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1 **Endurance exercise decreases protein synthesis and ER-mitochondria contacts in mice skeletal**  
2 **muscle**

3

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11

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14

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19

20 **Abstract**

21 Exercise is important to maintain skeletal muscle mass through stimulation of protein synthesis, which  
22 is a major ATP-consuming process for cells. However, muscle cells have to face high-energy demand  
23 during contraction. The present study aimed to investigate protein synthesis regulation during aerobic  
24 exercise in mouse hindlimb muscles. Male C57Bl/6J mice ran at 12 m/min for 45 min or at 12 m/min  
25 for the first 25 min followed by a progressive increase in velocity up to 20m/min for the last 20 min.  
26 Animals were injected I.P. with 40 nmol/g of body weight of puromycin and sacrificed by cervical

27 dislocation immediately after exercise cessation. Analysis of gastrocnemius, plantaris, quadriceps,  
28 soleus and tibialis anterior muscles revealed a decrease in protein translation assessed by puromycin  
29 incorporation, without significant differences among muscles or running intensities. The reduction of  
30 protein synthesis was associated with a marked inhibition of mTORC1-dependent phosphorylation of  
31 4EBP1, a mechanism consistent with reduced translation initiation. A slight activation of AMPK  
32 consecutive to the running session was measured but did not correlate with mTORC1 inhibition. More  
33 importantly, exercise resulted in a strong upregulation of REDD1 protein and gene expressions, while  
34 transcriptional regulation of other recognized exercise-induced genes (IL6, KLF15, and Rcan1) did not  
35 change. Consistently with the recently discovered role of REDD1 on mitochondria-associated  
36 membranes, we observed a decrease in mitochondria-ER interaction following exercise. Collectively,  
37 these data raise questions concerning the role of MAM disruption in the regulation of muscle  
38 proteostasis during exercise and, more generally, in cell adaptation to metabolic stress.

39

#### 40 **Key words**

41 AMPK, MAMs, protein synthesis, REDD1, running exercise

42

#### 43 **New & Noteworthy**

44 How muscles regulate protein synthesis to cope with the energy demand during contraction is poorly  
45 documented. Moreover, it is unknown whether protein translation is differentially affected among  
46 mice hindlimb muscles under different physiological exercise modalities. We showed here that 45 min  
47 of running decreases puromycin incorporation similarly in five different mice muscles. This decrease  
48 was associated with a strong increase in REDD1 protein expression and a significant disruption of the  
49 mitochondria and sarcoplasmic reticulum interaction.

50

51

52 **Introduction**

53 Regular physical activity exerts beneficial effects on health, which are largely related to muscle  
54 adaptations (17). For example, endurance training improves metabolic profile as illustrated by a  
55 reduced whole body adiposity and a greater insulin sensitivity (8, 17). At the muscle level, metabolic  
56 adaptations to repeated endurance exercises involve a transition in muscle typology from glycolytic to  
57 more oxidative fibers and an alteration of mitochondrial function and/or network (17, 27). Endurance  
58 exercise also prevents from skeletal muscle atrophy induced by disuse or various pathologies (41). The  
59 maintenance or the increase in muscle mass relies on protein balance and particularly on protein  
60 synthesis. While studies have often focused on resistance exercise, a stimulus known for promoting  
61 muscle protein accretion (28), knowledge about the regulation of protein synthesis with endurance  
62 exercise remains more limited. Considering that protein synthesis is an energy demanding process (34,  
63 45), one may suppose that it will be reduced proportionately to the increased metabolic disturbance  
64 occurring during exercise. However, it is unclear, whether muscle typology or exercise intensity  
65 influence protein synthesis in response to an endurance exercise bout.

66 The mammalian target of rapamycin complex 1 (mTORC1) is a central node regulating translation of  
67 capped mRNA into polypeptides through activation of initiation and elongation factors (14). For  
68 instance, phosphorylation of eIF4-binding protein 1 (4EBP1) by mTORC1 allows eIF4E-eIF4G  
69 interaction, which is a crucial event for mRNAs recruitment to ribosomes (34). Most of the studies (e.g.  
70 (24, 37)) report the activation of mTORC1 as a proxy for protein synthesis, partly due to methodological  
71 difficulties to directly measure mRNA translation. Among the negative regulators of mTORC1, AMP  
72 activated protein kinase (AMPK) and Regulated in Development and DNA Damage 1 (REDD1) have  
73 been proposed to inhibit protein synthesis during exercise (13, 31). AMPK activation depends on the  
74 cell energy status, notably the AMP:ATP ratio, and promotes both ATP generation and inhibition of  
75 biosynthetic pathways (23). During muscle contractions, AMPK activation depends upon exercise  
76 intensity (29, 37, 48). While the exact mechanisms remain to be uncovered, REDD1 protein expression  
77 is also enhanced by energetic stress and thus aerobic exercise (7, 19, 24). REDD1 reduces protein

78 synthesis and alters mitochondrial function in skeletal muscle (6, 7). The effect of REDD1 on  
79 mitochondrial activity might originate from its role on endoplasmic reticulum (ER)-mitochondria  
80 contact sites (called hereafter MAMs for mitochondria-associated membranes). MAMs are a key hub  
81 linking mitochondrial respiration, mTORC1 signaling and protein synthesis (4, 7). Recently, our team  
82 showed that REDD1 reduced MAMs formation in muscle cells, which was associated with a decrease  
83 in protein synthesis (7). However, it is still unknown whether exercise influences MAMs dynamic.  
84 Hence, our study aims to determine how exercise intensity and/or muscle typology alter protein  
85 translation and its regulation in mice. We hypothesize that protein synthesis will be inversely  
86 correlated to exercise intensity and that the decrease in protein synthesis will be associated with  
87 reduced mTORC1 activity and MAM formation.

88

89

## 90 **Materials and methods**

### 91 *Animals*

92 Experiments were carried out on 5-month old male C57Bl/6J mice from our own stock. Animals (3-4  
93 per cage) were housed in a 12-h/12-h light/dark cycle at 22°C with food and water ad libitum.  
94 Experiments were conducted in the morning ( $\approx$ 10 am) with mice having free access to food until the  
95 beginning of the exercise bout. Food was removed in the control group while the other groups started  
96 their running exercise. These experiments were performed according to the European directives  
97 (86/609/CEE) and approved by the national ethics committee (referral file APAFIS#9706-  
98 2,017,042,516,166,776 v3).

