Supplemental Material

ER Chaperone GRP78 Protects Heart from Ischemia/Reperfusion Injury through Akt Activation

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Detailed methods

Animals

To engineer inducible, GRP78-expressing transgenic mice, GRP78 cDNA was amplified from a mouse brain cDNA library, cloned into the pCR4TA vector (Thermo Scientific) and verified by sequencing (Genewiz). The CAG promoter was cloned from the pCAGEN vector[1] into the pBluescript vector. A transcriptional/translational "stop" cassette[2] flanked by lox P sites was inserted downstream of the CAG promoter between Xba I and Nru I sites. The GRP78 cDNA was then inserted downstream of the second lox P site between Sac I and Sph I sites. The transgene cassette was liberated by digestion with Sfi I and Sal I restriction enzymes, and the resulting 6kb fragment of CAG-loxP-STOP-loxP-GRP78 was separated and isolated for pronuclear injection. Transgene-positive lines were then crossed with αMHC-Cre mice to achieve cardiomyocyte-specific expression of GRP78. Genotyping PCR primers are provided: Online Table I.

Cardiac ischemia/reperfusion

Adult male mice 12-14 weeks of age were subjected to cardiac I/R surgery as described.[3] Briefly, animals were anesthetized with a cocktail of ketamine and xylazine and placed on a warming pad to maintain body temperature within a range of 34°C to 37°C. After intubating with a 19 G stump needle, animals were ventilated with 95% oxygen/5% CO_2 using a MiniVent mouse ventilator (stroke volume: 250 μL; respiratory rate: 210 breaths per minute). Next, an oblique incision was made from the left sternal border to visualize the fourth intercostal space. With the left anterior descending coronary artery exposed, a ligature was placed 1 mm below the tip of the left atrial appendage using a 6-0 silk suture. After 45 minutes of coronary occlusion, the wound was re-opened, and the ligation was released to allow for tissue reperfusion. The animals were then sacrificed 24 hours later for 2,3,5-triphenyltetrazolium chloride (TTC) staining or tissue harvesting. The infarcted area and area at risk were quantified and relative ratios were calculated. To harvest tissues, the left ventricle was divided into three regions based on previous TTC staining patterns: ischemic, border, and remote. Tissues were frozen separately in liquid nitrogen and stored at -80°C until use.

Stimulated I/R and cell death assay

For simulated I/R *in vitro*, NRVM were first washed twice with 1 X PBS to remove culture medium and then incubated with fresh ischemia Esumi buffer (4 mM HEPES, 117 mM NaCl, 12 mM KCl, 0.9 mM $CaCl_2$, 0.49 mM $MgCl_2$, 20 mM sodium lactate, 5.6 mM 2-deoxyglucose, pH 6.2).[4, 5] Cells were then placed into a hypoxia chamber (Billups-Rothenberg) and maintained at 37°C. After flushing with 95% N_2/5% CO_2 for 30 minutes, the chamber was sealed and maintained for an additional 2.5-5.5 hours. Then, cells were reperfused by restoration of standard culture medium. NRVM incubated in normoxia medium (4 mM HEPES, 137 mM NaCl, 3.8 mM KCl, 0.9 mM $CaCl_2$, 0.49 mM $MgCl_2$, 5.6 mM D-Glucose, pH 7.4) were used as controls. Cell death was assessed by LDH assay using the CytoTox96 cytotoxicity kit according to the manufacturer's recommendations (Promega).

Echocardiography

Cardiac contractile function was examined by echocardiography in conscious, gently restrained mice using a Vevo 2100 system as previously described (MS400C probe, VisualSonics).[3] M-mode recordings were captured and analyzed to calculate LVID-diastole (LVIDD) and LVID-systole (LVIDS). Further, ventricular fractional shortening (FS%) was calculated as (LVIDD-LVIDS)/LVIDD. All measurements were conducted at the level of the papillary muscles.

Histology

Hearts were harvested and immediately fixed in 4% paraformaldehyde for 48 hours. Paraffin sections of 5 μm thickness were studied. Hematoxylin and eosin staining and Masson's trichrome staining were performed by the Molecular Pathology Core at UT Southwestern.

Cellular fractionation

To prepare enriched membrane fractions, cardiac tissues were lysed in homogenization buffer (10 mM, Tris.HCl, pH 7.4) supplemented with protease and phosphatase inhibitors (Thermo, A32961). After low-speed centrifugation at 500 x *g* for 10 min, supernatants were removed for protein concentration determination. A discontinuous sucrose gradient was prepared with ultra-clear centrifuge tubes (Beckman Coulter, 344059) by laying down different concentrations of sucrose solutions from 0.2 M to 2 M. Cardiac tissue lysates of equivalent total protein abundances from sham and I/R hearts were then mixed with the 2 M sucrose solution for a final concentration of 0.2 M sucrose. The samples were loaded on the top of the sucrose gradient for ultracentrifugation using an SW41Ti rotor at 40,000 RPM for 8 hrs. Fractions of 500 μL each were carefully removed from the top to the bottom and subjected to immunoblotting analysis.

