RESEARCH ARTICLE

Proteomic analysis of mitochondria from *Caenorhabditis elegans*

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Mitochondria play essential roles in cell physiological processes including energy production, metabolism, ion homeostasis, cell growth, aging and apoptosis. Proteomic strategies have been applied to the study of mitochondria since 1998; these studies have yielded decisive information about the diverse physiological functions of the organelle. As an ideal model biological system, the nematode *Caenorhabditis elegans* has been widely used in the study of several diseases, such as metabolic diseases and cancer. However, the mitochondrial proteome of *C. elegans* remains elusive. In this study, we purified mitochondria from *C. elegans* and performed a comprehensive proteomic analysis using the shotgun proteomic approach. A total of 1117 proteins have been identified with at least two unique peptides. Their physicochemical and functional characteristics, subcellular locations, related biological processes, and associations with human diseases, especially Parkinson's disease, are discussed. An orthology comparison was also performed between *C. elegans* and four other model organisms for a general depiction of the conservation of mitochondrial proteins during evolution. This study will provide new clues for understanding the role of mitochondria in the physiological and pathological processes of *C. elegans*.

Keywords:
Animal proteomics / *Caenorhabditis elegans* / Development / Evolutionary conservation / Mitochondrial proteomics / Shotgun proteomics

1 Introduction

Mitochondria are one of the most important organelles in the cell. Known as the ''powerhouses'' of cells, they play essential roles in several cellular processes, including respiration and ATP production, fatty acid metabolism, oxidative stress, ion homeostasis, Fe-S cluster formation, apoptosis and aging [1]. Previous studies have revealed possible links between mitochondrial dysfunction and a wide variety of degenerative diseases, metabolic diseases and cancer [2]. To understand the roles that mitochondria play in health and disease, it is important to know the complete protein composition of this organelle. In the last few years, newly developed proteomic approaches have been successfully applied to explore mitochondrial proteins and their functions in several cell types and model organisms, including *Tetrahymena thermophila* [3], yeast [4–6], *Arabidopsis* [7, 8], rice [9], mice [10, 11], rats [12, 13] and humans [14–16]. Furthermore, labeling strategies have been

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Abbreviations: FA, formic acid; **FDR,** false discovery rate; **GOA,** Gene ontology annotation; **IAM,** iodoacetamide; **KEGG,** Kyoto encyclopedia of genes and genomes; **KOG,** eukaryotic orthologous groups; **NDUFS3,** NADH dehydrogenase (ubiquinone) Fe-S protein 3; **OXPHOS,** oxidative phosphorylation; **PD,** Parkinson's disease; **PHD1,** polyclonal anti-prolyl hydroxylase protein 1 **PDH E1** α, pyruvate dehydrogenase complex subunit E1 α; **TCA,** tricarboxylic acid; **TEM,** transmission electron microscopy

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employed to effectively identify mitochondrial proteins and to distinguish contaminant proteins and even multilocation proteins [17, 18]. These studies have contributed to our understanding of mitochondrial function and mitochondrion-related diseases.

The free-living nematode *Caenorhabditis elegans* is one of the best-studied multicellular model organisms. Owing to its short lifespan, fixed number of cells, transparent body, and its completed genome sequencing, it has been an ideal model system in basic biological research, especially in the fields of development, genetics, neurobiology, signal transduction and aging. Proteomic approaches are increasingly being used to complement genetic and RNA interference-based studies of gene function in *C. elegans* [19]. As early as 2003, 2D-LC-MS/MS was used for large-scale identification of *C. elegans* proteins [20]. Bantscheff *et al.* applied 2-DE and MALDI-TOF-MS for differential proteome analysis and identification of germ line development-related proteins in 2004 [21]. In 2006, Chu *et al.* performed a subtractive proteomic study using two mutants of *C. elegans* (*fer-1* and *him-8*) and identified several evolutionarily conserved fertility factors [22]. In 2003, Krijgsveld *et al.* developed a quantitative ^{15}N metabolic-labeling method for *C. elegans* and applied it to the comparison of protein expression levels in two *C. elegans* strains [23]; more recently, this labeling method was elegantly used to identify insulin-signaling targets in *C. elegans* [24].

The previous studies of *C. elegans* mitochondria have characterized the role of mitochondria in the life of the nematode and extended our understanding of mitochondrial dynamics, mitochondrial diseases, aging and life span [25]. However, to the best of our knowledge, there is still no comprehensive research focused on the mitochondrial proteome of *C. elegans*. In this study, we purified mitochondria from *C. elegans* and performed proteomic analysis using 2D-LC-MS/MS. We also analyzed the physicochemical characteristics and biological functions of the identified mitochondrial proteins and compared them with their counterparts in other model organisms.

2 Materials and methods

2.1 Materials

The wild-type *C. elegans* strain N2, initially obtained from the *Caenorhabditis* Genetics Center, was kindly donated by the Miao lab at the Institute of Biophysics, Chinese Academy of Sciences. Polyclonal antibody for prolyl hydroxylase protein 1 (PHD1) antibody was purchased from Abcam (ab52726, Cambridge, MA, USA). Monoclonal antibodies for NADH dehydrogenase (ubiquinone) Fe-S protein 3 (NDUFS3) and pyruvate dehydrogenase complex subunit E1 α (PDH E1 α) were the products of MitoSciences (MS112 and MSP07, Eugene, OR, USA). Mitochondrial activity (cytochrome c oxidase activity) assay kit was purchased from BioChain (FKC310100, Hayward, CA, USA). The density gradient medium Nycodenz® was purchased from Axis-shield (Oslo, Norway). Prestained protein standard was from Invitrogen™ (LC 5800, Carlsbad, CA, USA). Protease inhibitor cocktail was purchased from Roche Applied Sciences (cat04693132001, Mannheim, Germany). Sequencing-grade modified trypsin was purchased from Promega (V5113, Madison, WI, USA). Pure water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA). Super enhanced chemiluminescence detection reagents were purchased from Applygen Technologies (Beijing, China). ACN and formic acid (FA) were obtained from J. T. Baker (Phillipsburg, NJ, USA). All other chemicals used in *C. elegans* cultivation and biochemical experiments, including sucrose, sodium glucose 6-phosphate, sodium adenosine 5'-monophosphate, NaOH (sillico free), NH_4HCO_3 and urea, were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Culture conditions and harvesting procedures

All handling methods including synchronization, cultivation and harvest of the nematodes were performed according to the relevant chapters of WormBook [26]. Briefly, synchronized *C. elegans* were obtained from axenic eggs and maintained in S medium using concentrated *Escherichia coli* OP50 as the food source. The complete S Medium solution was prepared from stock solutions: to each liter of S basal medium (0.1 M sodium chloride, 0.05 M potassium phosphate, 5 mg/L cholesterol, pH 6), were added 10 mL 1 M potassium citrate (pH 6), 10 mL trace metal solutions (50 μM EDTA, 25 μM ferrous sulfate, 10 μM manganese chloride, 10 μM zinc sulfate, 1 μM copper sulfate), 3 mL 1 M calcium chloride and 3 mL 1 M magnesium sulfate. Worms growing in the liquid medium were limited to one generation to avoid overcrowding.

