

Proteomic changes in response to lipin1 overexpression in 293T human renal epithelial cells

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Lipin1, a member of the lipin family, serves as a phospholipid phosphatase or a co-transcriptional regulator in lipid metabolism. Recent studies also show that lipin1 is involved in many other cellular metabolism processes. However, the clear regulatory mechanism for lipin1 is unknown. The 293T human renal epithelial cell line represents a commonly used and well established expression system for recombinant proteins. Herein, we used two-dimensional polyacrylamide gel electrophoresis (2D-GE) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) to explore the changes in protein expression induced by lipin1 overexpression in 293T cells. Western blotting was used to confirm one of the expression changes of related proteins. Subsequently, the function and relationship of these proteins were analyzed by bioinformatics approach. By using 2D-PAGE, approximately 152 proteins were separated and eleven proteins were found to be significantly affected by lipin1 overexpression compared to the control. Among them, three proteins (eEF-1B γ , CCT1 and CCT3) were up-regulated and other eight proteins (NDKA, Stathmin, HNRNP A1, TK, KRT1, PKM, RanBP1 and LDHB) were down-regulated. These proteins were successfully identified with peptide mass fingerprinting using MALDI-TOF-MS after in-gel trypsin digestion. The bioinformatic analysis showed that these proteins are classified into seven protein species, including transferase, cleavage enzyme, cytoskeleton protein, chaperone protein, regulatory protein, structural protein and oxidoreductase. The results highlight the potential roles of lipin1 involved in many cellular metabolism processes, including myelin synthesis, extracellular domain formation, membrane bound vesicle synthesis and companion protein T complex synthesis.

Keywords: 293T cells, Lipin1, MALDI-TOF-MS, Overexpression, Proteomics, RanBP1

Lipin1, encoded by *LPIN1* gene, which belongs to the family of lipins, is a newly discovered key protein participated in regulation of lipid metabolism in recent years¹. Lipin1 not only performs as a phosphatidic acid phosphatase (PAP), which is necessary for triglycerides biosynthesis, but also exhibits transcriptional coactivator function which regulates the expression of lipid metabolism related genes²⁻⁴. As a PAP, lipin1 promotes the conversion of phosphatidic acid into triglycerides and phospholipids during adipocytes development³. Lipin1 also could act as a transcriptional coactivator in the liver and directly interact with nuclear receptor peroxisome proliferator-activated receptor γ (PPAR γ) and PPAR α

coactivator (PGC-1 α) to regulate the expression of genes related to fatty acid utilization and lipid synthesis^{5,6}.

In addition to the involvement of lipid metabolism, the increased expression of lipin1 could partially inhibit insulin signaling through PKC ϵ -mediated suppression of hepatic glucose production⁷ and the dysfunction of lipin1 could induce long-term syndrome such as noninsulin-dependent diabetes mellitus and atherosclerosis⁸. Lipin1 was also served as a macrophage proinflammatory factor⁹. A recessive *LPIN1* mutation was the cause of severe rhabdomyolysis in children¹⁰⁻¹². In myoblast differentiation, lipin1 was up-regulated and migrated to the nucleus¹³. Furthermore, lipin1 depletion could suppress cell proliferation of prostate and breast cancer cells and reduce cancer cell migration ability¹⁴. These previous studies show that lipin1 participates in many cellular metabolism processes.

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However, its molecular mechanism is still unclear. The 293T human renal epithelial cell line represents a commonly used and well established expression system for recombinant proteins since generated by Graham, Smiley, Russell, and Nairn in 1977¹⁵. Therefore, lipin1 was transiently overexpressed in 293T cells. Since proteomics has become a well-established technology^{16,17}, herein we used the technique to investigate differential expression of proteins in this cellular model. It is expected to find some proteins related to the regulation of lipin1 and explain the important role of lipin1 in the regulation of cell metabolism.

Materials and Methods

Materials

The 293T human renal epithelial cells were supplied by the Institute of Cell Biology (Shanghai, China). Lipofectamine[®]2000 was used for transfection pursuant to manufacturer's instruction (Invitrogen, Camarillo, CA, USA). Lipin1 (code number: ab92316), RanBP1 (code number: ab97625) and β -actin (code number: ab8227) antibody were from Abcam (Cambridge, UK). Goat anti-rabbit IgG-HRP (code number: 111-036-003) was purchased from Jackson Immuno Research Laboratories, Inc. (West Grove, PA, United States). Amersham Hybond-P polyvinylidene fluoride (PVDF) membrane was from GE Healthcare (Buckinghamshire, UK).

