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ORIGINAL ARTICLE

Proteomic differences between white and brown adipocytes

Wei-Qiang Chen · Lin Li · Gert Lubec

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Abstract Although a series of protein levels from several protein pathways have been shown to differ between white (WA) and brown (BA) adipocytes, proteomic work on this subject with the exception of mitochondrial protein differences is limited. It was, therefore, the aim of the study to compare WA with BA soluble protein levels. Proteins were extracted from WA and BA and the soluble fraction was run on two-dimensional gel electrophoresis. Quantification of spot volume was carried out and protein spots, statistically different between groups ($P < 0.01$), were in-gel digested with trypsin and peptides were identified using nano-LC–ESI–MS/MS in the CID and ETD mode. Differences between selected proteins were evaluated by immunoblotting. A network was generated using the ingenuity pathway analysis. Five proteins, protein DJ-1, dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, isocitrate dehydrogenase subunit alpha, electron transfer flavoprotein subunit alpha and immunoglobulin-binding protein 1, were increased in BA based on a gel-based proteomic method and differential expression was verified by immunoblotting. These individual proteins were represented by one spot each and sequence coverages were between 28 and 65 %. A network generated based on these results indicated a link to ubiquitination. Differential protein levels between WA and BA allow interpretation of previous work on adipocyte biochemistry and form the basis for future studies with genetic or pharmacological inhibition of these proteins accompanied by work on phenotype and adipocyte function.

Keywords White adipocyte · Brown adipocyte · LCMS · Ubiquitination

Introduction

The differentiation into white (WA) and brown adipocytes (BA) or white adipose and brown adipose tissue (BAT) is well known and documented (Hansen et al. 2004; Rodriguez de la Concepcion et al. 2005; Scime et al. 2005; Hansen and Kristiansen 2006; Seale et al. 2007; Cannon and Nedergaard 1996; Tai et al. 1996; Obregon 2008; Elabd et al. 2009; Perwitz et al. 2010; Morganstein et al. 2010; Jimenez et al. 2003). Work on the functional differences between WA and BA is still holding centre stage and there is consensus that BAT is primarily functioning in non-shivering thermogenesis mediated by the uncoupling protein 1 (Cancello et al. 1998; Watanabe et al. 2008; Wolf 2009; Swida-Barteczka et al. 2009; Ribeiro et al. 2010; Cinti et al. 2005; Kajimoto et al. 2004). White adipose tissue may be mainly for fat storage but white fat tissue is also proposed to be involved in detoxification of xenobiotics.

At the transcriptional level, a large series of genes are differentially expressed in WA and BA and at least some differences may be under the control of fat-specific protein 27 (Li et al. 2010). Pan and coworkers (2009) showed that Twist-1, a PPARdelta-inducible, negative-feedback regulator of PGC-1alpha in brown fat controls the expression of metabolic pathways.

Forner and coworkers (2009) carried out a comprehensive study that allows major insight into proteome

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differences between white and brown fat mitochondria. The authors applied high-resolution quantitative mass spectrometry to compare the mouse mitochondrial proteomes of WA and BA revealing that quantitative and qualitative differences are observed.

It was the aim of the current study to investigate the differences between WA and BA not limited to the mitochondrial organelles as it may well be the case that other than mitochondrial protein differences may contribute to the WA and BA proteomes.

Using a gel-based proteomic approach applying nano-LC–ESI–MS/MS for protein identification, higher protein levels of the multifunctional protein DJ-1 along with mitochondrial key enzymes were observed in BA, thus complementing/extending work published by Forner and coworkers (2009) while generating a network with a link to the ubiquitination system.

Materials and methods

Adipocyte cultures

Male NMRI mice, purchased from a local supplier (B&K, Stockholm, Sweden), were used for the preparation of primary cultures of brown and white adipocytes. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and all efforts were made to minimize suffering. Mice were kept at room temperature ($\sim$22 °C) for at least 24 h after arrival. At the age of 3–4 weeks, mice were killed by CO_2. Brown adipose tissue was isolated from the interscapular, cervical, and axillary depots and white adipose tissue was isolated from epididymal depots. The pooled tissue pieces were minced in DMEM and transferred to a digestion solution with 0.2 % (wt/vol) collagenase (type II; Sigma) in a buffer consisting of 0.1 M HEPES (pH 7.4), 123 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 4.5 mM glucose, and 1.5 % (wt/vol) BSA. The digestion was performed for 30 min at 37 °C with continuous vortex mixing. The cell suspension was filtered through a 250-µm pore-size nylon filter (Sintab, Oxie, Sweden) into sterile 15-ml tubes. The filtered suspension was kept on ice for 20 min to let the mature adipocytes float up. The top layer of the suspension was removed, and the rest of the suspension was filtered through a 25-µm pore-size nylon filter (Sintab) and centrifuged at 700×*g* for 10 min, to pellet preadipocytes. The pellet was then suspended in culture medium (0.5 ml/animal for brown preadipocytes, 0.4 ml/animal for white preadipocytes). The cells were cultured in 6-well plates (10 cm^2/well, Corning, Tewksbury, MA, USA); 2 ml of culture medium was added to each well before 0.2 ml of cell suspension was added. The culture medium was Dulbecco's modified Eagle's medium with 10 % (v/v) newborn calf serum (Invitrogen, Life Technologies Europe BV, Stockholm, Sweden), 2.4 nM insulin, 25 µg/ml sodium ascorbate, 10 mm HEPES, 4 mM glutamine, 50 units/ml penicillin, and 50 µg/ml streptomycin. The cells were grown at 37 °C in an atmosphere of 8 % CO_2 in air with 80 % humidity. The cells were washed in Dulbecco's modified Eagle's medium, and medium was changed on day 1 and then every second day. The cells were cultured for 7 days. On day 7, cells were washed three times with PBS and then scraped in residual PBS. These experiments were carried out at the Wenner-Gren Institute, Arrhenius Laboratories, Stockholm University, SE-106 91 Stockholm, Sweden, by Dr. Natasa Petrovic.

