Supplemental Material

Hyperactive Adverse Mechanical-Stress Responses in Dystrophic Heart Are Blocked by cGMP-PKG Modulation of TRPC6

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Supplemental Methods

Cardiomyocyte Isolation

Hearts were quickly removed from the chest after euthanasia. Retrograde perfusion is performed with Ca^{2+}-free HEPES buffer containing (in mmol/L) 140 NaCl, 5.4 KCl, 0.33 NaH_2PO_4, 0.5 $MgCl_2$, 11 glucose, 5 HEPES (pH7.4) at 1.0 ml/min for 3 min, followed by an enzyme solution containing collagenase (1.0 mg/ml collagenase type II, Worthington), protease (0.05 mg/ml, type XIV, Sigma), and 0.1 mmol/L Ca^{2+} for 7 min. The ventricular tissue was then cut into small pieces and filtered with 250-μm nylon mesh. The calcium concentration of Tyrode solution was gradually increased to 1.0 mmol/L for physiologic analysis.

Echocardiography

In vivo cardiac geometry and function were serially assessed by transthoracic echocardiography (Acuson Sequoia C256, 13 MHz transducer; Siemens) in conscious mice. M-mode LV end-systolic and end-diastolic cross-sectional diameter (LVESD, LVEDD), and the mean of septal and posterior wall thicknesses were determined from an average of 3–5 cardiac cycles. LV fractional shortening (%FS) and LV mass were determined using a cylindrical model as previously described[1].

Polymerase Chain Reaction (PCR)

Quantitative PCR was used to assess fetal and hypertrophic gene expression and TRPC1, 3, or 6 mRNA expression in cells and myocardial tissue. RNA was extracted with TRIzol (Invitrogen) and cDNA synthesized by Taqman Reverse Transcription Reagents and protocol (Applied Biosystems). Quantitative real-time PCR was performed with the 7900HT (Applied Biosystems) or the CFX384 PCR detection system (Bio-Rad) with sample duplicates. Taqman primers and probes for rat and mouse were obtained from Applied Biosystems. Gene expression was calculated using the $\Delta\Delta$Ct method, and normalized to GAPDH or 18s rRNA expression.

PDE5A, PDE1 and PKG activity

PDE5 and PDE1 activity was assessed by fluorescence polarization assay (Molecular Devices), following manufacturer's instruction and as previously described [1]. Briefly, heart tissue was homogenized in lysis buffer (Cell Signaling Technologies) and sonicated. Protein concentration was determined used the bichinchoninic acid (BCA) protein assay (Pierce). Complete reaction buffer containing 0.1 % BSA and 1 mmol/L DTT and cGMP substrate solution (100 nmol/L final concentration) were freshly prepared according to manufacturer's instruction and added to a 384 well plate (sample input ~3 μg total protein). Lung tissue lysates were used as positive control and to generate a standard curve. Sildenafil was added at 1μmol/L final concentration and samples incubated for 1h at 22 °C in the dark. IMAP binding buffer was prepared according to the manufacturer, added to the wells and incubated for another 60-120 minutes. Fluorescence polarization was measured at 485nm excitation and 525 emission on a SpectraMax M5 microplate reader (Molecular Devices). For determination of PDE1 activity Ca^{2+} (50 μmol/L) and Calmodulin (5U) were added and the PDE1 inhibitor PF4822163 (Pfizer, 1μmol/L) was used. PKG activity was assessed

by EIA colorimetric assay (CycLex). Briefly, kinase reaction buffer containing 2.5 mmol/L ATP and 500 μmol/L cGMP was prepared according to the manufacturer and added to protein lysates prepared from myocardial tissue in a 96 well reaction plate and incubated at 30°C for 20 minutes. After washing HRP conjugated anti-phospho specific antibody was added to the wells for 60 minutes at 22°C. After washing substrate reagent was added to the wells for 5-15 minutes and finally the reaction was halted using stop solution and absorbance at 450/550 nm was measured using the SpectraMax M5 microplate reader (Molecular Devices). cGK positive control was obtained from CycLex and used per manufacturer instructions.

