**Ganoderma microsporum** immunomodulatory protein, GMI, promotes C2C12 myoblast differentiation *in vitro* via upregulation of Tid1 and STAT3 acetylation

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**Abstract**

Ageing and chronic diseases lead to muscle loss and impair the regeneration of skeletal muscle. Thus, it’s crucial to seek for effective intervention to improve the muscle regeneration. Tid1, a mitochondrial co-chaperone, is important to maintain mitochondrial membrane potential and ATP synthesis. Previously, we demonstrated that mice with skeletal muscular specific Tid1 deficiency displayed muscular dystrophy and postnatal lethality. Tid1 can interact with STAT3 protein, which also plays an important role during myogenesis. In this study, we used GMI, immunomodulatory protein of *Ganoderma microsporum*, as an inducer in C2C12 myoblast differentiation. We observed that GMI pretreatment promoted the myogenic differentiation of C2C12 myoblasts. We also showed that the upregulation of mitochondria protein Tid1 with the GMI pre-treatment promoted myogenic differentiation ability of C2C12 cells. Strikingly, we observed the concomitant elevation of STAT3 acetylation (Ac-STAT3) during C2C12 myogenesis. Our study suggests that GMI promotes the myogenic differentiation through the activation of Tid1 and Ac-STAT3.

**1. Introduction**

Skeletal muscle, wrapped with the connective tissues, constitutes of myotubes that bundles into myofibrils. It comprises about 40% to 45% of our body weight and enables our body to maintain the posture and to perform a wide range of movements, motions and stability [1,2]. Skeletal muscle possesses regenerative capability against minor injury, where the satellite cells differentiate into myoblasts followed by fusion of myoblast into multinucleated myotubes to
replace the injury myofibers [1,3,4]. However, ageing or chronic diseases such as atrophy, cachexia and sarcopenia will lead to muscular loss and the deficiency to maintain or restore the normal structure and function of the impaired muscle [2,5,6]. Hence, muscle regeneration and transplantation of myogenic cells is an essential therapy for the muscular dystrophies.

Skeletal muscle differentiation is a multi-stage highly-regulated process that includes myoblast division, elongation, and fusion [7]. Along with myogenesis process the expression of stem cell markers such as Pax7 is gradually lost. Meanwhile, appearance of differentiation markers, such as myogenin, myosin heavy chain (MyHC) and muscle-regulatory factor 4 (Mrf4) are gradually increase [8]. Apart from this, previous studies demonstrated that interleukin 6 (IL-6) a pro-inflammatory cytokine, is elevated in response to muscle contraction [9]. IL-6 is found to participate in muscle regeneration in muscular dystrophy [10]. Additionally, it has been reported that IL-6 promote myogenic differentiation of C2C12 via signal transducer and activator of transcription 3 (STAT3) axis. Knockdown of IL-6 and STAT3 in C2C12 differentiating cells suppress the expression of the myogenic markers myogenin and MyHC, consequently, resulting the inhibition of myotube fusion. Furthermore, primary muscle cells isolated from IL-6 knockout mice feature a significant reduction of MyHCIIb positive cells [11].

Tumorous imaginal disc 1 (Tid1) is a mammalian mitochondrial DNAJ/HSP40 co-chaperone protein homolog to the Drosophila tumor suppressor protein Tid56 [12]. Tid1 protein contains a conserved J-domain by which to interact with heat shock protein 70 (HSP70) family members via stimulation of ATPase activity [13]. Tid1 transcripts are expressed in two alternatively splicing isoforms, Tid1-long form (Tid1-L) and Tid1-short form (Tid1-S). Interestingly, Lu et al. reveal that Tid1-L exhibit higher cytosolic stability and a slower rate of mitochondrial import in comparison with Tid1-S. Further, they report that Tid1 interacts with the HSP70 substrate proteins, STAT1 and STAT3 [14].

Tid1 has been identified as a tumor suppressor [15–19]. Previously, we have demonstrated that cell proliferation is inhibited in Tid1 overexpressed head and neck squamous cell carcinoma (HNSCC) cells. Conversely, Tid1 gene knockdown enhances cancer cell malignancy such as cell migration and invasion in vitro, and tumorigenicity in vivo [15]. Additionally, Tid1 abrogates the Galectin-7/TCF3/MMP9 axis to repress the cancer metastasis [16]. We also report the participation of Tid1 in early embryogenesis [20], T cells development [21], muscular development and mitochondrial biogenesis [22] in normal development. We observe the elevation of Tid1 protein in differentiated C2C12 myoblast. In opposite, Tid1 gene knockdown impairs the differentiation ability of C2C12 myoblasts. Concurrently, we perceive the reduction of intracellular ATP amounts and mitochondrial activity which results in energy imbalance and promotion of cells apoptosis. Moreover, our established HSA-Tid1<sup>f/f</sup> and HSA-Tid1<sup>f/+</sup> transgenic mice (mice with Tid1 deficiency specifically in skeletal muscle) show severe muscular dystrophy with reduced motor activity, accompanied with impairment of activity of ATP sensor (p-AMPK) and mitochondrial biogenesis protein, peroxisome proliferator activated receptor gamma coactivator-1 alpha (PGC-1α) [22]. Thus, activation of Tid1 is important to maintain the integrity of mitochondrial and myogenesis of skeletal muscle.

Ganoderma is a genus of polypore fungi widely used as medicinal purposes for centuries, particularly in China, Japan and Korea [23], and commonly called as Lingzhi. In accordance with the theory of traditional Chinese medicine, Ganoderma possesses the ability to strengthen body resistance and consolidate the constitution [24]. GMI, an immunomodulatory protein cloned from G. microsporum, is found to exhibit anti-inflammatory effect. GMI has also been studied in a broad-spectrum application for the anti-cancerous treatment through the regulation of immune system. GMI inhibits tumor necrosis factor alpha (TNFα)-mediated matrix metallopeptidase 9 (MMP-9) expression and migration in A549 cancer cells [25].
Furthermore, Hsin et al. shows that GMI has the ability to inhibit tumorigenicity and induce autophagy cell death in non-small lung cancer cell lines [26–29]. The anti-cancerous roles of GMI have also been studied in urothelial carcinoma cells [30], oral carcinomas stem cells (OCSRs) [31] and human fibrotic buccal mucosal fibroblasts (BMMFs) [32]. Despite these, the biological function of GMI is seldom been discussed in normal circumstances.

Inflammatory responses are critical in skeletal muscle myogenesis process. Previous study has reported that the transition of M1 to M2 macrophage releases the anti-inflammatory cytokines to promote skeletal muscle differentiation [33]. Moreover, for IL-10 knockout mice, with the deficiency of the well-known anti-inflammatory cytokine, display muscle loss and develops muscle weakness [34]. However, this frail model is reversed by up taking grape seed extract [35], which is known for its anti-oxidative and anti-inflammatory effect [36,37].

