MicroRNA-101-2-5p targets the ApoB gene in liver of chicken (Gallus Gallus)

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MicroRNA-101-2-5p targets the *ApoB* gene in liver of chicken (*Gallus Gallus*)

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Key words: Chicken; ApoB; miRNA; Expression regulation

Abstract

Apolipoprotein B (ApoB) is a major protein component of plasma lipoproteins, involved in many important biological processes such as lipid transportation, enzyme activity regulation and receptor recognition. Extensive studies have showed that the expression of the ApoB was regulated at multiple levels. However, the regulation of *ApoB* expression by microRNAs (miRNAs) still remains unknown. In present studies, miRNAs that interact with *ApoB* have been predicted in chicken. The predicted relationship between the predicted miRNAs and *ApoB* was verified through dual luciferase reporter assay in chicken DF1 cells, and the effect of miRNAs on *ApoB* expression was analyzed in chicken embryo hepatocytes stimulated by 17 β -estradiol. The results showed that miR-101-2-5p was predicted to interact with *ApoB*. Dual luciferase reporter assay together with the miR-101-2-5p mimics study demonstrated that *ApoB* is the target of miR-101-2-5p which suppresses the expression of *ApoB* through binding with the 3'UTR of *ApoB*. Our experiments suggested that miR-101-2-5p might be involved in lipid metabolism through binding with the 3'UTR of *ApoB* gene in liver of egg-laying chickens.

Key words: Chicken; *ApoB* gene; miRNA; Expression regulation

Introduction

Apolipoprotein B (ApoB) is a major protein component of plasma lipoproteins, involved in many important biological processes such as lipid transportation, enzyme activity regulation and receptor recognition. Due to the important biological roles that ApoB plays, its regulatory mechanism has been extensively studied. Some studies on the regulatory role of *ApoB* mRNA untranslated region (UTR) suggested that both 5'UTR and 3'UTR are likely to govern *ApoB* mRNA translation and protein synthesis (Pontrelli et al. 2004); other studies revealed that the *ApoB* was regulated at multiple levels, including gene transcription, mRNA editing, protein translation and translocation into the endoplasmic reticulum and intracellular degradation of nascent proteins (Wang et al. 2003). However, the possible role of microRNAs (miRNA) in regulating ApoB expression remains unknown yet.

miRNAs are a class of endogenous non-coding small regulatory RNAs, which are single-stranded RNA molecules with a length of 18-25 nucleotide (nt) (Kang et al. 2013; Shao et al. 2014). miRNAs regulate gene expression through binding to the specific sites in the 3'UTR of their target mRNAs. The sequence of the 2-8 nt of the 5' end in mature miRNA, known as the “seed region”, plays a critical role in miRNA binding and interacting with target mRNAs (Brennecke et al. 2005; Li et al. 2012). It has been confirmed that miRNAs can repress the expression of target mRNAs at a post-transcriptional regulation pattern in animals (Bartel 2009). Therefore, miRNAs down-regulation increases the expression of targeted mRNAs. In contrast, the expression of the targeted gene is decreased by miRNAs up-regulation. It has been estimated that up to 60% of all human genes are either directly or indirectly regulated by miRNAs (Friedman et al. 2009). The increasing evidences have showed that miRNAs are involved in multiple biological and metabolic processes, including skeletal muscle development (Chen et al. 2006; Zheng et al. 2009), cell proliferation (Haapa-Paananen et al. 2013; Hu et al. 2014), disease (Chai et al. 2016; Lewis & Esquela-Kerscher 2015), and lipid metabolism through modulating the expression of targeted genes (Esau et al. 2006; Iliopoulos et al. 2010).

