1 **Metabolite profile and mitochondrial energetics characterize poor early recovery of muscle**
2 **mass following hind limb unloading in old mice**

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29 **SUMMARY**

30 The progression of age-related sarcopenia can be accelerated by impaired recovery of muscle

31 mass following periods of disuse due to illness or immobilization. The molecular underpinnings

32 of poor recovery of aging muscle following disuse remain largely unknown. However, recent

33 evidence suggests that mitochondrial energetics may play an important role. Here, we report that

34 22-24 month old mice with low muscle mass and insulin resistance display poor early recovery

35 of muscle mass following 10 days of hind limb unloading. We took an unbiased approach to

36 identify changes in energy metabolism gene expression and metabolite pools and show for the

37 first time that persistent mitochondrial dysfunction, dysregulated fatty acid β-oxidation and

38 elevated H_2O_2 emission underlie poor early recovery of muscle mass following a period of disuse

39 in old mice. Importantly, this is linked to more severe whole-body insulin resistance. The

40 findings suggest that muscle fuel metabolism and mitochondrial energetics should be a focus for

41 mining therapeutic targets to improve recovery of muscle mass following periods of disuse in

42 older animals.

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52 **INTRODUCTION**

53 Skeletal muscle is a highly plastic tissue that can adapt its mass, structure and metabolic capacity

54 in response to changes in mechanical load. During aging, skeletal muscle exhibits a progressive

55 loss of mass and strength, also called sarcopenia, and an attenuated adaptive recovery following

56 atrophy induced by: disuse, immobilization, starvation and glucocorticoid treatment (Dardevet *et*

57 *al.* 1995; Zarzhevsky *et al.* 2001; Degens & Alway 2003; Hao *et al.* 2011; Magne *et al.* 2011).

58 The hypertrophic response to functional overload is also blunted with aging (Degens & Alway

59 2003). Poor recovery of muscle mass and strength, even with exercise training, a potent anabolic

60 stimuli, has also been reported in older humans following unilateral leg immobilization (Suetta *et*

61 *al.* 2013). Indeed, the lack of recovery from disuse has been suggested to accelerate sarcopenia in

62 older adults, a condition that can increase risk of frailty, morbidity and loss of independence

63 (English & Paddon-Jones 2010). Currently, there are no effective therapies to counteract disuse-

64 induced muscle atrophy and poor recovery of muscle mass, in part because a detailed

65 understanding of the mechanisms underlying loss of muscle plasticity with aging are unknown.

66 In addition, the older population demographic is growing and tends to experience more frequent

67 and prolonged periods of inactivity, increasing the need for the development of safe and effective

68 therapeutics.

69   Maintenance of skeletal muscle mass reflects a finely controlled balance between protein

70 synthesis and degradation. Hypertrophy results from a shift to protein synthesis and the primary

71 anabolic pathway involves the mammalian target of rapamycin complex 1 (mTORC1). mTORC1

72 is principally regulated by nutritional cues, insulin/growth factor signaling cascades and

73 mechanotransduction (Hoppeler 2016). Contractile inactivity induced muscle atrophy is due to

74 reduced basal and postprandial protein synthesis, also known as anabolic resistance, and elevated

75   protein degradation by ubiquitin/proteasome and autophagy/lysosome pathways (Bonaldo &

76   Sandri 2013). Protein breakdown is initiated by inflammatory, glucocorticoid, and

77   myostatin/TGFβ pathways (Egerman & Glass 2014). While the protein degradation/synthesis

78   pathways are relatively well characterized, upstream regulation, particularly in the context of

79   muscle plasticity in aging, is less well understood.

80   Separate lines of investigation have shown that mitochondrial dysfunction occurs in

81   aging muscle, as evidenced by decreased content, mitochondrial protein synthesis, elevated

82   reactive oxygen species (ROS) emission and apoptosis, and reduced ATP production, calcium

83   handling (Joseph *et al.* 2015). Recent evidence also points to mitochondria as an important nexus

84   of control for acute muscle atrophy during disuse (Powers *et al.* 2012). Rodent studies using

85   mitochondrial-targeted antioxidants support a crucial role for mitochondrial ROS in mediating

86   muscle atrophy, through upregulation of ubiquitin-protease enzymes (Min *et al.* 2011; Talbert *et*

87   *al.* 2013). In addition, a model of increased ROS production, the superoxide dismutase (Sod1)

88   knockout, shows exacerbated muscle atrophy during aging (Jang *et al.* 2010). Elevated

89   mitochondrial oxidative stress is purported to stimulate muscle protein breakdown by activating

90   lysosome-autophagy and ubiquitin-proteasome systems (Grune *et al.* 2003; Li *et al.* 2003;

91   Aucello *et al.* 2009; Smuder *et al.* 2010) and energetic stress (reduced ATP production) may also

92   activate the AMP kinase (AMPK)-FoxO3 pathways leading to increased protein degradation

93   (Greer *et al.* 2007; Romanello *et al.* 2010). Moreover, over-expression of PGC-1α, a key driver

94   of mitochondrial biogenesis, can protect muscle mass from acute atrophy due to immobilization

95   or disuse (Sandri *et al.* 2006; Brault *et al.* 2010; Cannavino *et al.* 2014). Key regulators of

96   autophagy and mitochondrial fission also regulate muscle mass (Masiero & Sandri 2010;

97   Romanello *et al.* 2010).

98 Taken together, this body of evidence implicates a central role for mitochondrial

99 energetics in regulating muscle mass and likely plays a vital role in recovery of muscle mass

100 following a period of disuse. The goal of this study was to determine whether age-associated

101 alterations in muscle mass (sarcopenia), mitochondrial function and gene/metabolic profile are

102 linked with responses to hind limb unloading induced atrophy and recovery during reloading in

103 adult (6 mo) and old mice (22-24 mo). Our data suggest that dysregulated fatty acid β-oxidation

104 (FAO) and elevated H_2O_2 emission from mitochondria likely contribute to compromised

105 recovery of soleus muscle mass following unloading in old sarcopenic mice.