Immunoblot

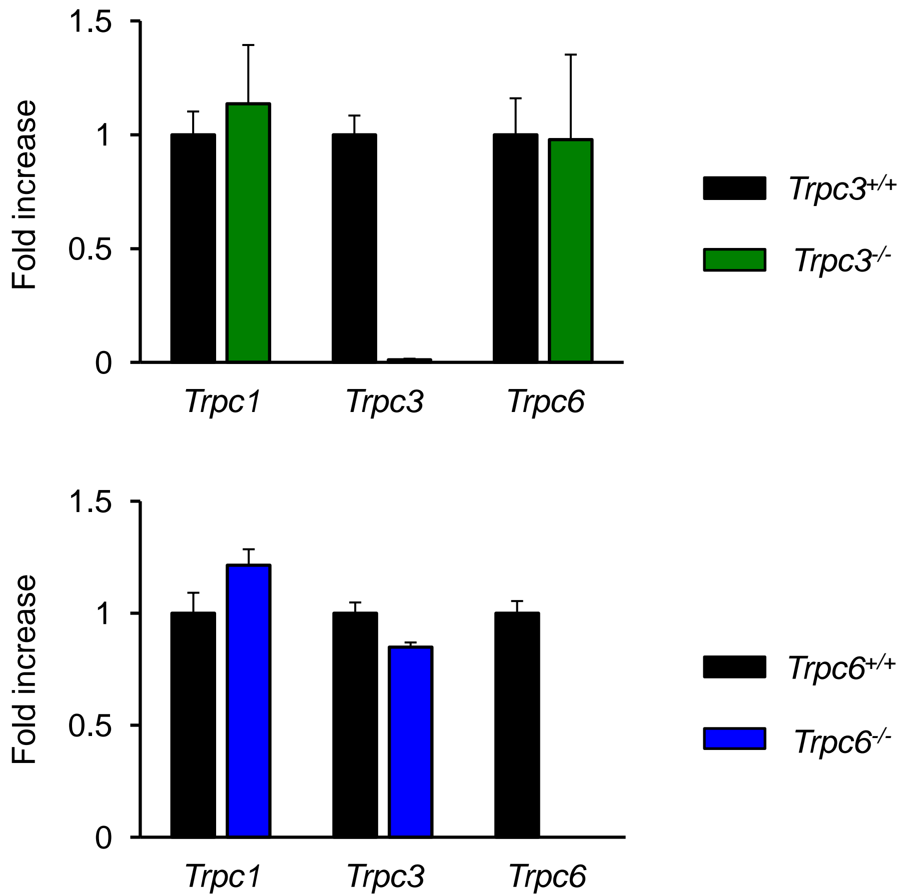
Heart tissue was lysed with RIPA buffer and subjected to SDS-PAGE using Nupage gels (Invitrogen) under reducing and denaturing conditions and transferred to nitrocellulose membranes. The antibodies against Pan-Calcineurin A and Gapdh were obtained from Cell Signaling Technologies (#2614) and Abcam (#ab9484), respectively. Fluorescence labeled secondary antibodies were obtained from Licor. Membranes were scanned on an infrared imaging system (Odyssey, Licor) and quantification of band intensity performed using Odyssey Application Software 3.0.

