**Cell Metabolism**

Lack of Glycogenin Causes Glycogen Accumulation and Muscle Function Impairment

**Graphical Abstract**

**Highlights**
- Glycogen synthesis does not require a protein primer
- Glycogenin depletion causes high glycogen accumulation in striated muscles
- Glycogenin depletion alters skeletal muscle functionality
- Over-accumulation of skeletal muscle glycogen affects oxidative metabolism

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**In Brief**
Although glycogenin is thought to be essential for glycogen synthesis, Testoni et al. show that glycogenin-deficient animals still make glycogen. Surprisingly, glycogen accumulates in striated muscle affecting functionality, including decreased exercise endurance. These findings impact our understanding of glycogen storage disease XV where patients lack glycogenin-1 and accumulate muscle glycogen.
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SUMMARY

Glycogenin is considered essential for glycogen synthesis, as it acts as a primer for the initiation of the polysaccharide chain. Against expectations, glycogenin-deficient mice (Gyg KO) accumulate high amounts of glycogen in striated muscle. Furthermore, this glycogen contains no covalently bound protein, thereby demonstrating that a protein primer is not strictly necessary for the synthesis of the polysaccharide in vivo. Strikingly, in spite of the higher glycogen content, Gyg KO mice showed lower resting energy expenditure and less resistance than control animals when subjected to endurance exercise. These observations can be attributed to a switch of oxidative myofibers toward glycolytic metabolism. Mice overexpressing glycogen synthase in the muscle showed similar alterations, thus indicating that this switch is caused by the excess of glycogen. These results may explain the muscular defects of GSD XV patients, who lack glycogenin-1 and show high glycogen accumulation in muscle.

INTRODUCTION

Glycogen is a branched polymer of glucose residues that is stored and later released to meet energy demands. Skeletal muscle comprises a spectrum of fast-twitch glycolytic fibers, which use glycogen as the main source of energy for anaerobic metabolism to fuel short and intense activity (the extensor digitorum longus [EDL] is a muscle rich in this fiber type), and slow-twitch oxidative fibers, which are used for prolonged low-intensity activity (abundant in the soleus) driven primarily by fuels such as blood glucose and fatty acids (Schiaffino and Reggiani, 1996). It is generally accepted that glycogen synthesis is mediated by the action of two enzymes: glycogenin, the primer of the reaction, and glycogen synthase (GS), the elongator of the glucose chain. Glycogenin is a glycosyltransferase that catalyzes the addition of glucose residues to itself. The first glucose is transferred from UDP-glucose to its Tyr195 residue. The following glucose residues are then bound sequentially to form a chain of 10–20 residues (Alonso et al., 1995; Cao et al., 1993; Krisman and Barengo, 1975; Smythe and Cohen, 1991; Whelan, 1989). Further chain elongation is performed by GS, and branches are introduced by the glycogen branching enzyme (BGE). GS interacts directly with the glycosyl-primer chain through the active site and also interacts with the 34 conserved amino acids of glycogenin’s C-terminal domain (Zeqiraj et al., 2014). The interaction between GS and glycogenin is considered essential for glycogen synthesis.

In humans and most mammals, glycogenin is present in two isoforms: GYG1, which is widely expressed, and GYG2, which is predominantly expressed in the liver and to a minor degree in cardiac muscle and the pancreas. Recently, a new form of glycogenosis (GSD XV) resulting from GYG1 loss of function has been described. Patients with this condition present with glycogen accumulation and muscle weakness (Malfatti et al., 2014). Differently from humans, rodents carry a single Gyg gene, which is expressed in all tissues (Mu et al., 1997; Zhai et al., 2014). This characteristic makes Mus musculus an ideal model in which to study the impact of glycogenin depletion on glycogen metabolism. To challenge the role of glycogenin, we generated a Gyg knockout mouse model (Gyg KO). Unexpectedly, rather than preventing the synthesis of glycogen, the absence of glycogenin caused glycogen over-accumulation in striated muscles. Remarkably, this glycogen was synthesized without the participation of a substitute protein primer. Furthermore, in spite of the higher muscle glycogen levels, these animals showed impaired endurance muscle performance, a phenotype similar to that of GDS XV patients.
Figure 1. Characterization of Gyg KO Mouse Model and Gyg KO Muscle Glycogen

(A) Immunoblot for glycogenin in the skeletal muscle, heart, liver, and brain of WT, Gyg +/−, and Gyg KO mice. Glycogenin protein is detectable only after treatment with α-amylase, used to degrade covalently bound glycogen and allow entrance in the polyacrylamide gel.

(B) Histological localization of glycogen by PAS staining in the absence or presence of diastase (PAS-D) on paraffin-embedded slides of skeletal muscle, heart, liver, and brain from 15- to 20-week-old males. Scale bar: 250 μm.

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RESULTS

Glycogenin-Deficient Animals Have the Capacity to Synthesize Glycogen

We generated homozygous Gyg KO mice by mating heterozygous mice (Gyg +/-) for the constitutive disruption on the Gyg gene. The genotype was confirmed by western blot and mRNA expression analysis of glycogenin (Figure 1A and Figure S1A). The number of pups per litter was lower than expected, and the proportion of Gyg KO mice was only 4% (lower than the 25% predicted by Mendelian genetics) (Figure S1B). However, the genetic ratio of embryos at E18.5 was in line with Mendelian proportions. Indeed, most of the Gyg KO pups died shortly after birth due to cardiorespiratory failure.