Neonatal rat ventricular myocyte isolation

Neonatal rat ventricles from 1-2 day old Sprague-Dawley rats were collected and subjected to cardiomyocyte isolation using a neonatal rat/mouse cardiomyocyte isolation kit (NC-6031, Cellutron). After clearance of fibroblasts by pre-plating for 2 hours, myocytes were plated at a density of 1,250 cells/mm^2 in culture medium containing 5% FBS, 10% horse serum and 100 μM bromodeoxyuridine. Immunostaining for α-actinin was conducted after each isolation to monitor the purity of preparation. We typically obtain >90% cardiomyocytes.

Adenovirus infection

To engineer adenovirus expressing GRP78, a Flag tag was inserted after the signal sequence (aa 1-19) in wild type mouse GRP78. The cDNA was then cloned into pShuttle and used for adenovirus production. For mutant GRP78, a Flag tag was inserted between aa 104 and 105 to engineer a protein

previously shown[6] to eliminate GRP78-dependent PI3K activation without disrupting surface migration. NRVM were exposed to GRP78-expressing adenovirus for 6 hours (MOI 10-50), followed by restoration of standard culture medium. We routinely obtained ≈ 70% infection rates.

Protein carbonylation detection

Cardiomyocytes were lysed with RIPA buffer, and protein concentration was determined by BCA assay (Thermo Scientific). Each sample was evenly divided into two tubes (20 μg each), and the protein was denatured by adding an equal volume of 12% SDS (final concentration 6%). DNPH (2,4-dinitrophenylhydrazine, Sigma) was dissolved in 10% (v/v) trifluoroacetic acid for a 20 mM stock. Trifluoroacetic acid (10%) was used as negative control. DNPH (10 μL) or equal volume of the negative control was added to the protein aliquots with 15 minutes of incubation. The reaction was then neutralized by adding Tris solution (7.5 μL, 2 M). A noticeable color change from light yellow to orange was observed. Both the DNPH-treated and negative control samples were subjected to polyacrylamide gel electrophoresis. Carbonyl groups were detected by Western blotting with anti-DNP antibody (Millipore, MAB2223).

Immunoblot analysis

Heart tissues (20-50 mg) or NRVM were lysed in RIPA buffer supplemented with protease inhibitors and phosphor-STOP (Roche). The lysates were then cleared by centrifugation (14,000 rpm, 10 min, 4°C). Equal amounts of protein (10-20 μg) were separated by SDS-PAGE (Criterion, Bio-Rad), followed by transfer to nitrocellulose membranes for immunoblot analysis (Odyssey scanner, Li-Cor). Except for GRP78 (BD Biosciences, 610919), p-IRE1α (Novus Biologicals, NB100-2323), ATF6 (Cosmo Bio., 73-505-EX), t-eIF2α (Thermo, AHO1182), p-eIF2α (Thermo, PA5-37800), O-GlcNAc (Thermo, RL2, MA1-072), Na-K ATPase (Thermo, MA3-929), and GAPDH (Fitzgerald, 10R-G109A), all antibodies were purchased from Cell Signaling Technology (GRP94, #2104; total-Akt, #9272; p-Akt (T308), #13038; p-Akt (S473), #4060; total-Erk, #4695; p-Erk, #4370; p-PERK, #3179; p85, #4292; t-IRE1α, #3294, Serca2, #9580). Goat anti-rabbit secondary antibody 800 CW (Li-Cor, 925-32211) and goat anti-mouse secondary antibody Alexa Fluor 680 (Thermo Scientific, A21057) were used to detect the fluorescence signal.

Immunofluorescence staining

NRVM were first infected with adenovirus expressing either Flag-tagged wild-type GRP78 or mutant GRP78, followed by fixation in 4% paraformaldehyde for 10 min. The cells were then washed with cold PBS three times and blocked with 5% BSA in PBS on ice for 30 min. Mouse anti-Flag antibodies (1:1000, Cell Signaling, #8146) were used to incubate with NRVM overnight at 4°C. After washing with cold PBS 3 times, the cells were stained with Alexa Fluor-594 goat anti-mouse antibodies in 1% BSA/PBS (1 hr at 4°C) without permeabilization. The cells were then permeabilized with 0.5% saponin (15 min at RT). After washing with 0.01% saponin in PBS three times, NRVM were blocked

again with 5% BSA in PBS/saponin (1 hr at RT). Primary rabbit anti-p85 antibodies (1:50, Cell Signaling, 4292) were diluted in 1% BSA in PBS/saponin and incubated with NRVM (2 hrs at RT). The cells were washed with PBS three times and incubated with Alexa Fluor-649 goat anti-rabbit antibodies (1 hr at RT). NRVM were then counterstained with DAPI, and images were obtained using a Nikon A1 confocal microscope.

