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Myostatin gene inactivation increases *post-mortem* calpain-dependent muscle proteolysis in mice

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ABSTRACT

Myostatin deficiency leads to extensive skeletal muscle hypertrophy, but its consequence on *post-mortem* muscle proteolysis is unknown. Here, we compared muscle myofibrillar protein degradation, and autophagy, ubiquitin-proteasome and Ca²⁺-dependent proteolysis relative to the energetic and redox status in wild-type (WT) and myostatin knock-out mice (KO) during early *post-mortem* storage. KO muscles showed higher degradation of myofibrillar proteins in the first 24 h after death, associated with preserved antioxidant status, compared with WT muscles. Analysis of key autophagy and ubiquitin-proteasome system markers indicated that these two pathways were not upregulated in *post-mortem* muscle (both genotypes), but basal autophagic flux and ATP content were lower in KO muscles. Proteasome and caspase activities were not different between WT and KO mice. Conversely, calpain activity was higher in KO muscles, concomitantly with higher troponin T and desmin degradation. Altogether, these results suggest that calpains but not the autophagy, proteasome and caspase systems, explain the difference in *post-mortem* muscle protein proteolysis between both genotypes.

1. Introduction

Skeletal muscle accounts for 30–40% of the total body weight, and is a key player in protein and energy metabolism. In livestock species, skeletal muscle is the precursor of meat, and the *post-mortem* maturation of muscle into meat is a critical event for meat tenderization and sensory quality (Ouali et al., 2006; Valin, 1988). This maturation process is characterized by energetic and oxidative stress, and by dynamic changes in the muscle architecture and integrity, including biochemical changes, myofibrillar protein degradation and oxidation, and high-energy compound breakdown (Huff Lonergan, Zhang, & Lonergan, 2010; Sierra & Olivan, 2013).

The conversion of muscle into meat after slaughter takes place in

three phases: i) pre-rigor during which muscle remains excitable, ii) installation of cadaveric rigidity or *rigor mortis*, iii) and ripening (tenderization). During the *rigor mortis* installation, the muscle energy reserves (ATP, phosphocreatine, glycogen) are depleted, whereas during the tenderization phase muscle proteins are degraded (Koohmaraie, 1996; Ouali et al., 2006). Meat tenderization results from the alteration of the muscle contractile structure (i.e. myofibrils and associated proteins) and has been largely attributed to endogenous proteolytic enzymes (Koohmaraie & Geesink, 2006; Ouali et al., 2006; Ouali et al., 2013; Sentandreu, Carbonell, & Sendra, 2002; Sierra & Olivan, 2013). Strong evidence has indicated that the calpain system plays a central role in *post-mortem* proteolysis. Degradation patterns of myofibrillar proteins incubated with calpains are the same as that seen in *post-mortem*

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muscle (Geesink & Koohmaraie, 1999; Huff-Lonergan et al., 1996). More directly, a reduction in *post-mortem* proteolysis of muscle proteins has been reported in transgenic mice overexpressing calpastatin, the endogenous inhibitor of the calpain enzymes and in μ -calpain knock-out mice (Geesink, Kuchay, Chishti, & Koohmaraie, 2006; Kent, Spencer, & Koohmaraie, 2004). It has been shown that the caspases can degrade and inactivate calpastatin (Kemp, King, Shackelford, Wheeler, & Koohmaraie, 2009; Kemp, Sensky, Bardsley, Buttery, & Parr, 2010) and that might affect *post-mortem* proteolysis. Moreover, the inhibition of the apoptotic factor caspase 3 has been shown to reduce muscle protein degradation (Huang, Huang, Xue, Xu, & Zhou, 2011).

Besides the fundamental role of calpain system in *post-mortem* proteolysis, more recently, a number of subsequent studies have provided evidence supporting the possibility that others catabolic systems, such as the ubiquitin proteasome system (UPS) and macroautophagy (autophagy hereafter) could also contribute to this proteolytic process (Cramer, Penick, Waddell, Bidwell, & Kim, 2018; Ouali et al., 2006; Sentandreu et al., 2002; Sierra & Olivan, 2013).

In the UPS, proteins or fragments of larger proteins are tagged through covalent attachment of an ubiquitin chain for their targeted degradation by the proteasome. Several studies have explored the contribution of UPS in muscle *post-mortem* proteolysis. For example, it has been reported that incubation of bovine myofibrils with the 20S proteasome induced the degradation of myofibrillar proteins (Robert, Briand, Taylor, & Briand, 1999). Another study reported that the UPS remained active during *post-mortem* conditioning period (Lamare, Taylor, Farout, Briand, & Briand, 2002). Additionally, it has been shown that the inhibition of the proteasome activity using an anti-proteasome inhibitor resulted in a lack of degradation of muscle structural proteins, and that proteomic/mass spectrometry data on meat samples where the UPS activity was inhibited revealed a different fragmentation pattern of sarcomeric proteins compared with the “normal” pattern described *in vivo* (Houbak, Ertbjerg, & Therkildsen, 2008). Conversely, it should be noted that incubation of myofibrillar proteins with proteasome fails to change the structure of myofibrils (Koohmaraie, 1992). The discrepancy among these studies may reflect differences in experimental conditions. Moreover, if all conditions are optimized and that the *in vitro* assay does not allow to reproduce the proteolysis pattern observed after *post-mortem* storage of meat; this may be explained by differences of environment factors between artificial incubation of enzymes treatment and *post-mortem* condition of muscle (Geesink et al., 2006; Houbak et al., 2008; Robert et al., 1999; Taylor, Geesink, Thompson, Koohmaraie, & Goll, 1995). The interpretation of these studies (based on an indirect assessment to estimate protein degradation products in meat) is thus complicated but the results from these studies are not sufficient to exclude the contribution of proteasome to the process of *post-mortem* proteolysis.

Autophagy is a critical mechanism for eukaryotic cell survival whereby cells degrade dysfunctional subcellular components in response to starvation and to other stressors (Coto-Montes et al., 2012). A recent study proposed that early activation of autophagy delayed cell degradation and ultimately meat maturation. Specifically, the expression of beclin1 (BECN1) and microtubule associated protein 1 light chain 3 (MAP1LC3/LC3), two specific autophagy markers, increased transiently at the onset of *post-mortem* beef ageing (Garcia-Macia et al., 2014), supporting the hypothesis of autophagy implication in the pre-rigor phase of meat maturation. Conversely, the ‘omic’ study by Longo et al., in which *Longissimus thoracis* muscle samples from eight different Piedmontese cattle breeds were analysed over time after death, did not find any indication of a cellular autophagic response (Longo, Lana, Bottero, & Zolla, 2015). Recently, it has been reported that the pre-slaughter autophagic status influenced the *post-mortem* changes in muscle proteins (Nakanishi, Nishimoto, Erickson, & Kawahara, 2017). A

deeper understanding of the mechanisms which govern the *post-mortem* muscle proteolysis is further required.

Although, it is recognized the importance of the calpain system in this process and the possible contribution of 20S proteasome and autophagy, it is not known which muscle factors affect *post-mortem* proteolysis. One of these factors is myostatin (MSTN), a member of the TGF β superfamily, which strongly inhibits the muscle mass development. *Mstn* genetic ablation in mice results in excessive skeletal muscle growth and increases muscle mass (up to 200%) (McPherron, Lawler, & Lee, 1997). Conversely, *Mstn* overexpression or its systemic administration causes muscle atrophy (Zimmers et al., 2002). Compelling evidence in the literature indicate that MSTN controls the muscle protein balance by regulating protein synthesis and degradation (Rodriguez et al., 2014). A mechanism by which MSTN inhibits muscle growth is the down-regulation of protein synthesis (W. E. Taylor et al., 2001). The data support that the down-regulation of Akt/mTOR/S6K signaling could mediate the negative effect of MSTN on protein synthesis (Amirouche et al., 2009). In addition to inhibit protein synthesis, MSTN might also stimulate muscle proteolysis. For example, *in vitro*, MSTN upregulates the expression of genes involved in the ubiquitin proteasome pathway including the atrogenes Atrogin-1, MuRF-1 (Muscle RING finger protein-1) (i.e. genes that encode ubiquitin ligases and the expression of which is increased in catabolic situations) mediated by the activation of Forkhead box O (FoxO1) via an inhibition of Akt pathway (McFarlane et al., 2006). Finally, MSTN could trigger protein degradation via the stimulation of the autophagy-lysosomal system. Indeed, MSTN stimulates autophagosome formation and expression of several autophagy-related genes *in vitro* (Lee, Hopkinson, & Kemp, 2011).

