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Mammalian mitochondrial proteomics: insights into mitochondrial functions and mitochondria-related diseases

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Mitochondria are organelles that are essential for cell life and death. A huge range of pathologies, including neurodegenerative diseases, cancer, diabetes and aging, have been reported to be associated with mitochondrial dysfunction. Therefore, identification of mitochondrial proteins that are differentially expressed in these pathologies will help to further our understanding of these diseases. In recent years, great achievements have been made in mammalian mitochondrial proteomics. Here we provide an overview of the current state of knowledge with respect to the whole mitochondrial proteome, the mitochondrial subproteome, mitochondrial complexes and mitochondrial post-translational modifications. Applications of comparative mitochondrial proteomics to various pathologies that have provided clues for understanding the relationship between mitochondrial dysfunction and pathogenesis are described. We conclude that mitochondrial proteomics can be used not only to map all the components of mitochondria, but can also provide information for discovering therapeutic targets for mitochondria-related diseases.

Keywords: 2-DE • BN-PAGE • comparative mitochondrial proteome • DIGE • ICAT • LC-MS/MS • mitochondrial proteome • mitochondrial-related diseases • SILAC

Mitochondria are important organelles that perform several fundamental cellular functions in higher eukaryotes, including oxidative phosphorylation (OXPHOS), substance metabolism, apoptosis and ion homeostasis [1]. It has been reported that defects in mitochondria can lead to a huge range of diseases, such as cancer, diabetes, neurodegenerative diseases and aging [2,3]. Given the central role of mitochondria in cell life and disease, several tools have been developed to study mitochondrial functions. Of these tools, proteomics offers great advantages, as it can profile all of the mitochondrial components and identify disease-associated mitochondrial proteins [4,5], greatly improving our understanding of mitochondrial functions and mitochondria-associated diseases.

Current mitochondrial proteomic research mainly focuses on two aspects: mitochondrial proteome profiling, which involves identifying all the mitochondrial components of a single sample and their post-translational modifications (PTMs) to create a complete mitochondrial database, and comparative mitochondrial proteomics, which involves identifying differentially expressed mitochondrial proteins in disease-altered samples, thus providing information for further understanding the molecular mechanisms of these diseases. Since the first mitochondrial proteome study in 1998 [6], great progress has been made in mitochondrial proteomics. This article focuses on the development of mammalian mitochondrial proteomics, including an overview of the whole mitochondrial proteome, the mitochondrial subproteome, mitochondrial protein complexes and mitochondrial protein PTMs, and a discussion of the applications of comparative mitochondrial proteomics for the study of various disease pathologies.

Mitochondrial proteomic technology

The most direct approach for studying the mitochondrial proteome is to purify mitochondria from tissues or cells and identify mitochondrial proteins using mass spectrometry (MS). A collection of proteomic approaches and

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technologies have been developed for purification of mitochondria and for mitochondrial protein separation, identification and quantification.

Purification of mitochondria

As the most critical issue for mitochondrial proteome analysis is to obtain mitochondria of high purity, different methods have been applied to purify mitochondria from tissues or cells, such as differential centrifugation, density gradient centrifugation with Percoll™ [7], Nycodenz [8], Metrizamide [9] or sucrose [10], free-flow electrophoresis [11] and kit-based methods [12]. Although free-flow electrophoresis has great advantages in terms of the purity and integrity of the isolated mitochondria, the low yield and special equipment requirement limit its application in mitochondrial proteome research. Compared with free-flow electrophoresis, density gradient centrifugation has great advantages both in obtaining functional mitochondria and good yields [12].

After purification, the purity of the mitochondria is assessed by measuring marker enzyme activity and/or western blotting, using markers specific for mitochondria and other common contaminating organelles.

Mitochondrial protein separation

Most protein separation approaches are based on electrophoretic or chromatographic methods. 2D electrophoresis (2-DE) [13], which separates proteins according to isoelectric point (pI) and molecular weight, was the first technology used in mitochondrial proteome research. Although this technique can separate thousands of proteins in one gel, it suffers from several disadvantages when separating mitochondrial proteins, as many mitochondrial proteins are hydrophobic, low-molecular-weight and basic proteins. Although paper bridge loading represents a great improvement to 2-DE for separating basic mitochondrial proteins [14], methodological limitations still exist in its application to the identification of hydrophobic and low-abundance mitochondrial proteins.

To avoid the limitations of 2-DE in the identification of specific classes of mitochondrial proteins, SDS-PAGE coupled with liquid chromatography tandem MS (LC-MS/MS) has been applied in large-scale mitochondrial proteome research [15]. This method takes advantage of the resolving power of electrophoresis in a single dimension and is the most applicable gel-based method for large-scale mitochondrial protein profiling.

Since conventional 2-DE cannot separate intact protein complexes, blue native gel electrophoresis (BN-PAGE) [16] has advantages over 2-DE as it preserves the integrity of these protein complexes and, therefore, is frequently applied in the analysis of mitochondrial protein complexes. In this technique, protein complexes are solubilized and resolved in native form in the first dimension. 2D-SDS-PAGE then denatures the complexes and resolves them into individual subunits.

Complementary gel-free methods for mitochondrial proteome analysis rely on LC for the separation of proteins or peptides. An important advance in LC is multidimensional protein identification technology (MudPIT) [17], which combines two stationary phases (usually strong cation exchange [SCX] and reversed-phase [RP]) to increase their separation power. Chromatographic methods are usually coupled on-line with MS to enable automated protein identification and quantification.

Mitochondrial protein identification

By virtue of its sensitivity and speed, MS has become the workhorse of protein identification. In combination with separation techniques, MS has been widely used in mitochondrial proteome research. The most frequently used mitochondrial protein identification strategies are 2-DE coupled with MALDI-TOF-MS or LC-MS/MS, SDS-PAGE coupled with LC-MS/MS, and 2D-LC-MS/MS.

Mitochondrial protein quantification

Strategies used in mitochondrial protein quantification include gel-based and MS-based (or gel-free) approaches. In gel-based strategies, proteins are separated by gel electrophoretic methods, including 2-DE and 2D difference gel electrophoresis (DIGE), coupled to MS. 2-DE is the classical approach taken for mitochondrial protein quantification, while DIGE is an improvement on 2-DE in which the proteins of two samples labeled with different fluorescent dyes are separated in the same gel. This technology overcomes inter-gel variability by using an internal standard thus allowing accurate relative quantification. However, 2D gel-based approach has many limitations, including limited dynamic range, difficulty in detecting hydrophobic mitochondrial proteins and proteins with extreme molecular weights and pI values.

In contrast to the gel-based approaches, in which quantification was conducted by comparing gel images, MS-based strategies, including stable isotope-labeling quantification and label-free quantification, rely on mass spectrometric data to perform comparison. In stable isotope-labeling strategies, relative quantification is performed by introduction of a chemically equivalent differential mass tag that allows comparative quantification of proteins in different samples. Proteins or peptides in different samples can be labeled metabolically with stable isotope amino acids (Stable isotope labeling by amino acids in cell culture [SILAC]) [18], chemically with stable isotope tags (Isotope-coded affinity tag [ICAT] [19] and isobaric tags for relative and absolute quantitation [iTRAQ]) [20] or enzymatically with ^{18}O during proteolytic digestion [21]. These stable isotope-labeling methods have great advantages in quantification accuracy and reproducibility when using quantitative proteomic techniques to study protein changes in complex samples. However, most labeling-based quantification approaches suffer potential limitations, including limited linear dynamic range, increased time and complexity of sample preparation and high cost of the labeling reagents. Furthermore, to date only iTRAQ allows the comparison of multiple (up to eight) samples at the same time. The other labeling methods can only compare protein changes between two and three different samples.

Mass spectrometry-based label-free quantitative proteomics falls into two major categories. The first one is based on the measurement and comparison of the mass spectrometric signal intensity of peptide precursor ions belonging to a particular protein [22],

and the second one is by counting the number of fragment spectra identifying peptides of a given protein [23]. Although these approaches are the least accurate among the MS-based quantification strategies, they provide a higher dynamic range of quantification than stable isotope labeling. Moreover, there is no limit to the number of experiments that can be compared in label-free quantification. Therefore, label-free quantification approaches are advantageous when investigating large and global protein changes between experiments [24]. The label-free quantitative proteomics technique has been used to systematically analyze tissue-specific mitochondrial proteome changes in normal and diabetic mice [25].

To summarize, different quantitative strategies have their advantages and limitations, choice of an appropriate strategy for a given situation will depend on the experimental design and aims.

