Proteomic analysis of murine testes lipid droplets

Weiyi Wang*, Suning Wei*, Linghai Li†, Xueying Su†, Congkuo Du†, Fengjuan Li†, Bin Geng†, Pingsheng Liu† & Guoheng Xu‡

Testicular Leydig cells contain abundant cytoplasmic lipid droplets (LDs) as a cholesteryl-ester store for releasing cholesterol as the precursor substrate for testosterone biosynthesis. Here, we identified the protein composition of testicular LDs purified from adult mice by using mass spectrometry and immunodetection. Among 337 proteins identified, 144 were previously detected in LD proteomes; 44 were confirmed by microscopy. Testicular LDs contained multiple Rab GTPases, chaperones, and proteins involved in glucuronidation, ubiquination and transport, many known to modulate LD formation and LD-related cellular functions. In particular, testicular LDs contained many members of both the perilipin family and classical lipase/esterase superfamily assembled predominately in adipocyte LDs. Thus, testicular LDs might be regulated similar to adipocyte LDs. Remarkably, testicular LDs contained a large number of classical enzymes for biosynthesis and metabolism of cholesterol and hormonal steroids, so steroidogenic reactions might occur on testicular LDs or the steroidogenic enzymes and products could be transferred through testicular LDs. These characteristics differ from the LDs in most other types of cells, so testicular LDs could be an active organelle functionally involved in steroidogenesis.

The testis consists of three major cell types: germ cells, Sertoli supporting cells within seminiferous tubules, and Leydig cells in the interstitium between the tubules. Leydig cells are particularly enriched with endoplasmic reticulum (ER), mitochondria, and cytoplasmic lipid droplets (LDs)1,2. This structure is associated with the androgen production function of Leydig cells.

Testosterone biosynthetic enzymes are generally located in the ER and mitochondrial membranes and the adjacent cytoplasm. The precursor substrate for steroidogenesis is cholesterol. An individual Leydig cell could secrete 20 ng of testosterone daily in humans3 and 0.5 ng in adult rodents2. To ensure such a high rate of steroidogenesis, the testis utilizes endogenous cholesterol rather than transported from the plasma4,5. The intracellular LDs of Leydig cells contain a large pool of cholesteryl ester that can be broken down into free cholesterol on demand for steroidogenesis5. In response to the varied androgen production during pubertal growth6 and breeding1, the size and number of LDs in Leydig cells may vary greatly, which reflects an altered demand for stored cholesterol-cholesteryl ester for testosterone biosynthesis1,6. Also, Sertoli cells contain a fair amount of small LDs that show cyclic variations throughout the spermatogenic cycle in rat7 and human8 and can transfer from Sertoli cells to spermatocytes8. Therefore, testicular LDs play functional roles in testes.

The LDs in all eukaryotes contain a core of neutral lipids, a monolayer surface of phospholipids, and a number of proteins that are embedded in the surface9. In contrast to biochemically inert neutral lipids, the protein components on the LD surface are biologically active and control LD storage and hydrolysis and LD-related cellular functions. A considerable number of LD proteins have been identified in many types of cells by immunodetection or proteomic approaches. The investigation of these LD proteins has greatly extended our understanding of the properties and functions of LDs in given cells.

The LDs in testicular cells are particularly small, with mean diameter 1 μm2, and thus are not easily detected by common immunodetection approaches. Only a few LD-associated proteins have been

1Department of Physiology and Pathophysiology, School of Basic Medical Sciences, Peking University, Beijing 100191, China. 2National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing, 100101, China. Correspondence and requests for materials should be addressed to P.L. (email: pliu@ibp.ac.cn) or G.X. (email: xug@bjmu.edu.cn)
identified in testicular cells. This insufficient information has long restricted the investigation of functional roles of testicular LDs.

This proteomic study aimed to identify protein components of testicular LDs of adult mice. We detected 337 proteins from testicular LD preparations; 144 were previously detected in LD proteomes and 44 were previously confirmed in LDs by microscopy. Testicular LDs contained almost complete sets of LD-related protein members of both the perilipin (Plin) family and lipase/esterase superfamily that assemble predominantly in adipocyte LDs and contain many enzymes that govern biosynthesis of steroids and hormonal steroids. These distinct characteristics are different from the LDs in most other cells. Testicular LDs are a unique, biologically active cellular organelle that might be regulated like adipocyte LDs and play important roles in the biosynthesis and metabolism of hormonal steroids.

**Methods and Materials**

**Animals and antibodies.** Polyclonal antibodies against Plin1–4 and hormone-sensitive lipase (HSL) were from C. Londos (US National Institutes of Health). Other antibodies were from Abcam, Cell Signaling, or Santa Cruz Biotechnology. The animal study was performed in accordance with the NIH guidelines for the care and use of laboratory animals and was approved by the animal care and utilization committee of Peking University Health Science Center.

**Purification of the LDs from mice testis.** For each individual preparation, 20 testes obtained from 10-week-old C57BL/6 mice were used. LDs were purified by the protocol we developed recently. Manipulations were performed at 4 °C or on ice, if required. After removal of blood vessels and connective tissues, 20 testes were grouped and homogenized by use of a Dounce glass homogenizer containing 10 ml buffer A (250 mM sucrose, 0.2 mM phenylmethylsulfonyl fluoride, 25 mM tricine, pH 7.6) by 20 strokes with a loose-fitting pestle and 40 strokes with a tight-fitting pestle. The homogenate was disrupted for 15 min at 750 psi in a nitrogen bomb chamber and cleaned by centrifugation at 3000 × g. The post-nuclear supernatant was transferred to a SW40 tube, then buffer B (20 mM HEPES, pH 7.4, 100 mM KCl and 2 mM MgCl2) was loaded on top of the supernatant. After centrifugation at 38,000 × g for 1 h, a white LD layer appeared on the top of the tube. The membrane was pelleted at the bottom, and the infranatant was the cytosolic fraction. All 3 fractions were collected. The LD fraction was transferred to a new tube and centrifuged for 4 min at 14,000 × g. After removal of the underlying liquid, LDs were washed 3 times, each with 200 μl buffer B and centrifuged at 14,000 × g for 4 min. The LD fraction on the top was collected.

**Protein in-gel digestion and mass spectrometry analysis.** Manipulations were performed as we reported recently. Protein components in the LD preparation were precipitated with 100% acetone. Proteins were separated by 10% SDS-PAGE followed by Coomassie Blue or silver staining. For the total proteome, 2 mM MgCl2) was loaded on top of the supernatant. After centrifugation at 38,000 × g for 1 h, a white LD layer appeared on the top of the tube. The membrane was pelleted at the bottom, and the infranatant was the cytosolic fraction. All 3 fractions were collected. The LD fraction was transferred to a new tube and centrifuged for 4 min at 14,000 × g. After removal of the underlying liquid, LDs were washed 3 times, each with 200 μl buffer B and centrifuged at 14,000 × g for 4 min. The LD fraction on the top was collected.

**Immunoblotting.** Proteins from the LD preparation were extracted with acetone, separated by 10% SDS-PAGE, and underwent immunoblotting analysis with primary antibodies, then horseradish peroxidase-conjugated IgG. The blots were developed with enhanced chemiluminescence detection reagents (Applygen Technologies, Beijing).

**Histology and immunofluorescence.** Mice testes were fixed with 4% paraformaldehyde and embedded in paraffin and cut. For routine histology, sections were stained with hematoxylin-eosin. For immunofluorescence staining, sections were incubated for 10 min with 3% H2O2 to eliminate endogenous peroxidase activity and underwent antigen retrieval with 0.3% sodium citrate and phosphate buffered saline, pH 7.4, for 15 min at 72 °C. Sections were blocked with 1% defatted albumin and immunostained with primary antibody, then FITC-labeled IgG. Signals were observed under a Nikon Eclipse 50i fluorescence microscope.

**LD staining.** LDs in frozen testicular sections were stained with Nile Red. Nuclei were stained with Hoechst 33258. For in vitro staining, LDs purified from testicular tissue were spread on glass slides, dried, and stained with Lipid-TOX Deep Red. Fluorescent signals were viewed under an Olympus FV1000 confocal microscope.

**Thin-layer chromatography.** LDs were purified from brown adipose tissue and testes of mice and from cultured Chinese hamster ovary (CHO) cells. Total lipids in different LD preparations were extracted in chloroform and acetone (1:2, v/v) and centrifuged at 14,000 × g for 10 min. The organic phase was collected and dried under nitrogen gas. Lipid extracts were dissolved in chloroform and loaded on silica gel
plates for analysis. Neutral lipids were separated on plates in a hexane:diethyl ether:acetic acid (80:20:1, v/v/v) solvent system and visualized by the iodine vapor method.

Data mining and bioinformatics. To obtain reliable results, we performed at least two biological replicates of proteomic analysis and results were combined for further analysis. The online database used to sort the proteomic table was http://genome.ucsc.edu/cgi-bin/hgNear. Protein associations were revealed by the website program String (http://string-db.org/).

Results
Testicular LD staining. Interstitial cells were located in the interstitium between the seminiferous tubules of mouse testicular tissue (Fig. 1A, panel a and b). Numerous small, concrete LDs stained with Nile Red were present in the interstitium between the seminiferous tubules. The merged images of boxed area are in panel b. B, LDs stained with Nile Red in frozen testis sections. Nuclei were stained with Hoechst 33258. Panel a and b, Nile Red stained LDs. Panel c and d, the merged images. C, LDs purified from mice testes were spread on slides and stained with Lipid-TOX Deep Red.

