

**Remodeling of calcium handling in skeletal muscle through the peroxisome proliferator-activated receptor  $\gamma$  coactivator 1 $\alpha$  (PGC-1 $\alpha$ ):**

**Impact on force, fatigability and fiber type**

**Serge Summermatter<sup>1</sup>, Raphael Thurnheer<sup>2</sup>, Gesa Santos<sup>1</sup>, Barbara Mosca<sup>3</sup>, Oliver Baum<sup>4</sup>, Susan Treves<sup>2</sup>, Hans Hoppeler<sup>4</sup>, Francesco Zorzato<sup>2,3</sup> and Christoph Handschin<sup>1</sup>**

<sup>1</sup>Biozentrum, Department of Pharmacology/Neurobiology, University of Basel, Klingelbergstrasse 50-70, CH-4056 Basel, Switzerland

<sup>2</sup>Departments of Anesthesia and Biomedicine, Basel University Hospital, Hebelstrasse 20, CH-4031 Basel, Switzerland

<sup>3</sup>Department of Experimental and Diagnostic Medicine, University of Ferrara, Via Borsari 46, 44100 Ferrara, Italy

<sup>4</sup>Institute of Anatomy, University of Bern, Baltzerstrasse 2, CH-3009 Bern, Switzerland,

Running head: Remodeling of muscle calcium handling by PGC-1 $\alpha$

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Address correspondence to: Christoph Handschin, Division of Pharmacology/Neurobiology, Biozentrum, University of Basel, Klingelbergstrasse 50-70, CH-4056 Basel, Switzerland, *Phone*: +41 61 267 2378, *Fax*: +41 61 267 2208, *Email*: [christoph.handschin@unibas.ch](mailto:christoph.handschin@unibas.ch)

**Regular endurance exercise remodels skeletal muscle, largely through the peroxisome proliferator-activated receptor  $\gamma$  coactivator 1 $\alpha$  (PGC-1 $\alpha$ ). PGC-1 $\alpha$  promotes fiber type switching and resistance to fatigue and intracellular calcium levels might play a role in both adaptive phenomena. Using mice with transgenic over-expression of PGC-1 $\alpha$ , we now investigated the effect of PGC-1 $\alpha$  on calcium handling in skeletal muscle.**

**We demonstrate that PGC-1 $\alpha$  induces a quantitative reduction in calcium release from the sarcoplasmic reticulum by diminishing the expression of calcium releasing molecules. Concomitantly, maximal muscle force is reduced *in vivo* and *ex vivo*.**

**In addition, PGC-1 $\alpha$  over-expression delays calcium clearance from the myoplasm by interfering with multiple mechanisms involved in calcium removal, leading to higher myoplasmic calcium levels following contraction. During prolonged muscle activity, the delayed calcium clearance facilitates force production in mice over-expressing PGC-1 $\alpha$ .**

**Our results reveal a novel role of PGC-1 $\alpha$  in altering the contractile properties of skeletal muscle by modulating calcium handling. Overall, our findings suggest that**

**PGC-1 $\alpha$  reduces maximal force, increases resistance to fatigue and drives fiber-type switching partly through remodeling of calcium transients in the adaptation to chronic exercise.**

### **Abbreviations**

CRU	calcium release unit
DHPR	1,4-dihydropyridine receptor
FFDM	fat free dry mass
Jnc	junctin
MICU	mitochondrial calcium uptake
NCX	sodium-calcium exchanger
PLB	phospholamban
PGC-1 $\alpha$	peroxisome proliferator-activated receptor $\gamma$ coactivator 1 $\alpha$
PVA	parvalbumin
RyR1	ryanodine receptor type 1
SERCA	sarcoplasmic/endoplasmic reticulum calcium ATPases
SLN	sarcoplipin
SR	sarcoplasmic reticulum
TBP	tata box binding protein
Tdn	triadin
TR $\alpha$	thyroid hormone receptor $\alpha$

## Introduction

More than six hundred different muscles are present in the human body and every skeletal muscle is composed of a distinct set of heterogeneous muscle fibers with various contractile properties (Bottinelli & Reggiani, 2000; Spangenburg & Booth, 2003). Based on their twitch characteristic, muscle fibers are broadly classified into two major groups: fast-twitch fibers, which are capable of strong, explosive contractions, and slow-twitch fibers, which are suitable for prolonged physical activities (Westerblad *et al.*, 2010).

A cardinal difference between different fiber types is their respective peak amplitude and rate of calcium transients (Baylor & Hollingworth, 2003). Compared to slow-twitch fibers, fast-twitch fibers express higher amounts of proteins involved in calcium release. The voltage sensor DHPR (1,4-dihydropyridine receptor) and the calcium channel ryanodine receptor1 (RyR1) are both abundant in the sarcoplasmic reticulum of fast-twitch muscles (Froemming *et al.*, 2000). Thus, more calcium can be released in response to motor neuron activation (Baylor & Hollingworth, 2003). Once released from the sarcoplasmic reticulum, calcium binds to troponin, thereby pulling away tropomyosin, exposing the myosin binding sites and allowing contraction. A direct quantitative relationship exists between calcium release and force generation (Moss *et al.*, 1985; Metzger & Moss, 1990). Moreover, fast-twitch fibers are endowed with a high amount of Sarcoplasmic/endoplasmic reticulum calcium ATPase 1 (SERCA1), a protein that pumps calcium back into the sarcoplasmic reticulum and with parvalbumin (PVA), a protein that sequesters calcium and enhances calcium re-uptake (Leberer & Pette, 1986; Froemming *et al.*, 2000). The interplay of these proteins allows rapid muscle relaxation. With respect to their amplitude and dynamic of calcium handling, the respective muscle fibers thus clearly differ with fast-twitch fibers displaying much higher peak amplitudes and faster rates of calcium turn-over.

The fiber type composition of muscle displays considerable plasticity even in the adult, differentiated state. In response to certain environmental demands (e.g exercise), skeletal muscle remodels accordingly to adapt its functional capacity (Fluck & Hoppeler, 2003; Bassel-Duby & Olson, 2006). A prominent hallmark of adaptation to chronic endurance exercise is the fiber type switching towards an increased proportion of slow-twitch fibers. The underlying molecular networks are complex and only partially elucidated. An interplay of

various, independent signaling pathways mediate exercise adaptation, many of which seem to converge ultimately on common key molecules where these signals are integrated (Bassel-Duby & Olson, 2006; Finck & Kelly, 2006). The peroxisome proliferator-activated receptor  $\gamma$  coactivator 1 $\alpha$  (PGC-1 $\alpha$ ) represents such a systemic hub in muscle plasticity (Lin *et al.*, 2002; Bassel-Duby & Olson, 2006; Handschin & Spiegelman, 2006, 2008).

The central importance of PGC-1 $\alpha$  in exercise adaptation is impressively exemplified by studies using PGC-1 $\alpha$  transgenic mice. Muscle-specific over-expression of PGC-1 $\alpha$ , even in the absence of physical activity, is sufficient to drive changes that are typical of endurance training. PGC-1 $\alpha$  increases the oxidative capacity by promoting mitochondrial biogenesis (Lin *et al.*, 2005), it improves oxygen supply to muscle by promoting angiogenesis (Arany *et al.*, 2008), increases peak oxygen consumption (Calvo *et al.*, 2008), lipid oxidation and energy refueling (Summermatter *et al.*, 2010). Most importantly, PGC-1 $\alpha$  has been shown to drive fiber-type switching from fast, glycolytic towards slow, oxidative fibers as defined by changes in myosin heavy chain composition and metabolic parameters (Lin *et al.*, 2002). Accordingly, muscle-specific PGC-1 $\alpha$  transgenic mice exhibit an increased endurance capacity in treadmill exercise tests (Calvo *et al.*, 2008).

