Materials and Methods

The authors declare that all supporting data are available within the article and its online supplementary files. All studies were conducted in accordance with protocols approved by the institutional review boards of National Taiwan University and Washington University in St Louis. All experimental animals were assigned unique identifiers to blind experimenter to genotypes and treatment. A block randomization method was used to assign experimental animals to groups on a rolling basis to achieve adequate sample number for each experimental condition.

2.1 Weighted co-expression network construction and analyses

A weighted co-expression network encompassing all confidently detected mRNAs (RPKM≥3, n=9477) from the human LV RNASeq dataset (GSE46224)[1] was constructed using WGCNA R package. In brief, the pairwise correlation coefficient of expression profiles between two genes was determined across all 24 human LV RNA samples, and the soft thresholding (β=6, based on scale-free topology criterion)[2] of the Pearson correlation matrix was utilized to determine the connection strengths between two genes. The connectivity measure (*k*) of individual genes was determined by summing of the connection strengths with all other genes. Genes with similar patterns of connection strengths (topological overlap) with all other genes inside the network were grouped as individual "modules". Each module consists of genes with similar expression profiles across all samples and their gene expression pattern is distinct from those of all the other modules; therefore, genes of the same module are considered highly correlated. Gene ontology analyses of genes of individual modules were performed using g:Profiler (http://biit.cs.ut.ee/gprofiler/).

2.2 Human primary cardiac fibroblast (hCF) cultures

Human ventricular tissues were obtained from non-failing (NF) donors (whose hearts were declined for transplantation, but whose next of kin had given consent for use in research). Left ventricular (LV) tissue samples were obtained in the operating room within minutes of excision and transported back to the laboratory in ice-cold cardioplegic solution. The tissue was washed 3x, 3 min each, in 45 µmol/L Ca^{2+} HEPES-buffered solution containing 5 mmol/L pyruvate, 20 mmol/L taurine and 5 mmol/L nitrilotriacetic acid (Sigma Aldrich, MO, USA). The tissue was minced, then incubated first in 0.036% proteinase in 200 µmol/L Ca^{2+} HEPES-buffered solution for 40 min and subsequently in 0.1% collagenase in 200 µmol/L Ca^{2+} HEPES-buffered solution for 45 min, each while being oxygenated with 100% O_2 at 37°C, shaking at a rate of 80/min. The supernatant was spun down in a centrifuge at 433xg to yield a cell pellet. The pellet was resuspended in Dulbecco's modified Eagle medium (DMEM, Thermo Fisher Scientific, MA, USA) containing 10% fetal bovine serum (FBS), 100 IU/ml penicillin G/100 µg/ml streptomycin/0.25 µg/ml amphotericin and plated in a culture flask maintained in an incubator at 37°C in a humidified atmosphere of room air supplemented with 95% O_2/5% CO_2. The next day, non-adherent cells were removed during a media change. Flasks containing fibroblasts were kept in culture and fed every 2-3 days until the cells

reached confluency. The cells were then passaged by trypsinization, re-plated, then frozen down and stored at -80°C so that multiple samples could be collected and studied together. Experiments were performed on subcultured cells that were from passage 5. Fibroblasts were plated at a density of 6.3 x 10^4 cells per well in 6-well plates, 1.5 x 10^5 cells per 60 mm dishes, 1.3 x 10^4 cells per chamber on four-chamber slides, and 2.2 x 10^3 cells per well in 96-well plates.

2.3 Murine primary cardiac fibroblasts (mCF) isolation

Four-week-old male WT (C57BL/6J) and *Txndc5*$^{-/-}$ mice were anaesthetized with Avertin (2'2'2-tribromoethanol, 0.25 mg/kg, intraperitoneally, i.p. Sigma Aldrich, MO, USA,) and the heart was harvested immediately using aseptic procedures. Hearts were washed clean in PBS (phosphate buffer saline). Atria were removed and ventricles were finely minced and digested with 3mg/ml of collagenase II (Worthington Biochemical Corp., NJ, USA) at 37°C for 1 hr with vortexing for 5 sec every 10 min. The reaction was stopped with DMEM-F12 (Thermo Fisher Scientific, MA, USA) containing 10% FBS. The samples were centrifuged at 300 rpm for 3 min to remove cardiomyocytes; the supernatant was then centrifuged at 4000 rpm for another 5min. Pellets were resuspended in DMEM-F12 containing 10% FBS, sodium bicarbonate, and 100U/ml penicillin-streptomycin. Freshly isolated and up to second passage of mouse cardiac fibroblasts were used in experiments.

2.4 NIH-3T3 mouse fibroblast culture

NIH-3T3 mouse fibroblast cell line was purchased from the American Type Culture Collection (ATCC) and cultured in DMEM with the addition of 10% FBS, 100 IU/ml penicillin G/100 µg/ml streptomycin/0.25 µg/ml amphotericin and plated in a culture flask maintained in an incubator at 37°C in a humidified atmosphere of room air supplemented with 95% O_2/5% CO_2.

2.5 TGFβ1 stimulation in fibroblasts

Cultured hCF and mouse fibroblasts (mCF and NIH-3T3) were treated with TGFβ1 (Sigma Aldrich, MO, USA) at 4 ng/ml and 2.5 ng/ml, respectively, for 48 hours, then processed for downstream experiments. For assaying SMAD3 activation in response to TGFβ1 stimulation, cell lysates were collected 3 hours after TGFβ1 treatment.

2.6 Human ventricular myocytes isolation

Transmural LV tissue wedges were obtained from NF donors (as consented above) and perfused with ice-cold cardioplegic solution in the operating room within minutes of excision and then transported back to the laboratory in cold cardioplegic solution. A diagonal off the left anterior descending coronary artery was cannulated for perfusion (76 cm pressure head) via a water-jacketed heating coil at 37°C first with 1.8 mmol/L Ca^{2+} Krebs solution (20 min) followed by Ca^{2+}-free Krebs solution (15 min), then 0.08% collagenase in 35 µmol/L Ca^{2+} Krebs solution (10-15 min, depending on the activity of the particular lot of

collagenase used). The tissue was then trimmed of fat, connective tissue and poorly perfused areas, minced and shaken (80/min) in fresh collagenase in 35 µmol/L Ca^{2+} Krebs solution in a heated water bath at 37°C (15 min). All Krebs solutions and shaking flasks were aerated with 95% O_2/5% CO_2 to maintain physiological pH. After shaking, the enzyme solution was filtered through 350-500 µm nylon mesh (Small Parts) into 50 µmol/L Ca^{2+}-HEPES-buffered wash solution (pH 7.3) enriched with taurine, sodium pyruvate, MEM amino acids and insulin and then spun down at 17xg (3 min). The cell pellet was resuspended in enriched (as above) 100 µmol/L Ca^{2+}-HEPES-buffered "storage" solution with 0.5% bovine serum albumin. Meanwhile, fresh collagenase solution was added to the softened tissue and returned to the shaking water bath for two to three additional harvests.

2.7 Lentiviral transduction for gene knockdown

Lentiviral particles containing shRNAs pLKO-sh*TXNDC5* (#TRCN000033258) and pLKO-sh*Txndc5* (#TRCN000009970) was used to knockdown *TXNDC5/Txndc5* in hCF and NIH-3T3 mouse fibroblasts, respectively. Lentiviral particles containing shRNAs pLKO-sh*VCP* (#TRCN0000004250) was used to knockdown *VCP* in hCF. Lentivirus containing scrambled shRNA, pLKO-shScr (#TRCN00001), was used as non-targeting control. NIH-3T3 and hCF were transduced with lentiviral vectors with MOI of 15, along with 8 µg/mL Polybrene (hexadimethrine bromide, Sigma Aldrich, MO, USA) in DMEM supplemented with 1% FBS for 24 hr and then replaced with fresh medium. Transduced NIH-3T3 and hCF were treated with culture media containing puromycin at final concentration of 3 ng/ml and 5 ng/ml, respectively, for selection of transduced cells.

2.8 Lentiviral transduction for *TXNDC5* overexpression in hCF

Lentiviral particles containing human *TXNDC5* gene (pLAS2w.pPuro-*TXNDC5*) were used to transduce hCF (MOI=15, along with 8 µg/mL Polybrene in DMEM supplemented with 1% FBS for 24 hr and then replaced with fresh medium) to overexpress *TXNDC5.* Transduced hCF were treated with culture media containing puromycin at final concentration of 5 ng/ml, for selection of transduced cells. Lentiviral particles packaged from an empty pLAS2w.pPuro vector were used as control.

2.9 siRNA for *NOX4 knockdown in hCF*

NOX4 was knocked down in hCF using human *NOX4*-targeting siRNA duplexes designed using the sequence from the open reading frame of human *NOX4* (5'-GGAAGAGCCCAGAUUCCAATT-3'). Scrambled oligo-ribonucleotide complex (siScr) not homologous to any mammalian genes was used as control. Cells were transfected with TransIT-X2 (Mirus Bio, WI, USA) according to manufacturer's instructions. Knockdown efficiency was assayed using immunoblotting.

2.10 Overexpression of *Atf6* in NIH-3T3 fibroblasts

Mouse *Atf6* open reading frame was cloned into pCMV6-Entry expression vector (pCMV6-Entry-m*Atf6*). The *Atf6*-expressing vector was transfected into NIH-3T3 fibroblasts using

TransIT-X2 (Mirus Bio, WI, USA) according to manufacturer's instructions.

2.11 Isoproterenol (ISO)-induced heart failure and cardiac fibrosis

To induce heart failure and cardiac fibrosis, 8-12-week-old male C57BL/6J mice and *Txndc5*$^{-/-}$ mice were subjected to isoproterenol hydrochloride (ISO) injection (30 mg/kg body weight per day subcutaneously) for ten days. Ten days after ISO treatment, mice were subjected to echocardiographic examination, followed by cardiac tissue harvesting.

2.12 Echocardiography

Mice were anaesthetized with 3% inhaled isoflurane and placed in supine position. Two-dimensional (2D) and M-mode transthoracic echocardiography were performed to determine cardiac function of the mice using Vevo2100 Imaging System (VisualSonic Inc., Toronto, Canada) equipped with a 30 MHz transducer. Endocardial margins were determined by B mode imaging. The interventricular septal thickness (IVS), LV internal dimension and LV posterior wall thickness (LV PW) during end systolic and diastolic phases were determined using M mode. LV ejection fraction (LVEF%) and LV fractional shortening (LVFS%) were calculated to determine LV systolic function of each individual animal. n=8-10 for each group of animals.

2.13 Histology

Mouse hearts were fixed in 4% formaldehyde, embedded in paraffin, and sectioned in 5µm thickness for Masson's trichrome staining. Measurement of fibrotic area was quantified using imageJ software (NIH, http://rsbweb.nih.gov/ij/).

