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Supplemental Information

GTF2H4 regulates partial EndMT via NF-κB activation through NCOA3 phosphorylation in ischemic diseases

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MATERIALS AND METHODS

Cell culture and chemical treatments

Human microvascular endothelial cells (HMEC-1) were obtained from Zhongqiaoxinzhou Biotechnology (Shanghai, China) and cultured in MCDB 131 medium (Sigma-Aldrich, cat#M8537), supplemented with 10% FBS (BI, cat#04-001-1A), 10 ng/mL epidermal growth factor (Zhongqiaoxinzhou, cat#CSP029), 1 μg/mL hydrocortisone (MCE, cat#HY-N0583) and 100U/mL penicillin-streptomycin (Gibco, cat#15140122) in a 37℃ humidified atmosphere of 5% CO_2. Mouse cardiac microvascular endothelial cells (MCMECs) were isolated from 1-week-old male Sprague Dawley (SD) rats following the procedure as previously reported[1]. Briefly, the pure ventricular tissues of rats were split into 1 mm^2 pieces before uniformly coating with FBS in dishes. After the incubation for 4 h, it was supplemented by high-glucose DMEM (Gibco, cat#11965092) containing 10% FBS. Then the pieces were removed after another 72 h of incubation, and the MCMECs were passaged. Both HMEC-1 and MCMECs were passaged 2-6 times prior to use. For hypoxic treatment accompanied by serum deprivation, culture dishes were placed in a humidified airtight chamber with inflow and outflow valves to control the inflow and retention of hypoxic gas mixture (5% CO_2, 1% O_2, and balance N_2) after a change for serum-free medium. Then the hypoxic cells were cultured in a 37 °C incubator for 1-3 days, while cells in the normoxic group were kept in an incubator containing 5% CO_2 and 21% O_2 after a change to fresh medium. 293T cells were purchased from Zhongqiaoxinzhou Biotechnology (Shanghai, China) and cultured in high-glucose DMEM containing 10% FBS and 100U/mL penicillin-streptomycin. MG132 (Sigma-Aldrich, cat#M7449) was stored in 10mM DMSO solution. Cycloheximide (Aladdin, cat#C112766) and 3-MA (MCE, cat#HY-19312) were stored in powder form and diluted in the medium when it was used. TNF-α (cat#abs04232) and Bay 11-7082 (cat#abs810013) were purchased from Absin (Shanghai, China). Bay 11-7082 was dissolved in DMSO as 50mM solution for stock, and TNF-α was dissolved in PBS as 10μg/mL. All the stock solutions were stored at -20 ℃ before use.

RNA extraction and real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA from cells was extracted with TRIzol (Invitrogen, cat#15596018) based on the manufacturer's instructions. By using PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, cat#RR047A), the genomic DNA was digested by gDNA Eraser, and then endogenous cDNA was generated by reverse-transcription of RNAs. For quantitative PCR analysis, aliquots of cDNA were amplified by using EXPRESS SYBR™ GreenER™ qPCR Supermix (Thermo, cat#11784200) on a CFX96 real-time Polymerase chain reaction (PCR) System (Bio-Rad Laboratories, Inc., CA, USA). 2-△△Ct method was employed to evaluate the relative expression of corresponding genes normalized to β-actin. The primer sequences used were as follows:

α-SMA, forward: 5'-CCGACCGAATGCAGAAGG-3', reverse: 5'-ACAGAGTATTTGCGCTCCGAA-3';

CD31, forward: 5'-AAGGAACAGGAGGGAGAGTATTA-3', reverse: 5′-

GTATTTTGCTTCTGGGGACACT-3';
VE-Cadherin, forward: 5'-GCACCAGTTTGGCCAATATA-3', reverse: 5'-GGGTTTTTGCATAATAAGCAGG-3';
GTF2H4, forward: 5′-GGTATTGGACCGATTGTATG-3′, reverse: 5'-CTTTCCTCCTGAGCCTTG-3';
β-actin, forward: 5'-GTTGTCGACGACGAGCG-3', reverse: 5'-GCACAGAGCCTCGCCTT-3'.

Western blot analysis

Protein lysates of cells or tissues were extracted by using RIPA (Beyotime, cat#P0013C) with PMSF (Beyotime, cat#ST506) or protease/phosphatase inhibitor cocktail (Beyotime, cat#P1010). Then equal amounts of protein lysates were separated by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Merck, cat#ISEQ00010). Blocked with 5% non-fat milk with TBST at room temperature for 1 h, then the membranes were sequentially incubated with primary antibodies at 4 °C overnight and secondary antibodies at room temperature for 1 h, separately. The proteins were visualized by using Immobilon Western HRP Substrate (Merck, cat#WBKLS0050). The density of protein bands was evaluated through NIH ImageJ software (1.50i, USA) normalized to GAPDH or β-actin.

