Profiling of RNA-binding Proteins Interacting With Glucagon and Adipokine Hormone mRNAs

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ABSTRACT

Objective: Glucagon in mammals and its homolog (adipokinetic hormone [AKH] in Drosophila melanogaster) are peptide hormones which regulate lipid metabolism by breaking down triglycerides. Although regulatory mechanisms of glucagon and AKH expression have been widely studied, post-transcriptional gene expression of glucagon has not been investigated thoroughly. In this study, we aimed to profile proteins binding with Gcg messenger RNA (mRNA) in mouse and Akh mRNA in Drosophila.

Methods: Drosophila Schneider 2 (S2) and mouse 3T3-L1 cell lysates were utilized for affinity pull down of Akh and Gcg mRNA respectively using biotinylated anti-sense DNA oligoes. Mass spectrometry and computational network analysis revealed mRNA-interacting proteins residing in functional proximity.

Results: We observed that 1) 91 proteins interact with Akh mRNA from S2 cell lysates, 2) 34 proteins interact with Gcg mRNA from 3T3-L1 cell lysates. 3) Akh mRNA interactome revealed clusters of ribosomes and known RNA-binding proteins (RBPs). 4) Gcg mRNA interactome revealed mRNA-binding proteins including Plekha7, zinc finger protein, carboxylase, lipase, histone proteins and a cytochrome, Cyp2c44. 5) Levels of Gcg mRNA and its interacting proteins are elevated in skeletal muscles isolated from old mice compared to ones from young mice.

Conclusion: Akh mRNA in S2 cells are under active translation in a complex of RBPs and ribosomes. Gcg mRNA in mouse precursor adipocyte is in a condition distinct from Akh mRNA due to biochemical interactions with a subset of RBPs and histones. We anticipate that our study contributes to investigating regulatory mechanisms of Gcg and Akh mRNA decay, translation, and localization.

Keywords: Glucagon; RNA-binding proteins; Mass spectrometry
INTRODUCTION

Glucagon in mammals and its homologues in invertebrates, adipokinetic hormone (AKH) are peptide hormones regulating lipid metabolism. They mainly function in lipid droplets of adipocytes by promoting break down of lipids from triglycerides (TGs) to a glycerol and three fatty acids. In Tobacco hornworm (Manduca sexta) fat body adipocytes, AKH is recognized by AKH receptor, activates cyclic adenosine monophosphate-dependent protein kinase (PKA), and then induces phosphorylation of lipid storage droplet protein-1 (LSD1) subsequently. Once phosphorylated, LSD1 activates TG-lipase hydrolyzing TGs in lipid droplet, a process called lipolysis. Similarly, Glucagon also influences lipolysis in human and mouse adipose tissues. Glucagon activates PKA to induce phosphorylation of hormone sensitive lipase (HSL) and perilipins (P). P activates adipose triglycerol lipase (ATGL) by dissociation of an adaptor protein, comparative gene identification 58. Activated ATGL and HSL induce lipolysis of TGs in lipid droplet.

Overall lipid metabolic pathways are similar to each other in mammals and invertebrates.

To keep the homeostasis of glucose levels, production and release of Glucagon and AKH are under active control in transcriptional and post-transcriptional levels by external stimuli including insulin in mammals and insulin-like peptide in Drosophila (DILP). Physical interaction between DILP1 and DILP2 regulates Akh messenger RNA (mRNA) and protein level in Drosophila. Similarly, glucagon gene (Gcg) expression is regulated by transcription factors such as PAX6, MAF and FoxA during α-cell differentiation in human. PAX6 forms heterodimer with cMAF or MAFB; PAX6/cMAF and PAX6/MAFB complex interact with the promoter region of G1 and induce Gcg mRNA transcription in mammals. FOX also hetero-dimerizes with Pax6; Pax6/FoxA complex regulates steady state level of Gcg mRNA and FoxA1 knockout mice died in 12 days after birth due to biosynthetic defect of gluconeogenic enzymes leading to hypoglycemia. Numerous studies of Glucagon and Akh, post-transcriptional regulatory mechanisms of Gcg and Akh mRNA are still under investigation. RNA-binding protein HuD was reported to bind 3′-UTR of Gcg mRNA in mouse pancreatic islet and mouse glucagonoma αTC1 cell line. Depletion of HuD using siRNAs decreased level of pro-glucagon in αTC1 cells whereas Gcg mRNA levels did not change significantly. Overall, there are a handful of studies in regulation of Gcg and AKH expression in transcription and post-transcription levels.

