Supplemental Information

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Thbs4 and Thbs1 DTG mice show protein expression in the ER/SR compartment of adult hearts from transgenic mice, Related to Figure 1

(A) Immunohistochemistry of Thbs4 protein from Thbs4 DTG hearts with co-staining for caveolin-3 (red) to show the sarcolemma (plasma membrane). The longitudinal sections show Thbs4 protein (green) almost exclusively in the ER/SR compartment without obvious protein aggregates and without localization to the outer sarcolemma. Nuclei are stained blue. Scale bars = 10 μm.
(B) Immunohistochemistry of Thbs4 protein (green) from Thbs4 DTG hearts with co-staining for calreticulin (red) to show the ER/SR compartment. Staining is overlapping and shows almost exclusively intracellular localization. Scale bars = 50 μm.
(C) Immunohistochemistry in the adult heart from a sham and TAC-pressure loaded Wt mouse which shows that endogenous Thbs4 (green) localizes inside the cell with minor extracellular areas, but only after induction by TAC stimulation. Scale bars=50 μm.
(D) Immunohistochemistry of Thbs1 from Thbs1 DTG hearts also shows nearly exclusive localization to the ER/SR compartment in the adult heart. Membranes are red (wheat germ agglutinin-TRITC) and Thbs1 is in green. Nuclei are blue. Scale bars = 10 μm.

Figure S2. Thbs4 DTG mice show no cardiac abnormalities with aging or after pressure overload stimulation, Related to Figure 1.

(A) H&E staining of cardiac histological sections from 40-week-old tTA and Thbs4 DTG mice show no differences or overt pathology with Thbs4 overexpression. Scale bars = 100 μm.
(B) Heart weight normalized to body weight (HW/BW) at 16 and 40 weeks of age in single transgenic Thbs4 mice, single transgenic tTA mice, or DTG mice that overexpress Thbs4 in the heart (N=6 or more mice per group, +/- SD). No differences were observed.
(C) Factional shortening percentage (FS%) as determined by echocardiography in tTA single transgenic controls or Thbs4 DTG mice before or 12 weeks after pressure overload induced by transverse aortic constriction (TAC) surgery (N=6 or more mice per group, +/- SD). Sham controls were also used. No loss of function was observed in either group, suggesting that Thbs4 overexpression did not predispose to disease. There were also nosignificant differences in lethality between the groups subject to TAC.
(D) Survival of Wt and *Thbs4*$^{-/-}$ mice after MI injury, which showed slightly greater lethality in the *Thbs4*$^{-/-}$ group. Sham mice showed no lethality (N=10 or more mice per MI group).
(E) Ventricular fractional shortening (FS) 4 weeks after MI in the indicated groups of mice. There was a trend towards a greater reduction in FS in *Thbs4*$^{-/-}$ mice that survived MI. *P<0.05 versus Sham.

Figure S3. Cardiac-specific Thbs4 overexpression increases expression of Atf6α-dependent ER stress genes, but Thbs4 did not affect other ER membrane-bound transcription factors that are also processed and cleaved for activation, Related to Figure 2.

(A) Immunohistochemistry for ER resident proteins (Calreticulin, Creld2, and Hyou1) in green, showing that expression is greatly enhanced in the Thbs4 DTG hearts versus wildtype (Wt)

hearts. Myocyte membranes are shown in red and nuclei are shown in blue. Scale bars = 10 μm.
(B) Western blots for other ER membrane bound Creb-related bZIP family members (and Srebp bHLH transcription factors) from Wt and Thbs4 DTG hearts shows only activation and upregulation of Atf6α (N-terminus, nuclear form), but no other factors examined. There were also no differences in the level of proteases (S1P and S2P) involved in Atf6α processing nor in other processing proteins (Insig1 and Insig2). Thus, the Thbs4 effect on ER stress signaling is highly specific to Aft6α. The Atf6α shown in the western blot corresponds to the cleaved/activated form that enters the nucleus. Atf6α-C refers to the C-terminal nuclear fragment of Aft6α.

Figure S4. Thbs1 and Thbs4 induce an adaptive ER stress response profile in heart and skeletal muscle of tissue-specific transgenic mice, Related to Figure 2.
(A) Schematic of the binary, inducible transgenic system used to express Thbs1 in the heart.
(B) Western blot for Thbs1 protein from the heart of a Thbs1 DTG mouse not on doxycycline (induced) versus a tTA single transgenic control heart.
(C) Western blots for expression of ER stress response proteins from the heart a single Thbs1 DTG mouse line versus a tTA control heart. FL=full-length Atf6α, N=N-terminal Atf6α fragment.
(D,E) Schematic of the transgene constructs used to make skeletal muscle-specific Thbs4 or Thbs1 transgenic mice and western blots from skeletal muscle of 2 different lines for each Thbs4 and Thbs1 versus a Wt control for the indicated ER stress proteins. Gapdh is a loading control. The arrowheads so the specific bands for the indicated proteins.