Double immunofluorescence staining of HMEC-1

After exposure to hypoxia, HMEC-1 incubated in glass bottom cell culture dish (Nest, cat#801001) were washed with phosphate-buffered saline (PBS) (Gibco, cat#10010023) 3 times, fixed in 4% paraformaldehyde (Boster, cat#AR1068) at room temperature for 20 minutes, and then permeabilized with 0.5% Triton X-100 (Solarbio, cat#T8200) for 20 min at room temperature. After being blocked with 5% BSA for 1 h, HMEC-1 was incubated with a monoclonal mouse anti-CD31 antibody (CST, cat#3528), monoclonal rabbit anti-α-SMA antibody (Abcam, cat#ab5694) or anti-VE-Cadherin antibody (CST, cat#2500S) overnight at 4°C. Cells were subsequently incubated with Alexa Fluor™ 488-conjugated goat anti-mouse IgG (Thermo, cat#A-11001), Alexa Fluor™ 594-conjugated goat anti-rabbit IgG (Thermo, cat#A-11012), or phalloidin-Alexa Fluor™ 594 (Beyotime, cat#C2205S), and nuclei were stained with DAPI (Beyotime, cat#C1005). Cells were visualized under a fluorescence microscope (Olympus), and fluorescence intensity was evaluated by using NIH ImageJ software (1.50i, USA).

Bioinformatics analysis of public database

The GEO database was utilized to explore the role of GTF2H4 in patients with ST-segment elevation myocardial infarction (STEMI) and post-PCI. GSE61144 Series were downloaded from the GEO database (http://www.ncbi.nlm.nih.gov/geo/). Gene expression values were log2 transformed and normalized by quantile normalization. Then the expression of GTF2H4 was analyzed in different tissues of the cardiovascular system, and age-associated differential expression was also examined by using the Genotype-Tissue Expression (http://gtexportal.org/) database.

Lentivirus construction and infection

GTF2H4 overexpression and GTF2H4-shRNA (short hairpin RNA) lentiviral particles and their lentiviral control particles were constructed by Hanbio Biotechnology (Shanghai, China). The sequence of shGTF2H4 was as followed:
forward primer: 5'-GATCCGTAGCTCTGTGGGTAAAGATTCAAGAGATCTTTACCCACAGAGCTACTTTTTTG-3'
and reverse primer: 5'-AATTCAAAAAAGTAGCTCTGTGGGTAAAGATCTCTTGAATCTTTACCCACAGAGCTACG-3'.
Similarly, ERCC3 overexpression and ERCC3-shRNA lentivirus were purchased from Hanbio Biotechnology (Shanghai, China). The sequence of shERCC3 was as followed:
forward primer: 5'-GATCCGCCAAGACTTCTTGGTGGCTATTGCACTCGAGTGCAATAGCCACCAAGAAGTCTTGGTTTTTTG-3'
and reverse primer:5'- AATTCAAAAAACCAAGACTTCTTGGTGGCTATTGCACTCGAGTGCAATAGCCACCAAGAAGTCTTGGCG-3'.
GTF2H4 mutation (E310K/R314E) overexpression lentivirus were also constructed after site-directed mutagenesis of GTF2H4 plasmid. Then HMEC-1 and 293T cells were infected by these lentiviruses to establish stable cell lines, while the control cell lines were infected by the respective control lentivirus. To establish the stable HMEC-1 combined with GTF2H4 overexpression and ERCC3 knockdown, HMEC-1 cells were co-infected by the two corresponding lentiviruses. Meanwhile, their control cell lines were constructed.

Cell viability assay and cell apoptosis analysis

The Cell counting kit-8 (CCK-8) (Beyotime, cat#C0043) assay was performed to detect the cell viability based on manufacturers' instructions. In brief, after being infected with GTF2H4 lentivirus, HMEC-1 was seeded into a 96-well plate at 5000 cells/well density with 100 μl of complete growth medium. After 24 h, cells were cultured in hypoxic conditions for 3 days after a change to a serum-free medium. Subsequently, 10 μl of CCK-8 solution diluted in 100 μl of complete culture medium replaced the original medium of each at different time points (1, 2, 3 d). After another 2 h incubation, the absorbance at 450 nm was measured by a microplate reader (Synergy H4; BioTek Instruments, Inc., USA). For apoptosis analyses, HMEC-1, either in normoxic or hypoxic conditions for 3 days was washed twice with PBS and digested by trypsin (Gibco, cat#15050057). After being resuspended at a concentration of 10^6 cells/ml in 1X Annexin V Binding Buffer (BD Biosciences, cat#556547), cells in 100 μl solution were transferred to a 5-ml culture tube prior to 5 μl of FITC Annexin V and 5 μl of Propidium Iodide Staining Solution (BD Biosciences, cat#556547) staining for 15min. Stained cells were then analyzed by flow cytometry (FACSCanto II; BD, San Jose, CA, USA).

