SUPPLEMENTAL MATERIAL

Online Materials and Methods Supplement

Transgenic mice

cDNAs encoding rat NKA-α1 and NKA-α2 (gift from Dr. Jerry Lingrel, University of Cincinnati) were cloned into the murine α-myosin heavy chain (MHC) promoter expression vector and used to inject newly-fertilized oocytes to generate transgenic mice (FVB/N background was used for all mice). We observed no baseline phenotypes in these mice by approximately four months of age. NFAT-luciferase mice were previously described [1]. Experiments involving animals were approved by the Institutional Animal Care and Use Committee of Cincinnati Children's Hospital.

Echocardiography and pressure overload induction

Mice were anesthetized with 2% isofluorane by inhalation. Echocardiography was performed in M-mode using a Hewlett Packard SONOS 5500 instrument with a 15 MHz transducer. For pressure overload induction, mouse littermates aged 8-11 weeks were subjected to transverse aortic constriction (TAC) or a sham surgical procedure, as previously described [1]. Doppler echocardiography was performed on mice subjected to TAC in order to determine pressure gradients across the aortic constriction. In Figure 1D, 12 out of 26 Wt mice survived 10 weeks of TAC (46% survival) and 14 out of 19 NKA-α2 mice survived 10 weeks of TAC (74% survival). In Figure 2E, 7 out of 8 Wt mice survived 12 weeks of TAC (88% survival) and 9 out of 14 NKA-α1 mice survived 12 weeks of TAC (64% survival).

Western blotting, immunoprecipitation and mRNA analysis

Immunoprecipitation and Western blotting of mouse heart homogenates was performed using antibodies against NKA-α1 (Millipore and Developmental Studies Hybridoma Bank), NKA-α2 (Millipore), total NKA (a5-s, Developmental Studies Hybridoma Bank), p-PLN S16 (Badrilla), p-PLN T17 (Badrilla), PLN (Pierce), p-RyR2 S2808 (Badrilla), p-RyR2 S2814 (Badrilla), RyR2 (Santa Cruz), $Ca_V1.2$ (Alomone), NCX1 (Swant Inc.), SERCA2 (Badrilla), p-PLM S63 (Abgent), p-PLM S68 (Abgent), PLM (Abcam), β-tubulin (Santa Cruz), GAPDH (Research Diagnostics Inc.), calcineurin B (Sigma-Aldrich) and calmodulin (Zymed Laboratories). Secondary antibodies for chemifluorescent detection were from Santa Cruz. Chemifluorescent detection was performed with the Vistra ECF reagent (Amersham Pharmacia Biotech). Secondary antibodies for fluorescent detection were from LI-COR and immunoblots were visualized using the Odyssey CLx imaging system (LI-COR). RNA was extracted from ventricles using the RNeasy Kit according to manufacturer's instructions (Qiagen). Reverse transcription was performed using the SuperScript III First-Strand Synthesis System (Invitrogen). Analysis of the hypertrophic markers was performed using individual Taqman gene expression assays (Applied Biosystems). Messenger RNA expression was quantified, normalized to GAPDH, and expressed relative to control.

Isolation of adult cardiomyocytes and Ca^{2+} measurements

Adult mouse ventricular cardiomyocytes were isolated as previously described [2]. Cardiac myocytes were loaded with 2 μM Fura-2 acetoxymethyl ester (Invitrogen) for 15 min in Tyrode solution containing: 131 mM NaCl, 4 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES (pH 7.4) . The Fura-2 fluorescence ratio was determined using a Delta scan dual-beam spectrofluorophotometer (Photon Technology International) operated at an emission wavelength of 510 nM and excitation wavelengths of 340 and 380 nM. The stimulation frequency for Ca^{2+} transient measurements was 0.5 Hz. For caffeine-induced Ca^{2+} release, cells were perfused with a control solution and stimulated at 0.5 Hz until stabilization of the transients. The electrical stimulation was interrupted and the cells were perfused for 1 min with either control solution or a solution containing 10 mM $NiCl_2$ to block NCX activity. 10 mM caffeine (dissolved in Tyrode solution) was added to induce Ca^{2+} store depletion. Baseline Ca^{2+} levels, transient amplitude, caffeine-induced Ca^{2+} release (estimated by the 340 nM/380 nM ratio), and Ca^{2+} decay kinetics were analysed using Felix 1.1 and Ion Wizard (IonOptix) software.

