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Irisin ameliorates age-associated sarcopenia and metabolic dysfunction

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Abstract

Background Age-associated sarcopenia is characterized of progressed loss of skeletal muscle power, mass, and function, which affects human physical activity and life quality. Besides, accompanied with sarcopenia, aged population also faces a series of metabolic dysfunctions. Irisin, the cleaved form of fibronectin type III domain-containing protein 5 (FNDC5), is a myokine induced by exercise and has been shown to exert multiple beneficial effects on health. The goal of the study is to investigate the alterations of Fndc5/irisin in skeletal muscles during ageing and whether irisin administration could ameliorate age-associated sarcopenia and metabolic dysfunction.

Methods The mRNA and protein levels of FNDC5/irisin in skeletal muscle and serum from 2- and 24-month-old mice or human subjects were analysed using qRT-PCR and western blot. FNDC5/irisin knockout mice were generated to investigate the consequences of FNDC5/irisin deletion on skeletal muscle mass, as well as morphological and molecular changes in muscle during ageing via histological and molecular analysis. To identify the therapeutic effects of chronic irisin treatment in mice during ageing, *in vivo* intraperitoneal administration of 2 mg/kg recombinant irisin was performed three times per week in ageing mice (14-month-old) for 4 months or in aged mice (22-month-old) for 1 month to systematically investigate irisin's effects on age-associated sarcopenia and metabolic performances, including grip strength, body weights, body composition, insulin sensitivity, energy expenditure, serum parameters and phenotypical and molecular changes in fat and liver.

Results We showed that the expression levels of irisin, as well as its precursor Fndc5, were reduced at mRNA and protein expression levels in muscle during ageing. In addition, via phenotypic analysis of FNDC5/irisin knockout mice, we found that FNDC5/irisin deficiency in aged mice exhibited aggravated muscle atrophy including smaller grip strength (-3.23%, $P < 0.05$), muscle weights (quadriceps femoris [QU]: -20.05%; gastrocnemius [GAS]: -17.91%; tibialis anterior [TA]: -19.51%, all $P < 0.05$), fibre size (QU: $P < 0.01$) and worse molecular phenotypes compared with wild-type mice. We then delivered recombinant irisin protein intraperitoneally into ageing or aged mice and found that it could improve sarcopenia with grip strength ($+18.42\%$, $P < 0.01$ or $+13.88\%$, $P < 0.01$), muscle weights (QU: $+9.02\%$, $P < 0.01$ or $+16.39\%$, $P < 0.05$), fibre size (QU: both $P < 0.05$) and molecular phenotypes and alleviated age-associated fat tissues expansion, insulin resistance and hepatic steatosis (all $P < 0.05$), accompanied with altered gene signatures.

Conclusions Together, this study revealed the importance of irisin in the maintenance of muscle physiology and systematic energy homeostasis during ageing and suggested a potent therapeutic strategy against age-associated metabolic diseases via irisin administration.

Keywords FNDC5; irisin; ageing; sarcopenia; metabolic dysfunction

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Introduction

Accompanied with increased life expectancy, human have stepped into the ageing society and faced challenges in public health globally. Ageing is the key risk factor for various diseases including sarcopenia, metabolic dysfunction, cardiovascular diseases and neurodegeneration caused by increased incidence of genetic material instability, protein homeostatic loss and cellular senescence during ageing.[1]

Skeletal muscle, including glycolytic (white) and oxidative (red) muscles, is the most abundant tissue accounting for 40% of the total mass in healthy individuals, which controls locomotion, energy expenditure and whole-body metabolic homeostasis.[2] During ageing, skeletal muscle undergoes functional decline, leading to ageing-associated sarcopenia characterized by a significant reduction in skeletal muscle mass and functionality, which inflicts majorly on life quality and longevity.[3] Muscle atrophy is associated with fibre-type specific senescence and it appears that ageing elicits preferential atrophy of glycolytic fibres.[4] Accompanied with age-associated muscle loss, aged population also faces a series of metabolic dysfunctions, including insulin resistance, nonalcoholic fatty liver disease or hepatitis.[5] Enhancement in the functionality of skeletal muscle by exercises has been shown to increase insulin sensitivity and lean mass, improve liver health, reverse age-related changes in protein homeostasis and extend the lifespan of elderly individuals. However, due to various pre-existing diseases and declined capability in exercise or even mobility during ageing, it is difficult to achieve sufficient exercise training in some elderly populations, which leads to a vicious cycle of exercise insufficiency and sarcopenia. Thus, novel interventions that could mimic the benefits of exercises hold great potential as alternative strategies against sarcopenia and its related defects.

Irisin, the cleaved form of fibronectin type III domain-containing protein 5 (FNDC5), is a newly identified myokine that features potent induction by exercise. Irisin has been shown to exert function in an autocrine fashion to promote skeletal muscle hypertrophy and rescue denervation-induced muscle wasting.[6] Besides, circulating irisin has versatile beneficial effects in multiple tissues and organs, that is, promoting energy expenditure and ameliorating diet induced obesity, hepatic steatosis and insulin resistance by stimulating the browning of subcutaneous white fat, improving cognitive function and synaptic plasticity, and is involved in cardiovascular functionality and bone remodelling.[7–10] Notably, aged population often characterizes dysregulated metabolism, declined cognition, heart diseases and loss of bone mass. Thus, although it has been reported that young Fndc5/irisin mutant mice have no obvious defect on developmental or postnatal growth and that Fndc5/irisin is dispensable for skeletal muscle growth, regeneration, and myofibre types distribution, as well as glucose and lipid metabolism in mice under both chow and high-fat diet.[11] It would thus be pragmatic to study the role of FNDC5/irisin during ageing.

In the present study, we aim to investigate the alterations of Fndc5/irisin in skeletal muscles during ageing and whether irisin administration ameliorates age-associated sarcopenia and metabolic dysfunction. Of note, we found that the mRNA and protein levels of Fndc5/irisin in muscles were reduced during ageing. Ageing FNDC5/irisin knockout mice exhibit accelerated loss in muscle mass and strength. Intraperitoneal delivery of recombinant irisin protein in ageing (14-month-old) and aged (22-month-old) mice alleviated ageing-associated sarcopenia and metabolic disorders, including adiposity, insulin resistance and hepatic steatosis. Overall, our study suggested that irisin supplementation may function as a potent therapeutic strategy to combat sarcopenia and ageing-associated metabolic dysfunction.

Materials and methods

Animal studies

C57BL/6J mice were purchased from Shanghai Laboratory Animal Center (Shanghai, China). Fndc5 KO mice were generated by Viewsolid Biotech via targeting Exon 3. The animals were housed in specific-pathogen-free conditions with a 12-h light/dark cycle and had free access to standard chow and water. All mouse studies were approved by the Animal Ethics Committee of East China Normal University. The 2-month-old and 24-month-old mice were used for the comparison of young and aged mice. Wild-type and FNDC5/irisin knockout mice (KO) at 22-month-old were analysed. To analyse the influence of recombinant irisin on metabolism, ageing mice at 14-month-old or aged mice at 22-month-old were subjected to intraperitoneal irisin or control His-tag

administration three times a week for 4 months or 1 month, respectively. Body composition was measured by AccuFat-1050 NMR system (MAGMED) and body weight was recorded weekly. Mice were kept in separate cages of CLAMS system (Columbus instruments) for 5 days. Oxygen consumption, carbon dioxide production and energy expenditure were recorded. A digital grip-strength meter (BIOSEB Research Instruments, BIO-GS3) was used to measure the limb muscular strength of mice as previously reported.[12] Briefly, the mice were acclimatized to the meter for 10 min before the grip strength test began. Mice were permitted to grab the metal pull bars and were pulled backwards by the tail. The force at the time of release was recorded as the peak tension. Each mouse was tested five times with a 30-s break, and the average data were used for statistical analysis. Investigators were blind to the animal groups.