Unexpectedly, Periodic acid-Schiff staining (PAS) and biochemical measurements revealed that Gyg KO mice were able to synthesize glycogen (Figures 1B and 1C). In fact, both the liver and brain contained normal levels of this polysaccharide, while skeletal and cardiac muscle contained four and seven times more glycogen, respectively. Gyg heterozygous mice (carrying one allele of the Gyg gene) showed a moderate increase. Interestingly, the accumulation of glycogen did not increase progressively with age in any tissue (Figure S1C).

To analyze the impact of glycogenin depletion on the two key enzymes of glycogen metabolism, GS and glycogen phosphorylase (GP), we measured their levels and activity in the KO model. In skeletal muscle, the mRNA levels of GS and GP were equal to those found in the controls (Figure S1D), while an increase in protein level and activity was detected (Figure S1E). This indicates that neither glycogen synthesis nor degradation was impaired.

In several glycogen storage diseases (GSDs), such as Lafora disease (LD; EPM2, OMIM254780) and Adult Polysaccharavan Disease (APBD, OMIM263570), glycogen is poorly branched and accumulates in the form of amylase-resistant aggregates. However, in the PAS staining of Gyg KO mouse muscle and heart tissue, we observed a uniform distribution of the polysaccharide (Figure 1B). Furthermore, amylase treatment resulted in the complete degradation of glycogen (PAS-D). We also measured the degree of branching by analyzing the spectra of the glycogen-iodine complex. Gyg KO glycogen gave the same absorbance peak as commercial glycogen and purified glycogen from WT animals (Figure 1D). This indicates that glycogen synthesized by Gyg KO animals shows a normal degree of branching. We also characterized the size of the particles using size-exclusion chromatography (SEC). This analysis revealed that the glycogen granules isolated from Gyg KO muscle were larger than those purified from WT muscle and that they extended over a wide range of sizes, reaching up to a 4-fold greater radius than the particles in the WT animal (Figure 1E).

Electron microscopy studies also showed large particles, which accumulated in the intermyofibrillar space of Gyg KO muscle (Figures 1F and 1G and Figures S1F and S1G).

Glycogen Synthesis Does Not Require a Protein Primer

The presence of glycogen in the absence of glycogenin implies one of two possibilities: (1) glycogen is synthesized without a priming protein; or (2) another unknown protein replaces glycogenin in the Gyg KO mice. To address these questions, we designed a mass spectrometry-based approach to identify proteins covalently bound to the polysaccharide.

Glycogen was purified from the skeletal muscle of Gyg KO mice and control littermates by digesting the tissues with 30% KOH under conditions in which the protein that was covalently bound to glycogen were not completely hydrolyzed. The resulting glycogen samples were repeatedly washed in order to remove the non-covalently bound peptides. Glycogen samples, which retained the covalently linked peptides, were then degraded with α-amylase or amyloglucosidase (Figure S1H). These two enzymes differ in their ability to act on the covalent bond between an amino acid and a glucose residue. While amyloglucosidase cleaves the covalent link (meaning that no hexose should be found in the treated peptides), α-amylase is not able to cut the covalent amino acid-sugar bond, meaning that all the peptides covalently bound to glycogen should still carry at least one hexose (Table S2). According to our experimental design, criteria for the identification of a glycogenin substitute in the glycogen purified from Gyg KO mice would be as follows: presence of peptide(s) originating from the primer in the samples treated with both amylase and amyloglucosidase, and at least one hexose residue in the sample treated with amylase. We identified the resulting peptides by mass spectrometry. The proteins to which they belong are listed in Table S3 and Figure 1H. As expected, in the analysis of WT samples, only glycogenin fulfilled the selection criteria (i.e., was present in the two series). Strikingly, in the glycogen extracted from skeletal muscle of Gyg KO mice, no protein corresponding to both selection criteria was identified, indicating that glycogen is synthesized without a protein primer in these animals (Table S3).

Glycogenin Depletion Alters Skeletal Muscle Functionality

To assess the consequences of glycogenin depletion on muscle performance, we subjected Gyg KO and WT mice to forced exercise on a treadmill increasing in speed, until exhaustion. A strong association between muscle glycogen levels and strenuous exercise performance has been previously described (Bergström et al., 1967; Hermansen et al., 1967). Unexpectedly, in spite of the higher glycogen level in Gyg KO muscle, these...
Figure 2. Gyg Depletion Impairs Skeletal Muscle Functionality

(A) Running time and distance were measured from the individual performances on the treadmill. WT: n = 9; Gyg KO: n = 8.

(B) Glycemia was measured just before and after treadmill exercise. Statistical analyses were performed in R using the Wilcoxon/Mann-Whitney U test (non-parametric analog for two-sample t test) to compare variables of interest between WT and KO groups. WT: n = 9; Gyg KO: n = 8.

(C) Blood lactate was measured just before and after treadmill exercise. Statistical analyses were performed in R using the Wilcoxon/Mann-Whitney U test (non-parametric analog for two-sample t test) to compare variables of interest between WT and KO groups. WT: = 9; Gyg KO: = 8.

