Comparative proteomic study of liver lipid droplets and mitochondria in mice housed at different temperatures

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(Received 21 March 2019, revised 11 June 2019, accepted 14 June 2019, available online 12 July 2019)
doi:10.1002/1873-3468.13509
Edited by Michael Brunner

Laboratory mice are standardly housed at around 23 °C, setting them under chronic cold stress. Metabolic changes in the liver in mice housed at thermoneutral, standard and cold temperatures remain unknown. In the present study, we isolated lipid droplets and mitochondria from their livers in a comparative proteomic study aiming to investigate the changes. According to proteomic analysis, mitochondrial tricarboxylic acid cycle (TCA cycle) and retinol metabolism are enhanced, whereas oxidative phosphorylation is not affected obviously under cold conditions, suggesting that liver mitochondria may increase TCA cycle capacity in biosynthetic pathways, as well as retinol metabolism, to help the liver to adapt. Based on proteomic and immunoblotting results, perilipin 5 and major urinary proteins are increased significantly, whereas mitochondrial pyruvate carrier is decreased dramatically under cold conditions, indicating their involvement in liver adaptation.

Keywords: chronic cold stress; lipid droplet; liver adaptation; mitochondrion; organellar proteomics; thermoneutral temperature

Currently the laboratory mouse, *Mus musculus*, serves as a powerful model system for studying metabolism and related disorders of human diseases [1,2]. For example, mouse genes can be easily manipulated to study their functions, and the relevant insights provided can be used to highlight the possible effect in humans. However, direct translation of any findings might be limited by differences of mouse and humans in their thermal physiology. The mammals apply a profound mechanism to conserve and utilize heat produced by metabolism to maintain a stable internal temperature in diverse environments. Thermoneutral temperature (\(T_N\)) is a zone at which the metabolic rate (energy expenditure) required to maintain core body temperature is the lowest [3]. For most used laboratory mouse, C57BL/6J, \(T_N\) is around 29–31 °C [2,4,5]. For naked human, \(T_N\) is around 28 °C [6–8]. For clothed humans (i.e. clothed animal handlers), \(T_N\) is about 20–22 °C [9]. Thus, for the comfort of handlers, laboratory mice are housed at standard temperature of around 20–23 °C [1]. Consequently, standardly housed mice undergo thermal stress constantly, resulting in a dramatic alteration of their physiology and immune responses [10]. Furthermore, mice housed at a standard temperature fail to mimic a number of human diseases [11–14]. Ganeshan *et al.* [9] commented that warming the laboratory mouse might allow for more predictive modelling of human diseases and therapies.

**Abbreviations**
- ER, endoplasmic reticulum;
- H&E, hematoxylin and eosin;
- LC-MS/MS, liquid chromatography-tandem mass spectrometry;
- LD, lipid droplet;
- MPC, mitochondrial pyruvate carrier;
- MT, mitochondrion;
- MUP, major urinary protein;
- PLIN5, perilipin 5;
- PNS, post-nuclear supernatant;
- TAG, triacylglycerol;
- TCA cycle, tricarboxylic acid cycle;
- TEM, transmission electron microscopy;
- TM, total membrane;
- TMT, tandem mass tag;
- \(T_N\), thermoneutral temperature.
The liver plays a key role in the modulation of whole-body energy balance and fuel availability. Dysfunction of liver metabolism can cause a series of metabolic diseases, such as nonalcoholic fatty liver disease, type 2 diabetes and cardiovascular diseases. From a metabolic perspective, the liver is expected to undergo profound metabolic changes under chronic cold conditions to meet the metabolic demands. In 2017, for the first time, Simcox et al. [15] identified the liver as an essential site for cold adaptation and found that it provides acylcarnitines as fuel for peripheral tissues, including brown adipose tissue (BAT), heart and skeletal muscle during cold exposure.

However, the exact physiological changes occurring in the liver in the laboratory mouse under chronic cold stress are unclear. Energy reservoirs in liver are composed of glycogen and triacylglycerol (TAG). TAG is stored in lipid droplets (LDs). LD comprises a metabolic active organelle, with a mono-phospholipid membrane surrounding a neutral lipids core, involved in multiple cellular processes [16,17]. For example, LDs are an active site for lipid metabolism [18]. Ectopic storage of lipids in LDs is linked to human metabolic syndrome [19]. The mitochondrion (MT) is another major organelle regulating metabolism. Mitochondria host the machinery for oxidative phosphorylation, the most efficient pathway to supply ATP for cell. Furthermore, mitochondria include all the proteins for the tricarboxylic acid cycle (TCA cycle), which plays a central role in the break down organic fuel molecules, such as glucose, fatty acids and amino acids. The TCA cycle is also important in the biosynthetic processes in which the intermediates leave the cycle to be synthesized as glucose, fatty acids or amino acids [20,21].

To understand how the mouse liver adapts to housing conditions, a bottom-up strategy, conducting organellar proteomics other than whole tissue proteomics, was performed. We compared LDs and mitochondria from liver in laboratory mouse living in their $T_N$, $T_30$, commonly housed temperature $T_{23}$, as well as extremely cold temperature $T_6$, respectively. The results showed that, under 4-week cold acclimation, glycogen in the liver was decreased compared to under $T_N$. Comparative proteomic results showed an increase in mitochondrial TCA cycle and retinol metabolism in liver of mouse under extreme cold temperature but no change in oxidative phosphorylation. We found that mitochondrial pyruvate carrier (MPC), major urinary protein (MUP) and perilipin 5 (PLINS) may play roles in the regulation of liver metabolism.

Materials and methods

Materials

The Triglyceride Kit (GPO-PAP Method) was purchased from BioSino Bio-Technology & Science Incorporated (Beijing, China). The EnzyChrom™ Glycogen Assay Kit was obtained from BioAssay Systems (Hayward, CA, USA). Twenty-five percent glutaraldehyde solution, 8% paraformaldehyde solution, EMbed 812 kit, uranyl acetate and lead citrate were all obtained from Electron Microscopy Sciences (Hatfield, PA, USA). Osmium tetroxide (EM grade) was obtained from Nakalai Tesque (Kyoto, Japan). Potassium ferrocyanide was obtained from Sigma-Aldrich (St Louis, MO, USA). Pierce™ BCA Protein Assay Kit, HCS LipidTOX™ Red Neutral Lipid Stain and MitoTracker™ Red CMXROs were obtained from Thermo Fisher Scientific (Waltham, MA, USA).

