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Phosphoproteome profiling reveals the role of mitochondrial proteins phosphorylation in beef color development

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ABSTRACT

This study investigated the effects of mitochondrial protein phosphorylation on beef color stability through a quantitative mitochondrial phosphoproteome analysis. The mitochondria from beef samples of high, medium and low color stability were extracted and the phosphorylation status of their protein profiles were investigated. In total, 376 identified phosphopeptides were assigned to 191 phosphoproteins in mitochondria. The phosphorylation status of 47 mitochondrial proteins were significantly differed between the compared groups and 70% of phosphosites were significantly upregulated. The mitochondrial phosphoproteins were involved in the pathway of oxidative phosphorylation, signaling transduction, transport, muscle contraction, etc. The phosphorylation of NADH dehydrogenase, cytochrome *c* oxidase in the mitochondrial inner membrane and the phosphorylation of voltage-dependent anion-selective channel protein in the mitochondrial outer membrane were positively correlated with meat color. Collectively, this study explored the important role of mitochondrial phosphoproteins in meat color development and broadens our knowledge on mitochondria-mediated meat color regulation.

1. Introduction

Mitochondria is regarded as energy plant in living tissues. However, glycolysis becomes the main way of energy metabolism due to hypoxia after slaughter. Whether mitochondria in postmortem meat are completely useless after slaughter? Absolutely not. The functional mitochondria can still be separated in beef muscle up to 60 days postmortem (Tang, Faustman, Mancini, et al., 2005). Mitochondria involves in postmortem energy metabolism, apoptosis, calpain system, etc. (Zou et al., 2023). Myoglobin is the main heme pigments in postmortem meat, which competes oxygen with mitochondria and thus greatly affecting meat color (Suman & Joseph, 2013). Recently, the role of mitochondria on the development of meat color has attracted a lot attention (Mancini & Ramanathan, 2014; McKeith et al., 2016). It has been demonstrated that mitochondria influence meat color through competition of oxygen with myoglobin or through affecting metmyoglobin reduction (Mancini & Hunt, 2005; Zhang et al., 2023). With the application of proteomics in the meat science field, many proteins located in mitochondria could be linked to color stability of dark cutting/normal beef (Wu et al., 2020; Yu et al., 2017b). It is suggested that specific properties of mitochondrial proteins have a considerable effect on meat color.

Protein phosphorylation is a reversible post-translational modification (PTM) contributing to changes of protein properties. Protein phosphorylation regulates meat quality through glycolytic enzyme activity, myofibrillar protein degradation, μ-calpain autolysis, actomyosin dissociation and myoglobin redox stability (Li et al., 2021). Some mitochondrial proteins like ADP/ATP translocase 1 and voltage-dependent anion channel are identified in previous study but they are not concerned a lot (Weng et al., 2022). The pyruvate dehydrogenase complex was the first mitochondrial protein studied from the

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protein phosphorylation point of view in beef kidney (Linn et al., 1969). Later studies proved that mitochondria not only had a vital role in energy production but also in signaling, apoptosis, etc. through protein phosphorylation that can regulate mitochondrial processes (Pagliarini & Dixon, 2006; Thomson, 2002). Protein phosphorylation of the outer membrane proteins in mitochondria influenced their activity and interaction with other proteins (Kerner et al., 2011). Moreover, phosphorylated proteins in mitochondria contribute to metabolic pathways, redox proteins and processes, and mitochondrial organization resulting in impacts on enzyme activity and electron transfer (Sunitha et al., 2020). The above studies about mitochondrial protein phosphorylation are mostly performed in life science. However, the role of mitochondrial protein phosphorylation in postmortem muscle has not been well explained. Here, quantitative mitochondrial phosphoproteomes of beef showing different color stabilities were analyzed in order to better understand the relationship between mitochondrial protein phosphorylation and meat color and to target critical mitochondrial phosphoproteins relating to color development.

2. Materials and methods

2.1. Sample collection, meat color parameters and grouping

The *longissimus thoracis et lumborum* (LTL) muscles from the left side of 60 Simmental cow carcasses (24-month-old, intensively reared) were collected. Cow were transported to the commercial abattoir of Fortune Ng Fung Food (Hebei) Co., Ltd. with a short distance. The slaughter procedure was according to the guidelines of the abattoir. About 8–10 cm LTL muscle sample of posterior were collected from every beef carcass and stored for 0, 2, 6 and 10 days at 4 °C by wrapping them in polyvinylchloride film under display. The ultimate pH values of all 60 LTL muscle samples were recorded at day 2 of storage with a calibrated pH meter (Testo205 pH meter, Lenzkirch, Germany). The pH meter was calibrated with buffers of pH 4.0, 7.0 and 10.0 at a refrigeration room about 4 °C. A 3 cm thick piece at the same location of each LTL muscle sample was prepared for meat color measurement. The surface color (CIE-$L^*a^*b^*$) of muscles' cross section was recorded at four random points by a Minolta CM-600D spectrophotometer (Konica Minolta Sensing Inc., Osaka, Japan) with illuminant D_{65}, 10° standard observer at day 0, 2, 6 and 10 of storage. Meat color of day 0 was recorded after being exposed to the air for 2 h.

The a^* (redness) is regarded as the main parameter of fresh meat because L^* and b^* always show slight changes during display (Rosenvold & Andersen, 2003; Li et al., 2018). The design of grouping is shown in Fig. 1. All 60 LTL muscle samples were ranked from highest a^* (No. 1) to lowest a^* (No. 60) according to the a^* at day 10 of storage. Six LTL muscle samples selected from No. 1 to No. 10 were regarded as the high color stability group. Six LTL muscle samples selected from No. 26 to No. 35 were regarded as the medium color stability group. Six LTL muscle samples selected from No. 51 to No. 60 were regarded as the low color stability group. Thus, 6 LTL muscle samples (n = 6) were included per group at each storage time and totally 18 beef carcasses were collected for final analysis. The reflectance at 630 nm divided by the reflectance at 580 nm (R630/580) was another measure to indicate color stability. The ratio of deoxymyoglobin (DMb), oxymyoglobin (OMb) and metmyoglobin (MMb) was calculated as described in a previous study (M. Li et al., 2018). Briefly, the reflectance/100 was regarded as R, and K/S was calculated by $(1\text{-}R)^2/2R$. The equations of $1.5 - (K/S_{474})/(K/S_{525})$, $1 - (K/S_{610})/(K/S_{525})$, $2 - (KS_{572})/(K/S_{525})$ were calculated for DMb, OMb and MMb, respectively. In addition, there were no significant differences of ultimate pH values between the three groups so there was no effect of pH on meat quality.