2.14 Immunohistochemical staining

Immunohistochemical staining was implemented using Novolink™ Polymer Detection System (Leica Biosystems, Wetzlar, Germany) according to manufacturer's instructions. In short, mouse cardiac tissues were embedded in paraffin, sectioned and deparaffinized followed by antigen retrieval in sodium citrate buffer (pH=6.0) with TWEEN-20 for 20 min at 95-100°C. Peroxidase activity was then blocked with 3% H_2O_2 for 10 minutes. Protein blocking was performed with 0.4% casein in phosphate-buffered saline for 10 min. Mouse cardiac sections were then incubated with primary antibodies including anti-COL1A1, anti-CTGF, anti-periostin (1:500, OriGene, MD, USA) and anti-TXNDC5 (1:500, Abcam, Cambridge, UK) overnight at 4°C. Sections were then washed and incubated with HRP-conjugated secondary antibodies for 1 hr at room temperature, followed by incubation with Novolink™ Polymer for 15 min. Sections were developed with DAB working solution and then counterstained with hematoxylin and mounted with mounting medium. Staining area quantification was performed using image J.

2.15 Immunofluorescence (IF) and immunocytochemical (ICC) staining

Frozen cardiac samples from vehicle- and ISO-treated *Col1α1-GFP*^{Tg} mice[3] (a gift from Dr. David Brenner at UCSD, n=3 in each group) were sectioned (short axis, 10µm), fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 30 min, permeabilized with 0.1%

Triton X-100 for 30 min, and blocked with 1% bovine serum albumin (BSA) for 1 h. For ICC staining in hCF grown on glass coverslips, cells were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature, washed three times with ice-cold PBS, permeabilized with 0.1% Triton X-100 for 30 min, and blocked with 1% BSA in BPS-T (PBS +0.1% Tween 20) for 30 min. Fixed cardiac sections and hCF were then incubated with the primary antibody, rabbit anti-TXNDC5 (1:500, Proteintech, IL, USA, 19834-1-AP) and/or mouse anti-αSMA (1:500, Genetex, CA, USA, GTX629702) at 4℃overnight. After washing with PBS (3 x 10 min), the sections/cells were incubated with Alexa Fluor 594-labeled anti-rabbit secondary antibodies and/or Alexa Fluor 488-labeled anti-mouse (1:200, room temperature 1 h). The sections were then washed with PBS (3 x 10 min) and mounted with ProLong Gold (Thermo Fisher Scientific, MA, USA). Fluorescence imaging was acquired using a Zeiss Axio Imager M1 fluorescence microscope (Carl Zeiss, Oberkochen, Germany) and analyzed using iVision image analysis software (BioVisions Technologies, Exton, PA).

2.16 RNA extraction and qRT-PCR

Total RNA was isolated from cells and cardiac tissue using TRIzol (Thermo Fisher Scientific, MA, USA) according to the manufacturer's recommendations. Total RNA was reverse transcribed with Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, MA, USA) and SYBR Green qRT-PCR was performed as described previously.[4] The expression level of each individual transcript was normalized to control gene HPRT and expressed relative to the mean expression values of control samples. n=5-6 for each target gene in each experimental condition.

2.17 Cellular ROS detection assay

Cellular ROS levels in hCF were determined using a dichlorofluorescein diacetate (DCFDA) cellular ROS detection assay kit (Thermo Fisher Scientific, MA, USA, D399) according to manufacturer's instructions. In brief, hCF were washed with warm PBS, loaded with 10 μmol/L DCFDA at 37℃ for 30 min, washed with warm PBS again, and then fluorescence (excitation 485 nm, emission 535 nm) was measured in a microplate reader. Representative photomicrographs of DCFDA stained cells were taken using a Zeiss Axio Imager M1 fluorescence microscope (Carl Zeiss, Oberkochen, Germany). n=10-12 for each condition.

2.18 Immunoblot analysis

Cells and cardiac tissues were homogenized using 1x Cell Lysis Buffer (Cell Signaling Technology, MA, USA) supplemented with protease inhibitor cocktail and HALT phosphatase inhibitors (Thermo Fisher Scientific, MA, USA), centrifuged at 4°C for 10 min at 10,000 x g, and the supernatant was mixed with 5x Protein Loading Buffer. Protein lysates were boiled at 60°C for 5 min and the concentration of protein lysate was determined using BCA protein assay. A total of 15 to 30 μg of each protein sample was fractionated on 10% SDS-PAGE gel, transferred onto PVDF membrane and then blocked in blocking buffer (PBS

with 5% BSA, 0.1% Tween 20). Membranes were incubated with primary antibodies against COL1A1 (1:1000, EMD Millipore, CA, USA, AB765P), TXNDC5, CTGF, NOX4, β-tubulin (1:1000, Proteintech, USA, 19834-1-AP, 23936-1-AP, 14347-1-AP, 66240-1-Ig), ELN, POSTN, CCN1, CCN3, ATF6, IRE1α, ATF4, XBP-1, VCP (1:1000, GeneTex, CA, USA, GTX37428, GTX100602, GTX103669, GTX103377, GTX104820, GTX130387, GTX101943, GTX113295, GTX101089), Troponin T, JNK, total/p-ERK, XBP-1s, BiP, Calnexin, Ero1α, CHOP, PDI, total/p-eIF2α (1:1000, Cell Signaling Technology, MA, USA, 5593, 9252, 9102, 4370, 12782, 3177, 2679, 3264, 2895, 3501, 3597, 2103), αSMA, p-JNK, total/p-SMAD3 (1:1000, Abcam, Cambridge, UK, ab5694, ab124956, ab52903, ab40854), GAPDH (1:1000, Thermo Fisher Scientific, MA, USA) overnight at 4°C. After washing (15 min x 3), the membranes were incubated with HRP-conjugated anti-mouse or anti-rabbit whole IgG secondary antibodies (1:5000, Thermo Fisher Scientific, MA, USA) for 1 hr at room temperature. After washing, WesternBright ECL HRP substrate (Advansta, CA, USA) was applied and protein band detection was performed using ChemiDoc MP system (BioRad Laboratories, CA, USA). Protein band intensity quantification analysis was performed with ImageLab software version 5.1. n=5-6 for each target protein in each experimental condition.

2.19 Fibroblast proliferation assay

Fibroblasts (hCF and mCF) were treated with 10 μmol/L bromo-deoxyuridine (BrdU, Roche, Basel Switzerland) in a 96-well plate for 16-24 hr at 37°C. DNA synthesis was assessed as a surrogate for cell proliferation using a colorimetric ELISA according to the manufacturer's instructions. Absorbance of the samples was measured in a spectrophotometer at 370 nm (reference wavelength 492 nm). n=10-12 for each condition.

2.20 Protein stability assay (cycloheximide pulse chase assay)

Control and *TXNDC5*-knockdown hCF were treated with 30 μg/ml cycloheximide to inhibit protein translation. The protein levels of ECM proteins including type 1 collagen, elastin and CTGF were determined in control and *TXNDC5*-knockdown hCF at 0, 5, 10 and 24 hours after cycloheximide treatment. GAPDH was used as a control protein. The ECM protein levels (relative to control proteins) remaining at each time point were expressed as percentages relative to time 0. Repeated measures ANOVA was used to determine if the ECM protein decay rates between control and *TXNDC5*-knockdown cells were significantly different. n=4-6 for each condition.

2.21 Collagen secretion assay

The amount of collagen secreted from control and TXNDC5-overexpressing hCF was determined using a Human Collagen Type I Alpha I (COL1A1) ELISA Kit (CSB-E13445h, CUSABIO, MD, USA) according to manufacturer's instructions. In brief, conditioned medium from control (Empty Vector) and TXNDC5-overexpressing (TXNDC5 Vector) hCF was centrifuged (1000 x g at 4°C) to remove cell debris. One hundred μl of conditioned medium from each sample was added to each well of a 96-well coated assay plate and incubated (at

37°C for 2hr), followed by incubation with 100 μl biotin-antibody (37°C for 1hr), wash buffer x3, 100 μl of HRP-avidin (37°C for 1 hr), wash buffer x5, 90 μl of TMB substrate (30 min at 37°C) and finally 50 μl of stop solution. The COL1A1 concentration in each sample was determined on a microplate reader by substracting readings at 540 nm from the readings at 450 nm. The COL1A1 concentration was then normalized to the total protein content determined in each sample using BCA assay. n=5-6 for each condition.

2.22 TXNDC5 promoter luciferase activity assay

To investigate the activity of *Txndc5* promotor with (pGL3-m*Txndc5*, +773 to +780 deleted) or without (pGL3- m*Txndc5*, -1000 to +1000) deletion of its AFT6 binding site, constructs were generated by the BioMed Resource Core of the 1st Core Facility Lab, NTU-CM. Luciferase assay was performed according to the Luc-Pair™ Luciferase Assay Kit 2.0 user manual (GeneCopoeia, MD, USA). NIH-3T3 cells were transfected with 200 ng of each plasmid for 24 hr followed by 48 hr TGFβ1 (2.5 ng/ml) treatment in a 96-well plate. The plates were then placed on a shaker with 50 μl of 1x lysis buffer and rocked at room temperature for 30 min. Luc buffer I and Luc buffer II were added at 25°C and the signal intensity was evaluated in a plate reader. n=6-8 for each condition.

2.23 Generation of TXNDC5 AAA mutant lacking PDI activity

To generate a mutant *TXNDC5* construct with cysteine-to-alanine mutations in both ends of each of its 3 thioredoxin domains (CGHC to AGHA, supplemental figure VIIA), which are required for the PDI activity of TXNDC5, a full-length WT TXNDC5 cDNA clone (pEZ-M45-hTXNDC5) was subjected to site-directed mutagenesis using the QuickChange II Site-Directed Mutagenesis kit (Agilent Genomics, CA, USA) according to the manufacturer's instructions. Transfection of hCF with WT *TXNDC5* and *TXNDC5-AAA* mutant constructs was conducted using TransIT-X2 (Mirus Bio, WI, USA) according to manufacturer's protocol.

2.24 Immunoprecipitation of TXNDC5

Immunoprecipitation (IP) of TXNDC5 was conducted using a magnetic IP kit (Thermo Fisher Scientific, MA, USA). In short, protein lysates from hCF (with 1000 μg total protein) were incubated with 10 μg of rabbit anti-TXNDC5 monoclonal antibodies (Proteintech, USA, 19834-1-AP) overnight at 4°C. The immune complex was bound to protein A/G magnetic beads and collected with a magnetic stand. Proteins co-immunoprecipitated with TXNDC5 were eluted and subjected to gel electrophoresis and immunoblotting using the antibodies described above.

2.25 FRET (fluorescence resonance energy transfer)-based protein folding assay

Plasmids encoding human COL1A1 fused with cyan fluorescent protein (CFP) at the N' terminus and yellow fluorescent protein (YFP) at the C' terminus were constructed (CFP-COL1A1-YFP). A plasmid encoding a fusion protein that connects CFP and YFP with a short connecting peptide of 15 amino acid residues (CFP-YFP) was used as positive control; a plasmid with a dicistronic operon for coexpression of isolated CFP and YFP (CFP-IRES-YFP)

was used as negative control. shTXNDC5- and shScr-transduced hCF were transfected with 2 µg of CFP-COL1A1-YFP for 24 hr to determine the protein folding efficiency of COL1A1 in TXNDC5-depleted or control hCF, respectively. Cells transfected with CFP-YFP or CFP-IRES-YFP were used as positive and negative control, respectively. A plasmid encoding CFP-mouse Eln-YFP was constructed and transfected in shTxndc5- and shScr-transduced NIH-3T3 mouse fibroblasts to determine the protein folding efficiency of mouse ELN in control and TXNDC5-depleted mouse fibroblasts.

