博士論文（要約）

Studies of the skeletal muscle atrophy-responsive factor and regulatory mechanism

（筋萎縮シグナル応答性分子の発現制御及び機能解析）

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Article outline

Abbreviation

FAK	Focal Adhesion Kinase
ERK	Extracellular Signal-Regulated Kinase
Akt	Protein Kinase B
MuRF1	Muscle RING Finger 1
Atrogin1	Muscle Atrophy F-box Gene
MCK	Muscle Creatine Kinase
TBG	Thyroxine-Binding Globulin
CSA	Cross-Sectional Area
PDGFα	Platelet-Derived Growth Factor Alpha
GLUT	Glucose Transporter
P62	Ubiquitin-binding protein p62
LC3	Microtubule-Associated Protein 1A/1B-Light Chain 3
shRNA	Short Hairpin RNAs
rAAV	Recombinant Adeno-Associated Virus
H&E	Hematoxylin and Eosin
PCR	Polymerase Chain Reaction
PIC	Protease Inhibitor Cocktail
PMSF	Phenylmethanesulfonyl Fluoride
PPIC	Phosphatase Inhibitor Cocktail

Chapter 1

Introduction

As the global aging population expands, age-related neurodegenerative impairments are increasingly exerting significant stress on both the medical and government systems [1]. These detriments encompass reduced physical fitness, cognitive decline, and a multitude of chronic illnesses. Therefore, it is crucial to make strides in the development of therapies aimed at slowing down or protecting against aging-related detriments, as they play a significant role in promoting both "normal aging" and "healthy aging."

Skeletal muscle, constituting the largest organ in nonobese individuals, accounts for approximately 40% of the body weight in lean individuals [2]. Skeletal muscle serves physical movement, sustains body posture and position, regulates body temperature, stores nutrients, and provides joint stability [3]. As individuals age, there is a tendency for a decline in the level of daily activities among elderly persons, largely attributed to the gradual reduction in muscle mass and strength, which may develop primary sarcopenia [4]. This decline typically amounts to an approximate decrease of 30%–50% between the ages of 30 and 80 years, and thus provoke skeletal muscle atrophy [5]. The age-dependent skeletal muscle dysfunction significantly contributes to the exacerbation of metabolic disturbances, which encompass insulin resistance, diabetes mellitus, dyslipidemia and hypertension [6]. A hypothesis has been proposed that good health maintenance in later life relies on skeletal muscle healthy aging. However, the concrete mechanism of skeletal muscle atrophy remains complex and has not been fully elucidated. The disordered imbalance between protein synthesis and proteolysis in skeletal muscle is widely recognized as a typical detrimental event during the pathogenesis of muscle atrophy [7]. The subsequent cascades provoke an inflammatory response, oxidative stress, and mitochondrial dysfunction, thereby contributing to the progression of skeletal muscle atrophy [8]. Notably, skeletal muscle has garnered

recognition as a vital secretory organ that actively generates and releases myokines and cytokines, facilitating crucial physiological communication with various organs, such as the brain, liver, adipose tissue, and pancreas [9]. For instance, myofiber-secreted myokines, such as myostatin and interleukin-6 (IL-6), are involved in muscle hypertrophy and myogenesis, whereas regulate adipose tissue function to maintain metabolic homeostasis [10]. Physical exercise and nutrient intake have been identified to strengthen muscle mass remodeling and modulate the bioactive secretion process [11]. Therefore, interventions focused on preserving muscle homeostasis or addressing muscle-related tissue senescence requires an urgent development, presenting challenges in their clinical application and translation.

1-1 Overview of skeletal muscle atrophy

The size of skeletal muscle mass and muscle fibers exhibits variation based on both physiological and pathological conditions. Physiological growth, strength training and pharmacological interventions increase the muscle fiber size and strengthen muscle function. On the other hand, a decline in both muscle mass and fiber size, termed as muscle atrophy, occurs due to factors such as aging, fasting, metabolic disorders, cancer cachexia, denervation-associated neuron loss, or catabolic hormonal stimulation [12]. Complex cellular stress underpins the insufficient use of skeletal muscle, characterized by factors such as a decline in muscle contractile activity or fatigue, and further contributes to mass loss and atrophy in the skeletal muscle [13]. In human subjects, studies have reported that step reduction [14], limb immobilization [15], and exposure to microgravity [16] result in muscle atrophy, attributed to the disuse induced by mechanical loading reduction. Notably, skeletal muscle is a highly dynamic tissue that establishes crucial communication with surrounding tissues. Consequently, muscle mass decline and atrophy prominently increase the risk of metabolic disorders, such as neurogenerative diseases, cardiovascular diseases, diabetes, and even higher mortality [17]. Nevertheless, endocrine and inflammatory cytokines in the catabolic states can