Natural mutations in the *Mstn* gene are the cause of a hypermuscular phenotype in cattle (Grobet et al., 1997; Kambadur, Sharma, Smith, & Bass, 1997) and sheep (Clou et al., 2006). It has been shown that in general these mutations result in improved tenderness, especially in double-musled cattle (Uytterhaegen et al., 1994) but to date, no study has investigated the *post-mortem* muscle proteolysis in response to *Mstn* inhibition. Metabolic studies in mice or bovine lacking MSTN have demonstrated an increased number of the fast glycolytic type IIB fibres and a concomitant loss of oxidative type I and type IIA fibres, and that such conversion is associated with a mitochondrial depletion and impaired activity of oxidative enzymes (Amthor et al., 2007; Bouley et al., 2005; Deveaux, Cassar-Malek, & Picard, 2001; Matsakas et al., 2012; Ploquin et al., 2012). Concerning oxidative stress, we previously showed that in *Mstn*-deficient mice, lipid peroxidation is reduced and the antioxidant glutathione system is upregulated in skeletal muscle (Ploquin et al., 2012). These data highlighted the importance of MSTN in protein balance and skeletal muscle metabolism.

Taken together, these data led us to hypothesize that *Mstn* inactivation might alter the muscle proteolysis kinetics in the pre-rigor mortis period (first 24 h *post-mortem*). The first aim of this study was to compare the skeletal muscle proteolytic characteristics with respect to energy and redox status in wild-type (WT) and *Mstn* knock-out mice (*Mstn* KO) during early *post-mortem* storage. The second aim was to analyse the proteolytic systems (autophagy, UPS, calpain and caspase systems) to elucidate their implication in the changes observed during early *post-mortem* storage.

2. Materials and methods

2.1. Ethics statement

Our animal facility is approved by the Departmental Veterinary Services (No. E34-172-10) and our Ministry of Research (No. 7053). These experiments were performed according to European directives (86/609/CEE) and approved by the national ethics committee (referral

file APAFIS#21675-201908011541816 v3). The study was carried out in compliance with the ARRIVE guidelines.

2.2. Animals and experimental design

The *Mstn* KO mice used in this study have been described previously (Grobet et al., 2003). In these mice, constitutive deletion of the third *Mstn* exon led to the deletion of the entire mature C-terminal region of MSTN. Therefore, MSTN was not functional (Grobet et al., 2003). *Mstn* KO mice were generated in the C57BL/6 J genetic background by mating homozygous animals. The parental genotype was determined by PCR analysis of tail DNA.

A total of 224 *Mstn* KO mice, and 224 WT mice (male and 6 months) were used in the present work. Mice were maintained on a 12:12-h dark–light cycle in a temperature-controlled room in individual cages with filter lids, and ad libitum access to food and water. The diet was standardized and identical for all groups (A03, safe diets, Augy, France).

The mice in the time course experiment comprised 64 mice/genotype.

2.3. Fasting experiment

The mice in the fasting treatment experiments were randomized into two groups/genotype: control fed group (Fed, $n = 32$), fasted group (Fasted, $n = 32$).

WT and *Mstn* KO mice were housed in individual cage and deprived of food for 24 h, with free access to drinking water.

2.4. Colchicine administration

Mice received 4 intraperitoneal (ip) injections of saline (Ctl: control group) or colchicine (Sigma-Aldrich, Saint Louis, MO, USA) (Col: colchicine group, 0.8 mg/kg/day) with an interval of 12 h during the 48 h before the slaughter.

For experiments in fasting condition, mice were deprived of food for 24 h after the second ip injection of saline or colchicine. Four hours after the last ip injection, mice were weighed, killed by cervical dislocation, and exsanguinated.

The mice in the colchicine treatment experiments were randomized into two groups/genotype: control group injected with saline (Ctl, $n = 32$), group injected with colchicine (4 ip in 48 h) (Col, $n = 32$).

The mice in the colchicine treatment and fasting experiments were randomized into four groups/genotype: control fed group injected with saline (Fed, $n = 8$), fed group injected with colchicine (4 ip in 48 h) (Fed + Col, $n = 8$), fasted group for 24 h injected with saline (Fasted, $n = 8$), and fasted group injected with colchicine (Fasted + Col, $n = 8$).

Autophagic flux was calculated by western blotting as the difference between LC3BII expression in skeletal muscle from colchicine-treated and saline-treated animals (LC3BII Col – LC3BII Ctl) at each time point (Mizushima & Yoshimori, 2007). Values were expressed as fold change relative to WT at $T = 0$.

2.5. Muscle sampling

All animals were sacrificed in the morning by cervical dislocation followed by exsanguination. To be as close as possible to the meat sampling conditions of livestock animals, mouse carcasses were kept in a temperature-controlled room (22 ± 1 °C) for 1 h and then placed at 4 °C for 4, 8 and 24 h (Fig. 1a). At the end of each storage time, the *Gastrocnemius*, *Tibialis anterior* and *Quadriceps* muscles were removed, weighed, quickly frozen in liquid nitrogen, and then stored at -80 °C

until analyses. The *Longissimus thoracis* muscle was removed, weighed, and then used for pH measurement.

To assess calpain activity effect on myofibrillar protein degradation, just after sacrifice, *Gastrocnemius* samples were minced and incubated with saline (10% (vol/weight)) or MDL-28170 (20 pmol/mg of minced muscle) (sc-201301, Santa Cruz Biotechnology, Dallas, TX, USA), a selective inhibitor of μ -calpain (calpain-1) and m-calpain (calpain-2), in a temperature-controlled room (22 ± 1 °C) for 1 h and then at 4 °C for up to 24 h.

2.6. pH measurement

Longissimus thoracis samples were collected at different time points after sacrifice (0, 30 min, and 1, 2, 3, 4, 8, 24 h) and pH measurement was performed as described (Becila et al., 2010) using a SevenEasy™ pH meter (Mettler Toledo, Columbus, OH, USA) equipped with a combined glass electrode (InLab Expert Pro). The pH meter was calibrated with pH 4.01, 7.01 and 10.01 buffer solutions (Hanna Instruments) at 25 °C.

2.7. Protein isolation and western blot analysis

Powdered *Gastrocnemius* samples were extracted as described (Pagano et al., 2015) and supernatants were used for protein analysis.

For evaluating troponin T and desmin degradation, pellets were directly dissolved in RIPA buffer, with protease inhibitor cocktail (P8340, Sigma-Aldrich, Saint Louis, MO, USA) and phosphatase inhibitor cocktail (78428, Pierce, Thermo Fisher Scientific, Waltham, MA, USA) incubated on ice for 1 h, and centrifuged at 15,000 xg for 2 min (4 °C) and supernatants were used for protein analysis.

The Western blot protocol was performed as previously described (Rodriguez et al., 2011) with some modifications. Western blots were immunoprobed with primary antibodies (Table 1) and revealed using a Santa Cruz ECL kit (sc-2048, Santa Cruz Biotechnology, Dallas, TX, USA). Proteins were visualized by enhanced chemiluminescence and signals were detected with the ChemiDoc Touch Imaging System (Bio-Rad, 1.1.0.4, 732BR1121). Band intensities were measured with the Image Lab software (Version 5.2.1 for Windows 7). For AMP-activated protein kinase (AMPK) detection, after exposure, X-ray films were digitized, and analysed with ImageJ. Ponceau S staining was used as loading reference. Protein expression was calculated as the protein band intensity normalized to Ponceau S staining. Protein activation was calculated as the ratio of phosphorylated protein to total protein expression.

