

Comparative iTRAQ Proteomics Identified Proteins Associated With Sperm Maturation Between Yak and Cattleyak Epididymis

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Abstract

Background: During maturation, spermatozoa acquire motility and fertilizing capacity as they transit through the epididymis. In recent years, two-dimensional gel electrophoresis has been employed in proteomics studies conducted in rat, boar and human. However, there has not been a complete information regarding the proteins associated with sperm maturation in the epididymis. In this study, we employed iTRAQ proteomics to investigate proteins associated with sperm maturation between yak and cattleyak epididymis.

Results: After successfully establishing the samples and protein extraction, the iTRAQ coupled with LC-MS/MS mass spectrometry and bioinformatics analysis was performed. We identified 288 DAPs between yak and cattleyak epididymis; 151 were up-regulated while 137 were down-regulated in cattleyak relative to yak. Gene Ontology analysis identified that down-regulated DAPs in cattleyak were mostly enriched in the acetylation of protein component, negative regulatory activity, and positive regulatory activity. iTRAQ proteomics data showed that the top down-regulated DAPs were predominantly associated with sperm maturation, long-term sperm storage, sperm forward motility, sperm-oocyte fusion, and regulatory functions, while, the top up-regulated DAPs were mainly enriched in cell communication, cell adhesion, cytoskeleton organization, stress response, post-translational modifications and metabolic functions.

Conclusion: These results provide insight into the molecular mechanisms underlying male cattleyak infertility.

1. Background

Cattleyak is the hybrid of cattle (*Bos taurus*) and yak (*Bos grunniens*) that demonstrates strong adaptability to harsh environmental conditions in the adjacent Alpine regions and the Qinghai-Tibetan Plateau in China [1]. Cattleyak provides higher meat and milk to the local people compared to cattle and yak. It is female fertile and male sterile, male cattleyak sterility may be due to spermatogenic arrest [2, 3] and/or some other factors such as genes or proteins expression dynamics along the epididymis. The infertile male cattleyak phenomenon poses a major challenge and has restricted the hybridization procedure for decades. In previous years, some proteomics studies have been conducted to investigate male cattleyak sterility [4, 5]. However, these previous studies focused on the testis and could not completely elucidate the exact cause of the infertility in male cattleyak. Until now, no study has explored epididymis as a means of identifying the infertility mechanism in male cattleyaks. Since testicular spermatozoa further undergo a mandatory epididymal maturation, the epididymis poses as a suitable organ for further investigation in male cattleyak infertility.

The epididymis is a long complex convoluted tube found on the surface of each testis, connects the efferent duct to the vas deferens in the male mammalian reproductive tract [6]. The epididymal epithelium consists of 5 main epithelial cell types: principal, clear, basal, narrow, and halo cells, which provide not only structural support to epididymis but also play significant role in the epididymal

spermatozoa maturation process [7]. The epididymis is the site where newly produced testicular spermatozoa acquire swimming, penetrating and complete fertilizing competency. These functions are carried out from the secretion by epididymal epithelial cells (EECs) of several key proteins to create an interactive and dynamic microenvironment in the lumen where the spermatozoa are stored, protected, and undergo maturation. Moreover, Skerget et al. (2015) reported that, during sperms passage through the epididymis, the sperms surface proteins must be acquired, lost, and modified to confer motility and fertilization competency to sperms [8]. With their significant functions in the process of sperm maturation and male fertility, proteins have received considerable attention and have been assumed to be prospective targets for identifying and treating infertility. However, until now, very limited knowledge is available about the exact roles of proteins in the regulation of the several important processes involved in the epididymal sperm maturation and fertilization.

Advances in bioinformatics have greatly helped in understanding of sperm proteome composition and function. In recent years, some proteome studies have been successfully conducted in rat, human, and boar [9–11] by employing two-dimensional gel electrophoresis. However, the gel-based studies may suffer from drawbacks such as representation of the protein types at low concentrations and very acidic/basic proteins [5]. Isobaric tagging for relative and absolute protein quantification (iTRAQ) is a proteomics technique developed to quantitatively investigate protein abundance changes in different biological samples with high accuracy and reproducibility and the advantage of this multiplexing reagent is that 4 or 8 analysis samples can be quantified simultaneously [12, 13]. In this study, we identify proteins associated with sperm maturation between yak and cattleyak epididymis employing iTRAQ proteomics.

2. Methods

2.1. Animals and epididymis sample collection

Sample collection was carried out under license in accordance with the Guidelines for Care and Use of Laboratory Animals of China and all protocols were approved by the Institutional Review Board of Southwest University of Science and Technology [14]. Male yaks (Maiwa yaks) (n = 3; age, 1 year; named M1, M2, and M3) and Male cattleyaks (Maiwa yak × Tibetan taurine) (n = 3; age, 1 year; named P1, P2, and P3) were sampled from Maiwa yak population fed on a farm in Hongyuan county, Sichuan province of China.

Epididymis of yaks and cattleyaks were obtained by veterinary surgical operation and fat and connective tissues were removed. Epididymis were then separated apart from testis by fine-scale dissection and were preserved in liquid nitrogen (-196 °C), transported to the laboratory and stored at -80 °C until analysis. The animals would not be killed after sample collections. The animals would rather be kept in a safe and contained place close to the farm-house and away from hazards.

2.2. Extraction of the total protein from epididymis tissue

For protein extraction, each epididymal tissue sample was ground in a mortar with liquid nitrogen; an appropriate amount of lysis buffer (7 M urea, 2 M thiourea, 0.1% CHAPS) was added to the samples and vortexed to mix. The lysates were then extracted with ultrasonication (60 s, 0.2 s on, 2 s off, amplitude 25%), and placed at room temperature for 30 minutes. Subsequently, the lysates were centrifuged at 15000 rpm at 4 ° C for 10 min, after which supernatant was carefully collected and stored at -80 °C. Concentration of the extracted protein was measured using the Bradford method. The sample was diluted with lysis buffer to obtain a final concentration within the range of the standard curve. BSA was dissolved with lysis buffer to a series of standard protein concentrations. A 10 µL diluted sample and standard product were collected separately, and reacted respectively with 300 µL protein quantitation dye under dark for 15 ~ 20 min. Simultaneously, the absorbance was read at 595 nm with a microplate reader (Thermo, model: Multiskan MK3) for the standard product and the sample. The standard curve of the relationship between absorbance and concentration of the standard product in each tube was obtained, and the protein concentration of each sample was calculated.

2.3. Enzymatic digestion of the proteins

For trypsin digestion, a 100 µg of total protein was used by a filter-aided sample preparation protocol (FASP). The samples were reduced with 10 µL of reducing reagent at 37 °C for 1 hour and followed by addition of 2 µL of cysteine-blocking reagent for 30 minutes at room temperature. The reductive alkylated protein solution was then added to a 10 K ultrafiltration tube (Merck Millipore, REF UFC501096), centrifuged at 12,000 rpm for 20 minutes, and bottom solution of the collection tube was discarded. Afterwards, 100 µL dissolution buffer was added in the iTRAQ kit and centrifuged at 12,000 rpm for 20 min. Before collection tube replacement, the bottom solution in the collection tube was discarded, and the process was repeated 3 times. Subsequently, 4 µg (1:50 compared to protein) trypsin (Promega REF V5111) in a volume of 100 µL was added to the ultrafiltration tube and reacted overnight at 37 °C. The next day, the sample was centrifuged at 12,000 rpm for 15 min after which the peptide solution after enzymatic digestion was retained at the bottom of the collection tube. 200 µL of ddH₂O was then added to the ultrafiltration tube and centrifuged again at 12,000 rpm for 15 min. Finally, 500 µL of the digested sample was collected from the bottom of the collection tube.