$[Na^+]_i$ measurements

Isolated myocytes were plated on laminin-coated coverslips and loaded with 10 μM SBFI-AM for 90-120 min (Invitrogen) as previously described [3]. SBFI was allowed to de-esterify for 20 min in Tyrode solution containing:

140 mM NaCl, 4 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 5 mM HEPES (pH 7.4). SBFI was alternately excited at 340 and 380 nm (F_{340} and F_{380}) using an Optoscan monochromator (Cairn Research, Faversham, UK) and fluorescence was collected at 535±20 nm. F_{340}/F_{380} was calculated after two background subtraction and converted to $[Na^+]_i$ by calibration at the end of each experiment in the presence of 10 μM gramicidin and 100 μM strophanthidin.

Na^+/K^+ ATPase activity measurements in adult rat cardiac myocytes

Adult rat cardiac myocytes were isolated and plated onto 22mm circular coverslips and infected with either NKA-α1, NKA-α2, or β-galactosidase-expressing adenovirus at 5 MOI. 24-34 hours post-infection, cardiac myocytes were loaded with 10 μM SBFI (Invitrogen) with 0.05% Pluronic F-127 (Sigma) in DMEM at room temperature for 45 minutes followed by two washes and 15 minutes of de-esterification in Tyrode solution containing: 140 mM NaCl, 4 mM KCl, 1 mM $CaCl_2$, 10 mM glucose, and 10 mM Hepes. Photometric measurements were performed on a Nikon Eclipse Ti-U inverted microscope. Cells were alternatively excited at 340nm and 380nm using a Delta Scan dual-beam spectrofluorophotometer (Photon Technology, Birmingham, NJ) at a rate of 1 Hz to avoid photo bleaching. Fluorescent emission was recorded by a CoolSnap ES2 camera connected to a computer running the EZ Ratio Pro software by PTI. An *in situ* NKA assay was performed as previously described [4]. After a short baseline recording, cells were perfused with Na^+ loading buffer that lacks K^+, containing: 145 mM NaCl, 2 mM EGTA, 10 mM HEPES, and 10 mM glucose, which completely inhibits NKA and forces $[Na^+]_i$ to rise. After 30 mintues Na^+ levels reached a steady maximal value and NKA was activated by perfusion of a solution containing 140 mM TEA, 4 mM KCl, 2 mM EGTA, 10 mM HEPES, and 10 mM glucose. Once Na^+ returned to a stable minimum level, a calibration was performed as previously described [4]. Solutions containing 0, 10, 20, 30, and 40 mM Na^+ were derived from a combination of solutions made up of: 30 mM NaCl, 115 sodium gluconate, and a solution containing 30 mM KCl and 115 mM potassium gluconate. Calibration buffers also contained 10 μM gramicidin D, 100 μM strophanthidin, 10 mM glucose, and 2 mM EGTA. Regions of interest were drawn inside the boundaries of individual cardiac myocytes and background fluorescence was subtracted from each image before calculating the ratio of 340/380 nm. Ratios were converted to Na^+ concentration by fitting a linear equation derived from the calibration experiments. A velocity of Na^+ efflux was then determined for each concentration of Na^+.

