ONLINE DATA SUPPLEMENT

Spliced X-box Binding Protein 1 Couples the Unfolded Protein Response to Hexosamine Biosynthetic Pathway

Zhao V. Wang[1], Yingfeng Deng[2], Ningguo Gao[3], Zully Pedrozo[1,4], Dan L. Li[1], Cyndi R. Morales[1], Alfredo Criollo[1], Xiang Luo[1], Wei Tan[1], Nan Jiang[1], Mark A. Lehrman[3], Beverly A. Rothermel[1], Ann-Hwee Lee[5], Sergio Lavandero[1,4], Pradeep P.A. Mammen[1], Anwarul Ferdous[1], Thomas G. Gillette[1], Philipp E. Scherer[2,6], and Joseph A. Hill[1,7]

[1]Department of Internal Medicine (Cardiology), [2]Touchstone Diabetes Center, [3]Department of Pharmacology, [6]Department of Cell Biology, [7]Department of Molecular Biology, University of Texas Southwestern Medical Center, Dallas, TX, 75390, USA,
[4]Advanced Center for Chronic Diseases (ACCDiS) & Centro Estudios Moleculares de la Celula, Facultad Ciencias Quimicas y Farmaceuticas & Facultad Medicina, Universidad de Chile, Santiago, Chile, [5]Department of Pathology and Laboratory Medicine, Weill Cornell Medical College, New York, NY, 10065, USA.

Figure S1. Activation of the unfolded protein response (UPR) and the hexosamine biosynthetic pathway (HBP) by ischemia/reperfusion in heart, Related to Figure 1

(A) Protein O-GlcNAc modification was increased in the infarct zone in I/R-stressed heart (left). Protein levels of the HBP genes, GalE and UPR markers were increased in the same region. Male wild-type mice were subjected to surgical cardiac ischemia for 45 min followed by reperfusion for 4 hrs. GAPDH was used as loading control. Quantification showed a significant increase in the infarct region of I/R mice compared to sham-operated animals (right). N = 3 for each group.

(B) Competition assay to verify the specificity of O-GlcNAc antibody. Neonatal rat ventricular cardiomyocytes (NRVM) were incubated with PUGNAc for 6 hrs to enhance O-GlcNAc modification. Protein lysate was used for immunoblotting with or without 1 M GlcNAc during incubation with the primary antibody.

(C) The HBP pathway. UDP-GlcNAc is synthesized through the HBP by a series of reactions. The rate-limiting step is catalyzed by GFAT1 to convert fructose-6-P to glucosamine (Gln)-6-P. GNPNAT is responsible for addition of an acetyl group to Gln-6-P to produce N-acetylglucosamine-6-P (GlcNAc-6-P). PGM3, a mutase, catalyzes the conversion from GlcNAc-6-P to GlcNAc-1-P. GalE, an epimerase, catalyzes conversion between UDP-GlcNAc and UDP-GalNAc, both of which are critical substrates for glycan synthesis. UDP-GlcNAc is the obligate substrate for O-GlcNAc protein modification.

(D) The protein levels of GFAT1 (upper) and BiP (bottom) were increased in the ischemic region after I/R by immunofluorescence staining. The specificity of antibodies was first verified using no-primary antibody as a negative control. Alpha-actinin (red) was used as a cardiomyocyte marker. The profile of signal intensity of GFAT1 or BiP was then recorded using Image J over a 20 μm distance and quantified as arbitrary units (A.U.). The expression of GFAT1 and BiP was similarly recorded and quantified in different regions of hearts after I/R. Arrow indicates cardiomyocytes in the ischemic zone. Scale bars: 50 μm. N = 3-5. Data are represented as mean ± SEM. *, $p < 0.05$. **, $p < 0.01$. NS, not significant.

Figure S2. Xbp1s is an upstream activator of the HBP, Related to Figures 2, 3

(A) The precipitation of Xbp1s by anti-Xbp1 antibody in the ChIP assay was verified by immunoblotting. Note the sizes of Xbp1s and IgG heavy chain are similar.

(B) Xbp1s over-expression in NRVM in vitro led to up-regulation of GNPNAT1, PGM3 and GalE as assessed by qRT-PCR. N = 6 for each group.

(C) Sequence analysis of the promoters of GNPNAT1, PGM3, and GalE uncovered a conserved region, which is similar to the consensus sequence of the UPRE, an established Xbp1s binding site.

(D) Xbp1s over-expression in NRVM led to increases in O-GlcNAc modification. NRVM were infected with lentivirus expressing LacZ or Xbp1s, and O-GlcNAc levels were detected by immunoblotting. GAPDH was used as loading control. Quantification of O-GlcNAc levels showed a significant increase upon Xbp1s overexpression in vitro. N = 3.

(E) Other genes of the HBP pathway and O-GlcNAc protein modification did not show significant induction by I/R in hearts. The relative mRNA levels of GLUL, GPI1, UAP1 and OGA were quantified by qRT-PCR in different regions after I/R. N = 4-8 for each group.

(F) Other genes of the HBP pathway and O-GlcNAc protein modification did not show dramatic induction by Xbp1s overexpression in transgenic hearts. Xbp1s expression was induced by removal of Dox for 1-3 weeks. Gene expression of GLUL, GPI1, UAP1 and OGA was examined by qRT-PCR. N = 3 per group. Data are represented as mean ± SEM. *, $p < 0.05$.

Figure S3. Cell stress treatment induces strong activation of O-GlcNAc modification and up-regulation of the UPR, Related to Figure 5

(A) COS7 cells were cultured in low glucose DMEM medium containing 10% FCS. After overnight serum starvation, cells were treated with vehicle, 100 mM NaCl, 50 μM $CoCl_2$ or 75 μM arsenite for 8 hrs. Cells were harvested for analysis of O-GlcNAc modification and BiP expression. Comparison was conducted between vehicle (veh) and treatment groups. N = 3 for each group.

(B) Xbp1 expression was first silenced by siRNA knockdown in COS-7 cells. Stress treatments were then conducted for 8 hrs. Total cell lysates were prepared for immunoblotting of O-GlcNAc modification, and nuclear fractions were extracted for Xbp1s detection. GAPDH and Lamin were used as loading controls for total cell lysates and nuclear extracts, respectively. N = 4 for each group.

(C) NRVMs were stressed by starvation. Serum-free medium without glucose/pyruvate/glutamine was used to induce starvation stress. O-GlcNAc protein modification and BiP expression were quantified and compared between groups. Comparison was conducted between control and starvation groups. N = 3 for each group.

