TIGAR suppresses ER stress-induced neuronal injury through targeting ATF4-signaling in cerebral ischemia/reperfusion

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Abstract

Abstract Background and Purpose Previous studies have shown that TIGAR (TP53-induced glycolysis and apoptosis regulator) protects against cerebral ischemia/reperfusion injury via the improvement of the redox and energy homeostasis of neurons. TIGAR is found in the endoplasmic reticulum (ER) and nucleus but its role in ER stress is unclear. This study aimed to investigate the ER and nuclear translocation of TIGAR during ER stress and the influences of the nuclear TIGAR in cerebral ischemia/reperfusion-induced ER stress. Experimental Approach Mice were subjected to the middle cerebral artery occlusion/reperfusion. Cultured neurons were treated with oxygen and glucose deprivation, tunicamycin or thapsigargin. Key Results The increases in ATF4 target genes and ER stress-induced neuronal apoptosis were reduced by overexpression of TIGAR. Furthermore, increases in the localization of TIGAR and ATF4 to the nucleus were observed after in vitro and in vivo cerebral ischemia/reperfusion or ER stress models. The nuclear TIGAR interacts with ATF4 and inhibits the transcription of downstream pro-apoptotic genes of ATF4, resulting in protection against cerebral ischemia/reperfusion injury. Intriguingly, the translocation of TIGAR to the ER and nucleus and inhibition of the transcription of ATF4 is depend on Q141/K145 of TIGAR instead of its phosphatase activity and mitochondrial localization domains. Conclusion and Implications These results suggest that TIGAR translocates to the ER and the nucleus to interact with ATF4 after cerebral ischemia-reperfusion induced ER stress via its Q141/K145. The study uncovered a novel neuroprotective mechanism of TIGAR through regulating ER stress via a ATF4-mediated signaling pathway.

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Abstract

Background and Purpose

Previous studies have shown that TIGAR (TP53-induced glycolysis and apoptosis regulator) protects against cerebral ischemia/reperfusion injury via the improvement of the redox and energy homeostasis of neurons. TIGAR is found in the endoplasmic reticulum (ER) and nucleus but its role in ER stress is unclear. This study aimed to investigate the ER and nuclear translocation of TIGAR during ER stress and the influences of the nuclear TIGAR in cerebral ischemia/reperfusion-induced ER stress.

Experimental Approach

Mice were subjected to the middle cerebral artery occlusion/reperfusion. Cultured neurons were treated with oxygen and glucose deprivation, tunicamycin or thapsigargin.

Key Results

The increases in ATF4 target genes and ER stress-induced neuronal apoptosis were reduced by overexpression of TIGAR. Furthermore, increases in the localization of TIGAR and ATF4 to the nucleus were observed after in vitro and in vivo cerebral ischemia/reperfusion or ER stress models. The nuclear TIGAR interacts with ATF4 and inhibits the transcription of downstream pro-apoptotic genes of ATF4, resulting in protection against cerebral ischemia/reperfusion injury. Intriguingly, the translocation of TIGAR to the ER and nucleus and inhibition of the transcription of ATF4 is depend on Q141/K145 of TIGAR instead of its phosphatase activity and mitochondrial localization domains.

Conclusion and Implications

These results suggest that TIGAR translocates to the ER and the nucleus to interact with ATF4 after cerebral ischemia-reperfusion induced ER stress via its Q141/K145. The study uncovered a novel neuroprotective mechanism of TIGAR through regulating ER stress via a ATF4-mediated signaling pathway.

Key words

TIGAR; cerebral ischemia/reperfusion; ATF4; nuclear translocation; endoplasmic reticulum stress; neuroprotection.

1. Introduction

Endoplasmic reticulum (ER) is a central organelle in eukaryotic cells, which is responsible for synthesis, folding and maturation of proteins, glycogen metabolism, lipid synthesis and calcium storage[1]. Under nutrient deprivation, microbial infection, calcium imbalance or other predisposing factors, ER homeostasis is disrupted with massive unfolded or misfolded proteins accumulation in the ER cavity. This process is called as unfolded protein response (UPR) or ER stress[2]. During ER stress, GRP78 dissociates from downstream effector protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK), inositol-dependent enzyme 1

(IRE1α) and active transcription factor 6 (ATF6)[3]. Eukaryotic initiation factor α (eIF2α) are phosphorylated by activated PERK and then reduce protein synthesis, thereby decreasing protein load in ER lumen and preventing abnormal protein aggregation[4, 5]. However, eIF2α phosphorylation also promotes activating transcription factor 4 (ATF4) translation and subsequent transcription of adaptive or pro-apoptotic genes[6-9].

Stroke, characterized by high disability and high mortality, is one of the leading causes of death in the world and China. Stroke is the most common cerebrovascular disease, among which the incidence of cerebral ischemic stroke accounts for 75-80%[10]. Clinically, cerebrovascular occlusion due to thrombosis is a major cause of ischemic stroke[11]. The main treatment measures of ischemic stroke include drug therapy, interventional therapy and measures to restore cerebral blood flow. However, cerebral ischemia/reperfusion (I/R) injury after blood flow recovery may cause a series of secondary events including hypoxia, ATP depletion, Ca^{2+} overload, oxidative stress, which are known to induce ER stress[12-14]. Previous studies have shown that ER stress is the pivotal pathological processes of cerebral ischemia/reperfusion injury, leading to irreversible neuronal damage[15, 16]. A better understanding of the mechanism of ER stress during cerebral ischemia/reperfusion will help to find a more effective treatment approach.

TIGAR, the TP53-induced glycolysis and apoptosis regulator, is involved in regulating cellular glycolysis and metabolic homeostasis. TIGAR hydrolyzes fructose 2,6-diphosphate and fructose 1,6-diphosphate to inhibit glycolysis, which is similar to Fru-2 6-Bpase[17]. TIGAR thus promotes glucose metabolism to the pentose phosphate pathway (PPP), increasing the production of NADPH[18]. NADPH is a coenzyme of glutathione reductase, which converts oxidized glutathione (GSSG) into reduced glutathione (GSH), reduces intracellular reactive oxygen species (ROS), thereby protecting cells from oxidative damage and apoptosis[19, 20]. In 2015, our laboratory first reported the neuroprotective effects of TIGAR in cerebral ischemia/reperfusion and cerebral preconditioning[18, 21]. Downregulation of TIGAR expression aggravates cerebral ischemia/reperfusion-induced neuronal damage and abolishes the neuroprotection of cerebral preconditioning, whereas TIGAR overexpression protects neurons from cerebral ischemia/reperfusion injury, thereby improving survival rate and resuming motor and cognitive function of ischemic stroke animals[18]. TIGAR also prevents the injury of brain endothelial tight junctions[22]. Moreover, TIGAR inhibits NF-κB-mediated astrocyte inflammation by reducing ROS[23]. These results suggest that TIGAR showed a powerful neuroprotective effect in cerebral ischemia/reperfusion injury. But we intend to know whether the neuroprotection of TIGAR is only related to its phosphatase activity, or whether other key unknown mechanisms are involved?

