ONLINE SUPPLEMENTARY INFORMATION

Increased Nox2 activity modulates cardiac calcium handling and contractility via phospholamban phosphorylation

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DETAILED MATERIALS AND METHODS

Animal procedures were performed in accordance with the Guidance on the Operation of the Animals (Scientific Procedures) Act, 1986 (UK Home Office), and the standards set forth by the University of Maryland, Baltimore Institutional Animal Care and Use Committee and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011).

Transgenic mice

The 1.8kb human *Nox2* cDNA was cloned downstream of the mouse myosin light chain-2 (MLC-2v) promoter (kind gift from Dr Jeffrey Robbins). The mini-transgene (approx 8.6kb) was excised by NotI digestion and used for microinjection into fertilized 1-cell CBA/C57BL/6 F1 oocytes. Offspring were screened with polymerase chain reaction to confirm successful transgene integration. Transgenic founders were backcrossed >10 generations onto a C57BL/6 background. Gender-matched wild-type littermates (WT) served as controls.

In vivo studies

Subpressor AngII (0.3 mg/kg/day, Sigma) or saline vehicle were infused via subcutaneous osmotic minipumps (Model 1002, Alzet, Cupertino, CA) for 2 weeks (1). Blood pressure was monitored using tail cuff plethysmography (Kent Scientific, XBP 1000; Torrington, CT) (2). In some studies, *Nox2*-null mice and WT controls were treated with pressor AngII (1.1mg/kg/day) or saline vehicle for 14 days. Pressure overload was imposed by suprarenal abdominal aortic banding for 3 weeks, as previously described (3). Sham constriction

involved identical surgery apart from band placement. There were no differences in mortality between mouse strains.

Echocardiography was performed under 2% isoflurane anesthesia at heart rates >400 bpm, using a Vevo2100 system with a 40 MHz linear probe (Visualsonics, Canada) (3). *In vivo* left ventricular (LV) function was determined by closed-chest pressure-volume (PV) analysis (3). Mice were anesthetized with 2% isoflurane/oxygen and the body temperature maintained at 37 °C. A 1.2F microconductance pressure catheter (model FT112, Scisense, Canada) was positioned in the LV via the right carotid artery. Live recordings of the signal throughout the experiment were obtained on an RPV-6 mouse PV system and analyses were conducted using Labscribe2 software (iWorx, USA). Cardiac function was recorded at baseline and for 20 min after administration of AngII (1.5mg/kg ip).

Cardiomyocyte isolation

LV cardiomyocytes were isolated from adult mice using collagenase digestion (4). Cardiomyocytes were stored in a normal Tyrode's solution containing (in mmol/L): NaCl 140, KCl 5, $CaCl_2$ 1.8, $MgCl_2$ 0.5, HEPES 5, Glucose 5, NaH_2PO_4 0.33. Experiments were performed at room temperature, 22°C.

Measurement of myocyte contraction and $[Ca^{2+}]_i$ transients

Experiments were performed in custom fabricated cell chambers mounted on an LSM 510 inverted confocal microscope with a 40x Oil 1.2 NA objective (Carl Zeiss, Jena, Germany). For measurements of contractility, sarcomere length was monitored with a high-speed video camera and Fourier transform analysis (Aurora Scientific, Ontario, Canada). The $[Ca^{2+}]_i$ transient was evaluated with Fluo-3 by 15 min incubation with 2 µmol/L Fluo-3-acetoxymethyl (AM) ester (Invitrogen) and 0.01% Pluronic® F127 (BASF, Florham Park,

N.J., USA). Cells were allowed an additional 10 minutes for de-esterification and then scanned using a 488 nm argon ion laser in confocal line-scan mode at 1.92 ms/line. Cells were paced to steady state using 1Hz field stimulation. After 20s of 1Hz stimulation, all cells showed steady state transients and contractions. At this point, either 5 Ca^{2+} transients or 5 contractions were assayed on alternating trials in the same cell. This protocol was repeated 2x in each myocyte, and data was pooled for analysis. In parallel studies, cells were imaged after 20 min of either Control or AngII treatment (1 μmol/L). For specific experiments AngII-treated cells were tested in the presence of N-acetyl cysteine (NAC, 10 mmol/L; Sigma-Aldrich St. Louis, MO) or pre-treated for 30 minutes (prior to adding AngII) with either the Nox2 inhibitory peptide gp91ds-tat (1 μmol/L; Anaspec, Fremont, Ca), or the PKA inhibitors H89 dihydrochloride (1 μmol/L; Tocris Biosciences, Bristol UK) or Rp-cAMPS (100 μmol/L; Enzo, Farmingdale, NY)

Voltage clamp studies

Cell membrane potential was controlled and measured by whole cell patch clamp using the EPC 10 amplifier by HEKA (Lambrecht/Pfalz, Germany). Micropipettes with a resistance of 1.5-2.2 MΩ were filled with pipette/internal solution. All measurements were done in cells perfused with normal Tyrode's solution. To measure Ca^{2+} transients, cells were loaded with 60 μmol/L Fluo-4 pentapotassium salt via the patch pipette (pipette solution in mmol/L; 110 CsOH, 110 aspartate, 5 Mg-ATP, 10 NaCl, 20 TEA-Cl, 10 HEPES and 60 μmol/L Fluo-4 pentapotassium salt). Depolarization and caffeine-induced Ca^{2+} transients were imaged using an inverted microscope (Axiovert 100M LSM-510; Zeiss) equipped with a water immersion objective lens (40X, 1.2 NA). All fluxes were normalized to the cell volume using cell capacitance (Cm), which was estimated by measuring the capacitance current induced by a 5 mV hyperpolarizing step from -80 mV to -85 mV.

SR load measurements

As Ca^{2+} removal from the cytosol is primarily achieved through extrusion via sodium/calcium exchange (NCX) and reuptake by SERCA, emptying the SR and preventing SERCA reuptake allows NCX to extrude all the Ca^{2+} stored in the SR. This then allows a precise measure of the SR Ca^{2+} load via NCX current (I_{NCX}). For these experiments, cells were voltage clamped and the SR was brought to steady state by depolarizing pulses to 0 mV for 10 s at 1 Hz. After the final pulse, cells were held at -80 mV and 10 mmol/L caffeine was applied for 10 seconds. The I_{NCX} elicited by caffeine application was integrated to measure the amount of Ca^{2+} removed, and this measurement was adjusted to account for other removal mechanisms according to the formula $((\int I_{NCX}/96{,}490C/mol)/(Cm/6.44pF/pL^{-1}\ cytosol)x10^{-12}x0.8)x10^{6}$ (5).