Scratch assays and transwell assays

In the scratch (wound-healing) assay, lentivirus-mediated GTF2H4 overexpression / knockdown HMEC-1 cells were seeded in a 6-well plate with a whole cell confluence, separately. After the scratch wounds were made by scraping the cell monolayer across

each culture plate with a 100 μL pipette tip and the debris removed by PBS washing, wounded cultures were incubated in a serum-free medium for an additional 24 h. The same area of the scratch wound in each group was visualized by microscopy to assess cell migration ability by using ImageJ software. The transwell migration assay was performed by using a 6.5 mm Transwell® with 8.0 μm pore polycarbonate membrane insert (Corning, cat#3422). In brief, after being exposed to normoxia or hypoxia for 3 days, cells were digested by EDTA-trypsin (Gibco, cat#25200072) and suspended in a serum-free MCDB 131 medium. Then, 200 μl (1.5×10^4 cells/well) of the cell suspension was placed onto the upper chambers, while the lower chambers were filled with 600 μL of MCDB 131 medium supplemented with 10% FBS. After incubation for 24 hours, the non-migrating cells in the upper chamber were removed with a cotton swab and migrated cells on the underside of the membrane were fixed with 4% paraformaldehyde (PFA) for 30 minutes and subsequently stained with 0.1% crystal violet (Sangon, cat#A100528) for 10 minutes. The migrated cells were photographed by microscopy and counted.

Aortic ring assay

6-week-old male C57 mice were used for aortic ring assay following the procedure as previously reported[2]. Briefly, the aorta was cut from the rat after cervical dislocation killing and transferred into Petri dish containing Opti-MEM (Gibco, cat#31985070). After removing extraneous tissues and fat, the aorta was cut into 0.5-1.0 mm rings with a scalpel. Then aortic rings were transferred into a 24-well plate (20 per well) containing 1ml of Opti-MEM and were infected by GTF2H4 overexpression or control adeno-associated virus (AAV). After serum-starve culture at 37 °C and 5% CO_2 overnight, the aortic rings were embedded in a 96-well plate (1 per well) coated with1 mg/mL type I collagen (Absin, cat#abs47014921) matrix (diluted in 1X DMEM). Afterward, aortic rings were fed with MCDB 131 complete medium and incubated under the hypoxic condition at 37°C for 5 days. Finally, aortic rings were stained by Thiazolyl Blue (MTT), and the sprouting vessels were visualized by microscope. The number of sprouts per ring was evaluated by using NIH ImageJ software. All animal experiments in this study were approved by the Ethics Committee of Zhongshan Hospital, Fudan University, China.

Tube formation assay

HMEC-1 in each group was cultured under normoxia or hypoxia for 3 days. The cells subsequently were digested by EDTA-trypsin and suspended in MCDB 131 complete medium. Meanwhile, a 96-well plate coated with 50 μL Matrigel Matrix (Corning, cat#356234) per well was allowed to polymerize at 37°C for 30 min. Afterward, 100 μl (3×10^4 cells/well) of the cell suspension were seeded into the plate and cultured for 6 h. A microscope visualized enclosed capillary networks of tubes. And the number of junctions or total length was measured by using NIH ImageJ software.

4D-label-free proteomics analyses

Lentivirus-mediated GTF2H4 overexpression or knockdown HMEC-1 and their control

cell lines were prepared for protein extraction, digestion, and SDS-PAGE electrophoresis. With the technical assistance of Shanghai Applied Protein Technology (Shanghai, China), liquid chromatography-tandem MS (LC-MS/MS) analysis was performed on a timsTOF Pro mass spectrometer (Bruker) that was coupled to Nanoelute (Bruker Daltonics). The mass spectrum data for identification and quantitation analysis were searched by MaxQuant (version 1.5.3.17) software. The filtering criteria of fold change > 2.0 and *P* value < 0.05 were adopted to identify differentially expressed proteins (DEPs) in pairwise comparison, and these proteins were identified in at least two of three replicates in one group.

Co-immunoprecipitation (Co-IP) assays

In co-immunoprecipitation (Co-IP) assays, 500 μg of HMEC-1 or 293T cell lysates were incubated with anti-HA magnetic beads (Thermo, cat#88838), anti-Flag magnetic beads (MCE, HY-K0207), or anti-His magnetic beads (CST, cat#8811S) overnight at 4°C with gentle rotation. The beads were washed three times with lysis buffer before incubation with cell lysates, and the immunoprecipitation complexes were boiled with loading buffer (Beyotime, cat#P0015) for 10 min and then subjected to SDS-PAGE. The samples were transferred onto PVDF membranes and used for immunoblotting analysis. The enhanced chemiluminescence (ECL) signals detection was performed as previously described.

4D-label-free phosphorylation proteomics analyses

Lentivirus-mediated GTF2H4 overexpression or knockdown HMEC-1 and their control cell lines were prepared for protein extraction, digestion and SDS-PAGE electrophoresis. The phosphopeptide enrichment was performed by using High Select™ Phosphopeptide Enrichment Kits (Thermo, cat#A52283) according to the manufacturer's instructions. After lyophilization, the phosphopeptide peptides were resuspended in 20 μL of 0.1% formic acid for MS analysis. With the technological assistance of Shanghai Applied Protein Technology (Shanghai, China), liquid chromatography-tandem MS (LC-MS/MS) analysis was performed on a timsTOF Pro mass spectrometer (Bruker) that was coupled to Nanoelute (Bruker Daltonics) for 60 min. The mass spectrum data were searched by MaxQuant software for identification and quantitation analysis. The filtering criteria of fold change > 2.0 and *P* value < 0.05 were adopted to identify differential phosphopeptides in pairwise comparison, and these peptides were identified in at least two of three replicates in one group.