Sample preparation

5 WA and 5 BA were used for the experiments, the supernatant was removed and cells were washed three times with phosphate buffered saline pH 7.4. Cell pellets were homogenized and suspended in 1.8 ml sample buffer (20 mM Tris, 7 M urea, 2 M thiourea, 4 % w/v CHAPS, 10 mM 1,4-dithioerythritol, 1 mM EDTA, 1 mM PMSF, 1 tablet Complete™ (Roche Diagnostics, Risch, Switzerland), and 0.2 % v/v phosphatase inhibitor cocktail from Calbiochem, Merck, Darmstadt, Germany). The suspension was sonicated on ice for approximately 30 s and centrifuged at 14,000×*g* for 60 min at 12 °C. Desalting was carried out with an Ultrafree-4 centrifugal filter unit at a cut-off molecular weight of 10,000 Da (Millipore, Bedford, MA, USA) at 4,400×*g* at 12 °C until the eluted volume was about 4 ml and the remaining volume reached 100–200 µl (Myung and Lubec 2006). The protein content of the supernatant was determined by the Bradford assay (Bradford 1976).

Two-dimensional gel electrophoresis (2-DE)

2-DE was performed essentially as reported previously (Bae et al. 2012). Samples of 700 µg protein (5 gels per group, total 10 gels) were subjected to immobilized pH 3–10 nonlinear gradient strips. Focusing started at 200 V and the voltage was gradually increased to 8,000 V at 4 V/min and kept constant for further 3 h (approximately 1,50,000 Vh totally). Prior to the second dimensional run, strips were equilibrated twice for 15 min with gentle shaking in 10 ml of SDS equilibration buffer (50 mM pH 8.8 Tris–HCl, 6 M urea, 30 % v/v glycerol, 2 % w/v SDS, trace of bromophenol blue). DTT (1 %) w/v was added at the first incubation for 15 min and 4 % iodoacetamide w/v instead of DTT at the second incubation step for 15 min. The second-dimensional separation was performed on

10–16 % gradient SDS-PAGE. After protein fixation for 12 h in 50 % methanol and 10 % acetic acid, the gels were stained with colloidal coomassie blue (Novex, San Diego, CA, USA) for 8 h and excess of dye was washed out from the gels with distilled water. Molecular masses were determined by running precision protein standard markers (Bio-Rad Laboratories, Hercules, CA, USA), covering the range of 10–250 kDa. Isoelectric point values were determined as given by the supplier (GE Healthcare, Buckinghamshire, UK) of the immobilized pH gradient strips.

Quantification of protein levels

Protein spots from each gel were outlined (first automatically and then manually) and quantified using the Proteomweaver software (Definiens, Munich, Germany). The percentage of the volume of the spots representing a certain protein was determined in comparison with the total proteins present in the 2-DE gel (Langen et al. 1999).

Analysis of peptides by nano-LC-ESI-(CID/ETD)-MS/MS (High capacity ion trap, HCT)

Twelve spots which showed different levels between white and brown adipocyte were manually excised and placed into 1.5 ml lobind Eppendorf tubes. In-gel digestion and sample preparation for HCT analysis were performed as described before (Chen et al. 2006). Gel plugs were washed with 10-mM ammonium bicarbonate and 50 % acetonitrile in 10-mM ammonium bicarbonate repeatedly. Addition of 100 % acetonitrile resulted in gel shrinking and the shrunk gel plugs were then speedVac dried in a Speedvac Concentrator 5301 (Eppendorf, Hamburg, Germany). The dried gel pieces were re-swollen, in-gel digested with 40 ng/µl trypsin (Promega, Madison, WI, USA) in digestion buffer (consisting of 5 mM Octyl b-D-glucopyranoside (OGP) and 10 mM ammonium bicarbonate) and incubated overnight at 37 °C. Peptide extraction was performed with 20 µl of 1 % TFA in 5 mM OGP for 30 min, and subsequently 0.1 % TFA in 4 % acetonitrile for 30 min. The extracted peptides were pooled for HCT analysis.

40 µl of the extracted peptides was analyzed by HCT. The HPLC used was an Ultimate 3000 system (Dionex Corporation, Sunnyvale, CA, USA) equipped with a PepMap100 C-18 trap column (300 µm × 5 mm) and PepMap100 C-18 analytic column (75 µm × 150 mm). The gradient was (A = 0.1 % formic acid in water, B = 0.08 % formic acid in acetonitrile) 8–30 % B from 0 to 105 min, 80 % B from 105 to 110 min, 8 % B from 110 to 125 min. A HCT ultra ETDII PTM discovery system (Bruker Daltonics, Bremen, Germany) was used to record peptide spectra over the mass range of m/z 350–1,500, and MS/MS spectra in information-dependent data acquisition over the mass range of m/z 100–2,800. Repeatedly, MS spectra were recorded followed by six data-dependent CID MS/MS spectra and three ETD MS/MS spectra generated from three highest intensity precursor ions. An active exclusion of 0.4 min after two spectra was used to detect low abundant peptides. The voltage between ion spray tip and spray shield was set to 1,600 V. Drying nitrogen gas was heated to 150 °C and the flow rate was 10 l/min. The collision energy was set automatically according to the mass and charge state of the peptides chosen for fragmentation. Multiple charged peptides were chosen for MS/MS experiments due to their good fragmentation characteristics.

