AMPKα is essential for acute exercise-induced gene responses but not for exercise training-induced adaptations in mouse skeletal muscle.

Joachim Fentz¹, Rasmus Kjøbsted¹, Caroline Maag Kristensen², Janne Rasmus Hingst¹, Jesper Bratz Birk¹, Anders Gudiksen², Marc Foretz³,⁴,⁵, Peter Schjerling⁶, Benoit Viollet³,⁴,⁵, Henriette Pilegaard², Jørgen F.P. Wojtaszewski¹

¹Section of Molecular Physiology, the August Krogh Centre, Department of Nutrition, Exercise and Sports, University of Copenhagen, Copenhagen, Denmark

²The Centre of Inflammation and Metabolism, Department of Biology, University of Copenhagen, Copenhagen, Denmark.

³INSERM, U1016, Institut Cochin, Paris, France.

⁴CNRS, UMR8104, Paris, France.

⁵Université Descartes, Sorbonne Paris Cité, Paris, France.

⁶Institute of Sports Medicine, Department of Orthopedic Surgery, Bispebjerg Hospital and Center for Healthy Aging, Faculty of Medical and Health Sciences, University of Copenhagen, Copenhagen, Denmark.

Short running title: AMPKα in skeletal muscle exercise adaptations

Key words: PGC-1α, wheel running, UQCRC1, mitochondria

Abstract word count: 249

Word Count: 6516

Number of figures: 7

Number of tables: 3

Number of references: 66

Corresponding author:

Jørgen F.P. Wojtaszewski, PhD

The August Krogh Centre
Abbreviation list

AICAR 5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside
AMPK 5’AMP-activated protein kinase
β-GPA β-guanadinopropionic acid
βHAD β-hydroxyacyl coenzyme A dehydrogenase
BC basal cohort
CD31 cluster of differentiation 31
CD36 cluster of differentiation 36
COX-I cytochrome C oxidase subunit I (subunit of ETC complex IV)
CS citrate synthase
Cyt C cytochrome C
ERD equal running distance
ETC electron transport chain
FABPpm plasma membrane fatty acid binding protein
FATP1 fatty acid transport protein 1
FATP4 fatty acid transport protein 4
GLUT4 glucose transporter 4
HDAC5 histone deacetylase 5
HKII hexokinase II
KD kinase-dead
KO knockout
| 57 | LKB1  | serine/threonine kinase 11 |
| 58 | mdKO  | muscle-specific AMPKα1α2 double KO |
| 59 | MEF2  | myocyte enhancer factor 2 |
| 60 | MuRF1 | muscle RING finger 1 |
| 61 | ND6   | NADH-ubiquinone oxidoreductase chain 6 (subunit of ETC complex I) |
| 62 | Nur77 | nuclear receptor subfamily 4 group A member 1 |
| 63 | PGC-1α| peroxisome-proliferator-activated receptor γ coactivator-1α |
| 64 | PKD   | protein kinase D |
| 65 | RTC   | running test cohort |
| 66 | SDHA  | succinate dehydrogenase complex subunit A (subunit of ETC complex II) |
| 67 | SIRT1 | silent information regulator T1 |
| 68 | TTE   | time to exhaustion |
| 69 | UQRC1 | ubiquinol-cytochrome-C reductase core protein 1 (subunit of ETC complex III) |
| 70 | WT    | wild type |
Abstract

Exercise training increases skeletal muscle expression of metabolic proteins improving the oxidative capacity. Adaptations in skeletal muscle by pharmacologically induced activation of 5’AMP-activated protein kinase (AMPK) are dependent on the AMPKα2 subunit. We hypothesized that exercise training-induced increases in exercise capacity and expression of metabolic proteins as well as acute exercise-induced gene regulation would be compromised in AMPKα1 and –α2 muscle-specific double knockout (mdKO) mice. An acute bout of exercise increased skeletal muscle mRNA content of cytochrome C oxidase subunit I, glucose transporter 4 and VEGF in an AMPK-dependent manner, while cluster of differentiation 36 and fatty acid transport protein 1 mRNA content increased similarly in AMPKα wild type (WT) and mdKO mice. During four weeks of voluntary running wheel exercise training, the AMPKα mdKO mice ran less than WT. Maximal running speed was lower in AMPKα mdKO than WT mice, but increased similarly in both genotypes with exercise training. Exercise training increased quadriceps protein content of ubiquinol-cytochrome-C reductase core protein 1 (UQCRC1), cytochrome C, hexokinase II, plasma membrane fatty acid binding protein and citrate synthase activity more in AMPKα WT than mdKO muscle. However, analysis of a subgroup of mice matched for running distance revealed that only UQCRC1 protein content increased more in WT than mdKO mice with exercise training. Thus, AMPKα1 and –α2 subunits are important for acute exercise-induced mRNA responses of some genes and may be involved in regulating basal metabolic protein expression, but seem to be less important in exercise training-induced adaptations in metabolic proteins.
Introduction

Exercise training has the potential to prevent or offset the development of many health disorders, including the rapidly growing life style related disease type II diabetes (6). Augmentation of muscle angiogenesis, mitochondrial biogenesis and metabolic enzyme expression are important mechanisms in the improved regulation of metabolic homeostasis with exercise training (63). A single bout of exercise increases the mRNA content of metabolic proteins in skeletal muscle (40; 50). It is believed that if physical exercise is repeated regularly for a prolonged period (i.e. exercise training), each single stimulus accumulates to create a measurable increase in protein content (56). However, the signaling pathways responsible for mediating the adaptive response of skeletal muscle to a single exercise bout and to exercise training are poorly defined.

Evidence indicates that the energy sensor 5’AMP-activated protein kinase (AMPK) is important in conveying the exercise stimulus to induce biochemical adaptations in skeletal muscles. The regulation of metabolic genes by AMPK is achieved by downstream modulation of deacetylases, transcription factors and transcriptional co-activators. Phosphorylation of histone deacetylase (HDAC)5 by AMPK increases HDAC5 binding to 14-3-3 protein and the subsequent nuclear export of the complex allows transcription factor-mediated regulation of glucose transporter (GLUT)4 gene expression (41). The gene encoding the glucose metabolizing protein hexokinase (HK)II contains a cAMP response element in its promoter region (49) and AMPK regulates the activity of the transcription factor cAMP response element binding protein through direct phosphorylation (53). Experiments performed in vitro and in cell culture have shown that AMPK phosphorylates peroxisome-proliferator-activated receptor γ coactivator (PGC)-1α (23), which is known as a major transcriptional regulator of genes encoding oxidative proteins in skeletal muscle (36). It has been suggested that phosphorylation of PGC-1α allows its subsequent deacetylation by silent information regulator T1 (SIRT1) (8), which is required for PGC-1α mediated gene expression of fatty acid utilization proteins (16). Interestingly, SIRT1 activity is increased through an AMPK-mediated increase in NAD+/NADH ratio (7). Additional studies support an AMPK – PGC-1α pathway mediating mitochondrial and metabolic gene expression/adaptations; both AMPK and PGC-1α regulate gene expression of mitochondrial proteins (cytochrome C (Cyt C), cytochrome C oxidase I (COX-I, subunit of electron transport chain (ETC) complex IV), citrate synthase (CS), ATP-synthase, δ-aminolevulinate synthase, mitochondrial transcription factor A) (22; 25; 35; 62), metabolic and angiogenic proteins (GLUT4, VEGF) (25; 33; 43; 66) and PGC-1α itself (19). A single injection of the AMP-mimetic compound, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) has been shown to activate AMPK and increase PGC-1α, HKII and VEGF mRNA levels in skeletal muscles of wild type (WT), but not AMPKα2 deficient mice (25; 66). Also, mice that carry an activating
AMPKγ mutation (R225Q) display increased basal (15; 37; 46) and exercise-induced mRNA levels (2) along with increased basal protein content of metabolic proteins in skeletal muscle (15). Thus, AMPK signaling seems to control skeletal muscle gene expression by direct and indirect regulation of transcription factors.

