**Hsp74/14-3-3σ Complex Mediates Centrosome Amplification by High Glucose, Insulin, and Palmitic Acid**

Yu Cheng Lu, Pu Wang, Qi Gui Wu, Rui Kai Zhang, Alice Kong, Yuan Fei Li,* and Shao Chin Lee*

It has been reported recently that type 2 diabetes promotes centrosome amplification via 14-3-3σ/ROCK1 complex. In the present study, 14-3-3σ interacting proteins are characterized and their roles in the centrosome amplification by high glucose, insulin, and palmitic acid are investigated. Co-immunoprecipitation in combination with MS analysis identified 134 proteins that interact with 14-3-3σ, which include heat shock 70 kDa protein 4 (Hsp74). Gene ontology analyses reveal that many of them are enriched in binding activity. Kyoto Encyclopedia of Genes and Genomes analysis shows that the top three enriched pathways are ribosome, carbon metabolism, and biosynthesis of amino acids. Molecular and functional investigations show that the high glucose, insulin, and palmitic acid increase the expression and binding of 14-3-3σ and Hsp74 as well as centrosome amplification, all of which are inhibited by knockdown of 14-3-3σ or Hsp74. Moreover, molecular docking analysis shows that the interaction between the 14-3-3σ and the Hsp74 is mainly through hydrophobic contacts and a lesser degree ionic interactions and hydrogen bond by different amino acids residues. In conclusion, the results suggest that the experimental treatment triggers centrosome amplification via upregulations of expression and binding of 14-3-3σ and Hsp74.

1. Introduction

Type 2 diabetes mellitus (T2DM) is a serious health problem worldwide, which can cause various chronic complications.[1] There is evidence that T2DM is associated with increased cancer risk and poor cancer prognosis. Currently, about 8–18% of all cancer patients have preexisting diabetes.[2] Individuals with diabetes who develop cancer have a 42% increased risk of death, a 21% increased risk of recurrence, and a significantly decreased 5-year overall and cancer-specific survival rate, as compared to individuals with cancer but free of diabetes.[3] However, little is known about the biological links between diabetes and cancer. It is speculated that deregulation of insulin and insulin-like growth factor signaling, obesity and inflammation, metabolic symbiosis, endoplasmic reticulum stress, and autophagy might play their roles.

Centrosome is the main microtubule organizing center. Each centrosome has two centrioles surrounded by the pericentriolar material, which plays important roles in cell division and the maintenance of genomic stability.[4] Centrosome amplification, acquisition of more than two centrosomes in each cell, is commonly found in various types of cancers, including solid tumors and hematological malignancies.[5] Recent experimental results suggest that centrosome amplification can initiate tumorigenesis[6] and increase cancer cell invasion potential.[7] Moreover, it is associated with poor cancer prognosis.[8]

T2DM presents typical pathophysiological features that include hyperglycemia, hyperinsulinenia, and increased level of free fatty acids. Palmitic acid, the most common saturated free fatty acid, is often used to investigate the effects of free fatty acids, in particular the adverse effects.[9] We have recently reported that T2DM promotes cell centrosome amplification via...
AKT-ROS-dependent signaling of 14-3-3σ and ROCK1,[10] and the pathophysiological factors in T2DM are the triggers. These results implicate that centrosome amplification is a candidate biological link between T2DM and cancer development. In a functional proteomic study, we identified nine proteins associated with centrosome amplification, which included 14-3-3σ, NPM, and PCNA, which were all confirmed to mediate the centrosome amplification by high glucose, insulin, and palmitic acid.[11] The results emphasize that 14-3-3σ and its binding partners play important roles in the occurrence of the diabetes-associated centrosome amplification.

14-3-3 Proteins are a group of highly conserved, acidic proteins that have diverse intracellular functions, including intracellular signaling, cell division, apoptosis, cell cycle, and mitogenetic signaling.[12] 14-3-3 Proteins of higher eukaryotes contribute to cell cycle regulation and/or centrosome-related functions by controlling protein binding dynamics at centrosomes.[13] In mammals, there are seven distinct isoforms of 14-3-3 (β, γ, ε, ζ, η, σ, and τ/θ).[14] 14-3-3σ binds to γ-Tubulin, localizes to the centrosome, and can prevent centrosome amplification.[15] 14-3-3σ is considered to be an important tumor suppressor and plays an important role in drug resistance.[16]

In this study, to better understand the mechanisms of how 14-3-3σ promotes the T2DM-associated centrosome amplification, we characterized the 14-3-3σ binding partners using co-immunoprecipitation (CoIP) followed by MS, and further investigated the roles of the binding partners of interest in the occurrence of the centrosome amplification triggered by high glucose, insulin, and palmitic acid.

2. Experimental Section

2.1. Chemicals, Antibodies, and Cell

All chemicals were purchased from Sigma (St. Louis, MO, USA). Anti-γ-tubulin antibody (no. ab27074; mouse antibody) was purchased from Abcam (Cambridge, UK). Anti-14-3-3σ antibody (no. PLA0201; rabbit antibody) was purchased from Sigma. Anti-hepatitis shock protein 70 kDa protein 4 (Hsp74) antibody (no. ab137631; rabbit antibody) was provided by Abcam. Other antibodies were provided by Cell Signaling Technology (Boston, MA, USA).

