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# Analysis of cashmere goat meat by label-free proteomics shows that MYL3 is a potential molecular marker of meat toughness

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**Abstract:** The Inner Mongolia cashmere goat is famous for its bright white cashmere fibre. However, little attention is given to the excellent characteristics of this breed's meat. We used label-free proteomics to analyse the total protein content in five different muscles, and 1 227 proteins were detected. Through sequential windowed acquisition of all theoretical fragment ions (SWATH), 16, 33, 49, 39, and 31 differentially expressed proteins were successfully detected in the five muscles. Protein–protein interaction network analysis of differentially expressed proteins revealed many strong interactions related to fatty acid beta oxidation and muscle development. Based on SWATH in five muscles, 25 differentially expressed proteins related to muscle development were detected, including seven muscle fibre structural proteins (ACTG2, ACTN4, TAGLN, MYL3, MYL1, MYL6B and MYH4). Finally, immunohistochemical analysis of MYL3 showed that the proportion of MYL3 may be a potential molecular marker for muscle toughness.

**Keywords:** SWATH; interaction network; muscle fibre type

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The Inner Mongolia cashmere goat is famous for its fine, soft, and bright white cashmere fibre (Wang et al. 2018; Zhang et al. 2019). However, little attention is given to the excellent characteristics of this breed's meat, such as its juiciness and easily absorbed nutrients. The quality of Inner Mongolia cashmere goat meat varies depending on the part of the carcass that the meat is derived from. For example, the physicochemical properties differ among the *gluteal* muscles (GM), *longissimus dorsi* muscle (LM), and *m. triceps brachii* (TB). The GM has significantly higher moisture and fat content than the other two muscles, while the LM contains the most protein and the TB contains the highest ash content (Zhao et al. 2014). To date, proteomic studies of meat quality have focused mainly on comparisons between or among the GM, LM and TB (Yu et al. 2015; Hwang et al. 2017). However, there has not been a systematic study including the TB, *biceps femoris* (BF), *intercostal* muscle (IM), LM, and GM.

Proteomics is a powerful tool to explain differences in meat quality. Research shows that differential protein expression is correlated with pH, meat colour, water-holding capacity and tenderness (Huang et al. 2014; Wu et al. 2015a). Muscle tissue is an important site of protein storage, and different proportions of constituent proteins in muscle fibres, also known as muscle fibre structural proteins, influence meat quality (Gao et al. 2019). Meat with higher proportions of type I and IIa muscle fibres is higher in quality than meat containing more type IIb muscle fibres (Kim et al. 2018). The tenderness or toughness of meat is mainly influenced by structural proteins such as actin, MHCs, MLCs, the troponin complex, and titin (Lana and Zolla 2016). The tenderness of meat is strongly correlated with muscle fibre structural proteins. When the 31 kDa actin fragment is upregulated, the meat became tender, while upregulation of MYLC3 and MYLC1 had the opposite effect. As the phosphorylation of MYHC 1, 2, 6, and 7 decreased, meat tenderness decreased. TNNT is another factor used to predict meat tenderness (Sun et al. 2014). Sequential windowed acquisition of all theoretical fragment ions (SWATH) is a rapid data-independent MS/MS acquisition method that allows complete and permanent recording of all detectable fragment ions from peptide precursors present in a biological sample (Liu et al. 2013). This technique has already been successfully used to compare the proteomic differences between the *longissimus lumborum* and *psoas major* muscles (Yu et al. 2018). Our research, based on proteomics, is the first to characterize differential protein expression in different muscles of the Inner Mongolia cashmere goat with SWATH technology. The objective of this study, based on the fact that muscle fibre type can influence meat quality, was to identify the structural proteins of muscle fibres that affect muscle fibre type and to discover molecular markers that can indicate meat tenderness.

## MATERIAL AND METHODS

### Animals

To obtain muscle samples from the Yiwei white cashmere goat breeding farm (Erdos, Inner Mongolia), animals were slaughtered after being electrocuted. Five muscles (the TB, BF, IM, LM, and GM) from three Inner Mongolia cashmere goats were collected according to the "Guidelines for Experimental Animals of the Ministry of Science and Technology" (Beijing, China). The Experimental Animal Ethics Committee of Inner Mongolia Agricultural University (GB 14925-2001) approved all the experimental procedures. All goats were of the same age (2.5 years old, wether) and came from the same farm, where they had consumed the same diet with free access to food and water.

### Extraction of total protein

Samples were ground in liquid nitrogen and then placed in 1% SDS and vortexed for 30 s/min at room temperature for 20 minutes. After sonication for 20 min, the suspension was centrifuged at 12 000 rpm for 30 min at 4 °C. After centrifugation, the supernatant was collected. The total protein concentration was measured using a bicinchoninic acid (BCA) kit (Beyotime, Shanghai, China).