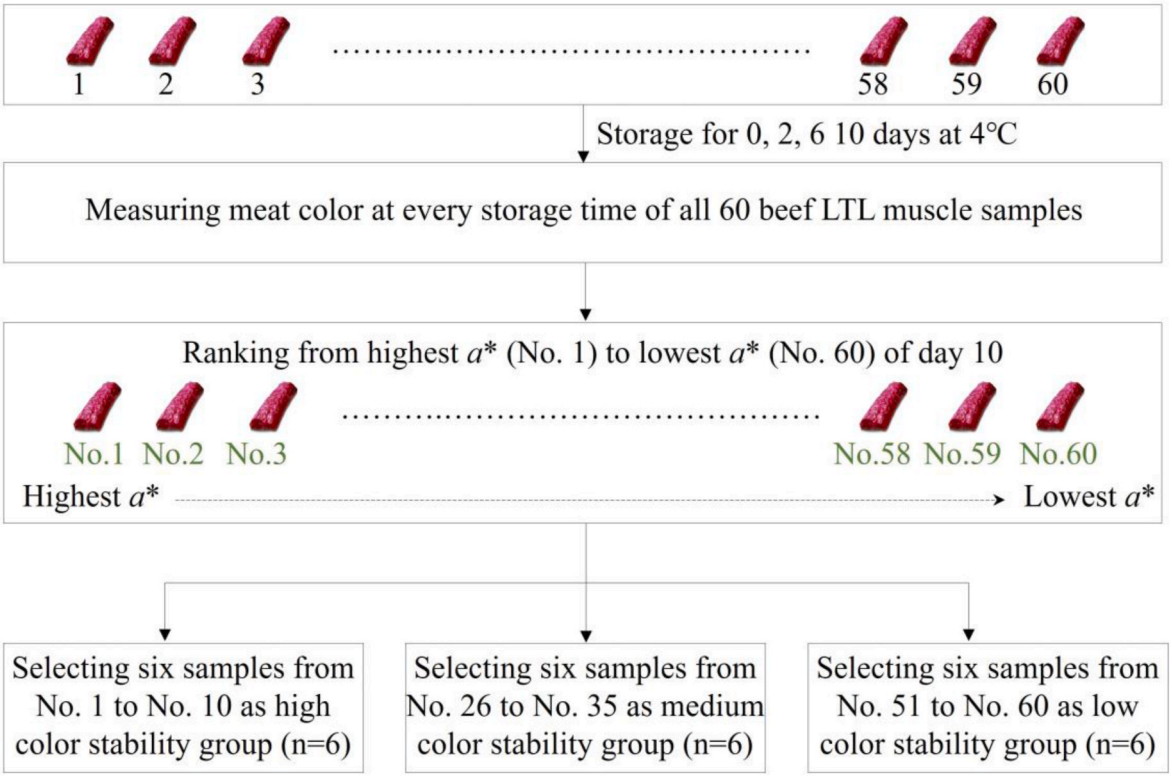

Fig. 1. The experimental design of this study. The *longissimus thoracis et lumborum* (LTL) muscles from of 60 beef carcasses were collected and stored at 4 °C for 0, 2, 6 and 10 days. The a^* values were measured and ranked from highest a^* (No. 1) to lowest a^* (No. 60) according to the a^* on day 10 of storage. High, medium and low color stability group (n = 6) were then selected. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2.2. Mitochondria extraction

The muscle samples of day 0 from the three groups were used for phosphoproteome analysis. Every two samples were mixed in each group to obtain triplicates within the same group (H1, H2, H3; M1, M2, M3; L1, L2, L3). The whole process of mitochondria extraction was performed at 4 °C. Approximately 2 g minced sample was homogenized with 4 mL lysis buffer from a tissue mitochondrial extraction kit (Solarbio Life Science, Bejing, China) and centrifugated for 5 min at 1000×*g*. The fat in the supernatant was removed with 8 layers of gauze and then centrifugated again. Crude mitochondria were sedimented after centrifugating for 5 min at 12000×*g*. The pellets were resuspended with 5 mL wash buffer and centrifugated for 5 min at 1000×*g*. High purity mitochondria were collected after 12000×*g* for 10 min. The mitochondria were stored at −80 °C after resuspending them in 600 μL store buffer.

2.3. Analysis of the quantitative phosphoproteomes

2.3.1. Trypsin digestion and iTRAQ labeling

The phosphoproteome was performed using tandem mass tag labeling by referencing the method of Li et al. (2018). The mitochondria were mixed with 1 mM DTT, 4% SDS and 100 mM Tris-HCl (pH7.6). Ultrafiltration with Microcon units of 10 kDa was performed with 8 M Urea and 150 mM Tris-HCl, pH 8.0 (UA buffer). Samples were incubated with 100 μL iodoacetamide for 30 min in darkness and then washed with 100 μl UA buffer and DS buffer (25 mM NH_4HCO_3), respectively. Four μg trypsin was added to digest protein samples overnight at 37 °C. Desalting of collected peptides was performed on C18 Cartridges (Empore™ SPE Cartridges C18, Sigma, St. Louis, MO, USA). The peptides were reconstituted in 40 μL of 0.1% (v/v) formic acid after vacuum centrifugation. One hundred peptides were labeled with iTRAQ reagent according to the manufacturer protocol (Applied Biosystems, Wilmington, USA).

2.3.2. Phosphorylated peptide enrichment and mass spectrometry analysis

Labeled peptides were resuspended with 500 μL buffer containing 2% glutamic acid, 65% ceric ammonium nitrate, and 2% TFA after vacuum concentration. Labeled peptides were mixed while shaking for 2 h with TiO_2 beads after which they were centrifugated at 5000×*g*. The precipitates were washed with a 50 μL buffer of 30% ACN (acetonitrile), 1% TFA (trifluoroacetic acid), and 80% ACN, 0.1% TFA for three times, respectively. Fifty μl of elution buffer (40% ACN, 15% $NH_3{\cdot}H_2O$) was used to elute phosphopeptides. The separation of phosphopeptides was done using a reverse phase trap column (Thermo Scientific Acclaim PepMap100, 100 μm*2 cm, nanoViper C18) connected to a C18-reversed phase analytical column (Thermo Scientific Easy Column, 10 cm long, 75 μm inner diameter, 3 μm resin). Q Exactive mass spectrometer in positive ion mode coupled to Easy nLC (Thermo Fisher Scientific) was performed to analyze phosphopeptides fractions. MASCOT engine (version 2.2, Matrix Science, London, UK) embedded into Proteome Discoverer 1.4 was used to identify the peptides.

2.3.3. Bioinformatic analysis

Blast2GO (version 3.3.5) and the online KEGG database (http://geneontology.org/) were used for GO and KEGG pathway annotation, respectively. Fisher' exact test was applied for functional enrichment analysis. Motifs were analyzed by MEME (http://meme-suite.org/tools/momo). The minimum number of occurrences required for a motif was set as 50. Other parameters were left as their defaults. A protein-protein interaction network was performed by STRING 11.5 (https://string-db.org/).