MS/MS spectra were interpreted and peak lists were generated by DataAnalysis 4.0 (Bruker Daltonics, Bremen, Germany). Searches were done using the MASCOT 2.3.02 (Matrix Science, London, UK) against latest UniProtKB database for protein identification. Searching parameters were set as follows: enzyme selected as trypsin with two maximum missing cleavage sites, species limited to human, a mass tolerance of 0.2 Da for peptide tolerance, 0.2 Da for MS/MS tolerance, fixed modification of carbamidomethyl (C) and variable modification of methionine oxidation and phosphorylation (Tyr, Thr, and Ser). Positive protein identifications were based on a significant MOWSE score. After protein identification, an error-tolerant search was done to detect unspecific cleavage and unassigned modifications. Protein identification and modification information returned from MASCOT were manually inspected and filtered to obtain confirmed protein identification and modifications.

The Modiro^{TM} software respected enzymes selected as used, with three maximum missing cleavage sites, species limited to human. Peptide mass tolerance was 0.2 Da for peptide tolerance, 0.2 Da for fragment mass tolerance, selecting modification 1 as carbamidomethyl and modification 2 of methionine oxidation. Protein identification was first of all listed by inspection of spectra and subsequently significant peptide identification was based on the ion-charge status of the peptide, b- and y-ion fragmentation quality, ion score >200 and a significance score >80 as suggested by the manufacturer's manual (http://www.protagen.com/customers_downloads/MAN_Modiro_v1.1.zip). Searches for unknown mass shifts, for amino acid substitution and calculation of significance were selected on advanced PTM explorer search strategies (http://www.protagen.com/customers_downloads/MAN_Modiro-Advanced-Search-Strategies.pdf).

Western blotting

Two-dimensional gels or 12 % SDS homogenous gels separating proteins from mouse adipocyte were electro-transferred onto PVDF membranes as described recently

(Sunyer et al. 2009). Primary antibodies used were as follows: rabbit antibody against protein DJ-1 (DJ1) (Abcam, Cambridge, UK, ab18257, in a dilution of 1:20,000); rabbit antibody against dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex (PDE2) (Abcam, Cambridge, UK, ab66511, in a dilution of 1:2,500); rabbit antibody against isocitrate dehydrogenase (NAD) subunit alpha (IDH3A) (Abcam, Cambridge, UK, ab58641, in a dilution of 1:20,000); rabbit antibody against electron transfer flavoprotein subunit alpha (ETFA) (Sigma, Deisenhofen, Germany, AV54290, in a dilution of 1:20,000); rabbit antibody against immunoglobulin-binding protein 1 (IGBP1) (Abcam, Cambridge, UK, ab70545, in a dilution of 1:10,000); rabbit antibody against poly(A)-binding protein (PABP) (Abcam, Cambridge, UK, ab21060, in a dilution of 1:10,000); mouse antibody against catalytic subunit of protein phosphatase 2A (PP2Ac) (Abcam, Cambridge, UK, ab33527, in a dilution of 1:2,000) and rabbit antibody against E3 ubiquitin ligase (EDD) (Abcam, Cambridge, UK, ab70311, in a dilution of 1:5,000). Secondary antibodies used were goat anti-rabbit IgG, HRP linked (Abcam, Cambridge, UK, ab6721, in a dilution of 1:20,000) and goat anti-mouse IgG, HRP linked (Abcam, Cambridge, UK). Membranes were developed with the Amersham ECL Plus Western Blotting Detection System (GE Healthcare, Buckinghamshire, USA). Densities of immunoreactive bands were measured by Image J software program (http://rsb.info.nih.gov/ij/). Differences were performed by unpaired Student's t test. In all proteomic studies, a probability level of $P < 0.01$ was considered as statistically significant. All calculations were performed using Excel (Microsoft Office, http://office.microsoft.com/en-us/excel/).

Pathway analysis

Differentially expressed proteins were analyzed further by bioinformatic pathways analysis [Ingenuity Pathway Analysis (IPA); Ingenuity® Systems, Mountain View, CA; www.ingenuity.com]. IPA constructs hypothetical protein interaction clusters on the basis of a regularly updated "Ingenuity Pathways Knowledge Base."

Results

A representative figure on light-microscopical appearance is shown in supplemental Fig. S1. A total number of well-separated 662 spots were observed on the 2DE gels (supplemental Fig. S2). A representative two-dimensional gel identifying proteins with differential levels and their corresponding UniProtKB/Swissprot numbers are shown in Fig. 1. Posttranslational modifications as well as protein modifications probably representing post-translational modifications or artifacts resulting from chemical modification by the analytical procedure are listed in the Supplementary Table 1 in alphabetical order. While deamidation, oxidations and methylations may be considered technical artifacts, hydroxylation and dihydroxylation (dioxidation) in isocitrate dehydrogenase (NAD) subunit alpha, aspartyl aldehyde formation on aspartic acid residues in dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex may reflect PTMs. "Sequence conflicts" in isocitrate dehydrogenase F211Y as indicated by Modiro searches may simply reflect hydroxylation of phenylalanine to tyrosine, while F332I and D353Y may indeed represent sequence conflicts.

