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

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Abstract

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

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

1. Introduction

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

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

2. Materials and Methods

2. 1. Animals

Three months old male transgenic myostatin knockout (*Mstn*$^{-/-}$) and C57/BL6 wild type littermates (*Mstn*$^{+/+}$) mice were bred in the biological resource unit, Reading University, UK. *Mstn*$^{-/-}$ mice were generously provided from Dr Lee (McPherron et al., 1997). All experimental procedures and animal use were performed according to the standard ethics and guides for the Care and Use of Laboratory Animals of the Institute for Research. The mice were divided into 4 subgroups: a normal diet group (N=8) *Mstn*$^{+/+}$ and *Mstn*$^{-/-}$ kept with a normal

diet with food and water provided *ad libitum* and a food restriction group (N=8) with an up to 60% reduction of the daily diet compared to normal mice for a 5 week period.

2. 2. Tissue isolation and processing

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

2. 3. Immunohistochemical staining

Frozen muscle sections were washed in PBS for 10 minutes, permeabilized using buffer solution containing 20 mM Hepes, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$ and 0.5% Triton-X100 (pH7) at for 15 minutes. Non-specific binding was blocked using wash buffer (5% fetal calf serum (v/v) and 0.05% Triton X-100 (v/v) in PBS) for 30 minutes at room temperature before the addition of antibodies. Myosin heavy chain (MHC) type I, IIA and IIB expressing myofibers were identified by incubating the slides with A4.840 mouse IgM (1:1), A.474 mouse IgG (1:4) and BFF3 mouse IgM (1:1) monoclonal primary antibodies (DSHB) respectively as previously described (Matsakas et al., 2009). MHC type IIA^+ and IIB^+ myofibers were stained together and MHC type I^+ myofibers were stained on consecutive section. All primary antibodies were pre-blocked in washing buffer 30 minutes prior to use and incubated on samples overnight at 4°C. Primary antibodies were highlighted via incubation with Alexa Fluor 633 goat anti-mouse IgM (Molecular Probes A21046, 1:200) for MHC I and MHC IIB and Alexa Fluor 488 Goat-anti-mouse IgG (Molecular probes A11029, 1:200) for MHC IIA secondary antibodies in dark at room

temperature for 45 minutes. Slides were mounted using a fluorescent mounting medium (Mowiol 4-88, Calbiochem) containing 2.5 μg/ml DAPI.

2. 4. Imaging and quantifications

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

2. 5. Statistical analysis

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

3. Results

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

In order to evaluate the effect of dietary restriction on skeletal muscle of $Mstn^{+/+}$ and $Mstn^{-/-}$ mice for a five week period, average body weight measurements (g) revealed that $Mstn^{-/-}$ND mice were significantly heavier compared to $Mstn^{+/+}$ND mice ($P<0.001$). However both genotypes showed approximately 33%

reduction in the body mass following dietary restriction (Fig. 1a). Individual muscle weight measurements showed that *Mstn*$^{-/-}$ND exhibited increases in muscle mass of T.lateral (P<0.0001), T.long (P<0.0001), T.medial (P<0.05), ECU (P<0.01) and FCU muscles (P<0.0001) compared to *Mstn*$^{+/+}$ND. To evaluate whether dietary restriction impact on different muscles mass. Our data showed that dietary restriction induced muscle specific weight loss e.g. T.lateral showed large reduction in muscle mass for *Mstn*$^{-/-}$FR and *Mstn*$^{+/+}$FR (P<0.0001 and P<0.001) respectively compared to genotype-matched ND as well as significant interaction (P<0.001) between diet and genotype (Fig.1b). Similarly, T.long muscle exhibited more pronounced reduction in the *Mstn*$^{-/-}$FR and *Mstn*$^{+/+}$FR (P<0.0001 and P<0.001) respectively compared to ND matched control also with significant interaction (P<0.0001) between the effect of diet and the genotype (Fig. 1c). Furthermore, ECU muscle showed more weight reduction following FR in *Mstn*$^{-/-}$ than *Mstn*$^{+/+}$ (P<0.001 and P<0.05) compared to genotype matched control (Fig.1e). On the other hand, T.medial and FCU muscles displayed a diet induced decreases in the muscle mass only in *Mstn*$^{-/-}$ mice (P<0.01 and 0.0001) compared to genotype-matched control (Fig.1d and f).

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

Based on previous experiments (Elashry et al., 2009) we examined whether a FR related muscle mass decrease was due to either loss in fiber number or loss of fiber size. Quantification of the average fibers number revealed that myostatin deletion increases muscle fiber number for T.lateral and T.long (P<0.0001), ECU and FCU (P<0.01) muscles on normal diet could be detected. In contrast, T.medial muscle displayed no fiber number change in *Mstn*$^{-/-}$. However dietary restriction for five weeks caused no change in the fibers number for all muscles of both genotypes (Fig. 2a, b, c, d and e). Representatives whole muscle reconstruction showed clearly the effect of *myostatin* deletion and dietary restriction on the cross section of the FCU muscle of *Mstn*$^{+/+}$ and *Mstn*$^{-/-}$ compared to normal diet control (Fig. 2f-i).

