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# **Novel role of TIEG1 in muscle metabolism and mitochondrial biogenesis**

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## **Abstract**

TIEG1 (TGF $\beta$  inducible early gene-1) is involved in multiple signaling pathways and human diseases and is highly expressed in skeletal muscle, yet its functions in this tissue are poorly understood. Here, we have utilized TIEG1 knockout mice to identify novel and important roles for this transcription factor in regulating skeletal muscle ultrastructure, metabolism and mitochondrial biogenesis. More specifically, we have found that loss of TIEG1 expression results in altered sarcomere organization, decreased mitochondrial numbers and respiratory activity and altered metabolic profiles. TIEG1 was also found to regulate the expression of genes known to play important roles in mitochondrial biogenesis and muscle function. Interestingly, these phenotypes were more pronounced in oxidative muscle compared to glycolytic muscle and resemble a number of human muscle diseases. Our findings lay the groundwork for better understanding the molecular basis of mitochondrial myopathies and identifying novel strategies for their treatment.

## Introduction

TGF $\beta$  inducible early gene-1 (TIEG1), also known as Krüppel-like factor 10 (KLF10), is a zinc finger-containing transcription factor known to be involved in multiple signaling pathways and disease processes<sup>1, 2</sup>. TIEG1 was originally discovered in human osteoblast cells as an early response gene following TGF $\beta$  treatment<sup>3</sup>. Clinically, altered expression levels of TIEG1, as well as single nucleotide polymorphisms (SNPs) in the TIEG1 gene, are associated with osteoporosis in humans<sup>4, 5</sup>. Further, SNPs in TIEG1 have been identified in patients with hypertrophic cardiomyopathy<sup>6</sup>. Finally, decreased expression of TIEG1 is observed during breast cancer progression<sup>7, 8</sup> and SNPs in the TIEG1 gene have been shown to predict risk of ovarian cancer<sup>9</sup>.

Many of these clinical findings have been corroborated through the use of a TIEG1 knockout mouse model system. Specifically, global deletion of TIEG1 in mice results in an osteopenic skeletal phenotype<sup>10, 11</sup> and attenuates estrogen signaling in bone tissues<sup>12, 13</sup>. Further characterization of this gene has revealed a central role for TIEG1 in regulating canonical Wnt signaling<sup>14, 15</sup>, TGF $\beta$  signaling<sup>16, 17, 18</sup> as well as BMP signaling<sup>19, 20, 21, 22, 23</sup> in multiple tissues and cell types.

As part of the original description and characterization of the TIEG1 gene, it was shown to be most highly expressed in skeletal muscle<sup>3</sup>. However, little is known about its involvement in mediating the development, repair or function of this tissue. Recent studies performed by our group have shown that deletion of TIEG1 in mice results in different functional responses of slow and fast twitch muscle fibers<sup>24</sup>. In parallel to these mechanical tests, imaging analyses (MRI, 9.4T) revealed changes in the texture profile between wild-type (WT) and TIEG1 knockout (KO) mouse skeletal muscles<sup>25</sup>. Histochemical analyses demonstrated hyperplasia and hypertrophy of all fiber types in TIEG1 KO muscles (soleus and extensor digitorum longus: EDL) with a significant increase in the percentage of glycolytic fibers and a decrease in the percentage of oxidative fibers<sup>24</sup>. At the cellular level, others have implicated TIEG1 in myogenic differentiation given its role in downstream regulation of myostatin and TGF- $\beta$  signaling in myoblasts<sup>26, 27</sup>.

Here, we expand upon our previous studies to comprehensively characterize the skeletal muscle defects observed in TIEG1 KO mice and to begin to elucidate the molecular mechanisms by which deletion of a single nuclear transcription factor results in a severe skeletal muscle phenotype that resembles multiple forms of mitochondrial-related myopathies in humans. Specifically, we have utilized RNA sequencing (RNAseq) to characterize the

TIEG1-regulated transcriptome in skeletal muscle and have identified mis-regulation of multiple genes implicated in mitochondrial biogenesis and muscle biology. Given these findings, we have further characterized the role of TIEG1 in skeletal muscle function and have identified significant defects with regard to mitochondrial biogenesis and metabolism in TIEG1 KO skeletal muscle. The present experiments shed light on the hierarchy of molecular changes occurring in TIEG1 KO muscles and enhance our understanding of muscle metabolism and mitochondrial biogenesis which are essential towards the goal of developing novel approaches for the treatment of myopathies and muscle-related diseases.