2.4. Statistical analysis

Meat color was analyzed with IBM SPSS Statistic software (version 19.0, SPSS Inc., Chicago, IL, USA) using a linear mixed model. Different color stability groups and storage times were regarded as fixed factors. Beef carcass was regarded as random factor. The data are shown as mean ± standard error and estimated by Duncan's multiple range tests ($P < 0.05$).

3. Results

3.1. Meat color

The a^* and R630/580 of selected LTL muscle samples were both significantly different between the three groups, between different storage times and their interaction ($P < 0.05$, Fig. 2B and D). Compared with the low color stability group, a^* in the high color stability group was higher on day 0, 6 and 10 ($P < 0.05$, Fig. 2B). The decreasing a^* in the low color stability group showed an inferior development of meat color. The R630/580 showed a significant decrease from day 0 to day 10 in the medium and low color stability group ($P < 0.05$, Fig. 2D). The L^* and b^* showed insignificant differences between the three groups ($P > 0.05$, Fig. 2) but storage time had a significant effect on them ($P < 0.05$, Fig. 2A and C). The L^* showed a slight increase from day 0 to day 10. The b^* in the low color stability group increased on day 2 and then decreased on day 6 and day 10. Compared with the medium and low color stability groups, the b^* was higher in the high color stability group on day 10 ($P < 0.05$, Fig. 2C). OMb and MMb were significantly affected by the three groups, the storage time and their interaction ($P < 0.05$, Fig. 2E and F), which suggested a pronounced change of different myoglobin statuses. There was a significant decrease of OMb on day 10 from the high to the low color stability group. MMb showed a huge increase from day 0 to day 10 especially in the low color stability group. There was a subtle decrease of DMb during the whole storage period (Fig. 2G).

3.2. Annotation and sequence properties of all identified phosphopeptides and phosphoproteins

A total of 2086 identified phosphopeptides was assigned to 961 phosphoproteins. The top 20 of GO terms and KEGG pathways were annotated to functions involved in meat color stability. The results of the GO analysis are divided according to biological process, molecular function and cellular component and shown in Fig. 3A. GO terms for the generation of precursor metabolites and energy, and oxidation – reduction processes were significantly enriched ($P < 0.05$). The molecular function of the phosphorylated proteins focused on the activity of transmembrane transporters such as proton transmembrane transporters, inorganic molecular entity transmembrane transporters and ion transmembrane transporters. Nineteen mitochondrial phosphoproteins were significantly annotated in cellular components. The top 20 of KEGG pathways are shown in Fig. 3B. The pathways related carbohydrate metabolism like glycolysis and gluconeogenesis, galactose metabolism, as well as starch and sucrose metabolism were enriched. Moreover, the AMPK signaling pathway, cGMP-PKG signaling pathway and calcium signaling pathway were identified as enriched. These results indicated that the pathways involved in energy metabolism and signaling took part in the development of meat color.

A total of 8 motifs were discovered to be significantly enriched through Motif-X software analysis (Fig. 3C). All of them included serine which suggested that the serine phosphorylation played a more important role than threonine and tyrosine phosphorylation in meat color development. In addition, the motif analysis provides the patterns of potential conserved sequences, which is one of the methods to predict the upstream kinase (Colaert et al., 2009). The sequences of ……S_P ….. were proline-directed kinase substrate motifs, which are always phosphorylated by mitogen-activated protein kinases (MAPKs). The sequences of … R._S_L ….., … RR._S_ ……, … R._S_ ……, and … K._S_ ….. were basophilic motifs, which are often phosphorylated by AGC kinases Akt (Humphrey et al., 2013).

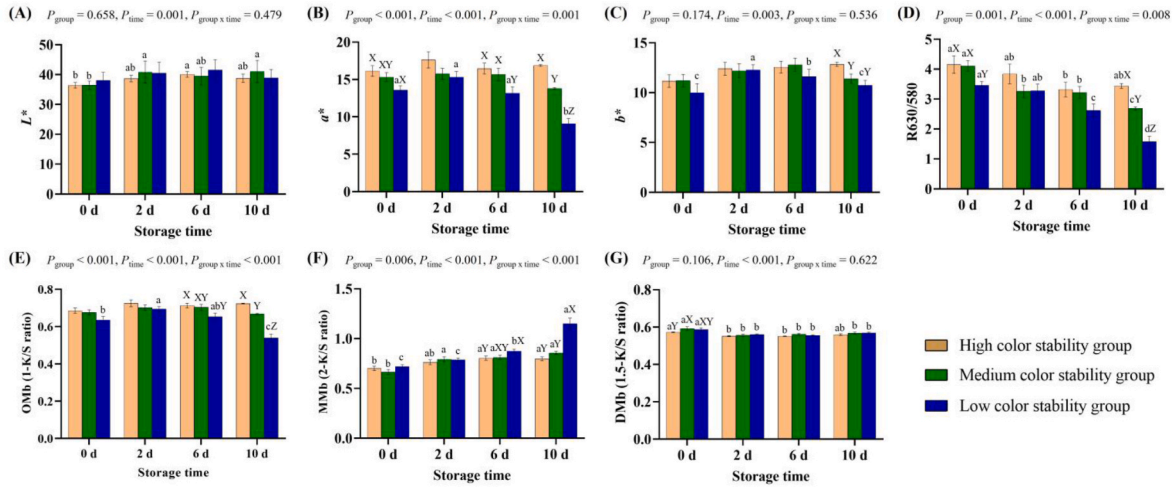

Fig. 2. The changes of meat color (A: L^*, B: a^*, C: b^*, D: R630/580) and myoglobin statuses (E: OMb, F: MMb, G: DMb) in beef LTL muscles of high, medium and low color stability groups stored at 4 °C for 10 days. The P values of the effect of groups, storage time and their interaction on each meat color trait were listed. The small letters of a-d represent significant differences on different storage times in the same color stability group ($P < 0.05$). The capital letters of X-Z represent significant differences in different color stability groups on the same storage time ($P < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