collaborate synergistically, aggravating the atrophy process and hastening its progression [13]. Hence, comprehending the underlying mechanisms and implementing measures to prevent muscle atrophy are pivotal for enhancing the quality of life among the elderly and promoting human health.

1-2 Mechanisms and degradative machineries involved in muscle atrophy

In general, the skeletal muscle mass is predominantly regulated by the equilibrium between two physiological processes: muscle protein synthesis and muscle proteolysis [17]. These two processes transpire incessantly and concurrently. Reduced muscle bulk or muscle atrophy takes place when protein degradation exceeds protein synthesis, leading to a reduction of the cross-sectional area of myofibers and decreased muscle strength. As shown in Figure 1-1, Muscle atrophy occurs when there is a disproportion between protein synthesis and breakdown, with the former surpassing the latter. This imbalanced protein homeostasis contributed to a diminished cross-sectional area of myofibers and a subsequent decline in muscle strength [18]. While age-related sarcopenia represents a fundamental physiological outcome of muscle atrophy, chronic protein degradation and muscle atrophy can also be attributed to inappropriate lifestyle choices, clinical cachexia, and various metabolic disturbances [19].

In the past two decades, potential molecular signaling networks associated with muscle atrophy and depletion have been elucidated. These networks primarily encompass the phosphoinositide 3-kinase (PI3K)/Akt-mediated protein synthesis pathway, as well as the ubiquitin–proteasome and autophagy–lysosome pathway-mediated protein degradation pathways. Insulin-like growth factor-1 (IGF-1) is widely recognized to assume a pivotal role as a critical anabolic factor in sustaining skeletal muscle protein synthesis and mitigating protein degradation via the activation of the PI3K/Akt pathway [20]. In this regard, the Akt-S6 kinase 1 (S6K1)-mammalian target of rapamycin (mTOR) pathway exhibited substantial activation during muscle hypertrophy, whereas its activity was attenuated during the progression of muscle

atrophy [21]. In skeletal muscle, Akt1 phosphorylation initiates the transcriptional activation of forkhead box O (FoxO) 1 and 3 [22]. This activation leads to the promotion of polyubiquitination of target proteins, especially two muscle-specific E3 ubiquitin ligase enzymes, Atrogin-1 (also known as MAFbx1) and MuRF1 (muscle RING-finger protein-1) [23]. The ubiquitination of muscle proteins during muscle atrophy involves a multistep reaction, including the activation of ubiquitin by ubiquitin-activating enzyme (E1), the transfer of activated ubiquitin to ubiquitin-conjugating enzyme (E2), and the catalyzation of ubiquitin ligation by E3 ubiquitin ligase enzymes [24]. In mammalians, Atrogin-1 and MuRF1 are muscle-specific ubiquitin ligases that play a crucial role in the ubiquitinated protein degradation of skeletal muscle, which represents classical muscle atrophy markers in the multiple in vivo and in vitro models [25]. Overexpression of either Atrogin-1 or MuRF1 was sufficient to cause muscle atrophy in the rodent's model [25], while knockout mice for them did not exhibit any discernible phenotypic abnormalities [25,26]. However, Atrogin-1 inhibition did not reverse muscle dysfunction and atrophy in the denervated rodents [27]. In addition, one comparative study demonstrated that aging-associated sarcopenia is not due to the suppression of IGF1/Akt pathway and inhibition of Atrogin-1 based on a cohort of old individuals and mice [28]. These observations imply an ambiguous direction when utilizing the measurement of Atrogin-1 and MuRF1 as an indicative measure of proteasome-dependent proteolysis in the progression of muscle atrophy.

