Neuromol Med (2017) 19:81–93
DOI 10.1007/s12017-016-8431-9

ORIGINAL PAPER

Identification of Novel SCIRR69-Interacting Proteins During ER Stress Using SILAC-Immunoprecipitation Quantitative Proteomics Approach

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Received: 15 April 2016 / Accepted: 29 July 2016 / Published online: 3 August 2016

Abstract Spinal cord injury and regeneration-related protein #69 (SCIRR69),also known as cAMP-responsive element-binding protein 3-like 2, belongs to the CREB/ATF family, some members of which play significant roles in ER stress. However, it is still not fully elucidated whether SCIRR69 involves in ER stress and its biochemical and functional roles during ER stress. In this study, we firstly treated fetal rat spinal cord neuron cells (SCN) and PC12 cells with ER stress activator thapsigargin (TG) or tunicamycin (TM) and then detected the expression pattern of SCIRR69 in response to ER stress at mRNA and protein levels using real-time PCR assay and immunoblotting. Results showed that the expression pattern of SCIRR69 was largely consistent with those of ER stress marker (ATF6, BIP and CHOP) at either mRNA level or protein level, implying that SCIRR69 may play important roles in ER stress. Subsequently, we used stable isotope labeling by amino acids in cell culture (SILAC)-immunoprecipitation quantitative proteomics to identify interaction partners of SCIRR69 during TG-induced ER stress in PC12 cells and found that transitional endoplasmic reticulum ATPase (TERA) and sideroflexin-1 (SFXN1) were potential SCIRR69-interacting proteins. The interaction between SCIRR69 and TERA or SFXN1 was validated using co-immunoprecipitation. Those results provide some clues for novel signaling nexuses that made by interactions between SCIRR69 and TERA or SFXN1. Our findings may facilitate a better understanding of the fundamental functions of SCIRR69 during ER stress.

Keywords Quantitative proteomics · SILAC · Protein interaction · SCIRR69 · ER stress

Introduction

Endoplasmic reticulum (ER) is the major site in cell for lipid synthesis, protein folding, and assembly, as well as cellular Ca^{2+} storage. Given its central roles in protein folding and its influence on Ca^{2+}-mediated signaling pathways, disruption of the ER homeostasis, also called ER stress, can cause severe damage to the cell (Engin and Hotamisligil 2010, Wang and Kaufman 2012). ER stress is characterized by increases in a number of transcription factors such as activating transcription factor 6 (ATF6), immunoglobulin heavy chain-binding protein/glucose-regulated protein 78 (BIP/GRP78), CCAAT/enhancer-binding protein homologous protein (CHOP), X-box-binding protein 1 (XBP1) and phosphorylation of protein kinase-like ER kinase (PERK), leading to translational attenuation and cell cycle arrest(Walter and Ron 2011).

Spinal cord injury and regeneration-related protein #69 (SCIRR69), also known as cAMP-responsive element-binding protein 3-like 2 (CREB3L2), belongs to the CREB/

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Electronic supplementary material The online version of this article (doi:10.1007/s12017-016-8431-9) contains supplementary material, which is available to authorized users.

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ATF family, which family also includes ATF6, old astrocyte specifically induced substance (OASIS), and cyclic AMP response element-binding protein H (CREB-H) (Ma et al. 2012; Liu et al. 2013). Each of these molecules contains a basic region for DNA binding, a leucine zipper motif for dimerization, and one or more transmembrane domains for membrane anchoring to the ER or Golgi (Ribeiro et al. 1994, Omori et al. 2001, Shen and Prywes 2005, Kehat et al. 2006). Our previous research indicated that following mechanical injury to neurons, SCIRR69 (81kD, p81-SCIRR69) and N-glycosylated SCIRR69 (83kD, p83-SCIRR69) could be proteolytically cleaved by site-1 (S1P) and site-2 (S2P) proteases into two fragments (60kD, p60-SCIRR69; 63kD, p63-SCIRR69). The p60-SCIRR69 is translocated to the nucleus where it binds to brain-derived neurotrophic factor (*BDNF*) gene promoter II (Liu et al. 2013). In response to ER stress, both ATF6 and OASIS are cleaved in a two-step process by S1P and S2P. The released N-terminus translocates to the nucleus and activates ER chaperone genes, such as BiP/GRP78 and glucose-regulated protein 94 (GRP94), through the ER stress response element and cAMP response element (Yoshida et al. 1998; Roy and Lee 1999, Yoshida et al. 2000; Murakami et al. 2006). However, it is not fully elucidated that whether SCIRR69, just like ATF6 and OASIS, involves in ER stress, and what biochemical and functional roles SCIRR69 plays in ER stress.

An essential step in understanding protein function is identification of relevant interacting proteins. There are a number of techniques available to screen unknown interacting proteins, including the yeast two-hybrid system, pull-down assays using recombinant protein, as well as tandem affinity purification or TAP tagging (Williamson and Sutcliffe 2010). However, these techniques suffer from high false positive and false negative rates, because the assay is usually performed under non-physiological conditions, and the posttranslational dynamics are not taken into account. Recently, stable isotope labeling by amino acids in cell culture (SILAC)-immunoprecipitation quantitative proteomics provides us a useful tool to tackle the obstacles mentioned above(Trinkle-Mulcahy 2012). In this method, cells containing an affinity tagged protein are grown in heavy isotopic medium, while wild-type cells are grown in light isotopic medium. After mixing equal quantities of these two populations of cells, an immunoprecipitation is performed against the affinity tag. In mass spectrum (MS), specific partners appear as isotopically heavy, while non-specific interaction partners appear as a mixture of isotopically light and heavy at a 1:1 ratio. Compared with the yeast two-hybrid approach and traditional TAP tagging, SILAC-immunoprecipitation quantitative proteomics has some significant advantages. For example, cell localization and posttranslational modifications are not perturbed using this method, and it is a quantitative rather than a qualitative approach allowing the user to readily distinguish non-specifically interacting proteins and contaminants (Emmott et al. 2013).

In this study, we firstly explored whether SCIRR69 involves in ER stress. Then, we identified the SCIRR69-interacting partners during ER stress in PC12 cells using SILAC-immunoprecipitation quantitative proteomics. These studies will facilitate a better understanding of the fundamental aspects of SCIRR69 during ER stress.

Materials and Methods

Plasmid Construction

The rat cDNA sequence encoding full-length *Scirr69* was amplified using following primers (sense: 5′-AAAG-GATCCATGGAGGTGCTGGAGAGCG-3′; antisense: 5′-AAACTCGAGTCAGAAGGTGGCGTTCACTC-3′) and then subcloned into the BamH I and Xho I sites of pCMV-tag-2B vector (Agilent Technologies, USA), which is modified to contain an N-terminal Flag tag.

Primary Cultivation of Fetal Rat Spinal Cord Neuron Cells

Spinal cord tissues isolated from 16 days old Wistar rat embryos were sheared into scraps after removal of meninges. The scraps were digested with 0.25 % trypsin (Sigma-Aldrich, USA) at 37 °C for 30 min and then washed twice with Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, USA) containing 10 % horse serum, 10 % fetal bovine serum (FBS) (Invitrogen, USA), and 2 mM glutamine (Sigma-Aldrich, USA). Following triturating with pipet, the trypsinized tissue scraps were dispersed into single cells. The cells were then seeded on polylysine-coated dish (35 mm × 10 mm) at a density of 5×10^5 cells/dish and cultivated in culturing medium mentioned above. After 24 h, the cells were cultured with medium without 10 % FBS for 3 days and then performed purified cultivation by adding of 1.5 μg/ml cytarabine (Upjohn, USA) for another 7 days until neural processes grown from the cultured primary neurons formed a dense network. Cells were stimulated with TG (1 μg/ml) (Sigma-Aldrich, USA), or TM (3 μg/ml) (Sigma-Aldrich, USA) for 0, 4, 8, 12 and 24 h, respectively. TG and TM are both ER stress activators.