(D) Xbp1 expression was first silenced by siRNA knockdown in NRVM. Starvation was similarly conducted. Total cell lysates were prepared for immunoblotting of GFAT1 and O-GlcNAc modification, and nuclear fractions were extracted for Xbp1s detection. GAPDH and Lamin were used as loading controls for total cell lysates and nuclear extracts, respectively. N = 4 for each group.

(E) NRVM were cultured in DMEM/M199 medium with serum for 48 hrs. Serum starvation was conducted by switching to serum-free DMEM/M199 overnight. ER stress inducers were then added to culture medium, and the treatment was terminated after 6 hrs. O-GlcNAc modification and BiP were detected by immunoblotting. The following drugs were used: thapsigargin (Tg), 1 μM; tunicamycin (TM), 0.5 ug/mL; DTT, 0.5 mM. Comparison was made between vehicle (veh) and treatment groups. N = 3 per group.

(F) Xbp1 expression was first silenced by siRNA knockdown in NRVM. ER stress inducers were used to trigger the UPR. Total cell lysates were prepared for immunoblotting of GFAT1 and O-GlcNAc modification, and nuclear fractions were extracted for Xbp1s detection. GAPDH and Lamin were used as loading controls for total cell lysates and nuclear extracts, respectively. N = 4 for each group.

(G) Tunicamycin (TM) administration induced the UPR in heart. TM was injected into mice at 1 mg/kg body weight. Hearts were harvested 24 hrs later for immunoblotting analysis. GAPDH was used as a loading control. Comparison was calculated between vehicle (veh) and TM groups. N = 5 for each group.

(H) The induction of UPR markers, HBP genes, GalE, and O-GlcNAc protein modification was diminished in hearts of Xbp1-deficient mice. The littermates of F/F and cKO mice were used for TM injection. Hearts were then harvested for Western Blotting. GAPDH was used as a loading control. N = 4 for each group. Data are represented as mean ± SEM. *, $p < 0.05$.

Figure S4. Inducible expression of Xbp1s in transgenic mice, Related to Figures 3, 4

(A) Xbp1s expression was induced exclusively in the hearts of double transgenic mice in the absence of Doxycycline (Dox), as assessed by RT-PCR. All possible combinations of transgenics and Dox were tested to confirm the inducibility and fidelity of Xbp1s expression. Primers used to amplify Xbp1 detect both full length (171 bp) and spliced Xbp1 (145 bp).

(B) Xbp1s protein was induced in double transgenic mice. Transgene expression was triggered by removal of Dox for 2 weeks. Hearts were harvested for nuclear extraction. Lamin was used as a loading control. * denotes a non-specific signal across all samples.

(C) Overexpression of Xbp1s in heart led to up-regulation of BiP transcription and protein levels. GAPDH was used as loading control. N = 6 for each group.

(D) The protein levels of GFAT1 (green) were increased in the transgenic heart compared to control by immunofluorescence staining. Alpha-actinin (red) was used as a cardiomyocyte marker. The profile of signal intensity of GFAT1 was then recorded using Image J over a 20 μm distance and quantified as arbitrary units (A.U.). Scale bars: 50 μm. N = 4.

(E) GFAT2 was not stimulated by Xbp1s over-expression. Neither transcript nor protein levels of GFAT2 were significantly increased by Xbp1s induction in hearts in the transgenic mice. GAPDH was used as a loading control. N = 3-4.

(F) Xbp1s overexpression in heart led to significant upregulation of UDP-Glc and UDP-GlcNAc compared to all other controls. Cellular free UDP-sugars were labeled, analyzed by FACE and quantified. Xbp1s expression was only induced in the TG/H_2O group. The presence of Dox suppressed transgene expression. SD, standard. N = 5-6 for each group.

(G) Xbp1s overexpression in heart led to significant up-regulation of O-monosaccharide levels as analyzed by FACE. Although mannose and GalNAc were not visible or barely detectable, O-linked monosaccharides of Glc and GlcNAc were significantly increased in the transgenic heart. Xbp1s expression was only induced in TG/H_2O group. The presence of Dox suppressed transgene expression. SD, standard. N = 5-6 for each group.

(H) Xbp1s over-expression led to induction of neutral N-Glycan in hearts. Adult male mice were placed on H_2O for 2 weeks to induce Xbp1s expression. Hearts were then harvested for analysis of neutral N-Glycan. N = 6 for each group. Data are represented as mean ± SEM. *, $p < 0.05$. **, $p < 0.01$.

Figure S5. Deficiency of Xbp1 does not affect HBP gene expression under basal conditions, Related to Figures 5, 6

(A) Excision of the Xbp1 allele as assessed by qPCR using DNA from whole heart. The fold change of intact Xbp1 genomic loci was normalized to GAPDH genomic loci. N = 8 per group. Data are represented as mean ± SEM. *, $p < 0.05$.

(B) Xbp1 allele was examined in isolated adult cardiomyocytes and non-cardiomyocytes. Genomic DNA was isolated and subjected to a series of dilutions as templates for PCR amplification. GAPDH was used as a control. Note the intensity of 1:10 dilution in cKO cardiomyocytes was comparable to 1:100 dilution of F/F cardiomyocytes, which indicates about 90% excision. No difference was detected in non-cardiomyocytes.

(C) Cardiac protein levels and mRNA expression of HBP genes, GalE, and UPR genes were compared between F/F and cardiomyocyte-specific knockout animals (cKO). No significant differences were detected. N = 4.

(D) Expression of the HBP genes and GalE was compared after Xbp1 knockdown in NRVM. No significant differences were detected. N = 3.

(E) Deficiency of Xbp1 did not affect cellular free UDP sugar levels or O-modifications under basal conditions. Heart tissues were harvested from F/F and cKO mice and subjected to FACE analysis to visualize cellular free nucleotide sugars (left). Cardiac proteins were then extracted and used for beta-elimination to detect O-linked sugar modifications (right). N = 8.

(F) Xbp1 knockout in cardiomyocytes did not alter basal levels of O-GlcNAc protein modification as shown by immunoblotting. GAPDH was used as loading control. N = 4.

(G) Xbp1 deficiency did not affect cardiac function at basal conditions as assessed by echocardiography and quantified as % fractional shortening, LVID-systolic, LVID-diastolic, and heart rate. N = 3 - 5.

(H) Cardiac Xbp1 knockout did not alter heart morphology. Scale bars, 2 mm.

