

1 Inducible deletion of skeletal muscle AMPK α reveals that AMPK is required for nucleotide balance but
2 dispensable for muscle glucose uptake and fat oxidation during exercise.

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24 Highlights:

- 25 • Inducible deletion of AMPK α in adult mice disturbs nucleotide balance during exercise, lowers
26 muscle glycogen content and reduces exercise capacity.
- 27 • Muscle mitochondrial respiration, glucose uptake and FA oxidation during muscle contractile
28 activity remains unaffected by muscle-specific deletion of AMPK α subunits in adult mice.

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49 *Abstract*

50 *Objective:* Current evidence for AMPK-mediated regulation of skeletal muscle metabolism during
51 exercise is mainly based on transgenic mouse models with chronic (lifelong) disruption of AMPK
52 function. Findings based on such models are potentially biased by secondary effects related to chronic
53 lack of AMPK function. In an attempt to study the direct effect(s) of AMPK on muscle metabolism
54 during exercise, we generated a new mouse model with inducible muscle-specific deletion of AMPK α
55 catalytic subunits in adult mice.

56 *Methods:* Tamoxifen-inducible and muscle-specific AMPK α 1/ α 2 double KO mice (AMPK α imdKO)
57 were generated using the Cre/loxP system with the Cre driven by the human skeletal muscle actin
58 (HSA) promoter.

59 *Results:* During treadmill running at the same relative exercise intensity, AMPK α imdKO mice showed
60 greater depletion of muscle ATP, which was associated with accumulation of the deamination product
61 IMP. Muscle-specific deletion of AMPK α in adult mice promptly reduced maximal running speed,
62 muscle glycogen content and was associated with reduced expression of UGP2, a key component of the
63 glycogen synthesis pathway. Muscle mitochondrial respiration, whole body substrate utilization as well
64 as muscle glucose uptake and fatty acid (FA) oxidation during muscle contractile activity remained
65 unaffected by muscle-specific deletion AMPK α subunits in adult mice.

66 *Conclusions:* Inducible deletion of AMPK α subunits in adult mice reveals that AMPK is required for
67 maintaining muscle ATP levels and nucleotide balance during exercise, but **is** dispensable for
68 regulating muscle glucose uptake, FA oxidation and substrate utilization during exercise.

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70 **Keywords:** AMPK, exercise, glucose uptake, muscle metabolism, fat oxidation, glycogen.

71 1. INTRODUCTION

72 Physical activity is associated with a marked increase in muscle metabolism and energy turnover [1].
73 Therefore, maintaining intracellular levels of adenosine triphosphate (ATP) during exercise represents
74 a major metabolic challenge for the muscle cell. The increased ATP turnover during exercise leads to
75 accumulation of intramyocellular adenosine monophosphate (AMP) in an exercise intensity- and
76 duration-dependent manner due to the adenylate kinase reaction ($2\text{ADP} \leftrightarrow \text{ATP} + \text{AMP}$) [2]. The
77 increased intramyocellular AMP/ATP ratio leads to activation of 5'-AMP-activated protein kinase
78 (AMPK) [3], which promotes catabolic processes and inhibits anabolic processes in order to normalize
79 the cellular energy status [4]. On this basis, skeletal muscle AMPK is proposed to function as a cellular
80 energy sensor that is activated during exercise and thus acts as a central mediator of cellular signaling
81 to maintain energy homeostasis.

82 Given its pivotal role in the regulation of muscle metabolism, AMPK activation provides a
83 putative therapeutic target for metabolic disorders such as type 2 diabetes (T2D) [5]. Acute
84 pharmacological activation of AMPK in rodent muscle by different pharmacological agents (e.g.
85 AICAR, PF739 and MK-8722) promotes glucose disposal and fatty acid (FA) oxidation [6–8]. Yet,
86 AMPK-deficient mouse models have provided conflicting results as to whether AMPK activation is
87 required for muscle metabolism to cope with the cellular energy stress during exercise. While reduced
88 muscle glucose uptake has been reported in AMPK deficient mice during *in vivo* treadmill exercise and
89 contraction of isolated mouse muscles in some studies [9–15], other studies have demonstrated intact
90 muscle glucose uptake during contractile activity [16–22]. Knockout (KO) of the two regulatory
91 AMPK β subunits (AMPK β 1 β 2M-KO) is associated with impaired muscle glucose uptake and
92 increased FA oxidation during treadmill exercise [9], while KO of both catalytic AMPK α subunits

93 (AMPK α 1/ α 2) in muscle (AMPK α mdKO mice) or KO of the AMPK upstream kinase liver kinase B1
94 (LKB1) (LKB1 KO mice) leads to increased reliance on glucose as a substrate during treadmill
95 exercise [16,17]. However, direct interpretation of these findings is confounded by disrupted
96 mitochondrial capacity [9,17] and changes in expression of key proteins/enzymes involved in lipid
97 metabolism (e.g. CD36 and FABPpm) in these models [16,17].

98 In most studies, maximal treadmill running speed is reduced in AMPK deficient mice compared to wild
99 type (control) littermates (see [23] for detailed review). During high metabolic stress, the muscle cell
100 prevents accumulation of AMP by converting it to inosine monophosphate (IMP) in a AMP-deaminase
101 (AMPD) dependent reaction that serves to maintain a homeostatic ATP/ADP ratio [24]. Accelerated
102 ATP degradation and reduced glucose uptake has been observed in muscle from mice overexpressing a
103 kinase-dead AMPK α 2 construct (AMPK α 2 KD mice) [10]. Whether these findings can be ascribed
104 directly to the lack of functional AMPK or rather should be seen as a consequence of the marked
105 impairment in mitochondrial function reported for this model remains unclear [10].

106 Taken together, previous findings in AMPK deficient mouse models indicate that the observed
107 phenotypes may be ascribed to secondary effects due to the lifelong lack of AMPK rather than acute
108 regulation of AMPK activity during e.g. exercise. In attempt to study the direct effect(s) of AMPK
109 activation during exercise, we developed a new mouse model where AMPK catalytic activity can be
110 deleted in a muscle-specific manner at a specific time point in adult mice. With this new model, we
111 sought to clarify the direct role of AMPK in exercise-stimulated regulation of muscle metabolism.

112 2. MATERIALS AND METHODS

113 2.1. Generation of the tamoxifen-inducible muscle-specific AMPK α double knockout mouse 114 model (AMPK α imdKO)

115 Inducible muscle-specific double AMPK α 1/ α 2 KO mice (AMPK α imdKO) were generated by breeding
116 double-floxed AMPK α 1 α 2 mice (AMPK α 1^{fl/fl}, AMPK α 2^{fl/fl}) [15] with mice expressing a tamoxifen-
117 inducible Cre-recombinase driven by the human skeletal actin promoter (HSA-MCM^{+/-}) [25]. Deletion
118 of AMPK α 1/ α 2 in skeletal muscle was achieved by intraperitoneal injection of tamoxifen (Cat. No.
119 T5648, Sigma-Aldrich) dissolved in 99% ethanol and re-suspended in sunflower seed oil (Cat. No.
120 S5007, Sigma-Aldrich). The tamoxifen treatment protocol consisted of three single injections (40
121 mg/kg body weight) each separated by 48 hours. Female double-floxed AMPK α 1 α 2 control mice
122 (AMPK α 1^{fl/fl}, AMPK α 2^{fl/fl}, HSA-MCM^{-/-}) and AMPK α imdKO (AMPK α 1^{fl/fl}, AMPK α 2^{fl/fl}, HSA-
123 MCM^{+/-}) on a mixed background (C57/Bl6 87.5% and SV129 12.5%) were used in all experiments.
124 Initially, a time course study was performed in order to determine the earliest time point for optimal
125 deletion of skeletal muscle AMPK α protein. For this time course experiment mice were investigated 1,
126 3 and 8 weeks after the final tamoxifen injection and compared to vehicle-injected control mice
127 (sunflower seed oil injections). 3 weeks after the last tamoxifen injection was the earliest time point
128 with optimal deletion of AMPK α protein and therefore all following experiments were performed ~3
129 weeks after the final tamoxifen injection. Both AMPK α imdKO mice and AMPK α double-floxed
130 control littermates aged 12 \pm 5 weeks (means \pm SD) were treated with tamoxifen. **The tamoxifen**
131 **administration protocol applied in the present study resulted in a substantial testicular swelling in male**
132 **mice (unpublished observations) and we decided for ethical and experimental reasons to perform all**
133 **following experiments in female mice only.** All mice had free access to water and rodent chow and

134 were maintained on a 12:12 hour light-dark cycle. All experiments were approved by the Danish
135 Animal Experiments Inspectorate (License #2013-15-2934-00911, #2014-15-2934-01037 and 2019-15-
136 0201-01659) and complied with the EU guidelines for the protection of vertebrate animals used for
137 scientific purposes.