Figure 1. Testicular lipid droplets (LDs) staining. A. Hematoxylin-eosin staining of mouse testicular tissue. The asterisk marks the interstitium between the seminiferous tubules in panel a. The amplified images of boxed area are in panel b. B, LDs stained with Nile Red in frozen testis sections. Nuclei were stained with Hoechst 33258. Panel a and b, Nile Red stained LDs. Panel c and d, the merged images. C, LDs purified from mice testes were spread on slides and stained with Lipid-TOX Deep Red.
Nile Red were observed in interstitial Leydig cells rather than in the cells located within the seminiferous tubules (Fig. 1B). Lipid-TOX staining showed that the LDs prepared for proteomic analysis were morphologically intact, with a diameter of about 1 μm, despite the presence of a few large droplets (Fig. 1C).

Lipid and protein patterns of testicular LDs. Thin-layer chromatography revealed that mice testicular LDs consisted of a fairly equivalent amount of cholesteryl esters and triacylglycerols and a small amount of ether lipid, similar to steroidogenic CHO cells; by contrast, adipose LDs contained a large amount of triacylglycerols but few cholesteryl esters and ether lipid (Fig. 2A). Equal amounts of protein extracted from different compartments were separated by SDS-PAGE. Silver staining revealed that the proteins in different LD preparations showed a highly consistent band pattern in gels (Fig. 2B), which indicated the reliability of the LD purification. In contrast, the protein band pattern of LD fractions differed from that of total membrane, cytosol, and post-nuclear supernatant fractions (Fig. 2C).

Proteomic analysis of testicular LD proteins. For the whole proteome of testicular LDs, the lane running testicular LD protein was excised into 23 gel slices (Fig. 2C). After in-gel digestion, tryptic peptides underwent mass spectrometry analysis. Only proteins with at least two unique peptides were accepted for identification. A total of 337 proteins were identified; at least 144 (42.7% of total) were previously reported in LD proteomes of other mammalian cells or tissues and 44 were previously confirmed in LDs by microscopy. Each identified protein and its encoding gene were searched in the UniProt and NCBI databases and PubMed. The 337 proteins were classified into 16 groups by known or putative functional annotation for identified proteins (Fig. 3 and Table 1).

Group 1 proteins represented vimentin and stomatin and particularly Plin1, Plin2/ADRP, Plin3/Tip47, and Plin4/S3-12, 4 classical LD proteins belonging to the perilipin family of 5 LD proteins (Plin1–5) conserved in their first ~100 amino-terminal residues. Plin1 binds and links vimentin to LDs, then vimentin filaments wrap the LDs tightly in a cage-like spherical structure surrounded by multiple ER tubules, thus facilitating LD formation. Plin2–4 widely express and localize at LDs and non-LD compartments, but Plin1 expresses exclusively in adipose and steroidogenic cells and localizes only at the LD surface. Plin1–4 provide a barrier and protect LDs against access by HSL and adipose triglyceride lipase (ATGL), but native Plin1 is more protective than Plin2–4. Interestingly, testicular LDs contained 4 variants of Plin1, termed Plin1a, 1b, 1c, and 1d, which share conserved N-terminal 198 residues and 11-mer regions. This was the first identification of Plin1d protein in the tissue (Table 1).

Group 2 included 7 lipases/esterases/thioesterases, which cover almost all currently known cellular lipases/esterases. HSL, ATGL and its co-lipase CGI-58 represent more than 95% of the lipolytic activity in adipocytes, with the remaining hydrolase activity contributed by triacylglycerol hydrolase/
Figure 3. Properties of murine testicular LD proteins. A. Protein categories of mouse testis LDs. All proteins identified by 2D-LC MS/MS were sorted by subcellular distributions and known functions based on literature or NCBI online sources. B. Network of function-related LD proteins. Lines in different colors represent functional association in various types of evidence. Red, fusion evidence; green, neighborhood evidence; blue, co-occurrence evidence; purple, experiment evidence; yellow, text-mining evidence; black, co-expression evidence.
| Symbol | Protein Name | Remarks | Expectation | GI number |
|--------|--------------|---------|-------------|-----------|
| Plin1  | Perilipin 1 (Perilipin) | &[^29]; #Adipocyte[^29,71]; Specific in adipose and steroidogenic cells | 3.33E-14 | 164698413 |
| Plin2  | Perilipin-2 (ADRP) | &[^29]; #Ubiquitous[^11,13,28-30,41,71] | 1.62E-12 | 11635489 |
| Plin3  | Perilipin-3 (TIP47) | &[^29]; #Ubiquitous[^28,39,41,71] | 1.37E-10 | 13385312 |
| Plin4  | Perilipin-4 (S3-12) | &[^29]; #Ubiquitous[^28-30] | 4.24E-8 | 157041252 |
| Vim    | Vimentin     | &[^29]; #Ubiquitous[^12,18,28,39,71] | 1.22E-10 | 31982755 |
| Stom   | Stomatin     | &[^13], #A431[^13], CHO[^20] | 2.82E-8 | 7710018 |

**Group 2: Lipases**

| Symbol | Protein Name | Remarks | Expectation | GI number |
|--------|--------------|---------|-------------|-----------|
| HSL    | Hormone-sensitive lipase | &[^15]; #Adipocyte[^29,71], Muscle[^31], Caco-2[^41]; Specific in adipose and steroidogenic cells | 6.23E-12 | 87239970 |
| ATGL   | Adipose triglyceride lipase | &[^29]; CHO[^11,24], Muscle[^31], Coca-2[^44] | 9.68E-7 | 254826780 |
| CGI-58 | CGI-58 (ATGL coactivator) | &[^16]; #Ubiquitous[^12,28-30,40], α/β-hydrolase | 3.29E-13 | 13385690 |
| Tgh/Ces3 | Triacylglycerol hydrolase | &[^29]; #Adipocyte[^11,31], Testis | 1.04E-6 | 117553604 |
| Mgl2   | Monoglyceride lipase | #Ovary[^39], Muscle[^31], Liver[^11], Caco-2[^41], Testes | 1.72E-9 | 261878509 |
| Ldah   | LD-associated hydrolase (C2orf43) | &[^21,22]; #Microphase[^22], Caco-2[^41]; Testes | 2.63E-12 | 268370116 |
| Lm2    | Lipase maturation factor 2 | Tests | 7.00E-9 | 30725786 |

**Group 3: Glycerolipid metabolism**

| Symbol | Protein Name | Remarks | Expectation | GI number |
|--------|--------------|---------|-------------|-----------|
| FATP-1 | Fatty acid transport protein 1 | &[^26]; #Ovary[^30], SLC27a1 | 5.09E-9 | 6755546 |
| Acsl1  | Long-chain acyl-CoA synthetase 1 | &[^29]; #CHO[^11], Adipocyte[^29], Muscle[^31] | 9.68E-11 | 31560705 |
| Acsl3  | Long-chain acyl-CoA synthetase 3 | &[^31,32]; #CHO[^31], Adipocyte[^29], Muscle[^31] | 7.04E-10 | 209977076 |
| Acsl4  | Long-chain acyl-CoA synthetase 4 | &[^29]; #Ubiquitous[^12,28-30,41], ω-3-hydrolase | 7.69E-13 | 46518528 |
| Acsl6  | Long-chain acyl-CoA synthetase 6 | Tests | 2.00E-10 | 75992911 |
| Acsl1g1 | Long-chain acyl-CoA synthetase Acsl1g1 | Tests | 5.03E-13 | 16716465 |
| Acsl2  | Very-long-chain acyl-CoA synthetase 2 | 1.76E-10 | 124487285 |
| Acsl3  | Very-long-chain acyl-CoA synthetase 3 | Tests | 4.59E-4 | 254553374 |
| Acadvl | Very-long-chain acyl-CoA dehydrogenase | #Muscle[^31] | 1.20E-9 | 23956084 |
| Fasn   | Fatty acid synthase | #Ovary[^39] | 4.20E-8 | 93102409 |
| Aldh3  | Fatty aldehyde dehydrogenase | &[^11,22]; #Yeast[^11], Microsomal | 1.74E-7 | 75677435 |
| Aldh2  | Aldehyde dehydrogenase | #Adipocyte[^11], Mitochondrial | 9.73E-9 | 6753036 |
| Gnpat  | Glycerone-phosphate O-acetyltransferase | Tests | 4.99E-8 | 160298207 |
| Gpat1  | Glycerol-3-phosphate acetyltransferase 1 | Mitochondrial | 1.43E-4 | 34536827 |
| Gpdh   | Glycerol-3-phosphate dehydrogenase | Mitochondrial; sperm capacitation | 1.51E-10 | 224922803 |
| Gk2    | Glycerol kinase, testis specific 2 | Testis specific | 4.74E-10 | 6754000 |
| Gk3    | Glycerol kinase 5 | Tests | 2.39E-9 | 28893497 |
| Cpt2   | Carnitine O-palmitoyltransferase 2 | #Muscle[^31] | 2.81E-10 | 162138915 |
| Crat   | Carnitine O-acetyltransferase | Tests | 2.18E-8 | 85662408 |
| Hadha  | Trifunctional enzyme subunit α | #Ovary[^39], Muscle[^31] | 7.94E-10 | 338543111 |
| Acox3  | Acyl-CoA oxidase 3, peroxisomal | Tests | 4.17E-5 | 134628334 |
| Alox12 | Arachidonate 12-lipoxygenase | #Muscle[^31] | 7.88E-10 | 31542127 |