### Tryptic digestion of total protein

Then, 100 µg of protein was added to 200 µl of a mixture of 8 M urea and 10 mM DL-dithiothreitol (DTT) and lysed for 1 h at 37 °C. The mixture was transferred into spin-x centrifuge tube filters

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(10 kb) and centrifuged for 40 min at 12 000 rpm. The filtrate was discarded. Then, 200 µl of 8 M urea was added to each spin-x centrifuge tube filters, and vortexed. Spin-x centrifuge tube filters were centrifuged at 12 000 rpm for 30 minutes. The filtrate was discarded. Then, 200 µl of 50 mM iodoacetamide was added to every spin-x centrifuge tube filters, and the tubes protected from light were incubated for 30 min. The filtrate was discarded. The following procedure was repeated three times: 100 µl of 50 mM ammonium bicarbonate was added to each tube, and the spin-x centrifuge tube filters were centrifuged for 20 min (12 000 rpm). 0.4 µg trypsin was added to each tube, and the spin-x centrifuge tube filters were incubated overnight at 37 °C and centrifuged for 30 minutes. Fifty microlitres of ammonium bicarbonate was added to each tube, and the samples were centrifuged for 30 min, after which the filtrate was collected and freeze-dried.

## Sample analysis by LC-MS/MS

LC-MS/MS (LC: Ekspert NanoLC 400, MS/MS: Sciex TripleTOF 5600; Sciex, Framingham, MA, USA) yielded a total of 45 files (three goats, five muscles, three replicates of each muscle). A C18 column (NanoLC 3 µm, ChromXP C18CL, 120 A, 15 cm × 75 µm) was injected with approximately 2 µg of enzymatic sample and 0.1% formic acid acetonitrile solution at a flow rate of 5 µl/min to separate the peptides. The eluent proportion was increased from 3% to 25% over 38 min, 25% to 32% over 5 min, and 32% to 80% over 2 min; kept at 80% for 3 min; reduced to 3% over 1 min; and kept at 3% for an additional 8 minutes. The mass spectrometry conditions were as follows: mass range between 150–1 800 m/z, resolution of 30 000 for $MS_1$ and 15 000 for $MS_2$.

## Data processing

For protein identification, we used Protein Pilot v4.5 software (Sciex, Framingham, MA, USA) and the UniProt/SWISS-PROT/*Capra hircus* database (https://www.UniProt.org). The results were filtered at a 1% FDR. The selected search parameters included the use of trypsin as the enzyme, allowing up to two missed cleavage sites. The peptide mass tolerance was ± 15 ppm, and the fragment mass tolerance was 20 mmu. Ion chromatograms were extracted using PeakView (Sciex, Framingham, MA, USA) software. Then, MarkerView (Sciex, Framingham, MA, USA) software was used for protein quantification and screening of differentially expressed proteins. The data for protein quantification were standardized using the sum of the total areas. To correct protein abundance variation, $\log_2$-values of the measured areas were normalized by the median values. We used Student's *t*-tests with Benjamini-Hochberg correction of the *P*-value to compare differentially expressed proteins among the five muscles ($P < 0.05$).

## GO and KEGG analyses

To clarify the biochemical metabolic and signalling pathways associated with meat, GO (Gene Ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) were used for functional analysis of differentially expressed proteins ($P < 0.05$) (Ebhardt et al. 2017).

## Construction of the PPI network

STRING (https://string-db.org/), a website containing a wealth of information about protein–protein interactions (PPIs), was used to build a PPI network. We uploaded the information of differentially expressed proteins to STRING v10.5 and obtained the PPI information. We set the minimum interaction score to 0.4. Finally, we created a visualization plot of the PPIs in Cytoscape v3.4.0 (http://manual.cytoscape.org/en/3.7.2/).

## Western blotting

The denatured proteins were separated using SDS-PAGE and transferred to PVDF membranes. PVDF membranes were incubated overnight in a dilution of mouse monoclonal MYL3 primary antibody (Abcam, Cambridge, MA, USA, dilution 1 : 1 000). The membranes were incubated with fluorescent goat anti-mouse secondary antibody (LI-COR Biosciences, Lincoln, NE, USA, dilution 1 : 30 000) for 1 h after washing three times. The results were finally observed with a LI-COR Odyssey 10 NIR imager (LI-COR Biosciences, Lincoln, NE, USA).

