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1 **The effect of caloric restriction on the forelimb skeletal muscle fibers of**
2 **the hypertrophic myostatin null mice.**

3 **Mohamed I. Elashry^{3, 4}; Antonios Matsakas²; Sabine Wenisch⁵; Stefan**
4 **Arnhold⁴ and Ketan Patel¹**

5 ¹School of Biological Sciences, Hopkins building, Whiteknights, University of
6 Reading, RG6 6UB, United Kingdom

7 ² Hull York Medical School, University of Hull

8 ³Anatomy and Embryology Department, Faculty of Veterinary Medicine,
9 University of Mansoura 35516, Egypt

10 ⁴Institute of Veterinary Anatomy, Histology and Embryology, Justus Liebig
11 University of Giessen 35392, Germany

12 ⁵Clinic of Small Animals, c/o Institute of Veterinary Anatomy, Histology and
13 Embryology, Justus Liebig University of Giessen 35392, Germany

14 Corresponding author:

15 Mohamed Ismail Elashry

16 Institute of Veterinary Anatomy, Histology and Embryology

17 Justus Liebig University of Gießen

18 Frankfurter Str. 98,

19 35392 Gießen

20 Email: mohammed.elashry@vetmed.uni-giessen.de

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24

25 **Abstract**

26 Skeletal muscle mass loss has a broad impact on body performance and
27 physical activity. Muscle wasting occurs due to genetic mutation as in muscular
28 dystrophy, age-related muscle loss (sarcopenia) as well as in chronic wasting
29 disorders as in cancer cachexia. Food restriction reduces muscle mass
30 underpinned by increased muscle protein break down. However the influence
31 of dietary restriction on the morphometry and phenotype of forelimb muscles in
32 a genetically modified *myostatin* null mice are not fully characterized. The effect
33 of a five week dietary limitation on five anatomically and structurally different
34 forelimb muscles was examined. C57/BL6 wild type (*Mstn*^{+/+}) and *myostatin* null
35 (*Mstn*^{-/-}) mice were either given a standard rodent normal daily diet *ad libitum*
36 (ND) or 60% food restriction (FR) for a 5 week period. *M. triceps brachii Caput*
37 *laterale* (T.lateral), *M. triceps brachii Caput longum* (T.long), *M. triceps brachii*
38 *Caput mediale* (T.medial), *M. extensor carpi ulnaris* (ECU) and *M. flexor carpi*
39 *ulnaris* (FCU) were dissected, weighted and processed for
40 immunohistochemistry. Muscle mass, fibers cross sectional areas (CSA) and
41 myosin heavy chain types IIB, IIX, IIA and type I were analyzed. We provide
42 evidence that caloric restriction results in muscle specific weight reduction with
43 the fast myofibers being more prone to atrophy. We show that slow fibers are
44 less liable to dietary restriction induced muscle atrophy. The effect of dietary
45 restriction was more pronounced in *Mstn*^{-/-} muscles to implicate the oxidative
46 fibers compared to *Mstn*^{+/+}. Furthermore, peripherally located myofibers are
47 more susceptible to dietary induced reduction compared to deep fibers. We
48 additionally report that dietary restriction alters the glycolytic phenotype of the
49 *Mstn*^{-/-} into the oxidative form in a muscle dependent manner. In summary our
50 study shows that calorie restriction alters muscle fiber profile of forelimb
51 muscles of Myostatin null mice.

52 **Keywords:** Skeletal muscle, Myostatin, food restriction, myosin heavy chain

53

54 **1. Introduction**

55 Skeletal muscle the main protein reservoir in the body, is a highly adaptable
56 tissue that changes its physical as well as composition based on physiological
57 demands. Mechanical and nutritional stimuli cause an increase in muscle mass.
58 In contrast undernutrition, aging and diseases as cancer cachexia reduces the
59 muscle mass. Unbalanced nutrient intake with increased energy requirements
60 enhances the muscle catabolism resulting in fiber atrophy and muscle mass
61 loss (Koskelo et al., 1990). Muscle loss also occurring in progressive wasting
62 diseases e.g. cancer cachexia (Bruggeman et al., 2016) and age-related
63 sarcopenia leads to a decrease in muscle mass and strength (Thomas, 2007).
64 Additionally the role of physiological and metabolic stimuli e.g. physical activity,
65 disuse and immobilization, food restriction, drugs and diseases cause
66 molecular and cellular dysregulation which results in loss of muscle mass
67 (Carmeli and Reznick, 1994). It has been reported that ageing induces muscle
68 mass loss of 16%, 18%, 37% and 38% for soleus, extensor digitorum longus,
69 plantaris and gastrocnemius muscles respectively with greater fiber area loss
70 compared to the decrease in body mass (Brown and Hasser, 1996). Similarly it
71 was reported that dietary restriction causes a decrease in muscle fiber size but
72 not fiber number in chicken and rabbits (Tanaka et al., 1992 and Timson et al.,
73 1983). In addition the glycolytic fibers were selectively decreased in the cross
74 sectional area lead to a proportional increase in the area of oxidative fibers in
75 bovine muscle (Greenwood et al., 2009). Muscle phenotype also changed
76 according to the dietary challenges, It has been reported that dietary deprivation
77 for 48 hours upregulates the expression of fast myosin heavy chain 2b mRNA
78 with no change in fiber type composition for EDL and soleus muscles in rat
79 (Mizunoya et al., 2013b) However 4 weeks fatty diet administration induced
80 reduction in the fast MHC2b and improved the oxidative metabolism in the EDL
81 of rat (Matsakas et al., 2013; Mizunoya et al., 2013a). Previously, it was shown
82 that fasting induces muscle proteolysis via glucocorticoid activation (Wing and
83 Goldberg, 1993) as well as causing an increase in the ATP-dependent
84 proteolysis and upregulation of ubiquitin conjugates and polyubiquitins in rat
85 skeletal muscle (Medina et al., 1991).

86 *Myostatin* is a TGF- β family member of secreted proteins that negatively
87 regulates skeletal muscle proliferation (Sharma et al., 2001). *Myostatin* protein
88 interference via knock out results in significant myofiber hyperplasia and
89 hypertrophy (McPherron et al., 1997). Furthermore, systemic administration of
90 *Myostatin* induced muscle atrophy in mice (Zimmers et al., 2002). Increased
91 myostatin expression was also associated with muscle wasting in cancer
92 cachexia, ageing, after HIV infection and in the course of chronic obstructive
93 pulmonary disease (COPD) (Costelli et al., 2008; Gonzalez-Cadavid et al.,
94 1998; Plant et al., 2010; Yarasheski et al., 2002). *Myostatin* knock out mice
95 showed a greater atrophy in response to unloading (McMahon et al., 2003).
96 Our previous study demonstrated a massive reduction in forelimb muscles
97 mass of aged *myostatin* null compared to wild type mice (Elashry et al., 2009).
98 These studies suggest that hypertrophic muscle has decreased adaptability
99 compared to WT. In this study we hypothesized that five weeks of 60% dietary
100 restriction would impact to a greater degree on the muscle of *Mstn*^{-/-} compared
101 to WT. We provide evidence that caloric restriction results in muscle specific
102 weight reduction with the fast myofibers being more prone to atrophy. We show
103 that slow fibers are less liable to dietary restriction induced muscle atrophy.
104 Furthermore, peripherally located myofibers are more susceptible to dietary
105 induced reduction. Additionally, we report that dietary restriction alters the
106 glycolytic phenotype of the myostatin null into more oxidative form in a muscle
107 dependent manner.

