SUPPLEMENTAL MATERIAL

Integrated Stress Response Couples Mitochondrial Protein Translation with Oxidative Stress Control

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Supplemental Methods

Animals

All mouse procedures were approved by the Institutional Animal Care and Use Committee of University of Texas Southwestern Medical Center (UTSW). Mice were on the C57BL/6 background and were maintained in 12:12 hours light:dark cycle in temperature-controlled rooms. Mice had free access to water and chow food (Teklad, #2916).

$PERK^{fl/fl}$ mice with two loxP sites flanking exons 7-9 were purchased from the Jackson Lab, Stock No: 023066. Generation of the cardiac-specific PERK knockout mouse model was achieved by crossing $PERK^{fl/fl}$ with αMHC-Cre transgenic mice.

To obtain cardiomyocyte-restricted inducible overexpression of PERK, we first generated a transgenic mouse model with FKBP-PERK fusion gene under the control of a universal CAG promoter. A transcriptional/translational stop cassette was inserted between the CAG promoter and FKBP-PERK. This transgenic construct backbone was generated previously and used to overexpress GRP78 in the heart.[18] The FKBP-PERK fusion protein contains two modified FK506-binding domains and the kinase domain of mouse PERK (amino acids 537-1,114). Activation of PERK was not dependent on protein-folding stress, rather relying on a chemical dimer (AP20187) to induce dimerization of FKBP and autophosphorylation of PERK.[19] A rabbit β-globin 3'UTR was inserted after FKBP-PERK to enhance transcription and translation of the transgene. The CAG-FKBP-PERK plasmid was then linearized by digestion with Pvu I and Avr II, and the fragment containing CAG-FKBP-PERK (7 kb) was purified for pro-nuclear injection by the Transgenic Core of UTSW. Multiple founders were received and screened for the transgene CAG-FKBP-PERK. We then crossed positive lines with αMHC-Cre transgenic mice. Single transgenic mice of CAG-FKBP-PERK and αMHC-Cre were used as controls. The following primers were used for genotyping: $PERK^{fl/fl}$, forward: 5'-TTGCACTCTGGCTTTCACTC-3', reverse: 5'-AGGAGGAAGGTGGAATTTGG-3'; αMHC-Cre, forward: 5'-GATTTCGACCAGGTTCGTTC-3', reverse: 5'-GCTAACCAGCGTTTTCGTTC-3'; CAG-FKBP-PERK, forward: 5'-GCCACCATGGGCGTCCAAGTCGAAACCATTAGTCC-3', reverse: 5'-TCTAGATTCCAGTTTTAGAAGCTCCAC-3'.

To induce FKBP-PERK autophosphorylation and activation, dimer AP20187 (ApexBIO, #B1274) was used. Dimer was dissolved in 100% ethanol at a concentration of 9.3 mM as stock. Then, 9.4 μL dimer stock was mixed with PEG-400 (100 μL) in 2% Tween 80 (890.6 μL). The final concentration was 0.125 μg/μL. Dimer was intraperitoneally (I.P.) injected at 0.5 mg/kg body weight. Vehicle was similarly prepared with ethanol only instead of dimer stock.

Ischemia/reperfusion (I/R) surgery

Mice of 12-16 weeks old were used for I/R as previously performed.[44,47] Briefly, mice are anesthetized with a cocktail of ketamine (100 mg/kg, I.P.) and xylazine (5 mg/kg, I.P.). A small, sagittal incision upon the anterior neck was made to reveal the trachea and facilitate cannulation of the upper airway. Intubation was achieved orally. An 8 mm oblique incision was made 2 mm from the left sternal border. Underlying muscles were gently separated, and the 4^{th} intercostals space was visualized. An axial incision was created in the chest wall, between the 4^{th} and 5^{th} ribs. A chest retractor was inserted and the heart

was visualized. The pericardium was gently dissected to reveal the left ventricle. With the left anterior descending artery (LAD) visualized, a ligature was placed 1 mm below the tip of the left atrial appendage, using 7-0 polypropylene sutures. At conclusion of the injury period, the wound was reopened and the suture around the LAD was removed. Chest cavity was then closed in layers — chest wall, musculature, and skin, using absorbable suture and skin glue. The animal was then returned to its housing cage and, subsequently, the vivarium. Sham mice underwent the same procedure without ligature constriction.

The left ventricular tissue of the heart was split into three regions: the ischemia region, 1 mm below the ligature to the apex; the remote region, 1 mm above the ligature, in parallel to the cutting of the ischemic region; the border region, between the ischemic and remote regions.

To detect serum LDH (lactate dehydrogenase) level, 50 μL blood was drawn from the tail vein 24 hours after reperfusion. Serum was collected after centrifugation at 4ºC for 10 minutes at 1,000 RPM and stored at -20ºC until use. The sample was diluted 1:10 with ddH_2O. LDH was then determined using an LDH assay kit (Promega, #G1780).

Echocardiography

Cardiac function was evaluated with unconstrained, conscious mice using echocardiography (Visual Sonics, #Vevo 2100, MS400C probe) as previously described.[44] The short-axis view of M-mode images at the level of papillary muscles was captured and analyzed to calculate various parameters. Heart rate was recorded. Left ventricular internal diameters at end diastole (LVID, diastolic) and at end systole (LVID, systolic) were measured from M-mode recordings. Fractional shortening = ((LVID, diastolic - LVID, systolic)/LVID, diastolic) $\times$ 100%.

Chemicals

PERK inhibitor (GSK2606414, Tocris Bioscience, #510710) was dissolved in DMSO to make a 0.5 mM stock and used at 500 nM. PERK activator (CCT020312, Millipore, #324879) was dissolved in DMSO to make a 1 mM stock and used at 1 μM. ISR inhibitor (ISRIB, Sigma, #SML0843) was dissolved in DMSO to make a 0.2 mM stock and used at 200 nM. eIF2α phosphatase inhibitor (salubrinal, Tocris Bioscience, #234710) was dissolved in DMSO to make a 50 mM stock, diluted with PEG 400 and Tween 80, and injected to mice at a concentration of 1 mg/kg body weight. Metformin (Sigma, #1396309) was used at 10 mM and Mito-TEMPO (Sigma, #SML0737) was used at 10 μM.

H9c2 cell culture and siRNA treatment

H9c2 cells were purchased from ATCC (#CRL-1446), which were derived from embryonic rat myocardium. H9c2 cells were cultured in 75 cm^2 flasks with DMEM medium (high glucose), supplemented with 10% fetal bovine serum (FBS). Mouse embryonic fibroblasts (MEFs) and HEK293 cells were similarly cultured.

Small interference RNAs (siRNAs) were purchased from Sigma. siRNAs were dissolved in Opti-MEM (Thermo Fisher Scientific, #31985070) at 40 μM. Approximately 80 pmol siRNAs was used for transfection in each well of 6-well plates with siLentFect lipid reagent (Bio-Rad, #1703361). After 6 hours, cells were replenished with fresh culture medium.

The following siRNA oligos were used: PERK, 5'-GUGGAAAGCUGAGGUAUAU[dT][dT]; NDUFAF2, 5'-ACCUACUAUGGAGGAAAUA[dT][dT].

Neonatal rat ventricular myocyte (NRVM) isolation and treatment

NRVMs were isolated from ventricles of 1 to 2 days old Sprague-Dawley rats (Charles River Laboratories) using a cardiomyocyte isolation kit (Cellutron, #NC-6031) as previously described.[48-50] After 2 hours of pre-plating to remove neonatal fibroblasts, NRVMs were plated at a density of 1,250 cells/mm^2 in plating medium (DMEM/M199 = 3:1) with 5% FBS, 10% horse serum, 1% penicillin/streptomycin, and bromodeoxyuridine (BrdU, 100 μM). BrdU was used to suppress fibroblast growth in NRVM culture. After 24 hours of plating, NRVMs were washed with PBS and cultured in serum-reduced medium (DMEM/M199 = 3:1, 1% FBS, 1% penicillin/streptomycin, and 100 μM BrdU). After another 24 hours, cells were switched to serum-free medium (DMEM/M199 = 3:1). NRVMs were then subjected to various treatments, including siRNA knockdown, adenovirus infection, simulated ischemia/reperfusion, etc. Adult mouse cardiomyocyte isolation was performed as previously described.[48]

Simulated ischemia/reperfusion (sI/R)

H9c2 sI/R was conducted as previous described.[44,47] Briefly, H9c2 cells were first washed twice with PBS and incubated with freshly made ischemia Esumi buffer (HEPES 4 mM, NaCl 117 mM, $CaCl_2$ 0.9 mM, $MgCl_2$ 0.49 mM, KCl 12 mM, 2-deoxyglucose 5.6 mM, sodium lactate 20 mM, pH 6.2).[51] Cells were then placed in a modular incubator chamber at 37°C (Billups-Rothenberg, #MIC-101). Mixed gas (95% N_2/ 5% CO_2) was used to flush the chamber for 30 minutes at pressure less than 2 psi. The chamber was then sealed for additional 3.5 hours. Esumi buffer was removed and H9c2 cells were replenished with DMEM culture medium. Cells were returned to a cell culture incubator (5% CO_2). Normoxia control medium contained HEPES 4 mM, NaCl 137 mM, $CaCl_2$ 0.49 mM, $MgCl_2$ 0.49 mM, KCl 0.9 mM, and D-glucose 5.6 mM at pH 7.4. H9c2 cell viability was determined by cell counting kit-8 (CCK8, Sigma, #96992).