Whether PGC-1 $\alpha$  changes calcium handling cell-autonomously in skeletal muscle is currently not known. Thus, we investigated whether PGC-1 $\alpha$  modulates calcium levels, whether it quantitatively and/or qualitatively affects force generation in skeletal muscle and which mechanisms might underlie such changes. To address these issues, we used a mouse model with physiological over-expression of PGC-1 $\alpha$  that is limited to skeletal muscle (Lin *et al.*, 2002).

## Materials and Methods

### ***Ethical approval***

All studies were performed according to criteria outlined for the care and use of laboratory animals and with approval of the Swiss authorities. MPGC-1 $\alpha$  TG mice (Lin *et al.*, 2002) and control littermates were maintained according to institutional guidelines in a conventional facility with a fixed 12-h light/dark cycle on a commercial pellet chow diet and free access to tap water.

### ***In-vivo and ex-vivo muscle strength assessment***

Maximal force was tested *in vivo* using a grip strength meter (Chatillon, DFE Series Digital Force Gauge). To test force *ex vivo*, EDL muscles were dissected and mounted into a muscle testing setup (Heidelberg Scientific Instruments). Muscle force was digitized at 4 kHz by using an AD Instruments converter. EDL tetanus was recorded in response to 400-ms pulses at 100 Hz. Specific force was normalized to the muscle cross-sectional area [CSA = wet weight (mg)/length (mm)  $\times$  1.06 (density mg/mm<sup>3</sup>)] (Delbono *et al.*, 2007). To test resistance to fatigue, repeated tetani were recorded in response to 350ms pulses at 100 Hz with intervals of 3.65secs (Gonzalez & Delbono, 2001).

### ***Electron microscopy***

Electron microscopy to determine muscle composition was performed as described previously (Hoppeler *et al.*, 1985). Glutaraldehyde-fixed samples were dehydrated in increasing ethanol concentrations and embedded in Epon. After cutting ultrathin sections (50–70 nm) on a LKB Ultratome III, uranyl acetate and lead citrate staining was performed. The sections were then photographed and examined. We analyzed 15 micrographs of one section per animal.

### ***RNA extraction and RT-PCR***

Frozen tissues were homogenized under liquid nitrogen and total RNA was isolated using Trizol reagent (Invitrogen). RNA concentrations were adjusted and reverse transcription was carried out using random hexamer primers (Promega). Real-time PCR analysis (Power SYBR Green Master Mix, Applied Biosystems) was performed using the ABI Prism 7000 Sequence Detector. Relative expression levels for each gene of interest were calculated with the  $\Delta\Delta C_t$

method and normalized to the expression of the Tata box binding protein (TBP). Primer sequences are listed in *Supplementary Table 1*.

### ***Body composition***

Body composition was determined with an EchoMRI qNMR (Echo Medical Systems).

### ***Calcium transients***

Flexor digitorum brevis (FDB) muscles were enzymatically dissociated at 37°C for 60 min in an incubator for cell culture in Tyrode's solution containing 0.20% collagenase I (Sigma C0130-16). The muscles were rinsed in DMEM 10% FCS, transferred to DMEM and mechanically dissociated with fire polished pasteur pipettes. The dissociated fibers were placed on glass coverslips previously coated with 1.5 microliter laminin (1 mg/ml) (Invitrogen cat. 23017-015). FDB fibers were loaded for 20 min at 20°C in Tyrode's solution ( NaCl 137 mM, KCl 5.4mM, MgCl<sub>2</sub> 0.5mM, CaCl<sub>2</sub> 1.8mM, HEPES-NaOH 11.8mM pH 7.4, Glucose 0.1%) containing 10 μM Mag-Fluo-4-AM and 50 μM BTS (4-Methyl-N-(phenylmethyl)benzenesulphonamide), and calcium transients was triggered by field stimulation with a 40 V pulse of 0.5 msec duration and tetanic stimulation at 100Hz and 300ms train pulse duration (Hollingworth *et al.*, 2009; Calderon *et al.*, 2010). Fluorescent signals were recorded with a Nikon EclipseTE2000-U fluorescent microscope equipped with a P101 photomultiplier and digitized at 10 kHz. Calcium transients were calculated as  $(F_{\max} - F_{\text{rest}})/F_{\text{rest}}$

### ***SERCA activity***

SERCA activity was determined as described by Simonides and Hardeveld (Simonides & van Hardeveld, 1990). In brief, fresh muscle homogenates were incubated in buffer A (1mM EGTA, 10mM phosphoenolpyruvate, 18 U/ml each of pyruvate kinase and lactate dehydrogenase, 0.2mM NADH, 20mM Hepes, pH 7.5) supplemented with 200mM KCl, 15mM MgCl<sub>2</sub>, 200mM NaN<sub>3</sub> and Triton X-100 (0.005%). The assay was started by addition of 4mM MgATP followed by calcium at various concentrations.

### ***Western blotting***

Protein extraction was performed as described previously (Summermatter *et al.*, 2008). In brief, frozen tissues were crushed under liquid nitrogen, homogenized in lysis buffer (20 mM Tris-HCl, 138 mM NaCl, 2.7 mM KCl, 5% (v/v) glycerol, 1% (v/v) NP-40, and various

hydrolase inhibitors) and incubated for 60 min. After centrifugation at 12 000 rpm for 15 min, protein concentration in the supernatant was quantified and equal amounts of protein extracts were separated by SDS-PAGE. The gels were then blotted on PVDF membranes and analyzed with the following antibodies: calsequestrin 1 and 2 (Sigma), SERCA 1, 2 and tubulin (Cell Signalling), thyroid hormone receptor  $\alpha$  (TR $\alpha$ ) (Abcam), sarcolipin, phospholamban and phospho-phospholamban (Thr17) (Santa Cruz Biotechnology).

#### ***Data analysis and statistics***

All data are presented as means  $\pm$  SE. The data were analyzed by 2-tailed, unpaired Student's *t* test or Mann-Whitney test when the difference between the two SDs was significantly different. Levels of significance are indicated as follows: \*\*\*  $p < 0.001$ , \*\*  $p < 0.01$ , \*  $p < 0.05$ .

## Results

### ***Reduced muscle strength, but unchanged muscle mass in MPGC-1 $\alpha$ TG mice***

PGC-1 $\alpha$  drives the formation of slow-twitch, oxidative fibers (Lin *et al.*, 2002). However, it is unresolved whether characteristic features of fast-twitch fibers are lost upon PGC-1 $\alpha$ -induced fiber-type transition. We thus first tested whether PGC-1 $\alpha$ -induced fiber type switching affects muscle strength *in vivo*. MPGC-1 $\alpha$  TG mice generated less force (fore limbs (-12%,  $p < 0.05$ ) (Fig. 1A) and hind limbs (-15%,  $p < 0.01$ ) (Fig. 1B)) than control animals in the absence of alterations in body weight (Fig. 1C) or fat free dry mass (Fig. 1D).