Cell culture

Cells were cultured in DMEM medium (Gibco, Camarillo, CA, USA) supplemented with 10% heat inactivated fetal bovine serum (FBS) (PAN-Biotech, Aidenbach, Germany) and penicillin-streptomycin (100 U/mL, 100 μ g/mL, Solarbio life science, China). The cells were cultured in 37°C under humidity atmosphere with 5% CO₂.

Plasmid construction and transfection

The ORF of lipin1 (NCBI: GI: 22027648) was shuttled in plasmid pcDNA3.1<+> from plasmid YEp51-lipin1 (a gift from Huang's lab¹⁸), by using *NheI* and *EcoRI* site. Lipin1 primers were as follows: forward: 5'-TAATGGTACCATGAATTACGTGGGG-3'; reverse: 5'-TATATCTAGACGCTGAGGCAGAA TG-3', for PCR amplification of ORF (Open Reading Frame) of *LPIN1* gene. On the day prior to transfection, the 293T cells were divided into empty vector (control) group and lipin1 overexpression

group (N=3/group), and cultured in 6-well plate to a final density of 5×10⁵ viable cells, overnight for transfection. In briefly, 4.0 μ g plasmid DNA (pcDNA3.1<+> for control group and pcDNA3.1<+>-lipin1 for lipin1 overexpression group) and 10 μ L Lipofectamine[®] 2000 were diluted by 200 μ L serum-free Opti-MEM, respectively. Mixed gently and incubated for 5 min at room temperature. Then diluted DNA and Lipofectamine[®] 2000 were mixed gently. Incubated Lipofectamine[®] 2000/plasmid DNA complexes at room temperature for 20 min. The mixture was added to cells, gently shook back and forth.

Reverse transcription PCR

For analysis of lipin1 mRNA, the total RNA of the 293T human renal epithelial cells were obtained by using TRIZOL reagent (Takara, Japan). Prime Script RT reagent kit (Takara, Japan) was applied to synthesize single-stranded cDNA with 1 μ g RNA. RT-PCR was conducted for analysis of lipin1 mRNA level. Primers used in the RT-PCR were as following. 18s rRNA, 5'-CGGCTACCACATCCAAGGAAG-3' (forward), 5'-AGCTGGAATTACCGCGGCT-3' (reverse); lipin1, 5'-CCGACCTTCAACACCTAA-3' (forward); 5'-GGGCTGGACTCTTTCATCTT-3' (reverse).

Western blotting

Cells were washed three times by PBS and lysed according to the procedure described previously¹⁹. By Bradford method²⁰, proteins were quantified. 50 μ g protein was separated by 10% SDS-PAGE and transferred to PVDF membranes. The proteins expression levels were examined by incubation with primary antibodies (RanBP1 and β -actin, respectively), following incubated with goat anti-rabbit IgG HRP secondary antibodies. The chemiluminescence-based kit (Tian Gen Biotech, China) was used for protein detection. And Tanon 6200 (Biotano, China) was used for detection of signal density.

Two-dimensional polyacrylamide gel electrophoresis (2D-GE)

The solubilized proteins were collected according to the previous method²¹. For isoelectric focusing, 150 μ g proteins were loaded onto a 7cm immobilized linear pH gradient (IPG) strip, pH 3-10 (GE Healthcare Life Sciences, Buckinghamshire, UK) at 18°C. The isoelectric focusing was performed with the Protein IEF cell (Bio-Rad) at 18°C as follows: active rehydration for 12 h at 50 V, 30 min

at 250 V in linear ramping mode, 30 min at 500 V in rapid ramping mode 3 h at 4000 V in linear ramping mode and 20 kVh at 4000 V in rapid ramping mode. Thereafter, the strips were equilibrated by using equilibration buffer (6 M urea, 50 mM Tris-HCl, pH 8.8, 2% SDS, 20% glycerol, 0.002% bromophenol blue) containing 2% (w/v) DTT and 2.5% (w/v) iodoacetamide, respectively. After that, SDS-polyacrylamide gel electrophoresis (PAGE) of 10% was used as the second dimensional electrophoresis. The 2D-GE experiments were repeated three times.