Since GMI is found to possess the anti-inflammatory effect; therefore, in this study, we explored the promotion of GMI in skeletal muscle myogenesis. Indeed, treatment of GMI in C2C12 myoblast promoted the differentiation of myoblast and fusion into myotubes. Additionally, we observed the elevation of Tid1, PGC-1α, and Acetylated-STAT3 (Ac-STAT3) proteins in the differentiated myoblast. Our findings provide a new insight of GMI treatment to promote C2C12 myoblast differentiation via activation of Tid1 mitochondria co-chaperon and Ac-STAT3.

2. Materials and methods

2.1 Myoblast cell line and myogenesis induction

Mouse C2C12 myoblasts (BCRC, 60083) were obtained from Bioresource Collection and Research Center (Hsinchu, Taiwan). The cells were expanded in growth medium of Dulbecco’s Modified Eagle Medium (DMEM), 10% fetal bovine serum (FBS), 1% L-glutamine and 1% Penicillin-Streptomycin Amphotericin (PSA) at 37˚C under 5% CO2. When cells reached 90% -100% confluence, the growth medium was replaced with differentiation medium consisting of DMEM, 2% horse serum (#16050–130, Thermo Fisher Scientific, New Zealand), and 1% PSA. Cell culture media and supplements were purchased from biological industries (BI, Israel).

2.2 Generation of the skeletal muscle specific Tid1 deletion mice

Tid1 floxed mice were generated according to previous study [20]. Mice with Tid1 gene homozygous or heterozygous deletion specifically in skeletal muscle were generated by crossing Tid1<sup>f/f</sup> or Tid1<sup>f/+</sup> mice with transgenic HSA-Cre mice. The genotyping of the HSA-Cre transgene and the Tid1-deficient mice (HSA-Tid1<sup>f/f</sup> or HSA-Tid1<sup>f/+</sup>) was performed by polymerase chain reaction (PCR) using genomic DNA isolated from the tail.

2.3 GMI protein

GMI protein was manufactured by MycoMagic Biotechnology Company Ltd (Taipei, Taiwan). During experimental conduction, the GMI sample was dissolved in differentiation medium to reach different concentrations.

2.4 Isolation and culture of murine primary myoblast

Primary myoblasts were isolated as described as previous study [38]. Skeletal muscle tissues were minced and digested with digestion medium (high glucose DMEM, 1% PSA, 2.5% HEPES, and 400 U/ml collagenase II). The digested tissues were centrifuged, and the muscle pellet was subsequently resuspended with neutralizing/isolation medium (NIM, high glucose...
DMEM, 10% FBS, and 1% PSA). The muscle pellet mixtures were filtered twice, firstly through the 70 μm strainer and followed by 30 μm strainer to get the cell mixture. The cell mixture was centrifuged and resuspended in myoblast growth medium (MGM, F-10 media, 20% FBS, 1% PSA, and 10 ng/ml fibroblast growth factor). After 72 hours cultured on a 10% matrigel-coated dish, the cells were trypsinized and passaged until the cells >95% myoblast purity was achieved.

2.5 Cell cytotoxicity assay

C2C12 myoblasts were counted and approximately 5.5 x 10^4 cells per well were seeded in a 24-well plate. The cells were expanded in growth medium of Dulbecco’s Modified Eagle Medium (DMEM), 10% fetal bovine serum (FBS), 1% L-glutamine and 1% Penicillin-Streptomycin Amphotericin (PSA) at 37˚C under 5% CO\textsubscript{2}. When cells reached 90% - 100% confluence, the growth medium was replaced with differentiation medium consisting a series concentrations of GMI (0, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 20 and 30 μg/ml). Three replicates were made for each measurement. The cells we pre-treated with GMI for 24 hours. 1/10 of the CCK-8 reagent (Dojindo, Japan) was added into each well, and O.D at 450 nm was measured using Spark Multimode Microplate Reader (Tecan Trading AG, Switzerland) after 2 hours incubation at 37˚C.

2.6 Immunoblot analysis

The cells crude proteins were extracted with RIPA buffer. The protein concentration was quantified through the Protein Assay Dye Reagent (Bio-Rad, USA). The extracted proteins were loaded onto SDS-polyacrylamide gels for electrophoresis and then transferred to nitrocellulose membranes. The nitrocellulose membranes were blocked with TBST containing 5% skimmed milk for 1 hour at room temperature followed by incubation with the corresponding primary antibodies and secondary antibodies. The signals were visualized by the enhanced chemiluminescence system as described by the manufacturer (Millipore, Germany) in conjunction with in LAS-4000 image analyzer (GE Healthcare, Japan). Mouse anti-MyoD (MA1-40117) and rabbit anti-Acetyl-STAT3 (Lys685) (PA5-17429) were purchased from Thermo Fisher Scientific (USA). Mouse anti-Tid1 (RS13) (sc18819) and rabbit anti-IL6 (M-19) (sc-1265) were purchased from Santa Cruz (USA).

Mouse anti-MyHC (05–716), mouse anti-PGC-1α (ST1202) were from Sigma-Aldrich, USA, rabbit anti-p-STAT3 (Tyr705) (Cell Signaling, USA). Rabbit anti-total STAT3 [C3] (GTX104616) and rabbit anti-beta actin (GTX109639) were purchase from GeneTex (USA). The signals collected from immunoblots were quantified by using Image Studio™ Lite Software.

2.7 Enzyme linked immunosorbent assay analysis

5.5 x 10^4 C2C12 myoblasts were seeded per well in a 24-well plate. When reached 90% -100% confluence, the myoblasts were pre-treated with differentiation medium consisting a series concentration of GMI (0, 0.01, 0.05, 0.1, 0.5, 1, 5, 10 and 10 μg/ml). The cultured medium were collected at different time points (24, 48 and 72 hours), and the levels of IL-6 were determined using ELISA kits (R&D Systems) according to the manufacturer’s instructions, and then quantified by Spark Multimode Microplate Reader (Tecn, Switzerland) at O.D 450 nm.

2.8 Statistical analysis

The statistical analysis was performed by using GraphPad Prism 6 (GraphPad Software, California, USA). The datasets with multiple groups were analyzed by One-way ANOVA.
presented data are mean± SEM of three independent experiments (n = 3) the probability values less than 0.05 (p < 0.05) were considered statistically significant.