Mitochondrial proteome profiling

Whole mitochondrial proteomes

The earliest research on the mitochondrial proteome was carried out 12 years ago on human placental mitochondria using differential centrifugation and 2-DE separation followed by protein identification with peptide mass fingerprinting (PMF) and N-terminal sequencing [6]. A total of 46 proteins were identified in this mitochondrial proteome study; however, most of these proteins were cytoskeleton contaminants. Since then, mitochondrial proteomes of different species (human [9,26], rat [27,28], mouse [29] and yeast [30,31]), and different tissues and cells have been reported, thus greatly enriching the mitochondrial protein database. Herein, we focus on the mammalian mitochondrial proteome and give a brief summary of methods for mitochondrial purification, mitochondrial protein separation and identification (Table 1).

In 2002, Fountoulakis and coworkers purified mitochondria from rat liver by differential centrifugation and identified proteins by 2-DE coupled with MALDI-TOF-MS [27]. A total of 192 proteins were identified, but 30% of them were annotated as contaminants. In order to remove contaminants from other organelles, they purified mitochondria from the neuroblastoma cell line IMR-32 by metrizamide gradient centrifugation [32]. Although 185 proteins were identified, most of them were abundant and high-molecular-weight proteins, while hydrophobic and low abundant proteins were still under-represented in the study. This under-representation of hydrophobic proteins may be due to the limitations of 2-DE. To overcome these limitations, alternative separation methods including SDS-PAGE coupled with LC-MS/MS and 2D-LC-MS/MS were developed to separate and identify mitochondrial proteins, thus greatly advancing mitochondrial proteome research. Taylor and coworkers purified human heart mitochondria using a metrizamide density gradient and analyzed proteins with SDS-PAGE coupled with LC-MS/MS [9]. A total of 615 proteins were identified in the purified mitochondria, most of which were related to signal transduction, ion transport, lipid metabolism and RNA, DNA and protein synthesis. In addition, 19% of the proteins listed in the study had unknown functions, indicating that novel components are present in mitochondria. This was the first large-scale mammalian mitochondrial proteome study. In a later study, Gaucher and coworkers expanded the number of human heart mitochondrial proteins to 680 by using both SDS-PAGE coupled with LC-MS/MS and multidimensional LC-MS/MS [33]. Their results suggest that combined use of different analytical strategies helps to increase mitochondrial proteome coverage.

Since mitochondria from different tissues have distinct morphologies, structures and functions, and mitochondrial proteins are expressed in a tissue-specific manner, characterizing mitochondrial components from different tissues will help in understanding tissue-specific mitochondrial functions [34]. The first integrated analysis of a tissue-specific mitochondrial proteome was carried out by Mootha and coworkers, who purified mitochondria from four mouse tissues (brain, heart, liver and kidney) with a Percoll density gradient and then identified mitochondrial proteins with SDS-PAGE and LC-MS/MS [35]. A total of 399 mitochondrial proteins were identified from the four tissues, of which 163 proteins had not previously been reported to be associated with mitochondria. Further investigation of this proteome data set revealed that only half of the proteins were expressed in all four tissues, indicating that a great number of mitochondrial proteins were expressed in a tissue-specific manner. Similar conclusions have also been drawn by mitochondrial proteome studies from different mouse and rat tissues [7,28,36].

The most comprehensive mammalian mitochondrial proteome to date was reported by Pagliarini and coworkers [7]. By purifying mitochondria from 14 mouse tissues with a Percoll density gradient and analyzing the mitochondrial proteins using MS, they identified 709 mitochondrial proteins. After integrated analysis with green fluorescent protein (GFP) tagging and bioinformatics, they created a mitochondrial compendium (MitoCarta) with 1098 proteins that may serve as a foundation for mitochondrial biology in the future.

Although in-depth study of the mitochondrial proteome from numerous tissues or cells has led to the identification of thousands of mitochondrial proteins, the number of mitochondrial proteins remaining to be identified is still an open question. Further study with different tools will give us some answers.

As shown in Table 1, nearly every mitochondrial proteome investigated so far includes several 'unknown proteins', that is, proteins that have not been previously reported to be associated with mitochondria. Therefore, one challenge faced in mitochondrial proteome research is to validate whether these unknown proteins are truly novel mitochondrial proteins or contaminants from other organelles. Several methods have been applied to solve this problem, one of which is based on confocal microscopy using antibodies or GFP tagging to determine the subcellular localization of the unknown proteins, and the other is based on *in silico* predictions of their locations using several bioinformatic tools (Table 2), such as TargetP1.1 [37], MitoProt [38], Predotar [39], SubLoc [40] and PSORT [41,42]. However, the specificity and sensitivity of these programs is still under discussion. A study conducted by Jiang and coworkers revealed that using a combination of TargetP and PSORT gave high specificity for mitochondrial protein prediction [43]. Another method for discriminating truly mitochondrial proteins from contaminants is based on quantitative proteome analysis of different subcellular fractions with SILAC [34] or ICAT [43].

Table 1. Progress in mammalian mitochondrial proteomics.

Species, tissues and cells	Publication (year)	Approaches		Proteins identified (n)	Unknown proteins	Ref.
		Mitochondrial purification method	*Analytical method*			
Human						
Placental	1998	Differential centrifugation	2-DE, PMF, N-terminal sequencing	46		[6]
Heart	2003	Metrizamide density gradient	1DE-LC-MS/MS	615	117	[9]
Neuroblastoma cell line IMR-32	2003	Metrizamide density gradient	2-DE, MALDI-TOF MS	185		[32]
Heart	2004	Metrizamide density gradient	1DE-LC-MS/MS and MDLC-MS/MS	680	107	[33]
Jurkat T cell	2005	Sucrose density gradient	1DE-LC-MS/MS	680	453	[10]
Skeletal muscle	2009	Differential centrifugation	1DE-LC-MS/MS	823		[26]
Rat						
Liver	2002	Differential centrifugation	2-DE, MALDI-TOF MS	192	10	[27]
Three tissues (muscle, heart and liver)	2006	Percoll density gradient	1DE-LC-MS/MS and 2D-LC-MS/MS	689		[28]
Mouse						
Four tissues (brain, heart, kidney and liver)	2003	Percoll density gradient	1DE-LC-MS/MS	399	163	[35]
Liver	2003	Nycodenz density gradient	2D-LC-MS/MS	182	20	[46]
Embryonic spinal cord uroblastoma cell line	2004	Percoll density gradient	2-DE, MALDI-TOF MS, MALDI-TOF-MS/MS and LC-MS/MS	470	75	[87]
Heart	2008	Percoll density gradient	1DE-LC-MS/MS	940	480	[29]
14 tissues	2008	Percoll density gradient	1DE-LC-MS/MS	1098		[7]
Brown and white adipose	2009	Percoll density gradient	1DE-LC-MS/MS	1404		[34]

1DE-LC-MS/MS: SDS-PAGE coupled with tandem mass spectrometry; 2-DE: 2D electrophoresis; 2D-LC-MS/MS: 2D liquid chromatography coupled by tandem mass spectrometry; MALDI-TOF MS: Matrix-assisted laser desorption ionization time of flight mass spectrometry; PMF: Peptide mass fingerprinting.

Future experimental evaluation with biochemical and molecular tools would provide more information regarding the subcelluar locations and functions of these unknown proteins.

Mitochondrial subproteomes

Mitochondria are unique organelles with two membranes, which separate the organelle into four compartments: the outer membrane, the intermembrane space, the inner membrane and the matrix. Each compartment has a different protein composition, for example, the matrix contains approximately two-thirds of the total mitochondrial proteins, but the mitochondrial outer and inner membrane proteins only account for 4 and 29% of the total mitochondrial proteins, respectively [44]. The high abundance of soluble proteins in the matrix makes detection of low abundance and hydrophobic mitochondrial membrane proteins difficult in proteomic analysis. Several studies have focused on the mitochondrial subproteome to detect low abundant mitochondrial proteins, especially mitochondrial membrane proteins. In 1967, Sottocasa and coworkers developed the method to separate the inner and outer mitochondrial membranes by sonication or swelling–shrinking of mitochondria followed by discontinuous density gradient centrifugation and this method is useful for mitochondrial subproteome studies [45]. Da Cruz and coworkers focused on liver mitochondrial inner-membrane proteins [46,47]. By purifying mitochondria using Nycodenz density centrifugation and extracting inner membrane proteins with organic acid, they identified 182 proteins with 2D-LC-MS/MS, most of which were annotated as participating in several biochemical processes, such as electron transport, protein import, protein synthesis, lipid metabolism and ion or substrate transport. In addition, they also identified 20 proteins that were not previously reported to be associated with the mitochondrial inner membrane. McDonald and coworkers expanded the rat liver inner mitochondrial membrane subproteome by using three separation methods (2D-LC with ProteomeLab™ PF 2D Protein Fractionation System, RP-HPLC and 2-DE) and identified 348 proteins [48]. Data analysis revealed that 82% of these proteins had not been observed in previous liver mitochondrial membrane inner proteome studies and 44% had not been identified in previous intact mitochondrial proteome studies. Both studies suggested that prefractionation

of mitochondrial subcompartments helps to enrich low-abundance membrane proteins, and thus facilitate their identification. Another study conducted by Distler and coworkers focused on the mitochondrial outer-membrane proteome and led to the identification of several mitochondrial outer membrane proteins (VDAC, CPT-I and LCAS) with exceptionally high sequence coverages (82–99%) using a two-step digestion with trypsin and proteinase K [49]. This newly improved protein digestion method will help to identify more membrane proteins. Although the focus of this review is placed on mammalian mitochondrial proteomics, it is worth noting that mitochondrial subproteomes in yeast are much more advanced. Zahedi and coworkers identified 112 outer membrane proteins in *Saccharomyces cerevisiae* with a coverage of approximately 85% known outer-membrane proteins [50]. Useful information regarding mitochondrial outer-membrane purification and hydrophobic protein separation and detection can be obtained from this study.