Figure S6. Xbp1s over-expression leads to improvements in cardiac function after I/R, Related to Figure 6

(A) Male mice of 8-12 weeks were split into four groups. Basal cardiac function was examined by echocardiography. N = 5 per group.

(B) Adult male mice of control and TG genotypes were placed on Dox water and subjected to I/R surgery. TTC staining was performed to assess cardiac injury. Note: The presence of Dox in water suppresses transgene Xbp1s expression. Blue, unaffected, viable tissue; red, area at risk; white, infarct area. The number of animals used is indicated. Scale bars: 1 mm.

(C) Cardiac function was followed up to three weeks after I/R by echocardiography. N = 4-5 for each group. Data are represented as mean ± SEM. *, $p < 0.05$.

Figure S7. GFAT1 is required for Xbp1s-dependent cardioprotection, Related to Figure 7

(A) The knockdown efficiency of Xbp1s was examined by immunoblotting. NRVM were transfected by control siRNA or siRNA against Xbp1 for 48 hrs and processed for nuclear extraction. Lamin was used as a loading control. N = 3 per group.

(B) Overexpression of Xbp1s by lentivirus led to up-regulation of Xbp1s. NRVM were infected by lentivirus expressing either LacZ or Xbp1s. Nuclear extracts were prepared for immunoblotting. Lamin was used as a loading control. N = 3.

(C) NRVM were transfected by control or Xbp1 siRNA and subjected to sI/R. Propidium iodide (PI) was added after reperfusion, and images were acquired for PI signal (red). The number of PI-positive cells was counted and compared between groups. N = 9-10 per group.

(D) NRVM were infected with lentivirus expressing LacZ or Xbp1s. After sI/R, PI was added to cells. Images were acquired for PI (red), LacZ or Xbp1s expression (green, GFP) and nucleus (blue). Scale bars: 50 μm. The number of PI-positive cells was counted and compared between groups. N = 9-10 per group.

(E) NRVM cell survival was examined by ATP content measurements. NRVM were transfected by control siRNA or Xbp1 siRNA and subjected to sI/R. ATP content was then assessed and compared between groups. N = 6 per group.

(F) NRVM cell survival was examined by ATP content measurements. NRVM were infected by lentivirus expression either LacZ or Xbp1 and subjected to sI/R. ATP content was then assessed and compared between groups. N = 6 per group.

(G) Knockdown of GFAT1 in NRVM was examined by immunoblotting. GAPDH was used as a loading control. N = 3 per group.

(H) Overexpression of GFAT1 in NRVM by lentivirus led to up-regulation of GFAT1. NRVM were infected by lentivirus expressing either LacZ or GFAT1 and subjected to immunoblotting. GAPDH was used as a loading control. N = 4 per group.

(I) Supplementation with GlcNAc protected NRVM from sI/R-induced cell death. NRVM were first transfected by control siRNA or Xbp1 siRNA. After simulated ischemia, GlcNAc (10 mM) was added in culture medium during reperfusion. Cell death was assayed by LDH release. N = 6 per group.

(J) OGT is required for Xbp1s-mediated protection against sI/R injury. NRVM were first infected with lentivirus expressing LacZ or Xbp1s. OGT was then silenced by siRNA knockdown. After sI/R, cell death was quantified by LDH release. N = 6 per group. Data are represented as mean ± SEM. *, $p < 0.05$. **, $p < 0.01$.

(K) Inhibition of GFAT1 diminished cardioprotection by Xbp1s over-expression. Supplementation of DON (50 μM) in perfusion solution significantly reduced the protection by Xbp1s in Langendorff analysis ex vivo. Cardiac function was assessed as left ventricular developed pressure (LVDP). N = 7 for control, n = 4 for TG, n = 3 for control + DON, and n = 3 for TG + DON. Data are represented as mean ± SEM. *, TG vs control, $p < 0.05$. #, TG vs TG + DON, $p < 0.05$.

(L) Model of Xbp1s action in heart during I/R.

Table S1. Primer sets used in this study

Table S2. Patient information, related to Figure 1

EXTENDED EXPERIMENTAL PROCEDURES

Animals

Mice were maintained on a 12 hr light/dark cycle from 6 AM to 6 PM with no more than 4 mice in each cage. Mice had unrestricted access to food (2916, Teklad) and water. All mouse surgeries and protocols were approved by the Institutional Animal Care and Use Committee of University of Texas Southwestern Medical Center.

Wild-type male mice (8-12 weeks old) of C57/B6 background were chosen for ischemia/reperfusion (I/R) surgery. Left coronary artery occlusion was performed for 45 min. The ligature was then released, and animals were sacrificed for TTC staining or tissue harvesting at various times following the procedure. The infarcted area and area at risk were then calculated and quantified. Left ventricular tissue was divided to three zones: ischemic, border, and remote zones according to previous TTC staining patterns. The tissues were then frozen in liquid nitrogen for subsequent processing.

To induce ER stress, tunicamycin was administrated intraperitoneally (1 mg/kg body weight). Hearts were harvested for immunoblot analysis 24 hrs later.

To generate the inducible mouse model, mouse Xbp1s cDNA was cloned into pTRE basic vector (Clontech). The transgenic mouse was generated in an FVB background by the Transgenic Core facility at UT Southwestern (Deng et al., 2013). A positive line was then backcrossed into the C57/B6 background for at least 9 generations. We then crossed the TRE-Xbp1s transgenic mouse with the αMHC-tTA mouse model in C57/B6 background (Yu et al., 1996). Doxycycline-containing water (0.1 mg/mL) was used to suppress Xbp1s expression during breeding, pregnancy, and weaning. Induction of the transgene was achieved by switching to regular water at 8-12 weeks for 1-3 weeks. αMHC-tTA transgenic mice were used as controls to exclude confounding effects due to tTA expression and activation (Yu et al., 1996). Xbp1s expression was tested at different times after induction (1 day to 3 weeks). No expression was observed until day 7 (data not shown). Unless specified, a 2-week induction was chosen for all experiments.

Conditional Xbp1 knockout mice (Kaser et al., 2008) were bred with αMHC-Cre mice to achieve cardiomyocyte-specific deletion of Xbp1. Both mouse models are in the

C57/B6 background. Double transgenic mice 8-12 weeks of age were used for I/R surgery, and age-matched αMHC-Cre or F/F animals were studied as controls.