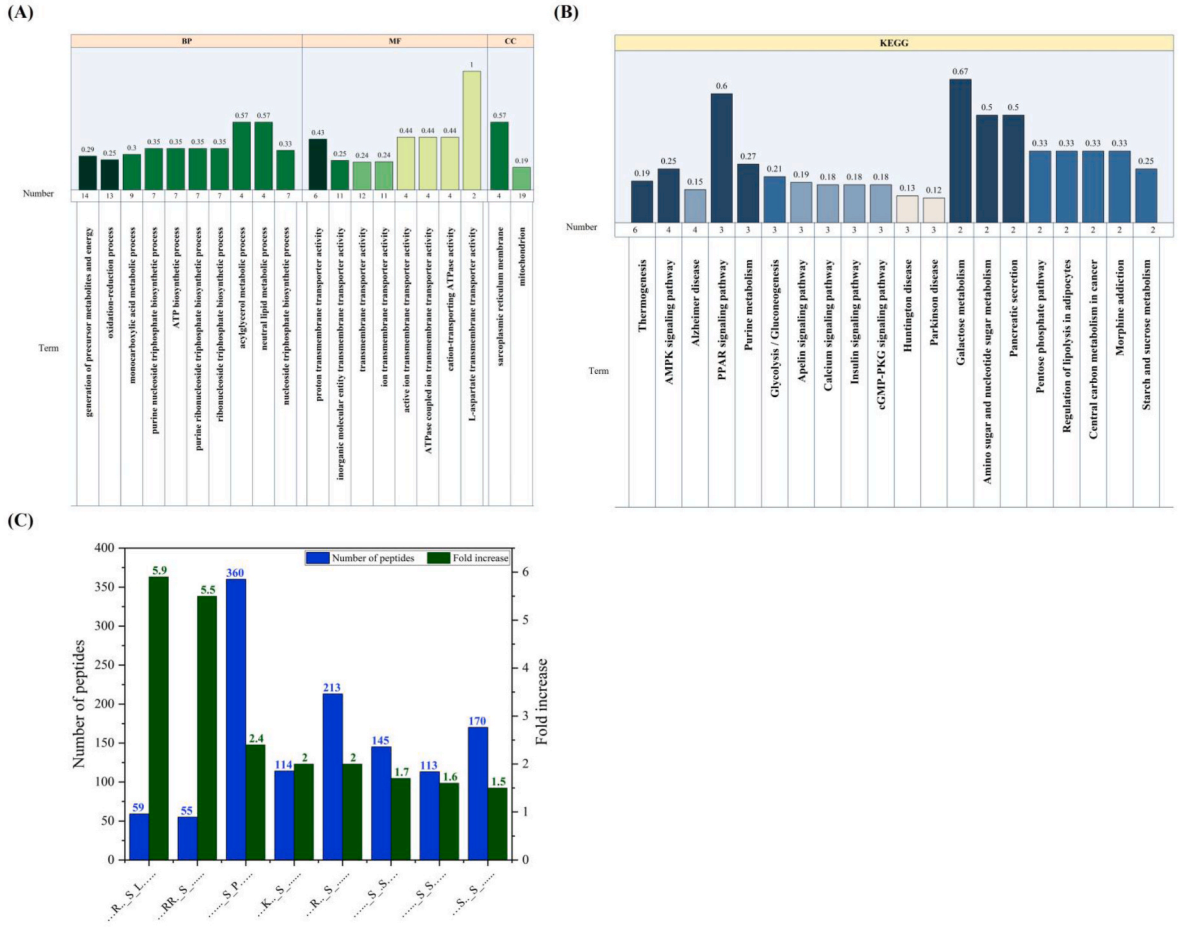

Fig. 3. The functional annotation of all phosphorylated proteins in beef muscle with different color stability. (A) The detail information of enriched GO terms (top 20) about biological process (BP), molecular function (MF) and cellular component (CC) of the phosphoproteins among the three groups. (B) The feature of enriched KEGG pathways (top 20) of the phosphoproteins among the three groups. The figures on the bars represented rich factor. (C) The information of over-enriched phosphorylation motifs identified from phosphopeptides. Fold increase represented the ratio of the number of identified phosphopeptides with the number of predicted phosphopeptides. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.3. Overview of phosphorylation profiles of mitochondrial proteins in beef with different meat color stability

Among all phosphorylation profiles, 376 phosphopeptides were assigned to 191 phosphoproteins in mitochondria according to subcellular location (Table S1). One hundred and nineteen mitochondrial phosphoproteins had only one phosphorylated site (Fig. 4B). Serine (Ser), threonine (Thr) and tyrosine (Tyr) are the three amino acids undergoing protein phosphorylation. There were 294, 57 and 25 of phosphosites at Ser, Thr and Tyr residues among mitochondrial phosphopeptides, respectively (Fig. 4A). The number of phosphorylated serine residues was most common and accounted for 78.2%.

The significant abundance of phosphopeptides was screened based on a fold change >1.2 or <0.833 and $P < 0.05$ (Fig. 4C). There were 24 phosphopeptides upregulated and 7 phosphopeptides downregulated between high and low color stability groups. Fifteen phosphopeptides were determined to be different between the high and medium color stability groups, of which 10 phosphopeptides were upregulated and 5 phosphopeptides were downregulated. Comparing the medium and low color stability groups showed 14 phosphopeptides differently enriched and the abundance of 8 phosphopeptides was increased and 6 phosphopeptides was decreased. Hierarchical clustering was performed on the phosphopeptides with significant enrichment to understand the regulation patterns of the different color stability groups (Fig. 4D–E). Every phosphopeptide was assigned to a row. The more yellow color represents a higher phosphorylation abundance and the more blue color means a lower phosphorylation abundance. Approximately, two different regulation patterns, I and II, were clustered among the compared groups. The regulation of pattern I showed a higher protein phosphorylation level in better color stability groups, while pattern II was opposite, i.e. low color stability beef samples showed a higher protein phosphorylation level.

3.4. Protein-protein interaction (PPI) networks of mitochondrial phosphoproteins

To better understand the phosphorylation of mitochondrial proteins and their link to color development in beef muscle, PPI networks were performed using STRING database. Forty-eight clusters were significantly enriched in PPI networks of mitochondrial phosphoproteins ($P < 0.05$, Table S2). The four largest clusters are labeled in Fig. 5. Among them, the cluster for oxidative phosphorylation and mitochondrial respiratory chain complex assembly (cluster ID: 13013) was the biggest one, which included 35 proteins. Besides, these proteins were also enriched in the cluster for respiratory chain complex, and proton-transporting ATP synthase complex (cluster ID: 13018), respiratory chain complex (cluster ID: 13019) and NADH dehydrogenase complex (cluster ID: 13024). The result of the PPI networks revealed that protein phosphorylation might be involved in meat color formation via the biological processes related with oxidative phosphorylation.

3.5. Functional analysis of mitochondrial proteins with differential expression between the color stability groups

A total of 47 mitochondrial phosphoproteins with significant enriched phosphosites were identified (Table 1). About 70% phosphosites were upregulated and 30% phosphosites were downregulated. There were 39 phosphorylated serine residues, 5 phosphorylated threonine residues and 3 phosphorylated tyrosine residues. The serine residues accounted for 83% of significant enriched phosphosites of mitochondrial proteins. NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 13 (NDUFA13), phosphoglycerate mutase (PGAM2), solute carrier family 25 member 12 (SLC25A12), target of myb1 membrane trafficking protein (TOM1) and upregulated during skeletal muscle growth protein 5 (USMG5) were identified with