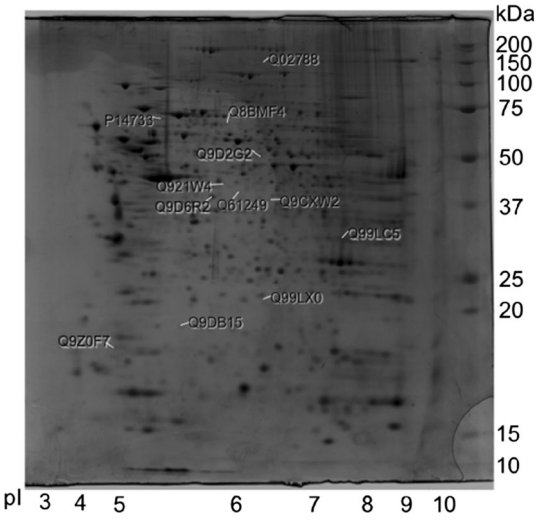

Fig. 1 Two-dimensional maps of mouse brown adipocyte proteins spots were quantified and statistically analyzed using Student t test. The 12 spots that were significantly different between white and brown adipocyte were identified using nano-LC–ESI–MS/MS

Western blot results were carried out and confirmed results from the gel-based proteomics approach for protein DJ-1, dihydrolipoyllysine- residue acetyltransferase component of pyruvate dehydrogenase complex, isocitrate dehydrogenase (NAD) subunit alpha, and electron protein 1. Immunoglobulin-binding protein 1 interacting with poly(A)-binding protein levels was increased in BA, whereas binding partners EDD and PP2Ac were comparable between WA and BA. Immunoblotting images for these proteins are given in Fig. 2. 2D-Western blotting of IGBP1 was performed along with 1D-Western blotting on the same membrane as shown in Fig. 3. There is only one spot detected on the 2D-Western blotting of GBP1. Results for significantly different protein levels as evaluated by immunoblotting are expressed as arbitrary units of optical density and are shown in Fig. 2.

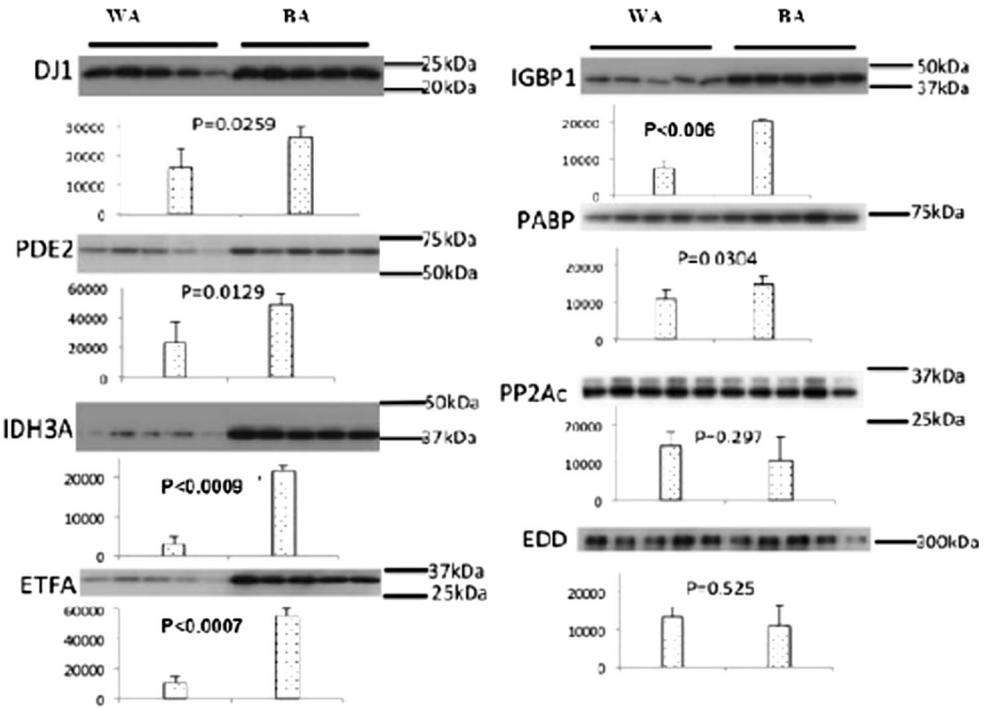

Fig. 2 Western blotting results of DJ1, PDE2, IDH3A, ETFA, IGBP1 and PABP between mouse white and brown adipocytes. The sample loaded on each lane is 10 μg for *DJ1* protein DJ-1, 5 μg for *PDE2* dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, *IDH3A* isocitrate dehydrogenase (NAD) subunit alpha, *ETFA* electron transfer flavoprotein subunit alpha, *IGBP1* immunoglobulin-binding protein 1 and *PABP* poly(A)-binding protein. The protein levels of DJ1, PDE2, IDH3A, ETFA, IGBP1 and PABP are significantly higher in mouse brown adipocytes compared to mouse white adipocytes. Unpaired Student's *t* test was used for the comparison of groups

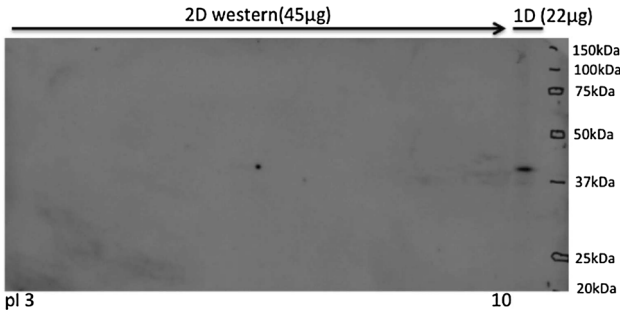

Fig. 3 2D-Western blotting of IGBP1 in mouse adipocyte 2DE was performed with 45 μg and when it applied to 10–16 % gradient SDS-PAGE as second dimension separation, 22 μg of protein was loaded at the edge for 1D-Western blot. 2D- and 1D-Western blots were performed on the same membrane with anti-IGBP1 (1:10,000)