**Group 4: Phospholipid metabolism**

| Symbol | Protein Name | Remarks | Expectation | GI number |
|--------|--------------|---------|-------------|-----------|
| Phb1   | Phospholipase B | &[^31]; Activated on sperm sterol removal | 5.41E-5 | 219440670 |
| Cpla2  | Cytosolic phospholipase A2 | &[^32,33]; #CHO[^11], Muscle[^31], LD formation | 2.06E-11 | 6679369 |
| Pcyt1a | Phosphocholine cytidylyltransferase A | &[^14,42]; #Muscle[^31], LD expansion | 1.88E-8 | 253683458 |
| Pgs1   | Phosphatidylglycerophosphate synthase 1 | #Muscle[^31], Testes | 4.91E-8 | 11062163 |
| Ddhd1  | Phospholipase Ddhd1 | PA-PLA1 | 1.20E-5 | 111955212 |
| Plaa   | Phospholipase A2-activating protein | Tests | 2.27E-5 | 114431250 |
| Sact1  | Phosphatidylinositol phosphate phosphatase Sact1 | Tests | 6.66E-9 | 13507622 |

Continued
| Symbol | Protein Name | Remarks | Expectation | GI number |
|--------|--------------|---------|-------------|-----------|
| Plp    | Phosphoinositide lipid phosphatase | Testes | 5.48E-8 | 23956130 |
| Pik3c3 | PI3-kinase type 3 | | 4.14E-7 | 42475974 |
| Pik3r4 | PI3-kinase regulatory subunit 4 | | 7.27E-7 | 124486789 |
| Sphk2  | Sphingosine kinase 2 | | 6.26E-8 | 289191399 |

**Group 5: Biosynthesis of sterols and hormonal steroids**

| Symbol | Protein Name | Remarks | Expectation | GI number |
|--------|--------------|---------|-------------|-----------|
| Lss    | Lanosterol synthase | &Yeast27; &Adipocyte27,28, Muscle28 | 3.96E-10 | 22122469 |
| Cyp51  | Lanosterol 14-α demethylase | &23,24; &Ubiquitous19,20,30,40 | 6.27E-11 | 71061451 |
| NsdhI  | NAD(P)H steroid dehydrogenase-like | &45,46; &Adipocyte29,30,40 | 3.33E-16 | 31982437 |
| Cyp17a1| 17α-hydroxyprogesterone aldolase | Testosterone synthesis | 4.74E-9 | 160948601 |
| Hsd3b1 | 3β-hydroxysteroid dehydrogenase 1 | &26,27; &Ovary20; Caco-220 | 6.12E-13 | 6880289 |
| Hsd3b7 | 3β-hydroxysteroid dehydrogenase 7 | &Muscle29; Caco-220 | 1.70E-6 | 100817048 |
| Hsd1b4 | 17β-hydroxysteroid dehydrogenase 4 | &Muscle29 | 3.66E-9 | 31982273 |
| Hsd1b7 | 17β-hydroxysteroid dehydrogenase 7 | &CHO28, Adipocyte29; Caco-220 | 1.22E-11 | 87162470 |
| Hsd1b11| 17β-hydroxysteroid dehydrogenase 11 | &41,42; &Muscle29; Caco-220 | 1.08E-11 | 16716597 |
| Hsd2   | Hydroxysteroid dehydrogenase-like 2 | | 2.93E-5 | 12565160 |
| Rdh14  | Retinol dehydrogenase 14 | &Caco-220 | 3.89E-6 | 12963791 |
| Rdh10  | Retinol dehydrogenase 10 | &25; &Muscle29; Caco-220 | 4.88E-14 | 25141231 |
| Aldh1a1| Retinal dehydrogenase 1 | Rdh10 counteracted | 3.38E-10 | 85861182 |
| Dhrs3  | Short-chain dehydrogenase/reductase 3 | &27,28; &Muscle29; Caco-220 | 1.05E-9 | 28906339 |
| Dhrs1  | Dehydrogenase/reductase SDR member 1 | #CHO28, Adipocyte29; Caco-220 | 4.64E-13 | 31980844 |
| Dhrx   | Dehydrogenase/reductase X-linked | | 7.04E-12 | 124424062 |
| MepH   | Epoxide hydrolase 1 | &Caco-220; Ephx1 | 1.60E-10 | 6573362 |
| Abcd3  | ATP-binding cassette transporter D3 | Sterol transport in testes | 1.54E-8 | 60218877 |
| Scarb1 | Cavenger receptor class B-1 | &Ovary20; Cholesterol uptake | 2.02E-5 | 14389423 |

**Group 6: Glucuronidation and glycosylation processes**

| Symbol | Protein Name | Remarks | Expectation | GI number |
|--------|--------------|---------|-------------|-----------|
| Alg5   | Dol-P-glucosyltransferase | &CHO11,28 | 1.71E-8 | 21728372 |
| Rpn1   | Ribophorin 1 | &CHO21, Adipocyte22,23, OST | 8.77E-14 | 282398108 |
| Stt3a  | Oligosaccharyltransferase Stt3a | | 1.40E-5 | 148747128 |
| Stt3b  | Oligosaccharyltransferase Stt3b | | 2.71E-9 | 61651673 |
| Uggt1  | UDP-Glcglycoprotein glucosyltransferase | | 1.38E-6 | 236664498 |
| Ugt1a6 | UDP-glucuronosyltransferase 1–6 | &Caco-220 | 4.36E-9 | 33186906 |
| Metl7a | Methyltransferase-like protein 7A | &25,26; &CHO28, Caco-220; AAM-B | 1.24E-10 | 33563290 |
| CGI-49 | CGI-49 | &Ubiquitous11,20,30,40 | 1.20E-11 | 30520019 |
| Pigt   | GPI transamidase component PIG-T | Glycolipid biosynthesis | 1.90E-6 | 120587021 |
| Pigs   | GPI transamidase component PIG-S | Complexed with Pigt | 2.06E-8 | 41351529 |
| Dpy19I2| Dpy-19-like protein 2 | Spermatogenesis | 5.69E-5 | 261245007 |
| Gcanb  | α-glucosidase 2 | &Ovary20; Caco-220 | 1.39E-7 | 6679891 |
| Man2a1 | α-mannosidase 2 | | 2.52E-7 | 226246610 |
| Mogs   | Mannosyl-oligosaccharide glucosidase | | 2.53E-8 | 31981106 |
| Gb1    | β-galactosidase | | 1.82E-5 | 6753190 |
| Gb1L3  | β-galactosidase 1-like protein 3 | | 2.05E-10 | 164519028 |
| Pycox1 | Prenylcysteine oxidase | &Caco-220; Testes | 9.00E-9 | 13385294 |

**Group 7: Carbohydrate process**

| Symbol | Protein Name | Remarks | Expectation | GI number |
|--------|--------------|---------|-------------|-----------|
| Slc2a3 | Glucose transporter 3 | | 8.33E-12 | 261862282 |
| Pkm2   | Pyruvate kinase 2/3 | &CHO21, Retina23 | 7.05E-9 | 31981562 |
| Hk1    | Hexokinase-1 | | 1.63E-8 | 225735584 |
| Hk2    | Hexokinase-2 | | 9.10E-7 | 7305143 |

Continued
| Symbol | Protein Name                  | Remarks                     | Expectation | GI number |
|--------|-------------------------------|-----------------------------|-------------|-----------|
| Ldha   | Lactate dehydrogenase A       | #Ovary, Retina, Sperm glycolysis | 4.60E-8     | 6754524   |
| Aldoa  | Fructose-bisphosphate aldolase A | #Caco-2, Sperm glycolysis     | 3.25E-8     | 293597567 |
| Ptkm   | 6-phosphofructokinase type A  |                             | 4.70E-10    | 25453346  |
| Pfkp   | 6-phosphofructokinase type C  |                             | 9.63E-10    | 9790051   |
| Pygb   | Glycogen phosphorylase        | Brain form                  | 4.59E-4     | 24418919  |

**Group 8: Tricarboxylic acid cycle**

| Symbol | Protein Name                  | Remarks                     | Expectation | GI number |
|--------|-------------------------------|-----------------------------|-------------|-----------|
| Cyb5r3 | NADH-cytochrome b5 reductase  | & ubiquitous, Diaphorase-1  | 2.33E-14    | 19745150  |
| P scaffold | NADH P450 oxireductase              | #Caco-2                  | 5.41E-12    | 6679421   |
| Ndufs1 | Complex 1-75kD                | #CHO, NADH dehydrogenase   | 5.21E-10    | 229892316 |
| Ndufs2 | Complex 1-49kD                |                            | 8.05E-9     | 23346461  |
| Ndufs8 | Complex 1-23kD                |                            | 3.20E-9     | 46195430  |
| Ndufa9 | Complex 1-39kD                |                            | 4.67E-9     | 254692859 |
| Ndufa10| Complex 1-42kD                |                            | 1.26E-9     | 13195624  |
| Me1    | NADP-dependent malic enzyme   |                             | 1.63E-7     | 162139827 |
| Usccr1 | Cytochrome b-c1 complex subunit 1 | Complex III              | 5.08E-12    | 46593021  |
| COXII  | Cytochrome c oxidase subunit II | Complex II              | 1.05E-13    | 34538601  |
| Dld    | Dihydrolipoamide dehydrogenase | Sperm capacitation          | 2.07E-6     | 31982856  |
| Dist   | Dihydrolipoamide S-succinyltransferase | #Ovary               | 4.69E-9     | 21313536  |
| Nampt  | Nicotinamide phosphoribosyltransferase |                     | 1.27E-6     | 257153454 |
| Sdha   | Succinate dehydrogenase subunit A | #Ovary                 | 5.88E-7     | 54607098  |
| Suclg1 | Succinyl-CoA synthase α      |                             | 8.05E-8     | 255958286 |
| Glad1  | Glutamate dehydrogenase 1    | #Retina                   | 4.83E-5     | 6680027   |
| Aco2   | Aconitate hydratase          | #Ovary                   | 6.93E-9     | 18079339  |
| Cs     | Citrate synthase             | #Ovary                   | 3.15E-6     | 13385942  |
| Fh1    | Fumarate hydratase           |                            | 2.00E-7     | 226823367 |
| Mdh2   | Malate dehydrogenase         | #Ovary, Retina, Caco-2    | 5.55E-15    | 31982186  |