HCT116 colon cancer cells were kindly provided by Dr. B. Vogelstein of the Johns Hopkins University School of Medicine. The culture medium and reagents were purchased from Gibco (Beijing, China). The palmitic acid stock was conjugated to fatty acid-free bovine albumin in a 3:1 molar ratio at 37 °C for 1 h before use. Anti-gamma tubulin antibody was used to detect centrosome by immunofluorescent staining.

2.2. Cell Culture and Experimental Treatment

HCT116 cells were maintained in the DMEM (glucose, 5 mM) supplemented with 10% fetal bovine serum, and 1% penicillin–streptomycin in a humidified incubator with 5% CO2 at 37 °C. Cells from the cultures at ≈70% confluency were used for all experimental treatments. Cells treated for 48 h were used for quantification of centrosome number. Time course assays were performed and the time point was chosen, since this time point produced the significant level of differences for centrosome amplification between the control and the treated samples. Cells treated for 30 h were used for CoIP and Western blot analysis. The experimental treatment included high glucose (15 mM), insulin (150 nM), and palmitic acid (150 μM).

2.3. Confocal Microscopy

A cover slip was placed in a well of a six-well plate. Cells were plated at a density of 50 000 cells per well. Cells grown on the cover slips were fixed in cold methanol and acetone (1:1; v/v) for 6 min at –20 °C, followed by three washes with PBS (10 min each time). Then, the cells were incubated with 0.1% Triton X-100 for 15 min and 3% BSA for 1 h. The cells were incubated with a primary antibody in 3% BSA in PBS overnight at 4 °C, washed twice with PBS, and incubated with an FITC-conjugated secondary antibody in 3% BSA in PBS for 1 h at room temperature in the dark. Finally, the cells were mounted with mounting medium. Confocal microscopy was performed using the Zeiss LSM880 microscope (Oberkochen, Germany) with a 1.4 NA oil-immersion lens, and image processing was performed with Zen software (Oberkochen, Germany).

2.4. CoIP Assay

The HCT116 cells were seeded in 10 cm dishes at a cell number of 5 × 10⁶. After treatment, cells were harvested and lysed in 500 μL precooled CoIP buffer (50 mM Tris/HCl pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride, 2 μg mL⁻¹ leupeptin, and 2 μg mL⁻¹ pepstatin A) for
30 min on ice. After centrifugation (14 000 rpm, 4 °C, 15 min), the supernatants were mixed with 30 μL protein A agarose bead slurry and incubated with rotation at 4 °C for 1 h. Following by centrifugation (14 000 rpm, 4 °C, 15 min), the supernatants were incubated with the primary antibody at 4 °C overnight. The next day, mixture was mixed with 10–30 μL bead slurry for 3 h at 4 °C. After three times of washing with CoIP buffer in 50 μL elution buffer with shaking (100 mM Gly-Cl pH 2.5, 500 mM NaCl, 0.05% Tween-20) for 1 min. After centrifugation (3000 rpm, 4 °C, 3 min), the supernatants were collected and 250 μL neutralization buffer (1 M Tris-Cl pH 8.0) were added. The samples were used for quantification of protein concentration then MS analysis.

2.5. Filter-Aided Sample Preparation

The eluted proteins were then digested according to the filter-aided sample preparation (FASP) procedure described. Briefly, 200 μg of proteins for each sample (supernatant) were incorporated into 30 μL SDT buffer (4% SDS, 100 mM DTT, 150 mM Tris-HCl pH 8.0) at 90 °C for 5 min. The detergent DTT and other low-molecular-weight components were removed using 200 μL UA buffer (8 m urea, 150 mM Tris-HCl pH 8.0) by repeated ultrafiltration (Microcon units, 30kDa). Then 100 μL 0.05% iodoacetamide in UA buffer was added to block reduced cysteine residues and the samples were incubated for 20 min in darkness. The filter was washed with 100 μL UA buffer three times and then twice with 100 μL 25 mM NH₄HCO₃. Finally, the protein suspension was digested with 2 μg trypsin (Promega) in 40 μL 25 mM NH₄HCO₃ overnight at 37 °C, and the resulting peptides were collected as a filtrate.

2.6. LC–ESI–MS/MS Analysis by Q Exactive

Experiments were performed on a Q Exactive mass spectrometer that was coupled to Easy nLC (Proxeon Biosystems, now Thermo Fisher Scientific). Six microliters of each fraction was injected for nanoLC–MS/MS analysis. The peptide mixture (5 μg) was loaded onto a the C18-reversed phase column (Thermo Scientific Easy Column, 10 cm long, 75 μm inner diameter, 3 μm resin) in buffer A (0.1% formic acid) and separated with a linear gradient of buffer B (80% acetonitrile and 0.1% formic acid) at a flow rate of 250 nL min⁻¹ controlled by IntelliFlow technology over 140 min. MS data were acquired using a data-dependent top 10 method dynamically choosing the most abundant precursor ions from the survey scan (300–1800 m/z) for high-energy collision-induced dissociation fragmentation. Determination of the target value is based on predictive automatic gain control. Dynamic exclusion duration was 60 s. Survey scans were acquired at a resolution of 70 000 at m/z 200 and resolution for HCD spectra was set to 17 500 at m/z 200. Normalized collision energy was 30 eV and the underfill ratio, which specifies the minimum percentage of the target value likely to be reached at maximum fill time, was defined as 0.1%. The instrument was run with peptide recognition mode enabled.