NRVM sI/R was similarly conducted, except that the ischemia time was 6 hours. NRVM cell death was assayed by measuring LDH release using a CytoTox96 non-radioactive cytotoxicity assay kit (Promega, #G1780).

MEFs were cultured with DMEM containing 10% FBS and 1% penicillin/streptomycin. sI/R was conducted similarly as for H9c2 cells. MEF viability was quantified by cell counting kit-8 (CCK8, Sigma, #96992).

Adenovirus production and infection

FKBP-PERK fusion gene was used for adenovirus generation with Adeno-X adenoviral system 3 (Takara, #632269). Adenoviruses expressing green fluorescent protein (GFP) were used as control. After infection, dimer AP20187 (1 μM) was used to treat the cells to stimulate PERK.

Immunoblotting

Total proteins were extracted from cells or cardiac tissues using RIPA lysis and extraction buffer (Thermo Fisher Scientific, #89900), supplemented with protease and phosphatase

inhibitors (Thermo Fisher Scientific, #88669). Protein concentration was determined with a BCA kit (Thermo Fisher Scientific, #23225). Equal amount of proteins for each sample was loaded on a Criterion TGX precast gel (Bio-Rad, 4-20%, #5671095) and transferred onto a nitrocellulose membrane (Bio-Rad, #1704157). After blocking for 1 hour, the membrane was incubated with primary antibodies overnight at 4°C, followed by incubation with fluorescent dye-labeled secondary antibodies for 1-2 hours and imaging with an Odyssey imager (Li-Cor). The following antibodies were used: GAPDH (Fitzgerald, #10R-G109A), PERK (Cell Signaling, #3192), p-Thr980-PERK (Cell Signaling, #3179), eIF2α (Thermo Fisher Scientific, #AHO0802), p-eIF2α (Thermo Fisher Scientific, #44-728G), puromycin (Millipore, clone 12D10, #MABE343), GCN2 (Cell Signaling, #3302), PKR (Santa Cruz Biotechnologies, #sc-6282), HRI (Sigma, #07-728), FKBP12 (Abcam, #ab2918), SDHB (Abcam, #ab14714), NDUFAF2 (Sigma, #SAB2900858), oxidative phosphorylation cocktail (Abcam, #ab110413), IRDye 800 CW goat anti-rabbit secondary antibody (Li-Cor, #925-32211), and Alexa Fluor 700-conjugated goat anti-mouse secondary antibody (Thermo Fisher Scientific, #A-21036).

Protein synthesis measurements

To measure protein synthesis *in vivo*, puromycin (Sigma, #P8833) was intraperitoneally injected at 0.04 μmol/g body weight and the heart was collected 30 minutes later. Protein lysates from cardiac tissues were then prepared for Western blotting. To determine protein synthesis in cultured cells, puromycin was included in culture media at 0.5 μg/mL for 15 minutes before cells were harvested for immunoblotting.

Protein carbonylation assay

H9c2 cells were subjected to either normoxia or sI/R. Total proteins were extracted and quantified with a BCA kit. Samples were adjusted to the same protein concentration (≥ 1 μg/μL). Each sample was then split into two tubes (10 μL each), one for carbonylation reaction and the other one for negative control reaction. SDS solution (10 μL, 12%) was added to each tube to denature all proteins. DNPH (2,4-dinitrophenylhydrazine, Sigma, #D199303) was dissolved in 10% trifluoroacetic acid (v/v) to make a 20 mM stock. Trifluoroacetic acid (10%) was used as negative control. DNPH (20 μL) or equal volume of trifluoroacetic acid was added. Samples were then left at room temperature for 15 minutes. The reaction was neutralized by adding neutralization solution (15 μL, 2 M Tris/ 30% glycerol). There was a noticeable color change from light yellow to orange. Samples were then used for immunoblotting with anti-DNP antibody (Millipore, #MAB2223).

Phos-tag gel electrophoresis

Phos binding reagent (Phosbind) acrylamide (ApexBIO, #F4002) was dissolved in methanol and diluted in water at a concentration of 5 mM. Phosbind was then added to resolving gel solution to prepare either 5% or 7% mini-gels using a Bio-Rad mini-gel casting system. The final concentrations of Phosbind in 5% and 7% resolving gel solution were 3.5 μM and 50 μM, respectively.[52] No Phosbind was included in stacking gel solution. Protein lysates were loaded at 30 μg/lane. Electrophoresis was done under the following conditions: 15 mA for 15 minutes, then 5 mA for 9.5 hours. After transferring at 95 V for 2 hours, immunoblotting was conducted.

Blue native gel assay

Mitochondria from either heart tissues or H9c2 cells were isolated using a mitochondria isolation kit (Thermo Fisher Scientific, #89801 and #89874). Digitonin at a final concentration 8 g/g mitochondrial proteins was used to solubilize electron transfer chain complexes, and 50 µg proteins per sample were applied and separated on a 3%-12% gradient blue native gel (Thermo Fisher Scientific, #BN1003BOX). After completion of the run, the gel was carefully removed and placed in ice-cold water and incubated in 20 mL of complex I substrate solution (Tris-HCl 2 mM, NADH 0.1 mg/mL, nitrotetrazolium blue chloride 2.5 mg/mL, pH 7.4). Appearance of violet bands was an indication of complex I activity. The reaction was stopped with 10% of acetic acid. The gel was then scanned and quantified by ImageStudio (Li-Cor).

Protein turnover assay

To detect protein turnover, cycloheximide (CHX, 100 ug/mL) was added to culture medium. For the sI-only condition, CHX was added to the normoxia and ischemia groups, respectively, at the same time upon ischemia. After ischemia for 4 hours, cells were harvested for Western blotting. For the sI/R condition, CHX was added to the normoxia and sI/R groups, respectively, upon reperfusion. After 30 minutes, cells were collected for immunoblotting.

Seahorse flux assay

Oxygen consumption rates (OCRs) were determined by a Seahorse XFe24 extracellular flux analyzer (Agilent) as previously described.[53] Briefly, 5×10^4 H9c2 cells were seeded in each well of a pre-coated XFe24 cell culture plate with 250 µL standard culture media, and treated with siRNAs or adenoviruses for 24 hours. After sI/R, cells were washed with XF media twice and incubated in a CO_2-free incubator at 37°C for 1 hour. The following reagents were used: Oligomycin (1 µM), FCCP (carbonyl cyanide p-trifluoromethoxyphenylhydrazone, 4 µM), and Antimycin A (10 µM) plus Rotenone (1 µM).

Proteomics

H9c2 cells were subjected to either normoxia or sI/R. Cells were lysed with RIPA buffer and protein concentration was determined with a BCA kit. A total of 1 mg proteins for each sample were precipitated with acetone. Mass spectrometry (MS) was conducted by the Proteomics Core of UTSW using the tandem mass tag (TMT)-labeling method. Data were analyzed with Proteome Discoverer 2.4 and hits were searched from the Uniprot rat protein database (www.uniprot.org) using MaxQuant.

Statistical and bioinformatics analyses were performed with Graphpad Prism 8.3.0, Microsoft Excel 16.39, and R statistical software. Differentially expressed proteins were defined as log2 fold change >1 or <-1, compared to the control group. Gene Ontology annotation was performed with DAVID 6.8 (david.ncifcrf.gov) for three categories: biological process (BP), cellular component (CC), and molecular function (MF). Kyoto Encyclopedia of Genes and Genomes (KEGG) was used to annotate signaling pathways.

The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE[54] partner repository with the dataset identifier PXD026679.

RNA-seq analysis

H9c2 cells were infected by adenoviruses expressing the FKBP-PERK fusion protein. Either vehicle or dimer was then used to treat the cells. After sI/R, total RNAs were extracted for RNA sequencing (RNA-seq) analysis (Psomagen). Differentially expressed genes (DEGs) were defined as |log2 (fold change)| > 1 and p(adjusted) value < 0.05.