138 **2.2. Body composition and morphological analyses**

139 Body composition was measured prior to and ~3 weeks after final tamoxifen injection by the use of
140 magnetic resonance imaging (EchoMRI 4-in-1; EchoMRI, Houston, TX). Skeletal muscle, heart, white
141 adipose tissue, liver, and kidney were carefully dissected from anaesthetized control and AMPK α
142 imdKO mice and visually inspected for any signs of disparity. Tissue mass was determined with 0.1 mg
143 accuracy (ED124S, Sartorius, Goettingen, Germany).

144 **2.3. Basal calorimetry**

145 Prior to measurements, mice were acclimatized for 3 days to individually-housed airtight calorimetric
146 cages connected to an indirect calorimetric system (Phenomaster/LabMaster system; TSE Systems, Bad
147 Homburg, Germany). O₂ consumption (VO₂), CO₂ production (VCO₂), food intake and physical
148 activity level (laser beam breaks) were recorded during a 48 hour period while the mice were receiving
149 a chow diet (Altromin no. 1324; Brogaarden, Horsholm, Denmark) followed by a high fat diet (60%
150 kcal derived from fat; no. D12492; Brogaarden, Horsholm, Denmark). After a 24-hour wash out period
151 on regular chow diet the effect of 24 hours of fasting was investigated. For all calorimetric
152 measurements mice were maintained on a 12 h:12 h light–dark cycle and housed at 20–21°C.

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154 **2.4. Treadmill acclimatization and maximal exercise capacity test**

155 On 3 separate days (Pre, 1 week and 3 weeks after final tamoxifen injection), mice were adapted to a
156 treadmill running system (TSE Systems). The adaptation protocol consisted of 5 min rest followed by 5
157 min running at 7.2 m/min and 5 min running at 9.6 m/min [16]. After one day of rest, a graded exercise
158 capacity test was performed. The test was initiated by 5 min rest followed by treadmill running at 4.8
159 m/min with treadmill speed increased 2.4 m/min every 2 min at a 5° incline. Mice were forced to run
160 by the use of pressurized air and an electric shocker grid at the back of the treadmill. Exhaustion was
161 reached when the mice stayed on the shocking grid despite repeated agitation with pressurized air. The
162 last passed speed level was defined as maximal running speed.

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164 **2.5. Substrate utilization during treadmill exercise**

165 Substrate utilization during treadmill exercise was investigated at least 48 hours after the maximal
166 exercise capacity test by measuring O₂ consumption (VO₂) and CO₂ production (VCO₂) in an airtight
167 treadmill running system (CaloSys apparatus; TSE Systems, Germany). Mice were placed on the
168 calorimetric treadmill and allowed to rest for 10 min. This was followed by a 5 min warm up at 40% of
169 maximal treadmill running speed and 30 min continuous running at 60% of individual maximal running
170 speed at a 5° incline. Measurements continued 15 min into exercise recovery.

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174 **2.6. Glucose uptake measurements during treadmill exercise**

175 Mice either rested or exercised for 30 min at 70% of individual maximal running speed at a 15° incline.
176 An intraperitoneal injection of saline containing [³H]-2-deoxyglucose ([³H]-2-DG) (8 ml/kg, 60
177 μCi/ml, Perkin Elmer, USA) was administered 20 min before the onset of exercise/rest. Blood samples
178 in combination with blood glucose measurements (Contour XT, Bayer, Germany) were obtained from
179 the tail vein immediately before and after exercise/rest to determine specific radioactivity in the blood.
180 Mice were euthanized by cervical dislocation immediately after the last blood sample was drawn and
181 tissues were quickly harvested and frozen in liquid nitrogen. Muscle 2-DG uptake was determined as
182 [³H]-2-deoxy-D-glucose-6-phosphate ([³H]-2-DG-6-P) content by Somogyi and perchloric acid
183 precipitations of muscle homogenate as previously described [16,26].

184 **2.7. Glucose and insulin tolerance tests**

185 For all tests, mice were individually housed. For the glucose tolerance test (GTT), mice were fasted for
186 5 hours in the morning before they were given an intraperitoneal injection of glucose (2 g/kg body
187 weight) dissolved in a 0.9% saline solution. For the insulin tolerance test (ITT), overnight fed mice
188 were fasted for 2 hours in the morning before they were given an intraperitoneal injection of insulin (1
189 U/kg body weight, Actrapid, Novo Nordisk, Bagsværd, Denmark). Blood was collected from the tail
190 vein at 0, 20, 40, 60, 90, and 120 min in the GTT and 0, 20, 40, and 60 min in the ITT. Blood glucose
191 concentrations were determined using a glucometer (Contour XT, Bayer, Germany). For the GTT,
192 plasma insulin concentrations were determined at 0, 20, and 40 min using an enzyme-linked
193 immunosorbent ELISA assay (Cat. No. 80-INSMSU-E10, ALPCO) according to the manufacturer's
194 instructions.

195 **2.8. Insulin- and contraction-stimulated glucose uptake in isolated muscles**

196 Fed mice were anesthetized by an intraperitoneal injection of pentobarbital (10 mg/100 g body weight)
197 before soleus and extensor digitorum longus (EDL) muscles were excised and suspended at resting
198 tension in incubation chambers (model 610/820M, DMT, Denmark) containing Krebs-Ringer buffer
199 (KRB) (117 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 0.5 mM
200 NaHCO₃, pH 7.4) supplemented with 0.1% bovine serum albumin (BSA), 2 mM Na-pyruvate, and 8
201 mM Mannitol. During the entire incubation period the buffer was maintained at 30°C and continuously
202 gassed with carbogen (95% O₂ and 5% CO₂). After ~30 min pre-incubation, basal, submaximal (100
203 μU/ml), and maximal (10,000 μU/ml) insulin-stimulated muscle glucose uptake was determined during
204 the last 10 min of a 30 min stimulation period by adding 1 mM [³H]-2-DG (0.028 MBq/ml) and 7 mM
205 [¹⁴C]-Mannitol (0.0083 MBq/ml) to the incubation medium. For contraction-stimulated glucose uptake,
206 the muscles were electrically stimulated to contract (1 s train/15 s, 0.2 ms pulses, 100 Hz, 30 V;
207 MultiStim System-D330, Harvard Apparatus) for 10 min. 2-DG uptake during contraction was
208 measured by adding 1 mM [³H]-2-DG (0.028 MBq/ml) and 7 mM [¹⁴C]-Mannitol (0.0083 MBq/ml) to
209 the incubation medium immediately before initiation of muscle contraction. After incubation, the
210 muscles were harvested, washed in ice-cold saline, blotted dry and quickly frozen in liquid nitrogen.
211 Uptake of 2-DG was determined as previously described [27].

212 **2.9. Contraction-stimulated FA oxidation in isolated soleus muscles**

213 Contraction-stimulated exogenous palmitate oxidation in isolated soleus muscle was measured
214 similarly as previously described [17]. In short, excised soleus muscles from anesthetized mice were
215 mounted at resting tension (~5 mN) in vertical incubation chambers (Radnoti, Monrovia, CA)

216 containing 30°C oxygenated (95% O₂ and 5% CO₂) KRB supplemented with 5 mM glucose, 2% fat-
217 free BSA, and 0.5 mM palmitate. After ~20 min of pre-incubation, the incubation buffer was replaced
218 with KRB additionally containing [1-¹⁴C]-palmitate (0.0044 MBq/ml). To seal off the incubation
219 chambers, mineral oil (Cat. No. M5904, Sigma-Aldrich) was added on top. Exogenous palmitate
220 oxidation was measured at rest and during 25 min contractions (18 trains/min, 0.6 s pulses, 30 Hz, 60
221 V). After incubation, incubation buffer and muscles were collected to determine the rate of palmitate
222 oxidation as previously described [17,28]. Palmitate oxidation was determined as CO₂ production
223 (complete FA oxidation) and acid-soluble metabolites (ASM, representing incomplete FA oxidation).
224 As there was no difference in complete and incomplete FA oxidation between genotypes, palmitate
225 oxidation is presented as a sum of these two forms.