NF-kB II signaling phospho antibody array analysis

Two hundred fifteen site-specific phosphoprotein array profiles of NF-κB-related proteins were detected with 6 replicates each by using an NF-kB II Signaling Phospho Antibody Array (Full Moon BioSystems, cat#PNK215). Lentivirus-mediated GTF2H4 overexpression or knockdown HMEC-1 and their control cell lines were cultured in the serum-free medium under hypoxia for 3 days. After being washed with cold PBS, cells were lysed in an extraction buffer. The lysis was then purified, and the extracted proteins were labeled by biotinylation. Afterward, the antibody microarray was blocked

for 45 min and was incubated with the biotin-labeled protein samples. With the addition of Cy3-streptavidin, the fluorescence of conjugation-labeled protein in the antibody array slides was detected with a microarray scanner (Axon Instruments, GenePix 4000B). Then GenePix Pro 6.0 software (Axon Instruments, USA) was used to analyze the results which were presented as the ratio of phosphorylation/unphosphorylation.

Luciferase reporter assay

In the Luciferase reporter assay, NF-κB luciferase reporter plasmid (pGMNF-KB-Lu) and Renilla luciferase reporter plasmid (pRL-TK) were obtained from Genomeditech (Shanghai, China). Among them, as an internal control vector, pRL-TK was used to normalize the variations in transfection efficiency. After lentivirus-mediated GTF2H4 or ERCC3 overexpression/knockdown performed in 293T cells, cells were seeded in 24-well plates and grew to approximately 70% confluency. Then transfection of pGMNF-KB-Lu (75ng/well) and pRL-TK (25ng/well) was performed by using Lipofectamine 3000 transfection reagent (Invitrogen, cat#L3000075) based on manufacturer's recommendations. To determine the role of SRC-3 and site-specific phosphorylation, 293 cells were co-transfected with luciferase reporter plasmids and the indicated expression plasmids. After 48 h post-transfection, TNF-α (10ng/mL) was added to stimulate NF-κB activation for 4h. Afterward, cells were harvested for luciferase activity detection with Dual-Luciferase® Reporter Assay System (Promega, cat#E1910) according to the manufacturer's instructions.

Nuclear protein extraction and electrophoretic mobility shift assay (EMSA)

The wild-type HMEC-1 or GTF2H4-associated stable cell lines were cultured in 6-cm dishes under the hypoxic condition in a time gradient. Once they were harvested, nuclear proteins were extracted from the cells according to the manufacturer's instructions for NE-PER nuclear and cytoplasmic extraction reagents (Thermo, cat#78833), and protein concentration was determined by a BCA Protein Assay Kit (Beyotime, cat#P0010). In electrophoretic mobility shift assay (EMSA), the biotin-labeled NF-κB consensus oligonucleotide sequences were used as the probe. The sequence of the NF-kB probe was as follows: 5′-AGTTGAGGGGACTTTCCCAGGC-3′ and 3′-TCAACTCCCCTGAAAGGG TCCG-5'. Then 5 μg of extracted protein extraction was incubated with NF-kB probe for 20min at room temperature, while the negative control reaction contained no nuclear extracts, and the cold competition reaction was supplemented with the unlabeled probe. The DNA-protein complexes were loaded on 4% polyacrylamide gels and electrophoretically separated in 0.5X Tris-borate (TBE) buffer at 100 V for 50 min. After that, the protein and nucleic acid mixture were transferred onto nylon membranes with 0.5 X TBE buffer at 300 mA for 40 min. The transferred DNA in the membrane was cross-linked for 20 min under UV light, blocked with 2000:1 streptavidin-HRP conjugate diluted blocking solution, and finally visualized by using BeyoECL Moon chemiluminescence reagent (Beyotime, cat#GS009).

Site-directed mutagenesis of NCOA3

The pCDNA3.1-Myc-HisA-NCOA3 plasmid was constructed by Tsingke Biotechnology (Beijing, China). To perform site-directed mutagenesis, 2 × Phanta® Max Master Mix (Vazyme, cat#P515) was used to extend the DNA strand corresponding to the SRC-3 mutants by using pCDNA3.1-Myc-HisA-NCOA3 as a template. The primers containing the mutations (S1330A, S1330D, and S1330E) were synthesized by Sangon Biotech (Shanghai, China). The primer sequences used were as follows:
S1330A, forward: 5'- GGCAGAGTGAGCGCGCCCCCTAACGC -3', reverse: 5'-AGGGGGCGCGCTCACTCTGCCAAAAGC -3';
S1330D, forward: 5'- GAGTGAGCGACCCCCCTAACGCCATG -3', reverse: 5′-GTTAGGGGGGTCGCTCACTCTGCCAAAAG -3';
S1330E, forward: 5'- GTGAGCGAACCCCCTAACGCCATGATG -3', reverse: 5'-GCGTTAGGGGGTTCGCTCACTCTGC -3'.
The amplification products were digested with DpnI (NEB, cat#R0176V) and underwent homologous recombination in a head-to-tail fashion by using ClonExpress II One Step Cloning Kit (Vazyme, cat#C112-01). Plasmid DNA was then extracted, and Sanger sequencing was performed by Sangon Biotech (Shanghai, China) to confirm all mutations.