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### Immunohistochemical analysis

Muscle samples were fixed in 4% paraformaldehyde and rinsed with running water overnight. After being dehydrated, transparentized, dewaxed, and embedded, the samples were embedded in hard wax and stored. Then, the tissue samples were sliced into 8 μm sections. Antigen retrieval was performed after dewaxing, hydration, and incubation in 3% $H_2O_2$. The samples were blocked in 5% BSA at room temperature for 1 h, after which murine MYL3 primary antibody (Abcam) was applied. Then, the samples were incubated at 4 °C overnight. At the same time, designated negative controls were incubated in 5% BSA. All samples were rinsed in PBS, after which the fluorescent goat anti-mouse secondary antibody (dilution 1 : 1 000) was added, and the samples were incubated at room temperature for 1 hour. The samples were rinsed in PBS again, and the colour was developed with haematoxylin. Finally, the slides were rinsed in water for 30 min, dehydrated, cleared, and sealed.

## RESULTS AND DISCUSSION

### Protein identification and functional annotations

In total, 1 227 shared proteins were detected in the five investigated muscles at an FDR of ≤ 1% (Figure 1A), and 707, 727, 647, 587, and 645 pro-

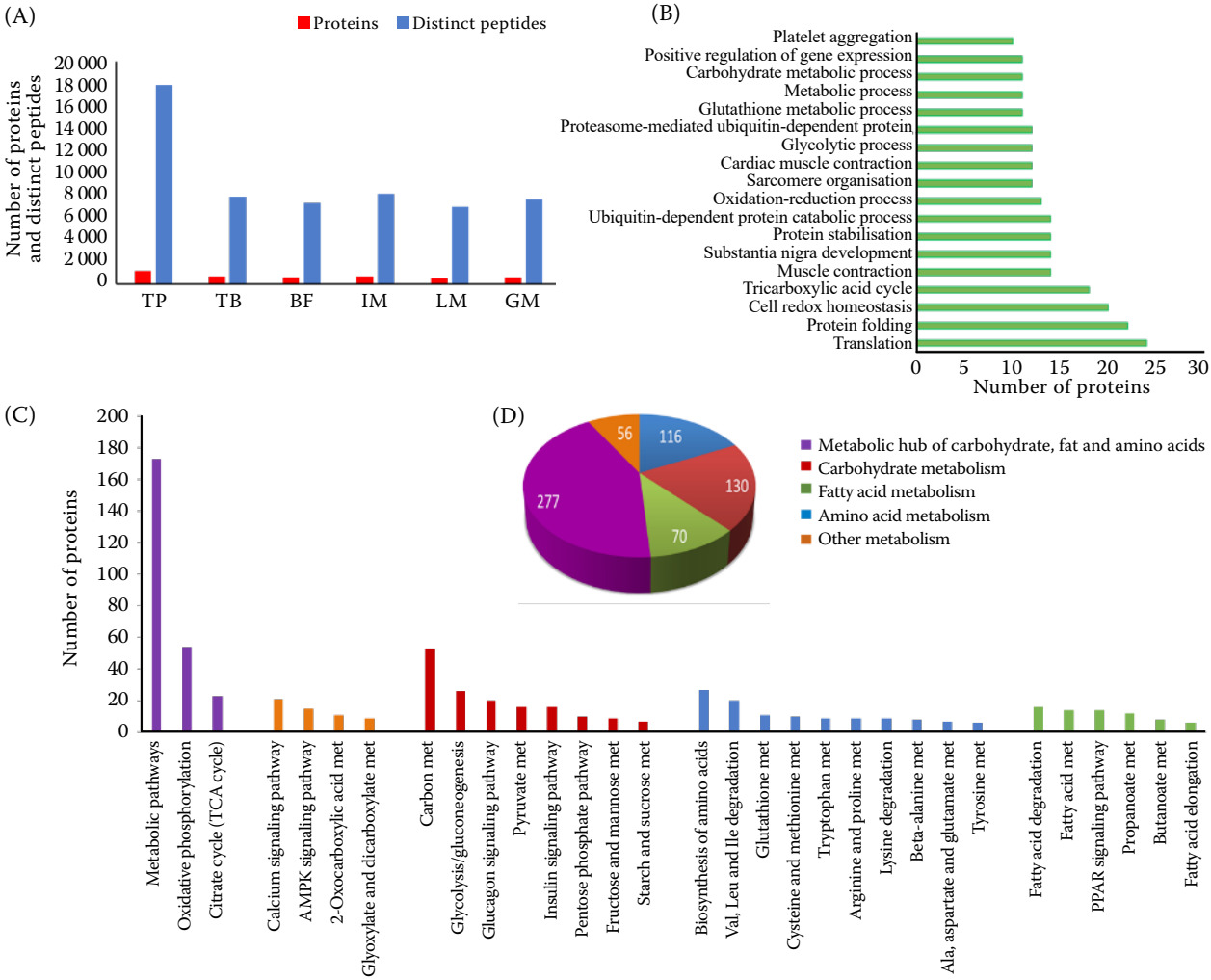


Figure 1. Protein identification and analysis in the *m. triceps brachii* (TB), *biceps femoris* (BF), *intercostal* muscle (IM), *longissimus dorsi* muscle (LM), and *gluteal* muscles (GM) of Inner Mongolia cashmere goats