226 **2.10. Mitochondrial respiration of permeabilized skeletal muscle fibers**

227 After excision, tibialis anterior (TA) muscles were immediately transferred to ice-cold BIOPS buffer
228 (10 mM Ca-EGTA, 0.1 μM free Ca, 20 mM imidazole, 20 mM taurine, 50 mM K-MES, 0.5 mM DTT,
229 6.56 mM MgCl₂, 5.77 mM ATP, 15 mM phosphocreatine, pH 7.1). Adipose and connective tissue were
230 removed, and muscle fibers were mechanically separated into small fiber bundles (~3 mg) with a fine
231 forceps to maximize surface area and minimize diffusion limitations. Permeabilization of fiber bundles
232 was ensured by 30 min saponin treatment (30 μg/mL in BIOPS) on a rotator at 4°C. After
233 permeabilization, fiber bundles were washed at least 30 min in MiR05 buffer (0.5 mM EGTA, 3 mM
234 MgCl₂, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH₂PO₄, 20 mM Hepes, 110 mM sucrose, 1
235 g/L BSA, pH 7.1) before analyses. Mitochondrial respiration was measured in duplicates in
236 permeabilized muscle fiber bundles under hyperoxic conditions ([O₂] ~400-200 μM) at 37°C in MiR05
237 medium using the Oxygraph-2k Oroboros Instruments, Innsbruck, Austria). Complex I supported leak

238 respiration was measured after addition of 5 mM pyruvate, 10 mM glutamate and 2 mM malate.
239 Maximal Complex I supported oxidative phosphorylation (OXPHOS) capacity (CI_P) was measured
240 after addition of ADP (4 mM). Complex I+II supported OXPHOS capacity ($CI+II_P$) was measured
241 after succinate addition (10 mM). Electron transfer system (ETS) capacity through Complex I+II was
242 measured after sequential addition of 0.5 μ M FCCP. Finally, ETS capacity through complex II (CII)
243 was achieved by adding 1 μ M rotenone to inhibit Complex I. After each respiration protocol,
244 permeabilized fibers were extracted from the respiration chamber and weighed after vacuum drying
245 and data are expressed as oxygen flux relative to muscle dry weight.

246

247 **2.11. Muscle glycogen, nucleotide, AMPD activity and lactate measurements**

248 Muscle glycogen content was determined by a fluorometric method as glycosyl units after acid
249 hydrolysis of 10-15 mg wet weight muscle samples [29]. Muscle specimens from quadriceps muscle
250 were extracted in perchloric acid and analyzed for nucleotides by reverse-phase HPLC. AMPD activity
251 was measured in quadriceps muscle by adding 2 μ l muscle homogenate to 1000 μ l of the reagent
252 solution containing 12.5 mM AMP and the formation of IMP was analyzed by HPLC. Kinetic
253 properties for AMPD (V_{max} and K_m) were analyzed in muscle homogenate in the presence of 15 mM,
254 0.1 mM, 0.06 mM, and 0.04 mM AMP and the formation of IMP was quantified by HPLC as
255 previously reported [30]. **Muscle lactate concentration was determined in crushed quadriceps muscle**
256 **by a fluorometric method as previously described [29].**

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259 **2.12. mRNA isolation, reverse transcription and real time PCR.**

260 A whole EDL muscle (~10 mg) and ~25 mg of quadriceps muscle were crushed in liquid nitrogen and
261 subsequently homogenized before RNA was isolated by the guanidine thiocyanate (GT) phenol-
262 chloroform method [31]. The pellet was washed twice in 75% EtOH (-20°C) and centrifuged for 5 min
263 at 12,000 g and 4°C between washes. The pellet was vacuum dried after last wash and resuspended in 1
264 µl/(mg initial muscle) 0.1 mM EDTA. RNA concentration was determined using a Nanodrop1000
265 spectrophotometer (Thermo Fischer Scientific, Waltham, USA) and RNA purity was ensured by the
266 260/280 nm ratio. Reverse transcriptase reaction was performed on 3 µg of total RNA using the
267 Superscript II RNase H- system (ThermoFisher Scientific, Waltham, USA) as previously described
268 [32]. The mRNA content of specific genes was determined by fluorescence-based real time PCR (ABI
269 PRISM 7900 Sequence Detection System, Applied Biosystems). The forward and reverse primers and
270 TaqMan probes were either designed from mouse specific sequence data (Entrez-NIH and Ensembl,
271 Sanger Institute) using computer software (Primer Express, Applied Biosystems) or purchased as kits
272 from ThermoFisher Scientific. PCR amplification was performed in triplicates of 10 µl with 10 ng of
273 cDNA as previously described [33] and with TATA Box Binding Protein (TBP) as endogenous control.
274 TBP has previously been described as an endogenous reference gene [34] and we found TBP to be
275 unaffected by the intervention and genotype in the present study. A detailed list of primer and probe
276 sequences is given in supplemental 1. **The cycle threshold (Ct) values of the unknown samples were**
277 **converted to an amount by use of a relative standard curve derived from a dilution series of a**
278 **representative pool. For each sample, the amount of specific genes analyzed was then divided by the**
279 **amount of housekeeping gene (TBP) for assay normalization.**

280

281 **2.13. Muscle processing**

282 Muscles were homogenizing in ice-cold buffer (10% glycerol, 20 mM Na-pyrophosphate, 150 mM
283 NaCl, 50 mM Hepes 1% NP-40, 20 mM β -glycerophosphate, 10 mM NaF, 2 mM PMSF, 1 mM EDTA,
284 1 mM EGTA, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 2 mM Na_3VO_4 , and 3 mM benzamidine, pH 7.5)
285 by the use of a TissueLyser II (QIAGEN, Hilden, Germany). Subsequently, homogenates were rotated
286 end-over-end at 4°C for 1 hour. Muscle lysate was obtained as supernatant from homogenate by
287 centrifugation for 20 min at 16,000 g and 4°C. Protein abundance in muscle homogenates and lysates
288 was determined in triplicate by the bicinchoninic acid method with BSA as protein standards (Thermo
289 Fisher Scientific, Waltham, USA).

290 **2.14 Glycogen synthase (GS)-activity**

291 GS-activity in muscle homogenates was measured in 96-well microtiter assay plates (Unifilter 350
292 plates; Whatman, Cambridge, UK) as previously described [35]. GS-activity is reported as percentage
293 of fractional velocity (% FV) and was calculated as 100 x activity in the presence of 0.17 mM glucose-
294 6-phosphate (G6P) divided by activity in the presence of 8 mM G6P (saturated).

295 **2.15 SDS-PAGE and Western blotting**

296 Muscle lysates were prepared in sample buffer and heated for 5 min at 96°C. Equal amounts of protein
297 were loaded on self-cast gels and separated by the use of SDS-PAGE. Gels were transferred to
298 polyvinylidene fluoride membranes (Merck, Darmstadt, Germany) using semidry blotting. Membranes
299 were incubated for 5 min in TBST containing either 2% skim milk or 3% BSA and subsequently
300 incubated overnight at 4°C with primary antibody. A detailed list of antibodies is given in the
301 supplemental material (Supplemental 2). Proteins with bound primary and secondary antibody were

302 visualized by chemi-luminescence and a digital imaging system (ChemiDoc MP System, BioRad,
303 California, USA). Linearity was assessed for all proteins in order to ensure that the obtained band
304 intensity was within the dynamic range.

305 **2.16 Statistical analyses**

306 Data are presented as means \pm SEM unless stated otherwise. Differences between AMPK α control and
307 imdKO mice were analyzed by a Students t-test or 2-way ANOVA with or without repeated
308 measurements as appropriate and specified in the figure legends. The Student-Newman-Keuls test was
309 used for post hoc testing. Statistical analyses were performed in Sigmaplot (version13.0 ; SYSTAT,
310 Erkrath, German) and $P < 0.05$ was chosen as significance level.