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We next tested the energy expenditure of the two genotypes in oxidative metabolism such as the EDL. Although higher levels of glycogen remained in the muscles of Gyg KO mice at exhaustion, skeletal muscle glycogen diminished in both control and Gyg KO mice upon exercise, indicating that the poorer exercise performance in Gyg KO mice is not due to an impaired ability to mobilize glycogen (Figure 2D). In fact, the consumption of this polysaccharide was almost 2-fold higher in Gyg KO than in WT animals (Figure 2D, insert). This suggests an overall glycolytic shift in muscle metabolism.

We next carried out ex vivo testing of the mechanical properties of isolated muscles representative of the two opposing metabolic and contractile types. Specifically, we tested the slow-twitch soleus muscle (rich in type I fibers, which use oxidative metabolism and serve to cover prolonged resistance activity) and fast-twitch EDL muscle (rich in type II glycolytic fibers, which use anaerobic metabolism and serve to sustain high-intensity activity). First, we verified that the muscle mass was maintained and that both soleus and EDL had increased glycogen in the Gyg KO model (Figures S2C and S2D). We also ruled out the existence of alterations in the proportion of fiber types in soleus and EDL by immunohistochemical analyses with antibodies specific for myosin heavy chain (MHC) I, Ila, and Iib (MHC IX were quantified by exclusion of positive staining) (Figures 2E and 2F).

Isometric force generation after applying trains of stimuli at increasing frequency (Figure 2G) was recorded in Gyg KO and control muscles. Remarkably, Gyg KO soleus generated a force approximately 2-fold greater than its paired WT control at all the stimulation frequencies tested, while Gyg KO EDL responded approximately 2-fold greater than its paired WT control at all the stimulation frequencies tested, while Gyg KO EDL was maintained and that both soleus and EDL had increased glycogen in the Gyg KO model (Figures S2C and S2D). We also ruled out the existence of alterations in the proportion of fiber types in soleus and EDL by immunohistochemical analyses with antibodies specific for myosin heavy chain (MHC) I, Ila, and Iib (MHC IX were quantified by exclusion of positive staining) (Figures 2E and 2F).

Forced Over-accumulation of Glycogen in Skeletal Muscle Recapitulates the Changes Observed in the Glycogenin-Deficient Muscles

The alterations observed in the Gyg KO mouse model could be a result of the absence of glycogenin or the increased accumulation of glycogen in muscles. To discern between the two possibilities, we generated an animal model with skeletal muscle-specific expression of a form of MGS that cannot be inactivated by phosphorylation (9A-GMS<sup>MTC</sup>) (Figure 4A). In resting conditions, these animals accumulated 7-fold more glycogen in this tissue compared to their WT littermates (Figure 4B). PAS staining of muscle sections showed that glycogen was uniformly distributed and was mostly degradable by amylase treatment (Figure 4C). Interestingly, western blot analyses showed an increase in the quantity of glycogenin, paralleling that of glycogen. To explore whether this increased muscle glycogen affects exercise performance, we subjected mice to forced exercise on a treadmill. 9A-GMS<sup>MTC</sup> mice reached exhaustion faster than their control littermates, despite the observed mobilization of muscle glycogen during exercise (Figure 4D). Indeed, as seen in Gyg KO animals reached exhaustion earlier and covered a shorter distance compared to WT animals (Figure 2A and Figures S2A and S2B).

Both groups showed a comparable increase in blood glucose and lactate levels at exhaustion (Figures 2B and 2C). Although higher levels of glycogen remained in the muscles of Gyg KO mice at exhaustion, skeletal muscle glycogen diminished in both control and Gyg KO mice upon exercise, indicating that the poorer exercise performance in Gyg KO mice is not due to an impaired ability to mobilize glycogen (Figure 2D). In fact, the consumption of this polysaccharide was almost 2-fold higher in Gyg KO than in WT animals (Figure 2D, insert). This suggests an overall glycolytic shift in muscle metabolism.

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**Glycogenin-Depleted Animals Show Changes in Muscle Oxidative Metabolism**

We next tested the energy expenditure of the two genotypes in a resting condition by measuring VO<sub>2</sub> and VO<sub>2</sub> (Figure 3A and Figure S3A). Indirect calorimetric measurements indicated lower energy consumption in adult Gyg KO animals. Oxygen consumption was lower over the whole day and was associated with a reduction in glucose oxidation (prominent in the dark phase when mice are awake and more active) and diminished lipid oxidation (especially during the light phase when mice are asleep). We confirmed that the differences observed were not due to anomalies in body weight or the proportion of fatlean body mass, which were both found to be equally maintained in the two animal models (Figures S3B and S3C). Moreover, food intake and locomotor activity were comparable (Figures S3A and S3D).

