Study on the Role of DNMT3A in Skeletal Muscle Physiology and Metabolism

By

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This two-year learning experience has been an incredible and unforgettable journey, only made possible by my mentors, collaborators, friends, and families, whose participation in this journey I am grateful for.

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I would also like to thank the members of my dissertation committee, Andreas Stahl and Danica Chen, for their guidance and support that have kept me on track.

And finally, special thanks to my family, for supporting and believing in me every step of the way. I'm completely overwhelmed with everything I could ever ask for and more.

INTRODUCTION

Skeletal muscle is one of the major tissues that are integral to the development of insulin resistance and serves as a reservoir for postprandial glucose storage as glycogen[1,2]. The glucose transporter 4 (GLUT4) expressed by the skeletal muscle allows the blood glucose to be transported out of the bloodstream and into tissues when stimulated by insulin[3]. Because of the large overall mass of skeletal muscle and its responsiveness to insulin in humans, around 80% of the blood glucose can be cleared from the blood with the presence of insulin and then be stored as glycogen in skeletal muscle[1]. However, when insulin-stimulated glucose transport into skeletal muscle is blunted, as it is in people with Type 2 Diabetes or insulin resistance[4], this results in an inability to maintain normal blood glucose concentrations. In fact, skeletal muscles of individuals with T2D also exhibit a decreased capacity to oxidize both glucose and fat[5], when compared with lean healthy individuals. Thus, skeletal muscle plays a primary role in the maintenance of normal blood glucose concentrations and overall metabolic homeostasis of the host. In addition to insulin-mediated glucose uptake, glucose uptake can also be induced by muscle contraction, for instance, a single bout of exercise[6]. This contraction signaling of glucose uptake occurs by facilitated diffusion, and is also dependent on the translocation of GLUT4 to the plasma membrane and transverse tubules[7,8]. Moreover, aerobic exercise has consistently been shown to help with weight management[9], to enhance insulin sensitivity[9–12], and to improve cardiovascular risk factors[13,14]. Therefore, It has been recommended by the American Diabetes Association (ADA) that individuals with T2Ds perform at least 150 min of moderate-intensity aerobic exercise and/or at least 90 min of vigorous aerobic exercise per week[15].

Insulin resistance in skeletal muscle or other tissues is believed to be caused by a number of factors, including an imbalance between caloric intake and output, a sedentary lifestyle, hyperinsulinemia, glucocorticoids, and a low-grade chronic inflammation, etc[16–19]. Although the interlinks between these stresses remains unclear, emerging evidence indicates that these disruptions in energy metabolism can be traced to mitochondria, organelles that generate the majority of a cell's needed ATP through cellular respiration and oxidative phosphorylation. Perturbations in mitochondrial oxidative capacity has been recognized in the development and progression of insulin resistance and T2D[20]. For example, as few as 3 days of high-fat feeding are sufficient to reduce muscle mitochondrial oxidative phosphorylation at the transcriptional level and induce insulin resistance in lean, sedentary individuals[21]. As a result of the oxidative capacity of mitochondria, it is well accepted that mitochondria play central roles both directly and indirectly in the regulation of skeletal muscle metabolism and functions. Mammalian skeletal muscle is composed of heterogeneous fibers with high variability in their morphology, biology, and metabolic phenotypes. Based on their properties, the skeletal muscle fibers can be classified into different categories. Type 1 fibers, also known as red muscle, have a high mitochondrial density, and thereby relying on aerobic metabolism as their fuel source. They are slow in contraction and exhibit a high resistance to fatigue owing to the sustainable oxidation in the mitochondria. On the other hand, Type 2B fibers are white in their appearance as they lack abundant myoglobin and mitochondria contents. As a result, they are glycolytic and are unable to continuously supply skeletal muscle fibers with ATP and fatigue easily.

Previously, it has been reported that epigenetic marks could partially explain some of the pathogenic mechanisms of complex disorders that involve the gene-environment interaction in tissues. For instance, the DNA methylation status of Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) promoter in the liver of patients with non-alcoholic fatty

live disease (NAFLD) is significantly correlated with plasma fasting insulin levels[22]. The patients with NAFLD also exhibit reduced mitochondrial biogenesis and mitochondrial DNA copy number in the liver[22]. DNA methylation is a reversible epigenetic mechanism involving the transfer of a methyl group onto the C-5 position of the cytosine to form 5-methylcytosine[23]. This process is mediated by the DNA methyltransferase (DNMT) family and can be reversed by ten eleven translocation (TET) enzymes[24]. Methylation can directly prevent the interaction of chromatin proteins and specific transcriptional factors with DNA, which usually results in transcriptional silencing[25].

In mammals, Dnmt1, Dnmt3a, and Dnmt3b are the only three DNMT members identified so far that possess DNA methyltransferase activity[26]. Their functions, however, are not exactly the same. Dnmt1, also known as the maintenance DNA methyltransferase, prefers to methylate the hemi-methylated DNA, whereas Dnmt3a and 3b usually act as de novo DNA methyltransferases[26]. A role for DNMT1 was suggested in a study where administration of a small molecule Dnmt inhibitor has proved to improve insulin sensitivity in the setting of obesity, in part by demethylating the Adiponectin promoter[27]. On the other hand, another study identified hypermethylation of PGC-1α in the skeletal muscle of diabetic subjects, which was negatively correlated with PGC-1α mRNA and mtDNA[28]. This process is only reversed by selective silencing of the DNMT3B, but not DNMT1 or DNMT3A[28]. Therefore, it is worth investigating and differentiating the slightly different roles played by each DNMT member in mediating the DNA methylation-related pathophysiological processes. Since our lab previously identified that only Dnmt3a expression was significantly induced by HFD in skeletal muscle[29], we propose that Dnmt3a, rather than other DNMT proteins may play a role in skeletal muscle. Previous studies in our lab also discovered that adipose-specific Dnmt3a knock-out mice are protected from diet-induced insulin resistance and glucose intolerance without accompanying changes in adiposity[29]. Hence, we are specifically interested in exploring whether Dnmt3a is necessary in mediating insulin resistance in skeletal muscle.

Meanwhile, epigenetic marks are known to be dynamic and are subject to external influences. Therefore, exercise, an event that activates multiple upstream signaling pathways, is associated with altered DNA methylation patterns in genes related to skeletal muscle metabolism and function. Acute and chronic exercise can induce both hyper- and hypo-CpG methylation of target loci[30–35]. For example, a single bout of aerobic endurance exercise in human subjects can transiently induce promoter hypomethylation in important mitochondria-related transcripts (e.g., PPARGC1A, PDK4, TFAM, and PPARD), followed by a time-lagged increase in their expression[30]. Despite the discovered links between modifications in DNA methylation and exercise, there is a lack of research elucidating the roles of individual DNMTs in muscle physiology. Thus, we propose that DNMT3a also plays a role in the physiological function of skeletal muscle in addition to regulating the glucose homeostasis, likely through mediating the expression level of genes related to mitochondria biology.

RESULTS

Our previous studies reported DNMT3A as a mediator of adipose insulin resistance *in vitro* and *in vivo*[29]. While exploring the role of adipose DNMT3A muscle in insulin sensitivity, we noted that DNMT3A levels were also significantly elevated upon high fat diet feeding in the quadriceps muscles of C57BL/6 mice. Given that skeletal muscle is another major tissue, in

addition to the adipose tissue, that helps maintain whole-body glucose homeostasis, we were prompted to investigate whether DNMT3A would have similar functions in skeletal muscle.

Considering the beneficial effect of exercise on the insulin sensitivity and overall glucose homeostasis, we first wanted to check whether DNMT3A is dynamically inducible by the skeletal muscle activity. In an attempt to answer this question, we examined the expression pattern of different DNMTs in various types of skeletal muscle fiber at resting and after a bout of endurance exercise in C57BL/6J wild-type mice. Strikingly, there is a 3-fold increase in both the Dnmt3a transcript and protein levels in the red soleus muscle following exercise (Fig. 1). On the other hand, no obvious changes in the Dnmt3b transcript levels was observed in any type of exercised muscles (Supplemental Figs. 1B-D). These observations further demonstrated that each individual DNMT protein member has its unique role. Meanwhile, we also measured the mRNA expression of PPARγ-coactivator 1α (Ppargc1a), whose expression is believed to be upregulated by exercise in skeletal muscle[30,36]. We noted that Ppargc1a levels increased after 50 minutes of treadmill running, especially in both soleus and gastrocnemius (GA) muscles, which are red and mixed muscle fibers, respectively (Supplemental Fig. 1A). This finding is consistent with the previous reports[30].