3. Results

3.1 Induced myogenesis of C2C12 cells and primary myoblasts in vitro

C2C12 cells and the isolated mice primary myoblast were grown under the differentiation medium and went on morphological change from mesenchymal cell type to extended stretched cell type as a myoblast along with cell fusion and elongation (Fig 1A). Immunoblotting analyses demonstrated that the expression profile of those known myogenesis markers, including MyoD, MyHC and STAT3 (both total- and phosphor-STAT3), which is similar to the previous findings [22] (Fig 1B). Strikingly, we observed the upregulation of acetylated-STAT3 in all the differentiated myoblasts (C2C12) and primary myoblasts (wild type and HSA-Tid1f/+), which has never been reported thus far (Fig 1B). Further, Tid1, a known mitochondrial protein acting as a tumor suppressor, was also up regulated in the differentiated C2C12 cells and the isolated primary myoblasts. However, the protein levels of Tid1 and MyHC in differentiated HSA-Tid1f/+ myoblasts were lower than that of wild-type differentiated myoblasts (Fig 1B). As shown in Fig 1A the number of isolated primary myoblasts from the HSA-Tid1f/+ mice were fewer than that isolated from the wild type mice. Of note, we observed that the expression level of MyoD and IL-6 of the C2C12 were less than that of wild type primary myoblasts (on both day 0 and day 4). In addition, we observed less total PGC-1α protein in the differentiated cell (C2C12 and primary myoblasts (wild type and HSA-Tid1f/+). This findings suggest that Tid1 protein is upregulated during myogenesis, and the Tid1 heterozygous deficiency leads to dysfunction of muscle tissue and these findings were consistent with our previous publication [22].

3.2 The cytotoxic effect of GMI on C2C12 myoblast

Cytotoxicity is defined as the toxicity caused due to the action of drugs on living cells. Therefore, we tested the cytotoxic effect of GMI on the cell viability of C2C12 myoblasts. The concentration of GMI applied to the C2C12 myoblast were 0, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 20 and 30 μg/ml, respectively. After treatment with GMI for 24 hours, we visualized the cells morphology changes under microscopic (Fig 2A). We also observed that high concentration (> 10 μg/ml) of GMI affected the cell viability. Consequently, we determined the cell viability after treatment with GMI by using CCK-8 assay. The analytical results were consistent with the cells morphology changes, at which the high concentration of GMI (20 μg/ml and 30 μg/ml) caused a dramatic cell death (Fig 2B). The cell viability ratio of C2C12 myoblasts at 20 μg/ml and 30 μg/ml were 32 ± 5.42% and 27 ± 6.00% respectively. Contrary, GMI at lower concentration (0.01, 0.05, 0.1, 0.5 μg/ml) induced cell proliferation.

3.3 GMI pre-treatment promoting induced myogenesis in myoblast

To assess the effects of GMI on promoting induced myogenesis in vitro, C2C12 cells of 90%-100% confluence were replaced with differentiation medium containing 0, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 20, 30 μg/ml of GMI for 24 hours. The GMI pre-treated C2C12 cells were set for further differentiation for 5 more days (Fig 3A). The morphology of C2C12 cells with GMI pre-treatment was recorded daily (data not shown). We observed that pre-treatment with GMI at high concentration, such as 20 and 30μg/ml, caused significant cell death (Fig 2). Nevertheless, pre-treatment of 0.05, 0.1, 0.5 and 1 μg/ml of GMI promoted the morphological change of C2C12 myoblast with cell fusion and elongation.
**Fig 1. Differentiation of C2C12 and primary myoblast.** (A) The representative phase contrast images of C2C12 cells and primary myoblasts under induced differentiation at different time intervals (Day 0, 4 and 6). White arrows.
Crude cell extract proteins of GMI pre-treated C2C12 cells were collected on day 6. Immunoblotting analyses showed that GMI pretreatment with low concentration (0.01, 0.05, 0.1 and 0.5 μg/ml) inhibited the expression of IL-6 in differentiated C2C12 cells whereas pre-treatment with high dose of GMI (1, 5, 10 and 20 μg/ml) enhanced the expression of IL-6 (Fig 3B). We also observed that the expression profile of phosphorylated STAT3 and PGC-1α displayed a similar pattern as that of IL-6 (Fig 3B). Interestingly, pretreatment with GMI enhanced the expression of mitochondrial protein Tid1 and acetylated STAT3 (Ac-STAT3) in a dose-dependent manner (Fig 3B), especially at the lower concentration of GMI (0.01, 0.05, 0.1 and 0.5 μg/ml). Additionally, the specific myogenesis marker, MyHC, was also up regulated within the GMI pretreated C2C12 cells in a dose-dependent manner. Overall, the above results demonstrated that pretreatment of GMI can vary the activity of IL-6 and phosphor-STAT3 along with the up regulation of mitochondrial protein (Tid1) and biogenesis marker (MyHC).

We next determined the IL-6 secretion from GMI pre-treated C2C12 myoblasts using ELISA assays. The secretions of IL-6 were assessed at 24, 48 and 72 hours after GMI treatment (GMI (0, 0.01, 0.05, 0.1, 0.1, 0.5, 1, 5 and 10 μg/ml). The results revealed that there was not significant IL-6 secretion from cells treated with GMI for 24 hours. Nevertheless, we observed significant increment of IL-6 secretion of cells in a dose-dependent manner after 48- and 72 hours treatment of GMI, particularly at 0.1, 0.5 and 1 μg/ml (p < 0.0001) (Fig 3C). These data supported the notion that secretion of IL-6 was enhanced during C2C12 myoblasts differentiation after 48- and 72 hours at low dose of GMI treatment.

4. Discussion

Mammalian adult skeletal muscle is defined as stable tissue and possesses a remarkable ability to initiate a rapid and extensive repairing process to prevent the loss of muscle mass during injury [1–3]. Currently, many studies have proposed the use of human recombinant growth factors to induce the regeneration of skeletal muscle [39–41]. Insulin-like growth factor-1 (IGF-1) is found highly mitogenic for myoblasts [42–44]. Overexpression the human IGF-1 display muscle hypertrophy [45]. In addition, basic fibroblast growth factors (bFGF) and platelet-derived growth factors (PDGF), demonstrated a potent stimulating effects on satellite cell proliferation [40,41]. However, these growth factors may induce the production of transforming factor-beta (TGF-β1) which acts as deleterious agent for skeletal muscle myogenesis [46]. Recently, Shin and colleagues have shown that Red Ginseng extract induced the mitochondrial biogenesis and ATP production, consequently, promoting the differentiation of C2C12 myoblast [47]. Red Ginseng, a medicine herb, has been reported to possess the ability to protect muscle damage after strenuous exercise, relief fatigue and to upregulate the energy metabolism [48,49]. GMI, an immunomodulatory protein from G. microsporum is a traditional medicine and has been used for thousand years. Recently, most of the studies report the use of GMI in anti-tumor progression, inhibition of the proliferation of cancer cells and anti-inflammation. GMI inhibits epidermal growth factor (EGF)-induced metastasis through autophagy signaling and cause the cell death in lung cancer cells. GMI also been reported as a suppressor agent for oral carcinomas stem cells and, inhibit migration and invasion of lung cancer cell [26,27,29]. Concurrently, a study shows GMI could induce apoptosis in urinary bladder urothelial carcinoma cells [30]. Although the functions of GMI are well define in anti-cancerous, the study of GMI is rarely been countered on normal cells condition. In this study,
Fig 2. The cytotoxicity of GMI on C2C12 myoblasts. (A) C2C12 cells were treated with different dosage of GMI (0, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 20 and 30 μg/ml) for 24 hours and the morphological changes were visualized. (B) The cell viability of GMI pre-treated C2C12 was determined by using CCK-8 assay. Statistical analyses were performed by One-way ANOVA. ***, p < 0.001 and **** p < 0.0001.