Table 1

List of primary antibodies used for western blotting.

Antibody	Supplier	Reference	Dilution
Phosphorylated Akt (Ser473)	Cell Signaling	4060	1/1000
Total Akt	Cell Signaling	9272	1/1000
Phosphorylated AMPK α (Thr172)	Cell Signaling	2535	1/1000
Total AMPK	Cell Signaling	2603	1/1000
Desmin	Sigma	D1033	1/300
MuRF1	Santa Cruz	sc-398608	1/1000
Phosphorylated FOXO1 (Ser256)	Cell Signaling	9461	1/500
FOXO1	Cell Signaling	9454	1/1000
LC3B	Sigma	L7543	1/1000
Troponin T	Sigma	T6277	1/2000
Ubiquitin	Santa Cruz	sc-8017	1/1000

Abbreviations: Akt: Protein Kinase B; AMPK: AMP-activated protein kinase; MuRF1: Muscle specific RING finger protein; FOXO1: Forkhead box O1; LC3B: Microtubule associated protein 1 light chain 3B.

2.8. Redox status

Lipid peroxidation levels or thiobarbituric acid reactive substances (TBARS) were measured in tissue homogenates (*Quadriceps* samples) according to the method of (Sunderman Jr., Marzouk, Hopfer, Zaharia, & Reid, 1985).

Protein oxidation was assessed by measurement of sulfhydryl groups according to Faure and Lafond (Faure P, 1995).

Reactive oxygen species (ROS) production was determined using the 2',7'-dichlorodihydrofluorescein-diacetate (H₂DCF-DA) probe as described (McMillan & Quadriatero, 2011). Muscle homogenates (50 µg) were transferred in duplicate to a 96-well plate with assay buffer (Chabi et al., 2008) with 5 µM H₂DCF-DA at 37 °C. Fluorescence emission (λ_{ex}: 485, λ_{em}: 530 nm) was monitored for 60 min with a microplate lumino- and fluoro-meter (Synergy2, Biotek France, Colmar, France). All data were analysed with the Gen5 software (version 1.04), and results were expressed as arbitrary fluorescent units (AFU).

2.9. ATP determination

For ATP extraction, *Tibialis anterior* muscles were homogenized, and ATP concentration was determined in collected supernatants as described (Chida, Yamane, Takei, & Kido, 2012) on a microplate lumino- and fluoro-meter (Synergy2, Biotek France, Colmar, France). Data were analysed with the Gen5 software (version 1.04), and ATP content was expressed as µmol per gram of wet muscle mass.

2.10. Determination of UPS, calpain and caspase activities

All proteolytic activities were measured in *Gastrocnemius* muscle.

UPS, calpain and caspase 3 assays. Proteins were extracted from muscle homogenates as described previously ((Gomes et al., 2012) for UPS and calpain assays). For caspase 3 activity assays, muscle samples were homogenized in extraction buffer (25 mM HEPES pH 7.4, 0.1% CHAPS, 0.1% Triton, 10% Glycerol, 1 mM EDTA, 2 mM DTT) and lysates were then centrifuged at 1000 xg for 10 min (4 °C). Muscle lysates (25 µg of protein) were incubated in the specific activation buffer in the presence of dimethylsulfoxide (vehicle) or inhibitor at room temperature for 20 min and then with the fluorogenic peptide substrate Suc-Leu-Leu-Val-Tyr-amino-4-methyl coumarin (LLVY-AMC, 4011369, Bachem, Bubendorf, Switzerland) (100 µM for UPS assay and 50 µM for calpain assay) or with the fluorogenic caspase-3-specific substrate Ac-DMQD-AMC (Ac-Asp-Met-Gln-Asp-7-amino-4-methylcoumarin; 25 µM) (ALX-260-078, Enzo Life Sciences, Villeurbanne, France). All assays were performed in a total volume of 100 µl in 96-well opaque plates.

To determine UPS activity, muscle lysates were incubated in extraction buffer with 100 µM ATP (Sigma-Aldrich, Saint Louis, MO, USA) in the presence or absence of 20 µM epoxomicin (HY-13821, MedChemTronica, Sollentuna, Sweden), a highly specific and irreversible proteasome inhibitor used to determine the contribution of non-proteasomal enzymes.

To determine calpain activity, muscle lysates were incubated in calpain activation buffer as described (Gomes et al., 2012) in the presence or absence of 0.1 mg/ml MDL-28170.

To determine caspase 3 activity, muscle lysates were incubated in extraction buffer without Triton and Glycerol in the presence or absence of 100 µM Ac-DMQD-CHO (ALX-260-077, Enzo Life Sciences, Villeurbanne, France).

After a preliminary incubation time at 37 °C (10 min for UPS assay, 45 min for calpain assay and 20 min for caspase 3 assay), AMC liberation

was measured with a microplate lumino- and fluoro-meter (λ_{ex}: 380 nm, λ_{em}: 460 nm; Synergy2, Biotek France, Colmar, France) at 2 min intervals for 20 min (UPS assay), 1 h (calpain and caspase assay). All assays were linear in this range, and each sample was tested in duplicate. The individual chymotrypsin-like (β5) activity of the 26S proteasome, calpain and caspase activities were determined by calculating the difference between the fluorescence units recorded in the absence and in the presence of epoxomicin, MDL-28170 or Ac-DMQD-CHO respectively, and expressed as arbitrary fluorescence units per minute and mg proteins.

2.11. Statistical analysis

Results are presented as mean ± standard error of mean. All data were analysed by two-way ANOVA (with genotype, time, and treatment (fasting and colchicine) conditions as the three factors). Significant effects and interactions between these factors were checked using Prism 6.01, GraphPad, San Diego, CA. When a main effect was identified, Sidak's multiple comparisons test was used to localise the differences. Statistical significance was set at *P*-values <0.05.

3. Results

3.1. Absence of functional myostatin results in faster muscle pH decrease and higher myofibrillar protein degradation during early post-mortem muscle ageing

To examine the effect of MSTN absence on the *post-mortem* muscle proteolysis, we harvested muscle samples from WT and *Mstn* KO mice at different time points during the first 24 h after death (Fig. 1A). Muscle pH decreased more rapidly in *Mstn* KO than in control mice (Fig. 1B), in agreement with the faster *post-mortem* pH drop observed in double-muscled cattle (Clinquart, Vaneenaeme, Mayombo, Gauthier, & Istasse, 1995). This effect could reflect the different muscle glycogen level at sacrifice (Alvarez, Moran, Keenan, Mullen, & Delgado-Pando, 2019). It is also in accordance with the higher proportion of fast glycolytic fibres in *Mstn* KO (Chelh et al., 2009) because these fibres are associated with faster meat ageing (Ouali et al., 2006).

We next monitored troponin T and desmin (two myofibrillar proteins that are usually studied in *post-mortem* muscle ageing) degradation during *post-mortem* ageing by western blot analysis. As previously reported (Ho, Stromer, & Robson, 1994; Huff-Lonergan et al., 1996), we detected bands ranging from 30 to 31 kD and from 37 to 50 kD in total *Gastrocnemius* muscle protein extracts that corresponded to troponin T (Fig. 1C) and desmin (Fig. 1D) degradation products, respectively. Moreover, accumulation of troponin T (Fig. 1C and 1E) and desmin (Fig. 1D and 1F) degradation products progressively increased and at 24 h *post-mortem* was significantly higher in *Mstn* KO than in WT muscle samples. Collectively, these results confirmed the degradation of myofibrillar proteins (troponin T, desmin) during the first *post-mortem* day, with a more pronounced degradation in *Mstn* KO mice.