In this study, we profiled proteins interacting with Gcg and Akh mRNA by RNA affinity pulldown assay and mass spectrometry. We identified numerous proteins, which interacted with Gcg and Akh mRNA from Drosophila Schneider 2 (S2) and mouse 3T3-L1 cell lysates. Their interactomes revealed RNA-binding proteins, carboxylase, ribosomal protein, and other regulatory proteins. These findings contribute to clarify regulatory mechanisms of Gcg and Akh decay, translation, and localization via interaction with Gcg and Akh mRNA.

MATERIALS AND METHODS

1. Culture of mouse 3T3-L1 and Drosophila S2 cells

3T3-L1 cell was obtained from ATCC (ATCC® CL-173™) and cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin. S2 cell was obtained from ATCC (Schneider’s Drosophila Line 2 [D. Mel. (2), SL2]) and cultured in Schneider’s Drosophila medium supplemented with 10% FBS and penicillin/streptomycin. The cells were grown in suspension culture at 25°C without CO2.
2. mRNA pull down using anti-sense DNA oligo

mRNA pull down using anti-sense DNA oligo was performed as described previously. The biotinylated DNA oligos against mouse Gcg mRNA and Drosophila Akh mRNA were designed and synthesized in IDT (Supplementary Table 1). 3T3-L1 and S2 cell lysates were prepared using protein extraction buffer containing 200 mM Tris-HCl pH 7.5, 100 mM KCl, 5mM MgCl₂, 0.5% NP-40 (1 mg per sample), incubated with 1 μg of three different anti-sense DNA oligos with or without biotin label for 4 hours at 4°C. Complexes containing Gcg and Akh mRNAs were isolated with streptavidin-coupled Sepahrose beads (GE Healthcare, Chicago, IL, USA). The proteins present in the pull-down material were profiled by mass spectrometry and the RNA presents were analyzed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

3. Mass spectrometry for protein identification

Gcg and Akh mRNA pull down materials were analyzed with mass spectrometry. The proteins were subjected into SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie Brilliant Blue. The gel bands were washed with 50 mM ammonium bicarbonate for 10 minutes. Next, the plugs were de-stained using 25 mM ammonium bicarbonate in 50% acetonitrile for 15 minutes, repeated twice. The bands were then reduced with DTT (Sigma-Aldrich, St. Louis, MO, USA) and alkylated using 55 mM Iodoacetamide (Sigma-Aldrich). The gel plugs were then washed with 50 mM ammonium bicarbonate for 10 minutes. The plugs were dehydrated with 100% acetonitrile for 15 minutes and dried in a speedvac. Each gel plug was covered with 100 ng of Proteomics Grade Trypsin (Sigma-Aldrich) and incubated at 37°C overnight. The supernatant was collected in a clean dry Eppendorf tube. Peptides were further extracted with one wash of 25 mM ammonium bicarbonate for 20 minutes and three washes of 5% formic acid, 50% acetonitrile for 20 minutes each. The supernatant was collected after each wash then dried down in a speedvac to ≤1 μL. Samples were reconstituted in 7 μL of mobile phase A (95% water, 5% acetonitrile, and 0.2% formic acid) and placed in auto-sampler vials.