## RESULTS

### RNA sequencing

Given the morphological and functional changes observed in the skeletal muscles of TIEG1 KO mice, we sought to elucidate TIEG1-dependent molecular mechanisms controlling skeletal muscle maintenance and functioning. Towards this goal, we assessed the transcriptome-wide effect of TIEG1 loss in the soleus of 12 week old mice (Figure 1A - 1B). In total, 279 genes were differentially regulated between the wildtype (WT) and TIEG1 knockout (KO) muscle (Figure 1C). Although the number of downregulated genes were substantially less, the pathway analyses indicated that a number of these genes are known targets of the Myod1 transcription factor (Supplementary data, S1) which was shown to be upregulated in the muscle of TIEG1 KO mice ( $\text{Log}_2\text{FC}=1.46$ ,  $p_{\text{adj}}=3.06\text{E}-16$ ). Interestingly, some of the downregulated genes (Pln, Tfcp2l1, Ide, Bcl2l1) are also known components of mitochondria (Supplementary data, S1) whereas most of the upregulated genes were associated with collagen and extracellular matrix organization (Supplementary data, S2).

### Histochemical staining analysis.

Given the importance of mitochondrial function in skeletal muscle, we next histologically assessed mitochondrial metabolism within the muscle biopsies of TIEG1 WT and KO mice. Remarkably, as shown in Figure 2A - 2B, SDH staining was completely absent in soleus muscle of TIEG1 KO mice while normal staining patterns were observed in WT littermates. In addition, COX (Figure 2C - 2D) and menadion (Figure 2E - 2F) staining was less intense in TIEG1 KO mice compared to WT controls. No significant differences in the staining for endoplasmic reticulum, connective tissue, glycogen content or lipids were observed (Figure 2G - 2L). Decreased staining for SDH, COX and menadion were also observed in the EDL muscle of TIEG1 KO mice, albeit to a lesser degree (Supplementary data, S3 A-F).

Given the observed differences in staining patterns for important mitochondrial enzymes, we next sought to analyze the ultrastructure of the soleus and EDL muscle fibers using TEM. Longitudinal sections revealed disorganization of the muscle structure in both TIEG1 KO soleus and EDL muscles (Figure 3) compared to WT littermates. Specifically, smaller sarcomere lengths ( $1.5\mu\text{m}$  for KO and  $2.5\mu\text{m}$  for WT), disappearance of the I band, and changes in the shape of mitochondria were observed in TIEG1 KO soleus (Figure 3B) and EDL muscles (Figure 3C).

The quantification of mitochondrial area (A) revealed a significant ( $P < 0.001$ ) decrease for soleus and EDL TIEG1 KO muscles ( $A_{WT\_soleus} = 0.079 \pm 0.003 \mu\text{m}^2$  vs  $A_{TIEG1\ KO\_soleus} = 0.054 \pm 0.002 \mu\text{m}^2$  and  $A_{WT\_EDL} = 0.08 \pm 0.003 \mu\text{m}^2$  vs  $A_{TIEG1\ KO\_EDL} = 0.045 \pm 0.002 \mu\text{m}^2$ ). Mitochondrial area was classified within different ranges based on Picard's study<sup>28</sup> and a non-Gaussian curve was observed in both muscles with smaller mitochondria ( $A_{soleus} < 0.04 \mu\text{m}^2$ : 37% vs 14% and  $A_{EDL} < 0.04 \mu\text{m}^2$ : 58% vs 23%), and a complete absence of mitochondria with an area greater than  $0.15 \mu\text{m}^2$ , in TIEG1 KO mice.

### **Mitochondrial Activity and Function.**

A significant decrease in citrate synthase (CS) and respiratory chain complex activities were observed in TIEG1 KO soleus muscle (Figure 4A - 4C). This result is in agreement with the decreased area occupied by mitochondria and the weaker COX and SDH staining (Figure 2) observed in TIEG1 KO soleus fibers. No significant changes were observed for COX and CS activities in TIEG1 KO EDL muscle (Figure 4D - 4F). However, NADH: ubiquinone oxidoreductase (Complex I) (Figure 4B - 4E) activity was decreased in both TIEG1 KO soleus and EDL muscles.

Respiration rates of total mitochondrial populations using substrates for complexes I, II and IV were markedly lower in permeabilized TIEG1 KO soleus muscle (Figure 5A) compared with WT controls. In contrast, mitochondria in TIEG1 KO EDL muscle did not exhibit any differences in respiration (Figure 5B) as compared with WT. These results are in agreement with the enzyme activities showing a significant decrease only for the TIEG1 KO soleus.

In soleus, protein content (Figure 6) of all complexes measured (I, II, III, V) was slightly lower in TIEG1 KO muscle though these changes did not reach statistical significance except for complex IV (COX). For the EDL muscle, no significant differences were observed for any of these proteins between WT and TIEG1 KO mice.

### **In vivo Magnetic Resonance Spectroscopy and <sup>1</sup>H-NMR metabolomics**

<sup>1</sup>H spectra showed no significant difference for choline, creatine, taurine and extramyocellular lipids between WT and TIEG1 KO muscle (Figure 7). However, <sup>31</sup>P spectra revealed a significant difference in the levels of phosphocreatine and ATP $\gamma$  (Figure 8). The PCA model obtained with the NMR data set containing WT soleus, TIEG1 KO soleus, WT EDL and TIEG1 KO EDL (Figure 9) revealed significant separation between soleus and EDL

muscles (PC1) explaining 50% of the total variability within the dataset. Mouse genotype (PC2) explained 24% of the total variability within the data. These results highlight differences between WT and TIEG1 KO metabolic profiles.