Recombinant irisin intraperitoneal injection

Mouse irisin recombinant protein was expressed and purified using pET expression system (Novagen). Briefly, mouse irisin complementary DNA (cDNA) was PCR-amplified and cloned into pET-32a expression vector in frame with 1 Flag and 6X histidine residues. The plasmid was transformed into *E. coli* strain BL21 and cultured in Lennox broth (LB) containing ampicillin (100 mg/L). The BL21 strain was induced to produce histidine-tag irisin by addition of 0.5-mM IPTG for 16 h. Lysates of BL21 strain were sonicated with 200-W power with 2 s interval for 20 min, then the lysates were centrifuged, and the supernatants were collected and run through the Ni-NTA agarose affinity column (GE) for recombinant irisin purification. The irisin proteins eluted from Ni-NTA agarose column were pooled and dialyzed in phosphate-buffered saline (PBS) buffers (pH = 7.4) with endotoxin examination. The purity of the recombinant irisin was examined by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot (*Figure S1*) and the concentration of irisin was identified via BCA protein assay kit (Beyotime Biotechnology, China).

For irisin administration, ageing mice at 14-month-old or aged mice at 22-month-old were subjected to intraperitoneal irisin administration at a dose of 2 mg/kg or control His-tag three times a week (Monday, Wednesday and Friday) for 4 months or 1 month, respectively.

Glucose and insulin tolerance tests

After irisin treatment, glucose and insulin tolerance tests were performed. Briefly, for glucose tolerance test (GTT) analysis, the animals were fasted 6 h and injected intraperitoneally with 1.5-g/kg body weight of glucose (Sigma, G8270) dissolved in PBS. Vein blood was obtained from the tail tip at 0, 15, 30, 60, 90 and 120 min for the measurement of plasma glucose levels via an automatic glucometer (OneTouch Ultra, Johnson's). For insulin tolerance test (ITT) analysis, mice received an intraperitoneal injection of insulin (Thermo Fisher Scientific, I9278) at a dose of 0.75 U/kg, and plasma glucose levels were measured at 0, 15, 30, 60, 90 and 120 min. A concentration–time curve was plotted, and the area under the curve (AUC) was calculated by GraphPad 7 software.

Histological analysis

Muscle tissues were freshly isolated and embedded in (OCT and frozen at −80°C. adipose tissues were fixed in a 10% neutral formaldehyde solution and embedded in paraffin according to standard procedures. The 10-μm transverse cryosections and 5-μm paraffin sections were prepared from muscle and fat tissues respectively for subsequent haematoxylin and eosin (H&E) staining. A Nikon camera was used to visualize sections. The cross-sectional areas (CSAs) of muscle fibre and adipocyte size were measured by Image-Pro Plus 6.0 software with five random fields per mouse.[13] The fibrotic quantifications of iWAT were analysed as previously described.[14]

Real-time PCR

Total RNA was isolated from the white adipose tissue and skeletal muscle using RNAiso Plus (Takara, 9108) according to the manufacturer's instructions. A 1 μg of total RNA was reverse transcribed to cDNA with the PrimeScript™ RT Master Mix (TaKaRa, RR036A). Quantitative real-time PCR was carried out with the Roche Light Cycler 480 system (Roche) using SYBR Green Master mix (Yeasen, 11143ES50). The primer sequences were listed in Table S1. *Gapdh* is used as reference gene in skeletal muscles, and *36b4* is used as reference gene in fat and liver.

Western blots

Protein extraction was performed with RIPA buffer containing 50-mM Tris (pH 7.4), 150-mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, sodium orthovanadate, sodium fluoride, EDTA, leupeptin, supplemented with 1-mM PMSF, 10-mM DTT and 10-μM protein kinase inhibitor. The protein concentrations were quantified using a BCA protein quantification kit (Beyotime) and the samples from the SDS-PAGE gels were transferred to a membrane of nitrocellulose by electrophoretic transfer. Membranes were blocked with 5% skimmed milk for 1 h at room temperature and then incubated with anti-FNDC5/irisin (Abcam, ab174833), anti-

MAFbx (Santa Cruz, sc-166806), anti-MuRF-1 (Santa Cruz, sc-398608), anti-PGC1α (Santa Cruz, sc-517380), anti-UCP1 (Abcam, ab234430), anti-mitochondrial complexes (Abcam, ab110413) and anti-β-actin (Santa Cruz, sc-47778) antibodies. The membranes were washed with TBST three times and incubated with IRDye secondary antibodies (926-68071 and 926-322210) from LI-COR Biosciences (NE, USA). The protein bands were detected by Odyssey® CLX imaging system (LI-COR Biosciences, NE, USA).

For serum irisin level analysis, 20 μL of mouse serum was precleared for albumin/IgG using the Albumin Erasin Reagent (Sangon Biotech, NO. C500065) following manufacturer's protocol. Samples were analysed using western blot against FNDC5 antibody. Ponceau S (Sangon Biotech, NO. A100860) was used as loading control.

Human muscle sample collection and analysis

To determine the Fndc5 expression levels in human muscle samples, we collected muscle biopsies from the vastus lateralis muscle from 25 human subjects aged between 20 and 80 years old that underwent orthopaedic surgery during 2012–2014 at Shanghai Jiao Tong University Affiliated Sixth People's Hospital for qPCR and statistical analysis. Human study was approved by Shanghai Jiaotong University Affiliated Sixth People's Hospital. Written informed consent was obtained from all subjects.

Enzyme activity

Citrate synthase (CS) activity was measured using a commercial kit from Solarbio (BC1060) as described previously.[15] Briefly, quadriceps skeletal muscle samples were homogenized with extraction buffer from the kit. The reaction was performed following the manufacturer's instructions, and the absorption at OD = 412 nm was measured to calculate CS activity.

Measurement of ATP contents

Skeletal muscle ATP contents were determined using a firefly luciferase-based ATP assay kit (Beyotime, S0027) following the manufacturer's instructions. Briefly, quadriceps muscle samples were lysed and subjected to centrifugation at 10 000 × *g* for 10 min at 4°C. The supernatant of lysates was incubated with ATP reaction mix for 5 min, and chemiluminescence was measured with a fluorescence microplate reader. ATP levels were calculated from standard curve and normalized to protein concentrations.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 7 software. Statistical comparisons between two groups were made by two-tailed unpaired Student *t*-test. The correlation between Fndc5 mRNA levels and human age was analysed by Pearson correlation analysis. All data are shown as mean ± SEM and $P < 0.05$ is considered statistically significant.

Results

The expression levels of FNDC5/irisin are reduced in muscles during ageing

Ageing-associated sarcopenia is manifested as reduced muscle strength and muscle mass, along with increased muscle dystrophic genes expression.[16] We used aged mice of 24-month-old as a naturally occurred ageing-associated sarcopenia model. We found that aged mice had significantly reduced grip strength and muscle weights with smaller muscle fibre sizes compared with young mice (*Figure 1A,B*). Consistently, muscles from aged mice showed increased atrophic (*Atrogin-1*, *MuRF-1* and *Mstn*) and inflammatory genes (*IL-1β* and *IL-6*) (*Figure 1C*), indicating the presence of sarcopenia in aged mice. To examine the role of irisin in aged muscles, we utilized GEO database (GSE75523) and found that Fndc5 mRNA expression was reduced in hind limb skeletal muscles of aged mice compared with young control mice (*Figure 1D*). Furthermore, we found significantly reduced Fndc5 mRNA levels in quadriceps femoris (QU), gastrocnemius (GAS) and tibialis anterior (TA) muscles from aged mice compared with young controls (*Figure 1E*). Using QU as a representative, we further demonstrated reduced FNDC5 and irisin protein levels in muscles from aged mice (*Figure 1E*), which is consistent with the reduced irisin levels in circulation in aged mice (*Figure 1F*). In addition, we found a significant decrease on *FNDC5* expression in aged human subjects compared with young subjects (*Figure 1F*), overall suggesting reduced FNDC5/irisin levels in aged mice and human, which is consistent with previous reports showing reduced irisin levels in circulation of aged population.[17,18]