TIGAR is widely distributed in cells including mitochondria, ER, cytoplasm and the nucleus[18]. Under hypoxia or stress, TIGAR translocates to mitochondria depending on mitochondrial hexokinase 2 (HK2) or ATP5A1[24, 25]. TIGAR then maintains mitochondrial function to reduce cell death. TIGAR translocates to the nucleus under the stimulation of genotoxic drugs or hypoxia, then regulates the expression of CDK5 to mediate ATM phosphorylation, thus arresting the cell cycle and promoting DNA damage repair and cell survival[26]. Intriguingly, our preliminary experiments found that TIGAR could translocate to the ER and nucleus during cerebral ischemia/reperfusion or neuronal glucose and oxygen deprivation.

It is known that the ER membrane is connected to the nuclear membrane. In addition to being responsible for protein folding and transportation, lipid synthesis, and maintenance of calcium homeostasis, ER can also transmit signals to the nucleus[27-29]. In the process of ER stress, nuclear transcription factors such as ATF4, CHOP and XBP-1 regulate the downstream signals of ER stress[30]. We thus presume that TIGAR may translocate to the ER and nucleus during cerebral ischemia/reperfusion, then to act on some transcription factors to regulate ER stress, thereby protecting against ischemic neuronal injury.

In this study, we established *in vivo* and *in vitro*cerebral ischemia/reperfusion or ER stress models, to study the ER and nucleus translocation of TIGAR during cerebral ischemia/reperfusion. We further explored the ER and nuclear translocation of TIGAR in regulating ER stress via ATF4/CHOP pathway. The results expand the neuroprotective mechanisms of TIGAR against cerebral ischemia/reperfusion injury.

2. Materials and methods

2.1 Materials

The information of materials is listed in supplementary Table 1 and Table 2.

2.2. Transient middle cerebral artery occlusion model in mice

ICR mice (male, 8-10 weeks, 25-30 g) and ICR pregnant mice (about 17 days of gestation) were provided by the Experimental Animal Center of Soochow University (Certificate No 20020008, Grade II) in the following conditions: temperature (20˜25), humidity (40˜70%), 12 h artificial day/night. *TIGAR* transgenic mice and matched WT mice were constructed by Model Animal Research Center of Nanjing University (Nanjing, China). Conditional KO mice carrying floxed alleles of *Tigar* (*Tigar* $^{flox/flox}$) and*Nestin* -Cre transgenic mice were also purchased from Model Animal Research Center of Nanjing University. We used *Tigar* $^{flox/flox}$, crossed with Cre mice to generate *Nestin* -Cre *Tigar* $^{flox/flox}$(Nes-cKO)[25] in SPF-class housing of laboratory of Soochow University. The identification of mice was performed by cutting the tail tip to extract genomic DNA. The primers used for quantitative PCR are listed in supplementary Table 3. All animals were kept in the Experimental Animal Center of Soochow University. The use of animals was approved by the ethical committee of Soochow University and animal procedures were followed the institutional guidelines for animal care and use. The cerebral ischemia-reperfusion model included two hours of transient middle cerebral artery occlusion (tMCAO) and subsequent restoration of blood flow which was conducted as described previously[31, 32]. Briefly, the origin of right middle cerebral artery was occluded by a 6-0 silicone-coated suture, and the suture was removed 2 h later to restore blood flow. Laser Doppler blood flow meter (LDFML191, Australia) was used to monitored cerebral blood flow. During the procedure, the mice were kept at their normal body temperature with a thermostatic blanket. Sham-operated mice were subjected to the same surgery without suture insertion. After cerebral ischemia/reperfusion, mice were sacrificed at different time points.

2.3. Cell culture

Primary culture cortical neurons were acquired from cerebral cortex of ICR embryos at 17 days of gestation. In brief, the fetal cortices were dissected under aseptic conditions, quickly placed in pre-cooled PBS and minced. Digestion was started with 2.5% trypsin and terminated with DMEM containing 10% fetal bovine serum after 15 min reaction at 37. Then the cell suspension was incubated with DNase I, blown for 3 min and centrifuged for 5min at 1500rpm. The pellets were resuspended in Neurobasal medium (NBM) containing 2% B27, 1% penicillin G and streptomycin, 0.5 mM L-glutamine, 25 μM L-glutamate. The cells were seeded on culture dishes coated with poly d-lysine after filtered through a 40-microm cell strainer. When the neurons grew out of antennae, they were cultured in NBM, 0.5 mM l-glutamine containing 2% B27, 1% penicillin G and streptomycin. Then half medium was replaced every 3 days. Generally, primary neurons were cultured *in vitro*for 7-10 days for experiments[33, 34].

HT22 mouse hippocampal neuronal cells (CVCL_0321) and human embryonic kidney 293 Tet-on (HEK293T) cells were purchased from Shanghai Institute of Cell Biology. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml streptomycin, 100 U/ml penicillin in 5% CO_2/95% air at 37 °C.

2.4. Oxygen and glucose deprivation-reperfusion and ER stress models

For Oxygen and glucose deprivation-reperfusion (OGD/R), the cells were changed medium with Hepes balanced salt solution (HBSS: 10 mM Hepes, 140 mM NaCl, 3.5 mM KCl, 12 mM $MgSO_4$, 5 mM $NaHCO_3$, 1.7 mM $CaCl_2$, 0.4 mM KH_2PO_4). Then the cells were put into an incubator chamber aerated with 5% CO_2/95% N_2 and were cultured at 37 °C for 4 h (primary neurons) or 6 h (HT22). To restore oxygen and glucose, cells were replaced with normal medium and transferred from hypoxia chamber to incubator containing 5% CO_2/95% air[33].

HT22 cells were treated with tunicamycin (TM) 12 μM or thapsigargin (Tg) 1 μM to induce ER stress[35, 36]. Cells were harvested at indicated time. HEK293T cells were treated with TM 12 μM to induce ER stress. Cells were harvested at 12 h after treatment. TM or Tg were first prepared with dimethylsulfoxide (DMSO) and deliquated with medium before use (the final DMSO concentration $< 0.1\%$).

2.5. Lentivirus or plasmids infection

Lentiviruses-TIGAR (Ubi-MCS-3FLAG-CBh-gcGFP-IRES-Puro, 2 × 10^8 TU/mL,Gene ID: 319801, NM 177003) and lentiviruses-ATF4 (Ubi-MCS-SV40-EGFP-IRES-Puro, 1 x10^8TU/mL, Gene ID: 11911, NM 009716) were synthesized by GENECHEM (Shanghai, China). The culture medium containing LV-TIGAR or LV-vector lentivirus was incubated with HT22 or HEK293T cells for 24 h. The virus-stably-infected cells were established by puromycin (2 μg/ml) treatment for two weeks and determined with GFP immunofluorescence and immunoblotting[32]. To overexpress exogenous ATF4, the culture medium containing LV-ATF4 or LV-vector lentivirus was incubated with control or LV-TIGAR-HT22 stably transfected cells for 24 h. After extra 48 hours of culture in normal medium, GFP immunofluorescence and immunoblotting were used to identified the expression of ATF4.

