Scriptaid/exercise-induced lysine acetylation is another type of posttranslational modification occurring in titin

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ABSTRACT

Titin serves important functions in skeletal muscle during exercise and posttranslational modifications of titin participate in the regulation of titin-based sarcomeric functions. Scriptaid has exercise-like effects through the inhibition of HDAC and regulatory acetylation of proteins. However, it remains mostly unclear if exercise could result in titin's acetylation and whether Scriptaid could regulate acetylation of titin. We treated C57BL/6 mice with 6-week treadmill exercise and 6-week Scriptaid administration to explore Scriptaid's effects on mice exercise capacity and whether Scriptaid administration/exercise could induce titin's acetylation modification. An exercise endurance test was conducted to explore their effects on mice exercise capacity and proteomic studies were conducted with gastrocnemius muscle tissue of mice from different groups to explore titin's acetylation modification. We found that Scriptaid and exercise did not change titin's protein expression, but they did induce acetylation modification changes of titin. In total, 333 acetylated lysine sites were identified. Exercise changed the acetylation levels of 33 lysine sites of titin, whereas Scriptaid changed acetylation levels of 31 titin lysine sites. Exercise treatment and Scriptaid administration shared 11 lysine sites. In conclusion, Scriptaid increased exercise endurance of mice by increasing the time mice spent running to fatigue. Acetylation is a common type of posttranslational modification of titin, and exercise/Scriptaid changed the acetylation levels of titin and titin-interacting proteins. Most importantly, titin may be a mediator through which Scriptaid and exercise modulate the properties and functions of

exercise-induced skeletal muscle at the molecular level.

Key words: acetylation; deacetylation; exercise; Scriptaid; titin.

New & Noteworthy statement

- Scriptaid administration increased mouse exercise endurance.
- Acetylation is another type of posttranslational modification of titin.
- Scriptaid/exercise changed acetylation levels of titin and titin - interacting proteins.
- Titin may mediate exercise-induced skeletal muscles properties and functions.

1. Introduction

Exercise promotes skeletal muscle health in many ways. It affects multiple physiological processes involved in molecular, cellular, and biochemical pathways regulating skeletal muscle gene expression and the metabolic process, thereby promoting the health of skeletal muscle (**11,20,27**). Sarcomeres are represented as the elemental contractile units of striated muscles and are composed of thick filaments, thin filaments, and titin filaments. Titin, a giant protein that spans half the sarcomere from the Z-disk to the M-line, has been recognized as a central mediator for exercise-induced mechanosignaling and skeletal muscle remodeling (**29,42**). Titin protein is composed of immunoglobulin (Ig)-like domains, fibronectin type 3 (FN3) domains, PEVK-domain (high abundance of proline [P], glutamic acid [E], valine [V], and lysine [K]) and unique sequences (us) (**28,44**). Human titin protein has up to 38,138 amino acids and is divided into Z-disk, I-band, A-band, M-band segments, and the alternatively spliced regions (**5,6,30**). Differential alternative splicing of the titin gene generates millions of titin expression variants, such as the cardiac N2B isoform (3.0 MDa) and the skeletal muscle N2A isoforms (3.3-3.7 MDa) (**28,51**). When skeletal muscle contracts, titin functions as a molecular spring by the (un)folding of the I-band Ig-domains during sarcomere stretching (**29**). As a key component in sarcomere, titin connects with thick/thin filaments and maintains the structural stability of the sarcomere. The Ig-domains and FN3 domains in titin A-band region tightly bind to myosin heavy chain (MHC) protein and myosin-binding protein C (MyBPC), which makes titin highly involved in thick filament

assembly (**32,37,49**). Moreover, titin participates in mechanochemical signaling events via binding other proteins, Ig-domains of N2A-domain in titin I-band part interact with muscle ankyrin-repeat proteins (MARPs). The activation of MARPs initiates exercise-induced skeletal muscle remodeling and has a significant role in transcriptional regulation, myofibrillar assembly, and myogenesis (**29**).

Serving as a molecular spring during skeletal muscle contraction, the compliance of a part of titin elastic can be affected by titin I-band stiffness. Current studies demonstrate that posttranslational modifications (PTMs), including phosphorylation and oxidation, have essential roles in regulating titin-based stiffness (**15,47**); however, few studies have examined the role of titin's PTM. Exercise regulates the stiffness of titin I-band through changing titin's phosphorylation. PEVK phosphorylation was increased in mice diaphragm after 3 weeks of exercise (**21**). Similarly, 15 minutes of a single eccentric exercise increased the phosphorylation of PEVK-domain of titin in rat muscle, which is expected to result in higher titin-based stiffness of vastus lateralis muscle (**45**). Another study showed that exercised mice had decreased PEVK phosphorylation and increased N2B phosphorylation, both of which are predicted to increase titin's compliance (**52**). However, it is unclear whether exercise modulates titin stiffness through other protein modifications except phosphorylation. Recently, Gaur et al. found that skeletal muscle class IIa HDAC (histone deacetylase) activity was reduced during exercise and chronic Scriptaid (a class IIa HDAC inhibitor) administration to mice can induce exercise-like adaptations (**13**). The study shows that Scriptaid disrupted the class IIa HDAC

97 corepressor complex and increased histone 3 lysine 9 acetylation (H3K9ac) and MEF2
98 (myocyte enhancer factor 2) transcription activity. Moreover, Scriptaid enhances skeletal
99 muscle insulin action and cardiac function in obese mice (**14**). In another study, Scriptaid
100 was shown to affect histone acetylation and expression of genes related to histone
101 acetylation (**54**). Gaur et al. showed that acetylation has important roles in
102 exercise-induced metabolic process indicating by increased exercise capacity and
103 oxidative activity in skeletal muscle (**13**). However, no study has reported that acetylation
104 modification occurs in titin, and it is still not clear whether titin's acetylation regulates
105 skeletal muscle gene transcription and the metabolic process.

106 Given the role of titin in exercise-induced mechanosignaling and skeletal muscle
107 remodeling as well as the Scriptaid-mimicking aspects of the exercise-adaptive response
108 in promoting expression of metabolism-related genes, we speculate that Scriptaid may
109 affect titin expression and/or modification. Therefore, we investigated exercise
110 performance and proteomic changes in mice gastrocnemius muscle resulting from
111 exercise intervention and Scriptaid administration. Our goal was to explore whether
112 Scriptaid affects titin expression and/or modification compared with exercise intervention.
113 In our study, Scriptaid-treated mice had increased exercise endurance in a treadmill-based
114 incremental test, increased grip strength, and increased mitochondrial citrate synthase
115 activity compared with those in the control mice. Surprisingly, we found that Scriptaid
116 and exercise did not alter the expression of titin protein. However, exercise and Scriptaid
117 increased acetylation of lysine residues on titin. Acetylation of titin has never been

reported before, and our study confirms that acetylation is another common type of PTM that occurs on titin. By further analyzing the acetylated lysine sites on titin, we found that exercise and Scriptaid have similar effects in influencing the location of titin acetylated lysine sites and the acetylation level of titin-interacting proteins. Our study indicates that titin may function as a mediator through which Scriptaid and exercise modulate properties and functions of exercise-induced skeletal muscles at the molecular level.