PLS-DA models obtained with <sup>1</sup>H-NMR datasets from soleus muscles (Supplementary data, S4 A, predictive ability  $Q^2=0.72$ , goodness of fit  $R^2Y(\text{cum})=0.99$ ) and EDL muscles (Supplementary data, S4 B, predictive ability  $Q^2=0.84$ , goodness of fit  $R^2Y(\text{cum})=0.99$ ) revealed a clear separation between WT and KO genotypes regardless of muscle type suggesting that deletion of TIEG1 results in significant changes in the metabolic profiles of skeletal muscle. The PLS-DA models allow for the identification of very important metabolites in projection (VIPs) whose changes are likely to be biologically relevant (Supplementary data, S5). Figure 10 depicts heatmaps (10A for soleus muscle, 10B for EDL muscle) generated for metabolites with a VIP > 1 in the PLS-DA models. For both TIEG1 KO soleus and EDL muscles, there were more down regulated metabolites compared to WT muscles. Of note, there were differences in the metabolites that were identified to be differentially regulated by loss of TIEG1 expression between the two muscle types.



## Discussion

We have previously shown that deletion of the TIEG1 gene in mice results in muscle fiber hypertrophy, texture profile changes and defects in the functional properties of skeletal muscle<sup>24</sup>. Here, we have expanded upon these original discoveries to further investigate the molecular and cellular basis for the observed skeletal muscle defects. Specifically, we have shown that TIEG1 plays a central role in regulating mitochondrial biogenesis and metabolism.

TIEG1 was originally identified as a primary response gene following TGF $\beta$  treatment in human osteoblasts<sup>3</sup>. Later, it was shown to regulate Smad signaling by inhibiting Smad7 and inducing Smad2 expression<sup>16, 17</sup>. In the present study, RNAseq analysis revealed a significant up-regulation of Smad7 in soleus of TIEG1 KO mice compared to WT controls. In addition, it has been demonstrated that increasing expression of Smad7 promotes skeletal muscle hypertrophy in mice<sup>29</sup>. Indeed, overexpression of Smad7 in muscles increases fractional protein synthesis rates and prevents muscle wasting by acting as an intracellular negative regulator. It prevents Smad2/3 activation and promotes degradation of ActRIIB (type IIB activin receptor) complexes, considered as an important mechanism underlying muscle wasting<sup>29</sup>. Thus, the muscle fiber hypertrophy and the observed increase in myosin diameter of TIEG1 KO mice could result in part from increased expression of Smad7. Miyake et al. (2011) have previously demonstrated a relationship between overexpression of Smad7 and muscle fiber hypertrophy<sup>30</sup>. This study further confirms the link between TIEG1, Smad7 and muscle structure. In addition, we observed up-regulation of the Myod1 “myogenic differentiation 1” gene in TIEG1 KO muscles. This gene is well known to be a regulator of myogenic differentiation<sup>31, 32, 33</sup>. In parallel to the hypertrophy that we observed, a disorganization of the muscle ultrastructure was found and was defined by smaller sarcomeres and the absence of I bands in TIEG1 KO muscle. Unger’s group showed that chaperone proteins were capable of aggregating and binding to titin filaments resulting in shorter sarcomeres in hereditary myopathies (Duchenne, Titinopathy, etc ...) <sup>34</sup>. However, RNAseq analysis did not show significant difference for HSP expression levels and it will be of interest to quantify the abundance of HSPs in TIEG1 KO muscle.

Pathway analyses of genes identified to be differentially expressed in TIEG1 KO skeletal muscle revealed alterations in the expression levels of 48 genes that are known to be related to muscle diseases (such as Duchenne muscular dystrophy, Becker muscular dystrophy, etc ...). Interestingly, the genes implicated in these muscle diseases were primarily collagens (Col1a1, Col1a2, Col5a1, Col6a2, etc ...) which were all upregulated in TIEG1 KO mice. A study by Crasten et al has previously shown that collagen VI-related myopathies encompass a spectrum

