1 **Muscle Atrophy: Different Strains of Mice Respond Differently to Single Limb**

2 **Immobilization**

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23 **Abstract**

24   To examine whether genetic variability plays a role in muscle adaption to its mechanical

25   environment, we examined hind limb immobilization in five different strains of mice: CAST/EiJ,

26   NOD/ShiLtJ, NZO/HILtJ, 129S1/SvImJ and A/J. Mice had one limb immobilized by a cast for

27   three weeks. The response to immobilization was dependent on the strain of mice examined.

28   A/J mice lost the most body weight following immobilization and displayed a significant

29   decrease in physical activity. None of the other strains displayed a significant decrease in

30   activity. Food consumption was significantly increased in NOD/ShiLtJ mice. All other strains

31   had non-significant changes in food consumption. Muscle mass/body weight was decreased by

32   immobilization, to varying degrees, in all strains except 1291/SvImJ. Casting decreased

33   absolute muscle mass in both quadriceps and gastrocnemius in NOD/ShiltJ and NZO/HILtJ

34   mice, two strains that can develop diabetes, but not in the other strains. Three weeks of

35   immobilization caused a significant increase in quadriceps levels of atrogenes in CAST/EiJ

36   mice but not in other strains. Immobilization caused a significant increase in quadriceps and

37   gastrocnemius levels of *Myh4* in CAST/EiJ mice but not in any other strain. A similar trend was

38   observed for *Myh7* in gastrocnemius muscle. Immobilization resulted in a decrease the p-

39   p70S6K1/total p706SK1 ratio in quadriceps of NOD/ShiLtJ mice and the gastrocnemius of A/J

40   mice, but not in other strains. Immobilization did not affect the p-4EBP1/total 4EBP1 ratio in

41   quadriceps of any of the strains examined. However, the p-4EBP1/total 4EBP1 ratio in

42   gastrocnemius was greater in immobilized, relative to control, limbs in CAST/EiJ mice. Muscle

43   mass normalized to body weight in both gastrocnemius and quadriceps displayed the greatest

44   degree of heritability. These results reveal remarkable variability in responsiveness to

45   immobilization across five different genetic strains of mice.

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47   **Key words**: Immobilization, muscle atrophy, genetic variation.

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1. Introduction

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The maintenance of muscle mass is critical for optimal quality of life, and mechanical loading plays an important role in maintaining muscle mass [1]. Different types of mechanical unloading, such as that resulting from bed rest, immobilization, spaceflight and decreased physical activity can result in disuse atrophy with serious consequences including increased propensity for falls, severe functional decline and eventually death [2]. Unfortunately, the mechanism underlying disuse atrophy is only partially understood.

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A better understanding of the mechanism underlying unloading-induced muscle loss, or disuse atrophy, could lead to the development of novel and more effective countermeasures and therapeutics not only for disuse atrophy but also age-related sarcopenia. In humans, heritability is approximately 66% for muscle mass and 80% for lean body mass. This suggests that genetic variability may contribute to muscle mass and therefore may also contribute to muscle adaptation to unloading. The role of genetics has also been studied in mouse models. For instance, Judex *et al.* [3] identified one Quantitative Trait Loci (QTL) on chromosome 5 for unloading-induced loss in muscle cross-sectional area in the F2 offspring of a double cross between BALB/cByJ22 and C3H/HeJ23 mice. This QTL accounted for 5% (p<0.001) of the variability in muscle loss in response to unloading in the F2 mice. We are not aware of other studies that have examined the genetics of unloading-induced muscle loss. Exploring how genetic variability contributes to unloading-induced muscle loss could provide mechanistic insight into disuse atrophy.

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To examine the effect of genetic variability on the response of muscle to unloading, we exposed five different strains of mice to hindlimb immobilization. The strains examined were A/J, 129S1/SvImJ, NOD/ShiLtJ, NZO/HILtJ, and CAST/EiJ. We chose to examine these strains because they are founder strains of Diversity Outbred (DO) mice. DO mice are a recently

3

77 developed high-resolution mapping population that enables gene discovery for traits such as the

78 response of muscle to unloading [4]. DO mice can be used to investigate the genetics of a wide

79 range of complex diseases. Examining these founder strains will not only enable us to examine the

80 role of genetic variability in the response of muscle to unloading but will also provide insight into

81 the usefulness of DO mice in future studies.

82

83 To assess the role of genetic variability in disuse atrophy we quantified unloading-induced changes

84 in muscle mass as well as proteins and genes critical to muscle turnover. Muscle atrophy results

85 from an imbalance of muscle protein breakdown (MPB) and muscle protein synthesis (MPS). MPB

86 and MPS balance is at least partially regulated by Akt which activates the protein kinase mTOR to

87 phosphorylate p70S6K1 and 4EBP1 resulting in increased MPS. Inhibition of Akt activates the

88 transcription factor FOXO. FOXO then activates transcriptions of the genes encoding two

89 atrogenes, Atrogin-1 (encoded by *FbXO32*) and MuRF-1 (encoded by *Trim63*), [5] leading to MPB.

90 [2,6,7] Thus, increasing levels of p70S6K1 and 4EBP1 reflect increases in MPS while increasing

91 level of Atrogin -1 and Murf-1 reflect increases in MPB.

92

93 Atrophy leads to changes in fiber type composition, and how those fibers change is dependent on

94 the type of atrophy that occurs. Unlike age-induced atrophy which results in reduced fast-twitch

95 fibers [8,9] , disuse atrophy can lead to increased fast-twitch myosin heavy chains. For instance,

96 Shen *et al.*[10] found different expression of *Myh4*, (encodes myosin heavy chain 4 and is

97 expressed in muscle with fast twitch fibers), and *Myh7* (encodes myosin heavy chain β and is

98 expressed in muscle with slow twitch fibers) in the *deltoid* and *supraspinatus* muscle. The deltoid

99 showed an increase in *Myh7*, in response to the microgravity of space, whereas the supraspinatus

100 displayed an increase in *Myh4*. The same increase in fast twitch fiber type was displayed in the

101 soleus, gastrocnemius and tibialis anterior muscles. Shenkman *et al.*[11] reported an increased

102 level of fast-twitch fibers in vastus lateralis and soleus muscles in monkeys after 12.5 days of

103 spaceflight. These data suggest that unloading may induce a shift from slow to fast-twitch fibers.