Assessment of SERCA function

To estimate SERCA function, we took advantage of the fact that the decay rate of the systolic Ca^{2+} transient is dependent on the activity of NCX and SERCA (with a minor contribution of plasma membrane Ca^{2+}-ATPase), while decay of the caffeine-induced transient depends mainly on NCX. The difference in the two rates can thus be ascribed to SERCA activity (6). Following steady state stimulation to produce systolic transients (sys), membrane potential was held at -80 mV and I_{NCX} current elicited by 10 mmol/L caffeine as described above. Systolic and caffeine transients were fitted with a single exponential $Y=a+e^{\kappa t}$, and the rate constant defined as $1/\kappa$. SERCA activity was estimated by subtracting the caffeine transient decay rate constant (κ_{caff}) from the systolic transient decay rate constant (κ_{sys}), ($\kappa_{sys} - \kappa_{caff}$).

Cultured cardiomyocyte studies

Primary cultures of neonatal rat cardiomyocytes (NRCM) were prepared as described (7). Cells were cultured for 48 hours and then transduced with adenoviral vectors expressing either β-Galactosidase as control (MOI 20), human Nox2 (MOI 20) or a short hairpin sequence targeted against Nox2 (MOI 5) (8) for an additional 24 hours. Cells were washed with PBS and starved overnight before treatment with AngII (100 nmol/L, 24 hours) in the presence or absence of PEG-SOD (50 U/ml, Sigma).

Real-time RT-PCR

An Applied Biosystems PRISM 7700 machine was used with SYBR Green and the comparative CT method. β-actin levels were used for normalization. Mouse oligonucleotide primers were (forward, reverse): procollagen 1 CCTCAGGGTATTGCTGGACAAC, TTGATCCAGAAGGACCTTGTTTG; procollagen 3 AGGAGCCAGTGGCCATAATG, TGACCATCTGATCCAGGGTTTC; fibronectin CCGGTGGCTGTCAGTCAGA, CCGTTCCCACTGCTGATTTATC; $p40^{phox}$ CTCGGCCTGGATCTGGTTG, ACATCGTCTGGAAGCTGCTC; iNOS TTCACTCCACGGAGTAGCCT, AGAGAAACTTCCAGGGGCAAG;
β-actin CGTGAAAAGATGACCCAGATCA, TGGTACGACCAGAGGCATACAG.

Western blotting

LV homogenates or cell lysates were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Antibodies used were: Nox2, eNOS and p-eNOS (S1177) (BD Transduction); Nox4 (3); $p22^{phox}$ (Santa Cruz); $p67^{phox}$, Rac1, SERCA2 (Abcam); $p47^{phox}$, total-phospholamban (PLN), phospho-PLN (S16) (Upstate); total-troponin I (TnI), phospho-TnI (Cell Signalling); RyR2 (Pierce Antibodies); phospho-RyR2 (S2808) (Badrilla);

phospho- and total-L-type calcium channel (LTCC, kindly provided by William Catterall); PP1α, PP1β, PP1γ, actin (Sigma). Protein levels were quantified by scanning densitometry. Actin was used as a loading control except for phospho-protein levels, which were normalized by the respective total-protein levels.

Cell staining

Adult cardiomyocytes were fixed, permeabilized, blocked, and incubated with a 1:100 dilution of primary antibodies to Titin-Z (rabbit polyclonal, courtesy of R. Bloch lab) and Nox2 (mouse monoclonal, BD Biosciences). Secondary antibodies were goat anti-rabbit AlexaFluor-647 and goat anti-mouse Atto-565 (Invitrogen) at 1:500 dilution. Images were acquired on an inverted confocal microscope (LSM 510 META, Carl Zeiss) using a 60x 1.2NA water immersion objective. Image processing and fluorescence intensity profiles were created in Image J.

Other assays

Cardiomyocyte cross-sectional area was measured from sections of paraffin-embedded hearts fixed in diastole and stained with FITC-conjugated wheat germ agglutinin (WGA, Vector RL-1022) (3). Interstitial fibrosis was assessed by blinded quantitative image analysis of Picrosirius red-stained sections imaged with polarized light. Apoptosis was assessed by TUNEL staining (Millipore S7110 kit). Myocardial NADPH oxidase activity was assessed by lucigenin-enhanced chemiluminescence (1). For protein phosphatase activity, heart extracts were prepared in Tris-HCl (100 mmol/L) containing protease inhibitor cocktail. 40 µl aliquots were assayed using a phosphorylated Thr peptide and a Malachite Green Assay kit (Millipore, #17-127) (9). PP1 activity was estimated by Calyculin A (20 nmol/L) inhibition, with recombinant PP1 (Sigma) used as a positive control.

Statistics

Data are shown as mean±SEM of at least 3 independent experiments. Comparisons were made by unpaired t-test, 1-way or 2-way ANOVA as appropriate, followed by Tukey post hoc analysis. $P<0.05$ was considered significant.

Reference List

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Supplementary Table 1. Basal characterization of *Nox2*-transgenic mice compared to WT littermates at age 3 months.

	WT (n=6)	TG (n=6)
Body weight (BW, g))	23.1 ± 1.1	23.6 ± 0.5
Atria (mg)	7.0 ± 0.6	6.1 ± 0.5
Right ventricle (mg)	19.1 ± 0.9	19.8 ± 0.5
Left ventricle (mg)	80.5 ± 3.2	86.1 ± 2.3
Heart weight (HW, mg)	106.6 ± 3.1	112.1 ± 2.8
HW/BW (mg/g)	4.64 ± 0.13	4.76 ± 0.08
Heart rate (beats/min)	459 ± 18	450 ± 13
IVSD (mm)	0.782 ± 0.012	0.788 ± 0.012
LVEDD (mm)	3.971 ± 0.072	4.085 ± 0.138
LVESD (mm)	2.861 ± 0.080	3.021 ± 0.099
ESV (µl)	30.6 ± 1.3	33.5 ± 3.2
EDV (µl)	62.0 ± 0.8	65.2 ± 4.7
EF (%)	50.7 ± 1.9	49.0 ± 1.6

Supplementary Table 2. Basal characterization of Nox2-transgenic mice compared to WT littermates at 1 year old.