To further characterize the metabolism in the two muscles, we measured mitochondrial respiration using high-resolution respirometry in isolated soleus and EDL. Once again, we identified a shift in the soleus of Gyg KO mice, which showed lower oxygen consumption affecting all mitochondrial states. These lower levels were comparable to those of fast-twitch muscles (e.g., WT EDL). On the other hand, EDL respiration values were not altered in the Gyg KO, giving oxygen consumption values comparable to those of control muscle (Figure 3B). However, the differences observed in the Gyg KO soleus were not due to a decreased number of mitochondria (Figures 3C and 3D) or to a specific decrease in OXPHOS proteins (Figures 3E and 3F and Figure S3E). We then quantified the adenylate energy charge in the two muscles. No differences were found for EDL, while Gyg KO soleus presented with lower AMP/ATP and ADP/ATP ratios than WT soleus, approaching those found in the EDL (Figure 3E).
mice, the net quantity of glycogen degraded after exercise was higher in 9A-MGS_{MLC1} animals than in controls (Figure 4B). We next analyzed mitochondrial respiration in fibers extracted from soleus and EDL muscles of 9A-MGS_{MLC1} mice. As in the Gyg KO animal, the soleus of 9A-MGS_{MLC1} mice showed reduced mitochondrial respiration compared to control littermates (Figure 4E). Taken together, the results from the 9A-MGS_{MLC1} mice are consistent with the changes observed in the Gyg KO animals and suggest that the over-accumulation of glycogen (rather than the absence of glycogenin) is the underlying cause of the glycolytic switch in oxidative muscles.

**DISCUSSION**

Our results unveil new and unexpected aspects of glycogen metabolism and challenge the concept that glycogenin is indispensable for glycogen synthesis (Zeqiraj et al., 2014). In addition to showing that glycogen is efficiently synthesized in animals lacking glycogenin, we further demonstrate that a priming protein is unnecessary for the synthesis of this polysaccharide. Furthermore, the finding that the striated muscles of Gyg KO mice display an over-accumulation of glycogen suggests that glycogenin depletion leads to a situation that is exactly the opposite of that expected.

One of our most striking findings is that glycogen synthesis in Gyg KO mice proceeds without a priming protein. In fact, this phenomenon can be seen in other organisms, such as bacteria, yeast, and plants (Szydłowski et al., 2009; Torija et al., 2005; Ugalde et al., 2003), in which GS homologs act as de novo initiators. Our hypothesis is that glycogen is synthesized by GS in Gyg KO animals, starting from free glucose. In support of this, Salsas and Larner demonstrated that, in the presence of UDP-glucose as a co-substrate, purified muscle GS converts glucose to maltose (Salsas and Larner, 1975). Other authors showed that GS can convert maltose into maltooligosaccharides with an increasing number of glucose residues, with the Km decreasing progressively alongside the length of the acceptor chain (Lerner et al., 1976).

Unlike the glycogen aggregates seen to accumulate in Lafora disease (Duran et al., 2014; Duran and Guinovart, 2015), the glycogen synthesized by the murine Gyg KO model did not form aggregates, demonstrated a regular degree of branching, and was hydrolyzed in vitro by amylase treatment. The latter is in accordance with it being metabolically active in vivo and degraded, at least in part, during exercise. Remarkably, glycogen particles from Gyg KO animals were larger than those from controls, as measured by chromatography and EM. The observation that Gyg KO muscle contains a higher quantity of glycogen than that of the WT control indicates that glycogenin may act as a regulator of glycogen content. There are various possible rationales for this. First, it is known that GS and Gyg interact strongly; therefore, glycogenin may modulate the amount of glycogen synthesized by acting on GS (Skrat et al., 2006). This idea is supported by our observation that the skeletal muscle and heart of Gyg heterozygous mice, which have reduced glycogenin expression, also accumulate more glycogen than WT animals. Second, Gyg could be important for the regulation of glycogen particle size, limiting the final volume of the particle. In favor of this hypothesis is our observation that the particles in Gyg KO muscle were larger than those present in WT animals. Furthermore, glycogenin overexpression in rat fibroblasts leads to a greater number of smaller molecules, rather than increasing glycogen production (Skrat et al., 1997).

In contrast to the prediction that glycogen accumulation in Gyg KO mice would confer greater resistance to fatigue (Bergström et al., 1967; Holloszy and Kohrt, 1996; Karlsson and Saltin, 1971), these animals reached exhaustion faster than controls when challenged with strenuous exercise. Importantly, simply generating an over-accumulation of glycogen in the muscle of 9A-MGS_{MLC1} mice caused a similar defect in muscle performance, consistent with the observation in absence of glycogenin. Although we cannot rule out the different glycogen particle size as a cause of the reduced exercise resistance in the Gyg KO model, the recapitulation of this phenotype in 9A-MGS_{MLC1} animals supports the key role of glycogen accumulation in the regulation of muscle function.

Our results also support the idea that the greater availability of glycogen in the muscles of both Gyg KO and 9A-MGS_{MLC1} mice induces oxidative fibers to preferentially use this polysaccharide. This renders them more glycolytic, similar to bona fide type II glycolytic fibers, which primarily use glycogen as a fuel. The aforementioned metabolic switch occurs despite the absence of changes in fiber type proportions (as revealed by MHC analysis) or levels of mitochondrial markers (such as porin, mitofilin 2, Tim44, OXPHOS proteins, and succinate dehydrogenase activity: demonstrated by SDH staining). Nevertheless, Gyg KO animals display lower energy expenditure and low oxygen consumption in a steady rested state, indicating a lower glucose and lipid oxidation capacity. Indeed, significant changes in mitochondrial respiration indicative of impaired functionality were detected by measuring oxygen consumption in permeabilized fibers.
Figure 4. Muscle Glycogen Over-accumulation Causes Skeletal Muscle Energetic Impairment
(A) Western blot characterization of the 9A-MGS<sup>M.C1</sup> mouse model: GS was detected in total tissue homogenate from skeletal muscle; loading control β-actin. Glycogenin was detected in α-amylase-treated samples from total homogenate of skeletal muscle; loading control β-actin.