Figure S5. Thbs4 reduces aggregates due to overexpression of a mutant CFTR protein In cultured MEFs, Related to Figure 3.
Immunocytochemistry for CFTR (green) in primary cultured MEFs with co-staining for phalloidin (red). MEFs were infected for 6 days with AdCFTR508 alone or with co-infection of AdThbs4 or Adβgal. The data show diffuse aggregation (arrows) of the mutant CFTR protein that is reduced with co-infection with AdThbs4. The CFTR antibody used shows non-specific nuclei staining. Scale bars = 50 μm.

Figure S6. Model for the proposed novel Thbs-dependent, beneficial ER stress response pathway through Atf6α, Related to Figures 6 and 7
Protein-folding based stress, protein aggregation sensing, or even more general stress to the heart induces Thbs4 expression, which directly controls Atf6α in the ER as it shuttles to the Golgi, where it then facilitates its processing and nuclear translocation. Once Atf6α activity is enhanced it secondarily induces protective chaperones and factors that augment ERAD and autophagy, as well as enhanced secretion, towards resolving stress associated with unfolded proteins or increased demands on protein production with hypertrophic or injury stress to the heart.

EXTENDED EXPERIMENTAL PROCEDURES

Animal models

Animal experiments were approved by the Institutional Animal Care and Use Committee of the Cincinnati Children's Hospital Medical Center. To over-express either Thbs1 or Thbs4 in the heart the tetracycline-inducible α-myosin heavy chain (α-MHC) promoter expression vector system was used as described previously (Sanbe *et al.*, 2003). This system requires crossing with a tetracycline transactivator (tTA) transgenic line to regulate expression of the first tet-inducible transgene, such that removal of doxycycline from the animals diet produces expression. Mouse Thbs4 cDNA was generated from RNA from the hearts of 6-month old calcineurin transgenic mice (Molkentin et al.,1998) and cloned into the Sal1 site of the α-MHC promoter expression vector (forward 5'-TTACGTCGACAAGCTTATGCCGGCCCCACG-3', reverse: 5'-TACGTCGACTCAATTATCCAAGCGGTCAAAAC-3). The α-MHC-Thbs4 plasmid backbone was remove using BamH1 and the α-MHC-Thbs4 fragment was gel purified followed by Elutip-D column purification (Cat. 10462617, Schleicher and Schwell Bioscience; Dassel, Germany) for oocyte injection.

The Thbs1 cDNA was obtained from Open Biosystems (Accession BC042422). Thbs1 cDNA was amplified by PCR and cloned into the Sal1 and HindIII sites of the α-MHC promoter vector (forward: 5'-CATGTCGACATGGAGCTCCTGCGGGGACTAGGTGTC-3', and reverse: 5'-GAGAAGCTTTAGGAATCTCGACACTCGTATTTCATGTC-3'). Not1 was used to remove the vector backbone before oocyte injection. Both Thbs1 and Thbs4 were also used to generate skeletal muscle-specific transgenic mice with the skeletal α-actin promoter construct as described previously (Goonasekera *et al.*, 2011), but these mice will be characterized more thoroughly in a subsequent publication. All transgenic mice were produced in the FVB/N background. Mice deficient for Thbs4 (*Thbs4*$^{-/-}$; C57BL/6) were purchased from Jackson Laboratories (Strain: B6.129P2-Thbs4$^{tm1Dgen/}$J). Other mice used in this investigation have previously been described and included αB-crystallin (CryAB) mutant (Wang *et al.*, 2001), desmin mutant (Wang et al., 2001a), myocyte enhancer factor 2a (MEF2) transgenic (Xu *et al.*, 2006), and *Csrp3*$^{-/-}$ (Arber *et al.*, 1997).

Cardiac performance was assessed by trans-thoracic echocardiography (Oka *et al.*, 2006) and/or hemodynamic analysis (Braz *et al.*, 2004). The trans-aortic constriction (TAC) surgical procedure to induce pressure overload stimulation (Wilkins *et al.*, 2004) and the myocardial infarction (MI) surgical procedure (Kaiser *et al.*, 2005) were performed as previously reported. Mice were sacrificed by CO_2 inhalation and organ weights recorded.