FNDC5/irisin deficiency in aged mice exacerbates skeletal muscle wasting

Given the declines of mRNA and protein levels of FNDC5/irisin in muscles in ageing, we further assessed the influences of FNDC5/irisin deficiency on sarcopenia in aged FNDC5/irisin knockout mice (KO) at 22-month-old, in which irisin was not detected in serum (*Figure S2A*). Of note, aged Fndc5 KO mice

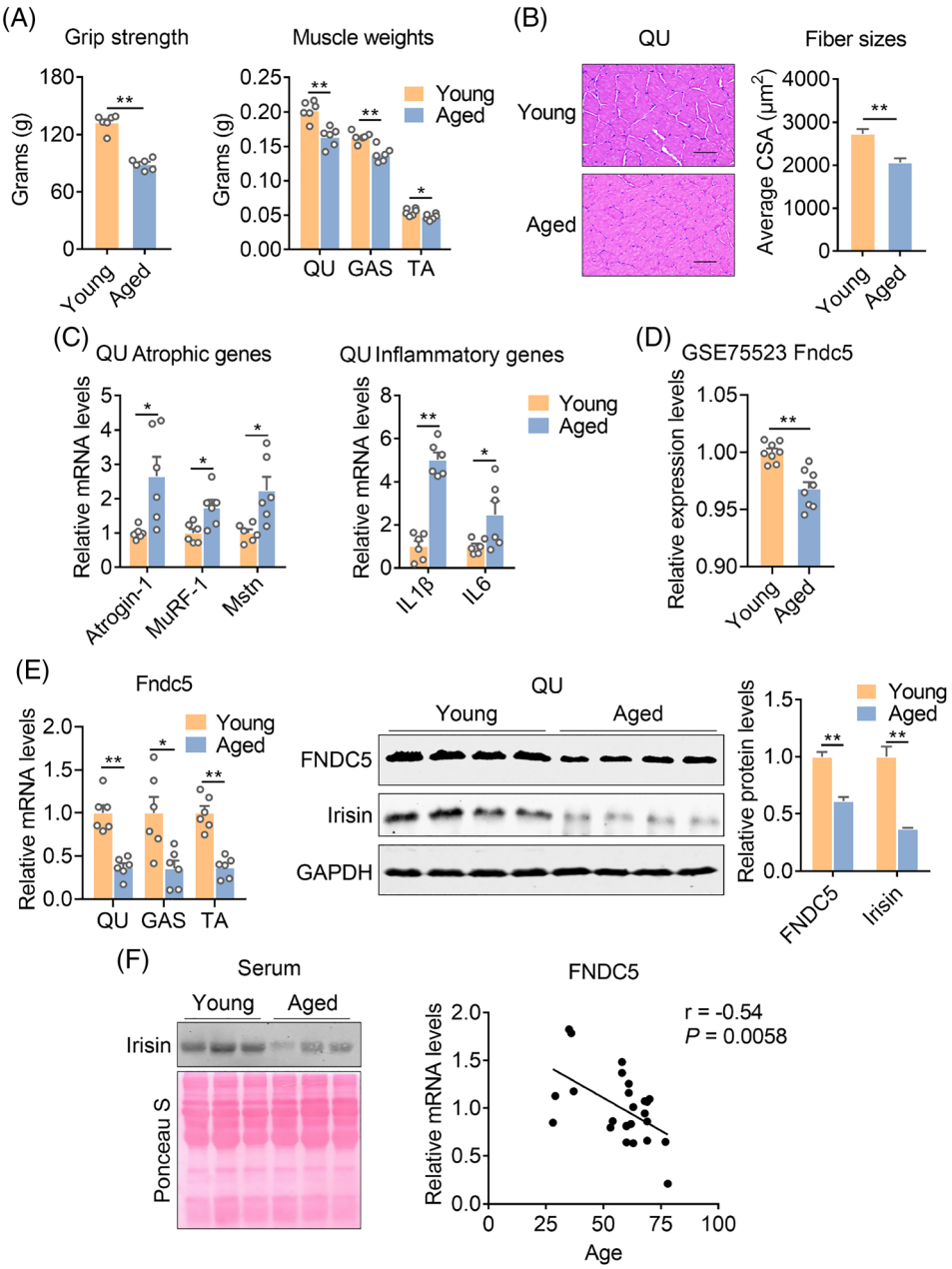

Figure 1 The expression levels of FNDC5/irisin are reduced in muscles during ageing. (A–F) Phenotypical and molecular analysis of muscles from young (2-month-old) and aged mice (24-month-old). *N* = 6 per group. (A) The forelimb grip strength and weights of quadriceps femoris (QU), gastrocnemius (GAS) and tibialis anterior (TA); (B) haematoxylin and eosin histological analysis and average cross-sectional area of fibre sizes and (C) gene expression profiles of atrophic and inflammatory genes from QU muscle. (D) Relative expression levels of Fndc5 in the hind limb muscle of young and aged mice from GSE75523 database. *N* = 8 per group. (E) Relative mRNA levels of Fndc5 in QU, GAS and TA muscle from young and aged mice (*n* = 6 per group), immunoblotting and quantification analysis for FNDC5 and irisin protein levels in the QU muscle from young and aged mice (*n* = 4 per group). (F) Immunoblotting analysis for irisin protein levels in the serum from young and aged mice (*n* = 3 per group) and Pearson correlation analysis for FNDC5 mRNA levels in the muscle biopsies from young and aged human (*n* = 25). Data are presented as mean ± SEM and $*P < 0.05$, $**P < 0.01$ compared with control group. Scale bar represents 100 μm.

exhibited decreased body weights (*Figure 2A*). Body composition analysis using nuclear magnetic resonance spectroscopy revealed that this change was majorly contributed by a reduction in lean mass, but not fat mass (*Figures 2B* and S2B). In detail, we found reduced grip strengths and significant decreases in QU, GAS and TA muscle weights in aged FNDC5 KO mice compared with their wild-type littermates (*Figure 2C*). Consistently, histological analysis showed smaller fibre sizes and quantified average CSA in QU muscle of aged FNDC5 KO mice (*Figure 2D*). Accordingly, the expression levels of

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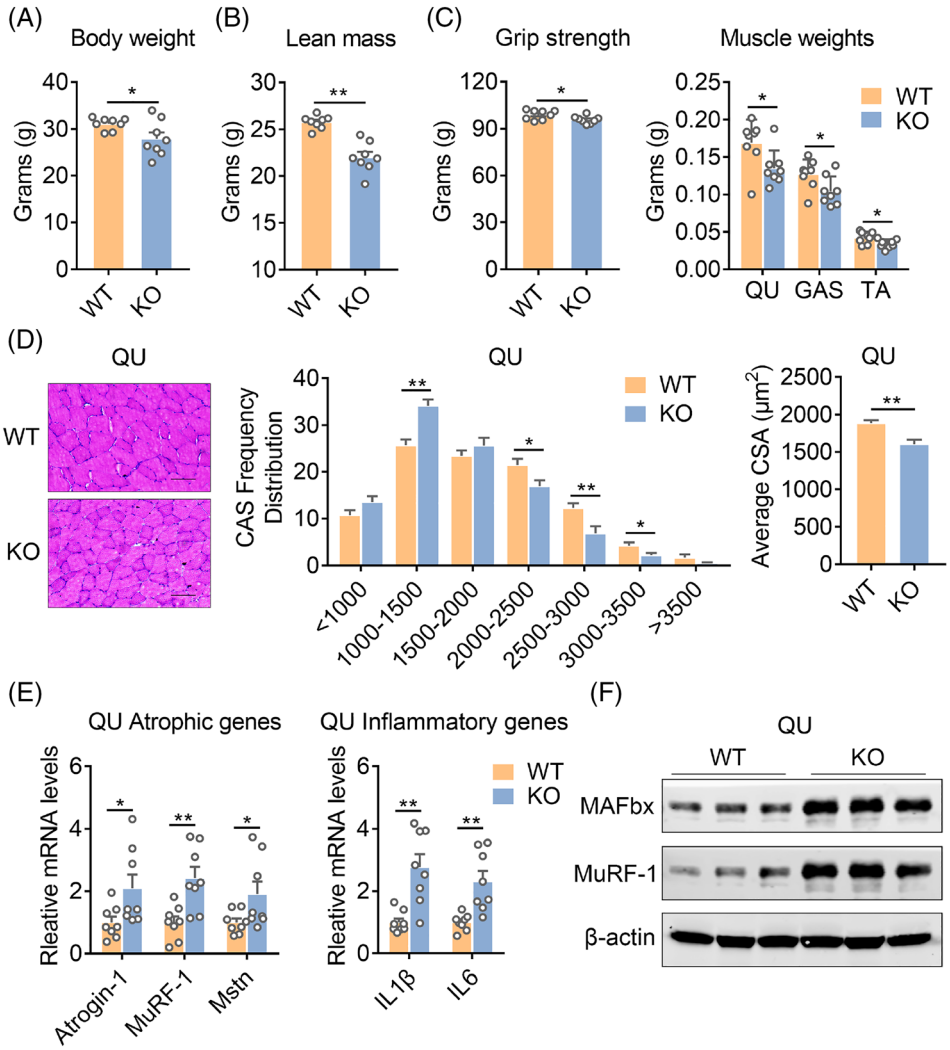