The 0.1% Coomassie brilliant blue R-250 was used to stain the gels. Images of each gel were acquired by Lab-Scan version 3.0 software (GE Healthcare) on Image Scanner III (GE Healthcare). The images were analyzed by using PDQuest TM 8.0 advanced software (Bio-Rad, California, USA).

Spot picking, in-gel digestion, protein identification

The differential proteins were excised from gels and digested with trypsin as previously described²². Mass spectra of the spots were acquired by 5800 MALDI-TOF/TOF mass spectrometer (AB SCIEX). Mass Spectrometry data were searched by using Mascot 2.2 software against known protein sequences in publicly available UniProt and NCBI databases with the Peptide program (<http://expasy.org/tools/peptide.html>). The parameters were set as protein taxon, Homo sapiens; one missed cleavage, carboxyamidomethyl modification; ion mode, [M+H]⁺; resolution, monoisotopic; molecular weight shift, max±0.4 Dalton¹⁸.

Statistical analyses

The gel maps of 2D-GE was analyzed by PDQuest TM 8.0 advanced software to find differential spots between control group and lipin1 overexpression group, based on their volume percentages in total spots volume of the whole gel images. Significantly altered spots were selected if their intensity had changed (increased/decreased) by at least two-fold. Statistical analysis was performed using the Student's *t*-test. *P*-values less than 0.05 were considered as statistically significant. All experiments were conducted no less than three times.

Results

Overexpression of lipin1 in 293T cells

For overexpressing lipin1, the human renal epithelial 293T cells were transfected with

plasmid pcDNA3.1<+>-lipin1. For plasmid transfection in mammalian cells, the mRNA level of target protein is usually detected at 24 h to determine the success of transfection, and then the expression of target protein is verified by WB after 48 h, which is helpful to confirm the overexpression of target protein. Herein, the mRNA level of lipin1 was detected by RT-PCR after transfection for 24 h, (Fig. 1A). And after transfection for 48 h, the level of lipin1 protein was analyzed by Western blotting (Fig. 1B). As shown in (Fig. 1A and B), the mRNA and protein level of lipin1 were both up-regulated compared to the control cells. Therefore, lipin1 was overexpressed successfully in 293T cells.

2D-GE map and differential protein identification

To find proteins regulated by lipin1, 2D-GE was conducted. Figure 2 showed a representative sample of 152 proteins separated on 2D-GE gels. Protein spots of cells transiently overexpressing lipin1 were selected if their intensities were changed (increased/decreased) by at least two-fold compared to the control cells. By using the PDQuest TM 8.0 advanced 2D analysis software, 11 protein spots were found to be significantly changed, which were marked in (Fig. 2). As shown in (Fig. 2), spots 1–8 were down-regulated and spots 9–11 were up-regulated induced by lipin1 overexpression. Figure 3A showed comparative close-up views of the representative protein spots which were altered by lipin1 overexpression. Figure 3B showed the MS and MS/MS (peak *m/z* 1034.5212) peptide mass fingerprint of protein spot 7. Table 1 presented the picked spots with its MW/pI, changed fold and biological function. Among these altered proteins, eight proteins of

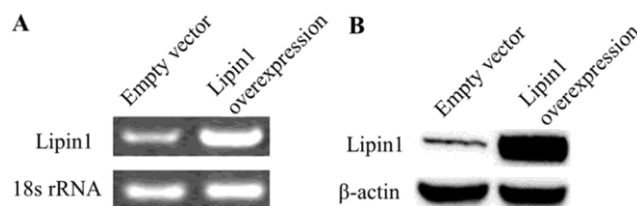


Fig. 1 — Analysis of lipin1 expression by using RT-PCR and Western blotting. After cells were transfected for 24 and 48 h, the expression level of lipin1 was analyzed by RT-PCR (for 24 h, A) and Western blotting (for 48 h, B). Empty vector, cells transfected with empty plasmid pcDNA3.1<+>; lipin1 overexpression, cells transfected with plasmid pcDNA3.1<+>-lipin1. 18s rRNA and β-actin were used as internal control, respectively