Plasmids

Vectors utilized in this study included pcDNA1 (Invitrogen), pShuttle-CMV (7.5kb, Stratagene), pEF/myc/ER (Invitrogen), pGEX-4T1 (GE Healthcare), and pCMV-Tag1 (Stratagene). PCR utilized Expand-long or high fidelity template polymerase (Roche, 11681842001). For all clones, restriction enzymes were from NEB, CIAP (18009-019) from Invitrogen, and T4 DNA ligase from NEB (M0202T). In all instances, PCR products were gel purified (Qiagen, 28706) and restriction enzyme digested cDNAs was also purified (Qiagen, 28106). Ligated cDNA was transformed into DH5α *E. coli* (18265-017 Invitrogen). To verify cDNA construction all plasmids were purified (Qiagen, 12165) and sequenced.

***pcDNA1 plasmids* -** Mouse Atf6α was generated from the hearts of 4-month old Thbs4 transgenic mice by mRNA isolation and cDNA production for subsequent cloning by PCR (forward: 5'-CATGCGGCCGCATGGAGTCGCCTTTTAGTCCG-3', reverse: 5'-GAACTCGAGCTACTGCAACGACTCAGGGATG-3'). This Atf6α cDNA was the template to make a constitutively-nuclear Atf6α (pcDNA1-Atf6α-CN, amino acids 1-364) plasmid cloned at Not1/Xho1 (above mentioned forward primer, and reverse: 5'-GAACTCGAGCTATCTTCGCTTTGGACTTGGGACTTTGAGC-3') Additional clones whose

cDNA was amplified by PCR to introduce restriction enzyme sites for cloning into pcDNA1 included: Thbs4 (NM_011582) at BamHI/Xho1 (forward: 5'-CATGGATCCATGCCGGCCCCACGCGC-3', reverse: 5'- GATCTCGAGTCAATTATCCAAGCGGTCAA-3'), and Atf4 (cDNA purchased from Open Biosystems, Accession BC085169) at BamH1/Xho1 (forward: 5'-CATGGATCCATGACCGAGATGAGCTTCCTG-3', reverse: 5'-GAACTCGAGTTACGGAACTCTCTTCTTCCC-3').

pCMV-Tag1 plasmids- *In vitro* transcription-translation (TNT) protein synthesis utilized the T3 promoter in a pCMV-Tag1-FLAG vector at Sac1/Not1 sites. For both Atf6α cDNAs, the reverse primer was the same (reverse: 5'-AGAGCGGCCGCACTGCAACGACTCAGGGAT-3') while the forward primer differed (amino acid 361-656 forward: 5'-CATGAGCTCATGCCAAAGCGAAGAGCTGTCTGTG-3', and amino acid 471-656 forward: 5'-CTAGAGCTCATGCGAGGCTGGGTTCATAGACAT-3').

Atf6β cDNA was purchased (Open Biosystems, Accession: BC013534) so that the luminal domain (amino acids 421-699) could be amplified by PCR (forward 5'-CATGAGCTCATGTCTCCTCGGATGAG-3', reverse: 5'-ATAGCGGCCGCTGGGGTGATTGAGGTAGAG-3') for cloning into pCMV-Tag1-FLAG at Sac1/Not1 sites.

GST plasmids – Thbs4 was cloned into pGEX-4T1 at BamH1/Xho1 (forward:5'-CTTGGATCCATGCCGGCCCCACGCGC-3', reverse: 5'-GTACTCGAGTCAATTATCCAAGCGGTCAAAACTCGGG-3') while Thbs1 was cloned into the Xho1/Not1 sites (forward: 5'-CTTCTCGAGGAGCTCCTGCGGGGACTAGG-3', and reverse 5'-GTAGCGGCCGCTTAGGAATCTCGACACTCGTAT-3'). Following sequencing, the GST-fusion plasmid was transformed into OneShot BL21 Star (DE3) *E. coli* (Invitrogen, C601003) for protein expression.

Adenoviruses – pShuttle-CMV (7.4kb) was the vector required to make adenovirus. The same primers used to amplify Thbs1 (Sal1/HindIII) and Thbs4 (Sal1) cDNA for the α-MHC promoter vector were utilized to amply the thrombospondin1 or 4 cDNA for cloning into the respective restriction enzyme sites of pShuttle. To make an ER-retained Thbs4, the Thbs4 cDNA was amplified by PCR for insertion into the pEF/myc/ER vector at Sal1/Xho1 sites (forward: 5'-CTTGTCGACATGCCGGCCCCACGCGCG-3', reverse 5'-GTACTCGAGATTATCCAAGCGGTCAAAACTC-3'). The ER-Thbs4-myc-KDEL plasmid was then the cDNA template amplified by PCR (forward: 5'-CATGGTACCATGGGATGGAGCTGTATCATCC-3', reverse: 5'-GATGGTACCCTACAGCTCGTCCTTCTC-3') for insertion into the pShuttle vector at Kpn1.