2.4. iTRAQ labeling

For the iTRAQ labeling, the iTRAQ reagent (8-plex; AB Sciex) was removed from the freezer and equilibrated to room temperature. 150 µL isopropanol was added to each tube of iTRAQ reagent, vortexed and centrifuged to the bottom of the tube. Thereafter, 50 µL of sample (100 µg of enzymatic product) was transferred to a new centrifuge tube, iTRAQ reagent was added to the sample, and then vortexed, centrifuged and reacted at room temperature for 2 hours. 100 µL of ddH₂O was added to the mixture to stop the reaction.

2.5. Offline pre-separation of enzymatically digested peptides and LC-MS/MS mass spectrometry

The samples, after the labeled lyophilized peptides, were dissolved in 100 μ L mobile phase A [98% ddH₂O, 2% acetonitrile (pH 10) (Merck, Cat. No: 100030, Germany)], centrifuged at 14,000 rpm for 10 min, and the supernatant was taken for use. 200 μ g enzymatically decomposed bovine serum albumin (BSA, Sigma-Aldrich, Cat. No. A2058, USA) was added to the column (Durashell-C18, 4.6 mm \times 250 mm, 5 μ m, 100 \AA) (Agela, Cat. No. DC952505-0) to test the conditions of isolation (column temperature: 45 $^{\circ}$ C, detection wavelength: 214 nm). Thereafter, 100 μ L of the prepared sample was loaded at a flow rate of 0.7 mL/minute. The separation gradient was as follows: 0 min: 5% mobile phase B (98 % acetonitrile, 2% ddH₂O) (ddH₂O adjusted the pH to 10 with ammonia); 5 min: 8% mobile phase B; 35 min: 18% mobile phase B; 62 min: 32% mobile phase B; 64 min: 95% mobile phase B; 68 min: 95% mobile phase B; and 72 min: 5% mobile phase B [15]. For protein analysis, the nanoscale reversed-phase chromatography connected to mass spectrometer (Thermo, Model: Fusion) was used. The segments obtained by high pH reversed-phase were reconstituted with 20 μ L (2% methanol, Sigma-Aldrich, article number: 14262, USA), (0.1% formic acid, Sigma-Aldrich, Cat. No. 56302, USA), and then centrifuged at 13,000 rpm for 10 min. Thereafter, a 10 μ L volume was loaded by a sandwich method with a loading pump flow rate 350 nL/min over 15 min. The isolation flow was 350 nL/min and isolation gradient was as follows: 0 min: 4% mobile phase B (100 % acetonitrile, 0.1 % formic acid); 5 min: 15% mobile phase B; 40 min: 25% mobile phase B; 65 min: 35% mobile phase B; 70 min: 95% mobile phase B; 82 min: 95% mobile phase B; 85 min: 4% mobile phase B; and 90 min: 4% mobile phase B [14]. The mass spectroscopy (MS) parameter settings were as follows: spray voltage 2.1 kV; capillary temperature: 250 $^{\circ}$ C, and Scan range: 350-1800m/z.

2.6. Data analysis

The selection of the database was based on the species required, the completeness of database annotations and the reliability of sequence. The used database was bovine protein sequence library, and the mass spectrometry analysis of iTRAQ was performed by Fusion mass spectrometry. The original mass spectrometry files were processed by Thermo's commercial software Proteome Discoverer 1.4. The search parameters were set as follows: enzyme, trypsin; static modification, C carboxyamidomethylation (57.021 Da); dynamic modification: M oxidation (15.995 Da), N terminal; species, *Acyrtosiphon pisum*; precursor ion mass tolerance, \pm 15 ppm; fragment ion mass tolerance, \pm 20 mmu; and max missed cleavages, 2.

The significant differentially abundant proteins (DAPs) were screened by T-test (t-test). Proteins with a p-value less than 0.05 and a fold difference greater than 1.5 or less than 0.67 were considered DAPs.

2.7. GO, KEGG and PPI analysis of DAPs

DAPs between yak and cattleyak epididymal tissues were mapped to Gene Ontology (GO) terms in the database (<http://www.geneontology.org/>). GO is a database established by the Gene Ontology Consortium, which is suitable for all species to define and describe the functions of genes and proteins. The calculated p-value was adjusted through Bonferroni Correction, taking enriched p-values as a threshold. Pathway analysis of identified proteins could deepen understanding of the metabolic capacity of the species, biological processes information, and related diseases. KEGG database (Kyoto

Encyclopedia of Genes and Genomes database) was used to enrich biological pathways of the DAPs. Using the String Protein Interaction Database, an interactive network analysis of significantly expressed proteins was performed and differential protein interaction network data files were directly imported into Cytoscape software for visual editing.

2.8. Enzyme linked immunosorbent assay (ELISA)

ELISA was carried out to confirm the differential abundances of eight proteins, namely CD63, ELP3, LSM5, GSTM1, GGH, ERAP1, GPX5 and MUC15. Briefly, total protein was taken out of each sample according to the manufacturer's protocol of DNA/RNA/protein co-extraction Kit (Tiangen Biotech (Beijing) Co., Ltd., China). Total protein concentration of each sample was detected by NanoDrop 3000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and adjusted to 0.1 mg/mL. ELISA detection was performed according to the manufacturer's instructions (MyBioSource, Inc., San Diego, CA, USA). The first antibodies against the proteins mentioned above were all originated from *Bos taurus* and all ELISA kits were provided by MyBioSource (San Diego, CA, USA). The standard sample provided by the manufacture was used to establish the standard curve and regression equation. Each sample was analyzed with triplicates and the OD value of each well was determined using microplate reader set to 450 nm within 15 min. The concentrations of the targeted proteins were calculated from the regression equation and the content of each protein was determined from each sample. Statistical analyses were performed using SPSS 22.0 standard version (IBM, Armonk, NY, USA). Student's t-test was employed to analyze differences of each protein expression level between the control (yak) and tested groups (cattleyak). For all tests, statistical significance was taken as $p < 0.05$.

3. Results

3.1. Identification of differentially abundant proteins (DAPs) between yak and cattleyak epididymis by iTRAQ

We have applied a quantitative iTRAQ-based proteomics approach for the determination of proteomic changes between yak and cattleyak epididymis. For the identification and relative quantification of proteins, an overview of workflow described by yak and cattleyak epididymis proteome comparison is shown in (Fig. 1). In 3 biological replicates, a total of 54088 spectrums were generated and 4596 proteins were identified from 23801 peptides between yak and cattleyak epididymis proteome. Normally, the number of proteins decreased from 1452 to 80, as the increase in the protein coverage ranges from 0–100% (Fig. 2A). For the identification of peptide segments distribution, the (Fig. 2B) shows most of the identified proteins contain < 25 peptide segments and the number of proteins segments decrease as the number of matching peptide segments increase. The distribution of the fold difference for each protein is shown in (Fig S1), and the Hierarchical clustering analysis results of DAPs are shown in the heat map (Fig S2).

3.2. Quantification of DAPs between yak and cattleyak epididymis by iTRAQ

Out of the total identified proteins between yak and cattleyak epididymis proteome, 288 met the DAPs selection criteria, out of which 137 were down-regulated (fold change ≤ 0.67 , $p \leq 0.05$) and 151 were up-regulated (fold change ≥ 1.2 , $p \leq 0.05$). Only 3 out of the 137 down-regulated proteins had fold change values ≤ 0.1 . These were Vitamin D-binding protein, DnaJ homolog subfamily C member 17, and Serotransferrin with fold changes of 0.06, 0.08 and 0.10 respectively. On the other hand, 34 out of the 151 up-regulated proteins had fold change values ≥ 2 ; among these were uncharacterized protein, A-kinase anchor protein 3, Uncharacterized protein, MHC class I antigen (Fragment), and Beta A4 crystallin with fold changes of 4.6, 4.2, 3.9, 3.8, and 3.8 respectively. We have listed top 30 up-regulated DAPs and top 30 down-regulated DAPs identified from cattleyak relative to yak (Tables 1 & 2, respectively).

