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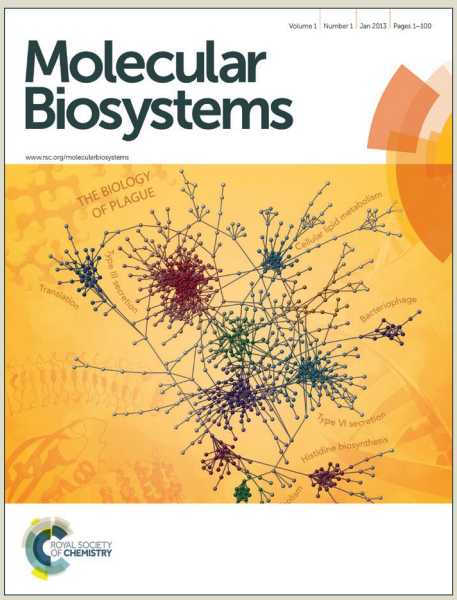



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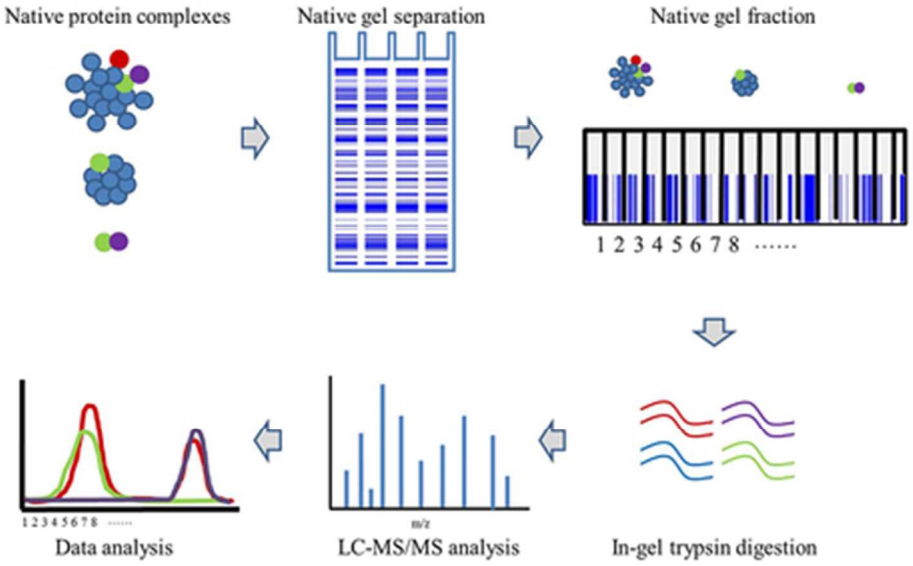


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# Identification of protein complexes of microsome in rat adipocyte by native gel coupled with LC-ESI-QTOF

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## Abstract

Study of the composition of microsome proteins/complexes/interactions in adipocyte provides useful information for researchers related to energy metabolism disorders. The native gel coupled with LC-ESI-QTOF approach was employed here for separating protein complexes. We found a series of proteins functionally clustered in biological processes of protein metabolic, cellular carbohydrate catabolic, response to stimulus and wounding, macromolecular complex subunit organization, positive regulation of molecular function, regulation of programmed cell death and biomolecule transport. According to clustering of proteins' electrophoresis profiles across native gel fractions and bioinformatics data retrieving, protein complexes/interactions involved in protein metabolic, cellular carbohydrate catabolic, macromolecular complex subunit organization and biomolecule transport were identified. Besides, the results also revealed some functional linkages, which may provide useful information for discovering previously unknown interactions. The interaction between SSAO and ALDH2 was verified by co-immunoprecipitation. The native gel combining mass spectrometry approach appeared to be a useful tool for investigating microsome proteins and complexes to complement the traditional electrophoresis approaches. The native gel strategy together with our findings should facilitate future studies of the composition of rat adipocyte microsome protein complexes in different conditions.


## Introduction



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Adipose tissue is important energy storage and metabolism organ, it is involved in the regulation of balance between energy intake and expenditure, whose disorder is related to diabetes mellitus, insulin resistance and disease[1-3]. Being endocrine organ adipose tissue is integrated with other peripheral organs and the CNS to maintain metabolic balance and normal physiological state. The appropriate glucose uptake, fat synthesis and decomposition are also necessary for maintaining the stable energy storage environment [3-5]. Study of the composition of microsome proteins/complexes/interactions may provide useful information for revealing related knowledge. Nevertheless, a practicable approach has yet to be performed.

The majority of proteins function as a portion of multiprotein complexes rather than isolated polypeptide [6]. In fact, the association of target unknown protein with partners belonging to a well-known protein complex involved in a particular mechanism would strongly suggest its biological function. Thus, the identification of interaction partners of a target protein would largely promote the understanding of its function and unravel related molecular mechanisms within the cell.

As very important communication bridge, membrane carries many important cellular processes, such as energetic metabolism, signal transduction and proteins trafficking [7]. Unfortunately, the highly lipophilic nature as well as the inclination to aggregation of protein complexes brings serious troubles to classical gel-based proteomic approaches [7, 8]. For this reason, the native polyacrylamide gel electrophoresis (native gel) approach was employed for the characterization of membrane protein complexes of rat adipose tissue in the presented study.



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Native gel is a nondestructive technique for separating native protein complexes by applying mild non-denaturing detergent (taurodeoxycholate (TDOC)) that are able to solubilize protein complexes with their native composition preserved, when it is dissolved in a proper concentration. TDOC provides the protein complex with a negative charge to enhance their mobility during electrophoresis[9]. Subunits belonging to a same complex would keep combining with each other during native gel electrophoresis, which could be validated by a second dimension SDS-PAGE. As SDS-PAGE separates individual subunits according to their own molecular mass in denaturing condition that forms vertical sets of spots. This newly developing technique would help researchers to isolate untagged, endogenous complexes in a wide scope[8, 10, 11].

In this study, we attempt to present the full picture of microsome protein complexes in rat adipocytes. We have visualized the separation results and characterized a number of protein complexes/interactions as well as some functional linkages. Furthermore, the interaction between SSAO and ALDH2 was further confirmed by immunoprecipitation and Western blotting.

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## Materials and methods

***Animals.*** Healthy male Wista rats were purchased from the Chinese Academy of Medical Sciences, Institute of Laboratory Animal. The rats were kept in normal cages with free access to food and drinking water, and were placed on a daily cycle of 12 hours of light followed by 12 hours of darkness without any special treatment, and sacrificed by cardio perfusion while they were 12 month old and weight 180–200 gram. Epididymal adipose was removed and preserved at –80 °C for further experiments. The experiments were approved by the Chinese Academy of Medical Sciences, Institute of Laboratory Animal. The license number is SCXK Beijing 2002–2001.