2. Material and Methods

2.1. Animals and experimental design

Thirty male 5-week-old C57BL/6 mice were obtained from Beijing HFK Bioscience Co., Ltd (Beijing, China). The mice were housed in a controlled environment with a 12:12-h light-dark cycle and free access to food and water. After 1 week of acclimation, the mice were randomly divided into a sedentary control group (n=10) (C), exercise group (n=10) (E), and Scriptaid administration group (n=10) (S). The mice from E group underwent 6 weeks of treadmill exercise for 12 m/min (at the intensity of 75% VO_2max) for 60 min/day, 5 days/week on a 0% grade. The mice from S group underwent 6 weeks of Scriptaid (Selleck) (dissolved in 1 × NS [normal saline] containing 5% DMSO) administration via intraperitoneal injection at the dose of 1 mg/kg body weight for 1 injection/day, 5 days/week, and performed no exercise. As a control, mice from C and E were injected vehicle (5% DMSO in 1 × NS) at the same dose (1 mg/kg body weight) for the same period (1 injection/day, 5 days/week, for 6 weeks). In the final week of the treatment, Grip strength was assessed 24 hours after the final treatment with the

YLS-13A Rat/mice grip strength test system (Yiyan Science and Technology, Jinan, China). In brief, mice was placed on measurement plate, and then pull the mouse backward by holding the tail when the mouse grasped firmly to the plate, each measurement was repeated 4 times. Exercise endurance test of mice from group C (n=3) and group S (n=3) was also assessed 24 hours after the final treatment by treadmill running at the speed of 12m/min. Then all mice were euthanized 24 hours following endurance and grip strength test. Skeletal muscles of the hind legs including the quadriceps femoris, gastrocnemius, and soleus, were dissected and snap frozen in liquid nitrogen and then stored at -80°C until analysis. All animal protocols were approved by the Tianjin Medical University Animal Care and Use Committee under the guidelines of the Chinese Academy of Sciences.

2.2. Citrate synthase activity

Quadriceps femoris samples (*n*=3 per group) were placed in ice-cold lysis buffer (50 mM Tris, pH 7.4, 0.15 M KCl) and homogenized and centrifuged at 13000 g for 10 min. The supernatants were taken, and the protein concentration was measured using Bradford assay. The reaction catalyzed by citrate synthase as follows: Acetyl-CoA + oxaloacetate + H_2O R citrate + CoA-SH (colorimetric reaction: CoA-SH + DTNB R TNB + CoA-S-S-TNB). Citrate synthase activity was determined by measuring the appearance of the yellow product (TNB), which is observed spectrophotometrically by measuring absorbance at 412 nm. The citrate synthase reagent consisted of 0.1 mM DTNB, 10% Triton X-100, 0.31 mM acetyl CoA (Sigma, USA), and muscle sample (5 μg). The

160 reaction regent (200 µl) also included 10 µl 10 mM oxaloacetate (Sangon Biotech, China),
161 which was added to start the reaction. Absorbance changes were measured every 20 s
162 over 3 min at 412 nm to determine citrate synthase activity (Biotek, Synergy HT
163 Multi-Mode Microplate Reader, USA). All assays were carried out at 30 uC. Citrate
164 synthase from porcine heart (C-3260, Sigma, USA) was used as a standard for assay
165 calibration.

166 **2.3. Protein extraction**

167 The skeletal muscle samples (gastrocnemius muscle from group C, E, and S) were
168 ground by liquid nitrogen and then dissolved in lysis buffer containing 8 M urea, 10 mM
169 dithiothreitol (DTT), 3 µM trichostatin (TSA), and 50 mM NAM and 1% protease
170 inhibitor cocktail, followed by sonication three times on ice using a high-intensity
171 ultrasonic processor (Scientz). The remaining debris was removed by centrifugation at
172 12,000 g at 4°C for 10 min. Finally, the supernatant was collected and the protein
173 concentration was determined with BCA kit according to the manufacturer's instructions.

174 **2.4. Trypsin digestion**

175 The protein solution was reduced with 5 mM dithiothreitol for 30 min at 56°C and
176 alkylated with 11 mM iodoacetamide for 15 min at room temperature in darkness. The
177 protein sample was then diluted by adding 100 mM TEAB to urea concentration less than
178 2 M. Finally, trypsin was added at 1:50 trypsin-to-protein mass ratio for the first digestion
179 overnight at 37°C and 1:100 trypsin-to-protein mass ratios for a second 4h-digestion.

180 **2.5 HPLC fractionation**

The tryptic peptides were fractionated into fractions by high pH reverse-phase HPLC using Thermo Betasil C18 column (5 μm particles, 10 mm ID, and 250 mm length). Briefly, peptides were first separated with a gradient of 8% to 32% acetonitrile (pH 9.0) over 60 min into 60 fractions. The peptides were then combined into 6 fractions and dried by vacuum centrifuging.

2.6. Affinity enrichment

Immunoprecipitation was used to enrich modified peptides, tryptic peptides were dissolved in NETN buffer (100 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl, 0.5% NP-40, pH 8.0) and then incubated with pre-washed antibody beads (Lot number 001, PTM Bio) at 4°C overnight with gentle shaking. The beads were then washed four times with NETN buffer and twice with H_2O. The bound peptides were eluted with 0.1% trifluoroacetic acid (TFA) and vacuum-dried. For LC-MS/MS analysis, the resulting peptides were desalted with C18 ZipTips (Millipore).

Peptide mixtures were first incubated with IMAC microspheres suspension with vibration in loading buffer (50% acetonitrile/6% trifluoroacetic acid). The IMAC microspheres with enriched phosphopeptides were collected by centrifugation, and the supernatant was removed. To remove nonspecifically adsorbed peptides, the IMAC microspheres were washed with 50% acetonitrile/6% trifluoroacetic acid and 30% acetonitrile/0.1% trifluoroacetic acid, sequentially. To elute the enriched phosphopeptides from the IMAC microspheres, elution buffer containing 10% NH_4OH was added and the enriched phosphopeptides were eluted with vibration. The supernatant containing

phosphopeptides was collected and lyophilized for LC-MS/MS analysis.

2.7 LC-MS/MS analysis

The tryptic peptides were dissolved in 0.1% formic acid (solvent A), directly loaded onto a home-made reversed-phase analytical column [15 cm length, 75 μm i.d. (inner diameter, i.d.)]. The gradient was comprised of an increase from 6% to 23% solvent B (0.1% formic acid in 98% acetonitrile) over 26 min, 23% to 35% in 8 min, climbed to 80% in 3 min, and then holding at 80% for the last 3 min, all at a constant flow rate of 400 nL/min on an EASY-nLC 1000 UPLC system.

The peptides were subjected to NSI source followed by tandem mass spectrometry (MS/MS) in Q ExactiveTM Plus (Thermo) coupled online to the UPLC. A 2.0kV electrospray voltage was applied. The m/z scan range was 350 to 1800 for full scan, and intact peptides were detected in the Orbitrap at a resolution of 70,000. Peptides were then selected for MS/MS using NCE setting as 28 and the fragments were detected in the Orbitrap at a resolution of 17,500. A data-dependent procedure that alternated between one MS scan followed by 20 MS/MS scans with 15.0s dynamic exclusion. Automatic gain control (AGC) was set at 5E4. Fixed first mass was set as 100m/z.