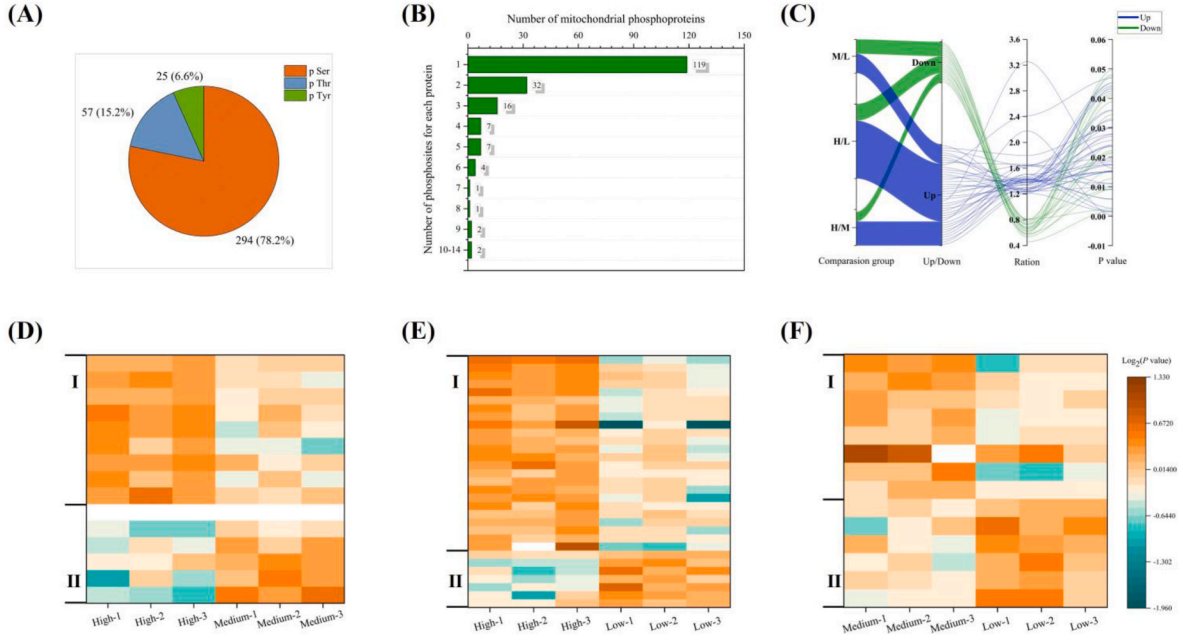

Fig. 4. Overview of phosphorylation profiling of mitochondrial proteins in beef muscle with different color stability. (A) Distribution of amino acid residues of phosphosites. "p Ser" represented phosphorylated serine residues, "p Thr" represented phosphorylated threonine residues, "p Tyr" represented phosphorylated tyrosine residues. (B) Number of phosphosites for mitochondrial proteins. (C) Phosphopeptides of mitochondrial proteins with significant upregulation and downregulation in comparison groups. "H/M" meant the comparison of high and medium color stability group, "H/L" meant the comparison of high and low color stability group, "M/L" meant the comparison of medium and low color stability group. Blue lines represented upregulation and green lines represent downregulation. Each line denoted a phosphopeptide. Ration represent the foldchange. (D–F) Hierarchical clustering analysis of significant enrichment phosphopeptides of mitochondrial proteins among each comparison group. The yellower or bluer represents the higher or lower enrichment of phosphopeptides assigned to each row. High-1, High-2 and High-3 were three replications in high color stability group. Medium-1, Medium-2 and Medium-3 were three replications in low color stability group. Low-1, Low-2 and Low-3 were three replications in low color stability group. The I and II represent two phosphorylation patterns of the muscle samples. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

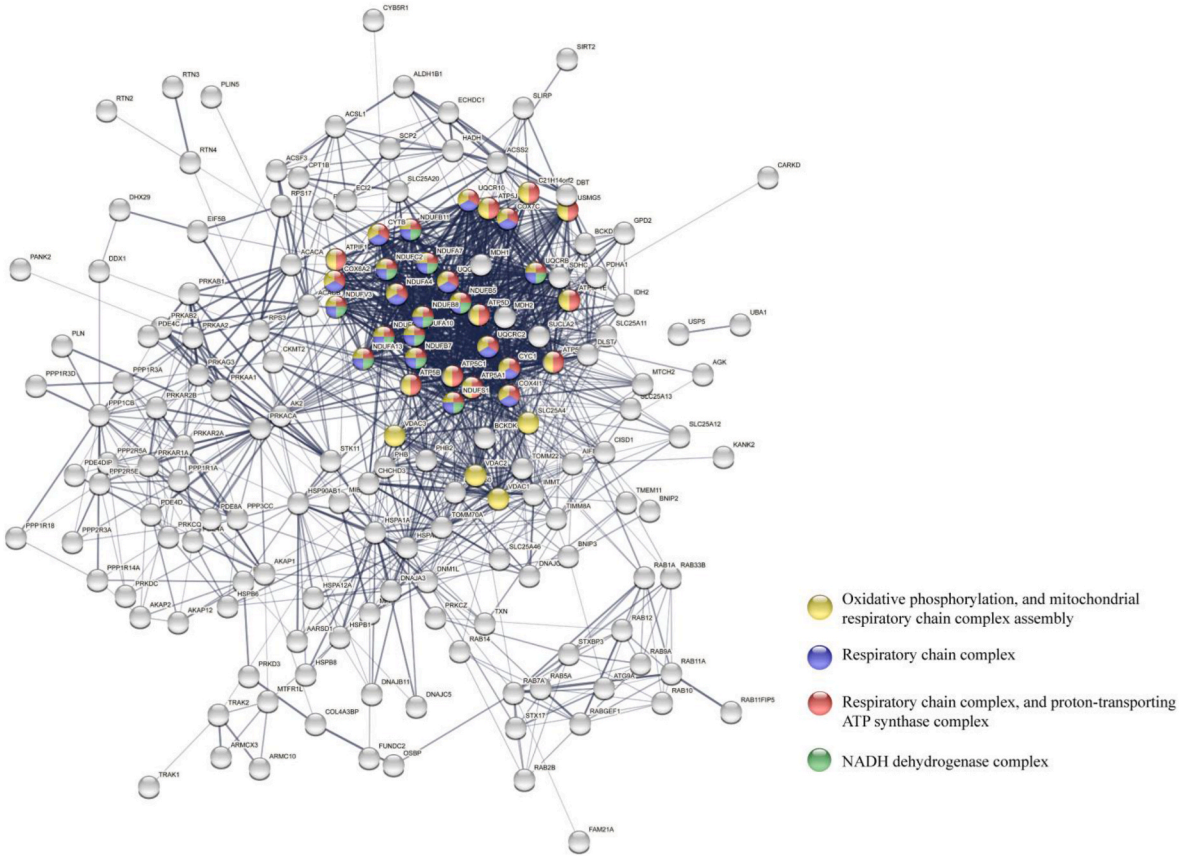

Fig. 5. Protein-protein interaction networks of mitochondrial phosphoproteins. The top four clusters were labeled with different color. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

significantly enriched phosphosites of threonine residues. Creatine kinase S-type (CKMT2), glutathione S-transferase (GSTM1) and an uncharacterized protein were identified with significantly enriched phosphosites of tyrosine residues. The 47 mitochondrial phosphoproteins mainly participated in biological processes like oxidative phosphorylation, carbon metabolism, muscle contraction, TCA cycle, glucagon signaling pathway, AMPK signaling pathway, etc. Besides, the mitochondrial phosphoproteins were also involved in the biological processes of transport, binding, catalytic activity and so on. This indicates that mitochondrial functions are related with many other biological processes through protein phosphorylation regulation.