Atf6α DN (amino acid 448-570) adenovirus was made by first amplifying the Atf6α luminal cDNA region by PCR (forward: 5'-CATCTCGAGATGCCTCCACCTCCATGTCAAC-3', reverse: 5'-ATAGCGGCCGCGTGGGTGGTAGCTGGTAATAGC-3') for insertion at Xho1/Not1 sites in the ER retained vector, pEF/myc/ER. Next, the ER-Atf6α DN-myc-KDEL plasmid was the cDNA template used to amplify by PCR using a universal primer set for pEF/myc/ER (forward 5'-CATAGATCTATGGGATGGAGCTGTATCATCC-3', reverse 5'-GAGAAGCTTCTACAGCTCGTCCTTCTCGCT-3') for insertion into pShuttle at BglII/HindIII sites.

The ER-Thbs4-repeat-myc-KDEL plasmid was made by amplifying the cDNA encoding the repeat domain of Thbs4 (amino acids 466-741) by PCR (forward: 5'-TATCTCGAGATGGATGTGGACATTGACAGTTACC-3', reverse: 5'-TATGCGGCCGCGTCCAGGACCACTGTCTG-3') for insertion into EF/myc/ER at Xho1/Not1 sites. This ER-Thbs4 repeat-myc-KDEL plasmid served as the template for PCR amplification using the universal BglII/HindIII primers previously mentioned.

BiP cDNA was purchased (Open Biosystems, Accession: BC050927) and Sal1/HindIII sites introduced by PCR (forward: 5'-CATGTCGACATGATGAAGTTCACTGTGGTGG-3', reverse: 5'-GAGAAGCTTCTACAACTCATCTTTTTCTGAT-3') for cloning into pShuttle.

CFTR mutant ΔF508 adenovirus (H5.040.CMV.DF508) was provided by the Penn Vector Core (Perelman School of Medicine, University of Pennsylvania).

Gal4-fusions- Full-length human Gal4-ATF6α (1-670 amino acids) cDNA has previously been described (Shen *et al*., 2002; Chen *et al*., 2002). We generated Atf6*α* luminal domain fusions to full-length Gal4 (Addgene; Plasmid 24345) by PCR of the Gal4 cDNA (forward: 5'-TATGAGCTCGGATCCCAACATGAAG-3', reverse: 5'-GAAGCGGCCGCACTCTTTTTTTGGGTTTGG-3') for cloning into pCMV-Tag1 at Sac1/Not1 sites. To target Gal4 to the ER, the ER signal sequence of pEF/myc/ER vector was amplified by PCR (forward: 5'-TATGAGCTCGGTACCATGGGATGGAGCTGTATC-3', reverse: 5'-GAAGAGCTCTGGGAGTGCGCGCCTGTGGA-3') and inserted 5' to the Gal4 cDNA in pCMV-Tag1. Next, deletion mutants of human ATF6α cDNA (NM_007348.3) were made by PCR using a single forward primer (5'-CTAAGATCTCTATGAAAGTCCCTAGTCCAAAGCG-3') along with different reverse primers corresponding to different locations along the ATF6α luminal domain. The ATF6α luminal domain PCR products were cloned in frame and 3' to the GAL4 cDNA at BglII/HindIII sites (reverse for amino acids 370-670: 5'-GAGAAGCTTTTGTAATGACTCAGGGATGGTG-3', reverse for amino acids 370-589: 5'-GAGAAGCTTTGGTCTTGTGGTCTTGTTATGG-3', and reverse for amino acids 370-468: 5'-GAGAAGCTTCTGACAAGGAGGTGGAGGAATG-3').

Immunohistochemistry and Immunocytochemistry

Hearts were fixed in 10% formalin and then dehydrated in ethanol (Wilkins et al., 2004). The hearts were processed and embed in paraffin. Global heart architecture was determined from longitudinal 5 μm deparaffinized sections stained with hematoxylin and eosin (H&E) (Woods *et al*., 1994). For immunohistochemistry (IHC) of paraffin-embedded hearts, 5 μm sections were processed and heated in antigen retrival cita (BioGenex, HK086-9K). The heart sections were blocked in a blocking buffer (0.1% cold water fish skin gelatin, 1% BSA, 0.1% Tween-20, 0.05% NaN_3) for 1 hour at room-temperature. Primary antibody incubations were over-night at 4^oC. Primary antibodies for Thbs4 were from R&D Systems (AF2390; 1:150 dilution) and Santa Cruz (sc-7657-R; 1:150 dilution). Other antibodies used for IHC (all at 1:100 dilutions) in this study included: Atf6α (Abcam, ab37149 at 1:100); BiP (Abcam, ab53068); calreticulin (Abcam, ab39818); Creld2 (R&D Systems, AF-3686); Hyou1 (LifeSpan Biosciences, LS-C46842), Thbs1 (R&D Systems, AF3074) and caveolin-3 (BD, 610421). Secondary antibodies (Invitrogen, Alexa Fluor) were applied for 2 hours at room temperature (1:400) along with counter-stains. Counter-stains included a membrane marker (Sigma-Aldrich, L5266; 500 μg/ml stock at 1:100 dilution) and/or a nuclear marker TO-PRO-3 iodine (Invitrogen; T3605; 2 μM).