99

### 100 *Exercise protocol*

101 Running sessions were performed on a motor-driven treadmill (Exer-6M Treadmill; Columbus  
102 Instruments). First, all mice were acclimatized to treadmill for 10-15 min at 12 m/min on three  
103 consecutive days. The last acclimatization session took place 48h before the exercise protocol. Then,

104 mild intensity exercise (M; n=8) consisted of a 45 min bout of running at a velocity of 12 m/min, which  
105 represents about 50% of maximal aerobic velocity in our conditions (7). Heavy intensity exercise (H;  
106 n=8) started by 25 min running at 12 m/min followed by increments of 2 m/min every 5 min. Exercise  
107 was stopped after a total duration of 45 min with the last 5-min performed at a speed of 20 m/min  
108 (approximately 75-80% of maximal aerobic velocity; (7)). We covered the front end of the treadmill to  
109 reduce the potential stress associated with exercise on treadmill and attract the mice to run forward.  
110 Animals from M and H groups were sacrificed immediately after the 45 min running bout by cervical  
111 dislocation. Mice from control group (C; n=8) were maintained in their cage. All animals were injected  
112 intraperitoneously (I.P.) with 40 nmol/g of body weight of puromycin (8  $\mu$ l/g of a solution at 5 mM in  
113 PBS) 20 min before cervical dislocation and tissue harvest. Exercise was interrupted (<15s) for the M  
114 and H groups to allow puromycin injection. Tibialis anterior (TA), gastrocnemius (GAS), soleus (SOL),  
115 plantaris (PLA) and quadriceps (QUA) muscles were removed in the same order consistently, within 10  
116 min following exercise cessation, and immediately snap-frozen in liquid nitrogen.

117

### 118 *Immunoblotting*

119 Frozen muscles were powdered and homogenized in lysis buffer containing 50 mM Tris-HCl, 100 mM  
120 NaCl, 5% glycerol, 1 mM EDTA, 1 mM EGTA, 50 mM  $\beta$ -glycerophosphate, 50 mM NaF, 1 mM  $\text{Na}_3\text{VO}_4$ ,  
121 1% Triton X-100, and protease inhibitor cocktail with Fastprep-24 (MP Biomedical, Santa Ana, CA).  
122 Total proteins (50  $\mu$ g) were loaded on a 4–15% acrylamide precast gel (Bio-Rad, Hercules, CA) before  
123 SDS-PAGE and transferred onto a nitrocellulose membrane with the Trans-Blot<sup>®</sup> Turbo<sup>™</sup> Transfer  
124 System (Bio-Rad). Membranes were blocked in 5% nonfat dry milk in Tris-buffered saline (TBS) for 1 h  
125 and then incubated overnight at 4°C with primary antibody at 1:1000 except for puromycin (1:2000)  
126 and REDD1 (1:500) in TBS 0.1% Tween and 5% of nonfat dry milk or BSA according to the  
127 manufacturer's recommendations. Antibodies 4EBP1 (#9452), 4EBP1 ser<sup>65</sup> (#9451), Acetyl-CoA  
128 Carboxylase (ACC; #3676), ACC ser<sup>79</sup> (#11818), AMPK (#5832), AMPK thr<sup>172</sup> (#2535), ERK1/2 (#4696),  
129 ERK1/2 Thr202/Tyr204 (#4370), p38 (#9212), p38 thr<sup>180</sup>/tyr<sup>182</sup> (#9211), S6 (#2317), and S6 Ser240/244

130 (#5364) were purchased from Cell Signaling Technology (Danvers, MA), puromycin antibody (MABE34)  
131 from Merck (Darmstadt, Germany), and REDD1 antibody (#10638-1-AP) from ProteinTech (Chicago,  
132 IL). Protein bands were visualized using peroxidase-conjugated secondary antibody and enhanced  
133 chemiluminescent (ECL) reagent with the Chemidoc™ Touch Imaging System and Image Lab™ Touch  
134 Software (Bio-Rad). Puromycin and REDD1 contents were normalized using stain-Free technology to  
135 loading controls on the membrane (Fig. 1). Phosphorylated protein 4EBP1, ACC, AMPK, ERK1/2, p38  
136 and rpS6 were normalized to total protein content.

137

### 138 *Duo-link proximity ligation in situ assay*

139 *In situ* duo-link proximity ligation assay was performed on paraffin-embedded sections of skeletal  
140 muscle to monitor MAM number as previously described (7). Soleus and plantaris muscles from C and  
141 M conditions (n=4/group) were fixed in 4% paraformaldehyde on ice for 4-5h. Longitudinal sections  
142 were stained for nucleus with haematoxylin and immunolabelled with rabbit anti-inositol  
143 trisphosphate receptor (IP3R; Santa Cruz Biotechnology, Inc., Dallas, TX; #sc-28614) and mouse anti-  
144 voltage-dependent anion channel (VDAC; Abcam, Cambridge, UK; #ab14734) primary antibodies.  
145 Then, samples were processed for proximity ligation assay according to the manufacturer protocol  
146 (Olink Bioscience, Uppsala, Sweden). Secondary anti-mouse and anti-rabbit IgG conjugated antibodies  
147 were added to complementary oligonucleotide extensions (probe MINUS and PLUS). If the distance  
148 between the IP3R and VDAC proteins was below 40 nm, the secondary antibodies conjugated  
149 oligonucleotides hybridized with a connector oligonucleotide allowing the formation of a circular DNA  
150 template. After ligation and amplification steps, this DNA template was detected following  
151 hybridization using an HRP coupled-oligonucleotide probe. Then, an immunohistochemistry HRP  
152 enzymatic reaction generated spots, each one resulting from a single endogenous IP3R/VDAC  
153 interaction. Finally, these spots were quantified with a 20x magnification lens using a bright-field  
154 microscopy device (10 fields per muscle).

155

156 *RNA isolation and RT-qPCR*

157 Total RNA was extracted from muscle samples using TRIzol Reagent (ThermoFisher Scientific,  
158 Courtaboeuf, France). RNA concentration was determined by spectrophotometry (Biodrop,  
159 Cambridge, UK) and purity was checked by the OD 260nm/OD 280nm ratio (>1.9). RNA quality was  
160 checked by electrophoresis on 1% agarose gel. Reverse transcription was performed with 2 µg of total  
161 RNA using the High-Capacity cDNA Reverse Transcription kit (4368813, Applied Biosystems,  
162 Courtaboeuf, France), and according to the manufacturer's instructions. Quantitative PCR (qPCR)  
163 analysis was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems) with 10 µl  
164 of Powerup SYBR Green Master Mix (A25742, Applied Biosystems), 10 nM of both forward and reverse  
165 primers (Table 1), and 5 µl of diluted cDNA (final volume of 15 µl). All PCR assays were performed in  
166 duplicate using the following cycling conditions: 50°C for 2 min, then 95°C for 2 min followed by 40  
167 cycles of 95°C for 3 s and 60°C for 30 s. The relative mRNA levels were normalized with the arithmetic  
168 mean of *α-tubulin* (*α-tub*) and *acidic ribosomal phosphoprotein P0* (*Arp*), as these housekeeping genes  
169 remained unaffected by the experimental conditions. Results were expressed using the comparative  
170 cycle threshold (Ct) method to generate  $\Delta\Delta Ct$  values with template dilutions ranging from  $10^1$  to  $10^6$   
171 copies. The PCR overall efficiency (E) was calculated from the slopes of the standard curves according  
172 to the equation  $E = [10^{(-1/\text{slope})}] - 1$  and this value was higher than 95% for all assays. The relative  
173 abundance of each sample was then normalized according to the equation: Relative Quantity (RQ) =  $2^{-\Delta\Delta Ct}$   
174 (39). All the experiments were conducted according to the minimum information for publication  
175 of quantitative real-time PCR experiments (MIQE) guidelines (9).