Figure 2 FNDC5/irisin deficiency in aged mice exacerbates skeletal muscle wasting. (A–F) Phenotypical and molecular analysis of muscles from aged wild-type (WT) and FNDC5 knockout (KO) mice at 22-month-old. $N = 8$ per group. Body weight; (B) lean mass; (C) forelimb grip strength and weights of QU, GAS and TA muscles; (D) representative haematoxylin and eosin staining, quantifications of muscle fibre sizes distribution and average cross-sectional areas (CSAs); (E) mRNA levels of atrophic and inflammatory genes and (F) protein levels of MAFbx and MuRF-1 in QU muscle. Data are presented as mean ± SEM and $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control group. Scale bar represents 100 μm.

atrophic genes (*Atrogin-1*, *MuRF-1* and *Mstn*) and inflammation-related genes (*IL1β* and *IL6*) were increased in QU muscle from aged FNDC5 KO mice (*Figure 2E*). Furthermore, FNDC5/irisin deficiency in aged mice resulted in dramatic increases in protein levels of atrophic ubiquitin ligase MAFbx and MuRF-1 in QU muscles (*Figure 2F*). In summary, FNDC5/irisin KO mice were featured with exacerbated skeletal muscle wasting in ageing.

Chronic irisin administration alleviates ageing-associated sarcopenia

Based on the observation that FNDC5/irisin deficiency in aged mice exacerbated muscle wasting, we decided to investigate the effects of irisin reintroduction in ageing-associated sarcopenia in vivo. We chronically delivered the recombinant irisin protein (irisin group) or control (His-tag group) via intraperitoneal injection into ageing mice (14-month-old) three times every week for a 4-month intervention as a prevention mice model. Of note, irisin-treated ageing mice showed enhanced grip strength and lean mass, without difference in body weight (*Figures 3A–C* and S3A). Among QU, GAS and TA muscles, QU was the most influenced muscle with increased weights, as well as larger fibre sizes of QU as shown by histological analysis and quantified CSA after irisin administration (*Figure 3D*). Besides, irisin treatment resulted in decreased mRNA levels of atrophic genes (*Atrogin-1*, *MuRF-1* and *Mstn*) and inflammatory genes (*IL1β* and *IL6*) as well as decreased MAFbx and MuRF-1 protein

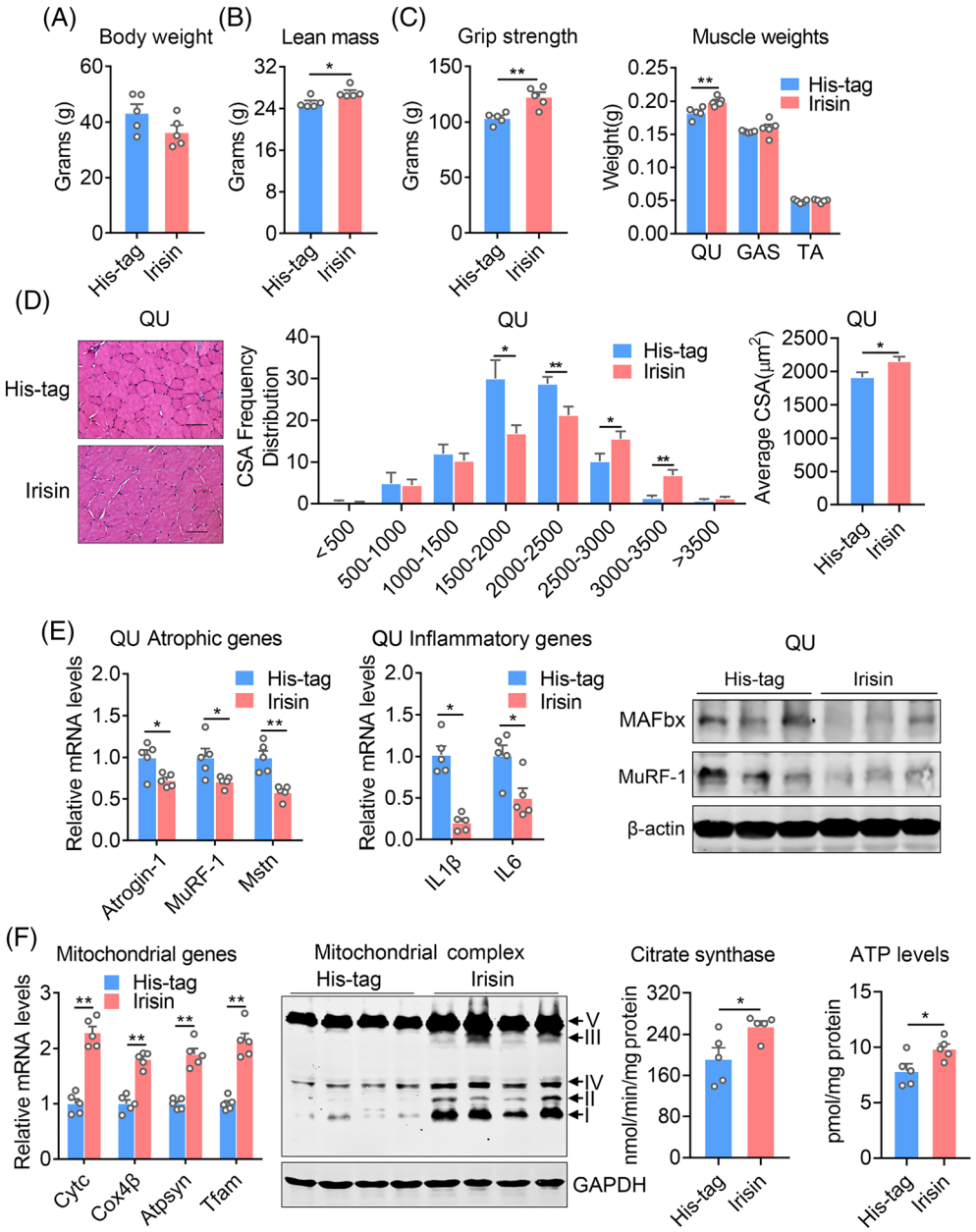

Figure 3 Chronic irisin administration alleviates sarcopenia in ageing mice. (A–F) Phenotypical and molecular analysis of muscles from middle-aged mice (14-month-old) intraperitoneally administrated with recombinant His-tag or irisin for 4 months. *N* = 5 per group. (A) Body weight; (B) lean mass; (C) forelimb grip strength and weights of QU, GAS and TA muscles; (D) representative haematoxylin and eosin staining, quantifications of muscle fibre sizes distribution and average cross-sectional areas (CSAs); (E) mRNA expression levels of atrophic and inflammatory genes and protein levels of MAFbx and MuRF-1; (F) mRNA expression levels of mitochondrial genes, protein levels of mitochondrial complex, citrate synthase activity and ATP levels in QU muscle. Data are presented as mean ± SEM and $*P < 0.05$, $**P < 0.01$ compared with control group. Scale bar represents 100 μm.

levels in vivo (*Figure 3E*). In addition, irisin treatment also improved mitochondrial functionality in mice as shown by increased mitochondrial gene programmes, OXPHOS complexes protein levels, CS activities and ATP levels (*Figure 3F*). In summary, these data demonstrated that intraperitoneally delivery of irisin recombinant protein ameliorated sarcopenia and improved muscle functionality in ageing mice.