Animals

All animal experiments were approved by the Committee of Biosafety, Ethics and Experimental Animal Management of Institute of Biophysics, Chinese Academy of Sciences, permit number SYKK (Jing) 2016-0026. All the procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals (8th edition). Ten-week-old male C57BL/6 mice were obtained from Beijing Vital River Laboratories (Beijing, China). They were randomly separated into three groups and housed at $T_N$ (30 °C, $T_{30}$), standard temperature (23 °C, $T_{23}$) and cold temperature (6 °C, $T_6$), respectively. The mice were all maintained in individual cages under 12:12 h light/dark cycles and fed standard laboratory chow for 4 weeks.

Histological and ultrastructural analysis of mouse liver

After being housed for 4 weeks at three temperatures, liver histology was analyzed by hematoxylin and eosin (H&E) staining. Briefly, the samples of liver were collected carefully and fixed immediately in 4% (w/v) paraformaldehyde for 24 h and washed with 70% ethanol solution. After dehydration in ethanol series, the samples were embedded in paraffin. The paraffin blocks were sectioned and then stained with H&E dyes for observation.

The ultra-structure of the liver was analyzed by transmission electron microscopy (TEM). Samples of mouse liver were quickly removed and fixed immediately in 2.5% (w/v) glutaraldehyde in 0.1 M phosphate buffer
buffer (pH 7.2) and then cut into small pieces (approximately 1 mm²). Then, the samples were fixed at 4 °C overnight and afterwards fixed in 1% (w/v) osmium tetroxide (with 0.8% potassium ferrocyanide) for 2.5 h at room temperature. After staining in 2% (w/v) uranyl acetate overnight at 4 °C, the samples were dehydrated in an ascending concentration series of ethanol at room temperature. Then they were embedded in EMBed 812 and prepared as 70 nm sections. After staining with uranyl acetate and subsequently with lead citrate, the sections were viewed with Tecnai Spirit (Thermo Fisher Scientific) electron microscope.

**Measurement of liver TAG content**

The content of liver TAG was measured using a Triglycerides Kit (GPO-PAP Method) in accordance with the manufacturer’s instructions. Approximately 20 mg of liver tissue was washed in cold PBS, and then suspended in 1% Triton X-100 and subsequently homogenized using a Dounce homogenizer. The homogenate was centrifuged and subsequently TAG in the sample was hydrolyzed and released glycerol was analyzed enzymatically. At 505 of the resulting product was read in a spectrophotometer and then the TAG content was calculated.

**Measurement of liver glycogen content**

The liver glycogen content was determined by using the EnzyChrom™ Glycogen Assay Kit in accordance with manufacturer’s instructions. Approximately 10 mg of liver tissue was washed in cold PBS, and then homogenized with a Dounce homogenizer. The homogenates were boiled for 10 min and centrifuged at 18 000 g for 10 min at 4 °C. The supernatant was transferred into a new tube for use.

The resulting supernatant was then mixed with the working reagent in the kit for 30 min to break down glycogen and oxidize glucose and finally produce a product generating color for detection. At 570 was read in a spectrophotometer. The glycogen content was then calculated by comparing with the standard reading values.

**Isolation of LDs from liver**

LDs were isolated from liver based on our previous method with some modifications [22]. Briefly, liver was carefully collected and rinsed in cold saline. Subsequently, the liver was transferred into 10 mL of Buffer A (25 mM tricine, pH 7.8, 250 mM sucrose) containing 0.2 mM PMSF and sliced into small pieces using tweezers. After being homogenized with a loose-fitting glass-Teflon Dounce, the homogenate was centrifuged at 1000 g for 10 min and the supernatant was the post-nuclear supernatant (PNS). Then PNS was transferred into a SW40 Ti tube and Buffer B (20 mM Hepes, pH 7.4, 100 mM KCl and 2 mM MgCl₂) was overlaid on the top. The gradient was centrifuged at 13 000 g for 1 h at 4 °C. The LD layer on the top was collected carefully and washed for use.

**Isolation of mitochondria from liver**

Mitochondria were isolated according to our previous method with some modifications [23]. The above PNS was centrifuged at 8000 g for 10 min at 4 °C. Then, the supernatant was discarded and the pellet was resuspended with Buffer B and washed three times. Then, the mitochondrial suspension was gently loaded on the top of a Percoll step gradient consisting of an upper 25% Percoll (8 mL) and a lower 50% Percoll (3 mL) layer. [Correction added on 5 August 2019, after first online publication: in the original publication, under the section “Isolation of mitochondria from liver”, it read “Then, the mitochondrial suspension was gently loaded on the top of a Percoll step gradient consisting of an upper 25% Percoll (3 mL) and a lower 50% Percoll (8 mL) layer.” This has been changed to “Then, the mitochondrial suspension was gently loaded on the top of a Percoll step gradient consisting of an upper 25% Percoll (8 mL) and a lower 50% Percoll (3 mL) layer.”] Subsequently, the gradient was centrifuged at 41 000 g for 1 h at 4 °C and then the mitochondria were recovered from the 50% to 25% Percoll interface and washed with Buffer B for use.

**Confocal microscopy analysis of isolated LDs and mitochondria**

The isolated LDs and mitochondria were stained on ice for 30 min using LipidTOX Red (dilution 1:500, v/v) (Thermo Fisher Scientific) and MitoTracker Red (dilution 1:500, v/v) (Waltham, MA, USA) respectively. Next, they were mounted onto glass slides and cover slips for visualization using a FV1200 Imaging System (Olympus, Tokyo, Japan).

**Preparation and analysis of proteins and lipids**

After collection, LDs and mitochondria were treated with acetone-chloroform (5:1, v/v) and strongly vortexed, respectively. The tubes were centrifuged at 20 000 g for 10 min and proteins were pelleted. The proteins were then analyzed by silver staining or western blotting.

For LDs, the supernatant lipid fraction was transferred to a new tube and evaporated under a stream of
nitrogen. Lipid was developed by TLC using solvent of hexane-diethyl ether-acetic acid (80 : 20 : 1, v/v/v). The TLC plate was stained by iodine vapor at room temperature.

**Mass spectrometry and data analysis**

For comparative proteomic study, two groups of mice (each with three mice), at each temperature were used. The liver LD or mitochondrial protein samples from those six groups were labelled by tandem mass tag (TMT) 6plex kit (Thermo Fisher Scientific) following the TMT 6plex Reagents Protocols. Detailed procedures are provided in Appendix S1. Briefly, the precipitated protein was reduced and digested with trypsin. For mitochondrial samples, the digested peptides were desalted. The peptides were labelled by TMT 6plex in accordance with the manufacturer's instructions. Then, the labelled peptides were separated by nano liquid chromatography-tandem mass spectroscopy (LC-MS/MS) using a Q Exactive equipped with an Easy-nLC 1000 HPLC system (Thermo Fisher Scientific). The raw data were extracted and quantitated using thermo proteome discoverer 2.2.0.388 (Thermo Fisher Scientific). The KEGG database was used for gene function categorization and pathway analysis. The STRING database was adopted to map the interaction (https://string-db.org). The DAVID database (https://david.ncifcrf.gov/home.jsp) was used to analyze proteins in KEGG pathways.