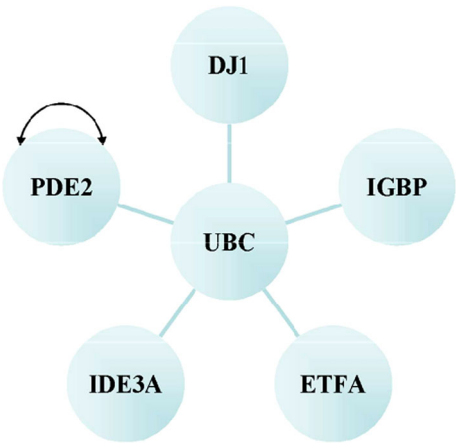

Fig. 4 A network pointing to the interaction of the differentially expressed proteins with the ubiquitination system is revealed. *DJ1* Protein DJ-1, dihydrolipoyllysine-residue acetyltransferase component of *PDE2* pyruvate dehydrogenase complex, *IDH3A* isocitrate dehydrogenase (NAD) subunit alpha, *ETFA* electron transfer flavoprotein subunit alpha, *IGBP* Immunoglobulin-binding protein 1, E3 isolated by differential display, EDD E3 ubiquitin ligase (UBC)

IPA networking

As shown in Fig. 4, there was a link between the ubiquitination system and the five proteins verified by western blotting. Quantification results of 12 protein spots with significantly different levels ($P < 0.01$) between WA and BA are listed in Table 1. Accession numbers, protein names, means and standard deviation, ratios and *P* values of the *t* test are provided. These 12 protein spots represented 12 individual proteins. Identification of these 12 protein spots along with Mascot scores, matched peptide

Table 1 Quantification result of 12 significantly different spots between white and brown adipocyte

No.	Acc. no.	Protein name	White adipocyte (mean ± SD)	Brown adipocyte (mean ± SD)	BA/WA ratio	*P* value of *t* test
532	Q99LX0	**Protein DJ-1**	0.362 ± 0.057	0.615 ± 0.070	1.699	0.0003
182	Q8BMF4	**Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial**	0.163 ± 0.097	0.391 ± 0.070	2.399	0.0035
401	Q9D6R2	**Isocitrate dehydrogenase (NAD) subunit alpha, mitochondrial**	0.890 ± 0.262	1.994 ± 0.520	2.240	0.0056
573	Q99LC5	**Electron transfer flavoprotein subunit alpha, mitochondrial**	0.953 ± 0.248	1.821 ± 0.437	1.911	0.0075
399	Q61249	**Immunoglobulin-binding protein 1**	0.044 ± 0.015	0.092 ± 0.024	2.091	0.0070
404	Q921W4	Quinone oxidoreductase-like protein 1	0.778 ± 0.177	0.166 ± 0.051	0.213	0.0009
599	Q9DB15	39S ribosomal protein L12, mitochondrial	0.180 ± 0.065	0.379 ± 0.062	2.106	0.0011
329	Q9CXW2	28S ribosomal protein S22, mitochondrial	0.097 ± 0.026	0.177 ± 0.031	1.825	0.0023
272	Q9D2G2	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial	0.203 ± 0.051	0.384 ± 0.073	1.892	0.0026
162	P14733	Lamin-B1	0.128 ± 0.030	0.222 ± 0.039	1.734	0.0030
112	Q02788	Collagen alpha-2(VI) chain	0.054 ± 0.030	0.186 ± 0.057	3.444	0.0038
600	Q9Z0F7	Gamma-synuclein	0.503 ± 0.130	1.339 ± 0.430	2.662	0.0100

Protein names given in bold were verified by WB

numbers, sequence coverage, peptide sequences including ion scores and mass errors, theoretical molecular weight and pI is given in Table 2.

Discussion

Mitochondrial proteins dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, isocitrate dehydrogenase (NAD) subunit alpha, electron transfer flavoprotein subunit alpha showed higher levels in BA confirming data published by Forner et al. (2009). Mitochondrial protein DJ-1 and 28S ribosomal protein S22 levels were higher in BA contradicting results from Forner et al. (2009). The use of different adipocytes as well as the use of a cell lysate in our methodology in contrast to the use of a mitochondrial preparation may account for these differences. It was the aim of the study herein, however, to compare partial proteomes of a lysate to show expression differences between WA and BA. Immunoglobulin-binding protein 1 (A4PP; syn.: alpha 4 phosphoprotein, CD79a-binding protein 1, lymphocyte signal transduction molecule alpha 4, p52) was twofold higher in BA. It represents the mammalian alpha4 phosphoprotein, the homolog of yeast Tap42, and is a component of the mammalian target-of-rapamycin (mTOR) pathway that regulates ribogenesis, initiation of translation and cell-cycle progression. This protein is a constituent of immune signaling pathways (Herzog et al. 2012) and binds to lactoferrin and this complex in turn interacts with the catalytic subunit of protein phosphatase 2Ac (PP2A), thus reducing the phosphatase activity of PP2Ac and triggering apoptosis (Li et al. 2011). Current results herein showed that increased A4PP levels were not accompanied by increased PP2Ac levels.

A4PP is also known to interact with E3 ubiquitin ligase (McDonald et al. 2010) as proposed by results from yeast two-hybrid analysis, thus showing a possible link to ubiquitination. The comparable EDD levels between WA and BA do not contradict interaction but on the other hand do not provide evidence for functional interaction. A4PP was reported to interact with (PABP) and indeed, increased A4PP levels were paralleling PABP levels, although no significant correlation was observed (data not shown). The biological meaning of increased PABP levels in BA may be represented by the essential role for recognition of polyadenylate RNA (Deo et al. 1999) via interaction with eukaryotic initiation factor eIF4G, a component of the eIF4F cap binding complex (Berlanga et al. 2006). PABP is able to destroy premature translation in the nonsense-mediated mRNA decay pathway (Behm-Ansmant et al. 2007) playing a crucial role for the regulation of translation and mRNA stability (Bag and Bhattacharjee 2010). In *drosophila*, PABP is one protein of the Hsp83 degradation element enhancing translation (Nelson et al. 2007). It is intriguing that DJ1, IGBP, IDE3A, ETFA and PDE2 are all linked to ubiquitination as revealed by the generation of a proposed network using IPA (Wagner et al. 2011; Kim