Chronic irisin administration ameliorates ageing-associated metabolic dysfunction

Irisin has been reported to exert beneficial effects to energy homeostasis, and we further analysed the effects of chronic irisin treatment on metabolic reprogramming of aged mice. Remarkably, chronic irisin administration in ageing mice improved serum lipid parameters with reduced triglycerides

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(TG), total cholesterol and low-density lipoprotein cholesterol levels, without worsening of liver and kidney function, suggesting the safeness of irisin administration (*Figure 4A* and *Table S2*). Besides, we observed enhanced insulin sensitivity as shown by better performances in glucose and insulin tolerance test in the irisin-treated group (*Figure 4B,C*). Furthermore, irisin treatment in mice increased energy expenditure as shown by enhanced oxygen consumption, carbon dioxide production and energy expenditure (*Figure 4D–F*), while no changes were observed in locomotor activity and food intake (*Figures S3B,C*).

Chronic irisin administration improves beige fat functionality and hepatic steatosis

Irisin has been shown to impact beige fat browning. Consistently, detailed analysis on adipose tissues revealed that

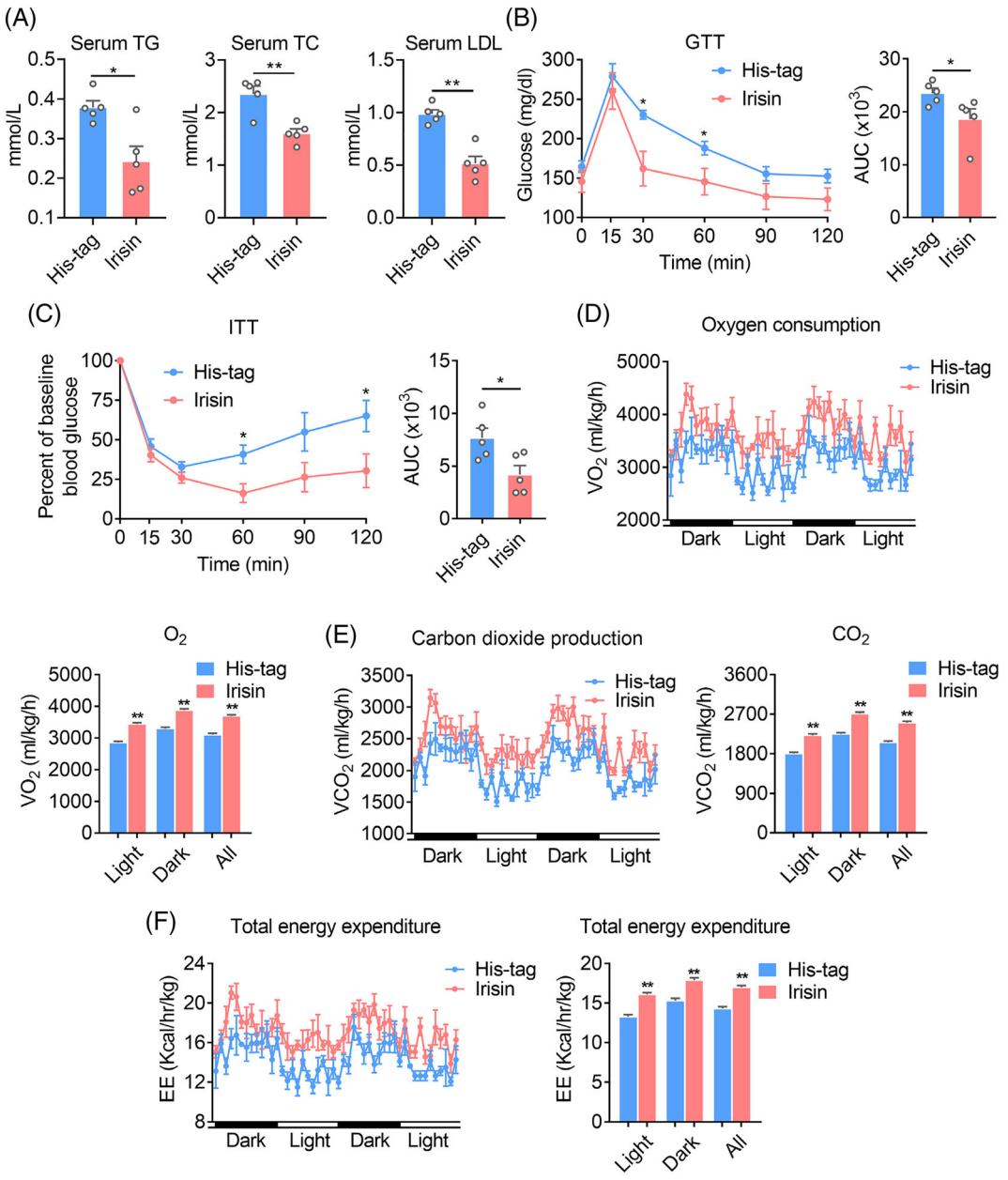

Figure 4 Chronic irisin administration ameliorates metabolic dysfunction in ageing mice. (A–F) Metabolic performances of middle-aged mice (14-month-old) intraperitoneally administrated with recombinant His-tag or irisin for 4 months. $N = 5$ per group. (A) Serum parameters analysis including total triglyceride (TG), total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C); (B) glucose tolerance test (GTT) and area under the curve (AUC); (C) insulin tolerance test (ITT) and AUC; (D) oxygen consumption and quantification; (E) carbon dioxide production and quantification; (F) total energy expenditure and quantification. Data are presented as mean ± SEM and $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control group.

chronic irisin administration in ageing mice decreased subcutaneous fat (iWAT) weight (*Figure 5A*) with smaller adipocytes as shown by H&E staining and adipocyte size quantification (*Figure 5B*). Molecular analysis demonstrated that mRNA levels of thermogenic and mitochondrial genes, as well as protein levels of PGC1α and UCP1 in iWAT were increased after irisin treatment (*Figure 5C,D*). Ageing is often associated with enhanced adipose tissue fibrosis; we thus analysed the fibrotic status of iWAT of mice treated with or without irisin. Interestingly, irisin treatment improved adipose tissue fibrosis as shown by Masson, Sirius red staining and reduced fibrotic gene programmes in iWAT (*Figure 5E,F*). In addition, irisin treatment also improved ageing-associated hepatic steatosis as shown by reduced liver weights, hepatic TG levels and lipid contents shown with Oil red staining, as well as increased β-oxidation genes and decreased inflammatory genes

