Quantitative Proteomic and Functional Analysis of Liver Mitochondria from High Fat Diet (HFD) Diabetic Mice*

Yurong Guo‡§, Manjula Darshi¶¶, Yuliang Ma§, Guy A. Perkins||, Zhouxin Shen**, Kristofer J. Haushalter§, Rintaro Saito‡‡, Ai Chen§§, Yun Sok Lee§§, Hemal H. Patel¶¶¶, Steven P. Briggs**, Mark H. Ellisman|||, Jerrold M. Olefsky‡‡, and Susan S. Taylor‡§¶b

Insulin resistance plays a major role in the development of type 2 diabetes and obesity and affects a number of biological processes such as mitochondrial biogenesis. Though mitochondrial dysfunction has been linked to the development of insulin resistance and pathogenesis of type 2 diabetes, the precise mechanism linking the two is not well understood. We used high fat diet (HFD)-induced obesity dependent diabetes mouse models to gain insight into the potential pathways altered with metabolic disease, and carried out quantitative proteomic analysis of liver mitochondria. As previously reported, proteins involved in fatty acid oxidation, branched chain amino acid degradation, tricarboxylic acid cycle, and oxidative phosphorylation were uniformly up-regulated in the liver of HFD fed mice compared with that of normal diet. Further, our studies revealed that retinol metabolism is distinctly down-regulated and the mitochondrial structural proteins—components of mitochondrial intermembrane space bridging (MIB) complex (Mitofilin, Sam50, and ChChd3), and Tim proteins—essential for protein import, are significantly up-regulated in HFD fed mice. Structural and functional studies on HFD and normal diet liver mitochondria revealed remodeling of HFD mitochondria to a more condensed form with increased respiratory capacity and higher ATP levels compared with normal diet mitochondria. Thus, it is likely that the structural remodeling is essential to accommodate the increased protein content in presence of HFD: the mechanism could be through the MIB complex promoting contact site and cristal junction formation and in turn facilitating the lipid and protein uptake. *Molecular & Cellular Proteomics 12: 10.1074/mcp.M113.027441, 3744–3758, 2013.

Obesity has become a global epidemic and in the United States alone more than one third of adults (34%) are obese and over 11% of the population over the age of 20 are diabetic (1, 2). Even though the precise mechanisms causing obesity are still being determined, it is well established that obesity induces insulin resistance leading to the pathogenesis of type 2 diabetes (T2D) (3, 4). Insulin resistance has been implicated in multiple organ damage such as liver, skeletal muscle, and adipose tissues (5). This is in view of the fact that cellular glucose homeostasis is tightly regulated by insulin secretion from the pancreatic β-cells and glucose uptake by muscle and output by liver. Thus, failure of insulin secretion by pancreatic β-cells to compensate for insulin resistance results in hyperglycemia (6, 7) and uncontrolled hyperglycemia has the potential to negatively impact a number of organ systems.

Mitochondrial dysfunction has been thought to play a critical role in insulin resistance and T2D (8–11), and the role of mitochondria in insulin resistance is highly tissue specific. Despite this the mechanisms of action is controversial: in skeletal muscle, oxidative metabolism of lipids is reduced in T2D patients (11, 12). However, it has been reported that carnitine palmitoyl transferase (CPT) activity is decreased or long-chain acyl-CoA dehydrogenase (LACD) is deficient, and these lead to an accumulation of intracellular lipids and insulin resistance in insulin-targeting cells (13). These excess metabolites eventually cause the decrease in glucose transport and other events downstream of insulin receptor signaling (14). In
adipose tissue, it has been shown that adiponectin expression and mitochondrial content in obese db/db mice were reduced (15). One of the favorable effects of adiponectin is the improvement of insulin action (16) and it has been reported that mitochondrial dysfunction in adipose tissue explains decreased adiponectin synthesis in obesity (15). In contrast to muscle, coordinated up-regulation of oxidative phosphorylation (OXPHOS) subunits in obese liver has been previously reported at mRNA (17) and protein (18) levels. Liver mitochondria also showed significant up-regulation of beta-oxidation, branched chain amino acid degradation, pyruvate metabolism, TCA cycle, and apoptotic pathways (18, 19).

In the fasting state, blood glucose levels are maintained by liver regulated gluconeogenesis. T2D is characterized by defects in insulin’s ability to inhibit gluconeogenesis. Hepatic insulin resistance and impaired fatty acid oxidation have been the major contributors for the development of hepatic steatosis and the progression of nonalcoholic fatty liver disease (NAFLD) (20). Mitochondrial abnormalities have also been associated with NAFLD, and hence liver insulin sensitivity and the potential relationship with mitochondria appear to be crucial.

In this study, we used a proteomic approach to investigate the biological pathways leading to insulin resistance in T2D in the liver and analyzed the protein expression profiles from mitochondria of mice fed on a high fat diet (HFD) for 30 weeks. Increased insulin resistance, elevated plasma insulin and impaired glucose tolerance were observed in mice after 16 weeks of HFD (21). Consistent with previous studies, liver mitochondria showed significant up-regulation of oxidation-phosphorylation (OXPHOS), beta-oxidation, branched chain amino acid degradation, pyruvate metabolism, TCA cycle, and apoptotic pathways (18, 19). Additionally, in our study we show that retinol metabolism was consistently down-regulated, and the proteins involved in the protein import into mitochondria, mitochondrial biogenesis and regulation of cristae morphology are up-regulated in HFD versus normal diet (ND) liver mitochondria. We further examined the ultrastructure of the mitochondria from the liver and show that under HFD conditions the mitochondria are often condensed with a remodeling of cristae and increased number of cristae. We suggest that the structural change in cristae is likely to be a necessary event to accommodate the increased OXPHOS gene expression and other proteins that are residents of the inner membrane. The remodeling of cristae is also accompanied by increased mitofilin expression and its interacting proteins, which include ChChd3 and OPAL, that are critical for cristae organization. Finally our oxygen consumption experiment showed increased activities of complex I and complex II in liver mitochondria of HFD versus ND mice and our ATP assay confirmed increased ATP production in HFD versus ND mice.

**Experimental Procedures**

**Animals and Animal Care**—Male C57BL/6J mice aged 12 weeks were purchased from The Jackson Laboratory and were fed with HFD (60% kcal from fat; D12492, Research Diets) or ND (12% kcal from fat; Purina 5001, LabDiet) for 30 weeks. Animals were maintained on a 12-h/12-h light/dark cycle with free access to food and water. All animal protocols were in accordance with the University of California, San Diego research guidelines for the care and use of laboratory animals.

**Mitochondria Isolation**—Mitochondria from mouse liver were isolated as described previously (22). In brief, livers were quickly excised and homogenized with a Potter-Elvehjem tissue homogenizer in ice-cold MSHE buffer (220 mM mannitol, 70 mM sucrose, 5 mM HEPES-KOH, pH 7.4, and 1 mM EGTA, pH 7.4) containing EDTA-free protease inhibitor mixture (Sigma) and PhoSTOP phosphatase inhibitor mixture (Roche). Nuclear material and unbroken cells were discarded by centrifugation for 10 min at 600 × g. Crude mitochondrial fractions were collected by centrifugation for 10 min at 15,000 × g and resuspended in 2% Rapigest, 50 mM HEPES, pH 7.3 with 2.5 mM sodium fluoride, 0.25 mM sodium p-mercuribenzoate, 0.25 mM sodium pyrophosphate, and 0.25 mM β-glycerophosphate. Mitochondria from each mouse (3HFD, 3ND) were isolated separately.

**Mitochondria Digestion**—Mitochondria samples were reduced with tris (2-carboxyethyl) phosphine (final concentration, 4 mM), alkylated with iodoacetamide (final concentration, 10 mM), and diluted 10× using 50 mM HEPES. Protein concentration was measured by using a bicinchoninic acid assay (BCA assay, Pierce) and samples were digested with sequence-grade modified trypsin (Promega, Madison, WI) at 1:100 enzyme to protein ratio overnight at 37 °C. Aliquots of the samples were analyzed on a 4–12% SDS-PAGE gel and stained using the SilverQuest SilverStain kit (Invitrogen, Carlsbad, CA) to confirm complete digestion. Toward the complete digestion, the BCA assay was again used to measure peptide concentration.

**Desalting and iTRAQ Labeling of Peptides**—An aliquot of each sample (100 μg) was treated with 50% trifluoroacetic acid to a final pH of 1.4, incubated at 4 °C for 1 h and centrifuged at 18,000 × g for 10 min. The supernatant was centrifuged through a 0.22 μm filter. The flow through was desalted on Sep-Pak Plus C18 cartridges, and reconstituted in 0.5 μl HEPES, pH 7.2. For iTRAQ labeling, each desalted sample was treated with one tube of different 4-plex iTRAQ reagents (ND: 114; HFD: 115) in 70% isopropanol at pH 7.2 for 2 h at room temperature (22). The samples were dried under speed vacuum to about 25 μl, combined and used for mass spectrometric analysis. Three pairs of HFD and ND samples were treated as three biological replicates.