Mitochondrial subproteomics is thus valuable for identifying low-abundance mitochondrial proteins and for discovering novel hydrophobic membrane proteins that perform important functions in mitochondria. However, the mitochondrial subproteome also faces the purity problem; a critical assessment of the purity of different fractions with western blot or measurements of marker enzyme activity would help to reduce contaminations of the subproteome.

Several studies have centered on mammalian mitochondrial ribosomes (mitoribosome) [51–53]. Mammalian mitochondria are responsible for the synthesis of 13 mitochondrial genome-encoded polypeptides that are components of the OXPHOS system. Mammalian mitochondrial ribosomes are composed of small (28S) and large (39S) subunits. The ribosome small subunit contains a 12S rRNA and approximately 30 proteins, whereas the large subunit contains a 16S rRNA and approximately 50 proteins [52]. Suzuki and coworkers identified 31 proteins in the bovine mitoribosomal large subunit [54] and 21 proteins in the bovine mitoribosomal small subunit [51]. Although these studies provide some information on mitochondrial protein synthesis, the number of proteins identified in these studies was lower than expected, possibly because the analytical method used was 2-DE, which results in loss of low-molecular-weight, acidic and basic ribosomal proteins.

Mitochondrial protein complexes

There are many protein complexes embedded in mitochondrial inner and outer membranes, which serve important roles in biological processes, such as OXPHOS and protein import. As mentioned earlier, BN-PAGE coupled with MS has played an important role in the analysis of some important mitochondrial complexes [55] and detection of novel protein complexes [56]. Devreese and coworkers separated the five OXPHOS complexes of human heart mitochondria with BN-PAGE and identified them with PMF and LC-MS/MS [57]. The authors not only detected 60% of the components of the known OXPHOS proteins, but also discovered a novel variant of a cytochrome c oxidase subunit – Vic (Glu40Asp). Similarly, Meyer and coworkers discovered two new ATP synthase-associated membrane proteins – AGP and MLQ – using BN-PAGE [58]. These two proteins were lost during purification in previous studies because of their small mass (smaller than 7 kDa) and extreme pIs (close to 10). These two studies suggest that BN-PAGE is a powerful tool for separating intact inner membrane OXPHOS protein complexes and for discovering novel complex-associated components. In addition, BN-PAGE has also been used to study mitochondrial outer-membrane complexes, such as the translocase of the outer membrane (TOM) complex [59] and the liver carnitine palmitoyltransferase 1(CPTI) complex [60]. In addition to its use in discovering novel complex components under physiological conditions, BN-PAGE has also served as an important tool for investigating mitochondrial OXPHOS-related defects involved in disease. LopeCampistrous and coworkers discovered an unknown assembly defect in mitochondrial OXPHOS complexes I, III, IV and V in the mitochondria of brain and brain stem tissues from hypertensive mice [61]. These assembly defects in the mitochondria impair the function of the mitochondrial respiration chain, providing new clues for understanding the molecular mechanisms of hypertension.

In addition to BN-PAGE and MS analysis, alternative methods, such as affinity fractionation and HPLC, have also been applied to discover novel complex components. Schilling and coworkers purified bovine and mouse heart mitochondrial OXPHOS complex II and III by immunoprecipitation, coupled with SDS-PAGE separation and MS analysis, and identified all the components of these two complexes except subunit 11 of complex III [62]. Several PTMs were also identified, since the MS identification sequence coverage was high. In another study, Carroll and coworkers discovered four novel nuclear-encoded subunits from bovine heart mitochondrial complex I using three independent methods: SDS-PAGE, 2-DE and RP-HPLC [63].

In summary, BN-PAGE and other separation methods are important tools not only for discovering novel complex components under physiological conditions, but also for identifying disease-associated mitochondrial protein complex defects.

PTM of mitochondrial proteins

As PTMs of mitochondrial proteins have been reported to control mitochondrial function, numerous studies involving investigation of the PTM of mitochondrial proteins using proteomic tools have been published.

The most frequent protein PTM is phosphorylation and several mammalian mitochondrial phosphoproteomes have been published. Schulenberg and coworkers detected 13 phosphoproteins from steady-state bovine heart mitochondria using Pro-Q diamond staining [64]. Hopper and coworkers identified 45 phosphoproteins from the pig heart mitochondrial matrix using methods similar to those of Schulenberg [65]. However, neither of these studies provided information on protein phosphorylation sites. Lee and coworkers identified 84 phosphorylation sites in 62 phosphorylated proteins from mouse liver mitochondria using improved immobilized metal ion affinity chromatography (IMAC) coupled with LC-MS/MS [66]. Recently, we identified 144 phosphorylation sites in 84 different mitochondrial proteins from INS-1β

Table 2. Useful tools for *in silico* predictions of mitochondrial proteins.

Tool	Website	Description	Ref.
TargetP	www.cbs.dtu.dk/services/TargetP	Predicts mitochondrial proteins based on N-terminal targeting signal analysis	[37,121]
Predotar	http://urgi.versailles.inra.fr/predotar/predotar.html	Predicts mitochondrial proteins based on N-terminal targeting signal analysis	[39]
MitoProt	http://ihg2.helmholtz-muenchen.de/ihg/mitoprot.html	Predicts mitochondrial proteins based on N-terminal targeting signal analysis	[38]
SubLoc	www.bioinfo.tsinghua.edu.cn/SubLoc	Predicts protein subcellular localization based on amino acid composition	[40]
PSORT	http://psort.ims.u-tokyo.ac.jp/form2.html	Predicts mitochondrial proteins based on N-terminal targeting signal analysis	[41,42]
Mitopred	http://bioapps.rit.albany.edu/MITOPRED	Predicts nuclear-encoded mitochondrial proteins based on the occurrence patterns of protein domains, amino acid composition and pI value	[122]
Cell-PLoc	www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc	Predicts subcellular localization of proteins in different organisms	[123]

cell mitochondria and constructed an interaction network of these mitochondrial phosphoproteins and their putative kinases [67]. Recent studies have revealed that some kinase signaling pathways, such as protein kinase A (PKA), protein kinase B/Akt, protein kinase C, Raf-MEK-ERK, JNK/SAPK and p38 MAPK, can be targeted to mitochondria, where they modulate mitochondrial activity and functions [68–71]. All these mitochondrial phosphoproteome results indicate the importance of reversible phosphorylation in the regulation of mitochondrial functions, but exactly how phosphorylation modulates mitochondrial functions still awaits further investigation.

Besides phosphorylation, other modifications of mitochondrial proteins, such as acetylation and oxidative modifications, have also been investigated with proteomic approaches. A study focusing on modifications of bovine heart mitochondrial complex I revealed that 14 of its 39 nuclear-encoded subunits had modified N termini; 13 out of these 14 modifications were N-α-acetylated and the remaining one was N-α-myristoylated [72]. As mitochondria are the site of reactive oxygen species (ROS) generation, some mitochondrial proteins are also subjected to ROS-induced oxidative damage, which is linked to aging, neurodegeneration and cell death. Murray and coworkers investigated peroxynitrite-induced tyrosine modification in mitochondrial OXPHOS complexes by coupling 2D-PAGE, immunological staining and mass spectrometric analysis [73]. With this approach, the authors showed that peroxynitrite-induced tyrosine modifications are predominately associated with some subunits of complex I. Taylor and coworkers investigated oxidative modifications of tryptophan in the normal human heart mitochondrial proteome and discovered that some subunits of complex I and complex V are targets of oxidative modifications [74]. Hunzinger and coworkers investigated bovine heart mitochondria using four types of 2D-PAGE and revealed multiple aconitase-2 isoforms with two N-formylkynurenine modifications (products of the dioxidation of tryptophan residues), which act as a protein biomarker signature for ROS damage [75]. Lin and coworkers focused on the modifications of mitochondrial protein thiols [76]. Combining (4-iodobutyl) triphenylphosphonium labeling with 2-DE, they found a number of matrix proteins with active thiols. This study provided a novel method for measuring the thiol redox state in individual mitochondrial proteins. Recently, Choksi and coworkers studied heart mitochondria from young, middle-aged and old mice and revealed an age-related increase in oxidative modifications of complex I and V subunits [77]. All these results suggest that characterization of the PTMs of mitochondrial proteins with proteomic tools will improve our understanding of mitochondrial functions. Recently, Vögtle and coworkers performed a first comprehensive analysis of the N-proteome of yeast mitochondria and identified the N-termini of 615 different proteins [78] which provided useful information for the characterization of mitochondrial presequences, thus serving as important foundations for the analysis of mitochondrial protein targeting, cleavage and turnover.