To scrutinize further the nature of the reduced muscle force generation, the experiments were extended to intact muscle preparations *ex vivo*. Isolated fast-twitch EDL muscle of MPGC-1 $\alpha$  TG mice showed reduced absolute (-25%,  $p < 0.001$ ) (Fig. 2A) and specific (-25%,  $p < 0.01$ ) (Fig. 2B) tetanic force compared to their control littermates. EDL weight (Fig. 2C) and length (Fig. 2D) were not different between control and transgenic animals, indicating that the reduced force was not due to loss in muscle mass. Despite the lower force in MPGC-1 $\alpha$  TG mice, kinetics of muscle force contraction (Fig. 2E) and relaxation (Fig. 2F) in response to tetanic stimulation remained similar in both groups.

### ***Altered muscle morphology in EDL of MPGC-1 $\alpha$ TG mice***

Since muscle mass was equal between transgenic and control mice, we speculated that the reduced force generation could conceivably result from altered muscle morphology. To test whether structural changes account for the reduced force, the composition of EDL muscle was determined by electron microscopy. Due to the over-expression of PGC-1 $\alpha$ , transgenic mice showed elevated mitochondrial mass (+187%,  $p < 0.001$ ), at the expense of total myofibrillar structures, which were reduced by 20% ( $p < 0.001$ ) (Fig. 3A).

Detailed quantitative analyses revealed that both mitochondrial sub-populations were elevated to a similar extent, the centrally located intramyofibrillar mitochondria, which are in close contact to the sarcoplasmic reticulum, by +198% ( $P < 0.001$ ) and the subsarcolemmal mitochondria by +175% ( $P < 0.01$ ) (Fig. 3B).



### ***Capacity for calcium release is diminished in EDL of MPGC-1 $\alpha$ TG mice***

Calcium release from the sarcoplasmic reticulum is an important determinant of muscle force generation. The skeletal muscle L-type Ca<sup>2+</sup> channel (1,4-dihydropyridine receptor; DHPR) serves as the voltage sensor for excitation-contraction (EC) coupling and activates Ca<sup>2+</sup> release from the sarcoplasmic reticulum via the type 1 ryanodine receptor (RyR1) (Sheridan *et al.*, 2006). RyR1 entertains a complex network with regulatory proteins, such as calsequestrin, triadin and junctin (Beard *et al.*, 2009).

While the mRNA expression of DHPR $\alpha$ 1s was similar between wild-type and transgenic animals, the mRNA expression levels of RyR1, triadin and junctin were reduced in EDL muscles of transgenic animals (Fig. 4A).

In addition, the mRNA expression of calsequestrin 1 was reduced, while calsequestrin 2 was elevated (Fig.4B and C, respectively). The same pattern was observed at the protein level (Supplementary Fig.1). This is in line with the fiber type switching towards oxidative fibers in the MPGC-1 $\alpha$  TG animals, as calsequestrin 1 and 2 are specific for fast-twitch and slow-twitch fibers, respectively (Beard *et al.*, 2009).

To test functionally whether reduced expression of the calcium release machinery impairs calcium release from the SR, single fibers were isolated from FDB muscle and stimulated electrically. The kinetic of the calcium rise was unaltered in the transgenic animals (Fig.4D). However, quantitatively less calcium was released into the myoplasm in transgenic animals compared to controls (Fig. 4E and Supplementary Fig. 2).

### ***Improved resistance to fatigue in EDL of MPGC-1 $\alpha$ TG mice***

Interestingly, when muscles of MPGC-1 $\alpha$  TG mice and control littermates were repeatedly stimulated, they showed different temporal development of force generation. The first tetanic stimulation generated less force in transgenic animals than in their control littermates. However, following repeated tetanic stimulation, transgenic animals displayed higher force generation than wild type animals over time (Fig. 5) and were therefore more resistant to fatigue, similar to previously published data that demonstrated an increased time of stimulation in muscles of MPGC-1 $\alpha$  TG animals until force generation dropped to 30% (Lin *et al.*, 2002).

### ***Calcium reuptake capacity is diminished in EDL of MPGC-1 $\alpha$ TG mice***

A possible explanation for the relative higher tetanic force in transgenic animals in response to repeated tetani could reside within a delayed decay of the calcium transient. Reduced calcium reuptake into the sarcoplasmic reticulum has been demonstrated in fatigue-resistant endurance-trained athletes (Li *et al.*, 2002). Sarcoplasmic/endoplasmic reticulum calcium ATPases (SERCAs) are largely responsible for post-contraction calcium clearance. In adult skeletal muscle, two major isoforms of SERCA exist: SERCA1, which is very abundant in glycolytic muscle and SERCA2, which has generally a low expression in all muscles, but which is the predominant isoform in slow oxidative fibers (Beard *et al.*, 2009).

EDL muscle over-expressing PGC-1 $\alpha$  showed reduced mRNA levels of SERCA1 (Fig. 6A), but unaltered levels of SERCA2 (Fig. 6B). These results were confirmed at the protein level (Supplementary Fig.1). Total SERCA activity was significantly reduced in transgenic animals (Figure 6C). In line with this finding, the half relaxation time of the calcium transient was increased in transgenic animals (Fig. 6D).

Besides calcium re-uptake by the sarcoplasmic reticulum, calcium binding to the buffering molecule parvalbumin (Leberer & Pette, 1986), mitochondrial calcium uptake through MICU1 (Perocchi *et al.*, 2010) and calcium export by sodium-calcium exchangers (NCX) (Bers & Bridge, 1989) are involved in muscle relaxation. Parvalbumin expression was significantly reduced in PGC-1 transgenic animals (Fig. 6E), while MICU1 was unaltered (Fig. 6F). Moreover, NCX levels were significantly reduced in MPGC-1 $\alpha$  TG mice (Fig. 6G).

### ***Potential inhibitory mechanisms of SERCA in MPGC-1 $\alpha$ TG mice***

The expression of SERCA is regulated by thyroid hormones (Simonides & van Hardeveld, 2008). We thus tested whether thyroid hormone receptor expression is altered in skeletal muscle of PGC-1 transgenic mice. We observed a significant reduction in thyroid hormone receptor mRNA expression in transgenic mice (Fig. 7A). Moreover, protein levels of the thyroid hormone receptor were reduced (Fig 7A). Finally, we tested the expression of inhibitors of SERCA activity. Relative mRNA and protein levels of sarcolipin were elevated in MPGC-1 $\alpha$  TG mice (Fig.7B). Furthermore, we found elevated mRNA and protein levels of phospholamban (un-phosphorylated and phosphorylated) in MPGC-1 $\alpha$  TG mice (Fig.7C).

## Discussion

Muscle fibers are distinguished based on their physiological features, such as appearance (red *vs.* white), predominant myosin heavy chain (MHC) isoform (MHC I and IIA *vs.* MHC IIB and X), metabolic parameters (oxidative *vs.* glycolytic) or contractile properties (slow- *vs.* fast-twitching) (Spangenburg & Booth, 2003; Bassel-Duby & Olson, 2006; Baar, 2010; Booth *et al.*, 2010; Handschin *et al.*, 2010). The latter concept is inextricably linked to calcium handling. The speed of calcium release, clearance and its peak amplitude determine the characteristic of the twitch (Baylor & Hollingworth, 2003; Trinh & Lamb, 2006).