***Preparation of Microsomes.*** Epididymal adipose microsomes were separated from male Wistar rats under non-denaturing condition as previously described[12] with all steps carried out at 4 °C. In brief, ~2 gram adipose tissues were excised into pieces less than 1 $mm^3$, then were homogenized in sucrose buffer containing 250 mM sucrose, 1 mM EDTA, and 20 mM Tris/HCl (pH 7.4), protease inhibitor (Roche Diagnostics, US) was added before use. Homogenate was centrifuged at 1000 × *g* for 15 min, the supernatant was decanted and saved as Cyto-I, the pellet was saved and labeled as Tiss (tissue and nuclei). The Cyto-I supernatant was then centrifuged at 6 000 × *g* for 15 min to pellet mitochondrion. The supernatant was decanted and saved as Cyto-II and the pellet was saved and labeled as Mito. Lastly, the Cyto-II supernatant was centrifuged at 100 000 × *g* for 1h to pellet microsome. The supernatant was saved as Cyto and the pellet was labeled as Micro. A fraction of Tiss,



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Mito, Micro and Cyto were resuspended in RIPA buffer, and the relative amount of SSAO (a typical membrane protein) and Calr (Calreticulin, an endoplasmic reticulum protein) were analyzed by Western blot with equal amount of total proteins loaded. The qualified microsome was dissolved in extraction buffer containing 1% TDOC, 10% glycerol and 50 mM Bis-Tris (pH 7.4), to extract native protein complex. Protein concentration was measured by using Bradford reagent (BioRad).

***Native Gel electrophoresis and In-Gel Tryptic Digestion*.** 12 cm length of 2–7% native acrylamide gradient mini gels were prepared following a previously described protocol[9]. 100 μg proteins/protein complexes were loaded onto native gel followed by the performing of electrophoresis at 4 mA per gel at 4°C for 3h. Native gels were developed by coomassie blue staining followed by distaining in 10% methanol contained buffer. To intuitively evaluate the native gel separation effects, a 2-D SDS-PAGE was performed (Figure 2b). Approximately 1.5 mm width of native gel strips were cut off along the vertical direction and then were equilibrated in equilibration buffer containing 20% Glycerol and 0.1 M Tris-HCl (pH 6.8) at 0-4 °C for 1h. Gel strips were transferred onto 10% SDS separating gels followed by electrophoresis at 8 mA per gel at room temperature for separating complex subunit. SDS gels were developed by silver staining (Figure 2b). The qualified native gel was cut into 3 mm width slices along the horizontal direction. Each gel slice was then cut into approximately 1$mm^3$ pieces followed by in-gel digestion in 1.5 mL centrifuge tubes as described in[13]. After digestion, peptides were dried by vacuum concentration and solubilized in 3% acetonitrile/0.1% formic acid aqueous solution.



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***LC-Q-Tof Acquisition.*** Peptides were analyzed on two Agilent HPLCs, 1200 series and1260 series, coupled to a 6538 series ESI-Q-TOF (Agilent, CA, USA). The system comprised a C18 RP trap column (Agilent, Zorbax, 300 Å , 5μm, 5mm×3μm), and one C18 RP capillary column (Agilent, Zorbax, 300 A° , 5 μm, 0.075×150 mm). In the 1200 series pump system, mobile phase A contained 0.1% FA, 3% ACN in water, and in the 1260 series pump system, mobile phase A contained 0.1% FA, 5% ACN in water, whereas mobile phase B contained 0.1% FA, 10% water in ACN. Firstly, the 1200 series pump was switched on to load samples into the trap column and desalt online at 20 μl/min for 10 min. Secondly, the 1260 series pump was switched on to driving peptides across the capillary column at 8μl/min using a linear 90 min gradient (0–20% B over 40 min, 20–40% B over 20 min, 40–80% B over 10 min, 80% B for 3 min, 80–0% B over 7 min, and 100% A for 10 min).An Agilent 1200 series six-way valve was connected to switch the system between loading and analyzing steps. The mass spectrometer parameters were set as previously described [14].

***Data analysis.*** The MS/MS data were searched with the Spectrumill software (Agilent Technologies) using the rat species sequences of the Swiss-Prot database (sequences downloaded in October 2013; 33563 entries). A maximum of two missed cleavages were allowed for tryptic digestion, carbamidomethyl (C) was used as a fixed modification, methionine oxidation was defined as variable modification, and mass tolerance values of 50 ppm and 0.1 Da were set for precursor and fragment ions, respectively. Data retrieval was executed under the following limits: Peptides were considered to be identified if the probability of a false discovery rate (FDR) was less



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than 1%, the FDR calculated through statistic method of protein identification result was limited to less than 1%, protein was considered to be identified while at least two peptides of which were matched. Later, cluster functional annotation based on GO [15, 16] was conducted using DAVID (database for annotation, visualization, and integrated discovery) [17], where the default parameters of DAVID were used and the ontology GO_CC_FAT was selected. The total protein intensity profiles across the gel fractions were normalized and then hierarchically clustered based on Pearson distance measurement using Cluster 3.0[6, 18-20]. The output of the clustering has been presented in a heat map using the TreeView java software. Consensus clustering implemented in Genepattern [21, 22] was performed. The parameters used were set as follows: k tested = from 2 to predicted value (e.g. 15); clustering algorithm = hierarchical clustering; *n* resamplings = 1,000; clustering metrics = (1–Pearson correlation) distance and average linkage; proportion of samples in each resampling = 80%. Finally, the STRING database was used to retrieve the protein components of each defined clusters to identify either previously known protein–protein interactions or other functional linkages within the cluster [23, 24].

***Co-immunoprecipitation*.** Adipose tissues were lysed by RIPA buffer, followed by pelleting tissular debris by centrifugation at 12,000 rpm for 15 minutes at 4°C. Protein concentrations of supernatant were measured by using Bradford reagent. Protein extracts were precleared by adding the appropriate control IgG and resuspended Protein A/G PLUS-Agarose (Santa Cruze) to incubate at 4° C for 30 minutes. After centrifugation at 2,000 rpm for 5 minutes at 4° C, supernatants were transferred to a



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fresh 1.5 ml centrifuge tube and incubated with primary antibody (anti-SSAO (abcam, UK) or anti-ALDH2 (Santa Cruze)) on ice for 12 h and then were incubated with resuspended Protein A/G-Agarose at 4 °C on a rotating device for 12 h. Immunoprecipitates were collected by centrifugation at 2,000 rpm for 5 min at 4 °C, and the pellets were washed 4 times with 1 mL RIPA buffer[25-27]. The negative controls were carried along by using an isotype normal antibody (Santa Cruze). SSAO and ALDH2 were detected by Western Blotting.