2.7. Sequence Database Searching and Data Analysis

All MS/MS spectra were searched using Mascot software v2.2.2 software (Matrix Science, London, UK), against the Homo sapiens UniProtKB database (www.uniprot.org). For protein identification, the following options were used: Peptide mass tolerance, 20 ppm; MS/MS tolerance, 0.1 Da; enzyme, trypsin; and missed cleavage, 2. Fixed modification was carbamidomethyl. Variable modification: oxidation.

2.8. Bioinformatic Analysis

To examine the biological and functional properties of the identified proteins, Gene ontology (GO) annotation was conducted by searching the GO database (http://www.geneontology.org). Functional category analysis was performed with protein2go and go2protein for annotation. Visualization and Integrated Discovery v6.7 was used for functional enrichment analysis of GO terms and KEGG pathways. A false discovery rate of <0.01 was selected as the cut-off criterion.

2.9. Small Interfering RNA and Transfection

For the siRNA studies, the pre-designed small interfering RNA (siRNA) oligonucleotides (Sangon Technology, Shanghai, China) were: 1) 14-3-3σ, ACCUGUCUCAGUAGCUATT (sense) and UAGGCUACUCAGAGCUGATT (anti-sense) and 2) Hsp74, AGGACGAGUUGAG CACAATT (sense) and UUGCGUC CAAACUGUUGCUTT (anti-sense). HCT116 cells (5×10⁶ cells per well) were seeded in six-well plates and cultured for 24 h, and then were transfected with 200 pm siRNA oligonucleotides using Lipofectamine 2000 transfection reagent (Invitrogen, California, USA), according to the manufacturer’s instructions. The protein level was evaluated by Western blot analysis.

2.10. Western Blot Analysis

The cells were lysed in RIPA buffer. Proteins were separated by PAGE and transferred onto polyvinylidene fluoride membrane. After blocking for 1 h at room temperature with TBST containing 0.05% (v/v) Tween-20 and 5% (w/v) non-fat milk, the membranes were incubated with primary antibodies overnight at 4 °C, followed by washes with TBST containing 0.05% Tween-20. The membranes were then incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. ECL reagents (Thermo Biosciences, Massachusetts, USA) were used to visualize the protein bands which were captured on X-ray film.

2.11. Homology Modeling and Molecular Docking

To further elucidate the functional relationships between 14-3-3σ and interacting proteins, a protein of interests was chosen, which is Hsp74. The crystallographic structure of the Hsp74 has not been published yet. In order to expose the binding mode between human 14-3-3σ protein and Hsp74 at the molecular level, the 3D structure of the Hsp74 was
built by means of modeler 9.19 homology modeling software (http://salilab.org/modeller/). The sequence in FASTA format of Hsp74 was retrieved from NCBI (Accession: P34932.4). The crystallographic structure of *Saccharomyces cerevisiae* Hsp110 (PDB ID: 3C7N) was selected as the templates for modeling. Molecular docking were performed to investigate the binding mode between the human 14-3-3σ protein and the Hsp74 using the ZDOCK server (http://zdock.umassmed.edu/). The 3D structure of the human 14-3-3σ (PDB ID: 6FCP) was downloaded from Protein Data Bank (http://www.rcsb.org/pdb/home/home.do), while the 3D structure of the Hsp74 was built by modeler 9.19. For docking, the default parameters were used as described in the ZDOCK server. The top ranked pose as judged by the docking score was using PyMoL 1.7.6 software (http://www.pymol.org/).

2.12. Statistical Analysis

All the experiments were performed in triplicate. Data were expressed as the mean ± SD. Student’s *t*-test was performed for comparison between two groups. The statistical analysis software package SPSS 21.0 was employed for the statistical comparisons. A *p*-value < 0.05 was considered significant.

3. Results

3.1. High Glucose, Insulin, and Palmitic Acid Induce Centrosome Amplification

We have recently reported that the level of centrosome amplification is increased in peripheral blood mononuclear cells from patients with type 2 diabetes. AKT-ROS-dependent upregulation of 14-3-3σ and ROCk1 as well as their binding and translocation to centrosome is the underlying signal transduction pathway for the diabetes-associated centrosome amplification.[10] In the present study, we further investigated the molecular basis of centrosome amplification associated with T2DM using colon cancer cells as an experimental model, which were treated with high glucose, insulin, and palmitic acid. As shown in Figure 1A,B, high glucose, insulin, and palmitic acid were able to induce moderate centrosome amplification in the cells. Under the experimental conditions, most cells with centrosome amplification had three to five centrosomes per cell (Figure 1A). Glucose, insulin, and palmitic acid were used at 15 mM, 5 nM, and 150 μM, respectively, which were close to their pathophysiological levels.

3.2. Identification of 14-3-3σ-Interacting Proteins

To characterize proteins attached to the 14-3-3σ, we performed CoIP using 14-3-3σ antibody and identified the partner proteins using MS. As shown in Table 1, a total of 165 proteins were identified, among which 134 protein were identified from the treated samples, 19 proteins were identified from the control samples and 12 proteins were identified from both control and treated samples (Figure 2). Thus, 153 proteins were responsive to the experimental treatment.