Co-staining for PIP3 was similarly conducted. Briefly, NRVM were incubated with rabbit anti-Flag antibodies (1:1000, Cell Signaling, 14739) overnight at 4°C and stained with Alexa Fluor-649 goat anti-rabbit antibodies. The cells were then permeabilized and incubated with mouse anti-PIP3 antibody (1:50, Echelon Biosciences Incorporated, Z-P345b). After staining with Alexa Fluor-594 goat anti-mouse antibodies, images were obtained using a confocal microscope. Colocalization quantification was conducted as previously reported.[7]

Cell surface protein biotinylation and detection

NRVM were first subjected to sI/R. Then, cells were rinsed twice with cold PBS. EZ-link® Sulfo-NHS-LC-Biotin (Thermo Scientific) at a concentration of 0.5 mg/mL was added to 6-well plates. As a control, the same volume of PBS was added to cover the surface of the cell layer. Plates were gently shaken (30 min at 4°C). Tris-Cl, pH 7.5 at 100 nM was used to extinguish the biotinylation reaction. After rinsing twice with ice cold PBS, the cells were lysed with radioimmune precipitation buffer (RIPA). To purify surface proteins, neutravidin agarose beads were mixed (overnight at 4°C) with the lysate. The beads were then washed six times with PBS. Cell surface proteins were released by mixing with SDS-PAGE loading buffer and heating (100°C for 5 min). Immunoblotting was then conducted to detect GRP78 biotinylation.

Flow cytometry

To quantify cell death, NRVM were harvested by digesting with 0.05% trypsin without EDTA after washing in cold PBS. Proteolysis was then neutralized with fetal bovine serum, and the lysates were concentrated and resuspended in 100 μL PBS. Annexin V-FITC and PI staining solution (Biotool) were added, and the suspension was subjected to flow cytometry. For ROS detection, cells were infected with control or GRP78 adenovirus. Next, either 10 μM LY294002 (Cell Signaling, 9901) or 20 μM Akt inhibitor VIII (STEMCELL technologies, 72942) was added for 24 hrs before sI/R. For ROS inhibition, NRVM were first incubated with 10 μM diphenyleneiodonium (Sigma, D2926) or 10 μM MitoTEMPO (Sigma, SML0737) for 30 min before sI/R. Dihydroethidium (Thermo Scientific, 50 μM) was then used to detect superoxide radicals. After 15 min incubation, 400 μL PBS with added, and the stained cells were analyzed by flow cytometry (BD FACSCalibur).

RNA isolation and PCR

Cardiac RNA was isolated using the total RNA Fatty and Fibrous Tissue kit (Bio-Rad). Approximately 5-10 mg of tissue were used for each sample. RNA from NRVM was harvested with the

Quick-RNA MicroPrep kit (Zymo research). Total RNA (150-250 ng) was subjected to reverse transcription using the iScript cDNA synthesis kit (Bio-Rad). The cDNA was then diluted 10-fold with ddH_2O, and real-time PCR was performed using 2 μL cDNA on a Lightcycler 480 (Roche). The fold change of relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method with 18S RNA serving as internal control.[8]

Statistics

Data are expressed as mean ± SEM. The Student's *t* test (2-tailed) was used to compare two groups. One-way or two-way ANOVA were used to compare differences among multiple groups. Tukey tests were used to evaluate differences between the mean values of paired groups within multiple groups. $P < 0.05$ was considered statistically significant.