Gastrocnemius muscle injection with adeno-associated virus (AAV)
To achieve the endothelia-specific GTF2H4 overexpression and knockdown in mouse gastrocnemius muscle, AAV-Tie-ZsGreen (negative control), AAV-Tie-GTF2H4-ZsGreen, AAV-Tie-EGFP (negative control) and AAV-Tie-shGTF2H4-EGFP viruses were purchased from Hanbio Biotechnology (Shanghai, China). Before intramuscular injection into 3-week-old male mice, the hair of the hind-limb was removed. Then 50 μl (1.0 × 1012 vg/mL) of AAV-GTF2H4 OE, AAV-shGTF2H4 or their control AAV were respectively injected into the gastrocnemius muscle in the left hind-limb of each mouse. The sequence of shGTF2H4 was as followed:
forward primer: 5'- GATCCGTTCTCCGAACGTGTCACGTAATTCAAGAGATT ACGTGACACGTTCGGAGAATTTTTTC-3'
and reverse primer: 5'- AATTGAAAAAATTCTCCGAACGTGTCACGTAATCTCTT GAATTACGTGACACGTTCGGAGAACG -3'.
The overexpression or knockdown efficiency in endothelial cells of the gastrocnemius muscle was confirmed by western blot 7 days after hind-limb ischemia.

The hind-limb ischemia surgery and the blood flow recovery scan assay
The protocol for the animal study was in accordance with our institutional guidelines and approved by the Institutional Animal Care and Use Committee at Zhongshan Hospital, Fudan University. Approximately 4 weeks after gastrocnemius muscle injection with AAV, all C57 mice (male, 8weeks) were anesthetized with 1% sodium pentobarbital 50mg/kg intraperitoneally, and the hair of the hind-limb was removed. Fixed in the supine position, the mouse was prepared to undergo a left hind-limb ischemia surgery. The surgery started with a ∼10 mm incision in the groin to expose the femoral artery, then the femoral vein and nerve were separated from the femoral

artery. After that, a ligation proximal to the outlet of the profundal femoris artery was made and another was made proximal to the outlet of the saphenous artery. Finally, the femoral artery segment between two ligations was excised, and the surgical wound was then closed by using 4-0 prolene sutures. The blood flow was detected by using a laser doppler ultrasound scanning system MoorLDI2-HIR (moor instruments Ltd, MoorLDI2-HIR, UK) pre- and post-hind-limb and the moorLDI software (V5.3, UK) was used to analyze the results.

Experimental myocardial (MI)

Anesthetized with 1% sodium pentobarbital 50mg/kg intraperitoneally, C57 mice (male, 8weeks) were fixed in the supine position. An open chest surgery was performed by using 10-0 nylon sutures to cross the myocardium into the anterolateral left ventricular wall and permanently ligate the left anterior descending artery.

Immunofluorescence staining of frozen tissue

The hearts of post-MI mice and gastrocnemius muscle extracted from mice after hind limb ischemia were embedded with optimal cutting temperature compound, frozen in liquid nitrogen and sectioned at a thickness of 5 μm. Sections were fixed and permeated with acetone for 15 min at -20℃. After being blocked with 5% BSA for 1 h at room temperature, the tissue sections were incubated with monoclonal mouse anti-CD31 antibody (CST, cat#3528) and monoclonal rabbit anti-α-SMA antibody (Abcam, cat#ab5694) overnight at 4℃. Heart tissue sections were subsequently incubated with Alexa Fluor™ 488-conjugated goat anti-rabbit IgG (Thermo, cat#A-11008) and Alexa Fluor™ 594-conjugated goat anti-mouse IgG (Thermo, cat#A-11005), while gastrocnemius tissue sections were incubated with Alexa Fluor® 647-conjugated goat anti-mouse IgG (Abcam, cat#ab150115) and Alexa Fluor™ 594-conjugated goat anti-rabbit IgG (Themo, cat#A-11012). Nuclei in the sections were stained by using the antifade mounting medium with DAPI (Beyotime, cat#P0131). Images were visualized under a fluorescence microscope (Olympus).

