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Systematic Characterization of the Murine Mitochondrial Proteome Using Functionally Validated Cardiac Mitochondria

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Abstract

Mitochondria play essential roles in cardiac pathophysiology and the murine model has been extensively used to investigate cardiovascular diseases. In the present study, we characterized murine cardiac mitochondria using an LC/MS/MS approach. We extracted and purified cardiac mitochondria; validated their functionality to ensure the final preparation contains necessary components to sustain their normal function; and subjected these validated organelles to LC/MS/MS-based protein identification. A total of 940 distinct proteins were identified from murine cardiac mitochondria, among which, 480 proteins were not previously identified by major proteomic profiling studies. The 940 proteins consist of functional clusters known to support oxidative phosphorylation, metabolism and biogenesis. In addition, there are several other clusters--including proteolysis, protein folding, and reduction/oxidation signaling-which ostensibly represent previously under-appreciated tasks of cardiac mitochondria. Moreover, many identified proteins were found to occupy other subcellular locations, including cytoplasm, ER, and golgi, in addition to their presence in the mitochondria. These results provide a comprehensive picture of the murine cardiac mitochondrial proteome and underscore tissue- and species-specification. Moreover, the use of functionally intact mitochondria insures that the proteomic observations in this organelle are relevant to its normal biology and facilitates decoding the interplay between mitochondria and other organelles.

Keywords

cardiac mitochondria; mass spectrometry; proteome; sample preparation; target validation

Introduction

Proteins populating an individual organelle contribute to, and often dictate, its biological functions. Compared to global cell proteomic analyses, studies targeting specific organelles offer the advantage of reduced sample complexity along with information about the spatial and

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functional relevance of the identified proteins [1−3]. Mitochondria have recently received extensive attention due to their importance in cellular function and known causative role in diseases [4−6]. Mammalian mitochondria are double-membrane organelles, serving as the metabolic powerhouses of eukaryotic cells. In addition to oxidative phosphorylation machinery and ATP synthesis, mitochondria coordinate functions including ionic homeostasis, apoptotic signaling, fatty acid metabolism and biogenesis.

Computational studies of the human genome have predicted that ~2,000 distinct gene products may constitute the mitochondrial proteome [1,2,4]. The first experimental mitochondrial proteomic survey was carried out in 1998 from human placenta using 2DE separation followed by MALDI analyses and led to the identification of 46 proteins [7]. In the intervening years, major advances in technology enabled subsequent large-scale proteomic investigations, which achieved the identification of a significant number of proteins from yeast [8,9], human [10, 11], and rodent [12−15] mitochondria. These studies substantially contributed to the experimental dataset of mitochondrial proteins. Recently, using an integrative genomic approach, a seminal study by Mootha and colleagues [16] experimentally and computationally expanded the current list of mammalian mitochondrial proteins, creating a mitochondrial protein repertoire across species and cell types. Thus far, three public mitochondrial databases are available: the MITOP.2 (http://www.mitop.de/) [17], the HMPDb (http://bioinfo.nist.gov/hmpd/index.html) and Mito-Proteome (http://www.mitoproteome.org). All three collect data from computational predictions and proteomic mapping of mitochondrial proteins from all organs. However, they lack information representing organ and species origin, as well as the distinct roles that mitochondria (and the associated proteins) play in diseased phenotypes [2,6,16]. In this regard, organ-specific mitochondrial proteomic data obtained by experimental approaches would be more informative.

Cardiac mitochondrial dysfunction has been causally linked to myocardial ischemic injury and cell death [6,18,19]. Because the mouse is an animal model used extensively to study multiple cardiovascular diseases, comprehensive murine cardiac mitochondrial proteomic profiling is a necessary step to understand the function of these organelles during various diseases. Accordingly, in the present study, we focused on the comprehensive characterization of murine cardiac mitochondria by an LC/MS/MS-based approach.

We functionally validated the physiological state of murine cardiac mitochondria prior to systematically characterizing the proteins localized to this organelle. This approach entails careful sample isolation and purification prior to measuring oxygen consumption and ATP generation; only after this validation step are the organelles characterized by LC/MS/MS, followed by annotation of the proteins as well as target validation. With this approach we have identified 940 distinct proteins that grouped into 11 different functional clusters. This study demonstrates that murine cardiac mitochondria host proteins with multiple biological functions and underscores tissue- and species-specification.

Materials and Methods

All procedures were performed in accordance with the Animal Research Committee guidelines at UCLA and the *Guide for the Care and Use of Laboratory Animals*, published by the National Institutes of Health.

1. Materials

Polyclonal anti-ANT1, LAMP1, GRP75, and GRP78 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); Polyclonal anti-DHPRα1 antibodies, Affinity Bioreagents (Golden, CO); Anti-NuMA, HSP60, cathepsin B, metaxin, flotillin-1, Tim50 and

Tim44, BD Pharmingen (San Diego, CA); tetratricopeptide repeat protein 11 (TTC11) and decorin antibodies, Abcam (Cambridge, MA); anti-Bcl-2-like 13 protein polyclonal antibodies, GenWay (San Diego, CA). Anti-VDAC1 protein monoclonal antibodies, EMD chemicals, Inc (San Diego, CA); glutathione peroxidase 1 antibodies, Biogenesis (Kingston, NH); ANT2 antibodies, GenWay Biotech, Incp; n-dodecyl β-D-maltoside (DDM), Avanti Polar Lipid, Inc (Alabaster, AL); HEPES, percoll, cytochrome *c* oxidase assay kit, lactate dehydrogenase (LDH) activity assay kit and all other chemicals, from Sigma-Aldrich (St Louis, MO); and ECL, GE Healthcare (Piscataway, NJ).

2. Isolation of cardiac mitochondria

Mitochondria were isolated from mouse hearts by differential centrifugation [20,21]. For each set of experiments, 45 hearts (8−10 week old, ICR strain) were collected and immediately pooled, minced, and homogenized in isolation buffer (250mM sucrose, 1mM EGTA, 20mM HEPES, pH7.5). The homogenates were centrifuged to remove the nuclear fraction and unbroken cells. The supernatant was then subjected to centrifugation at 4,000g for 20min and the pellet was taken as the crude mitochondrial fraction. Subsequently, the crude mitochondrial pellet was resuspended in 19% percoll in isolation buffer, and slowly layered on two layers of 30% and 60% percoll (v/v) [22]. After centrifugation at 10,000g for 15min, mitochondria were collected and washed (3x) with isolation buffer. The final purified mitochondria were divided into three parallel groups: Group 1 was used for validation including immunoblotting with subcellular location-specific protein markers, LDH activity assay (kit from Sigma), cytochrome c oxidase activity assay (kit from Sigma) (20), functional assays (O_2 consumption and mitochondrial swelling assay), and electron microscopic analyses; Group 2 was used for the LC/MS/MS analyses; and Group 3 for validation of identified proteins by immunoblotting. This entire process was repeated three independent times (each consisting of 45 hearts). All procedures were performed at 4°C.