Peptides were separated and analyzed on an EASY nLC 1200 System (Thermo Fisher Scientific, Waltham, MA, USA) in-line with the Orbitrap Fusion Lumos Tribrid Mass Spectrometer (Thermo Fisher Scientific) with instrument control software v. 4.2.28.14. Two μg of tryptic peptides were pressure loaded onto-C18 reversed phase column (Acclaim PepMap RSLC, 75 μm × 50 cm (C18, 2 μm, 100 Å) Thermo Fisher Scientific cat. #164536) using a gradient of 5% to 40% B in 180 minutes (solvent A: 5% acetonitrile/0.1% formic acid; solvent B: 80% acetonitrile/0.1% formic acid) at a flow rate of 300 nL/min.

Mass spectra were acquired in data-dependent mode with a high resolution (60,000) FTMS survey scan, mass range of m/z 375-1500, followed by tandem mass spectra (MS/MS) of the most intense precursors with a cycle time of 3 seconds. The automatic gain control target value was 4.0e5 for the survey MS scan. HCD fragmentation was performed with a precursor isolation window of 1.6 m/z, a maximum injection time of 50 ms, and HCD collision energy of 35%. Monoisotopic-precursor selection was set to “peptide”. Precursors within 10 ppm mass tolerance were dynamically excluded from resequencing for 15 seconds. Advanced peak determination was not enabled. Precursor ions with charge states that were undetermined, 1, or >5 were excluded. For protein identification and quantification, the LC-MS/MS data were searched using the MaxQuant (MQ) platform and the protein intensities were normalized using the label free quantification (LFQ) algorithm. Data were searched against a Drosophila melanogaster or Mus musculus SwissProt reviewed database (August 2020).
and a database of common contaminants. The false discovery rate (FDR), determined using a reversed database strategy, was set at 1% at the protein and peptide level. Fully tryptic peptides with a minimum of 7 residues were required including cleavage between lysine and proline. Two missed cleavages were permitted. The “Label Free Quantitation” (LFQ) feature was used with “Match between runs” enabled for those features that had spectra in at least one of the runs. The “stabilize large ratios” feature was enabled, and “fast LFQ” was disabled. A 4.5 ppm mass tolerance was used. A minimum ratio counts of 2 was required for quantification with at least one unique peptide. Parameters included static modification of cysteine with carbamidomethyl and variable protein N-terminal acetylation and oxidation of methionine. The protein groups text files from the MQ search results were processed in Perseus.\textsuperscript{27} Protein groups were filtered to remove those only identified by a modified peptide, matches to the reversed database, and potential contaminants. The normalized LFQ intensities for each biological replicate were log2 transformed.

4. RT-qPCR

RT-qPCR was performed as described previously.\textsuperscript{28} Acidic phenol (Ambion) was used to extract RNA from pull down materials. Reverse transcription was performed using random nonamers and reverse transcriptase (Maxima, Thermo Fisher Scientific) followed by qPCR using gene-specific primers (Supplementary Table 1) and SYBR green master mix (Kapa Biosystems, Wilmington, MA, USA) under an Applied Biosystems 7300 instrument. Primers against \textit{GAPDH} or \textit{RP49} mRNA were utilized to normalize total amount of RNAs.

5. Protein-protein interaction network analysis

Protein-protein interactions were sourced from BioGRID, build 4.3.194.\textsuperscript{29} Gene Ontology (GO) analysis utilized GOrilla software.\textsuperscript{30} GO term analysis of mass spectrometry hits was performed using all \textit{D. melanogaster} protein coding genes as a background control. Homolog information was extracted from BioMart database within Ensembl.org, release 103.\textsuperscript{31} Specifically, “one2one” homologs were used to build the network of hits for homolog networks. For the comparison looking for enrichment compared to all polyA mRNA-binding proteins, the hit list\textsuperscript{32} was used as the background control list. Networks were initially created using R software to select the appropriate protein-protein interactions from hit proteins, homologs, and BioGRID data. Output tables were uploaded into Cytoscape 3.8.0 to create visual figures.\textsuperscript{33}