## Results and discussion

***Strategy*-**On the whole, microsomes from adipose tissues were isolated, proteins of which were separated by native gel according to molecular mass of complexes they belonged. We then evaluated the clustering of protein electrophoresis profiles to show the probability of interaction happened.

The detailed strategy was as shown in figure 1, native protein complexes of microsomes were extracted by TDOC contained extraction buffer. For proteomic analysis, the resulting protein complexes were firstly separated by native gel, and then the gel was cut into slices along the horizontal direction, proteins in each slice were in-gel digested separately, followed by desalting and LC-Q-Tof analysis. Peptides were identified using Spectrumill (Agilent Technologies). The total protein intensity profiles across the gel fractions were normalized and then hierarchically clustered or k-means classified based on Pearson distance measurement (Fig. 1).

Three biological replicates were performed and analyzed identically. In total, 206



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proteins were identified and 86 proteins were detected more than in one replicate. However, only 76 proteins were preserved that produced 10 cluster groups after the data had been filtered such that proteins were clustered in same cluster at least twice out of the three biological replicates. Finally, the STRING database was used to retrieve the protein components of each defined cluster group to identify either previously known protein–protein interactions or functional linkages.

***Microsomes purification-***To characterize the purity of microsomes, we compared the distribution of two typical microsome proteins among differential centrifugation fractions by Western blot (Supplemental Fig. 1a, b), and analyzed the GO terms of identified proteins by the DAVID functional annotation tool for cellular component (GOTERM_CC_FAT)[17] (Supplemental Fig. 2a, b). It was observed that SSAO, a typical membrane protein and Calreticulin (Calr), an endoplasmic reticulum protein, were highly enriched in microsomes fraction (Supplemental Fig. 1a, b). Additionally, ~87% of identified proteins were associated with microsome components according to their GO terms annotation. These results indicated relatively high purity microsomes were obtained. It was also observed that 7 cluster groups were significantly enriched with EASE Scores (ES) more than 1.3 (Supplemental Fig. 2a, b). They were vesicle, extracellular region organelle, microsome & membrane fraction, membrane-enclosed lumen, secretory granule, organelle membrane and ribosome subunit.

***Native gel separation-***Protein complexes of microsomes were separated by native gel primarily according to their physical shape and size and visualized by coomassie blue staining (Fig. 2a). To intuitively evaluate the native gel separation effects, gel strips



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cut along the vertical direction were transferred onto SDS gels for the 2-D separation. Protein subunits of complexes were separated in SDS-PAGE according to their own molecular mass. The SDS-PAGE was visualized by silver staining (Fig. 2b). The vertical sets of spots in the final two-dimensional gel thus corresponding to subunits of different protein complexes separated by native gel were observed. In addition, we also observed that ~39% proteins were detected in only one gel fraction and ~88% proteins were detected in less than five fractions (49% in 2-4 fractions)(Supplemental Fig. 2c), which indicated well resolution of our native gel. The 2-D SDS-PAGE results together with the analysis of protein distribution across native gel fractions demonstrated the native gel separation was reliable.

***Protein functional annotation-***Following the separation of native protein complexes, proteins in the native gel slices were in-gel digested and identified by HPLC-ESI-Q-TOF system, where, in total, 86 proteins were identified in at least two out of the three biological replicates. According to functional annotation clustering of DAVID, clusters related to regulation of protein metabolic process, cellular carbohydrate catabolic process, response to stimulus and wounding, macromolecular complex subunit organization, positive regulation of molecular function, regulation of programmed cell death and biomolecule transport were found having ES more than 1.3 (Fig. 2c). This may suggest the active role of adipose tissues in these processes.

***Protein complexes/interactions identification-***In the expect of proteins interacted within complex would display similar electrophoresis profiles, the separation and characterization of protein complexes across the gel fractions were used to facilitate



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the identification of possible interacted proteins. Through the hierarchical clustering of proteins' electrophoresis profiles by Cluster 3.0 we were able to observe the classification relationship of identified proteins on the whole. One example of the three replicates was presented as heat map in Figure 3a. However, the exact number of protein clusters required was unascertained so far. Therefore, we tested a series number of clusters ($k$) performing Consensus Clustering in Genepattern. In Consensus Clustering, data were analyzed based on resampling techniques. For each tested $k$ value, consensus among the multiple sampling runs was assessed and summarized in a consensus matrix. Visual inspection of the consensus matrixes (Fig. 3b), and of the corresponding summary statistics (cumulative distribution function (CDF) plots) (Fig. 3c) can help determine the optimal number of clusters as described in Ref.[28]. An example of the three replicates was presented in Figure 3. As evident in the figure, $k$ = 15 led to a clean consensus matrix and no obvious increase in clustering stability was observed going from $k$ = 15 to $k$ = 16. Thus, all the identified proteins of this replicate were divided into 15 clusters.

Based on the classification calculation, proteins were clustered in same cluster at least twice out of the three biological replicates were preserved. This resulted in 76 proteins belonging to 10 cluster groups. Protein accession numbers within each cluster group were package inputted into the multiple names dialog box of STRING to retrieve known and predicted protein-protein interactions, where a medium confidence score of 0.400 was selected. The result was showed in table 1.

***Protein complexes or protein interactions***



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*1*. In protein metabolic, the most complete complex identified in native gels was proteasome (cluster group 1): an ATP-dependent multicatalytic proteinase complex involves regulation of protein metabolic that can cleave peptides with Arg, Phe, Tyr, Leu, and Glu adjacent to the leaving group. Eleven subunits of the core proteasome complex were identified, PSA1-7 and PSB1-3, 5, 8. The major function of proteasome is degrading unrequired or damaged proteins, which is the main mechanism of the cell to regulate particular proteins or to remove misfolded proteins. These subunits formed the strongest vertical sets of spots on the 2D SDS-PAGE gel suggesting adipose tissues as very active organs in biosynthesis and metabolism[29].

In addition, the interaction between Vdac2 and Ubb[30] and between Cav1 and Ubb [31] (cluster group 2) were previously identified by affinity capture-western assay, which was consistent with our findings that both Vdac2 and Cav1 were classified into the same cluster with Ubb. Both Vdac2 and Cav1 seem to interact with Ubb relating to its protein degradation functions for no other functional relations have been found so far.

*2*. Regarding carbohydrate catabolic, several protein interaction pairs involved in were found, e.g. Ogdh and Dld (cluster group 3), pyruvate dehydrogenase E1 (Pdha1 and Pdhb) and Ldha (cluster group 4).