**Group 9: Small GTPases**

| Symbol | Protein Name                  | Remarks                     | Expectation | GI number |
|--------|-------------------------------|-----------------------------|-------------|-----------|
| Rab1   | Rab1                          | #CHO, Muscle, Sperm flagella | 1.40E-10    | 6679587   |
| Rab1b  | Rab1b                         | #Muscle, Sperm flagella     | 1.79E-12    | 21313162  |
| Rab2a  | Rab2a                         | #CHO, Muscle                | 6.09E-9     | 10946940  |
| Rab2b  | Rab2b                         | #CHO, Muscle                | 9.39E-9     | 30520501  |
| Rab4a  | Rab4a                         | #Muscle                   | 6.63E-6     | 171184402 |
| Rab5a  | Rab5a                         | & CHO, Muscle              | 1.96E-5     | 13385374  |
| Rab5c  | Rab5c                         | #Ubiquitous                 | 9.77E-9     | 113866024 |
| Rab7   | Rab7                          | #CHO, Adipocyte, Muscle    | 9.09E-8     | 148747526 |
| Rab8a  | Rab8a                         | #Ovary, CHO, Muscle        | 6.51E-11    | 38372905  |
| Rab8b  | Rab8b                         | #Muscle                    | 1.58E-8     | 27734154  |
| Rab10  | Rab10                         | #CHO, Muscle               | 3.35E-6     | 7710086   |
| Rab11a | Rab11a                        | & CHO, Adipocyte, Muscle   | 2.80E-13    | 31980840  |
| Rab14  | Rab14                         | #CHO, Adipocyte, Muscle    | 2.86E-10    | 18390323  |
| Rab18  | Rab18                         | & CHO, Adipocyte, Muscle   | 7.15E-11    | 30841008  |
| Rab21  | Rab21                         | #Muscle                    | 4.84E-9     | 33859751  |
| Rab22a | Rab22a                        | #Muscle                    | 5.22E-9     | 148747177 |
| Rab31  | Rab31                         | #Muscle                    | 1.10E-7     | 225579124 |
| Rap1a  | Rap1a                         | #Ovary, Muscle             | 5.78E-8     | 21704066  |
| Rap1b  | Rap1b                         | #Muscle, Liver, HuH7       | 7.85E-9     | 33859753  |
| Igkap1 | Cdc42-Rac1 effector protein   | #Sebocyte                | 9.31E-8     | 242332572 |
| Arhgap1| Rhod GTPase-activating protein | #CHO, Adipocyte, Cdc42 activator | 1.93E-7     | 225543424 |

Continued
| Symbol | Protein Name | Remarks | Expectation | GI number |
|--------|--------------|---------|-------------|-----------|
| Cdc42  | Cdc42 GTPase  | #Muscle | 2.04E-4     | 6753364   |
| Arl8a  | ADP-ribosylation factor-like 8A | #Muscle | 6.76E-9     | 29596194  |
| Arl8b  | ADP-ribosylation factor-like 8B | #Muscle, Arf-like GTPase | 3.95E-5     | 13385518  |
| Elmo2  | ELMO domain-containing protein 2 | &#65150; #Muscle, Caco-2, Arf2 GTPase | 7.54E-7     | 283436077 |
| Ehd1   | EH domain-containing protein 1 | &#65150; Ehd2 in Muscle, Testilin; Testes | 1.31E-8     | 7106303   |
| Irgc1  | Interferon inducible GTPase 5 | | 5.64E-6     | 134031980 |
| Atl3   | GTPases atlastin-3 | #CHO | 5.51E-9     | 254826716 |

**Group 10: Protein chaperones**

| Symbol | Protein Name | Remarks | Expectation | GI number |
|--------|--------------|---------|-------------|-----------|
| Hsp1   | Heat shock protein 60kDa | #Ubiquitous | 4.06E-11 | 183396771 |
| Hspa1l | Spermatid-specific HSP70 | #Muscle, Spermatogenesis | 5.12E-10 | 124339838 |
| Hspa1b | Heat shock protein 70.1 | &Adipocyte; #Ovary, Caco-2 | 6.59E-5 | 124339826 |
| Hspa2  | Heat shock protein 70.2 | #Muscle, Testis specific | 7.77E-14 | 315606866 |
| Hspa4l | Heat shock 70kDa protein 4 L | | 2.20E-7 | 40254361 |
| Hspa8  | Heat shock protein cognate 70 | #Ubiquitous | 2.16E-13 | 31981690 |
| Hspa5  | Glucose-regulated protein 78kDa | #Ubiquitous, Grp78 | 1.39E-6 | 254540166 |
| Hsyu1  | Hypoxia upregulated protein 1 | #Liver | 4.61E-12 | 157951706 |
| Hsp90a1 | Heat shock protein 90-α | #Ovary | 7.44E-14 | 6754254 |
| Hsp90ab1 | Heat shock protein 90-β | #CHO, Muscle, Caco-2 | 1.81E-8 | 40556608 |
| Hsp90b1 | Heat shock protein 90-β member 1 | #Muscle, Caco-2 | 4.73E-8 | 6755863 |
| Hsp9a  | Heat shock protein cognate 74 | #Muscle, Caco-2 | 2.39E-9 | 162461907 |
| Dnajc7 | dnaJ (Hsp40) homolog c7 | #CHO | 1.24E-6 | 31980994 |
| Dnajc10 | dnaJ (Hsp40) homolog c10 | | 5.70E-5 | 119508443 |
| Dnajc13 | dnaJ (Hsp40) homolog c13 | | 8.54E-8 | 247494234 |
| Pdia1  | Protein disulfide-isomerase | #CHO, Liver | 3.51E-4 | 42415475 |
| Pdia3  | Protein disulfide-isomerase A3 | #Caco-2, Spermatogenesis | 1.11E-11 | 112293264 |
| Pdia4  | Protein disulfide-isomerase A4 | #Adipocyte, Caco-2 | 8.73E-8 | 86198316 |
| Pdil1  | Protein disulfide-isomerase Pdil1 | Testes specific, fertility | 2.23E-10 | 253735751 |
| Canx   | Calnexin | &18,22, #Ubiquitous | 6.01E-10 | 160333216 |
| Calr   | Calreticulin | #Liver, Caco-2, Chaperone | 4.47E-7 | 6680836 |
| Tcp1   | T-complex protein 1α | Chaperone complex | 5.62E-11 | 110625624 |
| Cct2   | T-complex protein 1(i) (TCP-1(i)) | #Ovary | 6.49E-12 | 126521835 |
| Cct3   | T-complex protein 1γ | | 1.04E-8 | 6753320 |
| Cct4   | T-complex protein 1 delta | | 2.23E-8 | 6753322 |
| Cct5   | T-complex protein 1 epsilon | | 2.39E-8 | 6671702 |
| Cct6a  | T-complex protein 1 zeta | | 1.22E-6 | 6753324 |
| Cct7   | T-complex protein 1 eta | | 1.33E-11 | 238814391 |
| Cct8   | T-complex protein 1 theta | Sperm capitacion | 1.32E-7 | 126723461 |
| Tcp11  | T-complex protein 11 | Spermatogenesis | 1.38E-4 | 148277067 |

**Group 11: Ubiquination process**

| Symbol | Protein Name | Remarks | Expectation | GI number |
|--------|--------------|---------|-------------|-----------|
| Atad3a | AAA ATPase Atad3a | Mitochondrial dynamics | 7.30E-5 | 239985513 |
| Afg3l2 | AAA ATPase Afg3l2 | AFG3-like protein 2 | 2.90E-12 | 110625761 |
| p97/Vcp | AAA ATPase p97 (Vcp) | &16,20, #Muscle, Binds Ubx8 | 2.22E-15 | 235543319 |
| Ubx8  | UBX domain-containing protein 8 | &16,20, #Ubiquitous, Binds Aup1 and Sell1 | 1.51E-10 | 158533976 |
| Ubx1d | UBX domain-containing protein 2 | &16, #CHO, Caco-2, Ubxn-2, Ubxn-4 | 9.05E-12 | 85861252 |
| Aup1  | Ancient ubiquituous protein 1 | &16, #Ubiquitous, | 3.14E-8 | 90403601 |
| Sell1 | Protein sel-1 homolog 1 | Binds Sell1, Aup1, Ubx8 and p97 | 8.53E-12 | 46309573 |
| Ube1  | Ubiquitin-activating enzyme E1 | | 6.61E-9 | 444189294 |