In chicken, in order to supply efficient nutritional requirements for embryo growth and development, hydrophobic lipids, including triacylglycerols, cholesteryl esters, cholestery esters and free fatty acids, are synthesized in the liver and assembled to form egg-yolk precursors such as very-low density lipoprotein (VLDL) and vitellogenin particles during the egg-laying stage. The molecules are then secreted into the blood circulation, transported to the developing oocyte and taken up by the growing oocytes through specific cell-surface receptors (Brady et al. 1976; Nikolay et al. 2013; Walzem et al. 1999; Wiskocil et al. 1980). The ApoB is the most important component of specialized VLDL molecules and plays a crucial role in the assembly and secretion of triglyceride-rich lipoproteins as well as lipid transportation (Nikolay et al. 2013). It has been widely accepted that the plasma estrogen level in female chicken is significantly elevated in egg-laying hens (Tanabe et al. 1981; Williams & Harvey 1986), which results in a dramatic increase in synthesis of *ApoB* in chicken liver during egg-laying stage (Chan et al. 1976; Kirchgessner et al. 1987). Our previous RNA-seq studies on livers of both pre- and peak-laying hens found that the expression level of *ApoB* in liver was significantly up-regulated in peak-laying hens in comparison with pre-laying hens (Li et al. 2015a). To investigate whether the expression of *ApoB* is also regulated by miRNAs in chickens, bioinformatics analytical tools were employed to predict the miRNAs that potentially interact with *ApoB*. A series of experimental studies demonstrated that miR-101-2-5p targets and regulates the expression of *ApoB* in the liver of chickens.

Material and Methods

Ethics statements

All the animal experiments were carried out according to the protocol approved by the Institutional Animal Care and Use Committee (IACUC) of Henan Agricultural University.

Animals and sampling

Animals used in this study were Lushi Green-shelled-egg hens obtained from the

core breeding farm of Henan Agricultural University. All the birds had free access to food and water under the same environmental conditions. Eight female birds at the age of 20 weeks old (pre-laying) and 30 weeks old (peak-laying) were humanely killed, respectively. The liver tissues were collected, snap frozen in liquid nitrogen, and stored at -80°C until use.

Computational prediction miRNAs interacting with ApoB

The miRDB (<http://mirdb.org/miRDB/>) as an online database for miRNA target prediction and functional annotations was used to predict the miRNAs that potentially interact with *ApoB*. The miRNAs which target to *ApoB* gene must be down-regulated in liver of peak-laying hens in comparison to pre-laying hens since the expression level of *ApoB* was significantly increased in peak-laying hens in comparison to pre-laying hens. To select the down-regulated miRNAs in the liver of peak-laying hens, the predicted miRNAs were screened against the previous miRNA expression profiles derived from the livers of pre- and peak-laying hens (Li et al. 2016). Meanwhile, the target genes of the miRNAs, which were predicted to interact with *ApoB* gene and shown to be down-regulated in the liver of peak-laying hens, were conversely predicted by the miRDB (Wong & Wang 2014). In additionally, the minimum free energy (mFE) between gga-miR-101-2-5p and 3'-UTR of the *ApoB* gene were analyzed by RNA hybrid (online website: <http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/>).

Construction of plasmids for dual luciferase reporter assay

To construct the vector for dual luciferase reporter assay, the fragment of chicken *ApoB* 3'-UTR encompassing the miRNA binding site was amplified by PCR with the cDNA synthesized using the chicken liver RNA. The primers used in the PCR were showed in Table 1. Each 25 μL reaction mixture contains 1.0 μL of first-strand cDNA, 12.5 μL of 2 $\times$ PCR Mix (Takara), 1.0 μL of the forward and reverse primers (10 μM) respectively, and 9.5 μL RNase-free water. The PCR condition was as following: 1 cycle of 94°C for 2 min, 30 cycles of 94°C for 40 s, 58°C for 40 s and 72°C for 1.5 min, followed by 72°C for 7 min. The amplified PCR products were digested with