Ogdh and Dld are components of 2-oxoglutarate dehydrogenase complex, which is a well described multicopy complex containing three enzymatic components, 2-oxoglutarate dehydrogenase (E1), dihydrolipoamide succinyltransferase (E2) and lipoamide dehydrogenase (E3) that catalyzes the overall conversion of 2-oxoglutarate



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to succinyl-CoA and CO2[32, 33]. In this study, Ogdh (E1) and Dld (E3) were detected together but not E2 component, suggesting the decomposition of this complex under our solubilization condition. However, it has been demonstrated that a high affinity interaction between E1and E3 that can form a stable subcomplex comprising single copies of E1 and E3[33], which is consistent with our results.

The pyruvate dehydrogenase complex catalyzes the overall conversion of pyruvate to acetyl-CoA and $CO_2$, and thereby links the glycolytic pathway to the tricarboxylic cycle [34, 35]. In this study, both pyruvate dehydrogenase E1 component subunit alpha and beta were detected together. Besides, an upstream lactate dehydrogenase [36, 37], Ldha was also detected.

Moreover, an obvious interaction network consists of three glycolysis related enzymes: Pklr, Pkm, and Ldhb, and a TCA cycle related enzyme: Mdh1, was found (cluster group 5). As Pklr and Pkm catalyze the synthesis of pyruvate from phosphoenolpyruvate[38], Ldhb uses pyruvate to produce lactate[39], and Mdh1 catalyzes the reaction of malate and $NAD^+$ to produce oxaloacetate and NADH[40]. This interaction network would probably largely facilitate the metabolism of carbohydrates, fats, and proteins, and therefore involves a wider range of biological metabolism processes.

*3*. The present study also found some protein interaction involved in macromolecular complex subunit organization. Three major constituents of microtubules: TBB4B, TBB5 and TBA1B were found together (cluster group 6). Microtubules are very



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important subcellular structures that are involved in maintaining the structure of the cell, intracellular transport, the movement of secretory vesicles, organelles, as well as intracellular macromolecular assemblies [41].

The interaction between Hspa5 and Hsp90b1 was identified in the same cluster (cluster group 7). Hspa5 is probably involved in multimeric protein complexes assembly inside the ER while Hsp90b1 seems to have two opposite functions: one is the molecular chaperone function in processing and transport of secreted proteins as the other one is involved in endoplasmic reticulum associated degradation[42]. Both molecules seem to interact inside the ER regulating synthesis of proteins.

*4*. As representatives of biomolecule transport group, four members of Ras-related GTPases were detected together (cluster group 8). There are four interactions among the four Ras-related GTPases: Rab2a and Rab1b, Rab1b and Rab6a, Rab1b and Rab11b, Rab6a and Rab11b. Rab2a and Rab1b are involved in regulation of protein transport from the endoplasmic reticulum (ER) towards the Golgi apparatus[43, 44], while Rab6a is required for protein transport from the Golgi apparatus to the ER and Rab11b is a regulator of membrane delivery during cytokinesis and endosomal trafficking[45-47].

Furthermore, four 14-3-3 proteins were detected together: Ywhag, Ywhab, Ywhaz and Ywhae (cluster group 8). This protein family is implicated in the regulation of a large spectrum signaling pathways which through binding to the phosphorylated serine motif results in the active/inactive of the interaction partner[48]. According to



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STRING retrieving result, these four proteins can bind to each other to form an integral interaction network suggesting active signal regulation in adipocyte.

On the other hand, unlike proteins clustered in protein metabolic, carbonhydrate catabolic, macromolecular complex subunit organization and biomolecule transport processes, proteins belonging to response to stimulus and wounding, positive regulation of molecular function and regulation of programmed cell death were either not clustered or seemed to have no interactional relations within cluster, which might be partially due to relatively lack of interaction information in Rattus norvegicus database or other factors. However, some functional linkages of these groups were discovered.

***Functional linkages of proteins within clusters-***Besides the previously known or predicted binding actions, we also found a lot of protein-protein functional linkages. Some examples were discussed below for their close functional relations and similar electrophoresis may bring new insights in discovering unknown interactions in Rattus norvegicus.

*1*. P4hb and Apoa1 were found to associate with each other in chylomicron assembly pathway (cluster group 8). P4hb is known as a microsomal triglyceride transfer protein (MTP) that is required for lipid addition in the second phase of chylomicron assembly in the lumen of the ER. Apoa1 is important lipoprotein component of chylomicron as a nascent chylomicron entering the lymphatic circulation contains ~60 copies of Apoa1. Moreover, it has been found that MTP binds to ApoB with high



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affinity through ionic interaction in human cells [49, 50].

*2*. Regulator of G-protein signaling 14 (Rgs14) and Rap1a (cluster group 8). Rgs14 functions either as a GTPase-activating protein, thereby driving G protein alpha subunits into their inactive GDP-bound form, or as a GDP-dissociation inhibitor that is involved in the regulation of Rap1a, a small GTPase that controls diverse processes, such as cell polarity, cell adhesion and cell-cell junction formation [51]. Intriguingly, functional linkages between Rap1a and 14-3-3 proteins were also retrieved (cluster group 8) but without any further information that could help to prove their direct connection. It is worth mentioning that they are both related to signal transduction, suggesting them to be connected through intermediate molecules. Similarly, linkages between Rab2a and 14-3-3 proteins were also presented by STRING. However, the confident scores were to some extent too low (less than 0.3 given by STRING) to consider their direct interaction.

*3*. SSAO/AOC3 and ALDH2 (cluster group 4). SSAO is a copper containing monoamine oxidase that catalyzes the oxidative deamination of amines and produce aldehyde, hydrogen peroxide, ammonia [52, 53]. ALDH2 is an aldehyde dehydrogenase that catalyzes oxidation of aldehyde and produce carboxylate [54]. To the best of our knowledge, the interaction between SSAO and ALDH2 has not been reported before. Thus, we performed co-immunoprecipitation assay [25-27] to verify whether SSAO interacts with ALDH2. Indeed, the co-immunoprecipitation result showed a conspicuous interaction between SSAO and ALDH2 (Fig. 4). These two proteins have very close relationship in beta-Alanine metabolism pathway and were clustered



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together in our data.

Similarly, Pdia3, a protein disulfide-isomerase[55], and Hsp90ab1, a molecular chaperone[56] (cluster group 5), both have functions in promoting the maturation of target proteins were detected together.

***Fragmented protein complexes or protein interactions-***There were still some well described complexes with subunits detected in different fractions/clusters, which were deviated from our desire. For instance, Both Atp5a1 (cluster group 5) and Atp5b (cluster group 9) are subunits of mitochondrial membrane ATP synthase, but were detected in separated fractions, and of course were divided into different clusters. The remaining independent had no information revealed. They might have homologous interaction to form homomultimer, or interact with unidentified partners, or interact with one/ some member(s) of the clusters that previously unknown. Since their molecular weights were too low to stay at their native gel locations. However, more detailed studies are still needed to figure out the exact causes.