For IHC of protein aggregate markers, hearts from 6- to 7-month-old mice that had been embedded in O.C.T. compound (Tissue Tek, 4583) and frozen were cut into 5 μm sections. Primary antibodies included: desmin (Biomeda, V1015; 1:200 dilution), αB-crystallin (Stressgen, SPA-223; 1:400 dilution) and A-11 (generous gift from Dr. Charles Glabe, University of California; 1:200 dilution). For detection of A-11, a biotinylated goat anti-rabbit (Vector Laboratories, BA-1000) secondary was required while Alexa Fluor 488nm was required for detection of both desmin and α-B-crystallin.

For immunocytochemistry (ICC) of CFTR, wt MEFs were isolated from E12.5 c57 mice and grown on cover-slips in 6-well plates. MEFs were infected with adenovirus and grown in DMEM

media supplemented with 1% BGS and 1% P/S for 6 days to allow time for mutant CFTR aggregate formation. Cells were fixed with 4% paraformaldehyde and blocked in the above mentioned blocking buffer before addition of the primary antibody (CFTR, Abcam, ab59354; 1:200)

For ICC of neonatal rat ventricular cardiomyocytes (NRVM), cells grown in Lab-Tek II chamber slides (ThermoScientific, 154461) were fixed for 5 minutes in 4% paraformaldehyde. The cells were then permeabilized in 0.3% triton/PBS for 20 minutes followed by blocking in 5% horse serum (Invitrogen, 26050-070). Primary antibody incubation was performed overnight at 4°C while the secondary antibody (1:400) along and counter-stains were applied for 2 hours at room-temperature. Coverslips were fixed with Vectashield Hardset (Vector Labs, H1400).

For ICC for Atf6α, neonatal rat ventricular cardiac fibroblasts grown in Lab-Tek II chamber slides were fixed for 10 minutes at -20°C in cold MeOH followed by 1-minute permeabilization in cold acetone. Primary antibody incubation (Santa Cruz Biotechnology, Inc., sc-166659; 1:100 dilution) was performed overnight at 4°C. Secondary antibody (1:400) and TO-PRO-3 counter-stain incubation was for 2 hours at room temperature. When multiple primary antibodies were required, Alexa Fluor 488 nm was used along with Alexa Fluor 568 nm.