The aim of present study

Muscle mass decline and dysfunction exhibits huge implications for human health, especially in elderly populations. Muscle atrophy and weakness manifest in various systemic stress conditions, associated with an imbalance between protein synthesis and protein break down in the skeletal muscle. We aim to identify effective factors and elucidate their underlying mechanisms in the development of muscle atrophy.

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Chapter 2

Factor X is increased upon muscle atrophy

2-1 Preface

As the contents of this chapter are anticipated to be published in a paper in a scholarly journal they cannot be published online. The paper is scheduled to be published within five years.

2-2 Materials and methods

Animals

The 4- or 7-week-old male C57BL6/J mice were purchased from Crea-Japan Inc. (Tokyo, Japan) and acclimated for 1 week in a standard animal cage before the experiments. Mice were housed with a 12-hr light-dark cycle (with the dark cycle occurring between 9:00 AM and 9:00 PM) at 22 ± 2℃ and 50 ± 10% relative humidity, respectively with food (Labo MR Stock, Nosan Corporation) and distilled water ad libitum. All experiments were conducted according to the guidelines set by the Animal Usage Committee of The University of Tokyo, which are based on the Law for the Humane Treatment and Management of Animals (Law No. 105, 1 October 1973, as amended on 1 June 2020).

Four-limb grip strength test

A grip strength meter (Muromachi, MK-380V) was used to measure grip strength. Mice were allowed to grab the device's metal grid with all four paws, and then they were gently pulled backward by their tails until they were released. Each mouse was tested five times at 30-second intervals. The highest and lowest values were not taken into account for calculation. The remaining three force measurements were averaged and normalized to body weight.

Cell culture

Human embryonic kidney cells (HEK293T) were maintained at 37 ℃ and 5% CO2 in Dulbecco's modified Eagle's medium (DMEM, High Glucose) containing 10% fetal bovine serum (FBS), 100 units/mL penicillin and 100 μg/mL streptomycin cocktail.

Murine myoblasts C2C12 cells were maintained at 37 ℃ and 5% CO2 in a growth medium containing Dulbecco's modified Eagle's medium (DMEM, High Glucose) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin and 100

μg/mL streptomycin cocktail. Cells are subcultured when they reach 50 to 60% of confluence. For myotube formation, cells were seeded at 7500 cells/cm^2 into collagen-pre-coated plates and proliferated in a growth medium (GM) until confluent. The culture medium was then replaced with a differentiation medium (DM) containing high glucose DMEM supplemented with 2% horse serum (HS), 100 units/mL penicillin, and 100 μg/mL streptomycin cocktail. The fresh medium was replenished every 2 days and cells were incubated 4 days for experiments. The passage number of C2C12 used in this research was no more than 15.

Plasmids amplification

Plasmids of interest were transformed in TG1 or Stbl3 competent cells and a single colony was cultured in LB medium containing ampicillin overnight at 37°C with shaking and purified with EndoFree Plasmid Midi Kit (Qiagen) or EndoFree Plasmid Mega Kit (Qiagen) for future use.

Transient transfection

For C2C12 cells transient transfection, cells were seeded on a 12-well plate in growth medium (GM) containing high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. When cells reached 70-90% density, e plasmid was transfected using HilyMax (Dojindo) following the manual instruction, respectively. The transfection complexes (for each well: 60 μL Opti-MEM, 1 μg DNA, and 6 μL HilyMax) were incubated at room temperature for 15 min and then the DNA-HilyMax complex was added to the cell culture well.

For HEK293T cells transient transfection, cells were seeded on a 12-well plate in a growth medium (GM). When cells reached 70-90% density, expression plasmid was transfected using PEIMAX (1 mg/mL, Polysciences). The transfection complexes (for each well: 111 μL Opti-MEM, 1 μg DNA, and 4 μL PEIMAX) were incubated at room

temperature for 15 min and then added to the cell culture well.

Histologic analysis

Muscles were dissected and snap-frozen in isopentane pre-cooled with liquid nitrogen and stored at -80℃ until processed. Serial 10 μm cryosections were cut by using a Cryostat. For hematoxylin and eosin (H&E) staining, cryosections were incubated with Mayer's Hematoxylin for 4 minutes at room temperature and then rinsed in tap water for 15 minutes, followed by staining in Eosin, with subsequent serial wash in 70%, 80%, 95%, 100% ethanol for 2 minutes, respectively. Finally, the sections were dehydrated twice with xylene for 2 minutes each and mounted with 20 μL of mounting medium, covered with a cover glass, and stored until observed with fluorescence microscopy (BZ X-800, Keyence). The cross-section area (CSA) was automatically calculated using Cellpose segmentation and Fiji plugin LablesToROIs as reported previously[29].