Chronic activation of AMPK by AICAR or the ATP-depleting creatine analog β-guanadinopropionic acid (β-GPA) results in numerous biochemical adaptations that are also induced by exercise training in skeletal muscle of rodents. These include increased protein content of GLUT4 and HKII in addition to mitochondrial enzyme content and activity (3; 20; 25; 32; 58; 65), as well as increased running endurance in mice (45). Importantly, the β-GPA- and AICAR-induced biochemical adaptations are not observed in mice lacking AMPKα2 catalytic activity (25; 65) proposing that AMPKα2 is essential in mediating these effects.

Acute exercise increases AMPK activation in human (9; 14; 61) and rodent skeletal muscle (57) and electrical stimulation of rodent muscle also potently increases the activity of AMPK (26; 55). Contraction and exercise primarily increase activation of the AMPKα2 isoform rather than the AMPKα1 isoform (14; 26; 55; 57; 61). However, both exercise-induced regulation of metabolic mRNA levels and exercise-induced regulation of metabolic proteins are essentially normal in AMPKα2 deficient mice (1; 25; 27). A heightened contraction-induced activity of AMPKα1 in AMPKα2 knockout (KO) mouse muscle could suggest that AMPKα1 may compensate for the lack of AMPKα2 to induce normal mRNA and protein responses (27). Supporting this, mice lacking LKB1 (serine/threonine kinase 11) or both of the β regulatory subunits of AMPK in skeletal muscle seem less capable of adapting to acute exercise and exercise training (48; 52). Thus, a more complete ablation of AMPK activity, either by deleting the primary upstream kinase LKB1 or by disrupting both β isoforms, appears to effectively inhibit muscle adaptations to contractile activity.

We recently revealed that skeletal muscle specific AMPKα1 and –α2 knockout (mdKO) mice have reduced mitochondrial oxidative capacity and reduced capacity for fatty acid utilization in skeletal muscle during exercise (13; 29). This suggests that the AMPKα subunits have important roles in acute regulation of metabolism. To investigate if AMPKα subunits are also involved in adaptations to exercise training by regulating gene and protein expression, AMPKα WT and mdKO mice were subjected to either an acute bout of exercise or to voluntary running wheel exercise training.
Methods

Mice

Female mice with skeletal muscle specific deletion of AMPKα1 and AMPKα2 (mdKO; AMPKα1^{fl/fl}, AMPKα2^{fl/fl}, HSA-Cre^{+/-}) and their wild type (WT; AMPKα1^{fl/fl}, AMPKα2^{fl/fl}, HSA-Cre^{+/-}) littermates were used in the study (mean (±SD) age, 14 ±2 weeks). The generation of the mice as well as the quality and specificity of the knockout has previously been described (13; 29). Offspring were obtained from mating AMPKα mdKO males with WT females to prevent any potential adverse effects that a pregnant mdKO female could have on the offspring during the gestation period. The mice were tested for genotype by PCR on tail DNA using the following primers (AMPKα1^{wt}: CCTCCAACTCTGTCATCGTTCTAATC and GCAGAGGAAGGACAAGCCAGACAC, AMPKα1^{fl}: CCTGAATTTGCCCTGCTTGACC and GGCCGCGAAGTTCCTATACTTTCTA, AMPKα2^{wt}: GTTATCAGCCCAACTAATTACAA and CGTTCCAAATGCATGCACCA, AMPKα2^{fl}: GGAAGCTTATAACTTCGTATAGCATACATT and CGTCTCCTGCTTCTGCAGTCCA, HSA-Cre: ACGGACAGAAGCATTTCAGG and CGGGTCGAGTCAACGGTGTAG). Only the last two primer sets (AMPKα2^{fl} and HSA-Cre) were used once it was established that the breeding mice were all AMPKα1^{fl/fl}, AMPKα2^{fl/fl} so only the presence of HSA-Cre was a variable. Mice were kept on a 12/12 hr light/dark cycle (light on 06:00 AM to 06:00 PM; room temperature, 22 °C) with free access to water and standard rodent chow at all times (#1319F, Altromin, Lage, Germany). All experiments were approved by the Danish Animal Experimental Inspectorate and complied with the EU convention for protection of vertebral animals used for scientific purposes (Council of Europe 123, Strasbourg, France, 1985).

Study design

Two cohorts of AMPKα WT and mdKO mice were randomly assigned to a sedentary control group or to a voluntary exercise training group (design shown in fig. 1). One cohort (n=13-15) was terminated after four weeks of exercise training (basal cohort, BC) to obtain tissues for analysis of biochemical adaptations. The second cohort (n=10-15) continued the exercise training in parallel with undergoing running performance tests to evaluate physiological adaptations to the exercise training (running test cohort, RTC). This design allows for assessing skeletal muscle biochemical adaptations to exercise training in the BC and at the same
time avoiding any confounding effects of the running test regimes. Glucose tolerance was however examined in both cohorts after 3 weeks.

The RTC mice were adapted to treadmill running on three consecutive days (starting on day 26) followed by one day of recovery before undergoing a maximal running speed test (described below) on day 30. On day 33 they were re-familiarized with the treadmill and on day 35 they performed a running endurance test (described below).

To investigate adaptive mRNA responses to exercise, a separate group of untrained AMPKα WT and mdKO mice performed an acute bout of treadmill exercise (described below).

In vivo protocols

Morphological analyses, MRI scan: The mice were weighed at the beginning of the study and thereafter once weekly along with registration of food consumption. The body composition of the mice was analyzed using magnetic resonance imaging (EchoMRI™ 4-in-1, EchoMRI, Houston, TX, USA) before and after the four weeks of exercise training.

Exercise training: AMPKα WT and mdKO mice were housed in single cages without (sedentary) or with (training) access to a running wheel (Techniplast activity cage, wheel Ø: 23 cm; Techniplast, Buguggiate, Italy). During the 4 week exercise training period voluntary running distance and duration were monitored with cycle computers (BC 1400; Sigma Sport, Neustadt, Germany). The running wheels were locked at 06:00 PM on the day before the mice were euthanized. Food was removed at 06:00 AM and mice were euthanized by cervical dislocation 6-8 hours later, and tissues were removed, instantly frozen in liquid nitrogen and kept at -80 °C until being further processed.

Oral glucose tolerance test: After 3 weeks of voluntary running wheel exercise training, the mice were subjected to an oral glucose tolerance test (OGTT). Running wheels of the exercise training groups were locked at 06:00 PM the day before the OGTT. On the experimental day the mice were fasted for 6 hours (starting 06:00 AM) before they were given an oral gavage of glucose (2 g/kg body weight) dissolved in a 0.9% saline solution (0.3 mg glucose/µl). Blood glucose concentration was measured in tail blood (Contour XT; Bayer, Leverkusen, Germany) shortly before mice were gavaged and again at 20, 40, 60, 90 and 120 minutes after the gavage.
Exercise capacity tests: The RTC mice underwent two exercise capacity tests. The tests were spaced at least three days apart to ensure proper time for recovery. At each occasion the running wheels were locked the evening before the test (at 06:00 PM). Maximal running speed of sedentary and trained AMPKα WT and mdKO mice was determined as previously described (13). In short; on day -4, -3 and -2, mice were acclimatized to treadmill running (5 minutes at 7.2 m/min followed by 5 minutes at 9.6 m/min) before the test (day 0). On the test day, treadmill speed started at 4.8 m/min and increased by 2.4 m/min every 2 minutes until exhaustion. The incline was kept constant at 5°. The running endurance test was conducted at 75% of individual mouse maximal running speed (as determined above). The mice ran at a 10° incline at their individually predetermined speed until exhaustion (time to exhaustion, TTE). Before and immediately after each test, blood glucose concentration was measured from tail blood using a glucometer (Contour XT; Bayer). In addition, blood lactate concentration was measured prior to and after the running endurance test (Accutrend Plus System; Roche, Basel, Switzerland).