Ala = alanine; Ile = isoleucine; Leu = leucine; met = metabolism; TP = total protein; Val = valine

(A) Basic information of protein and peptide identification. (B) Biological process terms for the total proteins. (C) KEGG pathway enrichment of total proteins associated with meat quality. (D) KEGG pathway classification of total proteins

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teins were successfully detected in the TB, BF, IM, LM, and GM, respectively. Biological processes of GO annotations showed that (Figure 1B) proteins participated in cell redox homeostasis, the tricarboxylic acid cycle and muscle contraction. KEGG pathway analysis of the total proteins showed that (Figure 1C) 1 098 proteins belonged to 31 signalling pathways associated with meat quality, including PPAR signalling pathways, fatty acid degradation, and biosynthesis of amino acids. The total set of proteins can be roughly subdivided into four categories (Figure 1D), including 130 overrepresented in carbohydrate metabolism, 116 in protein metabolism, 70 in lipid metabolism and 277 in pathways common to carbohydrate, lipid and protein metabolism. The metabolic processes for carbohydrates, lipids and proteins are interrelated. Overall, the proteins found in the five muscles were mainly associated with three major metabolic pathways: carbohydrate, lipid and protein metabolism. In this study, there were many signalling pathways involved in lipid metabolism, such as the PPARγ signalling pathway and fatty acid metabolism. The deposition of intramuscular fat is a key factor affecting meat quality (Calnan et al. 2017). Therefore, in the lipid metabolism pathway, some key genes related to intramuscular fat deposition have an important influence on muscle quality. Additionally, glycolysis/gluconeogenesis; the pentose phosphate pathway; alanine, aspartate and glutamate metabolism and cysteine and methionine metabolism are involved in carbohydrate and protein metabolism-related signalling pathways. Different mechanisms of carbohydrate oxidation affect proteins, enzymes and hormones in different metabolic pathways, further affecting the tenderness, pH, colour, and, ultimately, quality of meat (Luo et al. 2017; Malheiros et al. 2019).

## Comparison of the protein profiles of different muscles

We compared proteome signatures from each cut with those from the other four muscles, and fold change > 2 or < 0.5 and $P < 0.05$ were used as cut-off values to screen the most interesting proteins for further investigation. Volcano plots of differentially expressed proteins were prepared using the values of log (fold change) and $\log_{10}$ ($P$-value). There were 116 differentially expressed proteins in the Inner Mongolia cashmere goat samples overall (Figure 2), and in the TB, BF, IM, LM, and GM comparisons (Figure 2), 16, 33, 49, 39, and 31 differentially expressed proteins were identified. In the IM group, 38 proteins were upregulated, and 11 proteins were downregulated. PPI network analysis of differentially expressed proteins in the STRING database was used to identify the interrelations of differentially expressed proteins among the five muscles. The PPI network was constructed using Cytoscape software. Three strong interactions were found in relation to muscle development, myofibril assembly and the electron transport chain in the IM group (Figure 3): PPP2CA, PPP2R4, PPP2R2A, LMNA, LMNB2, CASQ2, CSRP3, MYL1, MYL3 and MYL6B had interactions related to muscle development; OGM, LUM, PRELP, BGM, ASPN, FMOD, DCN, KRT18, ANXA1 and ANXA2 had interactions related to myofibril assembly; and NDUFS1, NDUFA9, MT-CO1, MT-CO2, COX6A2 and ALDH9A participated in the electron transport chain. Two strong interactions were found among the BF group (Figure 3); one showed that MYOZ1, CSRP3, MYL3, CKMT2, ALDOA, CKM, ATPZA1, CS, ACADS, ETH1 and ETF had a pivotal role in myocyte production and energy supply by glycolysis, and the other showed that NDUFS3, NDUFS7, NDUFB1, NDUFA7, LONP1, TMEM14C had a pivotal role in fatty acid beta oxidation. The prediction of the protein interaction networks of the TB group related to long-chain fatty acid transport showed that FABP4, FABP3, and MB had pivotal roles in the network (Figure 3). The central interaction network of the LM group was related to the electron transport chain (Figure 3), and the interacting proteins of the GM group mainly included NDUFB7, NDUFA5, NDUFS7, MT-CO2, COX6C5, ACTN4, ACTG2, MYL6B, MYH4, VCL, MYOZ2, CASQ2, PLN, ATP2A1, ATP2A2, CAMK2A and PVALB, which are involved in muscle contraction accompanied by calcium transport (Figure 3). In conclusion, the 318-protein interaction network among the five muscles consists mainly of proteins involved in muscle fibre development, muscle fibre assembly, energy transfer and fatty acid oxidation.