NKA and NFAT activity assay

Na^+/K^+ ATPase activity was determined using an enzyme-linked assay measuring the rate of ADP production as linked to the rate of NADH fluorescence decrease in the absence or presence of 10 mM strophanthidin (Sigma Aldrich), as previously described [5]. Briefly, 2 μg of crude protein extract from heart homogenates was incubated for 20 min at 37°C in solution containing: 100 mM NaCl, 20 mM KCl, 8 mM $MgCl_2$, 40 mM Tris (pH 7.4), 1 mM EGTA, 25 mM choline chloride, 1 U/ml lactate dehydrogenase, 1 U/ml pyruvate kinase, 1 mM phosphoenolpyruvate, 80 μM NADH for a total reaction volume of 2.5 mL. The reaction was initiated with the addition of 1 mM ATP. NADH fluorescence was continuously monitored (excitation 340 nm, emission 460 nm) for 20 min using a spectrofluorimeter (PTI Delta Scan-1, Photon Technology International). Rates were calculated as μmoles ATP/minute/mg protein. NFAT activity assays were performed on heart homogenates as described previously [1].

CaMKII activity assay

CaMKII activity was determined using the SignaTect calcium/calmodulin-dependent protein kinase assay system (Promega) according to the manufacturer's instructions. For the assay, homogenates were made from WT or NKA-α2 hearts at baseline or after two weeks of TAC surgery using the following buffer: 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM dithothreitol, 20% glycerol, 0.1% Triton X-100, 1x protease inhibitor cocktail (Calbiochem); protein concentration was determined by Bradford assay and CaMKII activity was determined from 2 μg of protein.

Histology and immunohistochemistry

Analysis of cross-sectional areas was performed on histological sections from paraffin-embedded heart tissue. TRITC-conjugated wheat germ agglutinin (Sigma-Aldrich) was used to identify sarcolemmal membranes as described previously [6]. Isolated cardiomyocytes plated on laminin and fixed with 4% paraformaldehyde were treated with NKA-α2 (Millipore) or NKA-α1 antibody (Developmental Studies Hybridoma Bank) and FITC-conjugated antibody (Invitrogen) to visualize protein localization.

Statistics
Results are presented in all cases as mean ± SEM. Statistical analysis was performed using Prism 5 (Graphpad Software) for unpaired two-tailed t-tests. P-values less than 0.05 were considered significant.