3. Assessment of mitochondrial function

Mitochondrial O_2 consumption and membrane potential were measured as described [21,24]. Briefly, PO_2 in the buffer was continuously recorded via a fiber-optic oxygen sensor inserted through a hole in the cuvette cover. The tip of the oxygen sensor fiber was positioned in the center of the cuvette where it reacted to changes in PO_2. O_2 consumption was monitored during the addition of different concentrations of ADP. Mitochondrial membrane potential was recorded by including tetramethylrhodamine methyl ester (TMRM; 200nM) in the cuvette solution. $\Delta\Psi$ was estimated from TMRM fluorescence at 580nM and expressed as a percentage of the TMRM fluorescence in the presence of coupled mitochondria and substrates (100%) relative to that after the addition of alamethicin to fully depolarize mitochondria (0%). TMRM fluorescence emission was recorded simultaneously with PO_2.

Mitochondrial permeability transition (MPT) was determined by Ca^{2+}-induced swelling of isolated cardiac mitochondria, which is measured spectrophotometrically as a reduction in absorbance at 520 nm (A_{520}) [20]. Isolated cardiac mitochondria (250μg) were resuspended in swelling buffer (120mM KCl, 10mM Tris-HCl (pH 7.4), 20mM MOPS, and 5mM). MPT pore opening was induced by treatment of $CaCl_2$ to final concentration of 100μM and measured at A_{520nm}. In parallel, the effect of cyclosporin A (CsA) on calcium challenge was measured by preincubating cyclosporin A (final concentration 30nM) with mitochondria 5 min at room temperature followed by calcium treatment.

Mitochondrial suspensions were fixed in 2% glutaraldehyde and 2% folmaldehyde in isolation buffer at room temperature for 2 hours. After washing and post-fixation in 1% OsO_4 in 0.1M phosphate buffer for 1 hour, the suspensions were dehydrated by graded addition of ethanol, followed by treatment with propylene oxide and embeding in Epon. Sections approximately

65−75 nm thick were cut on a Reichert-Jung Ultracult E Ultramicrotome and picked up on formvar coated copper grids. The sections were stained with saturated uranyl acetate followed by Reynolds lead citrate and examined on a JEOL 100CX electron microscope at 80kV [25].

4. Proteomic survey of murine cardiac mitochondria

200μg of percoll-purified mitochondria were resuspended in isolation buffer with 0.5% DDM. After incubation for 30min on ice, the sample was centrifuged at 13,000g for 30min. Supernatant was collected, mixed with Laemmli buffer for 30min at room temperature without boiling, followed by separation with standard SDS-PAGE, and visualization by Commassie Brilliant Blue G250 staining.

Commassie-stained SDS-PAGE gels were sequentially cut into ~3mm strips and gel plugs were subjected to in-gel trypsin digestion following reduction/alkylation as described [26]. LC/MS/MS experiments were performed on an LTQ linear ion trap instrument (ThermoFisher, Waltham, MA) with a Surveyor LC pump system. The peptides were separated on a reversed phase column (75μm *i.d.* 10cm, BioBasic C18 5μm particle size, New Objectives, Woburn, MA, USA). Mobile phase A was 0.1% formic acid, 2% ACN in water, and mobile phase B was 0.1% formic acid, 20% water in ACN. The flow rate was 250nl/min and the following LC gradient was used: 5% B to 40% B in 70min, 40% to 100% B in 20min, and isocratic 100% B for 10min. The mass spectrometer was operated in data-dependent mode to switch between MS and MS/MS spectral acquisition. The normalized collision energy of linear ion trap was set up at 35% for ion fragmentation, the temperature of the ion transfer capillary was held at 190°C and the spray voltage was 1.8kV.

All MS/MS spectra were searched against the IPI mouse database (version 3.26) using the SEQUEST algorithm [27].The database search was performed using the following parameters: partial tryptic digest allowing 2 missed cleavages; differential modification of cysteine with carbamidomethylation (+57Da) and methionine with oxidation (+16Da), the peptide and fragment mass tolerances were set up at 1.5Da and 1.0 Da, respectively. Peptides matching the following criteria were used for protein identification: DeltaCN ≥0.1; Rsp=1; Xcorr ≥ 4.3, 4.7 for partially tryptic peptides with charge state +2 and +3, respectively and Xcorr ≥ 1.6, 2.4, 3.2 for fully tryptic peptides with charge state +1, +2 and +3, respectively [28]. All proteins were identified by more than two unique peptides and those identified with only two peptides were manually verified to minimize false-positive identification. Furthermore, a comprehensive BLAST analysis was performed to remove redundantly identified proteins. In addition, the reversed database search was performed as described [29].

5. Functional annotation

Functional annotation, including sequence features (MW, *pI*, GRAVY, transmembrane domain, transit sequences), gene distribution, gene ontology and InterPro families, were preformed as described [30−34].

6. Statistical Analysis

For swelling assays, data are reported as mean±SEM. Differences among the experimental groups were analyzed using one-way ANOVA. If the ANOVA showed an overall significance, *post hoc* contrasts were performed with Student t test [35].

Results

Part I. Isolation, purification and validation of murine cardiac mitochondria

Crude mitochondrial fractions were isolated from murine hearts by differential centrifugation and further enriched by percoll centrifugation. Subsequently, the purity, integrity and

functionality of mitochondria were determined, which ensured quality and accuracy in the subsequent proteomic studies (Supplemental Materials Figure S1).

First, subcellular marker proteins and LDH activity were used to determine the purity of mitochondria (Figure 1A). Note that the protein markers for non-mitochondrial fractions were undetectable in the post-percoll samples, indicating that this additional step effectively eliminated potential contaminations from lysosome, sarcrolemmal membrane, cytosol, and nucleus. Figure 1A also illustrate that the percoll step had no detectable effect on mitochondrial marker proteins, including HSP60 and GRP 75 (matrix), VDAC (outer membrane) and ANT (inner membrane). The ER protein marker GRP78 remained visible in the post-percoll gradient fraction, suggesting that the purification procedure removed some, but not all, ER proteins.

Next, mitochondrial integrity was examined by measuring cytochrome c oxidase activity (Figure 1B). The mitochondrial outer membrane acts as a barrier for the entrance of substrate into the organelle; therefore, where mitochondria are intact, no activity will be observed. DDM (0.1%) was added to break outer membranes of mitochondria, allowing the exogenous substrate ferrocytochrome c to be in contact with cytochrome c oxidase enzyme located in the inner mitochondrial membrane (thereby catalyzing the light-sensitive assay). Measurements following treatment with DDM were used to estimate the total enzyme activity. Our results showed that the percoll gradient significantly reduced the presence of broken mitochondria in the preparation (from 15.0±2.0% pre-percoll to 4.0±0.9% post-percoll), rendering a preparation with mostly intact outer membranes.