176

177 *Statistics*

178 Data are presented as mean + SEM. A two-ways analysis of variance (ANOVA) was performed to  
179 determine the respective effects of exercise intensity and muscle type on the dependent variables  
180 (protein and gene expressions) after confirming data normality using the skewness and kurtosis  
181 coefficients. Differences between groups were identified using the Bonferroni post-test for multiple



182 comparisons when ANOVA was significant. Mann-Whitney U test was used to analyze data differences  
183 from duo-link proximity ligation assay (n=4/group) and gene expressions on SOL muscles (n=3/group).  
184 The critical alpha level was set at 5%.

185

186

## 187 **Results**

188 Puromycin administration has been shown to be a reliable method to measure protein synthesis (18).  
189 Analysis of puromycin incorporation revealed a significant negative effect of exercise on protein  
190 translation (Fig. 1A). Contrary to our expectations, the 17 to 32% decrease in protein synthesis was not  
191 significantly different between mild (M) and heavy (H) exercise modalities and/or among the five  
192 studied muscles. mTORC1-dependent phosphorylation of 4EBP1 on ser<sup>65</sup>, an event required to  
193 promote cap-dependent translation initiation, displayed a marked decrease during exercise (from -47  
194 to -80%) with no difference between M and H groups (Fig. 1B). A significant, albeit weak, correlation  
195 between puromycin content and 4EBP1 phosphorylation was observed on the mean data from the five  
196 muscles ( $r^2=0.42$ ;  $p=0.033$ ). Phosphorylation of S6 ribosomal protein (rpS6) on ser<sup>240/244</sup>, a downstream  
197 target of mTORC1/S6 kinase signaling (32), was noted in three muscles (GAS, PLA and TA). Consistently  
198 with 4EBP1 phosphorylation changes, a significant decrease in rpS6 ser<sup>240/244</sup> phosphorylation during  
199 exercise (40-50% decrease except for TA in M modality, -19%) was observed, with no differences  
200 between M and H modalities (Fig. 1C).

201

202 Given the lack of differences in protein synthesis between the five muscles, we checked whether  
203 running exercise exerted differential strain on these muscles. Therefore, we measured p38 mitogen-  
204 activated protein kinase (MAPK) activation as it senses signals arising from mechanical strains and  
205  $Ca^{2+}_{intra}$  fluxes during aerobic exercise (27). Phosphorylation on thr<sup>180</sup>/tyr<sup>182</sup> was positively altered by  
206 exercise with no difference between M and H modalities (Fig. 2A). A main effect of muscle type and a  
207 positive interaction between intensity and muscle type were observed, SOL muscle displaying greater

208 responses to exercise (+1790 and +1970% in M and H condition vs. C, respectively) compared to other  
209 muscles ( $\approx$  +50% in GAS; +160% in PLA, and QUA vs. C) in agreement with the higher level of neural  
210 activation recorded for the SOL during running (38).

211 ERK1/2 MAPK activation was assessed in three muscles (GAS, SOL, and TA). Along with a p38 activation,  
212 we recorded a main positive effect of exercise, with muscles from H group exhibiting significant greater  
213 values compared to C group muscles (Fig 2B). In addition, exercise-induced ERK1/2 activation was  
214 higher in the SOL (+830 and +1300% in M and H vs. C groups, respectively) compared to GAS (+130%)  
215 and TA (+70%) muscles.

216

217 Then, we investigated known regulators of mTORC1 and protein synthesis. Running had a main positive  
218 effect on AMPK phosphorylation (Fig. 3A). Post-hoc analysis revealed that the H modality elicited  
219 greater AMPK thr<sup>172</sup> protein content compared to control (+15% in SOL; +30% in QUA; +74% in GAS; +  
220 166% in PLA and +210% in TA). There were no differences between muscles and no muscle x intensity  
221 interaction. Values from the M condition were not different from the two other groups (C and H).

222 We also analyzed the phosphorylation pattern of ACC, a recognized substrate of AMPK (47). AMPK-  
223 mediated phosphorylation of ACC was altered by exercise with M and H modalities displaying greater  
224 values compared to C group (Fig. 3B). Muscle type had a main effect on ACC phosphorylation while  
225 there was no interaction between muscle and intensity. Indeed, post-hoc analysis revealed that  
226 exercise-induced ACC phosphorylation tended to be greater in TA vs. GAS and SOL ( $p=0.013$  and  
227  $p=0.014$  respectively; with corrected  $\alpha$  risk set to 0.005).

228

229 Exercise strongly induced REDD1 mRNA and protein expression (Fig. 4A and B). REDD1 protein content  
230 was significantly greater in M (+110 to +500% depending on muscle type) and H conditions (+170 to  
231 +880% depending on muscle type) vs. C condition ( $p=0.0002$  and  $p<0.0001$ , respectively). We observed  
232 a main effect of muscle type on REDD1 protein expression with SOL tending to have lower values  
233 compared to GAS and PLA ( $p=0.019$  and  $p=0.022$ , respectively with Bonferroni corrected  $\alpha$  risk set to

234 0.005). Statistical analysis also showed a significant interaction between intensity and muscle type on  
235 REDD1 protein content. *Redd1* gene expression was enhanced by exercise, with no difference between  
236 M (+330 to +775% depending on muscles type) and H modalities (+250 to +1120% depending on  
237 muscle type). As for protein, we observed a main effect of muscle type and a significant interaction  
238 between muscle type and intensity on REDD1 mRNA level. Indeed, TA and PLA muscles exhibited  
239 greater increase in REDD1 mRNA (+650 to 1120%) compared to QUA muscle (+330 and +250% in M  
240 and H modalities, respectively, compared to C). All mRNA expression values from SOL were analyzed  
241 separately due to the lack of data for the H intensity. M condition induced a 170% increase in REDD1  
242 mRNA of SOL muscle. There was no significant correlation between REDD1 protein and mRNA (not  
243 shown).

244

245 Gordon et al. recently showed that the increase in REDD1 mRNA expression following running exercise  
246 was partially dependent on glucocorticoid receptor (19). Kruppel-like factor 15 (KLF15) mRNA  
247 expression shares common features with REDD1 since it is induced by glucocorticoids (40) as well as  
248 endurance exercise (21). In addition, both REDD1 and KLF15 suppress mTORC1 activity in muscle (40).  
249 However, in contrast to REDD1, there were no exercise or muscle type effects on KLF15 expression  
250 (Fig. 5A).