108 **2. Materials and Methods**

109 *2. 1. Animals*

110 Three months old male transgenic myostatin knockout (*Mstn*^{-/-}) and C57/BL6
111 wild type littermates (*Mstn*^{+/+}) mice were bred in the biological resource unit,
112 Reading University, UK. *Mstn*^{-/-} mice were generously provided from Dr Lee
113 (McPherron et al., 1997). The experiments were performed under a project
114 license from the United Kingdom Home Office in agreement with the Animals
115 (Scientific Procedures) Act 1986 (license number 7516). The University of
116 Reading Animal Care and Ethical Review Committee (AWERB) approved all
117 procedures. The mice were divided into 4 subgroups: a normal diet group (N=8)

118 *Mstn*^{+/+} and *Mstn*^{-/-} kept with a normal diet with food and water provided *ad*
119 *libitum* and a food restriction group (N=8) with an up to 60% reduction of the
120 daily diet compared to normal mice for a 5 week period. Animals were humanely
121 sacrificed via Schedule 1 killing.

122 2. 2. Tissue isolation and processing

123 Aged matched mice were sacrificed through schedule-1 killing following the
124 approval of the ethics committee. Five forelimb muscles were carefully chosen
125 to represent the arm and the forearm region *M. triceps brachii Caput longum*,
126 T.long; *M. triceps brachii Caput laterale*, T.lateral; *M. triceps brachii Caput*
127 *mediale*, T.medial; *M. extensor carpi ulnaris*, ECU; and *M. flexor carpi ulnaris*,
128 FCU. The muscles were dissected, snap frozen using isopentane pre-cooled
129 with liquid nitrogen and were embedded in tissue tech OCT (Sakura, VWR)
130 using dry ice cooled ethanol. 10 µm transverse mid-belly cryosections for each
131 muscle were obtained on a poly-L-lysine coated slides (VWR) and left to dry at
132 room temperature for 1 hour and stored at -80C°.

133 2. 3. Immunohistochemical staining

134 Frozen muscle sections were washed in PBS for 10 minutes, permeabilized
135 using buffer solution containing 20 mM Hepes, 300 mM sucrose, 50 mM NaCl,
136 3 mM MgCl₂ and 0.5% Triton-X100 (pH7) at for 15 minutes. Non-specific
137 binding was blocked using wash buffer (5% fetal calf serum (v/v) and 0.05%
138 Triton X-100 (v/v) in PBS) for 30 minutes at room temperature before the
139 addition of antibodies. Myosin heavy chain (MHC) type I, IIA and IIB expressing
140 myofibers were identified by incubating the slides with A4.840 mouse IgM (1:1),
141 A.474 mouse IgG (1:4) and BFF3 mouse IgM (1:1) monoclonal primary
142 antibodies (DSHB) respectively as previously described (Matsakas et al.,
143 2009). The primary antibodies were tested separately against the
144 corresponding MHC type on a muscle section and the following section with no
145 primary antibody was served as a negative control (Fig. 1g-l). Additionally, MHC
146 type IIA⁺ and IIB⁺ myofibers were stained together and MHC type I⁺ myofibers
147 were stained on consecutive section. All primary antibodies were pre-blocked
148 in washing buffer 30 minutes prior to use and incubated on samples overnight
149 at 4°C. Primary antibodies were highlighted via incubation with Alexa Fluor 633

150 goat anti-mouse IgM (Molecular Probes A21046, 1:200) for MHC I and MHC
151 IIB and Alexa Fluor 488 Goat-anti-mouse IgG (Molecular probes A11029,
152 1:200) for MHC IIA secondary antibodies in dark at room temperature for 45
153 minutes. Slides were mounted using a fluorescent mounting medium (Mowiol
154 4-88, Calbiochem) containing 2.5 µg/ml DAPI.

155 *2. 4. Imaging and quantifications*

156 Muscle sections were examined and photographed using a Zeiss Axioscop2
157 fluorescence microscope loaded with a digital camera and connected to a
158 computer equipped with the axiovision computer software (Zeiss). The whole
159 muscle cross sections was reconstructed, total number, number of IIB, IIA and
160 type I fibers quantifications were performed by manual counting using the
161 Photoshop CS6 extended software. Type IIX fibers were counted via
162 subtraction of all other fibers from the total fiber number. Cross sectional area
163 (CSA) for each fiber type for all muscles were measured using the axiovision
164 software (Zeiss).

165 *2. 5. Statistical analysis*

166 The effect of genotype (Mstn^{+/+} vs. Mstn^{-/-}) and diet regime (ND vs. FR) on total
167 muscle fiber numbers, CSA and the percentage of type IIB, IIX, IIA and I fibers
168 were analyzed by two-way ANOVA for T.lateral, T.long, T.medial, ECU and
169 FCU muscles. The effect of genotype, diet regime and region of myofiber
170 (peripheral vs central) on CSA for each muscle were analyzed via three-way
171 ANOVA. Multiple comparisons and the interactions were evaluated using
172 Tukey's Post Hoc test. Chi-square test was performed to examine frequency
173 distribution of fiber types CSA following FR. Statistical analysis was conducted
174 by using Graph Pad prism 6 software. All values are presented as mean ± SEM,
175 *P* values less than 0.05 were considered significant.

176 **3. Results**

177 ***3. 1. Dietary restriction induces body weight and muscle mass specific*** 178 ***reduction***

179 In order to evaluate the effect of dietary restriction on skeletal muscle of *Mstn*^{+/+}
180 and *Mstn*^{-/-} mice for a five week period, average body weight measurements (g)
181 revealed that *Mstn*^{-/-}ND mice were significantly heavier compared to *Mstn*^{+/+}ND
182 mice (P<0.001). However both genotypes showed approximately 33%
183 reduction in the body mass following dietary restriction (Fig. 1a). Individual
184 muscle weight measurements showed that *Mstn*^{-/-}ND exhibited increases in
185 muscle mass of T.lateral (P<0.0001), T.long (P<0.0001), T.medial (P<0.05),
186 ECU (P<0.01) and FCU muscles (P<0.0001) compared to *Mstn*^{+/+}ND. To
187 evaluate whether dietary restriction impact on different muscles mass. Our data
188 showed that dietary restriction induced muscle specific weight loss e.g. T.lateral
189 showed large reduction in muscle mass for *Mstn*^{-/-}FR and *Mstn*^{+/+}FR (P<0.0001
190 and P<0.001) respectively compared to genotype-matched ND as well as
191 significant interaction (P<0.001) between diet and genotype (Fig.1b). Similarly,
192 T.long muscle exhibited more pronounced reduction in the *Mstn*^{-/-}FR and
193 *Mstn*^{+/+}FR (P<0.0001 and P<0.001) respectively compared to ND matched
194 control also with significant interaction (P<0.0001) between the effect of diet
195 and the genotype (Fig. 1c). Furthermore, ECU muscle showed more weight
196 reduction following FR in *Mstn*^{-/-} than *Mstn*^{+/+} (P<0.001 and P<0.05) compared
197 to genotype matched control (Fig.1e). On the other hand, T.medial and FCU
198 muscles displayed a diet induced decreases in the muscle mass only in *Mstn*^{-/-}
199 mice (P<0.01 and 0.0001) compared to genotype-matched control (Fig.1d and
200 f). Representative immunofluorescent staining for T.long muscle of *Mstn*^{+/+}ND
201 show MHC types IIA, IIB and I expressing myofibers compared to the negative
202 control (Fig. 1g-l).