To elucidate the specific role played by Dnmt3a in regulating skeletal muscle metabolism and function, we generated muscle-specific Dnmt3a knock-out mice (MCK-KO) using muscle creatine kinase (MCK)-Cre (Fig. 2A-B). While we realize that Dnmt3a was also deleted in the cardiac muscle, MCK-Dnmt3a KO mice exhibited normal development and fertility. The mice were initially put on chow diet, when the KO mice did not display any significant differences in their body weight and body composition compared to their wild-type littermates (Fig. 2C-D). However, the KO male mice exhibit a trend to be more insulin resistant with intact glucose tolerance in comparison with the WT mice (Supplemental Fig. 2). Such trend towards insulin resistance in KO mice became significant after we challenged the mice with high-fat-diet feeding, which was not accompanied by hyperinsulinemia or interrupted insulin signaling pathways (Fig. 3).

Given that the muscle contraction has a beneficial effect on insulin sensitivity, we sought to determine the exercise capacity of mice. Physical exercise can be categorized as “endurance exercise” or “resistance exercise.” Endurance exercise is primarily aerobic and is characterized by high-frequency, long duration, and low power output activity, whereas resistance exercise is anaerobic and is characterized by activities of low frequency, high resistance, high intensity, and short duration[37]. We have employed two different regimens to investigate the effect of Dnmt3a depletion on the capacity of mice to perform endurance exercise. We performed a low-intensity regimen (Supplemental Fig. 3A) which tests the ability to run steadily at a relatively low speed (12 m/min) for an initial 40 mins, then at a gradually increased speed until exhaustion; We also did a high-intensity regimen (Supplemental Fig. 3B) where mice were placed on a treadmill with rapidly increased running speed (6 m/min and increased by 2 m/min every 5 min) to a maximal pace of 30 m/min until exhaustion[38]. We found that the exercise capacity of the MCK-KO mice was impaired in both regimens, but to more extent in the low-intensity regimen, as there was a nearly 50% reduction in both the distance and the duration the KO mice could run for (Fig. 4).

Since cardiac muscle plays an important role in exercise by supplying blood flow to peripheral tissues, and the Cre recombinase in the MCK-Cre line is also expressed in cardiac muscle[39,40], it is possible that there is a cardiac contribution to the exercise incapacity of the KO mice. To tease out this possibility, we thoroughly examined whether there are any cardiac defects in the KO mice that could have led to their exercise intolerance. We did not detect any

conspicuous changes in the overall cardiac appearance and morphology as indicated by the H&E staining (Supplemental Fig. 4A, B), and there was no change in the tissue mass of the heart (Supplemental Fig. 4C). No obvious fibrosis was detected with Sirius Red staining (Supplemental Fig. 4D), and there was no significant change in the expression of genes linked to inflammation, ER stress, fibrosis, and cardiac failure (Supplemental Fig. 4E). Together, these data preliminarily eliminate the possibility of cardiac contribution to the exercise intolerance in MCK-Dnmt3a KO mice. Meanwhile, we're also working on generating an independent line of muscle-specific Dnmt3a KO mice (ACTA-KO) using human alpha-skeletal actin (HAS or ACTA)-Cre-ERT, which exclusively derives the Cre expression in skeletal muscle in a tamoxifen-dependent manner to confirm the results.

As we observed that the KO exercise capacity was mostly affected in the low-intensity regimen, which primarily relies on the red "slow-twich" muscles, we initially thought that deletion of Dnmt3a might have a negative impact on the exercise-induced muscle fiber-type switching. However, despite the observation that the KO muscles seem paler in their appearance, this hypothesis was negated as we found that there was no discernable difference in the expression of muscle fiber type-specific myosin heavy chain isoforms between WT and KO muscles. Therefore, we then proposed that the red muscles are more susceptible to muscle Dnmt3a depletion. To pursue this question, we dissected out each muscle fiber distributed on mice hindlimbs, and found that the MCK-Dnmt3a KO mice on HFD showed a significant decrease in the mass of soleous muscle (Fig. 5). The soleous muscle is a red muscle highly enriched with myoglobin and mitochondria contents, and thus essential in maintaining the oxidative capacity that underpins the endurance exercise. On the other hand, the mass of mixed or white muscles remain unaffected (Fig. 5). Next, we employed various atrophy models to examine the Dnmt3a expression patterns in red soleous muscle specifically. Muscle atrophy, or muscle wasting, is defined as a loss of muscle tissue, and can sometimes be reversed by exercise[41]. A lack of mobility, extended fasting, aging, denervation, and various diseases are some common causes of muscle atrophy[42–45]. Firstly, by comparing the Dnmt3a levels in 2-year-old aged mice and 2-month-old young mice, we found that both the transcripts (Fig. 6A) and protein (Fig. 6B, C) levels of soleus Dnmt3a were greatly reduced in aged mice that have undergone progressive loss of skeletal muscle and declined functional performance[46]. Similarly, we fasted C57BL/6J mice for 24 hours to mimic the fasting-induced muscle wasting. We observed that soleus DNMT3A mRNA and protein expressions were reduced by more than 50% and 80%, respectively, after fasting, and was restored to the basal level upon refeeding (Fig. 6D-F). This finding is in accordance with a previous report that the expression of DNMT3A in skeletal muscle is repressed by 40-hr fasting in human subjects[44]. Taken together, the expression pattern of Dnmt3a in the red soleous muscle is associated with the maintenance of red muscle mass and activity.

Since nuclear mispositioning is a common feature routinely exhibited in muscle wasting conditions or other myopathies independent of muscle regeneration[47], we quantified the dislocated nuclei in the muscle after a single bout of low-intensity exercise as described earlier. In unaffected individuals, myonuclei are supposed to distribute throughout the periphery of the muscle fiber, whereas the nuclei are clustered within the center of the muscle fibers in diseased muscles[48]. Such nuclear mispositioning has also been linked to muscle dysfunction or muscular dystrophy, meaning progressive muscle weakness[49,50]. In accordance with the exercise incapacity of the KO mice, we observed an increased level of myonuclei dislocation in the KO red soleous muscle following exercise (Fig. 7A,B). Interestingly, KO soleus muscles also had decreased fiber

counts but increased fiber surface area (Fig. 7C, D), which might be a sign of sarcoplasmic hypertrophy defined by an increased volume of sarcoplasmic fluid and noncontractile components with no increased muscular strength[51,52].

Reduced mitochondrial density and mitochondrial dysfunction have been widely recognized as contributors to insulin resistance and exercise intolerance[46,53–55]. Therefore, we sought to determine if there's mitochondrial dysfunction upon Dnmt3a depletion. As a result, the mitochondrial biogenesis was reduced by 43% and 47% in the KO red soleus and mixed GA muscles, respectively (Fig. 8A). The overall mitochondrial contents also decreased in the KO red and mixed muscles after exercise, whereas no change was observed in the white muscle extensor digitorum longus (EDL) (Fig. 8A). We confirmed that reduced mitochondria biogenesis results in a reduced tissue-level oxygen consumption rate in the KO soleus muscle (Fig. 8B, C), indicating a mitochondrial dysfunction in Dnmt3a-deficient muscles. Succinate dehydrogenase (SDH) is located in the inner membrane of the mitochondrion and is responsible for oxidizing succinate to fumarate in the citric acid cycle[56]. Therefore, we conducted SDH staining to distinguish between oxidative and less-oxidative muscles and found that both KO soleus and GA muscles had a dramatic decline in the oxidative potential by more than 50% (Fig. 8D-G).

DISCUSSION

It has been reported by genome-wise studies that both acute and chronic exercise alter the DNA methylome of skeletal muscle, which is associated with changes in gene expression[30–35]. For instance, a single bout of aerobic exercise in human subjects transiently induces DNA hypomethylation of transcripts related to mitochondrial biology (e.g., PPARGC1A, PDK4, TFAM, and PPARD), resulting in a time-lagged increase in their expression level[30]. Likewise, another study reported that moderate-intensity exercise results in hypermethylation of FABP3 and COX4L1, which is inversely associated with their expression levels in healthy adult human subjects[34]. However, there is a lack of understanding in whether the DNA methylation events and the changes in gene expression it induces can reciprocally regulate the physiological capacity and metabolism of skeletal muscle.