Continued
| Symbol | Protein Name | Remarks | Expectation | GI number |
|--------|--------------|---------|-------------|-----------|
| Ube3b  | Piviquitin protein ligase E3B |         | 9.08E-10    | 68533242  |
| Ube4a  | Ubiquitination factor E4A |         | 2.33E-8     | 167736371 |
| Usp7   | Ubiquitin specific protease 7 |         | 8.83E-6     | 154146209 |
| Pum2d2 | 26S proteasome regulatory subunit S2 | #Ovary20 | 1.71E-8     | 19882201  |
| Uhl1   | E3 UFM1-specific ligase 1 | E3 ligase family | 6.63E-11 | 227330590 |
| Fbxl20 | F-box/LRR-repeat protein 20 | E3 ligase family | 2.10E-6  | 111494221 |
| Bat3   | Large proline-rich protein Bat3 |         | 2.47E-5     | 33147082  |
| Cand1  | TBP-interacting protein | Cullin-associated | 3.11E-14 | 188409138 |
| Cul3   | Cullin-3 | E3 ligase family | 1.55E-8   | 7770014  |
| Cul5   | Cullin-5 | E3 ligase family | 6.77E-9   | 239051067 |

**Group 12: Transport proteins**

| Symbol | Protein Name | Remarks | Expectation | GI number |
|--------|--------------|---------|-------------|-----------|
| Sec23a | Protein transport protein Sec23A | &e; COPII subunit | 1.17E-8 | 67906177  |
| Sec63  | Translocation protein Sec63 | Binds Ubxd2 | 5.26E-6 | 158937300 |
| Secf1  | Sec1 family domain-containing 1 | Vesicle transport | 6.41E-7 | 58037481  |
| Copa   | Coatamer (COP1) subunit α | &E; CHO1, COPI-α | 4.77E-8 | 226823359 |
| Copb   | Coatamer subunit β | &E; CHO1, Testes | 6.27E-10 | 15426055  |
| Copg1  | Coatamer subunit γ1 | &E; CHO1, Binds CDC42 | 6.59E-6 | 8567338   |
| Copg2  | Coatamer subunit γ2 | &E; CHO1 | 1.97E-5 | 8567340   |
| Cop6   | COG complex subunit 6 | Binds Zw10 | 3.05E-5 | 160333744 |
| Zwilch | Zwilch | Zwilch-Zw10 complex | 1.89E-6 | 257153357 |
| Zw10   | Zw10 | &CHO1, Sebocyte18 | 2.42E-8 | 22165349  |
| Rint1  | RAD50-interacting protein 1 | Zw10-Sec30-Rint1 complex | 1.40E-6 | 62899067  |
| Trappc8| Trappc8 | | 4.86E-7 | 291621688 |
| Trappc11| Trappc11 | Zw10-Trappc complex | 1.61E-6 | 62241019  |
| Slc18a1| Vesicular amine transporter 1 | | 8.50E-6 | 33859662  |
| Vps13a | Vacuolar protein sorting 13A | #Muscle20 | 1.78E-12 | 66392160  |
| Vps13c | Vesicle protein sorting 13C | #Muscle20 | 1.41E-11 | 122114537 |
| Vps13d | Vesicle protein sorting 13D | #Muscle20 | 5.01E-5 | 189491888 |
| Vps16 | Vesicle protein sorting 16 | | 9.95E-6 | 254939640 |
| Vps35 | Vesicle protein sorting 35 | #Ovary25 | 2.79E-8 | 13928670 |
| Cltc   | Clathrin heavy chain 1 | #CHO11, Muscle20 | 3.56E-9 | 51491845  |
| Ap1b1  | Clathrin adaptor Ap1b1 | | 8.90E-7 | 88853578  |
| Ap2a1  | Clathrin adaptor Ap2a1 | | 5.76E-6 | 116256510 |
| Ap2b1  | Clathrin adaptor Ap2b1 | | 9.22E-7 | 78711838  |
| Ap2b2  | Clathrin adaptor Ap2b2 | | 9.13E-10 | 163644277 |
| Ncstn  | Nicastrin | | 3.80E-4 | 224809376 |
| Ncln   | Nicastrin-like protein | | 2.60E-7 | 33469043 |
| Nomo1  | Nicalin-nodal modulator 1 | | 1.74E-8 | 22790803 |
| Wdr35  | WD repeat-containing protein 35 | | 2.23E-8 | 226958503 |
| Nup93  | Nucleoporin 93 | | 1.15E-7 | 27369533 |
| Nup98  | Nucleoporin 98 | | 4.75E-6 | 39930413 |
| Nup188 | Nucleoporin 188 | | 1.84E-4 | 38678526 |
| Nup210f| Nucleoporin 210 like | | 6.46E-9 | 254675162 |
| Kpna3  | Importin α4 (karyopherin α3) | | 2.22E-4 | 6680596 |
| Kpna6  | Importin α7 (karyopherin α6) | | 2.16E-8 | 227116300 |
| Kpnb1  | Importin β1 | #Caco20 | 6.26E-8 | 88014720 |
| Ip04   | Importin-4 | | 2.36E-6 | 19745156 |
| Ip05   | Importin-5 | | 1.75E-12 | 29789199 |

Continued
| Symbol | Protein Name | Remarksa | Expectationb | GI number |
|--------|--------------|----------|--------------|-----------|
| Xpo1   | Exportin-1   | #Ovary20, Sebocyte58 | 1.52E-7     | 38604071  |
| Xpo2   | Exportin-2   | #Sebocyte58 | 2.58E-9      | 12963737  |
| Xpo7   | Exportin-7   |          | 7.54E-6      | 12746422  |
| Axxa2  | Annexin A2   | #Ovary20, CHO11,28, Muscle30 | 4.09E-9     | 6996913   |
| Axxa6  | Annexin A6   | #Adipocyte71, Muscle30, Liver19 | 4.46E-9     | 15896670  |
| Snx25  | Sorting nexin-25 | Phospholipid binding | 8.97E-9     | 258613896 |

**Group 13: Nucleotide-catabolic process**

| Symbol | Protein Name | Remarksa | Expectationb | GI number |
|--------|--------------|----------|--------------|-----------|
| Atp5a1 | ATP synthase subunit α | #Ovary20, CHO11, Sperm flagella | 2.92E-10 | 6680748  |
| Atp5b  | ATP synthase subunit β | #Ovary20, Adipocyte29, Caco-20 | 1.33E-12 | 31980648 |
| Atp5f1 | ATP synthase subunit b | 2.21E-8 | 78214312    |
| Atp1a1 | Sodium pump subunit α1 | #Caco-20, Spermatogenesis | 1.02E-5 | 2145277  |
| Atp1a4 | Sodium pump subunit α4 | Spermatogenesis | 1.33E-4 | 226958351 |
| Ctps   | CTP synthase | 3.24E-11 | 172072613   |
| Gmps   | GMP synthase | 4.31E-7 | 85861218    |
| Umps   | UMP synthase | 3.43E-8 | 33859498    |
| Atp6va1| V-ATPase subunit A | #Ovary20 | 3.49E-7 | 31560731 |
| Atp6vh1| V-ATPase subunit H |          | 4.70E-6 | 31981588 |
| Atp13a1| Atp13a1 |          | 7.59E-5 | 283135194 |
| Atp13a2| Atp13a2 |          | 6.67E-6 | 256985106 |
| Atp2a1 | SR Ca(2+)-ATPase 1 | #Muscle30 | 3.07E-8 | 36031132 |
| Atp2a2 | SR Ca(2+)-ATPase 2 | #CHO11, Muscle30 | 1.54E-10 | 6806903 |
| Rent1  | ATP-dependent helicase Rent1 |          | 4.00E-8 | 170784813 |
| Eprs   | Glutamyl-tRNA synthase |          | 4.54E-7 | 82617575 |
| Iars2  | Isoleucyl-tRNA synthase |          | 6.26E-5 | 38490690 |
| hnRNPK | hnRNP K |          | 4.75E-6 | 13384620 |
| Pcbp1  | Poly(rC)-binding protein 1 |          | 1.95E-8 | 6754994 |
| Ruvbl1 | RuvB-like 1 (AAA ATPase) |          | 5.05E-8 | 9790083 |
| Eef1a1 | Elongation factor 1x1 | #CHO11, Caco-20 | 4.29E-10 | 126032329 |
| Eef2   | Elongation factor 2 |          | 5.43E-8 | 33859482 |
| Eif4a2 | eIF4A-II |          | 1.57E-9 | 176865998 |
| Gnb2   | G protein β2 | #Muscle30 | 1.39E-9 | 13937391 |
| Map2k2 | MAPK/ERK kinase 2 &30; #Muscle30, Testosterone synthesis | 4.88E-8 | 31560267 |
| Ide    | Insulin-degrading enzyme |          | 1.17E-6 | 121583922 |

**Group 14: Cytoskeletons**

| Symbol | Protein Name | Remarksa | Expectationb | GI number |
|--------|--------------|----------|--------------|-----------|
| Acta1  | α-actin |          | 4.11E-13 | 33563240 |
| Actn1  | α-actinin-1 | #CHO11 | 3.23E-5 | 61097906 |
| Myh9   | Myosin-9 | #Ovary20 | 7.18E-7 | 11432646 |
| Myh10  | Myosin-10 | #Ovary20 | 2.03E-7 | 33598964 |
| Myh11  | Myosin-11 |          | 2.96E-10 | 241982716 |
| Myo6   | Myosin-6 |          | 1.20E-10 | 261823961 |
| Myo1d  | Myosin-1d |          | 3.54E-4 | 118026911 |
| Myl1   | Myosin light chain A1/A2 |          | 2.89E-5 | 29789016 |
| Tubα1a | Tubulin α1A |          | 2.02E-7 | 6755901 |
| Tubα3a | Tubulin α3A | Testis specific | 1.51E-5 | 6678465 |
| Tubb2a | Tubulin β2A | #Caco-20 | 9.99E-15 | 33859488 |
| Tubb4b | Tubulin β4B |          | 6.27E-8 | 22165384 |
| Tubb3  | Tubulin β3 |          | 1.85E-8 | 12963615 |
| Tubb5  | Tubulin β5 | #Adipocytes30 | 1.40E-9 | 7106439 |