203 **3. 2. Caloric restriction does not alter muscle fiber number**

204 Based on previous experiments (Elashry et al., 2009) we examined whether a
205 FR related muscle mass decrease was due to either loss in fiber number or loss
206 of fiber size. Quantification of the average fibers number revealed that
207 myostatin deletion increases muscle fiber number for T.lateral and T.long
208 (P<0.0001), ECU and FCU (P<0.01) muscles on normal diet could be detected.
209 In contrast, T.medial muscle displayed no fiber number change in *Mstn*^{-/-}.
210 However dietary restriction for five weeks caused no change in the fibers

211 number for all muscles of both genotypes (Fig. 2a, b, c, d and e).
212 Representatives whole muscle reconstruction showed clearly the effect of
213 *myostatin* deletion and dietary restriction on the cross section of the FCU
214 muscle of *Mstn*^{+/+} and *Mstn*^{-/-} compared to normal diet control (Fig. 2f-i).

215 **3. 3. Fast fibers are more prone to caloric restriction-induced muscle** 216 **mass loss**

217 Next we evaluated the impact of dietary restriction on individual muscle fiber
218 size via quantification of the CSA (μm^2) of IIB, IIX, IIA and type I fibers per
219 muscle. A common trend that dietary restriction reduced the CSA of glycolytic
220 type IIB fibers in *Mstn*^{-/-} and *Mstn*^{+/+} ($P < 0.0001$) compared to controls was
221 observed (Fig. 3). Type IIB fibers CSA measurement of T.lateral muscle
222 displayed pronounced reduction (2074 ± 7 to $1232 \pm 3 \mu\text{m}^2$) in *Mstn*^{-/-} ($P < 0.0001$)
223 compared to (1605 ± 4 to $1246 \pm 3 \mu\text{m}^2$) in *Mstn*^{+/+} ($P < 0.0001$) following FR.
224 These results were supported with significant interaction ($P < 0.0001$) indicating
225 the effect of FR on *Mstn*^{-/-} compared to *Mstn*^{+/+} (Fig. 3a). Furthermore, T.long
226 muscle showed similar reduction in type IIB fibers CSA (3596 ± 52 to 1304 ± 3
227 μm^2) and (3118 ± 8 to $1085 \pm 3 \mu\text{m}^2$) for *Mstn*^{-/-} and *Mstn*^{+/+} respectively
228 ($P < 0.0001$) following dietary restriction and compared to genotype-matched
229 control (Fig. 3b). On the other hand, measurements of type IIB fibers CSA
230 showed more loss in the T.medial and ECU muscles of *Mstn*^{-/-} ($P < 0.0001$)
231 compared to *Mstn*^{+/+} following FR. E.g. T.medial muscle of *Mstn*^{-/-} demonstrated
232 1647 ± 42 to $1090.6 \pm 3 \mu\text{m}^2$ CSA reduction ($P < 0.0001$) compared to 1479.5 ± 4
233 $1257 \pm 3 \mu\text{m}^2$, ($P < 0.0001$) reduction in *Mstn*^{+/+} provided with significant
234 interaction ($P < 0.0001$) between the effect of diet and genotype (Fig. 3c and d).
235 Next, type IIX fibers CSA measurement for T.lateral, ECU and FCU muscles
236 displayed significant decreases in the CSA for both *Mstn*^{-/-} ($P < 0.05$, 0.01 and
237 0.0001) and *Mstn*^{+/+} ($P < 0.01$ and 0.0001) following dietary restriction compared
238 to control (Fig. 3a, d and e) however IIX fibers measurements showed more
239 loss in T.long and T.medial muscles in the *Mstn*^{-/-} ($P < 0.0001$ and $P < 0.01$)
240 compared to *Mstn*^{+/+} following FR (Fig. 3b and c). Additionally, we examined
241 whether the effect of dietary limitation involves the oxidative fibers, analysis of
242 type IIA fibers CSA demonstrated reduction in the T.long and T.medial muscles

243 (P<0.0001 and 0.05) respectively only in the *Mstn*^{-/-} compared to *Mstn*^{+/+} under
244 the FR regime (Fig. 3b and c). Surprisingly, IIA fibers CSA for ECU and FCU
245 muscles showed significant increase in *Mstn*^{-/-}FR (P<0.01 and 0.0001)
246 compared to *Mstn*^{-/-}ND (Fig. 3d and e). Furthermore, we tested the oxidative
247 capacity of the muscles following dietary restriction by analyzing type I (slow
248 oxidative) fibers CSA, our results displayed large reduction in T.long muscle of
249 *Mstn*^{-/-} (P<0.0001) compared to *Mstn*^{+/+} (Fig. 3b). These data demonstrated that
250 glycolytic fibers were more liable to dietary restriction followed by IIX, IIA and I
251 oxidative fibers. Furthermore the impact of dietary restriction was more
252 pronounced in some of the *Mstn*^{-/-} muscles to implicate the oxidative fibers (IIA
253 and I) compared to *Mstn*^{+/+}. In order to confirm our data, T.long fibers CSA
254 measurements were categorized into small (0-1000 and 1000-2000 μm²),
255 medium (2000-4000 μm²) and large (4000-6000 μm²) for type IIB, small (0-1000
256 μm²), medium (1000-2000 μm²) and large fibers (2000-4000 μm²) for IIX, IIA
257 and type I fibers. Frequency distribution assessment of type IIB, IIX, IIA and I
258 fibers of T.long muscle showed significant CSA shift from large size into the
259 small size population (P<0.0001) following dietary restriction compared to
260 genotype-matched control (Fig. 3f, g, h and i).

261 **3. 4. Dietary restriction causes peripheral myofiber atrophy**

262 Our results show that dietary restriction primarily target fast MHCIIB fibers. To
263 address whether the anatomical location of the fiber had an effect on the
264 myofibers CSA following caloric restriction, we analyzed the CSA of the
265 peripheral (PF) and central (CT) fibers within the mid-belly of the corresponding
266 muscle. Our results revealed that in T.lateral, T.long and ECU muscles but not
267 T.medial muscle showed a common observation that type IIB myofibers CSA
268 were larger in the PF (P>0.0001) compared to CT region for *Mstn*^{-/-} and *Mstn*^{+/+}
269 on normal diet (Fig. 4a, c, f and i). Furthermore, FCU muscles demonstrated
270 larger CSA of PF IIB compared to CT fibers (P<0.0001) only in the *Mstn*^{-/-} on a
271 normal diet (Fig. 4l). Furthermore, FR induced significant reduction in the CSA
272 of PF IIB fibers (P<0.0001) compared to CT fibers e.g. T.lateral muscle showed
273 (850±87 vs. 1247±67 μm²) and (609±1 vs. 1348±2 μm²) for *Mstn*^{-/-} and *Mstn*^{+/+}
274 respectively also significant interaction was detected (P<0.0001) indicating the