RNA extraction, quantitative real-time PCR analysis (RT-qPCR)

Total RNA was isolated from tissues by using ISOGEN (NIPPON GENE), according to the manufacturer's instructions. Briefly, tissues were homogenized with a TissueRuptor (QIAGEN) in 1 mL of ISOGEN for 45 seconds, and stored for 5 minutes at room temperature. 200 μL of chloroform was added to the mixture, shaken vigorously for 15 seconds, and allowed to stand for 3 minutes at room temperature. After centrifugation at 12,000 xg for 15 minutes at 4℃, 400 μL of the upper aqueous phase was collected and mixed with 400 μL of isopropanol, then incubated for 5 minutes at room temperature. After centrifugation at 12,000 xg for 10 minutes at 4℃, the RNA pellet was washed with 1 mL of 70% ethanol, followed by centrifugation at 7500 xg for 5 min at 4℃. After removal of the remaining ethanol, the RNA pellet was air-dried and resuspended in 30 μL of DPEC water and stored at -80 ℃.

For genomic DNA digestion, 2 μg of total RNA was digested with 4 μL of DNase (Roche) in a total volume of 20 μL, operated in the thermocycler with program settings

as 30 min at 37 ℃, 10 min at 75 ℃. Then, the DNase-treated RNA was reverse transcribed to complementary DNA (cDNA) with a high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific) in a total of 40 μL of the reaction mixture (4 μL of 10× RT Buffer; 4 μL of 10× random primers; 1.6 μL of 25× dNTP mix; 1μL of 200 U/μL RTase; 20 μL of RNA), followed by the operation in a thermocycler program for 10 min at 25 ℃, 2 h at 37 ℃, and 5 min at 85 ℃. After the reaction, samples were stored at -20℃.

Quantitative RT-PCR analysis was performed with FastStart Universal SYBR Green Master Mix (Roche) in a total volume of 10 μL (2.6 μL of DEPC water; 5 μL of SYBR Green Master Mix; 0.2 μL of 10 μM forward and reverse primer each; 2 μL of cDNA sample) and Applied Biosystems StepOnePlus real-time PCR system (Thermo Fisher Scientific) (for the 96-well plate) or Applied Biosystems QuantStudio 6 Flex (Thermo Fisher Scientific) (for the 384-well plate), with the reaction settings as: 2 min at 50 ℃, 10 min at 95 ℃, and 40 cycles of 15 seconds at 95 ℃ for denaturation, 1 min at 60 ℃ for annealing and extension. Relative gene expression was normalized to 18S ribosomal RNA expression and fold changes were expressed as relative mRNA levels using the $2^{-\Delta\Delta Ct}$ method. Primer sequences used for qPCR are summarized in **Table 2-3**.

Immunoblotting

Whole protein lysates were prepared by homogenizing tissues in radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, and 0.25% sodium deoxycholate) supplemented with phenylmethanesulfonyl fluoride (PMSF) (Sigma-Aldrich, MO), protease inhibitor cocktail (Nacalai Tesque) and phosphatase inhibitor cocktail (Sigma-Aldrich). Samples were left on ice for 10 minutes after 20 strokes with a 25G needle, and centrifuged at 14,000 rpm for 10 minutes at 4 ℃. The supernatant was collected, and the protein concentration was determined by BCA Protein Assay (Thermo Fisher). The required amount of protein was denatured with 6× Laemmli Sample Buffer (1 M Tris HCl (pH

6.8), 30% glycerol, 10% SDS, and 0.03% bromophenol blue) for 5 min at 95 ℃.