of disease ranging from severe Ullrich muscular dystrophy to mild Bethlem myopathy. Moreover, it has been demonstrated that *Colla1* is the most commonly evaluated collagen gene for fibrosis<sup>35</sup>, and our results are also in agreement with DiMario's study showing an increase of *Colla1* gene expression, and protein content, in TIEG1 KO muscle (tibialis anterior and diaphragm)<sup>36</sup>. In addition, this phenomenon is more accentuated in the TIEG1 KO/mdx (Duchenne muscular dystrophy mouse) mouse model<sup>36</sup>. Moreover, previous qRT-PCR experiments performed on fibroblasts extracted from TIEG1 KO flexor tendons were also shown to exhibit significant increases in the expression of *Colla2*<sup>37</sup>. Similarly, Gumez et al.<sup>38</sup> reported different X-ray diffraction patterns between WT and TIEG1 KO tendon fibers demonstrating that lack of TIEG1 expression results in a disorganization of the collagenous structure<sup>39</sup> which may be similar to some of the differences observed in the skeletal muscle ultrastructure reported here. TIEG1 KO mice also exhibit increased expression of TGF $\beta$  within the skeletal muscle. Similar results were observed after muscle rotator cuff tears in a rat model which results in a significant increase of fibrosis. To explain this phenomenon, Liu et al.<sup>40</sup> have linked TGF $\beta$  to the Akt/mTOR pathway and suggest that TGF- $\beta$  may be the primary factor regulating muscle changes after muscle rotator cuff tears. In general, TGF- $\beta$  pathways are highly conserved pathways that exert a potent level of control over muscle gene expression and are critical regulators of fibrosis in multiple organ systems.

In addition to muscular dystrophy, mitochondrial myopathy and mitochondrial encephalomyopathy pathways were also identified from the gene expression data. For instance, *Rmrp* (RNA component of mitochondrial RNAase P) was identified in both of these pathways and was significantly down-regulated in TIEG1 KO soleus muscle. The decreased expression of mitochondrial complex proteins have confirmed the involvement of TIEG1 with regard to mitochondrial biogenesis and may explain some of the structural and functional changes of the mitochondria observed in TIEG1 KO muscle. In addition, these mitochondrial disease are also related to another disease called "mitochondrial complex I deficiency". In the present study, complex I activity and respiration were found to be decreased in TIEG1 KO muscle, further demonstrating an important role for TIEG1 in mitochondrial activity and function. Such a specific impairment of complex I function has been observed in several diseases including cardiomyopathy and heart failure<sup>41, 42</sup>. Indeed, complex I specific and functional activities could be decreased, when other ETC (electron transport chain) complexes or total mitochondrial mass (usually estimated by citrate synthase activity) are normal. This is the case, for example, in generalized myopathy in advanced heart failure<sup>43</sup>. Similarly, complex I in skeletal muscle mitochondria is the primary target of the immunosuppressive

molecule cyclosporine toxicity<sup>44</sup>. A marked decrease in complex I activity was found in skeletal muscle in acute intermittent porphyria (a disease primarily affecting the nervous system), an autosomal dominant metabolic disease<sup>45</sup>. Such a specific inhibition of complex I has an important impact on the mitochondrial function because in striated muscle, this complex is the rate limiting step of the electron flux through ETC. Also, complex I is one of the main sites of reactive oxygen species production. The latter is known to be considerably augmented if the electron flux is blocked at the level of complex I.

Similarly, deficiency of SDH (Succinate dehydrogenase) has been reported in human mitochondrial diseases<sup>46, 47, 48</sup>. Haller et al. have reported on a 22 year old man with life-long exercise intolerance marked by premature fatigue<sup>47</sup>. Additionally, Kollberg et al. described a 77 year old woman with muscle fatigue and dyspnea resulting from SDH deficiency<sup>46</sup>. Moreover, Vladutiu et al. have assessed qualitative and quantitative SDH expression in muscle biopsies from 108 patients (age range: 1 to 68 years) with mitochondrial myopathy<sup>48</sup> exhibiting muscle pain, exercise intolerance and cardiomyopathy, etc. As is the case with TIEG1 KO mice as reported here, a decrease in all respiratory chain complexes was associated with a deficiency of SDH activity (23% of patients) showing an important reduction for 12 patients. The results reported in these clinical studies have described skeletal muscle disorders resulting from mitochondrial defects which are nearly identical to the muscle phenotype of the TIEG1 mouse model.

Interestingly, we have found that loss of TIEG1 expression has a strong muscle-type specific effect in that the soleus is more affected than that of the EDL. These observations are in agreement with DiMario's study where loss of TIEG1 expression resulted in a pronounced fibrotic phenotype in slow twitch (diaphragm) muscle compared to fast twitch muscle (tibialis anterior)<sup>36</sup>. However, through 1H-NMR metabolic profiling, we have shown that both slow and fast twitch muscles are affected by deletion of TIEG1. Metabolites such as fumarate, lactate and pyruvate were up-regulated in EDL TIEG1 KO muscle. These metabolites are involved in the Krebs cycle and reflect alterations in mitochondria metabolism. Nicotinurate was up-regulated, and beta-hydroxy-butyrate was down-regulated, in both muscles of TIEG1 KO mice. Nicotinurate is used to diagnose disorders associated with mitochondrial fatty acid beta-oxidation and beta-hydroxy-butyrate, a ketone body, which is produced by beta-oxidation as an energy source. Furthermore, in vivo 31P-NMR spectroscopy revealed decreased ATP (adenosine triphosphate) and PCr levels that are involved in energy metabolism, again implicating an important role for TIEG1 in mitochondrial function. The amino-acids valine, leucine, lysine and glutamate were also shown to be down-regulated in

both soleus and EDL muscles and are associated with protein metabolism. This metabolic imbalance in both TIEG1 KO muscle types can be related to mitochondrial pathways confirming the strong implication of the TIEG1 gene in the function and activity of mitochondria.