endonuclease *Xho I* and *Not I*, separated on 2% agarose gel, purified with a universal DNA purification kit (Tiagen Biotech Co. Ltd.) and linked into the luciferase reporter psi-CHECKTM-2-vector (Promega, Madison, WI, USA) between the *Xho I* and *Not I* sites to construct the vector-luc-ApoB-WT 3'UTR reporter vector. As control, bases deletion mutation in the seed region of the predicted miRNA binding sequence within the 3'UTR of chicken *ApoB* gene were generated using the overlap-extension PCR according to the method described previously (Aiyar et al. 1996). The generated plasmid was named vector-luc-ApoB-mut 3'UTR. All the generated vectors were confirmed by sequencing (BGI, China). Plasmids were extracted and prepared with EndoFree Plasmid Maxi Kit (Tiagen Biotech CO., LTD.) for further using.

DF1 cell culture and dual luciferase reporter assay

The DF1 cells used in the experiment are a type of immortalized chicken embryonic fibroblast cell line. The cells were cultured in Dulbecco's modified eagle medium (DMEM) (GIBCO, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin G and 100 µg/mL streptomycin. Cells were seeded into 24-well plate, and cultured in a humidified incubator at 37°C with 5% CO₂ overnight. When the cells reached 80% confluence, the cells were serum starved for 12 hours.

To investigate the interaction between *ApoB* and miR-101-2-5p, the serum starved DF1 cells were co-transfected with the luciferase reporter (psi-CHECKTM-2 or recombinant vector-luc-ApoB-WT 3'UTR or vector-luc-ApoB-Mut 3'UTR) and 100nM miR-101-2-5p mimics or the mimics negative control (mimics-NC) (Genepharma, China) alone or combined as indicated in figure 3 and 4 in triplicate for 24h. Cells were harvested and lysed in passive lysis buffer (Promega, USA), and the dual-luciferase activity assay was performed according to the manufacturer's instruction (Promega, USA). The firefly luciferase activity was normalized using the Renilla luciferase activity. All the transfection experiments were performed in triplicate.

Chicken hepatocytes culture and transfection

The fertilized eggs produced by the Lushi Green-shelled-egg hens were incubated in an incubator at 38°C with 97% humidity. When the eggs were incubated for 18 days, the liver tissue of 18-day old chicken embryo was dissected and hepatocytes were isolated by using an modified method based on the Fishcher and Marks (Fischer & Marks 1976). In briefly, the 18-day-old embryonic chicken livers were collected and washed with D-Hanks solution (Solarbio, Beijing, China). Cells obtained from chicken embryo livers tissue were suspended at a density of 5.0×10^5 cells/mL and seeded in 6-well plate with Williams'E complete medium (Sigma St Louis, MO, USA) consisted with 10 % fetal calf serum, 100 mg/mL streptomycin and 100 U/mL penicillin. Cells were incubated at 37 °C under a water-saturated atmosphere containing 95% air humidity and 5% CO₂ overnight.

When the hepatocytes cultured up to 80% confluence, the cells were serum starved for 12 hours, and the gga-miR-101-2-5p mimics or mimics-NC was transfected with concentration of 100 nM. The 17β-Estradiol (Sigma St Louis, MO, USA) with a final concentration of 25 nM was added into the treated and un-treated cell. The cells were collected after the transfection for 12 h, and stored at -80 °C for further experiments.

RNA extraction and the first strand cDNA synthesis

Total RNAs from liver tissue and cells were isolated using Trizol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). The quantity and quality of RNA were detected by using Nanodrop 2000 spectrophotometry (IMPLEN, CA) and denaturing agarose gel electrophoresis (Takaka, China), respectively.

To synthesize first strand cDNA for PCR amplification of genes, total RNA samples were reverse-transcribed into cDNA using random hexamer primers with the PrimeScript™ RT reagent kit with gDNA Eraser (TaKaRa, China) according to the manufacturer's instruction. To synthesize first strand cDNA for PCR amplification of putative miRNA, total RNA samples were reverse-transcribed into cDNA using the specific Hairpin primers ordered from company (Gene Pharma, China) and the same

reverse transcription kit as described above. The cDNA was stored at -20°C until use.