3.3. Gene Ontology (GO) analysis of differentially abundant proteins

Gene ontology (GO) analysis was conducted to better understand the functions of the all down-regulated and up-regulated DAPs identified by iTRAQ between yak and cattleyak based on biological process, cellular component, and molecular function categories. 35 GO terms were significantly enriched for down-regulated DAPs, including 22 biological process terms, 6 cellular component terms, and 7 molecular function terms. Majority of these down-regulated enriched GO terms relating to biological process were mainly associated with acetylation of protein component, negative regulatory activity, and positive regulatory activity, from which top three listed enriched GO terms were negative regulation of endopeptidase activity ($p = 0.002923$), negative regulation of peptidase activity ($p = 0.003519$) and histone H3 acetylation ($p = 0.005172$). According to the down-regulated cellular component, most of enriched GO terms were associated with acetyltransferase complex, among which the top three listed enriched GO terms were histone acetyltransferase complex ($p = 0.002818$), acetyltransferase complex ($p = 0.004352$) and protein acetyltransferase complex ($p = 0.004352$). From down-regulated molecular function, majority of enriched GO terms were involved in inhibitor activity and regulator activity, among which top three listed enriched GO terms were peptidase regulator activity ($p = 0.011867$), antigen binding ($p = 0.020786$), and endopeptidase inhibitor activity ($p = 0.021568$) (Fig. 3A), while, among the up-regulated DAPs, 48 GO terms were significantly enriched between yak and cattleyak epididymis, including 18 biological process terms, 7 cellular component terms, and 23 molecular function terms. Most of up-regulated DAPs enriched GO terms in particular to biological process were involved in metabolic process, protein localization and catabolic process, among which top three listed enriched GO terms were organonitrogen compound catabolic process ($p = 0.004521$), receptor-mediated endocytosis ($p = 0.005261$) and cellular modified amino acid metabolic process ($p = 0.006143$). With respect to cellular component, the most important up-regulated enriched GO terms were lysosome and sperm part, in which top three listed enriched GO terms were lysosome ($p = 0.001189$), lytic vacuole ($p = 0.001189$) and blood microparticle ($p = 0.005987$). Significantly up-regulated enriched GO terms based on molecular function

were associated with binding, inhibitor activity, and hydrolase activity, in which top three enriched GO terms were hydrolase activity ($p = 1.26E-05$), vitamin D binding ($p = 0.000388$) and steroid binding ($p = 0.001698$) (Fig. 3B).

3.4. KEGG pathway enrichment analysis of DAPs

Pathway analysis for identified proteins can deepen understanding of the metabolic capacity of the species, biological processes information, and related diseases. In this study, we have mapped 87 down-regulated DAPs and 136 up-regulated DAPs to the reference pathways in the KEGG database to determine biological pathways related to sperm function. In total, five up-regulated DAPs were significantly enriched in KEGG pathways whereas none of the down-regulated DAPs were significantly enriched in KEGG pathways. For the up-regulated DAPs, the enriched KEGG pathways were associated with Glutathione metabolism ($p = 9.75E-05$), Lysosome ($p = 0.000102$), Glycan degradation ($p = 0.000646$), Glycosaminoglycan degradation ($p = 0.01678$) and Thyroid hormone synthesis ($p = 0.021941$) (Fig. 4).

3.5. Protein-protein interaction (PPI) of DAPs

In this study, the Search Tool for the Retrieval of Interacting Genes /Proteins 11.0 (STRING 11.0) database was used for identifying protein-protein interaction network of DAPs. After removing unconnected and self-loops proteins, the resulting PPI network generated 64 protein nodes and 90 edges (Fig. 5). Furthermore, the network data file was directly imported into Cytoscape software for visual editing with a threshold value of a combined score > 0.7 . DAPs that connected higher than others in created network were considered as hubs (Fig. 6); these hubs may have significant roles in the regulation of the network. In the PPI network, the top four hub proteins were vesicle-associated membrane protein 8 (VAMP8), SERPINA3-3, GGH, and DNAJC3.

3.6. ELISA

ELISA was performed to verify the results obtained from iTRAQ proteomics. The up-regulation of GGH, RAP1, GPX5 and MUC15 in cattleyak in respect to yak determined by iTRAQ was confirmed by ELISA, respectively ($p < 0.05$). Meanwhile, ELISA also verified the decreased expression of CD63, ELP3, LSM5 and GSTM1 in cattleyak compared to yak identified by iTRAQ, respectively ($p < 0.05$) (Fig. 7). All these data validated the results obtained from iTRAQ.

4. Discussion

Proteomics technologies offer great opportunity to investigate the molecular mechanisms that regulate sperm functions [16]. Previously, the iTRAQ proteomics approach has been employed to investigate the infertility of male cattleyak focusing on the testis. Until now, no study has used the above technique to investigate the epididymis, which is known to be structurally and functionally associated with sperm maturation and fertility. In this study, iTRAQ proteomics is used to identify proteins associated with sperm maturation between yak and cattleyak epididymis.

4.1. Some down-regulated DAPs in cattleyak were involved in epididymal sperm maturation

The epididymis is a long convoluted tubule that functions in sperm maturation and sperm storage [17]. Epididymal maturation occurs when functional changes occur in the epididymis and render sperm the ability to capacitate in the female reproductive tract [18]. Biological function analysis identified a number of down-regulated DAPs involved in epididymal maturation in cattleyak (Table 1). In a previously published study, Phosphatidylethanolamine-binding protein 4 (PEBP4) was compared among fertile, low fertile and infertile bulls. It was observed that the novel seminal PEBP4 protein was expressed significantly higher in fertile bulls compared to low fertile and infertile bulls, suggesting that PEBP4 could play crucial role in spermiogenesis, epididymal sperm maturation, and sperm motility [19]. From the previous finding, it could be reported that, the down-regulation of PEBP4 in cattleyak may interrupt processes of sperm maturation and cause infertility. CD63 is the known marker of exosome localized throughout the apices of epididymal epithelial principal cells and microenvironment of the epididymis to allow long-term sperm storage [20], which is essential for each step of sperm maturation in the epididymis. The downregulation of CD63 in cattleyak may have drastic effects on the sperm storage as well as contribute to fertility related problems. Seminal plasma, a fluid medium that exists in semen, contains proteins related to sperm forward motility such as Zinc-alpha-2-glycoprotein (ZAG). ZAG has been demonstrated as a seminal plasma protein associated with forward motility of spermatozoa, and could play major roles during maturation of spermatozoa, from the epididymis through fertilization in the female reproductive tract [21]. Down-regulation of ZAG in cattleyak suggests that sperms possibly may not acquire motile ability after their ejaculation into the female reproductive tract.