The correlation analysis was performed to investigate the possible relationship between protein phosphorylation and meat color development (Fig. 6A). The phosphorylation levels of NDUFB7, SLC25A12, succinate–CoA ligase [ADP-forming] subunit beta (SUCLA2) and voltage-dependent anion-selective channel protein 2 (VDAC2) were significantly positively related with a^*. The phosphorylation levels of ATP5F1C, CYC1, DUSP27 protein (DUSP27), SLC25A12, SUCLA2 and VDAC2 were significantly positively related with b^*. The phosphorylation levels of SLC25A12, SUCLA2 and VDAC2 were significantly positively related with R630/580. Finally, the phosphorylation level of SUCLA2 was significantly positively related with OMb. Table 1 shows the functional information of all 47 mitochondrial phosphoproteins, which reveals that a multitude of them were involved in oxidative phosphorylation. Through searching the KEGG Pathway Database, some phosphoproteins involved in oxidative phosphorylation pathway were located in the mitochondrial membrane (Fig. 6B). Five complexes are part of the electron transport chain in mitochondria. The NDUFA13 and NDUFB7 belong to complex I. Succinate dehydrogenase (SDH) is included in complex II. UQCRC2 is one of the proteins in complex III. COX4I1 and COX7C are involved in complex IV. ATP5F1A and ATP5F1C belong to complex V. The phosphorylation of NDUFA13, NDUFB7, UQCRC2, COX7C, ATP5F1A and ATP5F1C increased distinctly in the higher color stability group (Table 1), while there was no difference of the phosphorylation status of SDH when comparing the three groups.

4. Discussion

4.1. Phosphorylation patterns of mitochondrial proteins in meat with different color stability

The a^*, R630/580, OMb and MMb were greatly affected by different groups, storage times and their interaction, which might contribute to meat color development. Compared with L^* and b^*, the value of a^* in postmortem muscle shows always larger changes (McKenna et al., 2005). The role of mitochondria in meat color regulation has been extensively focused on their effect on myoglobin redox status (Ramanathan et al., 2020; Suman & Joseph, 2013). The changes of OMb, MMb and DMb were all significantly affected by storage time (Fig. 2), which suggested an expected transformation from different statuses of myoglobin during display. Recently, mitochondrial proteomics revealed the importance of mitochondrial proteins on meat color development (Ranjith Ramanathan et al., 2021; Wu et al., 2020). It is known that phosphorylation alters protein function (Kokkinidis et al., 2020). Thus, this study performed a mitochondrial phosphoproteome analysis to better understand the relationship between mitochondria proteins phosphorylation and meat color development. The phosphoproteome profiling of day 0 was analyzed to predict differential meat color stability on final display. In total, 376 phosphosites of mitochondrial proteins were identified in this study (Fig. 4B). A number of 47 mitochondrial proteins were significantly enriched in beef samples of different color stability (Table 1). Quantitative analysis of differential

Table 1
The functional information of 47 mitochondrial phosphoproteins with significantly enriched phosphosites among the three comparison groups.

Protein Accessions	Protein Name	Gene Name	Phosphosites		Functions in biological process or pathway
			Up regulation	Down regulation	
Q58DN7	Acyl-CoA synthetase family member 3	ACSF3	Ser585		Metabolic pathways, fatty acid metabolism, fatty acid biosynthesis
E1BM36	A-kinase anchoring protein 1	AKAP1	Ser98		–
G3MWT9	A-kinase anchoring protein 12	AKAP12		Ser495	Regulation of protein kinase C signaling
F1MLB8	ATP synthase subunit alpha	ATP5F1A	Ser53		Metabolic pathways, oxidative phosphorylation
P05631	ATP synthase subunit gamma	ATP5F1C	Ser265		Metabolic pathways, oxidative phosphorylation,
Q2KJG8	[3-methyl-2-oxobutanoate dehydrogenase [lipoamide]] kinase	BCKDK	Ser31		Regulation of glucose metabolic process
F1MJT6	Creatine kinase S-type	CKMT2		Tyr368	Metabolic pathways, ATP binding, phosphocreatine biosynthetic process
P00430	Cytochrome *c* oxidase subunit 7C	COX7C	Ser17		Metabolic pathways, thermogenesis, oxidative phosphorylation, cardiac muscle contraction
Q7YRA7	Cytochrome *c*-1	CYC1	Ser117		Metabolic pathways, thermogenesis, oxidative phosphorylation, cardiac muscle contraction
Q0IIK5	ATP-dependent RNA helicase DDX1	DDX1	Ser481		ATP-binding, mRNA processing
P11179	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex	DLST	Ser82		2-oxoglutarate metabolic process, succinyl-CoA metabolic process
A6QNU6	DUSP27 protein	DUSP27	Ser425		Protein tyrosine/serine/threonine phosphatase activity
H9KUU8	Ethylmalonyl-CoA decarboxylase	ECHDC1		Ser13	Metabolic pathways
F1MCU7	Protein 4.1	EPB41	Ser345		–
E1BH17	Glutathione S-transferase	GSTM1	Tyr23		Glutathione metabolic process
F1N338	Hydroxyacyl-CoA dehydrogenase	HADH		Ser13	Metabolic pathways, fatty acid metabolism, fatty acid degradation
F1N614	Endoplasmic reticulum chaperone BiP	HSPA5	Ser590		–
Q04467	Isocitrate dehydrogenase [NADP]	IDH2	Ser301		Metabolic pathways, TCA cycle, carbon metabolism
Q95KV7	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 13	NDUFA13	Thr114		Metabolic pathways, thermogenesis, oxidative phosphorylation
Q02368	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 7	NDUFB7	Ser14		Metabolic pathways, thermogenesis, oxidative phosphorylation
F1MX42	Pantothenate kinase 2	PANK2		Ser87	Metabolic pathways, mitochondrion morphogenesis
A6QQQ0, F1N7Q1	Phosphodiesterase	PDE4A, PDE8A	Ser106	Ser393	Metabolic pathways, cAMP catabolic process
A7MB35	Pyruvate dehydrogenase E1 component subunit alpha, somatic form	PDHA1	Ser293		Metabolic pathways, TCA cycle, glucagon signaling pathway, carbon metabolism, pyruvate metabolism, glycolysis/gluconeogenesis
F1N2F2	Phosphoglycerate mutase	PGAM2	Thr152		Metabolic pathways, glucagon signaling pathway, carbon metabolism, glycolysis/gluconeogenesis
A6QLL0	Perilipin-5	PLIN5	Ser279		PPAR signaling pathway, mitochondrion localization
E1BLN7	Protein phosphatase 1 regulatory subunit 3A	PPP1R3A		Ser608	Insulin signaling pathway, regulation of glycogen biosynthetic process
F1MNS9	Protein phosphatase 1 regulatory subunit	PPP1R3D		Ser25	Insulin signaling pathway, glycogen metabolic process
F1MBG5	Non-specific serine/threonine protein kinase	PRKAA1		Ser508	Thermogenesis, insulin signaling pathway, glucagon signaling pathway, AMPK signaling pathway, FoxO signaling pathway, tight junction
E1B986	Protein kinase AMP-activated non-catalytic subunit beta 2	PRKAB2	Ser184		Thermogenesis, insulin signaling pathway, glucagon signaling pathway, AMPK signaling pathway, FoxO signaling pathway, tight junction
B0JYK4	Protein kinase cAMP-dependent regulatory type II beta	PRKAR2B	Ser114		Insulin signaling pathway
E1BNE5	Rab11 family-interacting protein 5	RAB11FIP5		Ser395	Protein transport
Q3ZBG1	RAB14 protein	RAB14		Ser204	AMPK signaling pathway
G3N2V0	Ras-related protein Rab-5A	RAB5A	Ser30		Regulation of protein localization
E1BKU2	Septin-2	SEPT2		Ser218	
A6QNM9	Solute carrier family 25 member 12	SLC25A12	Thr236		Calcium ion binding, transmembrane transport
F1MX88	Solute carrier family 25 member 13	SLC25A13	Ser54		Calcium ion binding, transmembrane transport
Q3SZA4	Solute carrier family 25 member 20	SLC25A20	Ser143		Thermogenesis, transmembrane transporter activity, mitochondrial transport
F1MGC0	Succinate–CoA ligase [ADP-forming] subunit beta	SUCLA2	Ser279		Metabolic pathways, TCA cycle, carbon metabolism
A5D7N3	Transmembrane protein 11	TMEM11		Ser13	Mitochondrion organization
Q5BIP4	Target of myb1 membrane trafficking protein	TOM1		Thr406	Protein transport
A6QPI6	Mitochondrial import receptor subunit TOM22 homolog	TOMM22	Ser13		Protein targeting to mitochondrion
P23004	Cytochrome *b*-*c*1 complex subunit 2	UQCRC2	Ser247		Metabolic pathways, thermogenesis, oxidative phosphorylation, cardiac muscle contraction
Q3ZBI7	Up-regulated during skeletal muscle growth protein 5	USMG5	Thr25		–
P45879	Voltage-dependent anion-selective channel protein 1	VDAC1	Ser104		Calcium signaling pathway, cGMP-PKG signaling pathway, necroptosis, cellular senescence
P68002	Voltage-dependent anion-selective channel protein 2	VDAC2	Ser115		Calcium signaling pathway, cGMP-PKG signaling pathway, necroptosis, cellular senescence
G3MXE1	Uncharacterized protein	–	Tyr32		–