Acute exercise: A separate group of untrained AMPKα WT and mdKO mice were randomized into either a rest or an exercise group (n=14-17). All mice were acclimatized to treadmill running and performed a maximal running speed test as described above. After three days of recovery, mice were re-familiarized with the treadmill once using the acclimation protocol described above. Five days after the maximal running speed test mice in the exercise groups performed an acute exercise bout consisting of 30 minutes of treadmill running up a 5° incline at 60% of individual mouse maximal speed. After exercise, mice recovered for 3 hours before they were euthanized by cervical dislocation and tissues were harvested and instantly frozen in liquid nitrogen. Work performed was calculated as follows; (J) = body weight (kg) · vertical ascent (m) · 9.8 m/s². Vertical ascent was determined using Pythagoras’ theorem (sin((5°/360°)·2π) = vertical ascent / (running speed (m/min) · running time (min)).

Muscle analyses

Muscle homogenization: Whole quadriceps muscles crushed in liquid nitrogen and whole soleus and EDL muscles were homogenized in ice cold homogenization buffer (10% glycerol, 20 mM Na-pyrophosphate, 150 mM NaCl, 50 mM Hepes, 1% NP-40, 20 mM β-glycerophosphate, 10 mM NaF, 2 mM PMSF, 1 mM EDTA, 1 mM EGTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2 mM Na3VO4, 3 mM benzamidine; final buffer adjusted to pH 7.5) with steel beads in a TissueLyser II (Qiagen, Maryland, USA) at 30 Hz for 2x1 min. Next, the samples rotated end-over-end for 60 min at 4°C and were then centrifuged at 18230g for 20 min at 4°C to obtain lysates (i.e. the supernatants).
Analysis of muscle protein and protein phosphorylation: Analysis of total protein concentration and preparation of lysates for SDS-PAGE were performed as previously described (13). Samples and controls were loaded onto self-cast Tris-HCl polyacrylamide gels (Mini-PROTEAN Tetra Cell Casting Module, BioRad, Hercules, CA, USA) or pre-cast Tris-HCl gels (Mini-PROTEAN, BioRad) in equal amounts as described (13). The sample protein amount loaded onto the gels was ensured to be within the linear range of the assays when using the specified antibodies. Control samples were loaded at each end of the gels to be able to correct for variations in gel-to-gel and in gel-to-membrane transfer efficiency. Resolved proteins were transferred to a polyvinylidene difluoride membrane (Immobilion-P Membrane, Millipore, Billerica, MA, USA) by semi-dry blotting. The membrane was blocked in a washing buffer (TBST) containing 2-5% skimmed milk and incubated with primary antibodies overnight at 4°C (TBST with 2-5% skimmed milk). The following primary antibodies were used: anti-cluster of differentiation (CD)36 (#AF2519, R&D Systems, Minneapolis, MN, USA), anti-FABPpm (kindly donated by Dr. Calles-Escandon, Wake Forest University, Winston-Salem, NC, USA), anti-HKII (#2867, Cell Signaling Technology), anti-GLUT4 (#PA1-1065, Thermo Scientific), anti-Cyt C (#556433, BD Pharmingen, Franklin Lakes, NJ, USA), anti-COX-I (#459600, Invitrogen, Carlsbad, CA, USA), anti-CD31 (#ab28364, Abcam, Cambridge, UK), anti-NADH-ubiquinone oxidoreductase chain 6 (ND6) protein: Complex I (#A31857, Molecular Probes, Invitrogen), anti-succinate dehydrogenase flavoprotein subunit (SDHA) protein: Complex II (#A11142, Molecular Probes, Invitrogen), anti-ubiquinol-cytochrome-C reductase Core protein 1 (UQCRC1) protein: Complex III (#A21362, Molecular Probes, Invitrogen).

Secondary antibody incubation and chemiluminescence detection were performed as described previously (13). VEGF protein was measured in muscle lysates using an ELISA kit (MMV00, R&D systems, Minneapolis, MN, USA).

Enzyme activities: The maximal activity of CS and β-hydroxyacyl-CoA dehydrogenase (βHAD) was measured in muscle lysates (1 μg/μl protein) as NADH production by spectrophotometric determination of NADH changes at 340 nm at 37°C, pH 7.0 using an automatic analyzer (Hitachi automatic analyzer 912; Roche, Basel, Switzerland). Acetyl-CoA and oxaloacetate were used as substrates for the CS mediated conversion of NAD to NADH and acetoacetyl-CoA as substrate for the βHAD-mediated production of NADH (38).

mRNA isolation: Approximately 25 mg of crushed quadriceps muscle was homogenized (Qiagen) and RNA was isolated by a guanidinium thiocyanate phenol-chloroform method as previously described (11; 50). The Superscript II RNase H- system (Invitrogen) was used for reverse transcription of 3 μg of total RNA using 0.5 μg oligo dT as described by Pilegaard et al. (50).

Real-time PCR: Fluorescence-based real-time PCR (ABI PRISM 7900 Sequence Detection System, Applied Biosystems, CA, USA) was used to determine mRNA content of specific genes as described (39). The mRNA...
content was analyzed using Taqman probes and forward and reverse primers as listed (Table 1), except for
plasma membrane fatty acid binding protein (FABPpm) (Mm00494703_m1), fatty acid transport protein
(FATP)1 (Mm00449511_m1) and FATP4 (Mm01327405_m1) mRNA that were analyzed using pre-made kits
(Applied Biosystems). Unknown samples were determined in triplicate and related to a standard curve
made by a serial dilution of a representative pooled sample. The target gene mRNA content of each sample
was normalized to the single stranded DNA content measured by OliGreen as described (39).

Statistics

All data are presented as means ±SEM. Statistical evaluation was performed by an unpaired t-test or by
Two- or Three-Way ANOVA with or without repeated measures as specified in figure legends. If significant
ANOVA interactions were detected, specific differences were identified by Student-Newman-Keuls post-hoc
analysis. Statistical significance was accepted at p<0.05. In the PGC-1α gene data set heteroscedasticity
persisted (Levene’s test) despite data transformation (square, natural log, reciprocal, exponential and
square root transformations were attempted), thus these data have been analyzed using multiple t-tests
applying Bonferroni correction to the p-values. Data were analyzed using SigmaPlot (version 13.0, Systat
Software, San Jose, CA, USA) and SPSS (version 20, IBM, Armonk, New York, USA).

Due to difference in total running distance of AMPKα WT and mdKO mice, an additional comparison of
protein adaptations was made. The comparison was made in subgroups of AMPKα WT and mdKO mice
having performed an equal exercise volume (ERD, equal running distance) during the four weeks of exercise
training. The inclusion criteria were defined as follows; the AMPKα WT mouse having run the shortest
distance set the lower limit of which mdKO mice to include (ERD mdKO, n=6; Fig. 7A). The AMPKα mdKO
mice having run the longest distance set the upper limit of which WT mice to include (ERD WT, n=8). The
resulting mean running distances were analyzed by an unpaired t-test showing no difference between the
two ERD subgroups (Fig. 7B). These ERD training groups were statistically analyzed together with the full
sedentary AMPKα WT and mdKO groups.
Results

Exercise-induced mRNA responses in skeletal muscle of AMPKα WT and mdKO mice

To examine the role of AMPKα on the mRNA response to a single bout of exercise we determined the mRNA content of proteins important in skeletal muscle metabolism and energy production. Mice were either rested or subjected to an acute bout of exercise as described in the methods after which they recovered for 3 hours before muscles were removed (maximal running speed; WT rested group, 33.4 ±1.2 m/min; mdKO rested group, 21.5 ±0.8 m/min; WT exercised group, 35.8 ±1.6 m/min; mdKO exercised group, 21.0 ±0.9 m/min; main effect of genotype, p<0.001. Work performed during the 30 min exercise bout at 60% of maximal running speed; WT rested group, 11.4 ±0.5 J; mdKO rested group, 7.1 ±0.3 J; WT exercised group, 12.1 ±0.6 J; mdKO exercised group, 7.1 ±0.4 J; main effect of genotype, p<0.001).