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Table 1. Identification of 14-3-3σ-interacting proteins in control and treated samples using MS.

| Source of proteins | Uniprot ID | Protein name | Theoretical MW [KDa] | Number of peptides | Number of unique peptides | Cover percent [%] |
|--------------------|------------|--------------|----------------------|--------------------|--------------------------|------------------|
| Proteins from treated samples | P09429 | High mobility group protein B1 | 24.893 | 2 | 2 | 11.6 |
| E9PAV3 | Nascent polypeptide-associated complex subunit alpha, muscle-specific form | 205.42 | 1 | 1 | 0.6 |
| B4DX78 | cDNA FLJ53484, highly similar to ATP-dependent RNA helicase DDX39 (EC 3.6.1.1) | 53.696 | 1 | 1 | 2.6 |
| O00299 | Chloride intracellular channel protein 1 | 26.922 | 1 | 1 | 3.7 |
| Q93008 | Probable ubiquitin carboxyl-terminal hydrolase FAF-X | 292.28 | 1 | 1 | 0.7 |
| Q8TAA3 | Proteasome subunit alpha type-7-like | 28.329 | 1 | 1 | 4.3 |
| O15143 | Actin-related protein 2/3 complex subunit 1B | 40.949 | 1 | 1 | 3.5 |
| O43175 | α-3-Phosphoglycerate dehydrogenase | 56.65 | 1 | 1 | 2.1 |
| O75368 | SH3 domain-binding glutamic acid-rich-like protein | 12.774 | 1 | 1 | 11.4 |
| O75390 | Citrate synthase, mitochondrial | 51.712 | 1 | 1 | 3.4 |
| O95433 | Activator of 90 kDa heat shock protein ATPase homolog 1 | 38.274 | 1 | 1 | 3.6 |
| P00505 | Aspartate aminotransferase, mitochondrial | 47.517 | 1 | 1 | 3.3 |
| P00538 | Phosphoglycerate kinase 1 | 44.614 | 4 | 4 | 9.6 |
| P02545 | Prelamin-A/C | 74.139 | 4 | 3 | 6.2 |
| P04406 | Glyceraldehyde-3-phosphate dehydrogenase | 36.053 | 1 | 1 | 8.7 |
| P05388 | 60S acidic ribosomal protein P0 | 34.273 | 1 | 1 | 8.8 |
| P06454 | Prothymosin alpha | 12.203 | 3 | 3 | 22.5 |
| P06576 | ATP synthase subunit beta, mitochondrial | 56.559 | 2 | 2 | 4.9 |
| P06733 | Alpha-enolase | 47.168 | 3 | 3 | 14.1 |
| P06748 | Nucleophosmin | 32.575 | 1 | 1 | 10.9 |
| B4DY90 | Tubulin beta chain | 52.048 | 3 | 2 | 8 |
| P07717 | Profilin-1 | 15.054 | 2 | 1 | 24.3 |
| P07900 | Heat shock protein HSP 90-alpha | 84.659 | 4 | 2 | 12.3 |
| P08238 | Heat shock protein HSP 90-beta | 83.263 | 9 | 6 | 19.6 |
| P08758 | Annexin A5 | 35.936 | 2 | 2 | 6.2 |
| A0A0C4DG17 | 40S ribosomal protein SA | 33.313 | 2 | 2 | 10 |
| P09382 | Galectin-1 | 14.716 | 1 | 1 | 8.9 |
| P09651 | Heterogeneous nuclear ribonucleoprotein A1 | 38.746 | 1 | 1 | 12.4 |
| P0DME0 | Protein SETSIP | 34.882 | 1 | 1 | 3.3 |
| P16402 | Histone H1.3 | 22.35 | 1 | 1 | 5 |
| P11021 | 78 kDa Glucose-regulated protein | 72.332 | 4 | 4 | 12.7 |
| P13639 | Elongation factor 2 | 95.337 | 2 | 2 | 3.8 |
| P13667 | Protein disulfide-isomerase A4 | 72.932 | 1 | 1 | 2.2 |
| P13796 | Plastin-2 | 70.288 | 3 | 3 | 5.7 |
| P13929 | Beta-enolase | 46.986 | 1 | 1 | 3.2 |
| P14174 | Macrophage migration inhibitory factor | 12.476 | 1 | 1 | 9.6 |

(Continued)
Table 1. Continued.