Western blotting

Mouse hearts were harvested and immediately frozen in liquid nitrogen for storage at -80°C. For western blot analysis, hearts were homogenized (Fisher TissueMiser) in a lysis buffer (20 mM Na phosphate, 150 mM NaCl, 2 mM $MgCl_2$, 0.1% NP-40, 10% glycerol, 1 mM DTT, 10 mM Na pyrophosphate, 10 mM NaF, and 0.1 mM Na_3VO_4; at pH 7.4) containing phosphatase inhibitors (Roche, #11873580001). Samples were sonicated (VirTis VirSonic 60, power setting 3 for 3x10 seconds) then centrifuged at 7500 rpm for 10 minutes at 4°C. Protein concentration was determined using DC Protein Assay Kit (Bio-Rad, #5000111). 5X SDS-PAGE loading buffer (0.25 M Tris, pH 7.0; 0.28 M SDS; 20% 2-mercaptoethanol; 0.1% Bromophenol blue) was added to 30 μg or 60 μg protein samples and placed in a boiling water bath for 5 minutes, loaded and subjected to SDS-PAGE. In all instances, wet transfer method was utilized with PVDF membranes (Millipore, IPVH00010). Several different primary antibodies were found to detect the Atf6α and verified to detect only Atf6α [Abcam, ab37149 at 1:1000; Pro-Sci Inc., 3681 and 3683 at 1:1000) while two antibodies detected Thbs4 [R&D Systems, AF2390 & MAB2390 at 1 μg/μl and 2 μg/μl, respectively] by western blot. Atf6α is present in heart as a full-length product (uncleaved) and an intermediate product, both of which are variably detected and somewhat difficult to reliably quantify. However, the cleaved form that resides primarily in the nucleus, which is approximately 50 kDa, is highly reliable for quantitation and it is the form representing the active species of Atf6α. This cleaved and active form of Aft6α is what is shown in all the western blots (except Figure 5G) and unless otherwise indicated in figure legends the Abcam Atf6α antibody was utilized. Other primary antibodies used in this study included: Akt (Cell Signaling Technology, 9272; 1:500); Armet (Abcam ab67271; 1:1000); Atf4 (LifeSpan BioSciences, Inc., LS-C30114; 1:1000); Atf5 (Abcam ab69935; 1:1000); Atrial natriuretic peptide (Millipore, AB5490; 1:1000); Atf6β (Santa Cruz, sc-30596; 1:200); BiP (Cell Signaling Technology, 3177; 1:1000); Calreticulin (Cell Signaling Technology, 2891; 1:1000); Creb3 (Novus Biologicals, H00010488-B01; 1:500); CD36 (Abcam; ab78054; 1:1000); CD47 (R&D System, AF1866; 1 μg/ml); CFTR (R&D Systems, MAB1660; 1 μg/ml); Creb3L1 (R&D Systems, AF4080; 1 μg/μl); Creb3L2 (Aviva Systems Biology, ARP34673_T100; 1:1000); Creb3L3 (Santa Cruz Biotechnology, Inc., sc-69375; 1:200); Creb3L4 (Abcam, ab58366; 1:1000); Creld2 (R&D Systems; AF-3686; 1 μg/ul); DAD1 (Abcam, ab23836; 1:1000); Derl1 (Abcam, ab93341; 1:1000); Edem1 (Abcam, ab67105; 1:1000); p-eIF-2α (Cell Signaling Technology, 3597; 1:1000); FLAG (Sigma, F1804; 1:1000); Grp94 (Cell Signaling Technology, 4875; 1:1000); Herpud1 (Abcam, ab73669; 1:1000); Hrd1 (Sigma,

H7790; 2 μg/ml); Hyou1 (LifeSpan BioSciences, Inc., LS-C46842; 1:500); Insig1 (Abcam, ab70784; 1:1000); Insig2 (Abcam, ab86415; 1:1000); IRE1α (Cell Signaling Technology, 3294; 1:500); Mthfd2 (LifeSpan BioSciences, Inc., LS-C32329); p97 (Abcam, ab36047: 1:1000); Pdi (Cell Signaling Technology, 2446; 1:1000); S1P (Sigma-Aldrich, HPA006239; 1:1000); S2P (Cell Signaling Technology, 2157; 1:1000); Sdf2L1 (Sigma, HPA005638, 1:200); Srebp1 (Abcam, ab3259; 1:1000); Srebp2 (Abcam, ab30682; 1:250); Thbs1 (LifeSpan BioSciences, LS-C26344; 1 μg/ml); Ub (Santa Cruz Biotechnology, sc-8017); VIMP (Abcam ab50354; 1:1000). In all instances, appropriate IgG-AP conjugated secondary antibodies (1:2000) were utilized (Santa Cruz Biotechnology) and all blots were exposed to ECF substrate (Amersham Biosciences, RPN5785) and visualized with a Gel-Doc XR with QualityOne Software. All membranes were stripped (25 minutes in 1X Re-blot Plus Strong Solution; Millipore, 2504) and blotted for Gadph (Fitzgerald, 10R-G109A; 1:10000), which served as a loading control.

Cell culture, adenovirus infection, and RT-PCR

Primary neonatal rat cardiomyocytes (NRVMs) were prepared from 1- to 2-day-old Sprague-Dawley rat pups as previously described (DeWindt *et al*., 2000). 1.5 x 10^6 cells per 10 cm plate were grown in HyClone Medium 199/EBSS (ThermoScientific, SH30253FS) supplemented with 2% bovine growth serum (ThermoScientific, SH3054103) and 1% penicillin-streptomycin (Cellgro 30-0002-CI). NRVMs were infected with adenovirus for 2 hours and then fresh media applied. Cells were harvested 24 to 72 hours post-infection.

When neonatal rat cardiac fibroblasts or MEFs were utilized, cells were grown in HyClone DMEM (ThermoScientific, SH30022FS) supplemented with 10% bovine growth serum and 1% penicillin-streptomycin. During the 2 hr adenoviral infection, fibroblasts were in 2% bovine growth serum supplemented media after which time they were switched back to 10% bovine growth serum. Other cell lines utilized in this study included COS7, NIH 3T3, and HEK293 and all these lines were grown in HyClone DMEM supplemented with 10% bovine growth serum and 1% penicillin-streptomycin. Also grown in the above supplemented DMEM media were c57 wt E12.5 MEFs as well as Atf6α wt and Atf6α-null MEFs (Yamamoto *et al*., 2007). When adult myocytes were studied, these cells were isolated from hearts of 16-week-old c57 wild-type and Thbs4-deficient animals as previously described (Maillet *et al*., 2009).