3.2. Post-mortem muscles from *Mstn* KO mice show evidence of protection against oxidative damage

As oxidative stress increases after death (Kanner, 1994), we then compared the ROS levels and oxidative damage in *Mstn* KO and WT muscles at 0 and 24 h after death (because myofibrillar degradation was higher at 24 h *post-mortem*). ROS levels were significantly lower in *Mstn* KO muscles than WT muscles (Fig. 2A). Then, we quantified TBARS

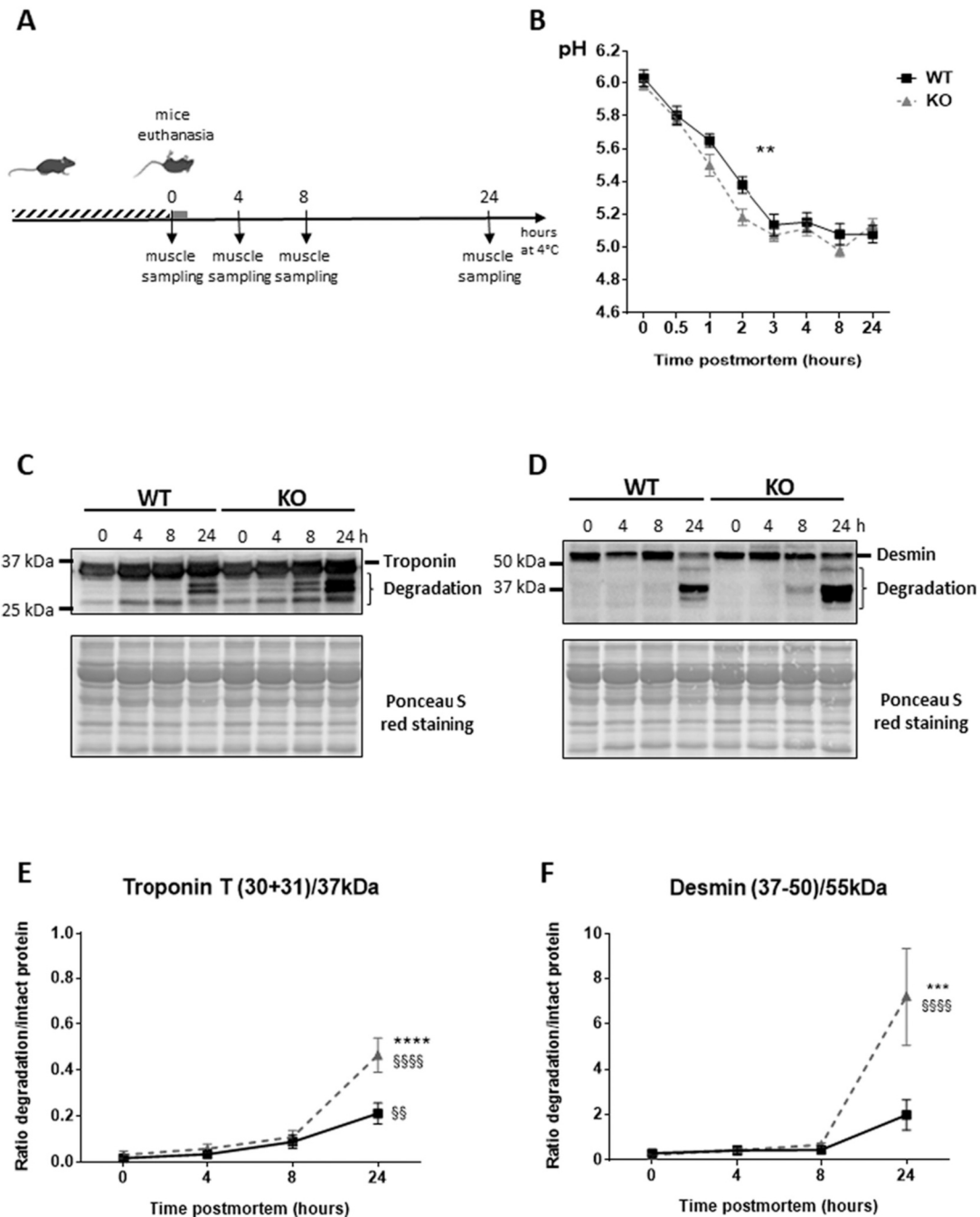


Fig. 1. In myostatin-deficient muscles, pH decreases faster and myofibrillar protein degradation is higher during *post-mortem* ageing.

A) Experimental design. Muscle samples from 6-month-old mice were collected at different time points after sacrifice by cervical dislocation and exsanguination. Carcasses were kept in a temperature-controlled room ($22 \pm 1^\circ\text{C}$) for 1 h (grey area) and then at 4°C for up to 24 h *post-mortem*. At each time point, muscles were removed, weighed and quickly frozen in liquid nitrogen until analysis, or used straightaway for pH measurement. Striped area indicates the 24 h period of food deprivation prior to euthanasia.

B) pH decrease in *Longissimus thoracis* muscles of WT and *Mstn* KO animals at different time points after death. Values are the mean \pm SEM ($n = 8$ mice for each time point *per* group); ** $P < 0.01$ vs. WT (main effect genotype by two-way ANOVA).

C) and D) Representative immunoblots of troponin T and desmin degradation, and Ponceau S staining (loading control) in *Gastrocnemius* muscle samples from WT and *Mstn* KO animals collected at different time points after death.

E) and F) Densitometric analysis of troponin T and desmin degradation in *Gastrocnemius* muscles from WT and *Mstn* KO animals collected at different time points after death. Results are expressed as the ratio of the signal intensity of the degradation bands (30–31 kDa for troponin T, and 37–50 kDa for desmin) over that of the intact protein band (37 kDa for troponin T; 55 kDa for desmin). Values are the mean \pm SEM ($n = 8$ mice for each time point *per* group); *** $P < 0.001$ and **** $P < 0.0001$ vs. relevant WT (specific effect for genotype by Sidak's multiple comparisons test); §§ $P < 0.01$ and §§§§ $P < 0.0001$ vs. relevant T0 (specific effect for time by Sidak's multiple comparisons test).

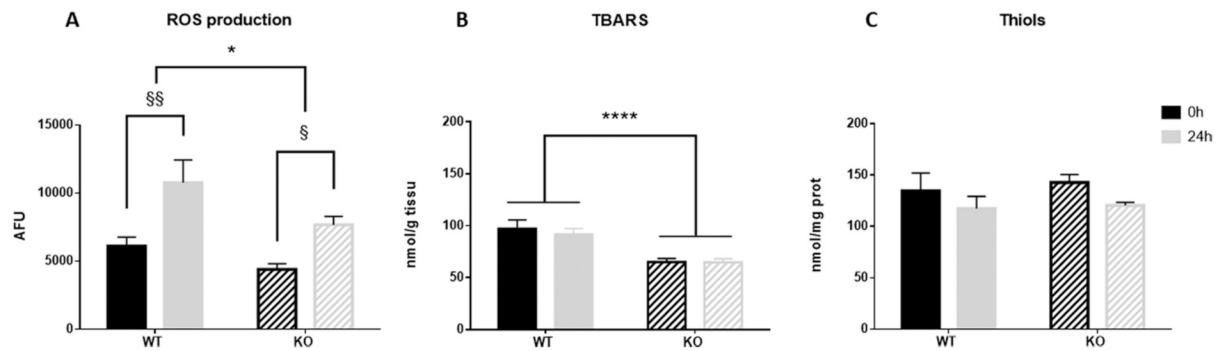


Fig. 2. Impact of myostatin deficiency on muscle oxidative stress markers during *post-mortem* ageing.

A) ROS production, B) TBARS and C) Thiols levels in *Quadriceps* muscle samples from WT (filled bars) and *Mstn* KO mice (striped bars) at 0 h (black) and 24 h (light grey). Values are the mean \pm SEM ($n = 5-8$ mice for each time point per group); * $P < 0.05$ and **** $P < 0.0001$ vs. WT (main effect for genotype by two-way ANOVA); § $P < 0.05$ and §§ $P < 0.01$ vs. relevant T0 (specific effect for time by Sidak's multiple comparisons test). AFU, arbitrary fluorescent unit.

production to assess lipid peroxidation, and found significantly lower TBARS levels in *Mstn* KO than WT muscles (0 h ($P < 0.001$) and 24 h ($P < 0.01$)) (Fig. 2B). Conversely, the level of muscle protein oxidation, evaluated by quantification of thiol groups, was not different in the two genotypes at 24 h *post-mortem* (Fig. 2C). Overall, these results indicate lower oxidative stress in *post-mortem* muscles from *Mstn* KO mice.