4

104   In this study muscle atrophy was induced by immobilization of the left hind limb through casting.

105   We hypothesized that there would be differential, strain-dependent effects of unloading on muscle

106   mass and expression of genes and proteins associated with muscle turnover in five strains of mice

107   – CAST/EiJ, NOD/ShiLtJ, NZO/HlLtJ, 129S1/SvImJ and A/J – after three weeks of unloading using

108   single limb immobilization.

109   **2. Methods**

110   **2.1. Animal model**

111   All the animal procedures were performed with the approval of the Virginia Commonwealth

112   University Institutional Animal Care and Use Committee. We used five Diversity Outbred (DO)

113   founder mouse strains: CAST/EiJ, NOD/ShiLtJ, NZO/HlLtJ, 129S1/SvImJ and A/J. Six male mice

114   of each strain (they were not littermates) were purchased from Jackson Laboratories (Bar Harbor,

115   ME, USA) and arrived at 4-14 weeks of age. The mice were given 2 to 12 weeks to acclimate. At

116   16 weeks of age mice of each strain had their left hind limb placed in a cast. The mice were

117   sacrificed after three weeks of casting.

118

119   **2.2. Casting protocol**

120   The left hind limb of the mice were casted as previously described [12]. Mice were placed under

121   anesthesia with isoflurane (2.5%), had the left hind limb shaved using clippers, and the skin

122   swabbed with 70% isopropanol. Surgical tape was tightly wrapped around the left hind limb from

123   the hip to the knee. A microcentrifuge tube was then glued on top of the tape. The tube had the

124   bottom end removed to allow air to flow into it. Right limbs were used as contralateral controls [13].

125   Mice were single housed and given access to food and water *ad libitum*. Casted mice were able to

126   freely move around the cages, dragging the immobilized limb.

127

128   **2.3 Physical Activity and Food Consumption**

129   Physical activity and food consumption were recorded over a 24-hour period, before and after

130   casting. Observations were made one week prior to casting surgery and two weeks after casting.

131   To asses individual mouse physical activity, video recordings captured for 24 hours were analyzed

132   using the OpenField Matlab function developed by Patel *et al* [14]. Food consumption was

133   measured by weighing food in the cages before and after the 24-hour observation period.

134

135   **2.4 RNA isolation and quantitative RT – PCR**

136   Total RNA from muscle samples was extracted using RNeasy Fibrous Tissue kit (Qiagen,

137   Valencia, CA, USA). The kit used a mixture of oligo (dT) and random primers to provide an

138   unbiased representation of the 5' and 3' regions of the target genes for freedom in qPCR primer

139   design. RNA was eluted from the column with RNase-free water, and 1µl was used for

140   quantification using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

141   Total RNA was reverse transcribed using iScript gDNA Clear cDNA Synthesis kit (Bio-Rad,

142   Hercules, CA) following the manufacturer's instructions. RT-qPCR was performed using 500 ng of

143   cDNA for genes encoding the atrogenes Atrogin-1 (encoded by *FbXO32*) and MuRF 1 (encoded by

144   *Trim63*), *Myh4*, (fast-twitch fiber), and *Myh7* (slow-twitch fiber). All primer sequences are shown in

145   Supplemental Table 1.

146

147   **2.5 Western Blot Analysis**

148   Approximately 20 mg muscle was used for protein isolation and before homogenization all fat was

149   removed. Muscle samples were homogenized in ice-cold buffer consisting of (mmol/L):20 HEPES

150   (pH 7.4), 2 EGTA, 50 sodium fluoride, 100 potassium chloride, 0.2 EDTA, 50 β- glycerophosphate,

151   1 DTT, 0.1 phenylmethane-sulphonylfluride, 1 benzamidine, and 0.5 sodium vanadate. Protein

152   concentration was quantified using a BCA kit (Thermo Fisher scientific), and an equal amount of

153   total protein (30 µg) per sample was subjected to standard SDS-PAGE using Mini-PROTEAN®

154   TGX™ 4-20% 12-wells gels (Bio-Rad, Hercules, CA). Gels were electroblotted onto PVDF

155   membranes (Bio-Rad, Hercules, CA). Western Blot analysis was performed for total and

156   phosphorylated 4EBP1 (T37/46, Cell Signaling Technology; Boston, MA) and total and

157   phosphorylated p70S6K1 (T389, Cell Signaling Technology; Boston, MA) with both antibodies

158   diluted 1:1000. The secondary antibodies were diluted 1:3000. The bound immune complexes

159   were detected using Clarity Max™ Western ECL Substrate (Bio-Rad, Hercules, CA).

160

2.6 Statistical analyses

Repeated measures two-way ANOVAs and post-hoc tests (Tukey's or Sidak's multiple comparisons tests) were used to test for significant differences among the strains after three weeks of casting. GraphPad Prism version 7.0 (GraphPad Software, La Jolla, CA) was used for statistical analysis. Heritability of the muscle phenotypic characteristics was also calculated as previously described [15].