The present study has demonstrated a new role for TIEG1 in the regulation of skeletal muscle energy metabolism, specifically in oxidative muscle which strongly depends on mitochondrial functions. Thus, the TIEG1 KO mice mimic human diseases associated with muscle fatigue and exercise intolerance and could allow for the study of mitochondrial myopathies associated with deficiency of SDH in skeletal muscle. For these reasons, TIEG1 KO mice represent a novel model for further understanding muscle dystrophies and mitochondrial myopathies and will enhance the understanding of the role of the TIEG1 gene in muscle. These findings have laid the groundwork for future efforts aimed at identifying specific genes and their associated pathways that can be pharmacologically manipulated to reverse/alleviate these defects in TIEG1 KO mice and to evaluate their efficacy for the treatment of muscle disorders in humans.

## **Methods**

### **Mice and Study design.**

The generation of TIEG1 KO mice has been previously described<sup>49</sup> and initial characterizations of TIEG1 expression and function in skeletal muscle were previously reported<sup>3, 24, 25</sup>. For the studies presented here, littermate female animals from heterozygous breeding pairs were utilized and sacrificed for analysis at approximately 3 months of age. All mice were maintained in a temperature controlled room ( $22 \pm 2^\circ\text{C}$ ) with light/dark cycle of 12 hours. Animals had free access to water and were fed with standard laboratory chow ad libitum. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the French ministry of higher education, research and innovation (Permit Number: DUO-4776) and the Mayo Clinic Institutional Animal Care and Use Committee (Permit Number: A9615).

### **RNA isolation and sequencing.**

For the RNA sequencing studies, a total of 6 WT and 6 TIEG1 KO mice were utilized. Immediately following sacrifice, the soleus muscle was dissected from mice, placed in trizol and sheared using an Ultra-Turrax homogenizer prior to extraction of total RNA using a miRNeasy Mini Kit (Qiagen, Germantown, MD). Total RNA was quantitated with a nanodrop and equal amounts of RNA from 2 individual WT or TIEG1 KO mice were pooled to generate a total of 3 replicates per genotype. All samples were tested for RNA integrity before submitting for sequencing. mRNA sequencing was performed by the Mayo Clinic Molecular Biology Sequencing Core Facility using standard procedures. RNA libraries were prepared using 200 ng of good quality total RNA according to the manufacturer's instructions for the TruSeq RNA Sample Prep Kit v2 (Illumina, San Diego, CA) employing poly-A mRNA enrichment using oligo dT magnetic beads. The final adapter-modified cDNA fragments were enriched by 12 cycles of PCR using Illumina TruSeq PCR primers. The concentration and size distribution of the completed libraries was determined using a Fragment Analyzer (AATI, Ankeny, IA) and Qubit fluorometry (Invitrogen, Carlsbad, CA). Libraries were sequenced at 40-50 million fragment reads per sample following Illumina's standard protocol using the Illumina cBot and HiSeq 3000/4000 PE Cluster Kit. The flow cells were sequenced as 100 X 2 paired end reads on an Illumina HiSeq 4000 using HiSeq 3000/4000 sequencing kit and HCS v3.3.52 collection software. Base-calling was performed using Illumina's RTA version 2.7.3.

### **Histochemical staining analysis.**

Soleus and EDL muscles were harvested from TIEG1 KO (N=10) and WT (N=10) mice and immediately frozen in an isopentane bath that was cooled by liquid nitrogen and subsequently stored at -80°C until the time of analysis. Ten µm serial sections were cut perpendicular to the muscle using a Microm HM 500M cryotome at -20°C. Analysis of succinate dehydrogenase (SDH), cytochrome c oxidase (COX) and menadione were performed to identify potential alterations in mitochondrial metabolism. Additionally, the endoplasmic reticulum (Dinitrophenylhydrazine), connective tissue (Gömöri trichrome), glycogen content (Periodic acid–Schiff) and lipid content (Oil Red O) were determined. Each staining procedure was performed on two independent tissue sections which were subsequently visualized under a Leica microscope using a 20 x objective. High-resolution images were captured with an Olympus cooled digital camera (DP-72) and interpretation of staining was based on the color histogram of the image using ImageJ software (US National Institutes of Health, Bethesda, MD, United States)<sup>50</sup>. This analysis separately measures the intensity in the red, green and blue channels of the image.