Again, two important proteins, Glutathione S-transferase Mu 1 (GSTM1-1) and Fetuin-B were also found to be down-regulated in cattleyak relative to yak. The potential function of Glutathione S-transferase Mu 1 (GSTM1-1) in epididymis is yet to be determined, although GSTM1-1 belongs to Glutathione S-transferase mu class (GSTM1) present on sperms. GSTM1 has been reported to play a significant role in sperm-oocyte fusion and believed to facilitate specific binding of sperm surface-active component with the zona pellucida (ZP) during sperm-egg fusion process [22, 23]. The decreased expression of GSTM1-1 could be speculated as a possible cause of impaired or interrupted fertilization in cattleyak. Fetuin-B has been reported in mice, as a plasma protein that inhibits ovastacin, which is a cortical granula protease known to trigger ZP hardening, resulting in premature ZP hardening and causing infertility [24, 25]. From this, it could be reported that downregulation of Fetuin-B in cattleyak may cause similar result. The biological functions of the above-mentioned proteins reveal that their down-regulation could negatively affect sperm maturation, long-term sperm storage, sperm forward motility and sperm-oocyte interaction.

4.2. Some DAPs in cattleyak may be associated with epididymal dysfunction.

Generally, epididymis contains a number of molecules and proteins that assist sperms to swim through the epididymis in the bid to fertilize oocytes. In this study, the biological function analyses identified some

up-regulated DAPs associated with epididymal dysfunction, which could possibly interfere sperm maturation during transit in the epididymis. In eukaryotic cells, communication between the nucleus and cytoplasm occurs through nuclear pores, which are involved in an exchange of macromolecules such as proteins and RNAs. This process of active nuclear protein transport is mediated by the nuclear localization signal (NLS), composed of 100 different proteins including Importin subunit alpha, which recognizes karyophile proteins to form a stable complex, termed the nuclear pore-targeting complex (PTAC) [26, 27]. The biological function of Importin subunit alpha suggests that increased expression of this protein might block the crucial communication between nucleus and cytoplasm in epididymal cells of cattleyak and hence adversely affect fertility. Mucins are heavily glycosylated proteins divided into two classes: secretory mucins and membrane-bound mucins. Mucin 15 was found as membrane-bound mucin expressed from epithelial cells considering that alterations in membrane-bound mucins could influence cellular growth, differentiation, transformation, invasion, and adhesion [28]. The up-regulation of Mucin 15 in cattleyak indicates that Mucin 15 could reinforce adhesion, which may negatively influence or interfere with sperm cells migration through the epididymis. GAS2 has been found as a component of the microfilament system, involved in growth arrest of cells via a phosphorylation mechanism during the $G_0 \rightarrow G_1$ transition [29]. The overexpression of GAS2 induces membrane ruffling and stops mitosis [30]. The up-regulation of GAS2 in cattleyak might have affected the essential molecules or cells that were involved in the normal development of sperms in the epididymis of cattleyak.

Aminopeptidase and Poly (ADP-ribose) polymerase (PARP) were also up-regulated in cattleyak and are involved in various stresses. Aminopeptidase, an enzyme encoded by Endoplasmic reticulum aminopeptidase 1 (ERAP1) gene was found to be involved in immune response [31]. Aminopeptidase is thought to behave similarly to acute-phase proteins, secretes into the blood in response to inflammatory stimuli, and are involved in enhancing NO synthesis as a host defense mechanism [32]. Poly (ADP-ribose) polymerase (PARP) was reported as a DNA repair enzyme found in various organs, including the epididymis and was activated when DNA strand breaks as a result of reactive oxygen species (ROS) or oxidative stress [33, 34]. PARP utilizes nicotinamide-adenosine dinucleotide (NAD) as a substrate for its activation resulting in the ADP-ribosylation of PARP. It has been reported that excessive PARP activation results in the depletion of NAD + and subsequent cellular ATP depletion ultimately leads to necrotic-type cell death [35]. It could be summarized that the increased expression level of Aminopeptidase and PARP indicate sperm cells may be in a constant stress state due to the acquisition of some toxic products produced by altered DAPs. Furthermore, the mammalian epididymal spermatozoa completes fertility acquisition via a process called post-translational modifications [36]. Polypeptide N-acetylgalactosaminyltransferase 6 (GalNAc-T6) belongs to polypeptide N-acetylgalactosaminyltransferases (GalNAc-Ts) family, which catalyzes the transfer of N-acetyl- α -D-galactosamine: polypeptide (GalNAc) from the sugar donor uridine diphosphate (UDP)-GalNAc to the serine and threonine residues of glycoproteins [37]. Previous studies reported that modification of spermatozoa plasma glycoproteins are important in the production of functionally mature sperms during migration through the epididymis [38, 39]. Therefore, the up-regulation of GalNAc-T6 could alter glycosylation, which may have severe negative impact on sperm motility and consequently on fertility.

Moreover, the interaction between spermatozoa and zona pellucida requires proteolytic cleavages of surface proteins by protease enzymes. These proteolytic cascades are, however, controlled by protease inhibitors, which stabilize the sperm membrane and inhibit premature capacitation [40]. Serpin A3-8 was found as a protease inhibitor in buffalos, where it was involved in the protease inhibition process [41]. Besides, an important Serine peptidase inhibitor-like protein (M/P = 3.339198), with Kunitz and WAP domains 1 (Eppin), were found in male monkeys, where these monkeys were immunized with Eppin and observed that high-titer monkeys were infertile [42]. Therefore, the up-regulation of these protease inhibitors suggest that, they could inhibit proteolytic cleavages, which are necessary for the early events of fertilization. Consequently, the biological functions of these proteins indicated that the increased expressions of these proteins were associated with cell communication, cell adhesion, cytoskeleton organization, stresses, and post-translational modifications.

4.3. Some DAPs in cattleyak may be involved in regulatory mechanisms

In the epididymis, different luminal environments exist, beginning with an undifferentiated epithelium that undergoes a series of changes to become fully differentiated with differences in terms of morphology, gene regulatory activities, and functions [43]. The process of gene expression is controlled at several points, from level of transcription to post-translational modification of protein. This highly complex process requires a number of important proteins responsible for the activation of certain vital mechanisms. GO biological function analyses revealed a number of down-regulated DAPs involved in regulation and acetylation. In gene expression machinery, the primary point of control is the transcriptional level where histones and their acetylation in nucleosome play functional roles in controlling gene expression. Histone H2A and its variant Core histone macro-H2A were demonstrated as members of histone octamer that formed a structural unit (nucleosome) of chromatin [44, 45]. Histone H2A has both N-terminal tail (H2ANtT) and C-terminal tail (H2ACtT). The H2ANtT was found to be critical to inter-nucleosome interaction [46], while H2ACtT recruits linker histone H1 to the nucleosome and stabilizes it [47]. Deletion of H2ACtT can increase H2A mobility and decrease nucleosome stability. Elongator complex protein 3 (ELP3) was found as a catalytic subunit of Elongator that encodes a histone acetyltransferase and helps in the process of histone acetylation [48]. Acetylation of histones allow chromatin to expose DNA binding sites and initiate transcription [49]. Therefore, it can be speculated that the down-regulation of Histone H2A and ELP3 in cattleyak might be a cause of transcriptionally silenced genes which encode important epididymal sperm maturational proteins.

Post-transcriptional regulation of genes can be controlled by the process of deadenylation and pre-mRNA splicing. CCR4-NOT transcription complex subunit 7 or CCR4-associated factor 1 (CAF-1) was found as a deadenylase enzyme involved in the mRNA degradation process, during which poly (A) tail was removed and the newly synthesized mRNA decreased from 200–250 nucleotides to 10–60 nucleotides [50, 51]. The length of the poly (A) tail was thought to play a significant role in determining mRNA stability, translation efficiency and in gene regulation [52], whereas U6 snRNA-associated Sm-like protein LSm5 was found as a major component of spliceosome that plays significant role in pre-mRNA splicing

process, resulting in the formation of matured mRNA [53]. In the pre-mRNA splicing process, a number of multiple protein isoforms can be produced from a single gene and can play a significant role in cellular differentiation and organism development [54]. Down-regulation of U6 snRNA-associated Sm-like protein LSm5 may lead to disruption of normal cellular function and lead to infertility. Moreover, Aspartyl-tRNA synthetase (AspRS) and Protein transport protein Sec61 subunit gamma were found to be involved in translational and post-translational processes respectively. Before starting the process of translation, the tRNAs are charged in the process called Aminoacylation. AspRS is an aminoacyl-tRNA synthetase, which charges its cognate tRNA with aspartate amino acid and play an important role in oxidative stress [55]. However, transport protein Sec61 subunit gamma, a member of the protein-conducting channel (PCC) is located in the membrane of the endoplasmic reticulum, where it binds to translating ribosomes for co-translational protein transport [56]. The biological function of these translational and post-translational proteins indicated that their down-regulation may result in proteins being unable to fold efficiently in the endoplasmic reticulum (ER).