## Differentially expressed proteins associated with muscle growth and development

Twenty-five differentially expressed proteins from five muscles related to muscle growth and

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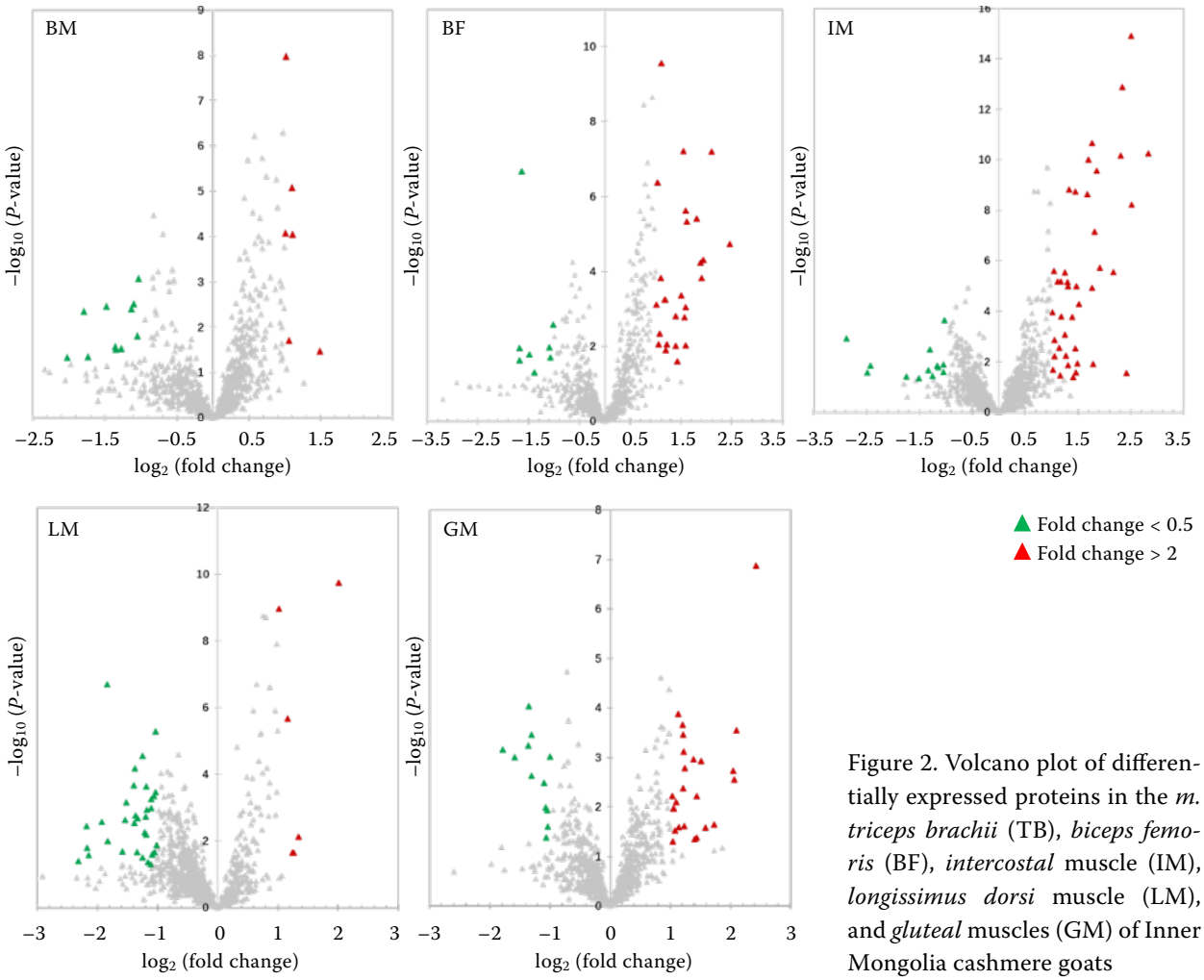


Figure 2. Volcano plot of differentially expressed proteins in the *m. triceps brachii* (TB), *biceps femoris* (BF), *intercostal* muscle (IM), *longissimus dorsi* muscle (LM), and *gluteal* muscles (GM) of Inner Mongolia cashmere goats

