Sirt6 reprograms myofibers to oxidative type through CREB-dependent Sox6 suppression

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Abstract

Expanding the exercise capacity of skeletal muscle is an emerging strategy to combat obesity-related metabolic diseases and this can be achieved by shifting skeletal muscle fibers toward slow-twitch oxidative type. Here, we report that Sirt6, an anti-aging histone deacetylase, is critical in regulating myofiber configuration toward oxidative type and that Sirt6 activator can be an exercise mimetic. Genetic inactivation of Sirt6 in skeletal muscle reduced while its transgenic overexpression increased mitochondrial oxidative capacity and exercise performance in mice. Mechanistically, we show that Sirt6 downregulated Sox6, a key repressor of slow fiber specific gene, by increasing the transcription of CREB. Sirt6 expression is elevated in chronically exercised humans and mice treated with an activator of Sirt6 showed an increase in exercise endurance as compared to exercise-trained controls. Thus, the current study identifies Sirt6 as a new molecular target for reprogramming myofiber composition toward the oxidative type and for improving muscle performance.

Introduction

Skeletal muscle is composed of two distinct fibers that are dependent upon the metabolic pathway for ATP production and the corresponding contraction pattern: type I (red-colored, slow-twitch) and type II (white-colored, fast-twitch) fibers. The former is rich in mitochondria and suited to performing long-lasting contraction, while the latter depends mainly on anaerobic glycolysis for its energy and is thus ideal for high-intensity contraction ¹. In mammalian skeletal muscles, multiple fiber types are generally intermingled within a single muscle, and each muscle has varying proportions of fiber types. Whereas soleus muscle is predominantly composed of type I oxidative fibers, extensor digitorum longus (EDL) muscle is rich in type II glycolytic fibers ². These compositions are plastic and muscles have the ability to remodel their fiber types to adapt to different conditions. Whereas in clinical situations, obesity and diabetes involve a shift toward type II fibers, with an inverse correlation between the proportion of type I fiber and intramuscular fat content ³, endurance exercise training increases the proportion of oxidative fibers ⁴. Despite the importance of muscle fiber type determination, however, the molecular basis of the regulatory process remains little understood.

Studies have identified several transcriptional regulators that control muscle fiber differentiation and fiber type switching ⁵⁻⁷. PPAR γ co-activator 1 α (PGC-1 α) is an activating regulator for oxidative muscle remodeling, which involves elevated mitochondrial biogenesis and a shift from glucose to fatty acid as the main energy source ^{8,9}. Specifically, the ectopic expression of PGC-1 α in skeletal muscle increases the composition of oxidative fibers ^{10,11}, whereas mice lacking PGC-1 α exhibit a decline in oxidative metabolism in muscle and exercise capacity ¹². Conversely, the transcriptional corepressor Sox6 suppresses mitochondrial oxidative capacity and exercise performance ¹³. Mechanistically, Sox6 cooperates with nuclear factor I X (Nfix) as a transcriptional repressor of the type I fiber-specific gene *Myh7* ¹⁴. PGC-1 α and Sox6 thus play opposing roles in the metabolic and contractile properties of skeletal muscle. The upstream regulatory mechanisms that determine these factors in fiber type

switching remain little known. Since aging is the main cause of fast-to-slow fiber type shift, in addition to loss of muscle mass¹⁵, we hypothesized that aging-related molecules may be associated with fiber type specification in skeletal muscle.

Sirtuins (Sirt1-Sirt7) have been implicated in aging and aging-related diseases by deacetylating histone and nonhistone proteins^{16–18}. In particular, Sirt6 has emerged as a therapeutic target for the treatment of metabolic diseases¹⁹. A recent study with a muscle-specific knockout (KO) mouse (*Sirt6^{flox/flox};Ckmm-Cre*) model has suggested that while Sirt6 is critical for glucose homeostasis and insulin sensitivity it does not affect PGC-1 α transcription or the associated mitochondrial biogenesis²⁰. However, Samant et al.²¹ have shown degenerating mitochondria with autophagic vacuoles in *Sirt6* deficient heart tissues. Thus, the precise roles of Sirt6 in skeletal muscle biology have thus far not been clearly elucidated, and its effect specifically on myofiber switching has not been previously explored. To investigate the role of Sirt6 in muscle fiber type specification and skeletal muscle adaptations to exercise, we used muscle-specific *Sirt6* KO (*Sirt6^{flox/flox};Myf1-Cre*) and *Sirt6* transgenic (Tg) mice, with a particular focus on mitochondrial biogenesis. We also treated mice with a small molecule Sirt6 activator to confirm and evaluate its value as a therapeutic target. Our findings provide clear evidence that activation of Sirt6 deacetylase offers an effective way of inducing myofiber switching to oxidative phenotype, thereby enhancing exercise performance in skeletal muscle.