WGA Staining for Myocyte Cross Sectional Area

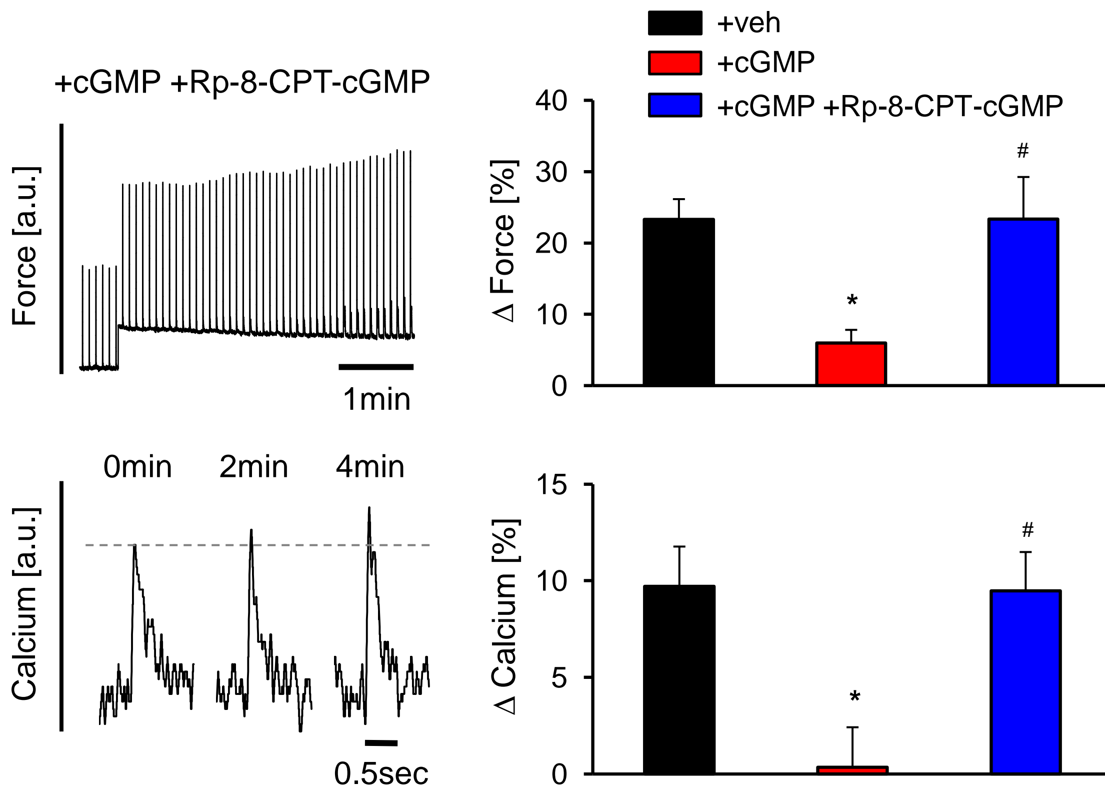
Myocardium was fixed with 4 % paraformaldehyde, paraffin embedded, sectioned into 4 μm slices, deparaffinized, rehydrated, and subjected to citrate-based head-mediated antigen retrieval. Slides were incubated with 5 μg/ml Alexa Fluor 488-conjugated wheat germ agglutinin (Invitrogen) overnight at 4 °C and mounted using Prolong Gold mounting medium (Invitrogen). Image acquisition was performed on a Zeiss LSM510-META laser scanning confocal microscope. Myocyte cross sectional area was analyzed blinded to the treatment group using an automated algorithm with NIH Image J 1.47i software. At least 1000 cells from 3-8 areas per heart were analyzed.

1. Takimoto E, Champion HC, Li M, Belardi D, Ren S, Rodriguez ER, Bedja D, Gabrielson KL, Wang Y, Kass DA. Chronic inhibition of cyclic gmp phosphodiesterase 5a prevents and reverses cardiac hypertrophy. *Nat.Med.* 2005;11:214-222