Application of comparative mitochondrial proteomics to disease pathology

Since mitochondrial dysfunction has been implicated in a large number of diseases, it is expected that comparing mitochondrial proteomes should help us find disease-related proteins and provide valuable information for gaining a better understanding of mitochondrial-related diseases.

Neurodegenerative diseases

Mitochondrial defects have been implicated in all the major neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD) and familial amyotrophic lateral sclerosis (ALS) [79,80]; however, the pathogenesis of these diseases is poorly characterized. Several comparative mitochondrial proteome studies have been conducted in cell and animal models

of these diseases to find differentially expressed mitochondrial proteins and to discover the relationships between mitochondrial protein alterations and the onset of disease.

Alzheimer's disease is a common neurodegenerative disease, characterized by neurofibrillary tangles composed of hyperphosphorylated tau proteins, neuropil thread formation and deposition of amyloid β peptides (Aβ) [81]; however, the exact molecular mechanism of this disease remains elusive. Proteomic methods have been applied to identify disease-associated mitochondrial proteins. Lovell and coworkers investigated the effects of Aβ on the mitochondrial proteome using ICAT and 2D-LC-MS/MS by comparing mitochondrial protein expression between primary neuron cultures and primary neurons exposed to Aβ for 16 h [82]. A total of 45 proteins were quantified and the expression of ten proteins was found to be significantly altered in Aβ-treated cultures. Among these significantly altered proteins, several proteins related to mitochondrial energy metabolism, neuronal survival and programmed cell death were upregulated after treatment with Aβ. Gillardon and coworkers compared protein changes in brain mitochondria of Tg2576 mice (a mouse model of AD), and discovered changes in respiratory chain complexes I and III and impaired respiration in mitochondria, suggesting that some links exist between mitochondrial dysfunction and AD progression [83]. Mitochondrial protein modifications are also known to be involved in the pathogenesis of AD [84].

Parkinson's disease is the second most common neurodegenerative disease after AD. Although exact mechanisms are unknown, evidence suggests that mitochondrial dysfunction is involved in the pathogenesis of this disease. Jin and coworkers used SILAC to investigate the effect of rotenone (a mitochondrial complex I inhibitor that induces neurodegeneration closely resembling PD) on mitochondrial protein profiles in dopaminergic MES cells [85]. Results showed that the expression of 110 mitochondrial proteins was significantly altered after stimulation with rotenone and the relationship of two of these proteins, SHH and SNX1, to PD pathogenesis was validated. In another study, Jin and coworkers used ICAT to compare mitochondrial protein expression in the substantia nigra of a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)/probenecid (prob)- induced PD mouse model (MPTP/prob-treated mice) [86]. More than 100 proteins displayed significant changes in MPTP/prob-treated mice and one protein, DJ-1, was shown to be involved in mitochondrial dysfunction and the pathogenesis of PD. This study suggests that MPTP-induced mitochondrial dysfunction results in decreased ATP production and increased production of ROS, and serves as the mechanism of MPTP-induced PD. These two models can thus make a contribution to our understanding of the pathogenesis of PD.

Comparative mitochondrial proteomic methods have also been used to identify biomarkers and elucidate the mechanisms involved in ALS [87].

Cancer

As mitochondrial dysfunction and mitochondrial DNA mutations have frequently been reported in many cancers, identification of mitochondrial proteins that are differentially expressed in cancer cells should lead to the discovery of new cancer biomarkers for early cancer detection, diagnosis and prevention [88,89]. Herrmann and coworkers used laser capture microdissection of cancer tissues and RP protein lysate arrays to investigate the relationship between the ratio of mitochondrial genome-encoded cytochrome c oxidase subunits to nuclear-encoded cytochrome c oxidase subunits and prostate cancer progression, and discovered an increased ratio of nuclear to mitochondrial genome-encoded cytochrome c subunits during the progression of prostate cancer [90]. Similar results have also been observed in prostate-derived cell lines and urothelial carcinoma cell lines [91]. Kim and coworkers compared mitochondrial alterations in the normal gastric cell line RGM-1 and the human gastric cancer cell line AGS, and discovered that not only were mitochondrial functions and morphology in an abnormal state in AGS, but also that the expression of four mitochondrial proteins was higher in AGS [92]. These results provide us with some clues for understanding the relationship between mitochondrial dysfunction and gastric cancer.

Besides differential protein expression in cancer cells, alterations in mtDNA, such as mutations, deletions and insertions, have also been associated with specific cancers [93,94]. Although the detailed mechanisms by which mitochondrial dysfunction or mtDNA mutations lead to cancers are still under investigation, mitochondrial proteome research should lead to the discovery of new biomarkers for the early diagnosis and prevention of cancer.

Diabetes

As mitochondrial dysfunction [95] was reported to be associated with the development of diabetes, comparative mitochondrial proteomes have been used to study the relationship between mitochondrial dysfunction and diabetes. Turko and coworkers compared heart mitochondrial protein alterations in streptozotocin-induced diabetic rats with light and heavy acrylamide labeling, 2-DE and MS [96]. They discovered upregulated mitochondrial fatty acid β-oxidation proteins and several downregulated electron transport protein subunits in mitochondria from the hearts of streptozotocin-treated diabetic rats. Bugger and coworkers investigated differences in the mitochondrial proteome across four tissues (liver, brain, heart and kidney) from wild-type and Type 1 diabetic Akita mice using label-free proteome expression analysis [25]. They discovered tissue-specific remodeling of the mitochondrial proteome in Akita mice: mitochondria from the heart were dysfunctional, while mitochondrial function in the kidney, brain and liver was unchanged. Both studies provide information on mitochondrial dysfunction and Type 1 diabetes.

Comparative mitochondrial proteomics has also been applied to the investigation of Type 2 diabetes. Deng and coworkers performed a comprehensive mitochondrial proteome study on liver mitochondria from spontaneous diabetic Goto-Kakizaki rats before and/or after they were rendered diabetic [97]. They identified 1091 mitochondrial proteins, 228 phosphoproteins and 355 hydroxyproteins in the liver mitochondria.

Semi-quantitative analysis revealed upregulated mitochondrial proteins involved in β-oxidation, the TCA cycle, OXPHOS and other bioenergetic processes, and downregulated anti-apoptosis and anti-oxidative stress proteins; all of these changes were correlated with the development of Type 2 diabetes. As mitochondrial function is central to pancreatic β-cell glucose-sensing and insulin secretion [98], mitochondrial dysfunction plays an important role in the disease progression of diabetes. We compared mitochondrial protein changes in high-glucose treated INS-1 β cells using SILAC and found that the expression of some proteins involved in OXPHOS, substance metabolism, mitochondrial protein synthesis and cell death were significantly decreased [Yang F, Unpublished Data].

In addition to mitochondrial protein expression changes, mitochondrial protein modifications also play an important role in the pathology of Type 2 diabetes. Højlund and coworkers identified potential biomarkers in the skeletal muscle of Type 2 diabetes patients, and found that the expression and phosphorylation of the ATP synthase β-subunit was altered, possibly accounting for the pathogenesis of Type 2 diabetes [99]. Kartha and coworkers detected an increase in mitochondrial protein tyrosine nitration in the kidney tissue of high calorie and fat diet-induced diabetic mice [100].

Aging

Oxidative damage to mitochondria caused by ROS has been implicated in the process of aging [101] but, the exact molecular mechanism of aging is not well understood. Liu and coworkers compared liver mitochondrial protein alterations between senescence-accelerated mouse prone/8 (SAMP8) mice and senescence-accelerated mouse resistant/1 (SAMR1) mice using both 2-DE and ICAT-based methods, and found that the expression and activity of mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase was decreased in the senescence-accelerated mouse model (SAMP8) [102]. In addition, they also observed an elevation of triglycerides and a reduction of acetyl-CoA. These results indicate abnormal fatty acid metabolism in the liver of SAMP8 mice. In addition, the expression of eight mitochondrial complex-related proteins were downregulated in SAMP8, and resulted in decreased energy output. These results suggest that mitochondrial alterations play an important role in the senescence-accelerated mouse. Yan and coworkers compared protein changes in monkey hearts from four groups (young and old, male and female) and revealed a gender-specific decrease in the expression of some proteins involved in electron transport, OXPHOS and the TCA cycle [103]. Recently, O'Connell and coworkers investigated mitochondrial protein changes in young and aged adult muscle tissue using DIGE and discovered an age-related change in 39 proteins [104]. Some proteins involved in OXPHOS, ATP production, fatty acid oxidation and antioxidant defense were upregulated, possibly acting as an anti-aging mechanism. Since different results and conclusions have been obtained in samples from different species (mouse, monkey and human) and tissues (liver, heart and muscle), a comprehensive analysis of the mitochondrial proteome in different tissues from the same species should provide further information regarding aging.