Results

Sirt6 ablation in skeletal muscle leads to a decrease in exercise performance

We first screened the sirtuin family members using the human GEO database to identify a key molecule for muscle fiber type determination and exercise capacity. The transcript level of *Sirt6* was the most upregulated in the skeletal muscle of individuals after exercise compared to sedentary subjects (Fig. 1A). This significant increase in Sirt6 protein was also confirmed in exercise-trained mice (Fig. 1B). To further explore the physiological role of Sirt6 in skeletal muscle, we generated skeletal muscle-specific *Sirt6* KO mice by crossing *Sirt6^{flox/flox}* mice with mice expressing Cre recombinase under the control of the myosin light chain 1 (*Myf1-Cre*), which is mainly active in postmitotic type II myofibers (Fig. S1A). To visualize the skeletal muscle tissues in which Cre-mediated recombination occurred, we mated *Myf1-Cre* mice with *R26R* reporter mice. X-gal staining revealed strong recombination in the quadriceps femoris (QF), gastrocnemius (GAS), tibialis anterior (TA), and soleus (SOL) muscles (Fig. S1B). Frozen section from GAS muscle tissue showed a specific β -gal expression in fast muscle cells (Fig. S1C). Western blot analysis confirmed the specific ablation of *Sirt6* in skeletal muscle tissues (Fig. S1D). *Sirt6* KO mice had a phenotypically normal gross appearance at birth (data not shown).

We subjected the *Sirt6* KO mice and their wild type (WT) littermates to involuntary exercise training on a treadmill. After one month of exercise training, the *Sirt6* KO mice exhibited significantly lower grip

strength compared to the WT mice (Fig. 1C). Similarly, endurance capacity—shown as average running time, average running distance, and run time to exhaustion—was significantly decreased in the *Sirt6* KO mice (Figs. 1D-1F). Intriguingly, indirect calorimetric analysis of energy metabolism revealed a significant increase in RER (VCO_2/VO_2) in the *Sirt6* KO mice (Fig. 1G), indicating a switch in mitochondrial substrate preference from fatty acid to glucose. All of these phenotypes were changed in opposite ways in the *Sirt6* Tg mice (Figs. 1H-1K).

Sirt6 ablation decreases oxidative myofiber relative to glycolytic myofiber

In general, impaired exercise endurance is associated with a decrease in the proportion of oxidative fibers¹. Accordingly, we next examined the fiber-type composition of several hind limb muscles. Upon visual inspection, each of the different types of muscles from the *Sirt6* KO mice were less red in color compared to those from the WT mice (Fig. 2A), due to a specific reduction in the number of oxidative fibers in the *Sirt6* KO mice. Muscle fiber-type assessment of GAS muscles by immunofluorescent staining for myosin heavy chain (MyHC) isoforms revealed that *Sirt6* ablation led to decreased oxidative fiber density in comparison with that of WT mice (Fig. 2B). In keeping with this, whereas the mRNA expression of slow fiber-specific genes such as MyHC-I (*Myh7*), MyHC-IIa (*Myh2*), troponin I (*Tnni1*), troponin C (*Tnnc1*), and troponin T1 (*Tnnt1*) were significantly downregulated, the fast fiber-specific gene MyHC-IIb/x (*Myh4*) was dramatically upregulated in muscle from the *Sirt6* KO mice (Fig. 2C). When muscle sections were immunostained for succinate dehydrogenase (SDH), a marker of mitochondrial activity, less than 50% of fibers were SDH positive in the muscle of the *Sirt6* KO mice, whereas over 70% of myofibers were SDH positive in the WT mice (Fig. 2D). Similar observations were made in the case of *Sirt6* KO EDL muscle with regard to the proportion of myofibers and the expression of fiber type-specific genes (Figs. S2A-S2C). The results obtained from *Sirt6* Tg mice also support the view that Sirt6 is required for myofiber switching to the oxidative phenotype (Figs. 2E-2H).

Sirt6 ablation reduces mitochondrial content and oxidative capacity

Electron microscopic analysis of GAS muscle revealed that the *Sirt6* KO mice showed lower numbers of subsarcolemmal and interfibrillar mitochondria relative to the WT mice (Figs. 3A and 3B). Significantly, abnormal mitochondria with destruction of cristae were also found in the *Sirt6* KO mice. These findings correlate well with the decreased mitochondrial DNA content and suppressed gene expression with regard to mitochondrial biogenesis and oxidative phosphorylation in muscle tissues from *Sirt6* KO mice (Figs. 3C-3E). Conversely, mitochondrial content and gene expressions were elevated in the *Sirt6* Tg mice (Figs. S3A and S3B). To determine whether the decrease in mitochondria numbers in the *Sirt6* KO mice was associated with decreased respiratory function, we isolated myofibers from GAS muscle and performed a Seahorse XF Mito Stress test (Fig. 3F). In conjunction with reduced mitochondrial abundance, we observed suppression of basal, ATP-linked, and maximal respiration in the *Sirt6* KO mice,

indicating that *Sirt6* ablation impairs the ability to meet cellular energy demands through oxidative phosphorylation.