Continued
| Symbol | Protein Name | Remarksa | Expectationb | GI number |
|--------|--------------|----------|-------------|-----------|
| Tln1   | Talin-1      |          | 2.57E-5     | 227116327 |
| Spna2  | Spectrin α2  | #Ovary19, Liver19 | 9.42E-9     | 115496850 |
| Cap1   | Adenylyl cyclase-associated protein 1 | Filament dynamic | 7.09E-10 | 157951604 |
| Ckap4  | Cytoskeleton-associated protein 4 |          | 3.95E-10     | 62526118  |
| Armc4  | Armadillo repeat-containing protein 4 | Outer dynein arms | 4.90E-5 | 124847093 |
| Dnahc8 | Dynein heavy chain 8 | Testis specific | 5.23E-6 | 153792273 |
| Dnchc1 | Dynein heavy chain, cytosolic 1 |          | 1.23E-13     | 134288917 |
| Dnchc2 | Dynein heavy chain, cytosolic 2 |          | 1.96E-8      | 72534792  |
| Dnm1I  | Dynamin-1-like protein | #Muscle30 | 2.30E-7 | 71061455  |
| Dnm2   | Dynein-2     | LD breakdown | 2.19E-6 | 87299637  |

**Group 15: Testis specific or spermatogenesis**

| Slc25a3 | Adenine nucleotide translocase 2 | #Ovary20, Spermatogenesis | 2.58E-7 | 22994075 |
| Slc25a31| Adenine nucleotide translocase 4 | Testis only, spermatogenesis | 3.15E-8 | 254692892 |
| Acr    | Acrosin | Sperm serine proteases | 1.54E-6 | 7304853  |
| Spam1  | Sperm-specific Sperm1 hyaluronidase | Sperm specific | 5.32E-7 | 120407035 |
| Gapdhs | Spermatogenic cell-specific GAPDH-2 | Spermatogenesis | 5.05E-9 | 6679939  |
| Spert  | Spermatid-associated protein | Spermatogenesis | 5.48E-9 | 256017220 |
| Spata20| Spermatogenesis-associated protein 20 | Spermatogenesis | 6.40E-11 | 46485467  |
| Tcam1  | Testicular cell adhesion molecule 1 | Testis specific | 1.05E-4 | 145279190 |
| Ihf122 | Intraflagellar transport protein 122 | Flagellar transport | 2.24E-5 | 268370099 |
| Clgn   | Celmegnin | Spermatogenesis | 3.10E-10 | 86262138 |
| Ace    | Angiotensin-converting enzyme | Sperm-zona binding | 1.23E-7 | 33468873 |
| Tfrc   | Transferrin receptor | Spermatogenesis | 4.35E-4 | 11596855 |
| Odf2   | Outer dense fiber of sperm tails 2 | Sperm tails | 1.77E-6 | 295054183 |
| Ddx1   | DEAD box protein 1 | Germ cell specific | 1.32E-9 | 19527256 |
| Ddx4   | DEAD box protein 4 | Germ cell specific | 9.11E-6 | 225007636 |
| Bpi    | Bactericidal permeability-increasing protein | Testis-specific | 2.73E-4 | 29244434  |
| Piwi1  | Piwi-like protein 1 | Spermatogenesis | 5.07E-9 | 10946612 |
| Tdrd1  | Testis antigen 41.1 | Testis-specific | 2.13E-8 | 50355696 |
| Stk31  | Serine/threonine-protein kinase 31 | Testis-specific | 6.56E-5 | 258613856 |
| Sh ebp1| Shc binding protein 1 | Testes | 8.81E-7 | 85701672 |
| Dpep3  | Dipeptidase 3 | Germ cell specific | 8.85E-13 | 2133683 |
| Adam6b | ADAM6b | Testis specific | 9.16E-4 | 57222276 |
| Ppm1j  | Protein phosphatase 1J | Germ cell specific. | 9.99E-15 | 114205424 |
| Akap3  | A-kinase anchor protein 3 | Germ cell specific | 9.17E-5 | 160358791 |
| Akap4  | A-kinase anchor protein 4 | Spermatid specific | 1.52E-5 | 110347483 |
| Akap12 | A-kinase anchor protein 12 | Germ cell protein | 5.12E-8 | 13626040 |

**Group 16: Miscellaneous**

| Alb    | Albumin | #Liver19 | 3.67E-8 | 163310765 |
| Slc3a2 | Solute carrier family 3 member 2 |          | 8.00E-7 | 238637277 |
| Pgc1p  | Plasma glutamate carboxypeptidase |          | 2.83E-8 | 28570174 |
| Ano10  | Anoctamin-10 |          | 5.95E-6 | 30794236 |
| Heatr2 | Dynein assembly factor 5 |          | 1.65E-9 | 124486915 |
| Cd109  | CD109 |          | 9.26E-8 | 23346525 |
| Aifm2  | Apoptosis-inducing factor 2 | #Caco-28, HuH730, Testes | 2.98E-9 | 85861162 |
| Api5   | Apoptosis inhibitor 5 |          | 8.42E-8 | 94158994 |
| Pdec6p | PDCD6-interacting protein | Apoptosis | 4.35E-8 | 258547154 |
| Bbs7   | Bardet-Biedl syndrome 7 protein |          | 1.30E-5 | 170650593 |

Continued
| Symbol | Protein Name | Remarks | Expectation | GI number |
|--------|--------------|---------|-------------|-----------|
| Ttc21b | Tetratricopeptide repeat protein 21B | Ciliary transport | 2.04E-8 | 114158711 |
| Ttc25  | Tetratricopeptide repeat protein 25 | 1.15E-4 | 124358957 |
| Ttc39b | Tetratricopeptide repeat protein 39B | 2.05E-5 | 58037187 |
| Tom70  | Mitochondrial import receptor Tom70 | Ttc domain | 9.95E-4 | 27552760 |
| Mtc2   | Mitochondrial carrier homolog 2 | 5.64E-7 | 9790055 |
| Lamp2  | Lysosome membrane protein 2 | 6.09E-9 | 6680878 |
| Ermp1  | ER metallopeptidase 1 | 2.27E-6 | 124487057 |
| Fam79a | Fam79a | 7.75E-14 | 21312776 |
| Fam91a1| Fam91a1 | 7.00E-7 | 112817622 |
| Fam129a| Fam129a | Niban | 3.82E-7 | 241982745 |
| Mic60  | Mic60 | 1.09E-9 | 70608131 |
| Stim1  | Stromal interaction molecule 1 | 3.04E-4 | 31981983 |
| Nbas  | Neuroblastoma-amplified protein | #CHO11,28 | 2.92E-10 | 255003837 |
| Lrc40  | Leucine rich repeat containing 40 | 4.43E-6 | 31541911 |
| Pdsc1  | Pdsc1 | 4.66E-8 | 88758582 |
| Genl11 | Genl11 | 1.28E-7 | 112807186 |
| Ilvbl  | ilvB-like protein | Acetolactate synthase | 4.24E-9 | 30424591 |
| Trim27 | Zinc finger protein RFP | 2.19E-6 | 125347389 |
| Srp68  | Signal recognition particle 68 | 2.64E-4 | 47271535 |
| Tm9s2 | Transmembrane 9 superfamily member 2 | 2.70E-8 | 188528994 |
| unknown| RIKEN cDNA 4732456N10 gene | 1.91E-7 | 269914154 |

Table 1. Proteins associated with testicular lipid droplets (LDs). A total of 337 proteins were identified from murine testicular LDs by mass spectrometry; 144 identified proteins had been previously detected in LD proteomic studies and are labeled with “#” and citations to annotate the tissue or cell source of the LDs. A total of 44 proteins had been previously confirmed in LDs by microscopy and are labeled with “&”. *Comparison with the reference data involved manual inspection of the GI number and then the standard names of proteins identified in the present and previous proteomic studies. The expectation value is a statistical term that allows for comparison of the reliability of results. Protein identifications were based on both the expectation value (< 10\(^{-4}\)) and the quality of MS/MS spectra of peptide fragments (> 3) identified. Low expectation values correspond to confident identifications.

carboxylesterase 3\(^{17,18}\) and monoglyceride lipase\(^{19,20}\). LD-associated hydrolase (C2orf43 protein) is a cholesteryl ester hydrolase that normally localizes to the ER but is translocated to LDs on lipid loading\(^{21,22}\). ATGL expresses specifically in adipose tissue\(^2\), but HSL expresses primarily in both adipose and steroidogenic tissues.