Cardiac diseases

Cardiac mitochondria are also targets for oxidative stress and cardiac mitochondrial dysfunction was proposed to be associated with specific cardiac states, such as ischemic heart damage [105], chronic stress-induced injury [106] and heart failure [107,108]. Kim and coworkers used 2-DE to investigate mitochondrial protein changes in ischemia–reperfusion, ischemic preconditioned (IPC) and control rabbit hearts [105]. They discovered that 25 mitochondrial proteins were differentially expressed in IR rabbit hearts compared with ischemic preconditioned hearts and the control, and that most of these proteins were related to the mitochondrial respiratory chain and energy metabolism. Liu and coworkers investigated chronic stress-induced heart injury

Table 3. Mitochondrial-related databases.

Database	Website	Description	Ref.
mtDB	www.genpat.uu.se/mtDB	Human Mitochondrial Genome Database	[124]
Mitomap	www.mitomap.org	A compendium of polymorphisms and mutations of human mitochondrial DNA	[125]
Mitoproteome	www.mitoproteome.org	Mitochondrial protein sequence database generated from experimental evidence and public databases	[114]
MITOP2	www.mitop.de:8080/mitop2	A database for mitochondrial-related genes, proteins and diseases	[116]
Human Mitochondrial Protein Database	http://bioinfo.nist.gov	Comprehensive data on mitochondrial and human nuclear-encoded proteins involved in mitochondrial biogenesis and function	
Arabidopsis Mitochondrial Protein Database	www.plantenergy.uwa.edu.au/ampdb	A database containing information on the predicted and experimentally confirmed protein complement of mitochondria from the model plant *Arabidopsis thaliana*	[126]
MitoMiner	http://mitominer.mrc-mbu.cam.ac.uk/release-1.1/begin.do	An integrated database for the storage and analysis of mitochondrial proteomics data	[117]

by comparing mitochondrial protein changes using 2-DE in chronic restraint stressed rats [106]. They discovered that five proteins involved in the TCA cycle and lipid metabolism were downregulated while two proteins were upregulated.

Damage to mitochondria of the heart was also investigated in two heart-failure animal models. Smith and coworkers compared heart mitochondrial protein changes in Dahl salt sensitive rats (hypertension, cardiac hypertrophy, and heart failure rat model) with ^{18}O labeling, and nine proteins were found to be differentially expressed [107]. Fountoulakis and coworkers investigated heart mitochondrial protein changes in the desmin-null heart-failure mice model and discovered that the expression of some proteins involved in ketone body and acetate metabolism, NADH shuttle, amino-acid metabolism and respiration was altered in desmin-null mice [108].

Other pathologies

Comparative mitochondrial proteome analysis has also been applied to identification mitochondrial proteins involved in hypertension [61], osteoarthritis [109], ethanol-induced stress [110,111] and endotoxic shock [112].

Summary

In summary, since mitochondrial dysfunction has been implicated in a variety of human disorders, comparative mitochondrial proteomics should provide valuable information for gaining a better understanding of these mitochondria-related diseases and pave the way to the development of new mitochondrial therapeutic drugs.

It should be mentioned that mitochondrial protein quantification in comparative proteomics is usually performed by combining equal amounts of mitochondrial proteins, but mitochondria are dynamic organelles [113], which can change their number, shape, structure and subcellular locations to fulfill specific requirements. Future work in comparative mitochondrial proteomics should, therefore, take into account the number of mitochondria in different tissues or situations.

Mitochondrial databases

With the reporting of large-scale mitochondrial proteomes from various tissues and cells, there is a growing need to create databases to facilitate the accessibility of such mitochondrial proteome data sets. Several databases that organize and store mitochondrial data sets have been published (Table 3), including Mitoproteome [114], MitoP2 [115,116], Human Mitochondrial Protein Database and MitoMiner [117]. MitoP2 provides a comprehensive list of mitochondrial proteins from yeast, mouse, human, *Arabidopsis thaliana* and *Neurospora*. It provides information about the functional annotation of proteins, their subcellular location, homologs and literature references. Recently, another comprehensive mitochondrial database called MitoMiner was released. This database integrates 33 sets of proteomic data from six species (human, rat, mouse, *Drosophila melanogaster*, *Caenorhabditis elegans* and *S. cerevisiae)* based on either MS or GFP tagging experiments together with protein annotation data from Uniprot, metabolic pathway data from Kyoto Encyclopedia of Genes and Genomes (KEGG), protein homology from HomoloGene and disease information from Online Mendelian Inheritance in Man (OMIM). This is the most complete mitochondrial database to date. In-depth analysis of these data sets has revealed that the number of mitochondrial proteins is approximately 3700, far more than the 1000–2000 that was estimated to be present in the mammalian mitochondrial proteome [118,119], indicating that more mitochondrial proteins, especially membrane proteins, may be discovered in subsequent mitochondrial proteome research with the development of proteomic technology. Despite the great progress that has been made in mitochondrial proteomics, data quality is still a major challenge in proteomics [120]. Therefore, careful handling with the available mitochondrial proteomic data and further data set validation are necessary to obtain more meaningful information regarding mitochondria.

Expert commentary & five-year view

Over the past 12 years, great progress has been made in mitochondrial proteomics research; mitochondrial proteomics now has applications beyond simple protein profiling and the production of protein lists. Mitochondrial proteomic technology offers great potential for discovering new mitochondrial proteins and identifying mitochondrial protein PTMs to provide functional information about the mitochondria. Current comparative mitochondrial proteomics research is mainly focused on finding disease biomarkers and providing insight into the molecular mechanisms of disease. Although great progress has been made, many challenges remain; for example, how to investigate the functions of the large number of mitochondrial proteins that do not have functional annotations, and how to build mitochondrial signaling networks and mitochondrial protein–protein interaction networks. Integration of various approaches, such as proteomics, transcriptomics, bioinformatics and biochemistry, should facilitate answers to these questions. In the next 5 years, the publication of mitochondrial proteomes from different mammalian tissues and cells will increase, and will provide useful information regarding tissue/cell-specific mitochondrial functions. Comparative mitochondrial proteomic research on various diseases will expand to provide profound insights into the molecular mechanisms of mitochondria-associated diseases.

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Key issues

- A collection of proteomic approaches and technologies have been developed for mitochondrial purification, mitochondrial protein separation, identification and quantification.
- Great progress has been made in mammalian mitochondrial proteomics since the first mitochondrial proteome study in 1998, providing more information to facilitate understanding of the function of mitochondria.
- Comparative mitochondrial proteomics has been applied to various diseases, such as neurodegenerative diseases, cancer, diabetes and aging, to determine the relationship between mitochondrial dysfunction and disease onset.
- Integration of proteomics with transcriptomics, bioinformatics and biochemical tools will provide novel insight into mitochondrial functions.

References

Papers of special note have been highlighted as:
• of interest
•• of considerable interest

1 Duchen MR. Mitochondria in health and disease: perspectives on a new mitochondrial biology. *Mol. Aspects Med.* 25(4), 365–451 (2004).

2 Lowell BB, Shulman GI. Mitochondrial dysfunction and Type 2 diabetes. *Science* 307(5708), 384–387 (2005).

3 Wallace DC. Mitochondrial diseases in man and mouse. *Science* 283(5407), 1482–1488 (1999).

4 Da Cruz S, Parone PA, Martinou JC. Building the mitochondrial proteome. *Expert Rev. Proteomics* 2(4), 541–551 (2005).

5 Reichert AS, Neupert W. Mitochondriomics or what makes us breathe. *Trends Genet.* 20(11), 555–562 (2004).

6 Rabilloud T, Kieffer S, Procaccio V *et al.* Two-dimensional electrophoresis of human placental mitochondria and protein identification by mass spectrometry: toward a human mitochondrial proteome. *Electrophoresis* 19(6), 1006–1014 (1998).

7 Pagliarini DJ, Calvo SE, Chang B *et al.* A mitochondrial protein compendium elucidates complex I disease biology. *Cell* 134(1), 112–123 (2008).

•• **The most complete mammalian mitochondrial proteome to date.**

8 Li J, Cai T, Wu P *et al.* Proteomic analysis of mitochondria from *Caenorhabditis elegans*. *Proteomics* 9(19), 4539–4553 (2009).