3 μg of protein and 3 μL of Prestained XL-Ladder (APRO Science) were loaded on the sodium dodecyl sulfates polyacrylamide (SDS-PAGE) gels (1 M Tris-HCl, 30%(w/v)-Acrylamide/Bis Mixed Solution, 10%(w/v)-Ammonium Peroxodisulfate Solution, N, N', N', N'-Tetramethylethylenediamine) at 90-150V in 1× Running Buffer (25 mM Tris, 192 mM Glycine, 0.1%(w/v) SDS). Then, the resulting gels were blotted onto a Polyvinylidene difluoride (PVDF) membrane by using 1× Transfer Buffer (100 mM Tris, 192 mM glycine, 5% methanol, 0.05% SDS) with semi-dry transfer cassette (Bio craft) (2 mA/cm^2, 90 min), blocked in 5% non-fat skim milk or 5% bovine serum albumin in Tris-buffered saline with 0.05% Tween-20 Detergent (TBS-T) for 1 h at room temperature, and incubated overnight at 4 °C with indicated primary antibodies.

Immunoblots were washed 3 times (5 min each) with TBS-T and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature. Blots were washed 3 times (5 min each) with TBS-T, developed in enhanced chemiluminescent reagent (Cat. No. WBKLS0500, MilliporeSigma), and visualized with the FUSION chemiluminescence imaging system (Vilber-Lourmat). Antibodies used are summarized in **Table 2-4**.

Enzyme-linked immunosorbent assay (ELISA)

The assay was conducted according to the manufacturer's instructions. The results from muscle tissues were normalized by protein concentration.

Statistical analysis

Data were expressed as mean ± standard deviation (SD) of the mean and analyzed by two-tailed, unpaired Student's *t*-test or one-way analysis of variance (ANOVA), followed by Tukey's multiple-comparison test as indicated. The significance was set at $P < 0.05$ for all cases.

2-3 Results

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2-4 Discussion

Skeletal muscle is the largest metabolic organ that dynamically maintains systemic homeostasis [30]. Skeletal muscles play a crucial role not only in movement but also in essential functions such as breathing, energy utilization, and the maintenance of homeostasis for glucose, amino acids, and lipids, which are fundamental to sustaining a high quality of life. It is widely recognized that the metabolic adaptations taking place in skeletal muscles are perceived as a factor influencing disease outcomes, and the overall quality of muscle mass serves as a significant predictor of mortality[30].

Muscle atrophy represents a debilitating systemic response that can result in diminished functional capacity, a decline in quality of life, and heightened morbidity and mortality[31]. Addressing muscle atrophy remains a formidable and unresolved challenge. Consequently, there is a pressing and unmet demand for the development of innovative therapies to counteract the loss of skeletal muscle mass[25,32,33].

One of the molecular mechanisms responsible for muscle atrophy has been identified as the ubiquitin-proteasome system (UPS), which involves activation of the muscle-specific E3 ubiquitin ligases, namely Atrogin1 (also known as MAFbx (muscle atrophy F-box protein), gene name Fbxo32) and MuRF1 (muscle-specific ring finger 1, gene name Trim63) for protein degradation[25,34,35]. The use of a proteasome inhibitor [36] or genetic deletion of each E3 ubiquitin ligase has been shown to mitigate denervation-induced muscle atrophy[25], underscoring the significance of UPS-mediated protein degradation as a primary pathway in this process.

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2-5 Reference

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Chapter 3

The potential driver of muscle atrophy

3-1 Preface

As the contents of this page are anticipated to be published in a paper in a scholarly journal they cannot be published online. The paper is scheduled to be published within five years.

3-2 Materials and methods

Animal care was performed as described in Chapter 2.

C2C12 cells were maintained and differentiated as described in Chapter 2.

RNA extraction and quantitative real-time PCR analysis (qPCR) were performed as described in Chapter 2.

qPCR primers used in this chapter were listed in **Table 3-2**.

Immunoblotting was performed as described in Chapter 2.

Antibodies used in this chapter are listed in **Table 3-3**.

ELISA assay was performed as described in Chapter 2.

RNA-sequencing (RNA-seq)

4 mice were sacrificed, and samples were snap-frozen in liquid nitrogen. Total RNA was isolated and the total RNA from the same group was mixed and submitted to Macrogen for analysis. Differentially expressed genes (DEGs) were screened by the following criteria: adjusted P value < 0.05 and |logFC| ≥ 1. Data analysis and visualization were conducted in R (v 4.3.1.).