**Results**

**Effects of thermoneutral temperature and chronic cold exposure on mouse liver**

Because the model animals are usually housed at a standard temperature of around 22 °C, which is lower than their \( T_N \) 30 °C, and the liver is a key metabolic organ, we wanted to determine how environmental temperature influences the metabolism in the mouse liver. Hence, laboratory mice C57BL/6, approximately 10 weeks old, were randomly allocated into three groups and then housed one per cage at temperatures of 30, 23 and 6 °C independently for 4 weeks. For each temperature, six mice were randomly separated into two groups (i.e. three for each).

Under three temperatures, all animals had been well. Compared to the morphology of the liver from standardly housed mice, which is the current well-known liver morphology, the livers from mice living in thermoneutral and extreme cold temperatures exhibited no anatomic changes, although their sizes differed (i.e. the colder, the bigger) (Fig. 1A). The livers under three temperatures showed no obvious differences in histological morphology as indicated by H&E staining. Ultra-thin sections of livers revealed that, when the environmental temperature declined, LDs became smaller and mitochondria showed no apparent changes. The body weights of mice housed at 6 °C were higher than those of the other two groups after 4 weeks of acclimation (Fig. 1B). The absolute liver masses differed among the three groups, with a tendency for heavier livers in colder environments. The relative liver mass (liver mass/whole body weight) in extreme cold temperatures was significantly increased compared to that at \( T_{30} \) and \( T_{23} \). These results are consistent with previous studies [10,24,25].

Because the liver is an essential organ with respect to providing fuel, we further tested the energy reservoir, glycogen and TAG content under the three temperatures. TAG contents under thermoneutral and extreme cold conditions were higher than that in the standard condition (Fig. 1C). Glycogen under extreme conditions was lower than that under thermoneutral conditions (Fig. 1D). All of these results indicate that the chronic cold temperature to which the laboratory mouse is always exposed can result in remarkable changes in the liver with respect to adapting to the whole-body demand. To determine the exact critical metabolic alteration, we carried out a comparative proteomic study on the key metabolic organelles, LDs and mitochondria involved in the changes and investigated how they help liver to adapt to distinct environments.

**Isolation and quality verification of lipid droplets and mitochondria from livers at three temperatures**

To study organelle proteomics, first we isolated the LDs and mitochondria from livers in mice living under three temperatures. LDs were isolated in accordance with previously established protocols (Fig. 2A) [22]. The quality of the isolated LDs was verified by LipidTOX Red staining and differential interference contrast (DIC) imaging analysis (Fig. 2Ba). The image showed the LipidTOX staining LDs and DIC imaging spherical structure were well overlapped, indicating a high-quality preparation of LDs. TLC analysis of lipids of isolated LDs showed a low phospholipid ratio, also suggesting no obvious membrane contamination (Fig. 2Bc). Also, the TLC result was in agreement with our above results regarding the liver TAG change pattern under three temperatures. Notably, the spot, validated to be retinyl ester [26], increased in the liver LDs from mice living under a cold temperature.
Cold adaptation at the level of the liver

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A

30 °C  23 °C  6 °C

4 weeks

B

a

Initial weight (g)

b

Body weight (g)

c

Liver mass (g)

d

Liver mass/Body weight

C

Liver triacylglycerol (μg mg⁻¹ protein)

D

Liver glycogen (μg mg⁻¹ tissue)
LD is the main storage site of retinyl esters in liver. Therefore, on exposure to cold, retinyl ester may play a role in helping the liver to adapt. The quality of isolated LDs was also verified by biochemical methods. As indicated by silver staining (Fig. 2C, upper), the protein pattern of isolated LDs was unique compared to the other three cellular fractions, cytosol, total membrane (TM) and PNS, indicating a great enrichment of LD-associated proteins. Two independent LD preparations were analyzed and they exhibited almost identical protein profiles, indicating a good reproducibility of LD preparation. The purity of LD fraction was further assessed by immunoblotting with the indicated antibodies (Fig. 2C, lower). Equal amounts of proteins from LD, cytosol, TM and PNS were blotted. The results show that PLIN2/ADRP, a marker protein of mammal LDs, was detected only in the LD fraction. ACSL5 and Rab18, the other two LD-associated proteins, were also enriched in the LD fraction. Marker proteins for mitochondria (VDAC, Tim23 and ATP5a), cytosol (GAPDH) and endoplasmic reticulum (ER) (BIP) were barely detected in the LD fraction. These results confirmed a high purity of isolated LDs.

Similarly, the purity of isolated mitochondria was analyzed. The results obtained showed that the MitoTracker Red staining and DIC imaging were merged well (Fig. 2Bb). Protein profile of the isolated mitochondria was significantly different from the other fractions (Fig. 2C, upper). A good reproducibility was confirmed by the identical protein profiles between two independent purifications. Immunoblotting analysis showed great enrichment of mitochondrial marker proteins in the isolated mitochondrial fraction, whereas other cellular proteins were barely detected (Fig. 2C, lower). The ER marker protein was also found in the mitochondrial fraction, indicating a tight association between ER and mitochondria, which is in agreement with the MAM structure [27]. These data confirmed a high purity of the isolated mitochondrial fraction.

**Proteomic profiling of mouse liver LDs and mitochondria**

After isolated LDs and mitochondria were shown to be high quality, a comparative proteomic study was carried out to gain insights into how the proteins of these two important organelles were altered under three housing temperatures. Figure 3A shows the schematic workflow. Briefly, LD proteins and mitochondrial proteins from two independent groups under each temperature were digested with trypsin. Then, the peptides were labelled with the indicated isobaric TMT. After mixing, the labelled peptides were subjected to nano LC-MS/MS analysis. The proteins were identified using Thermo Proteome Discoverer 2.2.0.388 and those with at least two peptides with 99% confidence (false discovery rate of 1%) were selected for further analysis.