3.3. *Post-mortem* profile of key determinants of the UPS and autophagy in *Mstn* KO and WT muscles

To mechanistically explain the difference in *post-mortem* muscle protein degradation between genotypes, we analysed the UPS status and first monitored the atrophy-related pathways. It is known that the Akt/FOXO pathway controls protein degradation, and that FOXO activity is suppressed by Akt-mediated phosphorylation, thus preventing the expression of atrogenes, such as atrogin-1 and MuRF-1 (Sandri et al., 2004; Stitt et al., 2004). Akt phosphorylation was strongly increased at sacrifice in *Mstn* KO mice compared with controls, reflecting the higher protein synthesis in these animals (Fig. 3A-B). However, after death, Akt phosphorylation levels progressively decreased and were comparable between genotypes. Quantification of FOXO1, a master regulator that activates catabolism through the UPS and autophagy (Milan et al., 2015; Sandri et al., 2004), did not highlight any difference in *post-mortem* expression and phosphorylation in *Mstn* KO and in WT muscles (Fig. 3C-D). Similarly, the abundance of MuRF1 (an E3 ubiquitin ligase involved in the UPS) did not change after death, whatever the genotype (Fig. 3C and 3E). In line with this observation, western blot analysis using anti-ubiquitin antibodies showed no significant change in the abundance of ubiquitinated proteins in muscle samples from both genotypes, although they tended to decrease over time (Fig. 3F-G, $P = 0.0721$). Last, analysis of proteasomal activity in *post-mortem* WT and *Mstn* KO muscle extracts using the fluorogenic peptide Suc-LLVY-AMC (Fig. 3H), in the presence or absence of the highly specific proteasome inhibitor epoxomicin to exclude the effects of other proteases, showed a stable activity during the first 24 h after death without differences between genotypes. These data suggest that the UPS activity is not increased in skeletal muscle immediately after death and therefore does not explain the increased *post-mortem* muscle protein degradation in *Mstn* KO mice compared with controls.

To determine whether other proteolytic systems were involved, we first analysed several key determinants of autophagy in muscles collected from *Mstn* KO and WT mice at different time points after death.

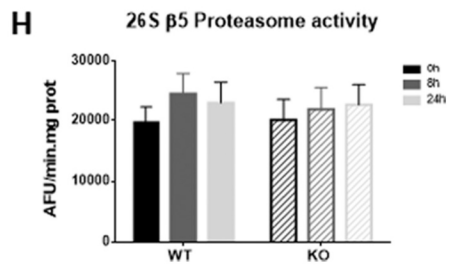
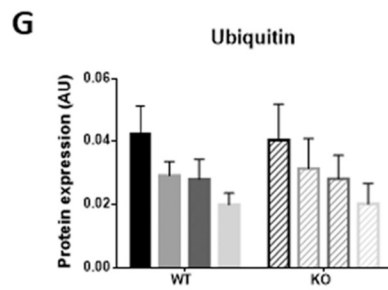
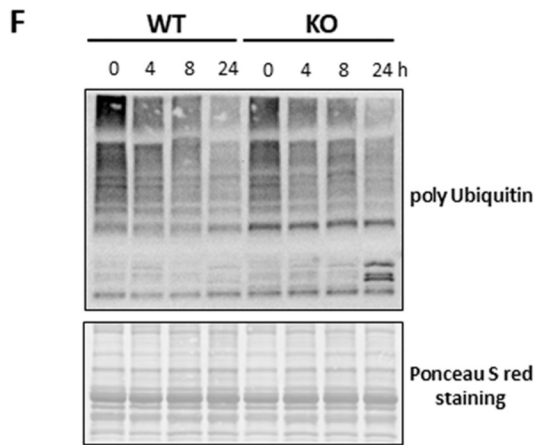
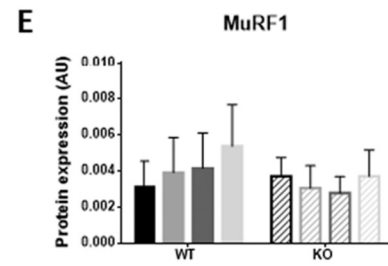
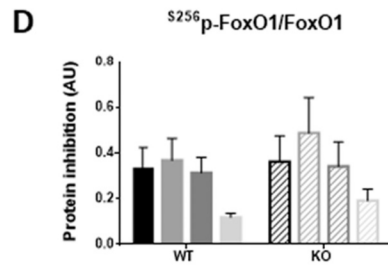
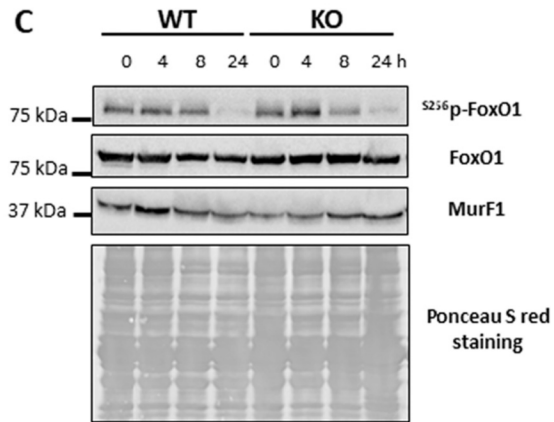
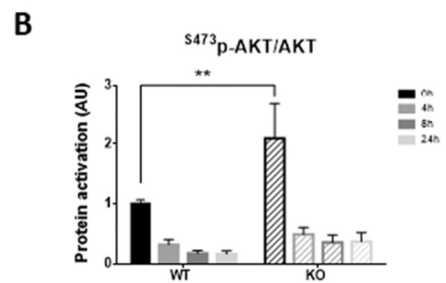
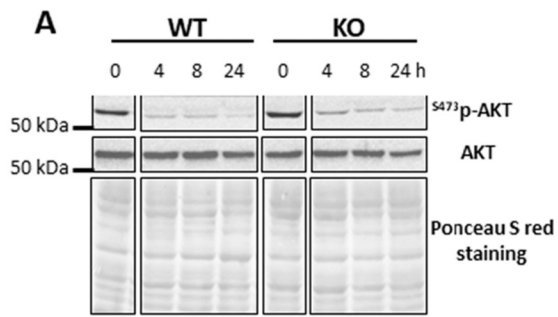
Analysis of AMPK phosphorylation status, a sensor of cell energy and upstream activator of autophagy, showed a transient increase of phosphorylated AMPK in WT mice at 4 h *post-mortem* (Fig. 4A-B). In addition, we confirmed that the *post-mortem* period was associated with energy exhaustion, as illustrated by the ATP content depletion in both WT and *Mstn* KO muscles starting at 4 h *post-mortem* (Fig. 4C). ATP content was lower in *Mstn* KO muscles at all *post-mortem* time points.

We then examined LC3-I conversion to LC3-II through lipidation, and therefore changes in LC3-II abundance, an indicator of autophagosome formation. We did not observe any change in WT and *Mstn* KO muscles during the *post-mortem* time course (Fig. 4D). As expected, treatment with colchicine (a microtubule depolarizing agent that blocks autophagosome maturation) resulted in LC3II accumulation in both genotypes (Fig. 4E). Calculation of the autophagy flux (LC3II Col - LC3II Ctl) revealed that autophagy flux was lower in *Mstn* KO than WT muscles (Fig. 4D and 4F), but did not change (both genotypes) over time.