C. elegans were harvested when they grew to the adult stage. The worms were collected by centrifugation at 1100 × *g*, washed three or four times using 0.1 M NaCl, resuspended in 30% pre-cooled sucrose solution as quickly as possible and centrifuged at 5400 × *g*. After centrifugation, the adult worms were collected from the upper layer of the centrifuge tube, thoroughly washed with 0.1 M NaCl and incubated in M9 buffer (0.3% KH_2PO_4, 0.6% Na_2HPO_4, 0.5% NaCl, 1 mM $MgSO_4$) for 30 min to digest the remaining bacteria in their guts. Finally, the worms were collected by centrifugation and prepared for mitochondria isolation.

2.3 Isolation of *C. elegans* mitochondria

The isolation and purification of the *C. elegans* mitochondria was performed as previously described with some modifications [27, 28]. The nematodes were resuspended in lysis buffer (LB: 250 mM sucrose, 10 mM HEPES, 1 mM EGTA, pH 7.4) with protease inhibitor cocktail. The samples were

kept on ice throughout the procedure. The nematodes were homogenized in 20 mL LB with a Kontes ground glass tissue grinder (Fisher Scientific, cat# K885450-0025) using ten strokes. The homogenate was centrifuged at $800 \times g$ for 10 min. The supernatant was reserved and another 20 mL LB with protease inhibitors was added to the pellet. The homogenization process and $800 \times g$ centrifugation were repeated twice more and the homogenates were microscopically examined to ensure that more than 95% of the nematodes were disrupted. Next, the supernatants were combined and centrifuged at $12\,000 \times g$ for 10 min and an aliquot of the resulting supernatant was saved as a post-mitochondrial fraction. The pellets were gently resuspended in 10 mL LB with a Dounce tissue grinder (Wheaton Industries, cat# 357544). The suspension was centrifuged at $800 \times g$ for 10 min, and the supernatant was collected and centrifuged at $12\,000 \times g$ for 10 min. The final pellets (crude mitochondria) were resuspended in 5 mL LB using a Dounce grinder and further purified over a step Nycodenz® gradient. The gradient was prepared from an 80% Nycodenz® stock (80% Nycodenz®, 10 mM HEPES, 1 mM EGTA, pH 7.4), which was subsequently diluted to 20, 23, 30, 34, 40 and 50% solutions with dilution buffer (10 mM HEPES, 1 mM EGTA, pH 7.4). Crude mitochondria were mixed with equal volume 50% Nycodenz® to make the 25% gradient. The gradient solutions (2 mL of 20%, 7 mL of 23%, 10 mL of crude mitochondria in 25%, 7 mL of 30%, and 5 mL of 34 and 40%) were carefully loaded in a centrifuge tube and centrifuged at 17 000 rpm for 90 min using the P28S rotor (CP100MX, Hitachi, Tokyo, Japan). After centrifugation, the mitochondrial fraction was collected from the brown band between 25 and 30% gradients. The 23%/25% band and 30%/34% band were also collected as upper band and bottom band, respectively. The samples were mixed with equal volumes of LB, centrifuged at $15\,000 \times g$ for 30 min and the pellets were resuspended in 500 uL LB. Protein concentrations were determined using Bradford assay [29]. The aliquots of purified mitochondria for transmission electron microscopy (TEM) examination and cytochrome c oxidase activity assay were processed immediately and the remaining aliquots were stored at $-80^{\circ}C$.

2.4 TEM work

Purified mitochondria were fixed in 2.5% glutaraldehyde in LB at 4°C for 2 h. After washing once with PBS, the pellet was post-fixed in 1% osmium tetroxide in PBS for 20 min at room temperature, dehydrated in a gradient series of ethanol, carefully transferred to propylene oxide and embedded in Epon-Araldite. Ultrathin sections (50–70 nm thick) were cut on a Leica EM UC6 Ultramicrotome (Leica, Milton Keynes, UK) with a diamond knife, picked up on formvar-carbon coated copper grids, stained with saturated uranyl acetate followed by lead citrate and examined on an electron microscope (FEI, Tecnai 20, Holland) at 120 kV.

2.5 Western blotting

Equal amounts of proteins (5 μg for the two mitochondrial markers NDUFS3 and PDH E1 α and 25 μg for PHD1) from total lysate, post-mitochondrial fraction and purified mitochondria were separated on 12% SDS-polyacrylamide gels, transferred to PVDF membranes (Millipore, Immobilon-P) in 25 mM Tris, 192 mM glycin, 20% methanol and blocked for 2 h with 5% nonfat milk in TBST (100mM Tris-HCl, 150mM NaCl, 0.05% Tween-20, pH 7.4). The proteins were probed with their primary antibodies for 2 h, washed three times in TBST and incubated with secondary antibodies for 1 h. All incubations were performed at room temperature. After washing again, the proteins were detected by enhanced chemiluminescence. Western blotting for each selected protein was repeated three times for every batch of mitochondria purification.

2.6 Enzyme assays

Glucose-6-phosphatase (ER) activity and 5′-nucleotidase (plasma membrane) activity were adapted from Stephenson and Clarke [30]. For glucose-6-phosphatase assay, enzyme fractions (20 μL) were mixed with 180 μL of 22.2 mM sodium glucose 6-phosphate, 19.4 mM histidine-HC1 and 1.1 mM sodium EDTA at pH 6.5 and were incubated at 37°C for 20 min. The reaction was quenched by adding 1 mL of phosphate assay reagent (a fresh 1:l mixture of 22.9 mM ammonium molybdate, 8 mM sodium EDTA, pH 6.5, and 180 mM hydroxylamine HCI, 145 mM H_2SO_4, 4.8% polyvinylpyrrolidone). After 2.5 min, 0.1 mL of the color developer (5.2 M NaOH, silicate free) was added and the samples were incubated for 10 min at room temperature. Samples were briefly spun down and the absorbance was read at 670 nm versus a control. The activity was determined by comparison with a standard curve prepared with inorganic phosphate. The procedure of 5′-nucleotidase assay is identical to that for glucose-6-phosphatase except that the enzyme assay reagent is 5.6 mM sodium adenosine 5′-monophosphate, 111 mM sodium glycine and 11 mM MgCl, at pH 9.1 in this case.

The cytochrome c oxidase activity assay and mitochondrial outer membrane integrity assay were performed using the assay kit purchased from BioChain. The cytochrome c oxidase activity assay is based on observation of the decrease in absorbance at 550 nm of ferrocytochrome c caused by its oxidation to ferricytochrome c by cytochrome c oxidase. The examination of the integrity of mitochondrial outer membranes was achieved by measuring cytochrome c oxidase activity in purified mitochondria in the presence and absence of the detergent *n*-dodecyl-β-D-maltoside. This assay

was performed according to the manufacturer's instructions (http://www.flowgen.net/pdfs/manual/page015.pdf). All enzyme assays were repeated three times using samples from different batches, and the final results were calculated by average.

2.7 In-solution digestion

Aliquots of purified mitochondria sample (~100 μg proteins) were dissolved in 20 μL of freshly made 8 M urea and reduced with 10 mM DTT at 56°C for 1 h. The sample was then treated with iodoacetamide at a concentration of 40 mM in the dark for 45 min to block the sulfhydryl group. Next, DTT was added to the final concentration of 40 mM to quench any remaining iodoacetamide. The sample solution was then diluted with 25 mM NH_4HCO_3 (pH 8.0) to bring the concentration of urea to below 1 M. Trypsin was added at a ratio of 1:60 to total protein content and the sample was incubated at 37°C. After 12 h, the same amount of trypsin was added and the sample was incubated in 37°C for another 6 h before adding 0.1% FA to end the digestion. The sample was then centrifuged at 14 000 × *g* for 10 min and the supernatant was collected and stored at −20°C until use.