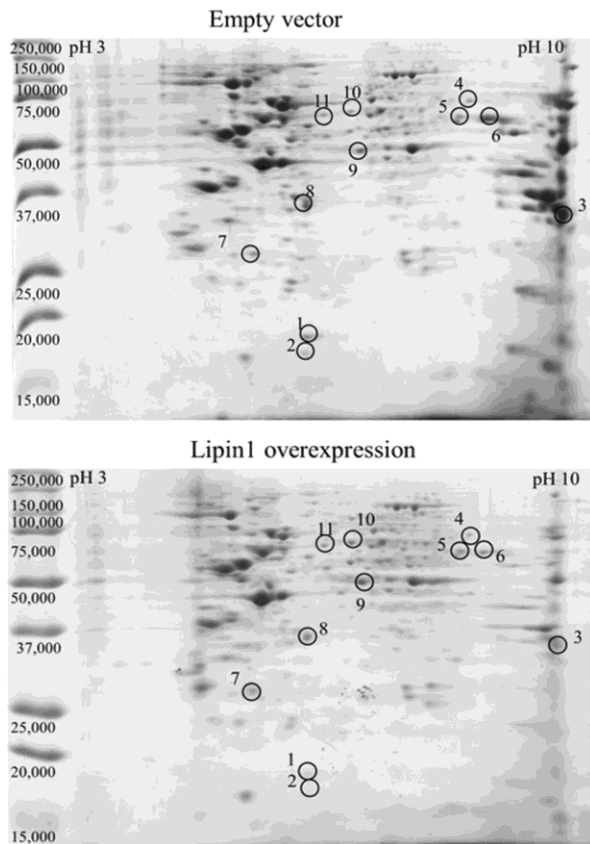


Fig. 2 — Two-dimensional electrophoresis maps of protein spots in 293T cells. Spots numbers represent the differential proteins induced by lipin1 overexpression

spots 1–8 (NDKA, Stathmin, HNRNPA1, TK, KRT1, PKM, RanBP1, LDHB) were down-regulated by lipin1 overexpression, while other three proteins of spots 9–11 (eEF-1B γ , CCT3, CCT1) were up-regulated by lipin1 overexpression.

As one of the eleven changed protein induced by lipin1 overexpression, RanBP1 was chosen to confirm the result of 2D-GE. As shown in (Fig. 4), the Western blotting result verified that the expression level of RanBP1 was reduced by lipin1 overexpression, which was consistent with the data of 2D-GE.

Classification of differential proteins

Subsequently, the eleven altered proteins induced by lipin1 overexpression were functionally classified using Panther 12.0 classification system. They were mainly grouped into four categories, including molecular function group, biological process group, protein specie group and cell component group (Fig. 5). In the classification of protein molecular function, 50% of proteins were the catalytically active protein, 25% of proteins were the binding protein, 12.5% of proteins were the constitutively active protein and 12.5% of proteins were the protein translation active protein. The classification of biological processes were consist of seven segments, including metabolic process (29.2%), cell process