All raw and processed RNA-seq data are available at the Gene Expression Omnibus under the accession number GSE177078.

Dihydroethidium staining

DHE (dihydroethidium, Thermo Fisher Scientific, #D11347) was dissolved in DMSO to make a 5 mM stock. Before using, the DHE stock were diluted with 10% FBS/ DMEM medium at 1:1000. After sI/R, culture medium of H9c2 cells was changed to DHE working solution (5 μM). After incubating at 37°C for 30 minutes, cells were washed for 3 times before imaging with a ZOE Fluorescent Cell Imager (Bio-Rad, #1450031).

SYTOX Green/Hoechst double staining

SYTOX Green (Thermo Fisher Scientific, #S7020) and Hoechst (Thermo Fisher Scientific, #H3570) were diluted in DMEM medium with 10% FBS at 1:1000 as working solution. After sI/R, culture medium of H9c2 cells was changed to SYTOX Green work solution. After incubating at 37°C for 30 minutes, cells were washed for 3 times before imaging with a ZOE Fluorescent Cell Imager (Bio-Rad, #1450031).

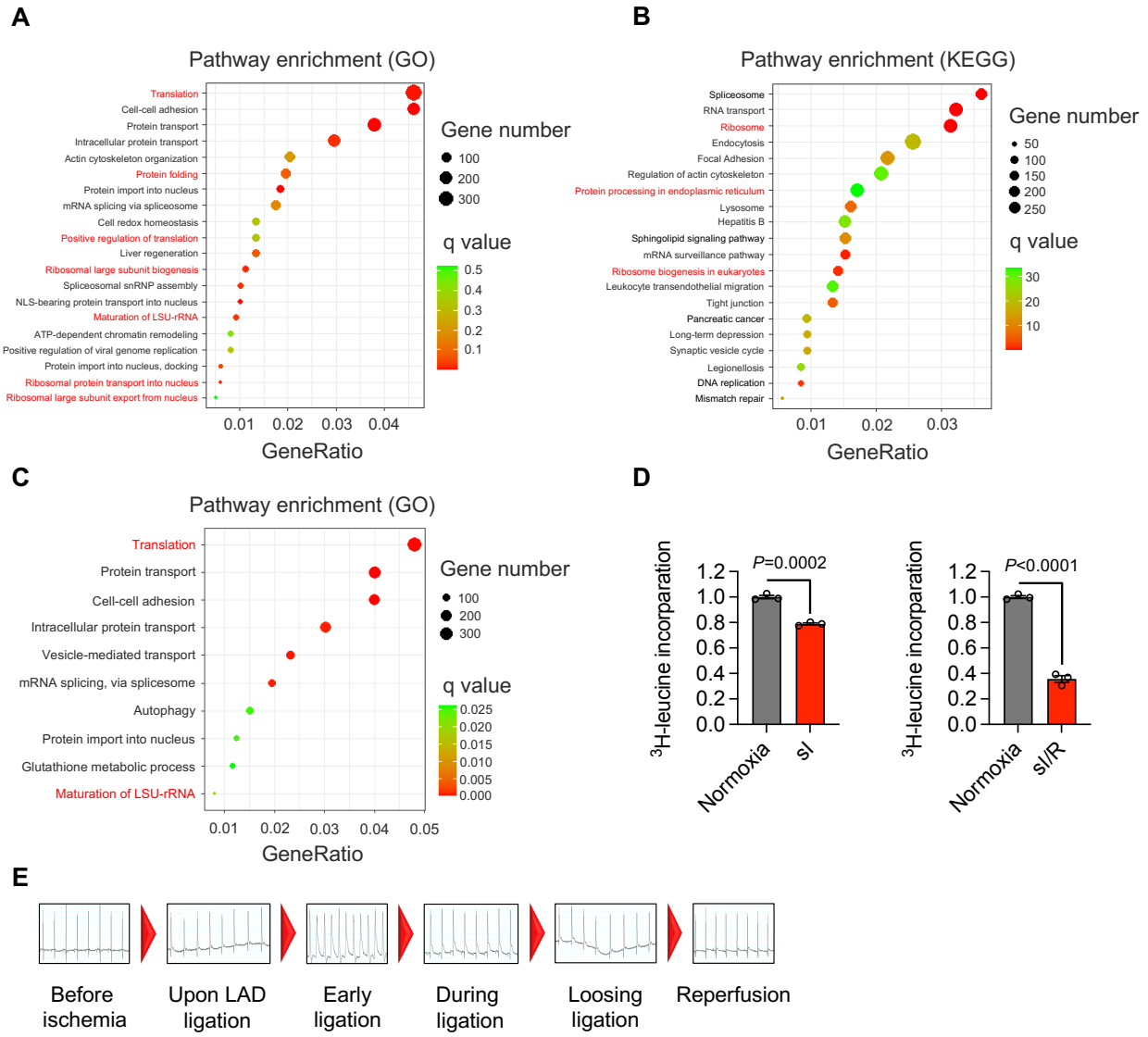
Histology

Mouse hearts were collected and fixed in 10% neutralized formalin for 48 hours at 4°C. Paraffin sections of 5-μm thickness were used. Hematoxylin and eosin (H&E) and Masson's trichrome staining was conducted by the Molecular Pathology Core of UTSW.

Statistical analyses

Data are represented as mean±SEM. Two-tailed Student's *t* test was performed to compare differences between two groups. For multiple group comparisons with one variable, one-way ANOVA was conducted, followed by either Tukey's or Dunnett's multiple comparisons test. For multiple group comparisons with more than two variables, two-way ANOVA was conducted, followed by either Tukey's or Sidak's multiple comparisons test. A *P* value of <0.05 was considered statistically significant. Statistical analyses were performed using Graphpad Prism software 8.3.0

Supplemental Figure I



Cardiomyocyte ischemia/reperfusion leads to a significant change in protein translation.

A. H9c2 cardiac myocytes were subjected to control treatment, simulated ischemia (sI) or simulated ischemia/reperfusion (sI/R). Total proteins were isolated for tandem mass tag (TMT)-based mass spectrometry (MS). Significantly changed proteins between control and treated (sI and sI/R) groups were used for pathway enrichment analysis (GO, gene ontology). The top 20 enriched pathways are shown. Translation-related processes are highlighted in red.

B. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of significantly changed proteins between control and treated (sI and sI/R) groups. Note that translation-related processes are highlighted in red.

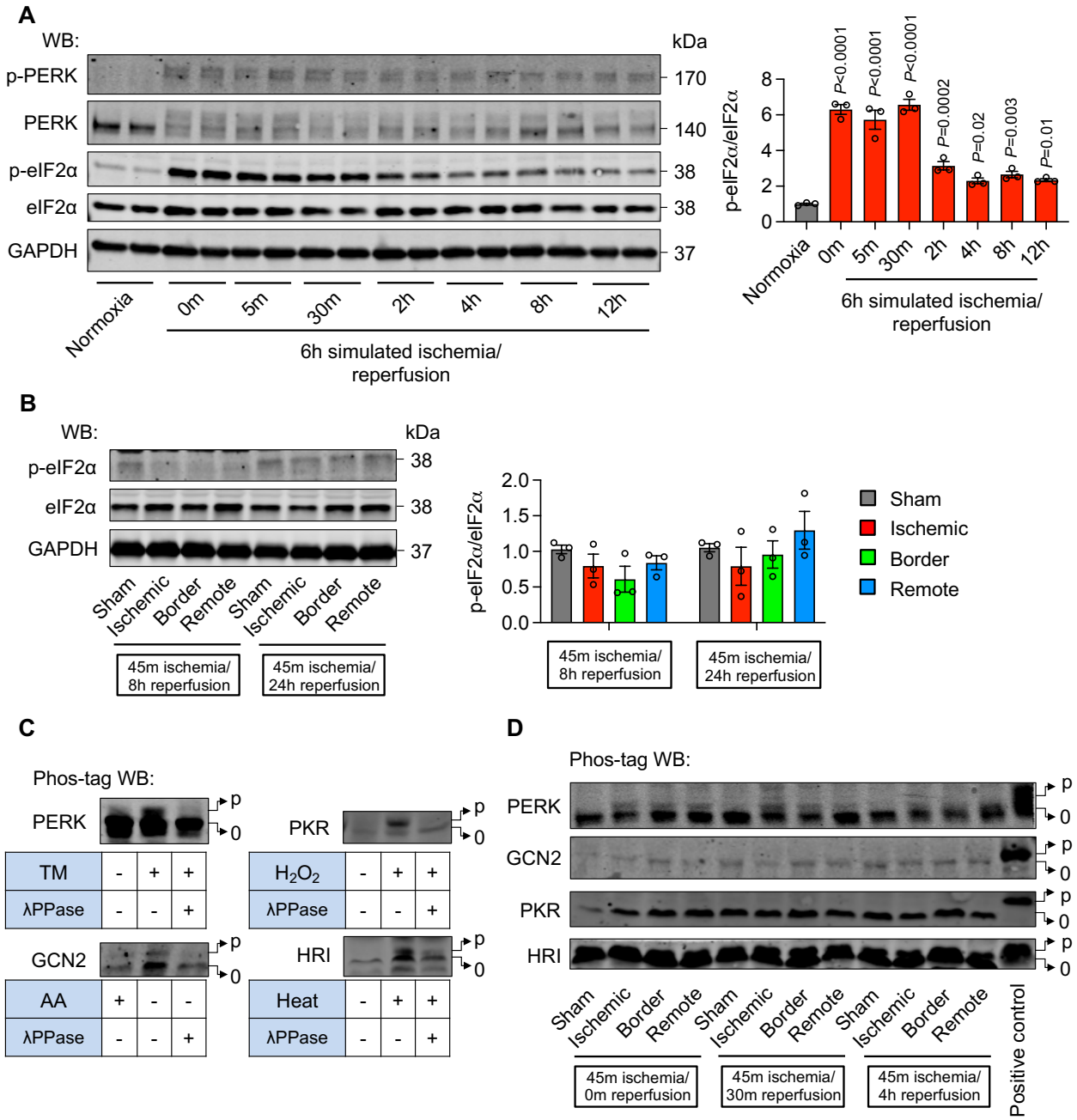
C. A comparison between sI and sI/R groups showed that translation remained the most enriched pathway.