**RP1/SCX/RP2-HPLC Separation of Peptides**—The peptides were separated on a three-phase column assembly adapted from Andreyev et al. (23) using an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA). Briefly, half of the iTRAQ-labeled peptide mixture was loaded to an RP1 column (RP1, 360 μm O.D. × 200 μm I.D., 5 μm Zorbax SB-C18 (Agilent Technologies), 20 cm packing) using a pressure chamber, rinsed with 0.1% formic acid and eluted to a SCX column (SCX, 360 μm O.D. × 200 μm I.D., 5 μm PolySULPHOETHYL A (PolyLC, Inc., 5 cm packing) online using a reverse phase gradient 0–80% B in 150 min (solvent A: 0.1% formic acid; solvent B: 90% acetonitrile in 0.1% formic acid). The peptides on the strong cation exchange (SCX) column were step eluted to a C18 analytical column with integrated tip (RP2, 360 μm O.D. × 200 μm I.D., 5 μm Zorbax SB-C18 (Agilent Technologies), 20 cm packing) with a series of salt gradients (38 steps). Following each salt elution, the peptides on the C18 analytical column were eluted with increased acetonitrile (0–40% in 120 min) to an LTQ-Orbitrap (Thermo Fisher Scientific, San Jose, CA). The flow rate out of the RP2 column was ~500 nl/min.
Tandem Mass Spectrometry Analysis—The LTQ-Orbitrap was operated under data-dependent mode with gas phase fractionation. Briefly, the full MS scan range was divided into three smaller scan ranges (400–805, 800–1055, 1050–2000). Each MS scan (Orbitrap, 7500R) was followed by four pairs of pulsed Q dissociation-collision activated dissociation (QD-CAD) scans on the top four most abundant parent ions in LTO (24). A dynamic exclusion of 1 min was used. The capillary temperature was 150 °C.

Data Analysis—The raw data were extracted and searched using Spectrum Mill v3.03 (Agilent Technologies). The PQQ and CAD spectra were merged together and searched against the International Protein Index mouse database (v3.31; 56,555 protein sequences). Trypsin was used as an enzyme, and the peptides were limited to fully tryptic with a maximum missed cleavage of two. Other parameters include: carbamidomethylation of cysteines, static; iTRAQ modification on N-term and lysine, differential; ± 20 ppm for precursor ions; ± 0.7 Da for fragment ions, and a minimum matched percent scored peak intensity (SPI%) of 50%. In situ, the false discovery rate (FDR) was calculated by searching the data against a concatenated forward-reverse database. Cutoff scores were dynamically assigned to each data set to maintain a FDR of 0.1% at the protein level. Only proteins with two or more unique peptides were selected for quantitative analysis. Proteins that share common peptides were grouped to address the database redundancy issue and the presence of parsimonious proteins. The proteins within the same group shared the same set of peptides and were represented by “protein group leader.” In the remaining text, the term “protein” refers to “protein group leader.”

For protein quantitation, protein iTRAQ reporter ion intensities were calculated by summing the iTRAQ reporter ion intensities of all peptide spectral matches (PSMs) for peptides for that protein and only unique peptides were considered. In our summing method, a peptide with high-intensity signal contributes more to the final calculation of the protein ratio than does a peptide with a low-intensity signal. This with high-intensity signal contributes more to the final calculation of the protein ratio than does a peptide with a low-intensity signal. This with high-intensity signal contributes more to the final calculation of the protein ratio than does a peptide with a low-intensity signal. This with high-intensity signal contributes more to the final calculation of the protein ratio than does a peptide with a low-intensity signal.

Gene Ontology Annotation—The DAVID 6.7 Bioinformatics tool (http://david.abcc.ncifcrf.gov/) (25, 26) was used for gene functional annotation and gene-gene ontology (GO) term enrichment analysis to highlight the most relevant GO terms associated with a given gene list.

Cytoscape Analysis—We mapped our protein p values into publicly available protein–protein interaction network using Cytoscape (27). This network provides global view of potentially relevant interacting partners of proteins whose abundances change in high-fat diet mice. We also searched for possible functional links among some significant proteins using computational prediction based on several criteria including co-expression of genes and colocalization of proteins. Protein–protein interaction (PPI) data were obtained from BioGRID database (http://thebiogrid.org). Because number of publicly available PPIs of mouse (~15,000) is far less than that of human (~130,000), we converted human PPIs into mouse PPIs using orthologous relationship (“interologs,”) (28) obtained from Imparanoid database (http://impanaroid.sbc.su.se/).

Immunoblot Analysis—To verify iTRAQ data, HFD/ND liver mitochondria samples (15 μg each) were subjected to immunoblot analysis. For this purpose, samples were lysed with ice-cold lysis buffer (50 mM tris, 150 mM NaCl and 1% Triton X-100) containing protease inhibitor mixture (Sigma) and separated on SDS-PAGE. Proteins were transferred onto PVDF membranes and probed with the corresponding antibodies. The following monoclonal (mAb) and polyclonal (pAb) antibodies were used: the mouse mAb Cyp1a2 (Abcam, Cambridge, MA), rabbit pAb ChChd3 (Life Technologies, Carlsbad, CA), mouse mAb mitoflin (Life Technologies), rabbit pAb Opa1 (Affinity BioReagents, Golden, CO), mouse mAb cytochrome C (Santa Cruz Biotechnology, Santa Cruz, CA), and rabbit pAb Tom40 (Santa Cruz Biotechnology). Protein expression was quantitated by densitometry using ImageJ 1.41o software (29). Student’s t test (tailed 2, type 1) was carried out using Prism 6 software and graph was then generated using Prism 6 software with outlier excluded.

Electron Microscopy (EM) of Liver Mitochondria, Isolated and In Situ—Isolated mitochondria prepared as above were pelleted and then fixed with 2% paraformaldehyde plus 2.5% glutaraldehyde. Anesthetized mice were whole-body perfused fixed with Ringer’s solution followed by this same fixative. The liver was extracted and immersed in the fixative on ice for 2 h. The tissue was chopped into smaller pieces about 2 mm × 2 mm × 2 mm cube. The following applies to both isolated mitochondria and tissue pieces. The samples were washed three times with buffer of 0.1 M sodium cacodylate plus 3 mM calcium chloride (pH 7.4) on ice and then post-fixed with 1% osmium tetroxide, 0.8% potassium ferrocyanide, 3 mM calcium chloride in 0.1 M sodium cacodylate (pH 7.4) overnight, washed three times with ice-cold distilled water, and then stained with 2% uranyl acetate at 4 °C for 1 h, dehydrated through graded ethanol solutions, and embedded in Durcupan ACM resin (Fluka, St. Louis, MO). Ultrathin (80 nm) sections were post-stained with uranyl acetate and lead salts before imaging using a JEOl 1200FX transmission EM operated at 80 kV. Images were taken on film at 6000 × magnification. The negatives were digitized at 1800 dpi using a Nikon CoolScan system, giving an image size of 4033 × 6010 pixels and a pixel resolution of 2.35 nm. Mitochondrial volume fractions were measured on the tissue images using steroereology as follows. A 9 × 13 rectangular grid (chosen for ease of use with Photoshop) was overlaid on each image and mitochondria and cytoplasm lying under intercepts were counted. The relative volume (volume fraction) of mitochondria was expressed as the ratio of intercepts coinciding with this organelle relative to the intercepts coinciding with cytoplasm and reported as a percentage. Mitochondrial lengths were measured on these same images using ImageJ (NIH). The cristae membrane surface area was also measured using ImageJ by tracing each crista in a mitochondrion and calculating its surface area. To normalize the measurement, the sum of cristae surface area was divided by the outer membrane surface area per mitochondrion. Mean and standard error of the mean (S.E.) were used throughout.

“Electron Flow” Respiration Analysis Using the XF24 Flux Analyzer—Methods for respiration analysis of isolated liver mitochondria were adapted from previously described methods (30). All steps were conducted on ice (4 °C) unless otherwise stated. On quantitation of protein concentration by Bradford assay, crude liver mitochondria from each subject group were diluted in 1× Mitochondrial Assay Solution (“MAS”): 70 mM succrose, 220 mM mannitol, 10 mM KH2PO4, 5 mM MgCl2, 2 mM HEPES, 1 mM EGTA, and 0.2% (w/v) fatty acid-free BSA, pH 7.4; supplemented with 10 mM pyruvate, 2 mM malate, and 4 μM FCCP to reach a normalized concentration of 0.1 μg/μL. Diluted
Mitochondria were isolated separately from each mouse (ND and HFD) and the mitochondria proteins were digested. The labeled mitochondria peptides from each pair were then pooled and separated with online 2D LC with PQD-CAD acquisition on LTQ-Orbitrap. Spectrum Mill was used to search the raw data for protein identification and quantitation. The proteins that were up- or down-regulated were then analyzed using the DAVID bioinformatics tool for over- or under-represented pathways.