### **Transmission Electron Microscopy (TEM).**

Prior to processing for TEM, the soleus (N=7) and the EDL (N=7) were dissected from WT and TIEG1 KO mice with both extremities pinned to avoid muscle contraction and to maintain original length. Muscles were then immediately placed in fixative [1% (vol/vol) glutaraldehyde and 4% (vol/vol) paraformaldehyde in 0.1 M phosphate buffer, pH 7.2]<sup>51</sup> and incubated overnight at 4°C. Subsequently, two pieces (1 mm x 2 mm) of tissue were dissected from the middle of the tissue and rinsed for 30 min in three changes of 0.1 M phosphate buffer, pH 7.2, followed by a 1h secondary fixation in phosphate-buffered 1% OsO<sub>4</sub> and 30 min in 1% uranyl acetate at room temperature. Following dehydration in a series of ethanol washes, tissue was embedded in EMbed 812 resin (EMS, Hatfield PA) and polymerized at 60°C for 18h<sup>52</sup>. Ultrathin (90 nm) sections were cut using a Leica UC7 ultramicrotome (Buffalo Grove, IL), placed on 200 mesh copper grids, and stained with lead citrate. Five micrographs of each specimen were randomly captured across the muscle tissue using a JEOL 1400Plus TEM (Peabody, MA), operating at 80 kV with a magnification of 42000x. ImageJ 1.46/Java 8 software (National Institute of Health, Bethesda, MD, United States)<sup>50</sup> was used to manually quantify the mitochondria and myosin areas.

### **Enzyme assays.**

Complete enzyme extractions from pieces of frozen soleus and EDL skeletal muscle tissues (N=7 WT, N=7 KO) were performed in an ice-cold buffer (50 mg ml<sup>-1</sup>; containing (in mM): HEPES 5 (pH 8.7), EGTA 1, dithiothreitol 1, and 0.1% Triton X-100) using a Bertin Precellys 24 homogenizer (Bertin, Montigny-le-Bretonneux, France). Protein concentrations were assessed using the bicinchoninic acid assay. Total enzyme activities of citrate synthase (CS), mitochondrial NADH: coenzyme Q oxidoreductase (Complex I), mitochondrial succinic dehydrogenase (SDH, Complex II) and cytochrome oxidase (COX, Complex IV) were determined (30°C, pH 7.5) using standard spectrophotometric protocols as previously described<sup>53, 54</sup>. Activities were reported as  $\mu\text{mol min}^{-1} / \text{g protein}$  ( $\mu\text{mol min}^{-1}$ , international units, IU).

### **Mitochondrial respiration.**

Respiratory parameters of total mitochondrial populations were studied *in situ* in saponin-permeabilized fibers isolated from soleus and EDL muscles (N = 7 WT, N = 7 KO). We followed the protocol described previously<sup>55, 56</sup> using a Clark electrode (Strathkelvin Instruments, Glasgow, Scotland) in a water-jacketed oxygraphic cell filled with 3 ml of respiration solution containing Complex I substrates (10 mM glutamate + 4 mM malate) at 22°C with continuous stirring. Respiration rates were determined in the absence of ADP (basal respiration,  $V_o$ ) and in the presence of a saturating (2 mM) ADP concentration. Functional activities of different complexes within the mitochondrial respiratory chain were estimated in the presence of succinate + amytal (or Amobarbital) to assess Complex II-supported respiration, and tetramethyl-p-phenylenediamine (TMPD) + ascorbate to assess the Complex IV-supported respiration. Respiration rates were expressed as  $\mu\text{mol O}_2 \text{ min}^{-1} \text{ g}^{-1}$  fiber dry weight.

### **Immunoblotting.**

Frozen soleus and EDL tissue samples from WT and TIEG1 KO mice (N=6) were homogenized (Bertin Precellys 24) in ice cold buffer containing (in mM): HEPES 50 (pH=7.1), KCl, ethylenediaminetetraacetic acid (EDTA),  $\beta$ -glycerophosphate, orthovanadate 1, dithiothreitol 1, sodium fluoride 50, Na pyrophosphate 5, phenylmethylsulfonyl fluoride 0.2 and an anti-protease cocktail (Calbiochem 539134) in the presence of Triton X-100 0.1%. Equal amounts of protein extracts were separated on SDS-polyacrylamide gels (10 to 12%)

and transferred to polyvinylidene difluoride membranes for Western blotting. After 1h of blocking in PBS containing TWEEN20 (0.1%) and nonfat milk (5%), the membranes were incubated overnight at 4°C with primary antibodies (mitochondrial OXPHOS (complex I subunit NDUFB8, complex II subunit CII 30, complex III subunit core 2 and complex V subunit alpha): MS604 (mitosciences), Citrate synthase : ab96600 (Abcam), COX IV: ab14744 (Abcam), MFN2 : H00009927-MO1 (Abnova), PGC-1 $\alpha$  : sc13067 (Santa Cruz)). After washing, the membranes were incubated with secondary antibodies coupled with horseradish peroxidase for 1h at room temperature and visualized using chemiluminescent substrate (Luminata™ Western Chemiluminescent HRP Substrates, Millipore). Light emission was detected by autoradiography and quantified using an image-analysis system (Bio-Rad).