Subsequently, mitochondrial membrane potential and O_2 consumption in response to ADP addition were determined (Figure 1C). Purified mitochondria (0.4 mg/ml) were added to KCl buffer (140mM KCl and 10mM HEPES, pH 7.4), and 5mM P_i and complex I substrates (pyruvate (Pyr), malate (Mal), and glutamate (Glu), each 1.5mM) were added to maintain the mitochondrial function, followed by addition of ADP at the different concentrations listed to stimulate O_2 consumption. As recorded on the top trace, mitochondrial membrane potential ($\Delta\Psi$) dissipated transiently after each ADP addition but fully recovered after a time delay proportional to the amount of ADP added. Simultaneously, mitochondrial O_2 consumption (i.e., buffer PO_2 decrease) was continuously recorded via an oxygen sensor fiber electrode. The O_2 consumption rate, which is represented by the slope of the bottom tracing in Figure 1C, also accelerated transiently during ADP addition, indicating that the mitochondria were indeed functional insofar as they were able to use substrates and consume oxygen to generate ATP from ADP (which ruptured, damaged, or non-mitochondrial organelles are incapable of).

The permeability transition is an organellar death response of mitochondria which causes the matrix to swell as long as the inner membrane is intact. Figure 1D showed that calcium challenge led to mitochondrial swelling, which was prevented by pretreatment with the mitochondrial permeability transition pore inhibitor, cyclosporin A (CsA) [20, 36]. This data indicates the mitochondrial preparation had intact inner membranes, consistent with their ability to generation membrane potential.

Finally, EM was used to verify the morphology of the isolated mitochondria (Figure 1E). As compared to the pre-percoll fractions, mitochondria purified by percoll gradient represented a more homogeneous population of organelles with intact membranes and orthodox cristae structure. Taken together, the studies in Part I demonstrate that the isolated mitochondria used in this study were pure, with intact membranes, and functionally complete metabolic and cell-death responses.

Part II. Characterization of murine cardiac mitochondrial proteome

II.1. Protein identification—Mitochondria were treated with 0.5%DDM to extract membrane proteins, separated by SDS-PAGE followed by Coomassie Brilliant G250 staining. Bands were sequentially cut from the continuum of the gel lane, proteins digested with trypsin, and peptides analyzed by LC/MS/MS (Supplemental Materials Figure S1 and S2). A total of 6732 unique peptides were identified in the present study, corresponding to 940 distinct proteins in murine cardiac mitochondria. The SEQUEST criteria used for peptide identification (see methods) have been shown to afford >95% confidence based on the reversed database search. A detailed list of total proteins identified in this study, together with their molecular weight, p*I*, GRAVY and sequence coverage are provided in the Supplemental Materials Table S1.

II.2. Gene/protein sequence analyses and biochemical features of identified proteins

a. Chromosomal distribution of genes for identified proteins: Among the identified proteins, genome distribution of genes encoding identified proteins was analyzed. Not surprisingly, the vast majority of proteins identified in our study were encoded by the nuclear genome, since only a small number of proteins are encoded by mitochondrial genome. However, we found 11 out of the 13 known mitochondrial-encoded proteins (Figure 2A), of which NADH-ubiquinone oxidoreductase subunit 6 and cytochrome *c* oxidase subunit 3 escaped our LC/MS/MS analyses. NADH-ubiquinone oxidoreductase subunit 6, one of the major proteins in the respiratory chain complex I, is obviously abundant but was not detected. Primary sequence analysis revealed that there are no tryptic cleavage sites on NADH-ubiquinone (molecular weight of 18.6 kDa), explaining why it escaped our analyses. Digestion with other enzymes (such as endoproteinase Glu-C) would likely make this protein detectable via LC/MS/MS in future studies.

We next analyzed the representation of identified proteins across 6 different databases based on the protein having at least one reference in the source databases in the order UniProtKB/Swiss-Prot, UniProtKB/TrEMBL, Vega, Havana, ENSEMBL, RefSeq (Figure 2B). Of all identified IPI entries, 85.88% had a corresponding entry in UniProtKB/Swiss-Prot, a very well-annotated protein database. Of those sequences not represented in UniProtKB/Swiss-Prot 7.18% had an entry in ENSEMBL. Only a very small percentage (0.32%) of identified protein sequences were based only on translations of transcript predictions (RefSeq_model/RefSeq_predicted).

b. Primary sequence features of identified proteins potentially related to function: Of the identified proteins, 469 are predicted to harbor an N-terminal transit sequence regulating mitochondrial import. Since mitochondria are unique double membrane organelles, many of their proteins presumably reside on or within these membranes, necessitating good recovery and identification ability for transmembrane proteins. The occurrence of transmembrane domains in identified proteins was predicted using TMHMM 2.0 [29,31]. Among 940 identified proteins, 195 proteins had at least one transmembrane domain, corresponding to 20.7% of the total identified proteins (Figure 3A), indicating our sample extraction method was reasonably effective in facilitating identification of membrane proteins.

c. Biochemical properties of mitochondrial proteins: We analyzed the biochemical proprieties of identified proteins including: molecular weight (MW, in kDa), isoelectric focusing point (*pI*) and average hydrophobicity (GRAVY) (Figures 3 B-D), as calculated using the online ProtParam tool available through ExPASY (www.expasy.org). As shown in Figure 3B, similar to the result of Taylor et al [11], we found that 50% of identified proteins had predicted molecular weight ≤40kDa, indicating that mitochondria may predominately host

lower molecular weight proteins, potentially because of the limitations of the import and export processes. Figure 3C displays the p*I* distribution of identified proteins, highlighting that more than half of the identified mitochondrial proteins had alkaline p*I* (≥8.0), again consistent with the work of Taylor and colleagues in human cardiac mitochondria. The hydrophobic index GRAVY is also an important parameter for protein characterization. In Figure 3D, the symmetric distribution of GRAVY values indicates a range of hydrophobic character in mitochondrial proteins.

II. 3. Functional annotation of identified proteins—We next sought to understand their function using functional annotations obtained from the Gene Ontology Annotation database (GOA) [37], the InterPro database [38] and Online Mendelian Inheritance in Man (OMIM) [39].

a. Functional and spatial distributions: The 940 mitochondrial proteins were assigned to 11 functional clusters including apoptosis (26 proteins), DNA/RNA/protein synthesis (141), metabolism (257), oxidative phosphorylation (96), protein binding/folding (72), proteolysis (40), redox (32), signal transduction (107), structure (41), transport (107) and cell adhesion (8). We were unable to assign functional group for 59 out of 940 proteins which were therefore classified as “unknown” (Figure 4).