Table 2 Identification results of 12 spots with $P < 0.01$ between white and brown adipocyte

No.	Acc. no.	Protein name	Total score	Matched peptides	Seq. cov. %	MS/MS peptides (ion score/mass error, Da)	Theor. MW	Theor. pI
532	Q99LX0	Protein DJ-1	655	13	65	6 R.ALVILAK.G 12 (46/-0.0233)	20,236	6.32
						13 K.GAEEM*ETVIPVDVM*R.R 2 Oxidation(M) 27 (63/-0.0387) 13 K.GAEEM*ETVIPVDVMR.R Oxidation(M) 27 (59/-0.0122)		
						13 K.GAEEMETVIPVDVMR.R 27 (66/0.0588) 33 K.VTVAGLAGK.D 41 (46/0.0008)		
						33 K.VTVAGLAGKDPVQCSR.D 48 (63/-0.0181)		
						49 R.DVM*ICPDTSLEDAK.T Oxidation(M) 62 (79/-0.0125)		
						49 R.DVMICPDTSLEDAK.T 62 (78/0.0076)		
						63 K.TQGPYDVVVLPGGNLGAQNLSESPM*VK.E Oxidation(M) 89 (58/0.0225)		
						63 K.TQGPYDVVVLPGGNLGAQNLSESPMVK.E 89 (62/0.058)		
						99 R.KGLIAAICAGPTALLAHEVGFGCK.V 122 (49/0.0722)		
						133 K.MMNGSHYSYSESR.V 145 (73/0.0046)		
						149 K.DGLILTSR.G 156 (43/-0.0829)		
182	Q8BMF4	Dihydrolipoyllysine- residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial	1,657	22	41	93 K.VPLPSLSPTM*QAGTIAR.W Oxidation(M) 109 (70/0.0478)	68,469	8.81
						93 K.VPLPSLSPTMQAGTIAR.W 109 (99/0.0325) 118 K.ISEGDLIAEVETDK.A 131 (100/0.0802)		
						132 K.ATVGFESLEECYMAK.I 146 (79/0.0596)		
						245 K.LSEGDLLAEIETDK.A 258 (112/0.0143)		
						259 K.ATIGFEVQEEGYLAK.I 273 (87/0.0405)		
						282 R.DVPLGAPLCIIVEK.Q 295 (57/0.0024)		
						364 K.GIDLTQVK.G 371 (49/0.0423)		
						383 K.DIDSFVPSK.A 391 (39/0.0031)		
						392 K.AAPAAAAAM*APPGPR.V Oxidation(M) 406 (63/0.0358)		
						407 R.VAPAPAGVFTDIPISNIR.R 424 (111/0.0535)		
						469 K.ISVNDFIIK.A 477 (53/0.0468)		
						486 K.VPEANSSWM*DTVIR.Q Oxidation(M) 499 (116/0.0115)		
						486 K.VPEANSSWMDTVIR.Q 499 (123/0.0336)		
						528 K.GLETIASDVVSLASK.A 542 (120/0.0461)		
						548 K.LQPHEFQGGTFTISNLGM*FGIK.N Oxidation(M) 569 (68/0.0578)		
						548 K.LQPHEFQGGTFTISNLGMFGIK.N 569 (69/0.0388)		
						570 K.NFSAIINPPQACILAIGASEDK.L 591 (65/0.0485)		
						600 K.GFDVASVMSVTLSCDHR.V 616 (42/0.071)		
						617 R.VVDGAVGAQWLAEFK.K 631 (99/0.0151)		
						617 R.VVDGAVGAQWLAEFKK.Y 632 (74/0.0775)		
						633 K.YLEKPITM*LL.- Oxidation(M) 642 (43/0.0221)		

Table 2 continued

No.	Acc. no.	Protein name	Total score	Matched peptides	Seq. cov. %	MS/MS peptides (ion score/mass error, Da)	Theor. MW	Theo r. pI
401	Q9D6R2	Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial	1,417	20	39	59 K.APIQWEER.N 66 (54/0.043) 67 R.NVTAIQGPGGK.W 77 (55/-0.0294) 101 K.TPIAAGHPSM*NLLLR.K Oxidation(M) 115 (65/0.0124) 101 K.TPIAAGHPSMNLLLR.K 115 (84/0.0381) 116 R.KTFDLYANVR.P 125 (49/0.0958) 116 R.KTFDLYANVRPCVSIEGYK.T 134 (47/0.0545) 117 K.TFDLYANVR.P 125 (64/0.0181) 117 K.TFDLYANVRPCVSIEGYK.T 134 (63/0.0064) 126 R.PCVSIEGYK.T 134 (55/-0.0256) 135 K.TPYTDVNIVTIR.E 146 (86/0.0008) 147 R.ENTEGEYSGIEHVIVDGVVQSIK.L 169 (81/0.0903) 170 K.LITEEASKR.I 178 (57/-0.0113) 178 K.RIAEFAFEYAR.N 188 (51/0.0344) 179 R.IAEFAFEYAR.N 188 (85/-0.0355) 206 R.M*SDGLFLQK.C Oxidation(M) 214 (69/0.0244) 206 R.MSDGLFLQK.C 214 (53/0.0117) 317 R.HM*GLFDHAAK.I Oxidation(M) 326 (44/0.0358) 317 R.HMGLFDHAAK.I 326 (55/-0.0679) 327 K.IEAACFATIK.D 336 (65/-0.0171) 351 K.CSDFTEEICR.R 360 (82/0.0084)	40,069	6.27
573	Q99LC5	Electron transfer flavoprotein subunit alpha, mitochondrial	846	11	42	47 R.LGGEVSCLVAGTK.C 59 (83/-0.0197) 76 K.VLVAQHDAYK.G 85 (57/0.0034) 102 K.QFSYTHICAGASAFGK.N 117 (82/0.0776) 127 K.LNVAPVSDIIEIK.S 139 (72/0.0133) 140 K.SPDTFVR.T 146 (48/-0.0093) 147 R.TIYAGNALCTVK.C 158 (70/0.008) 170 R.GTSFEAAATSGGSASSEK.A 187 (104/-0.0222) 188 K.APSSSSVGISEWLDQK.L 203 (103/-0.0381) 233 K.LLYDLADQLHAAVGASR.A 249 (149/-0.0351) 250 R.AAVDAGFVPNDM*QVGQTGK.I Oxidation(M) 268 (88/-0.0413) 250 R.AAVDAGFVPNDMQVGQTGK.I 268 (80/0.016)	35,330	8.62