In the LD proteome, 184 proteins were identified and categorized into 10 groups based on their cellular functions and subcellular locations according to the UniProt database (Fig. 3Ba and Table S1). Among the identified proteins, 114 (62% of the total) have been reported in previous LD proteomes or confirmed on LDs by imaging and the ratio is similar to the published LD proteomes [28,29], which indicates the reliability of the isolation and proteomic techniques (Table S1). Three perilipin family proteins, PLIN2, PLIN5 and PLIN1, were identified. The most abundant proteins identified were involved in lipid metabolism (25%; 46 proteins), which is consistent with previous reports on liver LD proteomics [30]. In addition, in our previous study, we found that isolated LDs, almost depleted of ER, were able to incorporate radiolabelled fatty acids into TAG and phospholipids [18]. Recently, we found adrenal LD may be important sites for steroid hormone metabolism [31]. All of these results confirmed the role of LD as a lipid metabolic organelle for maintaining the cellular lipid homeostasis [32]. Furthermore, ER, mitochondrial and peroxisome proteins constituted 16% (29 proteins), 7% (13 proteins) and 8% (14 proteins) of the total proteins, respectively. Proteins of those organelles were also observed in other LD proteomic studies, which suggested that LD dynamically interacts with other intracellular organelles [17,33]. Another major group consisted of 24 proteins involved in membrane trafficking (approximately 13% of the total) that exhibit important roles in LD dynamics and interactions with other cellular organelles [34,35]. Approximately 3% of the identified proteins (five proteins) were ribosome proteins, which have also been reported in other proteomic
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A

30 °C  23 °C  6 °C

4 weeks
Liver

LD → MT

LD purification  MT purification

LD proteins  MT proteins

B

a

LipidTOX  DIC  Merge
30 °C

LipidTOX  DIC  Merge
23 °C

LipidTOX  DIC  Merge
6 °C

b

MitoTracker  DIC  Merge
30 °C

MitoTracker  DIC  Merge
6 °C

C

a

LD  MT  Cyto  TM  PNS

kDa

170  130  95  72  55  34  26

ADRP  ACSL5  Rab18  VDAC  Tim23  ATP5a  GAPDH  BIP

b

LD  MT  Cyto  TM  PNS

kDa

170  130  95  72  55  34  26

ADRP  ACSL5  Rab18  VDAC  Tim23  ATP5a  GAPDH  BIP

C

LD  MT  Cyto  TM  PNS

kDa

170  130  95  72  55  34  26

ADRP  ACSL5  Rab18  VDAC  Tim23  ATP5a  GAPDH  BIP

2124  FEBS Letters 593 (2019) 2118–2138 © 2019 The Authors. FEBS Letters published by John Wiley & Sons Ltd on behalf of Federation of European Biochemical Societies.
The mitochondrial proteome of mouse liver was determined by comparative proteomic analysis of LDs and mitochondria from livers of mice at different temperatures. The results showed that mice housed at different temperatures for 4 weeks experienced changes in their mitochondrial proteins, indicating their involvement in energy production. Furthermore, significant changes in the proteome were observed, with up-regulation of proteins involved in energy metabolism. This suggests that mitochondria play a crucial role in energy production during cold adaptation.
environmental temperatures. The amount of the differential proteins (69 proteins) involved in metabolic pathways was the greatest when comparing mice living at $T_N$ with those in extreme cold conditions, indicating that mice had to increase the metabolic rate much more during conditions of extreme cold. In addition, the differential proteins related to carbohydrate, lipid and amino acid metabolism, the three main fuel molecules, differed greatly among the three groups, suggesting that possible changes in substrate selection as a fuel may take place at distinct temperatures. Ribosome proteins were also significantly enriched when comparing extreme cold conditions with thermoneutral conditions, which may provide a clue with respect to differences in liver mass between the two groups.

In addition to the above pathway enrichment analysis, the proteins enriched into pathways with $P < 0.05$ were further analyzed to obtain a global view of how they interact and form cellular networks to influence cellular processes. The STRING database was adopted to map the interaction (https://string-db.org). Several apparent interaction groups exerting diverse biological functions were displayed when the housing temperature changed (Fig. S1). The most complicated networks were formed in the $T_6$ vs. $T_{30}$ group, suggesting that most changes would take place when the environment was extremely cold, which is consistent with the above results. Proteins involved in oxidative phosphorylation, ribosomes, the TCA cycle, retinol metabolism and steroid hormone biosynthesis form the main

**Fig. 3.** TMT-based comparative proteomic analysis of liver LDs and mitochondria from mouse living at different temperatures. The LD and mitochondrial proteins were labelled with TMT for comparative proteomic study, respectively. (A) Flow chart of the experimental procedures. Briefly, the LD or mitochondrial proteins from six groups (two groups in each temperature) were digested. After labelling with TMT 6plex reagent and separation, THERMO PROTEOMES DISCOVERER 2.2.0.388 was used to search the raw data for protein identification and quantitation. (B) Overview of the identified proteins. (Ba) The identified LD proteins were analyzed and categorized by subcellular locations and functions according to the UniProtKB database. (Bb) The identified proteins in the mitochondrial preparations were subjected to enrichment analysis. The DAVID Bioinformatics tool was used and the enrichment factors were calculated for cellular organelles. (Bc) The identified mitochondrial proteins were categorized according to the KEGG pathway system. (C) Pathway enrichment analysis of the differential proteins in liver mitochondria under different conditions. The differential proteins were subjected to a KEGG pathway enrichment analysis. The ratio of the enriched differential proteins in a specific pathway to the total differential proteins was shown. The protein amount and the $P$ value are also shown. $P$ represents the significance of each pathway enriched by the mitochondrial proteins. $P < 0.05$ is considered statistically significant with respect to consideration.
Table 1. Significantly changed proteins revealed by comparative proteomic analysis of liver mitochondria from mice at different temperatures.