9 Taylor SW, Fahy E, Zhang B *et al.* Characterization of the human heart mitochondrial proteome. *Nat. Biotechnol.* 21(3), 281–286 (2003).

• **First large-scale mammalian mitochondrial proteome study.**

10 Rezaul K, Wu L, Mayya V, Hwang SI, Han D. A systematic characterization of mitochondrial proteome from human T leukemia cells. *Mol. Cell. Proteomics* 4(2), 169–181 (2005).

11 Zischka H, Weber G, Weber PJ *et al.* Improved proteome analysis of *Saccharomyces cerevisiae* mitochondria by free-flow electrophoresis. *Proteomics* 3(6), 906–916 (2003).

12 Hartwig S, Feckler C, Lehr S *et al.* A critical comparison between two classical and a kit-based method for mitochondria isolation. *Proteomics* 9(11), 3209–3214 (2009).

13 O'Farrell PH. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250(10), 4007–4021 (1975).

14 Kane LA, Yung CK, Agnetti G, Neverova I, Van Eyk JE. Optimization of paper bridge loading for 2-DE analysis in the basic pH region: application to the mitochondrial subproteome. *Proteomics* 6(21), 5683–5687 (2006).

15 Taylor SW, Warnock DE, Glenn GM *et al.* An alternative strategy to determine the mitochondrial proteome using sucrose gradient fractionation and 1D PAGE on highly purified human heart mitochondria. *J. Proteome Res.* 1(5), 451–458 (2002).

16 Schagger H, von Jagow G. Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. *Anal. Biochem.* 199(2), 223–231 (1991).

17 Washburn MP, Wolters D, Yates JR 3rd. Large-scale analysis of the yeast proteome by multidimensional protein identification technology. *Nat. Biotechnol.* 19(3), 242–247 (2001).

18 Ong SE, Blagoev B, Kratchmarova I *et al.* Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. *Mol. Cell. Proteomics* 1(5), 376–386 (2002).

• **Development of *in vivo* labeling technology (stable isotope labeling with amino acids in cell culture [SILAC]) for quantitative proteomic analysis.**

19 Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, Aebersold R. Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat. Biotechnol.* 17(10), 994–999 (1999).

• **Development of isotope-coded affinity Tags (ICAT)-labeling technology for quantitative proteomic analysis.**

20 Ross PL, Huang YN, Marchese JN *et al.* Multiplexed protein quantitation in *Saccharomyces cerevisiae* using amine-reactive isobaric tagging reagents. *Mol. Cell. Proteomics* 3(12), 1154–1169 (2004).

• **Development of isobaric tags for relative and absolute quantitation labeling technology for quantitative proteomic analysis.**

21 Miyagi M, Rao KC. Proteolytic ^{18}O-labeling strategies for quantitative proteomics. *Mass Spectrom. Rev.* 26(1), 121–136 (2007).

22 Ono M, Shitashige M, Honda K *et al.* Label-free quantitative proteomics using large peptide data sets generated by nanoflow liquid chromatography and mass spectrometry. *Mol. Cell. Proteomics* 5(7), 1338–1347 (2006).

23 Gilchrist A, Au CE, Hiding J *et al.* Quantitative proteomics analysis of the secretory pathway. *Cell* 127(6), 1265–1281 (2006).

24 Bantscheff M, Schirle M, Sweetman G, Rick J, Kuster B. Quantitative mass spectrometry in proteomics: a critical review. *Anal. Bioanal. Chem.* 389(4), 1017–1031 (2007).

25 Bugger H, Chen D, Riehle C *et al.* Tissue-specific remodeling of the mitochondrial proteome in Type 1 diabetic akita mice. *Diabetes* 58(9), 1986–1997 (2009).

• **Tissue-specific remodeling of the mitochondrial proteome in Type 1 diabetic mice.**

26 Lefort N, Yi Z, Bowen B *et al.* Proteome profile of functional mitochondria from human skeletal muscle using one-dimensional gel electrophoresis and HPLC-ESI-MS/MS. *J. Proteomics* 72(6), 1046–1060 (2009).

27 Fountoulakis M, Berndt P, Langen H, Suter L. The rat liver mitochondrial proteins. *Electrophoresis* 23(2), 311–328 (2002).

28 Forner F, Foster LJ, Campanaro S, Valle G, Mann M. Quantitative proteomic comparison of rat mitochondria from muscle, heart and liver. *Mol. Cell. Proteomics* 5(4), 608–619 (2006).

29 Zhang J, Li X, Mueller M *et al.* Systematic characterization of the murine mitochondrial proteome using functionally validated cardiac mitochondria. *Proteomics* 8(8), 1564–1575 (2008).

30 Prokisch H, Scharfe C, Camp DG *et al.* Integrative analysis of the mitochondrial proteome in yeast. *PLoS Biol.* 2(6), e160 (2004).

31 Sickmann A, Reinders J, Wagner Y *et al.* The proteome of *Saccharomyces cerevisiae* mitochondria. *Proc. Natl Acad. Sci. USA* 100(23), 13207–13212 (2003).

32 Fountoulakis M, Schlaeger EJ. The mitochondrial proteins of the neuroblastoma cell line IMR-32. *Electrophoresis* 24(1–2), 260–275 (2003).

33 Gaucher SP, Taylor SW, Fahy E *et al.* Expanded coverage of the human heart mitochondrial proteome using multidimensional liquid chromatography coupled with tandem mass spectrometry. *J. Proteome Res.* 3(3), 495–505 (2004).

34 Forner F, Kumar C, Luber CA, Fromme T, Klingenspor M, Mann M. Proteome differences between brown and white fat mitochondria reveal specialized metabolic functions. *Cell. Metab.* 10(4), 324–335 (2009).

•• **Comprehensive analysis of mitochondrial proteome differences between brown and white fat mitochondria with SILAC reveals cell-specific mitochondrial functions.**

35 Mootha VK, Bunkenborg J, Olsen JV *et al.* Integrated analysis of protein composition, tissue diversity, and gene regulation in mouse mitochondria. *Cell* 115(5), 629–640 (2003).

•• **First study on integrated analysis of a tissue-specific mitochondrial proteome.**

36 Johnson DT, Harris RA, French S *et al.* Tissue heterogeneity of the mammalian mitochondrial proteome. *Am. J. Physiol. Cell. Physiol.* 292(2), C689–C697 (2007).

37 Emanuelsson O, Nielsen H, Brunak S, von Heijne G. Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. *J. Mol. Biol.* 300(4), 1005–1016 (2000).

38 Claros MG, Vincens P. Computational method to predict mitochondrially imported proteins and their targeting sequences. *Eur. J. Biochem.* 241(3), 779–786 (1996).

39 Small I, Peeters N, Legeai F, Lurin C. Predotar: a tool for rapidly screening proteomes for N-terminal targeting sequences. *Proteomics* 4(6), 1581–1590 (2004).

40 Hua S, Sun Z. Support vector machine approach for protein subcellular localization prediction. *Bioinformatics* 17(8), 721–728 (2001).

41 Emanuelsson O, von Heijne G. Prediction of organellar targeting signals. *Biochim. Biophys. Acta* 1541(1–2), 114–119 (2001).

42 Nakai K, Horton P. PSORT: a program for detecting sorting signals in proteins and predicting their subcellular localization. *Trends Biochem. Sci.* 24(1), 34–36 (1999).

43 Jiang XS, Dai J, Sheng QH *et al.* A comparative proteomic strategy for subcellular proteome research: ICAT approach coupled with bioinformatics prediction to ascertain rat liver mitochondrial proteins and indication of mitochondrial localization for catalase. *Mol. Cell. Proteomics* 4(1), 12–34 (2005).

44 Distler AM, Kerner J, Hoppel CL. Proteomics of mitochondrial inner and outer membranes. *Proteomics* 8(19), 4066–4082 (2008).

45 Sottocasa GL, Kuylenstierna B, Ernster L, Bergstrand A. An electron-transport system associated with the outer membrane of liver mitochondria. A biochemical and morphological study. *J. Cell. Biol.* 32(2), 415–438 (1967).

46 Da Cruz S, Xenarios I, Langridge J, Vilbois F, Parone PA, Martinou JC. Proteomic analysis of the mouse liver mitochondrial inner membrane. *J. Biol. Chem.* 278(42), 41566–41571 (2003).

47 Da Cruz S, Martinou JC. Purification and proteomic analysis of the mouse liver mitochondrial inner membrane. *Methods Mol. Biol.* 432, 101–116 (2008).

• **Detailed protocol for mitochondrial inner membrane preparation and protein identification.**

48 McDonald T, Sheng S, Stanley B *et al.* Expanding the subproteome of the inner mitochondria using protein separation technologies: one- and two-dimensional liquid chromatography and two-dimensional gel electrophoresis. *Mol. Cell. Proteomics* 5(12), 2392–2411 (2006).