The cDNA of WT- *Tigar* (NM_020375,60-872, 813 bp), *Tigar*[?]tm (loss of Fru 2, 6-BPase activity)[17], *Tigar* [?]258-261 (unable to locate mitochondria)[24], *Tigar* ΔN, *Tigar* ΔC, *Tigar* 59-63 AAAAA and *Tigar* Q141A, K145A mutants were cloned into p3×FLAG plasmids. The plasmids of ΔN, ΔC, 59-63 AAAAA and Q141A, K145A mutants were constructed by Sangon Biotech (Shanghai, China). The HT22 or HEK293T cells were transfected with WT-TIGAR, [?]tm, [?]258-261 and Q141A, K145A for 24 h using JetOPTIMUS.

The 2188 bp region of the promoter and the first exon of *Atf4* was cloned into the pGL3-basic vector (Genewiz, Suzhou, China) as a luciferase reporter gene plasmid known as p-ATF4-luc. HEK293T were co-transfected with p-ATF4-luc and vector, WT, [?]tm or [?]258-261 TIGAR mutants for 24 h using JetOPTIMUS[35]. The information of TIGAR mutants and luciferase plasmids are listed in Supplementary Table 4.

2.6. Cell viability assay

After TM treatment for 12 h, the medium was changed to Cell Counting Kit-8 (CCK-8) diluent (CCK-8: DMEM = 1:10). After incubation at 37 for 2 h, the absorbance at 450 nm was detected by a microplate reader (ELX800, Bio-Tek).

2.7. Flow cytometry (FCM)

The cells were washed twice with precooled PBS 12 h after TM treatment. Cells were then harvested and suspended in Binding Buffer from Annexin V-PE/7-AAD Apoptosis Detection Kit, followed by incubation with Annexin V-PE and 7-AAD reagent at 25 for 15 min in dark. Then the suspension was diluted five times. Finally, cell apoptosis was detected by analytical flow cytometry (FACS Ca1ibur).

2.8. Western blot analysis

The ipsilateral cortex or cells cultured in vitro were lysed with the lysis buffer containing Tris-HCl (pH 7.4) 10 mM, NaCl 150 mM, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, EDTA 5 mM and EDTA-free complete protease inhibitor. Protein concentration of sample was measured by BCA assay. Polyacrylamide gels and nitrocellulose membranes were used to separated and blotted equal amounts of protein extracts respectively. Immunoblotting was performed to detect the levels of TIGAR (1:500), ATF4 (1:1000), CHOP (1:200). GRP78 (1:1000), caspase-12 (1:1000), GST (1:500). The β-actin (1:10,000), Calnexin (1:1000), α-tubulin (1:10000), GAPDH (1:10,000) or Lamin B (1:1000) were used to normalize the difference in loading.

2.9. Immunofluorescence

For immunofluorescence in frozen sections of mice brain, PBS and 4% paraformaldehyde were used to perfuse mice at 6 h after ischemia/reperfusion[18, 37]. Brains was placed in 4% paraformaldehyde for fixation followed by dehydrating with sucrose at 4 °C. Coronal sections of 30-μm-thickness were cut with a cryostat. After 1 h of blocking in 5% BSA, 0.4% Triton X-100, sections were incubated with ATF4 (1:100), TIGAR (1:100) or PDI (1:100), FLAG (1:100) for 48 h, followed by incubation with Coralite488-conjugated anti-Mouse IgG (1:500) and Coralite594-conjugated anti-Rabbit IgG (1:500) for 3 h. After stanning with DAPI (1:10,000), sections were visualized for immunofluorescence using a confocal microscopy (ZEIS S LSM710, Germany).

For immunofluorescence in vitro, HT22 cells were fixed with 4% paraformaldehyde for 15 min after 12 h of

TM treatment. The cells were then permeated with 0.1% Triton X-100 for 10 min and blocked with 1% BSA for 1 h, followed by incubation with TIGAR, ATF4 or PDI, FLAG antibodies at 4 °C overnight, and with Coralite488-conjugated anti-Mouse IgG (1:1000) and Coralite594-conjugated anti-Rabbit IgG (1:1000) for 2 h. Finally, a confocal microscopy (ZEISS LSM710, Germany) was used to observe immunofluorescence of the cells after DAPI (1:10,000) stanning.

2.10. Nuclear and ER extraction

The ipsilateral cortex was ground by glass homogenizer with buffer A containing 320 mM sucrose, 3 mM $CaCl_2$, 2 mM MgAc, 0.14mM EDTA, 1mM DTT, 0.5% NP40, EDTA-free complete protease inhibitor. HT22 cells were harvested and lysed in buffer A. The cytoplasmic (supernatant) and nuclear (pellets) fractions were obtained by centrifuging at 600 x g for 15 min. To purify the nuclear fraction, the pellets were resuspended in buffer A, and centrifuged at 600 x g for 10 min[38]. Lamin B and GAPDH were used as nuclear and cytoplasmic markers.

The fraction of ER was extracted with Endoplasmic Reticulum Isolation Kit. Briefly, the ipsilateral cortex was ground by glass homogenizer with Isotonic Extraction buffer. The lysate was centrifuged at 1000 g for10 min. Subsequently, the supernatant containing ER was centrifuged at 100,000 g for 1 h. The pellets were ER fractions. Calnexin was used as ER marker.

2.11. Transmission electron microscope and immunoelectron microscopy

Six hours after cerebral ischemia/reperfusion, male C57 mice were sacrificed. One cubic millimeter of parietal cortex in the ischemic center was rapidly dissected, fixed in 2.5% glutaraldehyde 0.1 M phosphate buffer (pH 7.0-7.5) for 30 min at room temperature and transferred to 4 degC for storage[39]. The samples were processed by Servicebio (Wuhan, China). A JEOL JEM-1230 electron microscope was used to observe the ultrastructure of endoplasmic reticulum and mitochondria.

ICR mice were sacrificed after cerebral ischemia/reperfusion. One cubic millimeter of parietal cortex in the ischemic center was rapidly dissected, fixed in 2.5% glutaraldehyde 0.1 M phosphate buffer (pH 7.0-7.5) for 2 h at 4 degC following rinse with 0.2 mol/L (68.5 g/L) sucrose solution. Tissues were dehydrated with graded alcohol, embedded in resin and sectioned. Sections were incubated with TIGAR (1:10) overnight at 4degC, followed by incubation with 10 nm colloidal gold secondary antibody (1:50) for 20 min at RT and 1 h at 37 . A JEOL JEM-1230 electron microscope was used to observe black 10 nm gold particles representing positive expression.