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Fig 3. Promoted C2C12 myogenic differentiation with GMI pretreatment. (A) The representative phase contrast images of induced differentiated C2C12 with pretreatment of GMI (0, 0.01, 0.05, 0.1, 0.5, 1, 5, 10 and μg/ml) on day 6. Immunoblot
we explored the promotion of GMI on myoblast myogenesis. We observed that pre-treatment with GMI successfully induced the C2C12 myoblast differentiation. We also observed the upregulating of specific myogenesis marker. Apart from that, we found upregulation of mitochondria protein Tid1, mitochondria biogenesis marker PGC-1α, and Ac-STAT3.

Regeneration of skeletal muscle is a highly synchronized process involving the activation of various cellular responses. Firstly, necrosis of damaged tissue and a cascade of inflammatory are activated, subsequently, followed by activation of myogenic cells to proliferate, differentiate and fuse to generate a new myofiber formation [2,3]. Many studies have reported that vary signaling factors are released during myogenesis of skeletal muscle. During skeletal muscle myogenesis, a continuous series of myogenic lineage is regulated by the positive or negative signals. Firstly, MyoD, the transcriptional activators of the myogenic regulatory factor family, is the upregulated [50–52]. Elevation of MyoD induces the proliferation of myoblast, subsequently follow by the downregulation of Pax7, a paired box transcription factor of satellite cells [4,53–55]. Kablar and colleagues have reported the total loss of skeletal muscle in MyoD /− and Myf-5 /− double knockout mice. Along with the myogenesis process, the proliferating myoblasts withdraw from the cell cycle and terminate the differentiation of myoblast. Consequently, the muscle-specific gene, myosin heavy chain (MyHC) is upregulated, where the terminally differentiated myoblasts fuse together and elongate to form a multinucleated muscle fiber [56–58]. Here, our data (Figs 1 and 3) showed the consistency with the previous publications.

In present study, we found that IL-6 signal was upregulated along with the myogenesis process (Fig 1B). Additionally, high dose GMI pre-treatment was able to increase the expression of intracellular IL-6 (Fig 3A) but low doses of GMI (0.1, 0.5 and 1 μg/ml) significantly induced the secretion of IL-6 (Fig 3B). Upregulation of IL-6 is essential for satellite cells proliferation [59]. Apart from that, IL-6 participates in myoblast differentiation and fusion. Hence, IL-6 is playing a dual role in myogenesis. For instance, the depletion of IL-6 reduces the extent of myoblast differentiation and fusion. In opposite, genetic overexpression of exogenous IL-6 induced the myogenesis with elevation of expression of muscle specific genes expression. Myoblasts derived from IL-6 null mice show inhibited differentiation and reduced fusion abilities. Although IL-6 is essentially needed in promoting differentiation, it also been reported that the activation of its downstream signaling molecule STAT3 is necessary to promote differentiation of myoblasts [11]. Wang et al. has reported the importance of JAK2/STAT2/STAT3 pathway in myogenic differentiation. They show that by individually knockdown the endogenous JAK2, STAT2, and STAT3, the differentiation of C2C12 myoblast is inhibited [60]. However, via the JAK1/STAT1/STAT3 pathway it could promote the myoblast differentiation [61]. In this study, we showed that the p-STAT3 is elevated when pre-treated with GMI. Nevertheless, upregulation of p-STAT3 was not dose-dependent. Surprisingly, we found the Ac-STAT3 signal was augmented with a dose-dependent manner along with GMI pre-treatment, particularly in low concentration of GMI (0.01, 0.05, 0.1 and 0.5 μg/ml), during C2C12 induced differentiation.

As abovementioned, the role of p-STAT3 in myogenesis is widely discussed. However, the role of STAT3 acetylation in myoblast myogenesis has never been determined. Acetylation of K685 STAT3 has been reported to facilitate the STAT3 dimerization and full transcriptional activity [62,63]. STAT3 acetylation is found to regulate the proliferation of cancer cell. CD44 is a type I transmembrane glycoprotein, through its C-terminal, to interact with N-terminal...
coiled-coil domain (NTD) of STAT3, then to alleviate the binding of p300 and to drive the STAT3 acetylation at K658. Subsequently, STAT3 acetylation activates Cyclin D1 promoter and induces the tumor proliferation [64]. STAT3 acetylation has been found to promote DNA methyltransferase 1 interactions (Dnmt1) expression in MEF cells [65]. In addition, DNMT1 is playing an important role in myogenic differentiation and cell fate transition [66]. A recent study shows that acetylated STAT3 is able to shuttle between cytosolic and mitochondria of lung cancer cells. Further, the constitutively acetylated STAT3 could translocate into mitochondrial and regulate the pyruvate metabolism for TCA cycle that helps to maintain the mitochondrial membrane potential and ATP synthesis [67].

Tid1 is expressed in two alternatively splicing isoforms, Tid1-L and Tid1-S which differ at the C- terminal tail. Interestingly, Lu et al. have highlighted the key differences between the subcellular localization dynamics of Tid1-L and Tid1-S. They also reveal that Tid1-L exhibit higher cytosolic stability and a slower rate of mitochondrial import compared with Tid1-S. They also reported that the interaction between Tid1 and STAT3 [14]. Furthermore, in our previous publication, we have reported the essential role of Tid1 in maintaining the integrity of mitochondrial and myoblast myogenic capacities. Tid1 deficiency can cause dysfunction of muscle tissue of transgenic mice in vivo. We also found that the 8-day old HSA-Tid1ff null mice showed Lordokyphosis phenotype and became postnatal lethality at postnatal day 8 to 10. Depletion of Tid1 suppresses C2C12 cell differentiation in vitro, which reduces the myotube formation. Moreover, Tid1 deletion impairs the mitochondria activity. The mitochondrial mass and membrane potential are abolished. Apparently, the mitochondrial biogenesis protein, PGC-1α is downregulated [22]. Above mentioned, isolated primary HSA-Tid1ff myoblasts were poor differentiated and the myoblast numbers were fewer compared to the wild-type primary myoblasts (Fig 1A). Together, we postulated that upregulation of Tid1 along with its interacting protein, STAT3, undergoing acetylation during the myogenesis induced by GMI. In this study, we observed the expression of mitochondrial protein Tid1 were upregulated in GMI pre-treatment differentiated myoblast. Hence, Tid1 plays a pivotal role in myogenic process and muscle regeneration.