3. Results

Body weight, mouse activity levels and food consumption

Body weights were recorded before and after three weeks of casting (**Table 1**). All strains displayed significant weight loss. A/J mice had the highest percentage of body weight loss compared with the other strains, and this loss was significantly more than in CAST/EiJ, and NOD/ShiLtJ mice. To analyze mouse activity, we video recorded movement around the cage for a period of 24 hours before and after casting. Immobilization significantly decreased activity only in A/J mice. All other strains had non-significant, slight decreases or large increases in distance travelled. Food consumption was significantly increased in NOD/ShiLtJ mice. All other strains had non-significant increases in food consumption except NZO/HlLtJ which had a non-significant decrease in food consumption.

188

189   **Table 1** Mouse body weight, activity and food consumption. * Significantly different than CAST/EiJ

190   and NOD/ShiLtJ, p<0.05. Values are mean±SD. Percent change is the percent change in mean

Mouse Strain	CAST/EiJ	NOD/ShiLtJ	NZO/HlLtJ	129S1/SvlmJ	A/J
Day 1 Weight (g)	17.0 ± 0.9	30.5 ± 2.9	48.9 ± 4.0	28.4 ± 1.8	27.5 ± 2.0
Day 21 Weight (g)	15.8 ± 0.8	27.7 ± 1.1	42.3 ± 3.4	25.3 ± 1.5	22.9 ± 1.8
% Change	-7.3%	-8.7%	-13%	-11%	-17% *
p-value	0.012	0.015	0.0002	0.001	0.0002
Baseline activity (km/day)	3.01 ± 1.09	1.65 ± 0.88	1.49 ± 0.59	1.91 ± 0.67	2.34 ± 0.52
Immobilized activity (km/day)	2.69 ± 0.70	2.11 ± 0.66	1.70 ± 0.79	2.10 ± 0.72	0.90 ± 0.74
% Change	-6%	+94%	+63%	+39%	-57%
p-value	0.827	0.486	0.416	0.530	0.024
Baseline food consumption (g/day)	3.7 ± 2.0	4.0 ± 0.4	5.0 ± 0.5	4.5 ± 1.1	3.3 ± 0.7
Immobilized food consumption (g/day)	4.4 ± 2.2	4.9 ± 0.6	5.0 ± 1.5	5.7 ± 1.6	4.4 ± 1.3
% Change	+22%	+24%	-1.6%	+33%	+43%
p-value	0.149	0.004	0.886	0.176	0.175

191   values.

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193

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195

196 *Muscle mass*

197 We assessed muscle mass both in absolute terms and as corrected for body weight. Assessing

198 absolute muscle mass is appropriate when evaluating the effects of unloading in one strain. This is

199 because in each mouse the contralateral limb serves as a control for the immobilized limb in the

200 same mouse. However, when evaluating muscle mass across strains it is necessary to normalize

201 to body weight. There was a significant immobilization and mouse strain interaction for absolute

202 gastrocnemius mass, absolute quadriceps mass, and quadriceps mass/body weight. There were

203 significant main effects of mouse strain and immobilization on gastrocnemius mass/body weight.

204 Interestingly, immobilization significantly decreased quadriceps and gastrocnemius absolute

205 muscle mass in only NOD/ShiltJ and NZO/HILtJ mice (**Fig. 1B**). CAST/EiJ, 129S1/SvImJ and A/J

206 quadriceps and gastrocnemius absolute muscle mass were not affected by casting. Quadriceps

207 muscle mass/body weight was significantly decreased by immobilization, to varying degrees, in all

208 strains except 129S1/SvImJ. Immobilization resulted in the most significant decrease in quadricep

209 muscle mass/body weight NOD/ShiltJ mice. Gastrocnemius muscle mass/body weight was

210 significantly decreased, to varying degrees, in all strains except 129S1/SvImJ and A/J, and again

211 the most significant loss was in NOD/ShiltJ mice. Finally, control muscle mass/body weight varied

212 across strains (**Fig. 1B**). Quadriceps mass/body weight was significantly different between 5 strain

213 pairs: CAST/EiJ vs. NOD/ShiLtJ (p=0.007); CAST/EiJ vs. A/J (0.036); NOD/ShiLtJ vs. NZO/HILtJ

214 (p<0.0001); NZO/HILtJ vs. 129S1/SvImJ (p=0.037) and NZO/HILtJ vs. A/J (p=0.0003). However,

215 gastrocnemius mass/body weight was significantly different in only one pair, CAST/Eij vs.

216 NZO/HILtj (p=0.031).

217

218 **Fig. 1. Effect of three weeks of casting on quadriceps and gastrocnemius muscle mass (A)**

219 **and muscle mass normalized to body weight (B).** Results for grey bars are data from control

220 mice and black bars are data from mice exposed to immobilization by casting. Values are

221 means±SD; n=6 for immobilized and control samples for five strains of mice. Numbers above bars

9

222   are the fold changes (immobilized/control) for each strain. &Significant immobilization and mouse

223   strain interaction (p<0.05); ^Significant main effect of mouse strain (p<0.05); #Significant main

224   effect of immobilization; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

225   .

226   *Atrogene levels*

227   To determine the level of muscle atrophy induced by casting, the levels of the atrogenes, *FbXO32*

228   (encodes Atrogin-1) and *Trim63* (encodes MuRF1), were quantified by RT-qPCR (**Fig. 2**). There

229   was a significant immobilization and mouse strain interaction for quadriceps and gastrocnemius

230   levels of *FbXO32* and gastrocnemius levels of *Trim63*. There was a significant main effect of

231   mouse strain on quadriceps levels of *Trim63*. Three weeks of immobilization resulted in a

232   significant increase in quadriceps and gastrocnemius levels of *FbXO32* and *Trim63* in CAST/EiJ

233   mice but not in any other strain. Control levels *FbXO32* and *Trim63* in both quadriceps and

234   gastrocnemius were similar across all strains.