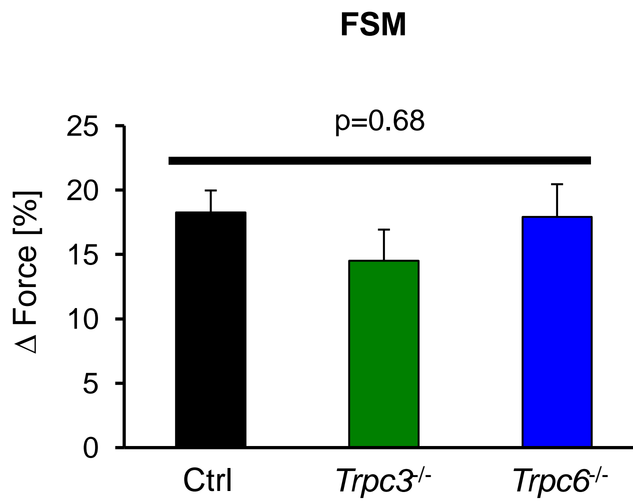
Supplemental Figures



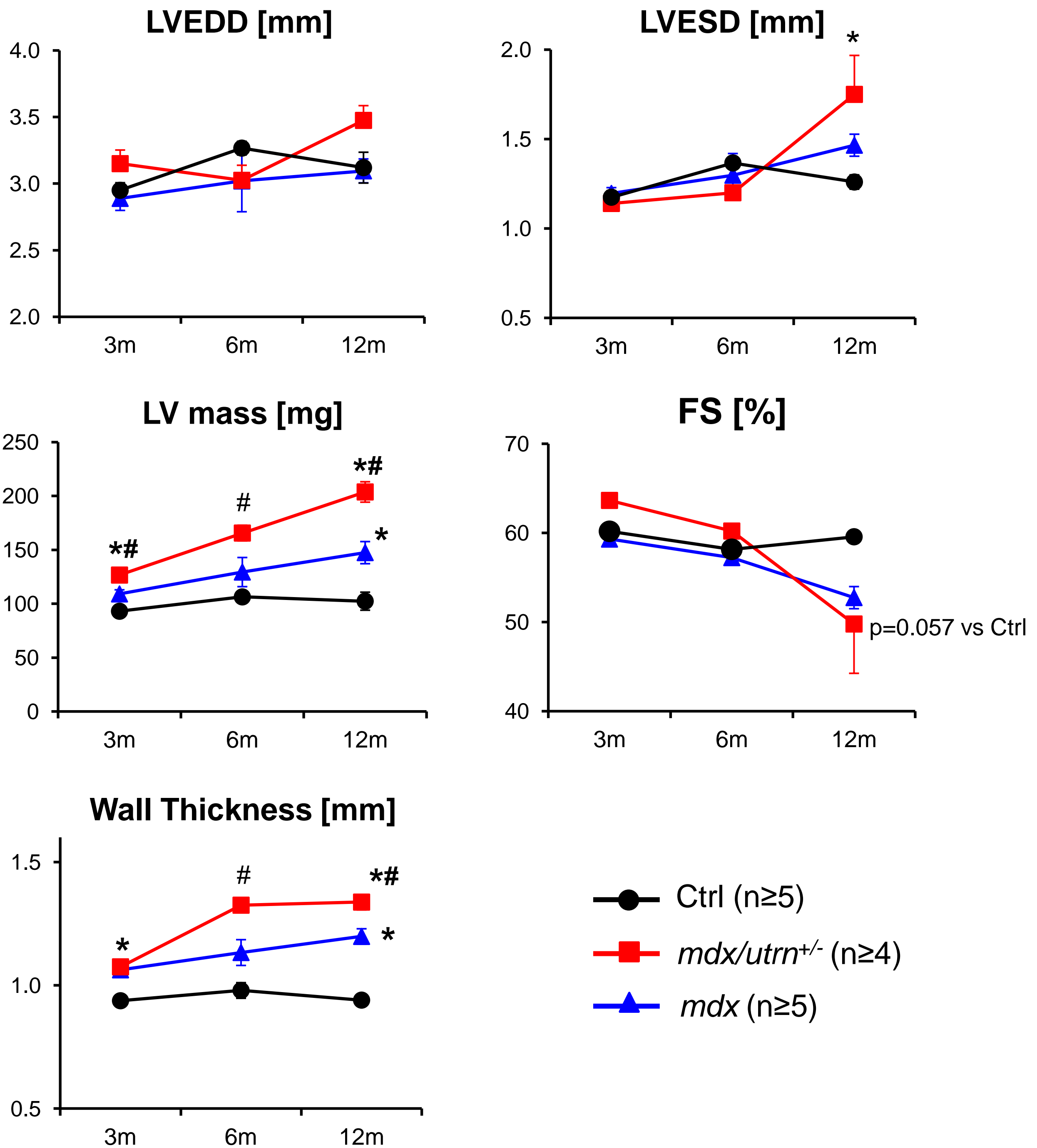
Online Figure I. Trpc1, 3 and 6 gene expression in TRPC knock-out mice and littermate controls. N=4-8 /group. Gene deletion in TRPC3 or 6 knock-out mice did not alter the expression of the other two TRPC channels.