These results suggest that the UPS and autophagy are not involved in the increased *post-mortem* muscle protein degradation observed in *Mstn* KO mice. As starvation is the most studied condition to induce the UPS and autophagy, we also checked the effect of acute starvation during the pre-slaughter period. Compared with fed animals, we did not detect any *post-mortem* change in myofibrillar protein degradation, or in the autophagy, proteasome, and oxidative stress marker expression after a 24 h pre-slaughter period of starvation in muscles from *Mstn* KO and WT mice (Supplementary Fig. S1, S2, S3).

3.4. Calpain activity and myofibrillar protein degradation during early *post-mortem* muscle ageing

Next, we investigated the implication of two calcium-activated proteases calpains and caspases in *post-mortem* proteolysis (Koochmariaie & Geesink, 2006; Ouali et al., 2006). Calpain activity was higher in *Gastrocnemius* muscles from *Mstn* KO than WT mice just after death ($T = 0$) (Fig. 5A). Conversely, caspase activity significantly decreased over time in both WT and *Mstn* KO mice (no between-group difference) (Supplementary Fig. S4). To confirm calpain involvement in the *post-mortem* myofibrillar protein degradation, we incubated *post-mortem* muscles with the calpain inhibitor MDL-28170. We found that the difference in *post-mortem* degradation of desmin and troponin T was abolished when *Mstn* KO and WT muscle samples were incubated with the calpain inhibitor (Fig. 5B-E).



(caption on next page)

Fig. 3. Effect of myostatin deficiency on proteolytic activity and UPS marker expression during *post-mortem* ageing.

- A) Representative immunoblot of Akt and Akt phosphorylated at Ser473 in WT and *Mstn* KO *Gastrocnemius* muscles collected at different time points after death. Ponceau S staining was used as loading control. Lane delineation by a black line shows lane rearrangement in the image relative to the sample deposition order on the SDS PAGE.
- B) Densitometric quantification of Akt activation (expressed as the ratio of phosphorylated over total protein expression) in WT (filled bars) and *Mstn* KO (striped bars) *Gastrocnemius* muscles during the *post-mortem* time course. Values are the mean \pm SEM ($n = 4-6$ mice for each time point per group); ** $P < 0.01$ vs. relevant WT (specific effect for genotype by Sidak's multiple comparisons test).
- C) Representative immunoblot of FOXO1, FOXO1 phosphorylated at Ser256, and MuRF1 protein expression in WT and *Mstn* KO *Gastrocnemius* muscles collected at different time points after death. Ponceau S staining was used as loading control.
- D) and E) Densitometric quantification of FOXO1 inhibition (expressed as the ratio of phosphorylated over total protein expression) (D) and MuRF1 protein expression relative to Ponceau S staining (E) in WT (filled bars) and *Mstn* KO (striped bars) *Gastrocnemius* muscles during the *post-mortem* time course. Values are the mean \pm SEM ($n = 4-6$ mice for each time point per group).
- F) Representative immunoblot of total protein ubiquitination in WT and *Mstn* KO *Gastrocnemius* muscles collected at different time points after death. Ponceau S staining was used as loading control.
- G) Densitometric quantification of total protein ubiquitination relative to Ponceau S staining in WT (filled bars) and *Mstn* KO (striped bars) *Gastrocnemius* muscles during the *post-mortem* time course. Values are the mean \pm SEM ($n = 4$ mice for each time point per group). Two-way ANOVA showed a trend towards protein ubiquitination decrease over time in both genotypes ($P = 0.0721$).
- H) Activity of the 26S $\beta 5$ proteasome in WT and *Mstn* KO *Gastrocnemius* muscles collected at different time points after death. Values are the mean \pm SEM ($n = 7$ mice for each time point per group).

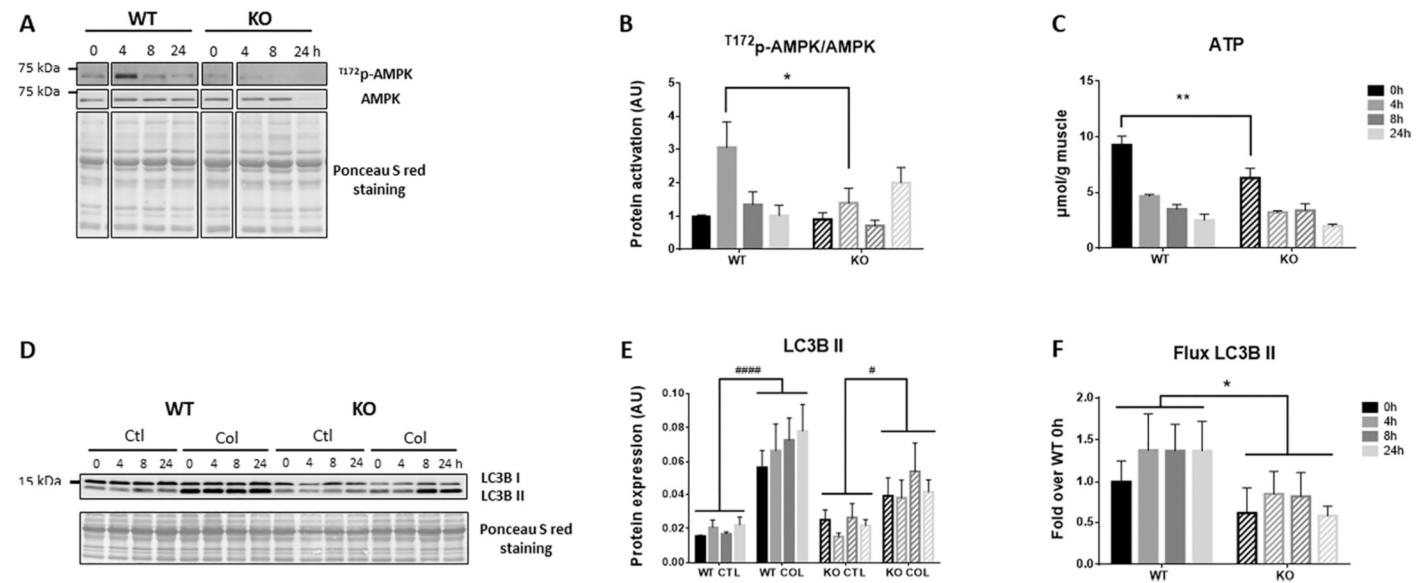


Fig. 4. Effect of myostatin deficiency on autophagy-related signaling proteins and markers, and on the autophagic flux during early *post-mortem* muscle ageing.

- A) Representative immunoblot showing total AMPK and phosphorylated (Thr172) AMPK levels in WT and *Mstn* KO *Gastrocnemius* muscles during the *post-mortem* time course. Ponceau S staining was used as loading control. Lane delineation by a black line shows lane rearrangement in the image relative to the sample deposition order on the SDS PAGE.
- B) Densitometric quantification of AMPK activation (calculated as the ratio of phosphorylated AMPK over total AMPK expression) in WT (filled bars) and *Mstn* KO (striped bars) *Gastrocnemius* muscles during the *post-mortem* time course. Values are the mean \pm SEM ($n = 6-8$ mice for each time point *per* group); * $P < 0.05$ vs. relevant WT (specific effect for genotype by Sidak's multiple comparisons test).
- C) ATP content in WT (filled bars) and *Mstn* KO (striped bars) *Tibialis anterior* muscles during the *post-mortem* time course. Values are the mean \pm SEM ($n = 4$ mice for each time point *per* group); ** $P < 0.01$ vs. relevant WT (specific effect for genotype by Sidak's multiple comparisons test).
- D) Representative immunoblot showing *post-mortem* LC3B lipidation changes in *Gastrocnemius* muscles from WT and *Mstn* KO mice injected with saline (Ctl) or colchicine (Col). Ponceau S staining was used as loading control.
- E) Densitometric quantification of *post-mortem* LC3B lipidation relative to Ponceau S staining in *Gastrocnemius* muscles from WT (filled bars) and *Mstn* KO (striped bars) mice injected with saline (Ctl) or colchicine (COL). Values are the mean \pm SEM ($n = 4-6$ mice for each time point per group); # $P < 0.05$ and #### $P < 0.0001$ vs. relevant Ctl group (main effect for treatment by Sidak's multiple comparisons test).
- F) LC3BII autophagic flux measurement in WT (filled bars) and *Mstn* KO (striped bars) *Gastrocnemius* muscles during the *post-mortem* time course. LC3BII autophagic flux was quantified as the difference in LC3B-II protein level between *Gastrocnemius* muscles from colchicine- and saline-injected mice at each time point and is expressed as fold change relative to the WT value at $T = 0$. Values are the mean \pm SEM ($n = 4-6$ mice for each time point per group); * $P < 0.05$ vs. WT (main effect for genotype by two-way ANOVA).