A decreased mRNA content was found in many (PGC-1α, Cyt C, GLUT4, CD36, FABPpm, FATP1, VEGF and HKII (p=0.054, power=0.369)) but not all (COX-I (complex IV) and FATP4) metabolic markers in muscle of AMPKα mdKO mice relative to WT (Fig. 2A-F). Three hours after the acute exercise bout an increase in mRNA content of COX-I, GLUT4 and VEGF was observed in AMPKα WT, but not mdKO muscle (Fig. 2B-C, F), and the PGC-1α mRNA three hours after exercise was significantly higher in WT than mdKO muscle (Fig. 2A). Further, the acute exercise bout increased the mRNA content of CD36 and FATP1 similarly in muscle of AMPKα WT and mdKO mice (Fig. 2D-E), although a tendency towards a larger increase in CD36 mRNA (p=0.096, power=0.253) was detected in muscle of WT mice compared with mdKO.

These data suggest that AMPKα is implicated in regulation of a diverse array of metabolic protein-encoding genes at basal and in response to exercise. A lack of gene responses to acute exercise in AMPKα mdKO mice could potentially result in absence of exercise training-induced increases in protein expression. To test this hypothesis AMPKα WT and mdKO littersmates underwent four weeks of exercise training.

Morphological data of sedentary and trained AMPKα WT and mdKO mice

There was no difference in weight gained between groups over the four weeks of exercise training (Table 2). To compare body morphology between genotypes, lean body mass, fat mass and the respective changes over time were expressed as percentage of body weight, correcting for a slightly higher body weight (~0.5 g) of AMPKα mdKO than WT mice. Lean mass percentage was not different between groups, although the
relative gain in lean mass tended towards being higher in the trained groups. Fat mass percentage was also similar in all groups, while there was a significantly larger decrease in fat mass in the trained groups than in the sedentary groups. Daily food intake related to body weight was higher in trained AMPKα WT mice (p<0.001) and tended towards being higher in trained mdKO mice (p=0.077, power of interaction=0.550), than in the respective sedentary groups. Within the trained groups AMPKα WT mice had a higher food intake than mdKO mice (p<0.05).

Reduced running distance and speed during voluntary running wheel exercise training in AMPKα mdKO mice

Over the 4 weeks of voluntary exercise training, AMPKα WT mice ran 135 ±7 km opposed to the mdKO mice, which only ran 56 ±7 km (p<0.001; Fig. 3A-B). While running distance increased over time in both groups, the AMPKα mdKO mice did at no time catch up with the WT mice (Fig. 3A). Also, when exercising in the running wheel AMPKα mdKO mice ran at a ~19% (p<0.001) lower running speed than the WT mice (Fig. 3C-D).

An OGTT was performed after three weeks of voluntary exercise. This revealed that glucose tolerance was slightly impaired in AMPKα mdKO compared with WT mice. Exercise training did not improve glucose tolerance in any of the genotypes (Fig. 3E-F).

AMPKα does not regulate exercise training-induced improvements in running performance

To investigate if lack of skeletal muscle AMPKα affects physiological adaptations to exercise training, AMPKα WT and mdKO mice were subjected to a maximal running speed test and a running endurance test on a motorized treadmill. Maximal running speed of sedentary AMPKα mdKO mice was ~34% lower than sedentary WT mice (WT sedentary, 30.4 ±2.0 m/min; mdKO sedentary, 20.2 ±0.8 m/min; Fig 4A). Exercise training increased the maximal running speed of both genotypes comparably (WT trained, 32.8 ±1.4 m/min; mdKO trained, 24.6 ±1.8 m/min).

Mice underwent a running endurance test at which they ran until exhaustion at a constant speed set to 75% of individual mouse maximal speed. Overall, the AMPKα WT mice ran for a longer time than the mdKO mice before reaching exhaustion and exercise training did not improve TTE of either WT or mdKO mice (Fig. 4B). For unknown reasons, TTE data variations were particular large in the AMPKα WT groups, decreasing
the power of the statistical analysis (power of interaction=0.05). Thus, despite being non-significant it is
worth noting that the TTE of trained AMPKα mdKO mice was increased by ~125% compared with sedentary
mdKO mice. At exhaustion blood lactate concentration had increased similarly in the two genotypes and
the increase was independent of training status (Fig. 4C). Blood glucose concentration decreased with
endurance exercise in the AMPKα WT mice, whereas it increased in the mdKO mice (Fig. 4D). This is in line
with previous observations in AMPKα mdKO mice (13).

In summary, the data suggest that the lack of skeletal muscle AMPK does not prevent exercise training-
induced improvements in running performance. Nonetheless, embryonic knock-out of AMPKα greatly
reduces exercise capacity regardless of training status.

Effects of exercise training on mitochondrial, cytosolic and angiogenic proteins

Molecular adaptations of skeletal muscles to exercise training were mainly examined in quadriceps muscle
and for some proteins also in the more oxidative soleus muscle and in the glycolytic EDL muscle.

Content of mitochondrial proteins succinate dehydrogenase complex subunit A (SDHA, subunit of ETC
complex II), Cyt C and COX-I (subunit of ETC complex IV) was generally reduced in muscles of sedentary
AMPKα mdKO mice compared with WT mice, while CS, β-HAD, NADH-ubiquinone oxidoreductase chain 6
(ND6, subunit of ETC complex I) and ubiquinol-cytochrome-C reductase core protein 1 (UQCRC1, subunit of
ETC complex III) activity/protein content was similar between genotypes (Fig. 5A-B and 6A-C). Exercise
training increased the activity/content of CS, UQCRC1 and Cyt C in quadriceps muscle of AMPKα WT mice
only (Fig. 5A-B and 6A). In contrast, the activity of β-HAD and the protein content of SDHA and COX-I
muscle were similarly induced in quadriceps of trained AMPKα WT and mdKO mice (Fig. 5A-B and 6A).

A similar protein content of GLUT4, HKII, CD36 and FABPpm was found in quadriceps muscle of sedentary
AMPKα WT and mdKO mice (Fig. 6D, G). The increase in HKII and FABPpm protein content with exercise
training was larger in AMPKα WT than mdKO mouse muscle, while exercise training increased GLUT4 and
CD36 comparably in quadriceps muscle of both genotypes (Fig. 6D, G).

To evaluate the role of AMPK in angiogenesis, protein content of VEGF and CD31 (marker of vascular
endothelium) was measured in quadriceps muscle. The level of VEGF protein was lower in sedentary
AMPKα mdKO mice than WT mice (Fig. 5C), whereas the CD31 protein level was similar in the two
genotypes (Fig. 5D). A trend towards a larger effect of exercise training on muscle VEGF protein content
was observed in AMPKα WT compared with mdKO mice (p=0.066, power=0.33), whereas exercise training induced a similar increase of CD31 protein in WT and mdKO mice (Fig. 5C-D).

In soleus muscle of sedentary mice, GLUT4 protein content was higher (Fig. 6E), while HKII, CD36 and FABPpm content was lower (Fig. 6E, H) in AMPKα mdKO mice than in WT mice. The EDL muscle of sedentary AMPKα mdKO mice also displayed lower levels of CD36 and FABPpm protein than WT (Fig. 6I), while there were no differences in EDL muscle GLUT4 and HKII content between genotypes (Fig. 6F).

Exercise training did not induce increased protein expression of GLUT4, HKII, CD36 or FABPpm in soleus and EDL in any of the genotypes (Fig. 6E-F, H-I).

Because we have previously shown that acute voluntary running wheel exercise increases AMPKα1 and AMPKα2 activity (also in 4 weeks trained animals) (25), these data suggest that AMPKα subunits play a role in exercise training-induced adaptations in quadriceps muscle.

Comparison of exercise training effects on protein content in a subgroup of AMPKα WT and mdKO mice having performed an equal training volume

Because the total training volume of AMPKα WT and mdKO mice differed, potential differences in muscle protein adaptations may simply be an effect of training volume rather than genotype. Taking this into account, we further performed a comparison in a subgroup of trained mice in which total running distance was equal (ERD: WT, n=8, 110 ±9 km; mdKO, n=6, 107 ±9 km; Fig. 7A-B). All mice from the sedentary groups have been included for this secondary comparison.