D. sI/R caused a more profound decrease in protein synthesis compared to sI only. H9c2 cells were subjected to sI and ^{3}H-leucine was included in culture media. After 4 hours of sI, cells were harvested to detect leucine incorporation as before[48-50]. In addition, H9c2 cells were subjected to sI for 4 hours. During reperfusion (4 hours), ^{3}H-leucine was added. Radioactive leucine was similarly determined. n=3.

E. Electrocardiogram (EKG) tracing of a mouse under cardiac ischemia/reperfusion (I/R). The elevation of ST-segment upon left anterior descending artery (LAD) ligation indicates a successful I/R surgery.

Unpaired Student's *t* test was conducted (**D**). Data are represented as mean±SEM.

Supplemental Figure II



I/R stimulates PERK/eIF2α signaling in cardiomyocytes.

A. Neonatal rat ventricular myocytes (NRVMs) were subjected to sI for 6 hours, followed by reperfusion for indicated times. Total proteins were isolated for immunoblotting. Quantification (at right) showed significant upregulation of eIF2α phosphorylation. Statistical difference was calculated between normoxia and individual time points post sI/R. n=3.

B. Prolonged reperfusion after ischemia did not affect eIF2α phosphorylation *in vivo*. Mice were subjected to I/R, followed by heart collection 8 hours and 24 hours post

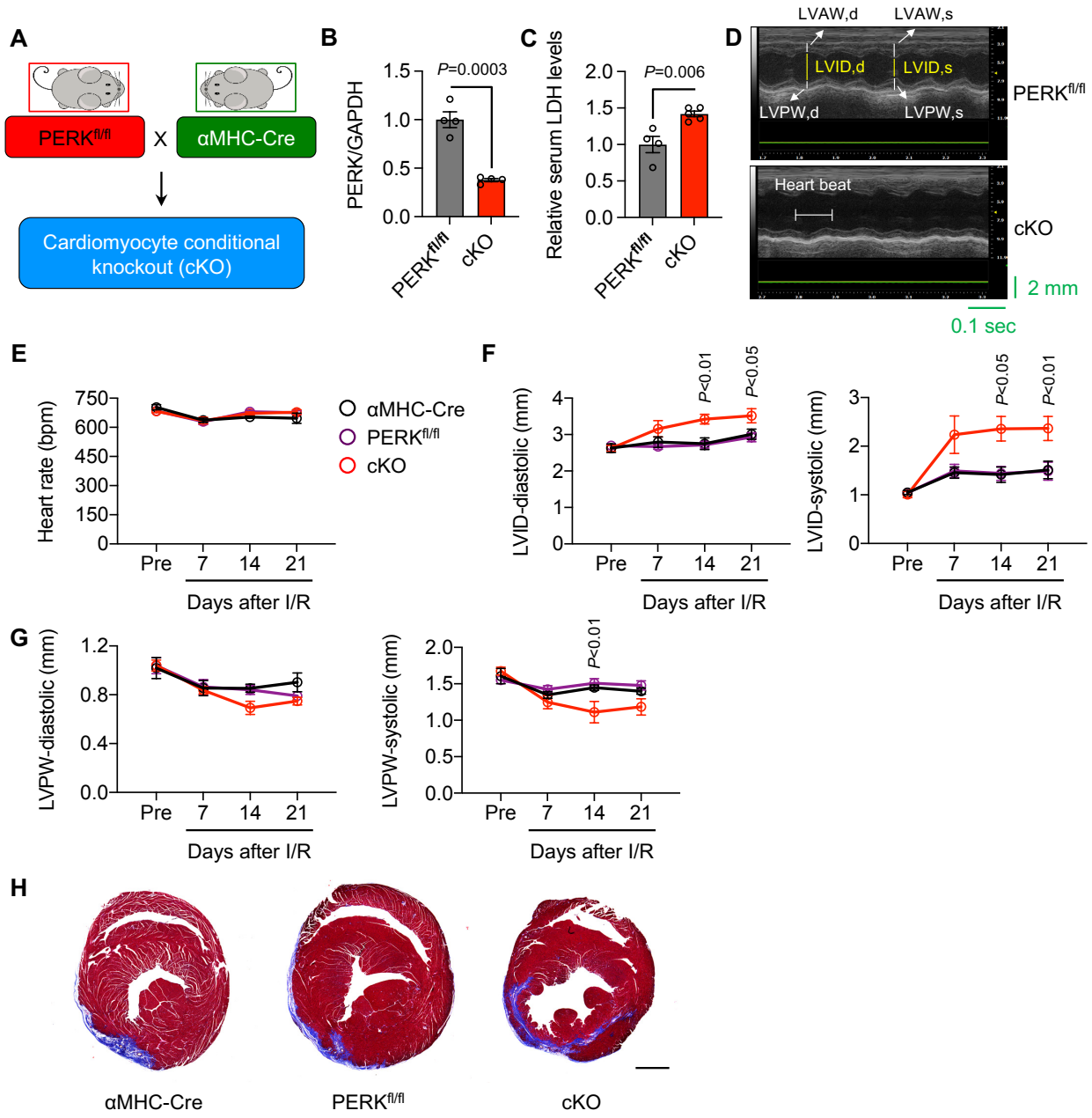
reperfusion, respectively. Total cardiac proteins were extracted and used for Western blotting. Note that there was no significant change of eIF2α phosphorylation between sham and ischemic samples. n=3.

C. Validation of antibodies for eIF2α upstream kinases. H9c2 cells were treated with various stimuli to activate PERK, PKR, GCN2, and HRI, respectively, as positive controls. After protein isolation, λ phosphatase was used to remove phosphate groups as a validation for phosphorylation. Phosbind was added during SDS-PAGE gel preparation. Western blotting was then conducted. p indicates phosphorylated protein; 0 indicate non-phosphorylated protein.

D. Phos-tag Western blotting showed that PERK was phosphorylated by I/R in the ischemic region in mice. A tunicamycin-treated H9c2 sample was used as positive control for PERK phosphorylation. p indicates phosphorylated protein; 0 indicate non-phosphorylated protein.

One-way ANOVA was conducted, followed by Dunnett's multiple comparisons test (**A, B**). Data are represented as mean±SEM.

Supplemental Figure III



Cardiac-specific deletion of PERK causes an exacerbated response to I/R.

A. Schematic of the cardiac-specific PERK conditional knockout mouse model (cKO).

B. Validation of PERK knockout. Adult cardiomyocytes were isolated from PERK$^{fl/fl}$ and cKO mice, respectively. Immunoblotting was conducted and PERK relative expression was quantified. n=4.

C. PERK deletion in the heart led to an increase of lactate dehydrogenase (LDH) release compared to PERK$^{fl/fl}$ control. I/R was conducted and serum was collected 24 hours after surgery for LDH measurement. n=4.

D. Representative echocardiography images are shown. Measurements for various parameters are labeled. LVID,d, left ventricular internal diameter at diastole; LVID,s, left ventricular internal diameter at systole; LVAW,d, left ventricular anterior wall thickness at diastole; LVAW,s, left ventricular anterior wall thickness at systole; LVPW,d, left ventricular posterior wall thickness at diastole; LVPW,s, left ventricular posterior wall thickness at systole.