(C) Quadriceps and Heart

(D) Time (min) and Distance (m)

(E) Oxidative respiration in different states

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muscle fibers from the soleus. Our current hypothesis is that the high availability of glycogen in Gyg KO soleus muscle affects energy production, impairing oxidative metabolism. This type of metabolism is particularly important when energy demands are high, such as during endurance exercise. In contrast, the high glycogen content found in the EDL muscle of this mouse model does not lead to any changes in metabolism, likely because these fibers normally utilize glycogen as their main energy source. Thus, a high degree of glycogen accumulation leads to an alteration of muscle function and oxygen consumption in a muscle that is predominantly reliant on oxidative metabolism. This translates into low resistance during prolonged, low-intensity activity.

Despite the high availability of degradable glycogen, both the Gyg KO and 9A-MGS\textsuperscript{MLC1} models showed a similar, low resistance to fatigue when forced to exercise on a treadmill until exhaustion. An alteration in mitochondrial respiration was also found in the 9A-MGS\textsuperscript{MLC1} mice, which may explain the poor endurance performance. On the basis of our findings, we conclude that the high glycogen content in the muscles of Gyg KO and 9A-MGS\textsuperscript{MLC1} mice decreases muscle endurance, against all expectations.

Humans and most mammals carry two isoforms of the glycogenin gene, namely GYG1 and GYG2. GSD XV is a recently described rare human disease caused by GYG1 depletion. As mice express only Gyg1, the Gyg KO mouse constitutively lacks the protein throughout the body. Comparison of GSD XV with Gyg KO mice is therefore beneficial to provide new insights into the role of glycogenin and the physiopathology of GSD XV. Like these patients (Akman et al., 2016; Colombo et al., 2016; Luo et al., 2015; Malfatti et al., 2014), Gyg KO animals also accumulate glycogen in skeletal muscle and show phenotypical muscle weakness. However, while the glycogen in Gyg KO mice is entirely amyrase sensitive, the glycogen that accumulates in GSD XV patients is accompanied by polyglucosans. This may contribute to the more severe muscle weakness phenotype in humans. Moreover, the Gyg KO model also shows glycogen accumulation in the heart. This may be of special interest regarding GSD XV patients, some of whom have been shown to suffer from cardiomyopathy and require a heart transplant (Hedberg-Oldfors et al., 2017).

In conclusion, a lack of glycogenin does not prevent the synthesis of glycogen, but rather causes an over-accumulation of the polysaccharide in striated muscle, reflective of GSD XV patients. Although the over-accumulated glycogen can be mobilized, it leads to functional impairment and metabolic rearrangement. These observations offer a new perspective on glycogen synthesis and the role of the glycogenin-glycogen relationship in muscle physiology.

**STAR METHODS**

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**SUPPLEMENTAL INFORMATION**

Supplemental Information includes three figure and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2017.06.008.

**AUTHOR CONTRIBUTIONS**

G.T. designed and performed experiments, analyzed experimental data, and participated in the manuscript preparation. J.D. and M.G.-R. performed experiments and analyzed experimental data. F.V. performed the SEC analysis and contributed to the discussion of the results. A.L.S. contributed to the performances of animal experiments. D.S. helped with respiration studies and advice. I.L.-S, M.A.S., and F.S. contributed to the design of the experiments and discussion of the results. M.V. advised, assisted, and supervised mass spectrometry analysis, P.M.-C. assisted in the myograph experiment, J.J.G. designed and supervised the whole project and revised the manuscript.

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REFERENCES


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KEY RESOURCES TABLE

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CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Joan J. Guinovart (guinovart@irbbarcelona.org), following an approval MTA between IRB Barcelona and the receiving institution.
EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals
ES cells (Gyg<sup>tm1a(KOMP)Wtsi</sup>) were generated by the trans-NIH Knock-Out Mouse Project (KOMP) and obtained from the KOMP Repository (www.komp.org). From ES cells, mice heterozygous for the glycogenin (Gyg) gene were generated (C57BL/6 background) and crossed to obtain Gyg KO animals. We also generated an mMGS-9A mouse model, as described in (Duran et al., 2012), and crossed it with the conditional transgenic strain MLC1-Cre (under the promoter of Myosin Light Chain 1) (Sala et al., 2014) to produce 9A-MGS<sup>MLC1</sup> mice, which express MGS that cannot be inactivated in skeletal muscle. Mice were maintained in the PCB-PRBB Animal Facility Alliance under a light–dark cycle (12 hr) and specific pathogen-free conditions, with access to food and water ad libitum. For experimental convenience, the animals analyzed were adult males (15-20 weeks old), unless otherwise stated. All procedures were approved by the PCB Animal Experimentation Committee, in accordance with National Institutes of Health and the European Community Council Directive guidelines for the care and use of laboratory animals. Experiments were conducted using littermates. Where necessary, experimental groups included multiple litters for statistical power.