For cell survival assays, NRVMs were plated in black 96-well plates (Fisher, 07-200-565) with 5000 cells per well and infected with adenovirus. An AlamarBlue (Invitrogen, DAL1025) assay was utilized according to manufacturer instructions and data was recorded using a BioTek Synergy 2 plate reader (BioTek Instruments, Inc.). MEF and adult myocytes survival assays were completed in 12 well plates with cells at 70 to 80% confluency. MEFs 12 hour post-infected with adenovirus were treated for 8-hours with 2 μg/ml tunicamycin or 2 mM DTT in low-serum conditions while adult myocytes were treated with 5 μg/ml TM for 7 hours before the addition of AlamarBlue.

Drugs used in culture during this study included classical ER stress inducers: tunciamycin (Santa Cruz Biotechnology, Inc., sc-3506) used at 1 to 10 μg/ml; DTT used at 1 to 5 μg/ml; Thapsigargin (Santa Cruz Biotechnology, Inc., sc-24017) used at 5 μg/ml); and Brefeldin A (BFA; Sigma, B7651) used at 10 μg/ml (Shen *et al*., 2002; Chen *et al*., 2002). The site-1 protease (S1P) inhibitor drug AESBF (R&D Systems, EI001) was used at 300 μM (Okada *et al*., 2003). Recombinant Thbs4 (R&D Systems, 2390-TH-050) was used at 2 μg/ml.

For reverse transcriptase PCR (RT-PCR), RNA was isolated from either tissue or cells using the Qiagen fibrous tissue kit (74704) coupled with the Qiagen QIAShredder (79656) according to manufacturer instructions. Invitrogen SuperScript III One-Step RT-PCR system (10928042) was utilized along with primers for either rat Thbs4 (forward 5'-CAGACAGAGATGGCATTGGAGAC-

3'; reverse 5'-GGTTACTGACATCAGGACAGCTG-3'; PCR product 338 bp), mouse Atf6α (forward: 5'-GATGCAGCACATGAGGCTTATG-3', reverse: 5'-GTTTCCAGGACCAGTGATAGGC-3'; 328 bp PCR product), or Gapdh primers (forward 5'-GCTCACTGGCATGGCCTTCCG-3'; reverse 5'-CTCTTGCTCTCAGTATCCTTG-3'). PCR reaction aliquots were removed at cycles 20, 25, 30, and 35. The samples were loaded onto a 1% agarose gel and visualized using a Typhoon 9400 Variable Mode Imager with Typhoon Scanner v.4.0 software.

Transfections and Luciferase assays

FuGENE6 (Roche, 1815075) was utilized for transfection of COS, NIH 3T3, and HEK293 cells according to manufacturer instructions (3:1, FuGENE:cDNA). 9xGAL4-luciferase reporter (Promega, E1370) was co-transfected with pSV-β-galactosidase control vector (Promega, E1081). Forty-eight-hours post-transfection cells were harvested in lysis buffer [1% Triton, 100mM Tris (pH 8.0), 2 mM EDTA, 2 mM DTT] and centrifuged at 7500 rpm for 10 minutes at 4°C to pellet cell debris. In 96-well plates (PerkinElmer, 6005290), 10 μL supernantant was placed with 100 μL reaction buffer [4 mM ATP, 15 mM $MgSO_4$, 30 mM Tricine (pH 7.8), 10 mM DTT] and 50 μM luciferin (Promega, E1605) was injected in a Victor X light 2030 Luminscence Reader (Perkin Elmer). Luciferase activities in cell extracts were normalized against β-galactosidase activity. β-galactosidase activity was measured by incubating 20 μl of cell lysate in a covered microtiter plate at 37°C for 1 hour, with 100 μl of ONPG solution (0.8 mg/ml) and the OD was measured at 405 nm (Waser *et al*., 1997) using a BioTek Synergy 2 plate reader. Data are reported as means ± SD of experiments performed in triplicate.

Secretion assays

NIH 3T3s were plated in 12-well dishes. Cells were co-transfected with pcDNA1 plasmids as well as pSV-β-galactosidase control vector and pCMV-GLuc plasmid (NEB, N8081S) using FuGENE6 (2μg cDNA, 3:1, FuGENE:cDNA). After 24-hour post-transfection, cells were washed several times with 1X PBS and serum-free DMEM. After 4-hours 10 μl aliquots of the media were removed for luciferase measurements according to manufacturer instructions with the Gaussia luciferase assay kit (NEB, E3300L). To ensure similar transfection efficiency, cells were harvested with lysis buffer and β-galactosidase activity measured.