To gain insights into mitochondrial dysfunction in liver under insulin resistance and T2D conditions, we analyzed the proteomic profiles of HFD and ND mice liver mitochondria. We carried out a quantitative proteomic analysis of HFD and ND mice of liver mitochondria. Fig. 1A shows the schematic of our work flow. We isolated mitochondria independently from each mouse (3 HFD and 3 ND) in three biological replicates and analyzed them using iTRAQ labeling technique. We identified and quantified the proteins using Spectrum Mill and used DAVID Bioinformatics to extract pathways that were over- or under-represented (25, 26) in HFD compared with that of ND samples.

We identified a total of 1508 proteins with a FDR of 0.1% at the protein level and with at least two unique peptides. To get the relative enrichment of mitochondria, we analyzed the full list of proteins using DAVID Bioinformatics and extracted the “cellular component” annotation information. Fig. 1B shows the enrichment of total proteins from different organelles. It is clear that even though we used crude mitochondria, mitochondrial proteins were greatly enriched. We compared our list of proteins with some existing studies using Deng et al. (18), the Mitoproteome database (http://www.mitoproteome.org/database/index.cgi), and mitochondria proteins from GO terms (25, 26) and found at least 649 identical mitochondrial proteins. The full list of proteins identified and their ratios for each biological replicate are displayed in supplemental Table S1.

We also evaluated the reproducibility of our approach. Fig. 1C illustrates the number of proteins in each biological replicate and the overlap between these replicates. A total of 1345, 1080, and 1299 proteins were obtained for set 1, set 2, and set 3 respectively, and combining all three data sets, a total of 1508 proteins were identified. Among the 1508 total proteins, 913 (60.5%) were common between all the three data sets. The protein overlap between set 1 and 2, set 2 and 3, and set 1 and 3 were 1169, 988, 972 respectively. The percentage of identifications by only one replicate totaled 13.6% ((101 + 33 + 71)/1508). Thus, these numbers confirm the reproducibility of our approach.

iTRAQ Quantitation Reveal Changes of Conserved Proteins Identified in Type 2 Diabetic Model—To identify proteins that have significant changes in HFD mitochondria, we first tried a fold change cutoff of >1.50 or <0.67 for all three biological replicates and a two-tailed paired Student’s t test with a p value cutoff of 0.05 (see Experimental Procedures). The results are summarized in supplemental Table S2A. Only 92 proteins were found to have significant changes with 72 proteins up-regulated and 20 proteins down-regulated in the liver mitochondria of HFD versus ND. Regarding the use of DAVID Bioinformatics for pathways that were up- or down-regulated, we found that retinol metabolism were significantly down-regulated, branched chain amino acid (BCAA) degradation and oxidative phosphorylation were significantly up-regulated. However this list excludes a large number of proteins that have been previously shown to change in HFD mitochondria such as Dci and Acadm which are involved in fatty acid oxidation, and Atp5o, Atp5a1, Cox5a, Ndufs1, Ndub6, and Sdhb involved in oxidative phosphorylation (18). Our studies failed to show fatty acid metabolism, pyruvate metabolism and TCA cycle that have been shown to be up-regulated in previous studies (18, 19). Our experience is consistent with the findings from O’Brien et al. (31) that the t test was not an adequate statistical measure of the reproducibility of protein changes using iTRAQ. The reason might be that isobaric tags.
Proteomic Analysis of Type 2 Diabetic Liver Mitochondria

in general suffer to some extent from compression of ratios in complex samples although the trends of up- or down-regulation are correct (32). Recently developed methods can be used to get around this issue and should help with future studies (33). In our final analysis, we considered the protein changes significant when a fold change cutoff of \(>1.50\) or \(<0.67\) is met for all three biological replicates. The q-values are also listed (supplemental Table S2A and S2B) (http://genomics.princeton.edu/storeylab/qvalue/) to give an understanding of the false discovery rate for reported changes.

Pathways Over- or Under-represented in HFD Mitochondria—The relative abundance of mitochondrial proteins was quantified by using the iTRAQ method. A total of 181 proteins showed abundance changes, of which 37 proteins were down-regulated and 145 proteins were up-regulated in HFD (supplemental Table S2B). We then analyzed these proteins using DAVID Bioinformatics for pathways that were up- or down-regulated.

With respect to molecular function, oxidoreductase activity (acting on NADH, NADPH, aldehyde, or oxo group of donors, NAD or NADP acceptors), cofactor binding, 3-hydroxyacyl-CoA dehydrogenase activity, hydrogen ion trans membrane transporter activity, protein transport, and membrane biogenesis were significantly enriched in HFD mice. In regards to biological processes, the fatty acid metabolic process, the generation of precursor metabolites and energy, the cellular respiration, the carboxylic acid catabolic process, the monocarboxylic acid metabolic process, and the carboxylic acid biosynthetic process were all significantly enhanced in HFD mice. Within the KEGG pathway, our results clearly showed that several processes were highly coordinated in HFD induced T2D. Because from the KEGG pathways, we can distinctly see the role of each protein, we used it as a start point to delve further into details on the key processes that were disturbed in HFD induced T2D mice.

From the KEGG pathways, our data indicated that 14 proteins involved in fatty acid oxidation are differentially expressed (Table I), and all these proteins are consistently up-regulated in HFD versus ND liver mitochondria. This is consistent with up-regulation of fatty acid beta-oxidation protein level in liver with obesity (34). supplemental Fig. S1 shows where these proteins are and the role of these proteins in fatty acid oxidation. The enzymes in fatty acid oxidation break fatty acids into small pieces and shuttle each piece through the TCA cycle. The proteins that are up-regulated in this pathway include two kinds of acyl-CoA dehydrogenases, the very long chain acyl-CoA dehydrogenase (Acadvl) and the medium chain acyl-CoA dehydrogenase (Acadlm), four kinds of hydroxyacyl-Coenzyme A dehydrogenases (Ehhad, Hadh, Hadha, and Hadhb), three kinds of aldehyde dehydrogenases (Aldh1b1, Aldh2, and Aldh7a1), two kinds of carnitine palmitoyltransferases (Cpt1a and Cpt2), acetyl-Coenzyme A acyltransferase 2 (Acaa2), acyl-CoA synthetase long-chain family member 6 (Acsl6), and dodecenoyl-Coenzyme A delta isomerase (Dci).

Branch chain amino acid (BCAA) degradation is significantly enhanced in HFD versus ND liver mitochondria. The degradation of BCAA has two steps: reversible transamination to yield branched chain keto-acid and irreversible oxidative decarboxylation of branched chain keto-acid, the product of which is the acyl-CoA derivative with one less carbon that can enter the TCA cycle. We have 14 significantly changed proteins involved in this pathway and all these proteins are coordinately increased (Table I and Fig. 2A). These proteins include four aldehyde dehydrogenases (Aldh1b1, Aldh2, Aldh6a1, and Aldh7a1), six Coenzyme A dehydrogenases (Acadm, Ehhadh, Hadh, Hadha, lvd, and Hadhb), 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2 (Hmgcs2), AU RNA binding protein/enoyl-Coenzyme A hydratase (Auh), Acaa2, and propionyl-Coenzyme A carboxylase (Pcc).
Proteomic Analysis of Type 2 Diabetic Liver Mitochondria