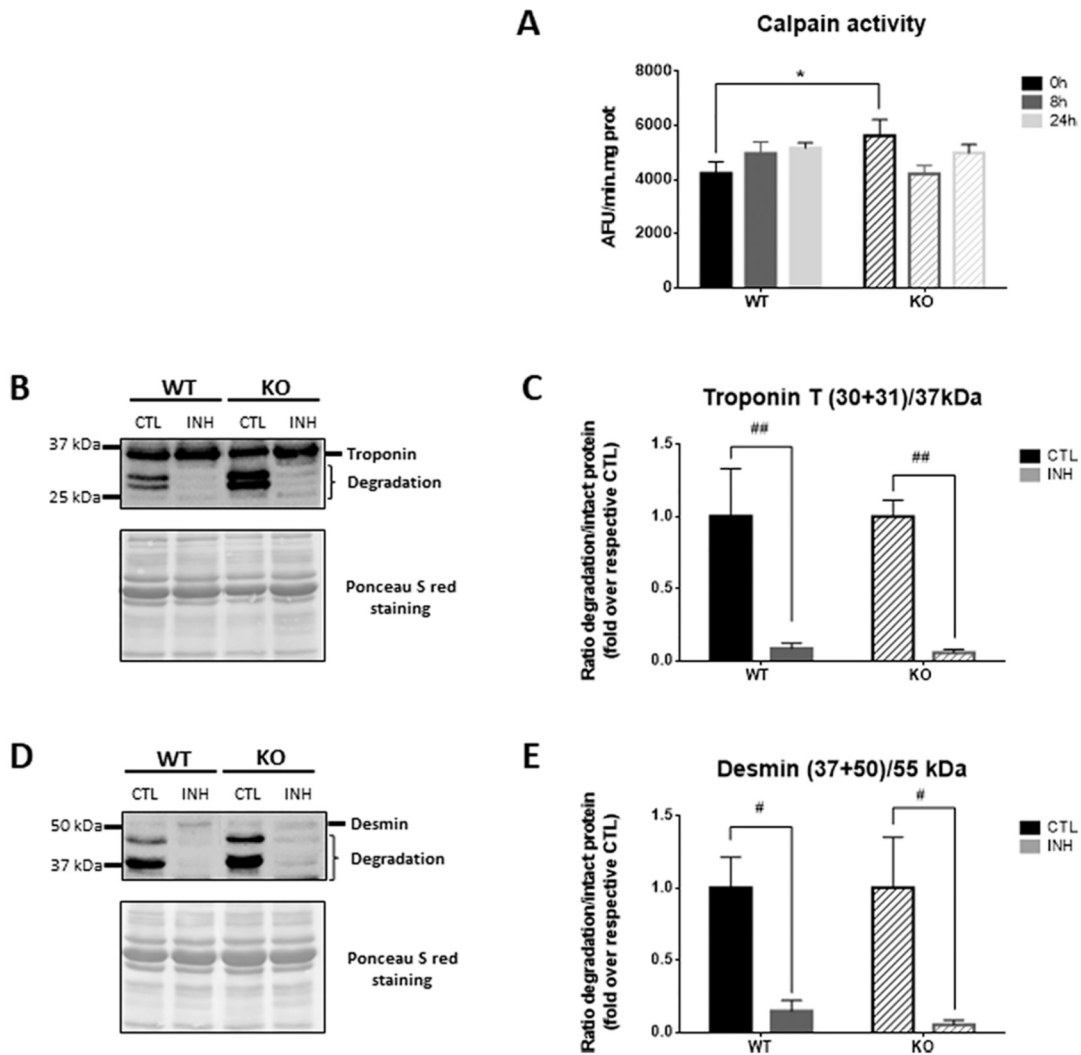


Fig. 5. Analysis of calpain activity during *post-mortem* ageing and effect of calpain inhibition on myofibrillar protein degradation.

A) Calpain activity in WT and *Mstn* KO *Gastrocnemius* muscles during the *post-mortem* time course. Values are the mean \pm SEM ($n = 8$ mice for each time point *per* group); * $P < 0.05$ vs. WT (specific effect for genotype by Sidak's multiple comparisons test).

B) Representative immunoblot showing troponin T degradation in WT and *Mstn* KO *Gastrocnemius* muscles incubated with saline (CTL) or MDL-28170, a calpain inhibitor (INH), for 24 h after death. Ponceau S staining was used as loading control.

C) Densitometric analysis of troponin T degradation in *Gastrocnemius* muscles of WT and *Mstn* KO animals incubated with saline (CTL) or MDL-28170, a calpain inhibitor (INH), for 24 h after death. Protein degradation ratios are expressed as the fold-change relative to the value in CTL animals (saline). Values are the mean \pm SEM ($n = 3$ per group); ## $P < 0.01$ vs. relevant CTL (specific effect for treatment by Sidak's multiple comparisons test).

D) Representative immunoblot showing desmin degradation profile in WT and *Mstn* KO *Gastrocnemius* muscles incubated with saline (CTL) or MDL-28170, a calpain inhibitor (INH), for 24 h after death. Ponceau S staining was used as loading control.

E) Densitometric analysis of desmin degradation in WT and *Mstn* KO *Gastrocnemius* muscles incubated with saline (CTL) or MDL-28170, a calpain inhibitor (INH), for 24 h after death. Protein degradation ratios are expressed as the fold change relative to the value in CTL animals (saline). Values are the mean \pm SEM ($n = 3$ per group); # $P < 0.05$ vs. relevant CTL (specific effect for treatment Sidak's multiple comparisons test).

4. Discussion

This study provides original observations on the effects of *Mstn* deficiency on the early *post-mortem* muscle proteolysis. By comparing the muscle protein degradation and changes in proteolytic systems (autophagy, UPS, calpains and caspases) in *post-mortem* muscles from WT and *Mstn* KO mice, we demonstrated that MSTN loss increased *post-mortem* protein breakdown associated with lower oxidative stress and lower basal ATP content. Moreover, the UPS and autophagy were active in *post-mortem* muscle (both genotypes), but the autophagy flux was lower in *Mstn* KO muscles. However, we did not find any difference in UPS and caspase activities between genotypes, whereas calpain activity was higher in *Mstn* KO than in WT muscle. These findings suggest that calpains might be driving the increased protein breakdown in *Mstn* KO

muscles during early *post-mortem* muscle ageing.

The involvement of the UPS and autophagy in *post-mortem* muscle proteolysis is still debated. Therefore, we wanted to precisely determine the implication of the UPS and autophagy in the *post-mortem* pattern of myofibrillar proteins degradation in WT and *Mstn* KO mice. First, we observed a different temporal profile of myofibrillar protein degradation between genotypes. Then, we found that the expression of FOXO1, a regulator of proteasome-mediated protein degradation and autophagy was not upregulated during the first 24 h of *post-mortem* muscle ageing. Accordingly, the expression of the MuRF1 E3 ubiquitin ligase, the profile of ubiquitinated proteins and proteasome activity remained unchanged during this period. Moreover, we did not detect any increase in LC3II level or autophagy flux changes in both genotypes, although the autophagy flux was lower in *Mstn* KO muscle. These results are consistent

with the fact that the UPS and autophagy are ATP-dependent processes, and soon after death, the ATP content in muscle is depleted in both genotypes. Altogether, these data suggest that the UPS and autophagy do not explain the *post-mortem* proteolysis differences between genotypes. However, they are in agreement with the previously published indirect evidence that these systems are active in *post-mortem* skeletal muscle (Lamare et al., 2002).