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121 **RESULTS**

122 **Old mice are resistant to recovery of muscle mass following unloading induced muscle**

123 **atrophy.** The overarching goal of the following experiments was to determine whether

124 alterations in fuel metabolism and mitochondrial energetics are linked to poor recovery of

125 muscle mass in old mice (22-24 mo), compared to adult mice (6 mo). To assess differences in the

126 atrophy recovery response of old mice, we used a modified version of the tail suspension hind

127 limb unloading model to induce hind limb muscle atrophy over a 10-day time course (Morey-

128 Holton & Globus 2002) as shown in Figure 1A, followed by 3 days of reloading and voluntary

129 cage ambulation. Body weight was greater in the old mice and decreased slightly during

130 unloading and reloading in adult and old mice (Figure 1B). There was no significant change in

131 daily food intake during unloading and reloading in adult and old mice (Figure 1C). As shown in

132 Fig. 1D, E & F, hind limb muscle groups in the old mice weighted less compared to young mice

133 consistent with a sarcopenic phenotype. While it is clear from previous reports that a full

134 recovery of muscle mass and strength may take up to 40 days after unloading (Magne *et al.*

135 2011), we examined the response following a reloading period of 3 days, a time that others have

136 shown to be important for induction of anabolic signal transduction and gene transcription (Akt,

137 p70S6K, 4E-BP1, STAT3) involved in hypertrophy and recovery of muscle mass (Childs *et al.*

138 2003; Baehr *et al.* 2016). Our goal was to understand the differences in response between adult

139 and old mice at this key early period during reloading. Unloading induced significant atrophy of

140 the soleus, gastrocnemius and quadriceps muscle groups in both adult and old animals. The

141 soleus is a postural muscle consisting predominantly of type IIA and I oxidative myofibers

142 making it particularly susceptible to unloading induced atrophy. For this reason, we focused our

143 analysis predominantly on the soleus. The magnitude of soleus muscle loss was similar between

144　adult and old mice. Following 3 days of reloading, significant recovery of soleus mass occurred

145　in the adult, but not the old mice (Figure 1D). Similar responses were observed for the

146　gastrocnemius and quadriceps (Figure 1E and 1F).

147　**Whole body insulin action remains suppressed in old mice following unloading and**

148　**recovery.** Periods of disuse can induce profound whole body and skeletal muscle insulin

149　resistance. We next examined whether differences in metabolic phenotype may link with the

150　poor recovery of muscle observed in old mice (Figure 2). For this experiment, we compared

151　control animals to reloaded animals. As expected, whole body insulin action (slope of blood

152　glucose response to ITT) was lower in the old compared to adult mice (ANOVA main effect for

153　age, Figure 2A&B). This was likely due to soleus and gastrocnemius glucose uptake being lower

154　in the old compared to adult mice (ANOVA main effect for age, Figure 2C & D). Significantly,

155　we found that whole-body insulin action following reloading was lower in old mice, when

156　compared to control. This was not the case for adult mice (Figure 2A & B). Insulin stimulated

157　muscle glucose clearance was similar between control and reloading in both adult and old mice.

158　However, following reloading, gastrocnemius glucose uptake was significantly greater in adult

159　compared to old mice which likely contributed to lower whole-body insulin action. Body

160　composition as assessed by NMR revealed that old mice had lower lean mass and greater fat

161　mass compared to adult mice (Figure 2 E&F, main effect for age), consistent with a sarcopenic

162　phenotype. Following unloading/reloading, old mice had greater fat mass and lower lean mass,

163　changes that again likely contributed to blunted whole body insulin action, and are consistent

164　with the lack of recovery of individual hind limb muscle mass.

165　　　　Muscle protein synthesis and insulin sensitivity are both very sensitive to muscle

166　contractile activity. To determine whether differences in ambulatory cage activity explained

167   differences in muscle recovery and insulin sensitivity between adult and old mice, we examined

168   cage activity over 3-days of the recovery period (Figure 2G). Generally, both the adult and old

169   mice similarly recovered night and day total ambulatory cage activity, albeit in some cases (Day

170   1, for example) the reloaded animals were more active compared to controls.

171   **Inhibition of the AKT/mTOR/FOXO3a pathway in hind-limb unloaded old mice was not**

172   **rescued by reloading.** Maintenance of skeletal muscle mass is a finely controlled balance

173   between protein synthesis and degradation. We next examined the basal phosphorylation status

174   of key mediators of protein synthesis and degradation in the soleus. The AKT/mTOR pathway

175   was inhibited following unloading in both adult and old mice as evidenced by reduced serine

176   phosphorylation of AKT (Ser473), mTOR (Ser2448) and S6 (Ser240/244). Strikingly, inhibition

177   of the AKT/mTOR pathway was rescued by reloading in the adult mice, but not in the old mice

178   (Figure 3A). Forkhead box O3 (FOXO3a) is a transcription factor that regulates the muscle

179   atrophy program. Under catabolic conditions, inhibition of AKT results in dephosphorylation

180   and nuclear translocation of FOXO3a, which activates the expression of Atrogin-1. Consistent

181   with this role, we found that FOXO3a was dephosphorylated (Ser253) during unloading in both

182   adult and old mouse soleus. Accordingly, the expression E3 ligases Atrogin-1 and Murf-1 were

183   also elevated during atrophy. However, FOXO3a remained dephosphorylated and Atrogin-1 and

184   Murf-1 tended to remain elevated in the old mice during recovery, compared to the adults

185   (Figure 3B).

186   The ratio of LC3BI/II, an index of LC3B lipidation and autophagy activation, was

187   elevated during unloading and reduced with recovery in adult and old mice (Figure 3C). Similar

188   trends were seen for ATG7 and PINK which are proteins involved in mitophagy. PINK1, a

189   protein thought to protect cells from stress-induced mitochondrial dysfunction, remained

190   elevated during reloading in the old mice (Figure 3C). Taken together, these data show that the

191   key regulators of protein synthesis and degradation respond as expected during unloading in both

192   adult and old mice. However, following 3 days of reloading, there is persistent activation of

193   protein degradation and inhibition of protein synthesis mediators in old mice.

194   **Mitochondrial function does not improve during recovery in old mice.** Our central

195   hypothesis is that alterations in mitochondrial energetics and fuel metabolism play a key role in

196   the poor recovery of muscle mass in older animals. Functional assays of soleus mitochondria

197   were conducted using saponin permeabilized myofiber bundles, an approach that preserves the

198   native reticular structure of the mitochondria (Picard *et al.* 2011). When compared to adult,

199   skeletal myofibers isolated from old control mice displayed lower respiration, OXPHOS content

200   and calcium retention capacity (CRC), an index of apoptotic susceptibility, while H_2O_2 emission

201   was elevated (Figure 4 A-G). Following unloading, LEAK, complex I, I&II and FAO supported

202   OXPHOS respiration and CRC in soleus were all reduced in both the adult and old mice. In

203   addition, mitochondrial H_2O_2 emission was elevated in both young and aged animals (Figure 4

204   A-F). Total cardiolipin and OXPHOS protein (measures of mitochondrial content) were both

205   reduced with unloading in the adult mice, but remained unchanged in the older animals

206   indicating that changes in function were independent of changes in mitochondrial content during

207   unloading in the old animals (Figure 4 G,H&J).