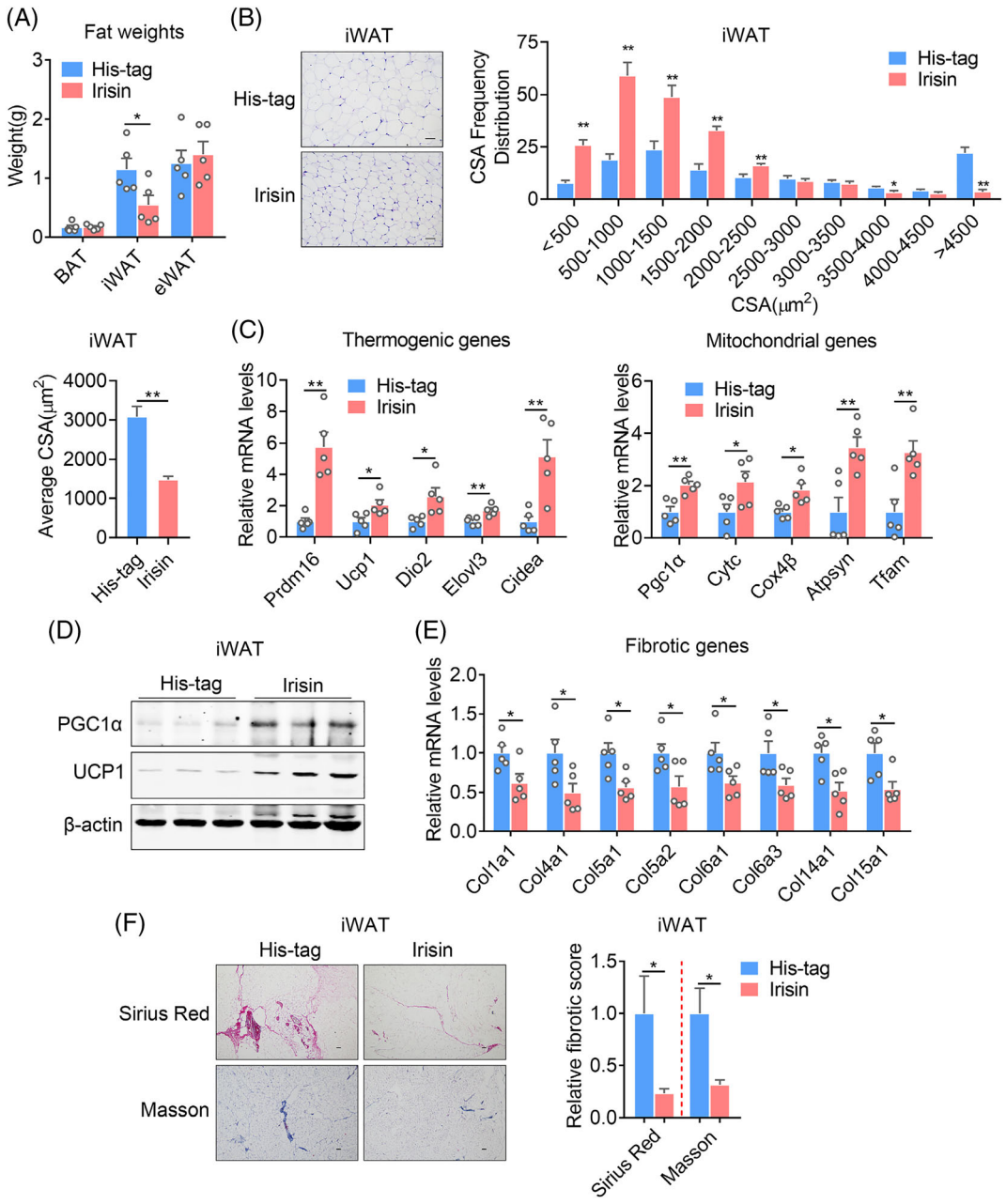

Figure 5 Chronic irisin administration improves beige fat functionality in ageing mice. (A–F) Analysis of adipose tissues from middle-aged mice (14-month-old) intraperitoneally administrated with recombinant His-tag or irisin for 4 months. *N* = 5 per group. (A) Weights of brown (BAT), inguinal (iWAT) and epididymal (eWAT) fat pads; (B) representative haematoxylin and eosin staining, quantification of adipocyte sizes and average adipocyte sizes; (C) mRNA levels of thermogenic and mitochondrial genes; (D) protein levels of PGC1α and UCP1; (E) mRNA levels of fibrotic genes and (F) mason, Sirius red staining in iWAT. Data are presented as mean ± SEM and $*P < 0.05$, $**P < 0.01$ compared with control group. Scale bar represents 100 μm.

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(*Figure S3D–F*). Taken together, these data demonstrated that the delivery of irisin recombinant protein intraperitoneally into ageing mice alleviated sarcopenia and ameliorated ageing-associated metabolic dysfunction, without obvious adverse effects, indicating that irisin administration represented a potent and safe strategy for healthy ageing.

Irisin administration in aged mice treats age-associated sarcopenia and metabolic dysfunction

In order to better examine the role of irisin in age-associated sarcopenia and metabolic dysfunction, we further established a treatment mice model by administrating 2mg/kg irisin or His-tag intraperitoneally three times per week for 1 month in aged mice (22-month-old). We found that similar to the beneficial effects of irisin in prevention mice model of 14-month-old ageing mice, irisin administration in treatment mice model of aged mice resulted in significantly alleviated muscle sarcopenia, as shown by increased lean mass, grip strength, muscle weights and fibre sizes, as well as reduced MAFbx and MuRF-1 protein levels and increased mitochondrial gene programmes and activities (*Figure 6A–F*).

In addition, similar to the prevention mice model, irisin treatment in aged mice also ameliorated metabolic disorders, including improved lipid parameters, insulin resistance and energy expenditure (*Figure 7A–F*) with reduced iWAT weights, adipocyte sizes and fibrotic scores and increased browning gene programmes (*Figure 8A–F*), as well as improved hepatic steatosis (*Figure S4*). Overall, these data suggested that irisin administration in aged mice could treat age-associated sarcopenia and metabolic dysfunctions.

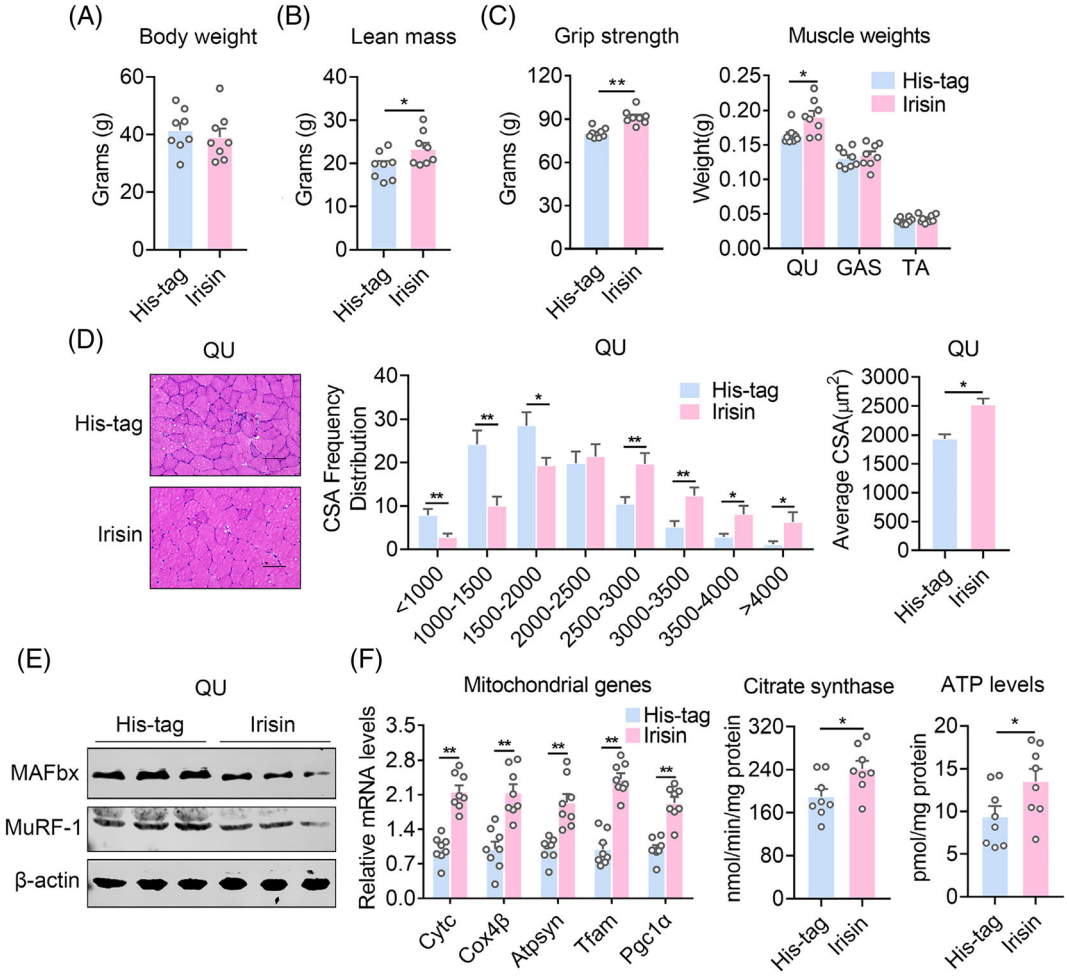

Figure 6 Chronic irisin administration treats sarcopenia in aged mice. (A–F) Phenotypical and molecular analysis of muscles from aged mice (22-month-old) intraperitoneally administrated with recombinant His-tag or irisin for 1 month. *N* = 8 per group. (A) Body weight; (B) lean mass; (C) forelimb grip strength and weights of QU, GAS and TA muscles; (D) representative haematoxylin and eosin staining, quantifications of muscle fibre sizes distribution and average cross-sectional areas (CSAs); (E) protein levels of MAFbx and MuRF-1; (F) mRNA expression levels of mitochondrial genes, citrate synthase activity and ATP levels in QU muscle. Data are presented as mean ± SEM and $*P < 0.05$, $**P < 0.01$ compared with control group. Scale bar represents 100 μm.