The role of DNMT3A has been reported in the context of multiple metabolic disorders, including diabetes and obesity[29,57]. In our previous study, we discovered that adipose-specific Dnmt3a knock-out mice are protected from high fat diet-induced insulin resistance and glucose intolerance with no accompanying changes in adiposity[29]. The notion that DNMT3A levels in the mouse quadriceps muscles were also significantly increased upon HFD feeding prompted us to investigate if there is a metabolic function for DNMT3A in skeletal muscle as well. To pursue this question, we first generated muscle-specific knockout mice (MCK-KO) using muscle creatine kinase (MCK)-Cre. We are now also creating an independent line of muscle-specific Dnmt3a KO mice using the human alpha skeletal actin (ACTA1)-Cre mice to confirm the phenotypes observed in the MCK-KO mice. The muscle-specific Dnmt3a KO mice (ACTA-KO) allows the DNMT3A to be depleted exclusively in skeletal muscle without affecting the cardiac muscles. Furthermore, since we will inject tamoxifen to 8-wk-old Dnmt3af/f/ACTACre+ mice to induce the deletion of Dnmt3a, this allows us to also eliminate the developmental defects as a potential explanation for the observed phenotypes.

We observed that mice lacking Dnmt3a in the muscle did not exhibit significant difference in their body weight or body composition as opposed to their WT littermate controls on both chow diet and HFD condition. However, the MCK-KO male mice developed insulin resistance

on chow diet, which was further exacerbated upon HFD feeding. Meanwhile, we also observed that the KO mice exhibited exercise intolerance particularly in the low-intensity exercise regimen. This was accompanied by an increased muscle damage, evidenced by the central-localized myonuclei, as well as decreased mitochondrial contents after a single bout of exercise. Additionally, we also observed a decreased mitochondrial respiration and oxidative capacity in KO mice after a single bout of endurance exercise. Given that the phenotypes of mitochondrial dysfunctions were observed in mouse soleus muscle, which is a red muscle enriched with type I and IIA MHC and mitochondria, this provides a possible explanation for the reduced capacity of the KO mice for low-intensity aerobic exercise during which mice primarily relies on the slow-twitch red muscles[58]. Previously, the link between exercise and mitochondrial biogenesis was mainly established based on the functions of PGC-1α, whose expression can be transiently induced in skeletal muscle by a single bout of exercise and is believed to increase mitochondrial contents[59]. However, there're accumulating evidence showing that PGC1A only has a mild effect on exercise capacity and does not alter fiber-type composition in muscle[60]. This indicates that PGC1A is not the only mediator of the metabolic adaptations in response to exercise. As we failed to detect any obvious changes in the expression of PGC1A upon DNMT3A depletion, we speculate that DNMT3A's function in the regulation of mitochondrial function and mass is likely to be PGC1A-independent. Moreover, it has been proposed that the proportion of slow-twitch fiber proportion in skeletal muscle correlates with insulin responsiveness[61,62]. There were reported to be fewer type I fibers in metabolic syndrome subjects, which is negatively associated with the severity of insulin resistance[61]. Therefore, we propose that the incapacity of the slow-twitch muscles, such as the soleous muscle, can potentially be involved in the onset of insulin resistance through declined mitochondrial contents and mitochondrial dysfunctions.

While whether perturbations in the functions of mitochondria per se are central to the pathophysiology of insulin resistance in skeletal muscle remains controversial[63,64], an increased mitochondrial oxidants seems a more consistent and robust feature of insulin resistance in multiple models[65–68]. The production of mitochondrial oxidants, also known as intracellular reactive oxygen species (ROS) naturally occurs as a consequence of aerobic metabolism[69]. When moderately produced, the reactive oxygen species are essential for modulating the singaling pathways of several physiological processes leading to adaptive cellular responses[70,71]. However, chronic ROS production, or oxidative stress, is suggested to be an important contributor to the pathogenesis of obesity-associated insulin resistance[65] and is associated with the endothelial, renal, and neural complications associated with hyperglycemia in late-stage diabetes[72,73]. Chronic exposure to high levels of ROS has also been associated with increased mitochondrial damage and muscle dysfunction, including muscular dystrophy, and age-related sarcopenia[74,75]. Numerous studies have proved that genetic or pharmacologic interventions to detoxify superoxide radical anion ($O2^{\cdot-}$) or hydrogen peroxide (H2O2) specifically in mitochondria overcome insulin resistance[68,76,77]. While mitochondria are the major sites of ROS production, extramitochondrial oxygen consumption can also produce ROS[78]. One primary sources of ROS include the NADPH oxidase (NOX) complex[79], which utilizes NADPH as an electron donor to produce the ROS superoxide (O2-)[80].

Our RNA-Seq data (not shown) profiling the transcriptomes of WT and KO soleus muscle identified Aldh1l1 as an upregulated gene in KO tissues. Aldh1l1 encodes Aldehyde Dehydrogenase 1 Family Member L1 (ALDH1L), a cytosolic enzyme that is involved in folate and one-carbon metabolism by oxidizing 10-formyltetrahydrofolate to tetrahydrofolate, producing NADPH as a byproduct[81]. The RNA-seq results also revealed that several antioxidant

genes such as SOD1, GPX were upregulated in KO Soleus muscle after a single bout of running, which is possibly a compensatory mechanism trying to mitigate the high ROS levels. Future studies are warranted to address whether the increased oxidative stress and muscle dysfunction were resulted from an elevated ROS levels in KO tissues and have eventually led to the insulin resistance observed in KO animals.

The rising prevalence of obesity and its comorbidities have become a global health concern. Considering the formidable capacity of skeletal muscle for energy expenditure through exercise and its indispensable role in maintaining the host glucose and lipid homeostasis, it is important to unravel the role for DNA methylation in mediating the exercise-induced metabolic outcomes. In the present study, we demonstrate that DNMT3A plays a vital role in skeletal muscle biology with impacts on whole-body glucose homeostasis and exercise capacity. It is of great importance to further understand how the targets of DNMT3A mechanistically contribute to the increased oxidative stress, mitochondrial dysfunction, and insulin resistance in the Dnmt3a-KO animals. Meanwhile, it is also noteworthy that, in addition to Dnmt3a, exercise also induced in soleus muscle the expression of Tet2 and Tet3, which are the enzymes that catalyze DNA demethylation. Therefore, it is conceivable that TET proteins are also involved in some exercise-induced gene expression alterations, and are likely working to wipe off some of the DNMT-mediated effects.

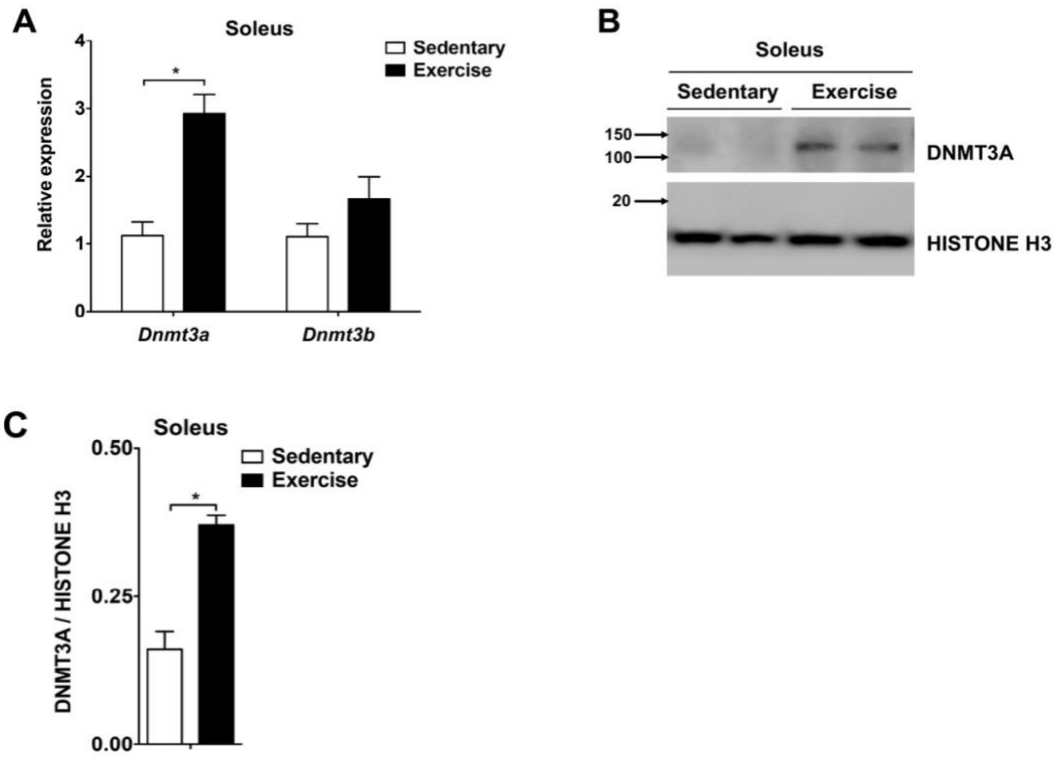


Fig. 1. Soleus Dnmt3a levels are upregulated by exercise. (A) Transcript levels of de novo Dnmts were measured in soleus from C57BL/6J mice at rest and after a bout of endurance exercise (n = 6 mice, $p < 0.05$, Student's t-test, mean ± s.e.m.). (B) Soleus DNMT3A protein level in the nuclear fraction from A was measured by immunoblotting and (C) normalized to Histone H3 using ImageJ.