In the ERD subgroups, the mean voluntary running speed tended to be reduced in AMPKα mdKO mice compared with WT mice (p=0.053, power=0.506; Fig. 7C). Morphological characteristics of the ERD subgroups (Table 3) only showed a few subtle differences compared with the full groups of mice (Table 2).

The analyses of protein adaptations in the ERD subgroups of mice showed that UQCRC1 (complex III) protein expression increased exclusively in trained AMPKα WT mice (Fig. 7D-P), suggesting that AMPK is an essential regulator of UQCRC1 in response to exercise training. The remaining markers found to increase with exercise training in a seemingly AMPK-dependent manner in the full groups of mice (CS, Cyt C, HKII and FABPpm), increased similarly in AMPKα WT and mdKO mice of the ERD subgroups. Thus, for the majority of exercise training-induced adaptations of metabolic proteins AMPKα is likely not essential.
Discussion

The present study reveals that AMPK\(\alpha\) is necessary for exercise-induced mRNA increments of several metabolic markers in mouse skeletal muscle (GLUT4, COX-I (complex IV) and VEGF). Further, exercise training-induced increases in protein activity/content of CS, UQCRC1 (complex III), Cyt C, HKII and FABPpm in quadriceps muscle of AMPK\(\alpha\) WT mice are partly or fully ablated in AMPK\(\alpha\) mdKO mice. However, lack of AMPK\(\alpha\)1 and –\(\alpha\)2 subunits in skeletal muscle does not compromise exercise training-induced improvements in running performance. Thus, while some molecular defects are evident in AMPK\(\alpha\) mdKO mice, physiological improvements in exercise capacity are not compromised.

Both AMPK and PGC-1\(\alpha\) are activated in response to exercise. Furthermore, both have been proposed to regulate GLUT4 expression. In myotubes that inherently express little or no PGC-1\(\alpha\) and GLUT4, adenoviral expression of PGC-1\(\alpha\) can drive the expression of GLUT4 in a myocyte enhancer factor (MEF)2-dependent manner (43). In addition, the binding of MEF2 to the GLUT4 promoter is mediated by an AMPK\(\alpha\)-dependent mechanism (17), and release of HDAC5 repression of the GLUT4 promoter is achieved by AMPK phosphorylation of HDAC5 directing it for nuclear export (41). The present observations that the PGC-1\(\alpha\) mRNA level in skeletal muscle was lower in AMPK\(\alpha\) mdKO mice than WT after acute exercise and that GLUT4 mRNA only increased in WT mice support the view of a coordinated AMPK\(\alpha\) – PGC-1\(\alpha\) pathway regulating GLUT4 gene expression in response to acute exercise. Interestingly, two previous studies in AMPK\(\alpha\)2 KO and AMPK\(\alpha\)2 kinase-dead (KD) mice did not report impaired induction of GLUT4 mRNA with exercise in the AMPK\(\alpha\)2-deficient mice (21; 27). Common to all three models (AMPK\(\alpha\)2 KO/\(\alpha\)2 KD/\(\alpha\)2 mdKO) is the almost total absence of AMPK\(\alpha\)2 catalytic activity in skeletal muscle. However, the AMPK\(\alpha\)2 KO mouse shows a compensatory increase in AMPK\(\alpha\)1 protein content (26), whereas the AMPK\(\alpha\)2 KD transgenic approach results in overexpression of a kinase-dead AMPK\(\alpha\)2 subunit leading to a severe reduction in endogenous AMPK\(\alpha\)2 protein but only a modest reduction in endogenous AMPK\(\alpha\)1 protein (31; 44). Thus, both the AMPK\(\alpha\)2 KO and KD mice do express AMPK\(\alpha\)-subunits in skeletal muscle, while the AMPK\(\alpha\) mdKO mice lack any form of AMPK\(\alpha\) expression in skeletal muscle (29). Hence, this may suggest that catalytic activity of AMPK\(\alpha\)2 is not necessary for an exercise-induced increase in GLUT4 mRNA.

Alternatively, the AMPK\(\alpha\)2 KO and KD mice may have developed different mechanisms to compensate for disrupted AMPK\(\alpha\) signaling during embryogenesis and/or in the early post-natal period, while the deletion of both AMPK\(\alpha\) subunits in the AMPK\(\alpha\) mdKO mouse may have prevented such successful compensatory mechanisms in developing.

In parallel to GLUT4, the VEGF and COX-I (complex IV) mRNA was also up-regulated with an acute bout of exercise in AMPK\(\alpha\) WT but not in mdKO mice. Exercise-induced angiogenesis and VEGF content have
previously been shown to be controlled by PGC-1α (10; 33). However, in AMPKα2 KD mice exercise results in higher VEGF mRNA levels than in WT mice (66). On the contrary, the increase seen in VEGF mRNA of WT mice with electrical stimulation is fully ablated in muscle of skmLKB1 KO mice (52). Again, the present results using the AMPKα mdKO mouse model in which both catalytic subunits of AMPK are ablated show that AMPKα is indeed required for exercise-induced increases in skeletal muscle VEGF mRNA.

The present observations in AMPKα mdKO mice suggest that AMPKα is important for maintaining basal protein expression of Cyt C, COX-I (complex IV) and SDHA (complex II), which is in line with previous observations in AMPKα2 KO mice (25) and with findings of reduced respiratory complex capacity in AMPKα2 KD mice (28; 30). Recently, we also reported a repressed respiratory capacity of complex I in AMPKα mdKO mouse muscle (29). Because the present results show a normal content of ND6 (complex I) protein in mdKO mice, the functional defect in complex I respiration may not be due to reduced protein expression, but perhaps related to acute regulation of complex activity.

CS activity is thought to be a good marker of mitochondrial content and exercise training increases both factors (4). The normal basal activity of CS in AMPKα mdKO compared to WT mice is in agreement with our previous data in these mice (29) and previous data from AMPKα2 KD mice (1). However, a decreased basal CS activity has been found in AMPKα2 KO (25), AMPKα2iTG (51), AMPKβ1β2M-KO (48), LKB1 MKO (24; 54) and skmLKB1-KO mice (52). Thus, while the majority of studies suggest that altered AMPK signaling plays a role in basal CS activity, it is clearly not the sole determinant. Habitual locomotor activity seems not to be an explanatory factor either, in that it is normal in AMPK/LKB1 deficient mouse models that at the same time show either unaltered (13) or decreased (48; 52) levels of basal CS activity.

Exercise training induced a higher CS activity and protein content of Cyt C, HKII and FABPpm in quadriceps muscle of AMPKα WT than in mdKO mice, although mRNA content of these markers was similarly regulated with acute exercise in WT and mdKO mice. Recognizing the unique temporal responses of different genes to acute exercise (34), the current analysis of a single post-exercise time-point may have missed later potential genotype differences in gene expression. This limitation may also overlook later compensatory effects in mRNA responses to exercise. Thus, it could perhaps also explain the discrepancy between the AMPKα-dependent increase in exercise-induced GLUT4, VEGF and COX-I (complex IV) mRNA levels and the AMPKα-independent increase in exercise training-induced GLUT4, VEGF and COX-I protein content. It has recently been shown that skeletal muscle of AMPKα2 KD mice shows a compensatory increase in protein kinase D (PKD) ser916 phosphorylation with exercise, and that this may help to preserve regulation of HDAC5 phosphorylation and subsequent expression of PGC-1α mRNA (42). This mechanism could be important in maintaining the adaptive responses in metabolic proteins to exercise training. Because VEGF is an activator
of PKD, it may further be essential to tightly control VEGF protein levels to secure PKD regulation of HDAC5 phosphorylation and nuclear export (18). Further downstream of PKD and HDAC5, VEGF stimulation induces MEF2 transcriptional activation and expression of the angiogenic orphan nuclear transcription factor Nur77 (nuclear receptor subfamily 4 group A member 1) (18; 64). Interestingly, it may be speculated whether the previously reported upregulation of the Nur77 pathway in AMPKα mdKO mice (13) could be a compensatory response to the reduced basal VEGF levels reported here. This might explain the seemingly similar basal vascularization of AMPKα WT and mdKO mice, as indicated from the content of the endothelial specific protein CD31.