275 interference of diet and genotype (Fig. 4a). Next we analyzed type IIX fibers,
276 larger IIX fibers CSA of both regions in T.lateral muscle of *Mstn*^{+/+} (P<0.0001)
277 and T.long muscle of *Mstn*^{-/-} (P<0.0001) on normal diet were observed. Also,
278 PF IIX fibers of *Mstn*^{-/-}FR showed more reduction in the CSA in T.lateral and
279 T.long muscles (P<0.05 and P<0.0001) compared to *Mstn*^{-/-}ND respectively
280 (Fig. 4b and d). However, reduction in the PF IIX fibers of both genotypes were
281 noticed in the T.medial (P<0.01), ECU and FCU muscles (P<0.0001) following
282 FR (Fig. 4g, j and m). Moreover, CT type IIX fibers displayed reduction in T.long
283 and T.medial muscles of *Mstn*^{-/-} (P<0.0001) as well as T.lateral muscle of
284 *Mstn*^{+/+} (P<0.0001) following FR. Additionally, PF type IIX fiber CSA
285 demonstrated further reduction in T.medial (P<0.001 and P<0.0001) and ECU
286 muscle (P<0.0001) of both genotypes compared to CT fibers (Fig. 4 g and j)
287 following FR. On the other hand, more reduction in the PF IIX fibers was
288 detected in T.lateral and FCU muscles of only *Mstn*^{-/-} (P<0.0001) compared to
289 CT fibers following FR (Fig. 4b and m), also significant interaction (P<0.01)
290 indicating the effect of diet on *Mstn*^{-/-}. In contrast, analysis of type IIA fibers
291 showed some variations, PF IIA fibers of T.medial muscle displayed more
292 reduction in the CSA (P<0.01) compared to CT fibers only in *Mstn*^{+/+} (Fig. 4h)
293 however PF fibers of ECU muscle showed more CSA reduction compared to
294 CT fibers (P<0.0001 and P<0.001) for *Mstn*^{-/-} and *Mstn*^{+/+} respectively following
295 dietary restriction (Fig. 4k). However, FCU muscle displayed larger CSA
296 reduction in PF IIA fibers only in the *Mstn*^{-/-} compared to CT fibers after dietary
297 restriction (Fig. 4n). Type I fibers was only localized in the central part of the
298 muscle therefore were excluded from this comparison.

299 **3. 5. Caloric restriction induces a slow phenotype conversion in a muscle-** 300 **specific manner**

301 Our previous study demonstrated that *myostatin* deletion induced various
302 degrees of slow to fast fiber type shift in the forelimb muscles (Elashry et al.,
303 2009). Therefore, we examined whether dietary restriction alters the muscle
304 phenotype of the *Mstn*^{-/-} compared to *Mstn*^{+/+}. Quantification of the average
305 percentage of each fiber type population per muscle demonstrated percentage
306 increases of type IIB fibers in the T.lateral, T.long, T.medial, ECU and FCU

307 muscles *Mstn*^{-/-}ND (P<0.0001) compared to *Mstn*^{+/+}ND. However FR altered
308 the fiber type composition for both genotypes (P<0.001) e.g. T.lateral muscle
309 demonstrated a reduction in the type IIB fibers (P<0.0001 and P<0.05) and
310 increases of type IIX fibers (P<0.0001 and P<0.05) for *Mstn*^{-/-}FR and *Mstn*^{+/+}FR
311 respectively compared to genotype matched control (Fig. 5a and b). Also FR
312 induced an increase in the type IIA fibers of *Mstn*^{-/-}FR compared to ND, however
313 *Mstn*^{+/+}FR showed a reduction in type IIA fibers compared to ND. These data
314 were confirmed by significant interaction between both genotypes (P<0.01, Fig.
315 5c). Furthermore, T.long muscle showed a reduction in type IIB fibers
316 (P<0.0001) with an increase in type IIX fibers (P<0.001 and P<0.0001) for *Mstn*^{-/-}
317 ⁻FR and *Mstn*^{+/+}FR respectively compared to genotype-matched ND (Fig. 5d
318 and e). Although there were no significant changes, *Mstn*^{-/-}FR demonstrated
319 marked increases in type IIA fibers compared to *Mstn*^{-/-}ND. However *Mstn*^{+/+}FR
320 showed significant increase in type IIA fibers (P<0.01) compared to *Mstn*^{+/+}ND
321 (Fig. 5f) as well as FR resulted in marked increase in type I fibers in both
322 genotypes (P<0.01) compared to ND control (Fig. 5g). On the other hand,
323 T.medial muscle analysis revealed fiber type population of 33%, 34%, 26% and
324 7% for IIB, IIX, IIA and I fibers respectively in the *Mstn*^{+/+}ND and showed almost
325 no fiber type shift following dietary restriction in the *Mstn*^{-/-} however *Mstn*^{+/+} FR
326 displayed fiber shift into type I fibers (IIB→IIX→IIA→I) following the same
327 treatment (P<0.05) compared to ND. These data was supported by significant
328 interaction (P<0.05) between diet and genotype effect (Fig. 5h, i and j and k).
329 On the other hand, ECU muscle displayed a different fiber shift pattern with
330 dietary restriction whereas IIB to IIX fibers transformation in the *Mstn*^{-/-}
331 (P<0.0001 and P<0.01) respectively (Fig. 5l and m) and exhibited type IIA to
332 type I fiber shift in *Mstn*^{+/+} (P<0.001) compared to ND (Fig. 5n and o). Similarly,
333 FCU muscle FR induced type IIB to IIX fiber shift for *Mstn*^{-/-} (P<0.0001)
334 compared to ND (Fig. 5p and q) however demonstrated fibers shift from IIB
335 directly into IIA fibers but not type IIX fibers in *Mstn*^{+/+} (P<0.0001 and P<0.05)
336 compared to *Mstn*^{+/+}ND (Fig. 5r and s). Representative double
337 immunofluorescent images of T.long muscle for *Mstn*^{+/+} and *Mstn*^{-/-} showed the
338 transformation of the glycolytic fibers type IIB and IIX into the oxidative type IIA
339 fibers following dietary restriction. A negative control without addition of primary

340 antibodies was performed on a consecutive muscle section (Fig. 5t, u, v, w and
341 x).

342 **4. Discussion**

343 Dietary intake plays a critical role to maintain the physiological composition of
344 the skeletal muscle. Previous studies revealed that *myostatin* deletion results
345 in an increase in muscle mass due to an increase in fiber number and fiber size
346 (McPherron et al., 1997). However, the impact of dietary restriction on
347 anatomically and structurally different forelimb muscles of the *myostatin* null are
348 not fully characterized. Our results suggest that the impact of dietary restriction
349 was greater in the *Mstn*^{-/-} than in wild-type muscle. In agreement with our
350 results, previous work showed that dietary limitation caused more muscle loss
351 in the hind limb muscles of the *Mstn*^{-/-} compared to *Mstn*^{+/+} suggesting higher
352 catabolic activities (Matsakas et al., 2013). These results suggest that the
353 hypertrophic muscle of *Mstn*^{-/-} is more prone to protein breakdown than that of
354 wild-types. In support of this notion is the finding that *Mstn*^{-/-} mice exhibited
355 more muscle mass loss during hind limb unloading (McMahon et al., 2003). Our
356 results indicate that muscle size (enlarged) and composition (fast) might play a
357 role in the degree of muscle loss following dietary limitation. This in line with
358 previous reports indicating that fast twitch IIB fibers of rodents and monkeys
359 are more sensitive to starvation compared to slow twitch fiber (Goldspink and
360 Ward, 1979; Li and Goldberg, 1976; Matsakas et al., 2013; McKiernan et al.,
361 2012). However we show that there is also a regional specificity of MHC types
362 in terms of atrophy.

363 We propose that the dietary restriction induced muscle loss is regulated via
364 inhibition of IGF-1 signaling which results in upregulation of atrogen-1 and
365 MuRF-1 expression. A similar study showed that undernutrition for 28 days
366 causes reduced growth rate via regulation of IGF-1 and myostatin expressions
367 (Jeanplong et al., 2015). We shows that the oxidative fibers are resistant to
368 dietary restriction. In agreement with our results, it has been shown that the
369 atrophy resistance of the oxidative fibers was due to a protection afforded by
370 peroxisome proliferator-activated receptor gamma coactivator-1 alpha
371 (PGC1 α) (Wang and Pessin, 2013). Unexpectedly, dietary restriction causes

372 CSA increases in type IIA fibers for ECU and FCU of *Mstn*^{-/-} which might be an
373 adaptive mechanism to compensate the decrease in glycolytic fibers number.