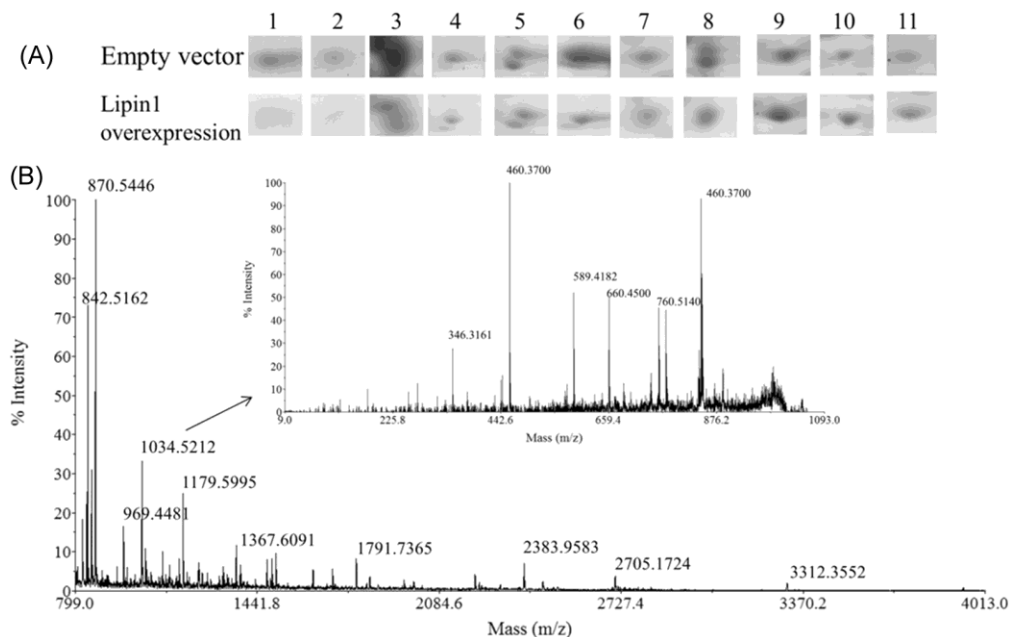


Fig. 3 — Spot pairs corresponding to differential proteins. (A) Spots represent the differential proteins. Spots 1–8 were down-regulated in cells transiently overexpressing lipin1 and spots 9–11 showed differential proteins, which appeared increase in cells transiently overexpressing lipin1; and (B) Peptide mass fingerprint of spot 7 excised from the two-dimensional electrophoresis gel. It showed the MS peptide mass fingerprint of spot 7 and the MS/MS peptide mass fingerprint of peak m/z 1034.5212

Table 1 — Identification of proteins differentially expressed in 293T cells transiently transfected with pcDNA3.1<+>-lipin1 for 48h

Spot Number	Protein Identity	UniProt	MW/pI	Pep. Count	Protein Score	Fold Change	Biological Function
1	NDKA	P15531	19869/5.42	8	428	0.28	cell proliferation, differentiation and development, signal transduction, G protein-coupled receptor endocytosis, and gene expression
2	Stathmin	P16949	17291.9/5.76	6	103	0.19	regulation of the microtubule filament system
3	HNRNP A1	P09651	34289.3/9.27	14	207	0.20	packaging of pre-mRNA, poly(A) mRNA export into cytoplasm, modulate splice
4	TK	P29401	68528/7.89	8	428	0.44	pentose phosphate pathway
5	KRT1	P04264	66170.1/8.15	23	214	0.28	fibrous structural proteins
6	PKM	P14618	58470.2/7.96	33	445	0.41	glycolysis
7	RanBP1	P43487	23466.6/5.19	9	76	0.48	proteins and nucleic acids import into nucleus
8	LDHB	Q4R5B6	36900.2/5.71	17	189	0.38	glycolysis
9	eEF-1B γ	P26641	50429.3/6.25	16	216	2.11	protein translation
10	CCT3	P49368	61065.5/6.1	26	169	2.17	molecular chaperone, folding of actin and tubulin
11	CCT1	P17987	60818.8/5.8	16	93	2.06	molecular chaperone, folding of actin and tubulin

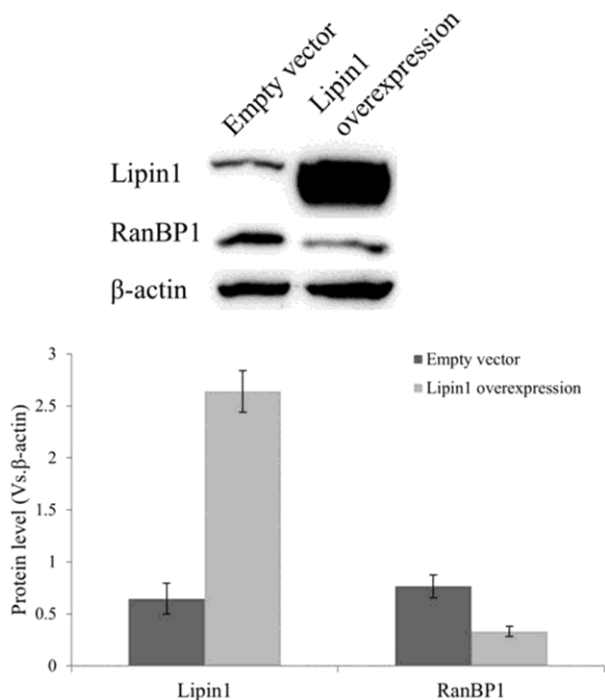


Fig. 4 — Lipin1 overexpression reduced the expression of RanBP1. Western blotting showed that the expression of RanBP1 was down-regulated in 293T cells transiently overexpressing lipin1. β -actin was used as internal control. Right panel showed the intensity level of lipin1 and RanBP1 after normalization with β -actin intensity. * $P < 0.05$, lipin1 overexpression vs. empty vector