Table 2 continued

No.	Acc. no.	Protein name	Total score	Matched peptides	Seq. cov. %	MS/MS peptides (ion score/mass error, Da)	Theor. MW	Theo r. pI
399	Q61249	Immunoglobulin-binding protein 1	243	5	16	23 K.LLEDVEVATEPTGSR.T 37 (76/-0.0236) 53 K.AAGMLSQLDLFSR.N 65 (80/0.0542) 53 K.AAGM*LSQLDLFSR.N Oxidation(M) 65 (79/0.0121) 66 R.NEDLEEIASTDLK.Y 78 (95/-0.0232) 79 K.YLM*VPALQGALTM*K.Q 2 Oxidation(M) 92 (44/0.0043)	39,118	5.81
404	Q921W4	Quinone oxidoreductase- like protein 1	592	11	35	23 K.ENVPVTEDNFVR.V 34 (49/-0.0113) 65 R.EVSGIVLEVGR.K 75 (68/0.0509) 117 K.VSWTEAAGVIR.D 127 (107/-0.015) 132 R.ACTALYYLSQLSPGK.S 146 (51/0.0883) 172 K.VISTAHSLEDK.Q 182 (44/0.0247) 172 K.VISTAHSLEDKQHLER.L 187 (47/0.0116) 228 R.LYSKDDEPAVK.L 238 (67/0.0492) 278 K.GATVAFLNDEVWNLSNAQQGK.Y 298 (78/0.1205) 310 K.LSAGVFRPLLDEPIPLYEAK.V 329 (74/0.1213) 317 R.PLLDEPIPLYEAK.V 329 (79/0.0744) 330 K.VSMEVVQK.N 337 (57/-0.028)	39,043	5.63
599	Q9DB15	39S ribosomal protein L12, mitochondrial	426	7	41	46 R.SEALAGAPLDNAPK.E 59 (68/-0.0375) 93 K.IQDVGLM*PM*GGMVPGPVSAAAPASEAA EEEDVPK.Q 2Oxidation(M) 126 (53/0.0171) 93 K.IQDVGLM*PM*GGM*VPGPVSAAAPASEAA EEEDVPK.Q 3Oxidation(M) 126 (70/-0.0385) 154 K.NYVQGINLVQAK.K 165 (66/-0.0263) 166 K.KLVESLPQEIK.A 176 (63/-0.006) 167 K.LVESLPQEIK.A 176 (40/0.0166) 189 K.AALEAVGGTVVLE.- 201 (49/0.0008)	21,809	9.34

Table 2 continued

No.	Acc. no.	Protein name	Total score	Matched peptides	Seq. cov. %	MS/MS peptides (ion score/mass error, Da)	Theor. MW	Theor. pI
329	Q9CXW2	28S ribosomal protein S22, mitochondrial	929	19	44	66 K.KPAFM*DEEVQR.I Oxidation(M) 76 (55/0.042) 66 K.KPAFMDEEVQR.I 76 (65/0.0063) 81 K.ITGLDLQK.T 88 (45/0.0151) 104 K.LM*TQAQLEEATR.L Oxidation(M) 115 (96/0.0237) 104 K.LMTQAQLEEATR.L 115 (83/0.0245) 127 K.M*PPVLEER.K Oxidation(M) 134 (64/0.049) 127 K.MPPVLEER.K 134 (49/-0.0235) 135 R.KPINDVLAEDK.I 145 (79/0.0156) 135 R.KPINDVLAEDKILEGTETNK.Y 154 (79/0.0337) 155 K.YVFTDISYNIPHR.E 167 (74/0.0315) 191 R.VIQIYFPK.E 198 (46/0.0519) 203 R.VLPPVIFKDENLK.T 215 (33/0.0651) 223 R.HADVLNLCVAQFEPDSAEYIK.V 243 (76/-0.0082) 244 K.VHHQTYEDIDR.H 254 (59/-0.0268) 266 R.HFGGM*AWYFVNK.K Oxidation(M) 277 (45/0.0345) 266 R.HFGGMAWYFVNK.K 277 (41/0.0871) 279 K.KIDGLLIDQIQR.D 290 (77/0.0443) 280 K.IDGLLIDQIQR.D 290 (69/-0.0022) 318 K.EQAAEGVDLIK.V 328 (48/0.029)	41,281	8.63
272	Q9D2G2	Dihydrolipoyllysine- residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial	1,353	13	35	69 K.NDVITVQTPAFAESVTEGDVR.W 89 (146/0.0517) 93 K.AVGDAVAEDEVVCEIETDK.T 111 (135/0.0903) 135 K.VEGGTPLFTLR.K 145 (61/-0.0046) 199 K.PVSAIKPTAAPPLAEAGAAK.G 218 (98/-0.0001) 241 K.EAQNTCAM*LTTFNEVDMSNIQEMR.A Oxidation(M) 264 (55/0.0081) 279 K.LGFM*SAFVK.A Oxidation(M) 287 (75/0.021) 279 K.LGFMSAFVK.A 287 (60/0.0321) 288 K.ASAFALQEQPVVNAVIDDATK.E 308 (145/0.0477) 327 R.GLVVPVIR.N 334 (54/-0.0184) 335 R.NVETM*NYADIER.T Oxidation(M) 346 (99/-0.0018) 335 R.NVETMNYADIER.T 346 (86/0.0413) 411 K.VEVRPM*M*YVALTYDHR.L 2 Oxidation(M) 426 (50/0.119) 411 K.VEVRPM*MYVALTYDHR.L Oxidation(M) 426 (85/0.0703)	49,306	9.11