Interconversion of different fiber types can occur due to the high plasticity of skeletal muscle. PGC-1 $\alpha$  confers a reddish appearance to skeletal muscle, increases slow MHC isoform expression and metabolically promotes a more oxidative phenotype (Lin *et al.*, 2002). We have now shown that PGC-1 $\alpha$  remodels calcium release by reducing the levels of several components of the sarcoplasmic reticulum calcium channel complex (namely RyR1, triadin and junctin). The ensuing lower concentration of calcium in combination with a decrease in contractile elements culminates in a diminished maximal force generation. Furthermore, PGC-1 $\alpha$  prolongs myoplasmic calcium transients by impairing SERCA expression and activity, thereby inhibiting calcium reuptake into the SR, by decreasing the levels of the cytosolic calcium buffer parvalbumin, and by reducing NCX levels and hence calcium export from the muscle fiber.

In contrast, the levels of the mitochondrial calcium importer MICU1 remains unaltered. Given the increase in mitochondrial mass, relatively lower calcium importers per mitochondria occur in MPGC-1 $\alpha$  TG mice.

Taken together, our findings demonstrate that PGC-1 $\alpha$  slows down calcium handling in skeletal muscle. Altered calcium transients secondarily influence force generation, fatigability and fiber type switching (Fig. 8).

Importantly, while PGC-1 $\alpha$  regulates the expression of post-synaptic genes in skeletal muscle (Handschin *et al.*, 2007), the modulation of calcium signaling by PGC-1 $\alpha$  is at least in part exerted in a cell autonomous manner. Thus, we observed altered calcium transients following electrical stimulation *ex vivo* in the absence of a motor neuron. Moreover, expression of the voltage dependent sensor DHPR that connects motor neuron activity with calcium release is

unaltered in MPGC-1 $\alpha$  TG mice. Thus, muscle PGC-1 $\alpha$  *per se* is able to change calcium handling in skeletal muscle.

PGC-1 $\alpha$  strongly promotes mitochondrial biogenesis in skeletal muscle and other tissues (Lin *et al.*, 2002). We now demonstrate that subsarcolemmal and intermyofibrillar mitochondria are both elevated to a similar extent. Intermyofibrillar mitochondria are often positioned adjacent to calcium release units (CRUs) and are even tethered to them (Vendelin *et al.*, 2005; Boncompagni *et al.*, 2009). This proximity facilitates the communication between mitochondria and sarcoplasmic reticulum (Dirksen, 2009). Emerging evidence suggest that mitochondria exert an inhibitory action on local SR calcium release presumably by controlling the local redox environment of CRUs (Isaeva & Shirokova, 2003; Isaeva *et al.*, 2005; Martins *et al.*, 2008). Support for this hypothesis derives from studies on isolated slow and fast twitch fibers. Mitochondria-rich slow-twitch fibers display diminished local SR calcium release compared to mitochondria-poor fast-twitch fibers (Isaeva & Shirokova, 2003; Isaeva *et al.*, 2005).

SERCA accounts for the majority of calcium removal in skeletal muscle and its expression is regulated by thyroid hormones (Simonides & van Hardeveld, 2008). We now show that PGC-1 $\alpha$  diminishes the levels of thyroid hormone receptor in skeletal muscle and thereby reduces SERCA transcription. Moreover, it has been demonstrated that calcium represses thyroid hormone dependent transcription of SERCA1 (Thelen *et al.*, 1997). The coordinated effect of reduced thyroid hormone receptor and sustained elevated myoplasmic calcium levels through PGC-1 $\alpha$  thus explains the transcriptional reduction in SERCA1. In addition, we observed elevated mRNA and protein levels of sarcolipin in MPGC-1 $\alpha$  TG mice. Sarcolipin inhibits SERCA activity and mice that over-express sarcolipin in muscle are resistant to fatigue but have weaker muscles compared to their control littermates (Tupling *et al.*, 2002), a phenotype that is remarkably similar to the PGC-1 $\alpha$  muscle-specific transgenic animals in that regard. Similarly, we found elevated mRNA and protein levels of the SERCA inhibitor phospholamban. Phospholamban is mainly expressed in slow-twitch muscle, where it interacts with SERCA2 and thereby inhibits calcium reuptake into the sarcoplasmic reticulum. Interestingly, the inhibitory effect of phospholamban can be amplified by sarcolipin (Asahi *et al.*, 2002). Paradoxically, the levels of phosphorylated phospholamban were simultaneously increased. Although the quantitative contribution of the different

inhibitors remains unclear, their interplay clearly results in inhibition of total SERCA activity and delayed calcium removal from the myoplasm.

Taken together, PGC-1 $\alpha$  interferes with multiple mechanisms that can lower myoplasmic calcium levels post contraction, but mainly affects SERCA expression and activity. Overall, PGC-1 $\alpha$  slows down calcium removal and thus induces features of calcium handling in fast-twitch fibers that are reminiscent of fatigue-resistant slow-twitch fibers. How PGC-1 $\alpha$  reduces the expression of these genes mechanistically remains unclear.

Beyond its key role in excitation-contraction coupling, calcium could be implicated in fiber type switching. Inhibition of the calcium release channel is crucial for fiber type transformation since RyR activity in fast muscle fibers contributes to the repression of slow muscle specific genes (Jordan *et al.*, 2004). Furthermore, calcium is involved in transcriptional regulation through calcium dependent enzymes such as calcineurin and calmodulin-dependent kinases. In adult mouse skeletal muscle fibers, the concerted action of the two pathways is required to accomplish a fast-to-slow fiber type transformation (Mu *et al.*, 2007). Our results suggest that modified calcium handling in PGC-1 $\alpha$  muscle-specific transgenic animals could relieve the repression on slow muscle specific genes and support sustained activation of calcium dependent enzymes that drive fiber type switching. Interestingly, PGC-1 $\alpha$  thus seems to be downstream and upstream of calcium signaling in muscle: the induction of PGC-1 $\alpha$  gene expression in endurance exercise is mediated to a large extent by calcium signaling (Handschin *et al.*, 2003) and in turn, PGC-1 $\alpha$  promotes a slow fiber type-specific calcium handling in muscle.

In conclusion, we have provided the first evidence that PGC-1 $\alpha$  drives changes in muscular calcium handling by reducing calcium release and clearance. Decreased calcium release accounts for reduced maximal force and relieves the inhibition of slow muscle specific genes, while diminished calcium clearance promotes resistance to fatigue and fiber type-switching. Our data suggest that for fiber type switching, a minimal amount of physical activity remains a prerequisite. PGC-1 $\alpha$  alters calcium handling capacity, but elevated calcium levels, which support fiber type switching, can be achieved only in response to contraction. Our findings provide new physiological insights into the role of PGC-1 $\alpha$  in skeletal muscle adaptation.

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#### **Author contributions**

C.H. and S.S. conceived and designed the experiments. R.T., G.S., B.M., O.B., F.Z. and S.S. performed the experiments. B.M., O.B., H.H. and S.S. analyzed data. C.H. and S.S. wrote the paper. O.B., S.T., H.H. and F.Z. revised the paper critically for important intellectual content. All authors approved the final version of the manuscript.