A standard acceptor photobleaching FRET protocol[5,6] was performed 24 hr after transfection, using a Carl Zeiss Confocal Laser Scanning Microscope 780 (LSM 780, Carl Zeiss, Oberkochen, Germany). Photobleaching was conducted by repeated laser scanning at a selected target cell using 514 nm at maximal intensity. An excitation wavelength of 458 nm and an emission wavelength of 470-500 nm were used for CFP, whereas an excitation wavelength of 514 nm and an emission wavelength of 525-545 nm were used for YFP. ZEN 2.3 software (Carl Zeiss, Oberkochen, Germany) was used to quantify and analyze the FRET signals. The FRET energy transfer efficiency was calculated as the difference between total fluorescence before and after photobleaching, divided by post-bleaching total fluorescence of the entire target cell. For each experimental condition, a non-bleached cell was used as control. n=8-10 for each condition.

2.26 Electrophoretic mobility shift assay (EMSA)

To confirm direct binding of ATF6 to the *Txndc5* promoter, electrophoretic mobility shift assay (EMSA) was performed using LifghtShift Chemiluminescent EMSA kit (Thermo Fisher Scientific, MA, USA) according to manufacturer's instructions. A biotin-labeled, double-stranded oligonucleotide containing the consensus-binding sequence for ATF6 (Biotin 5'-CGGGCCTCGCCGCTGACGTGGCGCTAGCCGC -3') from *Txndc5* promoter was used as a probe to assay for binding activity of nuclear extracts from NIH-3T3 cells overexpressing *Atf6*. A double-stranded oligonucleotide with the same sequence but without biotin labeling was used as a competitor (in 200-fold excess) to determine the specificity. Unlabeled *Txndc5* promoter DNA and biotin-labeled *Txndc5* promoter DNA with ATF6 binding site deletion were both used to confirm the specificity of ATF6-*Txndc5* promoter binding.

2.27 Generation of *Txndc5*$^{-/-}$ mice using CRISPR/Cas9 genome-editing

Cas9 mRNA and two single guide RNAs (sgRNA) targeting intron 1 and intron 3 of *Txndc5* were co-injected into C57Bl/6J mouse zygotes to generate mice with a large deletion spanning exons 2 & 3 of *Txndc5* (Supplemental Figure XIA). In short, the pX458 vectors expressing Cas9 and sgRNA targeting *Txndc5* genomic sequence were generated. The two sgRNA flanking exon2 (sgRNA1) and exon3 (sgRNA2) of *Txndc5* were designed using CRISPR tool website (http://tools.genome-engineering.org). The resulting sequences are sgRNA1: 5'ggaaacagaAATATCACACGTTTACTCGGaggtcaa3' and sgRNA2: 5'tcagaggttCAATCCA GTATCATCAAGGCaggaacatg 3'. T7 promoter sequence was then added to the Cas9 coding

region and to the sgRNAs by PCR amplification. T7-Cas9 and T7-sgRNA PCR products were used as templates for in vitro transcription with mMESSAGE mMACHINE T7 ULTRA kit (Thermo Fisher Scientific, MA, USA). Both the Cas9 mRNA and the sgRNAs were purified by MEGAclear kit (Thermo Fisher Scientific, MA, USA). Purified Cas9 mRNA, sgRNA1 and sgRNA2 were co-injected into one-cell mouse zygotes (C57BL6/J) in M2 media (Millipore Corp. MA, USA) using a Piezo impact-driven micromanipulator. Post-injected blastocysts were transferred into the uterus of pseudopregnant female mice at 2.5 dpc. The preparation of mouse zygotes, pronuclei microinjection of Cas9 mRNA/sgRNAs, blastocysts transfer, and initial breeding of the *Txndc5*$^{-/-}$ animals were performed by the Transgenic Mouse Core Laboratory in National Taiwan University. PCR primers were designed to identify founders harboring a large deletion of the intended target site, as indicated by the presence of a mutant PCR amplicon (350 base pair, 5F+3R), corresponding to the expected size of the region with exons 2 & 3 deleted and flanked by the 5F/3R primer pair (Supplemental Figure XIA). The potential off-target mutageneses of CRISPR were assayed by RFLP/sequencing analysis at off-target sites predicted by CRISPR Design Tool. PCR and DNA sequencing confirmed the presence of an allele with a 6.8 kb deletion encompassing exons 2 & 3 of Txndc5 in one of the founders (Supplemental Figure XIA). This founder was crossed to WT C57Bl/6J mice to obtain *Txndc5*$^{+/-}$ offspring; the F1 *Txndc5*$^{+/-}$ progeny were then crossed to generate homozygous *Txndc5*$^{-/-}$ mice. F2 *Txndc5*$^{-/-}$ mice were born at the expected Mendelian frequency and, at baseline, showed no detectable developmental defects or structural anomalies.

2.28 Apoptosis detection

The extent of cell apoptosis in control (shScr) and TXNDC5-knockdown (shTXNDC5) hCF was detected using BD Annexin V:FITC Apoptosis Detection kit (BD Biosciences, NJ, USA) on a BD FACSCalibur™ flow cytometer following the manufacturer's instructions. The data acquisition, analysis and reporting were performed using BD FACSuite software (BD Biosciences, NJ, USA). n=4-6 for each condition.

2.29 Statistical analyses

All experimental data are presented as means ± SEM. The statistical significance of differences between experimental groups was evaluated by Mann-Whitney *U* test or Fisher's exact test; repeated measures ANOVA was used to determine if the ECM protein decay rates between control and *TXNDC5*-knockdown hCF were significantly different. *P* values <0.05 are considered statistically significant.

2.30 List of pharmacological inhibitors and concentrations used in the present study

16F16: an inhibitor of protein disulfide isomerases, 20 μmol/L

4-phenylbutyrate (4-PBA): a general ER stress inhibitor, 0.5 mmol/L

Apocynin: an inhibitor of NA(P)DH oxidases, 2 mmol/L

Cycloheximide: an inhibitor of protein translation, 30 μg/ml

Eeyarestatin I (Eey I): an inhibitor of ER associated degradation, 0.5 μmol/L

GSK137831: an inhibitor of NOX1/4, 5 μmol/L

N-acetylcysteine (NAC): a ROS scavenger, 15 mmol/L

SP600125: an inhibitor of JNK, 10 μmol/L

Tauroursodeoxycholic acid (TUDCA): a general ER stress inhibitor, 0.5 mmol/L

Tunicamycin: an inducer of ER stress, 0.3 ng/ml

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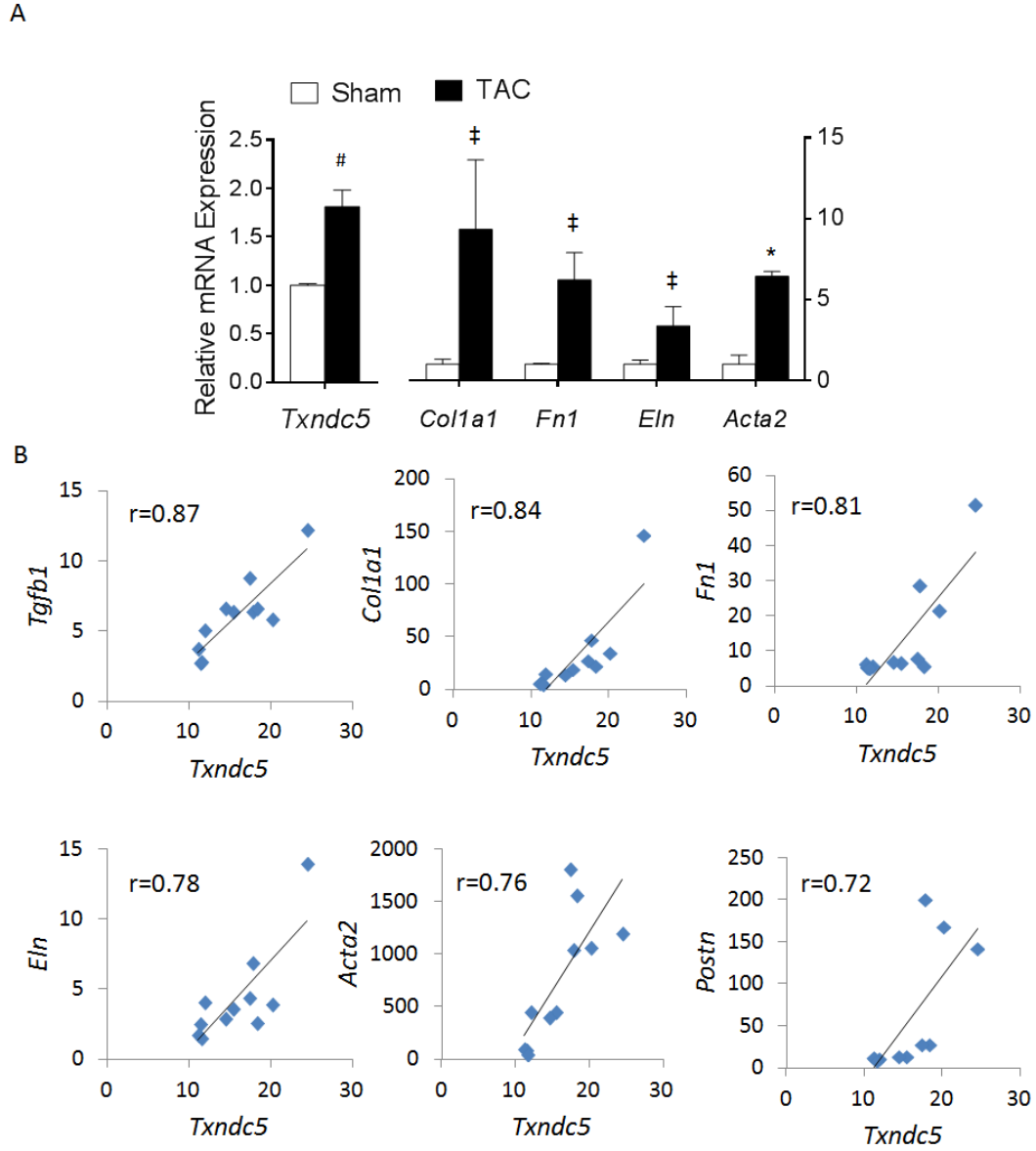
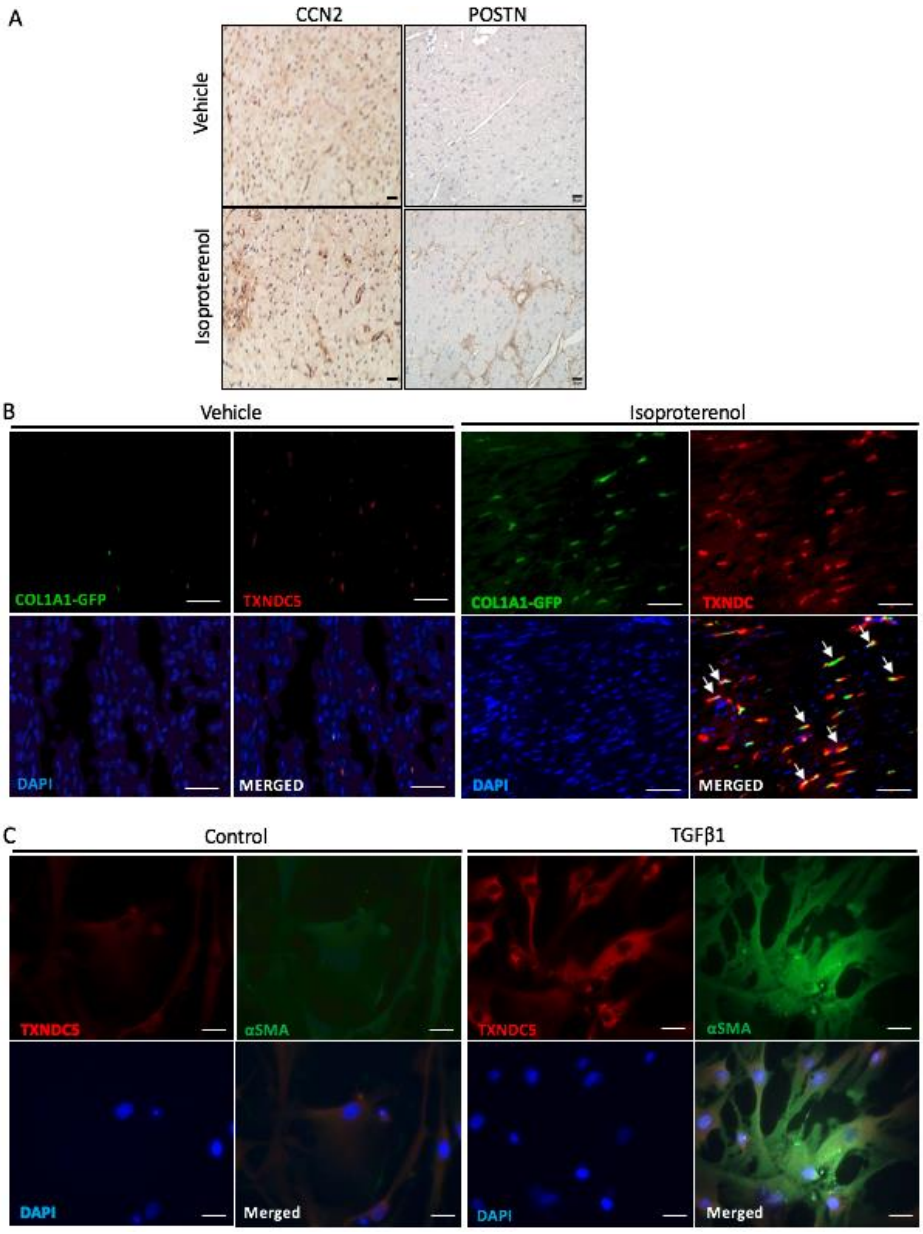
Supplemental Figures and Figure Legends