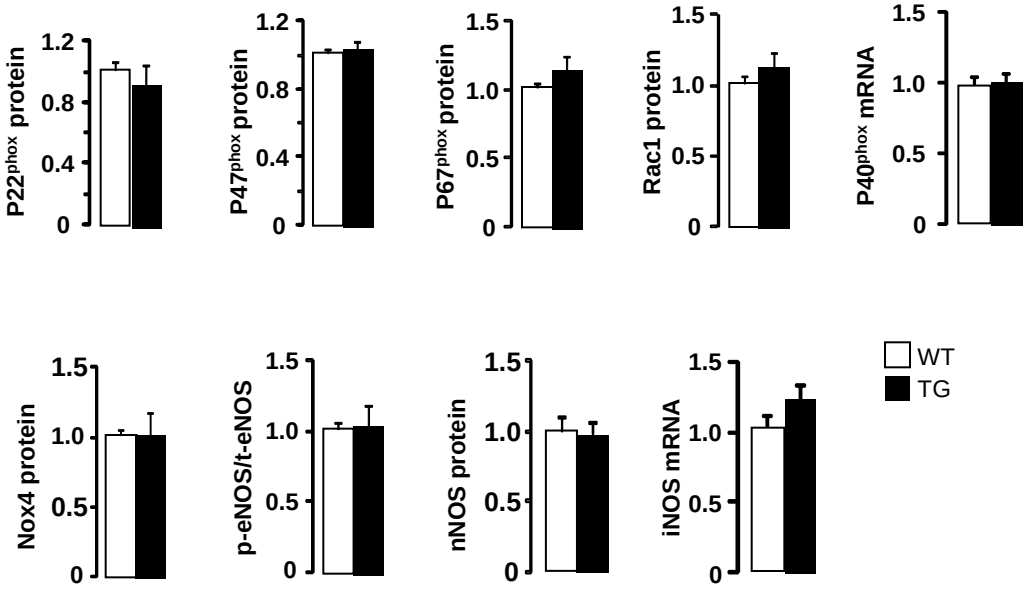
	WT (n=7)	TG (n=7)
Body weight (BW, g))	37.7±3.8	37.9 ± 1.5
Heart weight (HW, mg)	3.96 ± 0.41	3.96 ± 0.15
HW/BW (mg/g)	3.14 ± 0.33	3.14 ± 0.12
Heart rate (beats/min)	444 ± 17	453 ± 12
IVSD (mm)	0.788 ± 0.022	0.795 ± 0.011
ESV (μl)	39.6 ± 2.9	43.1 ± 1.5
EDV (μl)	76.6 ± 3.5	79.2 ± 2.7
EF (%)	48.4 ± 2.6	45.5 ± 0.5

Supplementary Table 3. Raw data of cardiomyocyte $[Ca^{2+}]_i$ and contractility

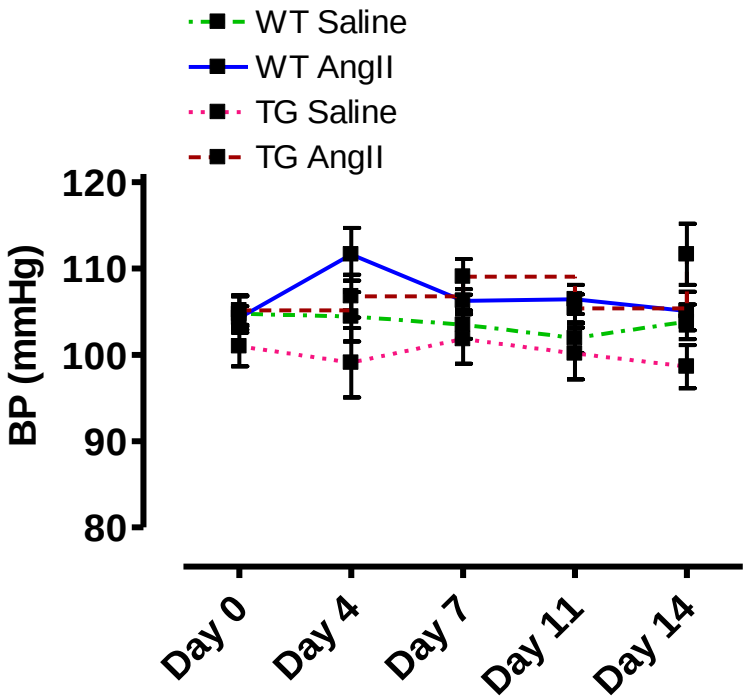
Single Cell Calcium Transients	**N**	**Rise Time 10-90% (ms)**	**Decay Time 10-50% (ms)**	**Decay Time 10-90% (ms)**	**CaT Peak (ΔF/ F_0)**
WT ctrl	15	18.7 ± 1.2	98.0 ± 3.6	399.2 ± 16.6	3.73 ± 0.21
WT +ATII	8	20.5 ± 2.7	111.0 ± 8.5	436.7 ± 29.7	3.78 ± 0.36
Nox2tg ctrl	23	19.0 ± 0.4	105.7 ± 3.0	421.5 ± 16.2	3.70 ± 0.21
Nox2tg+ATII	21	18.7 ± 1.1	**94.5 ± 3.3*, #**	**369.3 ± 14.1 *, #**	**4.56 ± 0.34***
Nox2tg+ATII+gp91ds	8	19.0 ± 2.7	106.4 ± 3.4	402.1 ± 14.6	3.28 ± 0.27
Nox2tg+ATII+NAC	12	26.3 ± 1.4	130.7 ± 2.2	464.3 ± 8.3	2.86 ± 0.21

Single Cell Contractility	**N**	**Rise 10-90% (ms)**	**Decay 10-50% (ms)**	**Decay 10-90% (ms)**	**Resting SL (μm)**	**ΔSL (μm)**	**ΔSL/ΔT (μm/s)**
WT ctrl	13	56.5 ±3.4	37.9 ± 3.8	175.7±17.6	1.82±0.01	0.12±0.01	2.1 ± 0.3
WT +ATII	10	62.3 ±5.0	40.0 ± 3.5	186.4±21.9	1.80±0.01	0.13±0.02	2.3 ± 0.3
Nox2tg ctrl	23	60.0 ±2.0	43.4 ± 3.4	180 ± 10.9	1.81±0.01	0.12±0.01	2.0 ± 0.2
Nox2tg+ATII	22	**53.8 ± 2.1***	**30.3 ± 1.4 **,##**	**136.6 ± 7.8**,##**	1.81 ± 0.01	**0.17 ± 0.01**,##**	**3.3 ± 0.2 **,##**
Nox2tg+ATII+gp91ds	8	65.5 ±3.3	46.7 ± 3.6	178.0±17.9	1.78±0.01	0.13±0.01	2.0 ± 0.2
Nox2tg+ATII+NAC	12	66.8 ±4.8	40.4 ± 3.0	155.4 ± 9.2	1.81±0.01	0.14±0.01	2.1 ± 0.1