Cell Culture and Stable Transfection

PC12 cells were cultured in DMEM (Invitrogen, USA) supplemented with 10 % horse serum and 5 % FBS

(Invitrogen, USA), 2 mM glutamine, 1 % penicillin/streptomycin (Sigma-Aldrich, USA) under 5 % CO_2 atmosphere at 37 °C. The pCMV-tag-2B-SCIRR69 and empty vector pCMV-tag-2B plasmids were transfected, respectively, into the PC12 cells by Lipofectamine 2000 (Invitrogen, USA). At 48 h after transfection, the media was replaced with G418-containing media (800 μg/ml). Individual colonies were picked following 2 weeks of selection. Transfection efficiency was confirmed by measuring the expression of SCIRR69 with immunoblotting assay. After initial selection, stably transfected cells were maintained in the media with 200 μg/ml G418.

Real-Time PCR

The expression levels of *Scirr69* gene and ER stress marker genes (*Atf6*, *Bip* and *Chop*) were detected by SYBR Green I assay using StepOnePlus Real-Time PCR Systems (Applied Biosystems, USA). Gene-specific primers were designed using Beacon Designer 7.91 software (Premier, Canada). The reactions contained 1 × SYBR Green Real-time PCR Master Mix-Plus (Toyobo, Japan), 1 μl diluted cDNA template and each primer at 400 nM in a 20 μl reaction volume. After an initial denaturation step at 95 °C for 10 min amplification was performed with 40 cycles of 95 °C for 15 s, 60 °C for 1 min; melting curve from 60 to 95 °C, read every 0.3 °C, hold for 1 min. For each sample, reactions were setup in triplicate to ensure the reproducibility of the results, and no-template controls were set for each gene. At the end of the PCR run, melting curves were generated and analyzed to confirm non-specific amplification, and then, the mean value of each triplicate was used for further calculations. Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) gene was used as the reference gene or internal control.

Stable Isotope Labeling

The PC12 cells stably transfected pCMV-tag-2B-*Scirr69* plasmid were grown in SILAC DMEM "heavy" media (Thermo, USA) without lysine and arginine, supplemented with 10 % dialyzed fetal calf serum (Thermo, USA), 1 % penicillin/streptomycin (Sigma-Aldrich, USA), 100 μg/ml L-arginine-HCl and 100 μg/ml $^{13}C_6$-L-lysine-HCl (both from Thermo, USA). The PC12 cells stably transfected empty vector pCMV-tag-2B plasmid were grown in SILAC DMEM "light" media (Thermo, USA) without lysine and arginine, supplemented with 10 % dialyzed fetal calf serum, 1 % penicillin/streptomycin, 100 μg/ml L-arginine-HCl and 100 μg/ml $^{12}C_6$-L-lysine-2HCl (Thermo, USA). This two cell populations were grown in corresponding culture medium for at least five cell divisions by changing medium every 2 or 3 days. After at least five cell divisions, cells were harvest and the incorporation efficiencies were detected, and then were stimulated with TG (1 μg/ml) for 12 h.

Immunoprecipitation

The two group cells (light and heavy) were harvested and lysed, respectively, in IP Lysis Buffer (Thermo, USA) supplemented with protease and phosphatase inhibitors (Roche, Switzerland). After determining protein concentration of each sample in triplicate using BCA Protein Assay Kit (Thermo, USA), equal protein amounts of each cell lysate were mix. The equally mixed sample (2 mg) was incubated with 10 μg rabbit anti-Flag polyclonal antibody (MBL, USA) in 1 ml IP Lysis Buffer for 8 h at 4 °C, and the immune complexes were precipitated with 20 μl protein A/G Plus agarose (Santa Cruz Biotechnology, USA). The immunoprecipitates were then separated by 12 % SDS–polyacrylamide gel electrophoresis. After the gel was stained with Coomassie Brilliant Blue G-250, each lane was cut into 10 gel slices (0.5 cm × 0.5 cm) for LTQ-MS analysis.

Liquid Chromatography Tandem Mass Spectrometry

Each slice of the Coomassie G-250-stained gel was in-gel digested using 0.1 μg of trypsin in 25 μl of 50 mM ammonium bicarbonate, pH 7.8. The samples were loaded on a C18 trap column (C18 PepMap, 300 μm ID × 5 mm, 5 μm particle size, 300 Å pore size; Dionex, Amsterdam, The Netherlands) and eluted onto a SCX capillary column (100 mm × 320 μm id). The ammonia acetate concentration of elution steps for SCX capillary column was 0 mM, 25 mM, 75 mM, 100 mM and 1 M. The eluted peptides were then transferred onto a RP capillary column (PepMap C18, 75 μm ID × 150 mm, 3 μm particle and 100 Å pore size; Dionex, Amsterdam, The Netherlands). The elution gradient for RP column was from 5 to 30 % buffer B (0.1 % formic acid, 99.9 % acetonitrile) over 3 h at a flow rate of 2 μl/minute. MS data were acquired in a survey scan from 400–2000 amu (1 μscans) followed by ten data-dependent MS/MS scans (10 μscans each, isolation width 3 amu, 35 % normalized collision energy, dynamic exclusion for 1.5 min) on an LTQ XL electrospray ion trap mass spectrometer (Thermo, USA).

Database Searching

Tandem mass spectra were extracted, charge state deconvoluted and deisotoped by Mascot Distiller version 2.4.3.3. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.4.1). Mascot was set up to search the SwissProt_2013_05 database (selected for Rattus, 7867 entries) assuming the digestion enzyme trypsin. Mascot was searched with a fragment ion mass tolerance of 0.80 Da and a parent ion tolerance of 10.0 PPM. Carbamidomethyl of cysteine was specified in Mascot as fixed modifications. Deamidated of asparagine and glutamine, label: ^{13}C (6) of lysine, oxidation of methionine, and acetyl of the N-terminus were specified in Mascot as variable modifications.