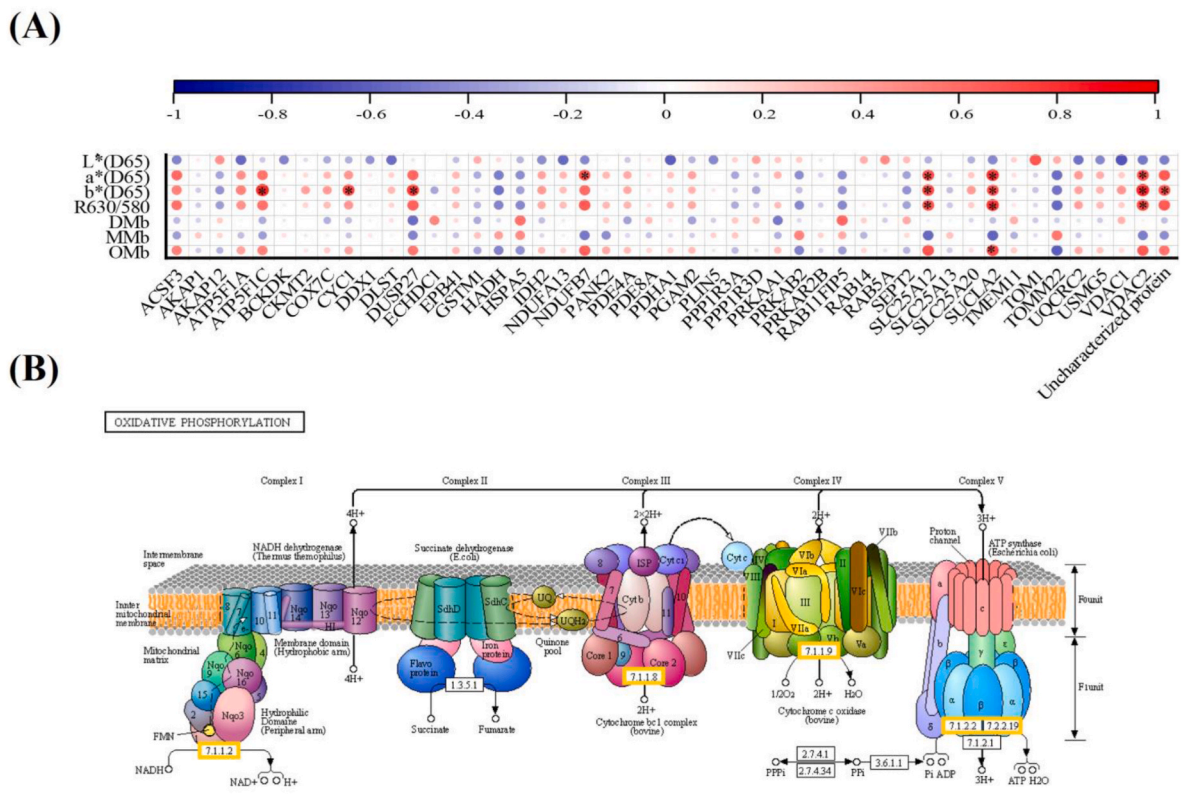

Fig. 6. Correlation analysis of 47 mitochondrial proteins phosphorylation and meat color (A) and mitochondrial phosphoproteins located in oxidative phosphorylation pathway (B). (A) The size of circles represented correlation coefficient. Red color represented a positive correlation. Blue color represented a negative correlation. *$P < 0.05$. (B) Yellow boxes represented the phosphoproteins with significant upregulation. The original picture (named map00190) can be downloaded at KEGG PATHWAY Database (https://www.kegg.jp/kegg/pathway.html). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

enrichment indicated that more upregulation sites than downregulation sites were identified, which suggested a possibly positive relationship between mitochondrial protein phosphorylation level and meat color development. The results of a previous study showed a potentially positive relationship between sarcoplasmic protein phosphorylation and meat color, but they mainly focused on the effect of glycolytic enzymes on meat color (Li et al., 2018). Thus, more validated tests are supposed to conduct to confirm the effect of mitochondrial protein phosphorylation on meat color.