311 **3. RESULTS**

312 **3.1 Time-course of skeletal muscle-specific deletion of AMPK α 1 and α 2**

313 In order to define the earliest time-point at which full deletion of the catalytic AMPK α 1 and α 2 protein
314 had occurred in myofibers, we investigated the AMPK subunit levels 1, 3 and 8 weeks after tamoxifen-
315 induced gene deletion. 1 week after the last tamoxifen injection, the mRNA content of AMPK α 1 and
316 AMPK α 2 in EDL muscle was reduced to ~58% and ~0%, respectively, compared to tamoxifen-treated
317 AMPK α double-floxed control mice (Fig 1A and 1B). These changes in AMPK α gene expression were
318 also present at the 8-week time-point and resulted in a marked reduction in AMPK α 1 and near
319 complete deletion in AMPK α 2 protein content (Fig 1C-D and Supplementary Fig S3F). Thus, the
320 AMPK α 1 protein level was reduced to ~60% in AMPK α imdKO compared to control EDL muscle 1

321 week after tamoxifen treatment and dropped further reaching ~30% at 3 weeks after ended tamoxifen
322 treatment (Fig 1C). The protein content of AMPK α 2 in EDL muscle from AMPK α imdKO was
323 reduced to ~30 and ~8% at 1 and 3 weeks following tamoxifen treatment, respectively (Fig 1D). A
324 similar pattern for tamoxifen-induced reduction in AMPK α 1 and AMPK α 2 protein levels were also
325 observed in quadriceps muscle (Supplementary Fig S3A-B). Importantly, protein levels of AMPK α 1
326 and AMPK α 2 in skeletal muscle from AMPK α imdKO mice measured 3 weeks after ended tamoxifen
327 treatment correspond to levels observed in the previously described conventional (chronic) AMPK α
328 muscle-specific double knockout (mdKO) mouse model [16] (right bar in Fig 1C-D). The remaining
329 amount of AMPK α 1 protein in whole muscle samples from AMPK α imdKO mice is also found in the
330 conventional AMPK α mdKO model and likely derives from non-muscle cells (e.g. blood cells,
331 adipocytes and endothelial cells) present in our crude muscle sample preparations. The expression in
332 non-muscle cells is not expected to be affected due to the skeletal muscle specificity of the HSA
333 promotor [16]. AMPK α 1 and AMPK α 2 protein levels in heart muscle remained similar between
334 genotypes following tamoxifen treatment (Fig 1E-F and Supplementary Fig S3G), verifying that KO of
335 the catalytic AMPK subunits indeed is specific for skeletal muscle myofibers. The AMPK β 2-associated
336 heterotrimer complexes account for ~95% of the total AMPK pool in mouse EDL muscle (α 2 β 2 γ 1
337 ~70%, α 2 β 2 γ 3 ~20% and α 1 β 2 γ 1 ~5%) [36], and in accordance the marked reduction in AMPK α
338 muscle protein levels observed in the present model is accompanied by a corresponding reduction in
339 protein levels of the regulatory AMPK subunits β 2 and γ 1, while AMPK γ 3 only tended to be reduced
340 (Supplementary Fig S3C-F). In absolute values, these protein levels resemble those seen in skeletal
341 muscle of the conventional AMPK α mdKO mouse model.

342 Collectively, these data suggest that a maximal reduction in AMPK α 1 and AMPK α 2 protein levels is
343 observed at the earliest 3 weeks after ended tamoxifen treatment. Based on these observations the 3-
344 week time-point was chosen for all following experiments.

345 **3.2. Body composition and resting metabolism remain unaffected by inducible KO of AMPK α 1** 346 **and α 2 in skeletal muscle**

347 Body composition of control and AMPK α imdKO mice was investigated before and 3 weeks after
348 ended tamoxifen treatment by use of magnetic resonance scanning (Table 1). Before tamoxifen
349 treatment no differences between genotypes were noticed, both having normal growth rates and born
350 with the expected mendelian ratio (data not shown). A decrease in body weight (~3%) and an increase
351 in fat mass (~10%) were observed in both genotypes 3 weeks after tamoxifen relative to before
352 tamoxifen treatment. A minor decrease in lean body mass (~3%) was observed in AMPK α imdKO but
353 not control mice following tamoxifen treatment. However, no significant difference between genotypes
354 in lean body mass was evident before and after tamoxifen treatment. Careful dissection and weighing
355 of individual organs revealed similar weight and appearance of muscle, heart, adipose tissue, liver, and
356 kidney from AMPK α imdKO and control littermates (Table 2).

357 Resting metabolism during the light and dark period on chow diet was found to be comparable between
358 control and AMPK α imdKO mice (Supplementary Fig S4A-B). We also investigated the ability to
359 switch substrate utilization towards FA oxidation by subjecting the mice to 2 days of high fat diet
360 (HFD) and a 24 hours fasting regimen, respectively. The HFD intervention lowered RER from ~0.95 to
361 ~0.80 in both genotypes during the light period, and RER increased similarly in the two genotypes
362 during the dark period (RER from ~0.80 to ~0.85) (Supplementary Fig S4A and C). Compared to the
363 chow diet, the 24 hours fasting intervention lowered RER markedly in the light period and RER

364 declined further during the dark period (RER ~0.73 to ~0.71 in both genotypes) with no difference
365 between genotypes (Supplementary Fig S4D-E). Together, these data suggest that resting metabolism
366 under these experimental conditions is similarly covered by FA oxidation in AMPK α imdKO and
367 control mice. Oxygen consumption, spontaneous physical activity **as well as food intake** during the
368 different diet interventions showed no difference between AMPK α imdKO and control littermates
369 (Supplementary Fig S4F-P).

370 Collectively these observations demonstrate that body composition, resting metabolism and metabolic
371 flexibility, i.e. the ability to adjust metabolism according to different feeding and fasting regimes,
372 remain unaffected by conditional deletion of AMPK α subunits in skeletal muscle of adult mice.

373

374 **3.3. Normal whole body insulin action and insulin-stimulated glucose uptake in isolated skeletal** 375 **muscle from AMPK α imdKO mice**

376 Whole body insulin action and muscle insulin sensitivity were investigated to determine whether
377 inducible deletion of muscle AMPK α in adult mice was associated with development of insulin
378 resistance. Following an intraperitoneal glucose tolerance test (GTT), blood glucose concentration
379 increased similarly and showed a comparable dynamic response in the two genotypes (Fig 2A). Plasma
380 insulin concentrations, determined before as well as 20 and 40 min after the glucose challenge, were
381 also similar in the two genotypes (Fig 2B). An intraperitoneal insulin tolerance test (ITT) revealed
382 comparable whole body insulin action in the two genotypes (Fig 2C). Collectively, these data
383 demonstrate that both whole body insulin- and glucose-tolerance remain intact in mice with acute
384 deletion of AMPK activity in skeletal muscle myofibers. Because skeletal muscle is responsible for the

385 majority of glucose uptake during *in vivo* insulin stimulation [37], 2-DG uptake in isolated skeletal
386 muscle was examined in the presence of a submaximal (100 μ U/ml) and maximal (10,000 μ U/ml)
387 insulin concentration. Both submaximal and maximal insulin-stimulated glucose uptake in soleus and
388 EDL muscles were comparable between AMPK α imdKO mice and control littermates (Fig 2D-E). In
389 addition, insulin-stimulated signaling in EDL muscle at the level of Akt (Akt Thr308) and its
390 downstream target TBC1D4 (TBC1D4 Thr642) were comparable between genotypes, suggesting that
391 insulin signaling to GLUT4 translocation was regulated similarly in muscles from the two genotypes
392 (Fig 2F-H). Overall, these findings demonstrate that acute deletion of AMPK catalytic activity in
393 skeletal muscle does not affect whole body insulin action or the ability of insulin to stimulate glucose
394 uptake in isolated skeletal muscle.

395 **3.4. Inducible deletion of AMPK α in adult mice impairs running performance and lowers muscle** 396 **glycogen content**

397 In accordance with previous observations in mouse models with embryonic and hence chronic deletion
398 of AMPK function in skeletal muscle [9,10,16,18], a reduction in maximal treadmill running speed was
399 found in AMPK α imdKO mice relative to control littermates (Fig 3A). Interestingly, a ~15% reduction
400 in maximal treadmill running speed was observed already 1 week after tamoxifen-induced AMPK α
401 gene deletion and was still present 3 weeks after tamoxifen treatment. This early response to AMPK α
402 gene deletion was also found for skeletal muscle glycogen content. Thus, glycogen concentration in
403 quadriceps muscle was decreased by ~25% in AMPK α imdKO already 1 week after ended tamoxifen
404 treatment and was still lower 3 and 8 weeks after tamoxifen treatment (Fig 3B). In order to evaluate
405 whether the metabolic stress induced by treadmill exercise differed between genotypes, we measured
406 muscle glycogen concentration before and after 30 min of treadmill exercise (Fig 3C). Although resting

407 muscle glycogen content was lower in AMPK α imdKO relative to control mice, the ability to utilize
408 muscle glycogen (glycogen degradation) during treadmill exercise was similar between the two
409 genotypes (Fig 3C). Moreover, activity of the glucose-incorporating enzyme - glycogen synthase (GS)
410 - was elevated in muscle from AMPK α imdKO mice compared to control mice (Fig 3D). The content
411 of key proteins involved in glucose uptake (GLUT4 and HKII) and glycogen degradation (glycogen
412 phosphorylase (GP)) were similar in muscle from AMPK α imdKO and control mice. Interestingly,
413 UDP-Glucose Pyrophosphorylase 2 (UGP2), but not GS protein content was lower in quadriceps
414 muscle from AMPK α imdKO mice compared to control mice (Fig 3E). UGP2 is essential for glycogen
415 synthesis as it generates UDP-glucose [38] to be incorporated into glycogen chains mainly catalyzed by
416 GS. Analyses of the time course experiment revealed that UGP2 gene expression was reduced by ~40%
417 in quadriceps muscle from AMPK α imdKO already 1 week after ended tamoxifen-induced AMPK α
418 gene deletion (Fig 3F). This observation indicates that AMPK is involved in the regulation of UGP-2
419 gene expression and we hypothesize that the concurrent lowering of muscle glycogen content in muscle
420 from AMPK α imdKO mice is related to this change in UGP-2 expression.