Supplemental References

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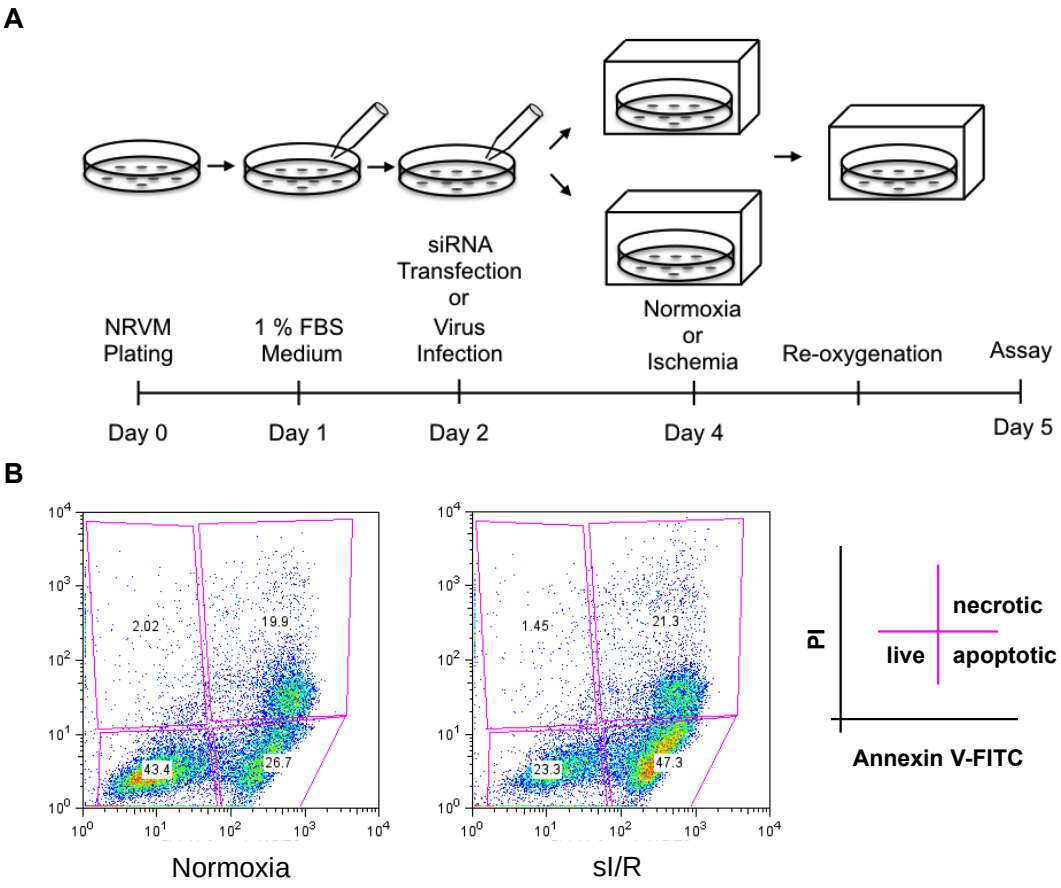
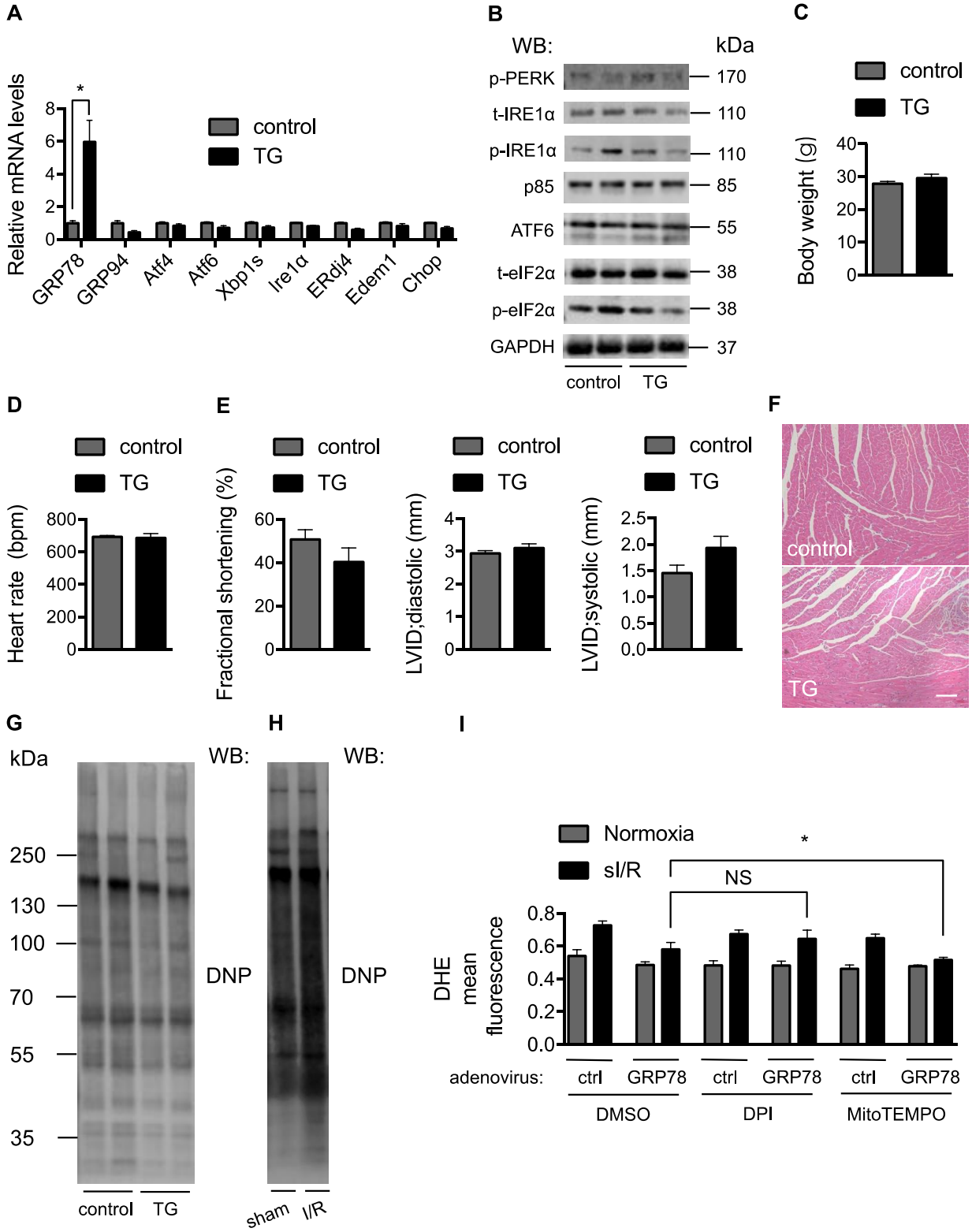
Online Figure I