49 Distler AM, Kerner J, Peterman SM, Hoppel CL. A targeted proteomic approach for the analysis of rat liver mitochondrial outer membrane proteins with extensive sequence coverage. *Anal. Biochem.* 356(1), 18–29 (2006).

50 Zahedi RP, Sickmann A, Boehm AM *et al.* Proteomic analysis of the yeast mitochondrial outer membrane reveals accumulation of a subclass of preproteins. *Mol. Biol. Cell.* 17(3), 1436–1450 (2006).

51 Suzuki T, Terasaki M, Takemoto-Hori C *et al.* Proteomic analysis of the mammalian mitochondrial ribosome. Identification of protein components in the 28S small subunit. *J. Biol. Chem.* 276(35), 33181–33195 (2001).

52 Koc EC, Burkhart W, Blackburn K, Koc H, Moseley A, Spremulli LL. Identification of four proteins from the small subunit of the mammalian mitochondrial ribosome using a proteomics approach. *Protein Sci.* 10(3), 471–481 (2001).

53 Koc EC, Burkhart W, Blackburn K, Moseley A, Koc H, Spremulli LL. A proteomics approach to the identification of mammalian mitochondrial small subunit ribosomal proteins. *J. Biol. Chem.* 275(42), 32585–32591 (2000).

54 Suzuki T, Terasaki M, Takemoto-Hori C *et al.* Structural compensation for the deficit of rRNA with proteins in the mammalian mitochondrial ribosome. Systematic analysis of protein components of the large ribosomal subunit from mammalian mitochondria. *J. Biol. Chem.* 276(24), 21724–21736 (2001).

55 Brookes PS, Pinner A, Ramachandran A *et al.* High throughput two-dimensional blue-native electrophoresis: a tool for functional proteomics of mitochondria and signaling complexes. *Proteomics* 2(8), 969–977 (2002).

56 Reifschneider NH, Goto S, Nakamoto H *et al.* Defining the mitochondrial proteomes from five rat organs in a physiologically significant context using 2D blue-native/SDS-PAGE. *J. Proteome Res.* 5(5), 1117–1132 (2006).

57 Devreese B, Vanrobaeys F, Smet J, Van Beeumen J, Van Coster R. Mass spectrometric identification of mitochondrial oxidative phosphorylation subunits separated by two-dimensional blue-native polyacrylamide gel electrophoresis. *Electrophoresis* 23(15), 2525–2533 (2002).

58 Meyer B, Wittig I, Trifilieff E, Karas M, Schagger H. Identification of two proteins associated with mammalian ATP synthase. *Mol. Cell. Proteomics* 6(10), 1690–1699 (2007).

59 Nakamura Y, Suzuki H, Sakaguchi M, Mihara K. Targeting and assembly of rat mitochondrial translocase of outer

membrane 22 (TOM22) into the TOM complex. *J. Biol. Chem.* 279(20), 21223–21232 (2004).

60 Faye A, Esnous C, Price NT, Onfray MA, Girard J, Prip-Buus C. Rat liver carnitine palmitoyltransferase 1 forms an oligomeric complex within the outer mitochondrial membrane. *J. Biol. Chem.* 282(37), 26908–26916 (2007).

61 Lopez-Campistrous A, Hao L, Xiang W *et al.* Mitochondrial dysfunction in the hypertensive rat brain: respiratory complexes exhibit assembly defects in hypertension. *Hypertension* 51(2), 412–419 (2008).

62 Schilling B, Murray J, Yoo CB *et al.* Proteomic analysis of succinate dehydrogenase and ubiquinol-cytochrome c reductase (complex II and III) isolated by immunoprecipitation from bovine and mouse heart mitochondria. *Biochim. Biophys. Acta* 1762(2), 213–222 (2006).

63 Carroll J, Fearnley IM, Shannon RJ, Hirst J, Walker JE. Analysis of the subunit composition of complex I from bovine heart mitochondria. *Mol. Cell. Proteomics* 2(2), 117–126 (2003).

64 Schulenberg B, Aggeler R, Beechem JM, Capaldi RA, Patton WF. Analysis of steady-state protein phosphorylation in mitochondria using a novel fluorescent phosphosensor dye. *J. Biol. Chem.* 278(29), 27251–27255 (2003).

65 Hopper RK, Carroll S, Aponte AM *et al.* Mitochondrial matrix phosphoproteome: effect of extra mitochondrial calcium. *Biochemistry* 45(8), 2524–2536 (2006).

66 Lee J, Xu Y, Chen Y *et al.* Mitochondrial phosphoproteome revealed by an improved IMAC method and MS/MS/MS. *Mol. Cell. Proteomics* 6(4), 669–676 (2007).

67 Cui Z, Hou J, Chen X *et al.* The profile of mitochondrial proteins and their phosphorylation signaling network in INS-1 β cells. *J. Proteome Res.* DOI: 10.1021/pr100139z (2010) (Epub ahead of print).

68 Pagliarini DJ, Dixon JE. Mitochondrial modulation: reversible phosphorylation takes center stage? *Trends Biochem. Sci.* 31(1), 26–34 (2006).

• **Focuses on the importance of phosphorylation in the regulation of mitochondrial functions.**

69 Horbinski C, Chu CT. Kinase signaling cascades in the mitochondrion: a matter of life or death. *Free Radic. Biol. Med.* 38(1), 2–11 (2005).

70 Yonekawa H, Akita Y. Protein kinase Cε: the mitochondria-mediated signaling pathway. *FEBS J.* 275(16), 4005–4013 (2008).

71 Feliciello A, Gottesman ME, Avvedimento EV. cAMP-PKA signaling to the mitochondria: protein scaffolds, mRNA and phosphatases. *Cell. Signal.* 17(3), 279–287 (2005).

72 Carroll J, Fearnley IM, Skehel JM *et al.* The post-translational modifications of the nuclear encoded subunits of complex I from bovine heart mitochondria. *Mol. Cell. Proteomics* 4(5), 693–699 (2005).

73 Murray J, Taylor SW, Zhang B, Ghosh SS, Capaldi RA. Oxidative damage to mitochondrial complex I due to peroxynitrite: identification of reactive tyrosines by mass spectrometry. *J. Biol. Chem.* 278(39), 37223–37230 (2003).

74 Taylor SW, Fahy E, Murray J, Capaldi RA, Ghosh SS. Oxidative post-translational modification of tryptophan residues in cardiac mitochondrial proteins. *J. Biol. Chem.* 278(22), 19587–19590 (2003).

75 Hunzinger C, Wozny W, Schwall GP *et al.* Comparative profiling of the mammalian mitochondrial proteome: multiple aconitase-2 isoforms including N-formylkynurenine modifications as part of a protein biomarker signature for reactive oxidative species. *J. Proteome Res.* 5(3), 625–633 (2006).

76 Lin TK, Hughes G, Muratovska A *et al.* Specific modification of mitochondrial protein thiols in response to oxidative stress: a proteomics approach. *J. Biol. Chem.* 277(19), 17048–17056 (2002).

77 Choksi KB, Papaconstantinou J. Age-related alterations in oxidatively damaged proteins of mouse heart mitochondrial electron transport chain complexes. *Free Radic. Biol. Med.* 44(10), 1795–1805 (2008).

78 Vogtle FN, Wortelkamp S, Zahedi RP *et al.* Global analysis of the mitochondrial N-proteome identifies a processing peptidase critical for protein stability. *Cell* 139(2), 428–439 (2009).

79 Gillardon F. Differential mitochondrial protein expression profiling in neurodegenerative diseases. *Electrophoresis* 27(13), 2814–2818 (2006).

80 Atamna H, Frey WH 2nd. Mechanisms of mitochondrial dysfunction and energy deficiency in Alzheimer's disease. *Mitochondrion* 7(5), 297–310 (2007).

81 Reddy PH, Beal MF. Are mitochondria critical in the pathogenesis of Alzheimer's disease? *Brain Res. Brain Res. Rev.* 49(3), 618–632 (2005).

82 Lovell MA, Xiong S, Markesbery WR, Lynn BC. Quantitative proteomic analysis of mitochondria from primary neuron cultures treated with amyloid β peptide. *Neurochem. Res.* 30(1), 113–122 (2005).

83 Gillardon F, Rist W, Kussmaul L *et al.* Proteomic and functional alterations in brain mitochondria from Tg2576 mice occur before amyloid plaque deposition. *Proteomics* 7(4), 605–616 (2007).

84 Sultana R, Butterfield DA. Oxidatively modified, mitochondria-relevant brain proteins in subjects with Alzheimer disease and mild cognitive impairment. *J. Bioenerg. Biomembr.* 41(5), 441–446 (2009).

85 Jin J, Davis J, Zhu D *et al.* Identification of novel proteins affected by rotenone in mitochondria of dopaminergic cells. *BMC Neurosci.* 8, 67 (2007).