| Source of proteins | Uniprot ID | Protein name | Theoretical MW [KDa] | Number of peptides | Number of unique peptides | Cover percent [%] |
|--------------------|------------|--------------|----------------------|--------------------|--------------------------|-------------------|
| Proteins from treated samples | P14625 | Endoplasm | 92.468 | 4 | 3 | 5.5 |
| | B4E1U9 | cDNA FLJ54776, highly similar to Cell division control protein 42 homolog | 26.528 | 1 | 1 | 4.7 |
| | J7M2B1 | Tyrosine-protein kinase receptor | 98.947 | 1 | 1 | 1.2 |
| | P15331 | Nucleoside diphosphate kinase A | 17.149 | 3 | 3 | 23 |
| | P17844 | Probable ATP-dependent RNA helicase DDX5 | 69.147 | 1 | 1 | 1.3 |
| | P17987 | T-complex protein 1 subunit alpha | 60.343 | 2 | 2 | 8.1 |
| | P18669 | Phosphoglycerate mutase 1 | 28.804 | 1 | 1 | 10.2 |
| | P19338 | Nucleolin | 76.613 | 1 | 1 | 5.1 |
| | B3KXY9 | cDNA FLJ46359 fis, clone TESTI4049786, highly similar to Hexokinase-1 (EC 2.7.1.1) | 106.25 | 1 | 1 | 1.2 |
| | P20700 | Lamin-B1 | 66.408 | 2 | 1 | 3.6 |
| | P22087 | rRNA 2-O-methyltransferase fibrillarin | 33.784 | 1 | 1 | 4 |
| | P22626 | Heterogeneous nuclear ribonucleoproteins A2/B1 | 37.429 | 3 | 3 | 10.5 |
| | P23246 | Splicing factor, proline- and glutamine-rich | 76.149 | 1 | 1 | 1.6 |
| | P23396 | 40S ribosomal protein S3 | 26.688 | 2 | 2 | 7.4 |
| | P23526 | Adenyllyhomocysteine | 47.716 | 1 | 1 | 2.8 |
| | E9PK25 | Cofilin-1 | 22.728 | 3 | 3 | 15.7 |
| | B4DRM3 | cDNA FLJ46492, highly similar to Eukaryotic translation initiation factor 4B | 69.725 | 1 | 1 | 1.8 |
| | P25398 | 40S ribosomal protein S12 | 67.877 | 1 | 1 | 1.4 |
| | P25705 | ATP synthase subunit alpha, mitochondrial | 76.149 | 1 | 1 | 1.6 |
| | P25788 | Proteasome subunit alpha type-3 | 28.429 | 1 | 1 | 3.9 |
| | P26641 | Elongation factor 1-gamma | 50.118 | 2 | 2 | 5.3 |
| | P27348 | 14-3-3 protein theta | 27.764 | 1 | 1 | 5.7 |
| | P27797 | Calreticulin | 48.141 | 2 | 2 | 4.8 |
| | Q53XS4 | Tyrosine-protein phosphatase non-receptor type | 67.178 | 1 | 1 | 1.5 |
| Proteins from treated samples | P29401 | Transketolase | 67.877 | 1 | 1 | 1.4 |
| | P29692 | Elongation factor 1-delta | 31.121 | 1 | 1 | 3.2 |
| | P30041 | Peroxiredoxin-6 | 25.035 | 1 | 1 | 5.4 |
| | P30086 | Phosphatidylethanolamine-binding protein 1 | 21.057 | 1 | 1 | 7.5 |
| | P30101 | Protein disulfide-isomerase A3 | 56.782 | 3 | 3 | 6.3 |
| | P34932 | Heat shock 70 kDa protein 4 | 94.33 | 2 | 1 | 3 |
| | P35268 | 60S ribosomal protein L22 | 14.877 | 1 | 1 | 10.2 |
| | Q13444 | Fus-like protein (Fragment) | 53.736 | 1 | 1 | 7.8 |
| | P36578 | 60S ribosomal protein L4 | 47.697 | 1 | 1 | 4.4 |
| | P37837 | Transaldolase | 37.54 | 1 | 1 | 3.9 |
| | P39023 | 60S ribosomal protein L3 | 46.108 | 1 | 1 | 3 |
| | P40227 | T-complex protein 1 subunit zeta | 58.024 | 1 | 1 | 2.1 |

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| Source of proteins | Uniprot ID | Protein name | Theoretical MW [KDa] | Number of peptides | Number of unique peptides | Cover percent [%] |
|-------------------|------------|--------------|----------------------|-------------------|--------------------------|------------------|
| P40926            | Malate dehydrogenase, mitochondrial | 35.503 | 2 | 2 | 7.4 |
| F6WQW2            | Ran-specific GTPase-activating protein | 31.904 | 2 | 2 | 7.2 |
| Q59CX9            | Ribosomal protein L5 variant (Fragment) | 35.203 | 1 | 1 | 3 |
| PS0990            | T-complex protein 1 subunit theta | 59.62 | 2 | 2 | 4 |
| PS0991            | T-complex protein 1 subunit delta | 57.924 | 1 | 1 | 7.2 |
| PS0993            | Sodium/potassium-transporting ATPase subunit alpha-2 | 112.26 | 1 | 1 | 1.2 |
| PS2597            | Heterogeneous nuclear ribonucleoprotein F | 45.671 | 1 | 1 | 6.5 |
| PS5072            | Transitional endoplasmic reticulum ATPase | 89.321 | 2 | 2 | 2.2 |
| F8YVS7            | Caspase | 46.452 | 1 | 1 | 2.4 |
| PS7721            | Poly(rC)-binding protein 3 | 39.465 | 1 | 1 | 3 |
| P60174            | Triosephosphate isomerase | 30.791 | 1 | 1 | 4.5 |
| B7Z6Z4            | Myosin light polypeptide 6 | 26.707 | 1 | 1 | 3.4 |
| P606842           | Eukaryotic initiation factor 4A-1 | 46.153 | 1 | 1 | 14.8 |
| P61155            | Actin-related protein 3 | 47.371 | 1 | 1 | 2.9 |
| P61160            | Actin-related protein 2 | 44.76 | 1 | 1 | 3 |
| P61247            | 40S ribosomal protein S3a | 29.945 | 1 | 1 | 11.4 |
| P61313            | 60S ribosomal protein L15 | 24.146 | 1 | 1 | 4.4 |
| K7ELC7            | 60S ribosomal protein L27 (Fragment) | 16.359 | 2 | 2 | 13.2 |
| Q5SEC54           | Heterogeneous nuclear ribonucleoprotein K transcript variant | 51.058 | 3 | 3 | 7.1 |
| P61981            | 14-3-3 protein gamma | 28.302 | 1 | 1 | 4.5 |
| P62241            | 40S ribosomal protein S8 | 24.205 | 1 | 1 | 3.4 |
| Q6IPX4            | 40S ribosomal protein S16 | 17.107 | 1 | 1 | 6.6 |
| G9K388            | YWHAEF/FAM22A fusion protein (Fragment) | 41.224 | 1 | 1 | 2.9 |
| P62269            | 405 ribosomal protein S18 | 17.718 | 1 | 1 | 7.2 |
| P62277            | 405 ribosomal protein S13 | 17.222 | 2 | 2 | 12.6 |
| P62280            | 405 ribosomal protein S11 | 18.431 | 1 | 1 | 7 |
| P62701            | 405 ribosomal protein S4, X isoform | 29.597 | 2 | 2 | 8.4 |
| J3KQES3           | CTP-binding nuclear protein Ran (Fragment) | 26.816 | 1 | 1 | 3.8 |
| P62906            | 60S ribosomal protein L10a | 24.831 | 1 | 1 | 6 |
| P62906            | Peptidylprolyl isomerase | 14.926 | 1 | 1 | 10.1 |
| I3L504            | Eukaryotic translation initiation factor 5A-1 | 20.303 | 1 | 1 | 18.8 |
| P63244            | Receptor of activated protein C kinase 1 | 35.076 | 3 | 3 | 10.4 |
| P63261            | Actin, cytoplasmic 2 | 41.792 | 14 | 1 | 48.3 |
| P63261            | tubulin beta-4B chain | 49.83 | 3 | 2 | 8.8 |
| K7E500            | Histone H3.3 (Fragment) | 16.621 | 2 | 2 | 10.6 |