Sirt6 ablation suppresses CREB transcription and its downstream genes

To trace the molecular mechanisms by which *Sirt6* modulates fiber-type specification, we performed RNA-seq analysis of GAS muscles. Upregulated (red colored) or downregulated (blue colored) genes in the muscles of the *Sirt6* KO mice relative to those of the WT mice were demonstrated in a volcano plot (Fig. 4A). Among the top 20 downregulated genes, multiple target genes downstream of cAMP response element-binding protein (CREB) were found, notably PGC-1 α (*Ppargc1a*), Nor1 (*Nr4a3*), and Nur77 (*Nr4a1*), which are related to muscle fiber specification and mitochondrial respiration (Fig. 4B). qPCR analysis and Western blotting further confirmed changes in the expression of these genes (Figs. 4C and 4D).

Since the role of *Sirt6* in CREB regulation has never been explored, we then investigated a possible relationship between *Sirt6* and CREB. Analysis of the human GTEx database revealed a strong positive correlation between *SIRT6* and *CREB* in the skeletal muscle tissues of 564 individuals (Fig. 4E). Consistent with this, CREB mRNA and protein levels were markedly downregulated in *Sirt6* KO muscles (Figs. 4F and 4G), while markedly upregulated in *Sirt6* Tg muscles (Figs. S4A-S4C), suggesting the *bona-fide* effect of *Sirt6* on the regulation of CREB. Results of ChIP and luciferase reporter assays revealed that *Sirt6* is enriched in the distal enhancer and proximal promoter regions of the *Creb1* gene (Figs. 4H and 4I), while recruitment of RNA polymerase II (RNAPII) to the promoter was significantly dampened in *Sirt6* KO muscles (Fig. 4J). In HEK293 cells transfected with increasing amounts of *Sirt6*, exogenous *Sirt6* dose-dependently increased the binding to RNAPII (Fig. 4K). Surprisingly, H3K9 acetylation status in the promoter region was increased by *Sirt6* ablation (Figs. 4L and 4M). Altogether, these results indicate that *Sirt6* increases CREB transcription through recruitment of RNA polymerase II, leading to its expression in target genes.

Sirt6 ablation induces Sox6 causality for oxidative myofiber suppression

Since transcriptional activators are counter-balanced by a number of transcriptional repressors in determining oxidative muscle fibers^{5,22}, we next measured expression levels of putative repressors in the *Sirt6* KO mice. Results showed that SRY-box transcription factor 6 (*Sox6*), but not chromobox protein homolog 1 (*Cbx1*), trans-acting transcription factor 3 (*Sp3*), or purine rich element binding protein B (*Purb*), was markedly increased in *Sirt6* KO muscle (Figs. 5A and 5B). Conversely, *Sirt6* Tg muscles showed decreased *Sox6* expression (Figs. 5C and 5D). Moreover, *Sox6* binding to the promoter of slow fiber specific gene *Myh7* was significantly increased by *Sirt6* ablation (Fig. 5E), supporting the inference of a functional increase of *Sox6* in the *Sirt6* KO mice. Since CREB is a negative regulator of *Sox6*²³ and given our prior observation that *Sirt6* enhanced CREB expression (Fig. 4), we measured whether CREB

occupancy on *Sox6* promoter was altered in *Sirt6* KO muscle. CREB enrichment on *Sox6* promoter was observed, which was decreased by *Sirt6* ablation (Fig. 5F), implying that Sirt6-CREB-*Sox6* axis regulates the expression of slow fiber-specific genes.

To demonstrate the causal relationship of *Sox6* to Sirt6-dependent muscle fiber type changes, we silenced *Sox6* in skeletal muscle using AAV-mediated sh*Sox6* delivery to mice (Fig. 5G). The reduction by *Sirt6* ablation of slow myofibers and corresponding increase in fast myofibers was efficiently restored by *Sox6* knockdown, which was confirmed by visual examination, immunostaining of MyHC isoforms, and qPCR analysis of fiber type-specific genes (Figs. 5H-5J). In addition, the reduction of *Sox6* restored mitochondrial oxidative capacity in *Sirt6* KO muscle (Fig. 5K). Strikingly, the decreased exercise performance in the *Sirt6* KO mice was completely reversed by *Sox6* silencing (Figs. 5L-5N). Altogether, these results clearly demonstrate that Sirt6 inhibits *Sox6* expression through CREB activation, thereby contributing to myofiber switching to oxidative phenotype and enhanced exercise performance.