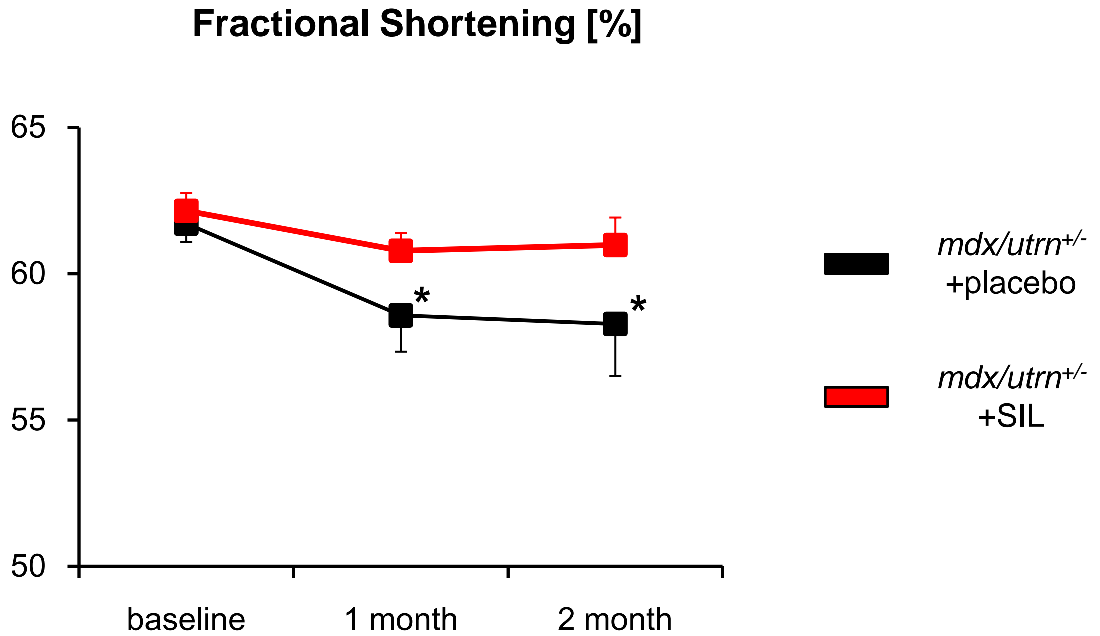
Online Figure II. Force/calcium in auxotonically contracting cardiomyocytes. Cyclic-GMP suppression of SSC is blocked by PKG-inhibition (Rp-8-CPT-cGMP, 10 µM, n=6-24/group, * $p<0.01$ vs +veh, # $p<0.05$ vs +cGMP)



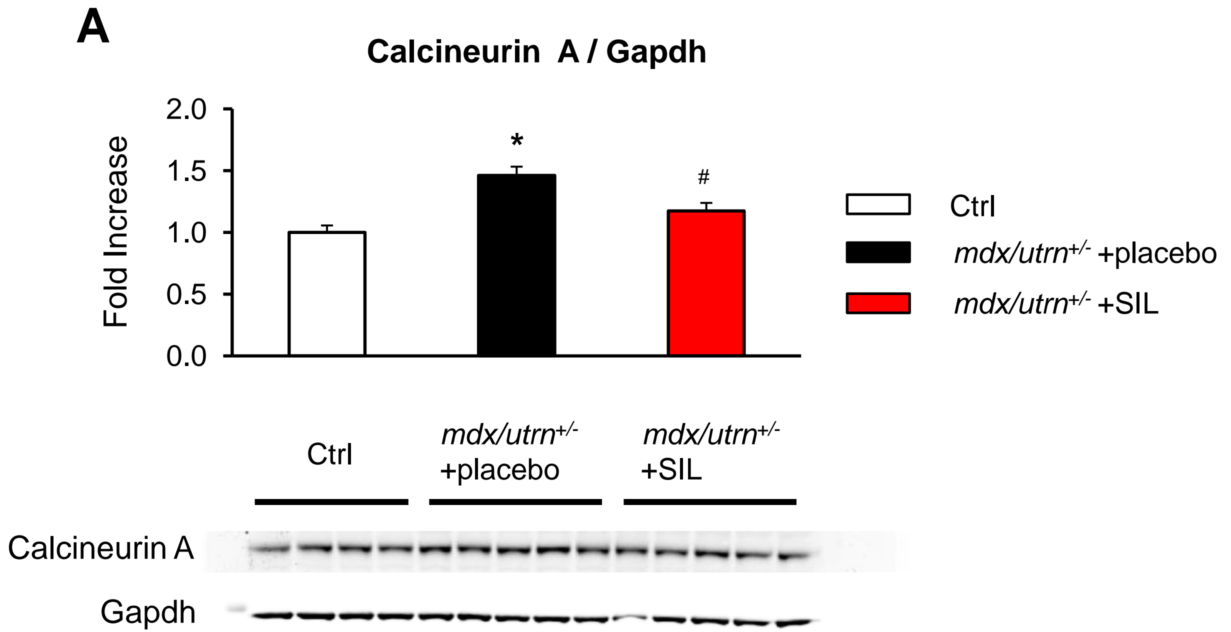
Online Figure III. FSM in muscle from control and mice lacking Trpc3 or Trpc6. N=4-13 /group. There is no difference among groups.