Real-time Fluorescent Quantitative PCR (qPCR)

The qPCR was carried out with specific primers (Table 1) and Power SYBR Green PCR Master Mix (TaKaRa, China) by using the LightCycler 96 Real-Time PCR System (Roche, Switzerland). The Ct values for target genes were normalized to that for the β -actin gene, and Ct values for miRNA were normalized to those for chicken small RNA U6. The relative expression levels were calculated using $2^{-\Delta\Delta\text{CT}}$ method (Schmittgen & Livak 2008).

Statistical Analysis

All the data is presented as the mean value $\pm$ standard error of at least three independent experiments. Unpaired Student's two-tailed t-test was used for statistical evaluation using GraphPad Prism 5 (Graph-Pad Software, La Jolla, CA). P -value < 0.05 was considered as statistically significant.

Results

Predicted interaction between the miRNAs and *ApoB* gene

To identify whether *ApoB* gene is regulated by miRNA, the 3'UTR sequences of *ApoB* were used to analyze the potential target binding sites of miRNAs through the online software miRDB. A total of 6 potential miRNAs were predicted to interact with *ApoB* (Table 2). Of them, only miR-101-2-5p was found to be significantly down-regulated in our miRNA-seq profile (Hong Li 2016), whereas *ApoB* gene was up-regulated in the mRNA-seq dataset derived from the liver of peak-laying hens. Therefore, miR-101-2-5p was selected as the putative miRNA interacting with *ApoB* (Fig. 1a). The mFE (minimum free energy) of the RNA duplexes between miR-101-2-5p and *ApoB* 3'-UTR was about -14.3 kCal/mole. It indicates that these two sequences are highly stable (Fig. 1b).

To further verify whether *ApoB* was the putative target gene of miR-101-2-5p, we reversely predicted the target genes of miR-101-2-5p by miRDB. The bioinformatics analysis on the target genes was conducted using KOBAS 2.0 software.

The functional annotation of the target genes showed that they were significantly enriched in Glycerophospholipid metabolism and phosphatidylinositol signaling system pathways. The GO terms were mainly enriched in metal binding molecular function, glycerol-3-phosphate dehydrogenase complex cellular component, glycerol-3-phosphate metabolic and lipoprotein transport process (Additional file 1: Table S1). As expected, *ApoB* was one of the potential target gene of miR-101-2-5p.

Verification of expression patterns of *ApoB* and *miR-101-2-5p* in liver of pre- and peak-laying chicken

To verify our previous miRNA-seq and mRNA-seq datasets (Li et al. 2016; Li et al 2015a), qRT-PCR was carried out by using cDNAs derived from the livers of pre- and peak-laying hens. The results confirmed that *ApoB* was significantly up-regulated ($P < 0.01$), and gga-miR-101-2-5p was down-regulated ($P < 0.01$) in liver of peak-laying chicken (Fig. 2 a & b).

Interaction between miR-101-2-5p and *ApoB* in DF1 cells

To further verify whether gga-miR-101-2-5p directly targets the *ApoB* gene, a luciferase reporter assay was conducted. The co-transfection of luciferase reporter vector-luc-*ApoB*-wt 3'UTR with gga-miR-101-2-5p mimics in DF1 cells resulted in a significant decrease of luciferase activity ($P < 0.01$), whereas co-transfection with a non-specific miRNA mimics-NC did not affect the luciferase activity (Fig. 3). When vector-luc-*ApoB*-mut 3'UTR was co-transfected with gga-miR-101-2-5p mimics or mimics-NC, the luciferase activity was not affected (Fig. 3). Compared to the vector-luc-blank group, only the group co-transfected with vector-luc-*ApoB*-wt 3'UTR and gga-miR-101-2-5p mimics had the luciferase activity significantly affected.