In summary, this native gel coupled mass spectrometry method employed here appeared to be helpful for separation and identification of microsome protein complexes of rat adipocytes. The clustering of protein electrophoresis profiles based on Pearson distance measurement helped us to objectively discover known/potential protein interactions. We have found a number of complexes related to a series of biological processes. Furthermore, we also found some protein pairs having very close relations co-electrophoresed in native gel, which may bring new insights in



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discovering unknown interactions. These findings as well as the presented native gel based pattern should facilitate future studies of the composition of rat adipocyte microsome protein complexes in different conditions such as obesity and diabetes mellitus.

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## Figure legends

**Figure 1. Native protein complex analysis applying native gel combining mass spectrometry workflow.** Workflow for native gel based protein complex separation.

**Figure 2. 2D Native/SDS-PAGE and gene ontology (GO) annotation clustering of identified proteins. (a)** Protein complexes of microsomes were separated by native gel. (**b)** Subunits of complexes were separated by SDS-PAGE. **(c)** DAVID functional annotation tool was applied for the GO analysis of identified proteins, and the significantly enriched clusters were plotted in bar graph with enrichment scores (ES). **(d)** Pie graph of the percent composition of enriched clusters.

**Figure 3. Example of hierarchical clustering of protein electrophoresis profiles and consensus clustering for evaluating the number of protein clusters required. (a)** Heat map of hierarchical clustering of protein electrophoresis profiles according to their Pearson correlation coefficients, where the normalized data were used. (**b)** Consensus matrices of identified proteins for k = 2 to k = 16. **(d)** Cumulative distribution function (CDF) plots corresponding to the consensus matrices for k = 2 to k = 16.

**Figure 4. Validation of newly identified interaction between SSAO and ALDH2 by co-immunoprecipitation.** reciprocal co-immunoprecipitation of endogenous SSAO and ALDH2 in rat adipocyte.

**Supplemental Figure 1. Ditribution of membrane proteins (SSAO and Calr) in differential centrifugation fractions.** Rat adipose tissues were continuously centrifuged and pelleted by 1 000 × g (Tissues), 6 000 × g (Mitochondria), 100 000 × g (Mirosomes), and cytoplasm fraction was



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collected in the 100 000 × g supernatant. Both SSAO and Calr were highly enriched in microsomes fraction according to Western blot (**a**) and quantitative analysis (**b**). Values were means ± SEM (n=3), **, $P < 0.01$.

**Supplemental Figure 2. Gene ontology (GO) annotation clustering for cellular component of identified proteins and protein frequency in the native gel fraction. a,** DAVID functional annotation tool was applied for the GO analysis for cellular component of identified proteins, and the significantly enriched clusters were plotted in bar graph with enrichment scores (ES). **b,** Pie graph of the percent composition of enriched clusters. **c,** protein frequency in the native gel fraction plotted as histogram..

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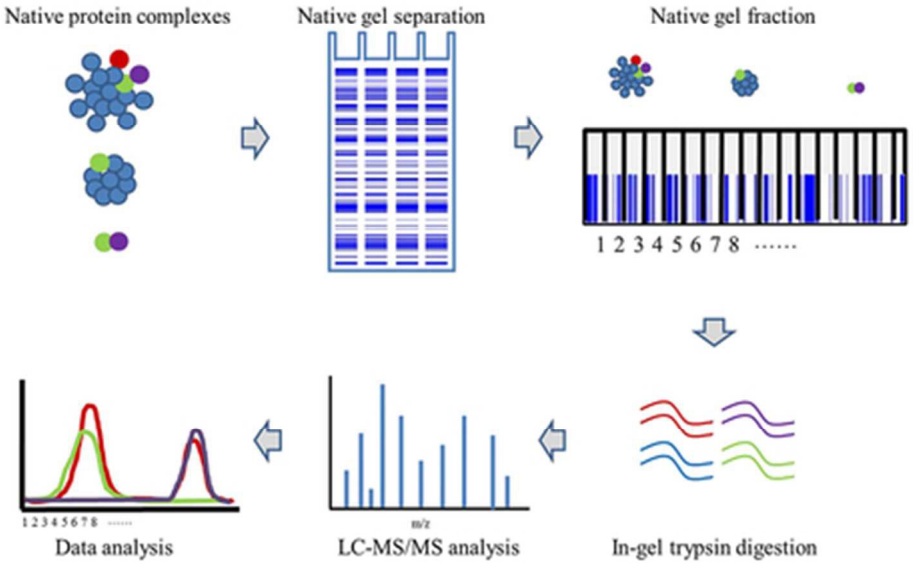


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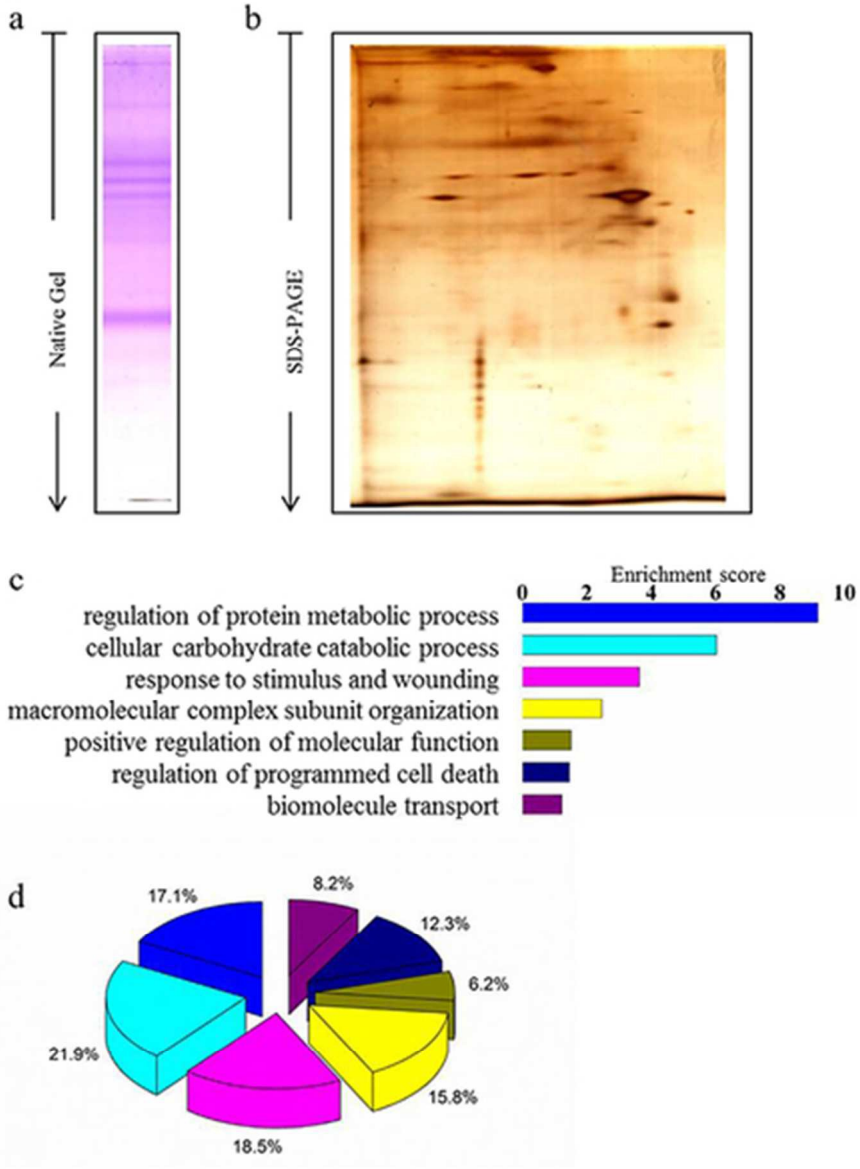