421 Taken together, these observations suggest that AMPK is required for treadmill running performance
422 and for maintaining resting muscle glycogen content potentially via regulation of UGP2. On the
423 contrary, the ability of skeletal muscle to use glycogen as an energy source during exercise remains
424 unaffected. Because maximal running performance differed between genotypes the following treadmill
425 running experiments were performed at a relative exercise intensity (% of maximal running capacity)
426 of each individual mouse.

427 **3.5 AMPK is required for maintaining muscle nucleotide balance during exercise**

428 Exercise increases the turnover of ATP in skeletal muscle and ATP regeneration through adenylate
429 kinase reaction is therefore important for maintaining cellular ATP levels. To avoid the accumulation
430 of intracellular AMP, the muscle cell deaminates AMP to IMP catalyzed by the enzyme AMPD (Fig 4).
431 Treadmill exercise for 30 min at the same relative intensity decreased muscle ATP levels in both
432 genotypes, but to a greater extent in AMPK α imdKO mice than in control littermates (36% vs. 14%
433 reduction in AMPK α imdKO and control mice, respectively) (Fig 5A). While muscle ADP content
434 remained unaffected and similar in the two genotypes (Fig 5B), a significant increase in muscle AMP
435 content was observed in response to treadmill exercise (Fig 5C). Interestingly, 30 min of treadmill
436 exercise at the same relative intensity was associated with a 7-fold increase in IMP in muscle from
437 AMPK α imdKO mice with no detectable increase in muscle from control littermates (Fig 5D). Lower
438 ATP levels during exercise accompanied by accumulation of IMP suggest a disturbance in muscle
439 energy balance when AMPK α imdKO mice perform treadmill exercise (Fig 4). Intracellular IMP,
440 formed during exercise, can be degraded to inosine (INO) and hypoxanthine (HX), which can pass
441 through the muscle cell membrane and hence can represent a potential loss of nucleotide precursors
442 from the exercising muscle [39]. Adenosine (ADO) increased similarly in both genotypes in response
443 to exercise ($p=0.16$ for main effect of exercise) (Fig 5E). However, both HX ($p=0.073$ for effect of
444 genotype) and INO ($p=0.077$ for interaction between genotype and intervention) tended to be elevated
445 in exercised muscle from AMPK α imdKO only (Fig 5F-G). Collectively, these findings suggest that
446 AMPK is required for maintaining the skeletal muscle nucleotide balance during exercise. The enzyme
447 adenosine monophosphate deaminase 1 (AMPD1) is highly expressed in skeletal muscle and catalyzes
448 the deamination of AMP to IMP and therefore plays a key role in the purine nucleotide cycle.
449 Interestingly, muscle AMPD enzyme activity (measured under saturated AMP concentrations) tended
450 to increase in response to exercise in AMPK α imdKO, while remaining unchanged in muscle from

451 control littermates ($p=0.08$ for interaction between intervention and genotype) (Fig 5H). However, this
452 could neither be explained by a genotype-dependent difference in kinetic properties in resting muscles
453 (Table 3 and Suppl. Fig. S3K) or AMPD1 protein content (Fig 5I).

454 Collectively these data demonstrate that AMPK is necessary for maintaining the cellular nucleotide
455 pool during exercise. Next, we investigated whether reduced ATP regeneration in muscle from
456 AMPK α imdKO mice was related to impaired substrate uptake and/or utilization during exercise and
457 muscle contraction.

458 **3.6. Similar substrate utilization during exercise and muscle contraction in control and AMPK α** 459 **imdKO mice**

460 Substrate utilization in control and AMPK α imdKO mice during treadmill exercise was investigated at
461 the same relative intensity (60% of individual maximal running speed). RER before exercise was
462 comparable in the two genotypes (~ 0.72) and increased similarly during 30 min of treadmill exercise
463 (averaged 0.78 ± 0.01 and 0.79 ± 0.01 for control and AMPK α imdKO mice, respectively) (Fig 6A).
464 Upon cessation of exercise, RER declined to pre-exercise levels with no difference between genotypes.
465 These findings suggest that exercise at the same relative intensity exerts a similar metabolic response in
466 control and AMPK α imdKO mice. In accordance, measurement of exogenous palmitate oxidation in
467 isolated soleus muscles revealed that FA oxidation was similar between genotypes at rest and increased
468 to the same extent in response to electrically-stimulated muscle contractions (Fig 6B). Impaired FA
469 oxidation in mice with chronic deletion of AMPK activity in skeletal muscle (AMPK α mdKO) has
470 been associated with reduced expression of FA handling transport proteins (CD36 and FABPpm)

471 [16,40]. However, protein content of these FA transporters was similar in controls and AMPK α imdKO
472 mice (Fig 6C and Suppl. Fig. S3H).

473 A number of previous AMPK-deficient mouse models have reported impaired mitochondrial function
474 and/or expression of mitochondrial proteins [9,15,41]. In order to investigate the effect of AMPK α
475 deletion in adult mice on mitochondrial function, we measured mitochondrial respiration rates in
476 permeabilized fibers from TA muscle. Mitochondrial respiration was similar in the two genotypes
477 when analyzed in the successive presence of malate + glutamate + pyruvate (Leak), ADP (CI_p),
478 succinate (CI+II_p), FCCP (ETS CI+II), and rotenone (ETS CII) (Fig 6D). These observations are in
479 line with similar protein levels of subunits in complex I-V of the mitochondrial electron transport chain
480 (Fig 6E and Suppl. Fig. S3I).

481 Collectively, these findings suggest that the phenotypic trait of the previously described AMPK-
482 deficient mouse models is a consequence of chronic deletion that likely does not reflect the actual
483 consequence of lacking AMPK activity acutely in skeletal muscle.

484

485 **3.7. Normal muscle glucose uptake during *in vivo* exercise and *ex vivo* muscle contractions in** 486 **AMPK α imdKO mice**

487 Treadmill exercise for 30 min increased AMPK phosphorylation at Thr172, a surrogate marker for
488 AMPK activation, in quadriceps muscle from control mice, while this effect was absent in AMPK α
489 imdKO mice (Fig 7A and Suppl. Fig. S3J). Similarly, treadmill exercise increased phosphorylation of
490 TBC1D1 Ser231 in quadriceps muscle from control but not in muscle from AMPK α imdKO mice (Fig
491 7B). This observation was not due to a difference in TBC1D1 protein levels as these were similar in

492 quadriceps muscle from control and AMPK α imdKO mice. In the present study, we observed generally
493 lowered phosphorylation of ACC Ser212 in muscle from AMPK α imdKO mice, while the ability to
494 increase ACC Ser212 phosphorylation in response to exercise still was present (Fig 7C and Suppl. Fig.
495 S3J). This may either suggest that other kinases than AMPK are capable of phosphorylating ACC
496 Ser212 during exercise or alternatively that AMPK-induced phosphorylation of ACC Ser212 takes
497 place in non-muscle cells that are present in our crude muscle sample preparations. Studies
498 investigating contraction- and exercise-stimulated glucose uptake in skeletal muscle from various other
499 AMPK-deficient mouse models suggest no or only a partial role of AMPK [9,12,14–16,42]. In
500 conventional AMPK α mdKO mice, exercise is associated with markedly elevated blood glucose levels
501 [16]. However, in the present study we found that 30 min of treadmill exercise resulted in a minor
502 increase in blood glucose levels in both control and AMPK α imdKO mice that occurred concomitant
503 with a similar increase in muscle lactate content (Fig 7D-E). Glucose uptake in TA, soleus, EDL and
504 quadriceps muscle during exercise was similar between control and AMPK α imdKO mice (Fig 7F).
505 These data demonstrate that muscle glucose uptake during *in vivo* exercise is not compromised by
506 muscle-specific deletion of AMPK catalytic activity in adult mice. To further illuminate this matter,
507 contraction-stimulated 2-DG uptake was assessed in isolated EDL and soleus muscles from control and
508 AMPK α imdKO mice. During tetanic muscle contractions glucose uptake increased to a similar extent
509 in isolated soleus and EDL muscles from control and AMPK α imdKO mice (Fig 7G-H). Muscle force
510 development during this electrical stimulation protocol showed no difference between genotypes in
511 EDL muscle (Suppl. Fig. S5A), but was significantly lower (~23% lower at all time points) in soleus
512 muscle (p=0.049) from AMPK α imdKO mice compared to control littermates (Suppl. Fig. S5B). Thus,
513 albeit force development was generally lower for soleus muscle from AMPK α imdKO, the decline in
514 force development over time (fatigue) showed a similar pattern in both genotypes. Force development