2.12. RNA analysis

The primer sequences for quantitative real-time (RT-qPCR) are listed in Supplementary Table 5. Tissue was isolated from ICR mice subjected to cerebral ischemia/reperfusion and HT22 cells were harvested at 12 h after TM treatment. Total RNAs were extracted by RNAiso Plus. Then PrimerScript RT Master Mix was used to synthesize cDNA. For RT-qPCR reaction, DNA template, primers and SyBR Green on an ABI Prism 7500 Sequence Detection system (Applied Biosystems, USA). The mRNA levels were analyzed with the comparative $\Delta\Delta$Ct method.

2.13. Co-immunoprecipitation (CoIP) and GST-pull down

Cortex were dissected 6 h after reperfusion and lysed in IP buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100 and EDTA-free complete protease inhibitor. HEK293T cells were harvested and lysed in IP buffer. To remove proteins that bind nonspecifically to Protein A/G -agarose, the lysates were precleaned with agarose for 1-3 h. Then the lysates incubated with antibody (ATF4, CST; TIGAR, Santa Cruz; FLAG, Santa Cruz;) or IgG overnight and incubated with protein G-agarose for 4-8 h[34]. Immunoblotting was performed with antibody against ATF4, TIGAR or FLAG to analyze the immunoprecipitates.

TIGAR GST fusion protein and GST were incubated with pre-washed Glutathione magnetic agarose beads for 1 h at 4 °C. Afterwards, the GST protein-beads complex washed away unbound proteins were incubated

with tissue lysates overnight[40]. The beads were eluted by 50 mM Tris (pH 8.0) containing 20 mM GSH. Finally, the bound proteins eluent was analyzed by immunoblotting using anti-TIGAR or anti GST antibody (1: 1000)[40].

2.14. Chromatin immunoprecipitation

Simple ChIP enzymatic chromatin IP kit with agarose beads was applied to perform the Chromatin immunoprecipitation (ChIP)-RT-qPCR. Twenty million LV-TIGAR-HEK293T cells were lysed and incubated with the FLAG antibody for generating each histone modification ChIP. Control for nonspecific immunoprecipitation of DNA was produced by rabbit IgG. For RT-qPCR assays, ChIP DNA was amplified was amplified for *CHOP* and*ATF3* primers listed in supplementary Table 6.

2.15. Luciferase assay

For the luciferase assay, transfected HEK293T cells were induced ER stress by TM and harvested at 12 h after treatment. The cells were lysed with the lysate in luciferase assay detected kit and centrifuged at 12500 x g for 10 min. The supernatant was added with an equal volume of luciferase assay solution. Fluorescence intensity of the mixture was evaluated at 560 nm with a Multiskan Spectrum (TECAN Infinite M1000 Pro).

2.16. Molecular docking

The Crystal Structure of TIGAR protein from Homo sapiens (PDB No. 3DCY) and ATF4 (PDB No. 1CI6) were referenced from the Protein Data Bank (http://www.rcsb.org). Protein docking of TIGAR and ATF4 was performed by the ZDOCK SERVER (https://zdock.umassmed.edu/, version ZDOCK 3.0.2). The structure of protein docking was visualized in Pymol program (version 1.8)[41, 42].

2.17. Statistical analysis

Data were expressed as mean +- S.D. All in vitro data represented 3 independent neuronal cultures with 2-3 replicates. In all animal experiments, n for statistical analysis was the number of mice for each condition. GraphPad Prism 5 was used to perform statistical analysis. The significance between two groups was compared with student's t-test, while multiple groups was determined with one-way or two-way repeated ANOVA. The post hoc analysis was performed by Newman-Keuls test or Bonferroni test. P value of ¡ 0.05 was considered as statistically significant.

3. Results

3.1 TIGAR inhibits ER stress-induced neuronal apoptosis during cerebral ischemia/reperfusion (I/R)

To determine the time-course of cerebral ischemia/reperfusion-induced ER stress and TIGAR upregulation, the expression of ER stress-related proteins and TIGAR was determined at different time points after oxygen and glucose deprivation/reperfusion (OGD/R) in primary cortical neurons and HT22 cells, and transient middle cerebral artery occlusion (tMCAO)/reperfusion (I/R) in mice. The expressions of GRP78, c-caspase12, CHOP and TIGAR in primary neurons (Fig. 1a) and HT22 cells (Fig. 1b) were remarkably upregulated at 3-6 h after OGD/R treatment. The peri-infarct cortex of the ipsilateral hemisphere was collected at indicated time points after I/R injury in mice. The protein levels of TIGAR and ER stress related proteins GRP78, c-caspase12, CHOP, and ATF4 were significantly increased in I/R group compared to sham group at 3 h and 6 h (Fig. 1c). The peri-infarct cortex was also collected for transcriptomic analysis at 6 h after I/R. The expression of ER stress related genes (*Grp78* , *Atf4* , *Chop* , *Gadd34* ,*Atf3* and *Trib3*) were also increased (Fig. 1d). Tunicamycin (TM) inhibits protein glycosylation, while thapsigargin (Tg) blocks the sarcoplasmic/ER calcium ATPase resulting in calcium depletion in ER. Both agents thus cause UPR and ER stress response[43]. HT22 cells were treated with TM (12 μM) or Tg (1 μM) to induce ER stress. The levels of ER stress proteins and TIGAR in HT22 cells were detected at indicated time after treatment. TIGAR expression was markedly upregulated at 12 h after TM treatment (Fig. 1e). Meanwhile, the levels of ER stress related protein GRP78, c-caspase12, CHOP and ATF4 were also significantly increased (Fig. 1e). Similarly, Tg treatment also increased the expression of TIGAR and ER stress related protein (Fig.

1f). The above data indicate that cerebral ischemia/reperfusion can upregulate the expression of TIGAR and activate ER stress in neurons synchronously.

To investigate the effect of TIGAR on ER stress induced by ischemia/reperfusion in neuronal cells, TIGAR-overexpressed HT22 cells (LV-TIGAR-HT22) were established, while *TIGAR* transgenic mice (TG) and neuron-conditional *Tigar* knockout mice (Nes-cKO, $Tigar^{flox/flox}$; *Nestin* -Cre) were bred as described. The overexpression and knockout of *TIGAR* in neurons was verified both *in vitro* (Fig. S1a) and *in vivo* (Fig. S1b, c). Immunoblotting was then performed to detect ER stress-related proteins. Consistent with previous data, I/R remarkably upregulated the protein levels of GRP78, CHOP and c-caspase12 in the cortex of the wild-type (WT) mice, whereas TIGAR overexpression (TG) significantly reduced these ER stress proteins compared with WT mice (Fig. 2a-c). Similarly, in LV-vector-HT22 (negative control, NC) cells, the expression of GRP78, CHOP, c-caspase12 and ATF4 were markedly increased with TM treatment (Fig. 2d-g). However, the overexpression of TIGAR significantly reversed the ER stress-related proteins in LV-TIGAR-HT22 (OV) cells compared with the NC group. We also investigate the effect of TIGAR on ER stress-mediated apoptosis. Cell viability evaluated with CCK-8 assay was significantly reduced (Fig. 2h), while apoptosis rate measured using flow cytometry was increased (Fig. 2i, j) in NC cells after TM-induced ER stress. However, TIGAR overexpression (OV) remarkably improved cell viability, and reduced apoptosis rate compared to the NC group. The above results indicate that overexpression of TIGAR can alleviate ER stress dependent-apoptosis in neurons.