Effect of gga-miR-101-2-5p on *ApoB* expression in chicken hepatocytes

The *ApoB*, as a component of specialized VLDL particles, is highly produced in liver in response to estrogen in chicken (Kirchgessner et al 1987). To demonstrate the roles of gga-miR-101-2-5p in regulating the expression of *ApoB*, we evaluated the interaction between miR-101-2-5p and *ApoB* in chicken hepatocytes. It was determined that miR-101-2-5p mimics could decrease the expression of *ApoB* in

primary chicken hepatocytes stimulated with exogenous *17β-Estradiol*. With transfection of gga-miR-101-2-5p mimics, the hepatocytes showed a significant increase in the miR-101-2-5p expression by almost five thousand folds ($P < 0.01$), in contrast, a decrease of miR-101-2-5p expression was observed when miR-101-2-5p mimics and *17β-estradiol* were co-transfected into the hepatocytes (Fig. 4a). The *17β-estradiol* significantly induced the expression of *ApoB* mRNA ($P < 0.05$), but miR-101-2-5p mimics could effectively suppress the *ApoB* expression (Fig. 4b).

Discussion

ApoB is primarily synthesized in hepatocytes and intestinal cells and plays a central role in lipoprotein metabolism (Wang et al. 2003). It was revealed that *ApoB* was regulated at multiple levels, such as gene transcription and the 5'UTR and 3'UTR of *ApoB* mRNA were likely to regulate *ApoB* mRNA translation and protein synthesis (Pontrelli et al. 2004). Meanwhile, miRNAs are proved to repress the translation of transcripts by degrading their target mRNA transcripts through binding to the 3'UTR (Bartel 2004). The miRNA-assisted mRNA degradation has emerged as a novel mode of post-transcriptional regulation of gene expression. Therefore, it is important to understand the potential interaction between miRNAs and *ApoB*. Until now, significant progress has been made to unveil the roles of miRNA in lipid metabolism. For instance, miR-33, an intron miRNA of *SREBPs*, was showed to regulate cholesterol metabolism through interaction with *FTO* gene (Najafi-Shoushtari et al. 2010; Shao et al 2014). The miR-27a was reported to regulate lipid metabolism by targeting lipid synthetic transcription factor and the lipid transporter in human hepatoma cells (Shirasaki et al. 2013). A previous study had suggested that gga-miR-101 was significantly differentially expressed between fat and lean broiler chicken (Wang et al. 2015), which implied that gga-miR-101 might be potentially important to the preadipocyte development. Here we reciprocally predicted that miR-101-2-5p binds to the 3'UTR of *ApoB*. Furthermore, our dual-luciferase reporter assays and site mutation analyses validated that *ApoB* was a target gene of miR-101-2-5p in chickens. These results suggested that miR-101-2-5p might

negatively regulate the expression of *ApoB* mRNA in liver of chickens.

It has been demonstrated that more than 90% of the *de novo* synthesis of fatty acid occurs in the liver of chickens (O'Hea & Leveille 1969) and *ApoB* is required for the assembly and secretion of both chylomicrons from the small intestine and very low-density lipoproteins (VLDL) from the liver (Davidson & Shelness 2000). Our recent RNA-seq study on the miRNA expression profile of the livers in pre-laying hens and peak-laying hens revealed that miR-101-2-5p was significantly down-regulated in the liver of peak-laying hen compared with the pre-laying hen (Li et al. 2016), and *ApoB* was found to be up-regulated in the liver of egg-laying hens (Li et al. 2015b). The negative correlation between the expression of miR-101-2-5p and *ApoB* gene in the liver of pre- and peak-laying hens implied that *ApoB* plays an important role in lipid metabolism (Nikolay et al. 2013) and miR-101-2-5p exerts *ApoB*'s function through regulating its expression.