TABLE I

Functional categorization of proteins differentially expressed in liver mitochondria of HFD vs. ND mice. +, fold changes (HFD/ND) of 1.5–2; ++, fold changes (HFD/ND) of 2–3; ++++, fold changes (HFD/ND) > 3; –, down-regulated in HFD

| Gene symbol | IPI number | Protein name | Changes | p values |
|-------------|------------|--------------|---------|----------|
| **Fatty acid metabolism** | | | | |
| Aldh7a1 | IPI00230084 | Aldehyde dehydrogenase family 7, member A1 | ++ | 0.01 |
| Aldh1b1 | IPI00113073 | Aldehyde dehydrogenase X | + | 0.02 |
| Cpt1a | IPI00330094 | Cpt1a Carnitine O-palmitoyltransferase I, liver isoform | + | 0.03 |
| Hadh | IPI00121105 | Hydroxacyl-Coenzyme A dehydrogenase | + | 0.04 |
| Cpt2 | IPI00131424 | Carnitine O-palmitoyltransferase 2 | + | 0.06 |
| Hadha | IPI00223092 | Hydroxacyl-Coenzyme A dehydrogenase, alpha subunit | + | 0.07 |
| Dci | IPI00331692 | Dci Dodecenoyl-Coenzyme A delta isomerase | + | 0.08 |
| Ehhadh | IPI00554834 | Ehhadh Peroxisomal bifunctional enzyme | + | 0.09 |
| Acadvl | IPI00119203 | Very-long-chain specific acetyl-CoA dehydrogenase | + | 0.09 |
| Aco2 | IPI00653158 | Aco2 Aconitate hydratase, mitochondrial precursor | + | 0.09 |
| Hadhb | IPI00111218 | Aldehyde dehydrogenase | + | 0.10 |
| Acsl6 | IPI00625955 | Acsl6 Long-chain-fatty-acid-CoA ligase 6 | + | 0.17 |
| Acadm | IPI00134961 | Medium-chain specific acyl-CoA dehydrogenase | + | 0.20 |
| **Branched chain amino acid degradation** | | | | |
| Aldh7a1 | IPI00230084 | Aldehyde dehydrogenase family 7, member A1 | ++ | 0.11 |
| Auh | IPI001214900 | Auh Isoform 1 of Methylglutaclyl-CoA hydratase | + | 0.12 |
| Ivd | IPI00471246 | Ivd isovaleryl-CoA dehydrogenase | + | 0.17 |
| Aldh1b1 | IPI00113073 | Aldehyde dehydrogenase X | + | 0.17 |
| Hmgcs2 | IPI00420718 | Hmgcs2 Hydroxymethylglutaryl-CoA synthase | + | 0.19 |
| Hadh | IPI00121105 | Hydroxacyl-Coenzyme A dehydrogenase | + | 0.20 |
| Hadha | IPI00223092 | Hydroxacyl-Coenzyme A dehydrogenase alpha subunit | + | 0.20 |
| Ehhadh | IPI00554834 | Ehhadh Peroxisomal bifunctional enzyme | + | 0.20 |
| Acad2 | IPI00119203 | Acad2 Very-long-chain specific acetyl-CoA dehydrogenase | + | 0.20 |
| Pccaa | IPI00330523 | Pccaa Propionyl-CoA carboxylase alpha chain | + | 0.20 |
| Aldh6a1 | IPI00419664 | Aldh6a1 Aldehyde dehydrogenase family 6, subfamily A1 | + | 0.20 |
| Hadh6 | IPI001115607 | Hadh6 Trifunctional enzyme subunit beta | + | 0.20 |
| Acadm | IPI00134961 | Medium-chain specific acyl-CoA dehydrogenase | + | 0.20 |
| **Pyruvate metabolism** | | | | |
| Aldh7a1 | IPI00230084 | Aldehyde dehydrogenase family 7, member A1 | ++ | 0.01 |
| Aldh1b1 | IPI00113073 | Aldehyde dehydrogenase X | + | 0.02 |
| Grhrp | IPI001130530 | Grhrp Glyoxylate reductase/hydroxypruvate reductase | + | 0.03 |
| Dlat | IPI00153660 | Dlat Dihydrolipoyllysine-residue acetyltransferase | + | 0.04 |
| Pdhb1 | IPI00337893 | Pdhb1 Pyruvate dehydrogenase E1 component alpha subunit, somatic form | + | 0.05 |
| Mdh2 | IPI00332552 | Mdh2 Malate dehydrogenase, mitochondrial precursor | + | 0.06 |
| Pdhb | IPI00132042 | Pdhb Pyruvate dehydrogenase E1 component subunit beta | + | 0.06 |
| Pcx | IPI00114710 | Pcx Pyruvate carboxylase | + | 0.06 |
| Aldh2 | IPI00111218 | Aldh2 Aldehyde dehydrogenase | + | 0.06 |
| **TCA cycle** | | | | |
| Dist | IPI00134809 | Dist Isoform 1 of Dihydrolipoyllysine-residue succinyltransferase | + | 0.01 |
| Diat | IPI00153660 | Diat Dihydrolipoyllysine-residue acetyltransferase component | + | 0.04 |
| Pdhb1 | IPI00337893 | Pdhb1 Pyruvate dehydrogenase E1 component alpha subunit, somatic form | + | 0.05 |
| Mdh2 | IPI00332552 | Mdh2 Malate dehydrogenase | + | 0.06 |
| Pdhb | IPI00132042 | Pdhb Pyruvate dehydrogenase E1 component subunit beta | + | 0.06 |
| Suc1g1 | IPI00406444 | Suc1g1 Succinyl-CoA ligase [GDP-forming] subunit alpha | + | 0.07 |
| Sdhb | IPI00338536 | Sdhb Succinate dehydrogenase [ubiquinone] iron-sulfur subunit | + | 0.07 |
| Idh3a | IPI00459725 | Idh3a Isoform 1 of Isocitrate dehydrogenase [NAD] subunit alpha | + | 0.09 |
| Pcx | IPI00114710 | Pcx Pyruvate carboxylase | + | 0.09 |
| Aco2 | IPI00116074 | Aco2 Acetyl-CoA hydrolase, mitochondrial precursor | + | 0.10 |
| Suc1g2 | IPI00495487 | Suc1g2 Isoform 1 of Succinyl-CoA ligase [GDP-forming] beta-chain Component of 2-oxoglutarate dehydrogenase complex | + | 0.13 |
beta subunit (Suclg2), which convert succinyl-CoA to succinate reversibly.

The OXPHOS pathway is significantly over-represented in HFD mitochondria. We have one or more subunits in each of the five complexes that are coordinately up-regulated, and not a single protein that is down-regulated (Table I and supplemental Fig. 4). These proteins include 7 NADH dehydrogenases from complex I (Ndufs1, Ndufs3, Ndufs7, Ndufs8, Ndufb2, Ndufb6, and Ndufb7), succinate dehydrogenase complex, subunit B (Sdhb) from complex II, three proteins from complex III (Cyc1, Uqrc2, and Uqcrb), four kinds of cytochrome c oxidases from complex IV (Cox4i1, Cox7a2, Cox6b1, and Cox5a), four kinds of ATP synthases (Atp5b, Atp5j, Atp5a1, and Atp5o) from Complex V.

Liver is the main source for gluconeogenesis. When we analyzed this pathway, we noticed an increase in several key enzymes in the liver mitochondria of HFD versus ND. These include pyruvate carboxylase (Pcx), mitochondrial phosphoenolpyruvate carboxykinase 2 (Pck2), and fructose-1, 6-bisphosphatase 1 (Fbp1). Gluconeogenic enzymes require ATP for their catalytic actions and because we noticed an increase in OXPHOS pathway under HFD conditions, it is likely that the ATP generated from OXPHOS contribute to up-regulation of gluconeogenesis and increased glucose levels in liver.

**Down-regulation of Retinol Metabolism**—Our analysis showed compelling changes of proteins involved in retinol metabolism. Although many of the changes listed above are consistent with previous studies (18, 19), in our analysis we found that the retinol metabolism pathway is significantly under-represented in HFD liver mitochondria. Retinol (Vitamin A) (all-trans retinol) and its metabolites, all-trans- and cis-Retinoic acid and 11-cis-Retinal, are involved in processes such as vision, reproduction, growth, development and immune function. In addition, retinoids have been shown to play important roles in obesity and insulin resistance (35, 36). We have nine significantly changed proteins involved in retinol metabolism and they are all consistently down-regulated (Table I and Fig. 2B). These proteins include retinol saturase (all-trans retinol 13, 14 reductase) (Retsat), UDP glucuronosyltransferase 2 family, polypeptide B1 (Ugt2b1), and 7 pro-
teins from the cytochrome P450 family (Cyp1a2, Cyp2a5, Cyp2c29, Cyp2c70, Cyp3a11, Cyp4a12b, and Cyp2c54).

**Increased Protein Expression of Mitochondrial Intermembrane Space Bridging (MIB) complex and Translocases of the Inner Membrane Proteins (TIMs)—** Recent studies from our lab and other groups have established that mitofilin together with ChChd3 (on the inner membrane) and Sam50 (on the outer membrane) exists in a complex at the crista junction as well as at the contact sites. Other proteins including OPA1 and metaxins have also been shown to be part of this complex—termed the mitochondrial intermembrane space bridging (MIB) complex or mitochondrial contact site (MICOS) complex. The MIB/MICOS integrity is required for maintaining cristae architecture, cristae junction formation, membrane organization and mitochondrial function (37–42). In our analysis, the majority of the components of this complex are significantly elevated in the liver mitochondria of HFD versus ND mice (Table II). All these proteins have roles in translocation of matrix targeted proteins with a cleavable presequence. Specifically, Tim17 and Tim44 are part of the Tim23 and PAM complex on the inner membrane and matrix respectively (43). Tim8 and Tim13, on the other hand, are intermembrane space proteins involved in the import of Tim23 under low membrane potential conditions (44). Heat shock proteins 60 (HSP60/Hspa9) and 70 (HSP70/Hspa9, associated with translocation and processing of the precursor proteins in the mitochondrial matrix, also increased in HFD liver mitochondria (Table II).