Since a major function of mitochondria is ATP production, it is not surprising that about 9.8% of identified proteins were related to the electron transport chain. Moreover, a significant proportion of proteins were involved in transporting metabolites or proteins (11%), and 26% of proteins were engaged in metabolism of amino acids, nucleotides, lipids, and carbohydrates, supporting the well-established metabolic role of this organelle in many metabolic tasks (Figure 4). We also found a significant umber of proteins involved in the biological processes of apoptosis (Supplemental Materials Table S2A), reduction/oxidation (Supplemental Materials Table S2B) and protein folding. Interestingly, 40 proteins identified from cardiac mitochondria had proteolytic and peptidolytic activities (Supplemental Materials Table S2C), which may play important roles in mitochondrial protein maturation and turnover.

Based on their GO association, 276 proteins were previously annotated to have sub-mitochondrial locations (Supplemental Materials Figure S4). Among them, 33 proteins were identified from the outer membrane of mitochondria (OMM) with multiple functions including apoptosis/signal transduction, metabolism and transport. From the intermembrane space of mitochondria, 12 proteins were found which are involved in apoptosis/signal transduction, electron transport, redox and metabolism. 145 proteins were found in the inner membrane of mitochondria (IMM), and they were preferentially involved in the electron transport chain and transportation; whereas 86 proteins were found in mitochondrial matrix, many of which belong to the function clusters of metabolism and DNA/RNA/protein synthesis.

The mitochondrial electron transport chain consists of a group of protein complexes located at the inner membrane of mitochondria comprising oxidative phosphorylation machinery. Among 90 different subunits within 5 different protein complexes, we identified about 90% of these proteins, including all subunits of complex II (4) and complex V (16), 42 out of 46 subunits of complex I, 9 out of 11 subunits of complex III, and 8 out of 13 subunits of complex IV. In addition, we identified all of the proteins involved in the tricarboxcylic acid cycle.

b. Protein families: The identified proteins were categorized into families based on information from the InterPro database. Protein families were ranked by the relative frequency of identified proteins associated with a particular InterPro family (Table 1) which was then compared to the overall ranking of protein families in the murine proteome represented by all IPI entries. The highest ranking protein family amongst proteins identified in the present study

was Ras GTPase (IPR001806), which was also ranked at #1 in the total murine proteome. The small GTPases have a low molecular weight and generally serve as molecular transducers for a variety of cellular signaling events. The mitochondrial substrate carrier protein family (IPR001993), mitochondrial carrier protein family (IPR002067), adenine nucleotide translocator 1 (ANT1) (IPR002113), mitochondrial import inner membrane translocase subunit Tim (IPR003397, IPR004217) and porin (IPR001925) were found to be the #3, #6, #7, #8, and #9 categories among identified proteins, participating in transporting proteins and metabolites across the mitochondrial double membranes. The other top ten protein categories were metabolism-related proteins, heat shock chaperone proteins, proteolytic proteins and transporters.

c. Disease association: We examined the potential relevance of the identified murine proteins for the study of human diseases using association of their human orthologs with entries in OMIM, a catalogue of human genes and genetic disorders developed by NCBI. Overall, 260 identified IPI entries were described by the known disease gene sequences. In addition, 125 IPI entries were related to disease phenotypes, of which 91 cases had known causative gene sequences and 34 cases had known molecular mechanisms. The specific disease phenotypes indicated for these proteins include Leigh syndrome, Naxos disease, Pheochromocytoma, trifunctional protein deficiency, myopathy due to carnitne palmitoyltransferase II deficiency and others.

II.4. Validation of identified proteins—To further confirm mitochondrial localization of protein identified by mass spectrometry, we carried out target validation by immunoblotting and immunocytochemistry using two independent samples. The top panels of Figure 5 confirmed the expression of five proteins in the mitochondria previously reported by others [11,12,15] including Hexokinase II, Bcl-2 like 13 proteins, tetratricopeptide repeat protein 11 and ANT2. The bottom panels displayed mitochondrial localization of five proteins identified in the present study but were not previously reported [11,12,15] including metaxin, TIM50, decorin, TIM44, and flotillin-1. We also validated the mitochondrial localization of proteins by confocal microscopy (data not shown).

Discussion

The present study presents a proteomic data repertoire of experimentally identified proteins of murine cardiac mitochondria. We find that among the 940 identified proteins; more than 400 were not previously reported to be associated with mitochondria by large scale proteomic screens [11,12,15]. The 940 proteins include a collection of protein functional clusters, implicating multiple biological functions of this organelle beyond their well-known role in biogenesis and metabolism. These investigations provide the basis for future studies in cardiac mitochondrial function and biomarker development for mitochondrial and cardiovascular diseases.

Global organellar proteomics and data discrepancies

Mitochondria are multifunctional organelles in eukaryotic cells, playing a critical role in energy metabolism and have been the focus of recent proteomic studies. Pflieger *et al* first adopted SDS-PAGE and LC/MS/MS to identify 179 proteins from yeast mitochondria in 2002 [8]. Subsequently, Taylor et al characterized human mitochondrial proteome, identifying a total of 615 distinct proteins [11]. More recently, Mootha *et al.* identified 591 mitochondrial proteins, 186 of which were identified from mouse hearts [12]. In another study by the same group [13], 689 proteins were identified from mitochondria of multiple organs including skeletal muscle, heart and liver. A further analysis showed that approximately 50% of these proteins were shared among different organs, suggesting a large number of mitochondrial proteins--the

other 50% or so—may be tissue specific. Recently, Kislinger and colleagues [15] used LC/MS/MS to characterize four organellar compartments including cytosol, membranes, mitochondria and nuclei in six organs (brain, heart, kidney, liver, lung, and placenta) of mice. Among 4768 proteins identified, 662 were from murine cardiac mitochondria. Additional studies characterizing mitochondrial subproteomes of either the inner or outer membranes [40−43] shed light on protein compartmentalization of this organelle. However, a comparison of cardiac mitochondrial proteins illustrated in the large scale proteomic studies by Kislinger *et al.*, Mootha *et al.*, and Taylor *et al*, indicated considerable variations among the reported datasets.

Using functionality and morphological validated mitochondria, we identified a total of 940 proteins. We compared these proteins with those published by other groups [11,12,15]; we treated two different proteins as non-distinct if they share more than 95% amino acid homology and had BLAST e-values less than 10^{-4}. If proteins from different species share 75% homologies, they were also considered to be of the same protein species. Accordingly, 302 out of 940 proteins in our studies were shared in the murine dataset reported by Kislinger *et al*; 147 proteins by the studies from Mootha *et al*. (also in mouse cardiac mitochondria); and 320 proteins by the human mitochondrial proteome reported by Taylor *et al* (Supplemental Materials Figure S5). The present investigation identified 480 murine cardiac mitochondrial proteins not previously reported by any of these three publications [11,12,15]. A comparison of all technology platforms utilized by the different studies was discussed in the Supplemental Materials and the Table S5.