3. 3. 3. Glycolytic fibers are more prone to caloric restriction-induced muscle mass loss

Next we evaluated the impact of dietary restriction on individual muscle fiber size via quantification of the CSA (μm^2) of IIB, IIX, IIA and type I fibers per muscle. A common trend that dietary restriction reduced the CSA of glycolytic type IIB fibers in *Mstn*$^{-/-}$ and *Mstn*$^{+/+}$ (P<0.0001) compared to controls was observed (Fig. 3). Type IIB fibers CSA measurement of T.lateral muscle displayed pronounced reduction (2074±7 to 1232±3 μm^2) in *Mstn*$^{-/-}$ (P<0.0001) compared to (1605±4 to 1246±3 μm^2) in *Mstn*$^{+/+}$ (P<0.0001) following FR. These results were supported with significant interaction (P<0.0001) indicating the effect of FR on *Mstn*$^{-/-}$ compared to *Mstn*$^{+/+}$ (Fig. 3a). Furthermore, T.long muscle showed similar reduction in type IIB fibers CSA (3596±52 to 1304±3 μm^2) and (3118±8 to 1085±3 μm^2) for *Mstn*$^{-/-}$ and *Mstn*$^{+/+}$ respectively (P<0.0001) following dietary restriction and compared to genotype-matched control (Fig. 3b). On the other hand, measurements of type IIB fibers CSA showed more loss in the glycolytic fibers of T.medial and ECU muscles (P<0.0001) compared to *Mstn*$^{+/+}$ following FR. E.g. T.medial muscle of *Mstn*$^{-/-}$ demonstrated 1647±42 to 1090.6±3 μm^2 CSA reduction (P<0.0001) compared to 1479.5±4 to 1257±3 μm^2, (P<0.0001) reduction in *Mstn*$^{+/+}$ provided with significant interaction (P<0.0001) between the effect of diet and genotype (Fig. 3c and d). Next, type IIX fibers CSA measurement for T.lateral, ECU and FCU muscles displayed significant decreases in the CSA for both *Mstn*$^{-/-}$ (P<0.05, 0.01 and 0.0001) and *Mstn*$^{+/+}$ (P<0.01 and 0.0001) following dietary restriction compared to control (Fig. 3a, d and e) however IIX fibers measurements showed more loss in T.long and T.medial muscles in the *Mstn*$^{-/-}$ (P<0.0001 and P<0.01) compared to *Mstn*$^{+/+}$ following FR (Fig. 3b and c). Additionally, we examined whether the effect of dietary limitation involves the oxidative fibers, analysis of type IIA fibers CSA demonstrated reduction in the T.long and T.medial muscles (P<0.0001 and 0.05) respectively only in the *Mstn*$^{-/-}$ compared to *Mstn*$^{+/+}$ under the FR regime (Fig. 3b and c). Surprisingly, IIA fiber CSA for ECU and FCU muscles showed significant increase in *Mstn*$^{-/-}$FR (P<0.01 and 0.0001) compared to *Mstn*$^{-/-}$ND (Fig. 3d and e). Furthermore, we

tested the oxidative capacity of the muscles following dietary restriction by analyzing type I (slow oxidative) fibers CSA, our results displayed large reduction in T.long muscle of *Mstn*$^{-/-}$ ($P<0.0001$) compared to *Mstn*$^{+/+}$ (Fig. 3b). These data demonstrated that glycolytic fibers were more liable to dietary restriction followed by IIX, IIA and I oxidative fibers. Furthermore the impact of dietary restriction was more pronounced in some of the *Mstn*$^{-/-}$ muscles to implicate the oxidative fibers (IIA and I) compared to *Mstn*$^{+/+}$. In order to confirm our data, T.long fibers CSA measurements were categorized into small (0-2000 μm^2), medium (2000-4000 μm^2) and large (4000-6000 μm^2) for type IIB, small (0-1000 μm^2), medium (1000-2000 μm^2) and large fibers (2000-4000 μm^2) for IIX, IIA and type I fibers. Frequency distribution assessment of IIB, IIX and IIA fibers of T.long muscle showed marked CSA shift from large size into the small size population following dietary restriction compared to genotype-matched control (Fig. 3f, g and h). Furthermore, Type I fibers of *Mstn*$^{-/-}$ displayed a larger shift into small CSA population ($P<0.0001$) compared to *Mstn*$^{+/+}$ following dietary restriction (Fig. 3i).

3. 4. Dietary restriction causes peripheral myofiber atrophy

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

fibers, larger IIX fibers CSA of both regions in T.lateral muscle of *Mstn*$^{+/+}$ (P<0.0001) and T.long muscle of *Mstn*$^{-/-}$ (P<0.0001) on normal diet. Also, PF IIX fibers of *Mstn*$^{-/-}$FR showed more reduction in the CSA in T.lateral and T.long muscles (P<0.05 and P<0.0001) compared to *Mstn*$^{-/-}$ND respectively (Fig. 4b and d). However, reduction in the PF IIX fibers of both genotypes were noticed in the T.medial (P<0.01), ECU and FCU muscles (P<0.0001) following FR (Fig. 4g, j and m). Moreover, CT type IIX fibers displayed similar reduction in (T.long and T.medial muscles) of *Mstn*$^{-/-}$ (P<0.0001) as well as T.lateral muscle of *Mstn*$^{+/+}$ (P<0.0001) following FR. Additionally, PF type IIX fiber CSA demonstrated further reduction in T.medial (P<0.001 and P<0.0001) and ECU muscle (P<0.0001) of both genotypes compared to CT fibers (Fig. 4 g and j) following FR. On the other hand, more reduction in the PF IIX fibers was detected in T.lateral and FCU muscles of only *Mstn*$^{-/-}$ (P<0.0001) following FR (Fig. 4b and m), also significant interaction (P<0.01) indicating the effect of diet on *Mstn*$^{-/-}$.Similarly, analysis of type IIA fibers showed some variations, PF IIA fibers of T.medial muscle displayed more reduction in the CSA (P<0.01) compared to CT fibers only in *Mstn*$^{+/+}$ (Fig. 4h) however PF fibers of ECU muscle showed more CSA reduction compared to CT fibers (P<0.0001 and P<0.001) for *Mstn*$^{-/-}$ and *Mstn*$^{+/+}$ respectively following dietary restriction (Fig. 4k). In contrast, FCU muscle displayed larger CSA reduction in PF IIA fibers only in *Mstn*$^{-/-}$ compared to CT fibers after dietary restriction (Fig. 4n). Type I fibers was only localized in the central part of the muscle therefore were excluded from this comparison.