235

236   **Fig. 2. Effect of three weeks of casting on Atrogenes (FbXO32 and Trim63) expression**

237   **levels in (A) quadriceps and (B) gastrocnemius muscles.** Values are means±SD; n=6 for

238   immobilized and control samples for five strains of mice. Grey bars are data from control limbs, and

239   black bars are data from limbs exposed to immobilization by casting. Numbers above bars are the

240   fold changes (immobilized/control) for each strain. &Significant immobilization and mouse strain

241   interaction (p<0.05); ^Significant main effect of mouse strain (p<0.05); *p<0.05; **p<0.01;

242   ***p<0.001; ****p<0.0001; immobilized vs control within the same strain.

243

244   *Muscle fiber type gene levels*

245   As previously mentioned, unloading also affects muscle fiber type composition. Levels of mRNA for

246   genes associated with fast-twitch fibers, *Myh4,* and slow-twitch fibers, *Myh7,* were quantified (**Fig.**

247   **3**). There was a significant immobilization and mouse strain interaction for quadriceps and

248   gastrocnemius levels of *Myh4* and gastrocnemius levels of *Myh7*. There was a significant main

249   effect of mouse strain on quadriceps levels of *Myh7*. Three weeks of immobilization resulted a

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250 significant increase in quadriceps and gastrocnemius levels of *Myh4* in CAST/EiJ mice but not in

251 any other strain. A similar trend was observed for *Myh7* in gastrocnemius muscle. However, *Myh 7*

252 levels were not affected by immobilization in quadraceps of any strain. Control levels *Myh4* and

253 *Myh7 FbXO32* in both quadriceps and gastrocnemius were similar across all strains.

254

255 **Figure 3. Effect of three weeks of casting on fiber type composition (Myh4 – fast twitch fiber**

256 **and Myh7- slow twitch fiber) gene levels in (A) quadriceps and (B) gastrocnemius muscles**

257 **in five strain of mice.** Grey bars are data from control mice and black bars are data from mice

258 exposed to immobilization by casting. Values are means±SD; n=6 for immobilized and control

259 samples for five strains of mice. Numbers above bars are the fold changes (immobilized/control)

260 for each strain. &Significant immobilization and mouse strain interaction (p<0.05); ^Significant main

261 effect of mouse strain (p<0.05); ****p<0.0001; immobilize vs control within same strain.

262

263 *Protein synthesis markers*

264 The mTOR pathway is related to the maintenance of MPS. A central regulator of protein synthesis

265 in skeletal muscle is mTORC1. Previous human and animal studies demostrated that the inibition

266 of mTORC1 by rapamycin associated with excercise did not significantly reduce the protein

267 synthesis rate [16–18]. However, by quantifying phosphorylation of p70S6K1 and 4EBP1, we

268 indirectly assessed mTOR activity in quadriceps and gastrocnemius muscles.

269

270 There was a significant immobilization and mouse strain interaction for quadriceps p-p70S6K1/total

271 p706SK1 ratio. There was a significant main effect of immobilization on quadriceps p-4EBP1/total

272 4EBP1 ratio. No other main effects were significant for p-p70S6K1/total p706SK1 and p-

273 4EBP1/total 4EBP1. Three weeks of immobilization resulted in a significant decrease the p-

274 p70S6K1/total p706SK1 ratio in quadriceps of NOD/ShiLtJ mice (**Fig. 4A**) and the gastrocnemius

275 of A/J mice (**Fig. 4A**). Immobilizing did not affect the p-p70S6K1/total p706SK1 in quadriceps or

276 gastrocnemius of any other strain. Immobilization did not affect the p-4EBP1/total 4EBP1 ratio in

277 quadriceps of any of the strains examined (**Fig. 4A**). However, the p-4EBP1/total 4EBP1 ratio in

278   gastrocnemius was significantly greater in immobilized, relative to control, limbs in CAST/EiJ mice

279   (**Fig. 4B**). The p-p70S6K1/total p706SK1 ratio in control quadriceps was similar in all strains,

280   except NOD/ShiLtJ in which levels were significantly greater than all other strains (NOD/ShiLtJ vs.

281   CAST/EiJ, p=0.0015; NOD/ShiLtJ vs. NZO/HILtJ, p=0.0028; NOD/ShiLtJ vs. 129S1/SvImJ,

282   p=0.0018; NOD/ShiLtJ vs. A/J, p=0.0009). The p-p70S6K1/total p706SK1 ratio in control

283   gastrocnemius was significantly greater in A/J mice relative to other strains (CAST/EiJ vs. A/J,

284   p=0.0174; NOD/ShiLtJ vs. A/J, p=0.0175; NZO/HILtJ vs. A/J, p=0.0442; 129S1/SvImJ vs. A/J,

285   p=0.0133). The p-4EBP1/total 4EBP1 ratio in in control quadriceps was similar in all strains

286   examined. However, the p-4EBP1/total 4EBP1 ratio in control gastrocnemius was significantly

287   reduced in A/J mice relative to the other strains examined.

288

289   **Fig. 4. Effect of three weeks of casting on phosphorylation of p70S6K1 and 4E – BP1 in (A)**

290   **quadriceps and (B) gastrocnemius muscles.** All western blots were quantified and bar graphs

291   represent mean ± SD; n=6 for immobilized and control samples for five strains of mice. Grey bars

292   are data from control limbs, and black bars are data from limbs exposed to immobilization by

293   casting. Numbers above bars are the fold changes (immobilized/control) for each strain

294   &Significant immobilization and mouse strain interaction (p<0.05); #Significant main effect of

295   immobilization (p<0.05); * p<0.05 immobilized vs control within the same strain.

296   .