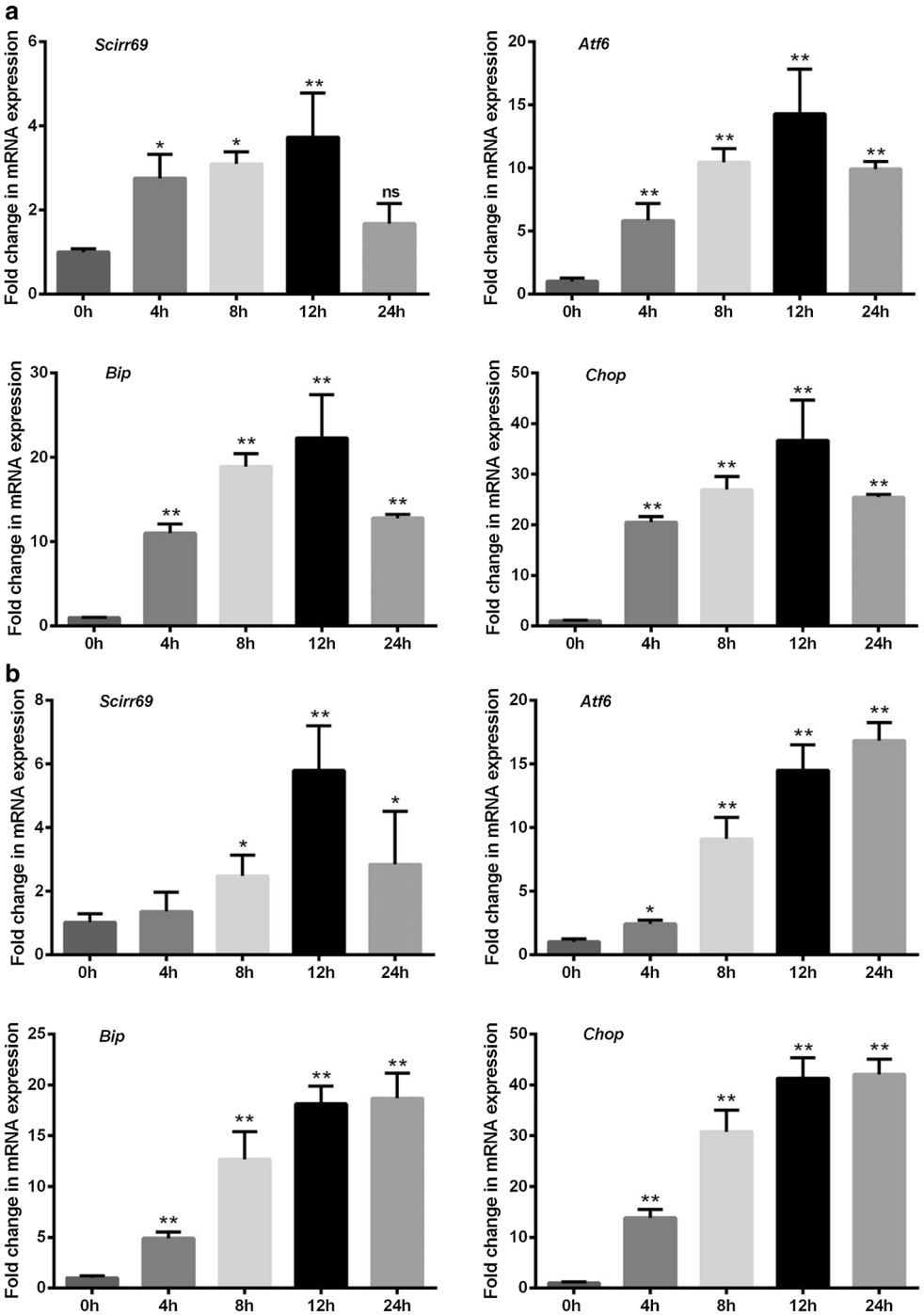


Fig. 1 Comparison of expression pattern of *Scirr69* gene with the ER stress marker genes during ER stress. SCN cells were treated with TG (1 μg/ml) **a** or TM (3 μg/ml) **b** for different time course (0, 4, 8, 12, 24 h), and then the expression patterns of *Scirr69* gene and the ER stress marker genes (*Atf6*, *Bip* and *Chop*) were detected using real-time PCR assay. PC12 cells were treated with TG (1 μg/ml) **c** or TM (3 μg/ml) **d** for different time course (0, 4, 8, 12, 24 h), and then the expression patterns of *Scirr69* gene, and the ER stress marker genes (*Atf6*, *Bip* and *Chop*) were detected using real-time PCR assay. *Y-axis* represents the fold change in transcript levels in treated cells compared with untreated cells (0 h). Results represent the mean ± SEM from $n = 3$ distinct experiments. *h* hours; $^{*}P < 0.05$, $^{**}P < 0.01$ versus untreated cells (0 h); *ns* not significant

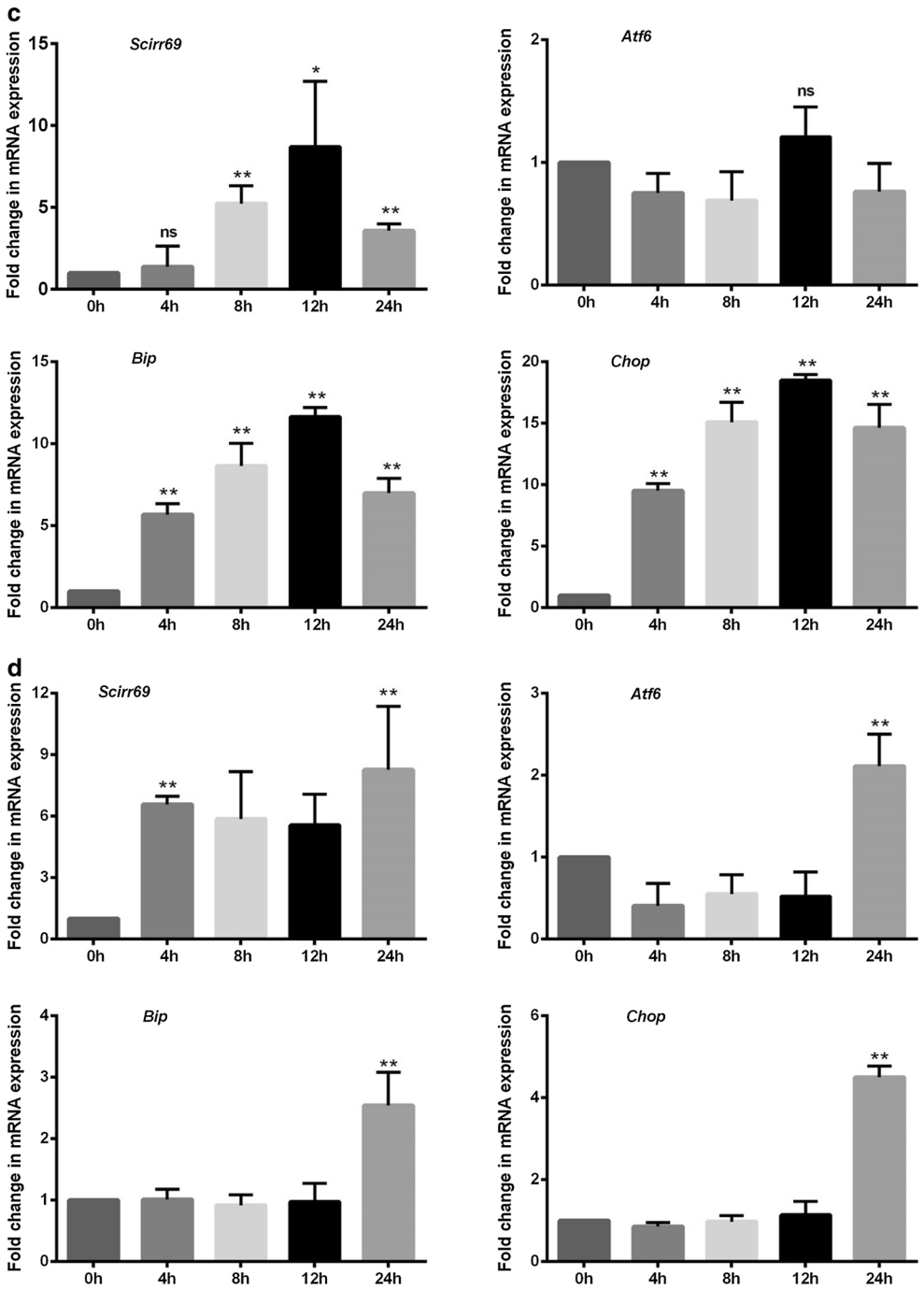


Fig. 1 continued

Criteria for Protein Identification

Scaffold (version Scaffold_4.0.5, Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0 % probability by the Peptide Prophet algorithm with Scaffold delta-mass correction(Keller et al. 2002). Protein identifications were accepted if they could be established at greater than 99.9 % probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al. 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were

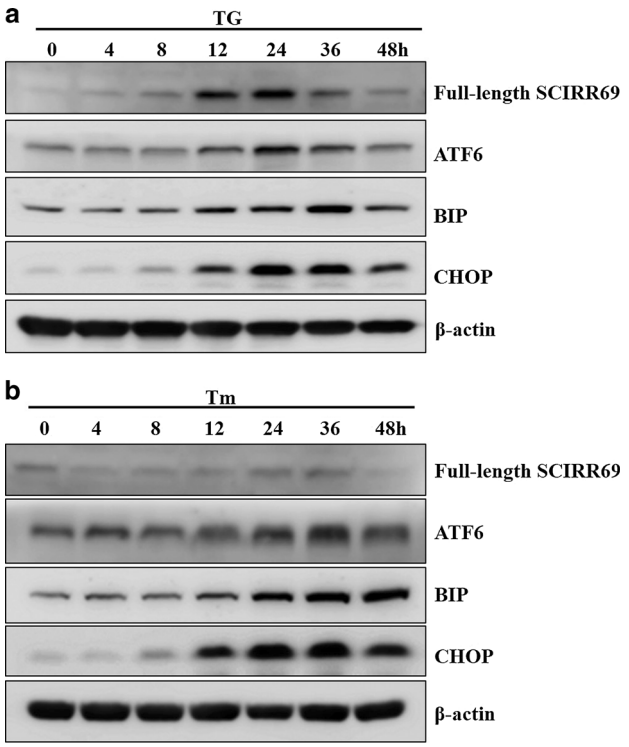


Fig. 2 Expression patterns of SCIRR69 protein and the ER stress proteins (ATF, BIP, CHOP) in response to ER stress. PC12 cells were treated with TG (1 μg/ml) **a** or TM (3 μg/ml) **b** for different time course (0, 4, 8, 12, 24 h), and then the expression patterns of SCIRR69 protein and the ER stress proteins (ATF6, BIP and CHOP) were detected by using immunoblotting. *h*, hours

grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters.