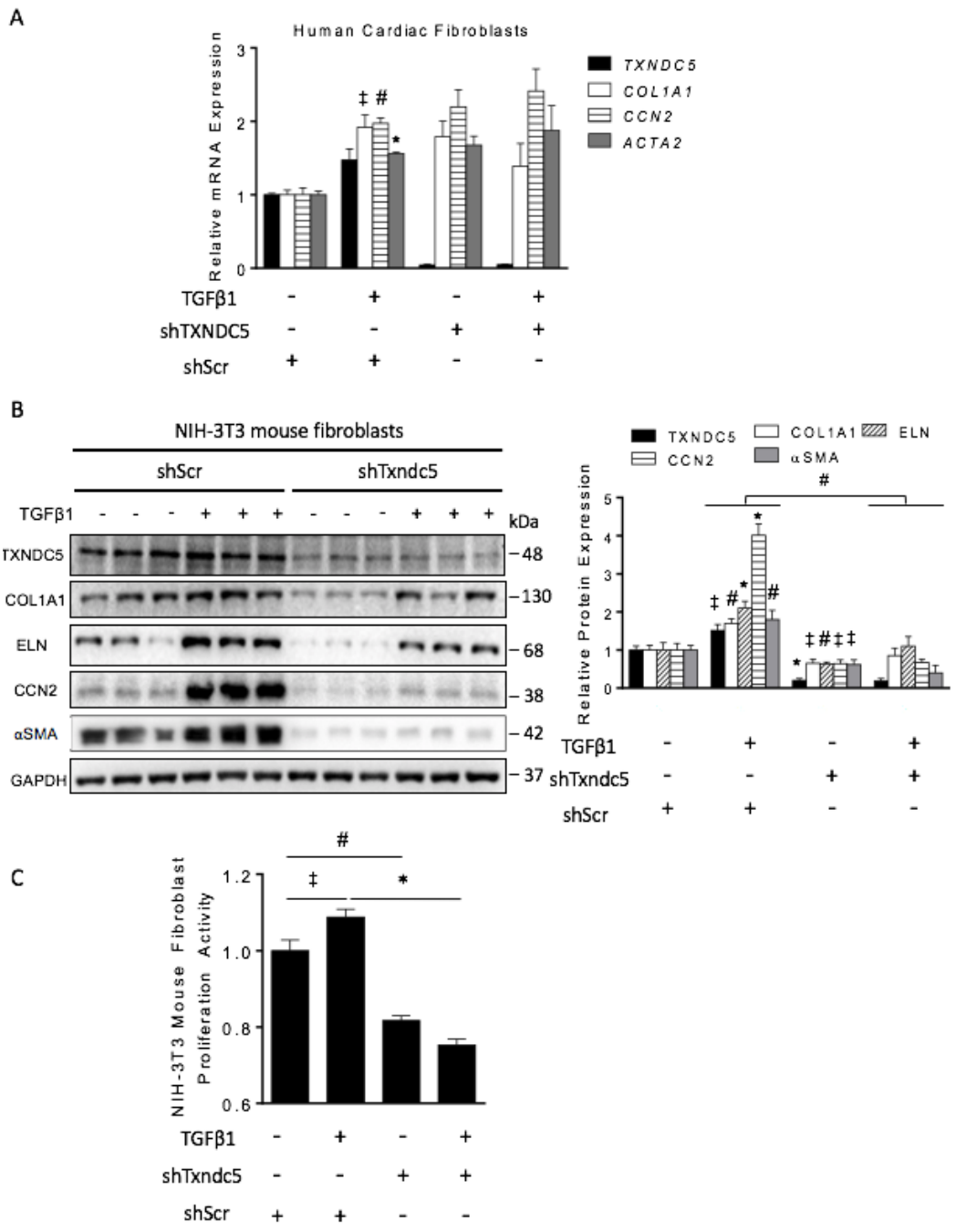


Supplemental Figure I. *Txndc5* expression showed strong positive correlation with fibrogenic protein genes in mouse heart with TAC-induced cardiac hypertrophy and fibrosis.

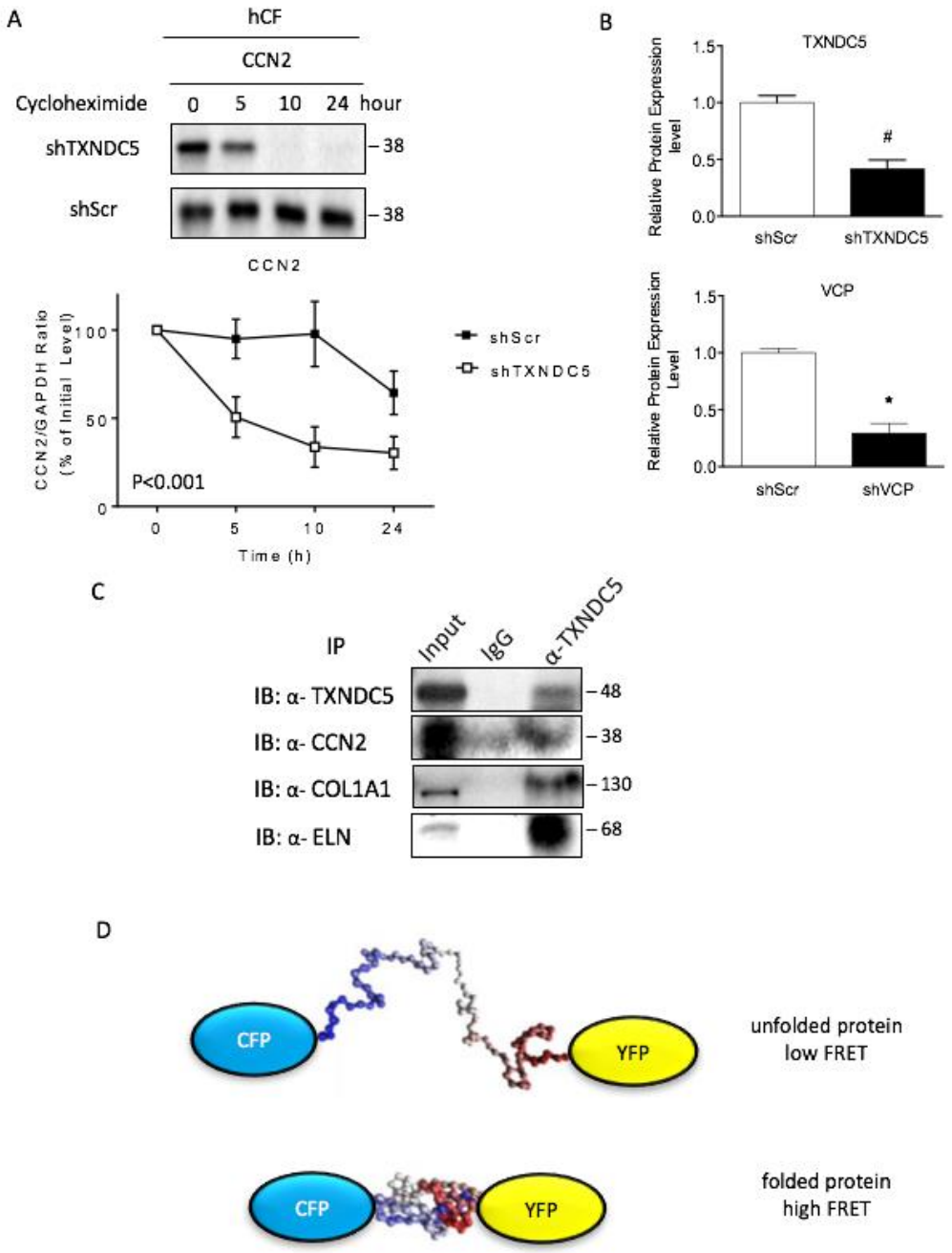
(A) RNASeq analysis (GSE35350) on LV samples from a mouse model of cardiac hypertrophy and fibrosis induced by TAC revealed significantly increased levels of *Txndc5* and fibrosis-related genes compared to sham-operated LV samples. (B) *Txndc5* expression in mouse LV also showed strong positive correlation with that of Tgfβ1 and fibrogenic protein genes including *Postn*, *Acta2*, *Col1a1*, *Eln* and *Fn1*.