208   Following 3-days of recovery, complex I & II supported mitochondrial respiration, CRC,

209   and H_2O_2 emission normalized in the young animals. Interestingly, FAO supported respiration

210   did not recover in the young animals. Old mice did not recover any aspect of mitochondrial

211   function as evidenced by sustained elevated H_2O_2 emission and suppressed CRC and respiration.

212   Taken together, these data indicate that mitochondrial function does not improve during the early

213   recovery phase in old mice.

214   We used shotgun lipidomics to quantify the cardiolipin pool (Han *et al.* 2006).

215   Cardiolipin is a phospholipid specific to the inner mitochondrial membrane and critical for ETC

216   integrity and mitochondrial function (Paradies *et al.* 2014). Cardiolipin species can be broadly

217   categorized based on acyl chain length and degree of saturation, into: immature, mature and

218   remodeled species. Immature cardiolipins are those that have heterogeneous acyl chain length

219   and degree of saturation and typically contain one or more 16:1 acyl chains. Mature cardiolipins

220   have more uniform acyl chain length and degree of saturation, for example, tetralinolyol

221   cardiolipin (18:2-18:2-18:2-18:2). Remodeled cardiolipin species contain greater PUFA

222   (typically 22:6) acyl chains and may have been remodeled due to oxidative insult (Chicco &

223   Sparagna 2007). The soleus of older animals had slightly lower content of many cardiolipin

224   species, again likely due to reduced mitochondrial content with aging (Figure 4I and Table 1S).

225   Unloading elicited a striking reduction in many immature, mature and remodeled species of

226   cardiolipin in adult mice. There was a partial recovery following reloading. The same dynamic

227   response of the cardiolipin pool was not evident in the older mice.

228   **Divergent metabolomic response between adult and old mice during unloading and**

229   **recovery.** Targeted metabolomic profiling was conducted on soleus samples to quantify

230   acylcarnitines, organic acids and amino acids in order to assess perturbations in mitochondrial

231   fuel metabolism that may occur with unloading and reloading. We show for the first time a

232   striking decrease in long-chain acylcarnitines during unloading in the adult animals (Figure 5A

233   and Table 2S), in-line with the observations of others who have shown that contractile inactivity

234   induces a switch to carbohydrate fuel source and reductions in fatty acid oxidation (Bergouignan

235   *et al.* 2011). Interestingly, following reloading, acylcarnitines remained suppressed in the adult

236   mice, consistent with lower FAO supported respiration. However, certain TCA cycle

237   intermediates (fumarate, malate, and α-ketoglutarate) and amino acids (Gln, Glu, Ala, and

238   Citrulline), increased during reloading in the adult soleus, consistent with increases in

239   TCA/CI&II supported respiration (Figure 5 B&C and Table 3&4S). These data suggest that FAO

240   remains suppressed during the early reloading phase in adult muscle and that recovery of

241   mitochondrial respiration is supported by glycolysis and TCA cycle anaplerosis via amino acids.

242   The increase in C4, and C5 acylcarnitines during reloading are also consistent with increased

243   amino acid catabolism and anaplerosis (Table 2S). In old mice, the metabolomic profile in

244   response to unloading and reloading was distinctly different from that of the adult animals. The

245   majority of long-chain acylcarnitines increased during unloading and reloading in the old mice

246   (Figure 5A). This, in concert with reduced TCA cycle intermediates and reduced capacity for

247   respiration, suggest that FAO outpaced TCA flux and resulted in accumulation of acylcarnitine

248   species. Interestingly, many of the amino acids that were increase upon reloading in the adult

249   animals did not rise in the older animals (Figure 5C).

250   **Transcriptomic response to unloading and reloading is similar in adult and old mice.** Gene

251   expression profiling was conducted on soleus samples from the 6 groups using RNA sequencing.

252   Our goal was to determine whether the divergent mitochondrial energetic and fuel metabolic

253   responses to unloading and particularly reloading in old mice were underpinned by a distinct

254   transcriptomic response. A summary of the number of differentially regulated genes is presented

255   in Table 7S. The database for annotation, visualization and integrated discovery (DAVID) v6.8

256   was used to identify enriched biological themes via functional annotation (Dennis *et al.* 2003).

257   The top significantly up- and down-regulated functional category terms for each group

258   comparison are presented in Supplemental Tables 8-11S. Terms related to mitochondrial

259   function and fuel metabolism were downregulated in during unloading for both adult and old

260   mice (Table 8&9S). Surprisingly, mitochondria related terms were also significantly

261   downregulated during reloading for adult and old mice (Table 10&11S). These findings were

262   confirmed by qRT-PCR assay for expression of key nuclear transcription factors that drive

263   mitochondrial energy metabolism, including: *Ppargc1a* (PGC-1α)*, Esrrg* (ERRγ), and *Esrra*

264   (ERRα) (Figure 6A).

265   We next compared the collective results of the transcriptomic and metabolomic profiling.

266   This comparative analysis is depicted by heat maps (Figure 6B&C) which display the direction

267   of differential expression. The analysis was restricted to a subset of genes involved in

268   mitochondrial fuel and energy metabolism relevant to the metabolites measured in the targeted

269   metabolomics profile. This comparison highlights striking differences in the metabolomic and

270   transcriptomic responses during the reloading condition for adult and old mice. In adult mice, the

271   recovery of mitochondrial respiratory capacity occurs along with partial normalization of

272   metabolites, particularly amino acids and organic acids, but not acylcarnitines. In contrast, FAO,

273   TCA and ETC genes remained repressed. In old mice, the metabolite response during recovery is

274   distinctly different. Namely, acylcarnitines were elevated while organic acids and amino acids

275   remained low. Moreover, FAO, TCA and ETC genes remained repressed. Taken together, these

276   results indicate that mitochondrial energetics gene and metabolite profiles are repressed in

277   concert with unloading and muscle atrophy. However, metabolite levels clearly distinguish the

278   early muscle hypertrophy response in adult animals versus the lack of recovery in old animals

279   upon reloading and to a far greater degree than mitochondrial energetics gene profiles.