| Groups       | Pattern          | Number of proteins | UniProtKB Accession | Protein description                           | Gene name | Ratio | P value |
|--------------|------------------|--------------------|---------------------|------------------------------------------------|-----------|-------|---------|
| $T_6$ vs. $T_{23}$ | Up-regulated     | 18                 | Q14DH7              | Acyl-CoA synthetase short-chain family member 3 | Acss3     | 1.46  | 0.06    |
|              |                   |                    | Q91X75              | Cyp2a4 protein                                  | Cyp2a5    | 1.38  | 0.10    |
|              |                   |                    | Q70400              | PDZ and LIM domain protein 1                    | Pdlim1    | 1.36  | 0.07    |
|              |                   |                    | Q8K0L3              | Acyl-coenzyme A synthetase ACSM2                | Acsm2     | 1.35  | 0.04    |
|              |                   |                    | Q505D7              | Optic atrophy 3 protein homolog                 | Opsi3     | 1.33  | 0.02    |
|              |                   |                    | P29758              | Ornithine aminotransferase                      | Oat       | 1.32  | 0.01    |
|              |                   |                    | Q9WVM8              | Kynurenine/alpha-aminoadipate                    | Aaadat    | 1.29  | 0.03    |
| Down-regulated | 59               |                    | Q9QZ40              | Carboxic anhydase 5B                             | Ca5b      | 1.25  | 0.07    |
|              |                   |                    | Q604M2              | MCG53595                                        | Pdp2      | 1.25  | 0.09    |
|              |                   |                    | D3ZVS6              | Monoglyceride lipase                            | Mgl       | 1.25  | 0.07    |
| $T_6$ vs. $T_{30}$ | Up-regulated     | 175                | P70670              | Nascent polypeptide-associated complex subunit alpha | Naca      | 2.44  | 0.04    |
|              |                   |                    | Q9CXV1              | Succinate dehydrogenase [ubiquinone] cytochrome b small subunit | Sdhb      | 2.23  | 0.02    |
|              |                   |                    | D3Y0W2              | Golgi integral membrane protein 4               | Golim4    | 2.16  | 0.01    |
|              |                   |                    | P22599              | Alpha-1-antitrypsin 1–2                         | Serpin1b  | 2.13  | 0.05    |
|              |                   |                    | Q8B0W3              | Eukaryotic peptide chain release factor subunit 1 | Etf1      | 1.97  | 0.10    |
| Down-regulated | 72               |                    | Q91X75              | Cyp2a4 protein                                  | Cyp2a5    | 1.95  | 0.003   |
|              |                   |                    | Q9ET30              | Transmembrane 9 superfamily member 3           | Tm9sf3    | 1.94  | 0.06    |
|              |                   |                    | Q3TGU7              | Proliferation-associated 2G4                    | Ps2g4     | 1.93  | 0.01    |
|              |                   |                    | Q9JMG1              | Endothelial differentiation-related factor 1    | Edf1      | 1.91  | 0.04    |
|              |                   |                    | Q9CZ08              | Elongation factor Ts                            | Tsfm      | 1.79  | 0.01    |
| $T_{23}$ vs. $T_{30}$ | Up-regulated     | 34                 | Q05816              | Fatty acid-binding protein, epidermal           | Fap1      | 0.36  | 0.004   |
|              |                   |                    | P63030              | Mpc 1                                           | Mpc1      | 0.44  | 0.006   |
|              |                   |                    | Q9DOB5              | Thiosulfate sulfurtransferase/rhodanese-like domain-containing protein 3 | Tstd3   | 0.46  | 0.03    |
|              |                   |                    | Q8VCF0              | Mitochondrial antiviral-signalling protein       | Mavs      | 0.51  | 0.07    |
|              |                   |                    | P62806              | Histone H4                                      | Hist1h4a  | 0.52  | 0.06    |
|              |                   |                    | A01W2P2768          | Histone H3.2                                    | Hist2h3c1 | 0.55  | 0.10    |
|              |                   |                    | P62996              | Transformer-2 protein homolog beta              | Tra2b     | 0.55  | 0.07    |
|              |                   |                    | A0A0N4SV8P8         | Predicted pseudogene 5580                      | Gm5580    | 0.56  | 0.01    |
|              |                   |                    | Q9D0D9              | ATP synthase subunit delta                      | Atp5d     | 0.58  | 0.01    |
|              |                   |                    | Q62425              | Cytochrome c oxidase subunit NDUFA4             | Ndufa4    | 0.58  | 0.01    |
|              |                   |                    | Q9CXV1              | Succinate dehydrogenase [ubiquinone] cytochrome b small subunit | Sdhb      | 1.83  | 0.08    |
|              |                   |                    | Q9ES51              | Stromal cell-derived factor 2-like protein 1    | Sdf2      | 1.61  | 0.09    |
|              |                   |                    | Q9CR21              | Acyl carrier protein                            | Ndufab1   | 1.61  | 0.09    |
|              |                   |                    | P00186              | Cytochrome P450 1A2                              | Cyp1a2    | 1.42  | 0.08    |
association networks, which revealed a significant influence on these intracellular processes as a result of exposure to cold. Proteins involved in steroid hormone biosynthesis form an interaction in all three groups, suggesting steroid hormone metabolism was particularly sensitive to cold.

Pathways over-represented or under-represented

Because metabolism was obviously affected by environmental temperature, we further analyzed the metabolic pathways that were over- or under-represented, aiming to obtain information on how the mouse liver adapts in various environmental temperatures. To enhance the reliability of the bioinformatic analysis, significantly affected pathways with a P value cut-off of 0.05 and with at least three differential proteins were considered. Under these criteria, in the T6 vs. T23 group, the steroid hormone biosynthesis process was under-represented in the T6 group (Fig. 4A). In the T6 vs. T30 group, several pathways were over-represented in the T6 group (Fig. 4B).

As described above, our analysis revealed compelling changes of proteins involved in TCA cycle and retinol metabolism in the T6 vs. T30 group (Figs 3 and S1). Further analysis showed that retinol metabolism pathway and the TCA cycle are over-represented in the liver mitochondria of mice living at a temperature of 6 °C (Fig. 4B). Retinol and its metabolites are shown to be involved in the regulation of metabolism in the liver and whole body. Eleven significantly changed proteins were involved in retinol metabolism and they were all up-regulated when comparing T6 with T30. These proteins include all-trans-retinol 13,14-reductase, two UDP-glucuronosyltransferases and eight proteins from the cytochrome P450 family. The pathway and those proteins were analyzed by the DAVID database (https://david.ncifcrf.gov/home.jsp) and are shown in Fig. 5. Seven significantly changed proteins were involved in the TCA cycle and they were all up-regulated when comparing T6 with T30. These proteins include aconitate hydratase, two isocitrate dehydrogenase, dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, succinate dehydrogenase, fumarate hydratase and malate dehydrogenase. The pathway and those proteins were analyzed by DAVID the bioinformatics database (https://david.ncifcrf.gov/home.jsp) and are shown in Fig. 5.

In both the T6 vs. T23 group and the T6 vs. T30 group, oxidative phosphorylation was not enriched as an over- or under-represented pathway (Fig. 4A,B). This result suggested that oxidative phosphorylation was not affected after 4 weeks of cold exposure, as reported previously [44]. The TCA cycle functions to degrade acetyl-CoA into CO2, yielding reducing power to be used in oxidative phosphorylation to produce...
ATP, and also to provide intermediates for biosynthetic processes. Under extremely cold conditions, the TCA cycle was shown to be enhanced, whereas oxidative phosphorylation showed no obvious change, hence suggesting that liver mitochondria may act to provide biosynthetic substances to help liver to adapt.

In the T\textsubscript{23} vs. T\textsubscript{30} group, no under-represented pathways in the T\textsubscript{23} group were enriched, whereas steroid hormone biosynthesis and retinol metabolism pathways were over-represented in the T\textsubscript{23} group (Fig. 4C). All of the enriched pathways are listed in Table S3.