Both sedentary and trained AMPKα mdKO mice displayed inferior voluntary and forced running capacity. However, exercise training increased maximal running speed similarly in AMPKα WT and mdKO mice. This may suggest that even though AMPK may be involved in exercise-induced regulation of some metabolic proteins, it is not essential for improving running speed.

The running endurance test showed no improvements in TTE with exercise training in either genotype, although it should be noted that trained AMPKα mdKO mice ran for twice as long as sedentary mdKO mice. An increase in TTE in the trained AMPKα mdKO mice may have been detected, if the statistical power of the analysis had been higher. If true, this would suggest that the AMPKα mdKO mice are highly responsive to exercise training. Still, the AMPKα mdKO would not have attained the same overall exercise capacity as the WT mice, as the absolute speed of the running endurance test was lower in both untrained and trained mdKO than in the corresponding WT groups, respectively.

Despite that the AMPKα mdKO mice ran less during the four weeks of training, they appear to run at the same or even higher relative intensity as WT mice when exercising voluntarily. This is indicated by the fact that forced maximal running speed was ~29% lower in AMPKα mdKO mice than WT mice (mean difference of AMPKα WT and mdKO untrained and trained mice, respectively), whereas the voluntary running speed of the mdKO mice was only ~19% lower than WT mice. If the running distance and speed of the AMPKα mdKO mice during the four weeks of voluntary exercise training actually corresponds to a similar relative work load (speed and distance) compared to WT mice, it would suggest that comparable adaptive responses of the genotypes to the exercise training could be expected. At least for PGC-1α, an exercise bout of a certain relative intensity induces a similar mRNA response despite differences in absolute exercise intensity in humans (12; 47). Whether the same conditions are valid for the remaining genes analyzed in the present study, and for the increases in exercise training-induced protein content, need further investigation.
Exercise training volume is an important determinant of the adaptive response to exercise training (4). Thus, due to the large difference in total running distance between the AMPKα WT and mdKO training groups, the results on protein adaptation could potentially be biased. In an attempt to circumvent this two subgroups of mice within the AMPKα WT and mdKO training groups, which had performed an equal running distance/exercise training volume during the four weeks (ERD subgroups) were compared. However, this approach decreases statistical power and may introduce unknown selection bias in that the AMPKα WT mice having run the least and the mdKO mice having run the most are compared. As none of the comparison options are optimal, we have chosen to show both the analysis of the full groups and that of the ERD subgroups. The major difference between the two analysis results is that exercise training-induced increases in CS activity and protein expression of GLUT4, Cyt C and UQCRC1 (complex III) are dependent on AMPK when all mice are included, while only the increase in UQCRC1 protein content with exercise training is dependent on AMPK when the ERD subgroups are analyzed. Because a considerable number of studies support a role of AMPK in regulation of exercise adaptations, the present study may suggest that redundant and/or compensatory mechanisms adequately make up for the loss of AMPKα1 and AMPKα2 in skeletal muscles. Whether such mechanisms develop during embryogenesis/early postnatal life and whether they involve stabilization/increased transcription of mRNA or up-regulation of other pathways engaged by exercise will remain for future studies to answer.

Interestingly, UQCRC1 (complex III) has been identified to interact with muscle RING finger (MuRF)1 in yeast two-hybrid screens of skeletal muscle cDNA libraries (59; 60), and we have recently reported MuRF1 mRNA content to be repressed in AMPKα mdKO mice (29). Because MuRF1 is known to induce muscle atrophy by ubiquitination of target proteins (5), a higher protein content of UQCRC1 could be expected in AMPKα mdKO than in WT mice. However, as we found a normal basal protein content of UQCRC1 in AMPKα mdKO muscle, additional studies will be required to elucidate the specific relationship between AMPK, MuRF1 and UQCRC1 expression in skeletal muscle. It may further be appealing to investigate if the reduction in MuRF1 mRNA in skeletal muscle of AMPKα mdKO mice slows the degradation of other metabolic proteins.

In summary, the data suggest that the AMPKα subunits mediate increases in skeletal muscle mRNA of key metabolic proteins in response to an acute bout of exercise. However, the catalytic subunits of AMPK are redundant to the majority of exercise training-induced adaptations in protein expression.
Grants

This work was carried out as a part of the research programs "Physical activity and nutrition for improvement of health" funded by the University of Copenhagen (UCPH) Excellence Program for Interdisciplinary Research; and the UNIK project: Food, Fitness & Pharma for Health and Disease (see www.foodfitnesspharma.ku.dk) supported by the Danish Ministry of Science, Technology and Innovation. The study was funded by the Danish Council for Independent Research Medical Sciences (FSS) and the Novo Nordisk Foundation. J.F. was recipient of a PhD fellowship from the Danish Diabetes Academy.

Disclosures:

The authors have nothing to disclose.

Author contributions:

Conception and design of research: J.F. and J.F.P.W.

Performed experiments: J.F., R.K. and J.R.H.

Analyzed data: J.F., C.M.K., J.B.B. and P.S.

Interpreted results: All.

Prepared figures: J.F.

Drafted manuscript: J.F.

Edited and revised manuscript: All.

Approved final version of the manuscript: All.

Acknowledgements

The authors acknowledge the assistance of Nicoline Resen Andersen, Betina Bolmgren, Irene Bech Nielsen, Maximilian Kleinert, Christian Pehmøller, Andreas Mæchel Fritzen, Lykke Sylow Hansen, Andreas Børsting Jordy and Jonas Møller Kristensen, Section of Molecular Physiology, the August Krogh Centre, Department
of Nutrition, Exercise and Sports, University of Copenhagen, Copenhagen, Denmark and of Nicolas Caesar Petersen, Section of Neural Control of Movement Department of Nutrition, Exercise and Sports, University of Copenhagen, Copenhagen, Denmark and of Helle Sørensen, Laboratory for Applied Statistics, Department of Mathematical Sciences, University of Copenhagen, Copenhagen, Denmark. The authors also appreciate the kind donation of antibodies by D.G. Hardie (Division of Molecular Physiology, College of Life Sciences, University of Dundee, Scotland, U.K.) and J. Calles-Escandon (Wake Forrest University, NC, USA).

Jørgen F.P. Wojtaszewski is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
Figures and tables

Figure 1:

**Study design.** Body composition of the mice was determined by magnetic resonance imaging (MRI) on day 0 and day 28. Right after the initial MRI-scan a basal cohort (BC) and a running test cohort (RTC) were provided with in-cage running wheels while control mice were housed in cages without running wheels. Mice underwent an oral glucose tolerance test (OGTT) on day 21. Between 05:00 and 06:00 pm on day 28 the running wheels in the BC were locked. On the following day the BC mice were euthanized and tissues were dissected and instantly frozen in liquid nitrogen. The RTC continued the voluntary exercise training and underwent a maximal running speed test on day 30 and an endurance running test on day 35, both performed on a motorized treadmill.

Figure 2:

**Impaired COX-I, GLUT4 and VEGF mRNA induction with acute exercise in skeletal muscle of AMPKα mdKO mice.** In quadriceps muscle of AMPKα WT and mdKO mice at rest or three hours after an acute treadmill exercise bout the mRNA content of (A) PGC-1α, (B) Cyt C, COX-I, (C) GLUT4, HKII (D) CD36, FABPpm, (E) FATP1, FATP4 and (F) VEGF was measured. Data are presented as means ±SEM (n=14-17, except for FATP4 in which analysis was only performed in n=5-7). Statistical tests; (A) Unpaired t-test with bonferroni correction; (B-F) Two-Way ANOVA. Significant effect of exercise, * p<0.05, ** p<0.01, *** p<0.001; Significant effect of genotype, # p<0.05, ## p<0.01, ### p<0.001; () indicates borderline significance 0.05<p<0.10; ——— indicates main effect.