Pharmacological Sirt6 activation enhances exercise performance

To provide a clinical proof-of-concept, we treated mice with the Sirt6 specific activator MDL801²⁴. The functionality of MDL801 as a Sirt6 activator was confirmed by H3K9 deacetylation in the muscle tissues (Fig. 6A). MDL801 treatment increased the expressions of *Creb1* and its target genes, while decreasing *Sox6* in mice (Fig. 6B). As the functional consequences, we observed that mice treated with MDL801 showed increases in slow fiber composition (Figs. 6C and 6D). When WT mice were treated with MDL801 for 4 weeks, enhanced oxygen consumption was observed, along with a shift in fuel preference toward fatty acids (Figs. 6E and 6F). Equally noteworthy was that running time, running distance, and run time to exhaustion were increased approximately 2-fold in MDL801-treated mice.

Discussion

In the current study, we have newly identified a previously unrecognized function of Sirt6 as a molecular switch for reprogramming myofibers to the oxidative type and as an enhancer of exercise endurance (Fig. S5). While skeletal muscle-specific *Sirt6* ablation attenuated the shift in fiber type composition toward slow fibers, *Sirt6* transgenic overexpression promoted it which was reflected in increased mitochondrial content and oxidative capacity. Through transcriptome analysis combined with physiological data, we identified CREB as a target of Sirt6 in determining fast-to-slow fiber composition. In line with previous findings that CREB plays a pivotal role in the metabolic functioning of skeletal muscle²⁵, slow fiber activating molecules downstream of CREB, including PGC-1 α and Nr4a3/1, were markedly upregulated by Sirt6. Conversely, *Sox6*, a potent repressor of slow muscle identity, was inhibited by Sirt6 and revealed to be downstream of CREB, thus specifying the Sirt6-CREB-*Sox6* axis as a novel pathway in determining myofiber type. Lastly, the present study shows for the first time that Sirt6 activation may offer a promising exercise-mimetic therapy, a conclusion corroborated by the evidence of Sirt6 upregulation by

exercise in humans and mice. Maintaining Sirt6 protein level or activity in skeletal muscle is therefore essential to switching to the slow myofiber type and enhancing exercise endurance.

Given that slow muscle fibers are rich in mitochondria and preferentially use oxidative phosphorylation, it was not surprising to observe noticeable derangements in mitochondrial structure and respiratory function in the *Sirt6* KO mice. When observed by EM, around 80% of the mitochondria in *Sirt6* KO muscle displayed morphological defects characterized by disrupted cristae. These findings were consolidated by results that the mitochondria from GAS muscles of *Sirt6* KO mice were functionally impaired, evidenced by higher RER and lower oxygen consumption during exercise training. Given the well-established role of PGC-1 α in the regulation of mitochondrial biogenesis in skeletal muscle^{8,9} and our observation of the downregulation of PGC-1 α in *Sirt6* KO mice (Fig. 4), we propose that downregulation of PGC-1 α is a potential molecular link to the mitochondrial dysfunction observed in *Sirt6* KO mice. PGC-1 α has emerged as a master regulator of mitochondrial biogenesis^{8,9}, modified by different upstream molecules including Sirt1 and AMP-activated protein kinase (AMPK)²⁶. Sirt1 has been shown to interact with PGC-1 α to regulate mitochondrial biogenesis and fatty acid oxidation in skeletal muscle^{27,28}. AMPK in skeletal muscle directly phosphorylates PGC-1 α , increasing fatty acid oxidation and mitochondrial biogenesis²⁹. Although Sirt6 has been found to regulate PGC-1 α in liver³⁰, our study is the first to report a link between PGC-1 α in skeletal muscle and Sirt6 in relation to mitochondrial biogenesis.

The determination of myofiber types is dynamically regulated and coordinated by several transcriptional activators and repressors³¹. To identify the molecules responsible for mediating the effects of Sirt6 on muscle fiber type specification, we first performed RNA-seq analyses, determining that CREB and its target genes are those most affected by *Sirt6* ablation. Further, GTEx analysis showed a strong correlation between Sirt6 and CREB in human skeletal muscle. CREB is activated by exercise in human skeletal muscle^{32,33} and drives gene expression changes capable of metabolic adaptation and muscle performance²⁵. In this study, CREB expression and its target genes responsible for the regulation of fiber types and mitochondrial function, including PGC-1 α and Nr4a3/1, were substantially repressed in *Sirt6* KO mice. As our study shows, Sirt6 promotes CREB transcription by enhancing RNA polymerase II recruitment to enhancers and promoters of *Creb1*, despite diminished histone acetylation. Although deacetylation of histone proteins by Sirt6 generally correlates with chromatin condensation and gene silencing³⁴, there is also evidence that Sirt6 can activate certain genes. For example, specific histone deacetylation by Sirt6 is required for gene activation of nuclear factor erythroid 2-related factor 2 in human mesenchymal stem cells, wherein Sirt6 was found to be in a protein complex with RNA polymerase II³⁵, in accordance with our results.