2.8 2D-LC-MS/MS analysis

The tryptic peptide mixtures were analyzed by 2D-LC coupled to a linear ion trap mass spectrometer LTQ (Thermo Electron, San Jose, CA, USA) [31]. For each experiment, the peptide mixtures (from about 100 μg proteins) were pressure-loaded onto a biphasic silica capillary column (250 μm id) packed with 3 cm of reverse phase C18 resin (SP-120-3-ODS-A, 3 μm, the Great Eur-Asia Sci&Tech Devolopment, Beijing, China) and 3 cm of strong cation exchange resin (Luma 5 μ SCX 100A, Phenomenex, Torrance, CA, USA). The buffers used were 0.1% FA (buffer A), 80% ACN/0.1% FA (buffer B), and 600 mM ammonium acetate/5% ACN/0.1% FA (buffer C). After sample loading, the biphasic column was first desalted with buffer A and then eluted using a 12-step salt gradient ranging from 0 to 600 mM ammonium acetate. After each salt gradient, a gradient of buffer B ranging from 0 to 100% was applied. Step 1 consisted of a 100-min gradient from 0 to 100% buffer B. For steps 2–11, after equilibrating with buffer A for the first 3 min, X% buffer C was applied for 5 min, and peptides were eluted using a linear gradient as follows: 0–10% buffer B in 5 min, 10–45% buffer B in 77 min, 45–100% buffer B in 10 min and 100% buffer B for 10 min, followed by re-equilibration with buffer A for 10 min. The 5-min buffer C percentages (X) were 5, 10, 15, 20, 25, 30, 40, 50, 60 and 80%. The gradient used in the final step contained 3 min of 100% buffer A, 20 min of 100% buffer C, a 5-min gradient from 0 to 10% buffer B, a 72-min gradient from 10 to 55% buffer B and a 5-min gradient from 55 to 100% buffer B. Then 100% buffer B was applied for 5 min, followed by a 5-min elution with buffer A and another 10-min elution with buffer B. The effluent of the biphasic column in each case was directed into an in-house-packed 10 cm C18 analytical column (100 μm id, SP-120-3-ODS-A, 3 μm) with a 3- to 5-μm spray tip. The flow rate at the tip was maintained at about 500 nL/min. Nano-electrospray ionization was performed at a spray voltage of 2.0 kV and a heated capillary temperature of 160°C. The MS instrument was set to the data-dependent acquisition mode with dynamic exclusion turned on, and maximum ion injection time was set to 100 ms. One MS survey scan, with mass range 400–2000 m/z, was followed by five MS/MS scans. All tandem mass spectra were collected using a normalized collision energy (a setting of 35%), an isolation window of 2 Da, and 1 micro-scan. The XCalibur data system (ThermoElectron, Waltham, MA, USA) was used to control the HPLC solvent gradients and the application of MS scanning functions.

MS data were searched using SEQUEST algorithm (Ver. 2.8) [32] against the WormBase database Wormpep195, which was released on October 25, 2008, and contains 23 906 protein sequences. The database was reversed and attached to estimate the false discovery rate (FDR). All searches were performed using a precursor mass tolerance of 3 Da calculated using average isotopic masses. Fixed modification was set for cysteine with the addition of 57.052 Da to represent cysteine carboxyamidation. Variable modification was set for methionine with the addition of 15.999 Da to represent methionine oxidation. A fragment ion mass tolerance of 1 Da was used. Enzyme cleavage specificity was set to trypsin and no more than two missed cleavages were allowed. The SEQUEST outputs were then analyzed using the commercial software Thermo Electron BioWorks (Rev.3.3.1). The filter settings for peptides were as follows – XCorr: 1.9 (+1 ions), 2.5(+2 ions), 3.75(+3 ions); Delta CN: 0.1; Sp: 500; Rsp: 1. The FDR is calculated by dividing the absolute number of reversed hits through the true hits against the actual database.

2.9 Physicochemical characterization and functional annotation

The physicochemical characteristics of the identified proteins were analyzed using free online tools. More specifically, protein molecular weights and IEF points were calculated using ExPASy tools [33]. The calculation of protein GRAVY (grand average of hydropathy) values and the prediction of transmembrane helices were performed using Protein GRAVY [34] and TMHMM Server v. 2.0 [35], respectively.

All identified proteins were first annotated according to Gene Ontology Annotation (GOA) database descriptions [36], Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways [37, 38] and NCBI Eukaryotic orthologous

groups (KOG) [39, 40] for both subcellular location and functional information. The proteins were also searched against the NCBI KOG database for orthologs and their subcellular locations were predicted using four website programs (TargetP 1.1 Server [41], MITOPROT [42], MITOPRED [43, 44] and SubLoc v1.0 [45]). The prediction of mitochondrial sublocation was accepted when at least two programs gave this result. The proteins without annotation would be assigned as mitochondrial protein candidates if they had orthologs that had previously been included in the MITOP2 database [46, 47] or were predicted to locate in mitochondria by the bioinformatic tools.

All of the proteins were manually classified according to their functional descriptions. The open source software GO::TermFinder (Ver. 0.82) [48] was employed to depict the related biological processes of the mitochondrial proteins and mitochondrial protein candidates. We examined the potential relevance of the mitochondrial proteins and candidate proteins to human diseases according to their entries in the KEGG disease database.

3 Results and discussion

3.1 Purification of mitochondria from *C. elegans*

When we searched PubMed using the keywords ''mitochondrial'' and ''proteome,'' more than 70 results were found, of which the earliest was published in 1998 [49]. There has not been any proteome-scale study of *C. elegans* mitochondria until now, possibly due to the difficulties in isolating intact mitochondria from *C. elegans*. The cuticle and collagen protect these nematodes against hypotonic conditions, detergents and even normal homogenization; however, under violent conditions, for example, high pressure, under which the nematodes come apart, their mitochondria also fall apart. We attempted to use the BeadBeater^{TM} according to the methods used by Grad *et al.* [50], which breaks the worms by collision with glass beads. We did purify some intact mitochondria in this way, but many of the mitochondria were broken. Then we turned to a Fisher Kontes ground glass tissue grinder, inspired by Clarke lab [27, 28], and found that it could greatly improve the yield of intact mitochondria, as compared with other methods. Furthermore, we noticed that fewer strokes for each single homogenization process and utilizing multistep homogenization-800 × *g*-centrifugation cycles helped to increase the yields. In our experiment, we isolated mitochondria from about 600 000 *C. elegans* (after sucrose floating) and the final yield was about 700–800 μg of mitochondrial proteins. Compared with larval worms and even dauers, the ''fat'' adults seemed to ''pop'' much more easily, so it is important to provide enough food during the cultivation stage. It should also be mentioned that operating the Kontes grinder is labor-intensive, so the electronic BeadBeater^{TM} is still useful if many mitochondria samples are needed.

3.2 Quality control of purified mitochondrial samples

As the purity and integrity of the organelles are of great importance in organelle proteomic studies, we used several different approaches for mitochondrial quality control.

3.2.1 TEM analysis

The morphology of the purified mitochondria was verified by obtaining TEM images (Fig. 1A). Mitochondria were found to be the predominant structures in the field of view, with intact membranes, clear cristae, diverse shapes and varying sizes. Because mitochondria form dynamic tubular networks that continually change their shape and move throughout the cell, we suspect that the mitochondrial dynamics [51, 52] and the tissue heterogeneity of the sample source may equally account for the observed morphological diversity.