Immunofluorescence and Laser Confocal Microscopy

C2C12 cells were seeded in a 35 mm film bottom dish. After differentiation for 3 or 4 days, the indicated treatments were conducted. For myosin heavy chain staining, cells were washed once with PBS and fixed in 4% paraformaldehyde in PBS for 10 min and permeabilized with 0.5% Triton in PBS for 5 min. After 3 washes with PBS, cells were blocked in 3% BSA in PBS for 60 min followed by incubation with primary antibody mouse anti-MHC (1:1000, MAB4470, R&D Systems) and secondary antibody Alexa

Fluro™ 488 (1:500, A11029, Invitrogen) for 1h, respectively. Cells were then washed 3 times with PBS and mounted with ProLong™ Glass antifade with NucBlue™ (Thermo Fisher Scientific). Samples were stored and protected from light until imaged with a ZEISS LSM 800 confocal laser scanning microscope (Carl Zeiss). The whole process was conducted at room temperature.

Measurement of myotube diameter

Myotube diameters were quantified at three points oriented along the longitudinal direction of the myotube based on immunofluorescent images of myotubes. Five fields per well were randomly chosen at a ×10 magnification and the diameter of 50-300 myotubes was measured by ImageJ software (National Institutes of Health, USA). Myotubes were defined as myosin heavy chain (MHC)-positive cells that contain a minimum of 3 nuclei.

Statistical analysis

Data were expressed as mean ± standard deviation (SD) of the mean and analyzed by two-tailed, unpaired Student's *t*-test or one-way analysis of variance (ANOVA), followed by Tukey's multiple-comparison test as indicated. The significance was set at $P < 0.05$ for all cases.

3-3 Results

As the contents of this page are anticipated to be published in a paper in a scholarly journal they cannot be published online. The paper is scheduled to be published within five years.

3-4 Discussion

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3-5 Reference

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Chapter 4

Functional analysis in vivo

4-1 Preface

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4-2 Materials and methods

Animal care was performed as described in Chapter 2.

Genomic DNA extraction

~5 mm of mouse tail was cut and boiled in 360 μL of 50 mM NaOH at 100 ℃ for 1 h. Next, 40 μL of 1 M Tris-Cl (pH 8.0) was added and vortexed, followed by centrifugated at 15,000 rpm for 10 minutes at 4 ℃. The supernatant containing mouse genomic DNA was used for subsequent genotyping analysis.

Four-limb grip strength test, histological analysis, and ELISA assay were performed as described in Chapter 3.

C2C12 cells were maintained and differentiated as described in Chapter 2.

RNA extraction and quantitative real-time PCR analysis (qPCR) were performed as described in Chapter 2.

qPCR primers used in this chapter were listed in **Table 4-1**.

Immunoblotting was performed as described in Chapter 2.

Antibodies used in this chapter are listed in **Table 4-2**.

Ponceau S staining

Ponceau S staining was performed as previously reported. After protein transfer, the PVDF membrane was rinsed briefly in DDW and incubated in Ponceau S solution (Sigma-Aldrich) for 5-10 minutes at room temperature with gentle shaking. Next, the membrane was destained in DDW until reddish-pink protein bands appeared and the image was captured. Finally, the membrane was completely destained with Tris-buffered saline with 0.05% Tween-20 (TBS-T) for 4-5 times, 5 minutes each until the

red color was gone and the membrane was continued to the blocking step.

In vivo protein synthesis assay

Protein synthesis was calculated by using the surface sensing of translation (SUnSET) assay as previously reported[37]. After anaesthetization with isoflurane, mice were intraperitoneally injected with 0.04 μmol/g puromycin (Wako) dissolved in phosphate-buffered saline (PBS). At exactly 30 minutes after the injection, tibialis anterior (TA) muscles were harvested and frozen in liquid N2 for immunoblotting analysis. Puromycin incorporation was used to indicate the protein synthesis.

Original RNA-sequencing (RNA-seq) data are from Chapter 2.

Statistical analysis

Data were expressed as mean ± standard deviation (SD) of the mean and analyzed by two-tailed, unpaired Student's *t*-test or one-way analysis of variance (ANOVA), followed by Tukey's multiple-comparison test as indicated. The significance was set at $P < 0.05$ for all cases.

4-3 Results

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4-4 Discussion

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4-5 Reference

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Chapter 5

Underlying Mechanism

5-1 Preface

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5-2 Materials and methods

Animal care, mice genotyping, and histological analysis were performed as described in Chapters 2, 3, and 4.