Immunoblotting

The immunoprecipitates or cells extract proteins were separated by 12 % SDS–polyacrylamide gel electrophoresis and blotted onto nitrocellulose membrane (Osmonics, USA) using a semidry blotting apparatus (Bio-Rad, USA). The membranes were first blocked with 5 % skimmed milk in washing buffer (20 mM Tris, 150 mM NaCl, 0.05 % Tween-20, pH 7.6) overnight at 4 °C and incubated with primary antibodies and then with appropriate horseradish peroxidase-labeled secondary antibodies (Santa Cruz Biotechnology, USA) after few washes. The blots were visualized using an enhanced chemiluminescence immunoblotting detection kit (Vigorous, China). Anti-SCIRR69 antibody is prepared in our laboratory; anti-His, anti-GFP, anti-ATF6, anti-BIP, and anti-CHOP antibodies are purchased from Proteintech (USA); anti-β-actin are from Cell Signaling Technology (USA).

Results

The Expression Patterns of SCIRR69 at mRNA and Protein Levels During ER Stress

To explore the expression change of *Scirr69* gene in cells in response to ER stress, we treated SCN cells and PC12 cells with TG or TM for different time course (0, 4, 8, 12, 24 h). TG and TM are both ER stress activators. TG is an inhibitor of ER Ca^{2+}-ATPase, while TM is an *N*-glycosylation inhibitor. Then, the expression pattern of *Scirr69* gene was detected using real-time PCR assay. At the same time, the expression changes in ER stress marker (*Atf6*, *Bip* and *Chop*) were also detected. The results revealed that the mRNA expression of *Scirr69* was gradually up-regulated and reached the highest expression levels at 12 h in TG-treated (Fig. 1a) and TM-treated SCN cells (Fig. 1b). Such expression pattern of *Scirr69* in response to ER stress was very similar to those of the ER stress marker genes (*Atf6*, *Bip* and *Chop*) (Fig. 1a, b). A similar result was collected in TG-treated PC12 cells (Fig. 1c). The mRNA expression pattern of *Scirr69* in TM-treated PC12 cells is different from that in TM-treated SCN cells, but was still similar to those of the ER stress marker genes (*Atf6*, *Bip* and *Chop*) (Fig. 1d). We think that PC12 cells are possibly less sensitive to TM (3 μg/ml) than SCN cells, so the mRNA expressions of *Scirr69* and the ER stress marker genes changed until 24 h after TM treatment in PC12 cells.

Meanwhile, immunoblotting was carried out to detect the expression patterns of SCIRR69 protein and the ER stress proteins (ATF, BIP, CHOP) in PC12 in response to ER stress (Fig. 2). Results showed that the expression of SCIRR69 was gradually up-regulated, reached the highest expression level at 24 h, and then decreased in TG-treated PC12 cells (Fig. 2a). The expression patterns of the ER stress proteins ATF6, BIP and CHOP were largely consistent with SCIRR69 and reached the highest expression level at 24 h, 36 h, and 24 h, respectively (Fig. 2a). The expression patterns of SCIRR69, ATF6, BIP, and CHOP in TM-treated PC12 cells were similar to that in TG-treated PC12 cells, except the highest expression levels appeared at 36 h, 36 h, 48 h and 36 h, respectively (Fig. 2b).

Dissection of SCIRR69-Interacting Proteins Formed in PC12 Cells During TG-Induced ER Stress

Firstly, we generated a PC12 cell line that stably expressed the Flag-tagged rat SCIRR69, and then we dissected the PC12 cells-specific SCIRR69 interactome formed during TG-induced ER stress, using an SILAC-based quantitative proteomic approach (Fig. 3a). Briefly, the Flag-SCIRR69-expressing PC12 cells were grown in the ‘‘heavy’’