3.2.2 Western blot analysis

Western blotting was applied to determine the purity of the isolated mitochondria (Fig. 1B). The mitochondrial membrane marker NDUFS3 and mitochondrial matrix marker PDH E1 α were largely enriched in the purified mitochondria fraction. PHD1, which is located in the cytoplasm and nucleus, was used as the marker of other organelles. The absence of the PHD1 band in the purified mitochondrial fraction indicated a fairly high purity of the purified mitochondria. A weak band of PDH E1 α in the post-mitochondrial fraction suggested that some mitochondria were broken during the homogenization process, which was expected considering the amount of grinding used to break down the *C. elegans*. Fortunately, the mitochondrial debris could be easily removed during purification, so it did not affect the integrity of purified mitochondria.

3.2.3 Enzyme assays

Due to the limited number of antibodies that can react with proteins from *C. elegans*, the activities of glucose-6-phosphatase and 5′-nucleotidase were assayed in all subcellular fractions to determine the extent of contamination of mitochondria with ER and plasma membrane, respectively (Table 1). Purified mitochondria showed a substantial decrease of activity for both of the enzyme assays compared

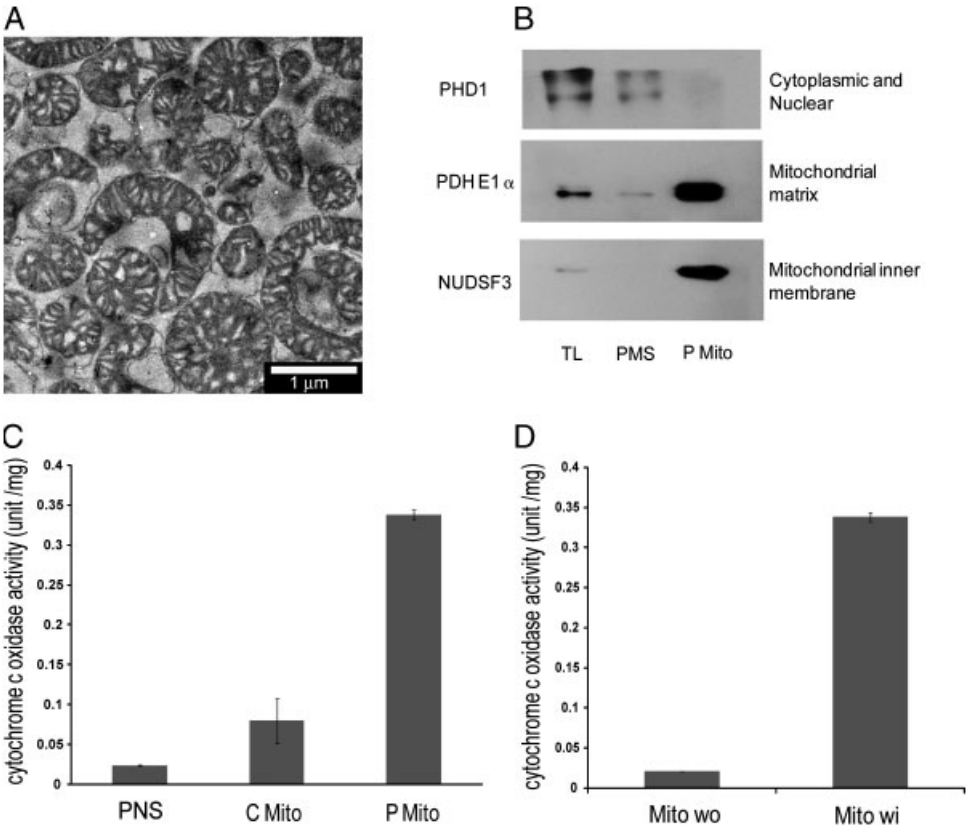

Figure 1. Quality control for the purified *C. elegans* mitochondria (A) Transmission electron microscopy (TEM) image of purified mitochondria. (B) Western blot results for three marker proteins. The names and their subcellular locations are listed on both sides of the picture. TL: total lysate; PMS: post-mitochondrial supernatant; P Mito: purified mitochondria. (C) Results of the cytochrome c oxidase activity assay of three subcellular fractions, showing the enrichment of mitochondria during the purification procedures. PNS: post-nuclear supernatant; C Mito: crude mitochondria. (D) Results of the mitochondrial integrity assay. Mito wo: mitochondria without any detergent, represents injured mitochondria; Mito wi: mitochondria with the detergent *n*-dodecyl-β-D-maltoside, represents total mitochondria.

Table 1. Enzyme assays

Subcellular fraction	Glucose-6-Phosphatase activity (ER) nmol phosphate produced/min/mg protein	5′-Nucleotidase activity (PM) nmol phosphate produced/min/mg protein
Post-nuclear supernatant	4.05±0.01	2.27±0.29
Post-mitochondrial supernatant	5.49±0.41	3.03±0.12
Crude mitochondria	4.54±0.39	1.32±0.42
Purified mitochondria	2.87±0.26	0.27±0.05
Upper band	2.18±0.11	0.36±0.01
Bottom band	7.87±0.18	0.98±0.19

to the post-nuclear supernatant, crude mitochondria and the bottom band.

A cytochrome c oxidase activity assay was performed to measure the efficiency of the enrichment of mitochondria. Figure 1C shows the measurement of the enzyme activity of the three fractions, indicating that 3.5-fold and 15-fold enrichment of mitochondria were obtained in the purified samples, as compared with the crude mitochondria fraction and the post-nuclear supernatant, respectively.

3.2.4 Analysis of mitochondrial outer membrane integrity

Mitochondrial outer membrane integrity was also verified using the cytochrome c oxidase activity assay utilizing the detergent *n*-dodecyl-β-D-maltoside. Cytochrome c oxidase localizes in the inner membrane of the mitochondria, so the added cytochrome c cannot access the cytochrome c oxidase when the outer membrane is intact. *n*-dodecyl-β-D-maltoside is one of the few detergents that can destroy mitochondrial outer membrane while maintaining the cytochrome c oxidase dimer in solution at low concentration, therefore maintaining the enzyme activity. The activity of cytochrome c oxidase observed without *n*-dodecyl β-D-maltoside represents the mitochondria with damaged outer membranes, while the activity measured with the detergent represents the total mitochondria. The ratio of intact mitochondria to total mitochondria $((Activity_{with\ detergent}–Activity_{without\ detergent})/Activity_{with\ detergent})$ could serve as a measurement of the integrity of the mitochondrial outer membrane. Figure 1D indicates that more than 93% of the purified mitochondria had intact outer membranes.

3.3 Proteomic analysis of mitochondria from *C. elegans*

The mitochondrial proteins were digested with trypsin and analyzed by 2D-LC-MS/MS. A total of six 12-step 2D-LC-MS/MS experiments were performed; for each of the three biological replicates (referred to as experiment I, II and III, respectively), two technological replicates were applied. For each 12-step 2DLC-MS/MS experiment, more than 4500 unique peptides were identified (see Supporting Information Table 1.1). With stringent criteria for peptide identification, the FDR was controlled below 0.5%. At the protein level, only proteins with at least two unique peptides were accepted. Figure 2 demonstrates the high reproducibility observed for the three biological replicates.