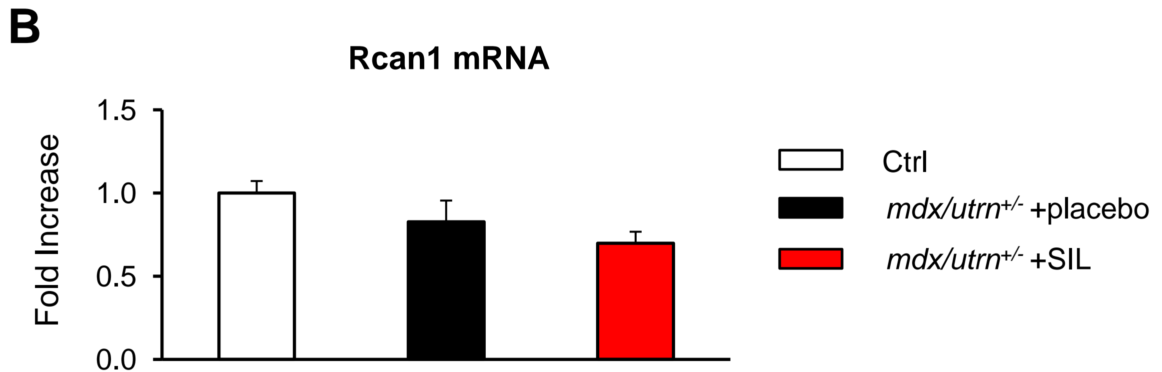


Online Figure IV. Echocardiographic analyses of cardiac morphology and function in control, *mdx/utrn*$^{+/-}$ and *mdx* mice up to 12 months of age. * $p<0.05$ vs Ctrl, # $p<0.05$ vs *mdx*. The mdx model which lacks dystrophin shows a modest increase in LV mass and wall thickness and no chamber dysfunction. However, the combined mdx – utrophin heterozygous deletion (*mdx/utrn*$^{+/-}$) develops more ventricular hypertrophy and chamber dilation (end-diastolic dimension: LVEDD, end-systolic dimension: LVESD, fractional shortening: FS).



Online Figure V. Echocardiographic analyses of cardiac function in *mdx/utrn$^{+/-}$* treated with sildenafil and placebo for 60 days. Chronic PDE5A inhibition by sildenafil prevents deterioration of cardiac function (fractional shortening) observed in *mdx/utrn$^{+/-}$* placebo group. n=14-17/group, *-$p<0.05$ vs baseline.





Online Figure VI. Calcineurin A and Rcan1 expression in hearts of *mdx/utrn*$^{+/-}$ mice treated with sildenafil and placebo for 60 days. A) Western blot of calcineurin A (n=4-5/group, * $p<0.01$ vs Ctrl, # $p<0.05$ vs placebo). **B**) mRNA expression of *Rcan1* (n=3-5/group, p=n.s. for 1-way ANOVA).