HEK293T cells were maintained and C2C12 cells were maintained and differentiated as described in Chapter 2.

RNA interference was performed as described in Chapter 3.

Plasmid amplification was described in Chapter 2.

Immunofluorescence and Laser Confocal Microscopy were performed as described in Chapter 3.

RNA extraction and quantitative real-time PCR analysis (qPCR) were performed as described in Chapter 2.

qPCR primers used in this chapter were listed in **Table 5-3**.

Immunoblotting was performed as described in Chapter 2.

Antibodies used in this chapter are listed in **Table 5-4**.

Cytoplasmic and nuclear fractions preparation

Nuclear protein extracted from tissues was performed according to a previous report[38]. 50 mg of Tibialis anterior (TA) muscles were homogenized in 500 μL of STM buffer (250 mM sucrose, 50 mM Tris–HCl pH 7.4, 5 mM $MgCl_2$) for 1 minute using a Tissue Raptor (Qiagen) and left for 30 minutes, vortexed vigorously for 15 seconds and then centrifuged at 800 xg for 15 minutes. The pellet (P0 fraction) was used for the following isolation of the nuclear fraction and the supernatant (S0 fraction) was used for the subsequent isolation of the cytosolic fraction. <u>For nuclear fraction:</u> the P0 fraction was

resuspended in 500 μL of STM buffer, vortexed vigorously for 15 seconds, and then centrifuged at 500 xg for 15 minutes. The supernatant was discarded, and the above process was repeated twice. The obtained pellet was resuspended in 500 μL of STM buffer, vortexed vigorously for 15 seconds, and then centrifuged at 1,000 xg for 15 minutes. The pellet was resuspended in 300 μL of NET buffer (20 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 0.5 M NaCl, 0.2 mM EDTA, 20% glycerol, 1% Triton X-100), completely resuspended by pipetting, vortexed vigorously for 15 seconds and left for 30 minutes. The suspension was stroked by an 18-gauge needle 20 times and then centrifuged at 9,000 xg for 30 minutes, the resultant supernatant was the nuclear fraction. For cytosolic fraction: the S0 fraction was centrifugated at 800 xg for 10 minutes and the obtained supernatant was again centrifugated at 11,000 xg for 10 minutes. Then, the supernatant was precipitated in four times the volume of pre-chilled acetone for a minimum of 1 h at −20°C followed by centrifugation at 12,000 g for 5 minutes. After the complete removal of acetone, the resultant cytosolic pellet was resuspended in 300 μL of STM buffer. All procedures were done on ice, protease and phosphatase inhibitor cocktails were added to each buffer right before use.

Isolation of nuclear protein from cells was performed as previously reported[39]. Cells from one well of a 6-well plate were rinsed with PBS and resuspended in 80 μL of Buffer A (10 mM HEPES pH 7.9, 0.1 mM EDTA, 0.1 mM EGTA,10 mM KCl, 1 mM DTT, 0.15% NP-40) and left for 15 minutes, followed by centrifugated at 12,000 xg for 2 minutes. The supernatant (s0) contained crude cytoplasm fraction and the pellet (p0) was the crude nuclear pellet. Then, the supernatant (s0) was centrifugated again at 22,000 xg for 10 minutes to get pure supernatant containing cytosolic fraction. The pellet (p0) was resuspended in 20 μL of Buffer B (10 mM Tris pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 400 mM NaCl, 0.4% Triton X-100), vortexed vigorously for 30 minutes, and centrifugated at 12,000 xg for 15 minutes. The resultant supernatant was nuclear extract. All procedures were done on ice, protease and phosphatase inhibitor cocktails were added to each buffer right before use.

Statistical analysis

Data were expressed as mean ± standard deviation (SD) of the mean and analyzed by two-tailed, unpaired Student's *t*-test or one-way analysis of variance (ANOVA), followed by Tukey's multiple-comparison test as indicated. The significance was set at $P < 0.05$ for all cases.

5-3 Results

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5-5 Reference

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Chapter 6

Comprehensive Discussion

Skeletal muscle is the largest organ of the body that plays a key role in metabolic regulation, which adapts to changes in exercise, nutrition, and stress by secreting various myokines and myometabolites, contributing to systemic homeostasis.

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Lirong Zheng
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