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281 **DISCUSSION**

282 During aging, skeletal muscle exhibits a progressive loss of mass and strength known as

283 sarcopenia (Rosenberg 1989), and an attenuated ability to recover muscle mass following acute

284 periods of disuse or unloading. Poor recovery of muscle mass following periods of disuse is a

285 significant health concern for older adults that leads to lower activities of daily living and

286 increased risk of frailty and mobility disability. The mechanisms responsible for the lack of

287 muscle mass recovery in old mice following disuse have not been fully elucidated but are likely

288 complex and multifactorial. Investigations to date have focused on attenuated protein synthesis

289 possibly due to ER stress and impaired neuromuscular transmission (Baehr *et al.* 2016). In

290 separate lines of investigation, aging has also been linked with insulin resistance and

291 mitochondrial dysfunction in skeletal muscle (Konopka & Sreekumaran Nair 2013). Here, we

292 report that 22-24 month old mice with low muscle mass and insulin resistance, display poor early

293 recovery of muscle mass following 10 days of hind limb unloading. We took an unbiased

294 approach to identify changes in energy metabolic gene expression and metabolite pools and

295 show for the first time that persistent mitochondrial dysfunction and a dysregulated metabolic

296 response underlie poor early recovery of muscle mass in old mice. Importantly, this is linked to

297 worsened whole-body insulin resistance. Our results demonstrate the power of using unbiased

298 molecular profiling to define signatures of complex physiological states combined with deep

299 phenotyping of mitochondrial energetics.

300 Physical inactivity results in attenuated mitochondria capacity along with a shift in fuel

301 selection to carbohydrate and suppressed FAO (Bergouignan *et al.* 2011). Here, in adult mice,

302 we show a distinct remodeling of soleus mitochondrial energetics and fuel metabolism during

303 unloading as evidenced by reduced respiration, elevated H_2O_2 emission and reduced long-chain

304   acylcarnitines and organic acids. This change in mitochondrial phenotype occurred along with

305   striking reductions in many individual molecular species of cardiolipin. Indeed, synthesis and

306   remodeling of cardiolipin is crucial for the structural and functional integrity of the

307   mitochondria. Our findings expand upon the work of Ostojic et al., others who reported

308   decreased cardiolipin during muscle atrophy (Ostojic *et al.* 2013).

309   Insulin signaling intersects with protein synthesis and degradation pathways via

310   AKT/FOXO/mTOR. Hence, a logical supposition is that baseline insulin resistance worsens

311   muscle atrophy response during disuse. Similarly, mitochondrial dysfunction and ROS emission

312   are linked to activation of protein degradation pathways and may worsen muscle atrophy due to

313   disuse (Romanello & Sandri 2010). Indeed, older adults with type 2 diabetes, a condition defined

314   by muscle insulin resistance and mitochondrial dysfunction, have an accelerated loss of muscle

315   mass over time (Park *et al.* 2009), indicating a propensity for catabolic metabolism. However, we

316   found that insulin resistant old mice with mitochondrial dysfunction did not have an exacerbated

317   muscle atrophy during disuse. The relative magnitude of muscle loss and alterations in the

318   activity (phosphorylation state) of AKT/mTOR/FOXO3a, and the majority of endpoint

319   measurements were similar in adult and old animals in response to unloading. These findings

320   suggest that the presence of insulin resistance or mitochondrial dysfunction in aging does not

321   influence the muscle atrophy response due to disuse or unloading.

322   Although impaired recovery by aged muscle is reasonably well documented, the

323   mechanisms responsible for the lack of growth are not clearly defined. We show for the first time

324   that impaired early recovery of muscle mass may be exacerbated by insulin resistance and

325   persistent mitochondrial dysfunction in old animals. In stark contrast to adult mice, there was no

326   change in the cardiolipin species of old mice that likely contributed to persistent mitochondrial

327   dysfunction. Cardiolipin synthesis and remodeling is critical for correct cristae folding and

328   functional integrity of the ETC complexes. The increase in acylcarnitines (long chain in

329   particular) in concert with reductions in particular TCA cycle organic acids (fumarate and

330   malate), and no change in pyruvate or lactate, suggest a bottleneck in carbon flux from ß-

331   oxidation into the TCA cycle which could reduce the capacity to generate the reducing

332   equivalents needed to produce ATP contributing to the anabolic response. The change in muscle

333   metabolite pools during reloading in old mice is similar to that observed with high fat feeding

334   and obesity (Koves *et al.* 2008). In addition, the metabolite profile served to robustly distinguish

335   the phenotype of poor muscle recovery in old mice. Our findings are also in line with

336   observations that insulin resistance, obesity, and elevated plasma free fatty acids are linked with

337   blunted amino acid stimulated muscle protein synthesis (Murton *et al.* 2015; Stephens *et al.*

338   2015). Further studies are needed to examine whether alterations in mitochondrial fatty acid

339   import or FAO may impact early recovery of muscle in old mice, potentially via improved

340   insulin sensitivity.

341   Interestingly, while metabolite profile distinguished the phenotype of poor muscle

342   recovery in old mice, transcriptomic profiles for energy metabolic pathways were similar

343   between old and adult mice during unloading and reloading. In addition, the recovery of

344   mitochondrial respiratory capacity in adult mice occurred along with partial recovery of

345   metabolites, while FAO, TCA and ETC genes remain repressed. Indeed, FAO genes appeared to

346   be further repressed during unloading in adult mice. These data suggest that the early recovery of

347   mitochondrial function following reloading is, seems to be independent of FAO and mediated by

348   cardiolipin remodeling and alterations in metabolite profile. This, in turn, could affect post

349   translational modification and activity of ETC, although this remains to be tested. Taken

350   together, these results indicate that mitochondrial energetics, gene and metabolite profiles are

351   repressed in concert with unloading and muscle atrophy.

352   In summary, our data suggest that dysregulated FAO and elevated H_2O_2 emission from

353   mitochondria likely contribute to the lack of recovery of soleus muscle mass following unloading

354   in sarcopenic mice. These findings suggest that pathways governing muscle fuel metabolism and

355   mitochondrial energetics might harbor promising therapeutic targets for improving the recovery

356   of muscle mass following periods of disuse in older patients.