374 Analysis of the muscles phenotypes revealed a general conversion toward slow
375 oxidative phenotype following dietary restriction. However, we observed only
376 the degree and the mechanism of fiber shift was dependent on the original fiber
377 type population e.g. T.long and T.lateral muscles displayed IIB→IIX→IIA in the
378 *Mstn*^{-/-} however only showed a IIB→IIX fiber type conversion in *Mstn*^{+/+}.
379 Moreover, ECU and FCU muscles showed a IIB→IIX fiber shift in *Mstn*^{-/-}
380 however demonstrated a IIA→I and IIB→IIA shift respectively in *Mstn*^{+/+}. These
381 data indicate that myostatin deletion and dietary limitation interactions alter
382 muscle adaptation and plasticity. Muscle oxidative conversion was previously
383 reported with ageing, although there was trophic activity in type II fibers,
384 additionally there was a relative increase in type I fibers (Kallman et al., 1990).
385 Furthermore, a recent study in muscular dystrophy showed that muscle atrophy
386 was accompanied with a myofiber oxidative shift perhaps due to an increase in
387 the expression of PGC1 α (Rocchi et al., 2016). On the other hand, soleus
388 muscle immobilization, hind limb unloading and spinal cord transection induced
389 muscle atrophy plus type I into type II fibers transformation see review by (Cho
390 et al., 2016). However in our model the physical performance and muscle
391 function were preserved compared to previous models which resulted in
392 improved phenotype particularly in *Mstn*^{-/-}. It could be explain that muscle
393 adaptability is dependent on the extend of the external stimuli therefore the high
394 demand for protein supplement in the *Mstn*^{-/-} induced fiber atrophy and fiber
395 type transformation. This is far greater compared to *Mstn*^{+/+} such a change in
396 muscle metabolism energy saving mechanisms are required.

397 Our data discussed the impact of caloric restriction in combination with
398 *myostatin* deletion on five muscles from different anatomical locations. We
399 provide evidence that 60% of dietary limitation results in body weight and
400 muscle specific mass reduction with fast myofibers more prone to atrophy
401 compared to slow myofibers. The effect of dietary restriction was more
402 pronounced in some of the *Mstn*^{-/-} muscles to implicate the oxidative fibers
403 compared to *Mstn*^{+/+}mice. Furthermore, peripheral myofibers are more

404 susceptible to dietary induced reduction compared to deep fibers. We provide
405 evidence that dietary restriction alters the fast phenotype of the *Mstn*^{-/-} into the
406 slower form in a muscle dependent manner. These results allow the conclusion
407 that caloric reduction alters the muscle fiber composition and the oxidative
408 pattern to compensate muscle mass loss which might impact on muscle
409 function particularly in the hypermuscular *myostatin* null animals.

410 **Competing interest**

411 All the authors have declared no conflict of interest regarding the publication of
412 this paper.

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528

529 **Figure legends**

530 **Figure 1 Dietary restriction induces body weight and muscle mass**
531 **specific reduction.**

532 **a** Average body weight (g) for normal diet (ND) and food restricted (FR) *Mstn*^{+/+}
533 and *Mstn*^{-/-} mice (N=4 for all groups). Significant increase in the *Mstn*^{-/-}
534 ($P<0.001$) compared to the *Mstn*^{+/+} on normal diet. FR decreases the body
535 weight for *Mstn*^{+/+} and *Mstn*^{-/-} compared to genotype-matched ND mice. **b-f**
536 Average muscle weight (g) for **b** T.lateral, **c** T.long, **d** T.medial, **e** ECU, and **f**
537 FCU muscles. Specific muscle mass increases in *Mstn*^{-/-} ND compared to
538 *Mstn*^{+/+} ND. FR induces more muscle mass reduction in T.lateral and T.long
539 muscles of *Mstn*^{-/-} (diet and genotype interaction $P<001$ and 0.0001)
540 respectively compared to genotype matched control. *Mstn*^{-/-} display more
541 muscle loss in T.medial, ECU and FCU muscles compared to *Mstn*^{+/+} following
542 FR. **g-l** Immunofluorescent images for T.long muscle of *Mstn*^{+/+}ND against
543 A.474, BFF3 and A.4840 specific MHC antibodies show **g** type IIA⁺ (green), **h**
544 type IIB⁺ (red) and **i** type I⁺ fibers (green) compared to **j**, **k** and **l** the
545 corresponding negative control (NC) respectively. DAPI was used to visualize
546 the myonuclei. All values displayed as mean \pm SEM. * = $P< 0.05$, ** = $P< 0.01$,
547 *** = $P< 0.001$ and ****= $P<0.0001$). Scale bar in **g-l** =20 μ m.

548 **Figure 2 Caloric restriction does not alter muscle fiber number.**

549 **a-e** Average total myofiber number for **a** T.lateral, **b** T.long, **c** T.medial, **d** ECU,
550 and **e** FCU muscles of ND and FR *Mstn*^{+/+} and *Mstn*^{-/-} mice. The muscles of
551 *Mstn*^{-/-} show increases in the myofiber number compared to *Mstn*^{+/+}. T.medial
552 muscle display no fiber number change in both genotypes. FR reveals no fiber
553 number loss compared to ND mice. **f-i** Reconstructive immunolabelled images
554 for FCU muscle of *Mstn*^{+/+} and *Mstn*^{-/-} ND and FR against MHCIIIB (red), MHCIIA
555 (green), MHCIIIX (unstained). *Myostatin* deletion increases the total CSA of the
556 muscle compare to wild type (**f** vs **g**). FR reduces the CSA of *Mstn*^{+/+} (**f** vs. **h**)
557 and *Mstn*^{-/-} (**g** vs. **i**). All values displayed as mean \pm SEM. *= $P< 0.05$, **= $P<$
558 0.01 and ****= $P<0.0001$).

559 **Figure 3 Fast fibers are more prone to caloric restriction-induced muscle**
560 **mass loss.**

561 **a-e** Average CSA measurements (μm^2) for type IIB, IIX, IIA and type I fibers of
562 **a** T.lateral, **b** T.long, **c** T.medial, **d** ECU and **e** FCU muscles of *Mstn*^{+/+} and
563 *Mstn*^{-/-} ND and FR mice. FR causes significant reduction in the CSA of type IIB
564 fibers in T.lateral, T.medial and ECU muscles of *Mstn*^{-/-} compared to *Mstn*^{+/+}.
565 FR induces significant type IIX reduction in T.medial muscle of only *Mstn*^{-/-},
566 T.lateral muscle of only *Mstn*^{+/+} and in T.long, ECU and FCU muscles for both
567 genotypes compared to ND. FR causes reduction in CSA of IIA fibers of T.long
568 and T.medial muscles of *Mstn*^{-/-} however increases the CSA of ECU and FCU
569 muscles. FR reveals no change in type IIA fibers for all *Mstn*^{+/+} muscles. FR
570 induces only significant reduction in type I fibers of T.long muscle but not
571 significant (NS) in T.medial muscle. **f-i** T.long muscle frequency distribution of
572 fiber CSA measurements (μm^2) for **f** type IIB, **g** type IIX, **h** type IIA and **i** type I
573 fibers for of *Mstn*^{+/+} and *Mstn*^{-/-} ND and FR mice. FR induces fibers shift into
574 small size population (arrow marked) compared to ND for both genotypes. All
575 values displayed as mean \pm SEM. *=*P*< 0.05, ** = *P*< 0.01 and ****=*P*<0.0001.