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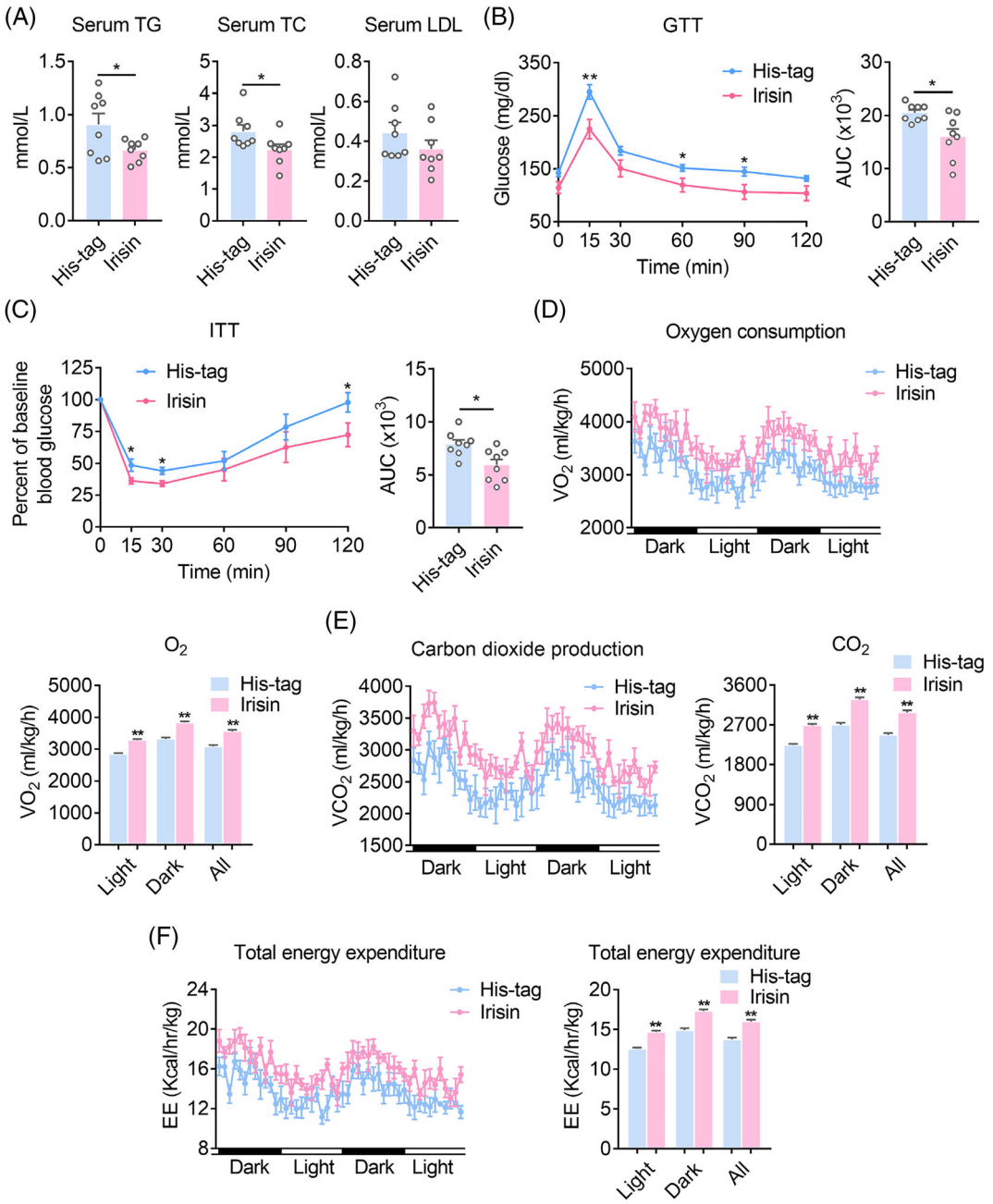

Figure 7 Chronic irisin administration treats metabolic dysfunction in aged mice. (A–F) Metabolic performances of aged mice (22-month-old) intraperitoneally administrated with recombinant His-tag or irisin for 1 month. $N = 8$ per group. (A) Serum parameters analysis including total triglyceride (TG), total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C); (B) glucose tolerance test (GTT) and area under the curve (AUC); (C) insulin tolerance test (ITT) and AUC; (D) oxygen consumption and quantification; (E) carbon dioxide production and quantification; (F) total energy expenditure and quantification. Data are presented as mean ± SEM and $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control group.

Discussion

Ageing-associated sarcopenia severely affected physical activity and life quality of elders. Meanwhile, due to the important contribution of skeletal muscle to systematic homeostasis, ageing-associated muscle wasting may also lead to metabolic dysfunction and cardiovascular and neurodegenerative diseases. In the present study, we found that the expression levels of myokine irisin and its precursor Fndc5 in glycolytic muscles were reduced in aged mice with sarcopenia. Besides, genetic FNDC5/irisin knockout mice showed exacerbated muscle wasting in ageing. Of note, we delivered recombinant irisin protein intraperitoneally in ageing mice chronically and alleviated ageing-associated sarcopenia and metabolic