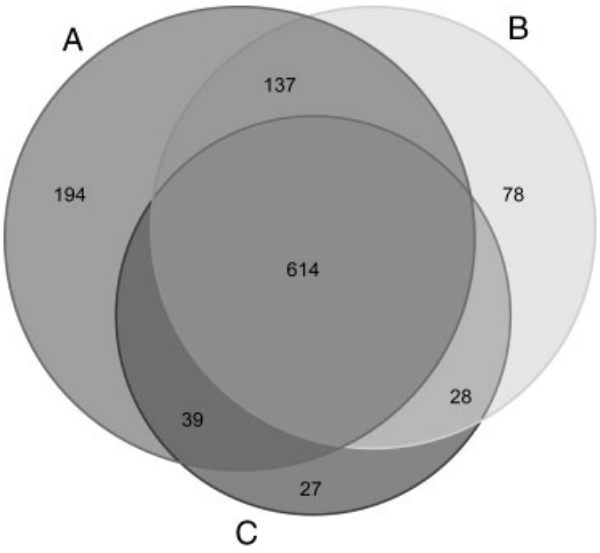

Figure 2. Distribution and overlap of the identified proteins in the three biological replicates: (A) experiment I; (B) experiment II; (C) experiment III.

3.4 Analysis of the physicochemical properties of identified proteins

We identified 1117 proteins in total and compared their physicochemical characteristics in Fig. 3 (also see Supporting Information Table 1.2). Most of the proteins are of middle-to-low molecular weight, consistent with previous studies of mitochondrial proteins [10, 14]. This may be explained by the fact that most mitochondrial proteins are synthesized in the cytoplasm and need to be transported into the organelle, a mechanism purported to prefer smaller proteins. The distribution of protein IEF points indicates that more than half of the proteins are alkaline proteins, which is also consistent with other studies [10, 14]. The protein GRAVY value, which was calculated by adding the hydropathy values for all residues and dividing by the

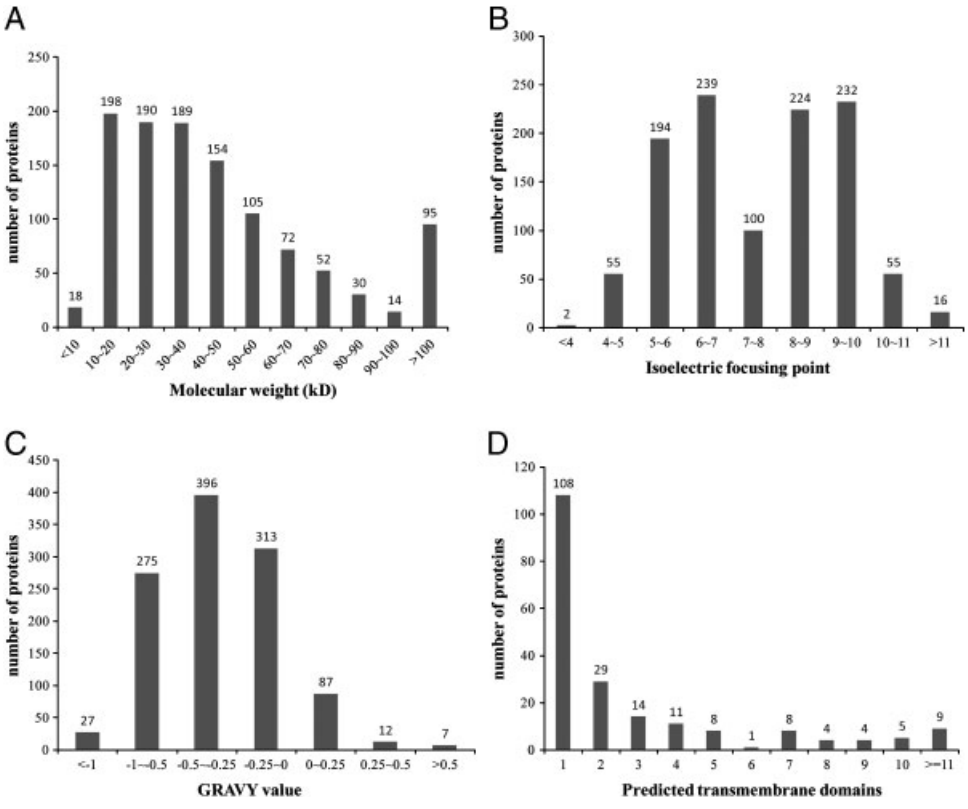

Figure 3. Analysis of physicochemical characteristics of identified proteins. (A) Distribution of the molecular weights of the identified proteins. (B) Distribution of IEF points of the identified proteins. (C) Distribution of the predicted GRAVY values of the identified proteins. (D) Distribution of the predicted numbers of transmembrane domains of the identified proteins.

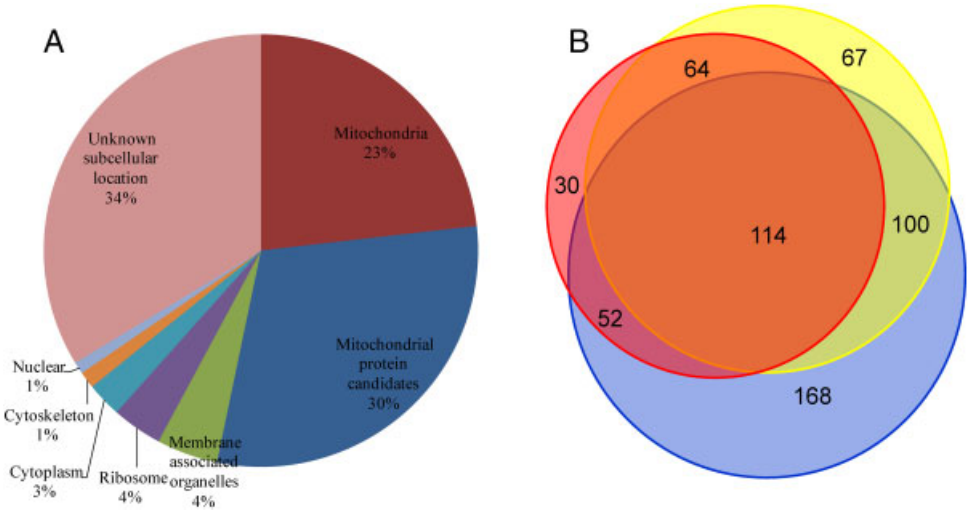

Figure 4. Analysis of protein subcellular locations. (A) Distribution of subcellular locations of identified proteins. (B) Venn diagram showing the overlap of annotated mitochondrial proteins and the mitochondrial protein candidates predicted by two approaches. Red: annotated mitochondrial proteins; yellow: mitochondrial protein candidates that have orthologs that are included in the MITOP2 database; blue: *in silico* predicted mitochondrial protein candidates.

number of amino acids in the sequence [34], is a useful parameter with which to evaluate the hydrophobicity of proteins. The GRAVY values of the identified proteins ranged from −2.013 to 1.188, indicating a wide range of hydrophobicity of mitochondrial proteins. More than 100 identified proteins had positive GRAVY values, including several mitochondrial proteins known to reside on the outer and inner membranes. In our study, of the 12 proteins encoded by the mitochondrial genome, which are generally thought to be highly hydrophobic [53], seven were confidently identified. In keeping with the double membrane structure of mitochondria, 201 proteins in this study were predicted to have transmembrane domains, of which 14 have more than ten predicted á helices.