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373 **EXPERIMENTAL PROCEDURES**

374 **Animals.** All animal experiments and euthanasia protocols were conducted in strict accordance

375 with the National Institute of Health guidelines for humane treatment of animals and approved

376 by the Institutional Animal Care and Use Committee at the Sanford-Burnham Medical Research

377 Institute at Lake Nona. 6-month and 22-24 month old male C57BL/6J mice were obtained from

378 the National Institute on Aging rodent colony (Charles River Laboratories, Madison, WI). Young

379 and aged animals were divided into the following experimental groups: 10-day of hind limb

380 unloading (UN), 10-day of hind limb unloading followed by 3 days of reloading recovery (RL),

381 and control (CON). Mice were fed ad libitum with a standard chow diet (#2916, Harlan-Teklad,

382 Houston, Tx) and water/hydrogel (ClearH$_2$O, Westbrook, ME, US) and housed at 22C with a 12-

383 h light/dark cycle.

384 **Tail suspension hind limb unloading**. Tail suspension is the one of the most commonly used

385 animal models of musculoskeletal non-weight bearing. Prior to tail suspension experiments the

386 individual mice acclimated in single cages and routine handling for 3 days. Tail suspension was

387 performed using a modified version of the Morey-Holton and Globus protocol (Morey-Holton &

388 Globus 2002). Briefly, a small metallic hook is taped to the base of the tail using non-abrasive

389 adhesive tape wrapped in a helical pattern. The hook is then attached to a small swivel key chain

390 that is attached to a metal rod that runs the length of the microisolator cage. The mice could

391 move on a y-axis and rotate 360 degrees and so had access to all areas of the cage. The hind

392 limbs are maintained just off the cage floor with the body of the mouse at ~30° angle from the

393 cage floor. The mice can move freely and the angle and height of the mice are checked daily.

394 The control mice were separated into the individual cages with the exactly the same conditions

395 as unloading groups, but without tail suspension.

396   After 10 days of UN, RL, or CON, the mice were fasted for 4 hours and sacrificed by

397   CO_2 asphyxiation and cervical dislocation, or anesthetized with sodium pentobarbital (IP

398   administration, 50mg/kg) for muscle harvested for metabolomics analysis. The soleus,

399   gastrocnemius, and quadriceps femoris muscle groups were immediately harvested and weighed.

400   The soleus muscle from right hind limb was used for fresh tissue assay immediately and other

401   muscles were snap-frozen in liquid nitrogen for other analysis.

402   **Cardiometabolic Phenotyping.** _Body Composition_. Measurement of fat and lean mass (g) is

403   accomplished using a LF90II TD-NMR (Bruker, Madison, WI). Conscious mice are placed in a

404   plastic restraint tube that is inserted into the instrument. Measurements are obtained in less than 1

405   minute. _Ambulatory cage activity_. Locomotor activity measurements are obtained using a

406   Promethion Mouse Multiplexed Metabolic System (Sable Systems International, Las Vegas,

407   NV). Body mass is collected using a housing cubby connected to a precision scale. Locomotor

408   activity along the X, Y and Z planes is measured using infrared beams that span the home cage.

409   Data are collected by a host computer. _Insulin-stimulated Tissue Glucose Uptake,_ was assessed

410   as described in the Supplemental Information section. Processing of tissues for glucose uptake

411   was determined as previously described (Ayala _et al._ 2007), and as detailed in the Supplemental

412   Information section.

413   **Myofiber Bundle Preparation.** Permeabilized fiber bundles (1-3mg) were prepared

414   immediately following tissue harvest, as previously described (Coen _et al._ 2015) and as detailed

415   in the Supplemental Information section.

416   **Mitochondrial respiration**. Respirometry assays were conducted using an Oxygraph-2k

417   (Oroboros Instruments, Innsbruck, Austria). The myofiber bundles were gently placed into the

418   respirometer chambers and after a stable baseline was reached, the assay protocol was run in

419   duplicate at 37°C and between 350-200 nmol of O_2 in Buffer Z with blebbistatin (25μM). The

420   assay protocols are described in the Supplemental Information section.

421   **Mitochondrial H_2O_2 Emission.** H_2O_2 emission was measured with Amplex Red reagent which

422   reacts with H_2O_2 to produce the stable fluorescent compound resorufin. Resorufin fluorescence

423   was monitored using a Fluorescence Spectrometer (Perkin Elmer LS50B, Waltham,

424   Massachusetts, USA) with temperature control (37°C) and magnetic stirring at

425   excitation/emission 563/587nm. The assay was run with buffer Z containing 5000U/ml CuZn-

426   SOD, 25μM blebbistatin, 50μM Amplex Ultra-Red, and 6U/ml horseradish peroxidase. Briefly,

427   the muscle fiber bundles were added to the reaction buffer with 10ug/ml oligmycin, 10mM

428   Glutmate, 2mM Malate and 10mM Succinate. The rate of emission of H_2O_2 (pmol) was

429   calculated from previously established fluorescence intensity standard curves with known

430   concentrations of H_2O_2, after correcting for the rate of change in background fluorescence.

431   Following each experiment, myofiber bundles were dried and weighed. Rate of H_2O_2 emission

432   was expressed as pmol/min/mg dry weight.

433   **Mitochondrial calcium retention capacity.** A continuous, spectrophotometric assay was

434   utilized to measure mitochondrial calcium retention capacity within soleus fiber bundles, as

435   detailed in the Supplemental Information section.

436   **RNA Sequencing and Informatics.** Details of sample preparation, library preparation

437   sequencing, mapping and differential gene expression analysis are described in the Supplemental

438   Information section. *Heat maps.* The normalized (z-score) mean of each cohort was visualized in

439   the heatmap, created with the "heatmap.2" function in the "gplots" package within the statistical

440   program R (http://www.r-project.org/). *Gene Ontology (GO) annotation.* The database for

441   annotation, visualization and integrated discovery (DAVID) v6.8 was used to identify enriched

442   biological themes via gene ontology (GO) annotation. The gene expression data discussed in this

443   publication have been deposited in NCBI's Gene Expression Omnibus and are accessible

444   through GEO Series accession number GSE102284.

445   **Real-time quantitative-PCR:** Details of sample preparation and gene expression analysis by

446   PCR are described in the Supplemental Information section.

447   **Western Blot.** Frozen soleus was prepared for immunoblot as described previous (Lee *et al.*

448   2017) and as detailed in the Supplemental Information section.

449   **Lipidomics Analysis.** Multi-dimensional mass spectrometry-based shotgun lipidomics (MDMS-

450   SL) was employed to measure and characterize the lipid patterns in mouse soleus muscle. The

451   analytical procedures for the lipidomics analysis are detailed in the Supplemental Information

452   section.