251 Therefore, we wanted to determine whether expressions of other previously identified exercise-  
252 responsive genes were induced in our conditions (i.e. immediately after exercise). We focused on  
253 calcium-dependent genes since it may mirror muscle recruitment. Indeed, activation of motoneurons  
254 during muscle contractions results in enhanced intracellular calcium ( $\text{Ca}^{2+}_{\text{intra}}$ ) flux. Increased  $\text{Ca}^{2+}_{\text{intra}}$   
255 then activates  $\text{Ca}^{2+}$ -dependent enzymes such as the phosphatase calcineurin. We measured mRNA  
256 expression of *regulator of calcineurin 1* (*Rcan1*, a.k.a *MCIP1*) and *interleukin-6* (*IL-6*), two genes with  
257 expressions related to calcineurin activity (1–3). Exercise had a main significant effect on *Rcan1* mRNA  
258 level (Fig. 5B). However, there was no effect of muscle type or muscle x intensity interaction. Post-hoc  
259 analysis revealed significantly greater values for M vs. H modality. As for *Rcan1*, we observed a main

260 effect of exercise intensity on IL-6 mRNA expression with no influence of muscle type or significant  
261 muscle x intensity interaction (Fig. 5C). In contrast to Rcan1 data, H group displayed greater IL6 gene  
262 expression compared to M group.

263

264 Since REDD1 can control MAM dynamic (7), we assessed MAM number via the interaction between  
265 two proteins that localize on the outer mitochondrial membrane (VDAC) and the sarcoplasmic  
266 reticulum membrane (IP3R). M condition induced a 30 and 44% decrease of VDAC/IP3R interaction in  
267 PLA and SOL muscles, respectively (Fig. 6). In addition, basal ER-mitochondria contacts tended to be  
268 higher in SOL vs. PLA muscle (+40%; p=0.083).

269

270

## 271 **Discussion**

272 In the present study, we measured puromycin incorporation, activation, and expression of relevant  
273 protein synthesis regulators immediately after an endurance exercise bout. We found a 20-30%  
274 inhibition of protein translation with no significant differences either between mild and heavy  
275 intensities or between the five muscles studied. These results are consistent with the similar reduction  
276 of mTORC1 activity regardless of muscle type or running intensity. The decrease in 4EBP1  
277 phosphorylation with exercise suggests a slower initiation of protein translation, which is in agreement  
278 with the impeded polysome formation previously observed by Williamson et al. in mice muscle  
279 following a 30 min running bout (46). Interestingly, these observations were concomitant with a  
280 decrease in muscle mitochondria-ER interaction. We further investigated the putative mechanisms  
281 involved in this regulation.

282

## 283 **Regulation of protein synthesis**

284 mTORC1 is considered a master regulator of protein synthesis in muscle cells following anabolic stimuli  
285 (28). While many studies reported similar variations between mTORC1 activity and protein synthesis

286 (6, 7, 13, 18, 30, 36, 46), Philp et al. observed an increase in myofibrillar and mitochondrial protein  
287 synthesis despite a reduction in mTORC1 activity 30 min after an endurance exercise bout (33). In  
288 addition, rapamycin administration, which inhibits mTORC1 activity, modestly reduced myofibrillar  
289 protein synthesis suggesting a marginal role of mTORC1 in endurance exercise-induced regulation of  
290 protein synthesis. However, one may hypothesize that the effect of rapamycin was modest because  
291 mTORC1 activity was already low. Moreover, it is important to note that our data may differ from  
292 Philip's due to a different muscle collection time (immediately vs. 30 min post exercise, respectively)  
293 as well as a different timing of protein synthesis tracer injection (during vs. after exercise, respectively).  
294 Although the precise role of mTORC1 in the protein synthesis response to endurance exercise remains  
295 to be elucidated, our data suggest that alteration of mTORC1 activity (but not in ERK1/2 or p38  
296 activities) could be responsible for the reduced protein anabolism during exercise.

297 AMPK and REDD1 are two negative regulators of mTORC1. AMPK acts through direct phosphorylation  
298 on Raptor (20) or tumor suppressor complex 2 (TSC2) (25), whereas REDD1 has been proposed to  
299 inhibit Akt activation (11) or to release TSC2 from its association with inhibitory 14-3-3 proteins (12).

300 In the present study, phosphorylation of AMPK and its downstream target ACC were not only slightly  
301 altered by exercise but also did not correlate with puromycin uptake or 4EBP1 phosphorylation. Similar  
302 to our study, others had previously reported an uncoupling between AMPK phosphorylation and  
303 protein synthesis or 4EBP1 phosphorylation (24, 37). In addition, genetic deactivation of AMPK does  
304 not alter inhibition of neither protein synthesis nor 4EBP1 phosphorylation in response to ex-vivo  
305 contraction (36). Altogether, it appears that AMPK is not the main nor the earliest repressor of protein  
306 synthesis during muscle contraction. Indeed, our results suggest that REDD1 could transiently inhibit  
307 muscle protein translation, even for mild intensity exercise. In addition, we previously showed that  
308 REDD1 deletion resulted in muscle AMPK overactivation following fasting, hypoxia and exercise (7).

309 Therefore, AMPK might be preferentially activated in conditions when muscle cells are in high energy  
310 demand.

311 The robust increase in both REDD1 protein (3 to 10-fold) and mRNA (3 to 12-fold) observed right after  
312 an exercise session reinforces the idea that REDD1 is important for muscle cell adaptation to  
313 contractions. *Redd1* gene induction could originate from several signals activated by exercise, among  
314 which glucocorticoids release (19), cellular hypoxia (15), adrenaline secretion (49), oxidative stress (22,  
315 26), and potentially heat stress (16, 43). In addition, exercise activates MEK pathway in skeletal muscle  
316 (44, 46), as attested here by the increase in ERK1/2 phosphorylation, and MEK stabilizes and protects  
317 REDD1 from proteasomal degradation in adipocytes and HEK cells (35). Thus, the rapid increase in  
318 REDD1 protein level during running could also be related to MEK signaling. Overall, our data provide  
319 evidences that REDD1 (mRNA and protein expressions) is a valuable biomarker to assess muscle stress  
320 during exercise.

321 We have previously demonstrated that REDD1 i) localizes in the crude mitochondrial fraction after  
322 endurance exercise, ii) interacts with some MAM components including glucose regulated protein 75  
323 (GRP75; also called mortalin/PBP74/mtHSP70), and iii) disrupts MAM (7). In turn, the reduced  
324 interaction between mitochondria and ER may decrease ATP availability for ER-dependent protein  
325 translation, which represents a major energy consuming process (34, 45). Indeed, we have showed  
326 that REDD1 silencing led to an increase in MAM interaction and protein synthesis in muscle cells (7).  
327 Accordingly, in our study, M exercise led to a clear decrease in ER-mitochondria interaction based on  
328 a proximity ligation assay performed on muscles. Furthermore, basal MAM formation tended to be  
329 greater in the SOL, a muscle with high protein turnover (10). These results support the hypothesis that  
330 protein synthesis in muscle cells could be tightly regulated by the connection between these two  
331 organelles, and that REDD1 might control this mechanism.