3.5 Analysis of protein subcellular locations

Figure 4 shows the illustrated results of the analysis of subcellular locations of identified proteins (also see Supporting Information Tables 1.2, 1.3, 1.4). Mitochondrial proteins and candidate proteins account for more than 50% of the total identified proteins. Ribosomes and other membrane-associated organelles (ER, lysosome, peroxisome, plasma membrane and vesicles) seem to be the major sources of contaminants. Although contamination is inevitable, we think most of these proteins may be ''mitochondria-associated'' proteins that have interaction with mitochondria. For example, some of the identified cytoskeletal proteins such as actin and myosin are likely to interact with mitochondria and are responsible for their movements and cellular locations [54], unlike simple contaminants. More than 60% of the predicted mitochondrial proteins were verified by database annotation and/or orthology comparison and approximately two-thirds of the annotated proteins were correctly predicted. These values were a little higher than the reported specificity and sensitivity of any single predicting program [46], possibly owing to the combinatorial use of four *in silico* predicting tools.

3.6 Classification of protein molecular functions

Based on descriptions from three databases (GOA, KEGG, NCBI KOG), all of the identified proteins were manually sorted into eight functional classes (Fig. 5A) including the following: metabolism related, tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS), proteolysis, protein biosynthesis, transport and locomotion, cell defense related, signal transduction and unknown function.

3.6.1 Metabolism related

This class includes proteins involved in the TCA cycle, OXPHOS, proteolysis and other metabolic processes and accounts for about 40% of the total identified proteins, reflecting the major function of mitochondria. About 80% of proteins in this class are enzymes, including synthetase, protease, catalase, hydrolase, dehydrogenase, oxidase and isomerase, taking part in the metabolic processing of amino acids, carbohydrates, lipids, nucleotides and cofactors (Fig. 5B).

The TCA cycle and OXPHOS are tightly related biochemical processes typical for mitochondria, which perform cell respiration and energy metabolism. Of the 73 protein subunits listed in the KEGG pathway as being involved in OXPHOS, we successfully identified 66 with more than two peptides and another three with a single peptide match. Specifically, we identified 31 out of 35 complex I subunits, three out of four complex II subunits, 12 out of 14 complex IV subunits and all the 8 subunits and 12 subunits of complex III and complex V, respectively . For the TCA cycle, 18 subunits were found out of 19.

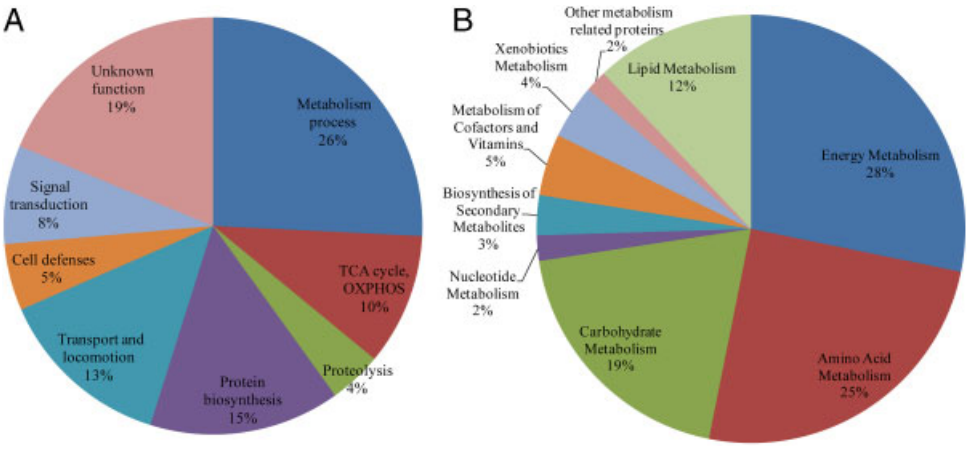

Figure 5. Analysis of protein molecular functions. (A) Functional distribution of proteins identified in purified *C. elegans* mitochondria. (B) Distribution of identified proteins involved in different metabolic processes.

Fe-S clusters are co-factors of numerous proteins with important functions in metabolism, electron transport and regulation of gene expression. The assembly of a Fe-S cluster is a complex process involving several components and is accomplished inside mitochondria. Previous genetic studies have identified seven *C. elegans* genes responsible for Fe-S cluster biosynthesis and assembly by searching for homologous genes described in *Saccharomyces cerevisiae* [55]. In this study, we successfully identified six of these proteins, along with two others that were not described in the genetic analysis: the mitochondrial Fe-S cluster biosynthesis-related proteins Isa-2 (Y54G11A.9) and Frh-1 (F59G1.7). Frh-1 is the *C. elegans* ortholog of human frataxin, deficiency of which has been reported to cause the symptoms of Friedrich's ataxia, a hereditary trinucleotide repeat disorder [56].

3.6.2 Protein biosynthesis

About 200 proteins were classified as related to protein biosynthesis. These proteins included 50 subunits of the mitochondrial ribosome, several transcription factors, coactivators, mitochondrial translation initiation factors, elongation factor Tu, and some mitochondrial aminoacyl tRNAs. They may represent the mitochondrial gene expression system that is a remnant of its prokaryote ancestor and take part in the biosynthesis of the 12 proteins encoded by the mitochondrial genome.

Many cytosolic ribosome proteins were also identified in this study, which seemed to be a large source of contaminants at first glance. However, more than 90% of mitochondrial proteins are encoded by the nuclear genome and are imported into mitochondria in both co-translational and post-translational manners [57]. The association between cytosolic ribosomes and mitochondria was discovered many years ago [58], and the discussion of coupling of protein synthesis to protein translocation remains a topic of interest [59]. It is also interesting to mention that some previous studies have suggested that mRNA populations encoding ancestral mitochondrial proteins tend to be translated by polysomes associated with the mitochondrial outer membrane [60, 61]. We hypothesize that rather than being simple contaminants, the identified cytosolic ribosome protein subunits are more likely to represent mitochondria-associated proteins.

3.6.3 Transport and locomotion

This class contains proteins involved in protein and ion transport, protein folding and sorting, and mitochondrial locomotion. Mitochondria perform a variety of energy-generating processes, and these metabolic activities require the rapid and highly specific exchange of molecules between the cytoplasm and the mitochondrial matrix. In eukaryotic cells, members of the mitochondrial carrier family (MCF) carry out the transport of metabolites, nucleotides and cofactors across the mitochondrial membranes, connecting cytoplasmic and matrix functions [62]. In this study, we identified several members of the mitochondrial carrier family that participate in ATP/ADP, ornithine/citrulline and carnitine/acylcarnitine exchange, uptake of phosphate, glutamate and folate, as well as the transport of ions, lipids, nucleotides and other substrates.

Mitochondria import hundreds of different precursor proteins from the cytosol and their correct positioning is of great importance to the implementation of their biological functions. The mitochondrial protein import machinery has been studied over the past 20 years, and more than 30 proteins have been identified as being involved in mitochondrial protein sorting mechanisms [59, 63], such as the subunits of the preprotein translocase of the outer membrane (TOM complex) and the inner membrane (TIM complex). In this study we identified 16 components of the import machinery, including Pam16, Tim8, Tim9, Tim10/Tim12, Tim13, Tim17, Tim21, Tim23, Tim44, Tim50, Tom7, Tom20, Tom22, Tom37, Tom40 and Mmp37. The porin/voltage-dependent anion-selective channel protein VDAC, a mitochondrial marker protein and the proton channel uncoupling protein Ucp4 were also identified. We

also identified more than 20 molecular chaperones, including several members of the mitochondrial DnaJ superfamily and HSP70 superfamily, which may facilitate the refolding and relocalizing processes of the imported mitochondrial proteins, and the folding and targeting of mitochondrial genome encoded proteins.