E. Heart rate was not altered by PERK cKO after I/R. Mice of 12-16 weeks old were used for I/R. Echocardiography was conducted before I/R (Pre) and 7, 14, and 21 days post I/R, respectively. n=5 to 7.

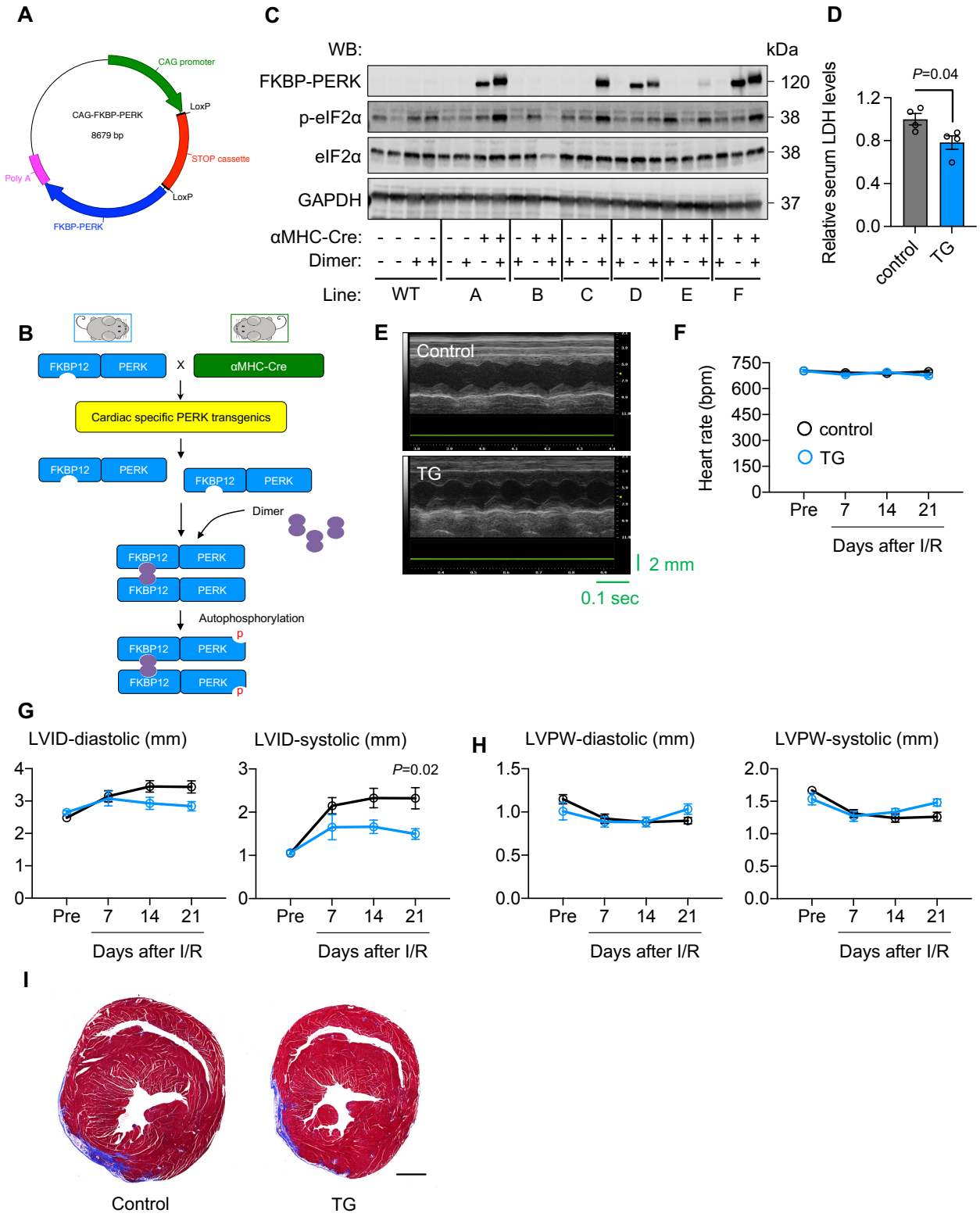
F. Both diastolic and systolic LVIDs were significantly elevated in cKO mice compared to $PERK^{fl/fl}$ and aMHC-Cre mice, respectively, indicating deteriorated cardiac function. n=5 to 7.

G. LVPW at systole showed a decline day 14 after I/R in cKO mice. n=5 to 7.

H. Cardiac-specific PERK deletion led to more severe cardiac damage, as revealed by an increase of fibrotic area 21 days after I/R. Scale bar: 1 mm.

Unpaired Student's *t* test was conducted (**B-C**). Two-way ANOVA was conducted, followed by Tukey's multiple comparisons test (**E-G**). Data are represented as mean±SEM.

Supplemental Figure IV

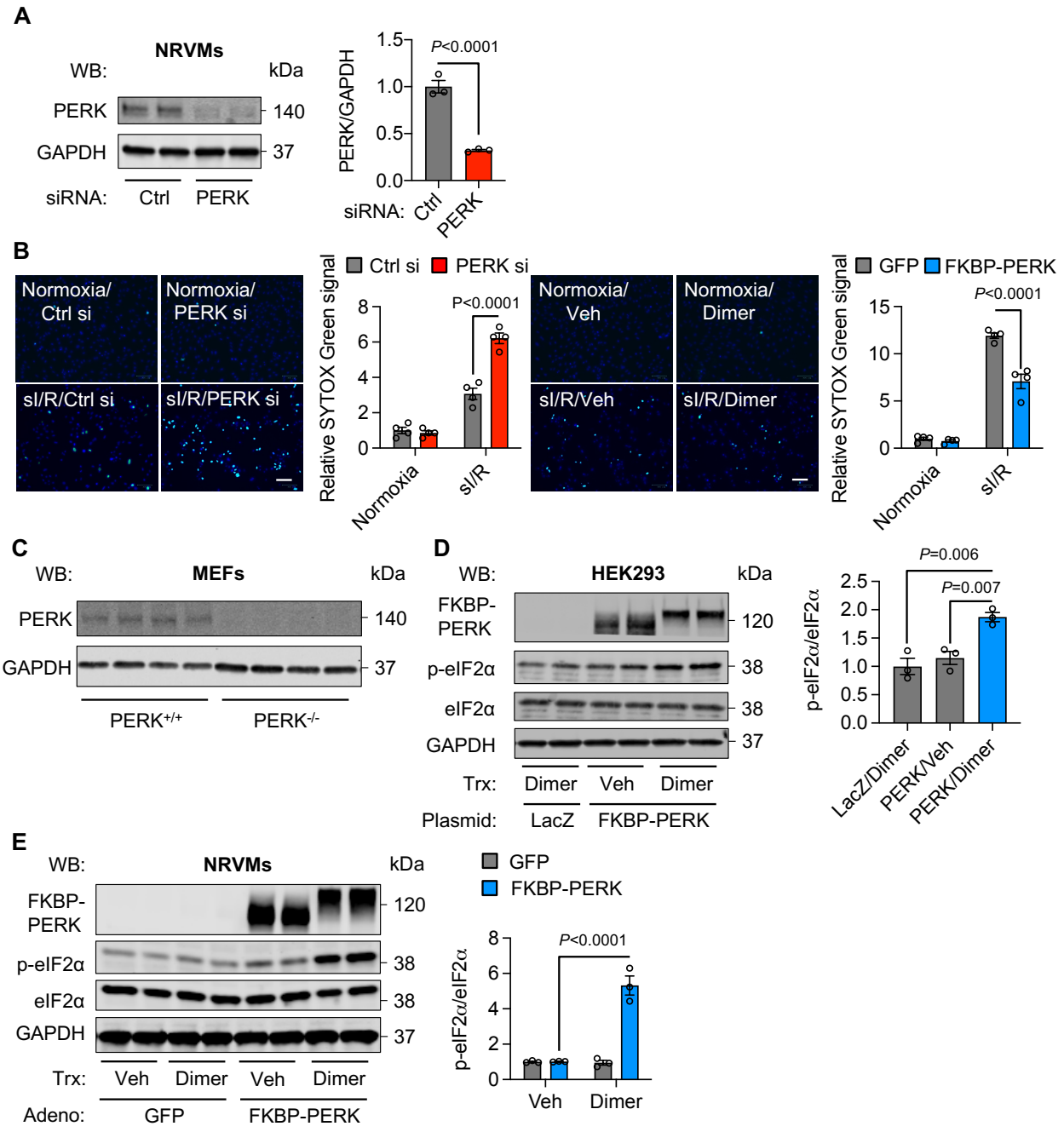


Cardiac-specific activation of PERK protects the heart from I/R injury.