Figure 3:

**Voluntary running distance and speed are decreased in AMPKα mdKO mice.** (A) Distances covered during 28 days of voluntary running wheel exercise training in AMPKα WT and mdKO mice. (B) Accumulated running distances computed from data in panel “A”. (C) Running speed of AMPKα WT and mdKO mice during 28 days of voluntary running wheel exercise training. (D) Mean running speed computed from data in panel “C”. Before the oral glucose tolerance test (OGTT) on day 21 the running wheels were locked. No measures of wheel running was obtained on that day and the corresponding data points in panel “A” and
“C” have been omitted. (E) OGTT time course of blood glucose concentrations in sedentary and trained AMPKα WT and mdKO mice. (F) AUC of data in “E”. Data are presented as means ±SEM (n=28-30). Statistical tests; (B, D) Unpaired t-test, (F) Two-Way ANOVA. Significant effect of genotype, # p<0.05, ### p<0.001; —— indicates main effect.

Figure 4:

**Forced treadmill running capacity is decreased in AMPKα mdKO mice and their blood profile is altered.**

(A) Maximal running speed in an incremental treadmill exercise test in sedentary and trained AMPKα WT and mdKO mice. (B) Time to exhaustion in a treadmill running endurance test in sedentary and trained AMPKα WT and mdKO mice. (C) Blood lactate concentration pre and post the treadmill running endurance test. (D) Blood glucose concentration pre and post the treadmill running endurance test. Data are presented as means ±SEM (n=10-15). Statistical tests; (A, B) Two-Way ANOVA, (C, D) Three-Way RM ANOVA. Significant effect of exercise training, * p<0.05, Significant effect of genotype, # p<0.05, ## p<0.01, ### p<0.001; Significant effect of time × genotype, ††† p<0.001; Significant effect of time (pre/post), ⌂iliary significance 0.05<p<0.10; —— indicates main effect.

Figure 5:

**Exercise training-induced adaptations in the activity and content of mitochondrial and angiogenic proteins in skeletal muscle of AMPKα WT and mdKO mice.** After twenty-eight days of voluntary running wheel exercise training, quadriceps muscle of AMPKα WT and mdKO mice were removed and assayed for enzyme activity of (A) citrate synthase (CS) and β-hydroxyacyl-CoA dehydrogenase (β-HAD) and protein content of (B) ND6, SDHA and UQCRC1. Protein content of (C) VEGF and (D) cluster of differentiation (CD)31 in quadriceps muscle of sedentary and trained AMPKα WT and mdKO mice were further analyzed. Representative immunoblots are shown below the figures. Data are presented as means ±SEM (n=13-15). Statistical test; (A-D) Two-Way ANOVA. Significant effect of exercise training, * p<0.05, ** p<0.01, *** p<0.001; Significant effect of genotype, # p<0.05, ### p<0.001; () indicates borderline significance 0.05<p<0.10; —— indicates main effect.

Figure 6:
Exercise training-induced adaptations in content of metabolic proteins in skeletal muscle of AMPKα WT and mdKO mice. Protein content of cytochrome C (Cyt C) and cytochrome C oxidase (COX)-I was analyzed in (A) quadriceps, (B) soleus and (C) EDL muscles of sedentary and trained AMPKα WT and mdKO mice. Protein content of glucose transporter (GLUT)4 and hexokinase (HK)II in (D) quadriceps, (E) soleus and (F) EDL muscles and protein content of cluster of differentiation (CD36) and plasma membrane fatty acid binding protein (FABPpm) in (G) quadriceps, (H) soleus and (I) EDL muscles of the sedentary and trained AMPKα WT and mdKO mice. Representative immunoblots are shown below the figures. Data are presented as means ±SEM (n=12-15). Statistical test; (A-I) Two-Way ANOVA. Significant effect of exercise training, * p<0.05, ** p<0.01, *** p<0.001; Significant effect of genotype, # p<0.05, ## p<0.01, ### p<0.001; () indicates borderline significance 0.05<p<0.10; —— indicates main effect.

Figure 7:

Activity and content of mitochondrial, metabolic and vascular endothelium-associated proteins in AMPKα WT and mdKO sedentary and trained ERD (equal running distance) subgroups. (A) Individual distances covered during 28 days of voluntary running wheel exercise training in AMPKα WT and mdKO mice (only mice from BC as no protein data on RTC mice exist). Observations that lie within the dashed box are those that have been included in the AMPKα WT and mdKO ERD subgroups. (B) Mean running distances of the ERD subgroups. (C) Mean running speed of the ERD subgroups. (D) Enzyme activity of citrate synthase (CS) and β-hydroxyacyl-CoA dehydrogenase (β-HAD) in quadriceps muscle. (E) Protein content of ND6 (complex I), SDHA (complex II) and UQRC1 (complex III) in quadriceps muscle. Protein content of cytochrome C (Cyt C) and cytochrome C oxidase (COX)-I in (F) quadriceps, (G) soleus and (H) EDL muscles. Protein content of glucose transporter (GLUT)4 and hexokinase (HK)II in (I) quadriceps, (J) soleus and (K) EDL muscles. Protein content of cluster of differentiation (CD36) and plasma membrane fatty acid binding protein (FABPpm) in (L) quadriceps, (M) soleus and (N) EDL muscles. Protein content of (O) VEGF and (P) cluster of differentiation (CD)31 in quadriceps muscle of sedentary and trained AMPKα WT and mdKO mice. Data are presented as means ±SEM (A, n=13-14; B-C, n=6-8; D-P, n=6-15). Statistical tests; (B-C) Unpaired t-test, (D-P) Two-Way ANOVA. Significant effect of exercise training, * p<0.05, ** p<0.01, *** p<0.001; Significant effect of genotype, # p<0.05, ## p<0.01, ### p<0.001; () indicates borderline significance 0.05<p<0.10; —— indicates main effect.
### Table 1: Primer and probe sequences used for real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Forward</td>
<td>5’ AGCCAAACAAACAACCTTTATCTCTTC 3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’ TTAAGGTTCGCTCAATAGTCTTGTGC 3’</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>5’ AGAGTCACCAAAATGACCCAAAGGGTCC 3’</td>
</tr>
<tr>
<td>PGC-1α</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GLUT4</td>
<td>Forward</td>
<td>5’ CGCGGGGCTCCTCTATGAGATAC 3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’ AGGCACCCGAAGATGAGT 3’</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>5’ TGGCGCTACTCAGGGCTAACATCA 3’</td>
</tr>
<tr>
<td>HKII</td>
<td>Forward</td>
<td>5’ CTGTCTCAAGAAAGACATCCCCATT 3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’ CACCGCCGTCAACATGC 3’</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>5’ CAGTGAGGAGGTCTGCTCGCCA 3’</td>
</tr>
<tr>
<td>Cyt C</td>
<td>Forward</td>
<td>5’ TGCCCAGTGCCACACTGT 3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’ CTGCTCTGCCCGCAACA 3’</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>5’ AGGCAAGCTAAAGACTGGACCAATCTCCA 3’</td>
</tr>
<tr>
<td>COX-I</td>
<td>Forward</td>
<td>5’ TGAACCTCACGAGGAGTAATA 3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’ ATGTATCGTAAAGCAGATGCTCA 3’</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>5’ TCTAACCAGAAATGTATTTATCAAACTCCCTCCA 3’</td>
</tr>
<tr>
<td>CD36</td>
<td>Forward</td>
<td>5’ CCAAATGAAGATGAGCATAGGACAT 3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’ GTGAACCTGAGTCGTTTTGC 3’</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>5’ AGATGTGGAAACCATACTGGATCTAAGAC 3’</td>
</tr>
<tr>
<td>VEGF</td>
<td>Forward</td>
<td>5’ ACCCGGCTTACTGCTGTAACCT 3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’ TCACTCTGGACTCTGCCTCTT 3’</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>5’ AAGTGGTCCCAGGCTGCACCCAC 3’</td>
</tr>
</tbody>
</table>
Table 2: Morphological parameters of sedentary and trained AMPKα WT and mdKO mice.