Cardiomyocyte isolation and treatment

Neonatal rat ventricular cardiomyocytes (NRVM) were isolated from 1-2 day old Sprague-Dawley rat pups. Ventricles were harvested, rinsed briefly in 70% ethanol and maintained in DMEM containing L-glutamine and sodium pyruvate. After mincing into small fragments, heart tissues were washed once using PBS and then transferred to digestion solution containing pancreatin (1 mg/mL in PBS without Ca^{2+}, Mg^{2+}). Repeated digestion was performed at 37°C for 20 min in a spinner flask (Wheaton) until most NRVM were dissociated into suspension. After each round of digestion, supernatant was collected, and the cells were pelleted by brief centrifugation. Cardiac fibroblasts were minimized by pre-plating for 2 hrs. Cardiomyocytes were then harvested and diluted in plating medium (DMEM:M199, 3:1, high glucose, with 10% horse serum, 5% fetal bovine serum and 100 μM BrdU). Cells were then cultured on gelatinized plates for 24-48 hrs before treatment. Cell preparations contained greater than 95% cardiomyocytes.

For simulated I/R (sI/R), 24 hrs after plating in a 12-well plate, NRVM were infected with lentivirus expressing either LacZ or Xbp1s. After another 24 hrs, siRNA treatment was performed. The cells were then subjected to simulated I/R 24 hrs later. NRVM were first washed twice with PBS and then incubated with I/R 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-deoxy-glucose, pH 6.2). NRVM were then placed in a hypoxia chamber (Billups-Rothenberg) and flushed with 95% N_2/5% CO_2 for 30 min. The gas flow was then extinguished, and the chamber was closed for an additional 3.5-4.5 hrs. The cells were then washed twice with PBS and replenished with culture medium and incubated overnight before harvesting. NRVM incubated with control buffer were used as controls (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). LDH assays were conducted using the CytoTox96 cytotoxicity kit (Promega) and calculated as (ischemia medium LDH + reperfusion medium LDH)/(ischemia medium LDH + reperfusion medium LDH + cell lysate LDH). Cell survival was also measured by ATP content using the CellTiter-Glo Luminescent cell

viability assay kit (Promega). In addition, cell death was visualized by propidium iodide (PI) staining. Briefly, PI (1 μg/mL) was added to culture medium after reperfusion. Cells were then imaged by fluorescence microscopy (Zeiss), and the number of PI-positive cells was quantified.

All siRNA constructs were purchased from Sigma and dissolved in Opti-MEM (Invitrogen). A total of 50-100 pmol siRNA was used for each well of a 12-well plate, and transfection was performed with Lipofectamine RNAiMAX reagent (Invitrogen).

For glucose deprivation experiments, NRVM were serum-starved overnight two days after plating. DMEM/M199 medium was switched to glucose-free DMEM (Invitrogen) with regular high glucose DMEM as control. After overnight incubation, cells were harvested by adding 1 x SDS-PAGE sample buffer and subjected to immunoblotting analysis.

For ER stress inducer treatments, NRVM were first starved of serum for 24 hrs following 2 days of plating. Various drugs were then used (6 hrs) to trigger the UPR, including thapsigargin (Tg), 1 μM; tunicamycin (TM), 0.5 μg/mL; DTT, 0.5 mM. Cells were then harvested for immunoblotting.

Adult mouse cardiomyocytes and non-cardiomyocytes were isolated from littermates of Xbp1 F/F and cKO hearts. Briefly, hearts from male mice of 8-12 weeks old were isolated and immediately retrograde perfused with Krebs-Ringer (K-R) solution (35 mM NaCl; 4.75 mM KCl; 1.19 mM KH_2PO_4; 16 mM Na_2HPO_4; 134 mM sucrose; 25 mM $NaCO_3$; 10 mM glucose; and 10 mM HEPES, pH 7.4) for 5 min (2 mL/min). The hearts were then perfused for another 15 min with K-R solution containing 0.8 mg/mL type II collagenase (Worthington). The left ventricle was removed and minced in KB solution (20 mM taurine; 100 mM glutamic acid; 25 mM KCl; 10 mM KH_2PO4; 10 mM glucose; 5 mM HEPES, 1 mM $MgSO_4$, and 0.5 mM EGTA, pH 7.2). After trituration and filtration, cardiomyocytes were precipitated by brief centrifugation and non-cardiomyocytes were then pelleted from supernatant. Cells were subjected to DNA isolation using DNeasy blood and tissue Kit (Qiagen).

COS7 cell culture and treatment

COS7 cells were cultured with DMEM/low glucose medium containing 10% serum. Before stress treatment, cells were serum-starved overnight and various treatments were conducted in serum-free medium for 8 hrs. Cells were then harvested by directly adding 1 x SDS-PAGE sample buffer. Xbp1 knockdown was achieved by siRNA transfection and stress treatment was then conducted 24 hrs later.

RNA isolation and PCR analysis

Total RNA was isolated from heart tissue or NRVM using the Aurum Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad). A total of 100-250 ng RNA was used for reverse transcription using iScript reagent (Bio-Rad). The RT product was then diluted 10-fold using ddH_2O, and 2 µL was used for either standard PCR or qPCR analysis (Roche). Primers for Xbp1 were described previously (Iwakoshi et al., 2003). All primer sequences are provided (**Table S1**).

Lentivirus production, purification, and infection

Mouse Xbp1s and GFAT1 were each subcloned into a lentiviral expression vector (Clontech), which was co-transfected into HEK293T cells (Clontech) together with pCD and VSVG constructs. Lentivirus was harvested from the culture supernatant and concentrated using Lenti-X Concentrator (Clontech) or ultracentrifugation. The viral titer was determined by qRT-PCR (Clontech) and stored at -80°C until use. To infect NRVM, an MOI of 2-10 was chosen in culture medium containing 3% FCS and polybrene (8 µg/mL). Gene expression was assayed 24-48 hrs post-infection.