### **In vivo Magnetic Resonance Spectroscopy.**

Twenty four mice were used (N=12 WT and N=12 TIEG1) for magnetic resonance (MR) acquisitions performed on a 9.4 Tesla Biospec 94/21 superconducting magnet (Bruker, Wissembourg, France) with a shielded gradient set (950 mT.m<sup>-1</sup> maximum gradient amplitude). A home built homogeneous scroll double tuned coil (10 mm inner diameter) was developed. The resonance frequencies were 400 MHz for the proton and 162 MHz for the phosphorus. Animals were anesthetized by inhalation of 2.5% isoflurane then maintained during MR experiments at 1.5% (0.5 L.min<sup>-1</sup> mixed in air and oxygen with 1:1 ratio). The physiological body temperature was maintained inside the magnet by circulating warm water. A pressure sensor was used to monitor the respiration cycle and obtain the respiration frequency.

Localized 1H spectroscopy was performed with a PRESS (point resolved spectroscopy) sequence (TE/TR, 16 ms/4000 ms, 256 accumulations, 17min) with VAPOR module (variable pulse power and optimized relaxation delays) for water suppression. A 22 mm<sup>3</sup> voxel was centered on the middle of the hindlimb.

31P spectroscopy was then performed with a PRESS sequence (TR = 1500ms, 1 pulse BP of 100 $\mu$ s 200 accumulations, DS = 2, TA = 5min). Due to the lack of sensitivity of 31P spectroscopy the entire hindlimb was considered for the acquisition (1000mm<sup>3</sup>).

Spectra were analyzed with a homemade (CRMBM, Aix Marseille university) CSIPO software used for quest interface<sup>57</sup> that works in time domain. On a 1H spectrum the assessment of choline, creatine, taurine and extramyocellular lipids was made (Figure 7A). On a 31P spectrum, different metabolites are quantifiable: Inorganic phosphate, phosphocreatine,



ATP $\alpha$ , ATP $\beta$  and ATP $\gamma$  (Figure 8A). Quantification of the area under peaks was performed in arbitrary units (AU). A Mann Whitney statistical test was used to compare those areas between both muscle genotypes.

### **<sup>1</sup>H-NMR Metabolomics.**

The protocol for extraction of metabolites from soleus (N=18) and EDL (N=18) muscles was adapted from the Folch type two-step procedure described by Wu et al.<sup>58</sup> for sample mass < 50 mg. Prior to NMR analysis, dried samples were reconstituted in 220 $\mu$ L of deuterated buffer containing TSP [3-trimethylsilylpropionic acid] at 145 $\mu$ M and transferred to conventional 3mm NMR tubes (CortecNet, Paris, France). <sup>1</sup>H-NMR spectra were obtained with a Bruker DRX-600 AVANCE-III HD spectrometer (Bruker SADIS, Wissembourg, France), operating at 14T, with a TCI cryoprobe. Standard water suppressed <sup>1</sup>H-NMR spectra were acquired at 298K using a “noesypr1d” pulse sequence with relaxation delay of 20s. Spectra were processed using Topspin version 3.2 software (Bruker Daltonik, Karlsruhe, Germany). <sup>1</sup>H-NMR spectra were automatically reduced to ASCII files using AMIX Software package (Analysis of MIXture, version 3.9.14, Bruker Biospin, Karlsruhe, Germany) as previously described<sup>59</sup>. Spectral intensities were scaled to the total spectral intensity and the resulting data (supplementary data S1 A-B) was analyzed by multivariate and univariate statistical analyses.

### **Multivariate and Univariate analysis.**

Principal Component Analyses (PCA) and Partial Least Square Discriminant Analyses (PLS-DA) were performed using SIMCA-P<sup>+</sup> Software (version 13.0, Umetrics, Umeå, Sweden) on the NMR dataset containing soleus and EDL as a function of genotype. All data sets were scaled to unit of variance allowing all metabolites to become equally important. PCA describes the total variability within the dataset and can be used as an informative indicator while the PLS-DA is used to predict the spectral features (metabolites) that define separation between groups (phenotypes)<sup>60</sup>. The overall quality of the models was judged by cumulative  $R^2$  (goodness of fit) and cumulative  $Q^2$  (goodness of prediction). Variable Importance in the Projection (VIP) > 1 were considered as the most contributive in the genotype separation. Student's *t*-tests were performed using MetaboAnalyst<sup>61</sup> for all VIP measures. A *p*-value < 0.05 was considered significant.

Following statistical analysis, heatmaps were generated to visually depict changes in metabolites ( $VIP > 1$  and a  $p\text{-value} < 0.05$ ) as a function of mouse genotype (Supplementary data S1 A-B).