METHOD DETAILS

Histochemistry
Histochemical analyses were carried out for PAS and PAS-D using an Artisanlink Pro machine (DAKO AR165 kit). SDH staining was performed manually in a pH 7.2-7.6 solution containing Nitroblue tetrazolium (Sigma-Aldrich). Soleus and EDL fiber types were characterized by MHC immunostaining using primary monoclonal antibodies against type I (BA-F8), Ila (SC-71), and Ilb (BF-F3) from the Developmental Studies Hybridoma Bank (DSHB). The secondary antibodies used were Alexa Fluor 488 goat anti-mouse IgG2b, Alexa Fluor 350 goat anti-mouse IgG1 (Invitrogen) and TRITC goat anti-mouse IgM (Millipore), respectively. Negative control slides with omission of the primary antibody were included in each immunostaining. Tissues were fixed in 4% PFA or OCT blocks in 2-methylbutane maintained in liquid nitrogen. Embedded tissues were cut into 3-μm sections. All histochemical analyses were performed successfully on a minimum of 6 animals per group, and blinded muscle fiber type counts performed.

Biochemical analysis
Tissues extracted for biochemical analysis were snap-frozen in liquid nitrogen and stored at −80°C until use. Enzymatic activity and western blot (WB) analyses were performed in frozen, pulverized tissues, which were homogenized at 4°C using a Polytron in 10 volumes of the homogenization buffer (10 mM Tris–HCl [pH 7], 150 mM KF, 15 mM EDTA, 15 mM 2-mercaptopethanol, 0.6 M sucrose, 25 mM okadaic acid, 1 mM sodium orthovanadate, 10 μg/ml Leupeptin, 10 μg/ml Aprotinin, 10 μg/ml pepstatin, 1 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride). Total GS activity was measured by measuring the incorporation of [U-14C]-glucose from UDP-[U-14C]-glucose into glycogen in the presence of 6.6 mmol/L of glucose-6-phosphate (G-6P) (Thomas et al., 1968). GP activity was determined by measuring the incorporation of [U-14C]-glucose from [U-14C]-glucose-1-phosphate into glycogen in the presence of 5 mmol/L AMP (Gilboe et al., 1972). Glycogenin determination was performed by western blots of homogenates treated with 13.2 U of α-amylase (A0521 from Sigma-Aldrich) per mg tissue, for 1 hr at 37°C. Western blots for mitochondrial proteins were performed in soleus and EDL muscle homogenates using the following lysis buffer: 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 50 mM NaF, 1% NP-40, 1 mM PMSF and a protease inhibitor mixture tablet (Roche). Homogenates were rotated for 1 hr at 4°C in an orbital shaker and centrifuged at 16,000 <i>g</i> for 15 min at 4°C. Proteins were resolved in 10% or 15% acrylamide gels for SDS-PAGE and transferred to Immobilon membranes (Millipore). The following antibodies were used: glycogenin (clone 3B5 from Novus Biological); GS (ref. 3886 from Cell Signaling); glyceraldehyde-3-phosphate dehydrogenase (GAPDH; ref. AM4300 from Thermo Fisher Scientific); GP (polyclonal antibody generated against the peptide 826-841 of muscle isoform of GP by Eurogentec, Cologne, Germany); porin (ab15895 from Abcam); Mfn2 (ref. 56889 from Abcam); mitochondrial import inner membrane translocase subunit TIM14 (ref. 612582 from BD Transduction Laboratories); Total OXPHOS Rodent Cocktail (ab110413 from Abcam); tubulin (clone DM1A from Sigma); and actin (clone AC-40 from Sigma). Proteins were detected by the ECL method (Immobilon Western Chemiluminescent HRP Substrate, Millipore). All protein detections were successfully performed on a minimum of 10 animals per group. Where otherwise specified, loading control of the WB membrane was performed using the REVERT total protein stain. Glycogen quantification was determined as previously described (Garcia-Rocha et al., 2001). Briefly, frozen tissue was homogenized in 4 volumes of 30% KOH at 4°C. Samples were heated at 100°C for 15 min and then precipitated on a 31ET paper (Whatman, Maidstone). After three washes with 66% EtOH, dried papers were incubated with amyloglucosidase (25 U/L Sigma) in 100 mM sodium acetate buffer at pH 4.8. Glucose content was determined by the reaction with hexokinase and G-6P dehydrogenase following the original method described by Chan (Chan and Exton, 1976). Results are expressed in μg of glycogen per mg of tissue.

Glycogen particle analysis
To determine the degree of glycogen branching, skeletal muscle was homogenized in 30% KOH, heated at 100°C for 15 min, and then precipitated in 66% EtOH (v/v). Dried, purified glycogen was re-suspended in NH<sub>4</sub>Cl (sat) plus H<sub>2</sub>O in a 1:2 ratio, and added to the iodine-iodide solution (1.5 M KI and 100 mM I<sub>2</sub>). Reference spectra were determined using glycogen type III from rabbit liver (Sigma G8876) and amylose (Sigma-Aldrich A7043). The absorbance spectrum was recorded from 400 to 650 nm.
SEC analysis was performed in skeletal muscle glycogen. Glycogen was obtained by KOH 30% treatment of the tissue followed by precipitation at 66% EtOH at −20°C and centrifugation at 16,000 × g at 4°C. The resulting pellet was re-suspended in H2O and freeze-dried. SEC was performed as previously described (Sullivan et al., 2014).