In recent years, it has been suggested that pre-slaughter management might influence the *post-mortem* muscle protein degradation. For instance, pig rearing strategies and stress related to animal transport affected *post-mortem* muscle autophagy (Rubio-Gonzalez et al., 2015). An increase in the pre-slaughter autophagic status following acute starvation results in *post-mortem* changes in muscle proteins (Nakanishi et al., 2017), specifically in the fraction of water-soluble proteins. As acute starvation is a potent autophagy and proteasome inducer (Finn & Dice, 2006; Rabinowitz & White, 2010), we hypothesized that changes in catabolic conditions during the pre-slaughter period might alter the muscle *post-mortem* proteolysis. However, we found that acute starvation prior to sacrifice had no effect on the *post-mortem* proteolysis in both genotypes. These discrepancies with previously published work might be explained by differences in the methods to analyse *post-mortem* proteolysis. Specifically, our study showed that *Mstn* KO muscles displayed lower autophagic flux consistent with a significant lower ROS level. This observation may be explained by metabolic differences between *Mstn* KO and WT muscles because the glycolytic metabolism is increased in *Mstn* KO muscles. Therefore, we cannot rule out that this difference in autophagy flux might have metabolic consequences during the conversion of muscle to meat. Additional studies are needed to address this point.

An important hallmark of the process driving the conversion of muscle to meat is the switch from aerobic to anaerobic metabolism. After slaughter, as a result of the oxygen supply disruption, ATP can be produced only through the glycolytic pathway, which is not very effective. Metabolized glycogen induces lactate production that causes the pH decrease from 7.2 to 5.4–5.7 in myofibrils. This pH decrease is faster in glycolytic fibres that use the anaerobic pathway for energy production (Clinquart, Hornick, Van Eenaeme, & Istasse, 1998). On the other hand, ATP reduction leads to a decreased activity of the sarco-plasmic ATPases, and consequently to increased Ca^{2+} concentrations in the endoplasmic reticulum. These metabolic changes lead to stress-induced activation of calcium-dependent proteolytic systems. Here, we found that the *post-mortem* pH decrease was faster in muscle of *Mstn* KO mice, in line with their predominant glycolytic muscle phenotype. Moreover, *post-mortem* degradation of myofibrillar proteins was more pronounced in *Mstn* KO muscle than WT muscle, associated with higher calpain activity. Altogether these data strongly support the hypothesis that *Mstn* deletion results in the conversion towards a more glycolytic phenotype that may be advantageous for inducing calcium-dependent proteolytic systems.

The calpain proteolytic system plays a central role in *post-mortem* proteolysis. Inhibition of micro-calpain reduces myofibrillar protein degradation in *post-mortem* muscles (Kent et al., 2004). Using *in vitro* models of *post-mortem* muscles, the degradation of desmin and troponin T has been linked to calpain activity (Bhat, Morton, Mason, & Bekhit, 2018). Our results indicated a greater activity of calpains in *Mstn* KO *post-mortem* muscles, but not of the other catabolic systems. This is in line with previous reports showing that desmin and troponin T are degraded faster in glycolytic type IIb muscle fibres than in oxidative type I fibres during *post-mortem* ageing, and that the proteolytic activity of Ca^{2+} -dependent micro-calpain is higher in fast glycolytic muscles (Muroya, Ertbjerg, Pomponio, & Christensen, 2010; O'Halloran, Troy, Buckley, & Reville, 1997). On the basis of all these data, we propose that the calpain system might explain the difference in *post-mortem* degradation of muscle proteins between WT and *Mstn* KO mice. In agreement, different studies in livestock animals have shown higher calpain activity in fast glycolytic muscles, associated with earlier *post-mortem*

degradation of myofibrillar proteins (Melody et al., 2004; O'Halloran et al., 1997). However, double-muscling breeds harbouring *Mstn* mutations display reduced calpain/calpastatin levels (Uytterhaegen et al., 1994). These discrepancies might be explained by differences in the species studied (bovine vs. mouse), muscle type, and analysis method. None of these studies investigated directly the degradation of myofibrillar protein products, a relevant parameter for the evaluation of *post-mortem* proteolysis.

Another hallmark of the muscle-to-meat conversion process is the progressive increase in ROS production and decrease of antioxidant capacity, leading to oxidative stress and cell damage (Lana & Zolla, 2015; Wang et al., 2018). By comparing ROS production and TBARS levels, we found that ROS production was lower in *Mstn* KO muscles than WT muscles. This is another evidence that *Mstn* deficiency is associated with a reduced redox state during the *post-mortem* period. This raises the question of whether the marked degradation of myofibrillar proteins in *Mstn* KO muscles is linked to the decreased redox state. Although the role of oxidative stress in *post-mortem* proteolysis is still debated, it is possible that myofibrillar proteins might change their susceptibility to proteolysis due to oxidative modifications (Zhang, Xiao, & Ahn, 2013). Alternatively or concomitantly, the activity of calpains, the major enzymes for the degradation of myofibrillar proteins, could also be affected. Indeed, it has been shown that oxidation induced by early *post-mortem* irradiation decreases calpain activity, slowing down the proteolysis rate during *post-mortem* muscle ageing and thereby, decreasing meat tenderization (Rowe, Maddock, Lonergan, & Huff-Lonergan, 2004). *MSTN* absence might induce a reducing environment that favours calpain activity in *post-mortem* muscle; however, we lack direct evidence to confirm the oxidation of calpains and myofibrillar proteins. Additional studies are needed to investigate the mechanisms underpinning the link between increased muscle proteolysis and redox status in *Mstn* KO muscle. Moreover, we cannot rule out the possibility that *MSTN* absence may affect, directly or indirectly, other parameters, such as Ca^{2+} concentration that in turn regulates calcium-dependent proteases.

4.1. Study limitation

It is acknowledged that the degradation of muscle myofibrils plays an important role in meat tenderness (Ouali et al., 2006). The proteolysis accounts for the variation in tenderness in some of the muscles but not of all the muscles (Rhee, Wheeler, Shackelford, & Koohmaraie, 2004). However, the tenderness is improved in all muscles with *Mstn* inactivation (Arthur, 1995; Fiems, 2012; Wheeler, Shackelford, Casas, Cundiff, & Koohmaraie, 2001). Given our results showing that proteolysis is increased in *Mstn* KO mice, we can speculate that similarly, increased proteolysis may increase meat tenderness in double muscling cattle. A limitation of our work is that we used *Mstn* KO mice to study the effect of *MSTN* absence on the *post-mortem* degradation of myofibrillar proteins. However, several previous studies have used mouse models to mimic slaughter and *post-mortem* storage conditions and they have shown that despite the species-specific physical and physiological differences, *post-mortem* mouse muscle exhibits similar patterns of protein degradation and redox status (Ehrenfellner, Zissler, Steinbacher, Monticelli, & Pittner, 2017; Morel et al., 2019). Nevertheless, it needs now to determine whether the regulation of *post-mortem* proteolysis by *MSTN* is correlated with tenderness in species of agronomical interest, and whether it depends on the muscle topology and intramuscular fat content.

In conclusion, the present work shows that *MSTN* absence affects the rate and extent of muscle protein breakdown during the early *post-mortem* period. It also found that the UPS and autophagy are active in the early *post-mortem* muscle and that the calpain system could explain the increased *post-mortem* degradation of muscle proteins in *Mstn* KO mice compared with WT controls. Furthermore, our data suggest that *post-mortem* degradation of myofibrillar proteins could be related to the *post-mortem* muscle redox state. These findings reinforce the well-established role of calpain proteolytic system in the *post-mortem* proteolytic

mechanisms implicated in meat ripening. Identifying the mechanisms that regulate *post-mortem* proteolysis in skeletal muscle is of economic importance because this is part of the process by which muscle is turned into meat.

Declaration of Competing Interest

The authors have declared no conflict of interest.

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