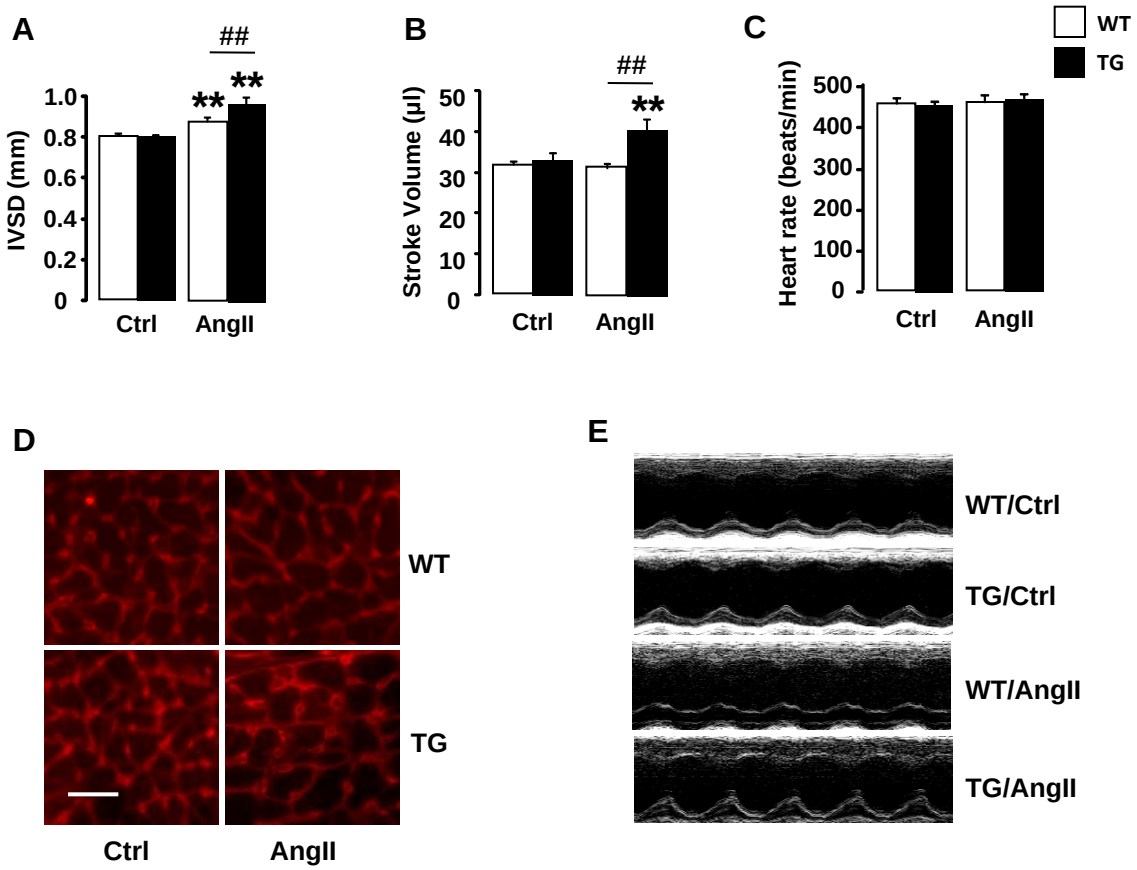
*** p<0.05 vs respective basal**
*** * p<0.01 vs respective basal**
 # p<0.05 vs WT/ATII,
p<0.01 vs WT/ATII



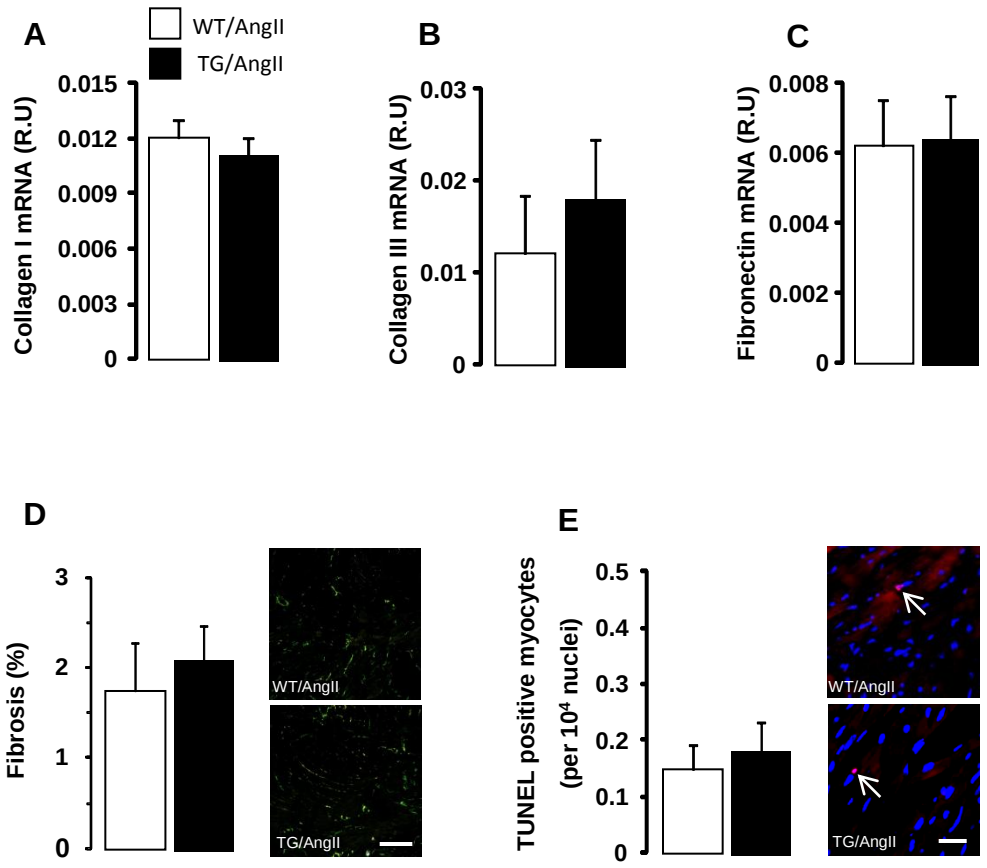
Supplementary Figure 1. Mean data of basal expression levels of Nox2 regulatory subunits, Nox4 and NOS isoforms in the hearts of Nox2-transgenic mice (TG) and wild-type littermates (WT). n=3-4.