development were detected (Table 1). Fourteen of those proteins can be divided into four categories according to function: MYOZ1 and MYOZ2 affect myofibril formation; PPP2R2A, PPP2CA, and PPP2R4 participate in microtubule polymerization and degradation; LMNA and LMNB2 are associated with skeletal muscle development; and ACTG2, ACTN4, TAGLN, MYL3, MYL1, MYL6B and MYH4 are muscle fibre structural proteins, among which MYL3, MYL1 and MYL6B are light-chain myosins. Through SWATH technology, seven different muscle fibre structural proteins were identified in the total protein samples. These seven proteins are involved in two strong protein networks related to myofibril assembly and muscle contraction with calcium transport. Among these seven proteins, there are few studies on meat quality in relation to ACTG2, ACTN4, and TAGLN. MYH4 is significantly associated with the number of type IIa muscle fibres and with water-holding capacity (Wu et al. 2015b; Cho et al. 2016). MYL1 is an early marker for differentiating fast muscle cells, negatively affecting the proliferation of myoblasts (Burguiere et al. 2011). With lower MYL3 expression levels during body growth, IMF deposition could be accomplished more effectively. MYL3 is a marker of the negative regulation of intramuscular fat development (Zhang et al. 2010). The proteins MYL1, MYL3 and MYL6B were identified in the present study and are known to be associated with the phenotype of meat tenderness (Polati et al. 2012; Rosa et al. 2018). Light-chain muscle fibre structural proteins have an important influence on the proliferation of myoblasts, IMF and tenderness, and among all light-chain muscle fibre structural proteins, MYL3 is the most important for meat quality.

## Confirmation of proteins by Western blot

Western blotting was performed to detect the expression levels of MYL3, which is a significantly

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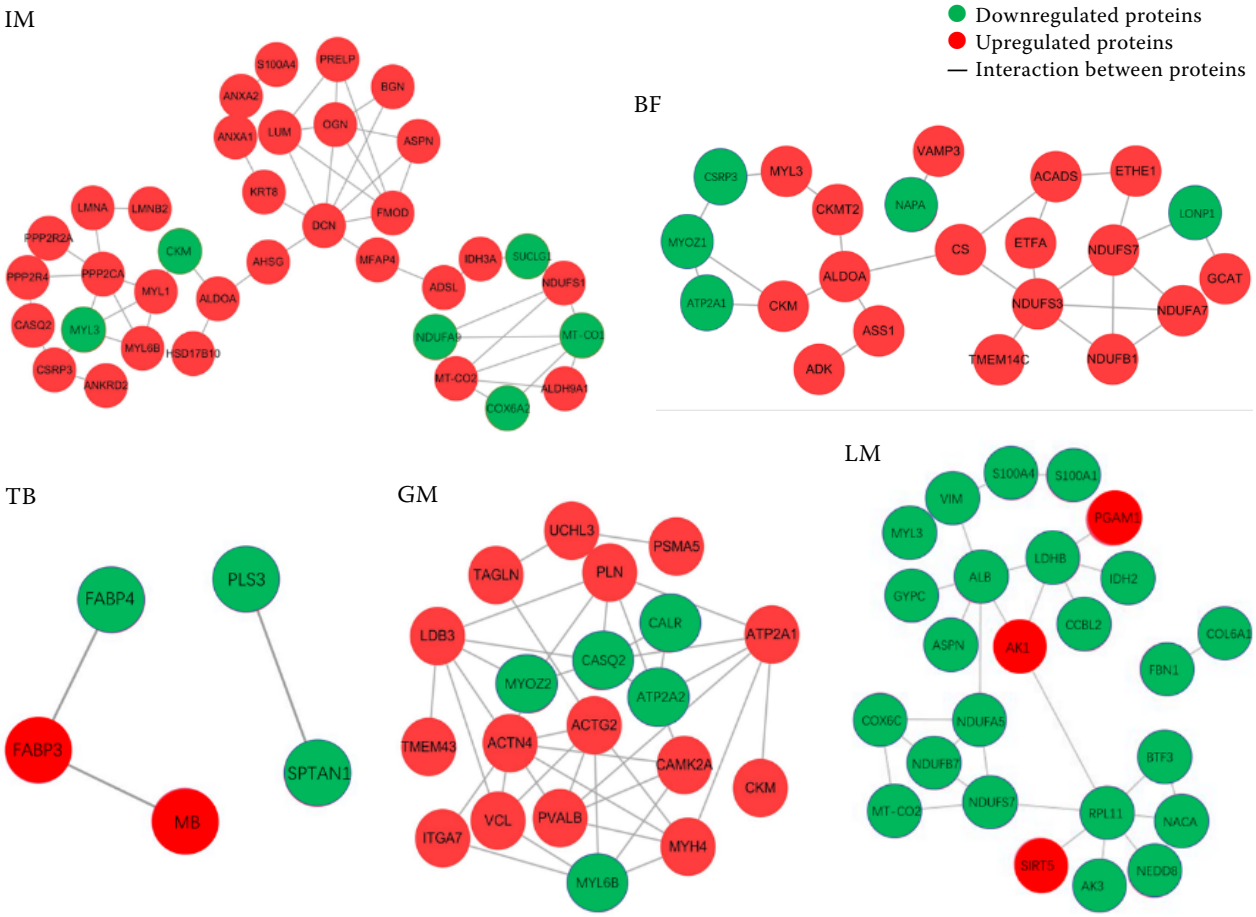