Importance of sample preparation and quality control

One of the challenges for organelle proteomic analysis is to distinguish contaminants (i.e. organelles other than the target) during sample preparations. Sample preparation remains the most critical step allowing for the subsequent functional validations to be conducted with confidence.

For mitochondria, the contaminants obviously arise from the biological milieu in which they reside. The mechanical procedure to release mitochondria from their host cells should be as gentle as possible to preserve the intactness of the mitochondria [44]. We assessed the level of sample contamination by immunblotting with various subcellular compartment-specific marker proteins. Morphological examination by electron microscopy, a traditional and straightforward method, was also applied. Mitochondrial functional assays such as mitochondrial membrane potential measurements and oxygen consumption also provided key aspects for functional validation of the isolated mitochondrial samples.

Although we were effective in removing the majority of contaminants during isolation of murine cardiac mitochondria, protein markers of ER remained prominent in the samples (Figure 1A). Further stripping the mitochondrial outer membrane proteins led to disruptions of mitochondrial function. In fact, there are about 59 proteins identified in the present study were originally annotated to the ER (Supplemental Materials Table S3). Montisano *et al*. [45] observed by electron microscopy that approximately 81% of isolated mitochondria were in contact with rough ER, demonstrating areas of close contacts between these two organelles. These data suggest these contacts may have cellular functions such as communication. Hajoncrky *et al*. [46] also found that the subdomains of the sarco-endoplasmic reticulum are in tight association with mitochondria, suggesting they may mediate calcium signaling to the mitochondria. These studies and our results support the concept that mitochondria may be physically associated with ER and that their interactions maybe critical to facilitate biological functions of these organelles.

Significance of functional clusters in the murine cardiac mitochondrial proteome

Of the 1,158 predicted murine mitochondrial proteins in the Mootha *et al* [16] (corresponding to 960 proteins from IPI v3.26 database), 444 proteins were experimentally confirmed by our study. Interestingly, these experimentally confirmed proteins concentrated on several functional clusters, such as the oxidative phosphorylation system, cell metabolism, and DNA/RNA/proteins biosynthesis (Supplemental Materials Table S4), representing the fundamental physiological functions of this preserved machinery in all cell/organ types. Therefore, it is possible that these differences in dataset of the predicted proteins and experimentally-identified proteins may be related to organ specificity (experimentally measured in the heart in this study versus predicted based upon genomic information in Mootha *et al.*). Among 940 experimentally identified proteins, 496 proteins were not predicted by the studies from Mootha *et al*. The functional annotation of these proteins revealed that these proteins primarily reside in clusters associated with signal transduction, proteolysis, apoptosis and protein-folding processes. These functional roles have been implicated in a diverse range of cardiac diseases, but not specifically related to mitochondrial dysfunction. (Supplemental Materials Table S4, ref.16).

Because of the necessity for interactions between the mitochondrial proteome and its surrounding cellular milieu, proteins/metabolites trafficking across the mitochondrial membranes are inevitable. This process may represent another factor that contributes to why different proteins are predicted based upon genomic information versus experimentally identified by proteomics. Moreover, due to the variety of roles that this organelle plays in many physiological and pathological processes, the mitochondrial proteome maybe highly regulated in cell- and organ-specific manners. Therefore, mitochondrial proteomes identified by experimental approaches are likely to be dynamic and heterogeneous. Expanding our understanding of the functional clusters of proteins in mitochondria (and populating them with new members) may shed light on the biological function of this critical organelle.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

DDM	N-dodecyl β-D-maltoside
COX	cytochrome *c* oxidase
CsA	cyclosporin A
TMRM	tetramethylrhodamine methyl ester
OMM	outer mitochondrial membrane
IMS	inter-membrane space
IMM	inner mitochondrial membrane
pI	isoelectric point
GRAVY	grand average hydrophobicity

ER endoplasmic reticulum

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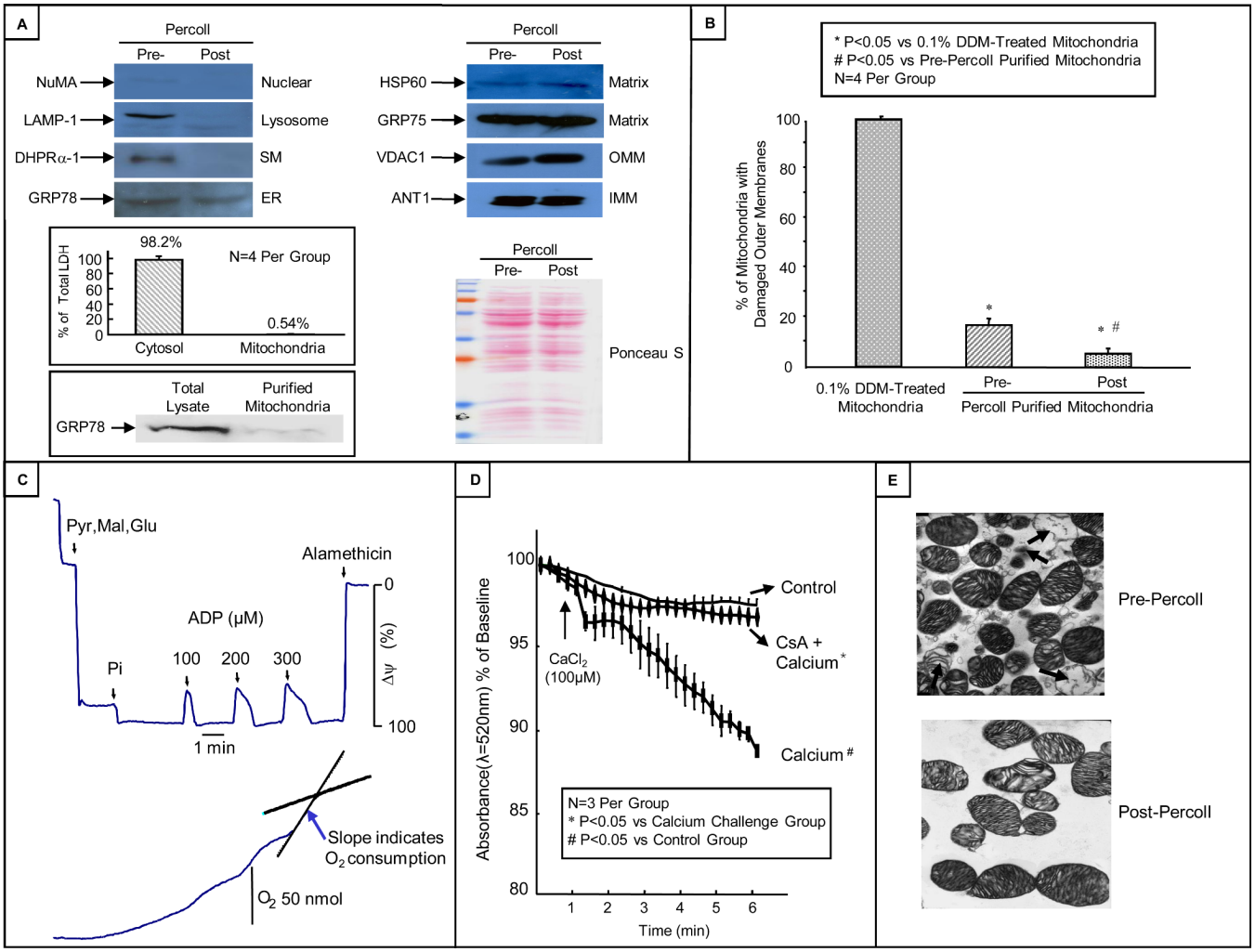