2.8. Database searches

The resulting MS/MS data were processed using the Maxquant search engine (v.1.5.2.8). Tandem mass spectra were searched against human uniport database concatenated with reverse decoy database. Trypsin/P was specified as cleavage enzyme allowing up to 4 missing cleavages. The mass tolerance for precursor ions was set as

20ppm in First search and 5ppm in Main search, and the mass tolerance for fragment ions was set as 0.02Da. Carbamidomethyl on Cys was specified as fixed modification and Acetylation modification and oxidation on Met were specified as variable modifications. FDR was adjusted to < 1% and minimum score for modified peptides was set at > 40.

2.9. Statistical analysis

Maxquant software (version 1.5.2.8) was used to carry out the label-free quantification in quantification of proteomic data. Briefly, normalization and the intensity-based absolute quantification (iBAQ) in MaxQuant were performed on the identified peptides to quantify protein abundance. Specifically, shared peptides that matched to different protein groups were excluded from quantification. The significant differences between replicates were determined using the t-test approach. Only proteins or peptides with a fold change > 1.20 (used to indicate upregulation) or < 0.83 (used to indicate downregulation) and a p value < 0.05 in at least two biological replicates were considered to exhibit a significant difference in protein abundance between the two stages. With the exception of identified titin peptides and acetylomes, all other experimental data were represented as means ± SEM and compared with an independent-samples t-test or one-way ANOVA using SPSS software. $P \leq 0.05$ was accepted as statistically significant.

3. Results

3.1. Scriptaid increases exercise capacity of mice

We tested the time mice spent running to the maximum fatigue point in a treadmill-

244 based incremental test. Our results show that after six weeks of Scriptaid administration,
245 mice in the Scriptaid group reached total exhaustion significantly later than the control
246 mice (Fig. 1A). Moreover, in order to explore whether Scriptaid could induce skeletal
247 muscle strength gain, we next tested the grip strength of mice. As shown in Fig. 1B,
248 Scriptaid mice had greater grip strength than that of the control mice. Next, the citrate
249 synthase activity assay revealed that Scriptaid could increase mitochondrial oxidative
250 capacity (Fig. 1C). In summary, Scriptaid can increase aerobic capacity of mice through
251 improving exercise endurance and enhancing mitochondrial oxidative capacity.

252 ***3.2. Scriptaid and exercise did not change titin protein expression but did increase***
253 ***acetylation modification of titin***

254 We next performed the proteomic and lysine acetylomes experiments of the mice
255 gastrocnemius samples to explore whether Scriptaid affects titin expression and/or its
256 posttranslational modification. In order to minimize the errors from muscle samples of
257 different groups, we conducted a sample quality control test by using 3 statistical
258 methods including PCA (principal component analysis), relative standard deviation
259 (RSD), and Pearson's correlation coefficient. Fig. 2A shows the results of modified
260 quantitative PCA analysis for all samples, and the more concentrative the aggregation of
261 repetitive samples in the circle is, the better the quantitative repeatability of samples will
262 be. As shown in Fig. 2B, the boxplot is plotted by the relative standard deviation (RSD)
263 of modified quantitative values between repetitive samples, the small RSD value
264 indicates that our samples have good quantitative repeatability. In addition, the thermal

chart drawn by calculating Pearson correlation coefficients between each two samples also confirms a good quantitative repeatability in our muscle samples during proteomic assay (Fig. 2C).

In our proteomic study, exercise and Scriptaid did not change the titin expression in mice gastrocnemius muscle by quantifying titin peptides in proteomic assay (Table 1). However, acetylated lysine sites were identified. A total of 333 titin acetylated lysine sites had been identified in mice from control (C), exercise (E), and the Scriptaid (S) groups. These 333 acetylated lysine sites are distributed in 6 different domains of titin protein (Fig. 2D), most of them located in Ig domains, FN3 domains, and the sequence insertion regions. Although 333 acetylated titin peptides were identified, many of them could not be further quantified in the next experiments steps or their fold change ratio did not satisfy the statistically significant threshold of 1.20 or 0.83. After getting rid off the statistically insignificant data of titin-acetylated sites, we found that exercise induced acetylation changes of 36 lysine sites in group E (Fig. 2E), Scriptaid-induced acetylation changes of 31 lysine sites in group S (Fig. 2F). The detailed information of these statistically significant acetylated lysine sites was collected in Table 2.

After further analyzing these acetylated lysine sites through their location distribution in titin and their up/downregulation changes, we concluded that: 1) most identified acetylated lysine sites of group E and S are located in Ig domains, FN3 domains, and the sequence insertion regions of titin, whereas only 1 site in group S is located in the PEVK region (Fig. 2F), in which exercise modulates titin spring's stiffness

through phosphorylation changes; 2) the effects of exercise and Scriptaid on titin lysine acetylation contain both upregulation and downregulation, but the up/downregulations of lysine acetylation in different titin domains are not fully the same between groups E and S. More specifically, in group E, most acetylated lysine sites in Ig domains, FN3 domains and the sequence insertion regions are downregulated (Fig. 2E). In group S, however, most acetylated sites in Ig domains and the sequence insertion regions are upregulated (Fig. 2F). Taken together, our results indicate that exercise and Scriptaid did not affect titin's protein expression but induced acetylation changes of lysine residues on titin.

3.3. Scriptaid and exercise changed acetylation levels of titin-interacting proteins

Studies have reported that exercise affects expression of some proteins that interact with titin, such as MARPs (**33,43,50**). Hence, we analyzed the changes of some titin-interacting proteins by protein-protein interaction (PPI) network. As a result, acetylation levels of 12 titin-interacting proteins were changed in group E; 3 of them were upregulated, 9 of them were downregulated (Fig. 3A). In group S, only 8 proteins were affected, with 5 downregulated and 3 upregulated (Fig. 3B). Moreover, acetylation levels of 6 proteins (Myh8 [Myosin-8], Ckmt2 [Creatine kinase S-type], Des [Desmin], Ckm [Creatine kinase M-type], Tnnc2 [Troponin C, skeletal muscle type], Mylpf [myosin regulatory light chain 2, skeletal muscle isoform]) were simultaneously affected in groups E and S. These results indicate that Scriptaid and exercise can also change the acetylation levels of some titin-interacting proteins, and specifically, Scriptaid and exercise also have shared effects on regulations of these proteins' acetylation levels.

3.4. Scriptaid and exercise have common effects on titin lysine residue acetylation

In our study, Scriptaid also induced titin's acetylation changes identical to those in the exercise group. By analyzing the exercised-induced 36 titin acetylated sites and the Scriptaid-induced 31 titin acetylated sites, we found that 11 acetylated sites are shared between exercise and Scriptaid and that their location distribution and up/downregulation conditions are exactly the same in groups E and S (Fig. 4A). In these 11 sites, most of them are downregulated, 5 sites are located in Ig domains, 3 sites are within FN3 domains, and the other 3 belong to the sequence insertion regions (Fig. 4A). Moreover, 4 acetylated lysine sites of Ig domains are located in the elastic I-band part; 1 site of Ig domains, 3 sites of FN3 domains, and 2 sites of sequence insertion are located in the A-band part; lastly, 1 site of sequence insertion is in the Z-repeats (Fig. 4B). However, due to the incomplete understanding of these acetylated lysine sites, whether the acetylation of these sites have functional roles on titin remain to be elucidated in further studies.