### **Bioinformatic analysis**

The FASTQ files were mapped to the mouse genome (assembly mm9) using TopHat Gapped-read mapper<sup>62</sup> with *--very-sensitive* Bowtie2 settings on Galaxy Platform (version 2.1.0)<sup>63</sup>. The read counting was performed via HTSeq<sup>64</sup> (version 0.6.1galaxy3) with following parameters: `-f bam -r name -s no -a 10 -t exon -m union`. The count files were subsequently subjected for differential analysis using the DESeq2 Package<sup>65</sup> on R (Bioconductor version 3.4.1). The threshold values to determine the differentially expressed genes were as follows:  $\text{abs}(\log_2\text{fold change}) \geq 0.8$  and  $\text{padj} < 0.05$ . The pathway analysis on the regulated genes was performed using the “Enrichr”<sup>66, 67</sup>.

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## **Author contributions**

S.F.B., M.K., J.R.H. and M.S. conceived the project



Y.L.F., M.H.N., F.S., M.K., S.F.B., M.S., J.R.H., S.J., Z.N., L.N.D., S.M., W.M, V.V., J.P., J.M.C. and P.P. designed and performed the experiments.

M.K., S.F.B., M.S., J.R.H., S.J., Z.N., L.N.D., S.M., W.M, V.V., J.P., J.M.C. and P.P. analyzed and interpreted data.

M.K., S.F.B., M.S., J.R.H., S.J., Z.N., V.V., J.P., J.M.C., S.M. and L.N.D. wrote and revised the manuscript.

All authors have read and approved the final manuscript.

## Figure Legends

**Figure 1.** Differential Gene Expression Analysis from TIEG1 KO and WT mice. The sample-to-sample variation of mRNA-Seq replicates was assessed via **A.** Principal Component Analysis (PCA) and **B.** Hierarchical clustering after variance stabilized transformation of sequencing counts. **C.** The volcano plot depicts the significance, i.e.  $-\log_{10}(\text{padj})$  versus the  $\log_2$ fold change (L2FCH) values of all the genes identified through mRNAseq. The upregulated genes, with  $\text{L2FCH} > 0.8$  and  $\text{padj} < 0.05$  are indicated in red, whereas the downregulated genes with  $\text{L2FCH} < -0.8$ ,  $\text{padj} < 0.05$  are shown in green.

**Figure 2.** SDH (**A-B**), COX (**C-D**), menadion (**E-F**), Periodic acid–Schiff (**G-H**), Oil Red O (**I-J**), and Gömöri trichrome (**K-L**) stainings for WT and TIEG1 KO soleus.

**Figure 3.** TEM of longitudinal and transverse sections of the WT (**A,D,G**), soleus TIEG1 KO (**B,E,H**) and EDL TIEG1 KO (**C,F,I**).

**Figure 4.** Mitochondrial enzyme activities.

\* :  $P < 0.05$  ; \*\* :  $P < 0.01$

**Figure 5.** Mitochondrial respiratory activities.

\* :  $P < 0.05$  ; \*\* :  $P < 0.01$  ; \*\*\* :  $P < 0.001$

**Figure 6.** Content of mitochondrial proteins in soleus and EDL muscle.

\*\* :  $P < 0.01$

**Figure 7.** (**A**) In vivo muscle  $^1\text{H}$  spectra.

1: Cre: creatine, 2: Tau: taurine, 3: Emcl: extracellular lipids

(**B\_C**) Quantification of muscle metabolites using  $^1\text{H}$  in vivo spectroscopy.

Cho: choline, Cre: creatine, Tau: taurine, Emcl: extracellular lipids

**Figure 8.** (**A**) In vivo muscle  $^{31}\text{P}$  spectra.

1: Pi: Inorganic phosphate, 2: PCr: Phosphocreatine, 3:  $\text{ATP}\gamma$ , 4:  $\text{ATP}\alpha$ , 5:  $\text{ATP}\beta$

(**B**) Quantification of muscle metabolites using  $^{31}\text{P}$  in vivo spectroscopy.

Ip: inorganic phosphate, PCr: phosphocreatine

\* :  $P < 0.05$  ; \*\* :  $P < 0.01$

**Figure 9.** Principal Component Analysis (PCA) score resulting from modeling the  $^1\text{H}$ -NMR spectral data matrix of WT Soleus (3 spectra, purple circle), TIEG1 KO Soleus (3 spectra, blue

circle), WT EDL (3 spectra, yellow circle) and KO EDL (3 spectra, red circle),  $R^2X(\text{cum}) = 0.74$ .

**Figure 10.** Heatmaps for soleus (**A**) and EDL (**B**) muscles generated from  $^1\text{H-NMR}$  spectral features (metabolites) showing up-regulated (red) and down-regulated (blue) metabolites in the TIEG1 KO and WT mice.