- **A.** CAG-FKBP-PERK plasmid map. A transcriptional/translational stop cassette was inserted between the CAG promoter and FKBP-PERK transgene. Two loxP sites were introduced to flank the stop cassette.
- **B.** Schematic for cardiomyocyte-specific, inducible activation of PERK *in vivo*. In the double transgenic mice of CAG-FKBP-PERK and αMHC-Cre, Cre expression in cardiomyocytes led to excision of the stop cassette, and FKBP-PERK fusion protein was only expressed in cardiomyocytes in the heart. After injection of dimer (AP20187), FKBP-PERK was forced to close proximity and PERK autophosphorylation was stimulated to activate downstream signaling.
- **C.** Transgenic mice were treated by either vehicle or dimer for 2 hours. Hearts were collected for immunoblotting. Lines A, C, D, and F showed strong induction of PERK phosphorylation after dimer treatment. Line D was chosen for further analysis since the increase of eIF2α phosphorylation was significant and within the physiological level.
- **D.** PERK overexpression and activation decreased LDH release after I/R *in vivo*. n=4.
- **E.** Representative echocardiography images from control and TG mice are shown. Control mice included αMHC-Cre single transgenics, CAG-FKBP-PERK single transgenics, and wild-type animals. TG included αMHC-Cre and CAG-FKBP-PERK double transgenics mice.
- **F.** Heart rate was not changed in control and TG mice after I/R. n=6 to 8.
- **G.** TG mice showed a significant decrease of LVID at systole after I/R. n=6 to 8.
- **H.** No significant difference in LVPW was discovered. n=6 to 8.
- **I.** PERK overexpression and activation ameliorated cardiac damage 21 days after I/R, as shown by a decrease of fibrotic area. Scale bar: 1 mm.

Unpaired Student's *t* test was conducted (**D**). Two-way ANOVA was conducted, followed by Sidak's multiple comparisons test (**F-H**). Data are represented as mean±SEM.

Supplemental Figure V



PERK activation improves cardiomyocyte survival against reperfusion injury.

A. PERK silencing in NRVMs. Quantification is shown at right. n=3.

B. SYTOX Green staining to visualize cell death in H9c2 cells after PERK knockdown and activation, respectively. Scale bar: 100 μm. n=4.

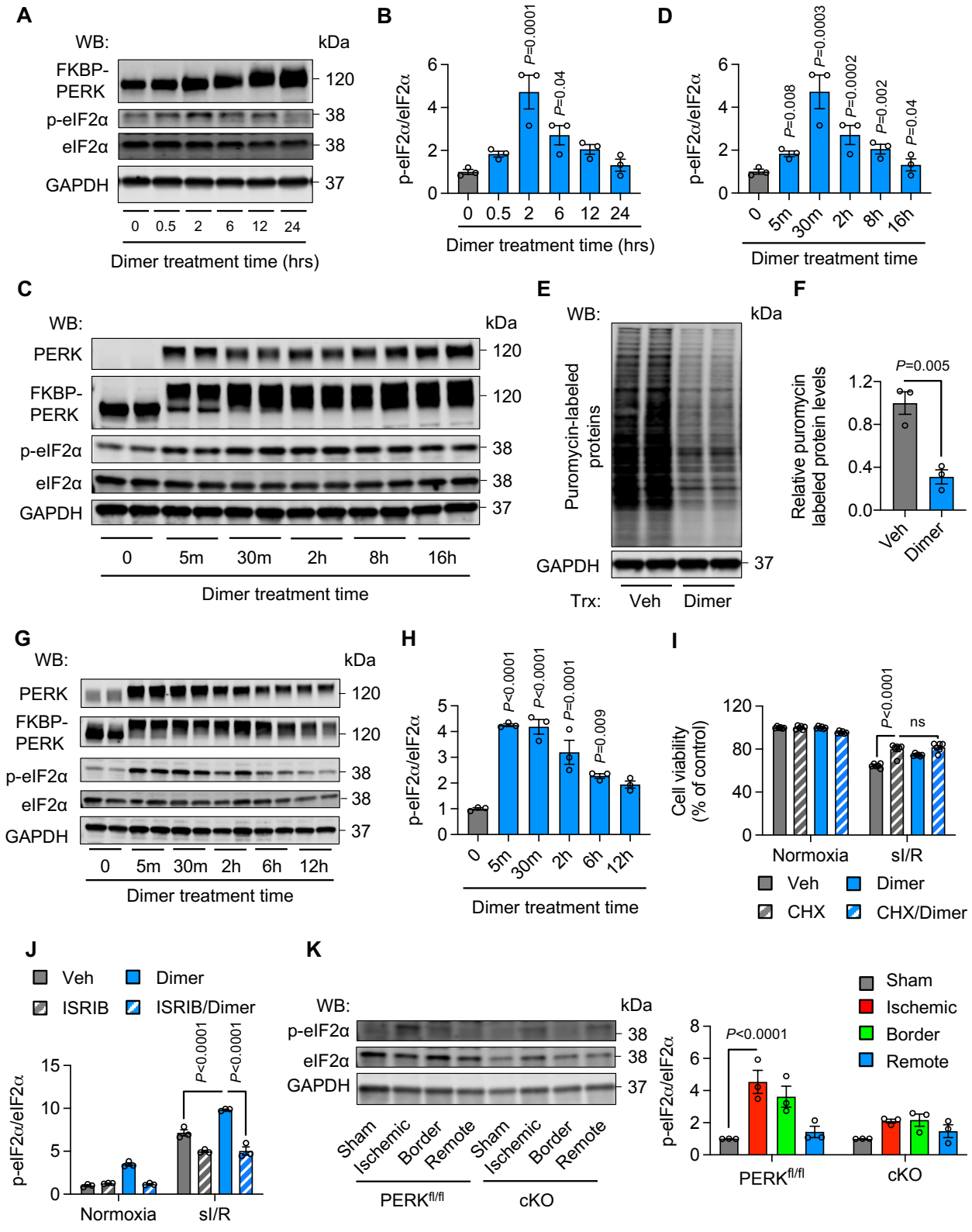
C. Validation of PERK deletion in PERK$^{-/-}$ mouse embryonic fibroblasts (MEFs).

D. Functionality validation of the FKBP-PERK fusion construct. Plasmid expressing either control LacZ or FKBP-PERK fusion protein was transfected to HEK293 cells. After 36 hours, either vehicle or dimer was used to treat the cells. Total proteins were extracted to detect FKBP-PERK expression and eIF2α phosphorylation. n=3.

E. PERK activation by dimer in NRVMs activated eIF2α. n=3.

Unpaired Student's *t* test was conducted (**A, D**). Two-way ANOVA was conducted, followed by Tukey's multiple comparisons test (**B, E**). Data are represented as mean±SEM.