### Verification of the proteomic results

To confirm the TMT-based comparative quantification results, immunoblotting analysis was conducted. In agreement with the proteomic study, the expression of LD resident protein, PLIN2/ADRP was decreased, whereas PLIN5 increased when the

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**Table 2.** Significantly changed proteins revealed by comparative proteomic analysis of liver LDs from mice at different temperatures.

| Groups     | Pattern    | Number of proteins | UniProtKB Accession Number | Protein description                                      | Gene name | Ratio | P value |
|------------|------------|--------------------|----------------------------|----------------------------------------------------------|------------|-------|---------|
| T\textsubscript{6} vs. T\textsubscript{23} | Up-regulated 21 | B5X0G2 | MUP 17                     | Mup17                                                   | 1.72      | 0.06  |
|           |            | P35980 | 60S ribosomal protein L18 | Rpl18                                                   | 1.53      | 0.01  |
|           |            | Q6ZWN5 | 40S ribosomal protein S9  | Rps9                                                    | 1.53      | 0.08  |
|           |            | Q88V21 | Perilipin-5                | Plin5                                                   | 1.46      | 0.06  |
|           |            | Q9CDM2 | Glutathione S-transferase kappa 1 | Gstk1                                               | 1.43      | 0.01  |
|           |            | Q9L2B | Dehydrogenase/reductase SDR family member 4 | Dhrs4                                               | 1.38      | 0.06  |
|           |            | Q99P30 | Peroxisomal coenzyme A diphosphatase NUDT7 | Nudt7                                               | 1.38      | 0.07  |
|           |            | P14170 | 40S ribosomal protein 16 | Rps16                                                   | 1.36      | 0.04  |
|           |            | Q18653 | Transitional ER ATPase     | Vcp                                                     | 1.36      | 0.03  |
|           |            | E9QKR0 | Guanine nucleotide-binding protein G(l)/(G(S)/(G(T) subunit beta-2 | Gnb2                                               | 1.34      | 0.04  |
| T\textsubscript{6} vs. T\textsubscript{30} | Up-regulated 29 | B5X0G2 | MUP 17                     | Mup17                                                   | 2.33      | 0.02  |
|           |            | P25688 | Uricase                    | Uox                                                     | 1.81      | 0.01  |
|           |            | P32202 | Non-specific lipid-transfer protein | Scp2                                                    | 1.76      | 0.01  |
|           |            | P24270 | Catalase                   | Cat                                                     | 1.70      | 0.01  |
|           |            | Q4Z368 | MUP 1                      | Mup1                                                   | 1.65      | 0.04  |
|           |            | Q9D2M2 | Glutathione S-transferase kappa 1 | Gstk1                                               | 1.57      | 0.03  |
|           |            | Q99Z72 | Peroxisomal trans-2-enoyl-CoA reductase | Pecr                                               | 1.56      | 0.02  |
|           |            | Q9VU19 | Hydroxacyl oxidase 1       | Hao1                                                   | 1.53      | 0.04  |
|           |            | Q0JK6 | Hypoxia up-regulated protein 1 | Hyou1                                               | 1.46      | 0.04  |
|           |            | Q91WG0 | Acylcarnitine hydrolase    | Ces2c                                                   | 1.44      | 0.04  |
| Down-regulated 10 |                 | P12770 | Fatty acid-binding protein | Fabp1                                                   | 0.74      | 0.09  |
|           |            | FT587H | Glutathione peroxidase      | Gpx4                                                   | 0.74      | 0.01  |
|           |            | P46338 | Ras-related protein Rab-11B | Rab11b                                               | 0.77      | 0.02  |
|           |            | Q3883 | Perilipin-2                | Plin2                                                   | 0.78      | 0.06  |
|           |            | Q8VC12 | 17-beta-hydroxy steroid dehydrogenase 13 | Hsd17b13                                         | 0.79      | 0.01  |
|           |            | P3279 | Ras-related protein Rab-6A  | Rab6a                                                   | 0.80      | 0.01  |
|           |            | Q9L16 | Ras-related protein Rap-1B  | Rap1b                                                   | 0.80      | 0.03  |
|           |            | P6282 | Ras-related protein Rab-1A  | Rab1A                                                   | 0.81      | 0.10  |
|           |            | Q9D1G1 | Ras-related protein Rab-1B  | Rab1b                                                   | 0.82      | 0.05  |
|           |            | Q3TL8 | RAS-related C3 butylin substrate 1 | Rac1                                               | 0.82      | 0.01  |
| T\textsubscript{23} vs. T\textsubscript{30} | Up-regulated 2 | A0AR3R4J110 | Iodothyronine deiodinase | Dio1                                               | 1.39      | 0.03  |
| Down-regulated 7 |                 | P24456 | Cytochrome P450 2D10       | Cyp2d10                                                 | 1.22      | 0.07  |
|           |            | A2AE89 | Glutathione S-transferase   | Gstm1                                                   | 0.58      | 0.07  |
|           |            | A01D8RMD4 | Kinesin-like protein Kif16B | Kif16b                                               | 0.72      | 0.08  |
|           |            | P46338 | Ras-related protein Rab-11B | Rab11b                                               | 0.78      | 0.03  |
|           |            | Q3TL8 | RAS-related C3 butylin substrate 1 | Rac1                                               | 0.78      | 0.003 |
|           |            | P56480 | ATP synthase subunit beta   | Atplb                                                   | 0.80      | 0.07  |
|           |            | P43883 | Perilipin-2                | Plin2                                                   | 0.80      | 0.08  |
|           |            | P84096 | Rho-related GTP-binding protein RhoG | RhoG                                               | 0.81      | 0.05  |
housing temperature decreased (Fig. 6A). The quantification results were also verified by the expression pattern of protein Rab18 determined using immunoblotting. Several proteins were also selected for the verification of the mitochondrial quantification results (Fig. 6B). Expression of MPC, MPC1 and MPC2 was decreased when the environmental temperature decreased (Fig. 6A). The quantification results were also verified by the expression pattern of protein Rab18 determined using immunoblotting.
Fig. 5. KEGG pathways for TCA cycle and retinol metabolism in the T₆ vs. T₃₀ group. In the T₆ vs. T₃₀ group, TCA cycle and retinol metabolism in mitochondria were enriched as over-represented pathways. The list of proteins that were up-regulated from proteomic analysis was further analyzed in the TCA cycle pathway (A) and retinol metabolism pathway (B) using the DAVID bioinformatics database (https://david.ncifcrf.gov/home.jsp). The red stars indicate the site where the differential proteins function.
decreased. MUP1 in mitochondria was identified as a protein that increased dramatically when the mouse was exposed to cold. Immunoblotting analysis indeed showed that the expression of MUP1 was enhanced significantly under extreme cold conditions. VDAC showed no obvious change, whereas prohibitin was decreased. The data show that a reliable proteomic database for liver LDs and mitochondria from mouse living at different temperatures has been set up. The mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium via the PRIDE [45] partner repository with the dataset identifier PXD014224.