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**Conflict of interest:** The authors have no conflict of interest.

## Figures and legends

### **Figure 1. Reduced maximal force in MPGC-1 $\alpha$ TG animals.**

(A and B) Maximal force of front legs (A) and hind legs (B) in MPGC-1 $\alpha$  TG and control mice.

(C and D) Total body weight (C) and FFDM (D) in MPGC-1 $\alpha$  TG and control mice. Abbreviations: FFDM, fat-free dry mass. All values are expressed as means  $\pm$ SE (n =7 per group); \* p<0.05; \*\* p<0.01

### **Figure 2. Reduced tetanic force, but unaltered kinetic of force generation in EDL muscle of MPGC-1 $\alpha$ TG animals.**

(A and B) Absolute (A) and relative tetanic force (B) in EDL muscle of MPGC-1 $\alpha$  TG and control mice in response to 100 Hz stimulation *ex vivo*.

(C and D) Muscle weight (C) and length (D) of EDL muscle of MPGC-1 $\alpha$  TG and control mice.

(E and F) Half contraction time (E) and half relaxation time (F) of EDL muscle of MPGC-1 $\alpha$  TG and control mice following tetanic stimulation at 100 Hz.

All values are expressed as means  $\pm$ SE (n =8 per group); \*\* p<0.01; \*\*\* p<0.001

### **Figure 3. Elevated subsarcolemmal and intermyofibrillar mitochondria, but diminished myofibrillar structures in EDL muscle of MPGC-1 $\alpha$ TG animals.**

(A) Quantification of morphometrical analysis of EDL muscle of MPGC-1 $\alpha$  TG and control mice.

(B) Detailed quantification of different mitochondrial subpopulations.

(C) Representative micrographs from electron microscopy of EDL muscle of MPGC-1 $\alpha$  TG and control mice. Arrows indicate intermyofibrillar mitochondria and arrowheads indicate subsarcolemmal mitochondria.

All values are expressed as means  $\pm$ SE (n =8 per group); \*\* p<0.01; \*\*\* p<0.001

**Figure 4. Decreased calcium release in muscle of MPGC-1 $\alpha$  TG animals.**

(A) Relative expression of genes involved in calcium release from the sarcoplasmic reticulum in muscle of MPGC-1 $\alpha$  TG and control mice.

(B and C) Relative mRNA expression of fast-twitch specific calsequestrin 1 (B) and slow-twitch specific calsequestrin 2 (C).

(D) Half rise time of myoplasmic calcium in response to electrical stimulation.

(E) Peak amplitude of calcium in muscle of MPGC-1 $\alpha$  TG and control mice.

Abbreviations: RyR1, ryanodine receptor1; DHPR, 1,4-dihydropyridine receptor; Tdn, triadin; Jnc, junctin.

All values are expressed as means  $\pm$ SE (n =8 per group); \*\* p<0.01; \*\*\* p<0.001

**Figure 5. Resistance to fatigue in MPGC-1 $\alpha$  TG animals.**

Absolute force of EDL muscle from MPGC-1 $\alpha$  TG and control mice in response to repeated tetanic stimulation at 100 Hz.

All values are expressed as means  $\pm$ SE (n =8 per group); \*\* p<0.01; \*\*\* p<0.001

**Figure 6. Diminished calcium removal in muscle of MPGC-1 $\alpha$  TG animals.**

(A and B) Relative mRNA expression of SERCA1 (A) and SERCA2 (B) in muscle of MPGC-1 $\alpha$  TG and control mice.

(C) Total SERCA activity in skeletal muscle of MPGC-1 $\alpha$  TG and control mice.

(D) Half decay time of calcium following electrical stimulation *ex vivo*.

(E) Relative expression of genes involved in calcium removal in muscle of MPGC-1 $\alpha$  TG and control mice. Abbreviations: SERCA, Sarcoplasmic/endoplasmic reticulum calcium ATPases; PVA, parvalbumin; MICU1, mitochondrial calcium uptake1; NCX, sodium-calcium exchanger.

All values are expressed as means  $\pm$ SE (n =8 per group); \*\* p<0.01; \*\*\* p<0.001

**Figure 7. Mechanisms of SERCA inhibition.**

(A) Relative mRNA expression and protein levels of thyroid hormone receptor in muscle of MPGC-1 $\alpha$  TG and control mice.

(B) Relative mRNA expression and protein levels of sarcolipin in muscle of MPGC-1 $\alpha$  TG and control mice.

(C) Relative mRNA expression, protein levels and phosphorylation of phospholamban in muscle of MPGC-1 $\alpha$  TG and control mice

Abbreviations: TR, thyroid hormone; SLN, sarcolipin; PLB, phospholamban.

All values are expressed as means  $\pm$ SE (n =8-10 per group); \* p<0.05

### **Figure 8. PGC-1 $\alpha$ slows down calcium handling in skeletal muscle.**

This model integrates the findings of the present study. PGC-1 $\alpha$  promotes mitochondrial biogenesis and exerts an inhibitory effect on the CRU, which consists of RyR1, Tdn, Jnc and calsequestrin. Subsequently lower levels of calcium are released from the sarcoplasmic reticulum and force generation is altered. Moreover, NCX, PVA and SERCA1, which are responsible for post-contraction calcium removal, are reduced in MPGC-1 $\alpha$  TG mice. Concomitantly the levels of SERCA inhibitors (SLN and PLB) are elevated. Thus calcium transients in the cytoplasm are slowed down and can influence muscle fatigability and fiber type switching.

Abbreviations: NCX, sodium-calcium exchanger; DHPR, 1,4-dihydropyridine receptor; PVA, parvalbumin; Ryr1, ryanodine receptor1; Tdn, triadin; Jnc, junctin; CRU, calcium releasing unit, MICU1, mitochondrial calcium uptake1; SERCA, Sarcoplasmic/endoplasmic reticulum calcium ATPases, SLN, sarcolipin; PLB, phospholamban.

### **Supplementary Figure 1: Western blots**

Western blot analyses of Calsequestrin 1 and 2, SERCA 1 and 2, and Tubulin in wt (left) and MPGC-1 $\alpha$  TG mice (right)

### **Supplementary Figure 2: Calcium trace in isolated FDB fibers**

Representative calcium trace in FDB fiber isolated from wt (black) and MPGC-1 $\alpha$  TG (red) animals.