We next applied electron microscopy to observe the organelle morphology and neuronal damage in TG and Nes-cKO mice after cerebral ischemia/reperfusion. In the sham group, the morphology of ER, mitochondria and other organelles in cortical neurons of WT, TG and Nes-cKO mice were basically normal. After WT mice underwent I/R, the neuronal ER were obviously dilated with medium densities in ER lumen (representing protein aggregates), and the mitochondria expanded with ruptured or disappeared cristae, indicating that I/R causes ER stress and mitochondrial damage in neurons. After I/R in *Tigar* Nes-cKO mice, the mitochondrial damage, ER dilation and protein aggregates in ER lumen were more obvious than in WT group. However, the morphology of ER and mitochondria in neurons of TG mice was significantly improved after I/R treatment (Fig. 2k). These results further demonstrate that TIGAR can alleviate ER stress and mitochondria damage in neurons induced by ischemia/reperfusion.

3.2 TIGAR translocates to the endoplasmic reticulum and nucleus during cerebral ischemia/reperfusion

Our preliminary data have shown that TIGAR was distributed in ER and the nucleus after cerebral ischemia/reperfusion. We assumed that the cellular distribution of TIGAR is related to its function of inhibiting ER stress. To determine the distribution of TIGAR in the ER and nucleus after cerebral ischemia-reperfusion, we extracted cellular fractions and analyzed the protein levels of TIGAR in whole cell lysate (WCL), cytoplasm, ER and the nucleus with Western blotting. Compared to the sham group, TIGAR expression was significantly upregulated in the ER (Fig. 3a) and nuclear fractions (Fig. 3b) after I/R injury. Immunofluorescence assay showed higher TIGAR intensity in ischemic cortex and HT22 cells suffering from OGD/R or TM- induced ER stress. Intriguingly, the distribution of TIGAR in the ER and the nucleus was significantly increased in neurons exposed to ischemia-reperfusion or ER stress (Fig. 3c, d, Fig. S2). Immunoelectron microscopy assay further confirmed that there were more TIGAR gold particles in the ER and nucleus of neurons in the ischemic cortex of I/R mice than sham animals (Fig. 3e). All these data suggest that TIGAR may translocate to the ER and nucleus during I/R or ER stress.

3.3TIGAR and ATF4 co-localization is increased in the nucleus after cerebral ischemia/reperfusion or ER stress

In the process of ER stress, transcription factor ATF4 translocates to the nucleus to regulate the downstream signals of ER stress[30]. We thus measured the protein levels of TIGAR and ATF4 in whole cell lysate (WCL), cytoplasm and the nucleus with Western blotting. As expected, compared to the non-treatment group, TIGAR expression was markedly upregulated in the nucleus of ischemic cortex and HT22 cells exposed to

TM-induced ER stress (Fig. 3b, Fig. 4a). Importantly, the levels of ATF4 in the nucleus as well as whole cell lysates (WCL) were also significantly increased in the *in vivo* I/R and *in vitro* ER stress models, (Fig. 4b, c). Consistently, immunofluorescence assay showed higher immunoreactivities of TIGAR and ATF4 in neuronal nucleus both *in vivo* and *in vitro* (Fig. 4d, e). Intriguingly, TIGAR and ATF4 showed obvious colocalization in the nucleus exposed to I/R or ER stress. These data suggest that the expression of TIGAR and ATF4 was elevated and translocated to the nucleus during ER stress induced by cerebral ischemia/reperfusion.

3.4 TIGAR interacts with ATF4 to inhibit ER stress during cerebral ischemia/reperfusion

The obvious colocalization of TIGAR and ATF4 in the nucleus make us speculate that TIGAR might interact with ATF4 and affect the downstream target gene transcription of ATF4. The interaction between TIGAR and ATF4 was investigated by co-immunoprecipitation and GST-pull down assay. The data showed that TIGAR and ATF4 were co-immunoprecipitated in the lysates from the ischemic cortex (Fig. 5a, b), and in the HEK293T cells transfected with FLAG-TIGAR (WT) and ATF4 (Fig. 5c, d). Moreover, by using GST-TIGAR or GST combined with glutathione-agarose, the specific interaction between ATF4 and GST-TIGAR in brain lysates was also confirmed in the GST pull-down assay (Fig. 5e).

As an active transcription factor, ATF4 modulates the transcription of*CHOP* and *ATF3* to regulate ER stress[44]. We thus investigated the effect of TIGAR on *ATF4* transcriptional activity by RT-qPCR. In WT mice, cerebral ischemia/reperfusion significantly increased the expression of *Atf4* and *Chop* mRNA in cortex (Fig. S3a, b). Compared with WT mice, the transcription of *Atf4* and *Chop* were decreased in *Tigar* TG mice after I/R. Similarly, in NC cells, TM treatment significantly increased the expression of *Atf4* and *Chop* mRNA (Fig. S3c, d), while overexpression of TIGAR impaired the enhancement of transcription of*Atf4* and *Chop* . We also performed ChIP-RT-PCR in LV-TIGAR-HEK293T cells using anti-FLAG-TIGAR antibody. The data showed an interaction of FLAG-TIGAR with the DNA of *CHOP* and *ATF3*(Fig. 5f), suggesting that TIGAR may bind to ATF4 to regulate the transcription of its downstream genes.

To further determine whether TIGAR regulates ER stress through ATF4, we applied lentivirus- mediated overexpression of ATF4 in HT22 cells (Fig. S1d). In accordance with the previous data, TIGAR overexpression significantly ameliorated TM-induced cell damage and increased cell viability, whereas ATF4 overexpression abolished the protection of TIGAR against TM-induced cell injury (Fig. 5g). Moreover, overexpression of TIGAR decreased TM-induced upregulation of CHOP, c-caspase12 and GRP78, whereas overexpression of ATF4 reversed the effect of TIGAR on CHOP and c-caspase12 (Fig. 5h-j). The above results demonstrate that TIGAR inhibits ER stress-dependent apoptosis by binding to ATF4.