Immunoblotting and immunostaining

Heart tissue (10-50 mg) was homogenized in 500 µL T-PER reagent (Thermo) containing protease inhibitors and phospho-STOP (Roche). When preparing lysates for O-GlcNAc analysis, PUGNAc (40 µM) was included to inhibit OGA activity and preserve O-GlcNAc levels. The lysate was then cleared by centrifugation at 4°C for 10 min. For NRVM, cells were washed twice in ice-cold PBS after treatment. T-PER reagent (150 µL) was added to each well, and the cells were quickly frozen at -80°C. To isolate nuclear extracts, the NE-PER kit was used according to manufacturer's recommendations (Thermo). Protein concentration was measured by BCA assay (Thermo). A total of 5 - 30 µg protein of each sample was loaded for electrophoresis

using 4-20% gels (Bio-Rad). After semi-dry transferring, the membrane was processed for immunoblotting and scanning using an Odyssey scanner (LI-COR). The following antibodies were used: BiP, BD Biosciences and Santa Cruz; GAPDH, Fitzgerald; GRP94, Abcam; CHOP, Santa Cruz; GFAT1, Santa Cruz; GFAT2, Santa Cruz; GNPNAT1, Sigma; OGT, Sigma; PGM3, Fitzgerald; GalE, Abcam; Xbp1, Santa Cruz; Lamin A/C, Cell signaling and Sigma; O-GlcNAc (CTD110.6), Covance; goat anti-rabbit IgG secondary antibody 800CW, LI-COR; goat anti-mouse IgG secondary antibody 680, Invitrogen; goat anti-mouse IgM (μ chain) secondary antibody 680.

For immunostaining, mouse hearts were fixed in 10% buffered neutral formalin for 48 hrs. Paraffin sections (5 μm) were used for staining overnight with primary antibody at 4°C after antigen retrieval (Vector labs). Alpha-actinin antibody was purchased from Sigma. Sections were then washed 3 times, and the secondary antibody was incubated for 1 hr at room temperature. The sections were then mounted using anti-fade reagent with DAPI (Invitrogen). All images were obtained using a confocal fluorescence microscope (Leica). To quantify fluorescent signal, we analyzed the level of fluorescence intensity along 20 μm in each photomicrograph. Representative profile and fluorescence intensity were obtained using Image J. The values were normalized and arbitrary units (A.U.) were calculated.

GFAT1 promoter cloning, luciferase assay, and ChIP assay

The mouse GFAT1 promoter (852 bp) was cloned into pGL3-TATA vector between Nhe I and Xho I sites (Promega). The construct was then co-transfected into HEK293T cells together with an Xbp1s expression plasmid. Luciferase assays were performed 36 hrs later (Promega). A β-galactosidase expressing construct was also co-transfected to normalize luciferase activity (ABI).

For ChIP assays, mouse C2C12 cells were transfected with an Xbp1s expression construct using Lipofectamine (Invitrogen). After 36 hrs, the cells were fixed with 1% formaldehyde for 15 min at RT. Nuclei were isolated using NE-PER reagent (Thermo). Chromatin was then sonicated and precipitation was performed using anti-Xbp1 antibody (Santa Cruz) or anti-TNP sera (Ferdous et al., 2007). The immunoprecipitated material was used for immunoblotting analysis to verify the precipitation of Xbp1s. DNA

was recovered using a QIAquick column after reverse crosslinking and protease K digestion. PCR was performed using primers spanning the Xbp1s binding site and of distal region in the GFAT1 promoter. Genomic DNA before immunoprecipitation was used as input controls.

Langendorff experiments

Langendorff experiments were performed as described previously (Bell et al., 2011; Reichelt et al., 2009). Briefly, mice (10-14 weeks old) were anesthetized, and the heart was quickly removed and submerged in ice-cold PBS. After carefully trimming connective tissues, the aorta was cannulated on a blunted 21 gauge needle, and perfusion was initiated. After 15-30 min equilibration, ischemia was initiated by arresting perfusion for 20 min followed by reperfusion for 40 min. Left ventricular pressure was recorded with a pressure transducer (AD Instruments) using a ventricular balloon. Hearts with initial developed pressure less than 60 mmHg were excluded. Azaserine (80 μM, Sigma) or 6-diazo-5-oxo-l-norleucine (DON) (50 μM, Sigma) was employed to inhibit GFAT enzymatic activity (Liu et al., 2007). Modified Krebs-Henseleit perfusion buffer was used: NaCl, 119 mM; Glucose, 11 mM; $NaHCO_3$, 22mM; KCl, 4.7 mM; $MgCl_2$, 1.2 mM; KH_2PO_4, 1.2 mM; $CaCl_2$, 2.5 mM; EDTA, 0.5 mM; sodium pyruvate, 1 mM.

Echocardiography

Echocardiograms were recorded on conscious, gently restrained animals using a Vevo 2100 system with a MS400C scanhead. M-mode recordings at the level of the papillary muscles were conducted to measure systolic and diastolic left ventricular internal dimensions. Fractional shorting was calculated as (LVIDd-LVIDs)/LVIDd and expressed as percentage.

Nucleotide sugar and protein glycosylation analysis in heart

Nucleotide sugar analysis was conducted as previously described (Gao and Lehrman, 2006). Briefly, snap-frozen heart tissue was pulverized with a polytron device directly in methanol and then dried. The powder was then sequentially extracted with chloroform:methanol, water, and chloroform:methanol:water. The water fraction was collected for free nucleotide sugar analysis. The remaining pellet was used to analyze

sugar modification on proteins. To analyze N-Glycan levels, N-Glycanase F (2 Units) was used to liberate N-Glycan from 1 mg cardiac proteins. The released N-Glycan was fractioned on an AG1-X2 column and labeled with ANDS for FACE gel analysis. For total O-Glycan analysis, the O-Glycan released by β-elimination were labeled with ANDS and resolved on a FACE oligosaccharide gel. For O-mono sugar analysis, the released sugars were further cleaned with ion exchange resin, then labeled with AMAC and resolved on a FACE monosaccharide gel.

Patient samples

Human heart tissue samples were obtained from patients with advanced heart failure of ischemic or non-ischemic etiology after informed consent was obtained under an IRB-approved protocol. The patients had been referred to the UT Southwestern Advanced Heart Failure, Ventricular Assist Device, and Heart Transplant Program for consideration of either implantation of a left ventricular assist device (LVAD) and/or heart transplantation. Paired ventricular tissue samples were obtained from each patient first at the time of LVAD implantation and then at the time of heart transplantation. Pre-LVAD samples were acquired from the LV apex while post-LVAD samples were obtained from the lateral wall of the LV. Heart tissues were immediately frozen in liquid nitrogen and stored at -80°C. Patient information is shown in (**Table S2**).