PGC-1α is required for the induction of many antioxidant-detoxifying enzymes and acts as a modulator to coordinate the skeletal muscle for adaption of exercise [68,69]. Skeletal muscle specific overexpression PGC-1α mice demonstrates enrichment of type I myofibers, a fast glycolytic muscle and facilitates the switch of oxidative metabolism, consequently, that promotes the muscle performance and reduces the muscle fatigue [35]. PGC-1α also has been reported to regulate skeletal muscle mass, particularly in condition of muscle atrophy [70]. Nevertheless, we did not observed the upregulation of PGC-1α protein level during C2C12 differentiation. Hence, we speculated that the limited detection of PGC-1α protein may be caused from unknown post-translational modification.

Sarcopenia, an age-related skeletal muscle loss and dysfunction is public health issue since the average life span is increasing these years. As ageing progresses, mitochondria dysfunction in skeletal muscle reduced the capabilities of muscle regeneration [71]. Thus ageing results the impairment of muscle contractile and muscular atrophy [72,73]. Moreover, during ageing, the abnormalities of metabolic or endocrines bring to chronic inflammation. The pro-inflammatory mediator such as TNF-α and nuclear factor-xB (NF-xB) induce the apoptotic cell death and reduce the myogenesis capabilities [74,75]. Above mentioned, GMI possess the anti-inflammatory effect, thus might suppress the ageing-related inflammation response. In additional, we had demonstrated that application of low concentration of GMI could induce myoblast differentiation and along with the upregulation of the mitochondrial protein Tid1. Collectively, our findings suggest that GMI may have the utility of skeletal muscle regeneration.
In summary, our results demonstrate that Tid1 might play a pivotal role in regulating GMI induced myoblast differentiation. We propose that pre-treatment of GMI promotes the myogenic differentiation via upregulation of Tid1 and Ac-STAT3 (Fig 4).

Fig 4. Schematic depicts that GMI pretreatment promotes C2C12 myogenic differentiation via activation of Tid1 and Ac-STAT3.

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Supporting information

S1 File. (RAR)

S2 File. (RAR)

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References

1. Bassel-Duby R, Olson EN. Signaling pathways in skeletal muscle remodeling. Annu Rev Biochem. 2006; 75:19–37. https://doi.org/10.1146/annurev.biochem.75.103004.142622 PMID: 16756483

2. Huard J, Li Y, Fu FH. Muscle injuries and repair: current trends in research. JBJS. 2002; 84(5):822–32. PMID: 12004029

3. Charge SB, Rudnicki MA. Cellular and molecular regulation of muscle regeneration. Physiological reviews. 2004; 84(1):209–38. https://doi.org/10.1152/physrev.00019.2003 PMID: 14715915

4. Forcina L, Miano C, Pelosi L, Musarò A. An overview about the biology of skeletal muscle satellite cells. Current genomics. 2019; 20(1):24–37. https://doi.org/10.2174/1389202920666190116094736 PMID: 31015789

5. Biolo G, Cederholm T, Muscaritoli M. Muscle contractile and metabolic dysfunction is a common feature of sarcopenia of aging and chronic diseases: from sarcopenic obesity to cachexia. Clinical Nutrition. 2014; 33(5):737–48. https://doi.org/10.1016/j.clnu.2014.03.007 PMID: 24785098

6. Lok C. Cachexia: The last illness. Nature News. 2015; 528(7581):182. https://doi.org/10.1038/528182a PMID: 26659165

7. Das M, Wilson K, Molnar P, Hickman JJ. Differentiation of skeletal muscle and integration of myotubes with silicon microstructures using serum-free medium and a synthetic silane substrate. Nature protocols. 2007; 2(7):1795. https://doi.org/10.1038/nprot.2007.229 PMID: 17641647

8. Burkly LC. Regulation of tissue responses: the TWEAK/Fn14 pathway and other TNF/TNFR superfamily members that activate non-canonical NFκB signaling. Frontiers in immunology. 2015; 6:92. https://doi.org/10.3389/fimmu.2015.00092 PMID: 25784914

9. Haddad F, Zaldivar F, Cooper DM, Adams GR. IL-6-induced skeletal muscle atrophy. Journal of applied physiology. 2005; 98(3):911–7. https://doi.org/10.1152/japplphysiol.01026.2004 PMID: 15542570

10. Kurek JB, Nouri S, Kannourakis G, Murphy M, Austin L. Leukemia inhibitory factor and interleukin-6 are produced by diseased and regenerating skeletal muscle. Muscle & Nerve: Official Journal of the
GMI promotes myogenesis

11. Hsin I-L, Ou C-C, Wu T-C, Jan M-S, Wu M-F, Chiu L-Y, et al. GMI, an immunomodulatory protein from mouse skeletal muscle cells: role of the STAT3 pathway. American Journal of Physiology-Cell Physiology. 2013; 304(2):C128–C36. https://doi.org/10.1152/ajpcell.00025.2012 PMID: 23114963

12. Kurzik-Dumke U, Gundacker M, Rentrop M, Gately E. Tumor suppression in Drosophila is causally related to the function of the lethal (2) tumor suppressor protein Tid56, can interact with the human papillomavirus type 16 E7 oncoprotein. Virology. 1998; 247(1):74–85. https://doi.org/10.1006/viro.1998.9220 PMID: 9683573

13. Schilling B, De-Medina T, Syken J, Vidal M, Münger K. A novel human DnaJ protein, hTid-1, a homolog of the Drosophila tumor suppressor protein Tid56, can interact with the human papillomavirus type 16 E7 oncoprotein. Virology. 1995; 16(1):64–76. https://doi.org/10.1002/dvg.1020160110 PMID: 7758246

14. Lu B, Garrido N, Spelbrink JN, Suzuki CK. Tid1 isoforms are mitochondrial DnaJ-like chaperones with unique carboxyl termini that determine cytosolic fate. Journal of Biological Chemistry. 2006; 281(19):13150–8. https://doi.org/10.1074/jbc.M509179200 PMID: 16531398

15. Chen CY, Chio SH, Huang CY, Jan CI, Lin SC, Hu WY, et al. Tid1 functions as a tumour suppressor in head and neck squamous cell carcinoma. The Journal of Pathology: A Journal of the Pathological Society of Great Britain and Ireland. 2009; 219(3):347–55.