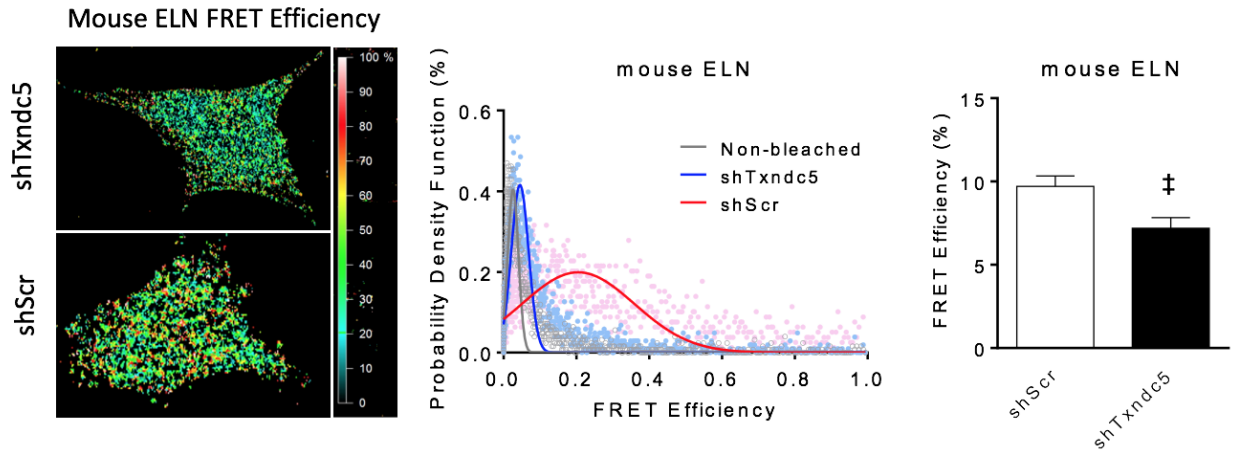
Supplemental Figure II. Immunohistochimical (IHC)/immunofluorescence (IF) staining in mouse heart and immunocytochemical (ICC) staining in hCF showed strong expression of TXNDC5 in activated, collagen-producing cardiac fibroblasts. (A) IHC staining of cardiac sections from a mouse model of ISO-induced heart failure showed strong upregulation of fibrogenic proteins CCN2 and periostin (POSTN), compared to vehicle-treated control mouse heart (Scale bar: 20 μm). (B) IF staining of the cardiac sections from *Col1a1-GFP*^{Tg} mice, where active cardiac fibroblasts are labeled with GFP, revealed that ISO treatment induced a strong increase in TXNDC5 expression that is highly co-localized with GFP-positive (arrows), collagen producing cardiac fibroblasts (Scale bar 50 μm). (C) ICC staining in control and TGFβ1-treated hCF showed that TXNDC5 (Red) is strongly upregulated in TGFβ1-activated, α-SMA-expressing (Green) hCF. The perinuclear staining pattern of TXNDC5 is consistent with its localization in the ER (Scale bar 10 μm).



Supplemental Figure III. *TXNDC5/Txndc5* knockdown prevented TGFβ1-induced fibroblast activation and upregulation of ECM proteins, but not of mRNA. (A) TGFβ1 treatment in hCF induced significant upregulation of ECM protein genes (*COL1A1*, *ACTA2* and *CCN2*), which was not affected by *TXNDC5* knockdown. (B) *Txndc5* knockdown in NIH-3T3 mouse fibroblasts also abolished TGFβ1-induced upregulation of αSMA and ECM proteins COL1A1, ELN and CCN2 (All significant symbols indicate comparisons to baseline shScr group without TGFβ1 treatment, except the symbol above the bars, which indicates the significant level of differences between groups of TGFβ1+shScr and TGFβ1+shTxndc5). (C) Knocking down *Txndc5* in NIH-3T3 fibroblasts prevented TGFβ1-induced cellular proliferation (n=6 for each group, $^{‡}P<0.05$, $^{\#}P<0.01$, $^{*}P<0.001$).

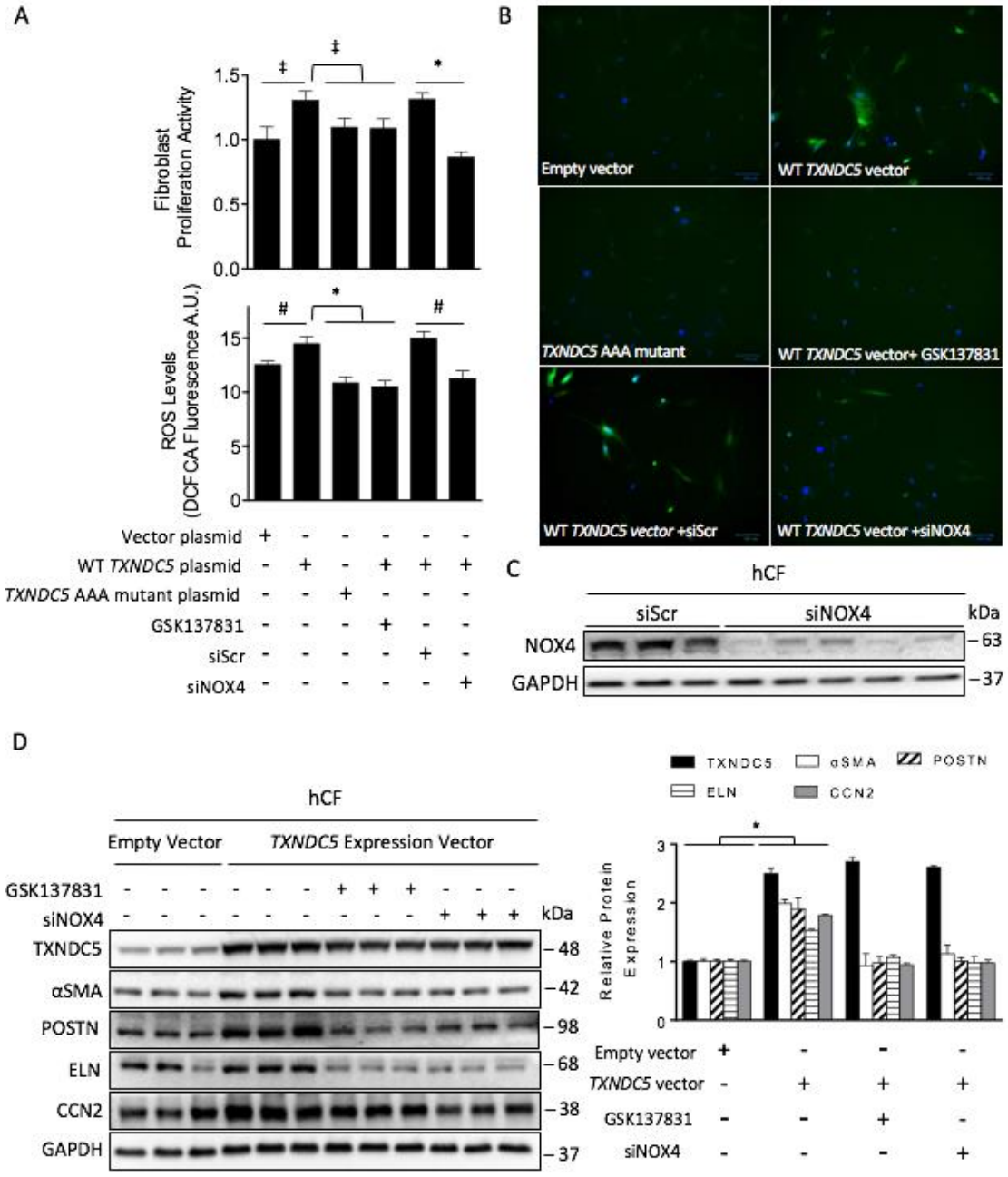


Supplemental Figure IV. *TXNDC5* knockdown accelerated degradation of matricellular protein CCN2 in hCF (A) Cycloheximide protein chase assay revealed significantly (P<0.001) accelerated degradation of matricellular protein CCN2 in hCF with *TXNDC5* knockdown, compared with shScr control. (B) Quantification of TXNDC5 and VCP protein knockdown efficiency by shRNAs shown in immunoblots presented in Figure 4B. (C) Protein co-immunoprecipitation experiments revealed strong physical interaction between TXNDC5 and fibrogenic proteins including CCN2, COL1A1 and ELN in hCF. (D) Schematic illustration of rationale for using a dual fluorescent protein-labeled construct (CFP-COL1A1-YFP or CFP-Eln-YFP) to determine the extent of COL1A1 or ELN protein folding in fibroblasts with FRET. When the COL1A1 or ELN proteins fold properly (bottom), the CFP and YFP chromophores will be brought in closer proximity, yielding a higher FRET efficiency.



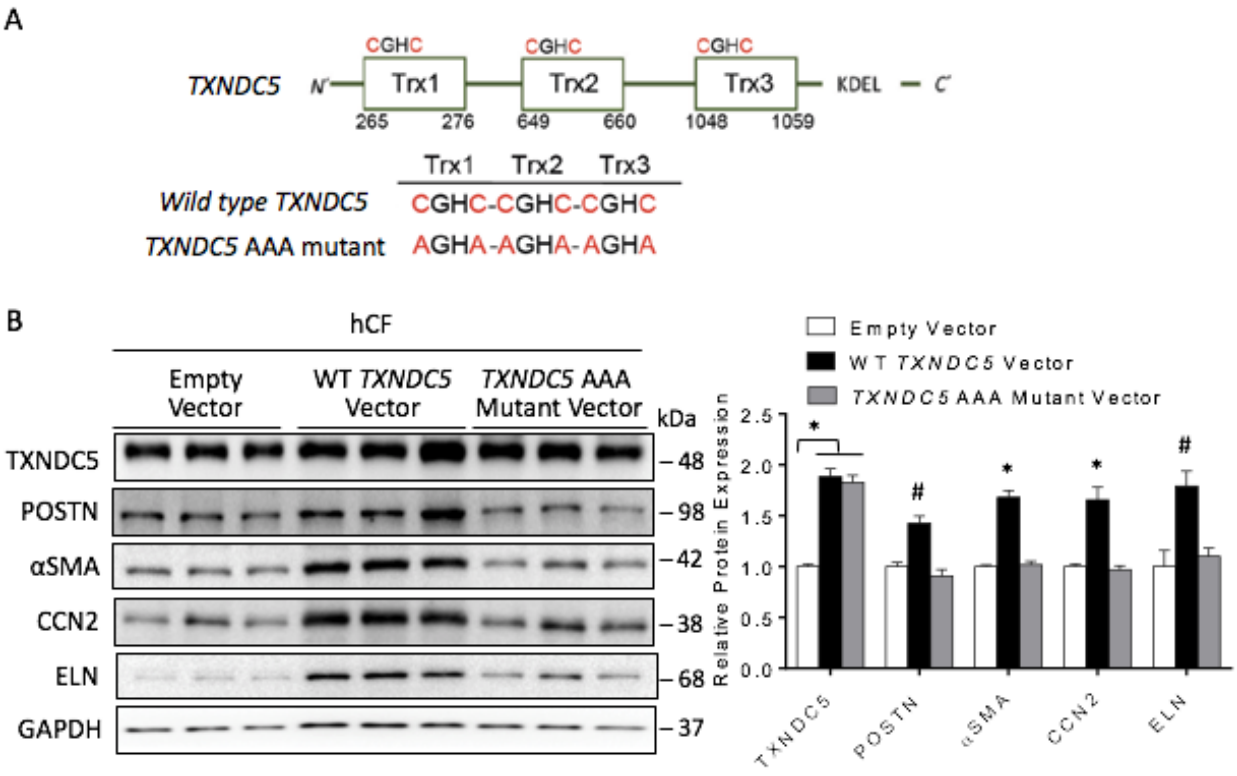
Supplemental Figure V. *Txndc5* knockdown reduced ELN protein folding in NIH-3T3 mouse fibroblasts