4. Discussion

It is widely accepted that aerobic exercise can increase exercise capacity such as exercise endurance (**9,12,26**). Scriptaid was shown to enhance skeletal muscle insulin action in obese mice (**14**), a recent report indicates Scriptaid induced adaptations similar to the effects of exercise (**13**). In our study, grip strength of exercise mice was not increased (Fig. 1B), which is consistent with the widely accepted recognition that aerobic exercise cannot increase muscle mass and muscle fiber size (**41,55**). However, Scriptaid

results in exercise-like results such as aerobic capacity improvement, an increased gain in skeletal muscle grip strength, and increased tricarboxylic acid cycle (TCA cycle)-based mitochondrial citrate synthase activity (Fig. 1A, 1B, 1C). Citrate synthase is mainly located in mitochondrial matrix and has always been regarded as a biomarker for mitochondrial oxidative capacity (**7**,**57**). In mitochondria, TCA cycle is the final pathway for the oxidation of acetyl-CoA generated from carbohydrates, fatty acids and amino acids. Acetyl-CoA oxidation in TCA cycle generates NADH/$FADH_2$ which will transport protons to electron-transport chain located in inner mitochondrial membrane to produce ATP through oxidation phosphorylation (**8**). Specifically, citrate synthesis from acetyl-CoA and oxaloacetate catalyzed by citrate synthase is the first rate-limiting step of TCA cycle (**39**).

Our results imply that Scriptaid and exercise share a common effect on exercise capacity, but the exact mechanism through which the effects of Scriptaid mimic exercise is still not clear.

Although previous studies reported that exercise affects titin expression, but exercise's ability to increase or decrease titin expression is in some dispute. Lieber et al. showed that titin staining increased in rabbits due to the loss of desmin after eccentric exercise (**36**). Another study similarly observed that titin expression increased in mice after an endurance-training exercise (**2**), but Li et al. demonstrated the conflicting result that aerobic and resistance exercise training did not alter the expression of titin (**35**). In our study, exercise and Scriptaid did not change titin's protein expression. The

349 inconsistent results from previous studies and our own study indicate that a lack of
350 understanding regarding the effects of exercise on titin expression persists, and it remains
351 to be elucidated in further research.

352 In another aspect, exercise was reported to modulate titin stiffness through PTM such
353 as phosphorylation (**21,45**) and oxidation (**11,20,52**). Acetylation of titin has not been
354 reported in previous studies. Our study showed that huge amount of lysine acetylation
355 could occur in titin. Scriptaid, a class IIa HDAC inhibitor, also induced titin's acetylation
356 changes in mice. 333 acetylated lysine sites were identified in control, exercise and
357 Scriptaid group in total (Supplemental Table S1 [https://figshare.com/s/0fd186b9570288e98c58
358 and https://doi.org/10.6084/m9.figshare.9916679]). Besides, 33 sites in group E and 31
359 sites in group S were found to up/downregulated greatly compared with those same sites
360 in control group. Except those greatly up/downregulated sites in group E and S, up to 200
361 acetylated lysine sites are within the sedentary control group, which indicates that even in
362 the sedentary condition, lysine acetylation still occurs in titin (Supplemental Table S1
363 [https://figshare.com/s/0fd186b9570288e98c58 and https://doi.org/10.6084/m9.figshare.9916679]).
364 Due to the huge amount of identified acetylated lysine sites in the sedentary mice, we
365 concluded that acetylation modification is definitely no accident in a condition even
366 without Scriptaid/exercise treatment. It indicates that these acetylated lysine sites must
367 have certain unknown roles to maintain titin's function or participate in other biological
368 processes involved in titin. Furthermore, those apparently up/downregulated acetylated
369 lysine sites in group E/S were found as a result of the 6-week exercise/Scriptaid treatment.

370 Specifically, the 33 sites in group E were also found in control group. However,
371 compared with control group, exercise greatly up/downregulated those sites in the context
372 of acetylation levels. The same goes for those 31 sites in group S. Based on this finding,
373 we hypothesized that these 33 and 31 sites might have certain roles in regulating titin's
374 function and are not stochastic PTMs. Those up to 200 acetylated sites in group C
375 indicate that, independent of exercise, acetylation is another common type of PTM in
376 titin.

377 Except for the function of being molecular spring, titin also plays key roles in
378 regulating the passive properties of skeletal muscle and in maintaining sarcomeric
379 structure by tethering to the thin and thick filaments (**51**). Early studies of titin showed
380 that in normal intact sarcomeres the thick filaments were kept at a more central position
381 due to the spring force developed by titin; but when myofibers were prestretched and
382 activated by Ca^{2+} under isometric conditions, the thick filaments move off - center toward
383 the Z-discs (**23,24**), indicating that titin-based elastic forces help to maintain high
384 Ca^{2+}-dependent tension development and to keep the thick filaments centered during
385 passive stretch. During the physiological straited muscle contraction, Ca^{2+} binding to
386 troponin C initiates the thin-thick filaments sliding. Length-dependent activation (LDA)
387 theory for titin's role in contraction has been established that tensile stretching increases
388 titin's elastic force in a radical direction which decreases the distance in interfilament
389 spacing and LDA is characterized by an immediate increase in the Ca^{2+} sensitivity of the
390 myofilaments upon stretching (**10**). Reduction in interfilament spacing caused by

391 stretching is considered to increase the Ca^{2+} sensitivity because the myosin heads of the
392 thick filaments would more easily reach over to actin of the thin filaments and thus
393 generate higher forces at the same Ca^{2+} sensitivity. Furthermore, the "winding filament"
394 hypothesis (**48**) suggests that titin is wound upon the thin filaments activated by Ca^{2+}
395 influx and the constitutively expressed PEVK segment of titin binds actin in a stronger
396 degree (**31,46**). In our proteomic analysis, we found that exercise and Scriptaid induced
397 acetylation changes of 36 and 31 lysine sites, respectively (Fig. 2E, 2F). Most of the 36
398 sites in group E and the 31 sites in group S are located in Ig domains and FN3 domains.
399 As lysine residue in peptide chains has only one free NH_3^+ which has only one positive
400 charge, acetylation modification leads to neutralization of this positive electrostatic
401 charge and result in the electrical neutrality of the lysine residues in titin. This
402 redistribution of charge in titin could lead to conformational changes which further
403 disturb the association of titin-interacting proteins and titin. These changes might affect
404 the titin-based stiffness, disturb the interaction of titin-interacting proteins with titin and
405 change the sarcomeric structural stability, especially considering the role of Ig domains in
406 maintaining titin's molecular spring function through folding/unfolding and the role of
407 Ig/FN3 domains in binding to titin-interacting proteins such as MHC and MyBPC of the
408 thick filaments (**16,40**).