3.5 Translocation of TIGAR to nucleus and inhibition of the transcription of ATF4is independent of its phosphatase activity and mitochondrial localization domains

TIGAR functions as a fructose-2,6-bisphosphatase is to promote the pentose phosphate pathway. However, the mitochondrial localization of TIGAR is independent of the phosphatase activity but is dependent on its C-terminal amino acids of 258-261[24]. To investigate the translocation of TIGAR to the ER and the nucleus in relation to its known functions, we applied a phosphatase functional region mutant (Δtm) and a mitochondrial localization mutant (Δ258-261) of FLAG-TIGAR, as well as FLAG-WT-TIGAR. HT22 cells were transfected with WT, Δtm and Δ258-261 mutants, and then exposed to OGD/R. Immunofluorescence assay showed that in untreated cells, FLAG-TIGAR was mainly distributed in the cytoplasm. The co-localization of FLAG-WT-TIGAR with PDI or DAPI was significantly enhanced after OGD/R, indicating that WT-TIGAR translocated to the ER and nucleus after OGD/R. Interestingly, the ER and nuclear distributions of Δtm and Δ258-261 mutants were not significantly different from WT-TIGAR, indicating that the translocation of TIGAR to ER and nucleus after ischemia/reperfusion is independent of its phosphatase activity and mitochondrial localization domains (Fig. 6a).

We further explored the effect of mutated TIGAR on the *ATF4*transcriptional activity by luciferase reporter assay. As expected, expression of WT-TIGAR reduced the luciferase activity of ATF4 in HEK293T cells treated with TM. Importantly, the luciferase activity of ATF4 was still significantly reduced in the cells transfected with the phosphatase functional region mutant ([?]tm) or mitochondrial localization mu-

tant ([?]258-261) of TIGAR (Fig. 6b). The results suggest that the inhibitory effect of TIGAR on *ATF4* transcription is independent of its phosphatase activity and mitochondrial localization domains.

3.6 TIGAR translocates to nucleus to interact with ATF4 depending on its Q141/K145

To investigate the key domains in TIGAR protein that mediate its translocation to ER and the nucleus and interaction with ATF4, we conducted molecular docking and simulation study using the crystal structures of TIGAR (PDB:3dyc) and ATF4 (PDB:1ci6). The molecular prediction indicated that Gln141, Lys145 of TIGAR may form hydrogen bonds with residues of ATF4 (Fig. S4). In addition,*PredictProtein* (https://predictprotein.org) predicted that the 59-63 of TIGAR might be involved in RNA binding[45]. We thus constructed truncated and mutated TIGAR plasmids, including FLAG-TIGAR ΔN, FLAG-TIGAR ΔC, FLAG-TIGAR Δ59-63AAAAA, FLAG-TIGAR Q141A/K145A (Fig. 7a). Consistent with previous data, WT- TIGAR translocated to the ER and nucleus after OGD/R. The ΔN, ΔC and Δ59-63AAAAA mutants of FLAG-TIGAR also showed significantly increased ER and nuclear localization of FLAG fluorescence after OGD/R. However, the ER and nuclear localization of Q141A/K145A mutant was significantly reduced after OGD/R compared with WT (Fig. 7b). As expected, WT-TIGAR reduced the cleavage of caspase 12 and CHOP upregulation induced by OGD/R, suggesting that TIGAR attenuated ER stress-dependent apoptosis (Fig. 7c, d). However, the Q141A/K145A mutant, but not the ΔN, ΔC and Δ59-63AAAAA mutants, failed to reduce the expression of c-caspase 12 and CHOP, suggesting that Q141/K145 is essential for TIGAR to rescue ER stress.

We further investigated the interaction of TIGAR mutants with ATF4. Consistent with previous results, WT-TIGAR co-immunoprecipitated with ATF4 and suppressed its expression. However, the co-immunoprecipitation of TIGAR and ATF4 was obviously decreased after TM- induced ER stress in the HEK293T cells transfected with Q141A/K145A mutant but not [?]tm and [?]258-261 mutants (Fig. 7e). These results suggest that TIGAR interacts with ATF4 depending on its Q141/K145, but not on the phosphatase activity and mitochondrial localization domains. All these results suggest that TIGAR translocates to the ER and the nucleus and interacts with ATF4 to reduce ER stress depending on its Q141/K145.

4. Discussion

The known function of TIGAR is to promote pentose phosphate pathway (PPP) via its Fru-2, 6-BPase activity, resulting in the production of NADPH and ribose 5-phosphate (R-5-P). NADPH maintains the reduced GSH, thioredoxin ($Trx(SH)_2$) and glutaredoxin, which contribute to scavenging ROS and preventing protein thiol oxidative damage[19, 20]. Our laboratory has previously demonstrated that the neuroprotection of TIGAR in cerebral ischemia and preconditioning is partly related to its antioxidative and anti-inflammatory effects[18, 21, 23, 46]. In the present study, we further investigated the neuroprotective mechanism of TIGAR regulating ER stress in cerebral ischemia/reperfusion. The results showed that TIGAR translocates to the ER and the nucleus and interacts with ATF4 to reduce the ER stress-dependent apoptosis, thus alleviating neuronal injury. This study revealed a new mechanism of TIGAR's neuroprotective action via regulating ATF4- mediated ER stress signaling.

Cerebral ischemia/reperfusion injury is a complex cascade involving oxidative stress[47], calcium overload[48], mitochondrial dysfunction[49], inflammatory reaction[50], blood brain barrier destruction[51]and neuronal apoptosis[15]. Recent studies have shown that ER stress is closely related with cerebral ischemia/reperfusion injury[52, 53]. In the context of ER stress, UPR is activated due to the aggregation of unfolded and misfolded proteins in the ER, which is triggered through signal proteins such as PERK, IRE1α and ATF6, to restore ER homeostasis[2]. In the early stage of ER stress, the function of ER can be recovered, while enduring and severe ER stress may induce apoptosis[54]. The expression of CHOP began increasing 3 h after cerebral ischemia/reperfusion and reached its peak at 24 h, which then mediated neuronal apoptosis. Hence, inhibition of CHOP shows a neuroprotective effect against cerebral ischemia/reperfusion injury[55]. In addition, caspase12 is also involved in ER stress-dependent apoptosis during cerebral ischemia[56]. Consistent with these reports, we found that ER stress related proteins increased after I/R. Importantly, TIGAR and ER stress responses were simultaneously upregulated during I/R. However, overexpression of TIGAR can

reverse the upregulated levels of ER stress-related proteins, and rescue ER stress-dependent apoptosis both *in vivo* and*vitro* . Moreover, the ER swelling with protein aggregates in ER lumen of neurons was more obvious in *Tigar* Nes-cKO mice after I/R, while this phenotype was significantly improved in *Tigar* TG mice. All these data demonstrate that TIGAR can inhibit ER stress-induced neuronal apoptosis during cerebral ischemia/reperfusion.