### **Supplementary Table 1: Primer list**

Fig 1

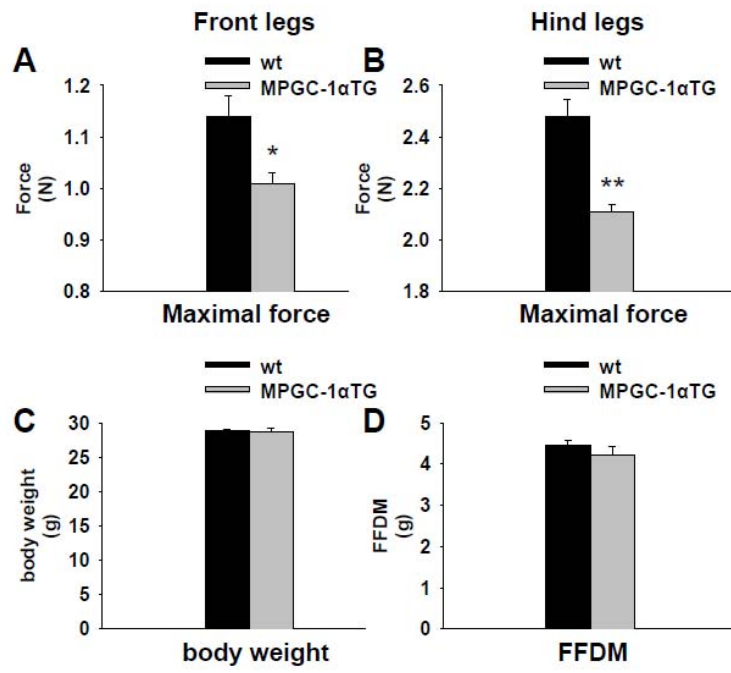
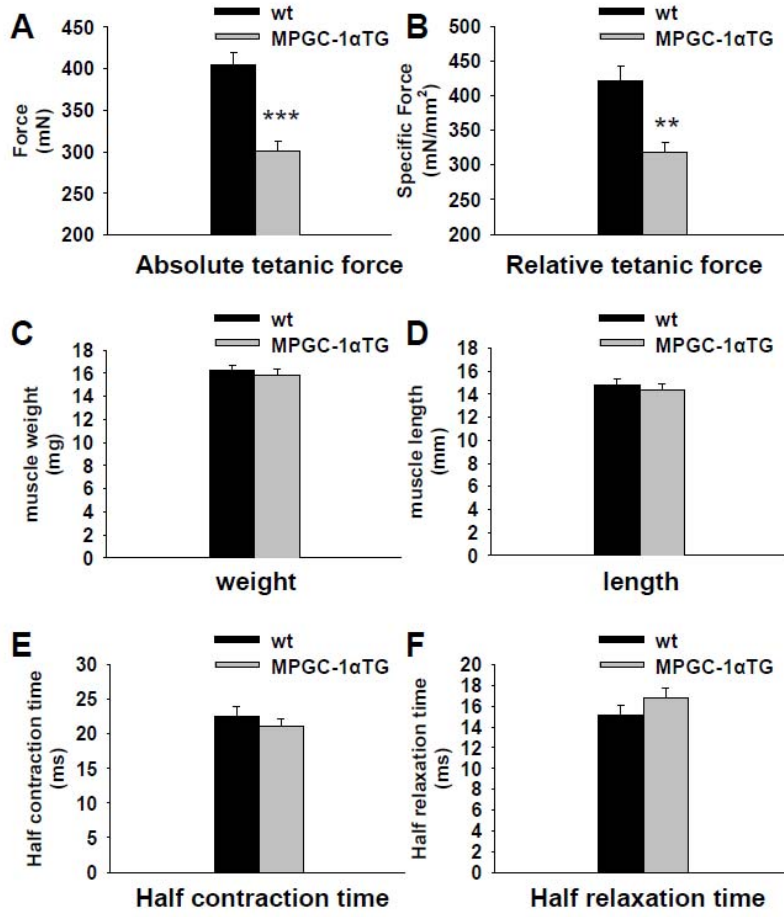
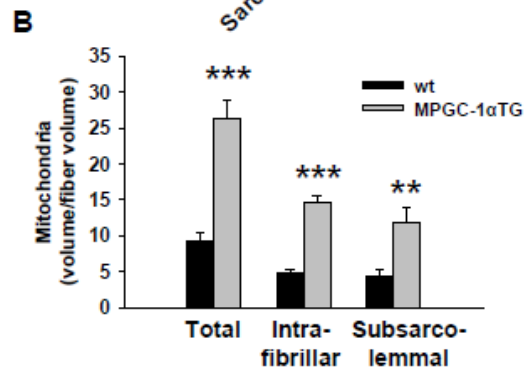
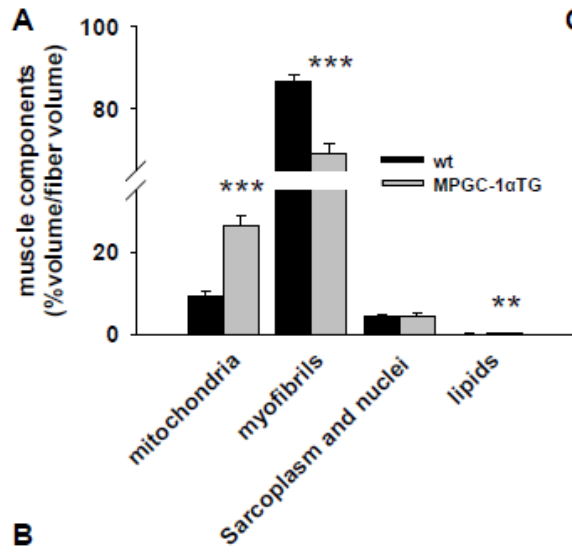