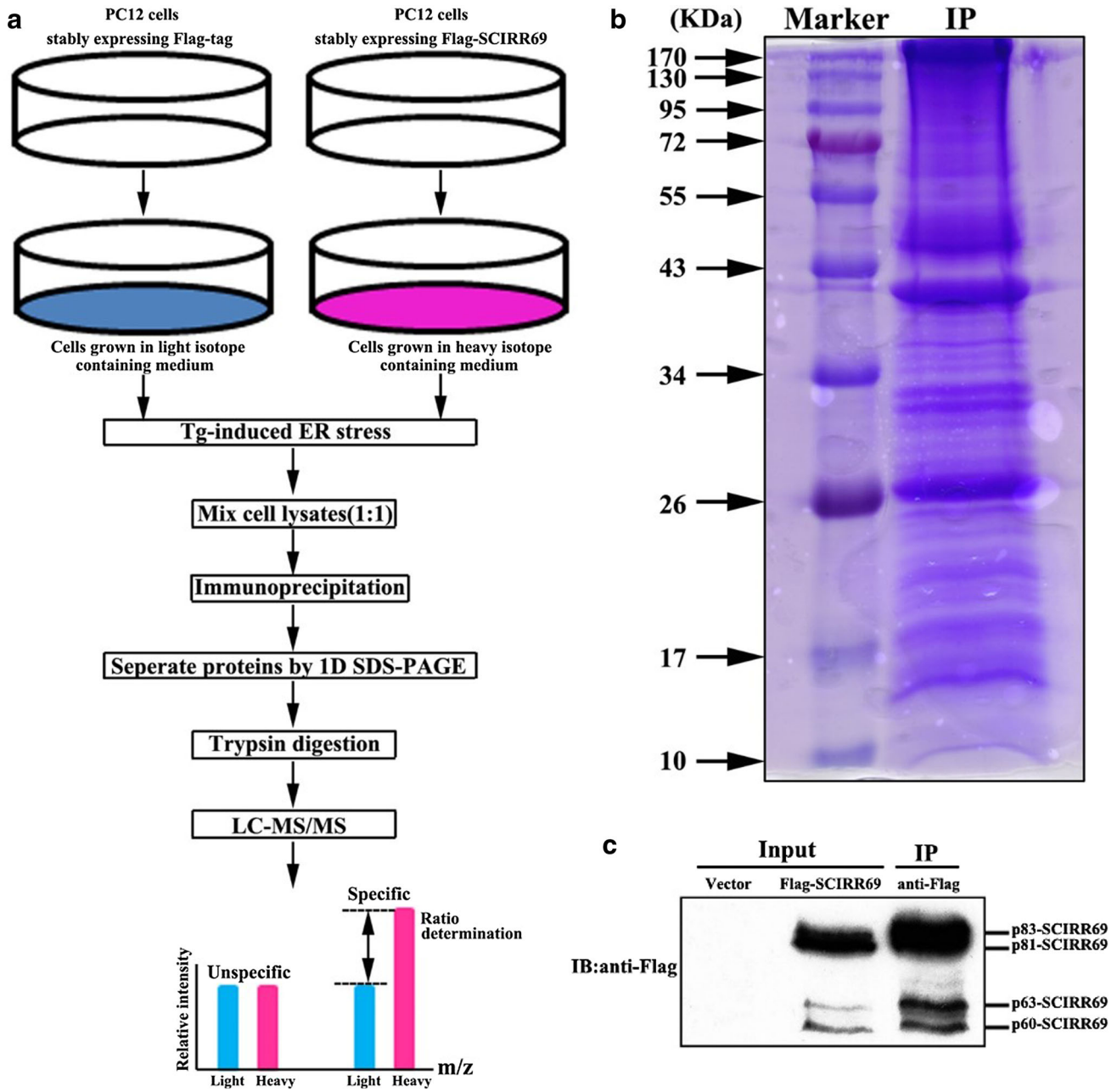


Fig. 3 Isolation of SCIRR69-interacting complex by immunoprecipitation. **a** Strategy to identify SCIRR69-interacting partners during TG-induced ER stress in PC12 cells. PC12 cells stably expressing Flag-SCIRR69 were maintained in "heavy" medium. In parallel, control cells stably expressing Flag tag were grown in ''light'' medium. After those cells were treated with TG (1 μg/ml) for 12 h, the whole cell lysates were derived from each cell pool and then mixed 1:1 based on the total protein mass. The SCIRR69-interacting complex was purified using anti-Flag beads, followed by SDS-PAGE separation, in-gel trypsin digestion, and LC–MS/MS analysis. **b** Isolation of the SCIRR69 complex by immunoprecipitation (IP). The immunoprecipitated proteins were separated on SDS-PAGE and stained with Coomassie brilliant blue. **c** Identification of Flag-SCIRR69 immunoprecipitated (IP) by anti-Flag beads in mixed cell lysates (1:1) with immunoblotting (IB). 25 μg pre-mixed whole cell lysate was used as input

medium-containing $^{13}C_6$ L—lysine, whereas the control cells (vector only) were maintained in ''light'' medium-containing $^{12}C_6$ L—lysine. After PC12 cells were induced ER stress with TG for 12 h, the proteins extracted from each group were mixed in a ratio of 1:1 based on the total protein mass, and anti-Flag beads were added to immunoprecipitate the SCIRR69 interacting complex. Immunoprecipitates were isolated by SDS-PAGE (Fig. 3b). After coomassie blue staining and in-gel trypsin digestion, protein bands were analyzed by LC–MS/MS.

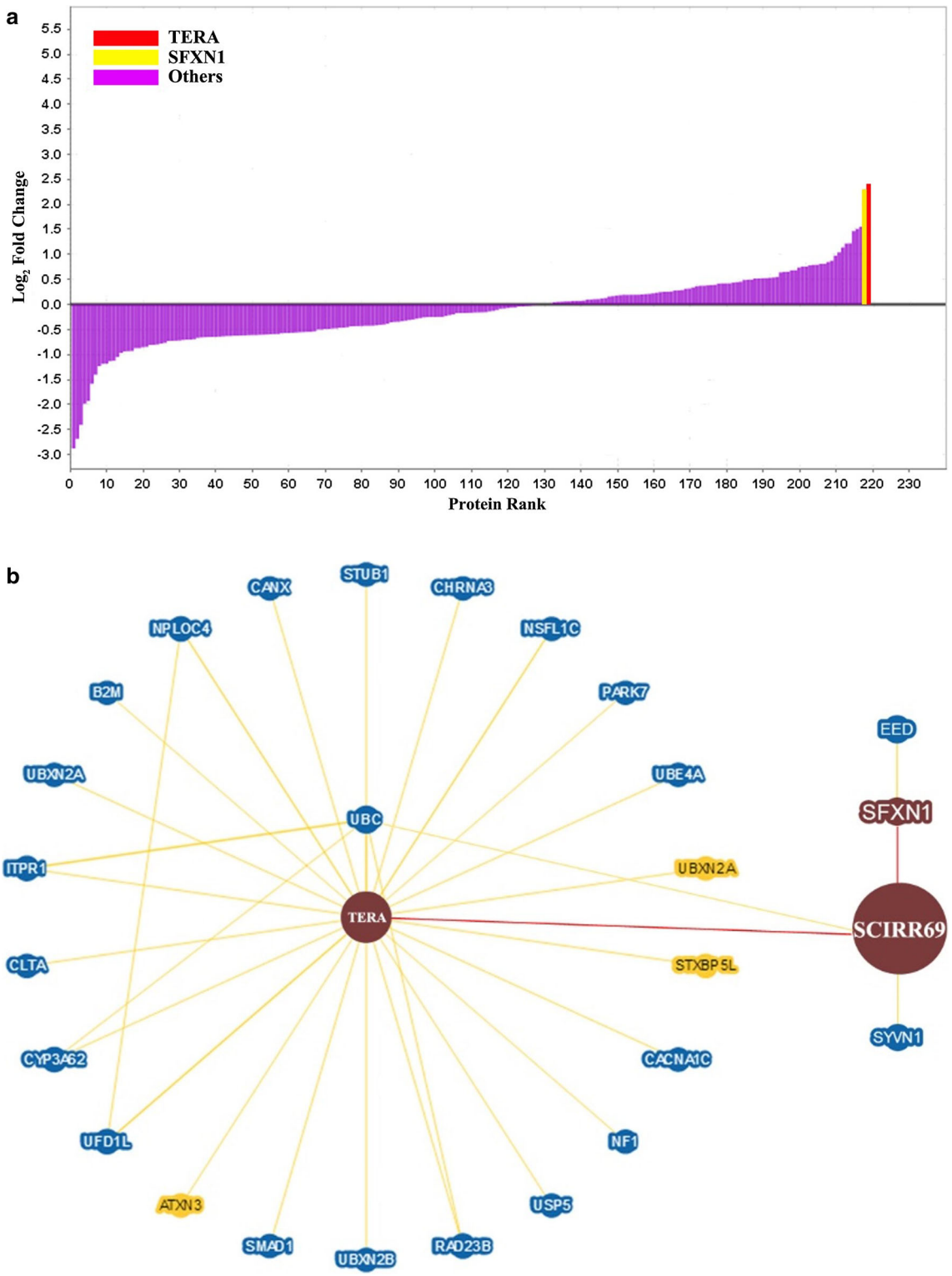


Fig. 4 Potential SCIRR69-interacting proteins. **a** SILAC analysis discriminates specific from unspecific binders. The bars represent the Log 2 base (Log_2) value of fold change heavy-to-light for each identified protein. Protein rank refers to the numbers of identified proteins. Protein SFXN1 and TERA are considered specific and other proteins unspecific. **b** A protein–protein interaction network was mapped by using bioinformatics method

Table 1 Functional clustering of SCIRR69-associated proteins with SILAC ratios having significant abundance changes

Protein name	ID (UniProt)	GO-term (molecular function)	$\log_2$ (ratio (H/L))
Cellular component organization			
Vimentin	P31000	Structural molecule activity	1.5
60S ribosomal protein L28	Q6J2U6	Structural molecule activity	1.2
Peripherin	P21807	Structural molecule activity	0.8
Tubulin beta-2A chain	P85108	Structural molecule activity	0.8
40S ribosomal protein S17	P04644	Structural molecule activity	0.8
Stress-70 protein, mitochondrial	P48721	Structural molecule activity	0.7
Transport			
Sideroflexin-1	Q63965	Transporter activity	2.3
Mitochondrial 2-oxoglutarate/malate carrier protein	P15999	Transporter activity	1.2
ADP/ATP translocase 2	Q09073	Transporter activity	1
Phosphate carrier protein, mitochondrial	E9PU28	Transporter activity	0.7
Metabolic process			
Non-POU domain-containing octamer-binding protein	Q5FVM4	Catalytic activity	1.6
Inosine-5′-monophosphate dehydrogenase 2	E9PU28	Catalytic activity	1.5
Serine/arginine-rich splicing factor 2	Q6PDU1	Catalytic activity	0.9
ATP synthase subunit alpha, mitochondrial	P15999	Catalytic activity	0.8
ATPase family AAA domain-containing protein 3	Q3KRE0	Catalytic activity	0.8
Protein-L-isoaspartate(D-aspartate) *O*-methyltransferase	P22062	Catalytic activity	0.8
RING finger protein 114	Q6J2U6	Catalytic activity	0.8
Vesicle-fusing ATPase	Q9QUL6	Catalytic activity	0.7
Translation			
Transcriptional activator protein Pur-alpha	P86252	Nucleic acid binding transcription factor activity	1.1
Eukaryotic translation initiation factor 5B	B2GUV7	Translation regulator activity	0.8
Unknown			
Transitional endoplasmic reticulum ATPase	P46462		2.4
Serine/arginine-rich splicing factor 5	Q09167		1
Leucine-rich repeat-containing protein 59	Q5RJR8		0.7

L/H ratios > 1.70, *i.e.,* Log_2 (L/H ratios) > 0.7

Immunoblotting was used to confirm whether Flag-SCIRR69 was immunoprecipitated from cell lysates. The result indicated that not only p83-SCIRR69 and p81-SCIRR69 proteins but also p63-SCIRR69 and p60-SCIRR69 proteins were immunoprecipitated by the anti-Flag antibody (Fig. 3c).