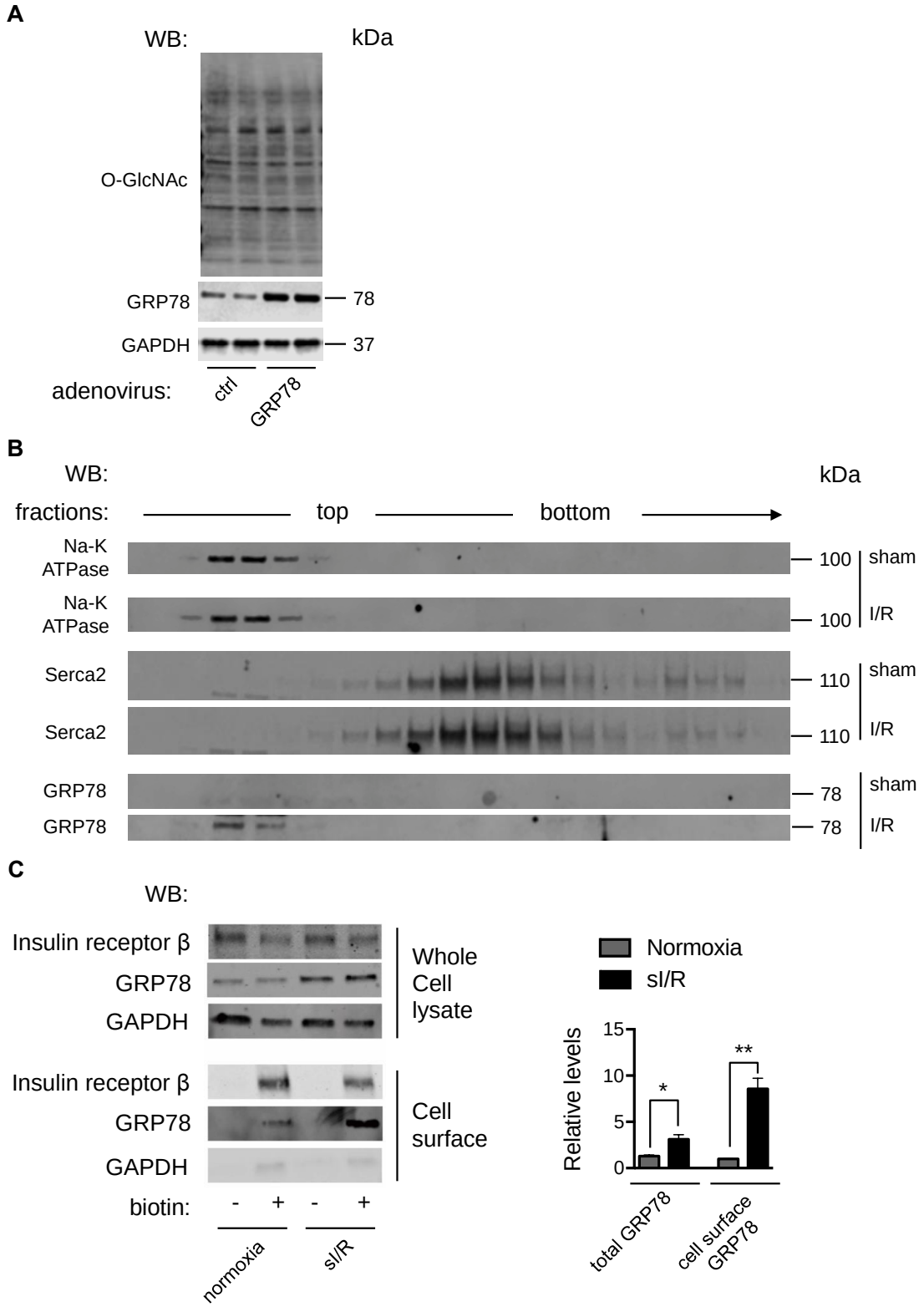
Online Figure I. Ischemia/reperfusion leads to cardiomyocyte death. **A,** Schematic of NRVM sI/R experiments. Cells were isolated from day 1-2 day old rat pups. Day 2 after plating in 6-well plates, adenoviral over-expression or siRNA knockdown was conducted. After 48 hours, cells were subjected to ischemia for 3-6 hours, followed by overnight reperfusion. NRVM were then harvested for cell death assays or gene expression profiling. **B,** Cardiac myocyte death as assessed by flow cytometry for Annexin V- and PI-positive cells. A representative histogram is depicted.