Antibodies

Anti-CD31: mouse monoclonal antibody (CST, cat#3528S), 1:1000 for immunoblotting and 1:400 for immunofluorescence; Anti-CD31: rabbit monoclonal antibody (Abcam, cat# ab182981), 1:400 for immunofluorescence; Anti-α-SMA: rabbit polyclonal antibody (Abcam, cat#ab55694), 1:1000 for immunoblotting and 1:300 for immunofluorescence; Anti-Fibronectin: rabbit monoclonal antibody (CST, cat#26836S), 1:1000 for immunoblotting; Anti-VE-Cadherin: rabbit monoclonal antibody (CST, cat#2500S), 1:1000 for immunoblotting and 1:400 for immunofluorescence; Anti-FSP-1: rabbit monoclonal antibody (CST, cat#13018S), 1:1000 for immunoblotting; Anti-Vimentin: rabbit monoclonal antibody (CST, cat#5741SS), 1:1000 for immunoblotting; Anti-GAPDH: rabbit monoclonal antibody (CST, cat#5174S), 1:1000 for immunoblotting; Anti-GTF2H4: mouse monoclonal antibody (Santa Cruz, cat#sc-514448), 1:1000 for immunoblotting; Anti-Bax: rabbit monoclonal antibody (CST, cat#41162S), 1:1000 for immunoblotting; Anti-Bcl-xL:

rabbit monoclonal antibody (CST, cat#2764S), 1:1000 for immunoblotting; Anti-MMP-9: rabbit monoclonal antibody (CST, cat#13667S), 1:1000 for immunoblotting; anti-ERCC3: mouse monoclonal antibody (CST, cat#8746S), 1:1000 for immunoblotting; anti-p65: mouse monoclonal antibody (CST, cat# 6956S), 1:1000 for immunoblotting; Anti-p-p65: rabbit monoclonal antibody (CST, cat#3033S), 1:1000 for immunoblotting; Anti-HA: rabbit monoclonal antibody (CST, cat#3724S), 1:1000 for immunoblotting; Anti-Flag: rabbit monoclonal antibody (CST, cat#14793S), 1:1000 for immunoblotting; Anti-His: rabbit monoclonal antibody (CST, cat#12698S), 1:1000 for immunoblotting; Anti-Snail: rabbit monoclonal antibody (CST, cat#3879S), 1:1000 for immunoblotting; Anti-PDGFR-β: Rabbit monoclonal antibody (CST, cat#3169), 1:100 for immunofluorescence; Anti-EGFP: mouse monoclonal antibody (CST, cat#ab184601), 1:100 for immunofluorescence; Anti-ZsGreen1 mouse monoclonal antibody (Sangon Biotech, cat#D199984), 1:100 for immunofluorescence; Anti-mouse 488-conjugated IgG: goat polyclonal antibody (Thermo, cat#A-11001), 1:500 for immunofluorescence; Anti-rabbit 594-conjugated IgG: goat polyclonal antibody (Thermo, cat#A-11012), 1:500 for immunofluorescence; Anti-rabbit 488-conjugated IgG: goat polyclonal antibody (Thermo, cat#A-11008), 1:500 for immunofluorescence; Anti-mouse 594-conjugated IgG: goat polyclonal antibody (Thermo, cat#A-11005), 1:500 for immunofluorescence; Anti-mouse 647-conjugated IgG: goat polyclonal antibody (Abcam, cat#ab150115), 1:500 for immunofluorescence; anti-rabbit HRP-linked IgG: goat polyclonal antibody (CST, cat#7074S), 1:5000 for immunoblotting; anti-mouse HRP-linked IgG: horse polyclonal antibody (CST, cat#7076S), 1:5000 for immunoblotting.

Statistics and reproducibility

Statistical analysis was performed by using Student's t-test or one-way ANOVA (multiple comparisons) in GraphPad Prism 6. Statistical significance was taken as $P < 0.05$. The composite data were expressed as mean ± SEM.

Reference

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2. Baker, M., Robinson, S.D., Lechertier, T., et al. (2011). Use of the mouse aortic ring assay to study angiogenesis. Nat Protoc ***7***, 89-104. DOI: 10.1038/nprot.2011.435.

Supplementary Figure Legends

Figure S1

(**A**) The histogram shows the mean fluorescence intensity of CD31 in each group (n=10). (**B**) The histogram shows the mean fluorescence intensity of α-SMA in each group (n=10). (**C**) The histogram shows the ratio of $CD31^+$α-$SMA^+/CD31^+$ cells (%) in each group (n=10). (**D**) The histogram shows the mean fluorescence intensity of VE-Cadherin in each group (n=8). (**E**) The histogram shows the mean fluorescence intensity of F-actin in each group (n=8). (**F**) The histogram shows the ratio of VE-$Cadherin^+Phalloidin^+$/VE-$Cadherin^+$ cells (%) in each group (n=8). (**G**) Quantitative western blot analyses of Fibronectin, VE-Cadherin, CD31, α-SMA, and FSP-1 protein expression to GAPDH in each group (n=3). (**H**) The histogram shows the number of junctions or total length in each group (n =5). (**I**) RT-qPCR analyses shows the GTF2H4 mRNA expression of HMEC-1 after the GTF2H4 overexpression lentivirus (GTF2H4 OE), overexpression control lentivirus (NC), GTF2H4 knockdown lentivirus (shGTF2H4) and knockdown control lentivirus (shCtrl) infection (n=3). (**J**) RT-qPCR analyses of GTF2H4, CD31, VE-Cadherin mRNA expression of HMEC-1 exposed to hypoxia for 3d in the NC group and GTF2H4 OE group (n =3). (**K**) RT-qPCR analyses of GTF2H4, CD31, VE-Cadherin mRNA level of HMEC-1 exposed to hypoxia for 3d in the shCtrl group and shGTF2H4 group (n =3). Data are represented as means ± s.e.m. P value were calculated by two-tailed t-test (**I-K**) or one-way ANOVA (**A-H**). NS indicates no significant difference.