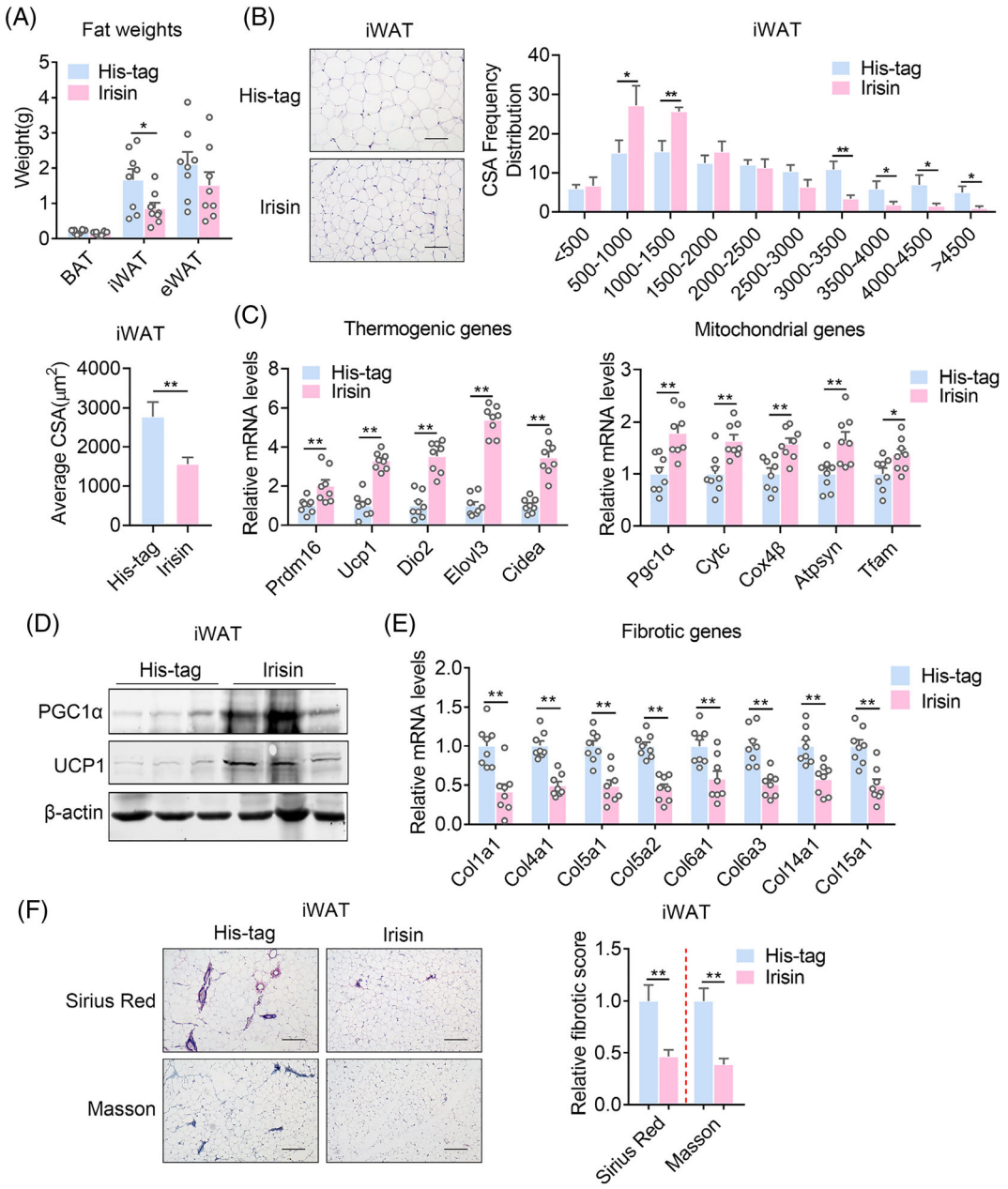

Figure 8 Chronic irisin administration improves beige fat functionality in aged mice. (A–F) Analysis of adipose tissues from aged mice (22-month-old) intraperitoneally administrated with recombinant His-tag or irisin for 1 month. $N = 8$ per group. (A) Weights of brown (BAT), inguinal (iWAT) and epididymal (eWAT) fat pads; (B) representative haematoxylin and eosin staining, quantification of adipocyte sizes and average adipocyte sizes; (C) mRNA levels of thermogenic and mitochondrial genes; (D) protein levels of PGC1α and UCP1; (E) mRNA levels of fibrotic genes and (F) mason, Sirius red staining in iWAT. Data are presented as mean ± SEM and $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control group. Scale bar represents 100 μm.

disorders, including adiposity, hyperlipidaemia, insulin resistance and fatty liver, overall suggesting that irisin supplement may be a potent therapeutic strategy to combat ageing-associated chronic diseases.

It is well accepted that regular exercise improves metabolism and tissue homeostasis via myokines and extracellular vesicles to combat ageing.[19] Irisin has been first defined as a myokine cleaved from its FNDC5 precursor in skeletal muscle under exercise and promotes browning of subcutaneous fat with subsequent metabolic improvements, for example, improved adiposity, insulin sensitivity and hepatic steatosis.[20] In addition, irisin has been further shown to be beneficial for combating neurodegenerative diseases, such as Alzheimer's disease (AD), which is also highly correlated with ageing. It

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has been reported that peripheral overexpression of FNDC5/irisin rescues memory impairment and exerts neuroprotective action on synaptic plasticity and astrocytes/microglia functionality in AD mice.[21] Meanwhile, the roles of irisin on cardiovascular system and bone remodelling are not certain. For example, during different stages of heart failure, irisin exhibits different influences on mitochondrial dysfunction, oxidative stress, metabolic imbalance, energy expenditure and heart failure prognosis.[22] Besides, irisin has been shown to both treat osteoporosis with intermittent administration while also exhibit adverse effects on bone mass with genetic evidences, suggesting that irisin targets both bone resorption and bone remodelling in a favourable manner with intermittent pulse dosing.[10] Therefore, further investigations to reveal the function of irisin on nervous, cardiovascular and bone systems under the context of ageing would be interesting.

As an exercise-induced myokine, in addition to its widely reported paracrine and endocrine functionalities towards other metabolic organs, irisin also exerts important function in muscles in an autocrine way. It has been shown that irisin improves muscle sarcopenia in different animal models, including muscle denervation, hindlimb suspension and genetic muscle dystrophic mdx mice, with its strong effects in enhancing satellite cell activation, reducing protein degradation, tissue fibrosis and necrosis and improving sarcolemmal stability.[6,23,24] Our study further extended the application of irisin in ageing-associated muscle sarcopenia and metabolic dysfunctions. In addition, although we demonstrated that irisin administration inhibits MuRF1, Atrogin-1 and myostatin, which were associated with muscle atrophy via the ubiquitin–proteasome system, the exact downstream of irisin signal transduction still needs further investigations. It has been reported that irisin exerts its biological functions on adipocyte browning, neural differentiation, osteoblast proliferation, myogenic differentiation and myoblast fusion via various intracellular signalling pathways, including MAPK, AMPK pathway, PI3K/AKT, STAT3 and IL-6 signalling pathway.[25] Besides, αV integrin receptors have been reported as irisin receptor in bone and fat and subsequently engages FAK-AKT-CREB signalling pathway[10] for downstream target genes modulation, such as Pgc1α and NR4A nuclear hormone receptors, for muscle functionality and hypertrophy. Future screening work with specific signalling inhibitors are warranted to provide comprehensive understanding of irisin-mediated signalling in muscle.

In the present study, we have applied the recombinant irisin protein for combating sarcopenia and ageing-induced metabolic disorders. However, one possible limitation to hinge the potential clinical usage of irisin is its relative short half-life time in vivo.[10] There are a few strategies to extend the durance of a protein drug. First, Fc fusion protein has been widely applied to extend half-time of secreting hormones.[26,27] For instance, Glucagon-like peptide-1 (GLP-1) analogue fused with Fc protein (LY2189265) has shown enhanced pharmacokinetics and prolonged half-life.[28,29] Besides, a long-acting FGF21 analogue PF-05231023 fused with Fc was shown to reduce body weight and improve circulating lipid profile in obese monkeys and humans.[30,31] Besides, other long-acting Fc-apelin fusion protein such as NRG1-Fc and IC7-Fc have been tested clinically for diabetes treatment.[32,33] Second, albumin is the most abundant plasma protein. It is very stable and has an extraordinarily long circulatory half-life, which is also a well-established strategy to extend half-life by joining albumin with proteins both noncovalently (by specific binding to albumin) and covalently (by conjugation and by direct genetic fusion).[34] For example, GLP-1 analogues are specifically designed to join with albumin for the improvement of half-life and pharmacokinetic profiles using both noncovalent and covalent strategies and have been approved for clinic or under clinical trials.[35,36] Lastly, recent developments of nanotechnology-based drug delivery strategies including hydrogels feature a prolonged therapeutic effects and low frequency of administration are also used to combating obesity.[37] For instance, Insulin loaded into hydrogel microparticles were achieved sufficient blood glucose control in fed diabetic rats and type 1 diabetic mice.[38,39] Thus, future studies are warranted to enhance irisin stability using these modifications for better treatment outcomes and clinical potential.

In summary, via genetic animal models and recombinant protein intervention, we demonstrated the importance of FNDC5/irisin in alleviating ageing-associated sarcopenia and metabolic dysfunction, which provides a basis for developing irisin intervention strategy for healthy ageing. However, when considering the metabolic phenotypic variation of ageing mice, as well as the influences of microbiota under different housing conditions in different facilities, the sample numbers for in vivo studies could be enlarged, and extra animal facilities could be included to further assess the effects of irisin in muscle physiology and metabolic homeostasis in aged mice.

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certify that they comply with the ethical guidelines for authorship and publishing in the *Journal of Cachexia, Sarcopenia and Muscle*.[40]

Online supplementary material

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Conflict of interests

The authors declare no competing interests.

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