**Discussion**

Mammals try to maintain a constant core temperature in diverse environments. Humans choose an easier way of meeting the goal by wearing clothing and/or using air-conditioners to regulate the ambient temperature. However, this condition set the laboratory mouse under chronic cold stress. When laboratory mice were housed at different temperatures, they regulated their body to adapt to distinct temperatures. For example, under extreme cold conditions, an increase in body weight was shown (Fig. 1). Liver mass showed an increase when the environmental temperature was lower than their $T_N$ (i.e. standard temperature or extreme cold temperature). These results confirm that the change in environmental temperature will have a profound influence on the metabolism in the whole body and individual tissues. Because we always aim to live within our thermal comfort via our clothing and/or by changing the surrounding temperature, when housing our model animals, the environmental temperature should be considered seriously. Indeed, thermoneutral housing is required to model diet-induced obesity in C57BL/6 nude mice [12]. Housing at thermoneutrality would initiate atherosclerosis in wild-type C57BL/6 mice [13]. Giles *et al.* [10] found that thermoneutral housing exacerbated mouse nonalcoholic fatty liver disease and allowed for the development of a more ‘human-like’ model. Therefore, housing laboratory mice at thermoneutrality is proposed to be an easier and better strategy for modelling human diseases and energy metabolism [9,46].

The liver is one of the largest and most metabolically active organs in mammals. However, how the
liver adapts itself at different temperatures remains unclear. Subsequently, we conducted a liver organelle proteomic study to investigate the involvement of LD and MT, two major metabolic organelles, when mice are raised at different temperatures. According to the LD comparative proteomic results, PLIN5 was screened in terms of obviously changed proteins showing the highest expression under extreme cold conditions. This was verified by immunoblotting (Fig. 6A). PLIN5 plays a central role in lipid homeostasis and is clearly required to limit the production of lipid intermediates and prevent disruption to tissue function [47]. PLIN5 may play a role in the regulation of lipid metabolism in the mouse liver when mice are exposed to chronic cold stress.

Eukaryotic cells must organize cellular metabolism well to adapt the status of environmental situations and hence they exhibit outstanding plasticity in bioenergetics. In the present study, we found that liver TAG would increase under $T_{30}$ housing compared to $T_{33}$ housing, although with no change in histology (Fig. 1). Liver glycogen content showed no obvious change under these two conditions. Small et al. [48] found the abundance of key proteins involved in liver lipogenesis was reported to be elevated, whereas the rate-limiting enzyme of gluconeogenesis showed no alteration in mice housed at $T_{N}$ for 13 weeks. Under $T_{N}$, the energy requirement for maintaining body temperature is reduced. Therefore, the liver would accumulate energy as occurred for the lipid under $T_{N}$.

When chronically exposed to cold, profound systemic metabolic changes take place to enable the organism to adapt to the environmental thermal challenge [49]. Cold exposure triggers energy expenditure. The current cellular metabolic patterns in mammals have evolved through the selective pressures of starvation and cold exposure [15]. In mammals, BAT is an organ known to play a major role in protecting against cold through non-shivering thermogenesis [50]. During cold exposure, glucose uptake in human BAT is increased by 12-fold [51]. Besides carbohydrates, activation of BAT by cold exposure increased the utilization of fatty acids from triglyceride-rich lipoproteins and fatty acids released by white adipocytes [52,53]. These studies suggest that BAT would input energy fuels, glucose and fatty acids from other organs, such as the liver, skeletal muscle and white adipose tissue, under cold exposure to help maintain thermogenesis [54].

Short-term cold exposure and long-term cold exposure elicit different liver responses [44]. Because laboratory mice are always kept under chronic cold conditions, we investigated how the liver reacts and helps the body to adapt to long-term cold exposure. Our results showed that, when confronted with chronic extreme cold, the C57BL/6 mouse would decrease liver glycogen significantly. In addition to the decrease of liver glycogen, 4-week cold-acclimated rats were reported to show a decrease of white adipose tissue mass by 20% compared to rats housed at a standard temperature [55]. Furthermore, their plasma glucose and cholesterol esters were not affected either by exposure to an extreme cold temperature. However, plasma TAG was obviously deceased. More importantly, it has been shown that the liver in cold acclimated rats has an increased capacity for gluconeogenesis [56,57]. The incorporation of tritium from $^3$H$_2$O into liver fatty acids was elevated 2.2-fold in 4-week cold-acclimated rats compared to warm-acclimated rats [58]. Liver from rats living under cold conditions (1–2 °C) for 14 days showed a decrease with respect to converting radiolabelled glucose into CO$_2$ compared to that from rats housed at 25 °C [59]. Hepatic mitochondrial oxidative phosphorylation showed no change after rats were exposed to cold (4 °C) for 30 days [44].

According to our comparative proteomic analysis of MT, when the surrounding temperature was lower, the mouse acted to enhance the mitochondrial TCA cycle in the liver (Fig. 4B). However, in agreement with previous results [44], hepatic oxidative phosphorylation under long-term cold acclimation was not obviously affected (Fig. 4A,B). Elevated TCA cycle flux was reported to be linked to the increase in gluconeogenesis and lipogenesis from TCA cycle precursors [20,60]. Hence, the liver mitochondria may increase the TCA cycle capacity to increase biosynthetic processes such as gluconeogenesis and lipogenesis, aiming to help the liver and whole body to adapt to long-term cold exposure.

Another point for consideration is how carbohydrate and fatty acid metabolism change under chronic extreme cold situations. Pyruvate is a key metaboite of glucose, the major simple carbohydrate. Our proteomic results showed a significant decrease of the abundance of MPC in the liver living at $T_{c}$, which was further verified by immunoblotting analysis (Fig. 6B). MPC, a mitochondrial inner member protein, transports pyruvate from the cytosol to the mitochondrial matrix and thus acts as a central node connecting carbohydrate, amino acid and fatty acid metabolism [21,61]. Is the decrease of MPC related to the change in metabolism in the liver of cold-acclimated mice?

A deficiency in MPC activity displayed a defect in glucose-derived pyruvate oxidation [61,62]. However, disruption of MPC activity was reported to promote
fatty acid oxidation and glutamine oxidation to sustain TCA cycle flux, as well as to increase lipogenesis [61,63–66]. In addition, a loss of MPC in the mouse liver resulted in reduced pyruvate-driven gluconeogenesis, which could be compensated for via pyruvate-alanine cycling [64,65]. Therefore, inhibition of mitochondrial pyruvate uptake (i.e. a reduction in glucose-derived pyruvate oxidation) was proposed to be able to rewrite the cellular metabolism [61].