Supplemental Figure VI

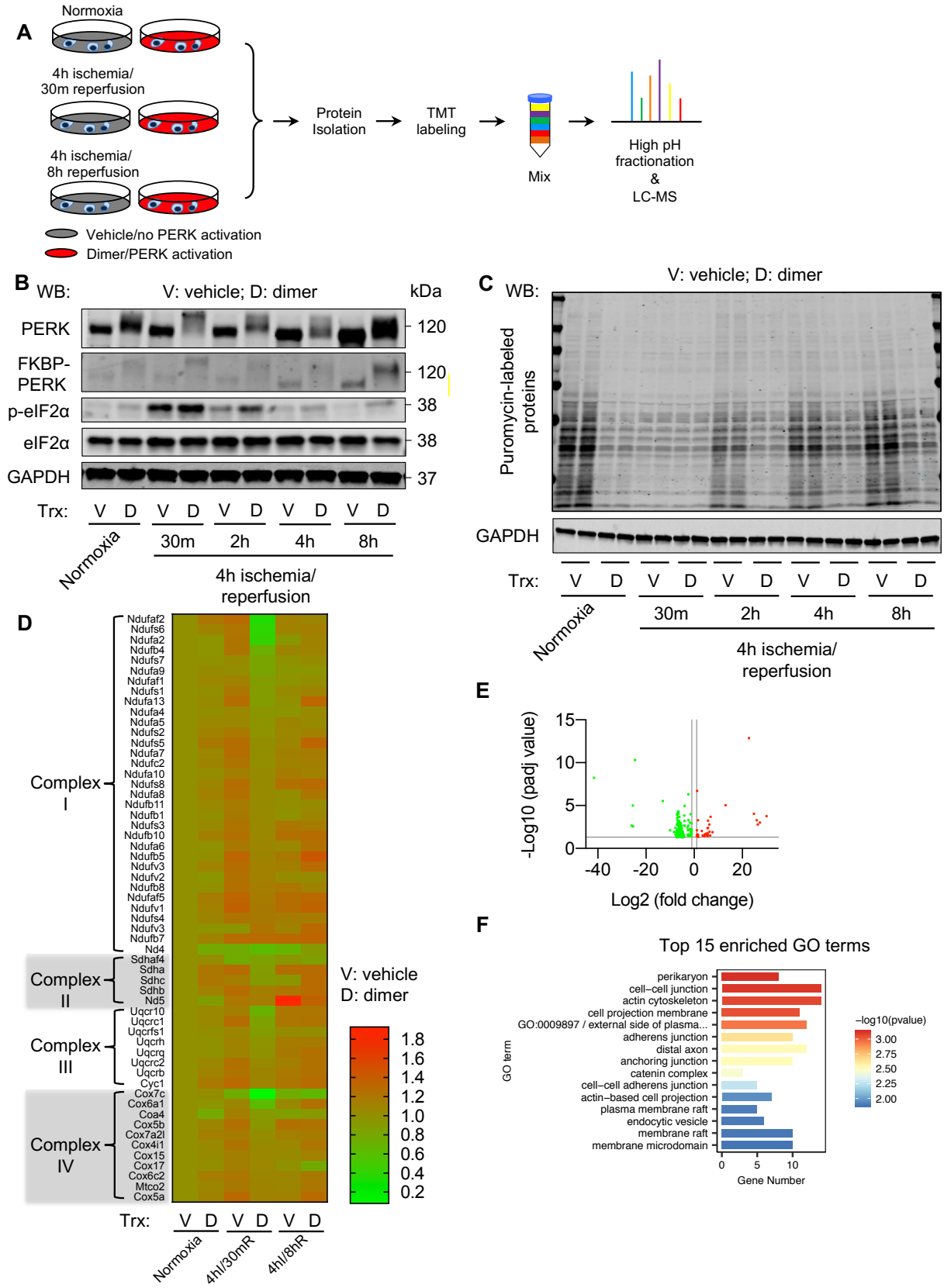


PERK activation suppresses protein synthesis.

- **A.** PERK activation by dimer injection in PERK transgenic mice led to phosphorylation of eIF2α. TG mice was injected by dimer and hearts were collected for Western blotting.
- **B.** Comparison was conducted between different time points and time 0 in PERK transgenic hearts of (**A**). n=3.
- **C.** PERK activation by dimer treatment in H9c2 cells caused eIF2α phosphorylation.
- **D.** Quantification of (**C**) showed a significant increase of eIF2α phosphorylation in H9c2 cells. Comparison was conducted between different time points and time 0. n=3.
- **E.** PERK activation in NRVMs led to a decrease of protein synthesis.
- **F.** Quantification of (**E**). n=3.
- **G.** PERK activation by dimer in NRVMs activated eIF2α phosphorylation.
- **H.** Quantification of (**G**). Comparison was conducted between different time points and time 0. n=3.
- **I.** Cycloheximide (CHX) treatment was sufficient to protect H9c2 cells from sI/R injury. It is important to note that the overexpression and activation of PERK did not confer further protection compared to CHX treatment alone. n=6. ns, not significant.
- **J.** ISRIB treatment caused a significant decrease of eIF2α phosphorylation in H9c2 cells. n=3.
- **K.** PERK deletion in the heart decreased eIF2α phosphorylation after I/R. n=3.

One-way ANOVA was conducted, followed by Dunnett's multiple comparisons test (**B, D, H, K**). Unpaired Student's *t* test was conducted (**F**). Two-way ANOVA was conducted, followed by Tukey's multiple comparisons test (**I, J**). Data are represented as mean±SEM.

Supplemental Figure VII



PERK activation leads to a decrease in mitochondrial complex proteins.

A. Schematic of proteomic analysis for PERK activation under normoxia and sI/R. H9c2 cells were infected by adenoviruses expressing FKBP-PERK. Either vehicle or dimer was included in culture medium. Cells were then subjected to various treatments. After protein isolation, TMT was used to label individual proteins for MS.

B. Dimer treatment for different times led to the activation of PERK and the phosphorylation of eIF2α in H9c2 cells. This experiment was used to identify the appropriate time points for MS. V, vehicle; D, dimer.

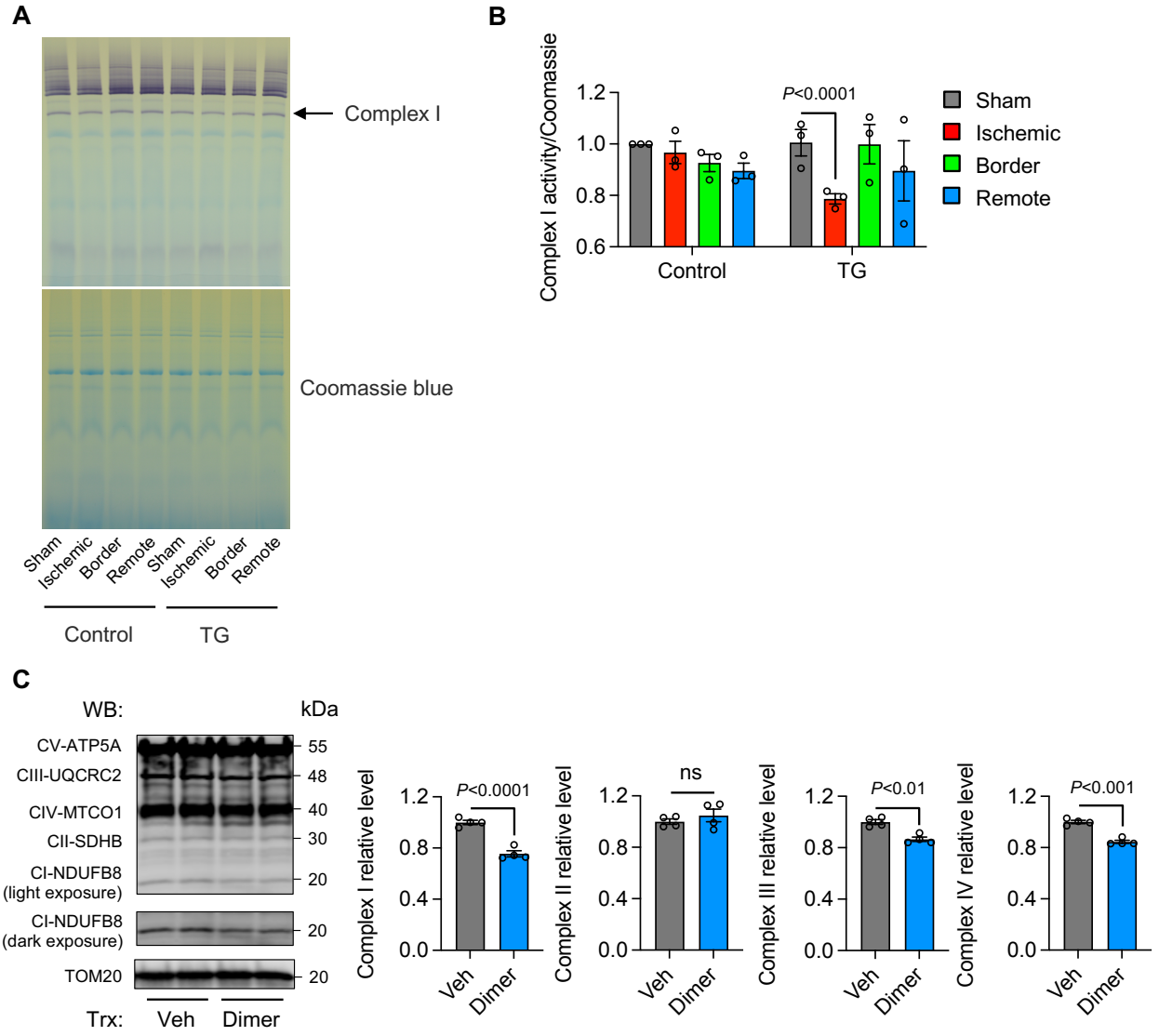
C. PERK activation caused a decrease in protein synthesis in a time-dependent manner in H9c2 cells. V, vehicle; D, dimer.

D. Differentially expressed mitochondrial complex proteins were compared between the vehicle/normoxia group and other treatment conditions. 4hI/30mR, 4 hours simulated ischemia/ 30 minutes reperfusion; 4hI/8hR, 4 hours simulated ischemia/ 8 hours reperfusion. V, vehicle; D, dimer.