With respect to the counterregulatory targets of Sirt6 against fast-to-slow fiber type shift, we observed that Sox6, a transcriptional repressor, was negatively regulated by Sirt6. While a previous study using Min6 pancreatic beta cells indicated that CREB binds to *Sox6* promoter to inhibit gene transcription²³, we reveal here a novel role of the CREB-Sox6 network in myofiber transition toward the slow fiber type. Sox6 belongs to the evolutionarily conserved Sox family, which is highly expressed in skeletal muscle³⁶.

Specifically, Sox6 suppresses the expression of slow fiber-specific genes by directly binding to their enhancer or promoter regions, thus indirectly inhibiting several transcriptional activators such as TEA domain family member 1 and 4 (TEAD1/4)³⁷. Skeletal muscle specific Sox6 KO mice exhibited an increased proportion of slow myofibers and mitochondrial activity, leading to improved muscular endurance³⁸. In this study, the altered proportion of slow fibers and concomitant repression of running endurance in *Sirt6* KO mice were completely reversed by AAV-mediated Sox6 knockdown in skeletal muscle. CREB mRNA level, however, remained unchanged by Sox6 silencing (data not shown), suggesting that CREB acts at the upstream level of Sox6.

Interestingly, we also determined that administering the Sirt6 activator MDL801 to mice dramatically increased the proportion of slow fibers, mitochondrial activity, and exercise endurance with adequate regulation of the CREB-Sox6 pathway, confirming that Sirt6 activation leads to reprogramming of myofiber composition to adapt to endurance exercise. Despite much interest, the exercise-mimetics that are currently known remain very limited: agonists of AMPK or PPAR δ have been considered to have this effect³⁹. In addition to the emerging value of Sirt6 as a therapeutic target for a variety of metabolic and inflammatory diseases³⁴, our findings add a further layer of clinical significance to the role of Sirt6 activators in enhancing exercise endurance.

Research Design And Methods

Animal studies

Sirt6^{flox/flox} mice (B6;129-*Sirt6*^{tm1Ygu}/J), *Myf1/MLC1f-Cre* mice (*Myf1*^{tm1(cresjb)}/J), and *Sirt6* Tg mice (C57BL/6-Tg(RP23-352G18)1Coppa/J) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). *Sirt6*^{flox/flox} and *Myf1-Cre* mice were crossed to generate skeletal muscle-specific deletion of *Sirt6*. For pharmacological activation, MDL801 (Chemscene, Monmouth Junction, NJ, USA) dissolved in 4% DMSO + 48% PBS + 48% PEG400 was administered to mice orally at a dose of 100 mg/kg for 30 days. Mice had free access to food and water and were maintained in a room with controlled humidity (50%) and temperature (22°C) on a 12-h light/dark cycle. All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85 – 23, revised 2011).

Muscle cell culture and differentiation

C2C12 cells were obtained from ATCC (Manassas, VA, USA). C2C12 cells were maintained in culture at < 80% confluence in DMEM supplemented with 10% FBS. Myotube differentiation was initiated by replacing 10% FBS by 2% horse serum (Gibco, Life Technologies, Waltham, MA, USA). Differentiation media were changed every two days. All studies were performed in differentiated myotubes.

Grip strength and exercise endurance

Forelimb grip strength of mice was measured using a digital grip-strength meter (Jeung Do Bio & Plant, Seoul, Korea) and normalized by body weight. For treadmill running, a single lane treadmill (Jeung Do Bio & Plant) was used. Prior to testing, mice were acclimatized to the treadmill with a daily 15 min run at 6 m/min for 5 days. The treadmill test was performed at 8 m/min for the first 20 min following by increases of 2 m/min every 10 min until exhaustion, which was defined as remaining on the treadmill for at least 5 min. Animals were removed after exhaustion.

Histology

Skeletal muscle tissues were immediately placed in 30% sucrose solution and embedded with liquid nitrogen-cooled isopentane. For succinate dehydrogenase (SDH) staining, frozen sections (10 μ m) were incubated in 0.2 M sodium phosphate buffer solution (pH 7.6) containing 0.6 mM nitro blue tetrazolium and 50 mM sodium succinate (Sigma-Aldrich, St Louis, MO, USA) for 30 min at 37°C. Slides were washed with DiH_2O and mounted with aqueous mounting media. For staining of myosin heavy chain isoforms, muscle sections were incubated overnight at 4°C with primary MyHC antibodies (DSHB, Iowa City, IA, USA). After washing, secondary antibodies (Alexa Fluor 350-conjugated goat anti-mouse IgG2b; Alexa Fluor 488-conjugated goat anti-mouse IgG1; and Alexa Fluor 594-conjugated goat anti-mouse IgM; Thermo Fisher Scientific, Waltham, MA, USA) were incubated for 1 h at 37°C. Images were acquired using a Leica DM750 microscope (Leica, Wetzlar, Germany). Image analysis was performed using iSolution DT 36 software (Carl Zeiss, Oberkochen, Germany).