4.4. Some DAPs in cattleyak may be associated with metabolic functions

Metabolism is a set of chemical and physical changes that occur in the animals' body and provide energy for the synthesis of new required materials and for life processes. GO biological function analysis identified many up-regulated DAPs involved in metabolic processes in cattleyak. Previously, it was reported that targeting sperm and energy metabolism have a closer relationship [57]. In this study, we have identified two important candidates of up-regulated DAPs that involved in the metabolic function; Biliverdin reductase A (BVR A) and Gamma-glutamyl hydrolase (GGH). In mammals, biliverdin (BV) together with its reduced form bilirubin (BR) has potential roles in the epididymis. BVRA was demonstrated as an enzyme that converted biliverdin to bilirubin and thought to play important role in scavenging reactive oxygen species (ROS), reactive nitrogen species (RNS) and nitric oxide through its substrate (BV), also by-product (BR) [58, 59]. The increased expression of BVRA in epididymis of cattleyak therefore could limit the biliverdin's ability and quantity to attenuate such oxidant activities. Proteins, micro and macro-nutrients play essential roles in successful fertilization; folate is a micronutrient and remains critical for sperm quality. Sperm aneuploidy has adverse effects on sperm quality and can be reduced by consumption of 400 mg to 700 mg total folate per day [60]. Gamma-glutamyl hydrolase (GGH) was found as a lysosomal enzyme that is involved in dietary folylpoly- γ -glutamate metabolism, allowing the folylmonoglutamate to enter the intestinal membrane and plays an important role in cellular homeostasis of folate [61]. The biological function of GGH indicates that the up-regulation of this protein may disrupt cellular maintenance of folate and could have a critical impact on sperm quality in cattleyak.

5. Conclusion

To understand the potential role of proteins involved in sperm maturation, we applied iTRAQ-based proteomics in the study of proteins associated with sperm maturation between yak and cattleyak epididymis. We identified total of 4596 proteins between yak and cattleyak epididymis. Out of the total,

288 were DAPs, of which 151 were up-regulated DAPs and 137 were down-regulated DAPs. Most of the identified proteins in cattleyak were involved in epididymal sperm maturation, epididymal dysfunction, regulatory and metabolic function. iTRAQ proteomics data identified several key proteins (PEBP4, CD63, ZAG, GSTM1-1, and Fetuin-B) that potentially play significant roles in the sperm maturation process in cattleyak. Our findings could be helpful in understanding the molecular mechanisms and genetics involved in sperm maturation process in cattleyak.

Abbreviations

AspRS- Aspartyl-tRNA synthetase

BVR A- Biliverdin reductase A

CAF-1- CCR4-associated factor 1

DAPs- Differentially expressed proteins

ddH₂O- Double distilled water

EECs- Epididymal epithelial cells

ELISA- *Enzyme linked immunosorbent assay*

ELP3- Elongator complex protein 3

ERAP1- Endoplasmic reticulum aminopeptidase 1

ERAP1- Endoplasmic reticulum aminopeptidase 1

FASP- Filter-aided sample preparation

GalNAc-T6- N-acetylgalactosaminyltransferase 6

GGH- Gamma-glutamyl hydrolase

GGH- Gamma-glutamyl hydrolase

GO- Gene ontology

GPX5- Glutathione Peroxidase 5

GSTM1- Glutathione S-transferase

iTRAQ- Isobaric tags for relative and absolute quantitation

KEGG Database -Kyoto Encyclopedia of Genes and Genomes database

MUC15- Mucin 15

NLS- Nuclear localization signal

PARP- Poly (ADP-ribose) polymerase

PCC- Protein-conducting channel

PEBP4- Phosphatidylethanolamine-binding protein 4

PTAC- Pore-targeting complex

RAP1- Ras- Proximate-1/ Ras- related protein 1

RNS- Reactive nitrogen species

ROS- Reactive oxygen species

SERPINA3- Serpin family A member 3

VAMP8- Vesicle-associated membrane protein 8

ZAG- Zinc-alpha-2-glycoprotein

Declarations

Ethics approval and consent to participate

Sample collection was carried out under license in accordance with the Guidelines for Care and Use of Laboratory Animals of China and all protocols were approved by the Institutional Review Board of Southwest University of Science and Technology.

Consent for publication

Consent is given for our names as authors to be published in this journal and also understand that the text, tables and figures published in the article will be freely available on the internet and may be seen by the general public.

Availability of Data and Material

All data included in this study are available upon request by contact with the corresponding author.

Competing interests

Not applicable.

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Authors' contributions

WSZ: Methodology, Funding acquisition, **SA:** Formal analysis, Writing-Original Draft, **JXL:** Investigation, Formal analysis, **SA:** Validation, Data Curation, **EQ:** Software, Formal analysis, **T HS:** Software, Validation, **YTW:** Visualization, Resources, **YLYL:** Investigation, Validation, **HMW:** Data Curation, Resources, **JJZ:** Writing-Review & Editing, Conceptualization, **XC:** Supervision, Project administration. All authors have read and approved the final version of manuscript.

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Tables

Table 1.

The top 30 down-regulated DAPs between yak and cattleyak epididymis.

Accession	Description of Protein	Protein symbol	Pvalue	FC (P/M)
F1N5M2	Vitamin D-binding protein	GC	4.65E-09	0.062
Q2KI83	DnaJ homolog subfamily C member 17	DNAJC17	0.000100554	0.081
G3X6N3	Serotransferrin	TF	0.006778114	0.106
A0JNP2	Secretoglobin family 1D member (LppAB)	SCGB1D	0.001735233	0.181
D4QBB3	Hemoglobin beta	HBB	1.06E-05	0.225
E1B9W6	Uncharacterized protein	ADCY10	0.000214338	0.241
A7Z033	T-complex protein 11-like protein 2	TCP11L2	0.007235083	0.256
P33433	Histidine-rich glycoprotein (Histidine-proline-rich glycoprotein) (HPRG) (Fragments)	HRG	7.60E-07	0.272
A5D7S6	PEF1 protein	PEF1	0.001584413	0.286
Q3MHV8	RBM15B protein (Fragment)	RBM15B	0.000115054	0.294
B0JYP6	IGK protein	IGK	0.002491825	0.295
G3N1U4	Serpin A3-3	SERPINA3-3	4.24E-07	0.306
Q9XSK2	CD63 antigen (CD antigen CD63)	CD63	7.98E-09	0.316
Q0IIG7	Ras-related protein Rab-5A	RAB5A	0.000214338	0.318
F6RF62	Uncharacterized protein	MYL4	1.97E-08	0.344
G9HQZ5	MHC class I antigen (Fragment)	BoLA	0.000559995	0.362
E1BND7	Uncharacterized protein	PPFIA2	0.007269613	0.373
Q58D67	Dynactin 4	DCTN4	2.36E-05	0.403
Q3SZK1	Angio-associated migratory cell protein	AAMP	0.004313819	0.409
E1BPI2	Non-specific serine/threonine protein kinase (EC 2.7.11.1)		0.006717303	0.412
Q3ZCH5	Zinc-alpha-2-glycoprotein (Zn-alpha-2-GP) (Zn-alpha-2-glycoprotein)	AZGP1	0.007264748	0.426
A6QQR0	WDR75 protein	WDR75	2.56E-05	0.427
Q2KJ61	Elongator complex protein 3 (EC 2.3.1.48)	ELP3	0.000855102	0.435
E1BIM7	Uncharacterized protein	CELF2	0.005137574	0.439