References

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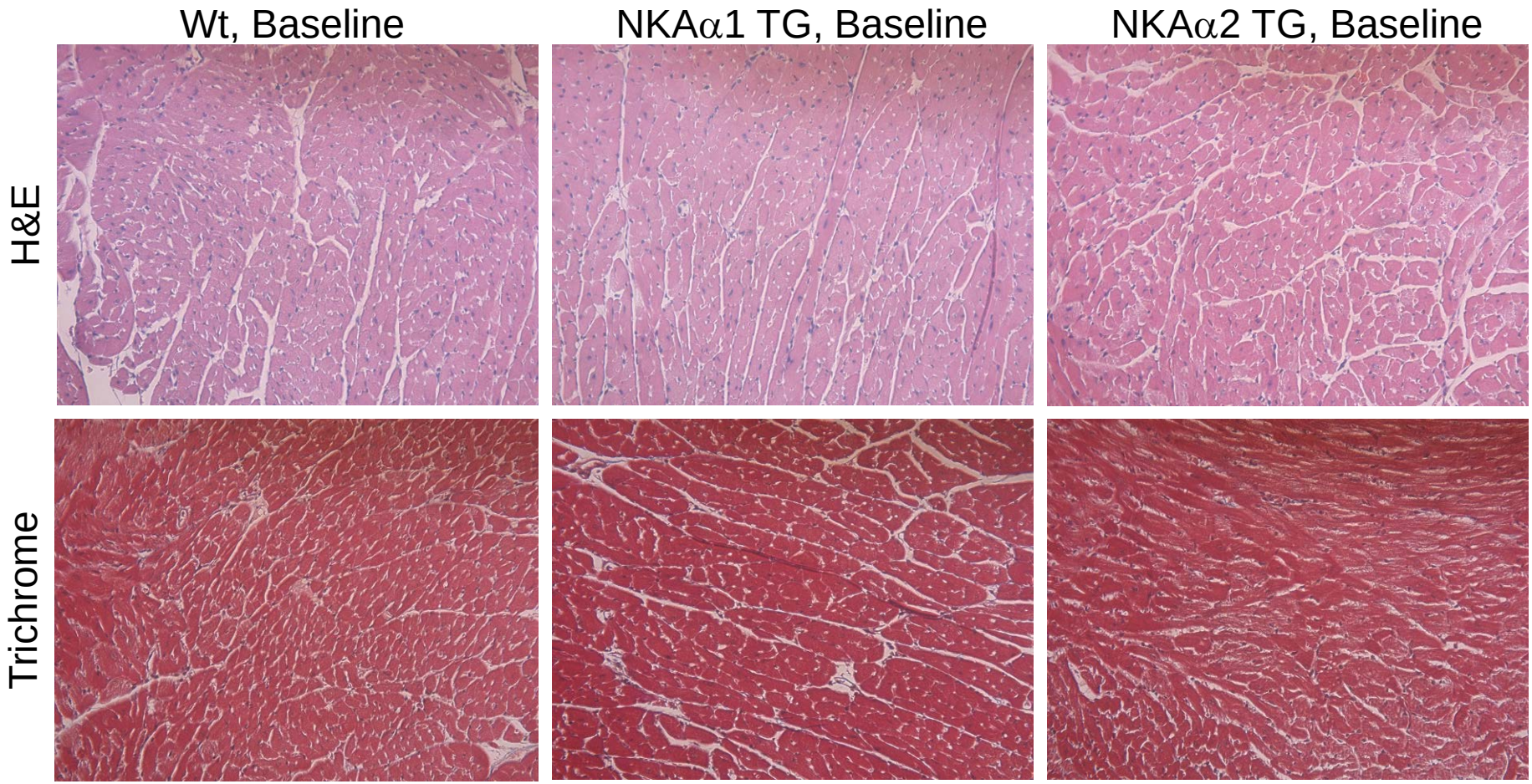
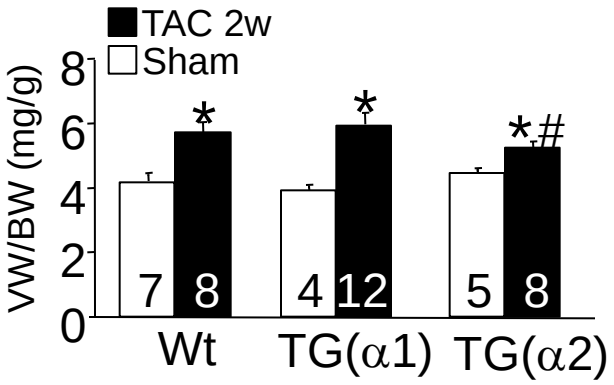
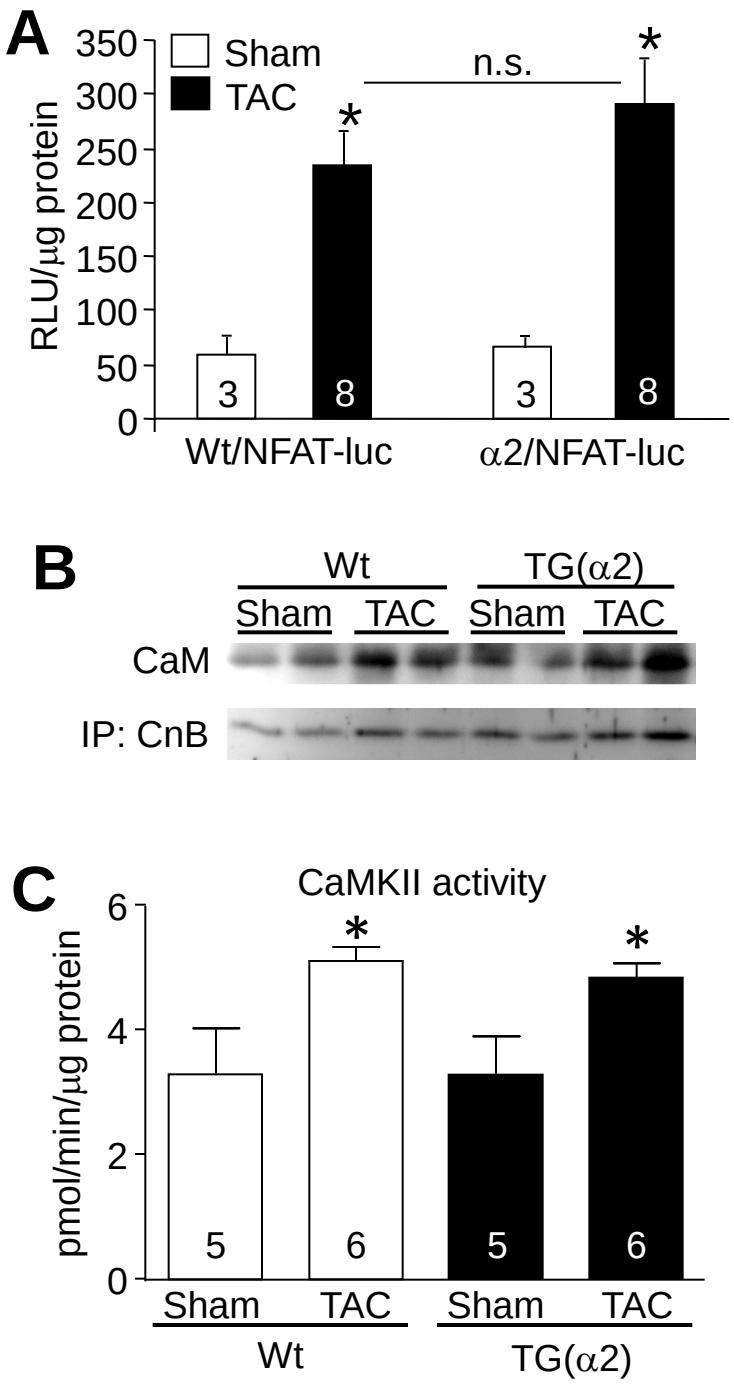
6. Xu J, Gong NL, Bodi I, Aronow BJ, Backx PH, Molkentin JD. Myocyte enhancer factors 2a and 2c induce dilated cardiomyopathy in transgenic mice. *J Biol Chem*. 2006;281:9152-9162