Based on the results obtained in the present study, as well as those of previous studies mentioned above, we propose a hypothesis concerning the role of the liver in adaptive metabolic responses when the mouse is confronted with extreme cold condition. When the environmental temperature is extremely cold, the liver degrades glycogen and increases gluconeogenesis to release glucose to maintain whole body glucose. Furthermore, the liver may shut down carbohydrate oxidation by turning off pyruvate transport into the reduction in pyruvate-derived gluconeogenesis would be compensated for by tissue cross-talk. For example, glycerol released from adipose tissue and lactic acid from muscle may be taken by liver to produce glucose. In addition, the liver may increase the mitochondrial TCA cycle capacity to increase biosynthetic processes, such as gluconeogenesis and lipogenesis. Accordingly, the liver changes its own metabolism to adapt to chronic cold stress and maintain blood glucose levels at the same time.

Noticeably, MUP17 and MUP1 were the most highly elevated proteins in the LD proteome when the environmental temperature was decreased in both the T6 vs. T23 group and the T6 vs. T30 group. According to the results of western blotting, MUP1 in mitochondria also showed a dramatic increase when the housing temperature decreased (Fig. 6B). MUPs comprise lipocalin family members synthesized predominantly in the liver and secreted into the circulation. The MUPs in the mouse are excreted into urine and account for more than 90% proteins in the urine [67,68]. MUPs in the mouse are traditionally proposed to act as a pheromone-binding protein in urine, playing a key role in chemical communication [69,70]. In addition, MUPs themselves can function as a protein pheromone to facilitate chemical information exchange [70]. In 2009, it was shown that MUP1 comprises a humoral metabolic regulator in mice [71] and that chronic elevation of circulating MUP1 in db/db mice could increase energy expenditure and raise the core body temperature. Subsequently, additional studies showed that MUPs are involved in the regulation of hepatic gluconeogenic and lipid metabolism [72]. In addition, MUPs belong to the lipocalin superfamily, sharing the characteristic eight β-strands forming a barrel to bind and transport small hydrophobic molecules, including steroid hormones, retinoids, odorants (e.g. pheromones) and lipids [71,72]. Lipocalin proteins play important roles in physiological processes by transporting molecules. For example, lipocalin 2 was recently reported to be a new adipose-derived cytokine and to play a critical role in regulating retinoid homeostasis and retinoid-mediated thermogenesis in adipose tissue [73,74].

The liver is the main site for the storage of retinyl ester. We found that the retinyl ester in the liver of mice living under a cold environment increased. Our proteomic data showed that, under a cold environment, the retinol metabolism in liver mitochondria was over-represented significantly. In addition, retinoids are known to be important physiological regulators of thermogenesis [75]. The requirements for retinoids were increased in the cold and vitamin A deficiency would lead to a reduced survival time for rats [76]. Therefore, MUP may be a metabolic regulator in the mouse liver by regulating retinol metabolism when mice were exposed to chronic cold.

As mentioned above, MUPs are mainly synthesized in the liver and secreted into serum. Because it is hard to exclude the existence of trace amounts of ER in isolated mitochondria, we analyzed the MUP1 distribution in the cellular component to determine whether the detection of MUP1 in mitochondria was a result of the mitochondrial associated ER. After cell fractionation, MUP1 distribution was analyzed by western blotting. With a similar amount of ER marker protein in mitochondrial and TM fractions, more MUP1 was detected in isolated mitochondria (Fig. S2). This result indicates that the detection of MUP1 in isolated mitochondria was not a result of contact between mitochondria and ER. Indeed, similar to our results, MUP1 was also found in isolated liver mitochondria via immunoblotting in another study [77]. Besides, MUP1 has been reported to enhance mitochondrial biogenesis [71]. Interestingly, when the environmental temperature decreased, we also observed a dramatic increase in the expression of PGC-1α, a major regulator of mitochondrial biogenesis [78], in the mouse liver (Fig. S2). Thus, MUP1 may be dynamically localized to mitochondria to help the liver to adapt when mice are confronted with cold conditions.

In addition, because MUP1 is secreted into serum, we also tested serum MUP1 in mice living at different temperatures. Circulating MUP1 was obviously enhanced in mice living under a cold temperature (Fig. S2). Impressively, lipocalin 2 was reported to be able to regulate BAT activation via a nonadrenergic
pathway [73]. Both MUP1 and lipocalin 2 belong to the lipocalin superfamily. We compared their structures and the results obtained demonstrated that they showed a high degree of structural similarity (Fig. S2). MUP1 may play a role similar to that of lipocalin 2 when the environment is cold. Certainly, the biological function of MUPs requires further investigation.

**Summary**

The liver acts as a hub with respect to controlling whole-body metabolism. The present study has revealed the characteristics of liver LD and MT of laboratory mice living in their $T_{SN}$ because they are always set in a cold room, resulting in metabolic alterations in the liver. Furthermore, the present study highlights the metabolic changes in the liver, an organ that is not taken seriously under conditions of exposure to chronic cold. We found that MPC, MUPs and PLIN5 may play a role in liver adaptation in laboratory mice living under chronic cold conditions.

**Acknowledgements**

We thank Mr Jifeng Wang for his help with the proteomic data analysis; Ms Chen Ye for her help with manuscript revision; and Mr Lianwan Chen for his help with TEM sample preparation. This work was supported by the National Key R&D Program of China (Grant No. 2016YFA0500100), National Natural Science Foundation of China (Grant No. 31671402, 91857201, 31571388, 31671233, 31701018 and U1702288). This work was also supported by the ‘Personalized Medicines – Molecular Signature-based Drug Discovery and Development’, Strategic Priority Research Program of the Chinese Academy of Sciences, Grant No. XDA12040218. Further support was provided by the CAS-Croucher Joint Laboratory Project, Project No. CAS16SC01.

**Author contributions**

SZ and PL designed the project. QL and SZ performed the experiments. ZZ helped with the bioinformatic analysis. SZ and PL wrote the manuscript. All authors have read and approved the final version of the manuscript submitted for publication.

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**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

*Fig. S1.* The association network of differential proteins in the liver mitochondria from mice living at different temperatures.

*Fig. S2.* Subcellular localization of MUP1 and its possible function in mice living in a cold environment.

*Table S1.* Categorization of the identified proteins and significantly changed proteins of liver LDs from mice at different temperatures.

*Table S2.* List of the identified proteins and significantly changed proteins of liver mitochondria from mice at different temperatures.

*Table S3.* Pathways over-represented or under-represented in liver mitochondria from mice at different temperatures.

*Appendix S1.* Supplemental information for mass spectrometry and Figs S1 and S2.