86 Jin J, Meredith GE, Chen L *et al.* Quantitative proteomic analysis of mitochondrial proteins: relevance to Lewy body formation and Parkinson's disease. *Brain Res. Mol. Brain Res.* 134(1), 119–138 (2005).

87 Fukada K, Zhang F, Vien A, Cashman NR, Zhu H. Mitochondrial proteomic analysis of a cell line model of familial amyotrophic lateral sclerosis. *Mol. Cell. Proteomics* 3(12), 1211–1223 (2004).

88 Verma M, Kagan J, Sidransky D, Srivastava S. Proteomic analysis of cancer-cell mitochondria. *Nat. Rev. Cancer* 3(10), 789–795 (2003).

89 Kagan J, Srivastava S. Mitochondria as a target for early detection and diagnosis of cancer. *Crit. Rev. Clin. Lab. Sci.* 42(5–6), 453–472 (2005).

90 Herrmann PC, Gillespie JW, Charboneau L *et al.* Mitochondrial proteome: altered cytochrome c oxidase subunit levels in prostate cancer. *Proteomics* 3(9), 1801–1810 (2003).

91 Krieg RC, Knuechel R, Schiffmann E, Liotta LA, Petricoin EF 3rd, Herrmann PC. Mitochondrial proteome: cancer-altered metabolism associated with cytochrome c oxidase subunit level variation. *Proteomics* 4(9), 2789–2795 (2004).

92 Kim HK, Park WS, Kang SH *et al.* Mitochondrial alterations in human gastric carcinoma cell line. *Am. J. Physiol. Cell. Physiol.* 293(2), C761–C771 (2007).

93 Copeland WC, Wachsman JT, Johnson FM, Penta JS. Mitochondrial DNA alterations in cancer. *Cancer Invest.* 20(4), 557–569 (2002).

94 Sanchez-Cespedes M, Parrella P, Nomoto S *et al.* Identification of a mononucleotide repeat as a major target for mitochondrial DNA alterations in human tumors. *Cancer Res*. 61(19), 7015–7019 (2001).

95 Mulder H, Ling C. Mitochondrial dysfunction in pancreatic β-cells in Type 2 diabetes. *Mol. Cell. Endocrinol*. 297(1–2), 34–40 (2009).

96 Turko IV, Murad F. Quantitative protein profiling in heart mitochondria from diabetic rats. *J. Biol. Chem.* 278(37), 35844–35849 (2003).

97 Deng WJ, Nie S, Dai J, Wu JR, Zeng R. Proteome, phosphoproteome and hydroxyproteome of liver mitochondria in diabetic rats at early pathogenic stages. *Mol. Cell. Proteomics* 9(1), 100–116 (2010).

• **Comprehensive analysis of the mitochondrial proteome in liver mitochondria of Type 2 diabetic rats.**

98 Maechler P, Wollheim CB. Mitochondrial function in normal and diabetic β-cells. *Nature* 414(6865), 807–812 (2001).

99 Hojlund K, Wrzesinski K, Larsen PM *et al.* Proteome analysis reveals phosphorylation of ATP synthase β-subunit in human skeletal muscle and proteins with potential roles in Type 2 diabetes. *J. Biol. Chem.* 278(12), 10436–10442 (2003).

100 Kartha GK, Moshal KS, Sen U *et al.* Renal mitochondrial damage and protein modification in Type-2 diabetes. *Acta Diabetol*. 45(2), 75–81 (2008).

101 Lambert AJ, Brand MD. Research on mitochondria and aging, 2006–2007. *Aging Cell* 6(4), 417–420 (2007).

102 Liu Y, He J, Ji S *et al.* Comparative studies of early liver dysfunction in senescence-accelerated mouse using mitochondrial proteomics approaches. *Mol. Cell. Proteomics* 7(9), 1737–1747 (2008).

103 Yan L, Ge H, Li H *et al.* Gender-specific proteomic alterations in glycolytic and mitochondrial pathways in aging monkey hearts. *J. Mol. Cell. Cardiol*. 37(5), 921–929 (2004).

104 O'Connell K, Ohlendieck K. Proteomic DIGE analysis of the mitochondria-enriched fraction from aged rat skeletal muscle. *Proteomics* 9(24), 5509–5524 (2009).

105 Kim N, Lee Y, Kim H *et al.* Potential biomarkers for ischemic heart damage identified in mitochondrial proteins by comparative proteomics. *Proteomics* 6(4), 1237–1249 (2006).

106 Liu XH, Qian LJ, Gong JB, Shen J, Zhang XM, Qian XH. Proteomic analysis of mitochondrial proteins in cardiomyocytes from chronic stressed rat. *Proteomics* 4(10), 3167–3176 (2004).

107 Smith JR, Matus IR, Beard DA, Greene AS. Differential expression of cardiac mitochondrial proteins. *Proteomics* 8(3), 446–462 (2008).

108 Fountoulakis M, Soumaka E, Rapti K *et al.* Alterations in the heart mitochondrial proteome in a desmin null heart failure model. *J. Mol. Cell. Cardiol.* 38(3), 461–474 (2005).

109 Ruiz-Romero C, Calamia V, Mateos J *et al.* Mitochondrial dysregulation of osteoarthritic human articular chondrocytes analyzed by proteomics: a decrease in mitochondrial superoxide dismutase points to a redox imbalance. *Mol. Cell. Proteomics* 8(1), 172–189 (2009).

110 Venkatraman A, Landar A, Davis AJ *et al.* Modification of the mitochondrial proteome in response to the stress of ethanol-dependent hepatotoxicity. *J. Biol. Chem.* 279(21), 22092–22101 (2004).

111 Shi L, Wang Y, Tu S *et al.* The responses of mitochondrial proteome in rat liver to the consumption of moderate ethanol: the possible roles of aldo-keto reductases. *J. Proteome Res.* 7(8), 3137–3145 (2008).

112 Miller I, Gemeiner M, Gesslbauer B *et al.* Proteome analysis of rat liver mitochondria reveals a possible compensatory response to endotoxic shock. *FEBS Lett*. 580(5), 1257–1262 (2006).

113 Detmer SA, Chan DC. Functions and dysfunctions of mitochondrial dynamics. *Nat. Rev. Mol. Cell Biol*. 8(11), 870–879 (2007).

114 Cotter D, Guda P, Fahy E, Subramaniam S. MitoProteome: mitochondrial protein sequence database and annotation system. *Nucleic Acids Res.* 32, D463–D467 (2004).

115 Elstner M, Andreoli C, Ahting U *et al.* MitoP2: an integrative tool for the analysis of the mitochondrial proteome. *Mol. Biotechnol*. 40(3), 306–315 (2008).

116 Elstner M, Andreoli C, Klopstock T, Meitinger T, Prokisch H. The mitochondrial proteome database: MitoP2. *Methods Enzymol*. 457, 3–20 (2009).

117 Smith AC, Robinson AJ. MitoMiner: an integrated database for the storage and analysis of mitochondrial proteomics data. *Mol. Cell. Proteomics* 8(6), 1324–1337 (2009).

• **Comprehensive mitochondrial protein database.**

118 McDonald TG, Van Eyk JE. Mitochondrial proteomics. Undercover in the lipid bilayer. *Basic Res. Cardiol*. 98(4), 219–227 (2003).

119 Meisinger C, Sickmann A, Pfanner N. The mitochondrial proteome: from inventory to function. *Cell*. 134(1), 22–24 (2008).

120 Wilkins M. Proteomics data mining. *Expert Rev. Proteomics* 6(6), 599–603 (2009).

121 Emanuelsson O, Brunak S, von Heijne G, Nielsen H. Locating proteins in the cell using TargetP, SignalP and related tools. *Nat. Protoc*. 2(4), 953–971 (2007).

122 Guda C, Fahy E, Subramaniam S. MITOPRED: a genome-scale method for prediction of nucleus-encoded mitochondrial proteins. *Bioinformatics* 20(11), 1785–1794 (2004).

123 Chou KC, Shen HB. Cell-PLoc: a package of web servers for predicting subcellular localization of proteins in various organisms. *Nat. Protoc*. 3(2), 153–162 (2008).

124 Ingman M, Gyllensten U. mtDB: Human Mitochondrial Genome Database, a resource for population genetics and medical sciences. *Nucleic Acids Res*. 34, D749–D751 (2006).

125 Ruiz-Pesini E, Lott MT, Procaccio V *et al.* An enhanced MITOMAP with a global mtDNA mutational phylogeny. *Nucleic Acids Res*. 35(Database issue), D823–D828 (2007).

126 Heazlewood JL, Millar AH. AMPDB: the Arabidopsis Mitochondrial Protein Database. *Nucleic Acids Res*. 33(Database issue), D605–D610 (2005).