**Cytoscape Analysis—** The cytoscape analysis (Fig. 3A) provides global view of potentially relevant interacting partners of proteins whose levels change in high-fat diet mouse. We also searched for possible functional links among eleven significant proteins in Table II using computational prediction based on several criteria including co-expression of genes and co-localization of proteins (Fig. 3B). We observed functional links among these 11 proteins.
Proteomic Analysis of Type 2 Diabetic Liver Mitochondria

List of proteins involved in mitochondrial protein transport, biogenesis and regulation of cristae morphology. The data shown are the average ratios (HFD vs. ND) from three biological replicates. Also shown are the number of unique peptides identified for each protein (unique_Pep) and number of PQD spectrum (Spectrum_num) used for protein quantitation. The protein coverage is shown as Protein_Coverage. ND: not detected

| Gene Symbol | Accession | set1_HFD/ND | set2_HFD/ND | set3_HFD/ND | Average_HFD/ND | p value | Unique_Pep | Spectrum_num | Protein_Coverage | Protein |
|-------------|-----------|-------------|-------------|-------------|----------------|--------|------------|-------------|----------------|---------|
| Chchd3      | IPI00754739| 2.10        | 1.10        | 3.68        | 2.29           | 0.18   | 6          | 18          | 0.38          | Coiled-coil-helix coiled-coil domain-containing protein 3 |
| Samm50      | IPI00221608| 0.96        | 3.08        | 3.90        | 2.64           | 0.20   | 6          | 24          | 0.10          | Sorting and assembly machinery component 50 homolog |
| Immt        | IPI00554845| 1.39        | 4.75        | 2.96        | 3.03           | 0.11   | 26         | 149         | 0.38          | Mitofilin Isoform 5 of Mitochondrial inner membrane protein |
| Mtx1        | IPI00112327| 4.26        | ND          | 8.21        | 6.24           | 0.20   | 4          | 7           | 0.14          | Optic atrophy 1 |
| Opa1        | IPI00403336| 2.15        | 1.76        | 2.61        | 2.17           | 0.02   | 12         | 22          | 0.13          | Metatrin-1 |
| Timm44      | IPI00135068| 1.22        | 2.67        | 7.03        | 3.64           | 0.18   | 9          | 21          | 0.18          | Import inner membrane translocase subunit TIM44 |
| Timm17b     | IPI00129504| 2.59        | ND          | 2.48        | 2.54           | 0.18   | 2          | 5           | 0.17          | Mitochondrial import inner membrane translocase subunit Tim17-B |
| Hspd1       | IPI00308885| 1.19        | 1.54        | 2.98        | 1.90           | 0.18   | 43         | 1003        | 0.55          | Hsp60 Isomorform 1 of 60 kDa heat shock protein |
| Hspat9      | IPI00133903| 1.62        | 1.60        | 3.96        | 2.39           | 0.12   | 34         | 947         | 0.49          | Hspa9 Stress-70 protein |
| Timm8a1     | IPI00125776| 0.88        | 1.62        | 4.57        | 2.36           | 0.32   | 2          | 33          | 0.23          | Mitochondrial import inner membrane translocase subunit Tim8 A |
| Timm13      | IPI00134484| 2.38        | 2.06        | 2.55        | 2.33           | 0.01   | 4          | 123         | 0.37          | Mitochondrial import inner membrane translocase subunit Tim13 |

**Immunoblot Analysis**—To further confirm our iTRAQ results, we performed immunoblot analysis on some of the significantly changed proteins (Fig. 4). Cyp1a2, the protein involved in retinol metabolism is clearly down-regulated in HFD mitochondria samples compared with that of ND. Mitofillin, ChChd3, and OPA1 were tested for the components of MIB/MICOS, and they were all clearly up-regulated in HFD samples. Consistent with the mass spectrometry results, cytochrome C is up-regulated and Tom40 has no change in expression level and hence was used as a loading control. Thus, the immunoblot results further validated our iTRAQ data. We also noticed that three out of the five proteins (ChChd3, mitofillin, and cytochrome C) were validated with immunoblot analysis, yet these proteins had p values ≥ 0.05 from our mass spectrometry analysis. If we limit our analysis to changes with a p value cutoff of 0.05, we would have missed these proteins. Therefore, there are certainly additional relevant changes beyond this significance threshold, but users should note the FDR rates at each p value cutoff.

**Liver Mitochondria of HFD are More Condensed**—We next analyzed the HFD-induced structural changes in liver mitochondria by using electron microscopy. The most significant structural difference between the mitochondria from HFD liver and their counterparts from ND liver was a conformational change to a more condensed state in the HFD mitochondria (Fig. 5). This change was found in both isolated mitochondria (Figs. 5A, 5B, 5C) and mitochondria in situ (Figs. 5D, 5E). The condensed mitochondria under HFD conditions were smaller, with a darker matrix, indicative of more densely packed proteins, and a preponderance of dilated cristae. In the isolated HFD samples, ultracondensed mitochondria were common and represent an extreme of the condensed configuration (Fig. 5C). Ultracondensation involves cristae remodeling with a reversal of the membrane curvature thus forming tubes that enclose the matrix instead of the intracristal space. More than half the HFD mitochondria (57%) in tissue showed a classically condensed conformation, whereas no ND mitochondria were condensed. Consistent with the condensed configuration, the mitochondrial volume fraction (density) was lower (Fig. 5F) and length was shorter (Fig. 5G) in the HFD liver tissue. The lowered mitochondrial density and smaller mitochondria are likely attributed to compacting of the matrix occurring during condensation. There was also an increase in the cristae abundance (Fig. 5H) in the HFD mitochondria.

**Oxygen Consumption Rate (OCR) and ATP Generation in Liver Mitochondria are Elevated with HFD Treatment**—To determine whether or not HFD treatment induces a significant difference in metabolic capacity, we have performed functional analyses of isolated liver mitochondria from both 30-week HFD treated groups as well as age-matched wild-type mice. First, we conducted oxygen consumption measurements using the XF24 metabolic flux analyzer to assess respiratory capacity among the two groups. As shown in one independent experiment with 10 technical replicates for each group (Fig. 6A), we observed a significant increase in respiratory capacity in HFD mice compared with wild-type mice as compared with baseline complex I activation as well as with succinate stimulus (i.e. complex II activation). After performing the experiment in two separate biological replicates, we see that the relative change in OCR % as compared with baseline complex I activity is also markedly increased in HFD mice as compared with ND mice (Fig. 6B). As a corroboration of these results, we also performed relative quantitation of ATP production in both groups using the CellTiter-Glo® cell viability kit (Promega). As seen in Fig. 6C, we observed that ATP production is also similarly increased in HFD mice as relative to ND mice given malate/pyruvate stimulus for activation of complex I driven respiration. In combination, these
findings indicate that diet with high fat content induces a change in metabolic phenotype, as manifested by overactive respiratory function in HFD mice as compared with ND mice.

DISCUSSION

Mitochondrial dysfunction is considered as one of the major underlying defects linking obesity to diabetes, through insulin resistance and β-cell dysfunction. Focusing on the changes in proteins and pathways can serve as a reference to better understand and ultimately provide therapeutic targets for the disease. With this purpose in mind, we used a quantitative proteomic approach to identify the protein expression changes in mouse liver mitochondria from an HFD induced obesity model. Our results illustrate that, in liver mitochondria, HFD triggers a coordinated increase in various metabolic pathways including OXPHOS, fatty-acid oxidation, gluconeogenesis, pyruvate metabolism, branched chain amino acid degradation and TCA cycle. In addition to this, the retinol metabolism pathway is distinctly down-regulated, and the proteins that are associated with cristae morphology and biogenesis, and mitochondrial protein import are up-regulated under HFD conditions. Electron microscopic analysis of the mitochondria revealed that HFD promotes remodeling to a condensed state with increased matrix density and cristae membrane. Furthermore, functional analysis of liver mitochondria demonstrated increased activities of complex I and
complex II, and increased ATP production in HFD versus ND mice. We propose that in HFD fed mice the liver mitochondria is able to up-regulate OXPHOS to synthesize ATP, and it is further used in gluconeogenesis causing hyperglycemia, consistent with previous findings (19, 45). Condensed mitochondria with higher cristae content and dense matrix in HFD may be a necessary structural remodeling event to accommodate the higher protein levels.