SILAC Analysis Discriminates Specific Binders from the Unspecific

According to the stringent criteria for protein identification and quantification (see Materials and methods), 219 proteins were quantified with L/H ratios (Fig. 4a and Table S1). Using significance B value ($p < 0.05$) as the threshold to distinguish the specific SCIRR69-interacting proteins (Cox and Mann 2008), 23 proteins were demonstrated as having significant abundance changes (L/H ratios > 1.70, *i.e.,* Log_2 (L/H ratios) > 0.7) (Table 1). A protein–protein interaction network was mapped by using bioinformatics method (Fig. 4b). However, certain highly abundant proteins, such as cytoskeletal proteins and ribosomal proteins, were also included in this list. Based on the previous report (Trinkle-Mulcahy et al. 2008), those proteins in the class of "beads proteome," *i.e.*, proteins that often stick to the agarose beads where the Flag antibody is conjugated to, co-purify with the "true" protein interacting partners during the process of immunoprecipitation. After excluding those proteins, transitional endoplasmic reticulum ATPase (TERA) and sideroflexin-1 (SFXN1) were considered to be the "true" SCIRR69-interacting proteins formed in response to ER stress in PC12 cells. Figure 5 shows the LC–MS/MS fragmentation spectrum identifies the peptide of TERA (Fig. 5a) and SFXN1 (Fig. 5b).

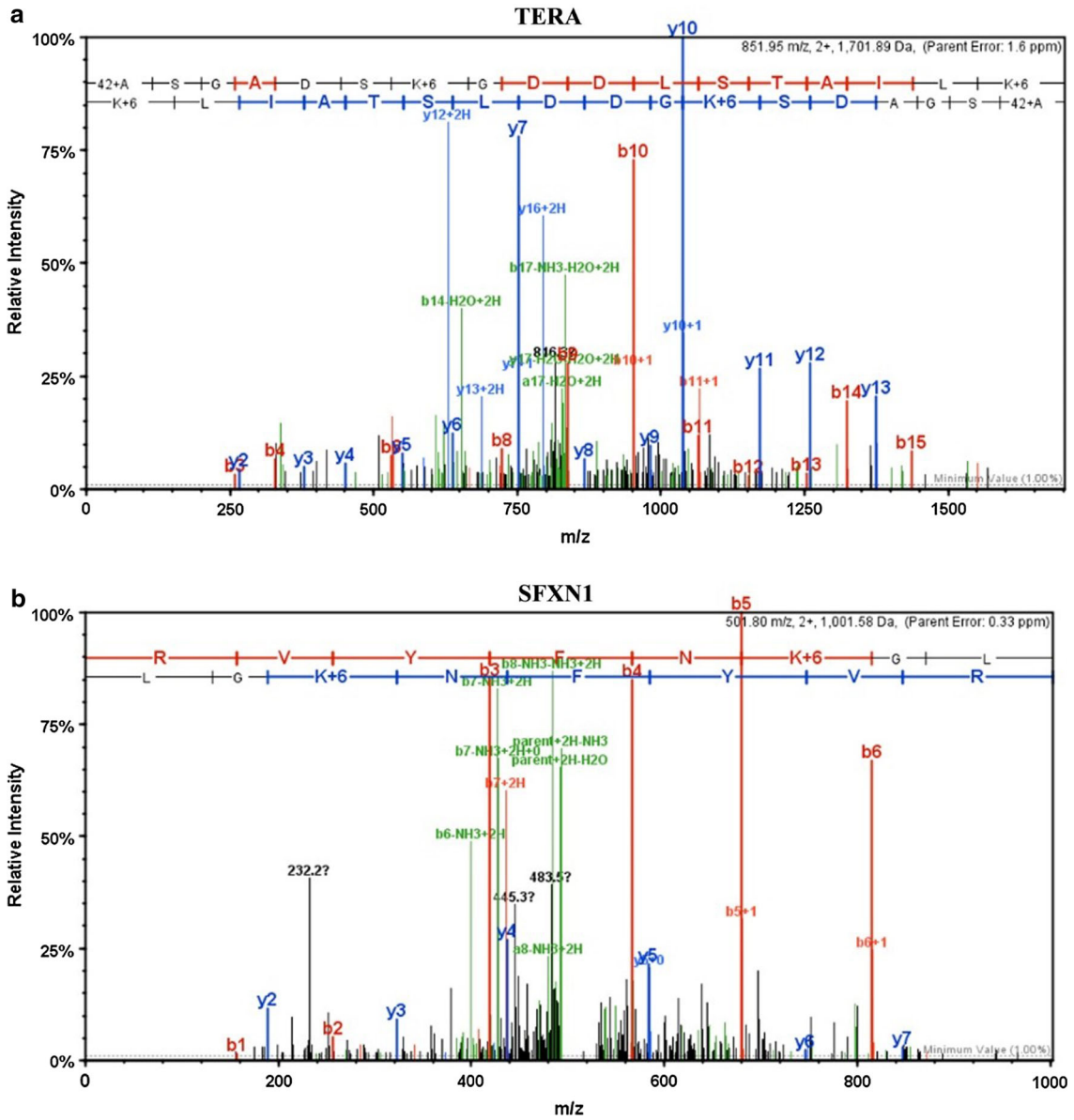
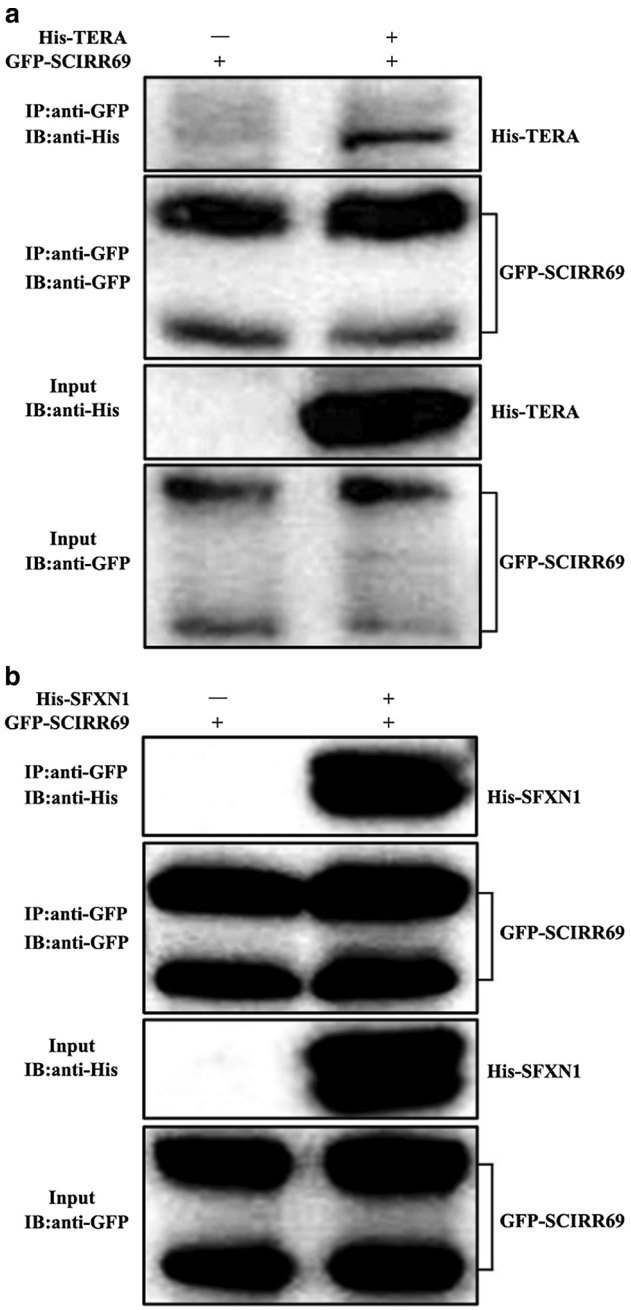