Figure S2

(**A**) The expression of GTF2H4 in ascending aorta from different ages (70-79 years and 20-29 years) was based on data obtained from the GTEx database. The x-axis indicated the age distribution of the 36 samples, and the y-axis indicated the log2 (TPM + 1) value of GTF2H4. (**B**) The line chart of GTF2H4 expression in HMEC-1 and MCMECs exposed to hypoxia (n=3). (**C**) Cell viability analyses of hypoxia-treated HMEC-1 by Cell Counting Kit-8 (CCK-8) assay (n =7). (**D**) Apoptotic cell measurement in each group exposed to hypoxia for 3d or under normoxia via the annexin V-FITC/ PI-PerCP-Cy5.5 double staining flow cytometric assay. (**E**) The histogram showed the apoptotic cell percentage (n =6). (**F, G**) Quantitative western blot analyses of Bax, Bcl-xL, cleaved-caspase3, and GTF2H4 protein expression to GAPDH in each group exposed to hypoxia for 3d (n=3). (**H, I**) The histogram showing the mean fluorescence intensity of CD31 or α-SMA in each group (n=8). (**J, K**) Quantitative western blot analyses of Fibronectin, α-SMA, FSP-1, VE-Cadherin, CD31, GTF2H4 protein expression to GAPDH in each group exposed to hypoxia for 3d or under normoxia (n=3). Data are represented as means ± s.e.m. P value by two-tailed t-test (**A, B, E, H, I**) or one-way ANOVA (**F, G, J, K**). NS indicates no significant difference.

Figure S3

(**A**) Venn diagrams showing the overlapping genes between each group identified by

using 4D-Label-Free proteomics. (**B**) Statistics of significantly changing genes in abundance and consistent presence/absence expression profile (GTF2H4 OE vs. NC; shGTF2H4 vs. shCtrl). (**C, D**) Heat map of the differentially expressed genes (DEGs) derived from 4D-Label-Free proteomics analyses of HEMC-1 infected by GTF2H4 overexpression or knockdown lentivirus. (Fold change ≥2.0 or ≤ 0.5, P <0.05). (**E, F**) Western blot analyses of ERCC3, GTF2H4 protein expression to GAPDH in each group (n=3). Data are represented as means ± s.e.m. P value by one-way ANOVA (**E, F**). NS indicates no significant difference.

Figure S4

(**A**) KEGG pathway enrichment analysis of DEGs between GTF2H4 OE and NC by using 4D-Label-Free proteomics (Fold change ≥2.0 or ≤ 0.5, P <0.05). Significance was ranked according to the rich factor. The top 20 pathways were listed in the diagram. (**B**) KEGG pathway enrichment analysis of DEGs between shGTF2H4 and shCtrl (Fold change ≥2.0 or ≤ 0.5, P <0.05). Significance was ranked according to the rich factor. The top 20 pathways were listed in the diagram.

Figure S5

(**A, B**) Quantitative western blot analysis of GTF2H4 protein expression to GAPDH in HEMC-1 transfected by ERCC3 overexpression or knockdown lentiviruses (n=3). (**C**) Western blot analyses of Fibronectin, VE-Cadherin, CD31, α-SMA, FSP-1, and ERCC3 protein expression to GAPDH in ERCC3 OE group and NC group exposed to hypoxia for 3d or under normoxia (n=3). (**D**) Quantitative western blot analysis of Fibronectin, VE-Cadherin, CD31, α-SMA, FSP-1, GTF2H4, and ERCC3 protein expression to GAPDH in shCtrl group and shERCC3 group exposed to hypoxia for 3d or under normoxia (n=3). (**E**) Western blot analysis of GTF2H4 protein expression to GAPDH in HEMC-1 transfected by GTF2H4 or E310K/R314E overexpression lentiviruses (n=3). (**F**) Quantitative western blot analyses of Fibronectin, VE-Cadherin, CD31, α-SMA, FSP-1, GTF2H4 and ERCC3 protein expression to GAPDH in GTF2H4 OE group and GTF2H4 OE+shERCC3 group exposed to hypoxia for 3d or under normoxia (n=3). Data are represented as means ± s.e.m. P value by one-way ANOVA (**A, B, D, F**). NS indicates no significant difference.