409 It has been reported that alteration of titin properties correlates with cardiomyocyte
410 fibrosis (**17**). Verdonschot et al. suggested that titin cardiomyopathy leads to increased
411 interstitial fibrosis (**56**). However, Scriptaid is known to regulate fibrosis through

412 modulating the renin-angiotensin system (RAS) in patients with autosomal recessive
413 renal tubular dysgenesis (RTD). In RTD models generated by mutation of genes in RAS
414 system, renal cortex exhibits a paucity of proximal tubules and abundant interstitial
415 fibrosis (**18**). The inhibition of HDAC with Scriptaid induces RAS system gene
416 expression in the intact metanephros and renal mesenchymal cells, and then recovers the
417 lack of angiotensin II-induced decrease in ureteric bud branching (**53**). A study in human
418 and murine cancer-associated fibroblasts showed that Scriptaid inhibits cancer-associated
419 fibroblasts - secreted abundant extracellular matrix (**25**). These results may provide hints
420 to put forth a hypothesis that Scriptaid might decrease titin alteration-induced skeletal
421 muscle fibrosis, but our proteomic data do not support this hypothesis because Scriptaid
422 did not decrease collagen protein levels statistically when downregulation criteria is set to
423 a fold change < 0.83 (0.83 = 1/1.20) (data not shown). In fact, skeletal muscle fibrosis is
424 a complicated process which can be regulated by many factors (**38**). The results from
425 current study are not enough to clarify how acetylation of titin affects skeletal muscle
426 fibrosis and whether Scriptaid is indeed related to decrease the titin alterations-induced
427 muscle fibrosis. Further studies are needed to prove this hypothesis.

428 More importantly, the 11 acetylated lysine sites (Fig. 4A) commonly shared by the
429 exercise and Scriptaid groups indicate Scriptaid and exercise have similar impacts on titin
430 acetylation at the molecular level, which may explain why Scriptaid has exercise-like
431 effects in mice at the individual level. As a key component of sarcomere structure and a
432 mediator in sarcomeric mechanosensing and hypertrophic signaling, titin interacts

directly or indirectly with many other proteins. Titin is reported to associate with more than 20 proteins involved in hypertrophy regulation, and the MARPs mentioned above are included (**29,30**). Our results show that, in titin's PPI network, 6 proteins and their acetylation levels were both affected by exercise and Scriptaid. Of these 6 proteins, Des, Ckm, Tnnc2, and Mylpf were regulated by the same direction, whereas Mybpc2 and Ckmt2 were regulated toward opposite directions by exercise and Scriptaid **(**Fig. 3A, 3B**)**. Mybpc2 binds MHC, F-actin, and native thin filament; thus, it has a key role in sarcomere assembly and optimal force generation at the cross bridge level (**34**). Ckm and Ckmt2 play a central role in energy transduction in skeletal muscle (**58**). Des is the muscle-specific type III intermediate filament that helps to maintain the structure of sarcomeres (**4,22**). Tnnc2, also known as troponin C, is the central regulatory protein of striated muscle contraction, and Mylpf binds calcium during skeletal muscle contraction. Of these 6 titin-interacting proteins, Ckm, Ckmt2, and Tnnc2 have important roles in energy-regulated striated muscle contraction. Des, Mybpc2, and Mylpf are the key components of sarcomere, which maintain the structure of sarcomeres. The effects of exercise and Scriptaid on acetylation regulations to these titin-interacting proteins shows that Scriptaid has significant impact on exercise- related changes of molecules in sarcomere, and titin maybe a key mediator during this process.

5. Conclusions.

This study demonstrated that Scriptaid has exercise-like effects at the level of individuals by increasing the exercise endurance performance and grip strength in mice. Lysine

454 acetylation is another type of PTM modification that occurs in titin. Moreover, the effects
455 of Scriptaid and exercise on titin/titin-interacting proteins acetylation are similar, which
456 indicates that titin may function as a mediator through which Scriptaid and exercise
457 modulate exercise-induced skeletal muscles properties and functions at the molecular
458 level, possibly explaining why Scriptaid has exercise-like effects. Besides, it remains to
459 be elucidated how Scriptaid/exercise modulate titin acetylation. This study raised a new
460 insight into titin's PTMs and provides a roadmap for further functional studies of titin and
461 titin-related biological processes in sarcomere.

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466 **DISCLOSURE**

467 No conflicts of interest, financial or otherwise, are declared by the authors.

468 **AUTHOR CONTRIBUTIONS**

469 S.H., and YM.N. prepared figures; drafted manuscript; SJ.L., and L.F. edited and revised manuscript;
470 S.H., SJ.L., YM.N., and L.F. approved final version of manuscript.

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FIGURE LEGENDS

Fig. 1. Scriptaid increases mice aerobic capacity and mitochondrial oxidative activity.

(A) The time mice from C (Control) and S (Scriptaid) group spent running to total exhaustion in a treadmill-based exhaustive training experiment, n = 3 mice/group. **(B)** Grip strength of forelimbs of mice from C (Control), E (Exercise), and S (Scriptaid) groups, n = 10 mice/group. **(C)** Citrate synthase activity levels in the quadriceps muscle of mice from C (Control), E (Exercise), and S (Scriptaid) groups, n = 3 mice/group. Values are shown as means ± SEM. **: $p<0.01$ *vs. C*; ##: $p<0.01$ *vs. S*. Student's t-test was used in SPSS.

Fig. 2. Scriptaid and exercise resulted in lysine acetylation changes in titin.

(A) Principal component analysis (PCA) result for modified quantitative values of all samples, a more concentrative distribution of the samples value indicates a better sample quantitative repeatability, **(B)** The relative standard deviation (RSD) result of modified quantitative values between repetitive samples. **(C)** Pearson correlation coefficients between each two samples, it measures the degree of linear correlation between two sets of data: when it is close to –1, it indicates a negative correlation; when it is close to 1, it indicates a positive correlation; the closer the coefficient is to 0, the more it indicates irrelevance. **(D)** Total collection of location distribution of the 333 acetylated lysine sites in titin identified in mice from C (control), E (exercise), and S (Scriptaid) groups. **(E–F)** Detailed information of location distribution and up/downregulations of the acetylated lysine sites with statistical significance identified in E group (E) and in S group (F). The outer ring represents the up/downregulation conditions, and the inner ring represents the location distribution. C: Control group; E: exercise group; S: Scriptaid group. Ig: Immunoglobulin; PEVK: high abundance of proline (P), glutamic acid (E), valine (V), and lysine (K). n = 3 mice/group. Data were analyzed by direct counting.

Fig. 3. Scriptaid and exercise changed acetylation levels of titin-interacting proteins.

(A–B) Protein-protein interaction network information of the titin-interacting proteins identified in E group **(A)** and in S group **(B)**, the black/gray color of the circle represents the up/downregulation of acetylation level, and the relative size of each circle is related to the number of the protein acetylation fold change, n = 3 mice/group. Data were analyzed by R package networkD3 (v.0.4).