Figure 1. Purity, integrity, functional and morphological validation of purified mitochondria

A. Percoll gradient improved sample purity. Equal amounts (50μg) of mitochondria either with or without further enrichment by percoll purifications were separated by SDS-PAGE followed by immunoblotting with subcellular marker proteins including, LAMP-1 (lysosome), DHPRα1 (sacrolemma [SM]), GRP78 (endoplasmic reticulum [ER]), NuMA (nucleus), as well as LDH activities (left middle panel). Note that percoll effectively eliminated the contaminations from lysosome, sarcrolemmal membrane, cytosol, and nucleus. Percoll step had no detectable effect on mitochondrial proteins (right panel), including HSP60 and GRP 75 (matrix), VDAC (outer membrane) and ANT (inner membrane). Although percoll removed some ER proteins, the ER marker GRP78 remained visible in the post-percoll gradient fraction (left bottom panel). Ponceau S-stained nitrocellulose membranes document equal loading (right bottom panel). **B**. Percoll-gradient removed mitochondria with broken outer membranes. The mitochondrial integrity was assessed by cytochrome c oxidase activity in the presence and absence of 0.1% DDM, which broke outer membranes and established maximal (100%) cytochrome c oxidase activity. The percoll gradient effectively reduced broken mitochondria from 15% to 4% of the total, rendering a preparation with improved integrity. **C**. Functional validation of purified mitochondria. Mitochondrial function was documented by respiratory ratio (>5) and intact mitochondrial membrane potential. Purified mitochondria (0.4 mg/ml) were added to KCl buffer (140 mM KCl and 10 mM HEPES, pH 7.4 with Tris). At the arrows, 5 mM P_i and complex I substrates [pyruvate (Pyr), malate (Mal), and glutamate (Glu), each

1.5 mM] were added, followed by addition of ADP at the indicated concentrations. The *top* tracing shows that membrane potential dissipated transiently after each ADP addition but fully recovered after a time delay proportional to the amount of added ADP. The *bottom* tracing shows that O_2 consumption (i.e., buffer PO_2 decrease) also accelerated transiently during ADP phosphorylation. At the end, alamethicin, a non-specific membrane permeabilizing agent (Ala; 5 μg/ml), was added to induce complete dissipation and maximum swelling for calibration purposes. **D.** Assessment of mitochondrial functionality by swelling assay. Calcium challenge leads to mitochondrial swelling, which could be prevented by pretreatment with cyclosphorin A (CsA). **E.** The morphology of the isolated mitochondria by electron microscope. Arrows in left panel indicate that ruptured mitochondria and non-mitochondrial membranes were virtually absent following percoll (magnification 19,000x).

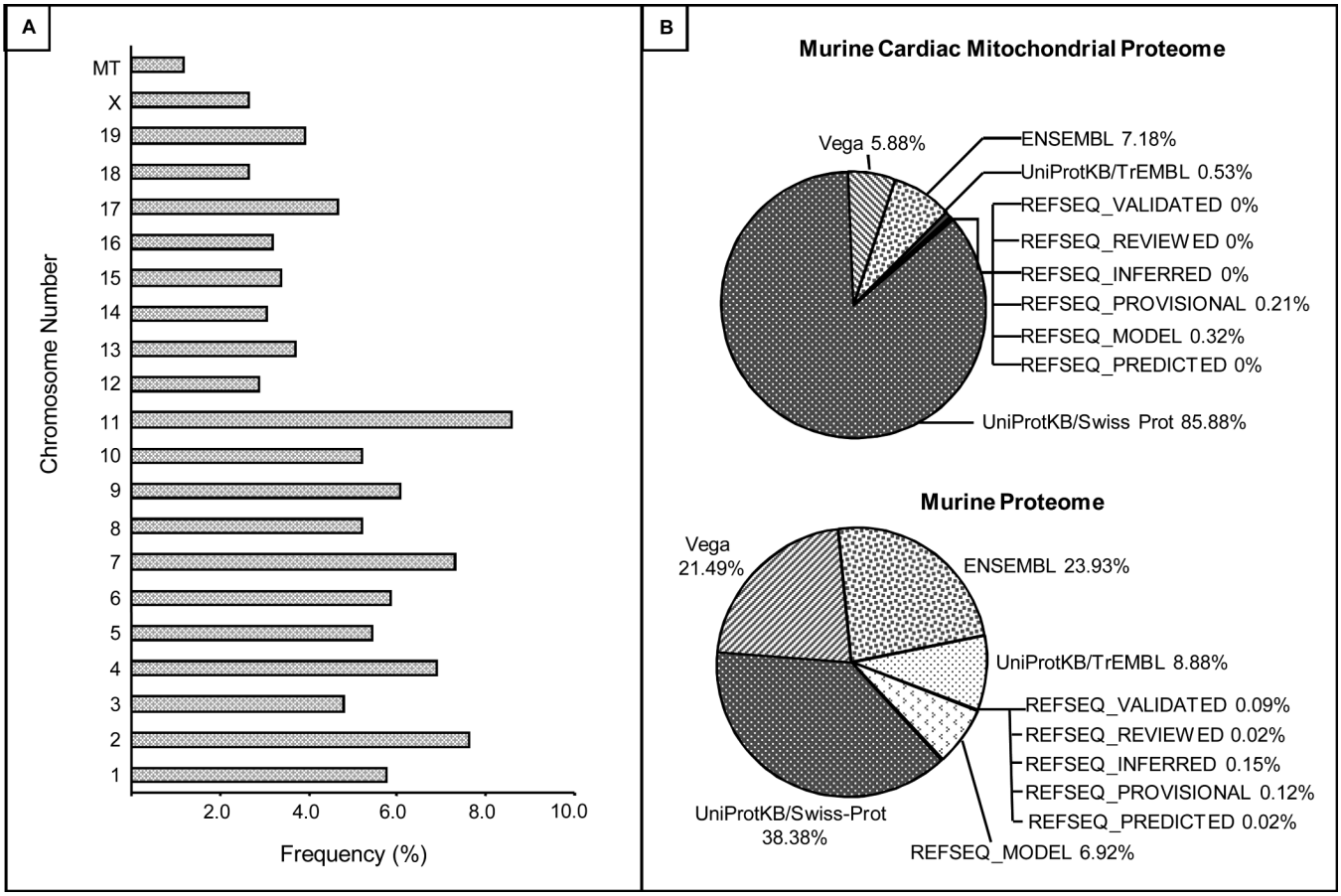

Figure 2. Sequence features of identified proteins in murine cardiac mitochondria