6. Protein and RNA purifications from skeletal muscle of young and old mice

“Young” (3-month-old) and “Old” (24-month-old) mouse tibialis anterior muscle were isolated (approved by KRIBB-AEC-16143), homogenized, and lysed with a buffer containing 20 mM HEPES (pH 7.2), 150 mM NaCl, 0.5% Triton X-100, 0.1 mM NaVO_4, 1 mM NaF, 1 mM 4-(2-aminoethyl)-benzene sulfonyl fluoride hydrochloride, and 5 mg/mL aprotinin (Sigma-Aldrich).\textsuperscript{34} The lysates were centrifuged at 15,000×g for 20 minutes at 4°C, and the supernatants were subjected to SDS-PAGE or acidic phenol extraction for RNA purification followed by immunoblot analysis.

7. Western blot analysis

Cell lysates or pull-down materials were separated by SDS-PAGE and transferred onto nitrocellulose membranes (iBlot Stack; Invitrogen, Waltham, MA, USA). Primary antibodies recognize Plekha7 (GTX131146; GeneTex, Irvine, CA, USA), Vimentin (sc-6260; Santa Cruz Biotechnology, Dallas, TX, USA), Pyruvate Carboxylase B (sc-365673), SRP54 (sc-393855), Plectin (sc-33649) and HSP70 (sc-7298). The horseradish peroxidase-conjugated secondary antibodies were from GE Healthcare.
RESULTS

1. Akh mRNA pull down enriches various proteins from Drosophila S2 cells

In order to profile proteins interacting with *D. melanogaster* Akh mRNA, we performed Akh mRNA pull down assay followed by mass spectrometry. First, we grew Drosophila S2 cells, extracted lysates using a buffer containing a mild detergent (0.5% NP-40), and then incubated them with biotinylated DNA oligos complementary to Akh mRNA (Fig. 1A). As a negative control, we utilized the same anti-sense DNA oligos without biotin label. Streptavidin-conjugated sepharose beads were utilized to pull down a complex containing Akh mRNA bound to biotinylated anti-sense DNA oligos. The resulting pellets were subjected either to RNA purification for RT-qPCR (Fig. 1B) or SDS-PAGE followed by Coomassie blue staining (Fig. 1B). The protein staining revealed enrichment of proteins around 75 kDa, 20 kDa, and 15 kDa, respectively. We also cut the corresponding lanes, extracted/digested proteins from each lane using Trypsin, and then profiled them using mass spectrometry. Our Mass Spectrum revealed that there are 18 major proteins including RNA helicase (bel and Upf1), mRNA translation elongation factors, heterogeneous nuclear ribonucleoproteins (hnRNPs), canonical RNA-binding proteins (lark, larp, and mod), RNA decay enzyme (Rat1), and ribosomal proteins (Fig. 1C). These findings demonstrate that Akh mRNA establishes molecular complexes containing RNA-binding proteins and ribosomes.

2. Akh mRNA interactome revealed clusters of ribosomes and RNA-binding proteins

GO term analysis of mass spectrometry hits indicated select major functions and processes were enriched for mRNA-binding (Supplementary Table 2). As expected, molecular function GO analysis showed that RNA-binding (FDR <1.8E-20), mRNA-binding (FDR <1.1E-7), translation regulator activity (FDR <2.2E-6), translation factor activity with RNA-binding (FDR <1.3E-4) were significantly enriched pathways. Biological process GO analysis showed that cytoplasmic translation (FDR < 1.9E-15), gene expression (FDR <2.6E-14), translation (FDR <4.3E-13), peptide biosynthetic process (FDR <5.1E-13), cellular biosynthetic process (FDR <3.2E-09), biosynthetic process (FDR <1.2E-08), regulation of gene expression (FDR <1.2E-05), regulation of alternative mRNA splicing, via spliceosome (FDR <1.3E-05), RNA processing (FDR <3.1E-05) were also significantly enriched pathways. The small ribosomal subunit was enriched (FDR <5.7E-3) whereas the large ribosomal subunit was not. The nucleolus pathway (FDR <6.6E-3) and ribonucleoprotein complex pathway (3.7E-12) were significant. Thus, we interpret mRNA-binding proteins and the small ribosomal subunit regulates Akh mRNA translation in Drosophila S2 cells.