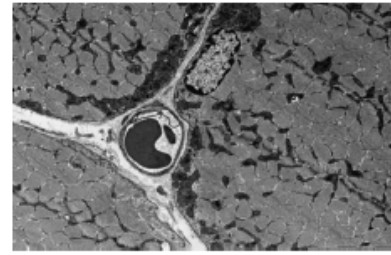


Fig 2

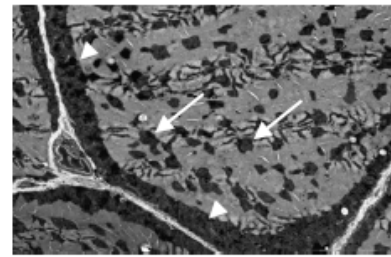




**C** Fig 3



wt



MPGC-1αTG

Fig 4

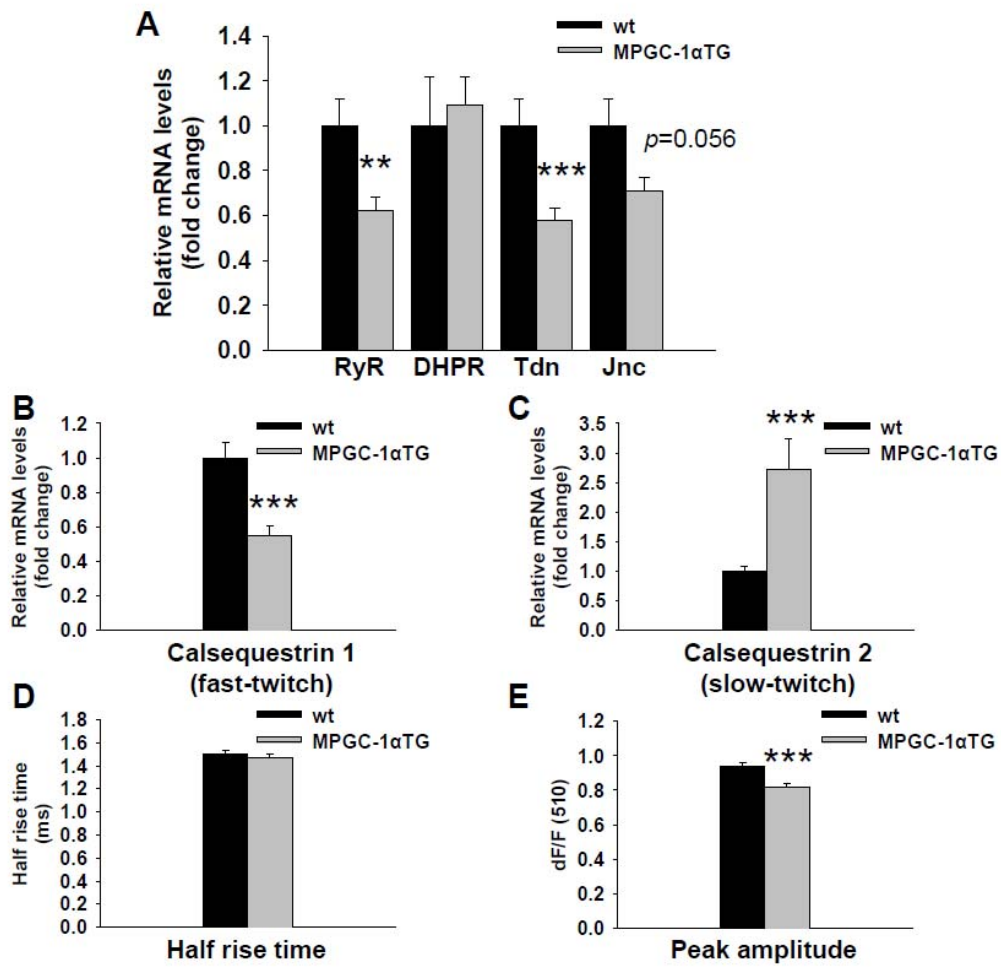


Fig 5

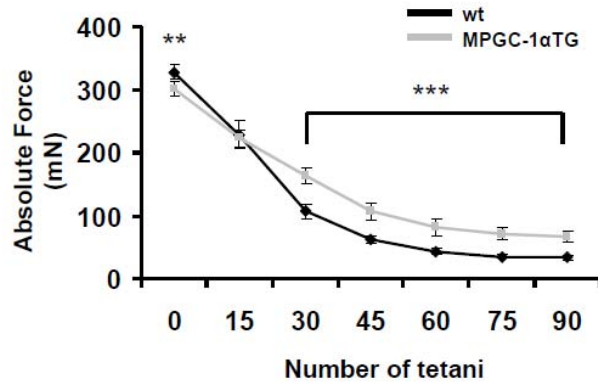


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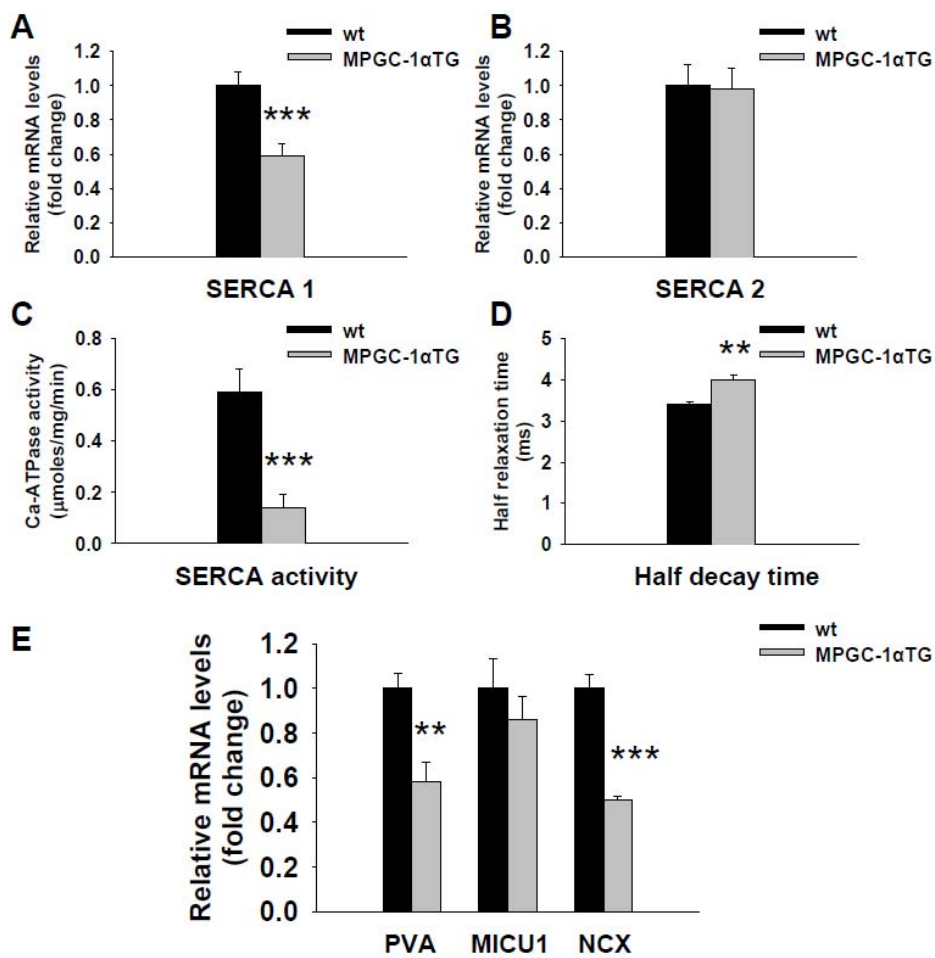