We first evaluated the criteria for defining protein changes and found that using fold change cutoff and \( p \) value cutoff of 0.05, we would miss some proteins and important pathways that have been shown to be changed previously. Some of these proteins which had \( p \) values \(< 0.05\) were validated with immunoblot. We then continued with fold changes only with the \( p \) values listed to give a sense of the false discovery rate. Among 1508 identified proteins 182 are differentially expressed in our analysis. We compared our data to a closely related recent study on mitochondria from T2D livers of GK rats (18). We noticed many similarities in our system including up-regulation of 15 proteins involved in OXPHOS, two proteins from the TCA pathway (Dlat, Sdhb), one protein involved in glucose metabolism (Gpd2), three proteins involved in fatty acid oxidation (Acadm, Dci, Hadh), and two proteins associated with ketone body metabolism (Bdh1 and Hmgcs2). With regards to KEGG pathways, OXPHOS, \( \beta \)-oxidation, pyruvate metabolism, TCA cycle, and gluconeogenesis are consistently up-regulated in HFD. Our data is consistent with a human

![Immunoblot analysis of mouse liver mitochondria.](image)

**Fig. 4.** Immunoblot analysis of mouse liver mitochondria. Proteins from isolated liver mitochondria (ND and HFD, 15 \( \mu \)g) were separated by SDS-PAGE (4–12%) and transferred to PVDF membranes. The membranes were incubated for 1 h at room temperature with blocking buffer (20 mM Tris-Cl, pH 7.5, 100 mM NaCl, 0.1% Tween 20 (TBS/Tween 20), and 5% nonfat milk powder), and then overnight at 4 °C with appropriate antibodies. Tom40 was used as a loading control. A, Immunoblot images from four biological replicates (4ND and 4HFD). B, Immunoblot bar graph. Protein expression was quantitated by densitometry using ImageJ 1.41o software, and statistical analysis was done using Prism 6 software. The ratios calculated from immunoblot were shown on the top, and the ratios calculated from iTRAQ data were shown on the bottom along with standard deviation. Note: ND immunoblot data from the second lane was omitted because of the high variation. (mean \( \pm \) S.E.; \( * p < 0.05 \)).

![EM analysis of liver mitochondria.](image)

**Fig. 5.** EM analysis of liver mitochondria. Mitochondria in HFD liver are more condensed and have more cristae membrane than in ND liver, indicating increased respiration. A–C, EM images of isolated mitochondria. A, typical mitochondrion from ND. Scale bar is 200 nm. B, condensed mitochondrion that is the most common form seen from HFD. The structure is classically condensed showing a darker matrix and enlarged cristae. C, ultracondensed mitochondrion, less commonly seen from HFD. D, typical mitochondrion in ND liver tissue. E, typical mitochondrion in HFD liver tissue. F, Mitochondrial volume fraction was significantly less in HFD liver (mean \( \pm \) S.E.; \( n = 9; * p < 0.01 \)). G, Mitochondrial lengths were also shorter in HFD liver (mean \( \pm \) S.E.; \( n = 100; * p < 0.05 \)). H, There was more cristae membrane surface area in the HFD mitochondria (mean \( \pm \) S.E.; \( n = 28; * p < 0.05 \)).
diabetic patient liver study by Takamura and coworkers (19). These authors showed a clear up-regulation of genes involved in OXPHOS, and glucose and lipid metabolism. Increase in OXPHOS was also accompanied by an increase in enzyme expression in the gluconeogenesis pathway. It has been suggested that hepatic insulin resistance contributes to an increase in gluconeogenesis through the ATP generated from fatty acid oxidation resulting in hyperglycemia in liver. Accordingly, we also noticed up-regulation of the enzymes involved in the gluconeogenesis pathway in HFD mitochondria.

Thus, our data from the T2D mouse model support the earlier work from other labs using different mammalian systems, and establish a key finding that increased protein expression of OXPHOS and other metabolic pathways is a common phenomenon observed in liver mitochondria under HFD induced obesity and diabetes. This is in contrast to the skeletal muscle studies where reduced OXPHOS genes/proteins and mitochondrial activity were reported (12, 14, 46, 47).

Electron microscopic analysis of muscle mitochondria in obese, T2D and HFD studies revealed smaller mitochondria along with reduced mitochondrial density (12, 48). The liver mitochondria on the other hand showed a remodeling to a condensed conformation (Fig. 5). The transition between the “orthodox” and “condensed” states is a reflection of the energy state of the mitochondria. In vitro and in situ studies have revealed that in state 4 (low respiratory activity), mitochondria are normally in the orthodox configuration, and in state 3 (high respiratory activity), they adopt a condensed configuration characterized by dense matrix and enlarged cristae (49–52).

Studies have shown that mitochondria in cancer cells switch from an orthodox to a condense conformation when the cells are shifted from glucose to galactose media (53). This conformational change was also accompanied by an increase in OXPHOS components. Thus, condensed mitochondria with increased cristae membrane and OXPHOS proteins in HFD liver, is a strong indicator that the mitochondria are energetically active. As we noticed this increase in proteins involved in fatty acid oxidation concomitant with increased cristae formation on OXPHOS protein expression, it can be predicted that the HFD liver mitochondria utilize fatty acids for synthesizing ATP.

There were contradictory findings in the literature regarding to mitochondria respiration. Several studies have reported increased respiration in the liver mitochondria of obesity models (17, 54), and other models of obesity have exhibited decreased respiration in liver (55). Therefore, we carried out oxygen consumption experiment in purified mitochondria from our HFD model. We see increased activities of complex I and complex II in liver mitochondria and this is consistent with more condensed mitochondria from our EM analysis and increased OXPHOS proteins from our proteomic analysis. Furthermore, our ATP assay confirmed increased ATP production in HFD versus ND mice.

One of the novel observations in the present study is the increased abundance of components from the MIB/MICOS complex in HFD mitochondria (Table II). We showed recently that ChChd3 is part of a complex that bridges the inner and outer mitochondrial membranes, and this was subsequently confirmed by several laboratories. This complex on the inner membrane is crucial for maintaining inner membrane architecture, cristal integrity and contact site formation (37–39, 42, 56). The inner membrane protein mitofilin is also a core component of this complex, and it interacts with several inner and outer membrane proteins at crista junctions and contact sites (41, 57). On the outer membrane, mitofilin associates with the SAM (sorting and assembly machinery) complex that is involved in biogenesis of outer membrane proteins (39, 58). On the inner membrane, ChChd3, ChChd6, and OPA1 have been shown to copurify with mitofilin (39, 59). Knockdown studies in cultured cells revealed similar phenotypes for many of these proteins including altered growth rates, impaired mitochondrial function and drastic alterations in cristae structure, along with fragmentation, rounding and loss of cristae (38, 39, 41, 59). In HFD mitochondria we noticed a significant increase in almost all the components of this complex including mitofilin, ChChd3, OPA1, Sam50, and metaxin1. Immunoblot analysis for mitofilin, ChChd3 and OPA1 confirmed the proteomic data and showed a clear increase in these proteins in all three liver mitochondria samples (Fig. 4). Mitochondrial contact sites are where the outer and inner membranes touch and function in maintaining structural integrity, coordinating fusion and fis-
sion events, and in transporting proteins, lipids, ions, and metabolites. As recent studies have shown that MIB/MICOS components are essential for contact site formation, the increase in the components of this complex likely facilitates the formation of these contacts under HFD conditions. Because lipid metabolism increased with HFD, it is likely that the MIB/MICOS complex can facilitate the transport of lipids through the contact sites into mitochondria and thus may have a role in fatty acid uptake and oxidation. We also observed an increased abundance of the contact site-specific proteins: voltage dependent anion channel (VDAC) and adenine nucleotide transporter (49). It is also likely that the MIB/MICOS complex is essential for the formation of condensed mitochondria where increased cristae and crista junctions were observed.

In a proteomic analysis from human skeletal muscle, a significant reduction in ChChd3 protein levels was observed in obese and T2D subjects (60). This is interesting mainly because the expression change in ChChd3 seems to directly correlate with changes in OXPHOS. It is unclear whether ChChd3, which is part of the MIB/MICOS complex, or the components of MIB/MICOS have a direct role in regulating OXPHOS function or whether the function is regulated through the scaffolding aspect of the complex. Studies toward understanding the function of MIB/MICOS components in glucose and lipid metabolism can provide clues to the role of these proteins in insulin signaling. Another key finding in this study is that the retinol metabolism pathway is significantly affected under HFD conditions. We identified a total of nine proteins that have changes in this pathway and all are significantly down-regulated with HFD. Retinyl esters, retinol, retinal, retinoic acid, and oxidized and conjugated metabolites of both retinol and retinoic acid are different forms of retinoids, and they are critical for development of the nervous system and notochord and many other embryonic structures, as well as for cell proliferation and differentiation (61). Retinoids have also been known to play an important role in glucose and lipid metabolism by regulating gene expression (62, 63). All-trans-retinoid acid (at-RA) functions as a ligand for nuclear retinoid acid receptor and they heterodimerize with PPARs, the main players in lipid and glucose metabolism, and significant regulation of retinol metabolism under HFD conditions. Future studies are warranted to address the specific role of these proteins in the pathogenesis of obesity and T2D.