(20.8%), biogenesis process (20.8%), cell composition (12.5%), development process (8.3%), multicellular biological process (4.2%) and positioning (4.2%). The classification of protein species included transferase (11.1%), cleavage enzyme (11.1%), cytoskeleton protein (11.1%), chaperone protein (22.2%), regulatory protein (11.1%), structural protein (11.1%), and oxidoreductase (22.2%). Regarding the classification of cell components, they contained the cell part (62%) and organelle (38%).

By using String 10.5 software, the protein-protein network of eleven altered proteins and lipin 1 were analyzed. Among these differential proteins, except STMN1 and KRT1, other nine proteins had direct or indirect links with each other. Although the expression of these proteins was affected by lipin1 overexpression, there was no direct interaction of lipin1 with these eleven proteins (Fig. 6). Based on KEGG pathway analysis, these altered proteins were involved in four signal pathways, including myelin synthesis, extracellular domain formation, membrane bound vesicle synthesis and companion protein T complex synthesis.

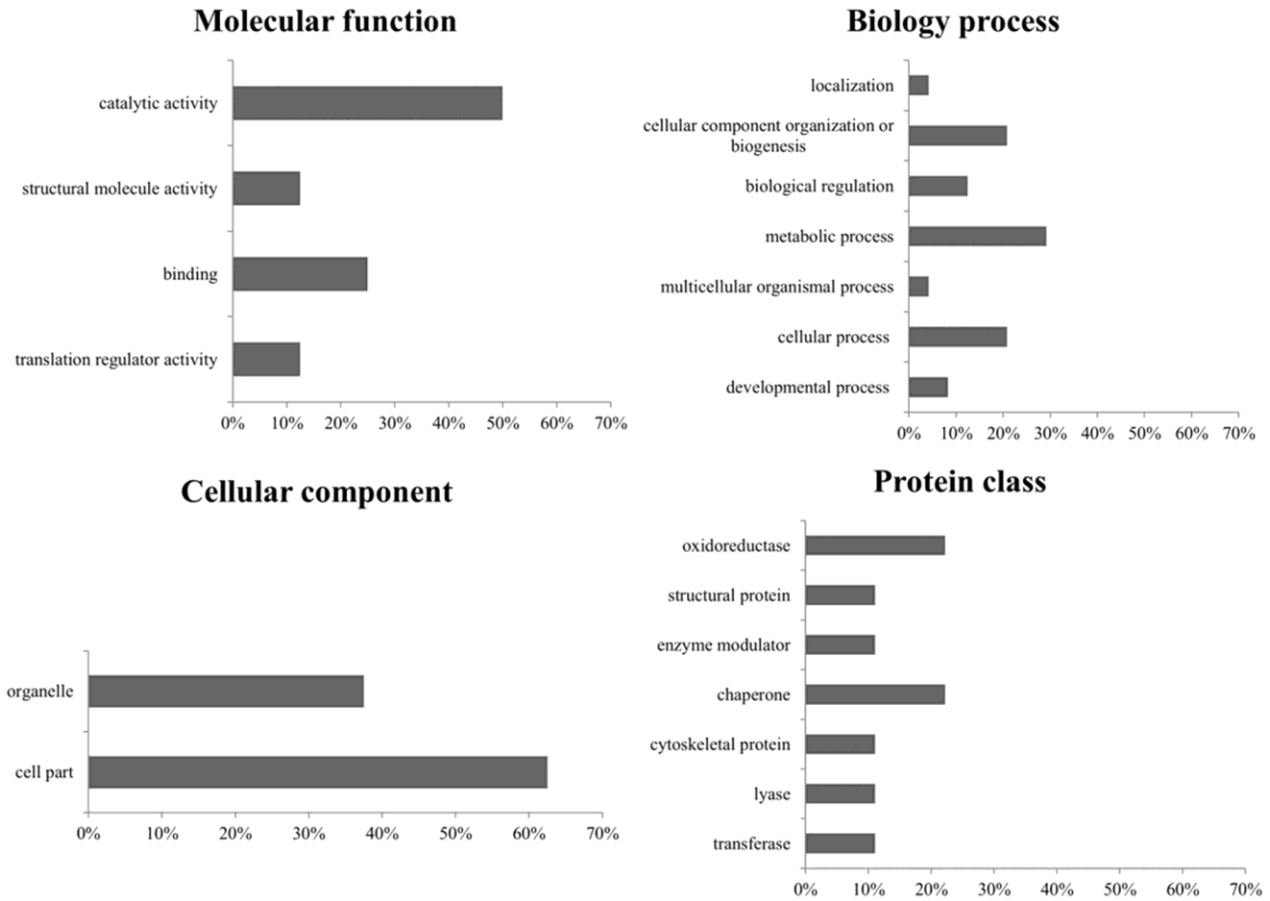