576 **Figure 4 Dietary restriction causes peripheral myofiber atrophy.**

577 **a-n** Average CSA (μm^2) measurements for peripheral (PF) and central fibers
578 (CT) type IIB, IIX and IIA fibers for the muscles T.lateral **a, b**; T.long **c, d, e**;
579 T.medial **f, g, h**; ECU **i, j, k** and FCU **l, m, n** of ND and FR *Mstn*^{+/+} and *Mstn*^{-/-}
580 mice. For all muscle except T.medial, PF IIB fibers show CSA increases in *Mstn*^{-/-}
581 *Mstn*^{+/+} ND compared to CT fibers. PF IIB fibers of T.lateral, ECU and
582 FCU muscles display smaller CSA compared to CT fibers following dietary
583 restriction (**a, c, f, i** and **l**). FR reduces the CSA of PF IIX fibers compared to
584 CT fibers for *Mstn*^{-/-} and *Mstn*^{+/+} (**b, d, g, j** and **m**). FR show larger CSA in the
585 CT IIA fibers of ECU and FCU muscles of *Mstn*^{-/-} (**k** and **n**). All values displayed
586 as mean \pm SEM. *=*P*< 0.05, **=*P*< 0.01, ***=*P*< 0.001 and ****=*P*<0.0001.

587 **Figure 5 Caloric restriction induces a slow phenotype conversion in a**
588 **muscle-specific manner.**

589 Average percentages of type IIB, IIX, IIA, and I fibers for T.lateral **a, b, c**; T.long
590 **d, e, f, g**; T.medial **h, i, j, k**; ECU **l, m, n, o**; and FCU muscles **p, q, r, s** of ND
591 and FR *Mstn*^{+/+} and *Mstn*^{-/-} mice. *Mstn*^{-/-} ND show increases in type IIB and IIX
592 fibers compared to *Mstn*^{+/+}ND for all muscles. FR induces an increase in type
593 IIX and type IIA fibers of T.lateral muscle in *Mstn*^{-/-} and only type IIX increases
594 in *Mstn*^{+/+}. T.long muscle exhibits type IIB to IIA fiber shift and IIB to IIX fiber
595 shift for *Mstn*^{+/+} and *Mstn*^{-/-} following FR respectively. FR causes an increase in
596 type I fibers for T.medial muscle of *Mstn*^{+/+}. ECU muscles show IIB to IIX fiber
597 type shift for *Mstn*^{-/-} compared to IIA to I fibers for *Mstn*^{+/+} following FR. FCU
598 muscle displays IIB to IIA fiber shift for *Mstn*^{+/+} following FR. **t-x** Representative
599 double immunofluorescent images of mid-belly section for T.long muscle in ND
600 and FR *Mstn*^{+/+} and *Mstn*^{-/-} mice. FR induces myofiber transformation from
601 glycolytic type IIB fibers (red) into oxidative type IIA. Type IIX fibers remained
602 unstained (black). A consecutive muscle section with only secondary antibodies
603 and DAPI was used as a negative control (NC). Scale bar =100 μm. All values
604 displayed as mean± SEM.

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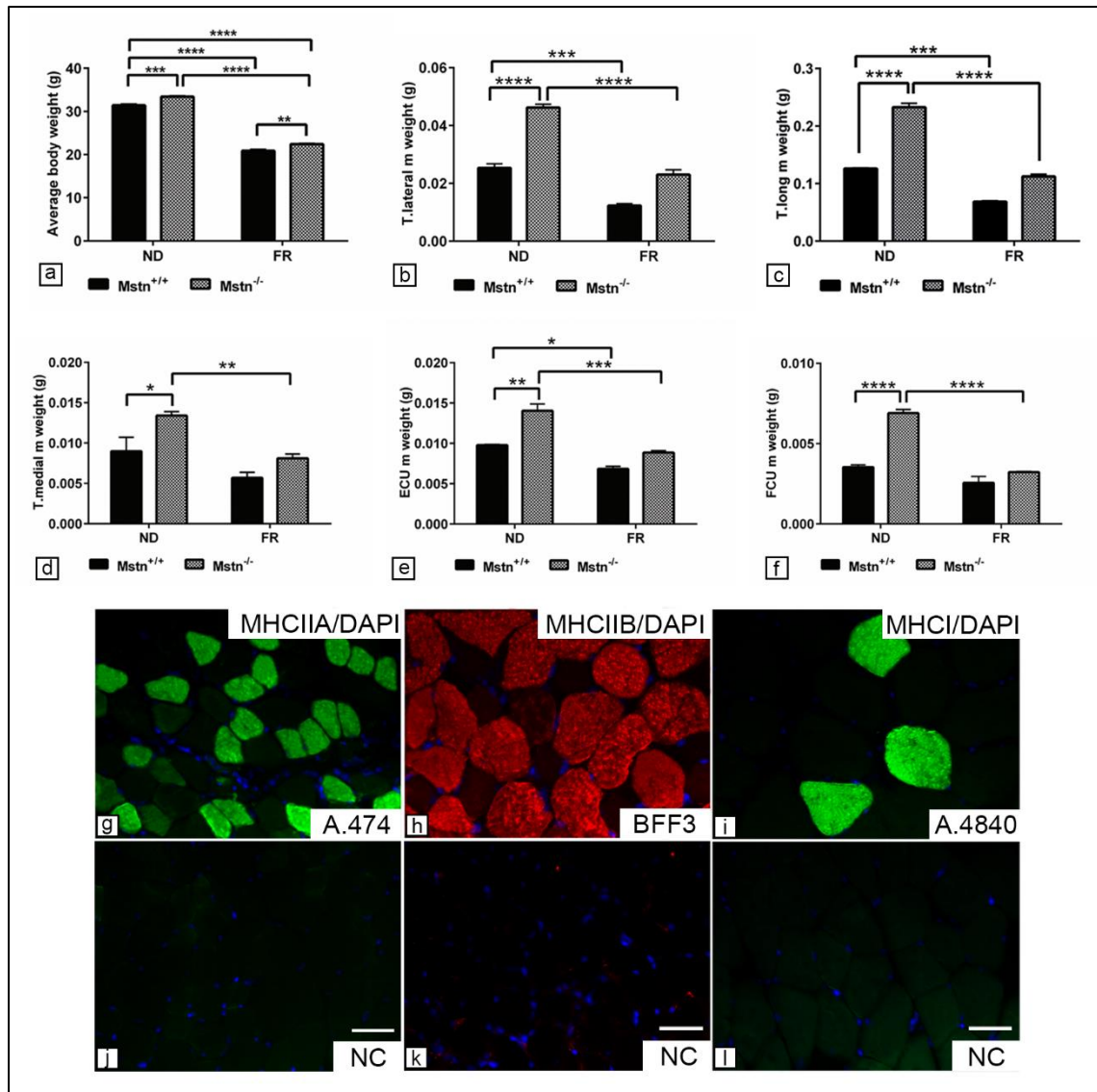
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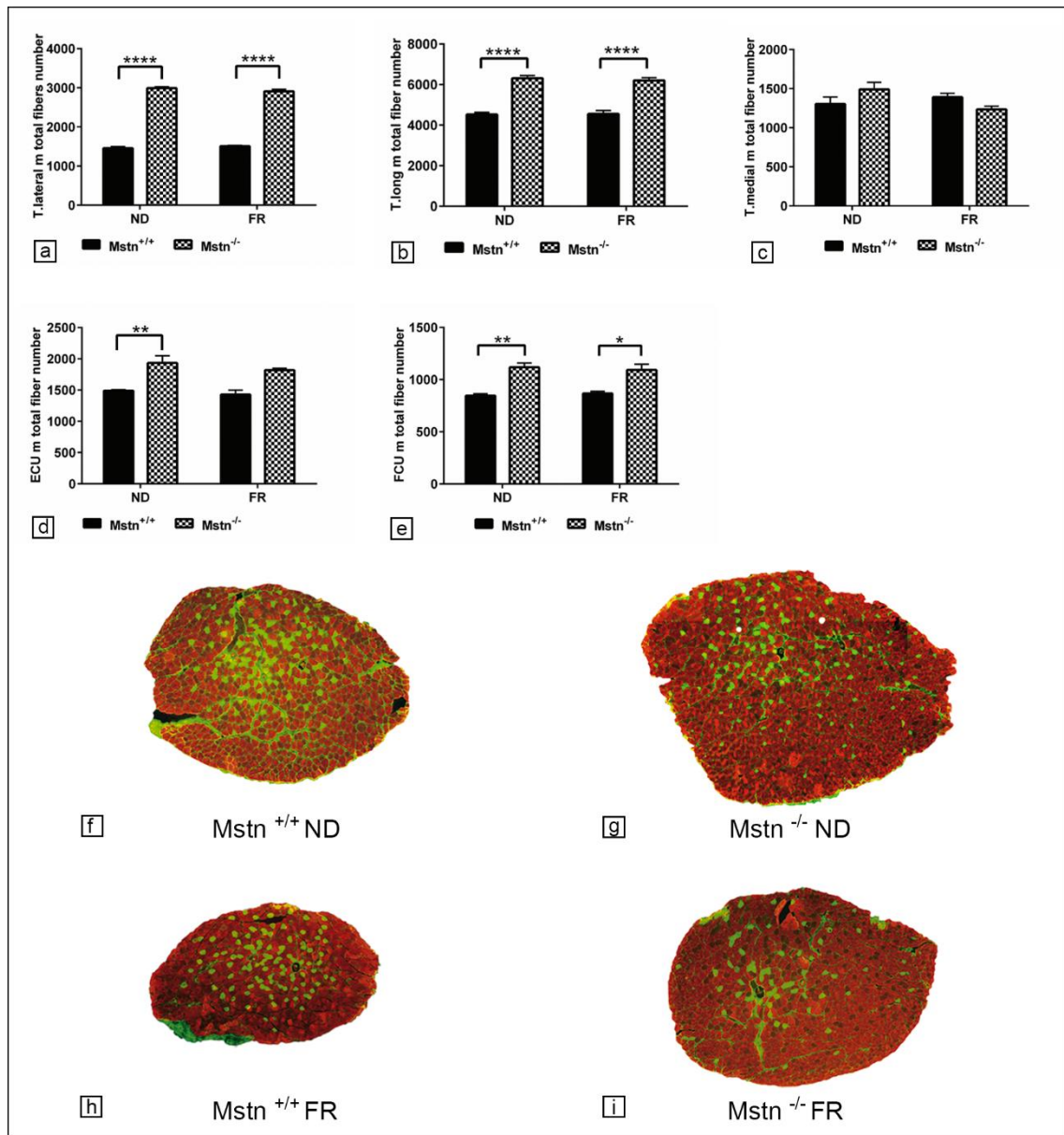
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617 **Figure 1 Dietary restriction induces body weight and muscle mass**
 618 **specific reduction.**