(Continued)
Table 1. Continued.

| Source of proteins | Uniprot ID     | Protein name                              | Theoretical MW [KDa] | Number of peptides | Number of unique peptides | Cover percent [%] |
|--------------------|----------------|-------------------------------------------|----------------------|---------------------|--------------------------|-------------------|
| P84103             | Serine/arginine-rich splicing factor 3 | 19.329                                    | 1                    | 1                   | 5.5                     |
| A0A087VVQ6         | Clathrin heavy chain               | 192.06                                    | 1                    | 1                   | 0.7                     |
| Q00839             | Heterogeneous nuclear ribonucleoprotein U | 90.583                                    | 1                    | 1                   | 1                       |
| Q01130             | Serine/arginine-rich splicing factor 2 | 25.476                                    | 1                    | 1                   | 7.7                     |
| Q01518             | Adenylyl cyclase-associated protein 1 | 51.901                                    | 1                    | 1                   | 1.5                     |
| Q02543             | 60S ribosomal protein L18a          | 20.762                                    | 1                    | 1                   | 7.4                     |
| Q02790             | Peptidyl-prolyl cis-trans isomerase FKBP4 | 51.804                                    | 1                    | 1                   | 2.8                     |
| Q0HBB3             | 60S ribosomal protein L6            | 32.891                                    | 1                    | 1                   | 3.1                     |
| Q04917             | 14-3-3 Protein eta                  | 28.218                                    | 1                    | 1                   | 4.1                     |
| Q0P6D2             | Protein FAM69C                      | 46.42                                     | 1                    | 1                   | 3.6                     |
| Q13162             | Peroxiredoxin-4                    | 30.54                                     | 2                    | 1                   | 7                       |
| Q14103             | Heterogeneous nuclear ribonucleoprotein D0 | 38.434                                    | 1                    | 1                   | 2.8                     |
| Q15084             | Protein disulfide-isomerase A6      | 48.121                                    | 2                    | 2                   | 6.4                     |
| Q8IYT4             | Katanin p60 ATPase-containing subunit A-like 2 | 61.252                                    | 1                    | 1                   | 2.2                     |
| B4DFR2             | cDNA FLJ59194, moderately similar to Dynein light chain 2A, cytoplasmic | 13.362                                    | 1                    | 1                   | 7.4                     |
| Q93045             | Statmin-2                          | 20.828                                    | 1                    | 1                   | 5.6                     |
| Q9BT56             | Spexin                             | 13.302                                    | 1                    | 1                   | 7.8                     |
| Q9BTT0             | Acidic leucine-rich nuclear phosphoprotein 32 family member E | 30.692                                    | 1                    | 1                   | 10.8                    |
| Proteins from treated samples | Q9P258 | Protein RCC2 | 56.084 | 1 | 1 | 3.3 |
| Q9UHV9             | Prefoldin subunit 2                | 16.648                                    | 1                    | 1                   | 9.1                     |
| A0A05Z2AZG4        | Tropomyosin 3 isoform 1 (Fragment) | 29.032                                    | 2                    | 2                   | 6                       |
| A0A0GC2J7A7        | Proteasome subunit beta type       | 20.941                                    | 1                    | 1                   | 8.2                     |
| B4DD86             | Heterogeneous nuclear ribonucleoprotein A3, isoform CRA_a | 37.029                                    | 2                    | 2                   | 6.5                     |
| D6RDG3             | Transcription factor BTF3 (Fragment) | 11.802                                    | 1                    | 1                   | 11.9                    |
| Q71V99             | Peptidyl-prolyl cis-trans isomerase | 17.971                                    | 4                    | 1                   | 28.7                    |
| Q9BZT5             | PNAS-26                            | 13.934                                    | 1                    | 1                   | 13                      |
| B2KLP9             | NADH-ubiquinone oxidoreductase chain 1 | 35.662                                    | 1                    | 1                   | 7.9                     |
| Proteins from control samples | A6NKL6 | Transmembrane protein 200C | 63.927 | 1 | 1 | 1.3 |
| A0A0T5B6H0         | Receptor-type tyrosine-protein phosphatase T | 163.97 | 1 | 1 | 1.6 |
| O75170             | Serine/threonine-protein phosphatase 6 regulatory subunit 2 | 104.94 | 1 | 1 | 0.7 |
| P06702             | Protein S100-A9                    | 13.242                                    | 1                    | 1                   | 11.4                    |