As previously reported, TIGAR is widely distributed in cells including mitochondria, ER, cytoplasm and nucleus[18]. Intriguingly, this study found that TIGAR could translocate to the ER and nucleus during I/R or ER stress. We extracted cellular components and found that the expression TIGAR protein in the ER and nucleus was significantly upregulated after I/R. Moreover, there is a distinct ER and nuclear relocalization of TIGAR in neurons subjected to I/R or ER stress. We thus hypothesized that the downstream effect of TIGAR's ER and nuclear localization may be related to its inhibitory effects on ER stress-dependent apoptosis.

ATF4, also known as cAMP response element binding protein 2 (CREB-2), is a member of ATF/CREB transcription factor family of basic leucine zipper domain proteins. In the ER stress pathway, ATF4 is an activating transcription factor of the downstream of PERK-eIF2α[57]. ATF4 translocates to the nucleus by its nuclear targeting KKLKK signal located within its basic region to regulate downstream genes expression such as *ATF3* , *CHOP* , cystine/glutamate transporter, asparaginase, hair bulb homologous body 3 (*TRB3*), etc[44, 58-61]. In accord with previous research, the present study found that the expression of ATF4 protein was low in the non-stress state, but significantly increased after I/R or ER stress. The transcriptome analysis showed that the*ATF4 -CHOP* pathway was activated after cerebral ischemia/reperfusion. In addition, nuclear extraction analysis found that the nuclear translocation of TIGAR and ATF4 occurred simultaneously. Importantly, there was an obvious colocalization of TIGAR and ATF4 in the nucleus after I/R or ER stress. Co-immunoprecipitation and GST-pull down assay further verified that TIGAR can interact with ATF4. ChIP assay also demonstrated that TIGAR may interact with the downstream genes of ATF4, and TIGAR can inhibit the transcriptional activity of *ATF4* after I/R or ER stress. Intriguingly, exogenous ATF4 negated the effect of TIGAR on ER stress-induced neuronal apoptosis. All these results indicate that TIGAR may interact with ATF4 to inhibit its transcriptional activity and downstream gene expression, thereby alleviating ER stress-dependent apoptosis.

In addition to maintaining metabolic homeostasis of cells by Fru-2, 6-BPase activity, TIGAR can also regulate some signaling proteins through non-enzymatic action. Specifically, TIGAR can translocates to mitochondria to interact with HK2, thus reducing mitochondrial ROS and promoting cell survival during hypoxia[24]. And the mitochondrial localization of TIGAR is independent of the phosphatase activity but is dependent on its C-terminal amino acids 258-261[24]. Interestingly, we found that TIGAR translocated to the ER and nucleus and inhibited the transcriptional activity of *ATF4* independently of its phosphatase activity and mitochondrial localization domains.

We next predicted through molecular docking simulation that TIGAR protein may bind to ATF4 via its Q141/K145 residues. These amino acids do not belong to phosphatase activity domain and two conserved pockets that can mediate dephosphorylation by binding to phosphate molecules on the substrate[62], suggesting that TIGAR may inhibit ER stress and promote cell survival through a non-enzymatic function. Importantly, the TIGAR Q141A/K145A mutation, instead of ΔN, ΔC, Δ59-63AAAAA mutations, abolished the TIGAR's translocation to the ER and nucleus and the interaction with ATF4. These results indicate that the Q141/K145 residues of TIGAR protein is required for its translocation to ER and nucleus and interaction with ATF4. Previous studies have shown that ATF4 protein enters the nucleus to bind and regulate the expression of downstream target genes through its DNA binding region (280-299 amino acid), while malfunction of its DNA-binding region blocks the downstream expression of ATF4[58]. Intriguingly, molecular docking data also showed that the binding to Q14/K145 of TIGAR was L281/L285 belonging to the DNA-binding domain of ATF4. This may further suggest that TIGAR binds to ATF4 to inhibit its downstream target gene expression.

In conclusion, this study found that TIGAR translocates to the ER and the nucleus to interact with ATF4

after cerebral ischemia-reperfusion induced ER stress via its Q141/K145. Then TIGAR inhibits the expression of ATF4 downstream pro-apoptotic genes, reduces ER stress-dependent apoptosis, and thereby alleviates neuronal damage. This study expands the mechanism of the neuroprotective effect of TIGAR in cerebral ischemia-reperfusion injury. The Q141/K145 structure is critical for the interaction of TIGAR with ATF4 and inhibition of ATF4 target genes.

Author contributions

R.S. conceived and supervised the study. L.C., J.T., Q.Q.L., Y.Y.L., and J.Y.L. carried out the experiments. L.C., J.T. and Q.Q.L. collected, analyzed and interpreted the data. L.C. wrote the manuscript. Z.H.Q., W.H.Z. and R.S. revised the manuscript. All authors discussed the results and commented on the manuscript.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

Declaration of interest

The authors declare no conflict of interest.

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Figure 1. ER stress-related proteins and TIGAR protein are increased synchronously after cerebral ischemia/reperfusion (I/R) or ER stress. Primary neurons (a) and HT22 neuronal cells (b) were subjected to oxygen glucose deprivation and reperfusion (OGD/R). Cells were harvested at indicated

time after reperfusion. (c) Mice were subjected to tMCAO/R (I/R) and cortex were dissected at indicated time after reperfusion. (d) ER stress related *Atf4-Chop* pathway and the downstream genes increased significantly after cerebral ischemia/reperfusion (I/R). Mice were subjected to tMCAO/R and cortex were dissected 6 h after reperfusion. The gene expression of ER stress related *Atf4-Chop* pathway and the downstream genes were analyzed by transcriptome sequencing. (e-f) HT22 cells were treated with tunicamycin (TM 12 μM, d) or thapsigargin (Tg 1 μM, e) to induce ER stress. Cells were harvested at indicated time. Protein levels were measured by Western blotting. n = 3 independent neuronal cultures with 2-3 replicates each *in vitro* or n = 3-6 mice *in vivo* . Bar represents mean ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ v.s. the control or sham group.