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

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

muscles *Mstn*$^{-/-}$ND (P<0.0001) compared to *Mstn*$^{+/+}$ND. However FR altered the fiber type composition for both genotypes (P<0.001) e.g. T.lateral muscle demonstrated a reduction in the type IIB fibers (P<0.0001 and P<0.05) and increases of type IIX fibers (P<0.0001 and P<0.05) for *Mstn*$^{-/-}$FR and *Mstn*$^{+/+}$FR respectively compared to genotype matched control (Fig. 5a and b). Also FR induced an increase in the type IIA fibers of *Mstn*$^{-/-}$FR compared to ND, however *Mstn*$^{+/+}$FR showed a reduction in type IIA fibers compared to ND. These data were confirmed by significant interaction between both genotypes (P<0.01, Fig. 5c). Furthermore, T.long muscle showed a reduction in type IIB fibers (P<0.0001) with an increase in type IIX fibers (P<0.001 and P<0.0001) for *Mstn*$^{-/-}$FR and *Mstn*$^{+/+}$FR respectively compared to genotype-matched ND (Fig. 5d and e). Although there were no significant changes, *Mstn*$^{-/-}$FR demonstrated marked increases in type IIA fibers compared to *Mstn*$^{-/-}$ND. However *Mstn*$^{+/+}$FR showed significant increase in type IIA fibers (P<0.01) compared to *Mstn*$^{+/+}$ND (Fig. 5f) as well as FR resulted in marked increase in type I fibers in both genotypes (P<0.01) compared to ND control (Fig. 5g). On the other hand, T.medial muscle analysis revealed fiber type population of 33%, 34%, 26% and 7% for IIB, IIX, IIA and I fibers respectively in the *Mstn*$^{+/+}$ND and showed almost no fiber type shift following dietary restriction in the *Mstn*$^{-/-}$ however *Mstn*$^{+/+}$ FR displayed fiber shift into type I fibers (IIB→IIX→IIA→I) following the same treatment (P<0.05) compared to ND. These data was supported by significant interaction (P<0.05) between diet and genotype effect (Fig. 5h, i and j and k). On the other hand, ECU muscle displayed a different fiber shift pattern with dietary restriction whereas IIB to IIX fibers transformation in the *Mstn*$^{-/-}$ (P<0.0001 and P<0.01) respectively (Fig. 5l and m) and exhibited type IIA to type I fiber shift in *Mstn*$^{+/+}$ (P<0.001) compared to ND (Fig. 5n and o). Similarly, FCU muscle FR induced type IIB to IIX fiber shift for *Mstn*$^{-/-}$ (P<0.0001) compared to ND (Fig. 5p and q) however demonstrated fibers shift from IIB directly into IIA fibers but not type IIX fibers in *Mstn*$^{+/+}$ (P<0.0001 and P<0.05) compared to *Mstn*$^{+/+}$ND (Fig. 5r and s). Representative double immunofluorescent images of T.long muscle for *Mstn*$^{+/+}$ and *Mstn*$^{-/-}$ showed the

transformation of the glycolytic fibers type IIB and IIX into the oxidative type IIA fibers following dietary restriction (Fig. 5t, u, v and w).

4. Discussion

Numerous external and internal stimuli regulate muscle composition based on functional demands. Dietary intake plays a critical role to maintain the physiological composition of the skeletal muscle. However, dietary imbalance or deficiency impairs muscle function with risk of muscle wasting. Previous studies revealed that *myostatin* deletion results in an increase in muscle mass due to an increase in fiber number and fiber size (McPherron et al., 1997). However, the impact of dietary restriction on anatomically and structurally different forelimb muscles of the *myostatin* null are not fully characterized. Our detailed analysis of muscle mass, fiber composition and cross sectional area of each myofiber highlight some key points of muscle response to dietary restriction in *Mstn*$^{-/-}$ mice. We showed that dietary restriction induces reduction in total body mass and specific muscle weight. T.long and T.lateral muscle demonstrated larger reduction, approximately of 53% and 33% respectively for both genotypes provided with significant interaction indicating the effect of diet was more pronounced in the *Mstn*$^{-/-}$. However more reduction was detected in the ECU muscle of *Mstn*$^{-/-}$ or even only the *Mstn*$^{-/-}$ in the T.medial and FCU muscles compared to *Mstn*$^{+/+}$ mice. These results suggest that the impact of dietary restriction was greater in the *Mstn*$^{-/-}$ considering the basal differences between both genotypes, which might refer to the myofiber composition and the glycolytic phenotype following myostatin deletion. Similar observation showed that dietary limitation caused more muscle loss in the hind limb muscles of the *Mstn*$^{-/-}$ compared to *Mstn*$^{+/+}$ suggesting higher catabolic activities (Matsakas et al., 2013). Furthermore, it could be speculated that dietary limitation enhances the process of protein turn over in the *Mstn*$^{-/-}$ compared to *Mstn*$^{+/+}$. In support of this notion is the finding that *Mstn*$^{-/-}$ mice exhibited more muscle mass loss during hind limb unloading (McMahon et al., 2003). In line with our observation, *Mstn*$^{-/-}$ mice displayed greater loss in muscle mass with ageing compared to age-matched WT (Elashry et al., 2009). As expected fast twitch muscles T.long