A FRET-based protein folding assay using a dual fluorescence-labeled mouse *Eln* construct (CFP-mouse Eln-YFP) in NIH-3T3 cells also showed significantly reduced FRET efficiency in cells with *Txndc5* knockdown, compared to shScr control, suggesting reduced folding of ELN in fibroblasts with TXNDC5 depletion (n=8-10 in each group, ‡ $P<0.05$).



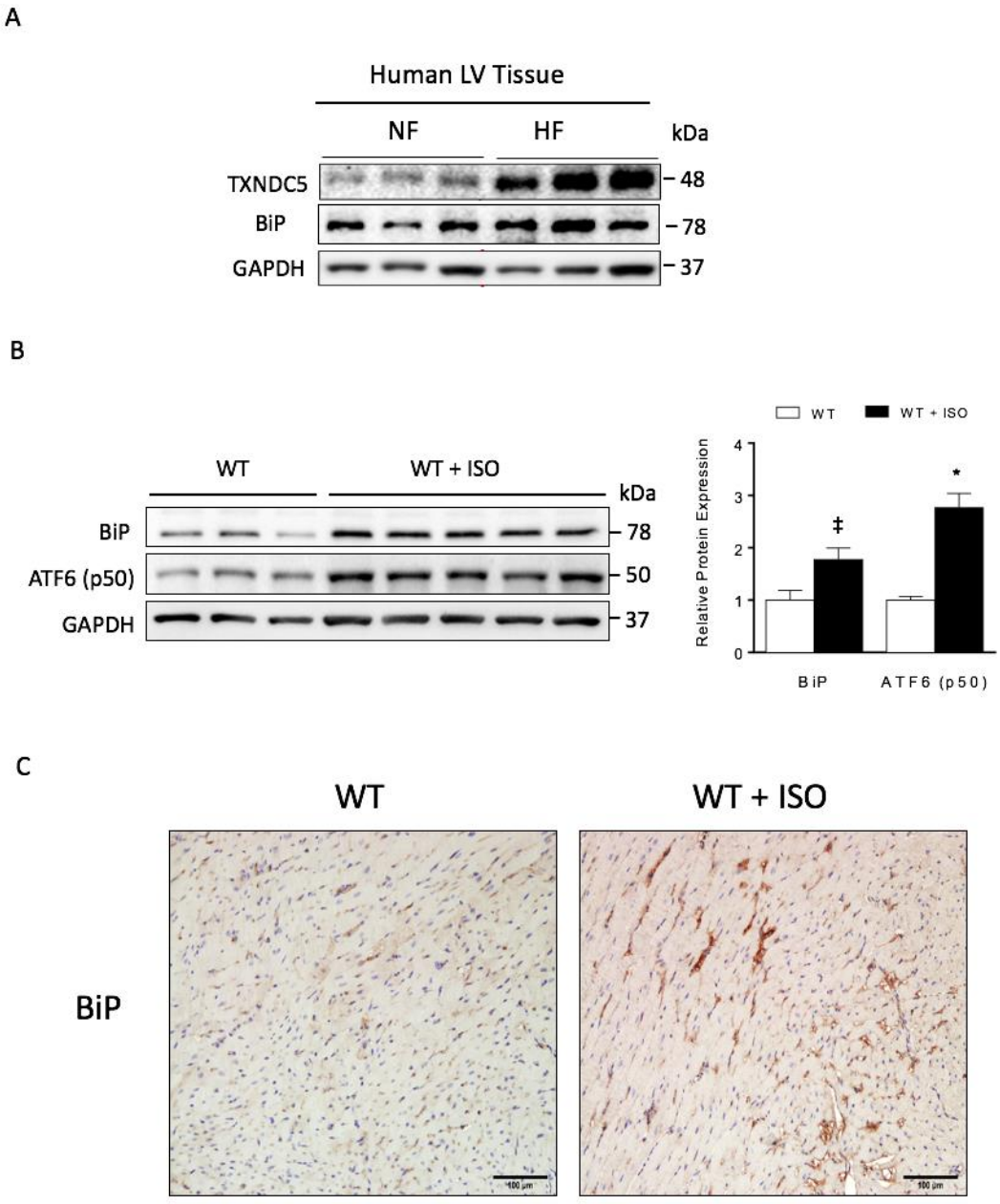
Supplemental Figure VI. NOX4 is required for TXNDX5-mediated pro-oxidant and fibrogenic effects

(A) Pharmacological inhibition (with NOX1/4 inhibitor GSK137831, 5μmol/L) or siRNA-mediated (siNOX4) knockdown of *NOX4* abolished *TXNDC5* overexpression-induced hCF proliferation and ROS production. Mutant *TXNDC5* (*TXNDC5* AAA, see text) lacking PDI activity failed to trigger ROS production and hCF proliferation. (B) Representative photomicrographs of DCFDA-stained hCF expressing WT *TXNDC5* (+/- GSK137831/siNOX4) or *TXNDC5* AAA mutant showing cellular ROS levels. (C) Representative immunoblots illustrating the knockdown efficiency of NOX4 by siRNA in hCF. (D) Immunoblots showing that NOX4 inhibition or depletion blocked *TXNDC5* overexpression-induced hCF activation (reflected by αSMA and POSTN levels) and ECM protein production. (n=6 for each group, $^{‡}P<0.05$, $^{\#}P<0.01$, $^{*}P<0.001$).



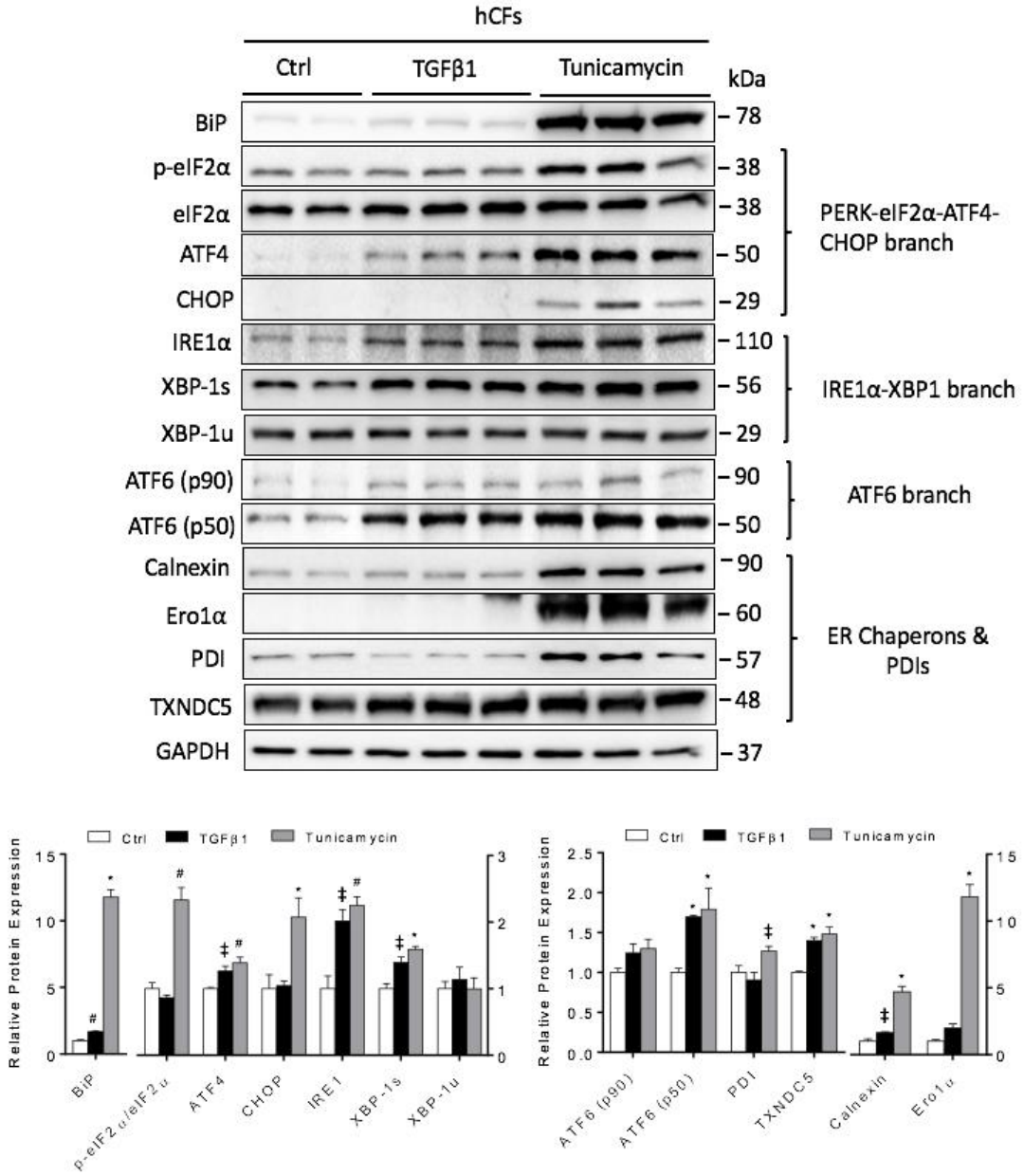
Supplemental Figure VII. The PDI function of TXNDC5 is required for its fibrogenic effects

(A) Scheme of WT and mutant TXNDC5 (*TXNDC5* AAA) proteins with Cys-to-Ala mutations in all three thioredoxin domains (Trx 1,2 and 3, which are required for its PDI function). (B) Immunoblots showing that WT *TXNDC5* overexpression led to increased hCF activation (reflected by increased POSTN and αSMA) and ECM protein production, which is not observed in hCF expressing *TXNDC5* AAA mutant. (n=6 for each group, $^{\#}P<0.01$, $^{*}P<0.001$).