A few unexpected pathways also contained select proteins, which led to marginal pathway enrichment. The mitochondrial proton-transporting ATP synthase complex was significantly enriched (FDR <1.8E-3) due to pull down of blw and ATPsyn-beta. Components of microtubule-mediated segregation of cellular components during mitosis were enriched, including spindle microtubule proteins (FDR <7.7E-6). DNA-binding proteins were enriched, particularly in the mitochondria DNA replication pathway (FDR <2.2E-2). These results are highlighted in a network in Fig. 2A. Many Akh interactions would be expected from non-specific mRNA-binding proteins. To discriminate pan-mRNA binding proteins to Akh mRNA-specific binding networks, a background of known Drosophila polyA mRNA-binding proteins was used (Supplementary Table 3). Mitochondrion organization (p<7.6E-4)
remained nominally enriched when compared to known polyA mRNA-binding proteins, as did microtubule component (FDR <8.6E-9) and DNA binding (FDR <2.4E-5). These results are highlighted in a network in Fig. 2B. Taken together, these results indicate DNA binding factors and mitochondria-microtubule protein networks interact with Akh mRNA at higher rates than expected by chance.
Fig. 2. Protein-protein interaction plot of Akh mRNA-interacting proteins. (A) Protein-protein interactions of hits identified in proteomic analysis. Edges are derived from BioGRID. Blue indicates DNA-binding proteins, red indicates RNA-binding proteins, and purple indicates dual RNA/DNA-binding proteins. Orange indicates ribosomal proteins. White fill indicates the other proteins, particularly microtubule-binding proteins and mitochondrial proteins. The circle size is proportional to mass spectrometry signal data. (B) Protein-protein interactions of hits identified in proteomic analysis after normalization with polyA mRNA-binding protein profiles. Edges are derived from BioGRID. Blue indicates DNA-binding proteins, red indicates RNA-binding proteins, and purple indicates dual RNA/DNA-binding proteins. Orange indicates ribosomal proteins. White fill indicates other protein classes, particularly microtubule-binding proteins and mitochondrial proteins. The circle size is proportional to mass spectrometry signal data. Gray node outlines indicate known polyA mRNA-binding proteins in Drosophila, black edges indicate previously unidentified hits. Hpo was manually added and were not a hit in the current proteomic profile.

mRNA, messenger RNA.
A complementary method of analyzing how Akh mRNA interacts with mRNA-bound proteins is to determine how Akh interactors relate to the overall polyA mRNA interactome. To do this, a network was generated from protein-protein interactions within the polyA mRNA-interactome and Akh mRNA-binding proteins were highlighted within the network. This is essentially the reverse of the network building strategy from Fig. 2B. As might be expected, the network of all known polyA-binding proteins was much more extensive than the Akh-specific binding protein network (Fig. 3). Nonetheless, the Akh mRNA-binding proteins were spread evenly throughout the pan-polyA binding network. Taken together with the networks in Fig. 2, these results indicate Akh mRNA is not handled as an “average” mRNA transcript from Drosophila S2 cells.