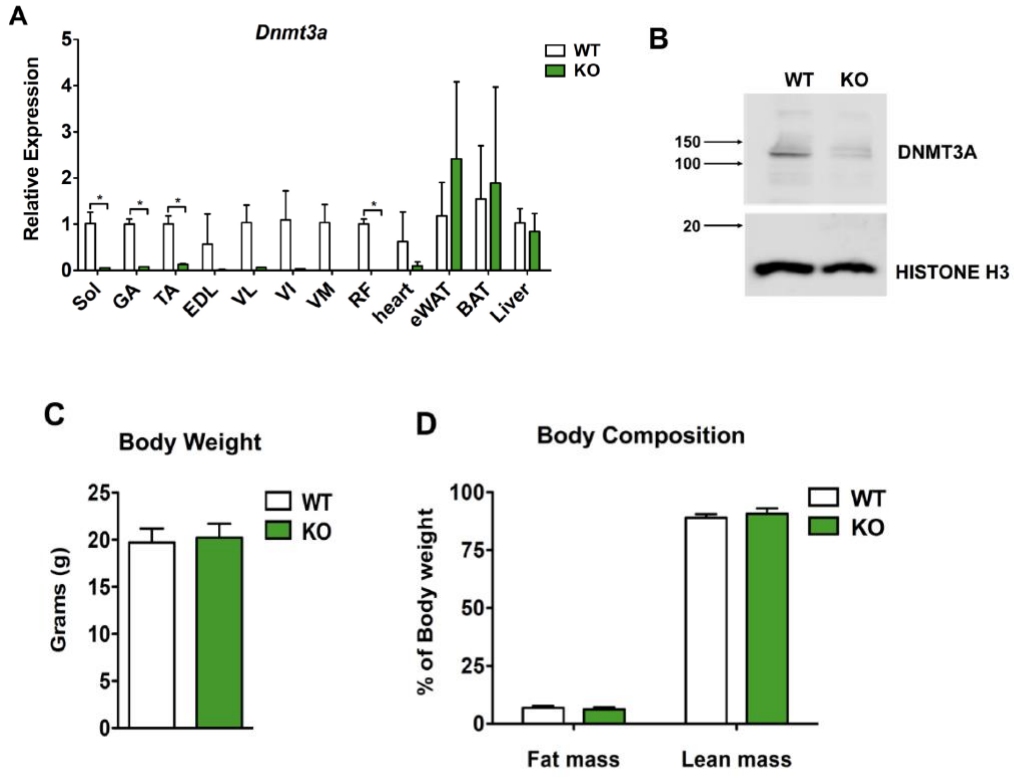


Figure 2. Muscle-specific knockout of Dnmt3a in MCK-Dnmt3a KO mice and basic physical characterization. (A) Dnmt3a mRNA expression was measured in various tissues from KO and WT mice by qPCR analysis (n = 5 mice, $p < 0.05$, Student's t-test, mean ± s.e.m.), (Sol: Soleus, GA: Gastrocnemius, TA: Tibialis anterior, EDL: Extensor digitorum longus, VL: Vastus lateralis, VI: vastus intermedius, VM: Vastus medialis, RF: Rectus femoris, eWAT: epididymal white adipose tissue, BAT; brown adipose tissue). (B) DNMT3A protein expression was assessed by immunoblotting using nuclear extract from KO and WT soleus muscle. (C) Body weight (n = 5 mice, $p < 0.05$, Student's t-test, mean ± s.e.m.). (D) Body composition (n = 5 mice, $p < 0.05$, Student's t-test, mean ± s.e.m.). (D) Muscle mass (n = 5 mice, $p < 0.05$, Student's t-test, mean ± s.e.m.).

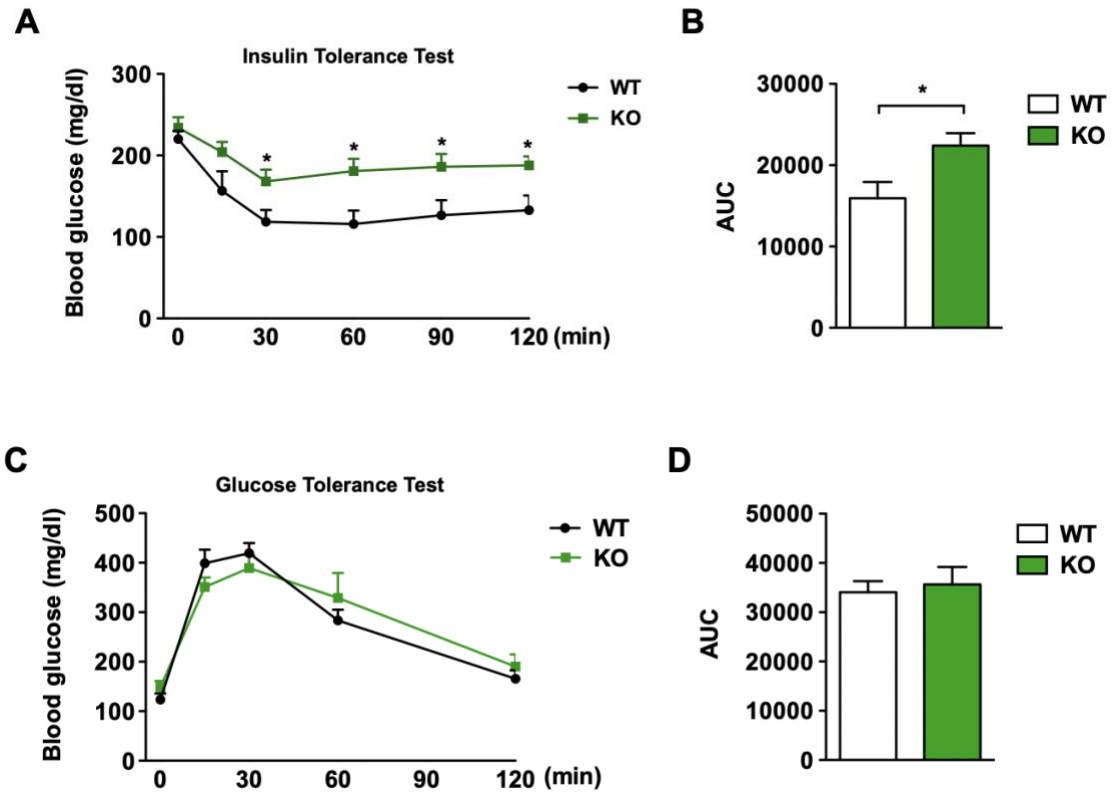


Figure 3. MCK-Dnmt3a KO male mice exhibit insulin resistance with intact glucose tolerance on HFD. (A) Insulin tolerance test after 5 weeks on HFD (n= 5 $p < 0.05$, Student's t-test, mean ± s.e.m.). (B) The area under curve (AUC) in (A). (C) Glucose tolerance test after 5 weeks on HFD (n=5, $p < 0.05$, Student's t-test, mean ± s.e.m.). (D) AUC in (C).

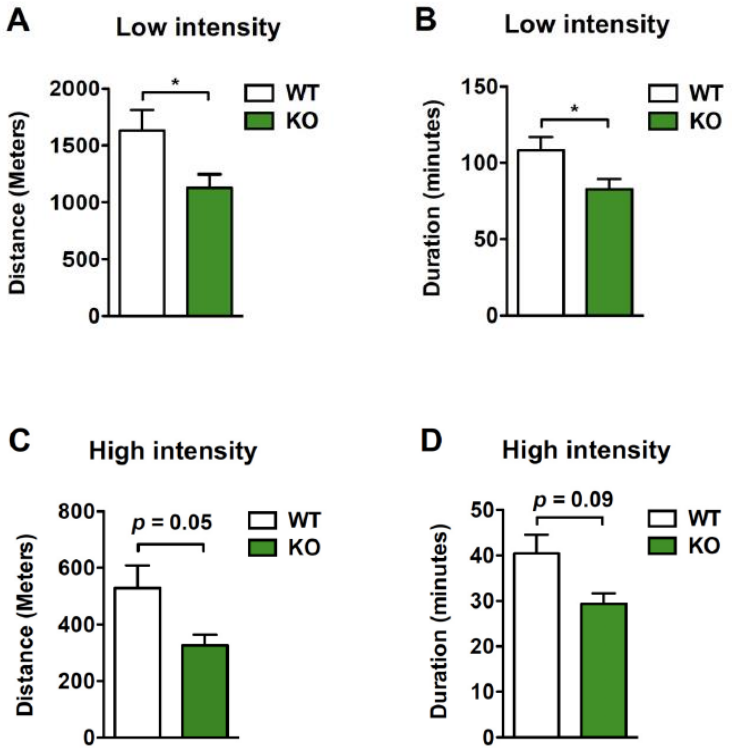


Fig. 4. MCK-Dnmt3a KO male mice have impaired exercise capacity. Low-intensity (A, B) and high-intensity (C, D) exercise were performed in chow-fed WT and KO male mice, and total distance and duration are presented (n = 5 mice, $p < 0.05$, Student's t-test, mean ± s.e.m.).