and T.lateral showed the biggest reduction for both genotype compared to slow twitch T.medial and ECU muscles following dietary restriction. These results indicate that muscle size and the myofibers composition might play a role in the degree of muscle loss following dietary limitation. It is well established that muscle wasting due to proteolytic activities in muscle disease or disuse included calcium-dependent calpain, caspase and lysosomal proteases as well as ubiquitin proteasomes (Malavaki et al., 2015). Thus, it can be assumed that dietary restriction enhances proteolytic activities in the *Mstn*$^{-/-}$ consequently amplifies protein break down compared to *Mstn*$^{+/+}$ mice. In this respect, a recent study revealed that *Mstn*$^{-/-}$ is strongly susceptible to dietary limitation which induces higher proteolytic activities and autophagy as well as switching off protein synthesis compared to WT mice (Collins-Hooper et al., 2015). Our data revealed that although *Myostatin* deletion causes various degrees of myofiber hyperplasia in most of the forelimb muscles, dietary restriction did not affect the total myofiber number following the experimental period excluding the possibility of myofiber loss. A similar observation was previously reported in the hind limb muscles (Matsakas et al., 2013) suggesting that loss of muscle mass is more likely due to protein break down rather than myofiber degradation. Furthermore, our results showed that FR induces selective type IIB glycolytic fibers atrophy followed by a lower extend in IIX and IIA fibers compared to type I oxidative fibers which displayed less liabilities to dietary restriction induced muscle atrophy. Furthermore, the degree of fiber atrophy was greater in the *Mstn*$^{-/-}$. These data suggest that FR primarily targeted the fast fibers which represent the main protein store and explain the massive reduction of muscle mass in T.lateral and T.long muscles due to the glycolytic phenotype of these muscles. This in line with previous reports indicating that fast twitch IIB fiber are more sensitive to starvation compared to slow twitch fiber (Goldspink and Ward, 1979; Li and Goldberg, 1976; Matsakas et al., 2013). Such selective effect on fiber type was observed in aged monkeys with an exclusive type II fiber atrophy instead of type I slow fibers (McKiernan et al., 2012). In agreement with our results, hypoxia induced preferential atrophy and activation of proteolysis in fast twitch EDL compared to slow twitch soleus muscle (Theije et al., 2015). On the

other hand, recent investigation revealed that *myostatin* inactivation via activin IIB receptor inhibition leads to preferential increase in the CSA of glycolytic fibers rather than oxidative fibers (Liu et al., 2016). Our data revealed that the impact of dietary restriction showed a regional specificity. We show that FR induced a larger loss in the PF glycolytic IIB and IIX fibers compared to CT fibers particularly in *Mstn*$^{-/-}$. These data are related to the localization of the glycolytic fibers at the most peripheral part of the muscle compared to the oxidative fibers, which are more centrally distributed in order to maintain energy storage and postural stability.

These data indicate a direct link between *myostatin* interference and protein metabolism. It has been described that IGF-1 signaling not only plays a key role to maintain protein synthesis via AKT/mTOR (mammalian target of rapamycin) pathway but also to inhibit protein degradation (Hitachi and Tsuchida, 2013). It has also been shown that immobilization induces muscle atrophy via activation of myostatin signaling which triggers the expression of atrogin-1 and muscle ring-finger-1 (MuRF-1) causing muscle protein break down (Tando et al., 2016). We propose that the dietary restriction induced muscle loss is regulated via inhibition of IGF-1 signaling which results in upregulation of atrogin-1 and MuRF-1 expression. A similar study showed that undernutrition for 28 days causes reduced growth rate via regulation of IGF-1 and myostatin expressions (Jeanplong et al., 2015). These data highlight that *myostatin* deletion and dietary limitation interaction interfere with the muscle metabolism, which resulted in effective proteolytic activities and rapid protein degradation. In the same line, previous report demonstrating that *myostatin* inhibition increased the muscle mass due to decreased expression of E3 ubiquitin ligase atrogin-1 (Mendias et al., 2011). Our data showed myostatin deletion amplify the effect of dietary restriction to involve the oxidative fibers CSA reduction e.g. type I fibers of T.long muscles which might be attributed to the mixed fibers composition of this muscle. On the other hand, T.medial muscle displays less change in the oxidative type I fibers suggesting that the oxidative fibers are resistant to dietary restriction. In agreement with our results, it has been shown that the atrophy resistance of the oxidative fibers was due to a protection

afforded by peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC1α) (Wang and Pessin, 2013). Unexpectedly, dietary restriction causes CSA increases in type IIA fibers for ECU and FCU of *Mstn*$^{-/-}$ which might be an adaptive mechanism to compensate the loss of the glycolytic fibers.

Analysis of the muscles phenotypes revealed a general conversion toward slow oxidative phenotype following dietary restriction. However, we observed only the degree and the mechanism of fiber shift was dependent on the original fiber type population e.g. T.long and T.lateral muscles displayed IIB→IIX→IIA in the *Mstn*$^{-/-}$ however only showed a IIB→IIX fiber type conversion in *Mstn*$^{+/+}$. Moreover, ECU and FCU muscles showed a IIB→IIX fiber shift in *Mstn*$^{-/-}$ however demonstrated a IIA→I and IIB→IIA shift respectively in *Mstn*$^{+/+}$. These data indicate that myostatin deletion and dietary limitation interactions alter muscle adaptation and plasticity. Muscle oxidative conversion was previously reported with ageing, although there was trophic activity in type II fibers, additionally there was a relative increase in type I fibers (Kallman et al., 1990). Furthermore, a recent study in muscular dystrophy showed that muscle atrophy was accompanied with a myofiber oxidative shift perhaps due to an increase in the expression of PGC1α (Rocchi et al., 2016). On the other hand, soleus muscle immobilization, hind limb unloading and spinal cord transection induced muscle atrophy plus type I into type II fibers transformation see review by (Cho et al., 2016). However in our model the physical performance and muscle function were preserved compared to previous models which resulted in improved phenotype particularly in *Mstn*$^{-/-}$. It could be explain that muscle adaptability is dependent on the extend of the external stimuli therefore the high demand for protein supplement in the *Mstn*$^{-/-}$ induced fiber atrophy and fiber type transformation. This is far greater compared to *Mstn*$^{+/+}$ such a change in muscle metabolism energy saving mechanisms are required.