Table 2 continued

No.	Acc. no.	Protein name	Total score	Matched peptides	Seq. cov. %	MS/MS peptides (ion score/mass error, Da)	Theor. MW	Theor. pI
162	P14733	Lamin-B1	1,454	30	45	16 R.ASAPATPLSPTR.L 27 (57/-0.0447) 44 R.LAVYIDK.V 50 (37/-0.0714) 53 R.SLETENSALQLQVTER.E 68 (93/0.04) 81 K.ALYETELADAR.R 91 (84/0.0252) 104 K.LQIELGK.F 110 (45/-0.0233) 111 K.FKAEHDQLLLNYAK.K 124 (87/0.0251) 113 K.AEHDQLLLNYAK.K 124 (70/0.0773) 136 K.LREYEAALNSK.D 146 (43/-0.0308) 138 R.EYEAALNSK.D 146 (37/-0.052) 147 K.DAALATALGDK.K 157 (76/-0.0031) 147 K.DAALATALGDKK.S 158 (60/0.0066) 183 K.KQLADETLLK.V 192 (55/-0.0107) 199 R.CQSLTEDLEFR.K 209 (72/0.0088) 211 K.NM*YEEEINETR.R Oxidation(M) 221 (87/0.0251) 211 K.NMYEEEINETR.R 221 (71/0.016) 260 R.LYKEELEQTYHAK.L 272 (62/-0.0964) 278 R.LSSEM*NTSTVNSAR.E Oxidation(M) 291 (88/0.0525) 278 R.LSSEMNTSTVNSAR.E 291 (103/-0.0634) 299 R.M*RIESLSSQLSNLQK.E Oxidation(M) 313 (44/0.0565) 301 R.IESLSSQLSNLQK.E 313 (102/0.0161) 322 R.IQELEDMLAK.E 331 (58/0.0329) 352 R.DQM*QQQLSDYEQLLDVK.L Oxidation(M) 368 (97/0.0403) 369 K.LALDM*EISAYR.K Oxidation(M) 379 (72/0.0019) 369 K.LALDMEISAYR.K 379 (83/0.0668) 459 K.NTSEQDQPM*GGWEMIR.K Oxidation(M) 474 (77/0.0617) 459 K.NTSEQDQPMGGWEMIR.K 474 (80/0.0008) 475 R.KIGDTSVSYK.Y 484 (41/-0.0065) 476 K.IGDTSVSYK.Y 484 (79/-0.0036) 493 K.AGQTVTVWAANAGVTASPPTDLIWK.N 517 (56/-0.0053) 518 K.NQNSWGTGEDVK.V 529 (52/-0.0192)	66,973	5.11

Table 2 continued

No.	Acc. no.	Protein name	Total score	Matched peptides	Seq. cov. %	MS/MS peptides (ion score/mass error, Da)	Theor. MW	Theor. pI
112	Q02788	Collagen alpha-2(VI) chain	933	15	18	111 R.YGGLHFSDQVEVFSPPGSDR.A 130 (60/0.0224) 167 K.GVVNFAVVITDGHVTGSPCGGIK.M 189 (101/0.0764) 218 R.DIANSPHELYR.N 228 (46/0.0541) 229 R.NNYATM*RPDSTEIDQDTINR.I Oxidation(M) 248 (76/-0.0462) 229 R.NNYATMRPDSTEIDQDTINR.I 248 (79/-0.0245) 651 K.NFVINVVNR.L 659 (43/0.0171) 675 R.VGVVQYSHEGTFEAIR.L 690 (104/0.0515) 696 R.VNSLSSFK.E 703 (58/-0.0058) 708 K.NLEWIAGGTWTPSALK.F 723 (104/0.0409) 724 K.FAYNQLIK.E 731 (40/-0.0003) 740 R.VFAVVITDGR.H 749 (80/0.0732) 846 R.PVDIVFLLDGSER.L 858 (107/-0.076) 981 R.KQNVVPTVVAVGGDVDMDVLTK.I 1002 (54/0.1324) 982 K.QNVVPTVVAVGGDVDM*DVLTK.I Oxidation(M) 1002 (48/0.1161) 1014 R.EKDFDSLAQPSFFDR.F 1028 (68/-0.0185)	1,11,406	6.01
600	Q9Z0F7	Gamma-synuclein	712	8	61	13 K.EGVVGAVEK.T 21 (39/0.0043) 44 K.TKENVVQSVTSVAEK.T 58 (108/-0.0297) 46 K.ENVVQSVTSVAEK.T 58 (77/-0.0331) 59 K.TKEQANAVSEAVVSSVNTVANK.T 80 (82/-0.0311) 61 K.EQANAVSEAVVSSVNTVANK.T 80 (83/-0.0024) 81 K.TVEEAENIVVTTGVVR.K 96 (104/-0.015) 97 R.KEDLEPPAQDQEAK.E 110 (63/0.0162) 98 K.EDLEPPAQDQEAK.E 110 (42/-0.0121)	13,152	4.68

et al. 2011; Danielsen et al. 2011; Zucchelli et al. 2010; McConnell et al. 2010; McCartney et al. 1997). The findings are relevant for interpretation of previous work and design of future studies on BA and WA biology and biochemistry.

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Conflict of interest The authors declare no conflicts of interest.

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