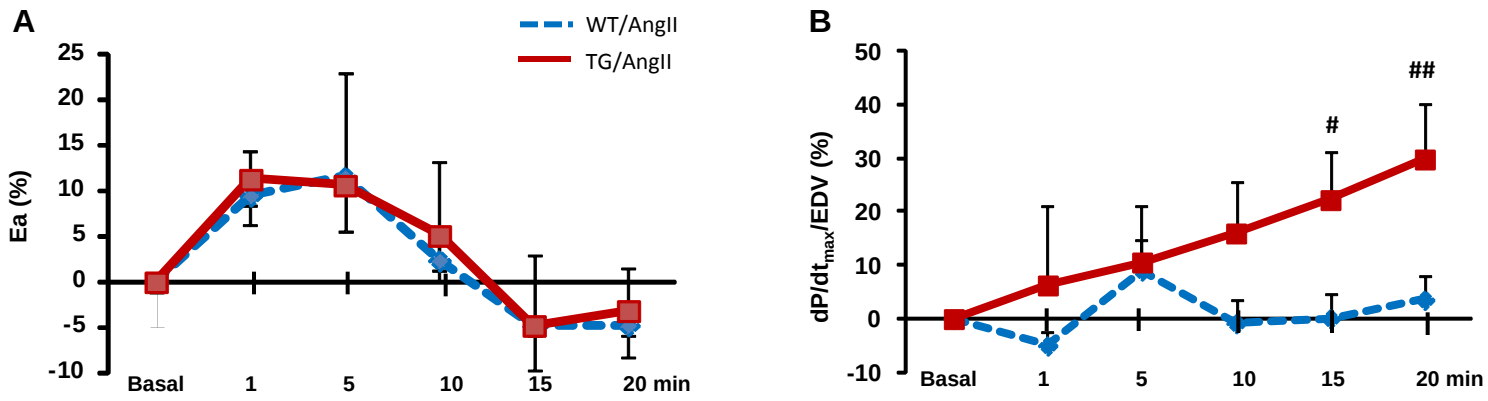
Supplementary Figure 2. Blood pressure measurements in TG and WT mice after AngII infusion (0.3 mg/kg/day) for 14 days. n=7.



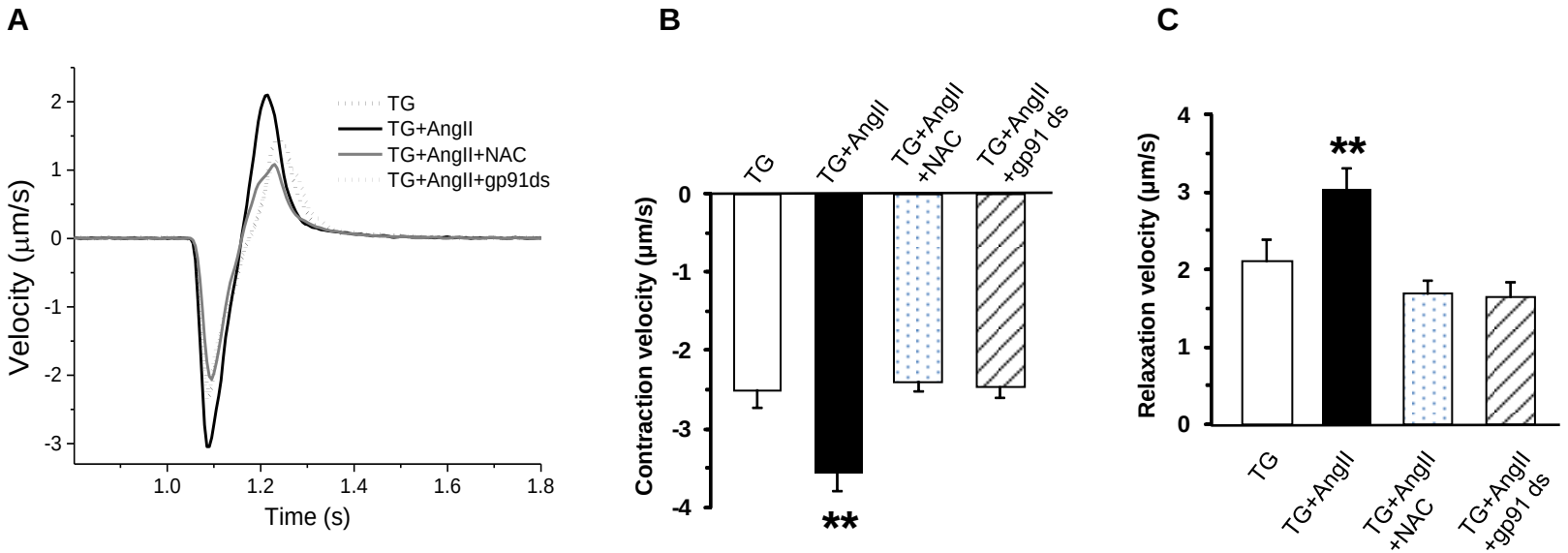
Supplementary Figure 3. Cardiac hypertrophy and *in vivo* cardiac function in TG and WT mice after AngII stimulation. A-C: Echocardiographic measurement of interventricular septal thickness in diastole, stroke volume and heart rate after 2 weeks AngII treatment (n=10). Ctrl = control. ** $p<0.01$ vs respective basal; ## $p<0.01$ vs WT/AngII. D: Representative WGA-stained LV sections. Scale bar: 20 µm. E: Representative M-mode traces of echocardiography.