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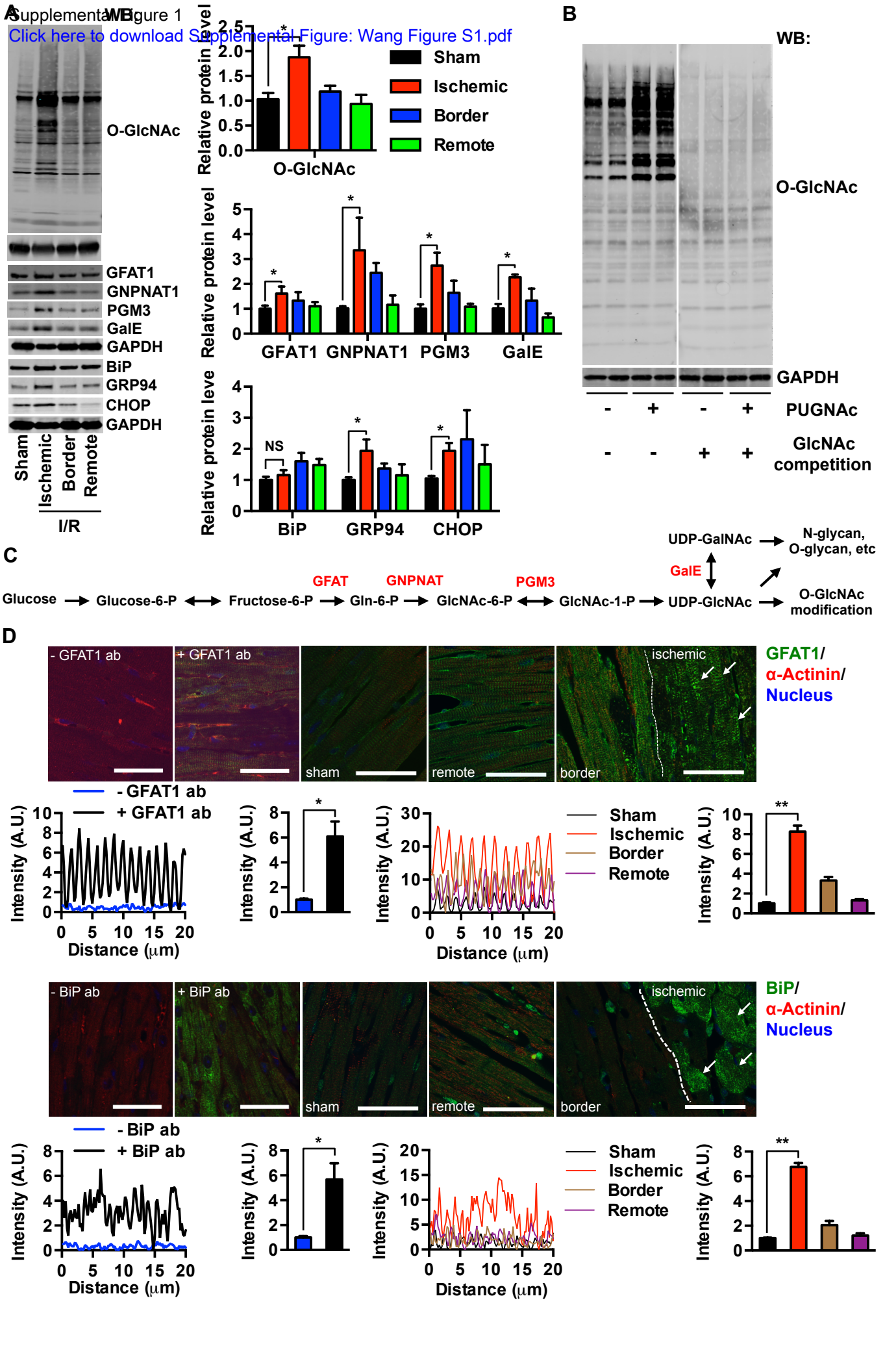
Supplemental Figure 1