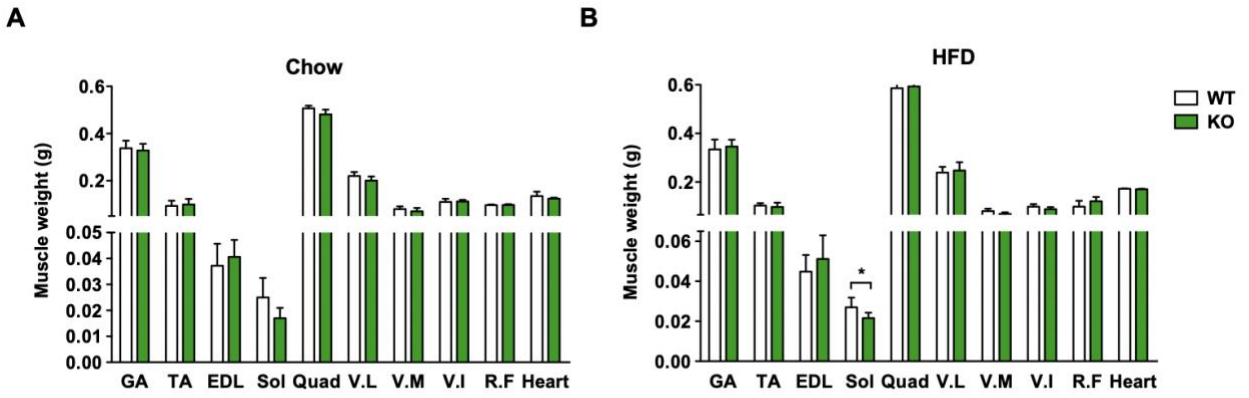


Fig. 5. MCK-Dnmt3a KO male mice on HFD have reduced mass of soleous muscle. (A) mass of each muscle fiber from WT and KO mice on Chow diet (B) mass of each muscle fiber from WT and KO mice on HFD for 12 weeks (n = 5 mice, $p < 0.05$, Student's t-test, mean ± s.e.m.).

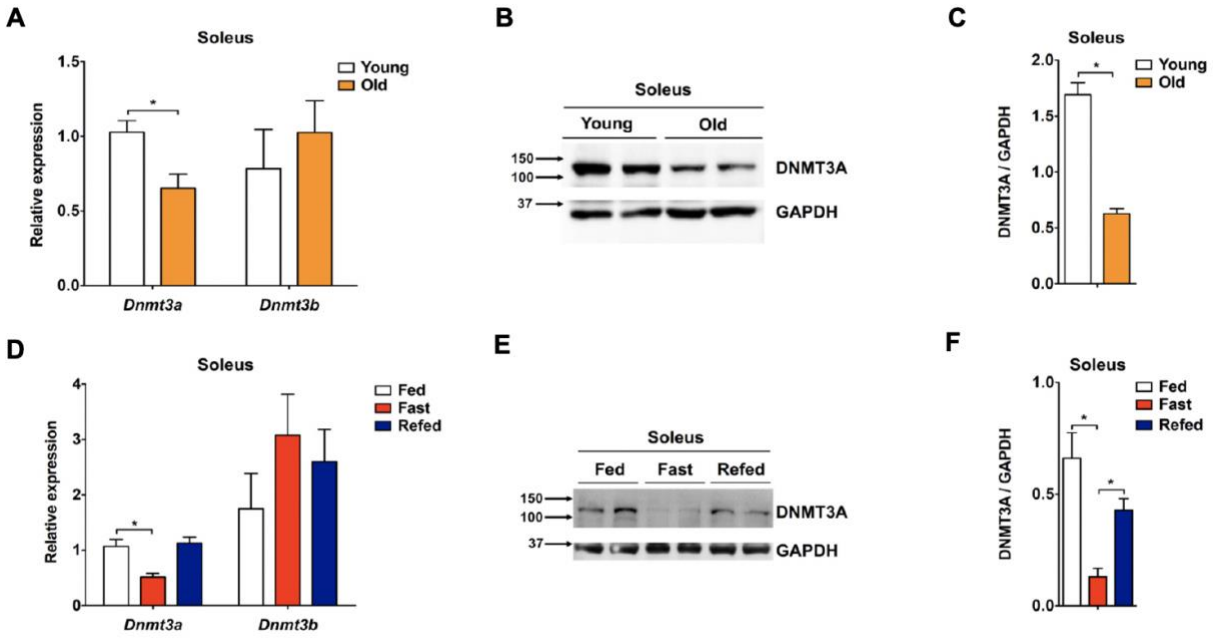


Fig. 6. Soleus Dnmt3a levels are regulated in atrophied conditions. (A) Transcript levels of de novo Dnmts were measured in soleus muscle from 2 month- and 2-year-old C57BL/6J mice (n = 10 mice, $p < 0.05$, Student's t-test, mean ± s.e.m.). (B) Soleus DNMT3A protein level from A was measured by immunoblotting and (C) normalized to GAPDH using ImageJ. (E) Transcript levels of Dnmts were measured in soleus from C57BL/6J mice at fed, after 24 hours fasting, and 24 hours of refed (n = 10 mice, $p < 0.05$, Student's t-test, mean ± s.e.m.). (F) Soleus DNMT3A protein level from E was measured by immunoblotting and (G) normalized to GAPDH using ImageJ.

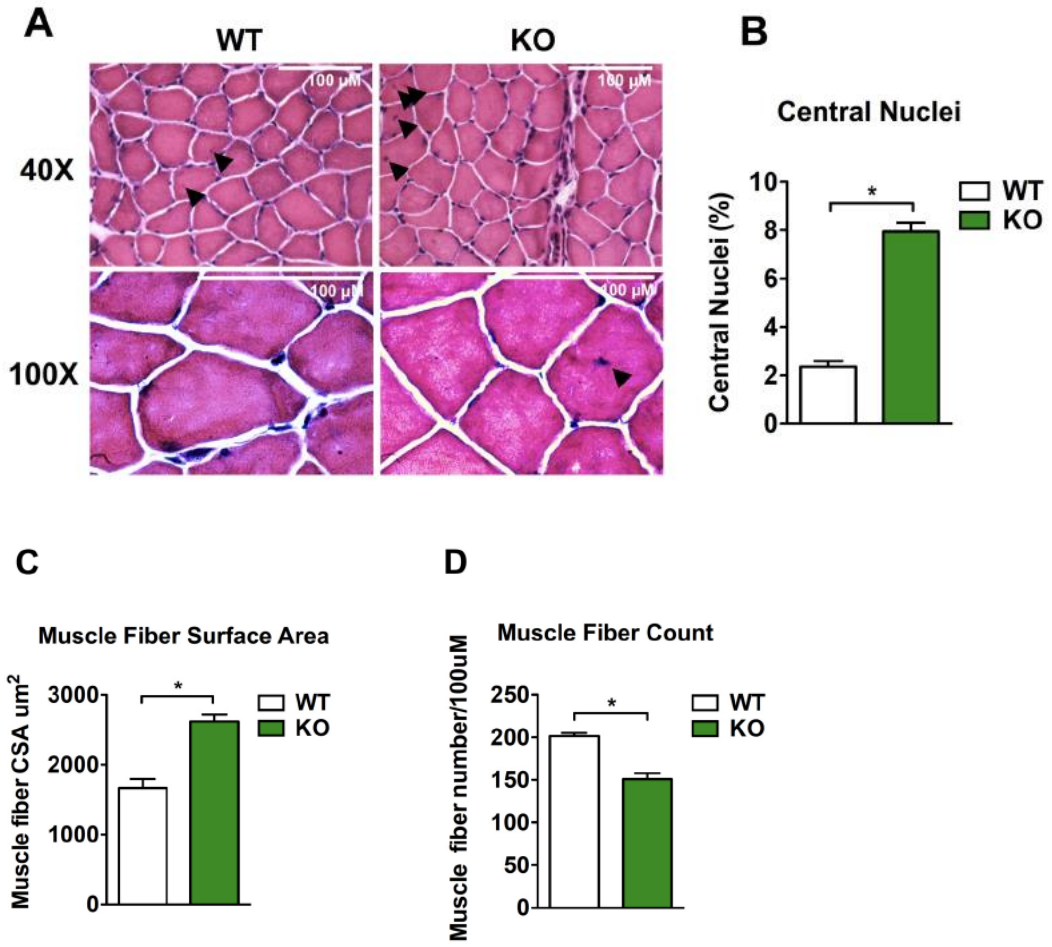


Fig. 7. Dnmt3a-KO muscle displays increased muscle damage and myopathy following exercise. (A) The H&E staining of KO and WT soleus after a bout of low-intensity treadmill running equally for 50 min (top; 40X, bottom; 100X magnification). Black arrow indicates nuclei that have migrated to the center of the myofiber. (B) The percentage of myofibers with centralized nuclei from A was determined by manually counting 100–150 myofibers. Muscle fiber surface area (C) and muscle fiber count (D) of KO and WT soleus was determined by measuring diameter and muscle fiber by ImageJ (CSA; Cross sectional area), (n = 3 mice, $p < 0.05$, Student's t-test, mean ± s.e.m.).