Our data discussed the impact of caloric restriction in combination with *myostatin* deletion on five muscles from different anatomical locations. We provide evidence that 60% of dietary limitation results in body weight and muscle specific mass reduction with fast myofibers more prone to atrophy

compared to slow myofibers. The effect of dietary restriction was more pronounced in some of the *Mstn*$^{-/-}$ muscles to implicate the oxidative fibers compared to *Mstn*$^{+/+}$mice. Furthermore, peripheral myofibers are more susceptible to dietary induced reduction compared to deep fibers. We provide evidence that dietary restriction alters the fast phenotype of the *Mstn*$^{-/-}$ into the slower form in a muscle dependent manner. These results allow the conclusion that caloric reduction alters the muscle fiber composition and the oxidative pattern to compensate muscle mass loss which might impact on muscle function particularly in the hypermuscular *myostatin* null animals.

Competing interest

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

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Figure legends

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

a Average body weight (g) for normal diet (ND) and food restricted (FR) *Mstn*$^{+/+}$ and *Mstn*$^{-/-}$ mice (N=4 for all groups). Significant increase in the *Mstn*$^{-/-}$ (*P*<0.001) compared to the *Mstn*$^{+/+}$ on normal diet. FR decreases the body weight for *Mstn*$^{+/+}$ and *Mstn*$^{-/-}$ compared to genotype-matched ND mice. **b-f** Average muscle weight (g) for **b** T.lateral, **c** T.long, **d** T.medial, **e** ECU, and **f** FCU muscles. Specific muscle mass increases in *Mstn*$^{-/-}$ ND compared to *Mstn*$^{+/+}$ ND. FR induces more muscle mass reduction in T.lateral and T.long muscles of *Mstn*$^{-/-}$ (diet and genotype interaction P<001 and 0.0001) respectively compared to genotype matched control. *Mstn*$^{-/-}$ display more muscle loss in T.medial, ECU and FCU muscles compared to *Mstn*$^{+/+}$ following FR. All values displayed as mean± SEM. * =P< 0.05, ** = P< 0.01, *** = P< 0.001 and ****=P<0.0001).

Figure 2 Caloric restriction does not alter muscle fiber number.

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

Figure 3 Glycolytic fibers are more prone to caloric restriction-induced muscle mass loss.

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

Figure 4 Dietary restriction causes peripheral myofiber atrophy.

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

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

Average percentages of type IIB, IIX, IIA, and I fibers for T.lateral **a, b, c;** T.long **d, e, f, g**; T.medial **h, i, j, k**; ECU **l**, **m**, **n**, **o**; and FCU muscles **p, q, r, s** of ND and FR *Mstn*$^{+/+}$ and *Mstn*$^{-/-}$ mice. *Mstn*$^{-/-}$ ND show increases in type IIB and IIX

fibers compared to *Mstn*$^{+/+}$ND for all muscles. FR induces an increase in type IIX and type IIA fibers of T.lateral muscle in *Mstn*$^{-/-}$ and only type IIX increases in *Mstn*$^{+/+}$. T.long muscle exhibits IIB to IIA fiber type shift and IIB to IIX fiber shift for *Mstn*$^{+/+}$ and *Mstn*$^{-/-}$ following FR respectively. FR causes an increase in type I fibers for T.medial muscle of *Mstn*$^{+/+}$. ECU muscles show IIB to IIX fiber type shift for *Mstn*$^{-/-}$ compared to IIA to I fibers for *Mstn*$^{+/+}$ following FR. FCU muscle displays IIB to IIA fiber shift for *Mstn*$^{+/+}$ following FR. **t-w** Representative double immunofluorescent images of mid-belly section for T.long muscle in ND and FR *Mstn*$^{+/+}$ and *Mstn*$^{-/-}$ mice. FR induces myofiber transformation from glycolytic type IIB fibers (red) into oxidative type IIA. Type IIX fibers remained unstained (black). Scale bar =100 µm. All values displayed as mean± SEM.