Supplemental Figure 2

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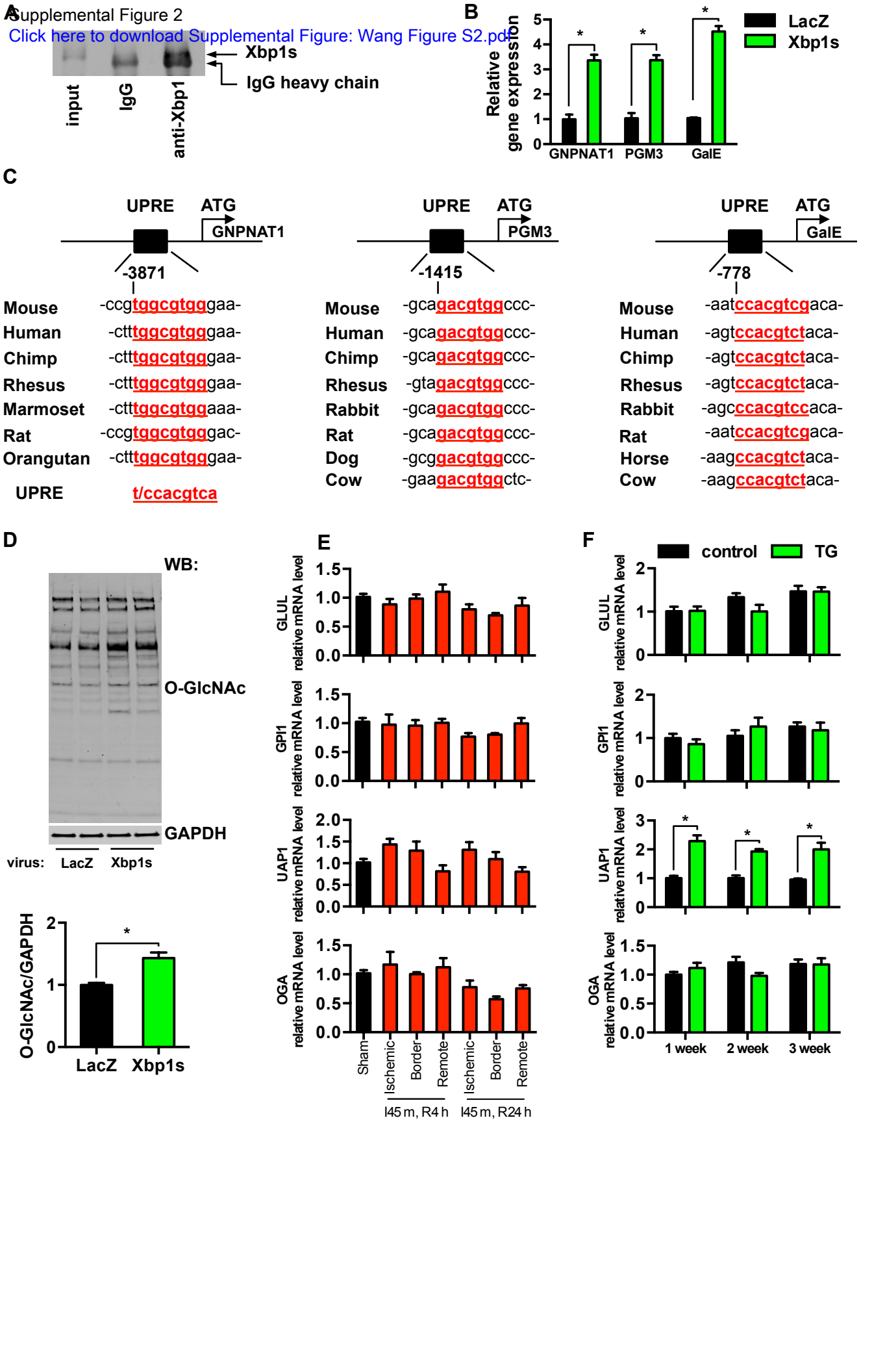
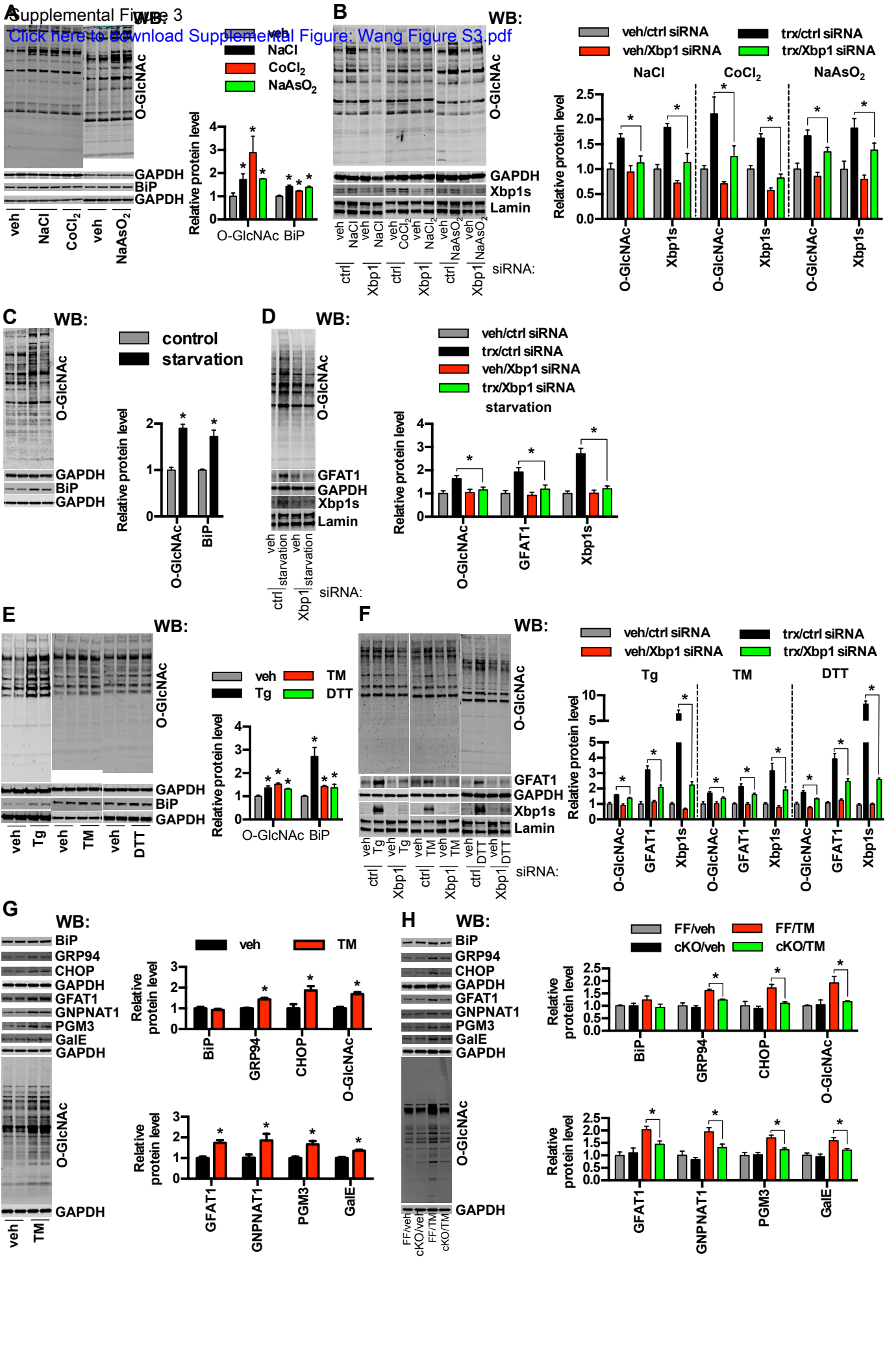