Online Figure II



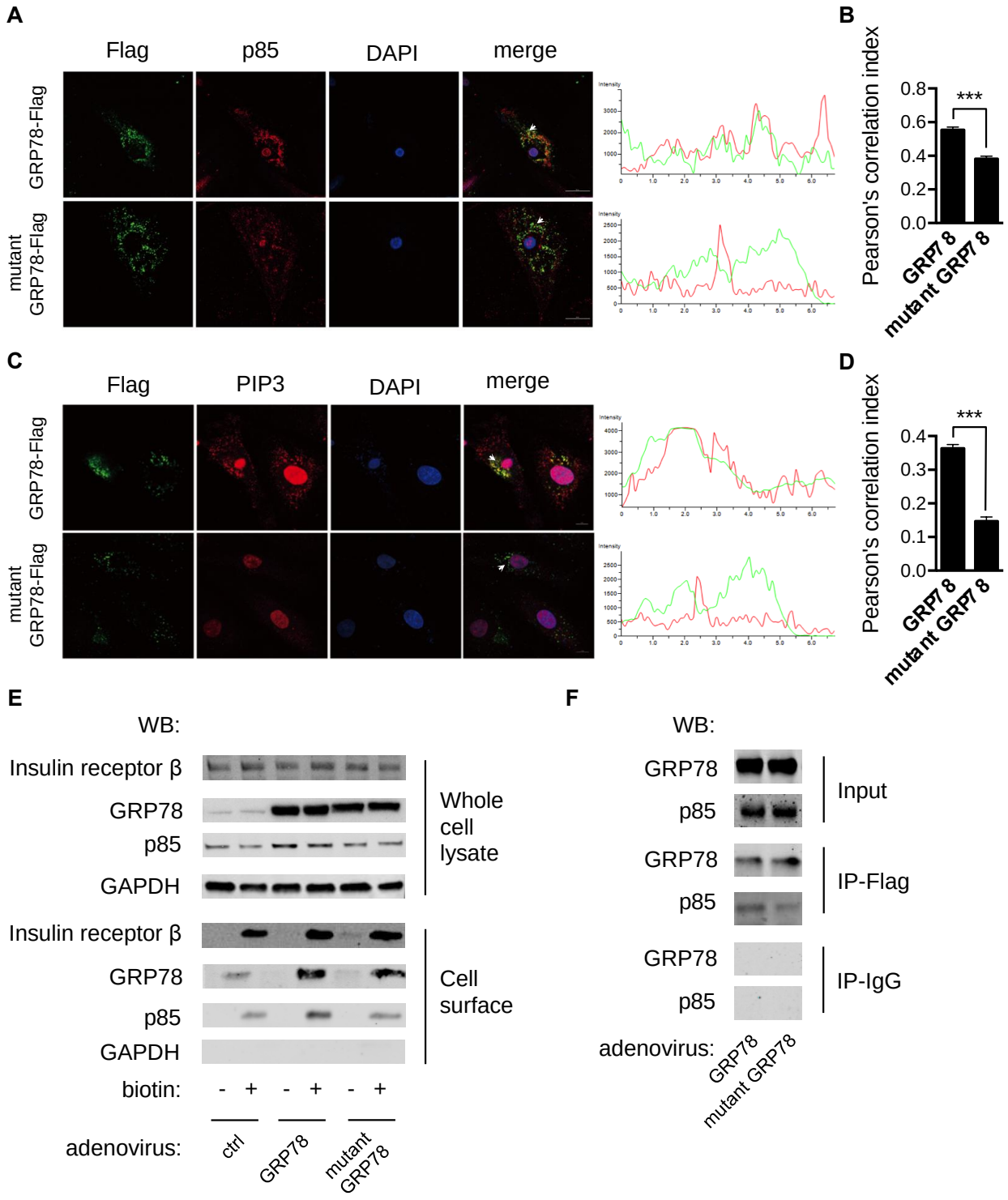
Online Figure II. Cardiomyocyte-specific over-expression of GRP78. **A,** GRP78 expression was induced specifically in cardiomyocytes. Total RNA was isolated from ventricular tissue of control and GRP78-expressing transgenic animals. Real-time PCR was conducted to assess genes involved in the UPR. N = 4-6 for each group. **B,** Cardiomyocyte-specific over-expression of GRP78 did not affect protein levels of UPR transducers. GAPDH was used as a loading control. **C,** GRP78 over-expression in the heart did not alter body weight in mice. N = 4-6 per group. **D,** No changes in heart rate were observed between control and GRP78 TG mice. N = 4-6 for each group. **E,** GRP78 over-expression in the heart did not affect baseline ventricular contractile function, as evidenced by lack of significant differences in ventricular fractional shortening, LVID-systole, and LVID-diastole. N = 4-6. **F,** No changes were observed at the level of tissue histology using H&E staining. Scale bar: 100 μm. **G,** Over-expression of GRP78 did not alter basal ROS levels as assayed by protein carbonylation assay. **H,** Myocardial ischemia/reperfusion (I/R) injury triggered an increase in protein carbonylation compared with sham-operated controls. **I,** GRP78-mediated quenching of ROS was largely dependent on NADPH oxidase. DPI was used to suppress NADPH oxidase, which did not manifest an additive effect over-and-above GRP78 over-expression. On the other hand, MitoTEMPO showed additional inhibition of ROS when NRVM were infected by GRP78 adenovirus, compared to GFP controls. N = 4. NS, not significant; *, $p < 0.05$.