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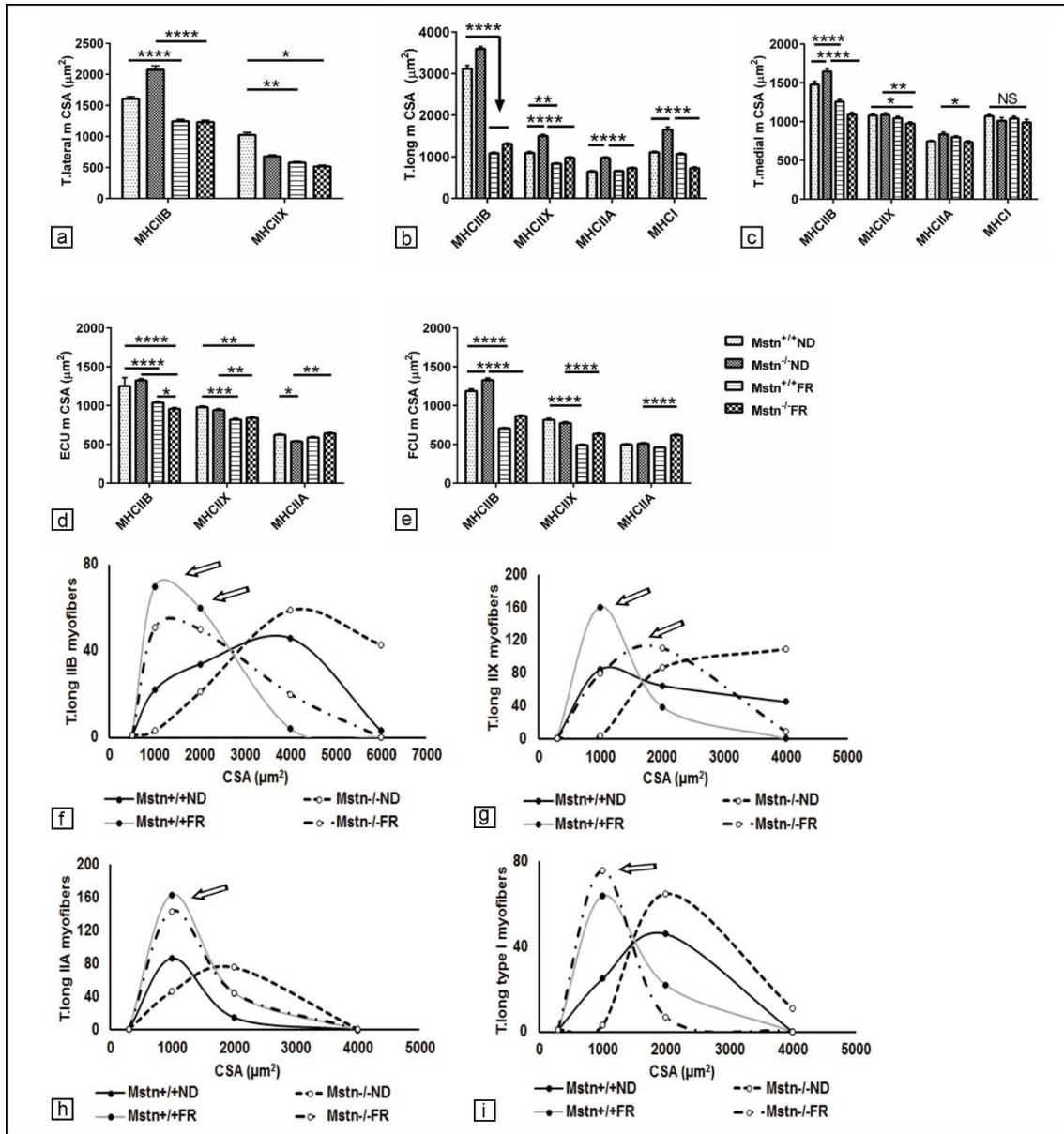
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623 **Figure 2 Caloric restriction does not alter muscle fiber number.**

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626 **Figure 3 Fast fibers are more prone to caloric restriction-induced muscle**
 627 **mass loss.**