According to the results of previous studies, a mean concentration of estradiol in plasma during sexual maturation in the female chickens was gradually increased (Tanabe et al. 1981; Williams & Harvey 1986), and *ApoB* gene expression was estrogen-responsive in the livers of both adult and embryonic bird (Laziera et al. 1995). Therefore, we examined the possibility that miR-101-2-5p targets *ApoB* in the hepatocytes of chicken under the condition of estradiol induction. The up-regulation of *ApoB* induced by estradiol could be suppressed by co-transfection gga-miR-101-2-5p mimics in primary chicken hepatocytes. This observation indicates that the *ApoB* is targeted by miR-101-2-5p in chicken hepatocytes.

In summary, gga-miR-101-2-5p negatively regulates the expression of *ApoB* gene by binding to its complementary sequences present in the 3'-UTR of *ApoB*. In other word, the expression of *ApoB* in chicken livers can be induced by estrogen and is likely negatively steered by miR-101-2-5p.

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Competing interests

The authors declare that they have no competing interests.

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Figure legends

Figure1. Prediction of the target gene of gga-miR-101-2-5p. (a) Sequence alignments of gga-miR-101-2-5p and the target site in the 3'-UTR of *ApoB*. **(b)** Secondary structure of the RNA duplex of gga-miR-101-2-5p and *ApoB* 3'-UTR target site (Red: Target sequence; Green: gga-miR-101-2-5p).

Figure 2 QRT-PCR validation of *ApoB* and miR-101-2-5p. (a) The expression level of *ApoB*, **(b)** The expression level of miR-101-2-5p. Note: ** means $P<0.01$.

Figure 3. The target gene verification of gga-miR-101-2-5p in DF1 cells. Statistically significant changes in luciferase activity were observed in comparison with controls (NC). Note: ** means $P<0.01$. The “+” means the corresponding component was added in the treatment, and “-” means the corresponding component was not added in the treatment.

Figure 4. The regulation of miR-101-2-5p and its target gene. (a) Transfection of the miR-101-2-5p mimics and 17β -Estradiol, the mRNA level of the miR-101-2-5p detected by qRT-PCR. **(b)** Transfection of the miR-101-2-5p mimics and 17β -Estradiol, the mRNA level of the *ApoB* detected by qRT-PCR. The “+” means the corresponding component was added in the treatment, and “-” means the corresponding component was not added in the treatment.

Tables

Table 1. The primers used for PCR or qPCR

Name	Primer Sequence (5'-3')	Accession No.
Primers for Vector construction		
ApoB 3'-UTR-F	cgc <u>CTCGAG</u> GAT GAACAACCTTGCCAC	NM_001044633
ApoB 3'-UTR-R	ataagaat <u>GCGGCCGC</u> CTT CCATTG ACCTTGAC	NM_001044633
ApoB 3'UTR-OF:	CTGTTTGAATCTGAATTTAAACTAAATAATCTACAGTAGG	NM_001044633
ApoB 3'UTR-OR:	CCTACTGTAGATTATTTAGTTTAAATTCAGATTCAAACAG	NM_001044633
Primers for RT-qPCR		
ApoB-F	ATGTTCAAAAGATGCGGCCC	NM_001044633
ApoB-R	GCAATGGCTCTTCTCTCACTG	NM_001044633
β-actin-F	CAGCCAGCCATGGATGATGA	NM_205518
β-actin-R	ACCAACCATCACACCCTGAT	NM_205518
gga-snoRNA-U6-F	GGGCCATGCTAATCTTCTCTGTATCG	RF00026
gga-snoRNA-U6-R	GTGCAGGGTCCGAGGT	RF00026
snoRNA-U6- RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCGATACA	RF00026
gga-miR-101-2-5p-F	AACGGCTCAGTTATCACAGTGCT	MIMAT0031077
gga-miR-101-2-5p-R	TATGGTTGTTCTCGTCTCCTTGTC	MIMAT0031077
snoRNA-miR-101-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCGATACA	MIMAT0031077

Note: Restriction sites were underlined, protective bases were dashed. *ApoB* 3'UTR-OF represents to forward primer for overlap PCR, *ApoB* 3'UTR-OR represents reverse primer for overlap PCR.