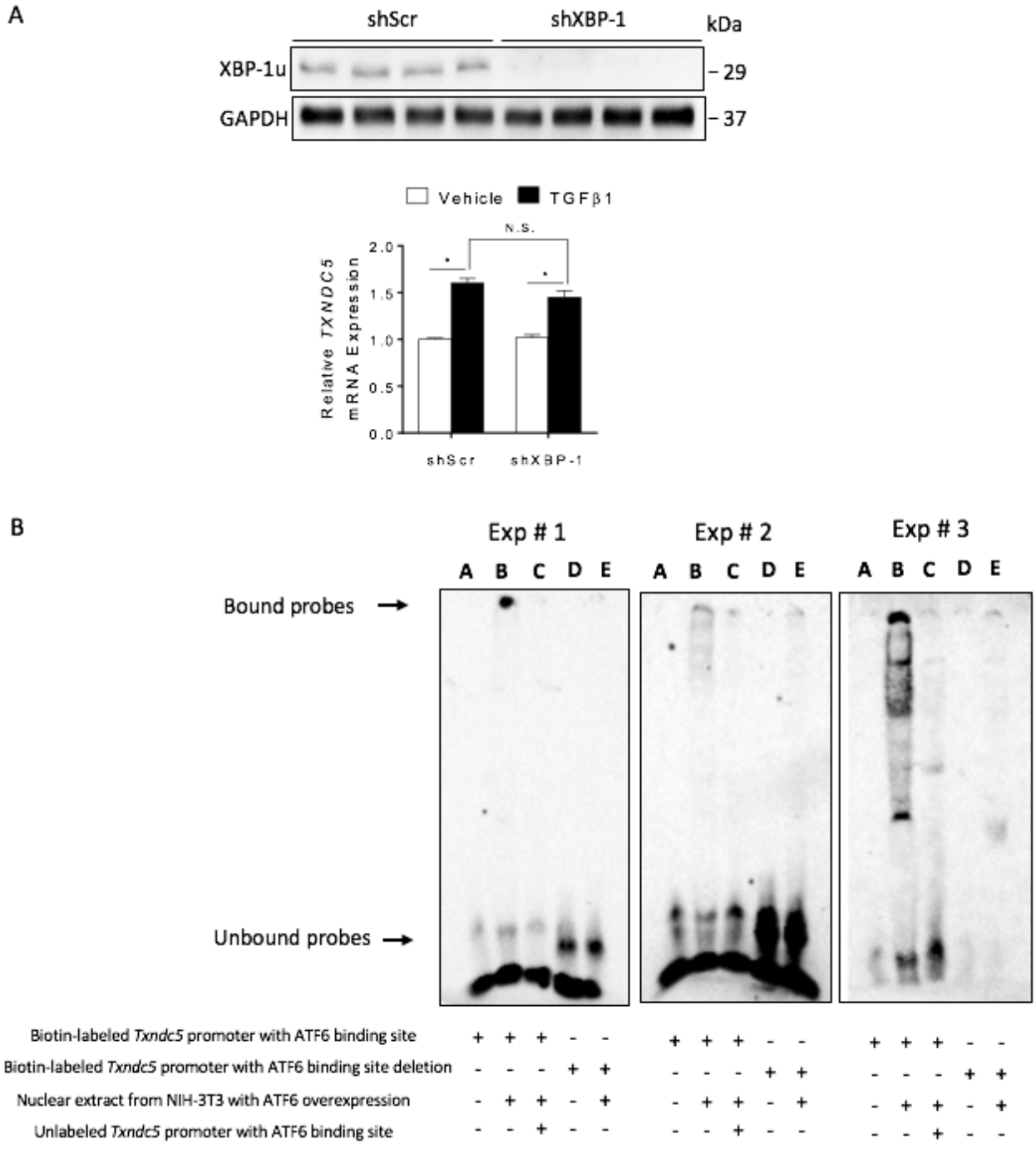
Supplemental Figure VIII. ER stress is increased in human failing LV and in mouse failing ventricular myocardium induced by ISO treatment

(A) Immunoblots showing that ER stress marker BiP was increased in human failing (HF), compared to non-failing (NF), LV. (B) ISO treatment led to significant increases in the protein expression levels of ER stress markers including BiP and ATF6 p50 in mouse LV. (n=5-6 for each group, $^{‡}P<0.05$, $^{*}P<0.001$). (C) IHC staining revealed increased ER stress protein BiP expression in ISO-treated mouse LV, particularly in the myocardial interstitium (Scale bar 100 μm).



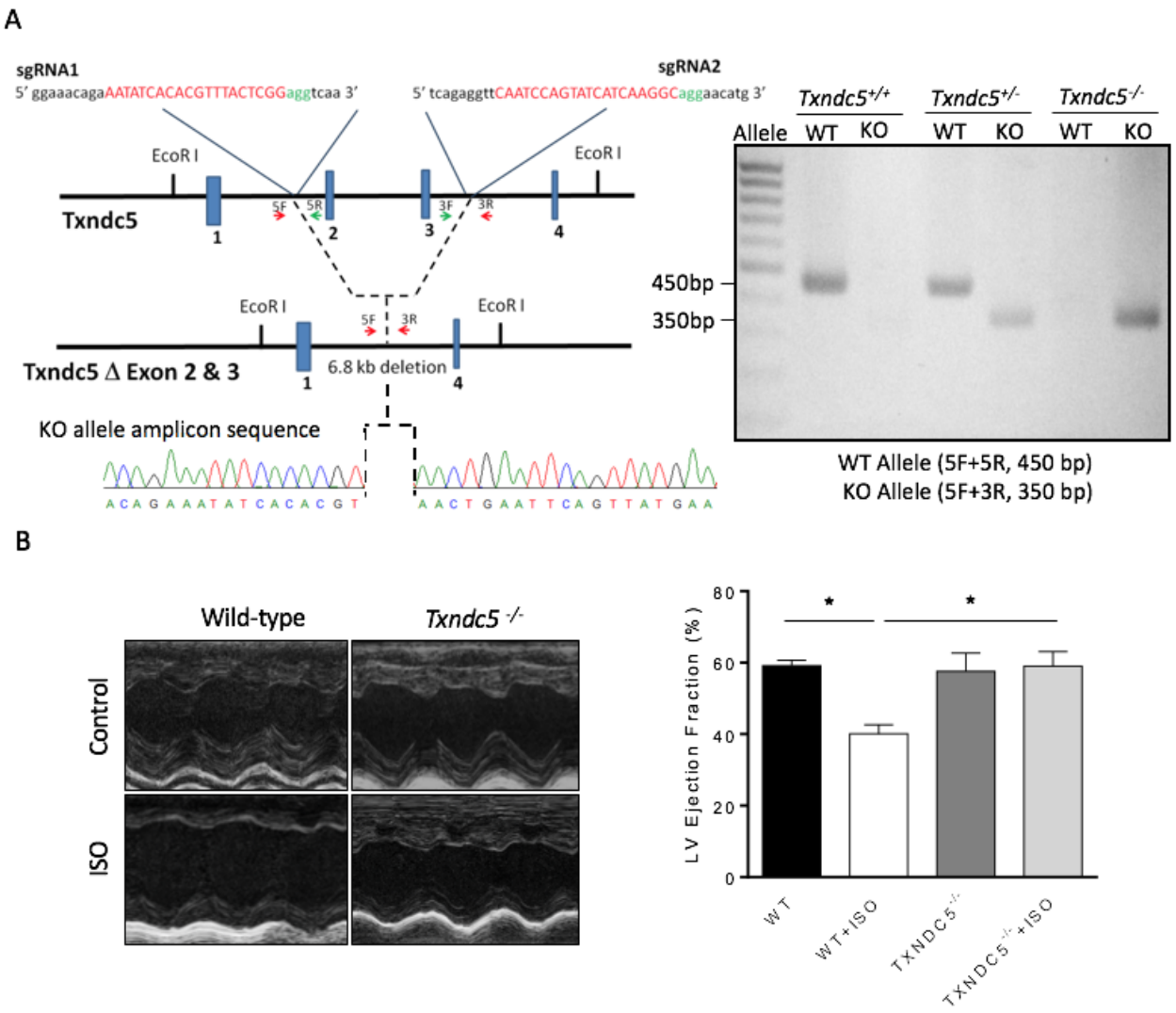
Supplemental Figure IX. TGFβ1 treatment in hCF triggered increased ER stress, especially in the ATF6 and IRE1α-XBP1 signaling branches

Immunoblots showing that TGFβ1 treatment in hCF led to significant increases in the expression levels of proteins involved in the ER stress pathway, including BiP, IRE1α, XBP-1s (IRE1α-XBP1 branch; XBP-1 exists in the transcriptionally inactive unspliced form XBP-1u and the spliced active form XBP-1s), ATF6 p50 (ATF6 branch; ATF6 p90 is the membrane-bound, precursor form of ATF6, which can be cleaved by proteases to become the soluble, active form ATF6 p50), calnexin and TXNDC5 (ER chaperons). Note that except for ATF4, proteins involved in the PERK-eIF2α-ATF4-CHOP branch of ER stress signaling were not altered by TGFβ1 treatment. In contrast, tunicamycin (0.3 ng/ml for 24 hr), a potent ER stress activator used as positive control, strongly induced ER stress proteins across all three signaling branches. (n=6 for each group, $^{‡}P<0.05$, $^{\#}P<0.01$, $^{*}P<0.001$).



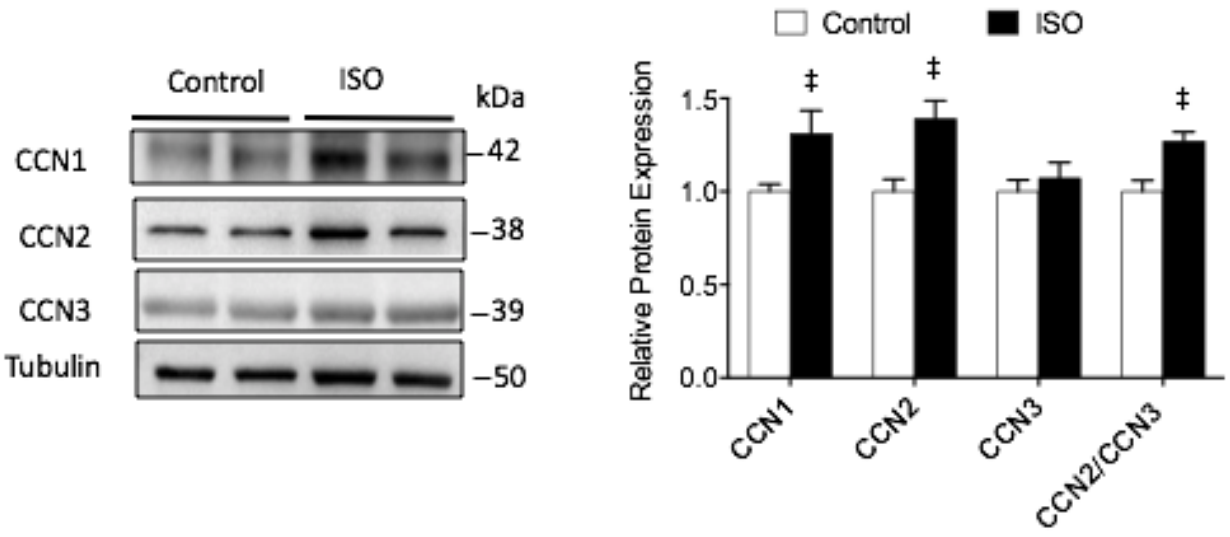
Supplemental Figure X. TGFβ1-induced *TXNDC5* expression in CF is independent of XBP-1

(A) shXBP-1 was effective in knocking down expression of XBP-1 in hCF (upper). TGFβ1-induced *TXNDC5* upregulation in hCF was not affected by XBP-1 knockdown (lower, n=6, $^{*}P<0.001$). (B) Results of the three repeats of electrophoretic mobility shift assay demonstrating the physical interaction between ATF6 and *Txndc5* promoter in NIH-3T3 fibroblasts.