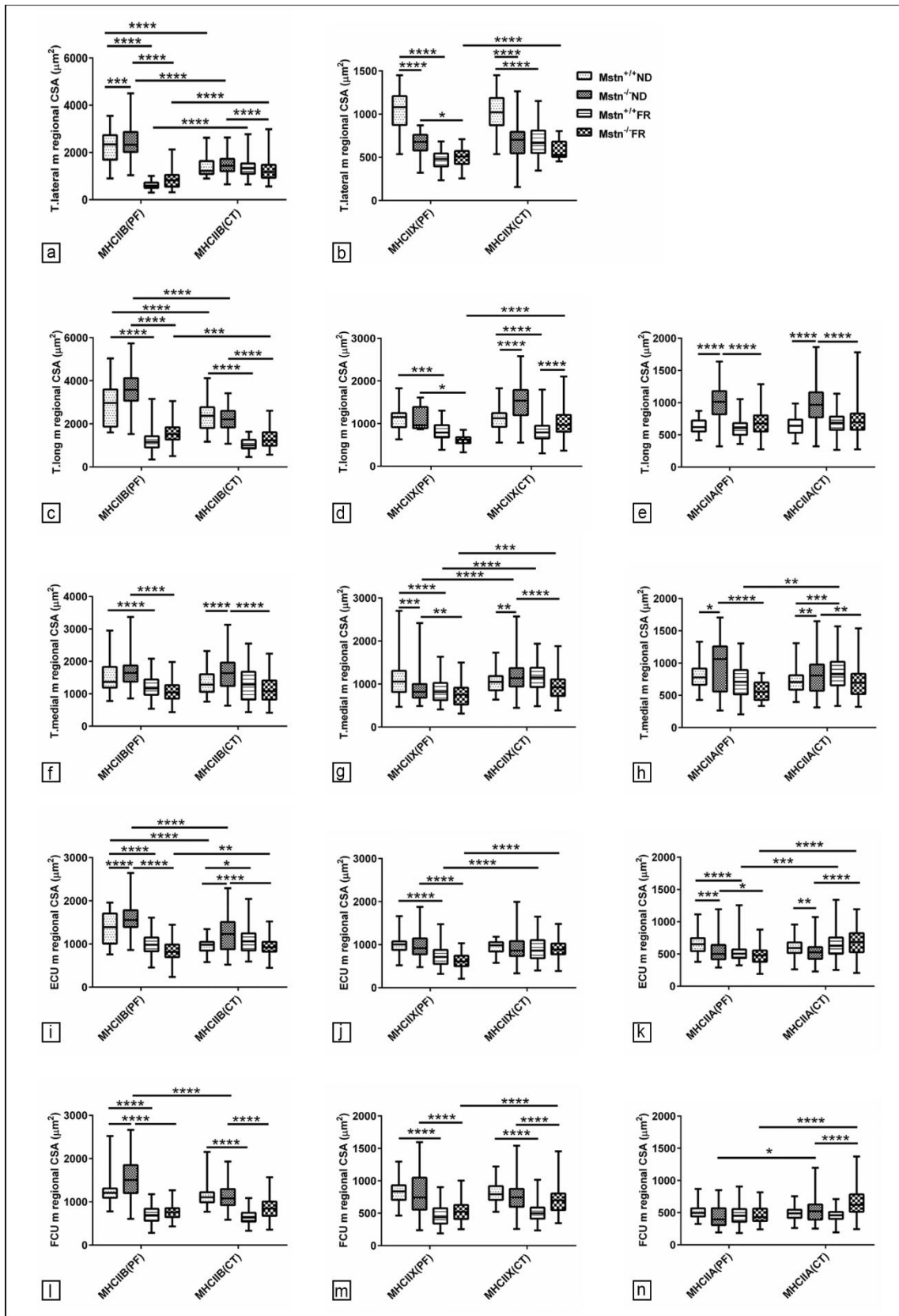
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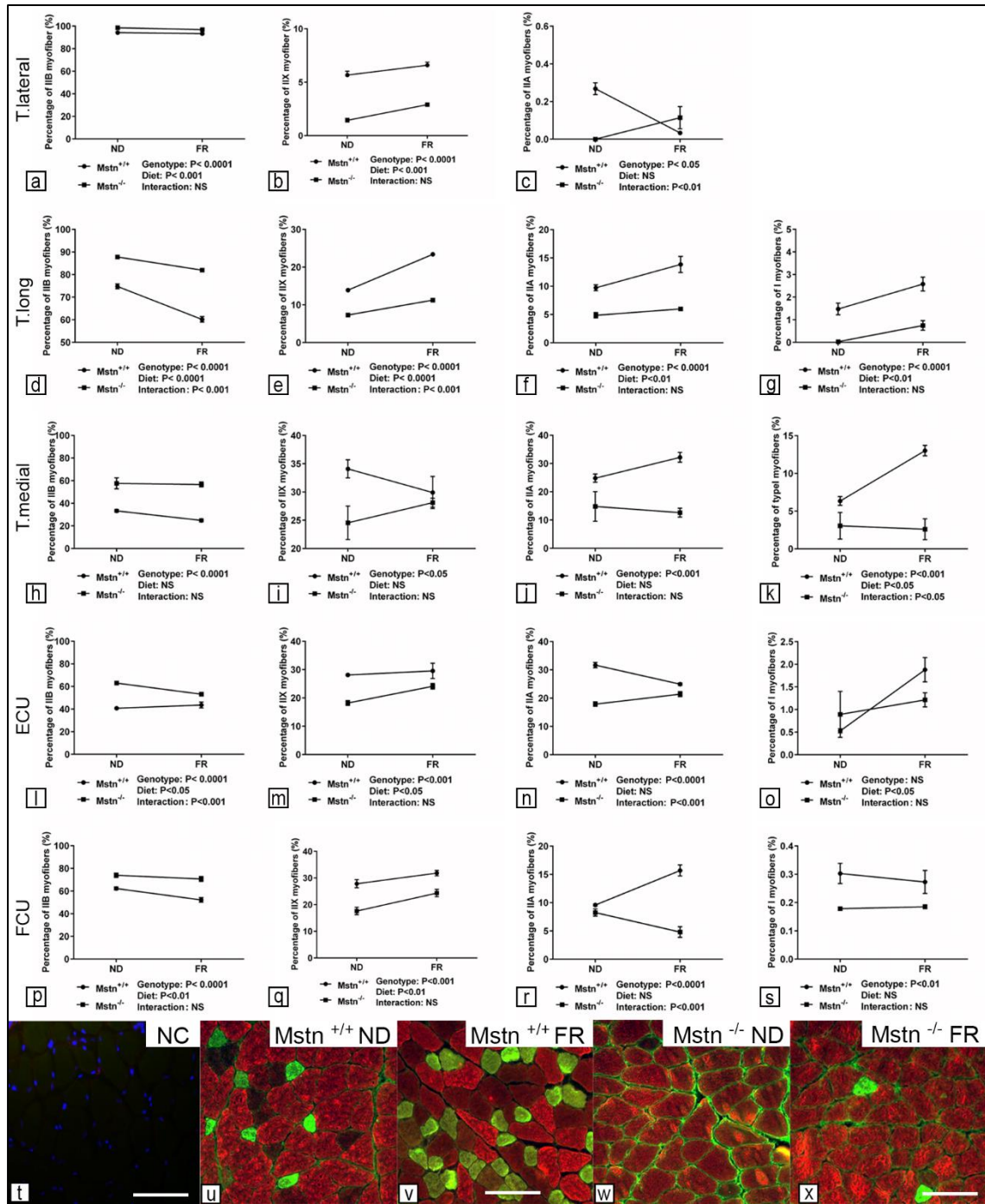
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635 **Figure 4 Dietary restriction causes peripheral myofiber atrophy.**



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638 **Figure 5** Caloric restriction induces a slow phenotype conversion in a
 639 muscle-specific manner.

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