A. Genomic distribution of genes encoding proteins identified in murine cardiac mitochondria. Frequency percentage was calculated by the number of identified proteins localized in individual chromosomes divided by the number of total proteins identified in this study. **B.** Distribution of database sources on identified murine cardiac mitochondrial proteins and the murine proteome. The distribution of identified IPI entries across the underlying databases was based on an entry having at least one reference in the source databases in the order UniProtKB/Swiss-Prot, UniProtKB/TrEMBL, Vega, Havana, ENSEMBL, RefSeq. The murine proteome is represented by all entries in IPI murine.

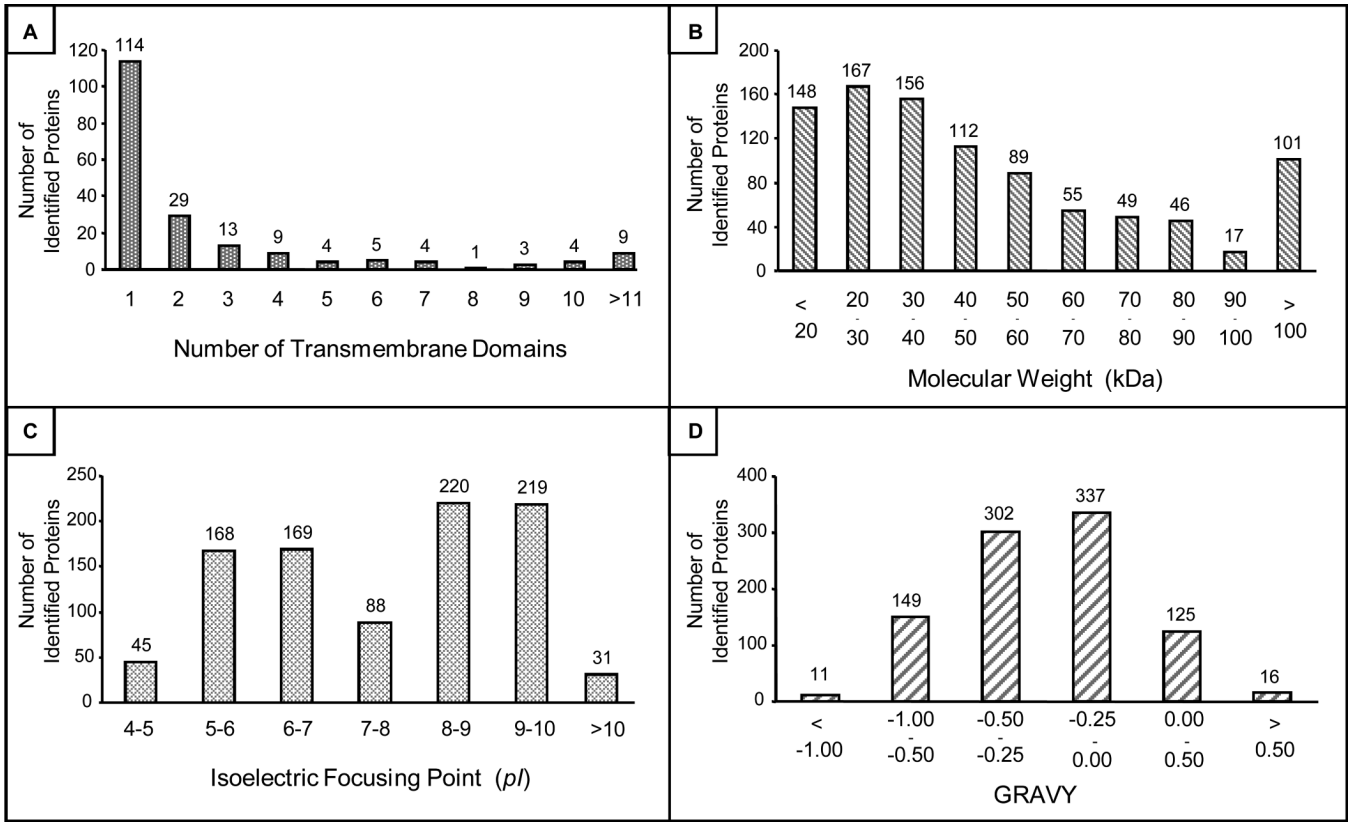

Figure 3. Chemical and physical properties of identified proteins

A. Transmembrane domain and transit peptides were predicted using TMHMM 2.0 and TargetP1.1. Among 940 identified proteins, 195 proteins (20.7%) had one or more transmembrane domains; in addition, 469 out of 940 proteins had mitochondrial transit peptide sequences (inlet). **B-D** Physiochemical property classifications of identified proteins. **B**. Molecular weight (MW, in kDa) analyses; **C**. Isoelectric focusing point (*pI*) distributions; **D**. Grand average hydrophobicity (GRAVY) distributions. Molecular weight, isoelectric point, and grand average hydropathicity value were calculated using the online ProtParam tool available through ExPASY.