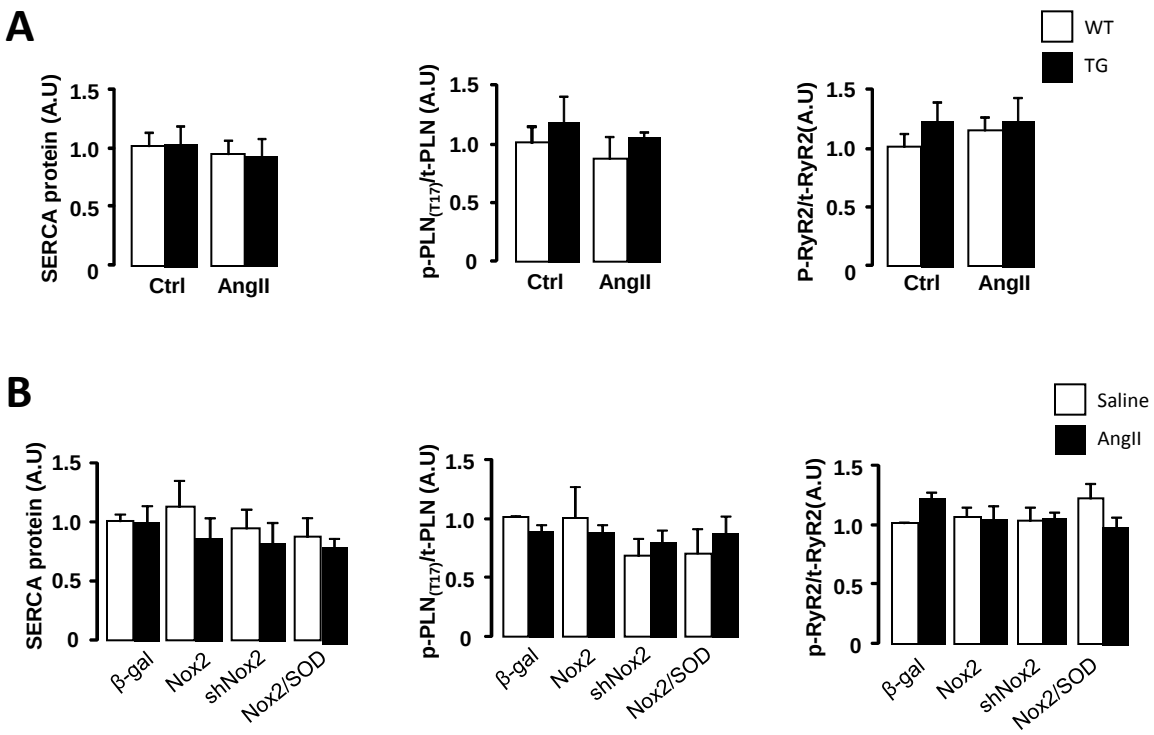
Supplementary Figure 4. Markers of interstitial fibrosis and myocyte apoptosis after 2 weeks of AngII infusion. (A-C) mRNA expression level of pro-fibrotic genes (n=5/group). (D) % interstitial fibrosis by Picrosirius Red staining (n=6 hearts/group). Representative images under polarised light on the right. Scale bar: 200µm. (E) TUNEL-positive cells in LV sections of TG and WT mice after AngII infusion (11-13 mice/group). Typical images on the right. Arrows indicate TUNEL positive cells. Scale bar: 25 µm.



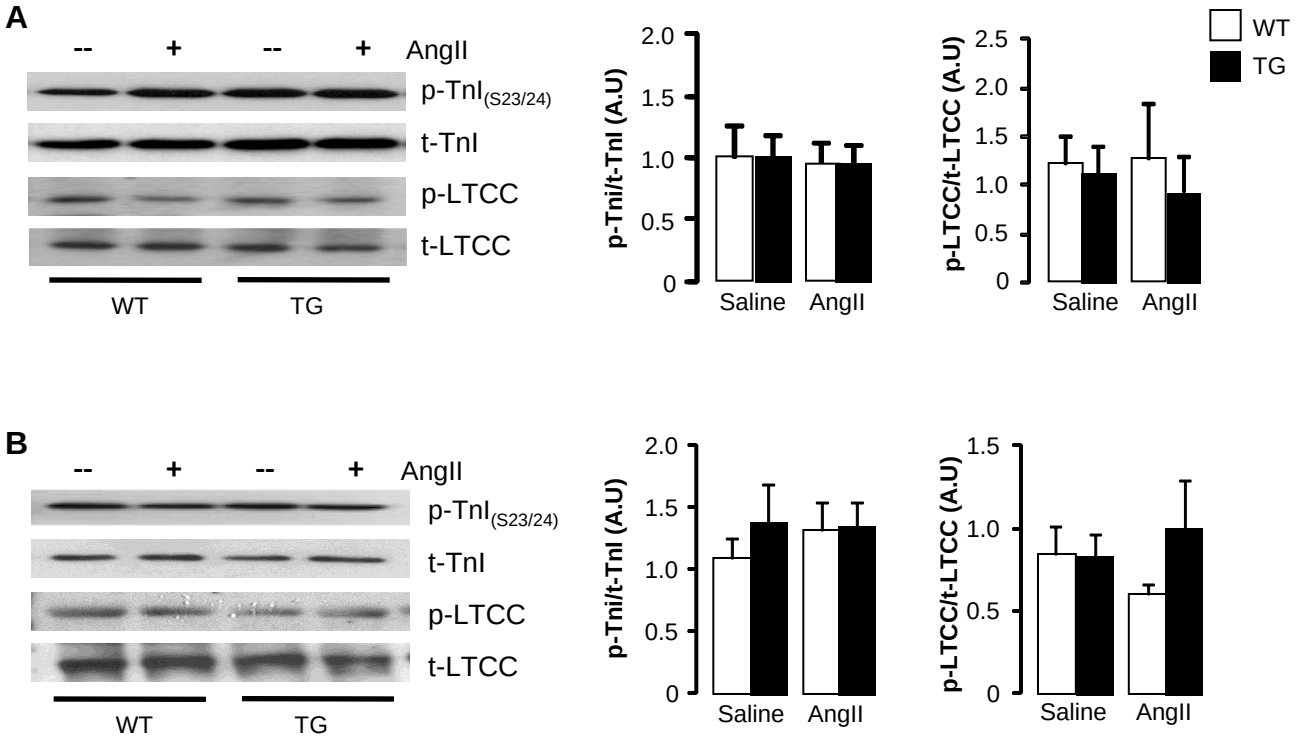
Supplementary Figure 5. Time-course of changes in (A) arterial elastance and (B) LV dP/dt_{max}/EDV after 1.5mg/kg i.p. AngII. #, $p<0.05$; ##, $p<0.01$ for WT vs. TG (n=9-11/group).