(Continued)
Table 1. Continued.

| Source of proteins | Uniprot ID | Protein name                        | Theoretical MW [KDa] | Number of peptides | Number of unique peptides | Cover percent [%] |
|-------------------|-----------|-------------------------------------|----------------------|--------------------|--------------------------|------------------|
|                   | P15924    | Desmoplakin                         | 331.77               | 3                  | 3                        | 1.4              |
|                   | P17066    | Heat shock 70 kDa protein 6         | 71.027               | 1                  | 1                        | 4.5              |
|                   | P29508    | Serpin B3                           | 44.364               | 1                  | 1                        | 2.6              |
|                   | P32969    | 60S ribosomal protein L9            | 21.863               | 1                  | 1                        | 14.1             |
|                   | Q8N1C8    | HSPA9 protein (Fragment)            | 73.853               | 2                  | 2                        | 7.5              |
|                   | Q01469    | Fatty acid-binding protein, epidermal | 15.164               | 1                  | 1                        | 13.3             |
|                   | F4ZW66    | NFI10b                              | 95.777               | 1                  | 1                        | 1.3              |
|                   | Q5T749    | Keratinocyte proline-rich protein   | 64.135               | 2                  | 2                        | 4.7              |
| Proteins from control samples | Q8N4B1    | Sesquipedalian-1                   | 27.215               | 1                  | 1                        | 4.0              |
|                   | Q8WTW4    | Nitrogen permease regulator 2-like protein | 43.658               | 1                  | 1                        | 3.4              |
|                   | Q9H165    | B-cell lymphoma/leukemia 11A        | 91.196               | 1                  | 1                        | 1.9              |
|                   | Q9HCR9    | Dual 3,5-cyclic-AMP and -GMP phosphodiesterase 11A | 104.75              | 1                  | 1                        | 2.6              |
|                   | B4DZQ0    | cDNA FLJ59289, highly similar to Retinoblastoma-binding protein 6 (Fragment) | 113.46 | 1 | 1 | 1.7 |
|                   | H7C2W5    | NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10, mitochondrial (Fragment) | 15.09 | 1 | 1 | 6.2 |
|                   | Q5T6K5    | Nuclear transcription factor Y subunit gamma (Fragment) | 34.212 | 1 | 1 | 3.5 |
| Common proteins from the control sample and treated sample | B4DR52    | Histone H2B                         | 18.041               | 2                  | 2                        | 24.7             |
|                   | J3KPS3    | Fructose-bisphosphate aldolase      | 39.817               | 1                  | 1                        | 10.9             |
|                   | P04908    | Histone H2A type 1-B/E              | 14.135               | 1                  | 1                        | 15.4             |
|                   | P10599    | Thioredoxin                         | 11.737               | 1                  | 1                        | 8.6              |
|                   | P10809    | 60 kDa Heat shock protein, mitochondrial | 61.054 | 1 | 1 | 12.7 |
|                   | P11142    | Heat shock cognate 71 kDa protein   | 70.897               | 3                  | 3                        | 23.5             |
|                   | P14618    | Pyruvate kinase PKM                 | 57.936               | 1                  | 1                        | 17.3             |
|                   | H01Y7A    | Calmodulin (Fragment)               | 20.762               | 1                  | 1                        | 15.5             |
|                   | P62805    | Histone H4                          | 11.367               | 2                  | 2                        | 31.1             |
|                   | P68104    | Elongation factor 1-alpha 1         | 50.14                 | 2                  | 2                        | 10.2             |
|                   | Q06830    | Peroxiredoxin-1                    | 22.11                 | 1                  | 1                        | 32.2             |
|                   | Q96SB8    | Structural maintenance of chromosomes protein 6 | 126.32                  | 1 | 1 | 0.6 |

categories (Figure 3A–C). KEGG pathway enrichment analysis revealed that the 14-3-3σ-interacting proteins were related to 149 pathways. The 20 highly enriched pathways are shown in Figure 3D. The five most significantly enriched were ribosome, carbon metabolism, biosynthesis of amino acids, PI3K–AKT signaling pathway, and protein processing in ER.

3.4. Hsp74 Mediates the Centrosome Amplification

From the proteins pulled down using 14-3-3σ antibody, we were interested in Hsp74. Why we targeted at Hsp74? The protein came to our attention, as several Hsp proteins are present in centrosome, which suggests that Hsp proteins may play roles in centrosome homeostasis. Moreover, Hsp proteins are known binding partners of 14-3-3 proteins. Thus, we investigated whether Hsp74 contributed to the centrosome amplification. Indeed, we found that the expression level of Hsp74 was increased by high glucose, insulin, and palmitic acid, which was inhibited by Hsp74 specific siRNA (Figure 4A). Knockdown of Hsp74 using their siRNA downregulated the treatment-induced centrosome amplification (Figure 4B).
3.5 14-3-3σ-Hsp74 Complex is Required for the Centrosome Amplification

We next performed experiments to confirm the binding between 14-3-3σ and Hsp74, and to examine whether 14-3-3σ and Hsp74 complex was required for the centrosome amplification. As expected, Hsp74 was pulled down by 14-3-3σ antibody (Figure 5A). Importantly, the binding between Hsp74 and 14-3-3σ was increased by high glucose, insulin, and palmitic acid (Figure 5A). If the Hsp74/14-3-3σ complex mediated the treatment-induced centrosome amplification, inhibition or disruption of the complex would inhibit the centrosome amplification. siRNA technology was used to inhibit or disrupt the protein complex via protein knockdown of Hsp74 or 14-3-3σ. Indeed, individual knockdown of Hsp74 or 14-3-3σ protein level (Figure 5B) attenuated the treatment-induced centrosome amplification (Figures 4A, B and 5C). In addition, when Hsp74 was knocked down using siRNA, although Hsp74 was pulled down by 14-3-3σ antibody, the level was very low (Figure 5D). These data proved that the complex was reduced using siRNA of Hsp74.