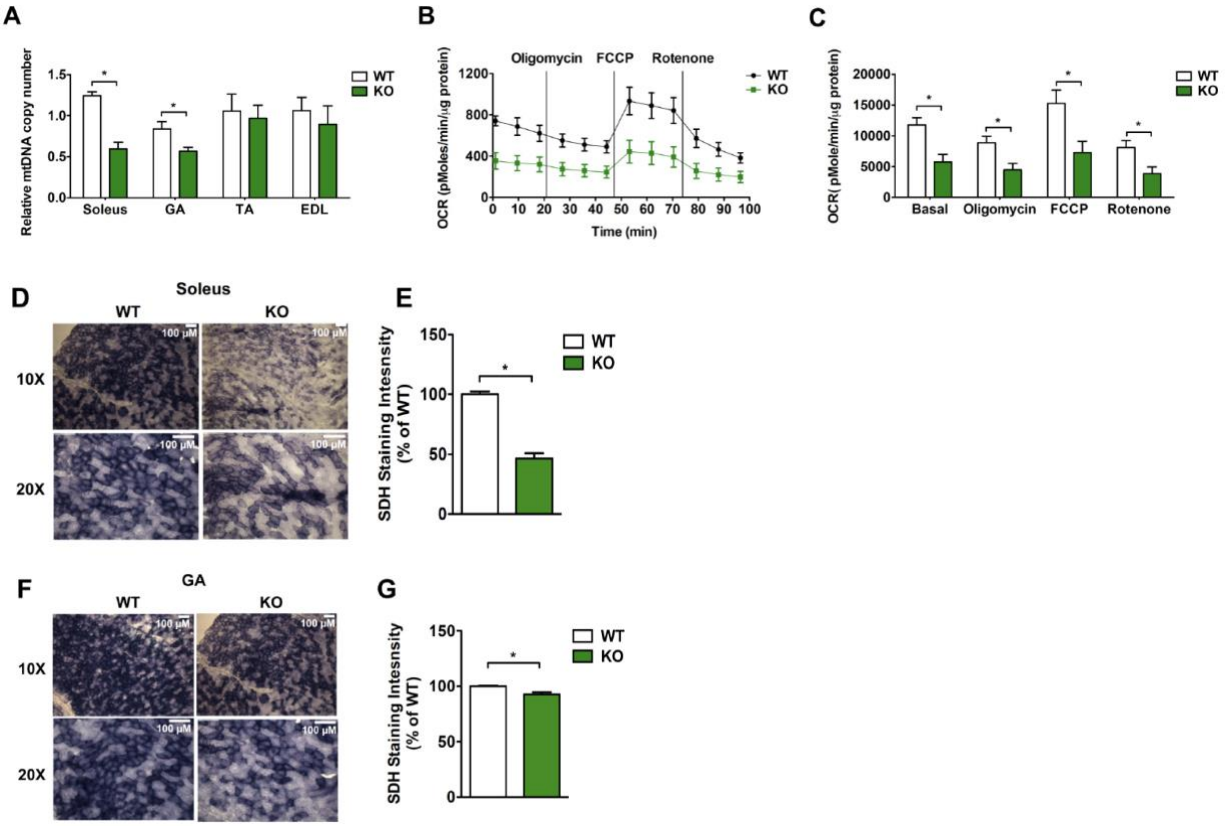
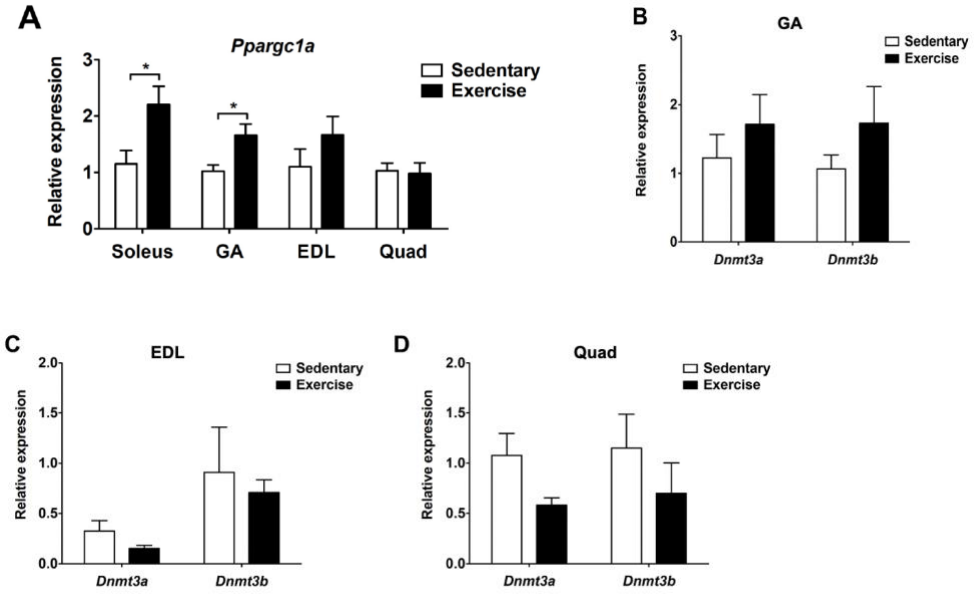
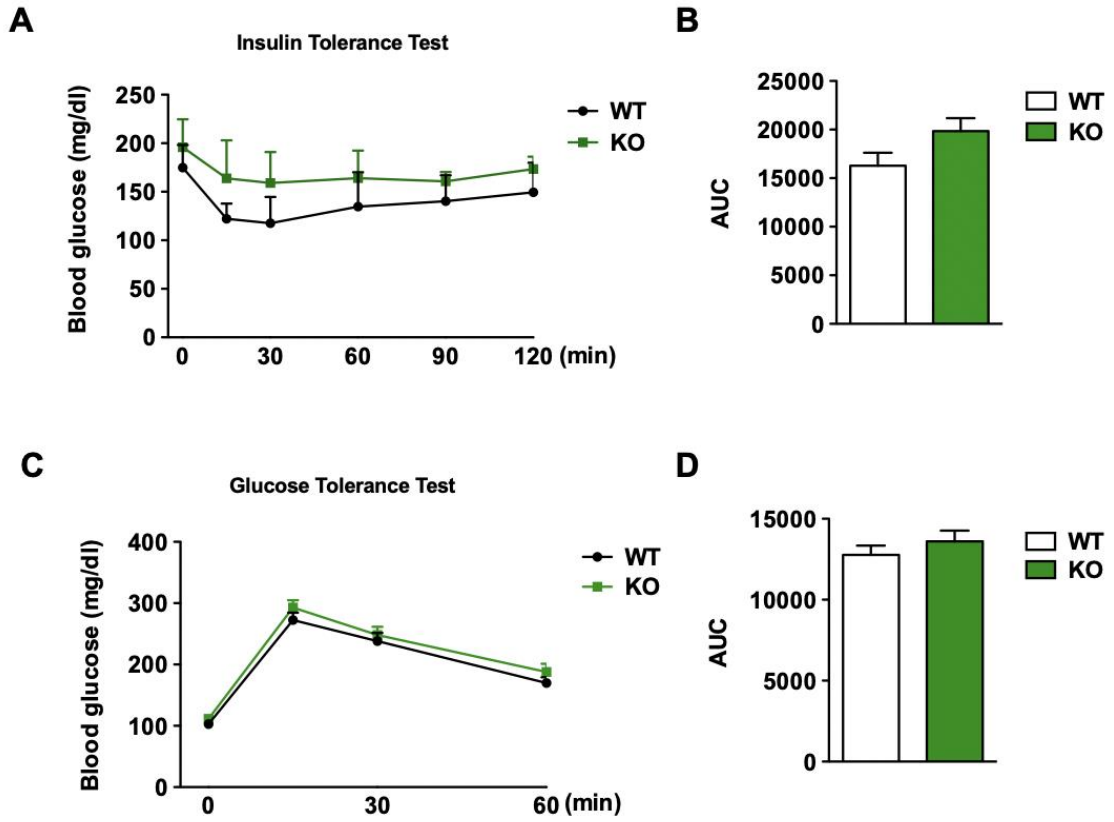