E1BGN3	Histone H3	HIST2H3D	2.16E-15	0.444
A1A4J3	Zinc finger, CCHC domain containing 3	ZCCHC3	0.000169658	0.449
E1BE33	Uncharacterized protein	ZEB2	1.58E-09	0.450
G3MZD8	Uncharacterized protein		0.002250709	0.452
E1BEP7	Uncharacterized protein	ELP2	0.00728467	0.459
Q08E58	Tubulin tyrosine ligase-like family, member 12	TTLL12	0.000147691	0.467

Table 2.

The top 30 up-regulated DAPs between yak and cattleyak epididymis.

Accession	Description of Protein	Protein symbol	Pvalue	FC (P/M)
E1BKY2	Uncharacterized protein		0.00881948	4.65
F1MJS8	A-kinase anchor protein 3	AKAP3	0.000526461	4.26
F1MIM1	Uncharacterized protein	LOC104976250	0.000889658	3.89
Q3YJL1	MHC class I antigen (Fragment)	BoLA	0.007469039	3.81
Q6DTZ8	Beta A4 crystallin (Beta-crystallin A4)	CRYBA4	8.45E-09	3.80
G3N2N9	Glutathione peroxidase	GPX5	7.28E-05	3.55
E1BE11	Uncharacterized protein	HMCN1	5.28E-09	3.53
F1MIM0	Uncharacterized protein		0.000618683	3.41
Q32KP8	Serine peptidase inhibitor-like, with Kunitz and WAP domains 1 (Eppin)	SPINLW1	1.30E-19	3.34
Q0VCG3	Parvalbumin alpha	PVALB	0.000112643	3.03
E1BGB7	Uncharacterized protein		1.07E-05	2.85
Q3MHJ9	Calcium/calmodulin-dependent protein kinase type II subunit beta (CaM kinase II subunit beta) (CaMK-II subunit beta)	CAMK2B	0.001572046	2.79
Q70IB2	Inactive ribonuclease-like protein 10 (Protein Train A)	RNASE10	0.000205134	2.77
F1MY32	Uncharacterized protein	LY6G5C	0.004856315	2.76
F1MSZ5	Uncharacterized protein	ADAM28	1.08E-09	2.72
P02192	Myoglobin	MB	3.62E-05	2.65
A6H742	Plastin-1	PLS1	7.90E-07	2.64
G3N2L2	Uncharacterized protein	RCN1	0.000161915	2.58
F1MTV5	Amino acid transporter	SLC1A5	0.004519988	2.56
F1N5W4	Uncharacterized protein	ENPP5	0.000276382	2.52
F1N2E1	Uncharacterized protein	WFDC8	0.0076419	2.45
Q2TBR5	Protein FAM166B	FAM166B	2.10E-08	2.42
Q0VCU3	Cathepsin F	CTSF	0.002189062	2.33
A7E340	Mucin 15, cell surface associated	MUC15	7.89E-33	2.32

E1BD73	Poly [ADP-ribose] polymerase (PARP) (EC 2.4.2.30)	PARP4	5.16E-14	2.31
F1MG20	17-beta-hydroxysteroid dehydrogenase type 6	HSD17B6	0.002590687	2.26
F1MV86	G1/S-specific cyclin-D3	CCND3	0.000102853	2.25
A6QQ08	SNCA protein (Fragment)	SNCA	0.009021835	2.24
F1MUC1	Uncharacterized protein	ABCC4	4.25E-06	2.21
E1BJ49	Uncharacterized protein	MASP2	0.000448626	2.18

Figures

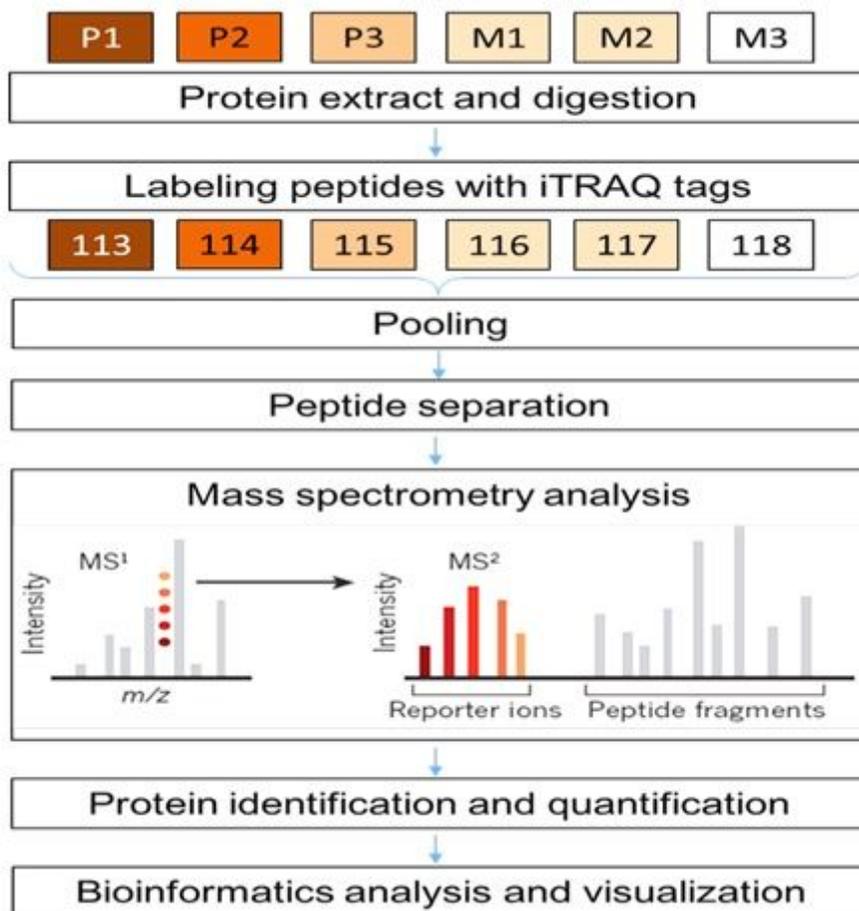
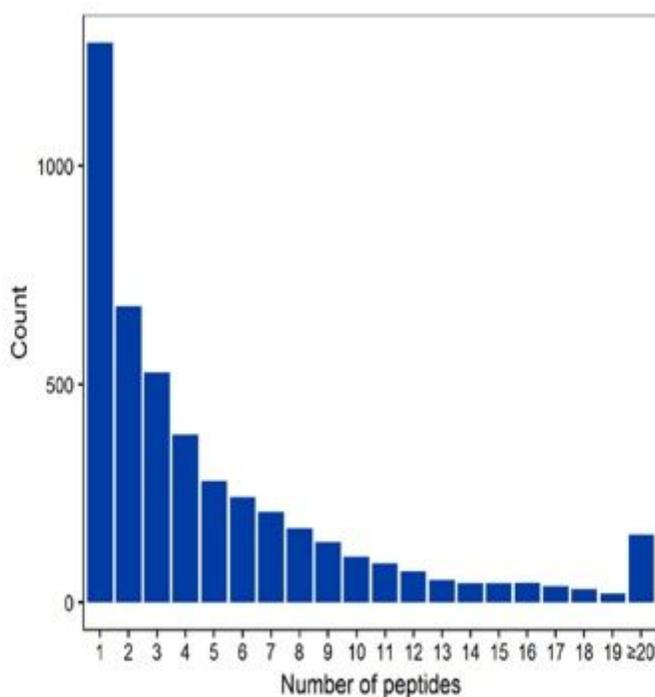
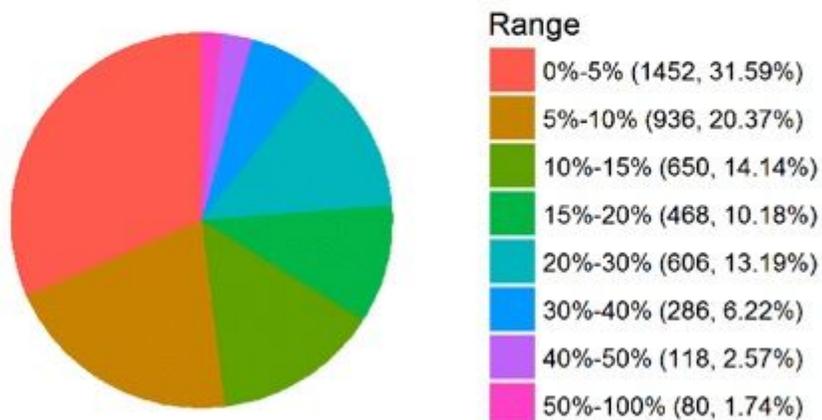