332

### 333 **Effect of muscle type**

334 We choose to analyze five hindlimb muscles that are commonly used in exercise physiology studies.  
335 Three of these muscles (GAS, QUAD and TA) are rich in type IIB (glycolytic) fibers while one (SOL)  
336 contains predominantly type I and IIA (oxidative) fibers. The last one muscle (PLA) displays a mixed

337 phenotype (5). Previous studies illustrated a different susceptibility of protein synthesis to repeated  
338 contractions between glycolytic and oxidative muscles, the latter being less affected by exercise (10,  
339 30, 36). However, these discrepancies with our results could be explained by two factors. Firstly, the  
340 level of muscle recruitment in the experimental models used in these studies (electrostimulation) is  
341 far greater compared to our conditions of exercise, thus contributing to the high levels of protein  
342 synthesis and/or mTORC1 inhibition recorded by others (10, 30, 36). Secondly, experiments on isolated  
343 muscles induced exogenous electrostimulation levels that are similar across the tested muscles, while  
344 during running session, muscles will be differentially recruited depending on the exercise modalities  
345 (e.g. SOL >> TA, (38)). The striking difference in p38 phosphorylation observed in this study between  
346 SOL and the four other muscles further confirms that running elicited various mechanical strains.  
347 Therefore, even if protein synthesis of glycolytic muscles is more affected by contraction than in  
348 oxidative ones (10, 30, 36), the expected stronger effect on protein synthesis could be balanced by a  
349 poorer recruitment of these fast twitch muscles during the running session.

350

### 351 **Methodological considerations**

352 We hypothesized that running velocity would impact protein synthesis and TORC1 activity as suggested  
353 by others (10, 37). Bylund-Fellenius et al. proposed that protein synthesis is inversely related to the  
354 energy state. Assuming that running at 20 m/min elicits greater metabolic changes compared to 12  
355 m/min, it seemed reasonable to expect higher variations of the energy status in H compared to M  
356 group. In fact, AMPK activation was significantly increased in H but not in M conditions compared to  
357 C. As AMPK negatively regulates TORC1 activity, a greater AMPK phosphorylation should have caused  
358 higher TORC1 inhibition in H modality. One may wonder if the duration of running at higher speeds  
359 (i.e. 18-20 m/min) during H exercise modality was sufficient to induce higher strenuous alterations in  
360 cell homeostasis compared to M modality. We also may have missed a difference in TORC1 activity  
361 between M and H intensity since the decrease in 4EBP1 phosphorylation could reach a plateau after

362 45 min of exercise, regardless of the running velocity. Indeed, 4EBP1 phosphorylation continues to  
363 decrease from 10 to 30 min of exercise in human and mouse muscles (37, 46).

364 One major difference in the measurement of puromycin uptake vs. 4EBP1 phosphorylation is that the  
365 former reflects the average protein translation during 20 min starting from puromycin injection  
366 whereas the latter is an endpoint marker. This emphasizes that the puromycin incorporation technique  
367 is likely less sensitive to acute variations than monitoring 4EBP1 phosphorylation. Moreover,  
368 puromycin incorporation is a global marker of protein synthesis, while 4EBP1 phosphorylation controls  
369 the translation of a subfamily of messengers depending of TORC1 signaling (TOP mRNAs; (42)).  
370 Therefore, these two methods can bring interesting and complementary information on translation  
371 dynamic during exercise.

372

373 In summary, our findings give new insights into how muscle cells face the high ATP need. Our results  
374 show that muscle protein building is impaired even with light intensity exercise. This work also  
375 highlights the fast and dynamic changes affecting mTORC1 activity and MAMs in the regulation of  
376 muscle proteostasis during running. Importantly, the present results may also apply to other conditions  
377 of cell adaptation in particular energetic context. For example, it would be interesting to determine  
378 whether such mechanisms are also effective in cancer, neurodegenerative disorders or during  
379 ischemia as MAMs number and REDD1 expression are both altered in these conditions.

380

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385

### 386 **Disclosures**

387 None.



388 **Bibliography**

389

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525

526

527 **Tables**

528

529 **Table 1.** Real Time PCR Primer-Characteristics for cDNAs Amplification

<b>Gene</b>	<b>GenBank Acc. No.</b>	<b>Forward primer</b>	<b>Reverse primer</b>	<b>Amplicon size (bp)</b>
<b><i>Arp</i></b>	NM_007475.5	5'-ACTGGTCTAGGACCCGAGAAG-3'	5'-CTCCACCTTGCTCCAGTC-3'	124
<b><i>α-Tub</i></b>	NM_011653.2	5'-CTGGAACCCACGGTCATC-3'	5'-GTGGCCACGAGCATAGTTATT-3'	114
<b><i>IL-6</i></b>	NM_001314054.1	5'-TGGTACTCCAGAAGACCAGAGG-3'	5'-AACGATGATGCACTTGCAGA-3'	128
<b><i>Klf15</i></b>	NM_023184.3	5'-TGTGCCCATTGCCGCCAACCTA-3'	5'-GTGCCTTGACAACCTCATCTG-3'	265
<b><i>Rcan1</i></b>	NM_019466.4	5'-ACCGTGTGGAATTGCCTTCTC-3'	5'-GGGACCCAGTAATACACATGCA-3'	70
<b><i>Redd1</i></b>	NM_029083.2	5'-CCAGAGAAGAGGGCCTTGA-3'	5'-CCATCCAGGTATGAGGAGTCTT-3'	112

530

531

532 **Figure legends**

533

534 **Figure 1. Exercise decreases puromycin incorporation, 4EBP1 and rpS6 phosphorylation in mouse**  
535 **hindlimb muscles.** ■ control, ■ mild and □ heavy exercise groups; GAS: gastrocnemius, PLA: plantaris,  
536 QUA: quadriceps, SOL: soleus, and TA: Tibialis anterior muscles; LC: loading control. Puromycin content  
537 (A) was normalized to loading control (stain-free method; n=6-8/group), whereas 4EBP1 (B; n=5-  
538 7/group) and rpS6 phosphorylation (C; n=6/group) were normalized to total 4EBP1 and rpS6  
539 expressions, respectively. Bonferroni post-hoc test revealed significant differences between M and H  
540 groups vs. C group for all three variables ( $p < 0.0167$ ).