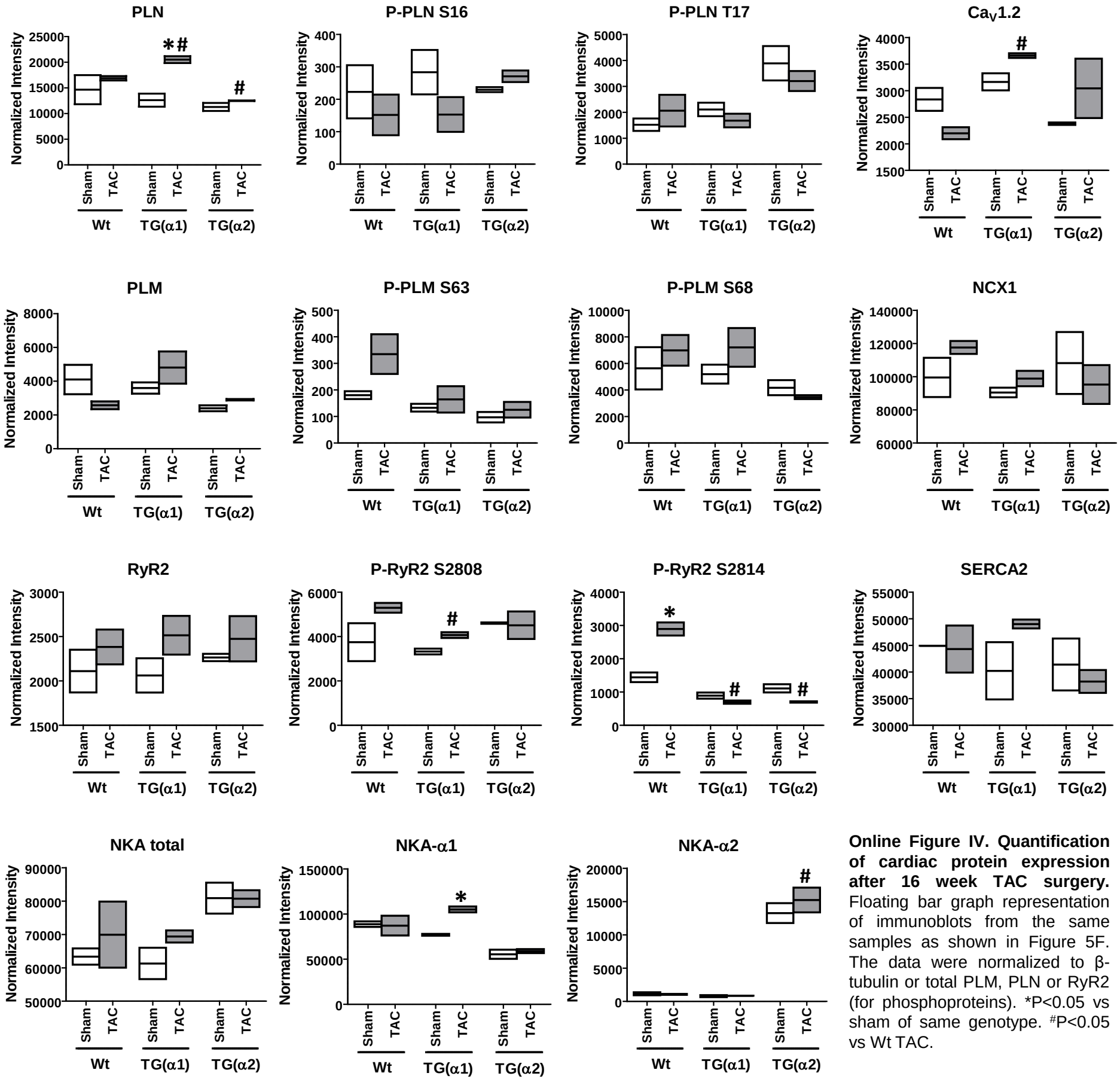
Online Figure I. Cardiac histological staining from baseline control mice. Representative H&E and Masson's trichrome-stained histological sections from hearts of Wt, high-line NKA-α1 transgenic or NKA-α2 transgenic mice at 24 weeks of age to show the state of hearts from these transgenic lines, which was normal. Cardiac histology from TAC-operated mice is found in Figure 3C and showed no differences in the degree of fibrosis induction between the transgenic lines and the Wt.