297   *Heritability*

298   Considering that genetics may play an important role in the response to unloading, heritability was

299   calculated for each property analyzed. The analysis was done for each muscle (**Table 2**). Genetics

300   had a greater impact on muscle mass/body weight than other traits. Heritability of muscle mass,

301   *FBXO32, Myh4 and Myh7* was greatest in the gastrocnemius. On the contrary, heritability

302   calculated for muscle mass/body weight, *Trim63,* p-p70S6K1/total p706SK1 and 4EBP1/total

303   4EBP1 was higher in the quadriceps than the gastrocnemius. Phosphorylated p-p70S6K1/total

304   p706SK1 and 4EBP1/total 4EBP1 were the traits least affected by genetics.

305

306

307 **Table 2.** Heritability of   different properties.

Property	Quadriceps	Gastrocnemius
Body weight	0.4497	0.4497
Muscle mass	0.2299	0.3133
Muscle mass/BW	0.6945	0.5074
FBXO32	0.2560	0.4749
Trim63	0.3898	0.2704
Myh4	0.2400	0.3697
Myh7	0.2807	0.3562
p-p70S6K1	0.1708	0.1490
p-4EBP1	0.07387	0.02344

4. Discussion

326 Our results reveal remarkable variability in responsiveness to immobilization across the five

327 different genetic strains of mice. NOD/ShiLtJ and NZO/HILtJ were the only strains that displayed

328 muscle loss in response to unloading. These two strains displayed similar changes in activity level,

329 lost similar amounts of body weight following immobilization and neither of these two strains

330 displayed an increase in atrogenes or changes in muscle fiber type following immobilization.

331 NOD/ShiLtJ mice displayed a decrease in the p-p70S6K1/total p706SK1 ratio in the quadriceps of

332 the immobilized limbs. Interestingly, the p-4EBP1/total 4EBP1 ratio levels were not affected by

333 immobilization in either NOD/ShiLtJ or NZO/HILtJ mice. These results are consistent with previous

13

334   data from our group showing p70S6K1 changes precede 4EBP1 changes following unloading

335   induced by hind limb suspension of C57BL/6J mice [19]. The decrease in p-p70S6K1/total

336   p706SK1 ratio, which is the downstream target of mTOR kinase, in NOD/ShiLtJ mice suggests that

337   changes mTOR kinase activity and decreased protein synthesis contribute to disuse atrophy in this

338   strain.

339

340   Our finding that only NOD/ShiLtJ and NZO/HILtJ mice displayed muscle atrophy in the immobilized

341   limb is especially interesting in light of the fact that males of both of these strains spontaneously

342   develop diabetes [20] while this has not been reported in the other strains examined in this study.

343   Disuse muscle atrophy is exacerbated in diabetes [21] suggesting that susceptibility of NOD/ShiLtJ

344   and NZO/HILtJ mice to disuse atrophy may be related to their diabetic phenotype.

345

346   CAST/EiJ mice displayed the least body weight loss during the experiment, were the most

347   physically active and displayed an intermediate muscle mass to body weight ratio in control limbs.

348   Interestingly, CAST/EiJ mice were the only mice that displayed changes in levels of atrogenes,

349   *Myh4* or *Myh7*. *Myh4* levels were increased in both the gastrocnemius and quadriceps of

350   immobilized limbs while *Myh7* levels were increased in the gastrocnemius of immobilized limbs.

351   These results suggest an unloading-induced switch from slow- to fast-twitch fiber type, at least in

352   the quadriceps. This is consistent with previous studies which revealed a switch from slow to fast

353   fiber type associated with loss of muscle mass and strength [22–28]. Additionally, unloading from

354   exposure to microgravity results in a switch from slow to fast fiber type [8].

355

356   Despite being resistant to disuse-induced muscle atrophy, CAST/EiJ mice displayed an increase in

357   both atrogenes in both quadriceps and gastrocnemius muscles in the immobilized limb. This was

358   accompanied by increases in p-4EBP1, a marker of protein synthesis, in gastrocnemius expose to

359   immobilization. These results suggest that, in response to immobilization, CAST/EiJ muscle display

360   increased muscle protein breakdown that is compensated for by an increase in protein synthesis,

361   resulting in no net muscle loss.

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362

363 A/J mice lost the most body weight throughout the experiment and were the only strain that

364 displayed decreased physical activity during casting. The reason for this is unclear. However, since

365 the A/J mice had the lowest physical activity after casting, it is possible that effectively both limbs

366 were immobilized. The decrease in activity may have caused control limb atrophy during the first 7-

367 14 days. Then the muscles reached homeostasis between days 15-21. Despite displaying a

368 decrease in p-p70S6K1/total p706SK1 ratio in the gastrocnemius of the immobilized limb, A/J mice

369 appeared, at least in the quadriceps, resistant to immobilization-induced muscle atrophy. This

370 suggests that increases in atrogene expression, which did not change in A/J mice, are necessary

371 but perhaps not sufficient, for immobilization-induced muscle atrophy. 129S1/SvImJ mice were

372 resistant to immobilization-induced muscle atrophy. They lost a modest amount of body weight

373 during the experiment but displayed no changes in physical activity as a result of casting. The

374 immobilized limbs had similar levels, relative to control limbs, of markers of fast- and slow- twitch

375 muscle fibers, atrogene levels, and markers of protein synthesis.

376

377 To better understand the role of genetics in the response to unloading, heritability for each property

378 was calculated based on the percent difference between immobilized and control limbs. The

379 results revealed a variety of impacts of genetics on these properties. Muscle mass normalized to

380 body weight in both gastrocnemius and quadriceps displayed the greatest degree of heritability

381 suggesting that this phenotype is the most affected by genetic variability. Interestingly the two

382 markers of protein synthesis p-4EBP1/total 4EBP1 ratio and p-p70S6K1/total p706SK1 ratio were

383 influenced the least by genetic variability. This suggests a disconnect between muscle mass/body

384 weight and protein synthesis, at least as regards the effects of genetic variability. We are unaware

385 of literature reports of the effect of disuse with casting in these strains of mice.