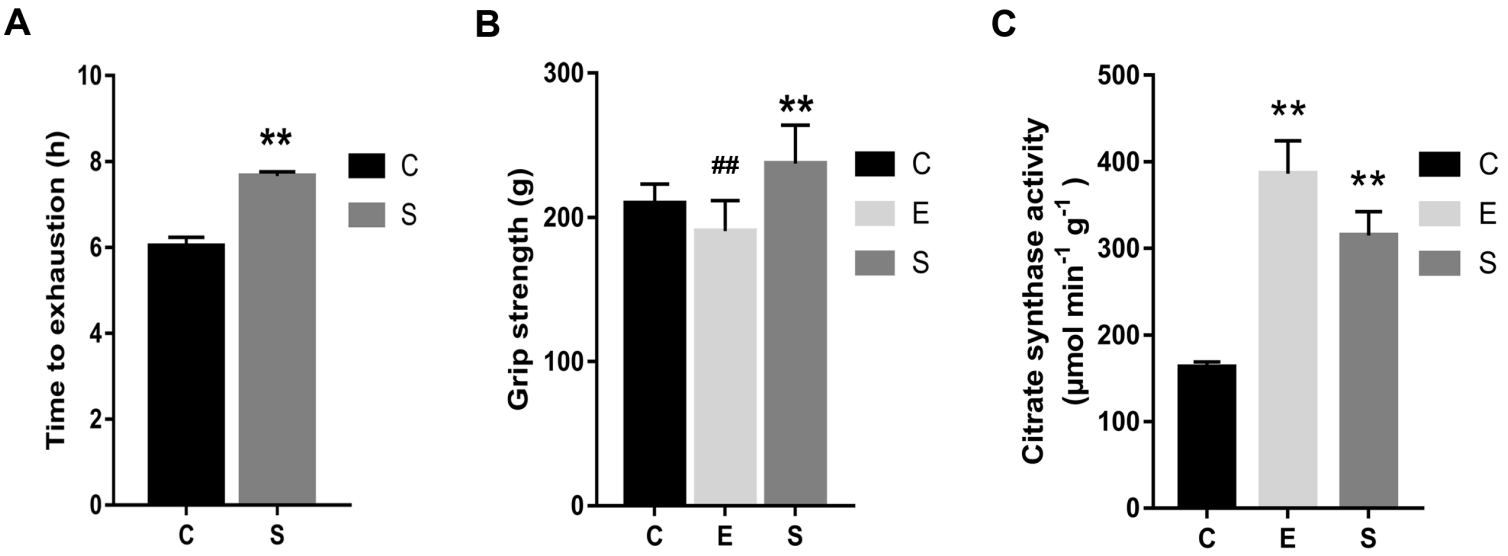
Fig. 4. Scriptaid and exercise have shared effects on titin lysine residue acetylation.

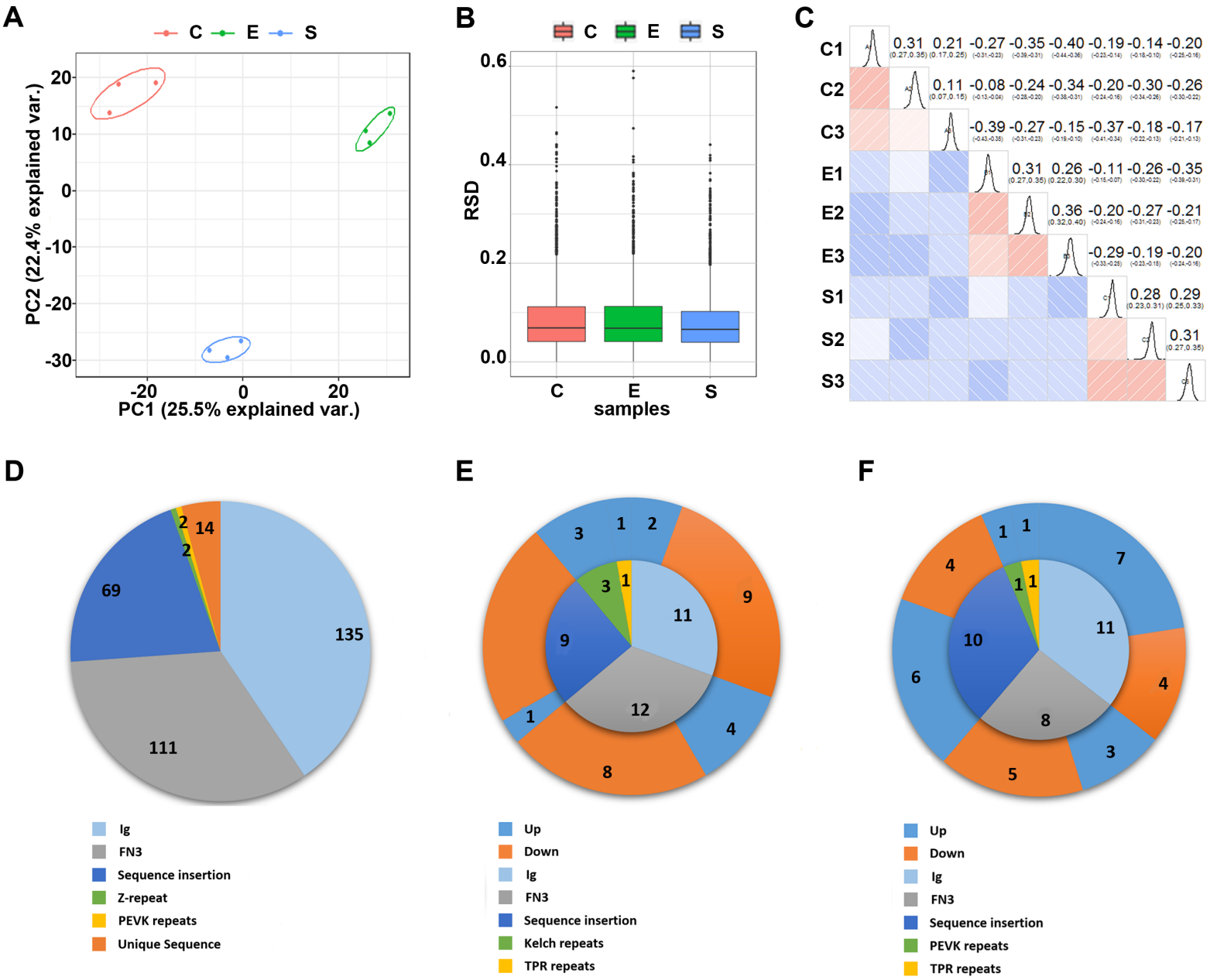
(A) 11 of the total 333 identified acetylated lysine sites are commonly shared by E (exercise) and S (Scriptaid) groups. **(B)** Detailed analysis of the location distribution of the 11 acetylated lysine sites in (A), red color represents the 5 sites located in Ig-domains, yellow color represents the 3 sites located in FN3 domains, green color represents the 3 sites located in sequence insertion regions. Ig: immunoglobulin. n = 3 mice/group. Data were analyzed by direct counting.