Acknowledgments—We thank Dr. Mira Sastri for editing this manuscript. The authors also wish to acknowledge the Reviewer 1 for his/her detailed and helpful comments.

* This work was partially supported by National Institute of Health grants P01 DK54441 (SST), 5P41RR004050 and P41GM103412-24 (MHE), HL107200 (HHP), DK-033651, DK-074868, T32-DK-007494, and DK-063491 (JMO), by the Eunice Kennedy Shriver National Institute of Child Health and Human Development/NIH through cooperative agreement of U54-HD-012303-25 as part of the specialized Cooperative Centers Program in Reproduction and Infertility Research, and by the National Resource for Network Biology (P41 GM103504), the San Diego Center for Systems Biology (P50 GM085764), and the Department of Veterans Affairs BX001963 (HHP).

This article contains supplemental Figs. S1 to S4 and Tables S1 and S2.

To whom correspondence should be addressed: staylor@ucsd.edu.

REFERENCES

1. Wang, Y. C., Colditz, G. A., and Kuntz, K. M. (2007) Forecasting the obesity epidemic in the aging U.S. population. Obesity 15, 2855–2865.
2. Zimmet, P., Alberti, K. G., and Shaw, J. (2001) Global and societal implications of the diabetes epidemic. Nature 414, 782–787.
3. Kahn, B. B., and Flier, J. S. (2000) Obesity and insulin resistance. J. Clin. Invest. 106, 473–481.
4. Kahn, S. E., Hull, R. L., and Utzschneider, K. M. (2006) Mechanisms linking obesity to insulin resistance and type 2 diabetes. Nature 444, 840–846.
5. Reaven, G. M. (1995) Pathophysiology of insulin resistance in human disease. Physiol. Rev. 75, 473–486.
6. Kahn, S. E. (2003) The relative contributions of insulin resistance and beta-cell dysfunction to the pathophysiology of Type 2 diabetes. Diabetologia 46, 3–19.
7. LeRoith, D. (2002) Beta-cell dysfunction and insulin resistance in type 2 diabetes: role of metabolic and genetic abnormalities. Am J Med 113, 35–115.
8. Wang, C. H., Wang, C. C., and Wei, Y. H. (2010) Mitochondrial dysfunction in insulin insensitivity: implication of mitochondrial role in type 2 diabetes. Ann. N.Y. Acad. Sci. 1201, 157–165.
9. Ma, Z. A., Zhao, Z., and Turk, J. (2012) Mitochondrial dysfunction and beta-cell failure in type 2 diabetes mellitus. Exp. Diabetes Res. 2012, 7035–7038.
25. Huang, da, W., Sherman, B. T., and Lempicki, R. A. (2009) Bioinformatics and molecular biology of large gene lists using DAVID bioinformatics resources. Nature Protocol, 4, 44–57

26. Shannon, P., Markiel, A., Ozier, O., Baliga, N. S., Wang, J. T., Ramage, D., Amin, N., Schiwkowski, B., and Ideker, T. (2003) Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res. 13, 2498–2504

27. Matthews, L. R., Vaglio, P., Rebolj, J., Ge, H., Davis, B. P., Garrelis, J., Vincent, S., and Vidal, M. (2001) Identification of potential interaction networks using sequence-based searches for conserved protein-protein interactions or “interologs”. Genome Res. 11, 2120–2126

28. Grimes, P. A., Carson, J. H., Hebert, A. S., Hubler, S. L., Niemi, N. M., Bologa, C. J., Jochem, A., Stapleton, D. S., Keller, M. P., Westphal, M. S., Yandell, B. S., Attie, A. D., Coon, J. J., and Pagliarini, D. J. (2012) Quantitative proteome analysis of pluripotent cells by iTRAQ mass tagging reveals post-transcriptional regulation of proteins required for ES cell self-renewal. Mol. Cell. Proteomics 9, 2238–2251

29. Chiu, H., Salim, M., Tichy, M. W., Rehman, I., and Wright, P. C. (2009) ITRAQ underestimation in simple and complex mixtures: “the good, the bad and the ugly”. J. Proteome Res. 8, 5347–5355

30. Ting, L., Rad, R., Gygi, S. P., and Haas, W. (2011) MS2 eliminates ratio distortion in isobaric multiplexed quantitative proteomics. Nat. Methods 8, 937–940

31. Firn, C. L., and Nadeau, J. H. (2011) Increased mitochondrial oxidation in skeletal muscle of patients with type 2 diabetes. J. Clin. Invest. 121, 1592–1599

32. Paschen, S. A., Rothbauer, U., Káldi, K., Bauer, M. F., Neupert, W., and Pfanner, N. (2004) The mitochondrial inner membrane protein mitofilin controls cristae morphology. EMBO J. 34, 4035–4045

33. Alkhaja, A. K., Jones, D. C., Nikolov, M., Vukotic, M., Lytovchenko, O., Ludewig, F., Schliebs, W., Riedel, D., Urlaub, H., Jakobs, S., and Deckers, M. (2012) MINOS1 is a conserved component of mitofilin complexes and required for mitochondrial function and cristae organization. Mol. Biol. Cell 23, 247–257

34. John, G. B., Shang, Y., Li, L., Renken, C., Mannella, C. A., Selker, J. M., Rangel, L., Bennett, M. J., and Zha, G. (2006) The mitochondrial inner membrane protein mitofilin controls cristae morphology. Mol. Biol. Cell 17, 1543–1554

35. Zerbes, R. M., van der Klei, I. J., Veenhuis, M., Pfluger, N., van der Laan, M., and Bohnert, M. (2012) Mitoflin complexes: conserved organizers of mitochondrial membrane architecture. Biol. Chem. 393, 1247–1261

36. Chacinska, A., Koehler, C. M., Milenkovic, D., Lithgow, T., and Pfanner, N. (2009) Importing mitochondrial proteins: machineries and mechanisms. Cell 138, 628–644

37. Paschen, S. A., Rothbauer, U., Káldi, K., Bauer, M. F., Neupert, W., and Brunner, M. (2000) The role of the TIM8–13 complex in the import of Tim23 into mitochondria. EMBO J. 19, 6392–6400

38. Griffin, T. J., Xie, H., Bandhakavi, S., Popko, J., Mohan, A., Carlis, J. V., Kelley, D. E., He, J., Menshikova, E. V., and Ritov, V. B. (2002) Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes. Diabetes 51, 2944–2950

39. Rasband, W. S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, U.S.A., http://imagej.nih.gov/ij/, 1997–2012

40. Nielsen, H., and Hojlund, K. (2007) Mitochondrial respiration is decreased in obese human skeletal muscle. Am. J. Physiol. Endocrinol. Metab. 279, E1039–E1044

41. Paschen, S. A., Rothbauer, U., Káldi, K., Bauer, M. F., Neupert, W., and Brunner, M. (2000) The TIM8–13 complex interacts with Complex 1 and DAP3 and regulates mitochondrial respiration and apoptosis. J. Biol. Chem. 284, 5414–5424

42. O’Brien, R. N., Shen, Z., Tachikawa, K., Lee, P. A., and Briggs, S. P. (2010) Quantitative proteome analysis of pluripotent cells by iTRAQ mass tagging reveals post-transcriptional regulation of proteins required for ES cell self-renewal. Mol. Cell. Proteomics 9, 2238–2251

43. Chiu, H., Salim, M., Tichy, M. W., Rehman, I., and Wright, P. C. (2009) ITRAQ underestimation in simple and complex mixtures: “the good, the bad and the ugly”. J. Proteome Res. 8, 5347–5355

44. Ting, L., Rad, R., Gygi, S. P., and Haas, W. (2011) MS2 eliminates ratio distortion in isobaric multiplexed quantitative proteomics. Nat. Methods 8, 937–940

45. Firn, C. L., and Nadeau, J. H. (2011) Increased mitochondrial oxidation in skeletal muscle of patients with type 2 diabetes. J. Clin. Invest. 121, 1592–1599

46. Paschen, S. A., Rothbauer, U., Káldi, K., Bauer, M. F., Neupert, W., and Brunner, M. (2000) The role of the TIM8–13 complex in the import of Tim23 into mitochondria. EMBO J. 19, 6392–6400