Group 3 proteins represented 22 enzymes involved in the metabolism of fatty acid and glycerolipids and as well as phospholipids and sterols. Five were previously observed in LDs by microscopy. Fatty acid transport protein 1 binds diacylglycerol acyltransferase 2 and colocalizes to the ER-LD interface to facilitate glycerolipid biosynthesis and LD expansion\(^24\). Long-chain acyl-CoA synthetase Acsl1\(^25\) and Acsl3\(^25,26\), along with glycerol-3-phosphate O-acyltransferase (Gpat4), are normally localized in the ER microdomain but effectively translocated to nascent LDs to facilitate LD biosynthesis on lipid loading\(^25,26\). Acsl4 and fatty aldehyde dehydrogenase were morphologically localized in yeast LDs\(^27\) and proteomically detected in LDs of CHO cells\(^28\), adipocytes\(^9\) and mouse muscle\(^30\). Carnitine O-palmitoyltransferase 2, very-long-chain acyl-CoA dehydrogenase, and mitochondrial trifunctional enzyme subunit \(α\) were detected from mouse muscle LDs\(^30\). Fatty acid synthase was detected from LDs of granulosa steroidogenic cells from rat ovary\(^20\). Many proteins in this group are known to specifically or highly express in testes (Table 1).

Group 4 proteins represented 11 phospholipid metabolic enzymes; 3 were previously physiologically confirmed in LDs. Phospholipase A2 is highly expressed in testis and activated by sterol removal in murine sperm membrane, which localizes at the LD surface and hydrolyzes glycerophospholipids to facilitate the LD structure\(^31\). Cytosolic phospholipase A2 (cPLA2) is activated by extracellular stimuli-hydrolyzed arachidonic acids from the sn-2 position of glycerophospholipids; in turn, released arachidonic acids induce the translocation of cPLA2 to the ER and LD interface to regulate lipid synthesis and nascent LD formation\(^32,33\). Phosphocholine cytidylyltransferase binds to growing LDs and then catalyzes phospholipid synthesis and promotes LD expansion\(^34,35\). Phosphatidylglycerophosphate synthase 1 and phosphoinositide
lipid phosphatase are highly expressed in testes, and phospholipase DDHD1 is required for spermatogenesis. The proteins in this group also participate in glycerolipid and sterol metabolism.

Group 5 contained 19 proteins that participate in biosynthesis and metabolism of cholesterol, retinol, and hormonal steroids; 6 were previously observed in LDs by microscopy and another 7 were previously detected in LD proteomes. Short-chain dehydrogenase/reductase 3 and retinol dehydrogenase 10 are reciprocally activated and on acyl ester biosynthesis, are translocated from the ER to LDs46–48. The key steroidogenic enzymes lanosterol synthase22, 3β-hydroxysteroid dehydrogenase (HSD) 1 and 7α, 3β-HSD-4, 7β, 11β-HSD-4, 7α-HSD-4, 7β-HSD-4, and 17β-HSD-4 were previously microscopically or proteomically detected in intracellular LDs. Many of these enzymes, such as 17α-hydroxyprogesterone aldolase and scavenger receptor class B-1,20, are highly expressed in testes and regulate cholesterol homeostasis.

Group 6 proteins represented 17 enzymes involving in glucuronidation and glycosylation. UDP-glucuronosyltransferase 1–6, Dolp-glucosyltransferase11,28, α-glucosidase20, and methyltransferase-like protein 7A were previously found in LD proteomes, and methyltransferase-like protein 7B was observed in LDs by microscopy19,45,46. CGI-49 proteins are frequently found in LD proteomes11,29,30,41.

Large oligosaccharyltransferase complexes contain ribophorin I, Stt3a, Stt3b, p97/Vcp, Sel1l, and Ubxd87 and may also interact with ancient ubiquitous protein 1 (Aup1), Acsl3 and stomatin48. Ubxd89,95,96, p97/Vcp49,50, Aup124, Acsl351,52 and stomatin23 have been verified in LDs by microscopy, which suggests that the present identification is reliable. Several enzymes in this group catalyze glucuronidation reactions of estrogens, testosterone, retinoic acids, and various metabolites of xenobiotics and endobiotics47.

Group 7 and 8 proteins included 29 enzymes involved in the metabolism of carbohydrate and tricarboxylic acid cycle. NADH-cytochrome b5 reductase was verified in LDs by microscopy45. Glutamate dehydrogenase, malate dehydrogenase, succinate dehydrogenase, lactate dehydrogenase, pyruvate kinase 2/3, and citrate synthase were previously reported in LD proteomes11,20,51. The identification of 17 other metabolic enzymes in testicular LDs is novel, which might reflect the close relationship between LDs and mitochondria in testicular cells52.

Group 9 proteins represented 28 small GTPases; 27 were previously reported in LD proteomes. In cells loaded with fatty acids, Rab5a53, Rab11a53, Arl2 GTPase Elmod254, and Rab1853,55 can localize to both the ER and LDs, where Rab18 recruits unknown effectors and microtubules to facilitate membrane trafficking and lipid exchange53,55. Testicular LDs might serve as a dock for various small GTPases for mediating Rab signaling.

Group 10 listed 30 protein chaperones; 18 were previously reported in LD proteomes. We previously showed that heat shock protein 70 (Hsp70) can translocate to adipocyte LDs on heat stimulation56. Spermatic-specific Hsp70, Hsp70.2 (Hspa2), T-complex protein 11, and protein disulfide-isomerase A3 (PDi3a) are testis-specific and play roles in spermatogenesis. PDI is a component of microsomal triacylglycerol transfer protein complex. T-complex protein 1 contains 8 distinct subunits to form a unique chaperone for escorting actin, tubulin, and numerous other proteins. In Leydig cells, the intermediate filaments of the cytoskeletons may bind to LDs52.

Group 11 listed 18 proteins involved in proteasome and membrane trafficking. Among them, p97, Atad3a and Afg3l2 are AAA ATPase family proteins that regulate ubiquination, membrane trafficking, and organelle biogenesis. p97, Ubxd2 and Ubxd8/Faf2) bind with each other and colocalize to LDs46,49,50. Aup1 localizes to the ER and LDs46,57. Aup1 may exist in several subcomplexes and associate with numerous other proteins48 such as Ubxd8, Ubxd2, Atad3a, RuvB-like 1, stomatin, ribophorin I and II, T-complex proteins, epoxide hydrolase 1, atlastin-3, Acsl3, pyruvate kinase 2/3, PDI, and ATP synthase48. Dozens of Aup1-associated proteins were also identified in testicular LDs, which might reflect the close association of these protein complexes with cellular LDs.

Group 12 contained 43 transport proteins; 16 were proteomically reported11,30,40,52 and 5 were microscopically confirmed in LDs59,60. Coatamer protein complex 1 (COPI) and clathrin adaptor complex mediate intra-Golgi transport and retrograde transport from the Golgi to ER. Arf1/COPII complexes localize between the ER and LDs for targeting the triacylglycerol synthesis enzyme Gpat4 to the LD surface and bud 60-nm nanodroplets from the LDs. In cells loaded with fatty acids, both COPI and COPII (Sec23) structures tend to localize to discrete foci surrounding LDs to create a membrane bridge for transporting ATGL and Plin2 to nascent LDs60.

Group 13 contained 26 proteins involved in nucleotide-catabolic processes, such as ion transport, transcription, translation, and cell signaling. Nine proteins were detected by previous LD proteomes. Some proteins might not easily fit into this single category because of the divergence of protein functions. MAPK/ERK kinase 2 is colocalized with CPLA2 in LDs, then rapidly activates cPLA2 for releasing arachidonate from LDs33; it is required for testosterone synthesis in Leydig cells. ATP synthase subunit α and sodium pump subunit α1 and α4 are expressed abundantly in testis and regulate spermatogenesis.

Group 14–16 included cytoskeletal proteins, testis-specific and miscellaneous proteins. Only 11 of these 81 proteins were previously reported in LD proteomes. The identification of albumin in the present and previous LD proteomes should represent a contamination because of its abundance in serum. The identification of testis-specific proteins could be due to the contamination or the difficult separation of these protein components from testicular LDs. For example, GAPDH2 and A-kinase anchor protein 3 and 4 participate in spermatogenesis, which can bind the cytoskeletal fibrous sheath and thus might be
Figure 4. Confirmation of LD proteins by immunodetection A. The fractions of LD, total membrane (TM), cytosol (Cyto), and post-nuclear supernatant (PNS) were prepared from mice testes. An equal amount of proteins extracted from different fractions was separated by SDS-PAGE and underwent immunoblotting with the primary antibodies indicated. A representative silver-stained gel showed equivalent protein loading. Plin variants Plin1a~1d were detected on a full-length blot. The blots of proteins were derived from the sample or different samples that were processed in parallel, and the corresponding full-length blots are shown in Supplementary Figure S1. Arom, aromatase; Cav, caveolin; Plin, perilipin; HSL, hormone-sensitive lipase; ATGL, adipose triglyceride lipase. B. Immunofluorescent staining of Plin1 (a,c) Plin2 (e), and 3β-hydroxysteroid dehydrogenase (HSD3B1) (g) in sections of mouse testis. The merged images are shown in panel b,d,f and h.

co-purified with LD-associated cytoskeletons. Also, these testis-specific or spermatogenesis-related proteins might exist in cellular subcomplex structures that associate with testicular LDs.

