTCTCGGAGAGACTAAAGGCGGGGA -497 |
| -496     | AACTGGACACGCACAGAGGAGGAGGGAGGACCCGAGAAACGCAACGTC -447 |
| -446     | TCTCTCTCTCTCTTCTAAGCTCAGAGGCGTCAGAAAGGAAGAAACGCAACGTC -397 |
| -396     | TGCGTACGGCGGAAATTTCTGCAGGAGATCTCCAGGGCTGGAGCCAAG -347 |
| -346     | GGTGAGCATACATCTTCGTCTCCAAGAAGAGGTCCTCCGCGGCGGTTCGAGATTGC -297 |
| -296     | GTGCACTAAGCGAGATTTGCTATTTCTCTCCCAAGGGGTTGAAAGGCGGT -247 |
| -246     | CGTGAGAGACCGGCAGACGACGCGCGCTGCTGGGACAGGAGGCCCTCGAA -197 |
| -196     | AAAAGAGGGACGCCGCCCGTGCGGTGACAGGAGGCCCTCGAA -147 |
| -146     | TCGTCTTTTTTCGGCCACCTGAGGTGCTCCAGGCGAGCGCAGCAGGCGGGG -97 |
| -96      | GTCCGTCGCGCGCAGCGCGCGCAGCGCGCGCGCGCGCGCGCGCGCGCGCGCGGAGG -47 |
| -46      | GGGCGCGCCTCGGAGGAGAGGAGAGGAGACGCACGACGGCGACGCAGC | 4

Fig. 9. Putative Sp1 binding sites (GGGGCGGGG) in the promoter of the mouse PGC-1β gene. Upstream of the PGC-1β gene from +4 to -546 and -1057 to -1306 is shown. The transcription start site is counted as +1. The Sp1 binding sites (GGGGCGGGG) are underlined (−99 to −107 and −1207 to −1215).
decreased MyoD expression in the skeletal muscles of FOXO1-Tg mice, compared with that in wild-type control mice (0.43-fold; Table 2). Additionally, we observed decreased MyoD levels in C2C12 cells expressing FOXO1-ER using tamoxifen treatment (Fig. 7B). FOXO1 may suppress PGC-1β gene expression via suppressing MyoD expression. Further work is required to clarify this issue.

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Conflict of interest

The authors declare no conflict of interest.

Data accessibility

The microarray data were submitted to the GEO database (https://www.ncbi.nlm.nih.gov/geo/). The records have been assigned GEO accession numbers as GSE146919.

Author contributions

SN and YH analyzed the data and undertook the statistical analyses. MO and YH performed cell experiment and collected the data. SA and SM performed enzyme assays and mtDNA experiments. TK performed FOXO1-KO experiments. YK prepared the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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