Fig. 5 MS/MS fragmentation spectrum identified the peptide of SCIRR69-interacted proteins. **a** The peptide sequence was determined from MS/MS fragmentation spectrum as (ASGADSKGDDLSTAILK) and identified as rat TERA peptide. **b** The peptide sequence was determined from MS/MS fragmentation spectrum as (RVYFNKGL) and identified as rat SFXN1 peptide

Validation of the Interaction Between SCIRR69 and TERA or SFXN1

To confirm the interaction between SCIRR69 and TERA, we analyzed the association of these two proteins in transfected mammalian cells. We prepared expression vectors that encoded a GFP-tag fused to the *Scirr69* (pGFP-N1-*Scirr69*) and a His-tag fused to the *Tera* (pcDNA3.1-His-*Tera*). These constructs were used to transiently co-transfect COS-7 cells. Meanwhile, transiently co-transfected empty vectors pcDNA3.1-His were used as a negative control. Immunoprecipitation and immunoblotting analysis were performed on cell lysates from these transfected cells. The result demonstrated that His-TERA was detected in the anti-GFP-immunoprecipitated cell lysates from pGFP-N1- *Scirr69* and pcDNA3.1-His-*Tera* co-transfected cells, but not from pGFP-N1- *Scirr69* and pcDNA3.1-His co-transfected cells (Fig. 6a). The interaction between SCIRR69 and SFXN1 was confirmed by using the same analyses as above (Fig. 6b).



◀**Fig. 6** Validation of the interaction between SCIRR69 and TERA or SFXN1 by co-immunoprecipitation experiments. **a** COS-7 cells were transfected with either pcDNA3.1-His-*Tera* plus pEGFP-*Scirr69* or pcDNA3.1-His empty vector plus pEGFP-*Scirr69* expression plasmids. 25 μg of whole cell protein lysate was used as input to confirm the expression of the His-TERA (with anti-His) or GFP-SCIRR69 (with anti-GFP) by immunoblotting (IB). The rest of cell lysates were incubated with anti-GFP-magnetics beads. The immunoprecipitated (IP) protein complex was resolved by SDS-PAGE and probed with related antibodies. **b** COS-7 cells were transfected with either pcDNA3.1-His-*Sfxn1* plus pEGFP-*Scirr69* or pcDNA3.1-His empty vector plus pEGFP-*Scirr69* expression plasmids. 25 μg of whole cell protein lysate was used as input to confirm the expression of the His-SFXN1 (with anti-His) or GFP-SCIRR69 (with anti-GFP) by immunoblotting (IB). The rest of cell lysates were incubated with anti-GFP-magnetics beads. The immunoprecipitated (IP) protein complex was resolved by SDS-PAGE and probed with related antibodies

Discussion

Our previous research indicated that SCIRR69 protein is a member of the CREB/ATF transcription factor family and functions as a transcriptional activator of *BDNF* gene (Ma et al. 2012, Liu et al. 2013). The SCIRR69 is proteolytically cleaved by S1P and S2P when neurons are injured, and two more peptides (p60-SCIRR69 and p63-SCIRR69) are detected in addition to full-length SCIRR69 (p81-SCIRR69 and p83-SCIRR69) that is primarily found in uninjured neurons (Liu et al. 2013). Interestingly, the above characteristics of SCIRR69 are similar to some members of CREB/ATF transcription factor family, such as ATF6 and OASIS. In response to ER stress, both ATF6 and OASIS are cleaved in a two-step process by S1P and S2P. The released N-terminus translocates into the nucleus and activates ER chaperone genes, such as *Bip/Grp78* and *Grp94* (Yoshida et al. 1998, Roy and Lee 1999, Yoshida et al. 2000, Murakami et al. 2006). All of those clues hint that SCIRR69 may be involved in ER stress. We treated SCN cells and PC12 cells with TG or TM to induce ER stress and found the expression patterns of *SCIRR69* at mRNA and protein levels under ER stress condition were very similar to those of ER stress marker genes (*Atf6*, *Bip* and *Chop*). Those results suggested that SCIRR69 may play similar roles in ER stress, like ATF6 and OASIS. In order to investigate the fundamental roles of SCIRR69 in ER stress, we used SILAC-immunoprecipitation quantitative proteomics to identify interaction partners of SCIRR69 during TG-induced ER stress of PC12 cells. After analysis and validation, TERA and SFXN1 were found to be interacted with SCIRR69 during ER stress of PC12 cells.

TERA, also known as valosin-containing protein (VCP) or p97, is a member of the ATPase associated with various activities AAA + protein family(Fujita et al. 2013). TERA contains ATPase catalytic domains and regulatory N-terminal and C-terminal domains; it forms a hexameric structure and couples ATP hydrolysis to cellular activities(Woodman 2003). TERA has a critical role in ER-associated protein degradation (ERAD) though interactions with Derlin-1, VCP-interacting membrane protein and UFD1/NPL4 complex (Ye et al. 2001, Ye et al. 2004). TERA supplies energy to this protein complex to extract misfolded proteins from the ER lumen and transport them to the proteasome for degradation. TERA may directly interact with gp78, a sensor of misfolded proteins, on the ER membrane(Zhong et al. 2004), and may also interact with small interfering RNA to affect cell division(Kittler

et al. 2004). Furthermore, a recent report revealed a role of TERA in DNA double-stranded break (DSB) repair, as TERA interacts with and promotes release of lethal malignant brain tumor-like protein 1 from DSBs and recruitment of p53-binding protein-1(Acs et al. 2011, Meerang et al. 2011). In this study, we found that TERA could be interacted with SCIRR69 during TG-induced ER stress in PC12 cells. Those findings imply that interaction of SCIRR69 with TERA may involve in regulating ERAD, membrane transport or DSB repair. However, those hypotheses need to be further investigated.

SFXN1, a member of evolutionarily conserved family of mitochondrial tricarboxylate carrier proteins, is a five-membrane spanning integral membrane protein predicted to be either a channel or carrier protein (Ye et al. 2003, Miotto et al. 2007, Li et al. 2010). It was found that the interaction of connexin 32 at the plasma membrane and SFXN1 at the outer mitochondrial membrane could serve to tether a pool of subplasmalemmal mitochondria to the plasma membrane and potentially facilitate transport of pyridoxine into the mitochondria(Fowler et al. 2013). Given the predicted structural features of SFXN1, it is likely that the SFXN1 facilitates the transport of a component, notably pyridoxine, required for iron utilization into or out of the mitochondria (Fleming et al. 2001). Therefore, SFXN1 may depend upon a network of channel transporters moving components through plasma membrane to mitochondria. SCIRR69 is an ER membrane-bound protein (Ma et al. 2012, Liu et al. 2013), and its interaction with SFXN1 may play roles in facilitating components transport through ER to mitochondria.

In summary, this work explores the roles of SCIRR69 in ER stress and describes the identification of SCIRR69-interacting proteins during ER stress in PC12 cells by using SILAC-immunoprecipitation quantitative proteomics and the validation of the interactions between SCIRR69 and TERA or SFXN1 by co-immunoprecipitation. Interactions between SCIRR69 and TERA or SFXN1 may provide some clues for novel signaling nexuses.

Acknowledgments This work was supported by grants from the Chinese National Natural Science Foundation (81471155 and 81370051).

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interest.