<table>
<thead>
<tr>
<th></th>
<th>Sedentary</th>
<th>Training</th>
<th></th>
<th></th>
<th>Main effect</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT (n=30)</td>
<td>mdKO (n=30)</td>
<td>WT (n=28)</td>
<td>mdKO (n=29)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight, day 0 (g)</td>
<td>22.2 ±0.3</td>
<td>22.4 ±0.3</td>
<td>21.7 ±0.3</td>
<td>22.9 ±0.3</td>
<td>#</td>
<td>N.S.</td>
</tr>
<tr>
<td>Body weight gain (g)</td>
<td>0.5 ±0.2</td>
<td>0.8 ±0.2</td>
<td>0.9 ±0.2</td>
<td>0.7 ±0.2</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>Lean mass, day 0 (% of BW)</td>
<td>81.6 ±0.5</td>
<td>82.2 ±0.5</td>
<td>81.9 ±0.5</td>
<td>81.9 ±0.5</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>Lean mass gain (% of BW)</td>
<td>3.2 ±0.7</td>
<td>4.4 ±0.8</td>
<td>5.2 ±0.7</td>
<td>5.0 ±0.8</td>
<td>(*)</td>
<td>N.S.</td>
</tr>
<tr>
<td>Fat mass, day 0 (% of BW)</td>
<td>9.3 ±0.4</td>
<td>9.3 ±0.3</td>
<td>8.9 ±0.3</td>
<td>9.8 ±0.3</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>Fat mass gain (% of BW)</td>
<td>-1.4 ±0.5</td>
<td>-1.2 ±0.3</td>
<td>-2.1 ±0.3</td>
<td>-2.4 ±0.3</td>
<td>N.S.</td>
<td>(p=0.06)</td>
</tr>
<tr>
<td>Daily food intake (g)</td>
<td>3.5 ±0.1</td>
<td>3.6 ±0.1</td>
<td>4.2 ±0.1</td>
<td>4.0 ±0.1</td>
<td>***</td>
<td>(p=0.09)</td>
</tr>
<tr>
<td>Daily food intake (% of BW)</td>
<td>15.7 ±0.5</td>
<td>16.2 ±0.5</td>
<td>19.5 ±0.7 ***</td>
<td>17.6 ±0.5 (*)</td>
<td>N.A.</td>
<td>p&lt;0.05</td>
</tr>
</tbody>
</table>

Data are presented as means ±SEM (n=28-30). Statistical test; Two-Way ANOVA. Significant effect of exercise training, * p<0.05, *** p<0.001; Significant effect of genotype, # p<0.05; () indicates borderline significance 0.05<p<0.10; N.S., non-significant; N.A., not applicable.
### Table 3: Morphological parameters of sedentary and trained (equal running distance subgroups) AMPKα WT and mdKO mice.

<table>
<thead>
<tr>
<th></th>
<th>Sedentary</th>
<th>Training (ERD subgroups)</th>
<th>Main effect</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT (n=30)</td>
<td>mdKO (n=30)</td>
<td>WT (n=8)</td>
<td>mdKO (n=6)</td>
</tr>
<tr>
<td>Body weight, day 0 (g)</td>
<td>22.2 ±0.3</td>
<td>22.4 ±0.3</td>
<td>21.6 ±0.7</td>
<td>22.6 ±0.5</td>
</tr>
<tr>
<td>Body weight gain (g)</td>
<td>0.5 ±0.2</td>
<td>0.8 ±0.2</td>
<td>0.9 ±0.4</td>
<td>0.8 ±0.2</td>
</tr>
<tr>
<td>Lean mass, day 0 (% of BW)</td>
<td>81.6 ±0.5</td>
<td>82.2 ±0.5</td>
<td>81.9 ±1.0</td>
<td>81.0 ±1.5</td>
</tr>
<tr>
<td>Lean mass gain (% of BW)</td>
<td>3.2 ±0.7</td>
<td>4.4 ±0.8</td>
<td>5.9 ±1.5</td>
<td>7.3 ±0.9</td>
</tr>
<tr>
<td>Fat mass, day 0 (% of BW)</td>
<td>9.3 ±0.4</td>
<td>9.3 ±0.3</td>
<td>9.2 ±0.8</td>
<td>10.5 ±0.9</td>
</tr>
<tr>
<td>Fat mass gain (% of BW)</td>
<td>-1.4 ±0.5</td>
<td>-1.2 ±0.3 (#)</td>
<td>-2.2 ±0.7</td>
<td>-3.7 ±1.1 **, (#)</td>
</tr>
<tr>
<td>Daily food intake (g)</td>
<td>3.5 ±0.1</td>
<td>3.6 ±0.1</td>
<td>4.1 ±0.2</td>
<td>4.4 ±0.2</td>
</tr>
<tr>
<td>Daily food intake (% of BW)</td>
<td>15.7 ±0.5</td>
<td>16.2 ±0.5</td>
<td>19.4 ±1.4</td>
<td>19.5 ±1.2</td>
</tr>
</tbody>
</table>

Data are presented as means ±SEM (n=6-30). Statistical test; Two-Way ANOVA. Significant effect of exercise training, * p<0.05, ** p<0.01, *** p<0.001; Significant effect of genotype, # p<0.05; () indicates borderline significance 0.05<p<0.10; N.S., non-significant; N.A., not applicable.


Day 21: OGTT
Day 0
MRI
BC+RTC
(n=28-30)
Day 21
OGTT
BC+RTC
(n=28-30)
Day 28
MRI
BC+RTC
(n=28-30)
Day 30
Treadmill maximal running speed test
RTC (n=15)
Day 35
Treadmill endurance test
RTC (n=10)

Day 29: BC mice were euthanized by cervical dislocation and tissues were dissected for subsequent analysis.
Fig. 2

A) Quadriceps

B) Quadriceps

C) Quadriceps

D) Quadriceps

E) Quadriceps

F) Quadriceps

- PGC-1α
- Cyt C
- COX-I
- GLUT4 HKII
- FATP1 FATP4
- CD36 FABPpm
Fig. 3

A) Voluntary running distance

B) Total running distance

C) Voluntary running speed

D) Mean voluntary running speed

E) OGTT

F) OGTT

WT - Sedentary
mdKO - Sedentary
WT - Training
mdKO - Training
**Fig. 4**

**A)** Max. running speed test

**B)** Running endurance test

**C)** Running endurance test

**D)** Running endurance test

---

**Max. running speed (m/min)**

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>mdKO</th>
<th>WT</th>
<th>mdKO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sedentary</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Training</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Blood glucose conc. (mmol/L)**

<table>
<thead>
<tr>
<th></th>
<th>Pre</th>
<th>Post</th>
<th>Pre</th>
<th>Post</th>
<th>Pre</th>
<th>Post</th>
<th>Pre</th>
<th>Post</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>mdKO</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mdKO</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Blood lactate conc. (mmol/L)**

<table>
<thead>
<tr>
<th></th>
<th>Pre</th>
<th>Post</th>
<th>Pre</th>
<th>Post</th>
<th>Pre</th>
<th>Post</th>
<th>Pre</th>
<th>Post</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>mdKO</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mdKO</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Time to exhaustion (min)**

<table>
<thead>
<tr>
<th></th>
<th>Pre</th>
<th>Post</th>
<th>Pre</th>
<th>Post</th>
<th>Pre</th>
<th>Post</th>
<th>Pre</th>
<th>Post</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>mdKO</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mdKO</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

**Maximal running speed test**

**Running endurance test**

---

**Running endurance test**

---

**Running endurance test**

---

**Running endurance test**
Fig. 5

A) Sedentary Training vs. Sedentary Training

B) Protein content

C) VEGF

D) CD31
Fig. 6
**Figure 7**

**A)** Individual running distance (total of 4 weeks)

**B)** ERD subgroups total running distance

**C)** ERD subgroups mean voluntary running speed

**D)** Quadriceps (equal running dist.)

**E)** Quadriceps (equal running dist.)

**F)** Quadriceps (equal running dist.)

**G)** Soleus (equal running dist.)

**H)** EDL (equal running dist.)

**I)** Quadriceps (equal running dist.)

**J)** Soleus (equal running dist.)

**K)** EDL (equal running dist.)

**L)** Quadriceps (equal running dist.)

**M)** Soleus (equal running dist.)

**N)** EDL (equal running dist.)

**O)** Quadriceps (equal running dist.)

**P)** Quadriceps (equal running dist.)