47. Buchner, D. A., Yaczek, S. N., Solinas, P., Burrage, L. C., Morgan, M. G., Ho, A., Rames, S. M., Götz, M., Vanoomans, C., Krischke, M., Mueller, M. J., Krohne, G., Rudel, T., and Kosjak-Pavlovic, V. (2012) Sam50 functions in mitochondrial intermembrane space bridging and biogenesis of respiratory complexes. Mol. Cell. Biol. 32, 1173–1188

48. Darshi, M., Mendiola, V. L., Mackey, M. R., Murphy, A. N., Koller, A., Perkins, G. A., Elliman, M. H., and Taylor, S. S. (2011) ChCh3δ, an inner mitochondrial membrane protein, is essential for maintaining cristae integrity and mitochondrial function. J. Biol. Chem. 286, 2918–2922

49. Alkhaja, A. K., Jones, D. C., Nikolov, M., Vukotic, M., Lytovchenko, O., Ludewig, F., Schliebs, W., Riedel, D., Urlaub, H., Jakobs, S., and Deckers, M. (2012) MINOS1 is a conserved component of mitofilin complexes and required for mitochondrial function and cristae organization. Mol. Biol. Cell 23, 247–257

50. John, G. B., Shang, Y., Li, L., Renken, C., Mannella, C. A., Selker, J. M., Rangel, L., Bennett, M. J., and Zha, G. (2006) The mitochondrial inner membrane protein mitofilin controls cristae morphology. Mol. Biol. Cell 17, 1543–1554

51. Zerbes, R. M., van der Klei, I. J., Veenhuis, M., Pfluger, N., van der Laan, M., and Bohnert, M. (2012) Mitoflin complexes: conserved organizers of mitochondrial membrane architecture. Biol. Chem. 393, 1247–1261

52. Chacinska, A., Koehler, C. M., Milenkovic, D., Lithgow, T., and Pfanner, N. (2009) Importing mitochondrial proteins: machineries and mechanisms. Cell 138, 628–644

53. Paschen, S. A., Rothbauer, U., Káldi, K., Bauer, M. F., Neupert, W., and Brunner, M. (2000) The role of the TIM8–13 complex in the import of Tim23 into mitochondria. EMBO J. 19, 6392–6400

54. Buchner, D. A., Yaczek, S. N., Solinas, P., Burrage, L. C., Morgan, M. G., Hoppel, C. L., and Nadeau, J. H. (2011) Increased mitochondrial oxidation in skeletal muscle of patients with type 2 diabetes. Diabetes 56, 1592–1599

55. Patti, M. E., Butte, A. J., Crouchman, S., Cusi, K., Berria, R., Kashyap, S., and
Proteomic Analysis of Type 2 Diabetic Liver Mitochondria

Miyazaki, Y., Kohane, I., Costello, M., Saccone, R., Landaker, E. J., Goldfine, A. B., Mun, E., DeFronzo, R., Finlayson, J., Kahn, C. R., and Mandarino, L. J. (2003) Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: Potential role of PGC1 and Nrf1. Proc. Natl. Acad. Sci. U.S.A. 100, 8466–8471

48. Bonnard, C., Durand, A., Peyrol, S., Chanseaune, E., Chauvin, M. A., Morlo, B., Vidal, H., and Rieusset, J. (2008) Mitochondrial dysfunction results from oxidative stress in the skeletal muscle of diet-induced insulin-resistant mice. J. Clin. Invest. 118, 789–800

49. De Martino, C., Floridi, A., Marcante, M. L., Malorni, W., Scorza Barcellona, P., Bellocchi, M., and Silvestrini, B. (1979) Morphological, histochemical and biochemical studies on germ cell mitochondria of normal rats. Cell Tissue Res. 196, 1–22

50. Hackenbrock, C. R. (1968) Chemical and physical fixation of isolated mitochondria in low-energy and high-energy states. Proc. Natl. Acad. Sci. U.S.A. 61, 598–605

51. Hackenbrock, C. R. (1968) Ultrastructural bases for metabolically linked mechanical activity in mitochondria. II. Electron transport-linked ultrastructural transformations in mitochondria. J. Cell Biol. 37, 345–369

52. Benard, G., and Rossignol, R. (2008) Ultrastructure of the mitochondrion and its bearing on function and bioenergetics. Antioxid. Redox Signal. 10, 1313–1342

53. Rossignol, R., Gilkerson, R., Appel, R., Yamagata, K., Remington, S. J., and Capaldi, R. A. (2004) Energy substrate modulates mitochondrial structure and oxidative capacity in cancer cells. Cancer Res. 64, 985–993

54. Raffaella, C., Francesca, B., Italia, F., Marina, P., Giovanna, L., Susanna, I. (2008) Alterations in hepatic mitochondrial compartment in a model of obesity and insulin resistance. Obesity 16, 958–964

55. Holmstrom, M. H., Iglesias-Gutierrez, E., Zierath, J. R., and Garcia-Roves, P. (2004) Energy substrate modulation by mitofilin and mitochondrial cristae morphology. J. Biol. Chem. 289, 7411–7426

56. Xie, J., Marusich, M. F., Souza, P., Whitelegge, J., and Capaldi, R. A. (2007) The mitochondrial inner membrane protein mitofilin exists as a complex with SAM50, metaxins 1 and 2, coiled-coil-helix coiled-coil-helix domain-containing protein 3 and 6 and DnaJC11. FEBBS Lett. 581, 3545–3549

57. An, J., Shi, J., He, Q., Lui, K., Liu, Y., Huang, Y., and Sheikh, M. S. (2012) CHCM1/CHCHD6, novel mitochondrial protein linked to regulation of mitofilin and mitochondrial cristae morphology. J. Biol. Chem. 287, 7411–7426

58. Miyazaki, Y., Kohane, I., Costello, M., Saccone, R., Landaker, E. J., Goldfine, A. B., Mun, E., DeFronzo, R., Finlayson, J., Kahn, C. R., and Mandarino, L. J. (2003) Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: Potential role of PGC1 and Nrf1. Proc. Natl. Acad. Sci. U.S.A. 100, 8466–8471

59. An, J., Shi, J., He, Q., Lui, K., Liu, Y., Huang, Y., and Sheikh, M. S. (2012) CHCM1/CHCHD6, novel mitochondrial protein linked to regulation of mitofilin and mitochondrial cristae morphology. J. Biol. Chem. 287, 7411–7426

60. Hwang, H., Bowen, B. P., Lefort, N., Flynn, C. R., De Filippis, E. A., Roberts, C., Smoke, C. C., Meyer, C., Hoijlund, K., Yi, Z., and Mandarino, L. J. (2010) Proteomics analysis of human skeletal muscle reveals novel abnormalities in obesity and type 2 diabetes. Diabetes 59, 33–42

61. Blomhoff, R., and Blomhoff, H. K. (2006) Overview of retinoid metabolism and function. J. Neurobiol. 66, 606–630

62. Rehe, E. J., and Plutzky, J. (2011) Retinoid metabolism and diabetes mellitus. Diabetes Metab. J. 36, 167–180

63. Zhao, S., Li, R., Li, Y., Chen, W., Zhang, Y., and Chen, G. (2012) Roles of vitamin A status and retinoids in glucose and fatty acid metabolism. Biochem. Cell Biol. 90, 142–152

64. Ross, A. C., and Zolfaghari, R. (2011) Cytochrome P450s in the regulation of cellular retinoic acid metabolism. Annu. Rev. Nutr. 31, 65–87

65. Schupp, M., Lefterova, M. I., Janke, J., Letnner, K., Cristancho, A. G., Mullican, S. E., Gat Watching, N., Steger, D. J., Curtin, J. C., Kim, R. J., Suh, M. J., Suh, M., Albert, M. R., Engelli, J. C., Gudas, L. J., and Lazar, M. A. (2009) Retinol saturation promotes adipogenesis and is downregulated in obesity. Proc. Natl. Acad. Sci. U.S.A. 106, 1105–1110

66. Acin-Perez, R., Hoyos, B., Zhao, F., Vinogradov, V., Fischman, D. A., Harris, R. A., Leitges, M., Wongsinroj, N., Blaner, W. S., Manfredi, G., and Hammerling, U. (2010) Control of oxidative phosphorylation by vitamin A illuminates a fundamental role in mitochondrial energy homeostasis. FASEB J. 24, 627–636

67. Nagaoka-Yasuda, R., Matsuo, N., Perkin, B., Limbaeck-Stokin, K., and Mayford, M. (2007) An RNAi-based genetic screen for oxidative stress resistance reveals retinol saturase as a mediator of stress resistance. Free Radic. Biol. Med. 43, 781–788