16. Chen Y-S, Chang C-W, Tsay Y-G, Huang L-Y, Wu Y-C, Cheng L-H, et al. HSP40 co-chaperone protein Tid1 suppresses metastasis of head and neck cancer by inhibiting Galectin-7-TF3-MMP9 axis signalling. Theraonotics. 2018; 14(8):1384. https://doi.org/10.7150/thno.25784 PMID: 30083263

17. Jan CI, Yu CC, Hung MC, Ham LJ, Nieh S, Lee HS, et al. Tid1, CHIP and ErbB2 interactions and their prognostic implications for breast cancer patients. The Journal of pathology. 2011; 225(3):424–37. https://doi.org/10.1002/path.2921 PMID: 21710689

18. Kim S-W, Chao T-H, Xiang R, Lo J-F, Campbell MJ, Fearns C, et al. Tid1, the human homologue of a Drosophila tumor suppressor, reduces the malignant activity of ErbB-2 in carcinoma cells. Cancer research. 2004; 64(21):7732–9. https://doi.org/10.1158/0008-5472.CAN-04-1323 PMID: 15520177

19. Kim S-W, Hayashi M, Lo J-F, Fearns C, Xiang R, Lazennec G, et al. Tid1 negatively regulates the migratory potential of cancer cells by inhibiting the production of interleukin-8. Cancer research. 2005; 65(19):8784–91. https://doi.org/10.1158/0008-5472.CAN-04-4422 PMID: 16204048

20. Lo J-F, Hayashi M, Woo-Kim S, Tian B, Huang J-F, Fearns C, et al. Tid1, a co-chaperone of the heat shock 70 protein and the mammalian counterpart of the Drosophila tumor suppressor (2) tid, is critical for early embryonic development and cell survival. Molecular and cellular biology. 2004; 24(6):2226–36. https://doi.org/10.1128/mcb.24.6.2226-2236.2004 PMID: 14993262

21. Lo J-F, Zhou H, Fearns C, Reisfeld RA, Yang Y, Lee J-D. Tid1 is required for T cell transition from double-negative 3 to double-positive stages. The Journal of Immunology. 2005; 174(10):6105–12. https://doi.org/10.4049/jimmunol.174.10.6105 PMID: 15879105

22. Cheng L-H, Hung K-F, Lee T-C, Huang C-Y, Chiu W-T, Lo J-F, et al. Mitochondrial co-chaperone protein Tid1 is required for energy homeostasis during skeletal myogenesis. Stem cell research & therapy. 2016; 7(1):1–10. https://doi.org/10.1186/s13287-016-0443-8 PMID: 27927223

23. Meng J, Hu X, Shan F, Hua H, Lu C, Wang E, et al. Analysis of maturation of murine dendritic cells (DCs) induced by purified Ganoderma lucidum polysaccharides (GLPs). International journal of biological macromolecules. 2011; 49(4):693–9. https://doi.org/10.1016/j.ijbiomac.2011.06.029 PMID: 21763719

24. Yue GG, Fung K-P, Tse GM, Leung P-C, Lau CB. Comparative studies of various Ganoderma species and their different parts with regard to their antitumor and immunomodulating activities in vitro. Journal of Alternative & Complementary Medicine. 2006; 12(8):777–89. https://doi.org/10.1089/acm.2006.12.777 PMID: 17034284

25. Lin C-H, Hsiao Y-M, Ou C-C, Lin Y-W, Chiu Y-L, Lue K-H, et al. GMI, a Ganoderma immunomodulatory protein, down-regulates tumor necrosis factor α-induced expression of matrix metalloproteinase 9 via NF-κB pathway in human alveolar epithelial A549 cells. Journal of agricultural and food chemistry. 2010; 58(22):12014–21. https://doi.org/10.1021/jf103068w PMID: 21028821

26. Hsin I-L, Ou C-C, Wu T-C, Jan M-S, Wu M-F, Chiu L-Y, et al. GMI, an immunomodulatory protein from Ganoderma microsporum, induces autophagy in non-small cell lung cancer cells. Autophagy. 2011; 7(8):873–82. https://doi.org/10.4161/auto.7.8.15698 PMID: 21490426

27. Hsin I-L, Ou C-C, Wu M-F, Jan M-S, Hsiao Y-M, Lin C-H, et al. GMI, an immunomodulatory protein from Ganoderma microsporum, potentiates cisplatin-induced apoptosis via autophagy in lung cancer cells. Molecular pharmaceutics. 2015; 12(5):1534–43. https://doi.org/10.1021/mp500840z PMID: 25811903

28. Hsin I-L, Wang S-C, Li J-R, Ciou T-C, Wu C-H, Wu H-M, et al. Immunomodulatory proteins FIP-gts and chloroquine induce caspase-independent cell death via autophagy for resensitizing cisplatin-resistant...
urothelial cancer cells. Phytomedicine. 2016; 23(13):1566–73. https://doi.org/10.1016/j.phymed.2016.09.003 PMID: 2783620

29. Hsin IL, Hsu JC, Wu WJ, Lu HJ, Wu MF, Ko JL. GMI, a fungal immunomodulatory protein from Ganoderma microsporum, induce apoptosis via β-catenin suppression in lung cancer cells. Environmental toxicology. 2018; 33(9):955–61. https://doi.org/10.1002/tox.22582 PMID: 29974605

30. Huang SY, Chien CC, Hseu RS, Huang VYJ, Chiang SY, Huang CJ, et al. Ganoderma microsporum immunomodulatory protein induces apoptosis and potentiates mitomycin C-induced apoptosis in urinary bladder urothelial carcinoma cells. Journal of cellular biochemistry. 2018; 119(6):4592–606. https://doi.org/10.1002/jcb.26616 PMID: 29240252

31. Wang TY, Yu C-C, Hsieh P-L, Liao Y-W, Yu C-H, Chou M-Y. GMI ablates cancer stemness and cisplatin resistance in oral carcinoma stem cells through IL-6/Stat3 signaling inhibition. Oncotarget. 2017; 8(41):70422. https://doi.org/10.18632/oncotarget.19711 PMID: 29050290

32. Lee PH, Hsieh PL, Liao YW, Yu CC. Inhibitory effect of GMI, an immunomodulatory protein from Ganoderma microsporum, on myofibroblast activity and proinflammatory cytokines in human fibrotic buccal mucosal fibroblasts. Environmental toxicology. 2018; 33(1):32–40. https://doi.org/10.1002/tox.22489 PMID: 28984080

33. Arnold L, Henry A, Poron F, Baba-Amer Y, Van Rooijen N, Plonquet A, et al. Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis. The Journal of experimental medicine. 2007; 204(5):1057–69. https://doi.org/10.1084/jem.20070075 PMID: 17485518