515 during electrically stimulated single twitch stimulation resulted in comparable force development
516 (Suppl. Fig S5C). *Ex vivo* contractions increased phosphorylation of AMPK α Thr172 in control EDL
517 and soleus muscles, while phosphorylation of AMPK α Thr172 in AMPK α imdKO muscle at rest was
518 reduced and did only increase modestly in soleus muscle in response to contractions (Suppl. Fig S5D-E
519 and S5J). Both basal and contraction-stimulated phosphorylation of AMPK downstream targets, ACC
520 Ser212 and TBC1D1 Ser231, were reduced in EDL muscle from AMPK α imdKO mice (Suppl. Fig
521 S5F-G and S5J). However, in response to contractions, phosphorylation of ACC Ser212 and TBC1D1
522 Ser231 in soleus muscle increased similarly in both genotypes (Suppl. Fig S5H-J). ACC and TBC1D1
523 protein levels in soleus were comparable in control and AMPK α imdKO mice (data not shown), while
524 TBC1D1 protein content was lower (~25%.) in EDL muscle from AMPK α imdKO mice compared to
525 control mice (data not shown).

526 Collectively, these data demonstrate that AMPK is dispensable for contraction-stimulated glucose
527 uptake in skeletal muscle and that the signaling axis leading to increased glucose uptake, at least in
528 glycolytic quadriceps and EDL muscles, can be mediated independently of TBC1D1
529 phosphoregulation.

530

531 **4. DISCUSSION**

532 The central role of AMPK as a gatekeeper in the regulation of skeletal muscle metabolism is mainly
533 based on AMPK-deficient mouse models with chronic lack of AMPK function. However, results
534 derived from such models are potentially biased by confounding adaptations due to the life-long
535 AMPK deficiency (including embryonic development), as emphasized by marked defects in metabolic

536 proteins, mitochondrial function and extreme exercise intolerance in some of these conventional
537 models. In an attempt to minimize the influence of these confounding factors and to study the direct
538 and acute effect of AMPK deficiency, we generated a new transgenic mouse model with tamoxifen-
539 inducible muscle-specific deletion of catalytic AMPK activity in adult mice (AMPK α imdKO).

540 It has previously been reported that whole body deletion of AMPK α 2 leads to a greater
541 degradation of ATP in skeletal muscle during exercise and is associated with accumulation of AMP
542 and IMP [43]. However, in that study the whole body AMPK α 2 KO mice performed exercise at the
543 same absolute intensity as the control littermates. Today, we know that these findings were biased, as
544 the maximal running capacity of this KO model is reduced compared to WT control mice. Therefore,
545 these observations may be related to a difference in exercise workload between genotypes rather than a
546 direct consequence of a lack in AMPK activity. Notably, Lee-Young and co-workers reported
547 accelerated ATP degradation but similar AMP concentrations during exercise in muscle from AMPK α 2
548 KD mice compared to control mice, although exercise was performed at the same relative exercise
549 intensity [10]. The study also reported a marked impairment in mitochondrial respiratory capacity (~32
550 and 50% impairment in complex I and IV activities, respectively) and it could therefore be speculated
551 that the accelerated ATP depletion in muscle from AMPK α 2 KD mice was the results of a reduced
552 capacity for aerobic ATP repletion due to mitochondrial dysfunction. In the present study, ATP
553 degradation was also accelerated in muscle from AMPK α imdKO mice despite intact mitochondrial
554 function and despite that these mice were running at the same relative exercise intensity. This clearly
555 suggests that AMPK activation during exercise is necessary for maintaining myocellular ATP levels
556 during exercise. The greater ATP degradation during exercise in combination with lower muscle
557 glycogen content in AMPK α imdKO mice may also explain the lower maximal running speed observed

558 in these mice. In contrast to previous studies, these observations were independent of muscle glucose
559 uptake, substrate utilization and mitochondrial function.

560 The role of AMPK in the regulation of substrate utilization during exercise has been
561 investigated in different mouse models with chronic deletion of AMPK activity [9,16,17,44].
562 AMPK β 1 β 2M-KO mice show increased reliance on FA oxidation during exercise (decreased RER) [9],
563 but direct interpretation of this observation is compromised by extreme exercise intolerance (~57%
564 reduction in maximal running speed) and hence dramatically absolute lower running speed during
565 treadmill exercise. In contrast, deletion of both AMPK α isoforms (AMPK α mdKO) or the upstream
566 kinase LKB1 (LKB1 KO) in skeletal muscle results in increased reliance on glucose utilization during
567 exercise (increased RER) [16,17]. This may be a consequence of impaired FA oxidation due to lowered
568 expression of fat transport proteins (e.g. CD36 and FABPpm) and lowered mitochondrial
569 capacity/enzyme activity reported for these mice [15–17]. In the current study, we observed normal
570 substrate utilization during *in vivo* exercise and *ex vivo* contractions of isolated muscles as well as
571 intact expression of fatty acid transporter proteins and mitochondrial respiratory function. Collectively,
572 this supports the notion that alterations in substrate utilization in mouse models with chronic deletion of
573 AMPK activity are due to persistent alterations in the protein expression profile, mitochondrial function
574 or extreme exercise intolerance rather than consequences of lacking acute AMPK-related regulation.

575 One of the proposed roles of AMPD is to prevent a large increase in ADP by removing AMP,
576 hereby favoring ATP formation by the adenylate kinase reaction [45]. Thus, we interpret the trend
577 towards increased AMPD activity and massive formation of IMP in skeletal muscle from AMPK α
578 imdKO mice during exercise as a protective mechanism to avoid accumulation of AMP. *In vitro* studies
579 have reported that AMPD activity is enhanced during muscle contractions in correspondence with

580 elevated H^+ as a result of lactate formation [46,47]. However, muscle lactate concentration increased
581 similarly in response to exercise in control and AMPK α imdKO mice, suggesting that other factors
582 than muscle H^+ accumulation contribute to the elevated AMPD activity in muscle from AMPK α
583 imdKO mice. For *in vitro* AMPD activity measurements, the muscle homogenate was diluted ~500 fold
584 for the assay procedure, likely eliminating possible influence of soluble factors present in the muscle.
585 This suggests the presence of a regulation *in vivo* during exercise in AMPK α imdKO mice that is
586 preserved during *in vitro* AMPD activity measurements. Phosphatase treatment has been shown to alter
587 affinity of AMPD for its substrate AMP while the V_{max} remains unchanged [30]. This suggests that
588 other allosteric regulations than protein phosphorylation have increased maximal AMPD activity in
589 muscle from exercising AMPK α imdKO mice in the present study.

590 While AMPK activation by pharmacological means demonstrates that AMPK is sufficient to
591 increase muscle glucose uptake [6–8], the proposed necessary role of AMPK in the regulation of
592 contraction-stimulated muscle glucose uptake has been studied intensively in various transgenic mouse
593 models with conflicting findings. Some studies have reported intact glucose uptake during *ex vivo*
594 muscle contractions [13,15,19,20,22], while other studies have reported a partially decreased ability to
595 increase muscle glucose uptake in response to contractile activity under some experimental conditions
596 [9,11–15]. Moreover, glucose uptake during exercise is impaired in AMPK β 1 β 2M-KO mice but
597 remains intact in AMPK α mdKO and LKB1-KO mice [9,16,17]. In the current study, we used an
598 inducible AMPK α KO mouse model and observed that AMPK is dispensable for regulating glucose
599 uptake in response to *in vivo* exercise and *ex vivo* muscle contractions.