Figure 2. TIGAR inhibits ER stress-induced neuronal apoptosis during cerebral ischemia/reperfusion. (a-c) Wild-type (WT) and TIGAR transgenic mice (TG) were subjected to tMCAO/R (I/R) and cortex were dissected 6h after reperfusion. (d-g). LV-vector-HT22 (NC) or LV-TIGAR-HT22 (OV) cells were treated with tunicamycin (TM, 12 μM) to induce ER stress and harvested at 12 h after treatment. Protein levels were measured with Western blotting. Note that overexpression of TIGAR can reverse the increase of GRP78 (a, d), CHOP (b, e), c-caspase12 (c, f) and ATF4 (g) *in vivo* and *in vitro* . (h) Cell viability was examined with Cell Counting Kit-8 assay. (i, j) Apoptosis was analyzed using flow cytometry, showing that overexpression of TIGAR can rescue neuronal apoptosis induced by ER stress. (k) Wild-type (WT), TIGAR transgenic mice (TG) or neuron-conditional TIGAR knockout mice (Nes-cKO, *Nestin-Cre* , $Tigar^{flox/flox}$) were subjected to tMCAO/R and cortex were dissected 6 h after reperfusion. The ultrastructure was examined with electron microscope. The bottom row shows the enlarged endoplasmic reticulum (ER) or mitochondria (M) taken from the boxed areas. N: nucleus. Scale bar = 1 μm or 500nm. n = 3-6 mice or 3 independent neuronal cultures with 2-3 replicates each. Bar represents mean ± S.D. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 3. TIGAR translocates to the ER and nucleus during cerebral ischemia/reperfusion. Mice were subjected to tMCAO/R and cortex were dissected. HT22 cells were subjected to oxygen glucose deprivation and reperfusion (OGD/R). (a, b). Endoplasmic reticulum (ER) fractions, whole cell lysate (WCL), nucleus and cytoplasm were extracted and subjected to Western blot analysis. (c, d). The brain sections or cells were labeled with the anti-TIGAR antibody (green), anti-PDI antibody (red) and DAPI (blue) to visualize TIGAR in ER and nucleus. Scale bar = 10 μm. Pearson correlation coefficients were calculated from these cells. N = 40 cells from three independent experiments. (e) Brain cortical tissues were stained with immunogold and examined with electron microscopy. Microphotographs showed immunoreactive elements (colloidal gold particles, red arrowheads) in the endoplasmic reticulum (ER) and nucleus (N). Scale bar = 200 nm. n = 3-6 mice or 3 independent neuronal cultures with 2-3 replicates each. Bar represents mean ± S.D. ** $P < 0.01$, *** $P < 0.001$.

Figure 4. TIGAR and ATF4 co-localization is increased in the nucleus after cerebral ischemia/reperfusion or ER stress. (a, c, e) HT22 cells were treated with tunicamycin (TM, 12 μM) to induce ER stress and handled at 12 h after treatment. (b, d) Mice were subjected to tMCAO/R (I/R) and cortex were dissected 6 h after reperfusion. (a, b, c) Nucleus, cytoplasm and whole cell lysate (WCL) were extracted and subjected to Western blot analysis. (d, e) The brain sections or cells were labeled with the anti-TIGAR antibody (red), anti-ATF4 antibody (green) and DAPI (blue). The immunofluorescence was examined in the peri-infarct region of cortex. Insets were the enlarged cells with obvious colocalization of TIGAR and ATF4 in the nucleus. Pearson correlation coefficients were calculated from these cells. N = 40 cells from three independent experiments. Scale bar = 10 μm. n = 3-6 mice or 3 independent neuronal cultures with 2-3 replicates each. Bar represents mean ± S.D. ** $P < 0.01$, *** $P < 0.001$.

Figure 5. TIGAR interacts with ATF4 to inhibit ER stress during cerebral ischemia/reperfusion. Mice were subjected to tMCAO/R (I/R) and cortex were dissected 6 h after reperfusion. (a, b) The brain lysates were immunoprecipitated with anti-ATF4 (a) or anti-TIGAR (b) antibodies. HEK293T cells were harvested at 24 h after transfected with p3×FLAG-TIGAR and ATF4. (c, d) The cellular lysates were immunopreciptitates with anti-ATF4 (c) or anti-FLAG (d) antibodies. Then total lysates

and immunoprecipitates were analyzed by Western blotting with anti-FLAG or anti-ATF4. (e) GST-TIGAR fusion protein or GST bound to glutathione-agarose were incubated with lysates of brain, and analyzed by Western blotting with anti-ATF4. (f) ChIP assay was performed by using anti-FLAG in LV-TIGAR-HEK293T cells. IgG served as a negative control. DNA enrichment of ChIP sample were measured with RT-qPCR using *CHOP* and *ATF3* primer. (g) Exogenous ATF4 negated the protective effect of TIGAR on tunicamycin (TM) induced cellular injury in HT22 cells. Control or LV-TIGAR stabled HT22 cells were infected with LV-ATF4 or LV-vector for 72 h. Then the cells were treated with TM (12 μM). The cell viability was examined with Cell Counting Kit-8 assay. (h-j) Exogenous ATF4 recovered the reduction in CHOP and c-caspase12 but not GRP78 upon TIGAR overexpression after TM treatment. Protein levels of CHOP (h), caspase12 (i) and GRP78 (j) were measured with Western blotting. n = 3-6 mice or 3 independent cell cultures with 2-3 replicates each. Bar represents mean ± S.D. * $P< 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 6. Translocation of TIGAR to the ER and nucleus and inhibition of the transcription of *ATF4* is independent of its phosphatase and mitochondrial localization domains. (a) HT22 cells were transfected with FLAG-WT-TIGAR, FLAG -TIGAR Δtm (H11A/E102A/H198A) or FLAG -TIGAR Δ258-261 plasmids before subjected to OGD/R. Cells were labeled with the anti- FLAG (green), anti-PDI antibody (red) and DAPI (blue). Scale bar = 5 μm. The FLAG-ER and FLAG-nucleus colocalization were quantified as Pearson's correlation coefficients of images. N = 40 cells from three independent experiments. (b) Analysis of ATF4 luciferase activity in HEK293T cells. HEK293T cells were co-transfected with ATF4 5'-UTR and FLAG-WT-TIGAR, FLAG -TIGAR Δtm or FLAG -TIGAR Δ258-261 plasmids for 24h. Then the cells were treated with TM to induce ER stress. n = 3 independent neuronal cultures with 3 replicates each. Bar represents mean ± S.D. * $P < 0.05$, *** $P< 0.001$.

Figure 7. TIGAR translocates to the nucleus to interact with ATF4 depending on its Q141/K145. (a) Schematic diagram of TIGAR and its mutants. (b-d) HT22 cells were transfected with FLAG-TIGAR ΔN, FLAG-TIGAR ΔC, FLAG-TIGAR Δ59-63AAAAA or FLAG-TIGAR Q141A/K145A plasmids before subjected to OGD/R. (b) Cells were labeled with the anti-FLAG (green), anti-PDI antibody (red) and DAPI (blue). Scale bar = 5 μm. The FLAG-ER and FLAG-nucleus colocalization were quantified as Pearson's correlation coefficients of images. N = 40 cells from three independent experiments. (c, d) The protein levels of CHOP and c-caspase 12 were measured by Western blotting. (e) HEK293T cells were transfected with Flag-WT-TIGAR, Flag-TIGAR [?]tm, Flag-TIGAR [?]258-261 or FLAG-TIGAR Q141A/K145A plasmids before treatment with TM. The cellular lysates were immunoprecipitated with anti-IgG or anti-Flag antibodies. Then total lysates and immunoprecipitates were analyzed by Western blotting with anti-Flag or anti-ATF4. n = 3 independent neuronal cultures with 3 replicates each. Bar represents mean ± S.D. ** $P < 0.01$, *** $P < 0.001$.