34. Walston J, Fedarko N, Yang H, Leng S, Beamer B, Espinoza S, et al. The physical and biological characterization of a frail mouse model. The Journals of Gerontology Series A: Biological Sciences and Medical Sciences. 2008; 63(4):391–8. https://doi.org/10.1093/gerona/d63.4.391 PMID: 18426963

35. Lin J, Wu H, Terr PT, Zhang C-Y, Wu Z, Boss O, et al. Transcriptional co-activator PGC-1α drives the formation of slow-twitch muscle fibres. Nature. 2002; 416(6899):797–801. https://doi.org/10.1038/ nature00904 PMID: 12181572

36. Wang B, Yang G, Liang X, Zhu M, Du M. Grape seed extract prevents skeletal muscle wasting in interleukin 10 knockout mice. BMC complementary and alternative medicine. 2014; 14(1):162. https://doi.org/10.1186/1472-6882-14-162 PMID: 24884473

37. Park J-S, Park M-K, Oh H-J, Woo Y-J, Lim M-A, Lee J-H, et al. Grape-seed proanthocyanidin extract as suppressors of bone destruction in inflammatory autoimmune arthritis. PLoS One. 2012; 7(12):e51377. https://doi.org/10.1371/journal.pone.0051377 PMID: 23251512

38. Hindi L, McMillan JD, Afroze D, Hindi SM, Kumar A. Isolation, culturing, and differentiation of primary myoblasts from skeletal muscle of adult mice. Bio-protocol. 2017; 7(9).

39. Delaney K, Kasprzycka P, Ciemerych MA, Zimowska M. The role of TGF-β1 during skeletal muscle regeneration. Cell biology international. 2017; 41(7):706–15. https://doi.org/10.1002/cbin.10725 PMID: 28035727

40. Doumit ME, Cook DR, Merkel RA. Fibroblast growth factor, epidermal growth factor, insulin-like growth factors, and platelet-derived growth factor-BB stimulate proliferation of clonally derived porcine myogenic satellite cells. Journal of cellular physiology. 1993; 157(2):326–32. https://doi.org/10.1002/jcp.1041570216 PMID: 8227164

41. Piñol-Jurado P, Gallardo E, de Luna N, Suárez-Calvet X, Sánchez-Riera C, Fernández-Simón E, et al. Platelet-derived growth factor BB influences muscle regeneration in Duchenne muscle dystrophy. The American Journal of Pathology. 2017; 187(8):1814–27. https://doi.org/10.1016/j.ajpath.2017.04.011 PMID: 28618254

42. Damon SE, Haugk KL, Bimbbaum RS, Quinn LS. Retrovirally mediated overexpression of insulin-like growth factor binding protein 4: Evidence that insulin-like growth factor is required for skeletal muscle differentiation. Journal of cellular physiology. 1998; 175(1):109–20. https://doi.org/10.1002/(SICI)1070-4652(199804)175:1<109::AID-JCP12>3.0.CO;2-6 PMID: 9491786

43. Saera-Vila A, Louie KaW, Sha C, Kelly RM, Kish PE, Kahana A. Extracellular muscle regeneration in zebrafish requires late signals from Insulin-like growth factors. Plos one. 2018; 13(2):e0192214. https://doi.org/10.1371/journal.pone.0192214 PMID: 29415074

44. Zhu H, Xue C, Yao M, Wang H, Zhang P, Qian T, et al. miR-129 controls axonal regeneration via regulating insulin-like growth factor-1 in peripheral nerve injury. Cell death & disease. 2018; 9(7):1–17. https://doi.org/10.1038/s41419-018-0760-1 PMID: 29915198

45. Coleman ME, DeMayo F, Yin KC, Lee HM, Geske R, Montgomery C, et al. Myogenic vector expression of insulin-like growth factor I stimulates muscle cell differentiation and myofiber hypertrophy in transgenic mice. Journal of Biological Chemistry. 1995; 270(20):12109–16. https://doi.org/10.1074/jbc.270.20.12109 PMID: 7744859
PLOS ONE | https://doi.org/10.1371/journal.pone.0244791 December 31, 2020 16 / 17

46. Liu D, Black BL, Derynck R. TGF-β inhibits muscle differentiation through functional repression of myogenic transcription factors by Smad3. Genes & development. 2001; 15(22):2950–66. https://doi.org/10.1101/gad.925901 PMID: 11711431

47. Shin EJ, Jo S, Choi S, Cho C-W, Lim W-C, Hong H-D, et al. Red Ginseng Improves Exercise Endurance by Promoting Mitochondrial Biogenesis and Myoblast Differentiation. Molecules. 2020; 25(4):865. https://doi.org/10.3390/molecules25040865 PMID: 32079067

48. Li X-T, Chen R, Jin L-M, Chen H-Y. Regulation on energy metabolism and protection on mitochondria of Panax ginseng polysaccharide. The American Journal of Chinese medicine. 2009; 37(06):1139–52. https://doi.org/10.1142/S0192415X09007454 PMID: 19938222

49. Voces J, Cabral de Oliveira A, Prieto JG, Vila L, Perez AdC, Duarte IDG, et al. Ginseng administration protects skeletal muscle from oxidative stress induced by acute exercise in rats. Brazilian journal of medical and biological research. 2004; 37(12):1863–71. https://doi.org/10.1590/s0100-879x2004001200012 PMID: 15558193

50. Rudnicki MA, Schnegelsberg PN, Stead RH, Braun T, Arnold H-H, Jaenisch R. MyoD or Myf-5 is required for the formation of skeletal muscle. Cell. 1993; 75(7):1351–9. https://doi.org/10.1016/0092-8674(93)90621-v PMID: 8269513

51. Hernández-Hernández JM, García-González EG, Brun CE, Rudnicki MA, editors. The myogenic regulatory factors, determinants of muscle development, cell identity and regeneration. Seminars in cell & developmental biology; 2017: Elsevier.