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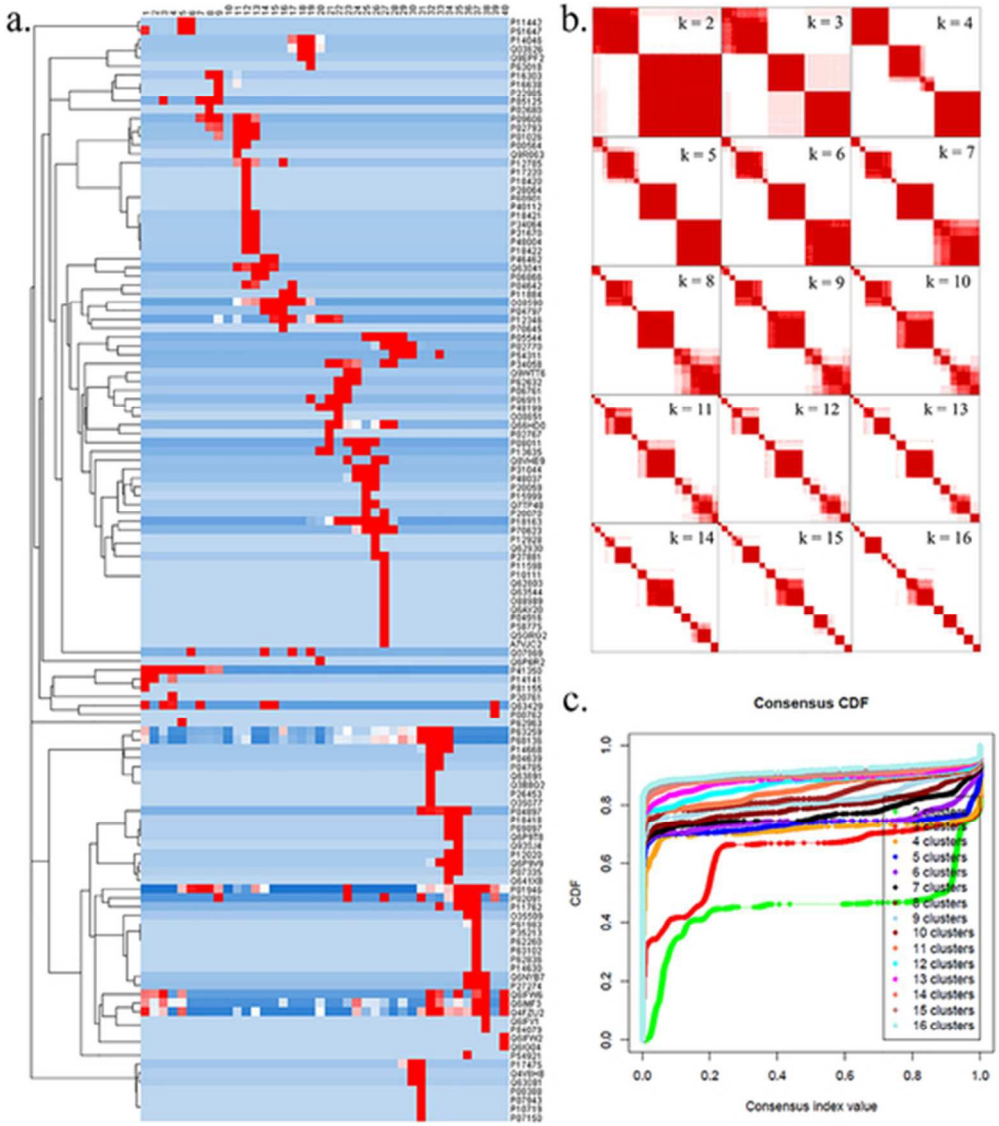


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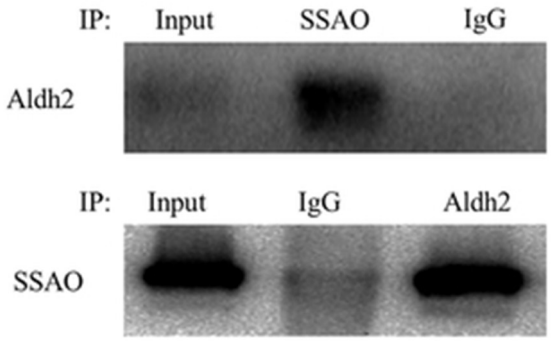


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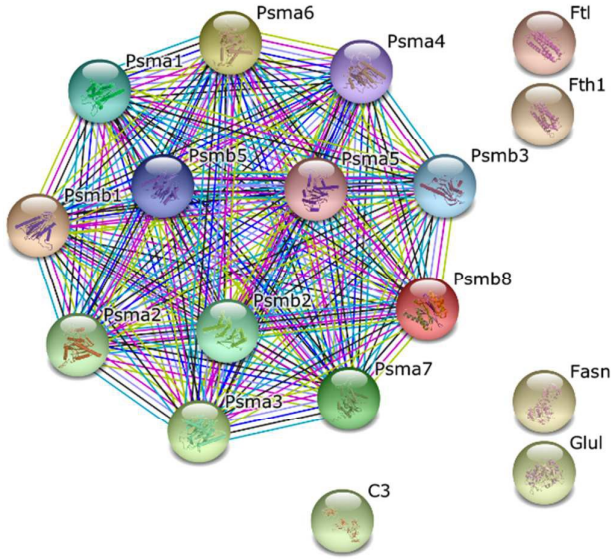
Table 1. Protein complexes or protein interactions or functional linkages within cluster groups