Adeno-associated virus (AAV) preparation and injection

Twenty-week-old *Sirt6* KO mice and age-matched wild type mice were treated with AAV9 scrambled control or AAV9-Sox6 shRNA (Genecopoeia, Rockville, MD, USA). AAV was singly injected into the tail vein (viral titer: 1×10^{11} particles/mouse). Three weeks after AAV injection, muscle tissues were collected and mRNA expression was measured by qPCR to confirm Sox6 expression.

Indirect calorimetry

Mice were housed in an Oxymax/CLAMS metabolic cage system from Columbus Instruments (Columbus, OH, USA). The study was carried out continuously for 72 h, in an environmental room set at 20–23°C with 12 h–12 h (7:00 pm–7:00 am) dark-light cycles. Respiratory exchange ratio (VO_2/VCO_2) was measured by the Oxymax system. Data collected from day 3 of the experiment were used for analysis.

Transmission electron microscopy (TEM)

For TEM analysis, GAS muscle samples were immediately fixed in 2% paraformaldehyde and 2% glutaraldehyde in 50 mM sodium cacodylate buffer (pH 7.4) overnight. The tissue samples were post-fixed for 1.5 h with 1% osmium tetroxide in 50 mM sodium cacodylate buffer, stained overnight with 0.5% uranyl acetate, and dehydrated in ethanol series. The samples were infiltrated with a mixture of propylene oxide and Epon 812 resin (EMS, Hatfield, PA, USA) for viewing and imaging under the Hitachi Bio-TEM

(Tokyo, Japan) at the Chonbuk National University Electron Microscopy facility. At least five fields of view of intermyofibrillar and subsarcolemmal mitochondria populations were captured for each section. iSolution DT 36 software (Carl Zeiss, Oberkochen, Germany) was used to calculate mitochondrial number.

RNA sequencing (RNA-Seq) and data analysis

RNA from GAS muscle of ~ 20-week-old mice was used. A TruSeq RNA sample preparation Kit v2 (Illumina, San Diego, CA, USA, #RS-122-2001) was used to convert the poly-A containing mRNA in total RNA into a cDNA library using poly-T oligo-attached magnetic bead selection. Following mRNA purification, the RNA was physically fragmented prior to reverse transcription and cDNA generation. The fragmentation step resulted in an RNA-Seq library that included inserts ranging in size from approximately 100–400 bp. The average insert size in an Illumina TruSeq RNA sequencing library was approximately 200 bp. The cDNA fragments then underwent an end repair process, with the addition of a single 'A' base to the 3' end followed by ligation of the adapters. The resulting products were then purified and enriched with PCR to create the final double-stranded cDNA library. Libraries were quantified using KAPA Library Quantification kits for Illumina Sequencing platforms according to the qPCR Quantification Protocol Guide (KAPA BIOSYSTEMS, KK4855), and qualified using TapeStation D1000 ScreenTape (Agilent Technologies, Santa Clara, CA, USA). Indexed libraries were then submitted to an Illumina HiSeq 4000, and paired-end (2×100 bp) sequencing was performed by Macrogen (Seoul, Korea). Statistical analysis of differential gene expression was performed using abundance estimates for each gene in the samples. Genes with FPKM values greater than 1 in the samples were excluded. To facilitate $\log_2$ transformation, 1 was added to each FPKM value for filtered genes. Filtered data were $\log_2$ -transformed and subjected to quantile normalization. The statistical significance of differential expression data was determined using fold change. Gene-enrichment analysis and KEGG pathway analysis for DEGs were also performed based on Gene Ontology (<http://geneontology.org/>) and KEGG pathway (<https://www.genome.jp/kegg/>) databases respectively.

Western blotting

Tissue homogenates (20 µg) were separated by 10% SDS-PAGE and transferred to PVDF membranes. After blocking with 5% skim milk, blots were probed with primary antibodies against Sirt6, CREB, p-CREB (Cell Signaling, Beverly, MA, USA), Sox6, T-OXPHOS (Abcam, Cambridge, UK), HSP90 (Enzo Life Sciences, Plymouth Meeting, PA, USA), PGC-1α (Millipore, Danvers, MA, USA), Ac-H3K9 (Sigma-Aldrich), Nr4a3, Nr4a1, and Lamin B1 (Santa Cruz Biochemicals, Dallas, TX, USA). For immunoprecipitation HEK293T cells were transfected with expression plasmids for Flag and Flag-Sirt6. After 24 h, 500 µg of protein precleared with protein G-agarose was incubated with anti-Sirt6 overnight at 4°C, then with protein G-agarose at 4°C for 2 h. Blots were probed with primary antibody against RNAPII (Covance, Emeryville, CA, USA), Sirt6 (Cell Signaling Technology), and signals were detected with a Las-4000 imager (GE Healthcare Life Science, Pittsburgh, PA, USA).