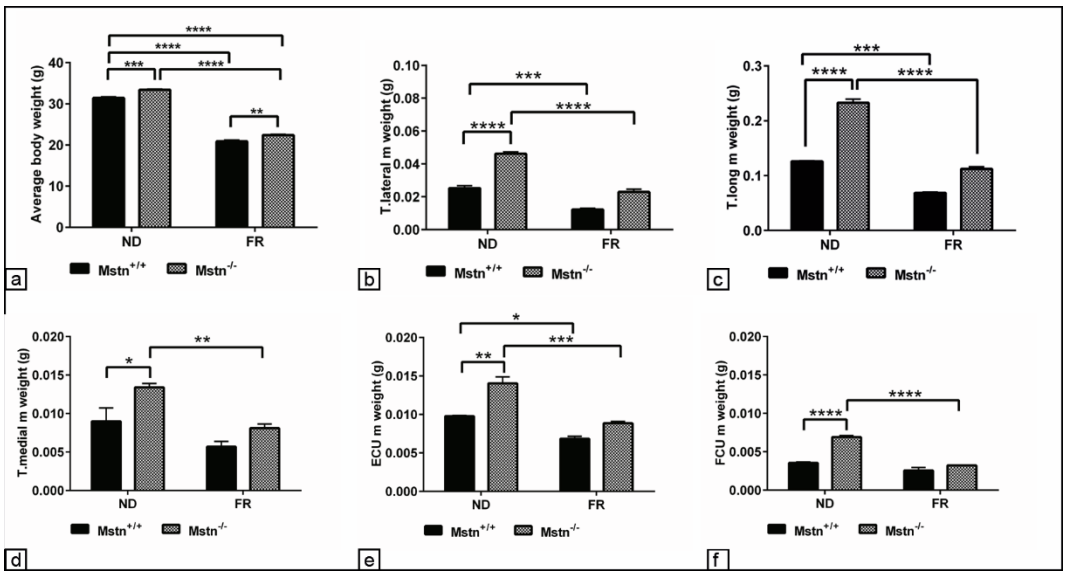


Figure 1 Dietary restriction induces body weight and muscle mass specific reduction.

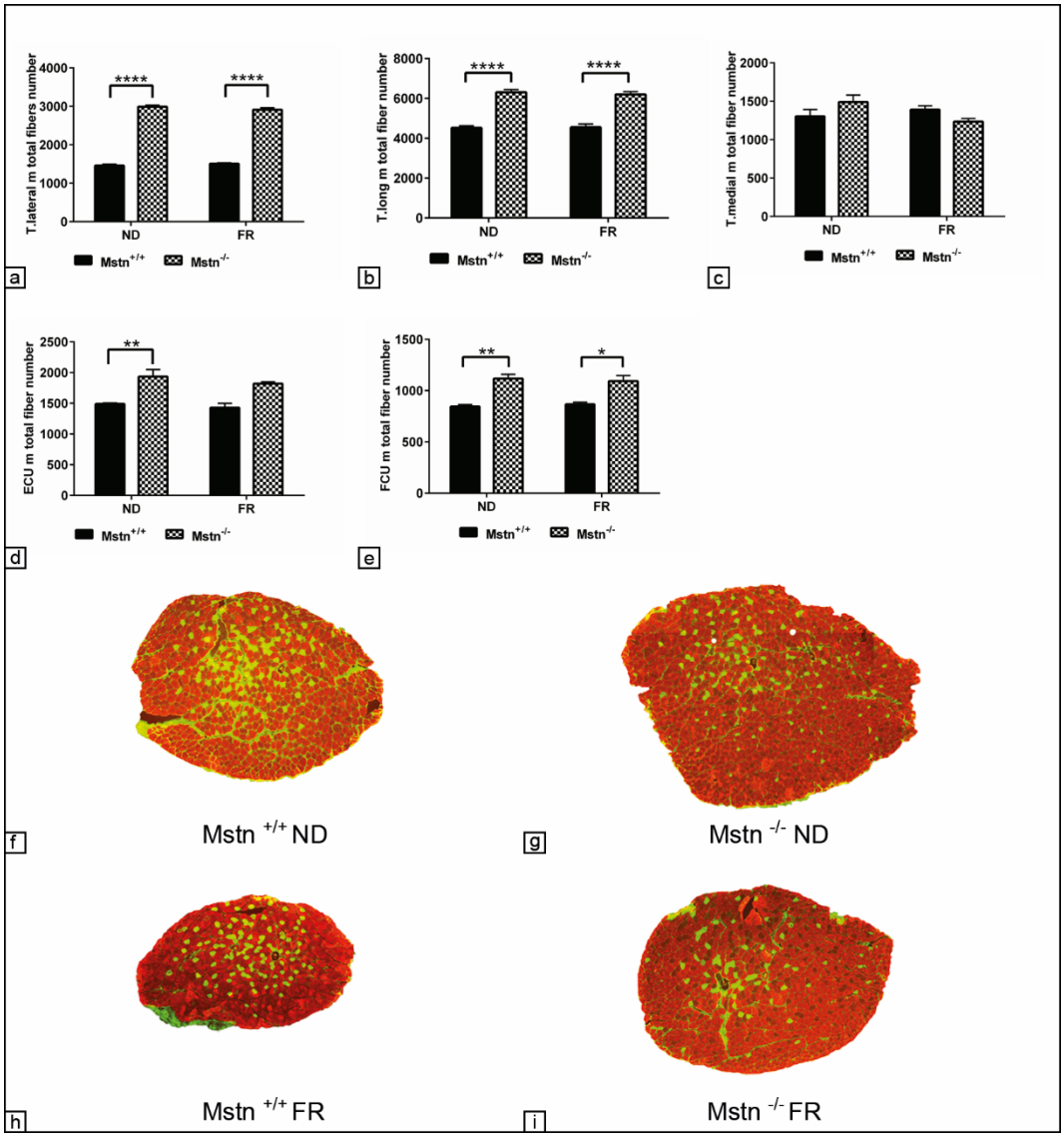


Figure 2 Caloric restriction does not alter muscle fiber number.