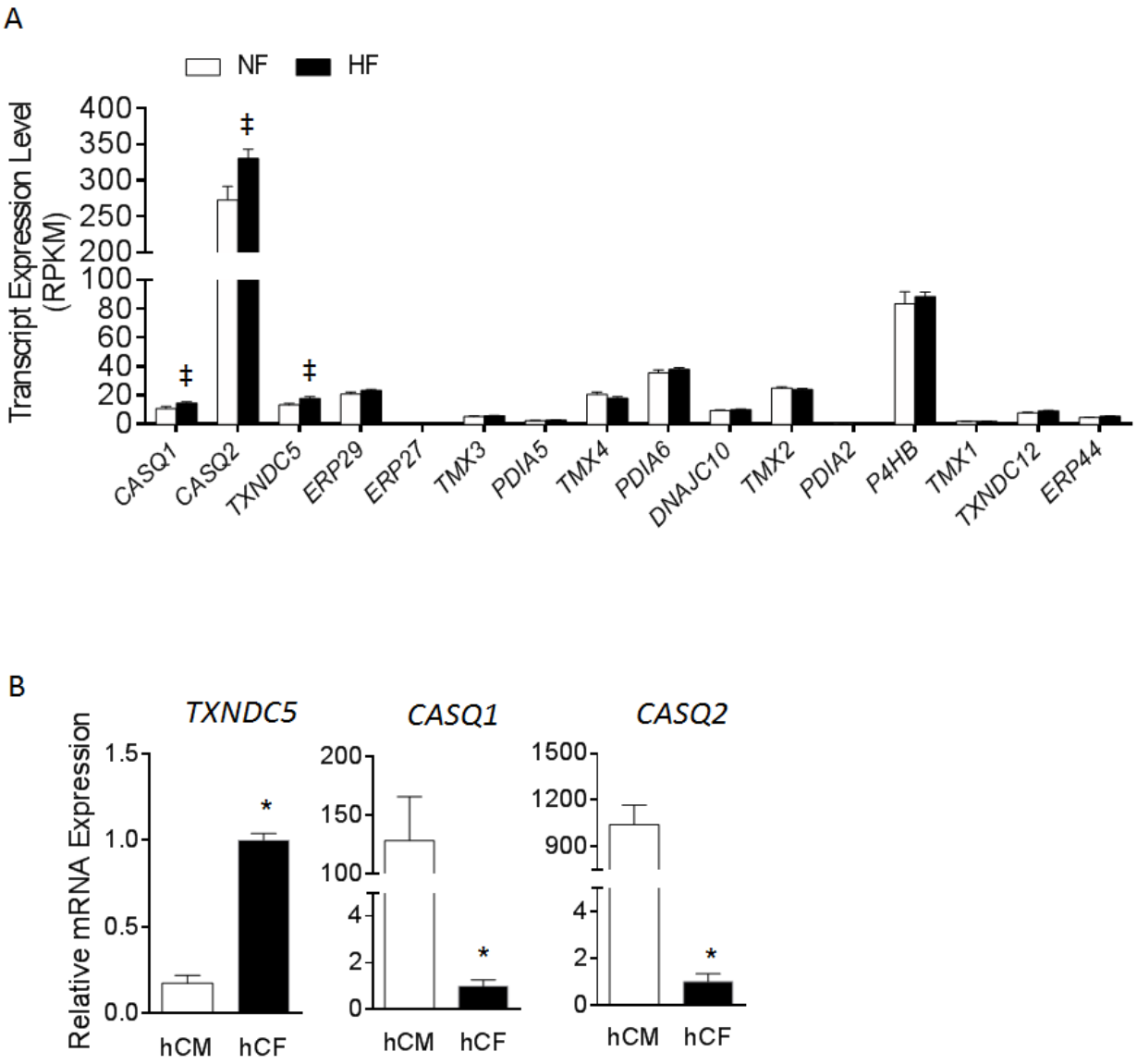
Supplemental Figure XI. Targeted deletion of *Txndc5* protects against ISO-induced myocardial dysfunction

(A) Schematic illustrating the design of the Cas9/single-guide RNA (sgRNA) sequences, which targeted DNA sequences flanking exon 2 and 3 of *Txndc5* (left). A PCR assay was designed to identify founders harboring a large deletion of the intended target site, as indicated by the presence of a mutant PCR amplicon (350 bp, 5F+3R) corresponding to the expected size of the region with exon 2/3 deletion flanked by the 5F/3R primer pair (right). (B) M-mode echocardiographic images taken from WT and *Txndc5*$^{-/-}$ mice (left). Isoproterenol (ISO, 30 mg/kg/day subcutaneously for 10 days) injection led to markedly impaired contractile function (reflected in the reduced LV ejection fraction) in WT, but not in *Txndc5*$^{-/-}$, mice (right). (n=8-10 for each group, $^{*}P<0.001$).



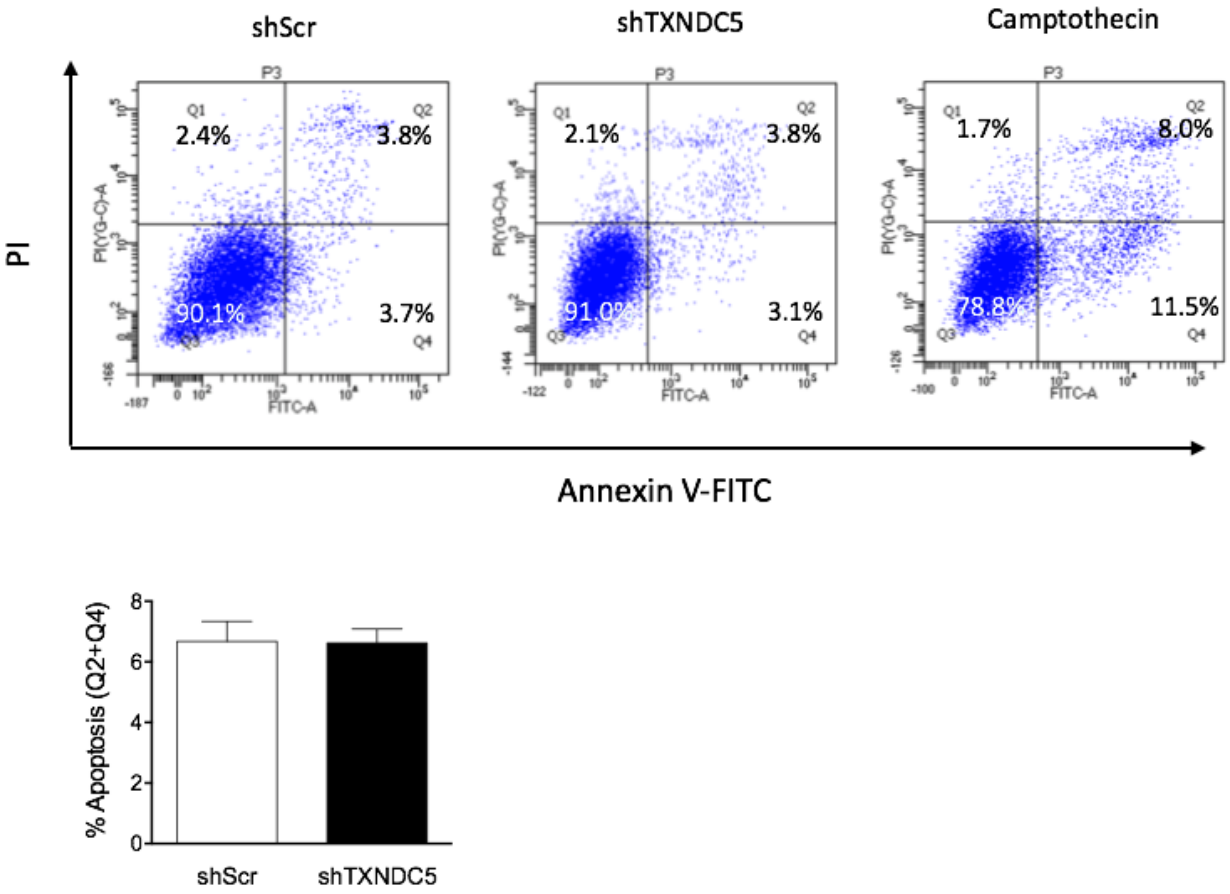
Supplemental Figure XII. ISO-induced mouse cardiac fibrosis is associated with increased expression of matricellular proteins CCN1 and CCN2

Representative immunoblots (left) and quantification (right) showing that the protein expression levels of matricellular proteins CCN1 (anti-fibrotic) and CCN2 (pro-fibrotic), but not CCN3 (anti-fibrotic), were significantly increased in ISO-treated, compared with control, mouse LV. The CCN2/CCN3 ratio, an indicator of the balance between pro- versus anti-fibrosis processes, was also significantly increased in ISO-treated mouse LV. (n=6 for each group, $^{‡}P<0.05$).



Supplemental Figure XIII. *TXNDC5* is the only PDI family gene that is enriched in hCF and upregulated in human failing heart

(A) RNASeq analysis on human failing (HF) and nonfailing (NF) heart revealed 3 PDI family genes that were significantly dysregulated in failing myocardium (*CASQ1, CASQ2* and *TXNDC5*) (‡ P<0.05). (B) Transcript expression analysis of *CASQ1, CASQ2* and *TXNDC5* showed that *TXNDC5* was highly enriched in hCF, whereas *CASQ1* and *CASQ2* were enriched in hCM (* P<0.001).



Supplemental Figure XIV. TXNDC5 depletion does not lead to increased hCF apoptosis

Flow cytometry was performed on control (shScr) and TXNDC5-depleted (shTXNDC5) hCF stained with annexin V (annexin V-FITC) and propidium iodide (PI). Camptothecin (20 μmol/L)-treated hCF was used as a positive control. The percentage of apoptotic cells (Q2+Q4) was similar between control and TXNDC5-depleted hCF (n=4-6 for each group).