For western blotting of proteins in cell media, plates of 48-hour post-adenovirus infected NRVMs (2x10 cm each) were washed several times with 1X PBS and then 7 ml HyClone Media 199 (serum-free) added to each plate. Six hours later the media was collected and concentrated (Amicon Ultra Centrifugal Filters, Millipore UFC801024) to 150 μl. 5X SDS-PAGE loading buffer was added to 40 μl of the concentrated media and samples were analyzed by western blot. Membranes were also exposed to Ponseau S (Sigma, P7170).

GST pull-down and *in vitro* translation

The mouse Thbs4 cDNA was cloned into the BamH1 and Xho1 sites of pGEX-4T1. The GST-Thbs4 fusion plasmid was transformed into BL21 competent *E. coli* (Invitrogen, C601003). cDNA for GST fusions of human THBS4 domains corresponded to the N-terminal domain [A(22)GAQAT…VKDL(240); type-3 repeat domain [D(464)VDID…VIDRI(716); C-terminal domain [D(717)VCPEN…DRFDN(961)] and transformed into BL21 *E. coli*. Cultures were grown at 34°C in LB-Amp to an OD_{550} of 0.6 at which time 1 mM IPTG was added. After 4 hours the culture was centrifuged and the pellet re-suspended in a suspension buffer (20 mM Tris, pH 7.6; 100 mM NaCl; 1 mM $MgCl_2$; used at 1 ml buffer per 50 ml culture), with 1 ml aliquots stored at -80°C. Tubes were thawed on ice and lysosome crystals were added as well as protease inhibitors (Roche, 11873580001), Triton X-100 (0.5%) and DTT (0.5 mM). Samples were sonicated and centrifuged (7500 rpm for 10

minutes at 4°C) and the supernatant saved. To the supernatant, 150 μl of hydrated GST-beads (GE Healthcare, S4506) were added and the sample was rotated at 4°C for 90 minutes. The beads were centrifuged (2000 x g for 5 minutes at 4°C) and washed several times with a modified suspension buffer (containing Roche Complete protease inhibitors, Triton X-100 and DTT,). Following the final centrifugation, the GST bead pellet was either: 1) re-suspended in crude protein lysate from Thbs4 adenoviral infected NRVM; or 2) re-suspended in buffer with TNT proteins. GST pull-downs using the TNT T3 coupled reticulocyte lysates (Promega, L4950) were performed according to the manufacturer's recommendations using pCMV-Tag1 plasmids of Atf6α or Atf6β. After addition of the lysate or TNT protein, samples were rotated at 4 °C for 90 minutes after which time the GST beads were centrifuged and washed as described. Following the final wash, the beads were re-suspended in 5X SDS-PAGE loading buffer and boiled for 5 minutes. Samples were analyzed by western blotting.

Immunoprecipitation

The protocol for immunoprecipation was from the Abcam website (http://www.abcam.com/index.html?pageconfig=resource&rid=11385). Heart ventricles from 6-month old tTA and Thbs4 DTG mice were homogenized in RIPA buffer containing protease inhibitors (Roche, #11873580001). Protein samples were pre-cleared using HA-probe rabbit IgG antibody (Santa Cruz Biotechnology, Inc., sc-805). The IP was preformed with an N-terminal Atf6α rabbit primary antibody (Abcam, ab37149) and Protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology, Inc., sc-2003). Final elution volume was 100 μl of which 20 μl was used for immunoblot with Thbs4 antibody (R&D Systems, AF2390; 1μg/μl) or Atf6α antibody to the C-terminus (Pro-Sci Inc., 3681; 1:1000).

Microarray analysis

RNA was isolated from heart ventricles of 4-month old tTA and Thbs4 DTG mice using the Qiagen fibrous tissue kit. RNA was biotinylated by the Gene Expression Microarray Core at CCHMC and applied to Affymetrix GeneChip Mouse Gene 1.0 ST Arrays. Data was analyzed by the Division of Biomedical Informatics at Cincinnati Children's Hospital.

Electron microscopy

Hearts of anesthetized mice were perfused with 1% glutaraldehyde/2% paraformaldehyde in cardioplegic buffer. Hearts were later fixed in cacodylate buffer and processed as previously described (Fewell et al., 1997). Ultra-thin sections counterstained with uranium and lead salts were examined using a Hitachi 7600 transmission electron microscope at accelerating voltage 80 Kv. Images were acquired with an AMT digital camera.

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