Table 2. The potential miRNAs binding with 3’UTR of ApoB.

Target Rank	Target Score	miRNA Name	Gene Symbol
1	80	gga-miR-6575-3p	ApoB
2	72	gga-miR-6663-5p	ApoB
3	71	gga-miR-101-2-5p	ApoB
4	67	gga-miR-1460	ApoB
5	55	gga-miR-20a-3p	ApoB
6	51	gga-miR-1712-3p	ApoB

ApoB gene is predicted to be targeted by 6 miRNAs in miRDB

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Additional files

Table S1 The significantly enriched pathways and GO terms of miR-101-2-5p target genes. Data are in Excel file format.

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Figure 1

Figure 2

Figure 3. The target gene verification of gga-miR-101-2-5p in DF1 cells. Statistically significant changes in luciferase activity were observed in comparison with controls (NC). Note: ** means $P<0.01$.

93x58mm (300 x 300 DPI)

Figure 4

Additional files

Table S1 The significantly enriched pathways and GO terms of miR-101-2-5p target genes.Data are in Excel file format.

Category	Term	PValue	Count	Genes
KEGG_PATHWAY	gga00564:Glycerophospholipid metabolism	0.0117	4	GPD2, GPD1L, DGKB, AGPAT3
GOTERM_MF_FAT	GO:0046872~metal ion binding	0.013	29	TSHZ3, FDX1, GLIS1, LMO7, RORA, ZNF365, CALB1, ENO4, ZRSR2, PLCB1, MLL3, RBM27, GPD2, KLF6, ZBTB46, RREB1, RBM20, ATP11B, MBNL2, WEE1, PJA2, ZDHHC14, ZDHHC17, EYA1, SP4, RHOT1, ADAM17, RNF138, CDH11

GOTERM_MF_FA T	GO:0008270~zinc ion binding	0.0141	19	KLF6, TSHZ3, ZBTB46, RREB1, RBM20, GLIS1, LMO7, MBNL2, RORA, ZNF365, PJA2, ZDHHC14, ZDHHC17, SP4, ADAM17, RNF138, ZRSR2, MLL3, RBM27
GOTERM_MF_FA T	GO:0043169~cation binding	0.0157	29	TSHZ3, FDX1, GLIS1, LMO7, RORA, ZNF365, CALB1, ENO4, ZRSR2, PLCB1, MLL3, RBM27, GPD2, KLF6, ZBTB46, RREB1, RBM20, ATP11B, MBNL2, WEE1, PJA2, ZDHHC14, ZDHHC17, EYA1, SP4, RHOT1, ADAM17, RNF138, CDH11
GOTERM_MF_FA T	GO:0043167~ion binding	0.0165	29	TSHZ3, FDX1, GLIS1, LMO7, RORA, ZNF365, CALB1, ENO4, ZRSR2, PLCB1, MLL3, RBM27, GPD2, KLF6, ZBTB46, RREB1, RBM20, ATP11B, MBNL2, WEE1, PJA2, ZDHHC14, ZDHHC17, EYA1, SP4, RHOT1, ADAM17, RNF138, CDH11
KEGG_PATHWAY	gga04070:Phosphatidylinositol signaling system	0.0172	4	DGKB, PLCB1, PIK3R1, ITPR2

GOTERM_CC_FA T	GO:0009331 glycerol-3-phosphate dehydrogenase complex	0.0296	2	GPD2, GPD1L
GOTERM_CC_FA T	GO:0044456~synapse part	0.0317	4	GABRA2, SYNPR, SYT11, CALB1
GOTERM_BP_FAT	GO:0006072~glycerol-3-phosphat e metabolic process	0.0421	2	GPD2, GPD1L
GOTERM_BP_FAT	GO:0042953~lipoprotein transport	0.0421	2	ZDHHC17, APOB