We found cytoskeleton-related proteins in this study, including tubulin, actin, myosin and dynein. It is now known that interactions between mitochondria and the cytoskeleton influence mitochondria fusion, fission, inheritance, localization at sites of high energy demand, respiratory activity and dissemination of mitochondrial apoptotic factors. It is also known that mitochondria interact with different cytoskeletal networks and use diverse cytoskeleton-based mechanisms for the control of their morphology, movement and anchorage within the cell [54, 64]. Therefore, like the cytosolic ribosome subunits, those cytoskeletal proteins may be ''mitochondrial-associated'' proteins too.

3.6.4 Cell defense related

Excess production of ROS may result from defects in electron transport complexes or other perturbations of mitochondria and have associations with a variety of pathologies and aging [1]. We classified the proteins responsible for cell redox homeostasis in this class, as they compose the main antioxidant defense mechanism against ROS, such as H_2O_2, $OH\cdot$, and $O_2\cdot$. We identified several antioxidant proteins and free radical scavengers, including superoxide dismutase SOD1, SOD2, and SOD3, ferredoxin, catalase, glutathione peroxidase, glutathione S-transferase and a few other related proteins.

Apoptosis is now recognized as an important biological process. Proteins associated with apoptosis were also assigned to this class, including proteins promoting apoptosis, such as wah-1, dap-3, mitochondrial endonuclease cps-6, and anti-apoptotic proteins icd-1 and car-1.

3.6.5 Signal transduction

There is increasing interest in intramitochondrial signaling pathways and also in the communication of mitochondria with the cytoplasm and other organelles, notably the nucleus, ER and cytoskeleton.

In line with the previous studies [11, 14], the inventory of this class includes several guanine-, GTP-, $Ca2^+$- and RAS-related proteins, kinases/phosphatases, protease inhibitors, receptors and other proteins that have roles in recognition and communication. Pyruvate dehydrogenase kinase was detected in two isoforms. Two isoforms of the mitochondrial inner membrane protein mitofilin, controlling cristae morphology [65], were also identified. Several proteins assigned to other functional classes also take part in signal transduction, such as the multifunctional cytochrome c and the mitochondrial channel protein VDAC, which is involved in the calcium signaling pathway.

The multifunctional molecular chaperones of the 14-3-3 family are usually thought to be cytosolic proteins; however, in this study we identified two members of this family orthologous with the human 14-3-3 protein epsilon included in the MITOP2 database. 14-3-3 proteins are a family of conserved acidic proteins expressed in all eukaryotic cells. They can interact with over 200 target proteins and control cell cycle, cell growth, differentiation, survival, apoptosis, migration and spreading, and it was reported that they are also a regulator of mitochondrial ATP synthase [66, 67].

It has been realized in the last few years that mitochondria are not only the passive recipients of signals from the other organelles, but are also active participants in signal transduction pathways. The anti-oxidative proteins may also take parts in regulation, as there is increasing evidence that ROS and reactive nitrogen species have effects on various biological process, such as OXPHOS, cellular redox signaling and mitochondrial gene expressing [68, 69].

Above all, as compared with other well-studied aspects of mitochondria, we still know little about what regulates mitochondria and the extent to which mitochondria control the whole cell. Because there is more and more evidence that mitochondria are not innocent bystanders in many human diseases, including cancer [2, 70], a fresh look should be taken at this organelle and comprehensive signaling pathways within mitochondria and between mitochondria and other organelles need to be established.

3.6.6 Unknown function

There are more than 200 proteins without clear descriptions of their molecular functions and this proportion (18.9%) is much higher than that of the recent proteomic study of mouse hearts (6.28%) [10]. This may be largely due to the incomplete database notes for *C. elegans*. As determined using the GO::TermFinder tools, of the identified 1117 proteins, less than half have GOA items for ''biological process'' and only 276 proteins have ''molecular function'' information (database updated to August 16, 2008). Further studies of these proteins may contribute to our better understanding of mitochondria, both about its functional mechanisms and its role in evolution, as more than 20 proteins in this class are described as ''uncharacterized conserved protein.''

3.7 Analysis of related biological processes of mitochondrial proteins and mitochondrial protein candidates

To focus on the roles of mitochondria in the life of *C. elegans*, the GO::TermFinder tools were used to analyze the distribution of mitochondrial proteins and candidate proteins at

various levels of biological processes based on their GO annotations (see Supporting Information Fig. 1 and Supporting Information Table 2). The biggest group was under the term ''metabolism process,'' and the well-studied mitochondrial metabolic pathways, such as ATP synthesis, amino acid metabolism, biosynthesis and organic acid metabolism were highlighted with significant *p*-values, consistent with the central role of mitochondria in the cellular metabolism processes. Besides the obvious predominance of the metabolism-related proteins, the other two largest groups were associated with development and growth.

More than half of the total development- and growth-related proteins are also metabolism-related proteins, including most components involved in the TCA cycle and OXPHOS, indicating a close relationship between metabolism and development. In fact, it has been reported that mitochondrial respiratory chain deficiency results in larval developmental arrest [71]. A previous study analyzed protein expression profiles during *C. elegans* development by 2-DE [72]; of the 76 proteins showing altered expression during different developmental stages, 14 were found in our data of mitochondrial proteins and candidate proteins. Most of them take part in metabolic processes; some of these proteins are Gpd-3, Mel-32, Let-721, Alh-1, Gta-1, Ech-6 and Atp-5.

When organisms' development and growth correlate with increases in cell volume and cell number, the demand for mitochondria grows accordingly. About 25 and 13% of the development- and growth-related proteins are involved in mitochondrial protein biosynthesis and translocation, respectively. There are also prohibitins, kinases, metallo-phosphoesterase and other factors regulating mitochondrial biogenesis, maintenance and their biological functions.

It is known that apoptosis is essential for the development and survival of most multicellular organisms and is required to ensure functional organ architecture and to maintain tissue homeostasis. During development of *C. elegans*, apoptosis claims over 10% of the generated somatic cells [73]. Several apoptosis related proteins, such as Icd-1 and Wah-1, were also classified in the development and growth groups. Icd-1 encodes the *C. elegans* ortholog of the β-subunit of the nascent polypeptide-associated complex (βNAC). During *C. elegans* development, its activity is required for prevention of Ced-4-dependent, but Ced-3-independent, programmed cell death and for normal embryonic development [74]. Wah-1 is expressed in most, if not all, cells of embryos and larvae and is required for apoptotic DNA degradation, rapid apoptosis during embryonic development, rapid engulfment of apoptotic cells, normal growth and large brood size [75, 76].