Online Figure II. NKA-α2 but not NKA-α1 transgenic mice show less cardiac hypertrophy after pressure overload for 2 weeks. Comparison of ventricle weight to body weight ratios measured from Wt, NKA-α2 and high-line NKA-α1 transgenic mice 2 weeks after TAC or a sham surgery. For each experiment, number of mice analysed is given within the graph. *P<0.05 versus sham; #P<0.05 vs Wt TAC.



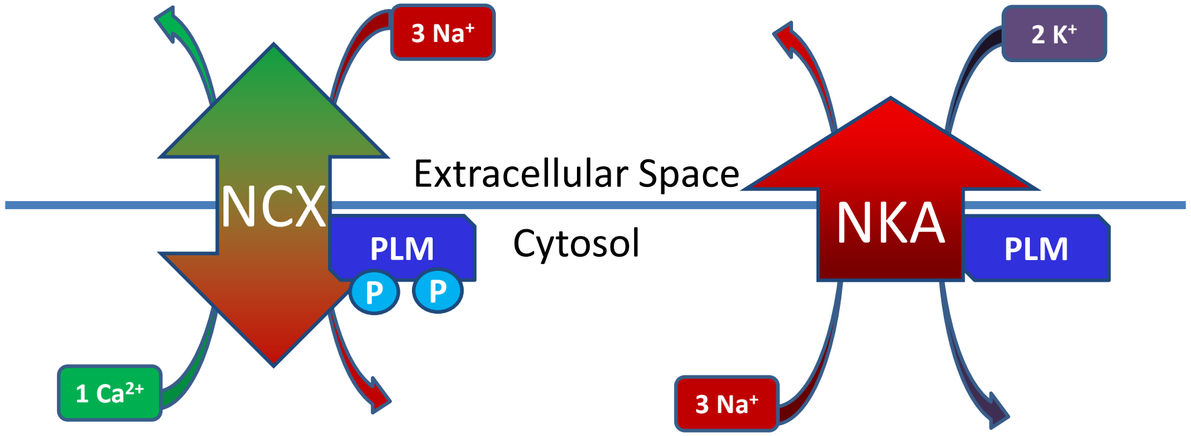
Online Figure III. NKAα2-mediated cardioprotection after pressure overload does not involve calcineurin/NFAT or CaMKII signalling. A, NFAT luciferase activity measured from NFAT-luciferase transgenic (control) and double NKAα2/NFAT-luciferase transgenic hearts after 2 weeks of TAC or a sham surgery. **B**, Immunoblots of calcineurin B and calmodulin (CaM) protein from calcineurin B immunoprecipitation fractions from Wt and NKAα2 heart homogenates after 2 weeks of TAC or a sham surgery. For each experiment, number of mice analysed is given within the graph. **C**, CaMKII activity measured from Wt and NKAα2 transgenic heart homogenates at baseline or after 2 weeks of TAC surgery. *P<0.05 versus sham. No significant differences observed between TAC treatments.



Online Figure IV. Quantification of cardiac protein expression after 16 week TAC surgery. Floating bar graph representation of immunoblots from the same samples as shown in Figure 5F. The data were normalized to β-tubulin or total PLM, PLN or RyR2 (for phosphoproteins). *P<0.05 vs sham of same genotype. #P<0.05 vs Wt TAC.

Online Figure V.

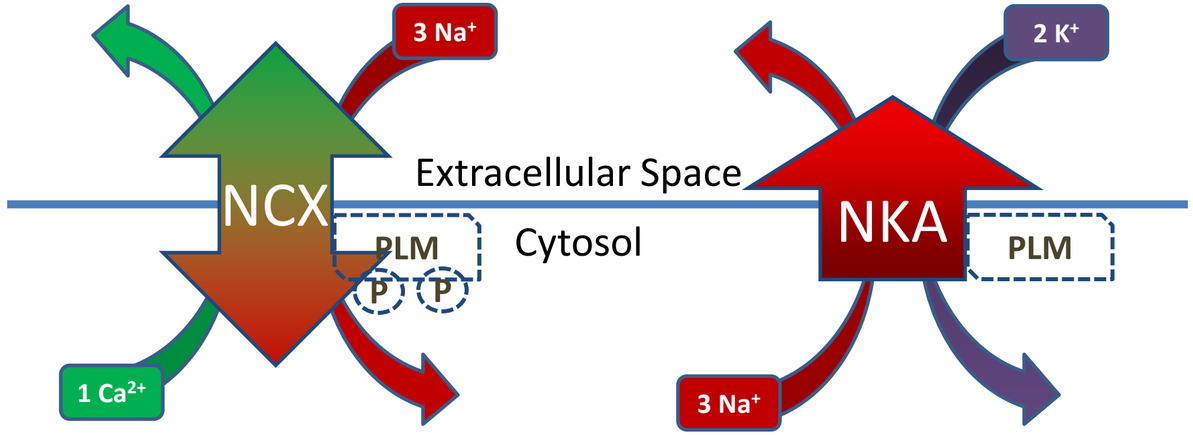
WT and NKA-α1 overexpression



Standard Ca^{2+} and Na^{+} Removal

Normal Hypertrophic Response

NKA-α2 overexpression



Enhanced Ca^{2+} and Na^{+} Removal

Resistant to Hypertropic Stimuli