3. **Mouse Gcg mRNA interactome revealed mRNA-binding protein, carboxylase and lipid metabolism related enzyme**

To profile proteins interacting with the AKH homolog in mouse, Gcg mRNA, we performed Gcg mRNA pull down assay and mass spectrometry. 3T3-L1 lysates were incubated with biotinylated or non-biotinylated DNA oligos complementary to Gcg mRNA (Fig. 4A). Streptavidin-conjugated sepharose beads enabled us to pull down a complex containing Gcg mRNA bound to the biotinylated DNA oligos. The pull-down materials were subjected either to RNA purification for RT-qPCR (Fig. 4B) or SDS-PAGE followed by mass spectrometry (Fig. 4C). Our Mass spectrum revealed that there are 34 protein hits including mRNA-binding protein (SERBP1), zinc finger protein (ZFP28), carboxylases (Acetyl-CoA carboxylase, Methylcrotonoyl-CoA carboxylase and Pyruvate Carboxylase), lipase (diacylglycerol lipase beta), histone proteins, signal recognition particle 54 (SRP54), cytoskeletal protein (Plectin, Plekha7, and Vimentin) and CYP2C44 (Fig. 4C). We verified that Plekha7 interacts with Gcg mRNA using western blot analysis (Fig. 4D). These findings demonstrate that Gcg mRNA establishes molecular complexes containing RNA-binding proteins, carboxylase, or lipid metabolism related enzymes.

4. **Gcg mRNA interactome revealed nucleosomal DNA-binding and protein-DNA complex enrichment**

GO term analysis of mass spectrometry hits did not show identical regulation of Gcg mRNA translation as might be expected from the Akh mRNA interactome. Few molecular functions, biological processes and cellular components of GO term analysis were significant FDR (Supplementary Table 4). Nucleosomal DNA-binding enriched nucleosomal DNA-binding (FDR <7.3E-3), nucleosome (FDR <7.1E-6) and DNA packaging complex (FDR <1.1E-5) were significantly enriched pathways. However, we could not find enriched GO term data of mRNA regulation, carboxylase or lipid metabolism related enzyme as we observed from Akh mRNA interactome. Both the Gcg and Akh datasets unexpectedly contained enrichment of DNA binding factors, suggesting these proteins may have additional non-canonical functions regulating mRNA as well.

5. **Network plot of Gcg mRNA-interacting proteins revealed cluster of histone proteins**

A network plot of Gcg mRNA interactions revealed that the most of the identified proteins clusters are in histone family proteins (Fig. 5A). They are connected each other with histone proteins both directly and indirectly, which are functionally connected with packaging DNA into chromatin in nucleus. GO analysis also revealed that nucleosomal DNA-binding (FDR <7.3E-3), nucleosome (FDR <7.1E-6) and DNA packaging complex (FDR <1.1E-5) were significantly enriched pathways. Another cluster centered with ryanodine receptor 2 or
ZFP28 were not connected with histone proteins in first-neighbor network analysis (Fig. 5A). Therefore, the network plot may be interpreted to show that most of Gcg mRNA-interacting proteins are possibly located in nucleus and involved in chromatin regulation.

6. Level of Gcg mRNA correlates with levels of Gcg mRNA-interacting proteins in aged mouse skeletal muscle

In order to connect our findings in Gcg mRNA-interactome with biological processes, we first
compared levels of Gcg mRNA in lysates of skeletal muscle isolated from young (3 months) and old (24 months) mice. Our RT-qPCR analysis revealed that levels of Gcg, Dlk1, and Pparg mRNAs are elevated in old muscle tissues while Fabp4 mRNA did not fluctuate (Fig. 5B). Western blot analysis of Gcg mRNA-interacting proteins showed that levels of Plekha7, Pyruvate Carboxylase
B, Vimentin, and SRP54 increased while the level of loading control HSP70 did not change as a loading control (Fig. 5C). These results implicate accumulation of Gcg mRNA in old skeletal muscle may originate from overexpression of Gcg mRNA-interacting proteins.