E. Volcano plot of differentially expressed genes (DEGs) between vehicle and dimer treatments. H9c2 cells were infected by adenoviruses expressing FKBP-PERK. Either vehicle or dimer was used to treat the cells, followed by sI/R. Total RNAs were isolated for RNA-seq analysis. A total of 221 genes were downregulated and 41 genes were upregulated in dimer-treated cells in comparison with vehicle-treated ones (defined as $|\log2 (\text{fold change})| > 1$ and p[adjusted] value < 0.05). n=3.

F. GO pathway analysis (cellular components, CC) of DEGs from RNA-seq.

Supplemental Figure VIII

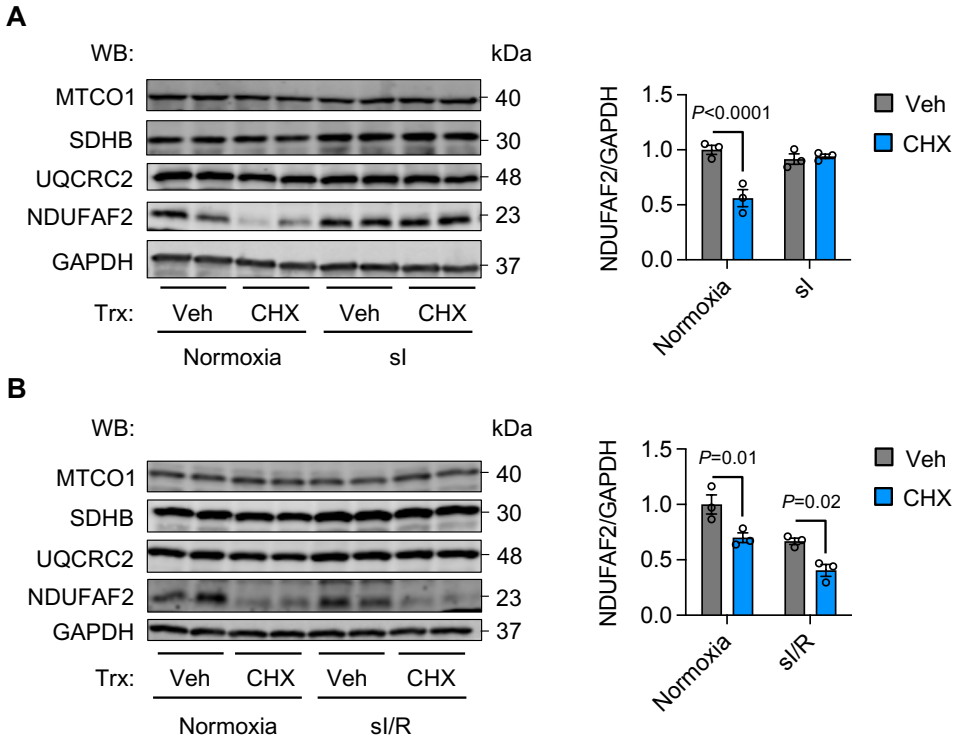


PERK activation reduces mitochondrial complex I activity.

- **A.** Control and PERK transgenic (TG) mice were used for I/R. Mitochondria were isolated from different regions of the heart. Mitochondrial complex I activity was determined by an in-gel activity assay. Coomassie blue staining was used as loading control.
- **B.** Quantification of (**A**). n=3.
- **C.** H9c2 cell mitochondria were isolated after sI/R and used for immunoblotting with the OXPHOS antibody cocktail. Representative components of individual complexes were quantified and compared between vehicle and dimer treatment groups. n=4.

One-way ANOVA was conducted, followed by Sidak's multiple comparisons test (**B**). Unpaired Student's *t* test was conducted (**C**). Data are represented as mean±SEM.

Supplemental Figure IX

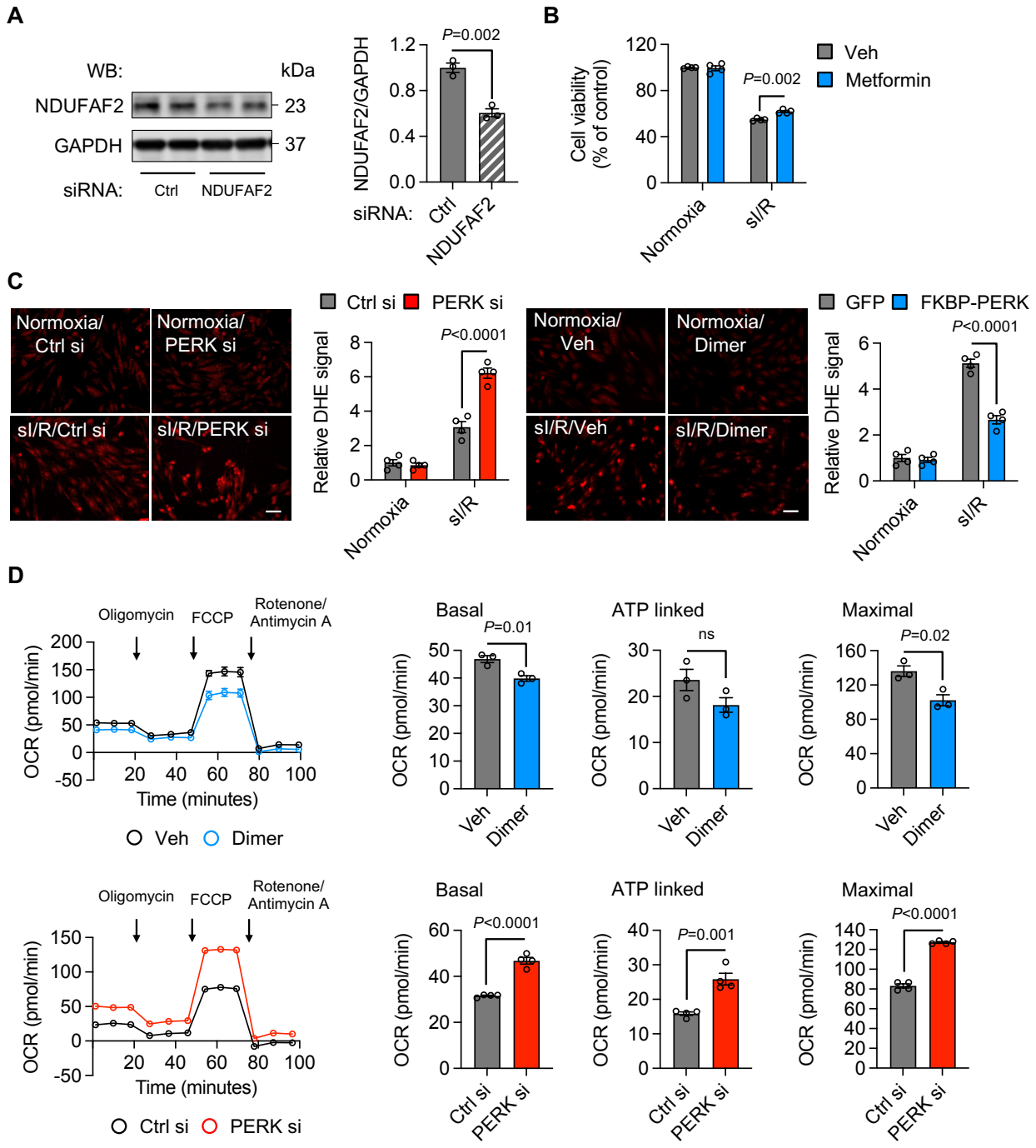


NDUFAF2 is differentially regulated by sI and sI/R.

- **A.** The turnover rate of NDUFAF2 was faster than components of other mitochondrial complexes. sI significantly decreased this rate. n=3.
- **B.** NDUFAF2 turnover rate was increased by sI/R compared to that under normoxia. n=3.

Two-way ANOVA was conducted, followed by Tukey's multiple comparisons test (**A-B**). Data are represented as mean±SEM.

Supplemental Figure X



PERK governs the production of mitochondrial ROS.

A. Validation of siRNA-mediated knockdown of NDUFAF2 at the protein level in H9c2 cells. n=3.

B. Metformin treatment improved cell survival under sI/R. n=4.

C. PERK silencing increased cellular ROS level in H9c2 cells. In contrast, PERK activation reduced ROS level, as assessed by DHE (dihydroethidium) staining. Scale bar: 100 μm. n=4.

D. PERK activation caused a decrease of OCR (oxygen consumption rate) in H9c2 cells. In contrast, PERK silencing led to an increase in OCR. n=3 to 4.

Unpaired Student's *t* test was conducted (**A, D**). Two-way ANOVA was conducted, followed by Tukey's multiple comparisons test (**B-C**). Data are represented as mean±SEM.