Online Figure III



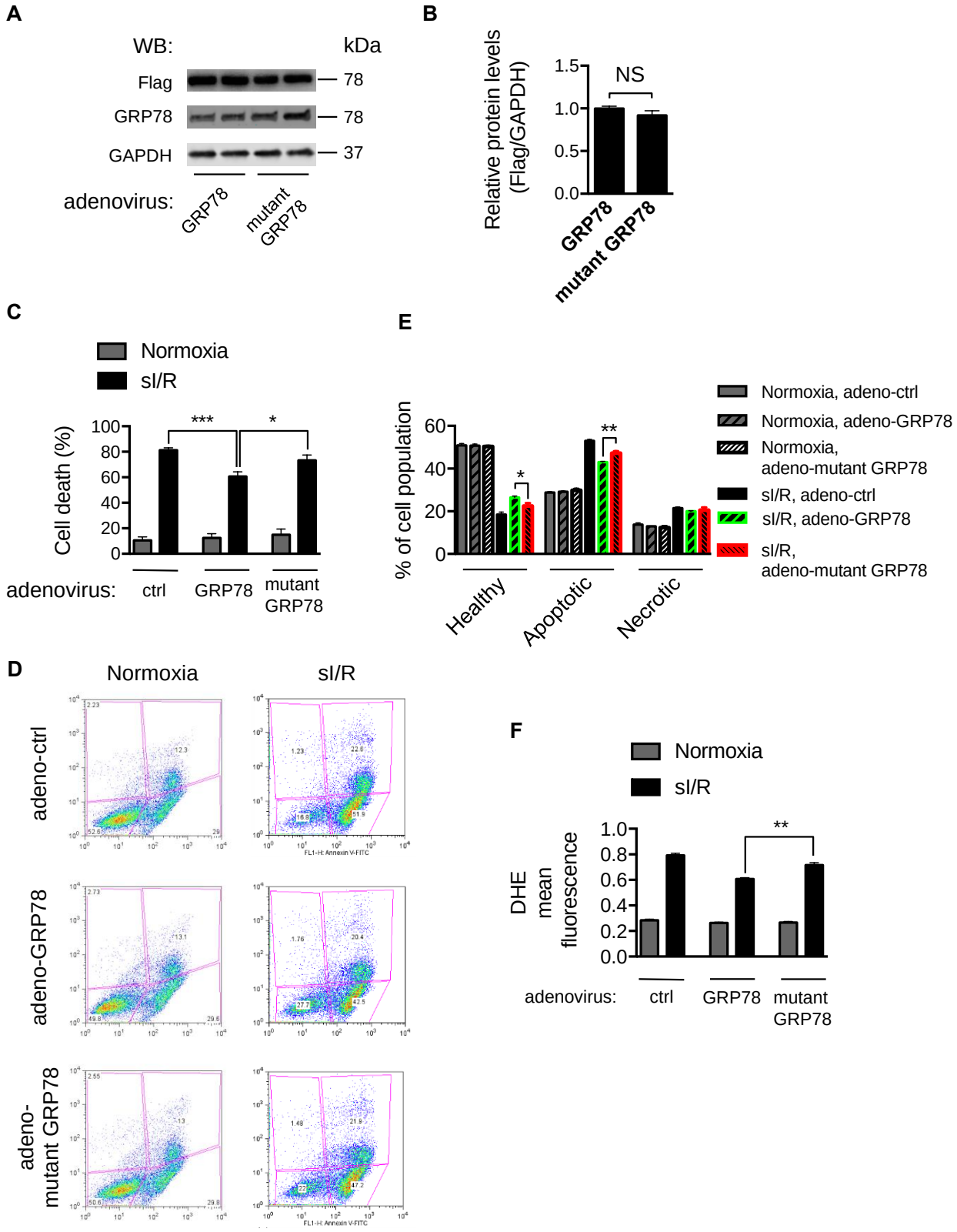
Online Figure III. GRP78 translocates to the cardiomyocyte plasma membrane. A, Over-expression of GRP78 did not alter O-GlcNAc post-translational protein modification. NRVM were infected by adenovirus expressing either control GFP or GRP78. Total proteins were isolated for immunoblotting for O-GlcNAcylation. GAPDH was used as a loading control. **B,** GRP78 was detected in the same sucrose gradient fractions as cardiac plasma membrane after I/R. The LAD coronary artery was ligated for 45 min followed by reperfusion for 24 hrs. The ischemic zone was harvested and cellular lysates were separated by a discontinuous sucrose gradient. Fractions were collected from the top to the bottom of the gradient and subjected for immunoblotting for Na-K ATPase (plasma membrane marker), Serca2 (SR/ER membrane marker), and GRP78. **C,** NRVM were subjected to sI/R and then placed on ice. EZ-link Sulfo-NHS-LC-Biotin was used to modify cell surface-localized proteins. After isolation of biotinylated surface proteins with neutravidin, immunoblotting was conducted to reveal GRP78. Insulin receptor β was used as a positive control. Quantification (right) revealed significant increases in both total GRP78 and cardiomyocyte surface-localized GRP78 after sI/R. N = 3 for each group. *, $p < 0.05$; **, $p < 0.01$.