Figure 1

The schedules of experimental treatment for the comparison of yak and cattleyak epididymal proteome. Proteins extracted from epididymis tissue samples were diminished, alkylated and enzymatically digested before labeling with iTRAQ, followed by LC-MS/MS mass spectrometry analyses. In the last section, proteins were identified and quantified before analysing and visualization.

A

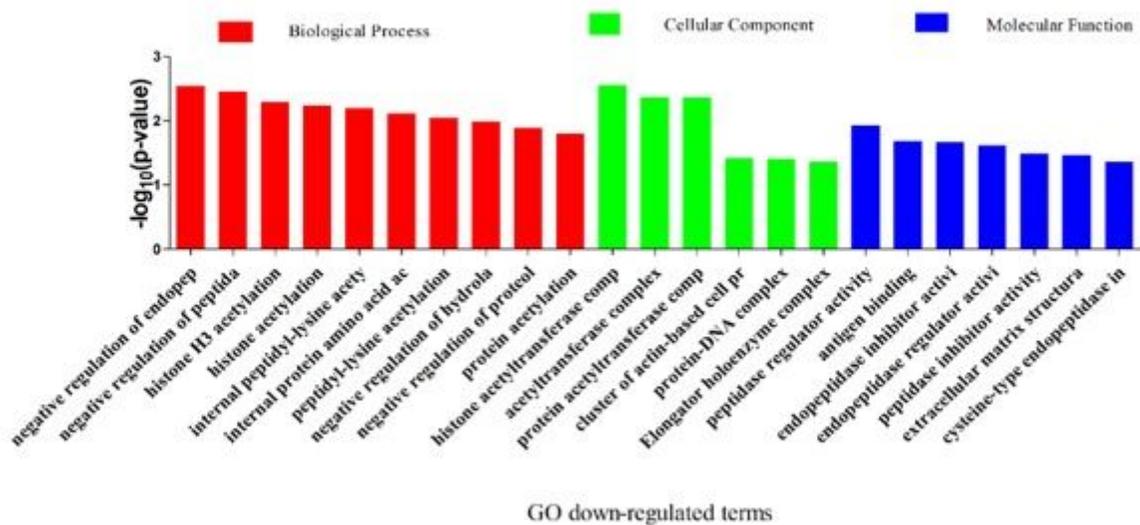
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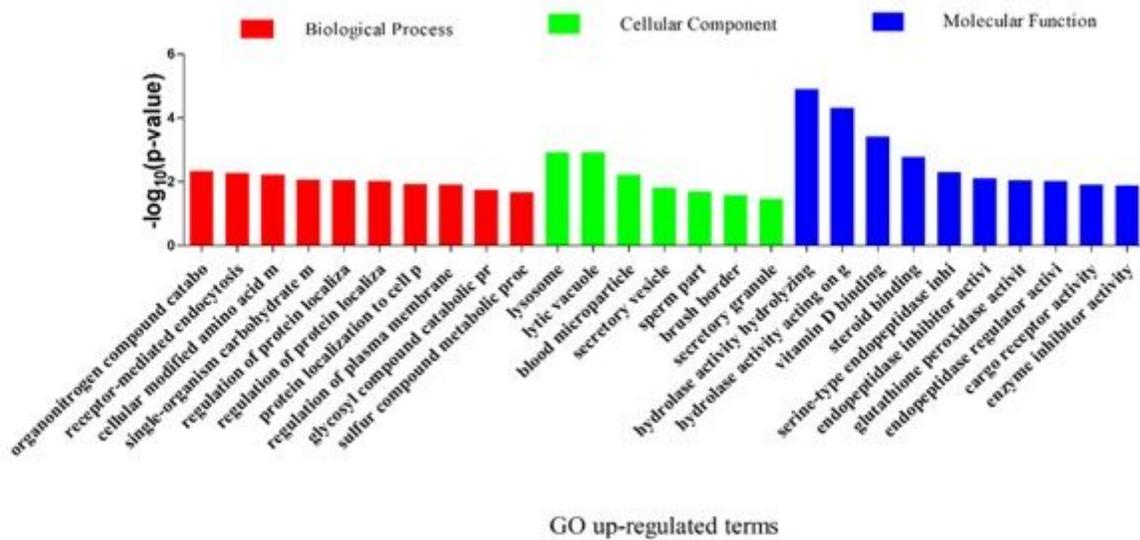
B

Figure 2

Peptide sequence coverage and identification of number of peptides. The (Fig 2A) shows the proportion of proteins in different coverage ranges. The different colors representing different ranges of sequence coverage. The brackets show the number of proteins in different coverage ranges and their proportion to the total protein. The (Fig 2B) shows the distribution of the number of peptides contained in the identified protein. The abscissa is the range of peptides covering the protein, and the ordinate is the number of proteins.



(A)



(B)

Figure 3

GO classification of DAPs in cattleyak compared to yak. The Fig 3A describes GO terms of down-regulated DAPs and the Fig 3B describes the GO terms of up-regulated DAPs. The x-axis represents the number of GO terms and y-axis represents p-value of each GO terms.

Figure 5

PPI of DAPs in cattleyak created by STRING database.