453   **Metabolomics Analysis.** Acylcarnitines, organic acids and amino acids were quantified using

454   internal calibration standards and mass spectrometry as described in detail in the Supplemental

455   Information section.

456   **Statistical Analysis**. All data are represented as the mean ± SEM. All the statistical analyses

457   were performed by GraphPad Prism. The differences between groups were conducted using

458   ANOVA or *t* -test (paired and unpaired) approaches with Tukey's/Bonferroni Multiple

459   Comparison Test. when appropriate $p < 0.05$, it was considered significant.

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AUTHORS CONTRIBUTIONS

XZ, MBT, TCL, DPK, XH, SJG, JA, RBV, PMC performed experiments, and analyzed and interpreted the data. XZ and RBV contributed to data interpretation and writing the manuscript. All co-authors reviewed and approved the manuscript. PMC contributed to the study concept and design, statistical analysis, interpretation of the data and wrote the manuscript. PMC is the guarantor of the data.

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635 **Figure 1. Old mice do not recover muscle mass following 10 days of hind limb unloading**

636 **and 3 days of recovery, compared to adult mice.** Panel A; image of the hind limb unloading

637 approach. Panel B; body weight was greater for the old mice and both adult and old mice lost

638 weight during the unloading and reloading periods. Panel C, average daily food intake was

639 similar for both old and adult mice and did not change over the unloading and reloading periods.

640 Panels D, E and F; Soleus, Gastrocnemius and Quadriceps atrophy during unloading in both

641 adult and old mice. However, after 3 days of reloading, muscle mass did not recover in the old

642 mice. $n = 11$ per group. Data are presented as mean ± SEM. ** $P < 0.05$ ANOVA/Bonferoni

643 Correction. * $P < 0.05$ v Control ANOVA/Bonferoni Correction.

644 **Figure 2. Old mice are insulin resistant and have lower muscle glucose clearance, a**

645 **phenotype that is exacerbated by unloading and reloading.** Grey bars represent control

646 conditions. Green bars represent animals that had 10 days of unloading and 5 days of recovery.

647 Panel A, blood glucose reductions (percent change from baseline) following intraperitoneal

648 insulin injection. Panel B, The slope of the fall in glucose levels from $t = 0$ to 15 min was used as

649 an index of whole-body insulin action. Panel C, Soleus glucose clearance. Panel D,

650 Gastrocnemius glucose clearance. Panel E, proportion of body fat mass determined by NMR.

651 Panel F, proportion of body lean mass determined by NMR. Panel G, day and night cage

652 ambulatory activity (x,y,z) using the Promethion Mouse Multiplexed Metabolic System. $n = 5$

653 per group. Data are presented as mean ± SEM. The letters A and B denote significant differences

654 between group/time points ($P < 0.05$, ANOVA/Tukey Correction).

655 **Figure 3. Mediators of protein synthesis are inhibited and breakdown are activated during**

656 **unloading, a response that does not return to baseline following reloading in the old mice.**

657 Panel A, protein synthesis signaling: pAKT/AKT, pmTOR/mTOR, and pS6/S6 ratios. Panel B,

658   protein breakdown signaling: pFOXO3a/FOXO3a ratio, Atrogin 1, and Murf 1 expression. Panel

659   C, autophagy/mitophagy protiens: LC3B I/II ratio, ATG7, Pink 1, Beclin 1 expression. n = 5 per

660   group. Data are presented as mean ± SEM. ** P <0.05 ANOVA/Bonferoni Correction. * P <0.05

661   v Control ANOVA/Bonferoni Correction.

662   **Figure 4. Mitochondrial function is impaired during unloading and improves during**

663   **recovery only in adult mice.** Panel A, Complex I supported LEAK (CI_L or State 4) respiration.

664   Panel B, Palmitoylcarnitine supported OXPHOS (State 3) respiration. Panel C, Complex I

665   supported OXPHOS respiration. Panel D, Complex I&II supported OXPHOS respiration. n = 8-

666   10 per group for respiration experiments. Panel E, Mitochondrial H_2O_2 emission. n = 8 − 10 per

667   group. Panel F, Mitochondrial calcium retention capacity. n = 8 − 10 per group. Panel G, Total

668   OXPHOS protein content by immunoblot. n = 5 per group. Panel H, Total cardiolipin content by

669   tandem mass spectrometry. Panel I, heat map depicting change in individual immature, mature

670   and remodeled cardiolipin species. n = 7 per group. Panel J, Representative immunoblot for

671   OXPHOS proteins. Data are presented as mean ± SEM. ** P <0.05 ANOVA/Bonferoni

672   Correction. * P <0.05 v Control ANOVA/Bonferoni Correction.

673   **Figure 5. Acylcarnitine, organic acid and amino acid profiles during unloading and**

674   **reloading.** Panel A, Acylcarnitine profile in soleus of adult and old mice during control,

675   unloading and reloading conditions. Acyl chain length and degree of saturation are denoted by

676   the numbers. Panel B, metabolite profiles showing lactate and pyruvate and organic acid

677   intermediates of the tricarboxylic acid (TCA) cycle. Panel C, amino acids in soleus of adult and

678   old mice during control, unloading and reloading conditions. BLQ = below limit of

679   quantification. n = 6 per group. Data are presented as mean ± SEM. ** P <0.05

680   ANOVA/Bonferoni Correction. * P <0.05 v Control ANOVA/Bonferoni Correction.

681 **Figure 6. Comparative analysis of transcriptomic and metabolomics profiles during**

682 **unloading and reloading.** Panel A, qRT-PCR assay for expression of key nuclear transcription

683 factors that drive mitochondrial energy metabolism. Panel B, heat map containing RNA

684 sequencing data set representing the level of expression of genes with reads per kilobase per

685 million mapped reads (RPKM) >1, plotted as a centralized Z-score for each gene in fatty acid

686 metabolism, tricarboxylic acid (TCA) cycle, and electron transport chain/oxidative

687 phosphorylation pathways defined by Ingenuity Pathway analysis. Panel C, heat map containing

688 the levels of acylcarnitines, organic acids, amino acids, pyridine and adenine nucleotides and

689 total level of free fatty acids (FFA), diacylglycerol (DAG) and triacylglycerol (TAG). For all