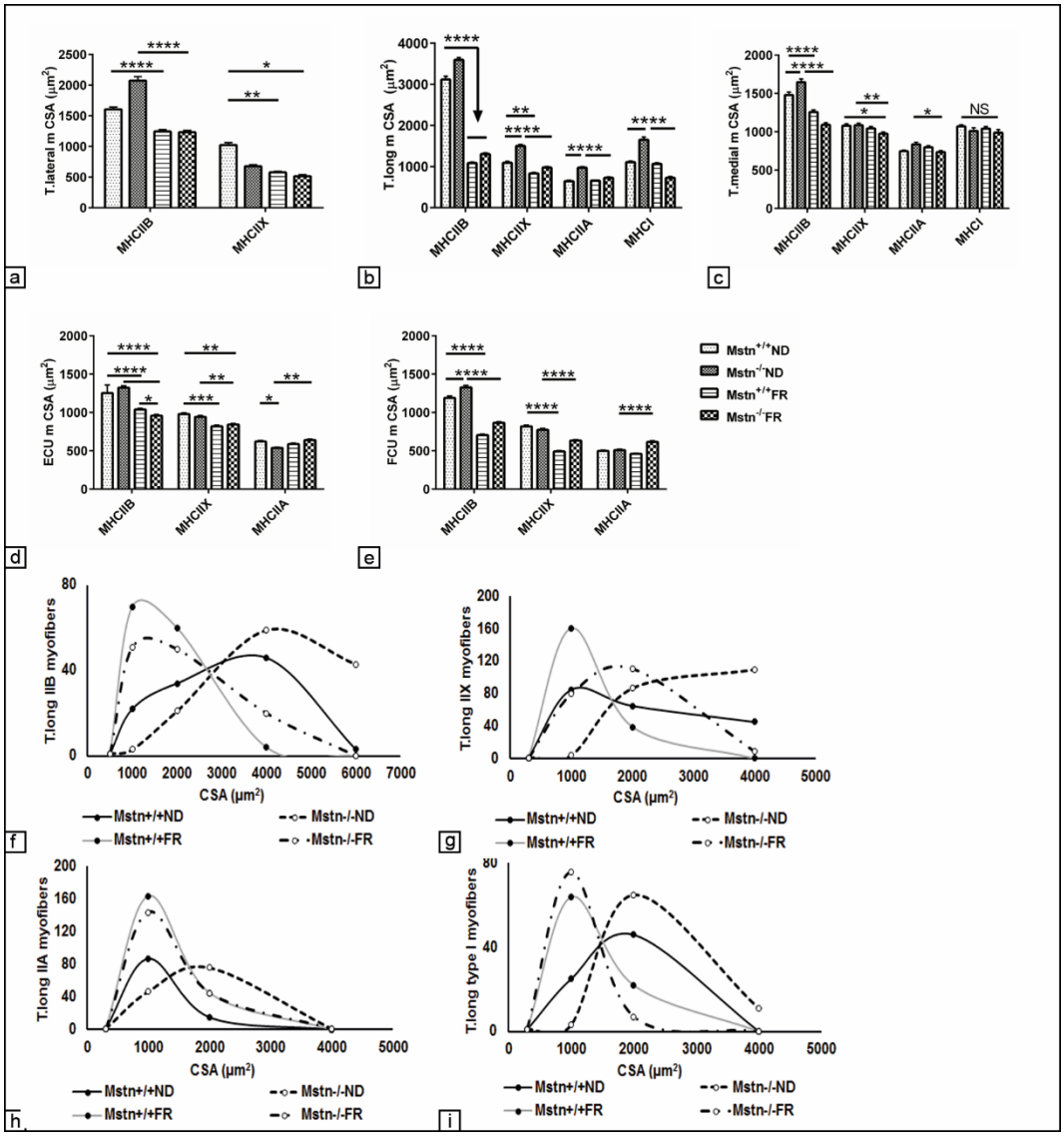
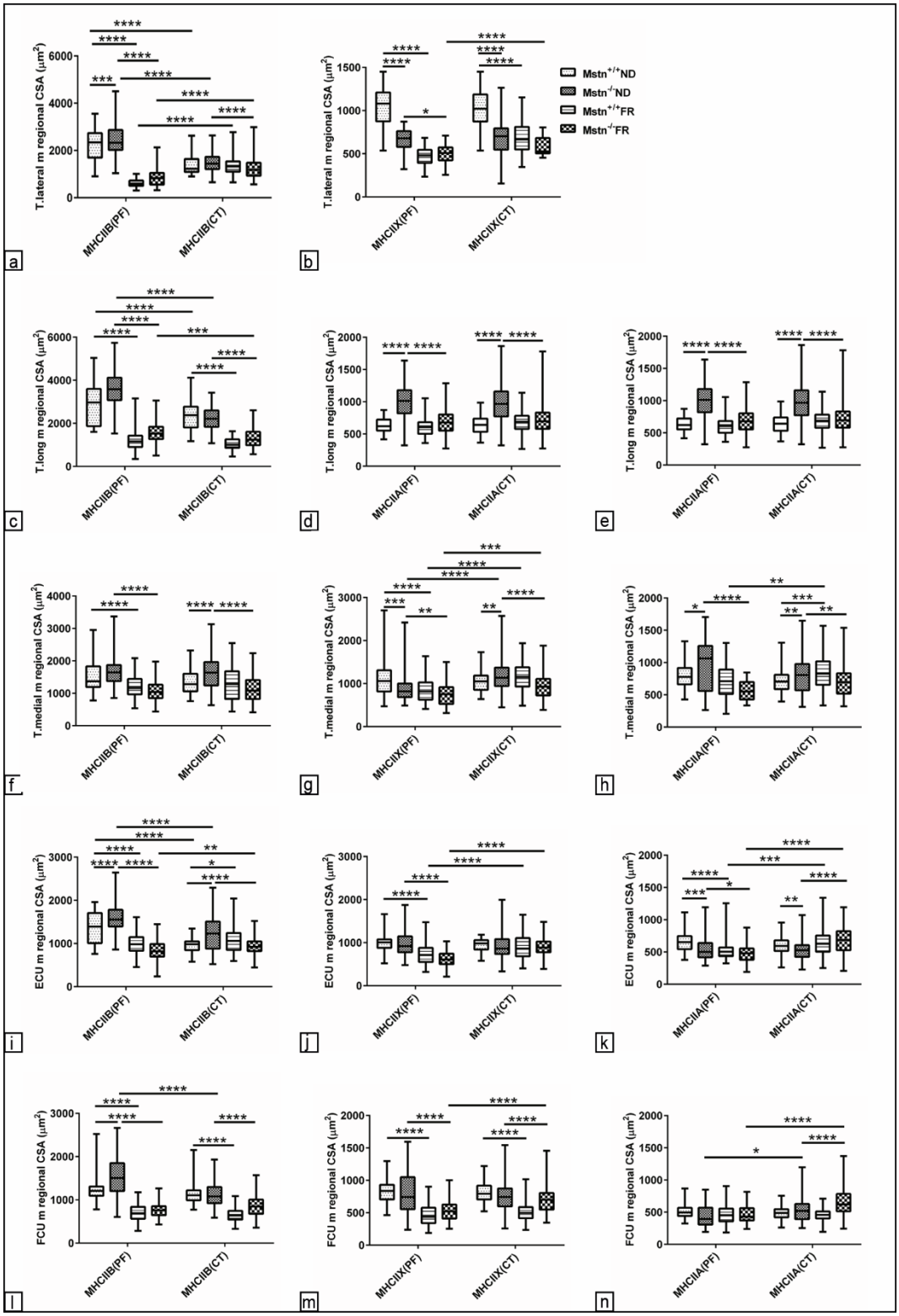