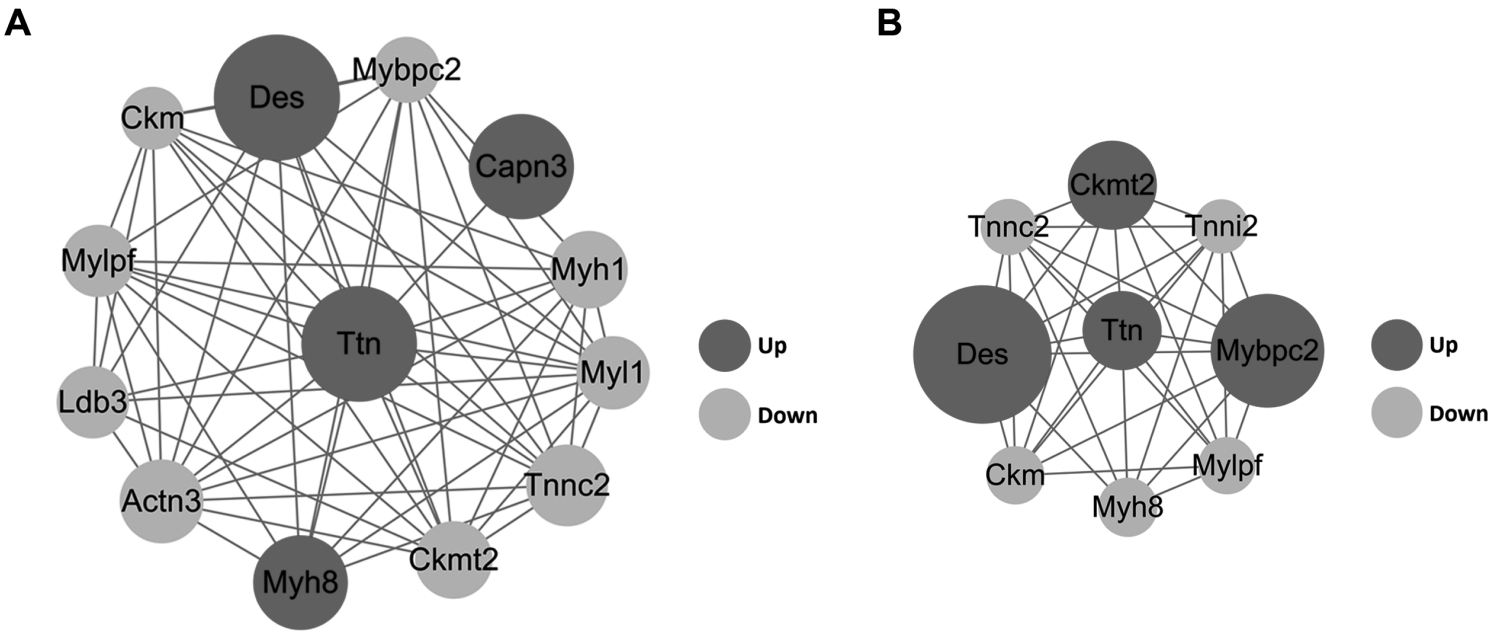
Table 1

Titin protein (peptides) relative content was measured in muscle samples of mice from C (Control), E

711 (Exercise), and S (Scriptaid) groups, *n* = 3 mice/group. Student's t-test was used in SPSS, no
712 significant differences between groups.
713
714 **Table 2**
715 Information of acetylated lysine sites in titin from group exercise and Scriptaid. All sites are found in
716 titin (Gene name: Ttn) (Protein accession: A2ASS6) and amino acid type of all sites is K (Lysine).
717 Positions: modification site localization in protein. Change ratio: the acetylation fold change ratio of
718 identified lysine sites. Regulated type is shown as Up (*change ratio > 1.20*) / Down (*change ratio <*
719 *0.83*). PEP: The maximal posterior error probability for peptides. Score: A simple rule to be used to
720 judge whether a result is significant or not. Modified sequence: Identified peptide sequence marked
721 with modification sites localization probabilities. *n* = 3 mice/group. Student's t-test was used in SPSS.







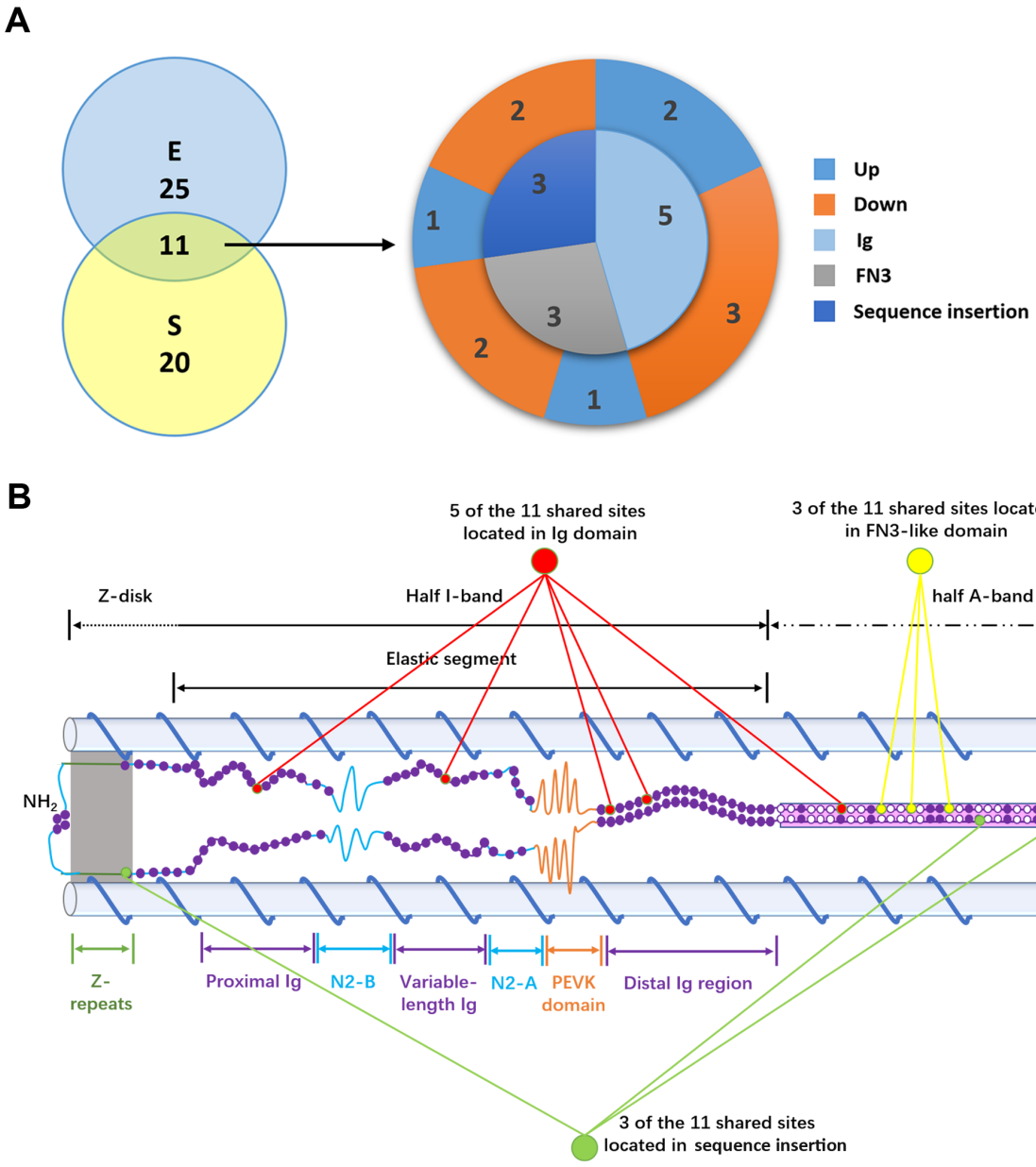


Table 1. Identified titin protein expression in proteomic assay

Identified titin protein relative content	Control (C) group (n = 3)	Exercise (E) group (n = 3)	Scriptaid (S) group (n = 3)
Sample 1	1.063	1.025	0.908
Sample 2	1.056	1.029	0.908
Sample 3	1.070	1.031	0.905

Note: Titin protein (peptides) relative content was measured in the skeletal muscle samples from C (Control), E (Exercise), and S (Scriptaid) group. n = 3 in each group.