386

387 This study reveals differences in the response to unloading among the five mouse strains and

388 suggests that DO mice, derived from these founders and three additional strains, would be

15

389   powerful tools in identifying the mechanisms underlying disuse atrophy. However, this study

390   has limitations that can be addressed with future studies. For instance, an examination of

391   which genes are responsible for protecting muscle from unloading in the strains that showed

392   minimal response to unloading would be insightful. This could be accomplished using RNA

393   sequencing of muscle tissue samples. Additionally, our finding that immobilization did not

394   reduce muscle mass in every strain but did affect at least one endpoint in all strains except

395   129S1/SvImJ, suggests that the duration of exposure to immobilization may differentially affect

396   strain-dependent response to immobilization. Another limitation is that we used only male mice

397   and it is well known that sex differences exist between male and female mice. Therefore, one

398   of the next steps could be to study the response to unloading in female mice of the DO founder

399   strains.

400

401   5. CONCLUSIONS

402   This study is the first to report a differential muscle response of five DO founder strains of mice

403   to immobilization. The results confirm our hypothesis that genetic variability plays an important

404   role in the development of muscle atrophy, leading to differential, strain-dependent effects of

405   unloading on muscles in these strains of mice. Additionally, we now have the basis for using

406   DO mice in order to identify specific gene changes that protect muscle from disuse atrophy. In

407   this way, a therapeutic strategy to limit the development of unloading-induced sarcopenia could

408   be developed.

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410   **ACKNOWLEDGMENTS**

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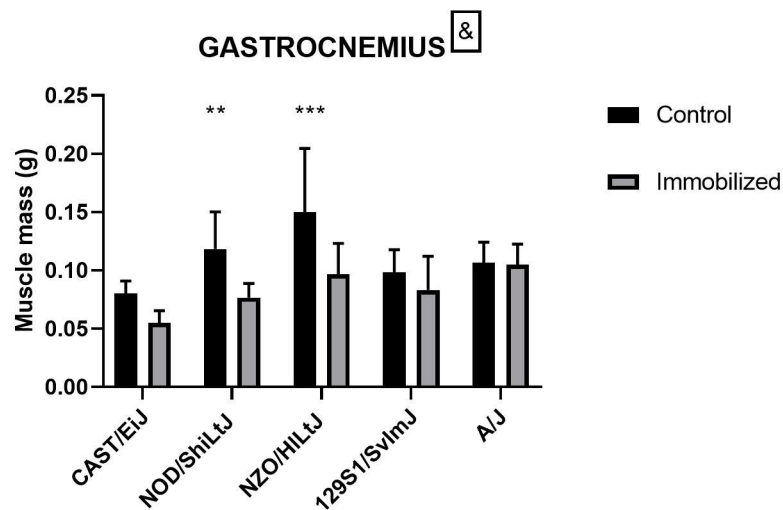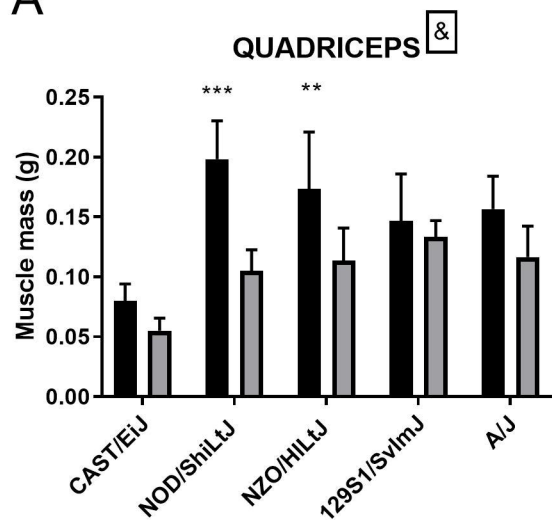492   18. doi:10.1016/j.jprot.2015.11.005