References

Acs, K., Luijsterburg, M. S., Ackermann, L., Salomons, F. A., Hoppe, T., & Dantuma, N. P. (2011). The AAA-ATPase VCP/p97 promotes 53BP1 recruitment by removing L3MBTL1 from DNA double-strand breaks. *Nature Structural & Molecular Biology, 18*(12), 1345–1350.

Cox, J., & Mann, M. (2008). MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nature Biotechnology, 26*(12), 1367–1372.

Emmott, E., Munday, D., Bickerton, E., Britton, P., Rodgers, M. A., Whitehouse, A., et al. (2013). The cellular interactome of the coronavirus infectious bronchitis virus nucleocapsid protein and functional implications for virus biology. *Journal of Virology, 87*(17), 9486–9500.

Engin, F., & Hotamisligil, G. S. (2010). Restoring endoplasmic reticulum function by chemical chaperones: an emerging therapeutic approach for metabolic diseases. *Diabetes, Obesity & Metabolism, 12*(Suppl 2), 108–115.

Fleming, M. D., Campagna, D. R., Haslett, J. N., Trenor, C. C, 3rd, & Andrews, N. C. (2001). A mutation in a mitochondrial transmembrane protein is responsible for the pleiotropic hematological and skeletal phenotype of flexed-tail (f/f) mice. *Genes & Development, 15*(6), 652–657.

Fowler, S. L., Akins, M., Zhou, H., Figeys, D., & Bennett, S. A. (2013). The liver connexin32 interactome is a novel plasma membrane-mitochondrial signaling nexus. *Journal of Proteome Research, 12*(6), 2597–2610.

Fujita, K., Nakamura, Y., Oka, T., Ito, H., Tamura, T., Tagawa, K., et al. (2013). A functional deficiency of TERA/VCP/p97 contributes to impaired DNA repair in multiple polyglutamine diseases. *Nat Commun, 4*, 1816.

Kehat, I., Hasin, T., & Aronheim, A. (2006). The role of basic leucine zipper protein-mediated transcription in physiological and pathological myocardial hypertrophy. *Annals of the New York Academy of Sciences, 1080*, 97–109.

Keller, A., Nesvizhskii, A. I., Kolker, E., & Aebersold, R. (2002). Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. *Analytical Chemistry, 74*(20), 5383–5392.

Kittler, R., Putz, G., Pelletier, L., Poser, I., Heninger, A. K., Drechsel, D., et al. (2004). An endoribonuclease-prepared siRNA screen in human cells identifies genes essential for cell division. *Nature, 432*(7020), 1036–1040.

Li, X., Han, D., Kin Ting Kam, R., Guo, X., Chen, M., Yang, Y., et al. (2010). Developmental expression of sideroflexin family genes in Xenopus embryos. *Developmental Dynamics, 239*(10), 2742–2747.

Liu, Y., Que, H., Ma, Z., Yang, S., Ni, Y., Luo, Z., et al. (2013). Transcription factor SCIRR69 involved in the activation of brain-derived neurotrophic factor gene promoter II in mechanically injured neurons. *Neuromolecular Med, 15*(3), 605–622.

Ma, Z., Que, H., Ni, Y., Huang, H., Liu, Y., Liu, T., et al. (2012). Cloning and characterization of SCIRR69: a novel transcriptional factor belonging to the CREB/ATF family. *Molecular Biology Reports, 39*(7), 7665–7672.

Meerang, M., Ritz, D., Paliwal, S., Garajova, Z., Bosshard, M., Mailand, N., et al. (2011). The ubiquitin-selective segregase VCP/p97 orchestrates the response to DNA double-strand breaks. *Nature Cell Biology, 13*(11), 1376–1382.

Miotto, G., Tessaro, S., Rotta, G. A., & Bonatto, D. (2007). In silico analyses of Fsf1 sequences, a new group of fungal proteins orthologous to the metazoan sideroblastic anemia-related sideroflexin family. *Fungal Genetics and Biology, 44*(8), 740–753.

Murakami, T., Kondo, S., Ogata, M., Kanemoto, S., Saito, A., Wanaka, A., et al. (2006). Cleavage of the membrane-bound transcription factor OASIS in response to endoplasmic reticulum stress. *Journal of Neurochemistry, 96*(4), 1090–1100.

Nesvizhskii, A. I., Keller, A., Kolker, E., & Aebersold, R. (2003). A statistical model for identifying proteins by tandem mass spectrometry. *Analytical Chemistry, 75*(17), 4646–4658.

Omori, Y., Imai, J., Watanabe, M., Komatsu, T., Suzuki, Y., Kataoka, K., et al. (2001). CREB-H: a novel mammalian transcription factor belonging to the CREB/ATF family and functioning via the box-B element with a liver-specific expression. *Nucleic Acids Research, 29*(10), 2154–2162.

Ribeiro, A., Brown, A., & Lee, K. A. (1994). An in vivo assay for members of the cAMP response element-binding protein family of transcription factors. *Journal of Biological Chemistry, 269*(49), 31124–31128.

Roy, B., & Lee, A. S. (1999). The mammalian endoplasmic reticulum stress response element consists of an evolutionarily conserved tripartite structure and interacts with a novel stress-inducible complex. *Nucleic Acids Research, 27*(6), 1437–1443.

Shen, J., & Prywes, R. (2005). ER stress signaling by regulated proteolysis of ATF6. *Methods, 35*(4), 382–389.

Trinkle-Mulcahy, L. (2012). Resolving protein interactions and complexes by affinity purification followed by label-based quantitative mass spectrometry. *Proteomics, 12*(10), 1623–1638.

Trinkle-Mulcahy, L., Boulon, S., Lam, Y. W., Urcia, R., Boisvert, F. M., Vandermoere, F., et al. (2008). Identifying specific protein interaction partners using quantitative mass spectrometry and bead proteomes. *Journal of Cell Biology, 183*(2), 223–239.

Walter, P., & Ron, D. (2011). The unfolded protein response: from stress pathway to homeostatic regulation. *Science, 334*(6059), 1081–1086.

Wang, S., & Kaufman, R. J. (2012). The impact of the unfolded protein response on human disease. *Journal of Cell Biology, 197*(7), 857–867.

Williamson, M. P., & Sutcliffe, M. J. (2010). Protein-protein interactions. *Biochemical Society Transactions, 38*(4), 875–878.

Woodman, P. G. (2003). p97, a protein coping with multiple identities. *Journal of Cell Science, 116*(Pt 21), 4283–4290.

Ye, Y., Meyer, H. H., & Rapoport, T. A. (2001). The AAA ATPase Cdc48/p97 and its partners transport proteins from the ER into the cytosol. *Nature, 414*(6864), 652–656.

Ye, Y., Shibata, Y., Yun, C., Ron, D., & Rapoport, T. A. (2004). A membrane protein complex mediates retro-translocation from the ER lumen into the cytosol. *Nature, 429*(6994), 841–847.

Ye, X., Xu, J., Cheng, C., Yin, G., Zeng, L., Ji, C., et al. (2003). Isolation and characterization of a novel human putative anemia-related gene homologous to mouse sideroflexin. *Biochemical Genetics, 41*(3–4), 119–125.

Yoshida, H., Haze, K., Yanagi, H., Yura, T., & Mori, K. (1998). Identification of the cis-acting endoplasmic reticulum stress response element responsible for transcriptional induction of mammalian glucose-regulated proteins. Involvement of basic leucine zipper transcription factors. *Journal of Biological Chemistry, 273*(50), 33741–33749.

Yoshida, H., Okada, T., Haze, K., Yanagi, H., Yura, T., Negishi, M., et al. (2000). ATF6 activated by proteolysis binds in the presence of NF-Y (CBF) directly to the cis-acting element responsible for the mammalian unfolded protein response. *Molecular and Cellular Biology, 20*(18), 6755–6767.

Zhong, X., Shen, Y., Ballar, P., Apostolou, A., Agami, R., & Fang, S. (2004). AAA ATPase p97/valosin-containing protein interacts with gp78, a ubiquitin ligase for endoplasmic reticulum-associated degradation. *Journal of Biological Chemistry, 279*(44), 45676–45684.