Fig. 5 — Gene Ontology analysis of the eleven proteins. The protein categorizations of molecular functions, biological processes, protein class and cellular components were shown by using Panther software

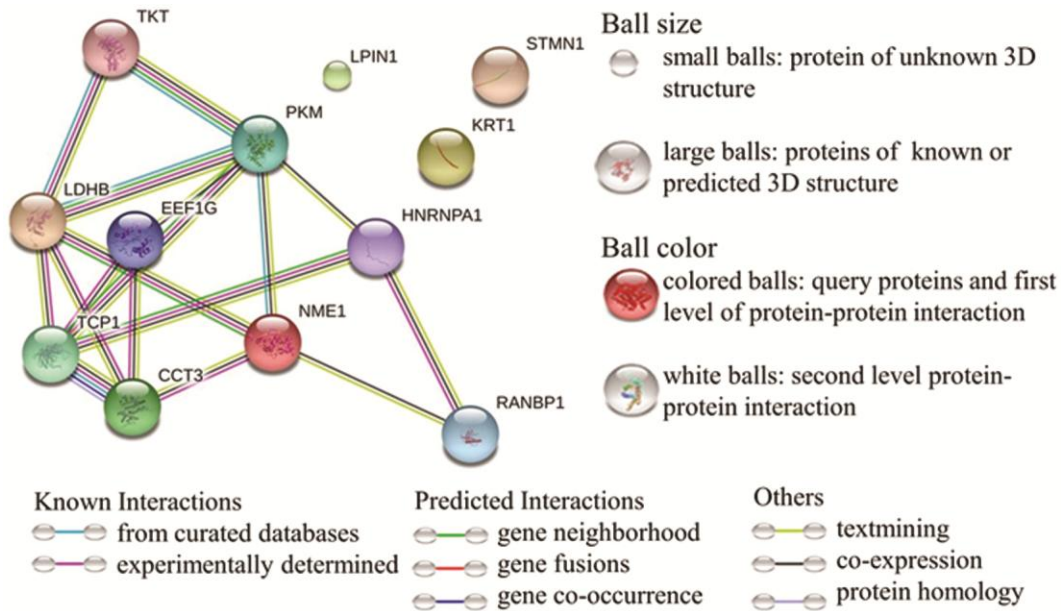


Fig. 6 — Visualization of protein-protein interaction networks of the eleven proteins and lipin1 by String 10.5. Among these differential proteins, except STMN1 and KRT1, other nine proteins had direct or indirect links with each other. Although the expression of these proteins was affected by lipin1 overexpression, there was no direct interaction of lipin1 with these eleven proteins

Discussion

Since generated by Graham, Smiley, Russell, and Nairn in 1977, the 293T human renal epithelial cell line is commonly used to be a well established expression system for recombinant proteins¹⁵. Although tissue-specific protein changes are very important in associated health and diseases, herein this cell model was only used to overexpress lipin1. Therefore, when we studied the differential expression of proteins induced by overexpression of lipin1, the relationship between these differential proteins and renal epithelial cell-related diseases was not considered.