Table 2. Information of acetylated lysine sites in titin from exercise and Scriptaid groups

Position	Change ratio	Regulated type	*p* value	PEP	Score	Modified sequence
Exercise group						
20753	0.8	Down	0.014584	3.56427E-28	126.09	K(1)DSGYYSLTAENSSGSDTQK
19160	1.223	Up	0.0184607	2.25295E-14	107.35	SHMAK(1)HLTEGNQYLFR
30276	1.669	Up	0.026379	0.000289148	91.853	IHHYVIEK(1)R
5378	0.805	Down	0.0094412	0.00208505	91.318	EPPSFIK(1)K
14538	0.797	Down	0.0080002	0.0172859	72.2	K(1)SVTFWCK
2845	0.752	Down	0.0122221	0.00134223	67.252	NVEIK(0.004)PSDK(0.996)HR
2841	1.485	Up	0.043941	0.00904906	57.785	NVEIK(1)PSDK
13415	0.75	Down	0.0125554	4.07763E-06	87.667	TVLMSSEGK(1)TYK
746	0.665	Down	0.040541	0.00107881	69.314	EHISTTK(1)VPEQPR
31952	0.806	Down	0.0036829	0.00724821	76.064	GK(1)PFPVCK
21620	1.239	Up	0.042255	2.56181E-07	90.412	K(1)SWSTVTTECSK
14763	0.761	Down	0.0076412	1.54342E-08	126.07	LQICDIK(1)PR
18975	1.407	Up	0.00158093	0.0306831	57.785	DGQSYK(1)FR
30598	0.724	Down	0.0131959	0.0342675	69.598	YK(1)EYCFR
34304	0.631	Down	0.0196432	5.39425E-05	91.313	EVK(1)SQMTETR
18045	0.763	Down	0.032541	0.00144792	76.228	ITNYVIEK(1)R
13517	0.778	Down	0.0140797	2.67591E-33	139	DK(1)GEYVCDCGTDTTK
17444	0.817	Down	0.033424	0.0312353	45.022	APITK(1)VGLK
15851	1.75	Up	0.0046803	2.31672E-14	101.17	AVNK(1)AGESEPSEPSDPVLCR
15734	1.235	Up	0.0165429	0.0134679	71.342	VK(1)GLTNK
18962	0.653	Down	0.0095393	1.3953E-09	97.203	ANHTPESCPETK(1)YK
17739	0.641	Down	0.0050211	0.0332035	93.374	GYVIEK(1)K
13592	0.77	Down	0.0160441	5.74374E-60	158	LKGEPLTASPDCEIIEDGK(1)K
24095	0.714	Down	0.0026951	8.33752E-06	105.65	LK(1)TGCEYQFR
19903	0.78	Down	0.0125043	0.00375226	94.309	GK(1)TFVYLK
7959	1.629	Up	0.028605	0.000217656	78.814	MQFK(1)NNVASLVINK
22784	1.312	Up	0.032196	3.15272E-06	87.429	HDGGSK(1)ITGYVIEAQR
794	0.759	Down	0.02888	0.0060058	78.342	K(1)TTDISTER
13647	0.708	Down	0.02568	8.03146E-24	130.2	DEAK(1)FECEVSR
32488	1.303	Up	0.02576	0.0102727	77.058	THAGK(1)YK
17367	0.817	Down	0.02348	6.66959E-12	114.7	AVNK(1)YGISDECK
13482	0.775	Down	0.0040212	4.59695E-05	115.71	DVPVK(1)WFK
15543	0.778	Down	0.00175747	9.45049E-34	163.9	STVTITDSK(1)R
25881	0.748	Down	0.0063419	0.00128228	87.184	NDAGK(1)YILK
2703	1.286	Up	0.0131791	0.000860899	94.767	VATSK(1)TSAK
16655	0.688	Down	0.00122256	3.12481E-07	117.2	YSVTK(1)LIEGK
Scriptaid group						
12868	2.614	Up	0.0023637	0.000241192	93.623	K(1)TPSPIEAER
9726	1.221	Up	0.0075808	5.50898E-05	86.288	IEAEPIQFTK(1)R
21188	1.373	Up	0.0181958	0.0136504	56.434	INK(1)MYADR
30099	0.818	Down	0.00106491	2.99661E-05	98.407	VNTEPCVK(1)TR
7959	1.52	Up	0.0169982	0.000217656	78.814	MQFK(1)NNVASLVINK
22784	1.439	Up	0.0109042	3.15272E-06	87.429	HDGGSK(1)ITGYVIEAQR
794	0.728	Down	0.023601	0.0060058	78.342	K(1)TTDISTER
13647	0.794	Down	0.0125409	8.03146E-24	130.2	DEAK(1)FECEVSR

25299	0.765	Down	0.0029957	5.40502E-08	105.95	ASK(1)NSECYVAR
35157	1.246	Up	0.0140649	0.000363426	83.182	K(1)IQNQEQQGR
19453	0.781	Down	0.037517	0.0019736	89.355	LK(1)VPHLQK
32488	1.312	Up	0.0072024	0.0102727	77.058	THAGK(1)YK
26335	0.83	Down	0.038821	0.000940126	93.345	EK(1)NSILWVK
17367	0.801	Down	0.0126005	6.66959E-12	114.7	AVNK(1)YGISDECK
13482	0.816	Down	0.0186422	4.59695E-05	115.71	DVPVK(1)WFK
15543	0.801	Down	0.021398	9.45049E-34	163.9	STVTITDSK(1)R
30225	1.404	Up	0.049162	0.000737606	70.089	YTLTVENNSGK(1)K
9605	1.253	Up	0.038521	0.0230665	77.282	VK(1)NCQPK
9679	1.324	Up	0.0037232	0.00317192	76.228	VK(1)TEVEHK
5449	1.22	Up	0.0022645	4.54854E-48	169.17	YVCQAK(1)NDAGIQR
25881	0.768	Down	0.0169008	0.00128228	87.184	NDAGK(1)YILK
9705	1.377	Up	0.0128352	2.3397E-102	189.57	AEDQGQYTCK(1)HEDLETSAELR
2703	1.266	Up	0.042296	0.000860899	94.767	VATSK(1)TSAK
26345	1.251	Up	0.042222	0.00006987	95.094	LNK(1)IPIQDTK
16655	0.761	Down	0.0067962	3.12481E-07	117.2	YSVTK(1)LIEGK
10124	1.303	Up	0.0022628	3.33593E-06	120.15	VPAVHTK(1)K
31315	0.826	Down	0.00043715	0.00994005	93.096	K(1)SATVLVK
10492	1.253	Up	0.012104	0.0169593	73.208	RPVPEK(1)R
1136	1.311	Up	0.038023	7.87155E-07	98.942	VSYNK(1)QTGECR
34140	1.861	Up	0.00183707	0.0222162	78.516	AALK(1)TQK
13678	0.784	Down	0.038699	2.77626E-09	88.555	GTQEITGDDRFELIK(1)DGTR

Note: Information of acetylated lysine sites in titin from exercise and Scriptaid groups. All sites are found in titin (Gene name: Ttn) (Protein accession: A2ASS6) and amino acid type of all sites is K (Lysine). Positions: modification site localization in protein. Change ratio: the acetylation fold change ratio of identified lysine sites. Regulated type is shown as Up (*change ratio* > *1.20*)/Down (*change ratio* < *0.83*). PEP: The maximal posterior error probability for peptides. Score: A simple rule to be used to judge whether a result is significant or not. Modified sequence: Identified peptide sequence marked with modification sites localization probabilities.