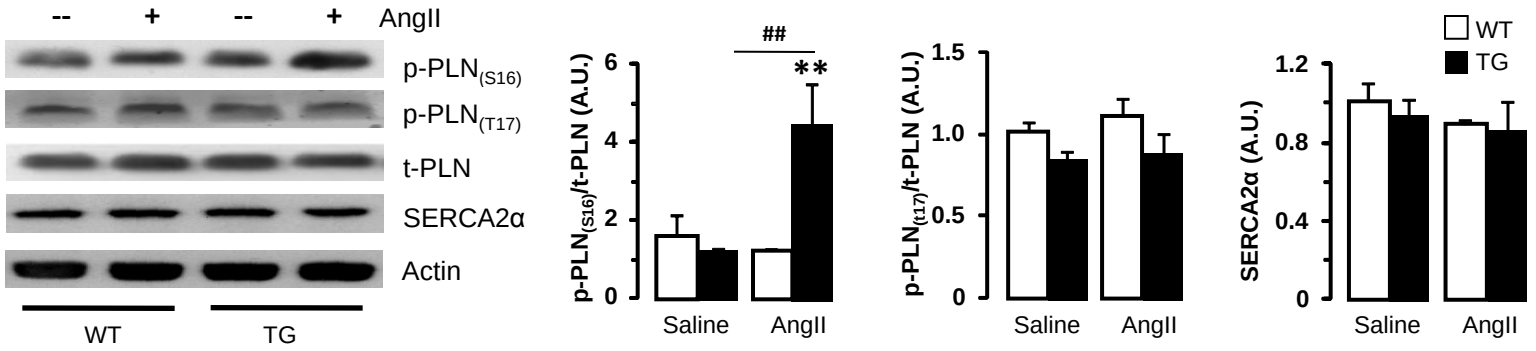
Supplementary Figure 6. Increased contractility of TG myocyte is Nox2-dependent. A: Representative raw data traces showing AngII-induced contraction velocity changes of TG cardiomyocytes with or without NAC or gp91 ds-tat. B, C: Average data show that the inotropic and lusitropic effects of AngII in TG cells are completely blocked by NAC or gp91 ds-tat. ** $p<0.01$ vs all other groups. n=8-12.



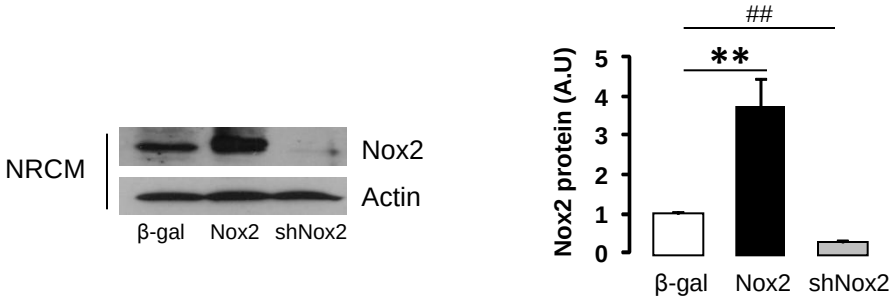
Supplementary Figure 7. A: Mean data of protein levels of SERCA2α, Thr^{17}-phosphorylated PLN/ total PLN and Ser^{2808}- phosphorylated RyR2/total-RyR2 in LV of TG and WT mice after acute AngII stimulation or saline control. B: Mean data of protein levels of SERCA2α, Thr^{17}-phosphorylated PLN/ total PLN and Ser^{2808}-phosphorylated RyR2/total-RyR2 in NRCM with Nox2 overexpression (Nox2), Nox2 knockdown (shNox2), control (β-gal) and ROS inhibitor peg-SOD. n=4.



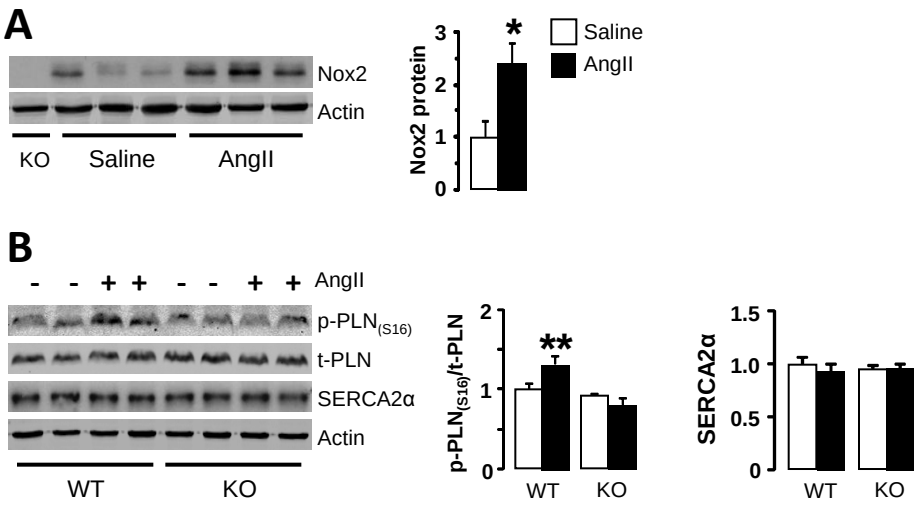
Supplementary Figure 8. Protein levels of total and phosphorylated troponin I (TnI) and L-type calcium channel (LTCC) in LV after acute (A) or chronic (B) AngII stimulation or saline control. n=3-4.



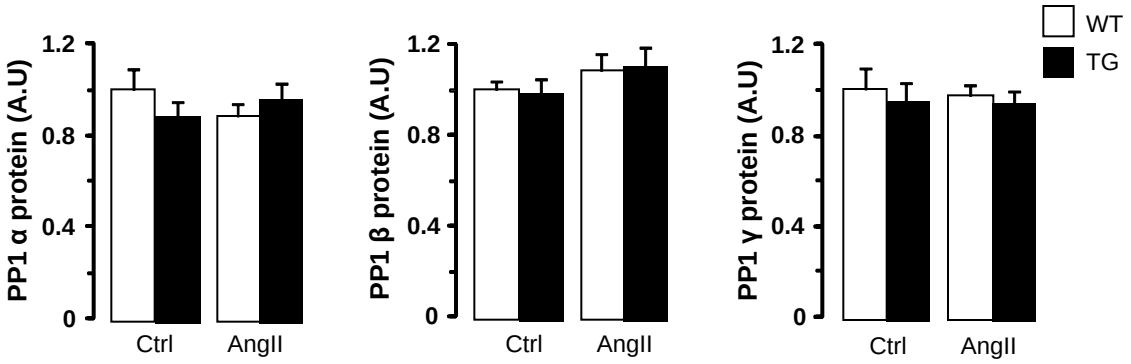
Supplementary Figure 9. Western blots for Ser[16]- and Thr[17]-phosphorylated phospholamban (PLN), total PLN and SERCA2α in LV of TG and WT mice after 2 weeks AngII stimulation or saline control. Mean data shown to the right. ** p<0.01, vs TG/Saline; ## p<0.01, vs WT/AngII. n=5-7.