Figure 3 Glycolytic fibers are more prone to caloric restriction-induced muscle mass loss.



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

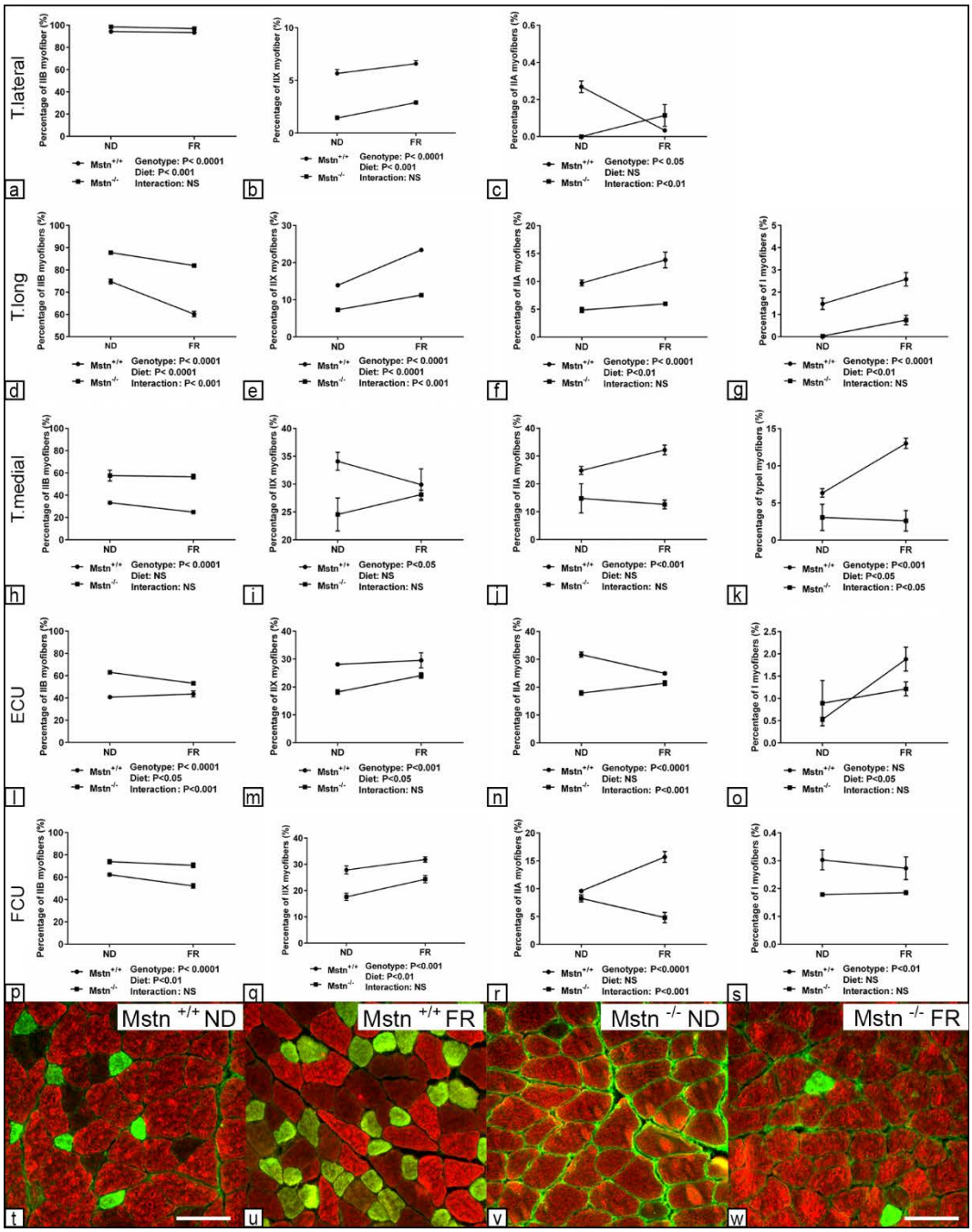

Figure 5 Caloric restriction induces a slow phenotype conversion in a muscle-specific manner.

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