600 TBC1D1 has been suggested to be involved in AMPK-mediated signaling that regulates
601 GLUT4 translocation to increase muscle glucose uptake during muscle contractions [48]. A number of

602 studies have reported reduced phosphorylation of TBC1D1 at Ser231 in muscle of AMPK-deficient
603 mice in response to muscle contractions [9,16,49–51]. Moreover, muscle from TBC1D1-deficient
604 mouse models show reduced contraction- and exercise-stimulated glucose uptake [52–54], suggesting
605 that the AMPK-TBC1D1 signaling axis is required for regulating glucose uptake during exercise.
606 However, the TBC1D1 KO mouse model is associated with impaired GLUT4 expression in skeletal
607 muscle, complicating the overall interpretation of those findings. Overexpression of TBC1D1 mutated
608 to alanine at four phosphorylation sites in skeletal muscle (Ser231Ala, Thr499Ala, Thr590Ala and
609 Ser621Ala) seems not to affect GLUT4 protein content and is still associated with a marked reduction
610 (22%) in contraction-stimulated glucose transport [55]. In contrast, mutation of a single
611 phosphorylation site on TBC1D1 (Ser231Ala) does not compromise contraction- and exercise-
612 stimulated glucose uptake [56]. In the present study, contraction-stimulated TBC1D1 phosphorylation
613 at Ser231 did not increase in glycolytic muscles (quadriceps and EDL) from AMPK α imdKO mice,
614 albeit muscle glucose uptake increased similarly in both genotypes. Recently, our research group has
615 clarified these seemingly discrepant findings of AMPK-TBC1D1 signaling in regard to contraction-
616 stimulated muscle glucose uptake. Thus, we convincingly show that AMPK and TBC1D1 are
617 necessary and important for maintaining glucose uptake elevated in skeletal muscle in the immediate
618 period after, but not during, exercise and contraction [57]. Altogether, these findings support the
619 concept that glucose uptake can be regulated independently of TBC1D1 phosphorylation at Ser231, and
620 it could be speculated that muscle contractile activity leads to an activation of a broad range of
621 intracellular signaling events that promote glucose uptake and fat oxidation during exercise
622 independently of AMPK.

623 All experiments in this study were performed at room temperature and hence below mouse
624 thermoneutrality. Recent evidence suggests that mild cold stress induced by ambient housing may
625 confound the experimental outcome in mice [58]. However, McKie and colleagues observed that
626 exercise-induced gene-expression and AMPK activation in muscle were comparable between
627 experiments performed at room temperature and thermoneutrality [59]. This indicates that at least the
628 acute exercise responses in skeletal muscle in the current study likely also would have been observed at
629 thermoneutrality.

630 Interestingly, the promptly reduced muscle glycogen content (1 wk after ended tamoxifen
631 treatment) in AMPK α imdKO mice was accompanied by a corresponding reduction in UGP2 gene
632 expression. Support for a direct link between AMPK activation and UGP2 gene expression has been
633 provided by transcriptomic profiling (microarray analysis) of muscles from AMPK γ 3^{R225Q} transgenic
634 mice, AMPK γ 3^{-/-} knockout mice, and AICAR-treated wild-type mice [60]. Thus, increased AMPK
635 activity, either by introducing an activating AMPK γ 3 mutation (AMPK γ 3^{R225Q}) or AMPK activation by
636 AICAR, induced an increase in UGP2 gene expression while genetic deletion of AMPK γ 3
637 (AMPK γ 3^{-/-}) induced a corresponding reduction. Furthermore, the AMPK γ 3 gain of function
638 mutation in pig muscle (AMPK γ 3^{R200Q}) leads to a 3-fold increase in UGP2 protein levels [61]. Based
639 on mutational cell studies, GS is considered rate-limiting for glycogen synthesis and UGP2 has
640 generally been considered to play a minor role for glycogen storage [62]. However, we speculate that
641 UGP2 may contribute to glycogen storage capacity by promoting glucose flux towards GS and explain
642 the lower muscle glycogen content in AMPK α imdKO mice.

643

644 In conclusion, we have generated a new mouse model with inducible skeletal muscle-specific
645 deletion of the catalytic AMPK α subunits in adult mice that allows us to study the direct effect(s) of
646 AMPK in muscle metabolism. Acute deletion of AMPK activity in adult mouse muscle reveals that
647 intracellular mediators other than AMPK are sufficient to regulate glucose uptake and substrate
648 utilization in response to exercise and muscle contractions. However, AMPK is central for maintaining
649 cellular nucleotide balance during exercise, as an increased deamination of AMP to IMP is observed in
650 muscle from AMPK α imdKO mice during exercise. Moreover, acute deletion of muscle AMPK α in
651 adult mice promptly reduces muscle glycogen content and lowers UGP2 expression. These
652 observations likely explain the lower maximal treadmill running capacity in AMPK α imdKO observed
653 already 1 week after AMPK α gene deletion.

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678

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876 **Figure legends:**

877

878 **Figure 1: Tamoxifen-induced deletion of AMPK α subunits in adult mice**

879 A-F: Muscle-specific deletion of AMPK α 1 and α 2 was obtained by expressing a tamoxifen-inducible
880 Cre-recombinase construct driven by the human skeletal muscle actin promoter. The tamoxifen
881 treatment protocol consisted of three single injections (40 mg/kg bw) each separated by 48 hours and
882 mice were investigated 1, 3 and 8 weeks after the last tamoxifen injection. For the vehicle experiment,
883 all mice received injections containing sunflower oil. Gene-expression of AMPK α 1 and α 2 subunits
884 was measured in EDL muscle. Protein levels of AMPK α 1 and α 2 were measured in EDL and heart
885 from control and AMPK α imdKO mice. These data from the AMPK α imdKO model were compared to
886 conventional AMPK α double KO model (AMPK α mdKO) with chronic lack of AMPK function.
887 Protein levels were measured by immunoblotting and gene-expression was measured by real time PCR
888 and presented relative to TATA-Box Binding Protein (TBP). Data have been normalized to control
889 mice (=100%). Data are given as means \pm SEM (n=5-6 within each group).
890 One-way ANOVA was used for comparing 1 wk, 3 wks and 8 wks to vehicle control within AMPK α
891 imdKO mice. Additional t-test was applied for comparison of AMPK α imdKO with control mice
892 within each given time point. * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$ for difference from corresponding
893 control mice. ## $p \leq 0.01$ and ### $p \leq 0.001$ for difference from corresponding AMPK α imdKO vehicle.
894 ††† $p \leq 0.001$ for difference from 1 week AMPK α imdKO.

895 **Figure 2: Normal insulin action despite acute deletion of catalytic AMPK function in skeletal**

896 **muscle.** A-B: 3 weeks after the last tamoxifen injection, control and AMPK α imdKO mice were fasted
897 for 5 hours before they were given an intraperitoneal injection of glucose (2 g/kg body weight)
898 dissolved in a 0.9% saline solution. Blood was sampled from the tail vein and analyzed for glucose
899 concentration by a glucometer before (0 min) and 20, 40, 60, 90 and 120 min after injection. Plasma
900 insulin levels were determined at 0, 20, and 40 min using an insulin ELISA assay (n=10-12). C: For the
901 insulin tolerance test (ITT), mice were fasted for 2 hours and insulin was injected intraperitoneally (1
902 U/kg body weight, Actrapid, Novo Nordisk, Bagsværd, Denmark). Tail vein blood glucose
903 concentration was measured before (0 min), 20, 40 and 60 min after injection (n=10-12). D-E: Isolated

904 EDL and soleus muscles from control and AMPK α imdKO mice were incubated for 30 min in the
905 absence (basal) or presence of 100 μ U/ml and 10,000 μ U/ml insulin and muscle glucose uptake was
906 determined by measuring the accumulation of intracellular [³H]-2-deoxyglucose (2DG) (n=6-8). F-H:
907 Key insulin signaling intermediates in EDL muscle from control and AMPK α imdKO mice were
908 investigated by immunoblotting and are given as representative immunoblots.
909 Data are given as means \pm SEM. Two-way RM ANOVA was used to investigate the effect of genotype
910 and time (GTT and ITT) or genotype and insulin concentrations (2DG uptake). #### p \leq 0.001 for
911 significantly different from basal (0 min). ** p \leq 0.01 and *** p \leq 0.001 for significantly different
912 compared to basal. §§ p \leq 0.01 and §§§ p \leq 0.001 for significantly different from 100 μ U/ml. Line
913 indicates main effect.