**DISCUSSION**

Glucagon and insulin are key hormones regulating lipid metabolism and glucose levels in blood. Dysregulation of these hormones induces obesity, hyperlipidemia, diabetes and even cancers. Although there is much published research regarding transcriptional regulation of AKH, DILP, glucagon and insulin expression, post-transcriptional regulation of Akh, and Gcg mRNA...
In this study, we found that numerous proteins interact with Akh and Gcg mRNA in Drosophila S2 and mouse 3T3-L1 cells. (Figs. 1 and 4). Akh mRNA interactomes revealed clusters of ribosomes and RNA-binding proteins (Figs. 2 and 3) whereas mouse Gcg mRNA interactomes revealed RNA-binding protein, carboxylase and lipid metabolism.

Eighteen major proteins include RNA helicase (bel and Upf1), mRNA translation elongation factors, hnRNPs, RNA-binding proteins (lark, larp and mod), RNA decay enzyme Rat1 and ribosomal proteins (Fig. 1D). RNA-binding proteins lark, larp and mod were reported to activate post-transcriptional regulation of translation. Therefore, Akh mRNA post-transcriptional regulation of translation is possible to relate with RNA-binding proteins of lark, larp and mod. RNA-binding proteins larp6 activates mRNA translation via recruitment of ATP-dependent RNA helicase A, which might function similarly with RNA helicase bel and Upf1 on Akh mRNA. On the other hand, RNA decay enzyme Rat1 may degrade Akh mRNA via 5’ to 3’. We expected RNA-binding proteins of lark, larp and mod and RNA decay enzyme Rat1 could function in regulation of Akh mRNA decay and translation, respectively. Detailed information is provided in the schematic in Fig. 6A.

GO term analysis also supported regulation of Akh mRNA translation. Molecular function of GO term analysis showed their proteins highly related with mRNA-binding and translation (Supplementary Table 2). Biological process of GO term analysis also showed their proteins highly related with translation and biosynthetic process (Supplementary Table 2). In addition, cellular component of GO term analysis highlighted that Akh mRNA was enriched with both small and large ribosomal subunits (Supplementary Table 2). The cytosolic ribosome pathway was enriched largely due to binding of ribonucleoproteins and small and large ribosomal subunits (Fig. 1D, Supplementary Tables 2 and 3). We interpret that Akh mRNA translation are under regulation with various proteins in cytosolic ribosome. Further study is needed for clarifying the interaction between Akh mRNA and our identified interacting proteins. However, our study showed the possibility of Akh mRNA interaction and regulation with 18 proteins.

Thirty-four major proteins include mRNA-binding protein (SERBP1), ZFP28, carboxylase (Acetyl-CoA carboxylase, Methylcrotonoyl-CoA carboxylase and Pyruvate Carboxylase), lipase (diacylglycerol lipase beta), histone proteins, SRP54, cytoskeletal protein (Plectin, Plekha7, and Vimentin) and Cyp2c44. We also found mRNA-binding protein of SERBP1 involved in gene expression regulation. It is reported that SERBP1 binds with CtIP mRNA and regulates polysome-associated CtlP mRNA translation. As a 54 kDa subunit protein of signal recognition protein, SRP54 has a methionine-rich RNA-binding domain directly interacting with 4.5S RNA, which is conserved in mouse and human. Vimentin also co-precipitates with a long noncoding RNA, BC088259, mouse mu opioid receptor mRNA, and collagen mRNAs, implicating its direct interaction with RNAs. In addition, we verified that Plekha7 interacts with Gcg mRNA using RNA pull down and western blot analysis (Fig. 4D). Plekha7 was also implicated in direct or indirect association with mature miRNAs. Interaction of SERBP1, SRP54, Plekha7 and Vimentin with Gcg mRNA require further study to define biochemical regulatory mechanisms. A previous study reported proglucagon mRNA-binding protein of HuD was not found in our study possibly due to low expression of HuD in undifferentiated 3T3-L1 cells. More details are provided in the schematic in Fig. 6B.