541

542 **Figure 2. Running exercise markedly enhanced p38 and ERK1/2 phosphorylation in mouse hindlimb**  
543 **muscles.** ■ control, ■ mild and □ heavy exercise groups; GAS: gastrocnemius, PLA: plantaris, QUA:  
544 quadriceps, SOL: soleus, and TA: Tibialis anterior muscles. A) p38 phosphorylation (n=6-8/group except  
545 for GAS and SOL of C group and SOL of H group, n=5); \* significantly different from the corresponding  
546 control group and † significantly different from the four other muscles for the corresponding intensity  
547 according to Bonferroni correction for multiple comparisons ( $p < 0.001$ ). B) ERK1/2 phosphorylation  
548 (n=5-7/group) in SOL muscle is significantly higher compared to the four other muscles according to  
549 Bonferroni correction for multiple comparisons ( $p < 0.0167$ ).

550

551 **Figure 3. Heavy intensity exercise activates AMPK in mouse hindlimb muscles.** ■ control, ■ mild and  
552 □ heavy exercise groups; GAS: gastrocnemius, PLA: plantaris, QUA: quadriceps, SOL: soleus, and TA:  
553 Tibialis anterior muscles. A) AMPK phosphorylation (n=4-6/group) is significantly increased in H vs. C  
554 group according to Bonferroni correction for multiple comparisons ( $p < 0.0167$ ). B) ACC phosphorylation  
555 (n=5-8/group) is greater in H and M groups compared to C group according to Bonferroni correction  
556 for multiple comparisons ( $p < 0.0167$ ).

557

558 **Figure 4. REDD1 protein and mRNA are strongly induced by endurance exercise in mouse hindlimb**  
559 **muscles.** ■ control (C), ■ mild (M) , and □ heavy (H) exercise groups; GAS: gastrocnemius, PLA:  
560 plantaris, QUA: quadriceps, SOL: soleus, and TA: Tibialis anterior muscles; A) REDD1 protein expression  
561 was normalized to loading control (LC) using the stain-free technology (n=6-8/group). \* and \$  
562 significantly different from the corresponding C group and the corresponding M group, respectively,  
563 according to Bonferroni correction for multiple comparisons ( $p < 0.0167$ ). B) REDD1 mRNA levels were  
564 analyzed in GAS, PLA, QUA, and TA muscles (n=5-7/group) by a two-way ANOVA followed by multiple  
565 comparisons with the Bonferroni post-hoc test. \* significantly different from the corresponding C  
566 group ( $p < 0.0167$ ). A Mann-Whitney test was used to analyze SOL values (n=3); \*  $p < 0.05$  vs. C group.

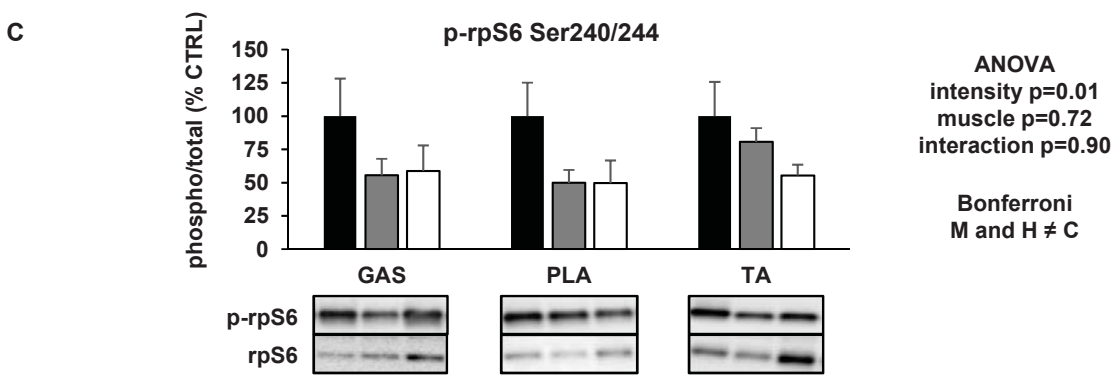
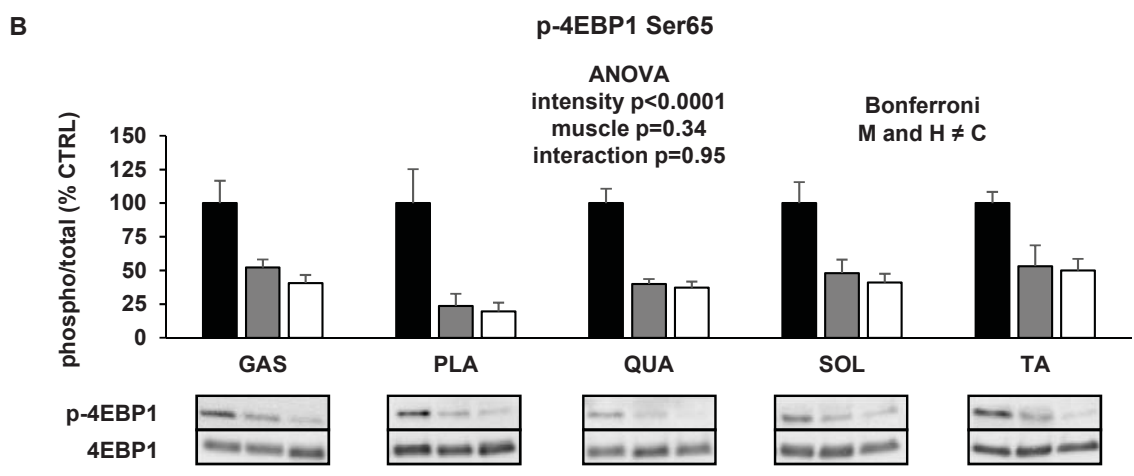
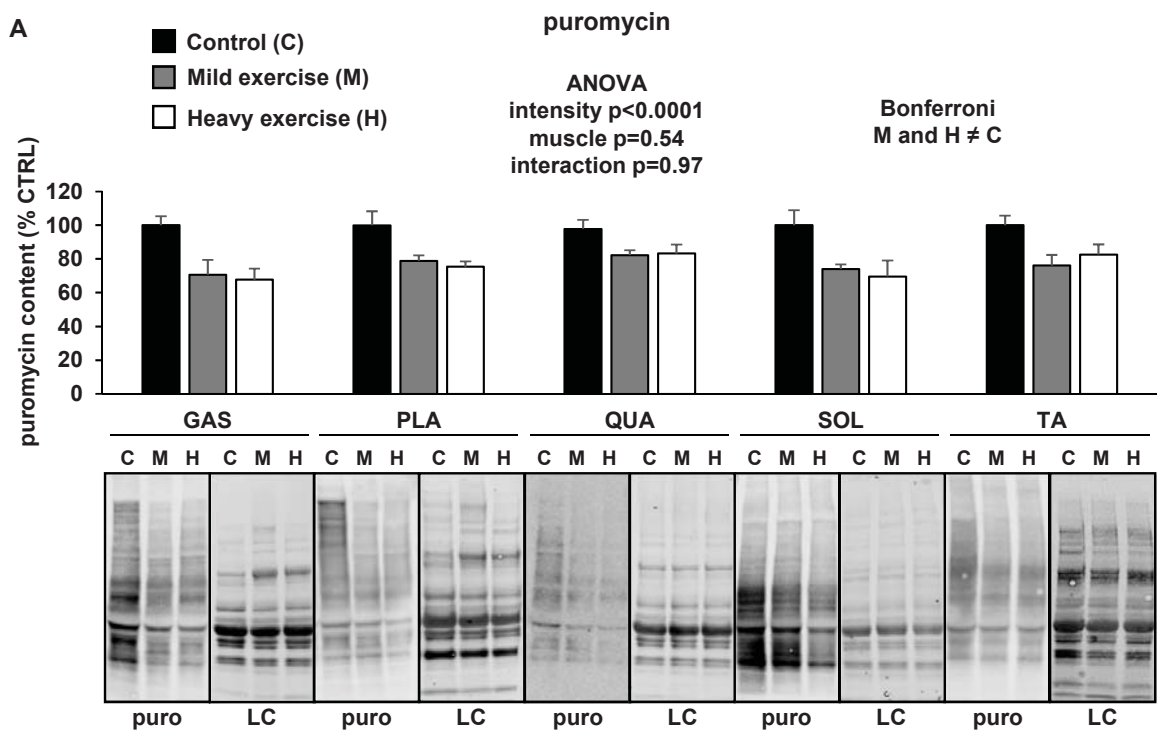
567