**Transmission electron microscopy**

Animals were perfused and tissues were fixed with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer. Tissue slices were post-fixed in 1% osmium tetroxide, stained with 0.8% potassium ferrocyanide, dehydrated, and embedded in EPO resin. Ultrathin sections collected on copper grids were stained with 2% uranyl acetate in water and lead citrate solution. Electron micrographs of skeletal muscle were taken using a Tecnai G2 F20 (FEI) 200 kV FEG with CCD Eagle 4k×4k transmission electron microscope. Thiery staining was performed with 1% periodic acid, 0.2% thiocarbohydrazyde (TCH) in 20% acetic acid and 1% silver proteinate (PATAg reaction). Electron microscopy images of the heart were taken using a Tecnai Spirit transmission electron microscope. TEM of glycogen purified particles was conducted on a 400-mesh grid, glow-discharged before use. Droplets of approx. 0.01% glycogen dispersed in distilled water were placed on the grid for ~1.5 min before staining with 2% aqueous uranyl acetate. The particles were imaged on a JEOL 1010 TEM (Tokyo, Japan) operating at 80 kV at the University of Barcelona, Spain. Particle size was measured by ImageJ software. All TEM analyses were successfully performed on samples from 3 animals per group, with a minimum of 15 pictures per sample analyzed by a blinded investigator.

**Quantitative RT-PCR**

Frozen tissues were homogenized by Polytron in TRIzol (Invitrogen), and mRNA was isolated using the RNeasy Mini kit (QIAGEN). Reverse transcription was performed using the Superscript one step TM RT-PCR System (Invitrogen). The following housekeeping genes were used: 18S and RPL13. Total DNA from tissues was extracted with the DNeasy Blood and Tissue Kit (QIAGEN). We quantified mitochondrial DNA by real-time PCR. Total DNA was used as a template and was amplified with specific oligodeoxynucleotides for mitochondrial DNA or Sdha (nuclear gene). We calculated the mitochondrial DNA copy number per cell by using Sdha amplification as a reference for nuclear DNA content. The primers used for qPCR are indicated in the supplemental information (Table S1).

**Metabolite determination**

Blood glucose levels were determined using a glucometer (Contour Next, Bayer Healthcare). Blood lactate was measured spectrophotometrically using a commercial kit (HORIBA ABX, Montpellier, France). The intracellular concentrations of ATP and other adenylates were measured from perchloric acid extracts of skeletal muscle tissue. They were quantified after HPLC in a Brisa column LC2 C18 (Teknokroms).

**Respiration studies in permeabilized muscle**

Soleus and EDL muscles were removed from the mice and placed in ice-cold isolation solution BIOPS (10 mM Ca-EGTA buffer [2.77 mM of CaK2EGTA + 7.23 mM of K2EGTA], 20 mM imidazole, 20 mM taurine, 50 mM K-Mes, 3 mM K2HPO4, 6.5 mM MgCl2, 5.7 mM ATP, 15 mM phosphocreatine, and 0.5 mM DTT [pH 7.1]). The addition of saponin (50 μg/mL) favored the permeabilization of single fibers that had previously been separated mechanically. After transferring the muscle bundles to a respiration medium (0.5 mM EGTA, 3 mM MgCl2-6H2O, 20 mM taurine, 10 mM KH2PO4, 20 mM HEPES, 1 g/L BSA, 60 mM K-lactobionate, and 110 mM sucrose [pH 7.1]), high resolution respirometry was measured at 37°C by Oxygraph-2K (Oroboros instruments), as described (Sebastián et al., 2016). The following protocol was used: resting respiration (absence of adenylates, state 4), 10 mM glutamate and 2 mM malate (complex I), 2.5 mM ADP (state 3), 10 μM cytochrome c (integrity of the outer mitochondrial membrane), 10 mM succinate (state 3, complex I and II), and protonophore carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone (FCCP) (maximal O2 flux).

**Indirect calorimetry**

An 8-chamber Oxymax system (Columbus Instruments) was used to measure O2 consumption, CO2 production, and other calorimetric parameters, as described in (López-Soldado et al., 2015). Briefly, measurements were taken after two days of acclimation for 2 cycles of 24 hr. Energy expenditure was calculated using the formula (3.185 + 1.232 × RER) × VO2, where the respiratory exchange ratio (RER) = VO2/VE2, Glucose oxidation in g/min/kg0.75 was calculated using the formula [(4.545 × VO2) − [3.205 × VO2]]/1000, and lipid oxidation in g/min/kg0.75 was calculated using the formula (1.672 × [VO2 − VO2])/1000. Locomotor activity was monitored by an infrared photocell beam interruption method.

**Food intake, body temperature and weight**

Food intake was monitored for five days in individually caged animals that were acclimatized for one week prior to the study. Total body fat and lean mass were monitored by EchoMRI-100 (EchoMRI, Houston, Texas).