Figure 3. Protein–protein interaction networks of identified differentially expressed proteins in the *intercostal* muscle (IM), *biceps femoris* (BF), *m. triceps brachii* (TB), *gluteal* muscles (GM), and *longissimus dorsi* muscle (LM) of Inner Mongolia cashmere goats

Table 1. Differentially expressed proteins related to muscle growth and lipid metabolism

| Approved symbol | Approved name | Function | Fold change |
|---|---|---|---|
| MYOZ1 | Myozenin 1 | Skeletal muscle tissue development | 0.313 |
| MYOZ2 | Myozenin 2 | Skeletal muscle tissue development | 0.405 |
| MYH4 | Myosin heavy chain 4 | Muscle contraction | 2.188 |
| LMNA | Lamin A/C | Muscle organ development | 2.479 |
| TAGLN | Transgelin | Actin filament binding | 2.606 |
| ACTN4 | Alpha actinin 4 | Actin binding | 2.708 |
| MYL6B | Myosin light chain 6b | Structural constituent of muscle | 2.727 |
| ACTG2 | Actin gamma 2, smooth muscle | Muscle protein | 2.997 |
| CSRP3 | Cysteine and glycine rich protein 3 | Actin binding, developmental protein | 3.513 |
| MYL3 | Myosin light chain 3 | Motor protein, muscle protein, myosin | 3.675 |
| MYL1 | Myosin light chain 1 | Motor protein, muscle protein, myosin | 5.338 |
| ACADS | Acyl-CoA dehydrogenase short chain | Fatty acid metabolism, lipid metabolism | 3.734 |
| ETFA | Short-chain specific acyl-electron transfer flavoprotein subunit alpha | Fatty acid metabolism, lipid metabolism | 4.290 |
| FABP3 | Fatty acid binding protein 3 | Lipid binding | 2.090 |
| FABP4 | Fatty acid binding protein 4 | Long-chain fatty acid transport | 0.301 |

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differentially expressed myofibrillar structural protein in the BF. The results showed that (Figure 4A) the MYL3 protein was expressed in the TB, BF, IM, LM, and GM. Figure 4B presents the relative levels of the target proteins. The results showed that the expression of MYL3 was higher in the BF than in the other groups, consistent with the trend from the SWATH analysis. Inner Mongolia cashmere goats are kept on pasture all year, and their legs are muscular. Typically, the percentage of type I muscle fibres is highest in muscles that are frequently used, which may be the main reason for the high expression of MYL3 in the BF (a muscle of the hind leg).

## Immunohistochemical analysis of MYL3

Observations were performed under a light microscope, and brown cells were considered to be positive. The background staining in the control group was colourless or light blue. Compared with the negative control (Figure 4C), a positive signal for the MYL3 protein was present in the TB, BF, IM, LM, and GM. However, the MYL3 signal was present in some muscle fibres and absent from others. The percentage of yellow muscle fibres, counted under uniform magnification, was highest in the TB and BF. The reason for the differential expression of the MYL3 protein among different muscles is still under investigation. Overall, MYL3 was most highly expressed in the TB and BF. The type of muscle fibre influences several features that affect meat quality, such as colour, tenderness, pH value, water-holding capacity and levels of intramuscular fat, type IIa fibre (fast-twitch, red muscle, oxidative), and type IIb/IIx fibre (fast-twitch, white muscle, glycolytic) (Schiaffino and Reggiani 2011; Wang et al. 2017). The percentage of type IIa and I fibres is related