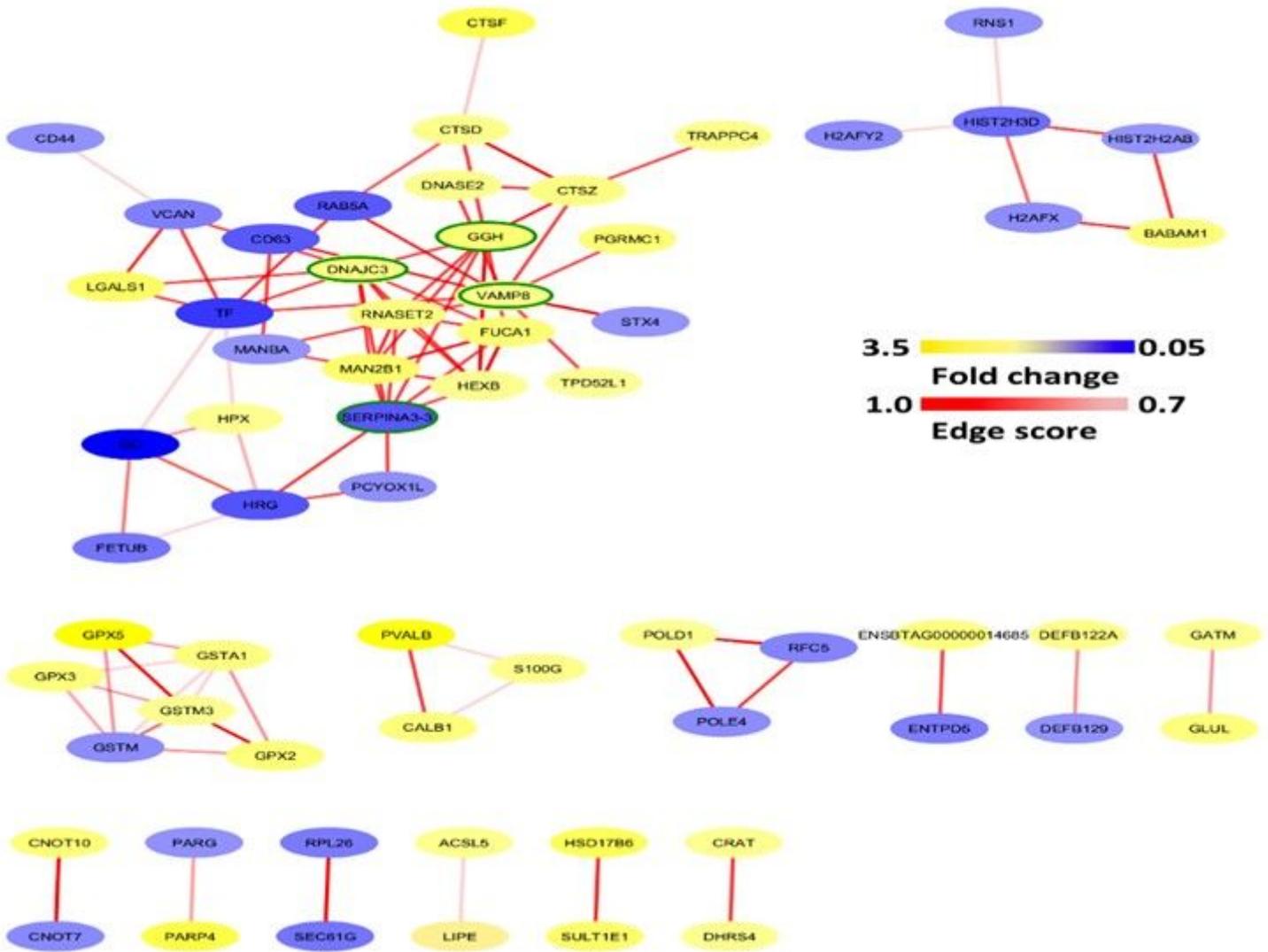


Figure 6

PPI network of DAPs created with STRING database and visualized with Cytoscape. Proteins in blue were down-regulated and those in yellow were up-regulated. The line that connected the proteins with each other is called an edge. The color of the edge indicated the combined interaction score (edge score). The nodes with green borders described hub proteins.

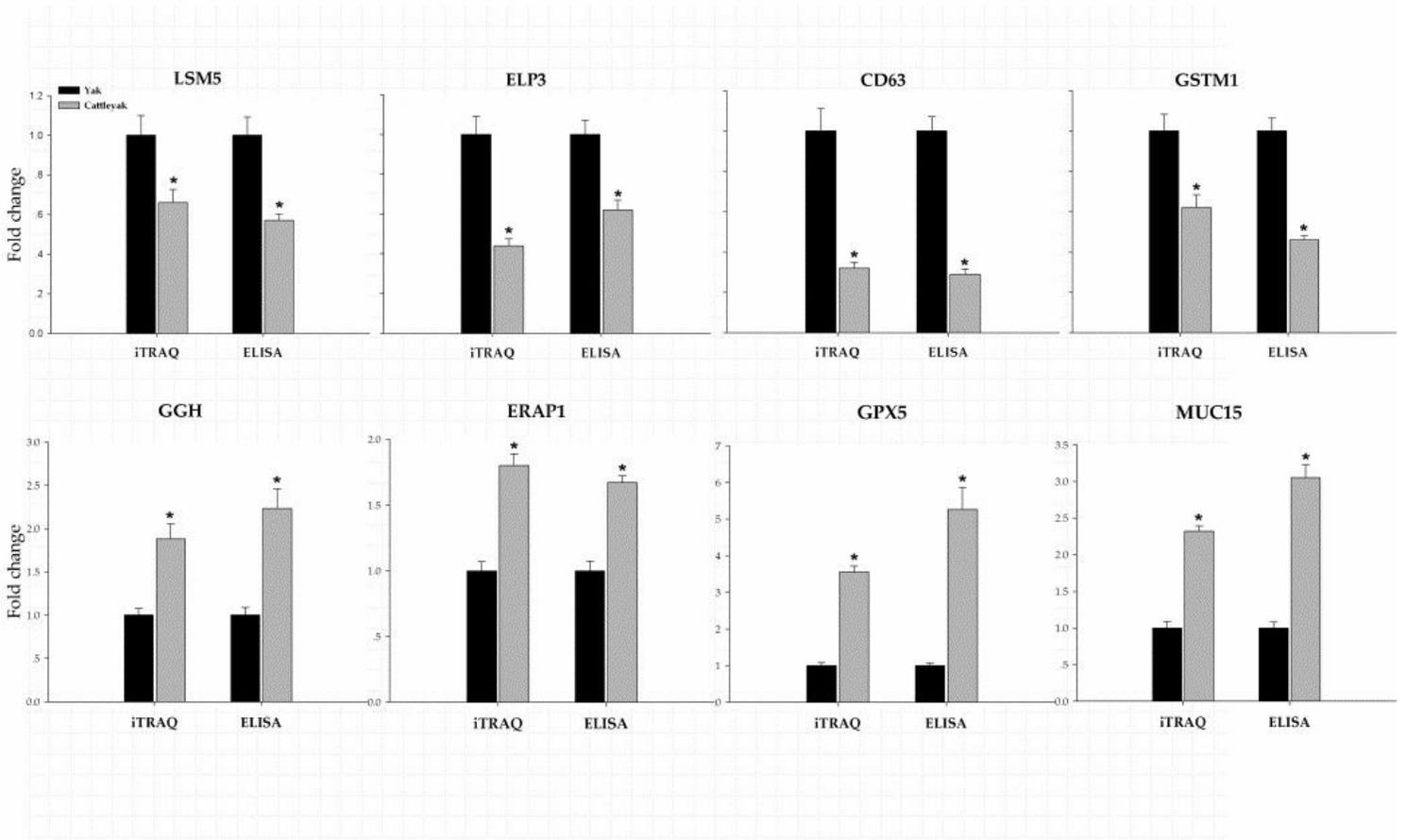


Figure 7

Validation of iTRAQ data with ELISA. The vertical axis indicates the fold change of protein abundance. The horizontal axis indicates the methods for ELISA and iTRAQ. In each method, the protein expression level of yak was the control and designated as 1 to determine the expression level of cattleyak. Expression level of each protein is the mean of three replicates for cattleyak and plotted (mean±SD) in relation to yak as control (Y=1). The expression pattern of the protein CD63, ELP3, LSM5, GSTM1, GGH, ERAP1, GPX5 and MUC15 was validated respectively. * Protein expression significantly different from control (p < 0.05, Student's t-test)