4.2. Phosphorylation of mitochondrial inner membrane proteins

Mitochondria consume oxygen and regulates meat color in postmortem muscle, even though oxygen supply at this stage is interrupted (Tang, Faustman, Hoagland, et al., 2005). Using functional analysis, it was revealed that the mitochondrial phosphoproteins belonged to a cluster of oxidative phosphorylation in this study (Fig. 5, Table 1). The phosphorylation of complexes in oxidative phosphorylation pathway affects their function (Huttemann et al., 2007). NDUFA6 and NDUFC2 were identified in Holstein beef muscle and showed a positive relationship with *a** in previous study (Yu et al., 2017a). The result of the present study also showed that the phosphorylation level of NDUFB7 (different kind of subunits of NADH dehydrogenase) in complex I was positively correlated with *a** (Fig. 6A). NDUFB7 phosphorylation might be involved in the electron movement via complex I and thus affect *a** through changing myoglobin status.

Cytochrome *c* oxidase (complex IV), one of the most important enzymes in mitochondria, control oxygen consumption rate and myoglobin reduction in meat (Yu et al., 2017b). Two types of beef muscle, superficial *semimembranosus* and deep *semimembranosus*, were collected by Seyfert et al. (2006) to evaluate the relationship between color stability and activity of cytochrome *c* oxidase. Their results showed that the color stability and activity of cytochrome *c* oxidase were higher in superficial *semimembranosus* than in deep *semimembranosus*. However, the phosphorylation level of COX7C in LTL beef muscle in the present study was insignificantly correlated with *L**, *a**, *b**, R630/580, DMb, MMb and OMb (Fig. 6A), which might be due to the various postmortem conditions in the different types of muscles. Moreover, the phosphorylation of Thr114 in NDUFA13, Ser14 in NDUFB7, Ser247 in UQCRC2, Ser58 in COX4I1, Ser17 in COX7C, Ser53 in ATP5F1A and Ser265 in ATP5F1C were all significantly upregulated in the samples with the high or medium color stability groups compared to the low color stability group in this study, which suggested a possible positive effect of mitochondrial protein phosphorylation on meat color through regulating the process of oxidative phosphorylation. In summary, phosphorylation of proteins located in the mitochondrial inner membrane had a great influence on mitochondrial functions related to meat color stability.

4.3. Phosphorylation of voltage-dependent anion-selective channel protein (VDAC) in the mitochondrial outer membrane

Phosphorylation of the outer membrane proteins in mitochondria regulated the bond to other proteins (Kerner et al., 2011). The VDAC is an outer membrane protein in mitochondria and its phosphorylation affects apoptosis. In the present study, the phosphorylation level of VDAC2 was positively correlated with *a**, *b** and R630/580 (Fig. 6A), suggesting a possible effect of meat color regulation through the effect of VDAC2 phosphorylation. A previous study reported that the phosphorylation of VDAC impacted the interaction of VDA and Bcl-2 family proteins, which activated apoptosis through release of cytochrome *c* (Banerjee & Ghosh, 2006). The phosphorylation of VDAC might be one of the ways linking apoptosis and meat color development. In addition,

when VDAC was phosphorylated by glycogen synthase kinase 3β, its association with hexokinase was broken and caused dissociation from the mitochondria (Pastorino et al., 2005). Cytoskeleton proteins like actin and desmin also can bind to VDAC and then change the function of mitochondria (Guzun et al., 2012). Actin is the primary part of thin filaments and desmin surrounds the myofibrillar Z-disk. The changes of cytoskeleton proteins have proved to control meat color through water holding capacity and myoglobin permeability (Kim et al., 2011). Thereby, mitochondria might also participate in meat color regulation through the influence of mitochondrial protein phosphorylation on apoptosis, glycolysis and muscle contraction.

4.4. Phosphorylation of other mitochondrial proteins

The releasing of cytochrome *c*-1 (CYC1) is a responsible step of apoptosis (Kalpage et al., 2020). The phosphorylation of CYC1 takes part in the activity of electron transport chain and activation of apoptosis (Almeida et al., 2004). The phosphorylation of CYC1 was significantly correlated with *b** in the present study (Fig. 6A), suggesting CYC1 phosphorylation might affect meat color through apoptosis regulation. Succinate-CoA ligase exists in mitochondrial matrix and convert succinate to succinyl-CoA. Adding succinate into postmortem meat decreased metmyoglobin reducing activity and made beef color deterioration under high oxygen (Krauskopf et al., 2024). SUCLA2 phosphorylation showed a significant relationship with meat color parameters (*a**, *b**, R630/580, OMb) in Fig. 6A. However, there is no related study focusing on the effect of SUCLA2 phosphorylation on meat color yet. Moreover, other mitochondrial proteins like PANK2 and ANT showed significantly-regulated phosphorylated sites in the present study, but the effect of phosphorylation on their function is not reported until now. PANK2 catalyzes the first step of coenzyme A biosynthetic by phosphorylating pantothenic acid to phosphopantothenate (Yun et al., 2000). ANT is an ADP/ATP transporter in mitochondria, which is not only responsible to ATP transfer from mitochondria to cytosol but also is involved in mitochondrial permeability regulation of cytochrome *c* release (Atlante et al., 2006). Although the effects of phosphorylation of these important mitochondrial proteins have not been revealed in previous studies, there might be a potential influence on meat color through biological pathways regulation. Further study should be performed to investigate the phosphorylation of mitochondrial proteins on mitochondrial function and meat color development.

5. Conclusions

Forty-seven phosphoproteins were significantly expressed among 191 mitochondrial proteins in beef meat with different color stability. The phosphosites showing a significant upregulation accounted for 70% and the phosphorylation level of 8 specific proteins all showed significantly positive correlations with meat color, which suggested a possible positive regulation pattern of mitochondrial protein phosphorylation on meat color development. Especially, some proteins located in the mitochondrial inner and outer membrane might have a more important role on regulating meat color through oxidative phosphorylation and apoptosis pathways. This study described protein phosphorylation profile in mitochondria and revealed the potential impacts of the mitochondrial phosphoproteome in meat color development.

CRediT authorship contribution statement

Xin Li: Writing – original draft, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation. **Chi Ren:** Formal analysis, Investigation, Visualization, Writing – original draft. **Guangjing Tian:** Formal analysis, Investigation. **Ying Wang:** Investigation. **Huawei Su:** Writing – review & editing. **Xinglong Zhang:** Writing – review & editing. **Chengli Hou:** Writing – review & editing. **Martine Schroyen:** Writing – review & editing. **Dequan Zhang:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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