Fig 7

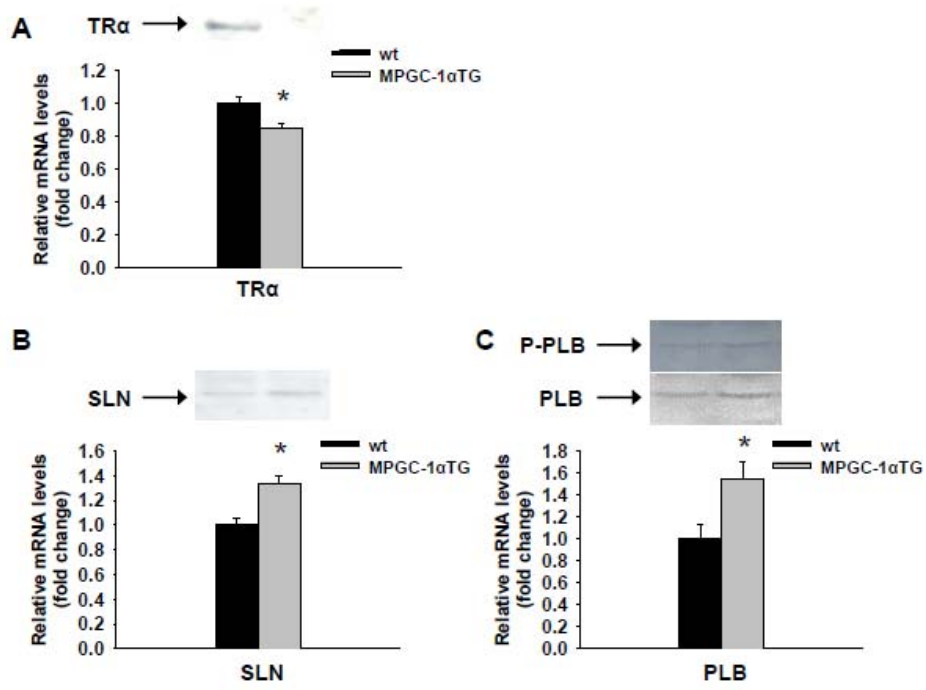
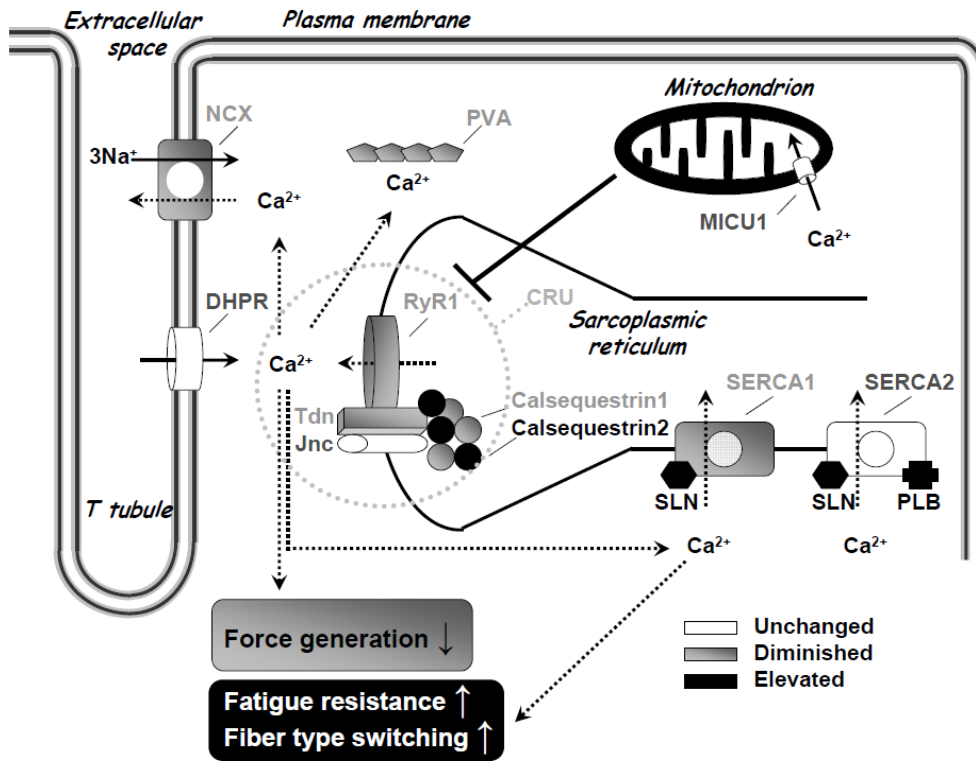
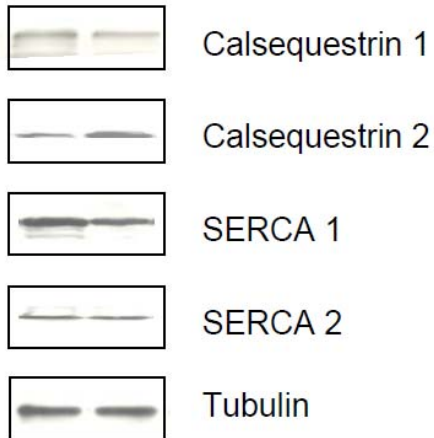


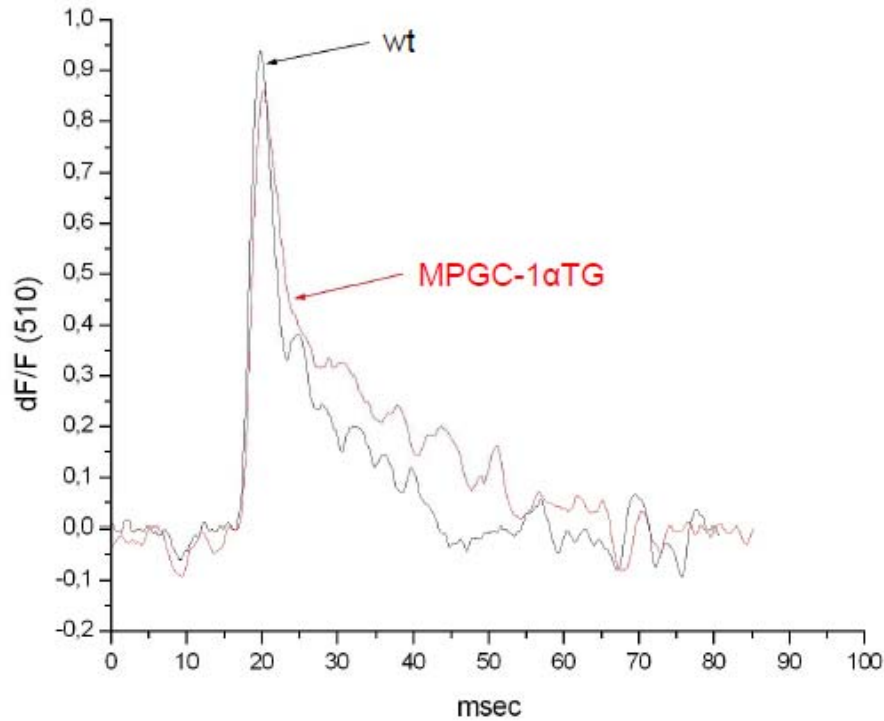
Fig 8



Supplementary Fig 1



Supplementary Fig 2



Supplementary Table 1

<i>mRyR1</i>	GCACACAGTCGTATGTACCTG	CCTCCCCTGTTGCGTCTTC
<i>mTBP</i>	ATATAATCCCAAGCGATTTGC	GTCCGTGGCTCTTATTCTC
<i>mDHPR</i>	TCAGCATCGTGGAAATGGAAAC	GTTCCAGAGTGTGTTGTCATCCT
<i>mTdn</i>	ATGACTGAGATCACTGCTGAAGG	ATGTTGTCACAATGTCTTCGGT
<i>mJnc</i>	CAGCTCTGACAAGAGTTCCAAG	CTGGCAGCCACTTTACTAGAAA
<i>mCalsequestrin1</i>	ACTCAGAGAAGGATGCAGCT	CTCTACAGGGTCTTCTAGGA
<i>mCalsequestrin2</i>	AGCTTGTGGAGTTTGTGAAG	GGATTGTCAGTGTGTGCC
<i>mSERCA 1</i>	AGCCAGTGATGGAGAACTCG	CACCACCAACCAGATGTCAG
<i>mSERCA 2</i>	GAGAACGCTCACACAAAGACC	CAATTCGTTGGAGCCCCAT
<i>mParvalbumin</i>	ATCAAGAAGGCGATAGGAGCC	GGCCAGAAGCGTCTTTGTT
<i>mMICU1</i>	ACACCCTCAAGTCTGGCTTAT	TTCCCATCTTTGAAGTGCTTCT
<i>mNCX</i>	CTTCCCTGTTTGTGCTCCTGT	AGAAGCCCTTTATGTGGCAGTA
<i>mTRa</i>	TTCTCTCCTTCTCCATCCTT	GGCTGGAGGGTCTGAGGG
<i>mSLN</i>	TGTGCCCCTGCTCCTCTTC	TGATTGCACACCAAGGCTTG
<i>mPLB</i>	ATGACGACGATTCAAATCTCTGG	TGGGTTTGCAAAGTTAGGCATAA