From our proteomic studies, eight proteins were down-regulated in the 293T human renal epithelial cells transiently overexpressing lipin1, including NDKA, Stathmin, HNRNPA1, TK, KRT1, PKM, RanBP1 and LDHB. Among them, NDKA (nucleoside diphosphate kinase A), also named NME1, is a wide-spectrum suppressor in tumor metastasis, which can prevent a variety of growth factors to binding cell membrane receptor resulting in disturbance signaling in cancer cells²³. Moreover, NME1 is also considered as a biological molecular marker of stroke²⁴. Stathmin is an actin-binding protein and a microtubule-regulated protein. It plays a key role in cell division, cell motility and cell migration²⁵. It is highly expressed in a variety of human cancers cells, such as cells of leukemia, breast cancer, ovarian cancer, prostate cancer, lung cancer and liver cancer. Therefore, Stathmin become a molecular target of cancer treatment²⁶. HNRNPA1 (heterogeneous nuclear ribonucleoprotein A1), one of the most abundant proteins in the HNRNPs family, could regulate its target genes expression by regulation of mRNA and microRNAs synthesis. Furthermore, HNRNPA1 could regulate activity of transcription factors and maintain telomere length during development of body and cell differentiation²⁷. And HNRNPA1 could also interact with KRAS, which plays a main role in pancreatic ductal adenocarcinoma (PDAC) tumorigenesis, to affect invasion of pancreatic cancer cells²⁴. The key element of this regulatory circuit is HNRNPA1, which can alter structure of transcriptional regulatory region in *KRAS* gene to make it easier to transcript²⁸. Interestingly, HNRNPA1 can be involved in regulation of protein expression of thymidine kinase (TK)²⁹. Therefore, lipin1 may regulate expression of other proteins by affecting expression of HNRNPA1.

Thymidine kinase (TK) is a kinase in pyrimidine remedy pathway which could catalyze deoxythymidine phosphorylation into thymidine, a small molecule which is closely linked to cell cycle and cell proliferation³⁰⁻³². In addition to TK, there is an another kinase, pyruvate kinase (PKM), which was undergone a similar change to TK, that was down-regulated by lipin1 overexpression. PKM catalyzes phosphoenolpyruvate and ADP into ATP and pyruvate, and is one of the major rate limiting enzymes in glycolysis³³. Our studies also found a structure protein, KRT1, which was reported to be associated with inflammatory response³⁴. Therefore, lipin1 may participate in macrophage inflammatory response *via* KRT1, which needs further study⁹.

As a regulator of Ran, RanBP1 plays a key role in early and final stages of eukaryotic cell mitosis, spindle assembly, correction of chromosomes distribution, nuclear membrane rupture and reorganization. It is known for functions in behavioral disorders including autism and schizophrenia³⁵. Lactate dehydrogenase B (LDHB), one of the eight down-regulated proteins, could influence proliferation by affecting lysosomal activity and autophagy in cancer cells³⁶. Interestingly, among the down-regulated proteins induced by lipin1 overexpression, although some proteins belong to different categories, their relationship between each other was found in our proteomic study, for instance NDKA and Stathmin³⁷, TK and HNRNPA1³⁸, KRT1 and PKM³⁹.

Three proteins up-regulated by lipin1 overexpression were eEF1B γ , CCT1 and CCT3, respectively. They are cell cycle-related proteins. EEF1 (formerly named EF-1) of higher eukaryotes is consisted of four different subunits (eEF1A, eEF1B α , eEF1B β and eEF1B γ). The subtype eEF1B γ is reported to be a part of the eukaryotic translation elongation factor-1 complex (eEF1) and plays a central role in extension phase of protein biosynthesis⁴⁰. The chaperonin containing TCP-1 complex (CCT), also known as Tri C, which is located in the cytoplasm, regulates protein folding and assembly⁴¹. CCT is a 16 subunit complex. And each of eight subunits forms a stacking ring and the eight subunits are different, such asg CCT1 (α), CCT2 (β), CCT3 (γ), CCT4 (δ), CCT5 (ϵ), CCT6 (ζ), CCT7 (η) and CCT8 (θ)⁴¹. Studies have shown that CCT1 could affect G1 to S phase overgrowth, meanwhile CCT3 can also affect mitosis⁴². Thus, it is obvious that lipin1

overexpression is associated with cell cycle regulation *via* these related proteins.

Conclusion

We found eleven changed proteins induced by lipin1 overexpression using proteomic technique, which includes cell cycle related proteins, cell structural proteins and metabolic enzymes and so on. Although so far there are no reports of these 11 proteins associated with lipin1, our results for the first time showed the effect of lipin1 on these proteins. It indicates that lipin1 may participate in different biological processes *via* some unreported proteins. It is expected to expand insights on new features of lipin1.

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