914

915 **Figure 3: Acute deletion of muscle AMPK impairs maximal running speed and reduces muscle**
916 **glycogen content and UGP2 expression.** A: Maximal running speed during an incremental running
917 test on a treadmill was assessed in control and AMPK α imdKO mice 1 and 3 wks after last tamoxifen
918 injection and compared to before tamoxifen treatment (pre) (n=10-20 within each group). B: Muscle
919 glycogen content in quadriceps muscle (normalized to control mice) was measured 1, 3 and 8 weeks
920 after tamoxifen-induced deletion of AMPK α and compared to vehicle control groups (n=5-6 within
921 each group). C: 3 wks following last tamoxifen injection muscle glycogen in quadriceps muscle from
922 control and AMPK α imdKO mice was measured in the rested state and following 30 min of treadmill
923 exercise at the same relative intensity (n=8-13). D: Glycogen synthase activity was measured as
924 fractional activity in the presence of 0.2 mM G6P and given relative to saturated conditions (8 mM
925 G6P) (n=8-13). E: Protein levels of GLUT4, HKII, GP, GS and UGP2 in quadriceps muscle were
926 measured by immunoblotting in control and AMPK α imdKO mice 3 wks following last tamoxifen
927 injection (n=5-6). UGP2 mRNA content in quadriceps muscle was determined 1, 3 and 8 weeks after
928 the last tamoxifen injection and compared to the vehicle group (sunflower oil) (n=5-6).
929 One-way ANOVA was used for comparing 1 wk, 3 wks and 8 wks to vehicle control within AMPK α
930 imdKO mice. Additional t-test was applied for comparison of AMPK α imdKO with control mice
931 within each given time point. The effect of exercise was investigated by a two-way ANOVA (C and
932 D). Data are given as means \pm SEM. * p \leq 0.05, ** p \leq 0.01 and *** p \leq 0.001 for effect of genotype

933 within a given time-point. ### p \leq 0.001 for difference from vehicle in AMPK α imdKO mice. §§ p \leq 0.01
934 for main effect of exercise. Line indicates main effect.

935
936 **Figure 4: Regulation of myocellular nucleotide pool during exercise.**

937 Overview of cellular processes regulating cellular nucleotide balance. The increasing ATP utilization
938 during exercise leads to ATP regeneration through the adenylate kinase reaction (2 ADP \rightarrow AMP +
939 ATP), which increases accumulation of AMP. In order to avoid a large accumulation of AMP in the
940 cell, AMP is deaminated to IMP via the enzyme AMP deaminase (AMPD). Intracellular IMP, formed
941 during exercise, can be degraded to inosine (INO) and hypoxanthine (HX), which can leave the muscle
942 cell potentially causing a nucleotide loss. cN-II: Cytosolic nucleotidase II, cN-IA: Cytosolic
943 nucleotidase IA, PNP: Purine nucleoside phosphorylase, P_i: Inorganic phosphate, NH₃: Ammonia.

944 **Figure 5: AMPK is necessary to maintain the cellular nucleotide pool during exercise.**

945 A-G: Control and AMPK α imdKO mice performed 30 min of treadmill exercise at the same relative
946 intensity and were compared to corresponding resting mice. Concentration of adenosine triphosphate
947 (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), inosine monophosphate
948 (IMP), hypoxanthine (HX), adenosine (ADO) and inosine (INO) were measured in quadriceps muscle
949 (n=6-8). H-I: AMPD activity and AMPD1 protein content was measured in quadriceps muscle (n=6-8).
950 Data are given as means \pm SEM. Two-way ANOVA was used for statistical analyses of genotype and
951 exercise. ** p \leq 0.01 and *** p \leq 0.001 for significant effect of genotype. §§ p \leq 0.01 and §§§ p \leq 0.001 for
952 difference from resting. Line indicates main effect.

953
954 **Figure 6: AMPK is dispensable for regulation of muscle substrate utilization and mitochondrial**
955 **function.**

956 A: RER before, during and after 30 min of a single treadmill exercise bout at 60% of individual
957 maximal running speed (n=18-20). B: Palmitate oxidation was measured *ex vivo* in resting or
958 contracting soleus muscles from control and AMPK α imdKO mice (n=15-18). C. Protein levels of
959 plasma membrane fatty acid binding protein (FABPpm) and cluster of differentiation (CD) 36 were

960 analyzed in TA muscle by immunoblotting (n=8-13). D: Mitochondrial respiration rates measured
961 during cumulative addition of substrates in permeabilized TA fibers (n=9-12). Abbreviations: CI_p:
962 Maximal complex I respiration, CI+II_p: Maximal complex I+II linked respiration (capacity for
963 oxidative phosphorylation), ETS (CI+II): Electron transport system capacity (uncoupled respiration)
964 through complex I and II, ETS (CII): Electron transport system capacity through complex II. E: Protein
965 levels of mitochondrial subunits for complex I, II, III, IV and V in TA muscle were determined by
966 immunoblotting (n=17-18). Data are given as means ± SEM. The effect of exercise and genotype was
967 investigated by two-way ANOVA. §§§ p≤0.001 for difference from resting. Line indicates main effect.

968

969 **Figure 7: AMPK is not required for exercise and contraction-stimulated glucose uptake in**
970 **skeletal muscle.**

971 A-C: Control and AMPK α imdKO mice were either rested or performed 30 min treadmill exercise at
972 the same relative intensity. Phosphorylation of AMPK α Thr172, TBC1D1 Ser231 and ACC Ser212
973 was determined in quadriceps muscle by immunoblotting (n=8-13). D-E: Blood glucose concentration
974 and muscle lactate concentration in quadriceps muscle was measured under resting conditions and
975 following 30 min of treadmill exercise (n=8-13). F: Muscle glucose uptake during 30 min of treadmill
976 exercise was measured in TA, soleus, EDL and quadriceps muscle from control and AMPK α imdKO
977 mice (n=8-13). G-H: Isolated EDL and soleus muscle from control and AMPK α imdKO mice were
978 electrically forced to contract and glucose uptake was measured in resting and contracting muscles
979 (n=4-8). Data are given as means ± SEM. The effect of genotype and exercise/muscle contraction was
980 investigated by two-way ANOVA. ** p≤0.01 and *** p≤0.001 for significant effect of genotype. §
981 p≤0.05, §§ p≤0.01 and §§§ p≤0.001 for difference from resting. Line indicates main effects.

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987 **Tables:**

988

989 **Table 1: Body composition of control and AMPK α imdKO mice**

	Pre tamoxifen treatment		3 wks post tamoxifen treatment		Main effect	Interaction
	control	imdKO	control	imdKO		
Weight (g)	26.3 \pm 0.4	26.3 \pm 0.5	25.7 \pm 0.5	25.3 \pm 0.4	***	-
Fat mass (g)	2.0 \pm 0.2	1.8 \pm 0.2	2.2 \pm 0.1	2.1 \pm 0.1	*	-
LBM (g)	21.9 \pm 0.3	22.3 \pm 0.4	21.8 \pm 0.4	21.5 \pm 0.3	-	p = 0.003

990 Body composition was investigated before (pre) and 3 weeks after (post) tamoxifen treatment. Data are
991 given as means \pm SEM (n=20). * p < 0.05 and *** p < 0.001 for significant difference from pre
992 treatment independently of genotype.

993

994 **Table 2: Tissue weight in control and AMPK α imdKO mice.**

Tissue	Weight (mg)		p-value
	control	imdKO	
Tibialis anterior	42 \pm 1	43 \pm 1	0.49
Heart	101 \pm 3	98 \pm 3	0.52
WAT	200 \pm 14	245 \pm 37	0.28
Liver	991 \pm 79	988 \pm 78	0.98
Kidney	134 \pm 6	136 \pm 10	0.90

995

996 Weight (mg) of tibialis anterior muscle, heart, white subcutaneous adipose tissue (WAT), liver, and
997 kidney was investigated 3 weeks after (post) tamoxifen treatment. Data are given as means \pm SEM
998 (n=8-12).

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1002

1003 Table 3: AMPD kinetic properties

	WT	imdKO	p-value
V_{\max} ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$)	479 ± 47	541 ± 83	0.35
K_m (mM)	0.27 ± 0,05	0.37 ± 0,13	0.46

1004 Kinetic properties for AMPD (V_{\max} and K_m) in basal quadriceps muscle were analyzed in homogenate
1005 in the presence of 15 mM, 0.1 mM, 0.06 mM, and 0.04 mM AMP and the formation of IMP was
1006 quantified by HPLC. Data are given as means ± SEM (n=6-8).