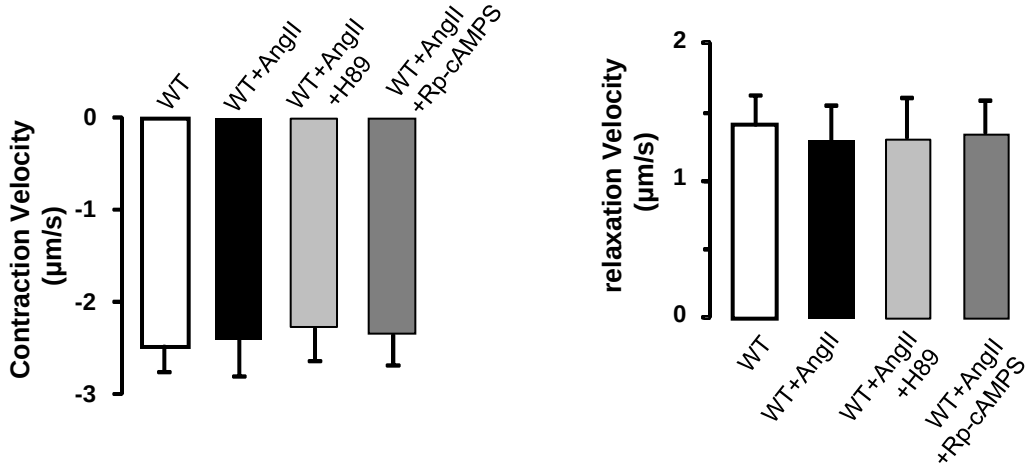
Supplementary Figure 10. Western blots showing Nox2 expression in cultured neonatal rat cardiomyocytes (NRCM) transduced with adenoviral vectors expressing either β-Galactosidase (β-gal) as control, Nox2 or a short hairpin sequence targeted against Nox2 (shNox2). Mean data to the right. ** p<0.01, ## p<0.01 vs β-gal. n=5.



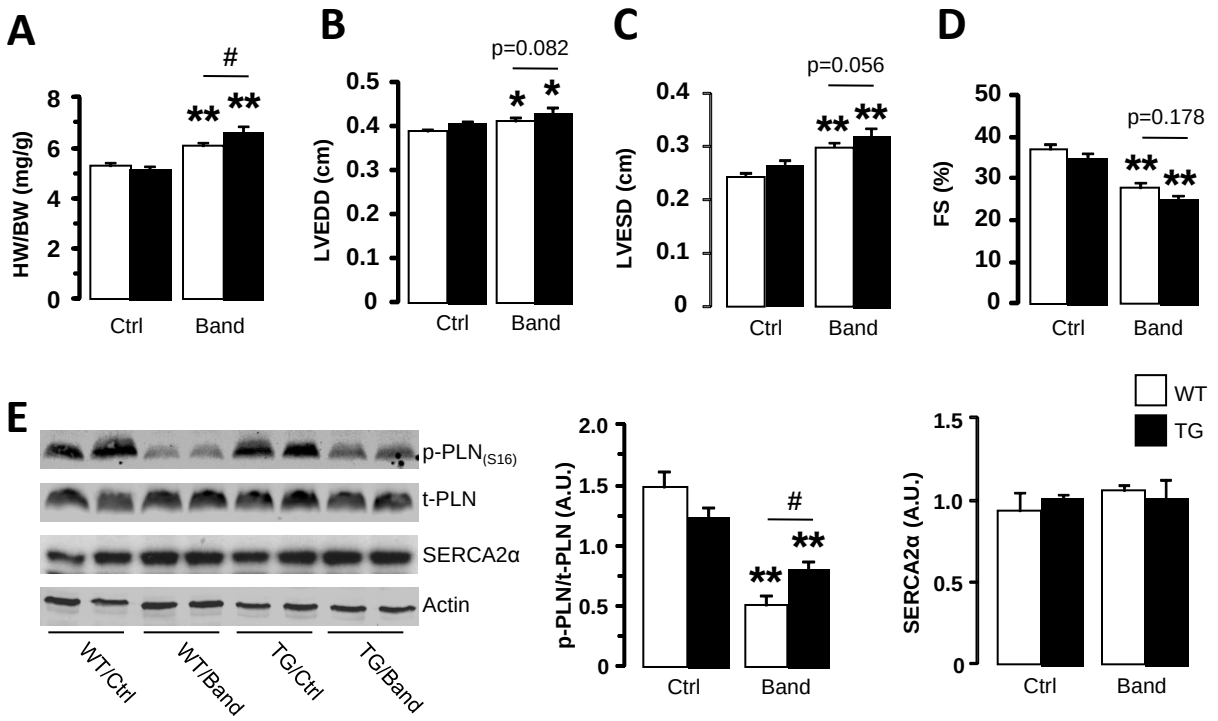
Supplementary Figure 11. Phospholamban phosphorylation in WT and *Nox2*-null (KO) mice after chronic AngII treatment (1.1mg/kg/day, 2 weeks) or saline control. A. LV protein levels of Nox2 after AngII treatment. * $p<0.05$, n=3/group. B. Western blots for Ser^{16}-phosphorylated phospholamban (PLN), total PLN and SERCA2α in LV of WT and KO mice. ** $p<0.01$ vs all other groups. n=4/group.



Supplementary Figure 12. Mean data of protein levels of PP1 isoforms in LV of TG and WT mice after AngII treatment. n=3-4.



Supplementary Figure 13. The effects of PKA inhibitors H89 or Rp-cAMPS on cell contraction and relaxation of WT cardiomyocytes. n=10.



Supplementary Figure 14. Effect of chronic pressure overload in TG mice. A: Cardiac hypertrophy assessed by heart/body weight ratio (HW/BW; n=10/group) B-D: Echocardiographic measurements of cardiac function (n=10/group); LVEDD, LV end-diastolic diameter; LVESD, LV end-systolic diameter; FS, fractional shortening. E: Ser^{16}-phosphorylated PLN, total PLN and SERCA2α in LV of TG and WT mice after 3 weeks banding or sham surgery (n=4/group). Representative blots shown to the left and mean data to the right. * $p<0.05$, ** $p<0.01$ vs respective controls; # $p<0.05$ vs WT/Band.