3.6. Molecular Docking between 14-3-3σ and Hsp74

The interaction between the 14-3-3σ (green) and the Hsp74 (rose red) is shown in Figure 6A. Detailed analysis (Figure 6B) showed that one hydrophobic interaction was observed between the residues Leu-174, Val-178, Leu-218, Leu-222, Leu-223, and Leu-229 of the 14-3-3σ and the residues Phe-440, Tyr-445, and Tyr-446 of the Hsp74. Another hydrophobic interaction was observed between the residues Tyr-19, Val-51, Gly-54, Ala-57, Ala-58, Val-61, and Val-88 of the 14-3-3σ and the residues Leu-452, Pro-453, Tyr-454, Pro-457, Ala-458, Ile-459, Ala-460, and Phe-462 of the Hsp74, forming a strong hydrophobic binding. In addition, the residue Asp-225 of the 14-3-3σ formed anion–π interactions with the side chain of the residues Phe-440 and Tyr-446 of Hsp74.
the Hsp74. Moreover, the anion–π interaction was observed between the residue Glu-182 of the 14-3-3σ and the side chain of the residue Tyr-445 of the Hsp74. The residues Arg-56 and Arg-60 of the 14-3-3σ formed cation–π interactions with the residue Tyr-445 of the Hsp74. Importantly, six hydrogen bond interactions were shown between the residue Glu-182 of the 14-3-3σ and the Tyr-445 of the Hsp74 (bond length: 2.3 Å), the residue Asp-225 of the 14-3-3σ and the Ser-447 of the Hsp74 (bond length: 2.9 Å), the residue Arg-60 of the 14-3-3σ and the Asp-451 of the Hsp74 (bond length: 3.5 Å), the residue Tyr-19 of the 14-3-3σ and the Asp-456 of the Hsp74 (bond length: 3.5 Å), the residue Val-88 of the 14-3-3σ and the Gln-461 of the Hsp74 (bond length: 3.5 Å), and the Arg-18 of the 14-3-3σ and the Glu-499 of the Hsp74 (bond length: 3.4 Å). These were the main binding affinity between the 14-3-3σ and Hsp74.

4. Discussion

In the present study, we showed that high glucose, insulin, and palmitic acid increased the protein level of Hsp74, which was inhibited by Hsp74 specific siRNA; B) knockdown of Hsp74 downregulated the centrosome amplification. Glu: glucose, 15 mm; Ins: insulin, 5 nm; Pal: palmitic acid, 150 μM. **p < 0.01, compared with that in the control group; ##p < 0.01, compared with that in the treatment group with siRNA.

Figure 4. Hsp74 mediates the centrosome amplification. A) High glucose, insulin, and palmitic acid increased the protein level of Hsp74, which was inhibited by Hsp74 specific siRNA; B) knockdown of Hsp74 downregulated the centrosome amplification. Glu: glucose, 15 mm; Ins: insulin, 5 nm; Pal: palmitic acid, 150 μM. **p < 0.01, compared with that in the control group; ##p < 0.01, compared with that in the treatment group with siRNA.
Figure 5. 14-3-3-σ-Hsp74 complex is required for the centrosome amplification. Total cell lysates were used to validate the interaction between 14-3-3-σ and Hsp74 by CoIP. A) CoIP of Hsp74 and 14-3-3-σ. The level of Hsp74 in cell lysate was used as negative control. The Hsp74 protein was immunoprecipitated with anti-14-3-3-σ antibody, and the presence of Hsp74 protein was detected by immunoblot analysis with anti-Hsp74 antibody. B) High glucose, insulin, and palmitic acid increased protein level of 14-3-3-σ, which is inhibited by 14-3-3-σ siRNA. C) Knockdown of 14-3-3-σ downregulated the centrosome amplification. D) Knockdown of 14-3-3-σ or Hsp74 disrupted the 14-3-3-σ/Hsp74 complex. Glu: glucose, 15 mm; Ins: insulin, 5 nm; Pal: palmitic acid, 150 μm. **p < 0.01, compared with that in the control group; ##p < 0.01, compared with that in the samples treated with Glu, Ins, and Pal.

In conclusion, high glucose, insulin, and palmitic acid promote centrosome amplification by increasing expressions of 14-3-3-σ and Hsp74 in HCT116 cells. The results from CoIP assay, proteomic analysis, and functional studies show the experimental treatment increase the formation of 14-3-3-σ/Hsp74 complex that mediates the centrosome amplification.

Conflict of Interest
The authors declare no conflict of interest.

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Figure 6. Homology modeling and molecular docking. A) Total view of the interaction between the 14-3-3-σ and Hsp74. B) Detailed view of the interaction between the 14-3-3-σ and Hsp74. Green, 14-3-3-σ; rose red, Hsp74.

Keywords
14-3-3-σ, Hsp74, centrosome amplification, high glucose, insulin, palmitic acid

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