Online Figure IV



Online Figure IV. Membrane-bound GRP78 colocalizes with PI3K. **A,** GRP78 localization overlapped significantly with that of p85, the regulatory subunit of the PI3K complex. NRVM were infected with either wild-type or mutant GRP78-expressing adenovirus. GRP78 was revealed by confocal immunofluorescence staining for the Flag tag. An insertion mutation of GRP78 did not alter plasma membrane translocation, but colocalization with p85 was strongly impaired. Scale bar: 20 μm. The areas of interests were labeled by white arrows. Signaling intensity for both channels were scanned and recorded at right. **B,** Colocalization of GRP78 and p85 was quantified by Pearson's correlation index. N = 20. **C,** GRP78 over-expression in NRVM led to increases in PIP3 production. Confocal staining for GRP78 (Flag tag) and PIP3 was conducted after adenovirus infection. The insertion mutation of GRP78 triggered less production of PIP3. Scale bar: 10 μm. The channels for GRP78 and PIP3 were scanned at the arrow-depicted areas, and results are shown at right. **D,** Pearson's coefficient indices were calculated for both GRP78 and PIP3 signaling. The comparison suggests that mutant GRP78 significantly disrupts GRP78 and PIP3 colocalization. N = 20. **E,** Wild-type or mutant GRP78 were over-expressed in NRVM. A biotinylation experiment for cell surface proteins was conducted. Western blotting revealed that over-expression of wild-type GRP78 increased cell membrane localization of p85, and the translocation was significantly impaired in mutant GRP78-overexpressing cells. Insulin receptor β was used as a control. **F,** After biotinylation and neutravidin isolation, membrane-bound proteins were eluted by free biotin competition. Next, a second immunoprecipitation was conducted using Flag antibodies or control IgG. Western blotting showed that association of p85 with GRP78 was decreased in mutant GRP78-overexpressing cells, highlighting a direct interaction of GRP78 and PI3K. ***, $p < 0.001$.

Online Figure V



Online Figure V. Cell surface GRP78 confers protection against reperfusion injury in cardiac myocytes. **A,** NRVM were infected by adenovirus expressing either wild type or mutant GRP78. Immunoblotting was conducted to evaluate expression levels. GAPDH was used as a loading control. **B,** Quantification of GRP78 revealed similar levels of expression of wild-type and mutant GRP78. N = 4. **C,** Over-expression of GRP78 led to significant protection against sI/R-induced cardiomyocyte death as assayed by relative LDH release. This beneficial effect was attenuated in mutant GRP78-expressing cells. N =3. **D,** Flow cytometry was conducted to determine the mode of cell death in NRVM after sI/R. Representative histograms are shown. **E,** Quantification of **D)** showed that over-expression of wild-type GRP78 significantly inhibited apoptotic cell death by sI/R; mutant GRP78 conferred less protection. N = 3. **F,** Over-expression of GRP78 led to decreases in ROS accumulation in sI/R cells. This effect was significantly diminished when mutant GRP78 was expressed. N = 3. NS, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Online Table I

Gene	Species	Primer sequence	Test
GRP78	mouse	CCTCTCTGGTGATCAGGATA CGTGGAGAAGATCTGAGACT	genotyping /qPCR
Cre		GATTTCGACCAGGTTCGTTC GCTAACCAGCGTTTTCGTTC	genotyping
18s	rat	AAACGGCTACCACATCCAAG CCTCCAATGGATCCTCGTTA	qPCR
GRP78	rat	CTTCTCAGCATCAAGCGAGG GTAGATCCGCCAACCAGAAC	qPCR
GRP94	rat	GACAGAAGCACAAGAAGACG GTTCCACGACCTAGTGTGTT	qPCR
Atf4	mouse	CCACCAGACAATCTGCCTTC CTAGCTCCTTACACTCGCCA	qPCR
Atf6	mouse	GCCGACTGTGGTTCAACTTC TCCTCAGCACAGCGATATCC	qPCR
Xbp1s	mouse	GGTCTGCTGAGTCCGCAGCAGG GAAAGGGAGGCTGGTAAGGAAC	qPCR
Ire1a	mouse	GCGATGGACTGGTGGTAACT TCTTGGCCTCTGTCTCCTTG	qPCR
Erdj4	mouse	CAGAATTAATCCTGGCCTCC ACTATTGGCATCCGAGAGTG	qPCR
Edem1	mouse	CTGCAATGAAGGAGAAGGAG TAGAAGGCGTGTAGGCAGAT	qPCR
Chop	mouse	GTATGAGGATCTGCAGGAGG CTGACTGGAATCTGGAGAGC	qPCR
18s	mouse	AGGGTTCGATTCCGGAGAGG CAACTTTAATATACGCTATTGG	qPCR