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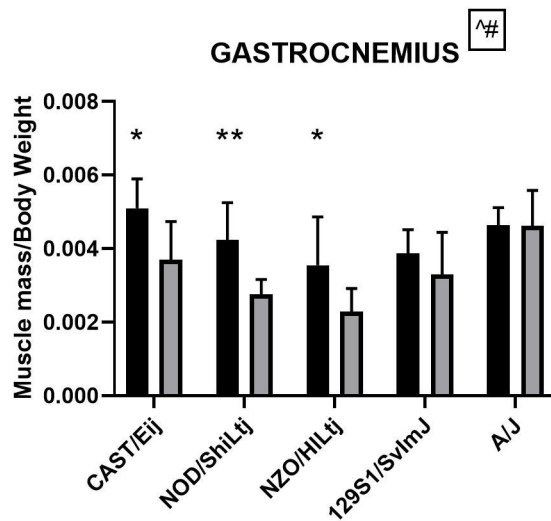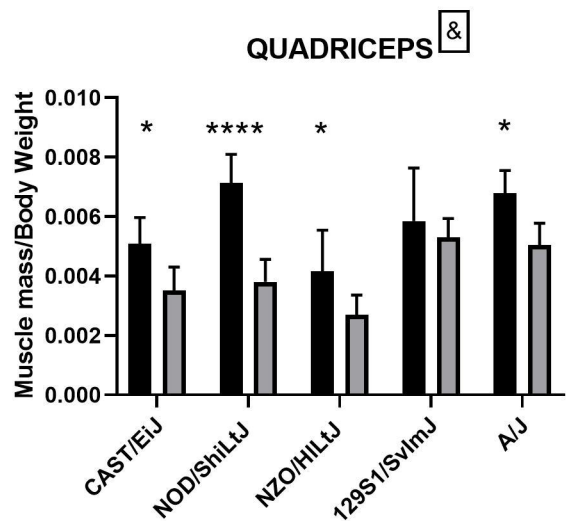
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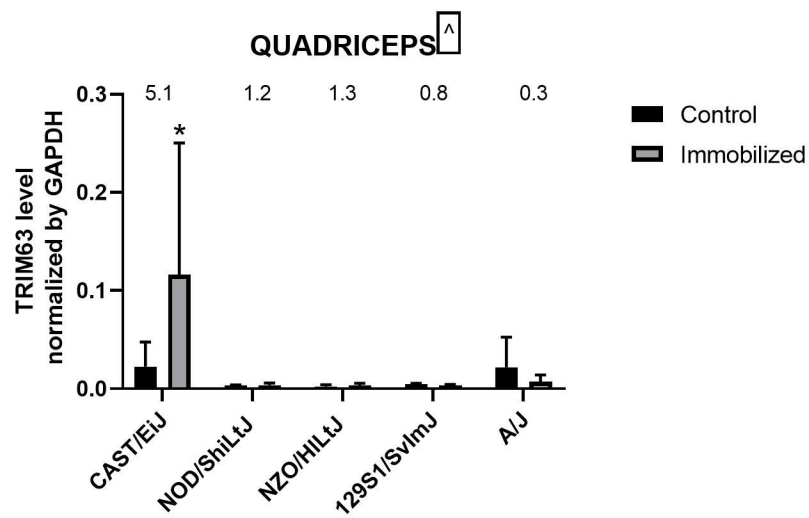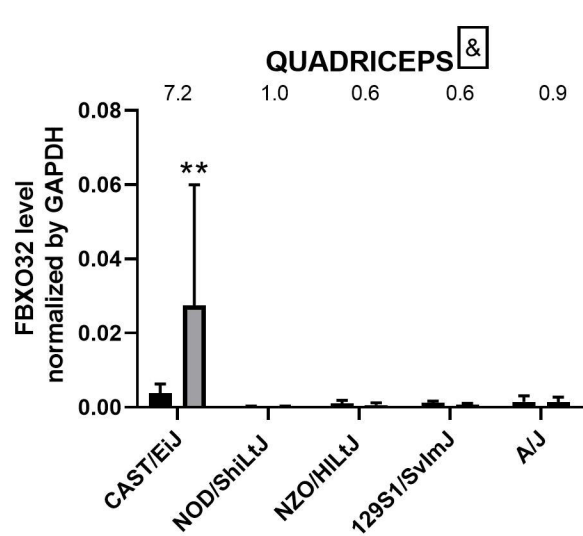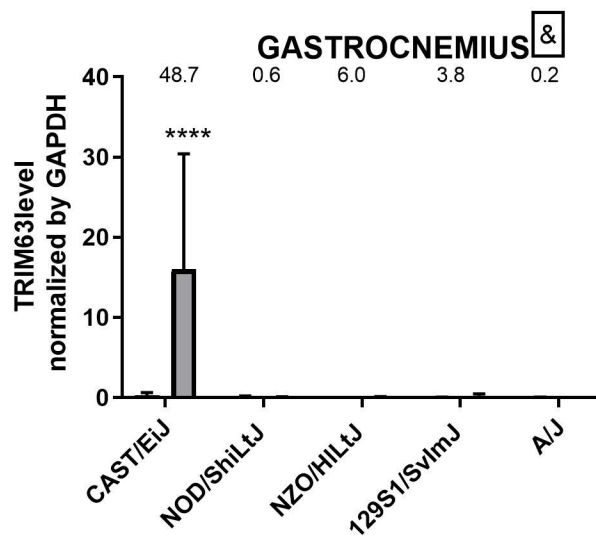
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501

A

A

QUADRICEPS &

7.2   1.0   0.6   0.6   0.9

** (CAST/EiJ)

FBXO32 level normalized by GAPDH

CAST/EiJ   NOD/ShiLtJ   NZO/HILtJ   129S1/SvImJ   A/J

QUADRICEPS ^

5.1   1.2   1.3   0.8   0.3

* (CAST/EiJ)

TRIM63 level normalized by GAPDH

CAST/EiJ   NOD/ShiLtJ   NZO/HILtJ   129S1/SvImJ   A/J

■ Control
■ Immobilized

B

GASTROCNEMIUS &

7.8   3.3   2.0   1.1   0.6

*** (CAST/EiJ)

FBXO32 level normalized by GAPDH

CAST/EiJ   NOD/ShiLtJ   NZO/HILtJ   129S1/SvImJ   A/J

GASTROCNEMIUS &

48.7   0.6   6.0   3.8   0.2

**** (CAST/EiJ)

TRIM63 level normalized by GAPDH

CAST/EiJ   NOD/ShiLtJ   NZO/HILtJ   129S1/SvImJ   A/J

A

QUADRICEPS &

CAST/EiJ	NOD/ShiLtJ	NZO/HlLtJ	129S1/SvlmJ	AJ
10.7	1.9	1.1	1.7	0.4

Myh4 level normalized by GAPDH

****

QUADRICEPS ^

CAST/EiJ	NOD/ShiLtJ	NZO/HlLtJ	129S1/SvlmJ	AJ
0.6	1.0	0.3	0.4	0.2

Myh7 level normalized by GAPDH

■ Control
■ Immobilized

B

GASTROCNEMIUS &

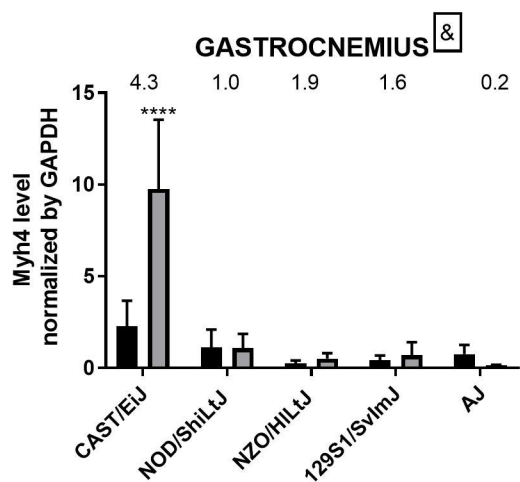| CAST/EiJ	NOD/ShiLtJ	NZO/HlLtJ	129S1/SvlmJ	AJ
4.3	1.0	1.9	1.6	0.2