| Cluster group | Accession Number | Protein ID | Gene ID | Protein Name | Mass (kD) | Electrophoresis distance (mm) | Intaractions/Fucyional linkages |
|---|---|---|---|---|---|---|---|
| 1 | P12785 | FAS_RAT | Fasn | Fatty acid synthase | 273 | 30-39, 45-48 | |
| | P17220 | PSA2_RAT | Psma2 | Proteasome subunit alpha type-2 | 26 | 33-36 | |
| | P18420 | PSA1_RAT | Psma1 | Proteasome subunit alpha type-1 | 30 | 33-36 | |
| | P18421 | PSB1_RAT | Psmb1 | Proteasome subunit beta type-1 | 26 | 33-39 | |
| | P18422 | PSA3_RAT | Psma3 | Proteasome subunit alpha type-3 | 28 | 33-39 | |
| | P19132 | FRIH_RAT | Fth1 | Ferritin heavy chain | 21 | 24-42 | |
| | P21670 | PSA4_RAT | Psma4 | Proteasome subunit alpha type-4 | 29 | 33-39 | |
| | P28064 | PSB8_RAT | Psmb8 | Proteasome subunit beta type-8 | 31 | 33-36 | |
| | P28075 | PSB5_RAT | Psmb5 | Proteasome subunit beta type-5 | 29 | 33-39 | |
| | P34064 | PSA5_RAT | Psma5 | Proteasome subunit alpha type-5 | 26 | 33-39 | |
| | P40112 | PSB3_RAT | Psmb3 | Proteasome subunit beta type-3 | 23 | 33-39 | |
| | P40307 | PSB2_RAT | Psmb2 | Proteasome subunit beta type-2 | 23 | 33-39 | |
| | P48004 | PSA7_RAT | Psma7 | Proteasome subunit alpha type-7 | 28 | 33-39 | |
| | P60901 | PSA6_RAT | Psma6 | Proteasome subunit alpha type-6 | 27 | 33-36 | |
| | P01026 | CO3_RAT | C3 | Complement C3 | 186 | 24-27, 30-39 | |
| | P02793 | FRIL1_RAT | Ftl1 | Ferritin light chain 1 | 21 | 21-27, 30-39 | |
| | P09606 | GLNA_RAT | Glul | Glutamine synthetase | 42 | 18-27, 30-39 | |
| 2 | P41350 | CAV1_RAT | Cav1 | Caveolin-1 | 21 | 0-27 | |
| | P81155 | VDAC2_RAT | Vdac2 | Voltage-dependent anion-selective channel protein 2 | 32 | 0-3 | |

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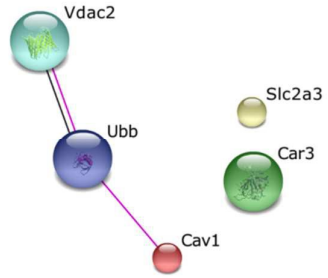
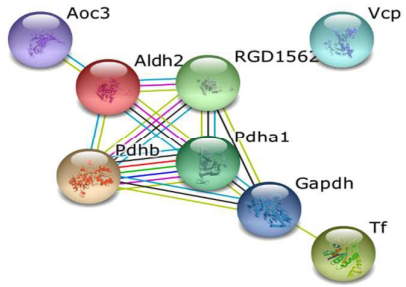
| Cluster group | Accession Number | Protein ID | Gene ID | Protein Name | Mass (kD) | Electrophoresis distance (mm) | Intaractions/Fucyional linkages |
|---|---|---|---|---|---|---|---|
| | Q63429 | UBC_RAT | Ubc | Polyubiquitin-C | 91 | 0-3, 6-12, 18-21, 39-45, 114-117 |   |
| | Q07647 | GTR3_RAT | Slc2a3 | Solute carrier family 2, facilitated glucose transporter member 3 | 54 | 0-3 | |
| | P14141 | CAH3_RAT | Ca3 | Carbonic anhydrase 3 | 29 | 0-6, 9-12 | |
| 3 | Q5XI78 | ODO1_RAT | Ogdh | 2-oxoglutarate dehydrogenase, mitochondrial | 116 | 57-60 |   |
| | Q6P6R2 | DLDH_RAT | Dld | Dihydrolipoyl dehydrogenase, mitochondrial | 54 | 57-60 | |
| 4 | P49432 | ODPB_RAT | Pdhb | Pyruvate dehydrogenase E1 component subunit beta, mitochondrial | 39 | 42-45 |   |
| | P04642 | LDHA_RAT | Ldha | L-lactate dehydrogenase A chain | 36 | 33-39, 48-51 | |
| | P26284 | ODPA_RAT | Pdha1 | Pyruvate dehydrogenase E1 component subunit alpha, somatic form, mitochondrial | 43 | 45-48 | |
| | P11884 | ALDH2_RAT | Aldh2 | Aldehyde dehydrogenase, mitochondrial | 56 | 45-51 | |
| | O08590 | AOC3_RAT | Aoc3 | Membrane primary amine oxidase | 85 | 30-57 | |

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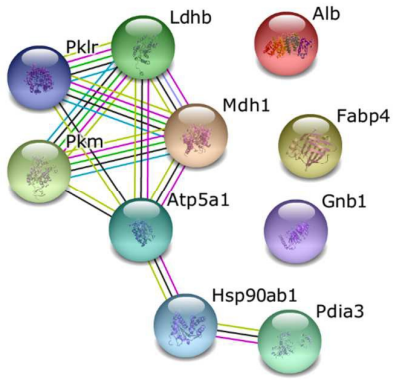
| Cluster group | Accession Number | Protein ID | Gene ID | Protein Name | Mass (kD) | Electrophoresis distance (mm) | Intaractions/Fucyional linkages |
|---|---|---|---|---|---|---|---|
| | P04797 | G3P_RAT | Gapdh | Glyceraldehyde-3-phosphate dehydrogenase | 36 | 39-48 | |
| | P12346 | TRFE_RAT | Tf | Serotransferrin | 76 | 24-27, 30-33, 42-66 | |
| | P46462 | TERA_RAT | Vcp | Transitional endoplasmic reticulum ATPase | 89 | 39-45 | |
| 5 | P42123 | LDHB_RAT | Ldhb | L-lactate dehydrogenase B chain | 37 | 63-66, 72-75 |  |
| | P11980 | KPYM_RAT | Pkm | Pyruvate kinase PKM | 58 | 15-18, 72-78 | |
| | P12928 | KPYR_RAT | Pklr | Pyruvate kinase PKLR | 62 | 75-78 | |
| | O88989 | MDHC_RAT | Mdh1 | Malate dehydrogenase, cytoplasmic | 36 | 78-81 | |
| | P34058 | HS90B_RAT | Hsp90ab1 | Heat shock protein HSP 90-beta | 83 | 60-72, 75-84 | |
| | P11598 | PDIA3_RAT | Pdia3 | Protein disulfide-isomerase A3 | 57 | 78-81 | |
| | P15999 | ATPA_RAT | Atp5a1 | ATP synthase subunit alpha, mitochondrial | 60 | 72-75 | |
| | P02770 | ALBU_RAT | Alb | Serum albumin | 69 | 75-93 | |
| | P54311 | GBB1_RAT | Gnb1 | Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1 | 37 | 81-99 | |
| | P70623 | FABP4_RAT | Fabp4 | Fatty acid-binding protein, adipocyte | 15 | 69-84 | |