3.8 A primary analysis of the conserved properties of mitochondrial proteins

The origin of mitochondria has long been discussed, and it is widely believed now that mitochondria originated from a single endosymbiotic event in which an amitochondriate pro-eukaryotic host took up a á-proteobacterial endosymbiont [77, 78]. It is interesting to investigate the conserved properties of mitochondrial proteins during evolution. Taking advantage of the NCBI KOG database as a natural framework for comparative genomes, we compared the mitochondrial proteins of *C. elegans* with their orthologs from the higher animal *Homo sapiens*, the green plant *Arabidopsis*, the fungi *S. cerevisiae*, and the intracellular microsporidian parasite *Encephalitozoon cuniculi*. Mitochondrial proteins and candidate proteins were used to search for orthologs; a total of 595 proteins were searched against the database and more than 400 proteins had entries. The presence and absence of orthologs of these proteins in other species are depicted in Fig. 6. We noticed that except for five proteins, 405 of the *C. elegans* mitochondrial proteins have human counterparts, possibly indicating a relatively high conservation of mitochondrial proteins in higher eukaryotes' evolution. Three hundred and four and 276 orthologs were found for the green plant *Arabidopsis* and the budding yeast *S. cerevisiae*, respectively. Only 62 of the identified *C. elegans* mitochondrial proteins have orthologs in the parasite *E. cuniculi*, as this species has the most distant genetic relationship with the nematode of the four species.

From the perspective of functional classification, it is interesting to note that proteins taking part in protein transport and localization are more conserved than proteins in the other functional classes. For the molecular chaperones involved in protein folding and sorting, six proteins have orthologs in all five species while the other six have counterparts in four species, showing a fairly high conservation. These results are consistent with the previous molecular and biochemical studies, which suggested the conservative properties of the mitochondrial transporter proteins [79] and the chaperone 14-3-3 family [80, 81].

High conservation was also found in some proteins responsible for cell redox homeostasis, such as superoxide dismutase SOD3, glutathione peroxidase, glutaredoxin and related proteins. As organisms began to fight against oxidative stress when they began aerobic respiration, these anti-oxidative proteins may represent components of the ancient cell defense systems of eukaryotic organisms.

3.9 *C. elegans* mitochondrial proteins and associated diseases

Because of its high conservation of genes and metabolic pathways with mammals, the availability of genetic strategies to identify novel proteins, protein interactions and drug targets, and the ease of large-scale cultivation, *C. elegans* has became an ideal model with which to study the pathology of various human diseases and substantial works have been conducted [82]. In this study, we used our dataset of mito-

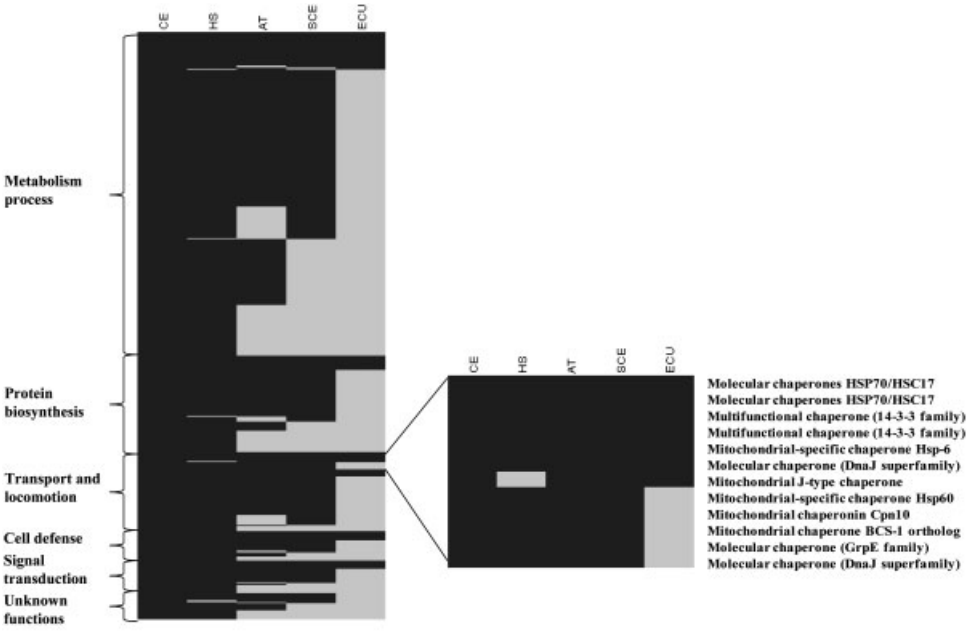

Figure 6. Presence and absence matrix for the 410 *C. elegans* mitochondrial proteins and mitochondrial protein candidates across five model organisms. The highly conserved mitochondrial chaperone proteins are listed on the side. Blue oblongs indicate orthologs of the *C. elegans* protein (row) in a target species (column). CE, *Caenorhabditis elegans*; HS, *Homo sapiens*; AT, *Arabidopsis thaliana*; SCE, *Saccharomyces cerevisiae*; ECU, *Encephalitozoon cuniculi*.

chondrial proteins and candidate proteins to search the KEGG disease database for any potential links between the identified mitochondrial proteins and human diseases. Intriguing results were obtained. For instance, cytochrome c is involved in the pathology of colorectal cancer and small cell lung cancer, and glyceraldehyde 3-phosphate dehydrogenase is involved in three types of neurodegenerative diseases, including Alzheimer's disease, Huntington's disease and Dentatorubropallidoluysian atrophy. In addition to these results, we were surprised to find a possible relationship between mitochondrial proteins and Parkinson's disease (PD).

PD is characterized by the degeneration of dopaminergic neurons in the substantia nigra pars compacta. With a complete cell lineage map and a wiring diagram of all 302 neurons, *C. elegans* has been used as a model system for PD in the past few years [83]. In this study, we found 75 proteins possibly involved in the pathology of PD (see Supporting Information Table 3). More than 90% of these proteins are subunits of the five complexes of the mitochondrial respiratory chain, highlighting the essential role of OXPHOS in the physiological and pathological processes of the organism. Furthermore, human orthologs were found for 56 out of the 75 proteins through orthology comparison. Although the association between mitochondria and PD has long been discussed, and there is evidence that mitochondrial point mutations are involved in PD pathogenesis; the exact role of mitochondria in the pathology of this neurodegenerative disease remains unresolved [84]. We hope our study will help researchers in this area, providing them some clues about which proteins may be responsible for the development of PD.

4 Concluding remarks

In this study, we performed a large-scale identification of mitochondrial proteins of *C. elegans* for the first time and discussed their physicochemical properties, molecular functions and related biological processes, as well as their associations with some human diseases. However, there are also shortcomings in our study, of which the biggest one stems from the tissue heterogeneity of our purified mitochondria. Mitochondria play diverse roles in many physiological and pathological processes, meeting different functional requirements for various tissues. Therefore, mitochondrial proteomes are dynamic and regulated in cell- and tissue-specific manners. In fact, there have been proteomic studies on the heterogeneity of mitochondrial proteins of mice [11, 61] and rats [12, 13], and it was estimated that only about 75% proteins are shared by any two tissues [11]. As functional consequences of mitochondrial heterogeneity are confirmed, including GABA metabolism in the brain, urea synthesis in the liver, and the domination of OXPHOS in the heart [85], we expect that by combining genomic and biochemical strategies, the study of mitochondrial proteins' distribution in various tissues and their expression during different developmental stages will possibly lead to new insights.

C. elegans is a well-studied model organism in the areas of development, aging and some metabolic diseases. Although a great deal of knowledge has been accumulated, much more remains unknown. It is of great importance to increase the depth of knowledge about *C. elegans* mitochondria. With our optimized method of mitochondrion purification, proteomics will surely make significant

progress in this field. Assisted by quantitative technologies, such as N-15 labeling, it is now possible to perform systemic comparisons of *C. elegans* mitochondria, either between mutants and wild type or during different developmental stages, and it is anticipated that further significant contributions to our understanding of this essential organelle are forthcoming with the *C. elegans* model system.

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