**Mass spectrometry**

Frozen skeletal muscle was rapidly homogenized in 30% KOH at 4°C for 18 s at increasing polytron speed. Samples were boiled for 3 min at 100°C and quickly cooled in ice. These conditions were optimized to achieve an incomplete digestion of the glycogen bound proteins, so that the covalently bound peptides remain linked to glycogen.
Glycogen was precipitated with 66% EtOH (v/v) centrifuged, and re-suspended in water. This step was repeated five times. Samples were extracted using a 1:1 solution of acetonitrile and 1% formic acid, then sonicated for 5 min and centrifuged in order to extract the non-covalently bound peptides. The supernatant was then removed. These steps were repeated three times. Glycogen samples (which still contained the covalentlylinked peptides) were re-suspended in water for mass spectrometry applications and parallel glycogen measurement. Samples were added to a FASP filer unit (50-kDa cutoff) and glycogen was digested in the filter by amyloglucosidase for 96 hr at 37°C or by amylase for 72 hr at 37°C. Eluted peptides were analyzed by nanoLC-MS/MS. The sample volumes were reduced to approximately 15 µl in a vacuum centrifuge and were cleaned with PolyLC C18 pipette tips. The nanoLC-MS/MS was set up via the following steps. Digested peptides were first diluted in 1% FA. Samples were loaded onto a 180 µm x 2 cm C18 Symmetry trap column (Waters) at a flow rate of 15 µL/min using a nanoAcquity Ultra Performance LC chromatographic system (Waters, Milford, MA). Peptides were separated using a C18 analytical column (BEH130TM C18 75 µm x 25 cm, 1.7 µm, Waters) with a 130 min run, comprising three consecutive steps with linear gradients from 1 to 35% B in 90 min, from 35 to 50% B in 15 min, and from 50 to 85% B in 2 min, followed by isocratic elution at 85% B in 10 min and stabilization to initial conditions (where A = 0.1% FA in water, B = 0.1% FA in CH3CN). The column outlet was directly connected to an Advion TriVersa NanoMate (Advion) fitted on an LTQ-FT Ultra mass spectrometer (Thermo). The spectrometer was working in positive polarity mode and in a data-dependent acquisition (DDA) mode. Singly charged precursors were rejected for fragmentation. Survey MS scans were acquired in the FT with the resolution (defined at 400 m/z) set to 100,000. Up to six of the most intense ions per scan were fragmented and detected in the linear ion trap. The ion count target value was 1,000,000 for the survey scan and 50,000 for the MS/MS scan. Target ions already selected for MS/MS were dynamically excluded for 30 s. Spray voltage in the NanoMate source was set to 1.70 kV. The capillary voltage and tube lens on the LTQ-FT were tuned to 40 V and 120 V, respectively. The minimal signal required to trigger MS to MS/MS switch was set to 1,000 and activation Q was 0.250. LC-MS/MS/MS data analysis was performed with Proteome Discoverer software v1.4 (Thermo Scientific) using the Sequest HT search engine and SwissProt analysis was performed with Proteome Discoverer software v1.4 (Thermo Scientific) using the Sequest HT search engine and SwissProt database (Mus musculus, amyloglucosidase from aspergillus niger and amylase [release 2014_07] and the common Repository of Adventitious Proteins [crap database]). Database searches were run against targeted and decoy databases. Search parameters included no-enzyme specificity, methionine oxidation, and 1 to 3 hexoses in serine, threonine, and tyrosine as dynamic modifications. Peptide mass tolerance was 10 ppm and the MS/MS tolerance was 0.6 Da. Peptides with a FDR < 1% were considered as positive identifications with a high confidence level. Detected peptides were validated manually.

**Treadmill exercise**

The animals were acclimatized to treadmill running (Columbus Instruments) for 5 min per day over 5 days at a speed between 10 and 14 cm/s and a slope of 0°. On the day of the experiment, animals ran on a treadmill with a 10° inclination, starting at a speed of 10 cm/s for 3 min. Every 2 min, the speed was increased by 2 cm/s until the mice were exhausted or a maximal speed of 46 cm/s was reached. A shock grid at the back of the treadmill was activated to prevent mice from stopping spontaneously. Exhaustion was defined as the inability of the animal to remain on the treadmill, and animals were removed from the treadmill after the following observations: 5 consecutive seconds on the shock grid without attempting to reengage the treadmill; spending more than 50% of the time on the shock grid; and willingness to sustain 2 s or more of shock rather than return to the treadmill on three occasions. Work was calculated as the product of body weight (kg), gravity (9.81 m/s²), vertical speed (m/s; angle), and time (s). Power is the product of body weight (kg), gravity (9.81 m/s²), and vertical speed (m/s; angle). On the day of the experiment, blood from the tail tip was taken for glucose and lactate measurements before and after exercise.

**Myo-mechanical analysis of isolated muscles**

Muscle mechanical properties were quantified ex vivo, as previously described (Pessina et al., 2014). Briefly, mice were sacrificed and muscles were rapidly excised into a dish containing oxygenated Krebs-Ringer solution. The commercially available 1200A isolated muscle test system (Aurora Scientific, ON, Canada) was used to assess muscle force. The optimum muscle length was determined by administering single electrical pulses while lengthening the muscle until the maximum isometric twitch force on single twitches was attained. Isometric-specific force was determined for each stimulation frequency (Hz) ranging from 1 to 200 Hz for 450 ms, with 1 min of rest between stimuli. Force was normalized to muscle weight and muscle fiber cross-sectional area in vivo, thereby calculating the specific net force (mN/mm²).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

**Statistical Analysis**

Results are presented as mean ± SEM of independent experiments. Unless otherwise indicated, significance between two variables was analyzed using a Student’s t test, performed with the GraphPad Prism software (La Jolla, CA, USA). The following p values were considered to be statistically significant: p value < 0.05 (*), p value < 0.01 (**), and p value < 0.001(***).

**DATA AND SOFTWARE AVAILABILITY**

The mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium via the PRIDE (Vizcaino et al., 2016) partner repository, with the dataset identifier ProteomeXchange: PXD006377.