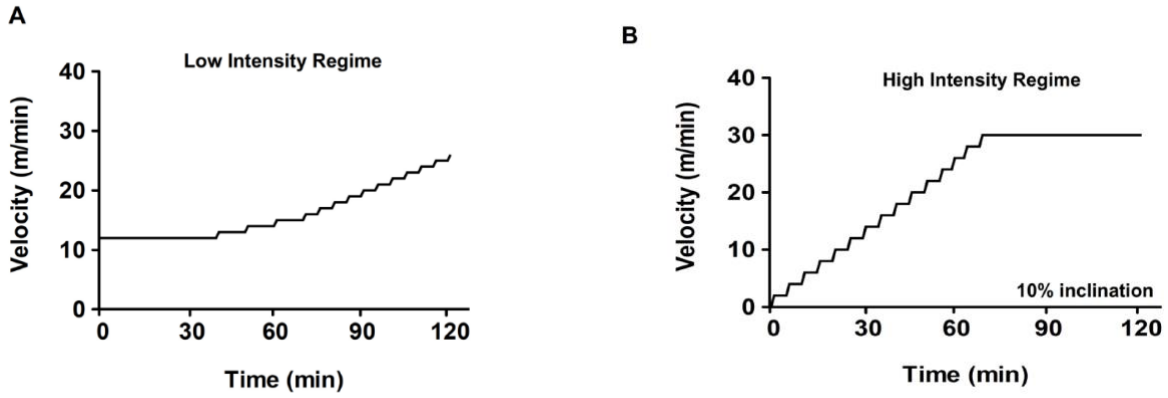
Fig. 8. Dnmt3a-KO soleus muscle displays reduced mitochondrial content and mitochondrial respiration with reduced oxidative capacity. (A) Genomic DNA was extracted from various muscle types from WT and KO mice after a bout of low-intensity exercise for 50 min. Mitochondrial DNA copy-number was calculated from the ratio of mitochondria-encoded COXII to nuclear-encoded cyclophilin A (n = 6 mice, $p < 0.05$, Student's t-test, mean ± s.e.m.). (B, C) Mitochondrial respiration was measured in WT and KO soleus tissue after a bout of low-intensity exercise for 50 min under basal conditions and in response to 1.5 mM oligomycin (complex V inhibitor), 4 mM FCCP (uncoupler), or 2 mM rotenone (complex I inhibitor) (n = 3, $p < 0.05$, Student's t-test, mean ± s.e.m.). (D-G) Succinate dehydrogenase staining was performed in WT and KO soleus and GA muscles after a bout of low-intensity exercise for 50 min (10X, 20X magnifications), and the staining intensity was quantified using ImageJ.



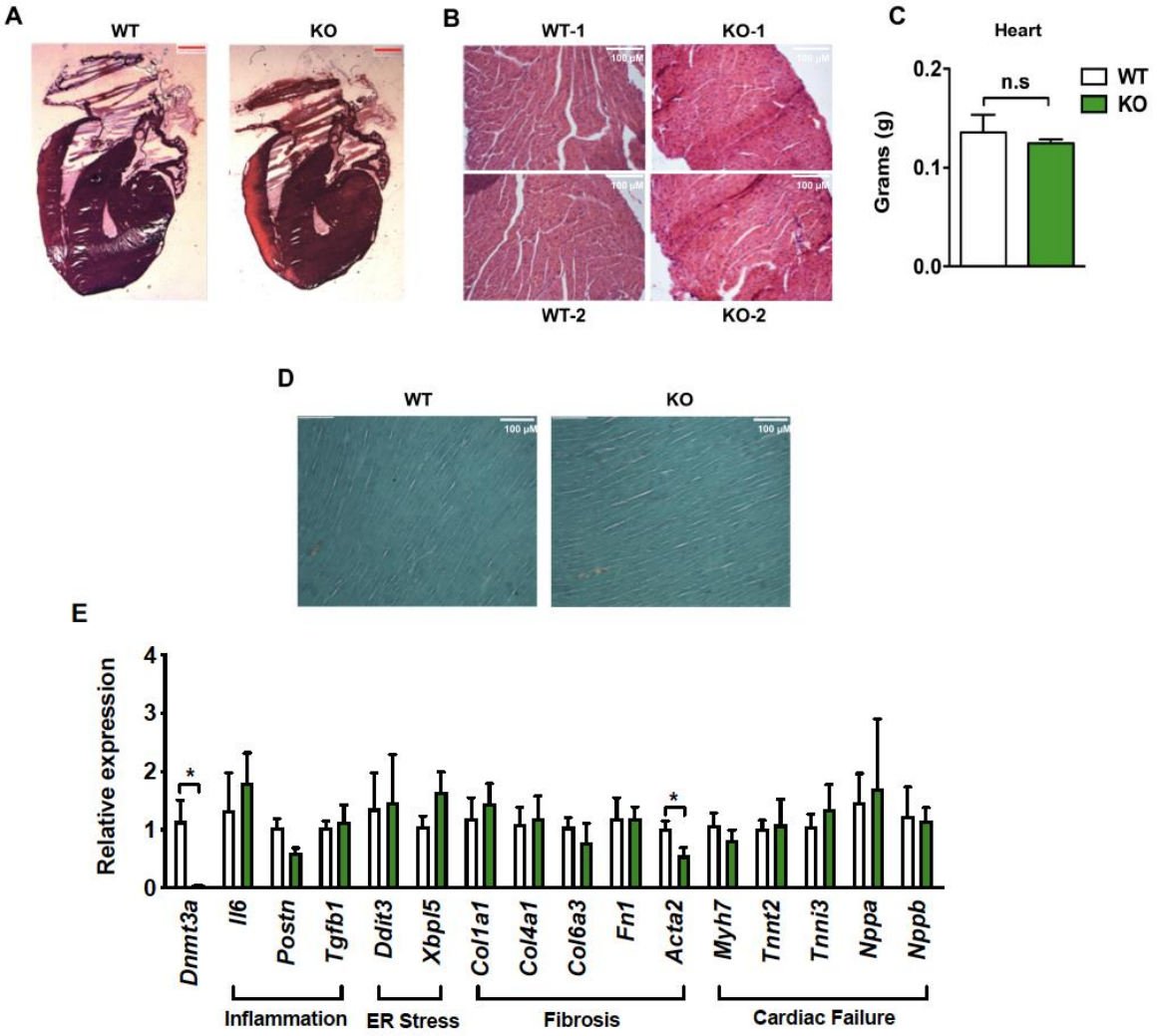
Supplemental Fig 1. (A) Ppargc1a was measured in various muscle types from C57BL/6J mice at rest and after a bout of low-intensity exercise (n = 6 mice, $p < 0.05$, Student's t-test, mean ± s.e.m.), GA: Gastrocnemius; EDL: Extensor digitorum longus; Quad: Quadraceps). (B-D) The mRNA expression of de novo Dnmts was measured in GA (B), EDL (C), and Quad (D) from C57Bl/6J mice at rest and after a bout of low-intensity exercise (n = 6 mice, $p < 0.05$, Student's ttest, mean ± s.e.m.).



Supplemental Fig 2. MCK-Dnmt3a KO male mice exhibit a trend towards insulin resistance with intact glucose tolerance on chow diet. (A) Insulin tolerance test for 7-week old mice on chow diet (n= 5 $p < 0.05$, Student's t-test, mean ± s.e.m.). (B) The area under curve (AUC) in (A). (C) Glucose tolerance test for 7-week old mice on chow diet (n=5, $p < 0.05$, Student's t-test, mean ± s.e.m.). (D) AUC in (C).



Supplemental Fig 3. Schematic representation of exercise regimens. (A) Low-intensity and (B) high-intensity exercise regimens.



Supplemental Fig 4. MCK-Dnmt3a KO mice exhibit no apparent cardiac defects. (A, B) H&E staining of the whole heart (A, whole tissue view) and cardiac sections of WT and KO mice (B, 10X magnification). (C) Tissue weight of heart from chow-fed WT and KO mice. (n = 5 mice, $p < 0.05$, Student's t-test, mean ± s.e.m.). (D) Sirius Red staining that assesses fibrosis (20X magnification). (E) Relative mRNA expression of genes involved in indicated biological pathways. (n = 5 mice, $p < 0.05$, Student's t-test, mean ± s.e.m.).