In contrast to Akh mRNA-interacting proteins GO analysis, Gcg GO analysis did not support specific regulation of Gcg mRNA translation in undifferentiated 3T3-L1 cells (Fig. 5, Supplementary Table 4). The mass spectrometry data enriched nucleosome DNA-binding
because of histone family proteins (Supplementary Table 4). We interpret the results both Gcg precursor and mature mRNAs were pull down with chromatin undergoing active transcription but still processing precursor mRNAs. Furthermore, we attempted Gcg mRNA pull down assay using 3T3-L1 cells after differentiation into adipocyte-like cells. However, we did not obtain enough amount of proteins for mass spectrometry from Gcg mRNA pull down materials. If this is the case, we can explain why GO analysis didn’t show RNA-binding proteins and ribosomes under active translation.

Interestingly, Acetyl-CoA carboxylase, Pyruvate Carboxylase and Methylcrotonoyl-CoA carboxylase interacted with Gcg mRNA (Fig. 4C). Downstream signaling of glucagon and its
receptor inactivates Acetyl-CoA carboxylase and Acetyl-CoA carboxylase mRNA transcription as a result of blocking synthesis of fatty acid. Methylcrotonoyl-CoA carboxylase also relates synthesis of fatty acid with Acetyl-CoA carboxylase. Contrary to the previous research indicating that two enzymes in this reaction are inhibited by glucagon and interact with Gcg mRNA, we speculate that these enzymes might be involved in inhibition of Gcg mRNA translation or activation of Gcg mRNA decay. Although there are no studies showing that these carboxylases bind RNA directly, they may interact with Gcg mRNA via other RNA-binding proteins. On the other hand, Pyruvate Carboxylase relates glucose synthesis by converting pyruvate to oxaloacetate. Glucagon affects flux of Pyruvate Carboxylase for carboxylation of pyruvate. Pyruvate Carboxylase might be involved in activation of Gcg mRNA translation or inhibition of Gcg mRNA decay. Interaction of Acetyl-CoA carboxylase, Pyruvate Carboxylase and Methylcrotonoyl-CoA carboxylase with Gcg mRNA need further investigation to explore post-transcriptional regulation of Gcg mRNA.

Diacylglycerol lipase beta and Cyp2c44 interacted with Gcg mRNA in this study. They relate with synthesis of arachidonic acid and prostaglandin, respectively. Arachidonic acid and prostaglandin are key factors of lipid metabolism. We speculate that lipid metabolic pathways could be related to translation or decay of Gcg mRNA. Interaction diacylglycerol lipase beta and Cyp2c44 with Gcg mRNA are not known previously, but arachidonic acid and prostaglandin pathways are involved in glucagon-mediated lipolysis. Further studies on the relationship between lipid metabolism and glucagon are needed in terms of Gcg mRNA decay and translation.

Our findings in young and old skeletal muscle implicate relevance of adipogenesis and skeletal muscle biology. Glucagon modulates proliferation and differentiation of human adipose precursors cells, which might happen similarly in intermuscular adipose tissues. Cytoskeletal proteins such as Vimentin, Plectin, and Plekha7 are also involved in lipogenesis, lipid droplet assembly and direct binding with phosphatidylinositol lipids, respectively. Molecular mechanisms of how Gcg mRNA-interacting proteins stabilizes Gcg mRNA in skeletal muscle should be investigated thoroughly.

In summary, we profiled various proteins interacting with Gcg and Akh mRNA. These newly identified proteins could be the start points for identifying the post-transcription regulatory mechanisms of Gcg and Akh mRNA.

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**SUPPLEMENTARY MATERIALS**

**Supplementary Table 1**
Sequences of primers used in this study

Click here to view
Supplementary Table 2
Drosophila melanogaster gene ontology analysis
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Supplementary Table 3
Drosophila melanogaster gene ontology analysis normalized with polyA mRNA-binding proteome
Click here to view

Supplementary Table 4
Mus musculus gene ontology analysis
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