Supplemental Figure 3



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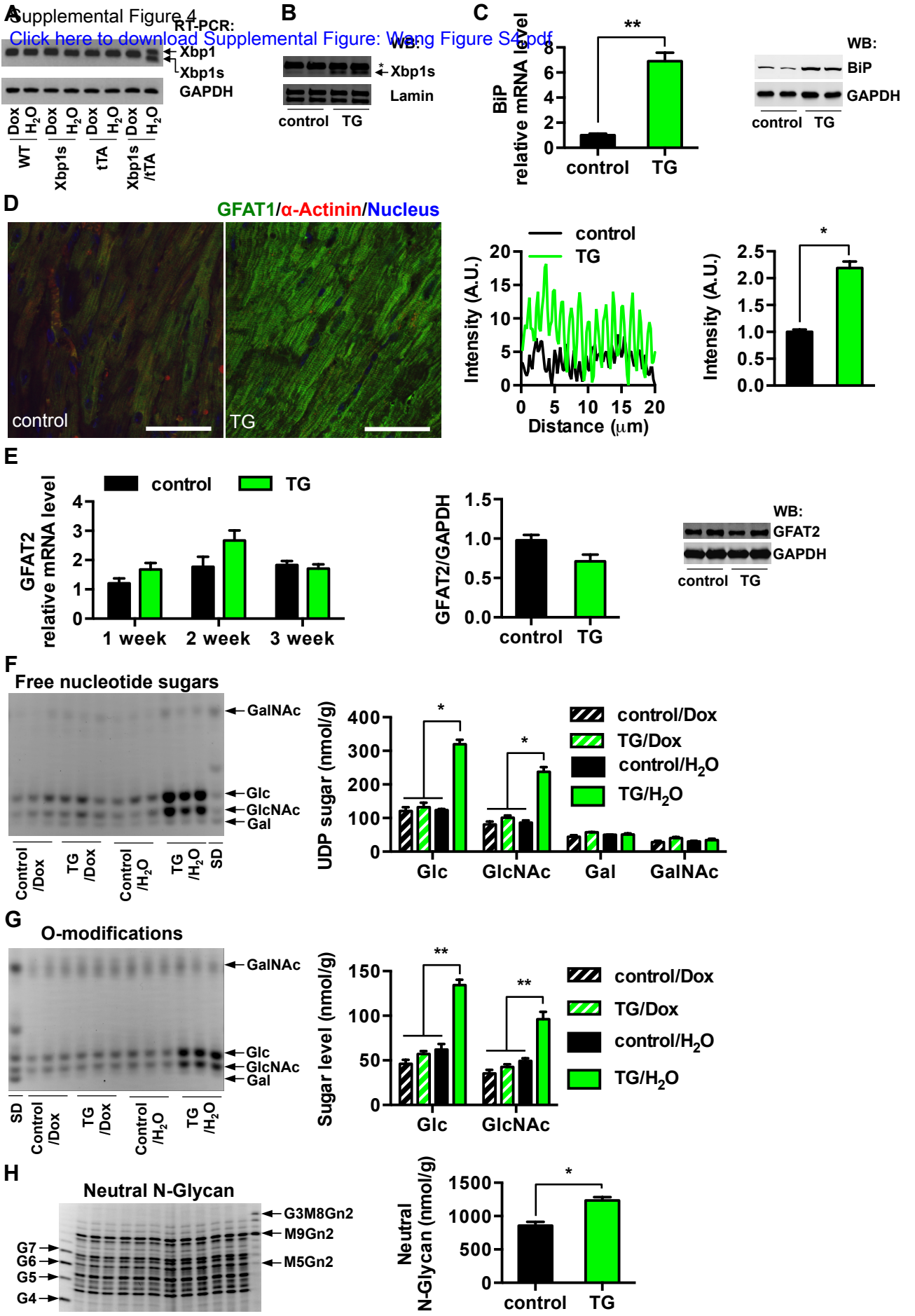
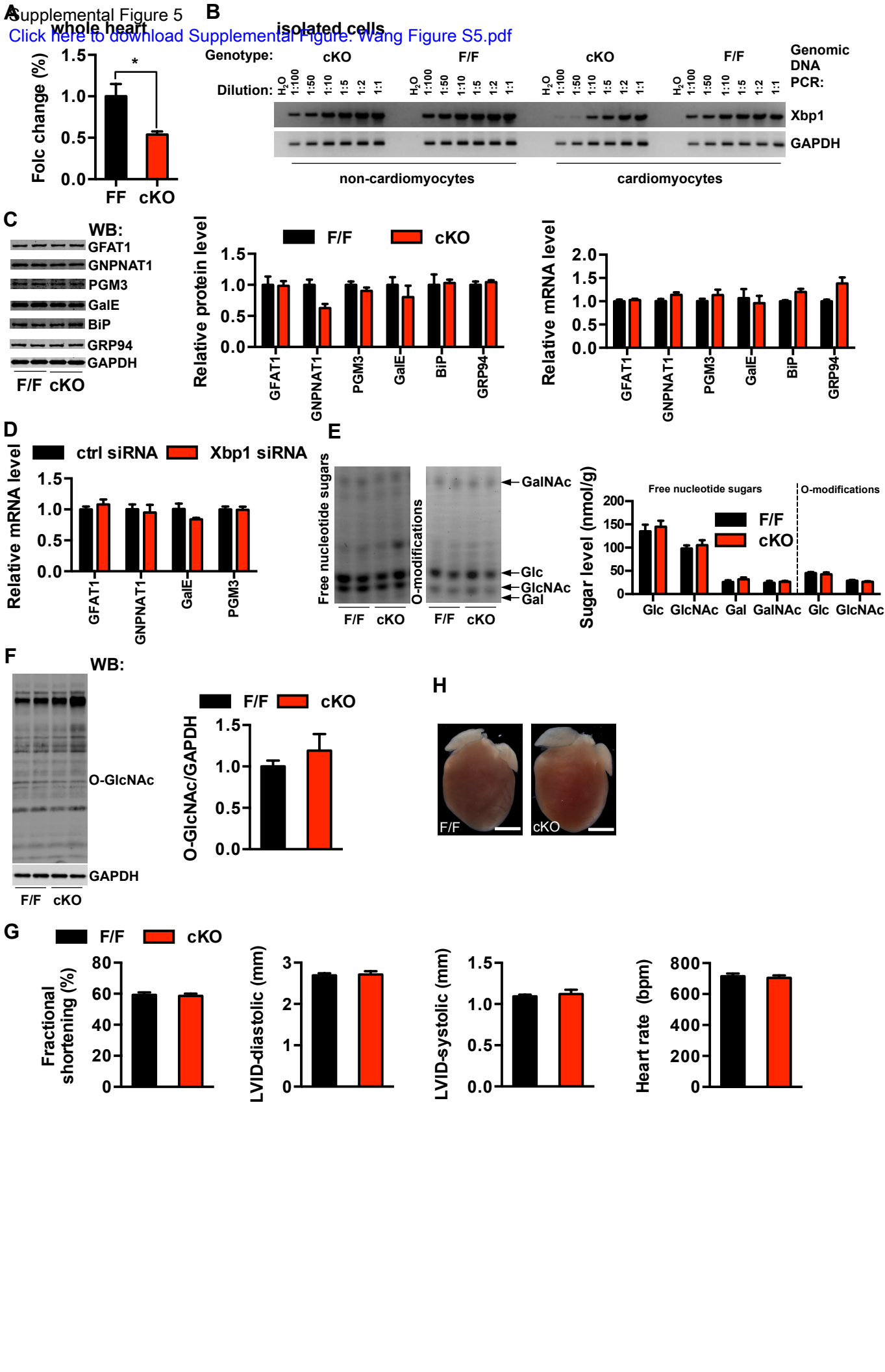


Supplemental Figure 5



Supplemental Figure 6

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