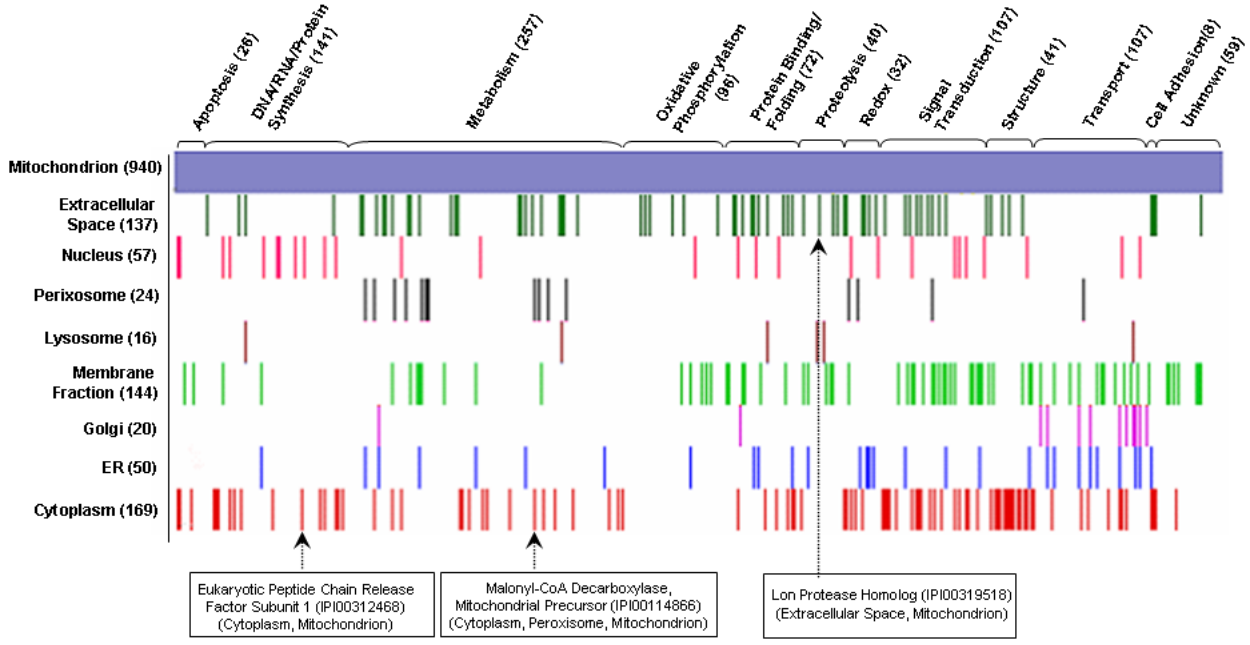

Figure 4. Function annotation and spatial distribution of identified proteins

Individual protein was assigned to their respective function cluster(s) and spatial location(s) according to the Gene Ontology Annotations database (GOA), the InterPro database, OMIM, and author initiated Pubmed search. The *y* axis represents the subcellular compartment(s) where a protein resides, whereas the *x* axis indicates the function cluster it belongs to. Our proteomic approach identified a total of 940 unique proteins residing in the cardiac mitochondria. In addition to mitochondria, many proteins reside in one or more other cellular locations. Among 940 proteins, 169 were also found in cytoplasm, 50 in ER, 20 in Golgi, 144 in sacrolemma or other membrane fractions, 16 in lysosome, 24 in perixosome, 57 in nucleus, and 137 in extra-cellular space. Furthermore, 47 out of 940 proteins were assigned to more than one function cluster(s). Arrows point to three examples, including the eukaryotic peptide chain release factor that is experimentally identified in the cardiac mitochondria; this protein was also previously found in the cytoplasm; and this protein belongs to the function cluster of DNA/RNA/Protein synthesis.

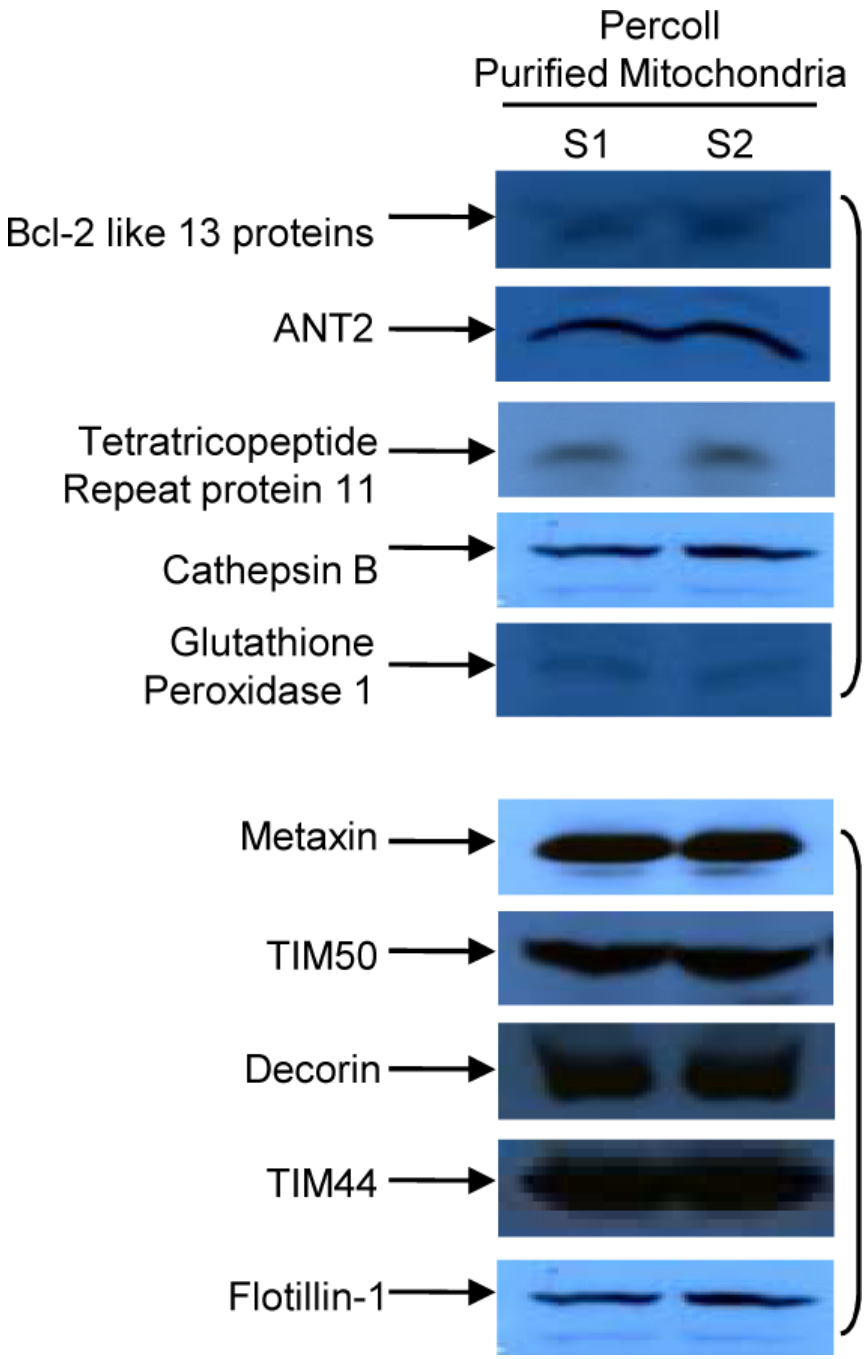

Figure 5. Target validation for identified proteins in murine cardiac mitochondria

Target validation using immunoblottings was conducted using two independent samples (S1 and S2). The upper panels confirm the expression of five proteins in mitochondria documented by previous studies [11,12,15]; the bottom panels validate the mitochondrial localization of five proteins identified in the present study but none of them was previously reported as mitochondria proteins [11,12,15]. Validations of identified proteins were also carried out by confocal immunochemistry, data not shown.