Chromatin immunoprecipitation

Muscle tissues (100 mg) were chopped and cross-linked by incubating cells in 1% formaldehyde for 15 min at room temperature. Cross-linking was arrested by 5 min of incubation with 125 mM glycine. ChIP assay was performed using ChIP Enzymatic Chromatin IP Kits (Cell Signaling Technology). Chromatins were immunoprecipitated overnight at 4°C with antibodies to Sirt6, nonspecific IgG (Cell Signaling Technology), RNAPII (Covance, Emeryville, CA, USA), CREB, Ac-H3K9 (Sigma-Aldrich), and Sox6 (Abcam). Data was normalized to input. All primer sequences are listed in Table S1.

Luminescence assay

Creb1 promoter activity was measured using the Secrete-Pair Dual Luminescence Assay Kit (GeneCopoeia, Rockville, MD, USA), which enables analysis of the activities of *Gaussia* Luciferase (GLuc) and Secreted Alkaline Phosphatase (SEAP) in cell culture media. While the *Creb1* promoter controls GLuc reporter gene expression, SEAP is controlled by a cytomegalovirus (CMV) promoter. SEAP expression was used as a normalization factor. Briefly, HEK293T cells containing *Creb1*-Gluc-ON Promoter Reporter construct were deprived of FBS for 5 h, then refed with full media containing 2% FBS for 24 h. Culture media were collected and luminescence was measured by Multi-Mode Microplate Reader. *Gaussia* luciferase activity was normalized with SEAP within each sample.

Mitochondrial respiration

For Seahorse analysis (XF96, Agilent Technologies), isolated myofibers and C2C12 myoblasts were seeded in XF24 plates. After six days of differentiation, C2C12 cells were treated overnight with vehicle (DMSO) or MDL801 (5 or 10 μ M). One hour prior to beginning the assay, myofibers and C2C12 myotubes were changed to DMEM containing with 5 mM glucose and 1 mM pyruvate. Oxygen consumption rate (OCR) was then measured according to manufacturer instructions with the injection of the Seahorse XF Cell Mito Stress Test Kit (Agilent Technologies). Respiration was measured three times by injection of oligomycin (1 μ M), FCCP (0.5 μ M) and rotenone/antimycin A (1 μ M). Data were normalized to protein content.

RNA isolation and real-time quantitative RT-PCR (qPCR)

Total RNA was extracted from skeletal muscle tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was generated using the random hexamer primer provided in a first-strand cDNA synthesis kit (Applied Biosystems, Foster City, CA, USA). Specific primers for each gene (Table S1) were designed using qPrimerDepot (<http://mouseprimerdepot.nci.nih.gov>). qPCR reactions were conducted in a final volume of 10 μ l containing 10 ng of reverse-transcribed total RNA, 200 nM of forward and reverse primers, and PCR master mix. qPCR was performed in 384-well plates using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems).

For mitochondrial DNA content analysis, total DNA was extracted using RNAiso Plus (Takara, Tokyo, Japan). Relative mtDNA was quantified by qPCR using primers for the mitochondrially encoded gene

cytochrome oxidase 2 (*Cox2*), normalized to the nuclear-encoded gene cyclophilin A (*Ppia*).

Statistical analysis

Data are expressed as the mean $\pm$ Standard Error of the Mean (SEM). Statistical comparisons were made using one-way analysis of variance followed by Fisher's *post hoc* analysis. The significance of differences between groups was determined using Student's unpaired *t*-test. A *p* value of less than 0.05 was considered significant.

Declarations

Competing interest: The authors declare no competing interests.

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Figures

Figure 1

Exercise performance in Sirt6 KO and Sirt6 Tg mice. (A) Heatmap visualizing expression of sirtuin family members in sedentary or exercise-trained subjects (extracted from GSE9103). (B) Expression of Sirt6 in GAS muscle was compared in sedentary or exercise-trained mice. (C) Forelimb grip strength test at three different time points. Grip strength values were normalized against mouse body weight (BW) (n=9-10). (D-F, H-J) Average running time, average running distance, and run time to exhaustion after four weeks of treadmill exercise training. (G, K) Forty-eight-hour light cycle and dark cycle respiratory energy expenditure (RER) were measured by indirect calorimetry. Values are mean $\pm$ SEM. *, $p < 0.05$ and **, $p < 0.01$ vs. WT.

Figure 2

Oxidative fiber density in GAS muscle of Sirt6 KO and Sirt6 Tg mice. (A) Representative photographs showing skeletal muscles from 20-week old WT and Sirt6 KO mice. (B, F) Immunofluorescence staining for MyHC-I, MyHC-IIa, and MyHC-IIb/x. Composition of each myofiber was quantified (n=4-6). (C, G) Expression of markers of slow and fast fibers was compared by qPCR. (D, H) Succinate dehydrogenase (SDH) staining and quantification of SDH-positive fibers. (E) Representative photographs showing skeletal muscles from 15-week old WT and Sirt6 Tg mice. Values are mean $\pm$ SEM. *, $p < 0.05$ and **, $p < 0.01$ vs. WT.