Myh4 level normalized by GAPDH

****

GASTROCNEMIUS &

CAST/EiJ	NOD/ShiLtJ	NZO/HlLtJ	129S1/SvlmJ	AJ
2.8	0.4	4.7	0.8	0.04

Myh7 level normalized by GAPDH

***

A

B

	CAST/EiJ		NOD/ShiLtj		NZO/HILtj		129S1/SvImJ		A/J	
	Immob.	CTRL	Immob.	CTRL	Immob.	CTRL	Immob.	CTRL	Immob.	CTRL

Gastrocnemius p S6K1 (T389)

Gastrocnemius total S6K1

Gastrocnemius p 4 E-BP1 (T37/46)

Gastrocnemius Total 4 E-BP1

GASTROCNEMIUS

0.2   0.2   0.1   2.0   0.2

p-p70S6K1(T389) level normalized by Total p70S6K1

*

CAST/EiJ   NOD/ShiLtJ   NZO/HILtJ   129S1/SvImJ   A/J

GASTROCNEMIUS

2.8   0.6   0.8   0.7   1.4

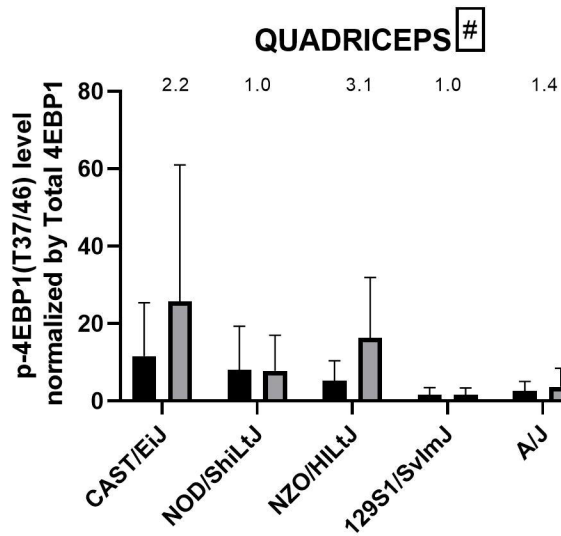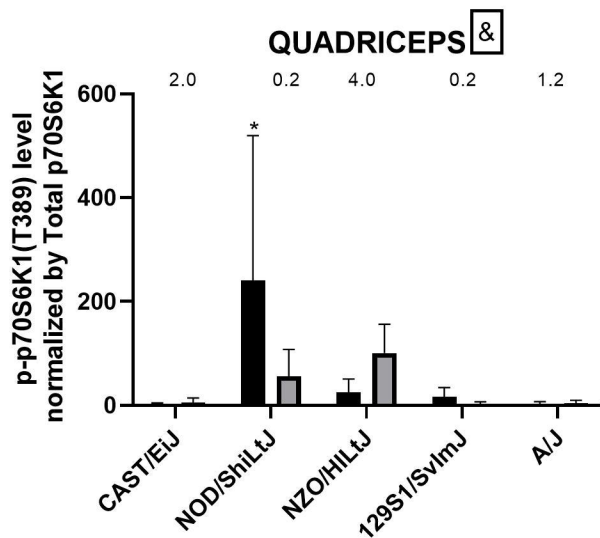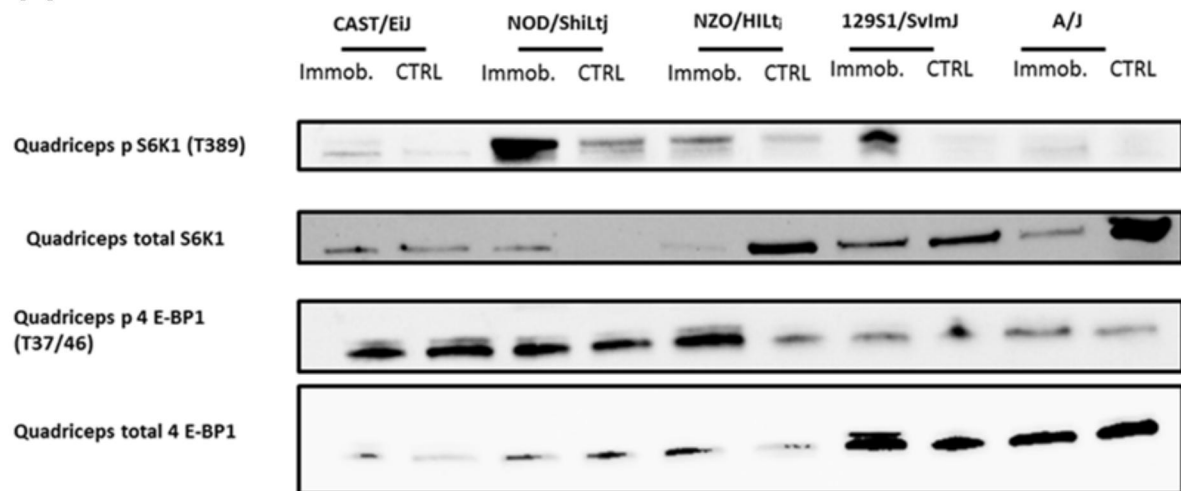p-4EBP1(T37/46) level normalized by Total 4EBP1

*

CAST/EiJ   NOD/ShiLtJ   NZO/HILtJ   129S1/SvImJ   A/J

■ Control
■ Immobilized