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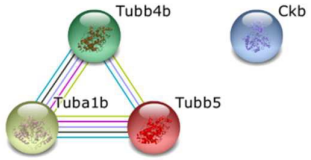
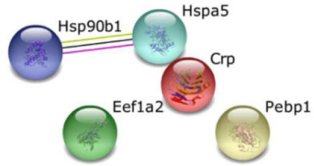
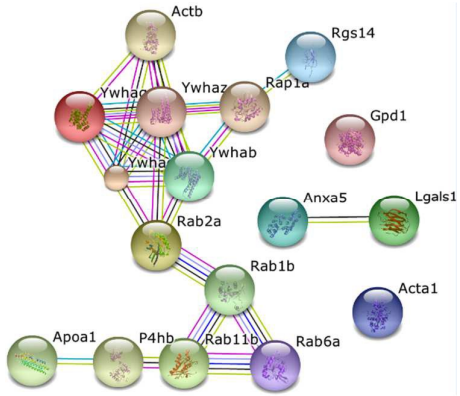
| Cluster group | Accession Number | Protein ID | Gene ID | Protein Name | Mass (kD) | Electrophoresis distance (mm) | Intaractions/Fucyional linkages |
|---|---|---|---|---|---|---|---|
| 6 | P69897 | TBB5_RAT | Tubb5 | Tubulin beta-5 chain | 50 | 99-105 |  |
| | Q6P9T8 | TBB4B_RAT | Tubb4b | Tubulin beta-4B chain | 50 | 99-105 | |
| | Q6P9V9 | TBA1B_RAT | Tuba1b | Tubulin alpha-1B chain | 50 | 93-105 | |
| | P07335 | KCRB_RAT | Ckb | Creatine kinase B-type | 43 | 99-105 | |
| 7 | Q66HD0 | ENPL_RAT | Hsp90b1 | Endoplasmin | 93 | 60-72, 75-84 |  |
| | P06761 | GRP78_RAT | Hspa5 | 78 kDa glucose-regulated protein | 72 | 63-69 | |
| | P31044 | PEBP1_RAT | Pebp1 | Phosphatidylethanolamine-binding protein 1 | 21 | 69-78 | |
| | P48199 | CRP_RAT | Crp | C-reactive protein | 25 | 57-69 | |
| | P62632 | EF1A2_RAT | Eef1a2 | Elongation factor 1-alpha 2 | 50 | 63-72 | |
| 8 | Q6NYB7 | RAB1A_RAT | Rab1A | Ras-related protein Rab-1A | 23 | 105-114 |  |
| | P05712 | RAB2A_RAT | Rab2a | Ras-related protein Rab-2A | 24 | 108-111 | |
| | Q9WVB1 | RAB6A_RAT | Rab6a | Ras-related protein Rab-6A | 24 | 105-114 | |
| | O35509 | RB11B_RAT | Rab11b | Ras-related protein Rab-11B | 24 | 105-111 | |
| | P63102 | 1433Z_RAT | Ywhaz | 14-3-3 protein zeta/delta | 28 | 108-111 | |
| | P61983 | 1433G_RAT | Ywhag | 14-3-3 protein gamma | 28 | 105-111 | |
| | P62260 | 1433E_RAT | Ywhae | 14-3-3 protein epsilon | 29 | 108-111 | |
| | P35213 | 1433B_RAT | Ywhab | 14-3-3 protein beta/alpha | 28 | 108-111 | |
| | P04785 | PDIA1_RAT | P4hb | Protein disulfide-isomerase | 57 | 93-99 | |
| | P04639 | APOA1_RAT | Apoa1 | Apolipoprotein A-I | 30 | 93-99 | |
| | P62836 | RAP1A_RAT | Rap1a | Ras-related protein Rap-1A | 21 | 108-111 | |

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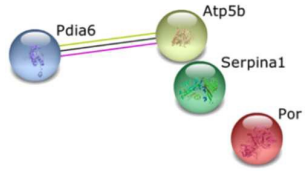
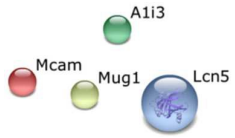
| Cluster group | Accession Number | Protein ID | Gene ID | Protein Name | Mass (kD) | Electrophoresis distance (mm) | Intaractions/Fucyional linkages |
|---|---|---|---|---|---|---|---|
| | O08773 | RGS14_RAT | Rgs14 | Regulator of G-protein signaling 14 | 59 | 99-102, 105-108 | |
| | P11762 | LEG1_RAT | Lgals1 | Galectin-1 | 15 | 96-99, 102-111 | |
| | O35077 | GPDA_RAT | Gpd1 | Glycerol-3-phosphate dehydrogenase [NAD(+)], cytoplasmic | 37 | 93-96 | |
| | P14668 | ANXA5_RAT | Anxa5 | Annexin A5 | 36 | 90-102 | |
| | P63259 | ACTG_RAT | Actg1 | Actin, cytoplasmic 2 | 42 | 0-3, 6-9, 15-27, 30-36, 42-57, 60-102 | |
| | P68136 | ACTS_RAT | Acta1 | Actin, alpha skeletal muscle | 42 | 0-3, 6-9, 15-27, 30-36, 42-57, 60-102 | |
| 9 | P00388 | NCPR_RAT | Por | NADPH--cytochrome P450 reductase | 77 | 90-93 |  |
| | P17475 | A1AT_RAT | Serpina1 | Alpha-1-antiproteinase | 46 | 85-93 | |
| | P10719 | ATPB_RAT | Atp5b | ATP synthase subunit beta, mitochondrial | 56 | 90-93 | |
| | Q63081 | PDIA6_RAT | Pdia6 | Protein disulfide-isomerase A6 | 48 | 87-93 | |
| 10 | P06911 | LCN5_RAT | Lcn5 | Epididymal-specific lipocalin-5 | 21 | 54-57, 60-69 |  |
| | P14046 | A1I3_RAT | A1i3 | Alpha-1-inhibitor 3 | 164 | 48-60 | |
| | Q03626 | MUG1_RAT | Mug1 | Murinoglobulin-1 | 165 | 48-60 | |
| | Q9EPF2 | MUC18_RAT | Mcam | Cell surface glycoprotein MUC18 | 71 | 51-57 | |

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