Confirmation of testicular LD protein identification by immunoblotting and immunofluorescence. Some of the identified testicular LD proteins were confirmed by immunoblotting by using marker proteins corresponding to different cellular compartments (Fig. 4A). Four members of the perilipin family, Plin1–4, including the 4 variants of Plin1, Plin1a, 1b, 1c and 1d, were detected only in the LD fraction. This was the first immunodetection of Plin1d in tissue (Fig. 4A). Plin5 signal was not detectable in testicular LD extracts (data not shown), which is consistent with its low level of expression in non-oxidative tissues. ATGL and CGI-58 appeared only in the LD fraction; HSL and 3β-HSD1 were
highly enriched in the LD fraction but also detectable in the membrane and cytosol compartments (Fig. 4A). Caveolin-1 and -3, caveolae marker proteins, were not identified in the testicular LD proteome (Table 1) but were immunodetected in the LD fraction or other cellular compartments (Fig. 4A). Aromatase, a cytochrome P450 enzyme that converts androgen to estrogen in seminiferous epithelium, was marginally detected in the testicular LD fraction but appeared mainly in the membrane fraction (Fig. 4A). Lysosome protein Lamp-1, ER protein p62, and cytoplasmic enzyme GAPDH were not detected in the LD fraction. The ER chaperone GRP78 and mitochondrial protein Tim 23 were detected predominately in the membrane and post-nuclear supernatant fractions, but a small amount appeared in the LD fraction (Fig. 4A). Clearly, the isolated LD fraction of mice testes was largely free of other organelle contamination, although a small amount of the ER and mitochondria components might be introduced, likely because of their abundance or general interactions with LDs. Furthermore, immunofluorescent signals of Plin1 appeared strongly in the interstitium of mice testis (Fig. 4B, panel a and c), and the fluorescent signal pattern was consistent with that of interstitial LDs stained with Nile Red (Fig. 1B, panel a and b). Immunofluorescent signals were weaker for Plin2 and 3β-HSD1 than Plin1 but still detectable in interstitial locations (Fig. 4B, panel e and g). The immunofluorescent signal for 17β-HSD11 was not detected (data not shown).

**Discussion**

We report the first proteomic analysis of LDs purified from adult mice testes. Testicular LDs contained 337 proteins; 144 were previously detected in the LD proteomes and 44 were verified by microscopy. From the functions of identified proteins, testicular LDs showed several characteristics different from the LDs of most other cell types. Testicular LDs may be unique, biologically active cellular organelles that might have functional roles in the biosynthesis of hormonal steroids.

First, testicular LDs featured most Plin family and lipase/esterase superfamily proteins and various enzymes for biosynthesis and metabolism of glycerolipids and phospholipids. The classical LD proteins, Plin1–4 and 4 variants of Plin1, are crucial for regulating LD formation. During LD expansion in differentiating adipocytes, nascent small LDs are coated with Plin3 and Plin4, medial-size LDs require both Plin2 and Plin1, and finally, Plin1 replaces Plin2 as a major coat of large LDs in mature adipocytes. We previously revealed that Plin2 is degraded by the proteasome with the induction of Plin1 and if Plin1 is null for replacing Plin2, LD growth and adipocyte differentiation are retarded. Different Plins target different types of LDs and have unique functions to govern triacylglyceride−cholesterol ester balance. Plin1a and Plin1b favor triacylglyceride-rich LDs, Plin1c and Plin4 prefer cholesteryl ester-rich LDs, but Plin2 and Plin3 show less specific localization to LDs. Plin1 expresses exclusively in adipose and steroidogenic cells. Thin-layer chromatography revealed that the LD of adipocytes was triacylglyceride-rich, so it associates mainly with Plin1a and Plin1b. In contrast, the testicular LD had a relatively equivalent proportion of triacylglycerides and cholesteryl esters. The accumulation of triacylglycerides promotes and stabilizes storage of cholesteryl esters within Leydig cells. Likely, the coats of Plin1–4, including Plin1a–1d, could cooperatively manipulate the appropriate balance of cholesteryl ester−triacylglycerides in steroidogenic cells of testes.

Also, testicular LDs contained most of the known lipases/esterases/phospholipases and enzymes of glycerolipid and phospholipid metabolism. HSL and ATGL represent ~95% of the lipolytic activity in adipocytes and the remaining activity is contributed by triacylglycerol hydrolase and monoglyceride lipase. We and others previously revealed that Plin1 phosphorylation induces the translocation of HSL from the cytosol to LDs and also indirectly activates ATGL by unsequestering the ATGL coactivator CGI-58, hence conferring a full lipolytic reaction in adipocytes. HSL is stimulated by catecholamine, thyroxine, and glucocorticoid, and in testes, HSL is activated by chorionic gonadotropin. Inactivation of ATGL causes diacylglyceride accumulation in testes, but HSL ablation disables spermatogenesis and causes male infertility. Despite these crucial roles of lipases, the control of lipolysis and even the catalog of lipases (except HSL) are largely unknown in testes. Although lipases can act on broad lipid substrates (e.g., glycerolipids in adipocytes), in Leydig cells, they predominately hydrolyze cholesterol esters to cholesterols for steroidogenesis. Unlike testicular LDs, the LDs in other types of cells including adipocytes were not found to contain so many lipases/esterases and enzymes for glycerolipid and phospholipid metabolism. Likely, testicular LDs need to be accurately modulated by these different enzymes, to facilitate the biosynthesis and hydrolysis of cholesteryl esters and thereby ensure cholesterol supply for steroidogenesis in testes.

The second unique feature is that testicular LDs contained a large number of steroidogenic enzymes such as lanosterol synthase and demethylase, various hydroxysteroid and retinol dehydrogenases, and various glucuronidation enzymes. Currently, steroidogenic enzymes are known to locate in the ER and mitochondria membranes and in the adjacent cytoplasm, where they catalyze different reactions, their substrates and products being shuttled between these compartments. The enzymes identified in testicular LDs, such as short-chain dehydrogenase, retinol dehydrogenase, 3β-HSD1, 17β-HSD11, 3β-HSD19, and NAD(P)H steroid dehydrogenase-like protein, another 3β-HSD, can translocate from the ER membrane to the LD surface on acyl ester biosynthesis. The substrates, products and metabolites of steroidogenic reactions are mostly insoluble and cannot distribute and move freely in the cytoplasm but instead could be chaperoned and escorted by hydrophobic LDs. Thus, considering that testicular LDs are spatially close to the ER and mitochondria and contain so many steroidogenic
enzymes at the oil–water interface, the present data suggests that testicular LDs could be a new compartment for carrying out steroidogenic reactions, more than just a simple pool of cholesterol substrates. At least, testicular LDs could be a chaperone vehicle to facilitate the biosynthesis of hormonal steroids, by transferring insoluble intermediate substrates and products between the mitochondria and the adjacent cytoplasm.

Third, testicular LDs contained large numbers of proteins involved in cellular signaling, chaperon, ubiquitination, transport, cytoskeleton and spermatogenesis. Proteins in the GTPase superfamily and Rab GTase subfamily were particularly abundant. Rab18,33,35 can recruit microtubules and localize between the ER and LDs to facilitate membrane trafficking and lipid exchange33,35. Ubxd8 and p97/VCP colocalize at the ER-LD interface and promote LD expansion by binding ATGL and inhibiting ATGL-mediated LD lipolysis36. Similarly, the vesicle transporters COPI and COPII are membrane bridges between the ER and LDs to deliver and modulate ATGL, Plin2 and Plin3 levels at nascent LDs30. Because many of these proteins may exist in large multicomponent complexes, their simultaneous identification from testicular LDs was not surprising. For example, Aup1 localizes to the ER and LDs and contributes to the formation of LDs that may temporarily store misfolded ER proteins under certain conditions8,37. Actually, Aup1 is a component of the Hrd1–Sel11 ER quality-control complex and physiologically associates with a hundred other proteins48. In comparison, testicular LDs contained at least dozens of Aup1-associated proteins48, such as Ubxd8, Ubxd2, p97/VCP, Atad3a, Sel11, Ruvb-like 1, stomatin, ribophorin I, T-complex proteins, epoxide hydrolase, atlastin-3, Acsl3, pyruvate kinase 2/3, and PDI3a. In addition, testicular LDs contained many cytoskeletal proteins, which might not be simply considered contamination. In steroidogenic cells, the LDs and mitochondria are known to tightly attach to the cytoskeleton and intermediate filaments that are thought to mediate transport of cholesterol49. An example is vimentin filaments, which bind Plin1 and wrap LDs52. Vimentin ablation results in defective steroidogenesis in adrenocortical and granulosa cells49. Overall, these findings suggest that testicular LDs could participate initially in cellular signaling, chaperon, ubiquitination, transport, cytoskeleton and spermatogenesis.

In summary, testicular LDs could be considered active cellular organelles participating in the regulation of multiple testicular functions. Plins and lipases/esterases/phospholipases could govern accurate control of the biosynthesis and hydrolysis of cholesteryl esters, thus ensuring appropriate cholesteryl ester-triacylglyceride balance and cholesterol supply for steroidogenesis. Notably, the association with various kinds of steroidogenic enzymes suggests that steroidogenic reactions might occur in testicular LDs or the steroidogenic enzymes and products could be transferred through testicular LDs. Because little was known about testicular LD proteins, the investigation of the roles of testicular LDs has been largely restricted to morphological observations. The present finding uncovers the full set of testicular LD proteins, for further examination of the functional roles of testicular LDs and their proteins in steroidogenesis and spermatogenesis in testes.

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Author Contributions
G.X. and W.W. conceived and designed the experiments. W.W., S.W., L.L., X.S., C.D., F.L., B.G. and P.L. performed experiments and prepared Figs 1–4. W.W., G.X. and P.L. analyzed the data and wrote the paper. All authors reviewed the manuscript.

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