METHODS

Animals
Animal Care Mice were maintained under a 12-hr light /12-hr dark cycle at constant temperature (23°C) with free access to food and water. All mice were extensively back-crossed onto a C57Bl/6J background. All animal work was approved by UC Berkeley and the University of IOWA ACUC. In vivo assays were done with 7- to 20-week-old littermate male mice.

Measurement of exercise capacity
All mice were acclimated to the treadmill prior to the exercise test session. For each session, food was removed 2 hr before exercise. Acclimation began at a low speed of 5 to 8 meters per minute (m/min) for a total of 10 min on Day 1 and was increased to 5 to 10 m/min for a total of 10 min on Day 2. The experiments were performed on Day 3. For the low intensity treadmill test, the treadmill began at a rate of 12 m/min for 40 min. After 40 min, the treadmill speed was increased at a rate of 1 m/min every 10 min for a total of 30 min, and then increased at the rate of 1 m/min every 5 min until the mice were exhausted. The high intensity treadmill test was conducted on the same open-field six-lane treadmill set at a 10% incline. Following a 5-min 0 m/min acclimation period, the speed was raised to 6 m/min and increased by 2 m/min every 5 min to a maximal pace of 30 m/min until exhaustion. Mice were considered exhausted when they were unable to respond to continued prodding with a soft brush.

RNA extraction and quantitative PCR
Total RNA was extracted from tissues using TRIzol reagent according to the manufacturer's instructions. cDNA was reverse transcribed from 1 μg of RNA using the cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative PCR (qPCR) was performed with SYBR green qPCR master mix (AccuPower 2X, Bioneer) using a CFX96 Touch (Bio Rad). The relative amount of mRNA normalized to cyclophilin B was calculated using the delta–delta method. Primer sequences are listed in Supplemental Table 1.

Western blot analysis and antibodies.
Whole-cell protein lysates were prepared according to the manufacturer's protocol using RIPA lysis buffer and protease inhibitor cocktail. Proteins were size fractionated by SDS-PAGE and then transferred to polyvinylidene difluoride membranes. After blocking with 5% nonfat dried milk in TBS-Tween (0.25%), the membranes were incubated with the appropriate primary antibodies against antiDNMT3A antibodies (#2160S). The loading control included anti-GAPDH (#2118) and anti-Histone H3 (#14269). Immunoblots were quantified by ImageJ.

Histological analysis of skeletal and cardiac muscle fibers
Harvested skeletal muscles were immediately embedded in T.F.M. compound (Tissue-Tek) and snap frozen using a Stand-Alone Gentle Jane (Instrumedics Inc.). We then prepared 10 μm sections from the muscle mid-belly using a Leica cryostat. Hematoxylin and eosin (H&E) staining was performed following fixation in ice-cold zinc formalin (Anatech Ltd. #175) for 60 min. Image analysis was performed using ImageJ software. For ATPase stains, slow-type fibers were dark, whereas fast-type fibers were lightly stained following preincubation at pH 4.35. Fiber type was identified on the basis of color for each myofiber. For Sirius Red staining, cardiac sections were then incubated in Bouins' solution (5% acetic acid, 9% formaldehyde, and 0.9%

picric acid) at room temperature for 1 hr. Next, after washing, slides were incubated in 0.1% Fast Green (Fisher, F-99) for 3 min, then in 0.1% Sirius Red (Direct red 80, Sigma, 0-03035) for 2 min. After three washes, slides were dehydrated with ethanol and xylene based on standard procedures.

Muscle tissue respiration

Mice were allowed to run for 50 min on the low-intensity regime. Following this, WT and MCK-KO soleus tissues were isolated and seeded in XF24 plates (catalog #100777-004, Seahorse Bioscience). Measurement of intact tissue respiration was performed using the Seahorse XF24 analyzer (Seahorse Bioscience). Oxygen consumption rates (OCRs) (picomoles of O2 per minute) were measured under basal conditions after three consecutive injections of the following: (1) oligomycin (ATP synthase inhibitor; 4 μM); (2) the electron transport chain accelerator ionophore FCCP (4 μM; FCCP treatment gives the maximal OCR capacity of the cells); and (3) the electron transport chain inhibitor Rotenone(2uM)+Antimycin A (2uM), which stops respiration.

Metabolic cages

Indirect calorimetry and food intake, as well as locomotor activity, were measured using the Comprehensive Lab Animal Monitoring System (CLAMS) (Columbus Instruments). The calorimetry system is an open-circuit system that determines O2 consumption, CO2 production, and RER. Data were collected after 3 hr of adaptation in acclimated singly housed mice.

Supplemental Table 1. Oligonucleotide sequences

Q-PCR	Cyclophilin_F	GGTGGAGAGCACCAAGACAGA
Q-PCR	Cyclophilin_R	GCCGGAAGTCGACAATGATG
Q-PCR	Dnmt1_f	GAGGACTGCAACGTGCTTCT
Q-PCR	Dnmt1_r	GAGCGTCTGTAGGACACGAA
Q-PCR	Dnmt3a_f	GTGGAGCCTGAAGCAGCTG
Q-PCR	Dnmt3a_r	CTGGCACATGCCTCCAATGAA
Q-PCR	Dnmt3b_f	CCATGGTGGTGTCCTGGAAA
Q-PCR	Dnmt3b_r	CAGGACTGCTGGAGAAGGTCT
Q-PCR	Tet1-f	CCCAGACTCCTTAACTTGCA
Q-PCR	Tet1-r	CTCGTCCTGGATATTATGTGTAC
Q-PCR	Tet2-f	AGAGCCTCAAGCAACCAAAA
Q-PCR	Tet2-r	ACATCCCTGAGAGCTCTTGC
Q-PCR	Tet3-f	CCGGATTGAGAAGGTCATCTAC
Q-PCR	Tet3-r	AAGATAACAATCACGGCGTTCT
Q-PCR	Ppargc1a_f	AGCCGTGACCACTGACAACGAG
Q-PCR	Ppargc1a_r	GCTGCATGGTTCTGAGTGCTAAG
Q-PCR	CoxII_f	CAGGCCGACTAAATCAAGCAAC
Q-PCR	CoxII_r	CTAGGACAATGGGCATAAAGCT
Q-PCR	Il6_f	CTCTGGGAAATCGTGGAAAT
Q-PCR	Il6_r	CCAGTTTGGTAGCATCCATC
Q-PCR	Ddit3_f	TATCTCATCCCCAGGAAACG
Q-PCR	Ddit3_r	TGGGCCATAGAACTCTGACTG
Q-PCR	Postn_f	CCTGCCCTTATATGCTCTGCT

Q-PCR	Postn_r	AAACATGGTCAATAGGCATCACT
Q-PCR	Acta2_f	GTCCCAGACATCAGGGAGTAA
Q-PCR	Acta2_r	TCGGATACTTCAGCGTCAGGA
Q-PCR	Tgfb1_f	CAACAATTCCTGGCGTTACCTTGG
Q-PCR	Tgfb1_r	GAAAGCCCTGTATTCCGTCTCCTT
Q-PCR	Col4a1_f	CCATGTCCATGGCACCCATC
Q-PCR	Col4a1_r	ACCGCACACCTGTTTTCCAA
Q-PCR	Col6a3_f	AACCCTCCACATACTGCTAATTC
Q-PCR	Col6a3_r	TCGTTGTCACTGGCTTCATT
Q-PCR	Myh7_f	GATGTTTTTGTGCCCGATGA
Q-PCR	Myh7_r	CAGTCACCGTCTTGCCATTCT
Q-PCR	Tnnt2_f	CTGAGACAGAGGAGGCCAAC
Q-PCR	Tnnt2_r	TTCTCGAAGTGAGCCTCGAT
Q-PCR	Tnni3_f	GAAGCAGGAGATGGAACGAG
Q-PCR	Tnni3_r	TTAAACTTGCCACGGAGGTC
Q-PCR	Nppa_f	CAACACAGATCTGATGGATTTCA
Q-PCR	Nppa_r	CCTCATCTTCTACCGGCATC
Q-PCR	Nppb_f	GTCAGTCGTTTGGGCTGTAAC
Q-PCR	Nppb_r	AGACCCAGGCAGAGTCAGAA
Q-PCR	Fn1_f	GCAAACCTATAGCTGAGAAGTG
Q-PCR	Fn1_r	CAAGTACAGTCCACCATCATC
Q-PCR	Tgfb1_f	CAACAATTCCTGGCGTTACCTTGG
Q-PCR	Tgfb1_r	GAAAGCCCTGTATTCCGTCTCCTT

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