Figure 3

Decrease in oxidative capacity in Sirt6 KO mice. (A) Electron microscopy of GAS muscles from WT and Sirt6 KO mice. (B) Percentages of normal and degenerating mitochondria shown in panel a. Values were calculated from a total of 4 different samples. (C) Mitochondrial DNA (mtDNA) was quantified by qPCR using nuclear DNA (nDNA) as a standard. (D) qPCR analysis of genes related to mitochondrial biogenesis and oxidative phosphorylation (OxPhos) in GAS muscles. (E) Western blotting of OxPhos complex. (F) Oxygen consumption rate was measured using Seahorse XF analyzer in isolated adult myofibers from GAS muscle. Basal respiration, respiration related to ATP production (uncoupled, difference between OCR before and after oligomycin injection), and maximal respiration (difference between OCR after FCCP and

rotenone/antimycin A injection) were determined. Values are mean $\pm$ SEM. *, $p < 0.05$ and **, $p < 0.01$ vs. WT.

Figure 4

Decreased CREB pathway in GAS muscle of Sirt6 KO mice. (A) Volcano plot showing the $\log_2$ fold-difference in mRNA expression in GAS muscle of WT and Sirt6 KO mice, assessed by StringTie analysis of RNA-seq data. Red and blue dots represent upregulated and downregulated DEGs with > 2 -fold change and $p < 0.05$, respectively. Each sample was analyzed in triplicate. (B) Heat map of RNA-seq analysis of top 20 downregulated genes. (C, D) qPCR and Western blotting analyses of CREB target genes. (E)

Genotype tissue expression (GTEx) analysis of human skeletal muscle. Pearson correlation coefficients between SIRT6 and CREB in human skeletal muscle were calculated. TPM, transcripts per million. (F, G) mRNA and protein levels of CREB. (H) Occupancy of Sirt6 on Creb1 promoters or enhancers was evaluated by ChIP-qPCR assay in WT and Sirt6 KO mice. (I) Creb1-luciferase activities in HEK293T cells overexpressing or deleting Sirt6. (J) ChIP-qPCR assay showing Sirt6-dependent recruitment of RNA polymerase II (RNAPII) on Creb1 promoter. (K) HEK293T cells were transfected with increasing amount of Sirt6 and the binding of Sirt6 to RNAPII was determined. (L) Acetylation of H3K9 in WT and Sirt6 KO mice was compared by Western blotting. (M) Enrichment of Ac-H3K9 on promoter regions of Creb1 was evaluated by ChIP assay. Values are mean $\pm$ SEM. *, $p < 0.05$ and **, $p < 0.01$ vs. WT or mock.

Figure 5

Sox6 expression in GAS muscle of Sirt6 KO and Sirt6 Tg mice. (A-D) mRNA and protein levels of transcriptional repressors of slow muscle fiber genes in Sirt6 KO and Sirt6 Tg mice. (E) ChIP-qPCR assay showing binding of Sox6 to promoter of Myh7. (F) ChIP-qPCR assay showing binding of CREB to promoter of Sox6. (G-N) WT and Sirt6 KO mice were injected with AAV expressing a scrambled shRNA (AAV-Scr) or shSox6 (AAV-Sox6). Three weeks later, muscle tissues were collected. (G) mRNA levels of Sox6 was measured by qPCR. (H) Gross observation of GAS muscles. (I) Immunofluorescence staining for MyHC-I, MyHC-IIa, and MyHC-IIb/x. Composition of each myofiber was quantified (n=4). (J) Expression of markers of slow and fast fibers was compared by qPCR. (K) Succinate dehydrogenase (SDH) staining and quantification of SDH-positive fibers. (L-N) Average running time, average running distance, and run time to exhaustion after four weeks of treadmill exercise training. Values are mean $\pm$ SEM. *, $p < 0.05$ and **, $p < 0.01$ vs. WT or AAV-Scr.

Figure 6

Increase in exercise performance in mice treated with Sirt6 activator. Twenty five-week old mice were treated every day with MDL801 (100 mg/kg) via oral gavage for four weeks during treadmill exercise program. (A) Western blot analysis of H3K9 acetylation in GAS muscles. (B) qPCR analysis of muscle fiber-type specification genes. (C) Representative immunofluorescence staining for MyHC-I, MyHC-IIa, and MyHC-IIb/x. Composition of each myofiber was quantified (n=6-7). (D) Succinate dehydrogenase (SDH) staining and quantification of SDH-positive fibers. (E-I) Oxygen consumption rate, RER, running time, running distance, and run time to exhaustion were compared between MDL801 (M)- and vehicle (V)-treated mice. Values are mean $\pm$ SEM. *, $p < 0.05$ and **, $p < 0.01$ vs. vehicle.

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