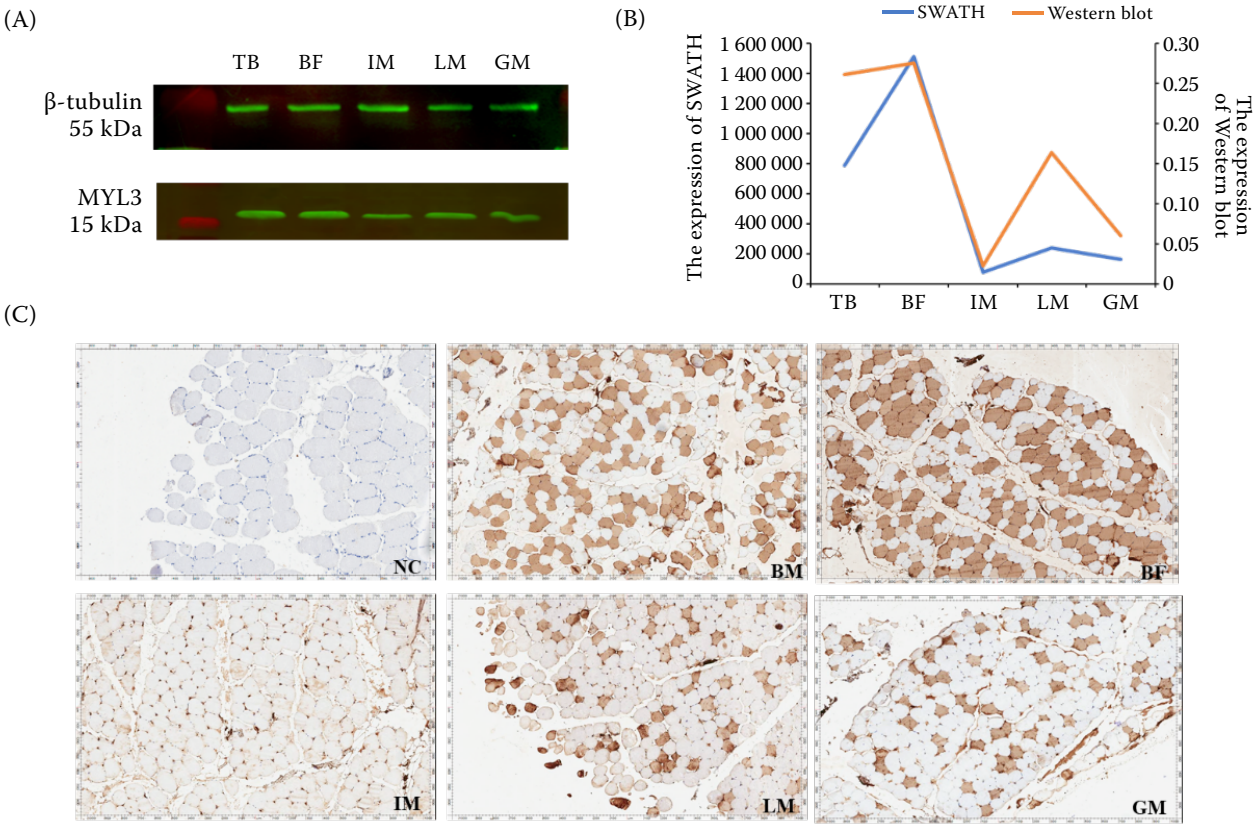


Figure 4. MYL3 expression in the *m. triceps brachii* (TB), *biceps femoris* (BF), *intercostal* muscle (IM), *longissimus dorsi* muscle (LM), and *gluteal* muscles (GM) of Inner Mongolia cashmere goats

(A) Western blot profiles of MYL3. Samples were subjected to SDS-PAGE followed by incubation with a monoclonal anti-MYL3 antibody. Bands corresponding to MYL3 were detected between 15 and 25 kDa, and protein levels were normalized to tubulin beta class I (TUBB), which was used as a loading control. (B) Expression trend of tubulin beta class I and MYL3 detected by Western blot and SWATH analyses. (C) Histological analysis of the muscle tissues. NC is the negative control by injection of BF. Every figure shows a 4-fold magnification

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to the majorization of meat quality (Ebhardt et al. 2017; Joo et al. 2017). Type I and IIa muscle fibres are oxidative fibres with thinner diameters, whereas type IIb/IIx muscle fibres are glycolytic fibres with thicker diameters (Sun et al. 2014). A higher content of glycolytic fibre will decrease the water-holding capacity of meat, causing it to become dry (Hwang et al. 2010; Koomkrong et al. 2017). Additionally, the muscle shear force and toughness decrease when the proportion of IIa muscle fibres increases (Shen et al. 2015; Koomkrong et al. 2017). In other words, the type and proportion of muscle fibres affect muscle tenderness. Approximately one-third of total muscle protein is myosin, which is the most abundant protein in muscles. Type IIx fibres consistently contain myosin heavy chains 1, 2, and 4 and myosin light chain 1. Type I fibres always contain myosin heavy chains 6 and 7 (MYH6 and MYH7) and myosin light chain 3 (MYL3), whereas MYH6, MYH7, and MYL3 are nearly absent from type IIx fibres (Stuart et al. 2016). Therefore, the yellow-stained fibres were type I or type IIa fibres. In summary, MYL3 was localized in type I or type IIa muscle fibres. In this study, the expression of MYL3 was highest in the fibres of commonly used muscles (the TB and the BF). That is, the proportion of MYL3 may be a potential molecular marker for the toughness of muscle.

## CONCLUSION

Overall, the proteins in the meat of the Inner Mongolia cashmere goat were mainly associated with three major metabolic pathways: carbohydrate, lipid and protein metabolism. Among the differentially expressed proteins, those related to meat quality were mainly muscle fibre structural proteins and proteins involved in fat deposition. The muscle fibre structural proteins include ACTG2, ACTN4, TAGLN, MYL3, MYL1, MYL6B, and MYH4. After localization as well as quantitative and bioinformatic analyses, we found that the muscle fibre structural protein MYL3 may be a potential molecular marker for muscle toughness.

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### Conflict of interest

The authors declare no conflict of interest.

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