690 heat maps, relative downregulation is in blue and upregulation in red. The intensity of color
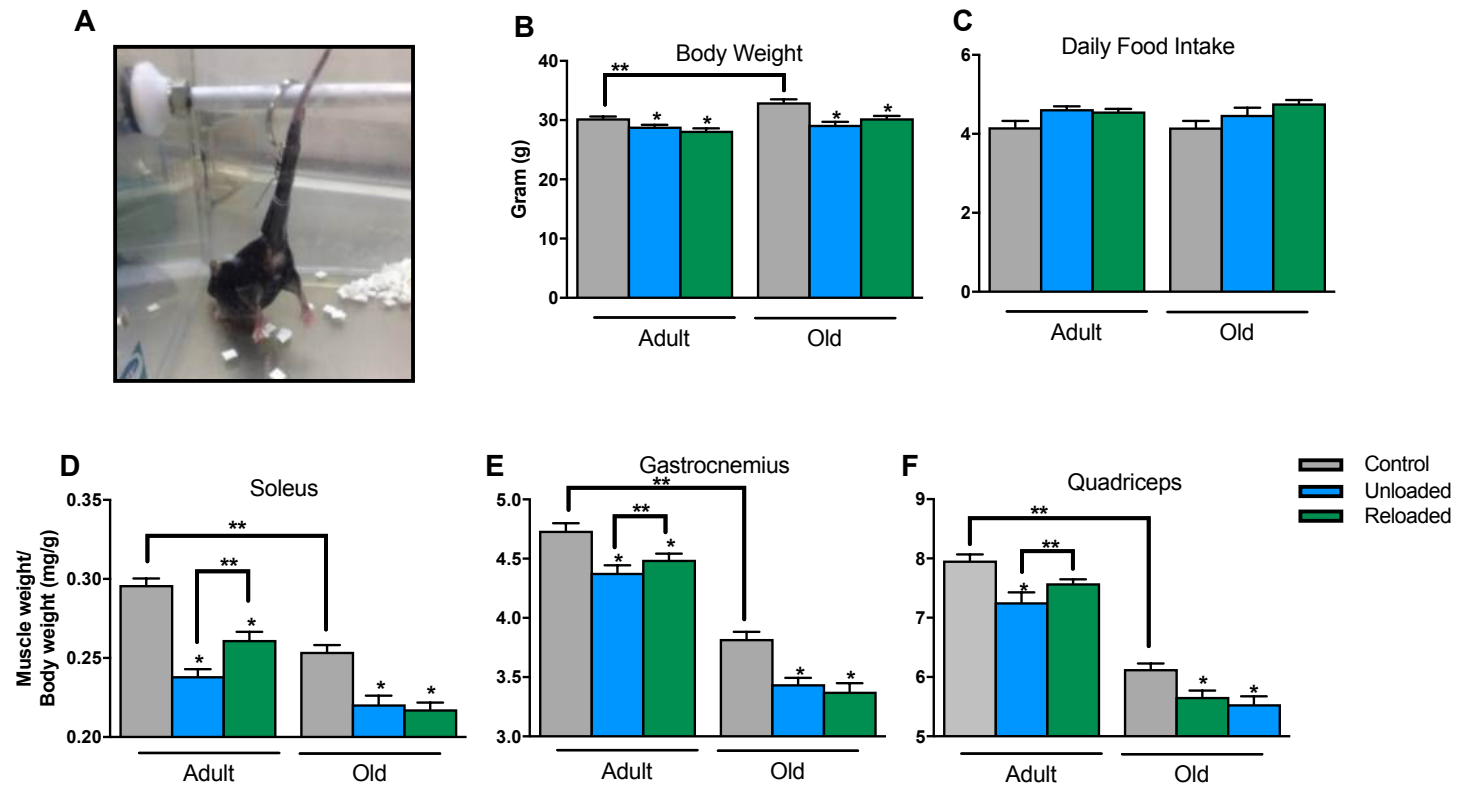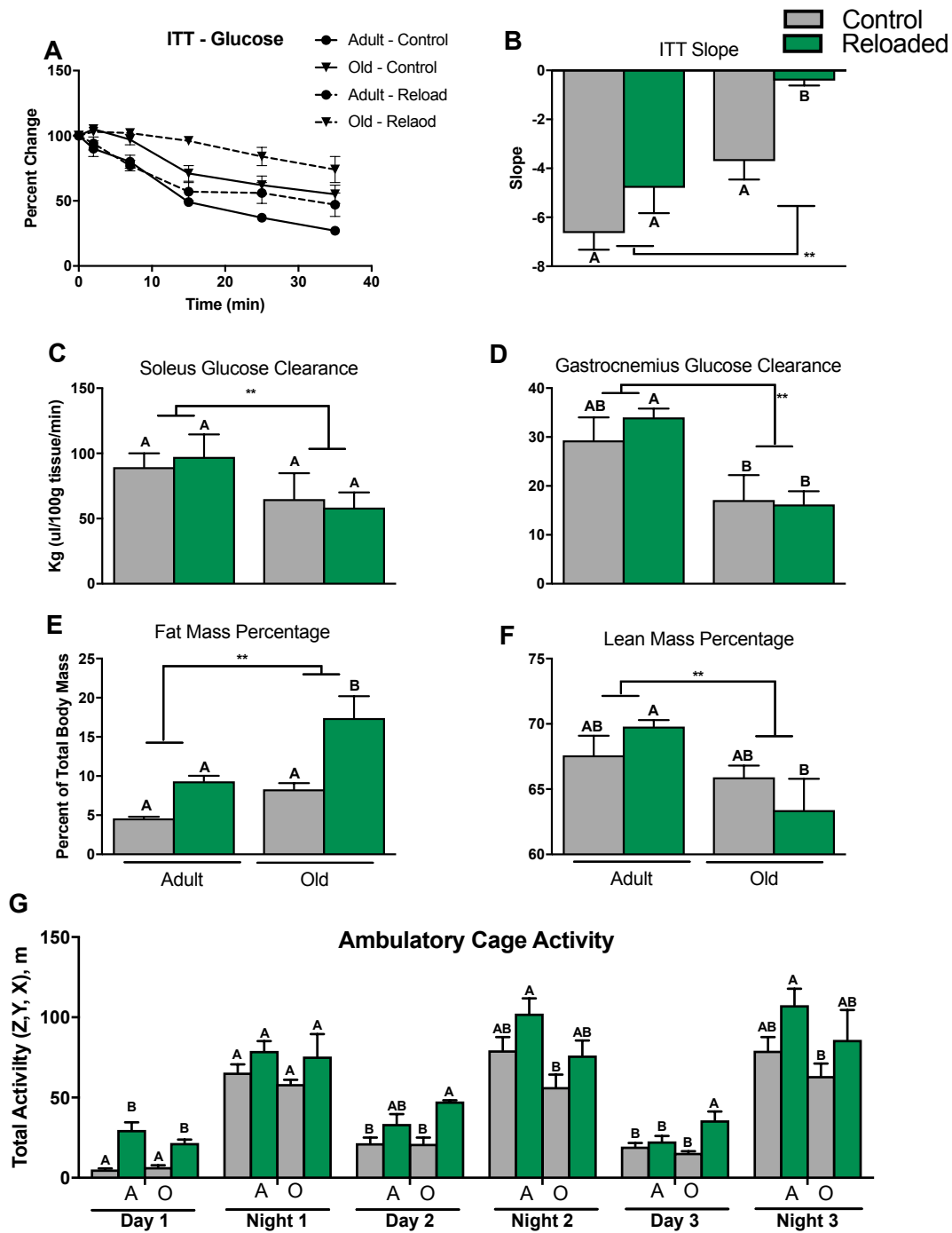
691 indicates the magnitude of the change.
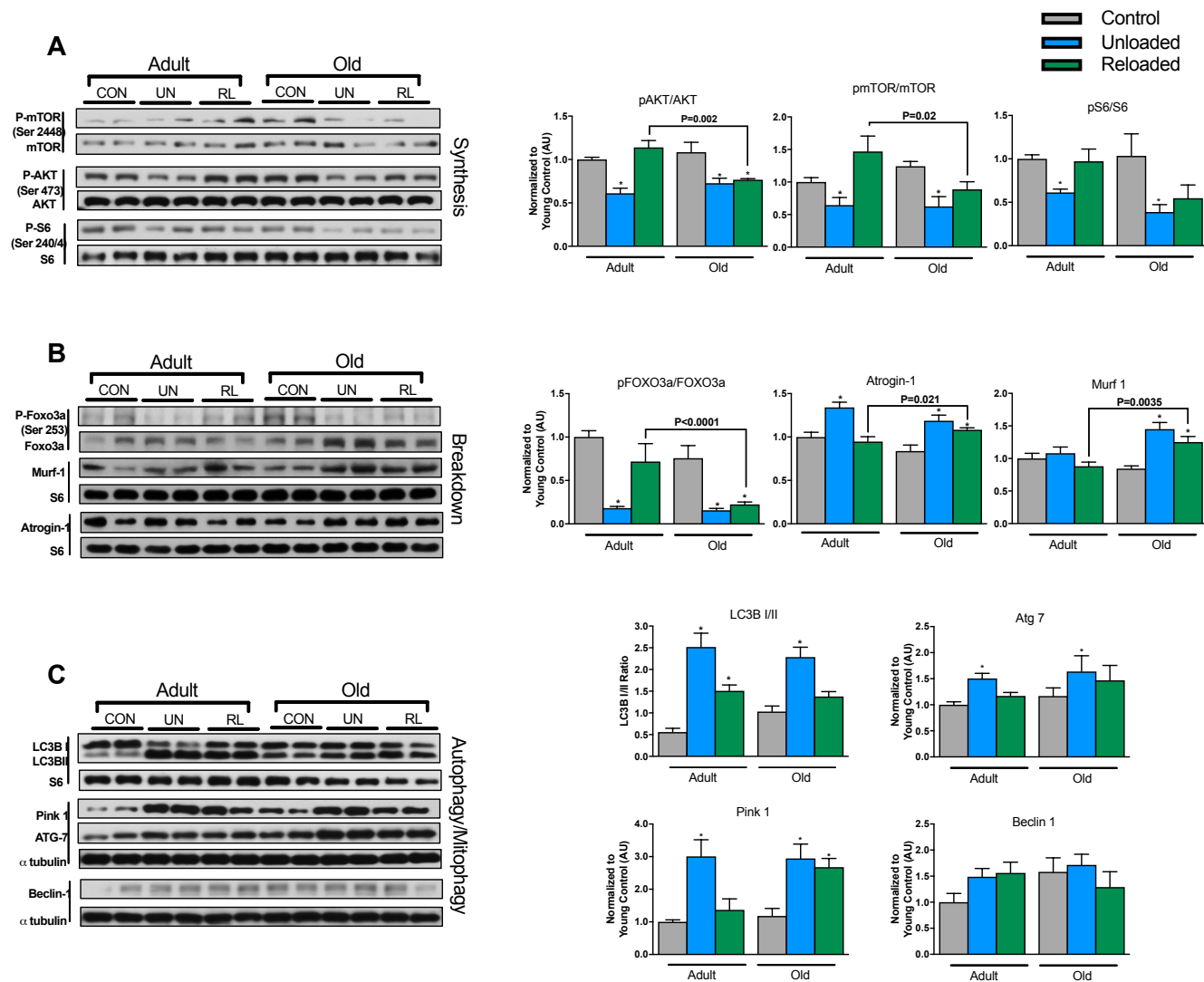
FIGURE 1