Figure S6

(**A**) Venn diagrams showed the overlapping proteins between each group identified using 4D-Label-Free phosphorylation proteomics. (**B**) Venn diagrams showed the overlapping peptides between each group identified using 4D-Label-Free phosphorylation proteomics. (**C**) Statistics of significantly changing peptides in abundance and consistent presence/absence expression profile (GTF2H4 OE vs NC; shGTF2H4 vs. shCtrl). (**D**) KEGG pathway enrichment analysis of DEGs between GTF2H4 OE and NC by using 4D-Label-Free phosphorylation proteomics (Fold change ≥2.0 or ≤ 0.5, P <0.05). Significance was ranked according to the rich factor. Top 20 pathways were listed in the diagram. (**E**) EMSA (electrophoretic mobility shift assay) for detecting the DNA-binding activity of NF-κB (p65/p50) in HMEC-1 under

hypoxia in a time gradient. (**F, G**) Quantitative western blot analysis of Snail and p-P65/P65 expression to GAPDH in HMEC-1 exposed to hypoxia for 1-3 days (n=3). Data are represented as means ± s.e.m. P value by one-way ANOVA. NS indicates no significant difference.

Figure S7

(**A**) Western blot analysis of GTF2H4 protein expression to GAPDH in 293T transfected by GTF2H4 overexpression or knockdown lentivirus (n=3). (**B**) EMSA (electrophoretic mobility shift assay) for detecting the DNA-binding activity of NF-κB (p65/p50) in shCtrl and shGTF2H4 exposed to normoxia and hypoxia for 1-2d. (**C, D**) Quantitative western blot analysis of GTF2H4, Snail and p-P65/P65 expression to GAPDH in each group exposed to hypoxia for 3d or under normoxia (n=3). (**E**) Western blot analysis of ERCC3 protein expression to GAPDH in 293T transfected by ERCC3 overexpression or knockdown lentivirus (n=3). (**F, G**) Luciferase reporter assays for NF-κb p65 transcriptional activation activity detection with TNF-α (10 ng/mL; NF-κB activation) stimulation for 4 hours in each group (n= 6-8). (**H**) Western blot analyses of ERCC3, Snail and p-P65/P65 expression to GAPDH in ERCC3 OE group and NC group exposed to hypoxia for 3d or under normoxia (n=3). (**I, J**) Quantitative western blot analysis of ERCC3, Snail and p-P65/P65 expression to GAPDH in shERCC3 group and shCtrl group exposed to hypoxia for 3d or under normoxia (n=3). (**K**) Quantitative western blot analyses of CD31, VE-Cadherin, α-SMA, and snail expression to GAPDH in each group exposed to hypoxia for 3 days (n=3). Data are represented as means ± s.e.m. P value by two-tailed t-test (**F, G**) or one-way ANOVA (**C, D, J, K**). NS indicates no significant difference.

Figure S8

(A) Luciferase reporter assays for NF-κb p65 transcriptional activation activity detection affected by ERCC3 knockdown, with TNF-α (10 ng/mL; NF-κB activation) stimulation for 4 hours in each group after treatment with the expression of WT NCOA3, S1330A, S1330D or S1330E NCOA3 mutant (n =6-9). (**B**) Western blot analyses of NCOA3 and GTF2H4 protein expression to GAPDH in GTF2H4 OE group and NC group exposed to hypoxia for 3d or under normoxia (n=3). (**C**) Western blot analyses of NCOA3 and GTF2H4 protein expression to GAPDH in shGTF2H4 group and shCtrl group exposed to hypoxia for 3d or under normoxia (n=3). (**D**) Western blot analyses of NCOA3 and ERCC3 protein expression to GAPDH in ERCC3 OE group and NC group exposed to hypoxia for 3d or under normoxia (n=3). (**E**) Western blot analyses of NCOA3 and ERCC3 protein expression to GAPDH in shERCC3 group and shCtrl group exposed to hypoxia for 3d or under normoxia (n=3). (**F**) Western blot analyses of NCOA3 and GTF2H4 protein expression to GAPDH in E310K/R314E OE group and NC group exposed to hypoxia for 3d or under normoxia (n=3). (**G, H**) Western blot analyses of NCOA3 and GTF2H4 protein expression to GAPDH in each group 7 days after hind-limb ischemia surgery (n=6). Data are represented as means ± s.e.m. P value by two-tailed t-test.

Figure S9

(A) Endothelia-specific AAV (AAV-Tie-ZsGreen, AAV-Tie-GTF2H4-ZsGreen, AAV-Tie-EGFP, and AAV-Tie-shGTF2H4-EGFP) was utilized to infect mouse gastrocnemius muscle 4 weeks before hind-limb ischemia surgery. The location of ZsGreen/EGFP with endothelial cells, pericytes, or skeletal muscle cells in gastrocnemius muscle was identified by immunofluorescence staining to determine the targeting capacity of AAV towards endothelial cells. Endothelial cells, pericytes, and skeletal muscle cells were respectively labeled by CD31, PDGFR-β, and F-actin. ZsGreen and EGFP respectively indicated GTF2H4 overexpression and knockdown. The nucleus was stained by DAPI. Representative immunofluorescence staining images are shown. **(B, C)** The histogram shows $CD31^+/\alpha\text{-}SMA^+$ capillary density (Number/mm^2) in each group (n=6). **(D, E)** Quantitative western blot analyses of GTF2H4, ERCC3, Vimentin, α-SMA, snail, and p-P65/P65 protein expression to GAPDH in each group 7 days after hind-limb ischemia surgery (n=6). Data are represented as means ± s.e.m. P value by two-tailed t-test (**B-E**).