52. Asfour HA, Allooh MZ, Said RS. Myogenic regulatory factors: The orchestrators of myogenesis after 30 years of discovery. Experimental Biology and Medicine. 2018; 243(2):118–28. https://doi.org/10.1177/1535370717749494 PMID: 29307280

53. Seale P, Sabourin LA, Girgis-Gabardo A, Mansouri A, Gruss P, Rudnicki MA. Pax7 is required for the specification of myogenic satellite cells. Cell. 2000; 102(6):777–86. https://doi.org/10.1016/s0092-8674(00)00666-0 PMID: 11030621

54. Saber J, Lin AY, Rudnicki MA. Single-cell analyses uncover granularity of muscle stem cells. F1000Research. 2020; 9. https://doi.org/10.12688/f1000research.20856.1 PMID: 32025292

55. Dei P, Baadners D, Schlipmann K, Velders M, Schwarz J. C2C12 myoblastoma cell differentiation and proliferation is stimulated by androgens and associated with a modulation of myostatin and Pax7 expression. Journal of molecular endocrinology. 2008; 40(5):231–42. https://doi.org/10.1677/JME-07-0175 PMID: 18434429

56. Brown DM, Parr T, Brameld JM. Myosin heavy chain mRNA isoforms are expressed in two distinct cohorts during C2C12 myogenesis. Journal of muscle research and cell motility. 2012; 32(6):383–90. https://doi.org/10.1007/s10974-011-9267-4 PMID: 22012579

57. Cooper S, Maxwell A, Kizana E, Ghoddusi M, Hardeman E, Alexander I, et al. C2C12 co-culture on a fibroblast substratum enables sustained survival of contractile, highly differentiated myotubes with peripheral nuclei and adult fast myosin expression. Cell motility and the cytoskeleton. 2004; 58(3):200–19. https://doi.org/10.11506/bbrc.2011.04.002 PMID: 15146538

58. Eom YW, Lee JE, Yang MS, Jang IK, Kim HE, Lee DH, et al. Red Ginseng Improves Exercise Endurance by Promoting Mitochondrial Biogenesis and Myoblast Differentiation. Molecules. 2020; 25(4):865. https://doi.org/10.3390/molecules25040865 PMID: 32079067

59. Begue G, Douillard A, Galbes O, Rossano B, Vernus B, Candau R, et al. Early activation of rat skeletal muscle IL-6/STAT1/STAT3 dependent gene expression in resistance exercise linked to hypertrophy. PloS one. 2013; 8(2):e57141. https://doi.org/10.1371/journal.pone.0057141 PMID: 23451164

60. Wang K, Wang C, Xiao F, Wang H, Wu Z. JAK2/STAT2/STAT3 are required for myogenic differentiation. Journal of biological chemistry. 2008; 283(49):34029–36. https://doi.org/10.1074/jbc.M803012200 PMID: 18935816

61. Sun L, Ma K, Wang H, Xiao F, Gao Y, Zhang W, et al. JAK1–STAT1–STAT3, a key pathway promoting proliferation and preventing premature differentiation of myoblasts. Journal of cell biology. 2007; 179(1):129–38. https://doi.org/10.1083/jcb.200703184 PMID: 17908914

62. Wang R, Cherukuri P, Luo J. Activation of Stat3 sequence-specific DNA binding and transcription by p300/CREB-binding protein-mediated acetylation. Journal of Biological Chemistry. 2005; 280(12):11528–34. https://doi.org/10.1074/jbc.M413930200 PMID: 15649887

63. Yuan Z-L, Guan Y-J, Chatterjee D, Chin YE. Stat3 dimerization regulated by reversible acetylation of a single lysine residue. Science. 2006; 307(5707):269–73. https://doi.org/10.1126/science.1105166 PMID: 15653507

64. Lee J-L, Wang M-J, Chen J-Y. Acetylation and activation of STAT3 mediated by nuclear translocation of CD44. Journal of Cell Biology. 2009; 185(6):949–57. https://doi.org/10.1083/jcb.200812060 PMID: 19506034
65. Lee H, Zhang P, Herrmann A, Yang C, Xin H, Wang Z, et al. Acetylated STAT3 is crucial for methylation of tumor-suppressor gene promoters and inhibition by resveratrol results in demethylation. Proceedings of the National Academy of Sciences. 2012; 109(20):7765–9. https://doi.org/10.1073/pnas.1205132109 PMID: 22547799

66. Liu R, Kim K-Y, Jung Y-W, Park I-H. Dnmt1 regulates the myogenic lineage specification of muscle stem cells. Scientific reports. 2016; 6:35355. https://doi.org/10.1038/srep35355 PMID: 27752090

67. Xu YS, Liang JJ, Wang Y, Xiang-zhong JZ, Xu L, Xu Y-y, et al. STAT3 undergoes acetylation-dependent mitochondrial translocation to regulate pyruvate metabolism. Scientific reports. 2016; 6(1):1–16. https://doi.org/10.1038/s41598-016-0001-8 PMID: 28442746

68. Akimoto T, Pohnert SC, Li P, Zhang M, Gumbs C, Rosenberg PB, et al. Exercise stimulates Pgc-1α transcription in skeletal muscle through activation of the p38 MAPK pathway. Journal of Biological Chemistry. 2005; 280(20):19587–93. https://doi.org/10.1074/jbc.M408862200 PMID: 15767263

69. Baar K, Wende AR, Jones TE, Marison M, Nolte LA, Chen M, et al. Adaptations of skeletal muscle to exercise: rapid increase in the transcriptional coactivator PGC-1. The FASEB journal. 2002; 16(14):1879–86. https://doi.org/10.1096/fj.02-0367com PMID: 12468452

70. Brault JJ, Jespersen JG, Goldberg AL. Peroxisome proliferator-activated receptor γ coactivator 1α or 1β overexpression inhibits muscle protein degradation, induction of ubiquitin ligases, and disuse atrophy. Journal of Biological Chemistry. 2010; 285(25):19460–71. https://doi.org/10.1074/jbc.M110.113092 PMID: 20404331

71. García-Prat L, Sousa-Victor P, Muñoz-Cánoves P. Functional dysregulation of stem cells during aging: a focus on skeletal muscle stem cells. The FEBS journal. 2013; 280(17):4051–62. https://doi.org/10.1111/febs.12221 PMID: 23452120

72. Alway SE, Mohamed JS, Myers MJ. Mitochondria initiate and regulate sarcopenia. Exercise and sport sciences reviews. 2017; 45(2):58. https://doi.org/10.1249/JES.000000000000101 PMID: 28098577

73. Huang D-D, Fan S-D, Chen X-Y, Yan X-L, Zhang X-Z, Ma B-W, et al. Nrf2 deficiency exacerbates frailty and sarcopenia by impairing skeletal muscle mitochondrial biogenesis and dynamics in a age-dependent manner. Experimental gerontology. 2019; 119:61–73. https://doi.org/10.1016/j.exger.2019.01.022 PMID: 30690066

74. Perandini LA, Chimin P, Lutkenmeyer DdS, Câmara NOS. Chronic inflammation in skeletal muscle impairs satellite cells function during regeneration: can physical exercise restore the satellite cell niche? The FEBS journal. 2018; 285(11):1973–84. https://doi.org/10.1111/febs.14417 PMID: 29473995

75. Tchkonia T, Zhu Y, Van Deursen J, Campisi J, Kirkland JL. Cellular senescence and the senescent secretory phenotype: therapeutic opportunities. The Journal of clinical investigation. 2013; 123(3):966–72. https://doi.org/10.1172/JCI64098 PMID: 23454759