FIGURE 2

FIGURE 3

FIGURE 4

FIGURE 5

Acylcarnitine Profile

Organic Acids

Amino Acids

FIGURE 6

A

Ppargc1a

Essrg

Essra

Cox4il

Adult   Old

B Energy Metabolic Pathways

Fatty Acid Metabolism

TCA Cycle

Electron Transport Chain

CON   UN   RL   CON   UN   RL

Adult   Old

C Metabolomic Profile

Acylcarnitines
C2
C3
C3-DC
C3-OH
C4 Butyryl
C4-DC Succinyl
C4-OH Isobutyryl
C5-OH
C6
C10
C14
C14:1
C16
C16:1
C18
C18:1

Organic Acids
Lactate
Pyruvate
Citrate
a-KG
Succinate
Malate
Fumarate

Amino Acids
1-Methylhistidine
Alanine
Arginine
Aspartate
Citrulline
Glutamate
Glutamine
Glycine
Histidine
Isoleucine
Leucine
Lysine
Methionine
Serine
Threonine
Tyrosine
Valine

Pyridine Nucleotides
NMN
NAD
NADH
NAD/NADH

Lipids
Total FFA
Total DAG
Total TAG

Adenine Nucleotides
AMP
ADP
ATP
Energy Charge*

CON   UN   RL   CON   UN   RL

Adult   Old

Color Key

-2   -1   0   1   2
Row Z-Score