568 **Figure 5. No transcriptional induction of Kruppel-like factor 15 (KLF15), regulator of calcineurin 1**  
569 **(Rcan1) and interleukin-6 (IL-6) by endurance exercise in mouse hindlimb muscles.** ■ control, ■ mild  
570 and □ heavy exercise groups; GAS: gastrocnemius, PLA: plantaris, QUA: quadriceps, SOL: soleus, and  
571 TA: Tibialis anterior muscles. GAS, PLA, QUA and TA mRNA expressions (n=5-7/group) were analyzed  
572 by a two-way ANOVA followed by multiple comparisons with the Bonferroni post-hoc test. Bonferroni  
573 post-hoc test revealed significant differences between M vs. H group for Rcan1 and IL6 mRNA  
574 expressions ( $p < 0.0167$ ). A Mann-Whitney test was used to analyze SOL mRNA expressions (n=3).

575

576 **Figure 6. Mild intensity exercise reduces ER-mitochondria contacts in PLA and SOL muscles.** ■ control  
577 (C), and ■ mild (M) exercise groups; PLA: plantaris and SOL: soleus muscles. \* $p < 0.05$  compared to  
578 control group following a Mann-Whitney test (n=4/group).

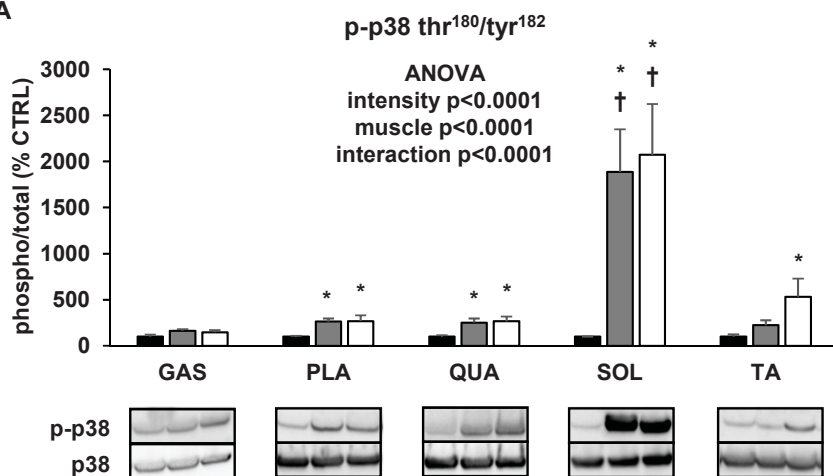
**Figure 1**





**Figure 2**

**A**



**B**

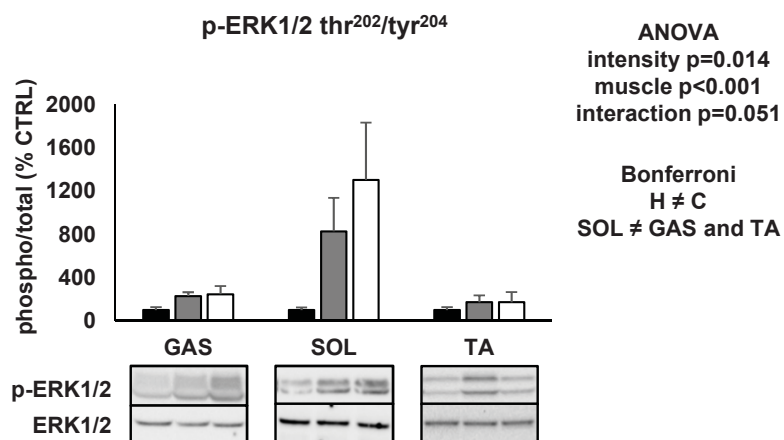


Figure 3

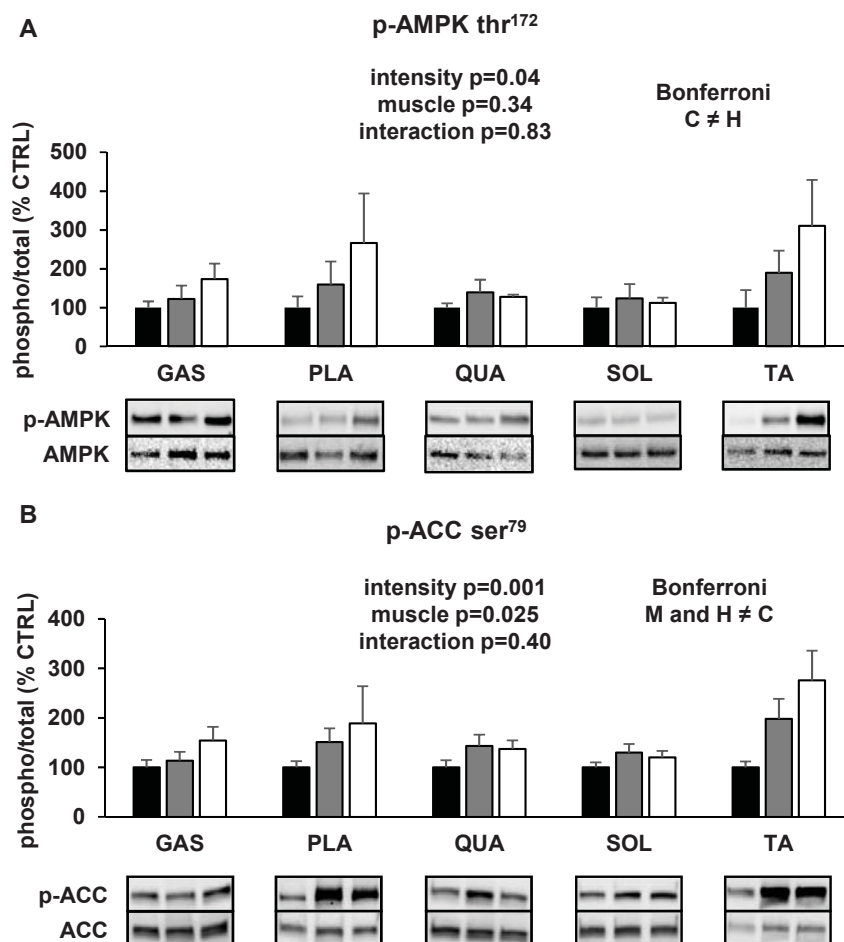


Figure 4

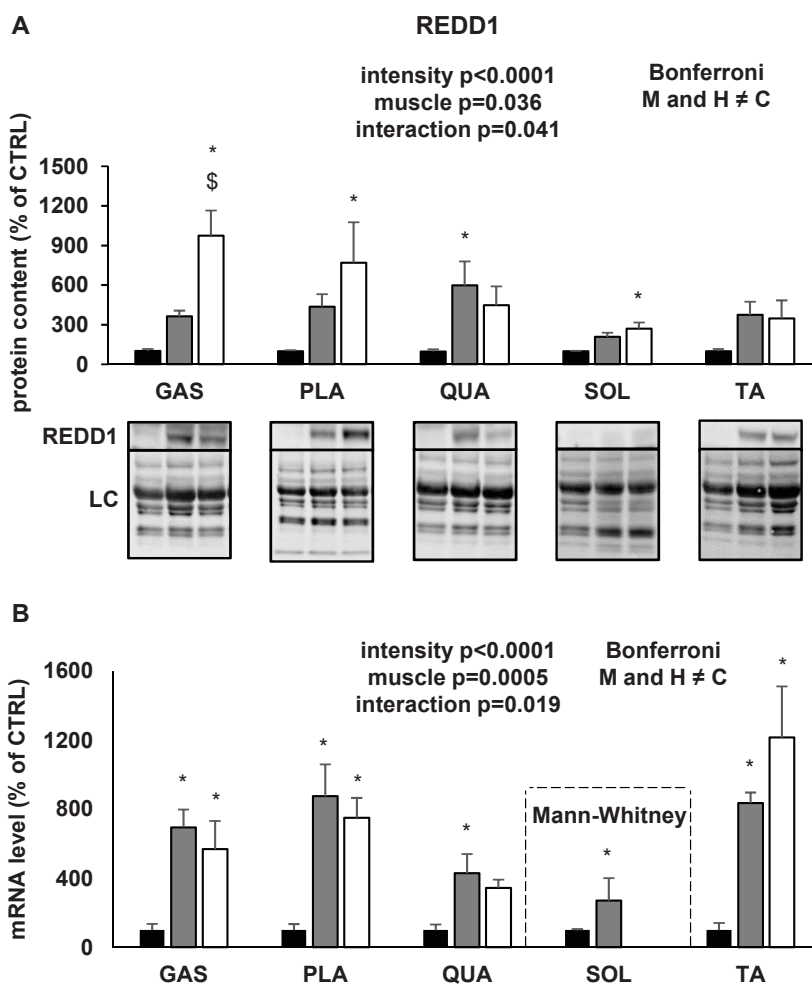


Figure 5

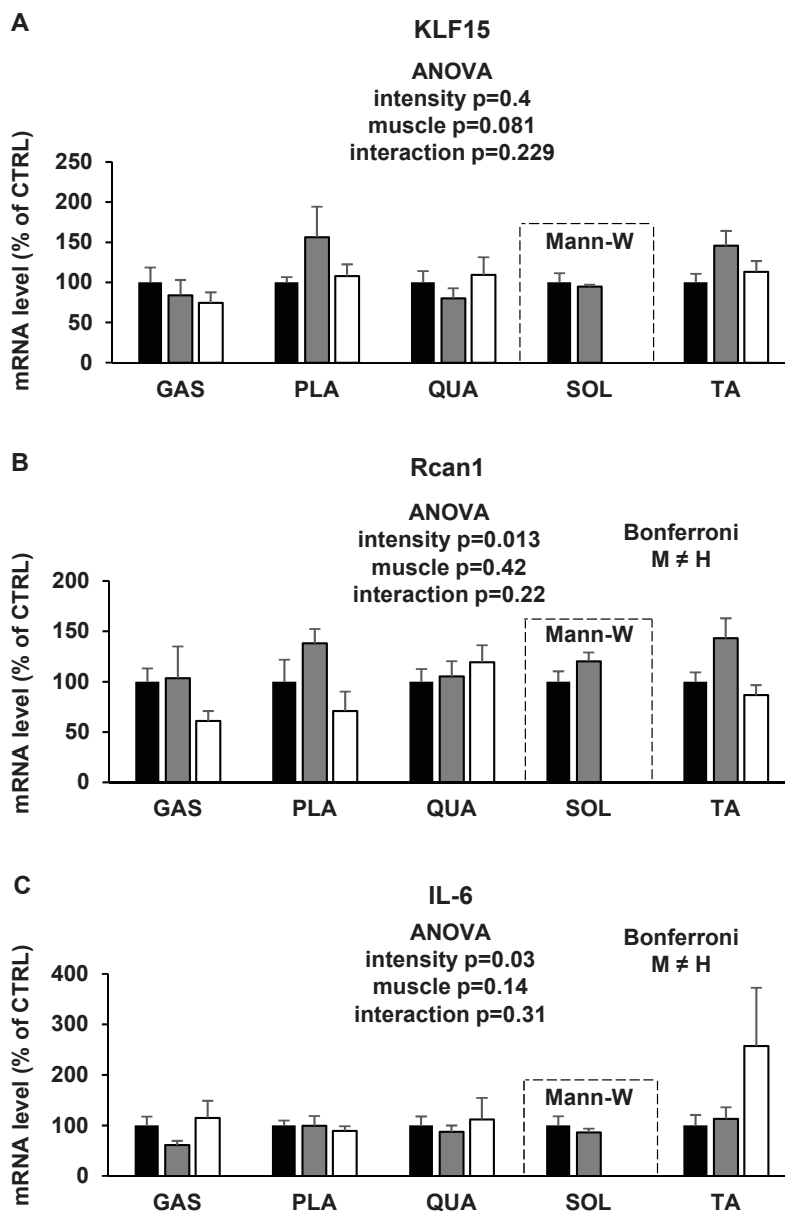


Figure 6

