# Supplemental Material

## Online Methods

### Animals and cardiomyocytes isolation

Several types of adult mice aged from 10 to 12 weeks were used, including: WT C57b/6J (Jackson Lab), WT C57b/6N (Jackson Lab), Ser280Ala KI,[56,57] MMVV-KI,[58] CaMKIIδ cardiac-specific and global KO,[59,60] NOX2$^{-/-}$ mice (Jackson Lab) and Grx1-roGFP2 (targeted either to the cytosol or mitochondria).[61] All the genetically modified mouse lines were bred on the C57b/6J background, except for the C57b/6N line. The cardiac-specific CaMKIIδ KO mouse was originally generated in the Black Swiss background, but mice used here had been crossbred into C57b/6J for more than 9 generations.[62]

The Ser280Ala KI mice were generated using a CRSPR/cas9 approach[57] using
CAMK-CRISPR-F (5'-CACCGCTGTTGCCTCCATGATGCAC-3') and
CAMK-CRISPR-R (5'-AAACGTGCATCATGGAGGCAACAGC-3')
and the sgRNA reporter plasmid pX330-U6-Chimeric_BB-CBh-hSpCas9 (addgene # 42230). Mice were crossbred for > 8 generations and the CaMKIIδ mRNA was sequenced to confirm the amino acid substitution in the KI. Subsequent genotyping of the mice was performed with rhPCR and primers WT
forward 5'-GCAACGCTCTACTGTTGCCrUCCATT/3SpC3/-3',
mutforward 5'-GCAACGCTCTACTGTTGCCrGCCATC/3SpC3/-3' and
reverse 5'-GGAGAGAAAGCCCAGCACAT-3'.

Cardiac ventricular myocytes were isolated using previously described methods[63] which were approved by the UC Davis Institutional Animal Care and Use Committee (IACUC). Freshly isolated myocytes were plated on laminin-coated glass cover slips for 30 min before dye loading. All experiments were performed at room temperature (RT) (22-23°C) unless otherwise stated with pH 7.4.

### Cardiomyocyte ROS production measurement

Freshly isolated ventricular cardiomyocytes were plated on laminin-coated glass cover slips in normal Tyrode's buffer. Intact cells were loaded with 1 μmol/L $H_2$DCFDA (Thermo Fisher) for 10 min to measure reactive oxygen species. DCF loaded cells were imaged at Ex= 488nm, with Em=530nm using confocal 2D-scanning microscopy with (Nikon, ×40 objective) ~20 nW laser power to minimize laser induced artifacts. Baseline linearity was checked with no treatment recording. For pacing experiments, cells were stimulated at 0.5 Hz and perfused on the microscope stage at 2 ml/min with normal Tyrode's solution (below) adding different drugs. For 35°C experiments, perfusing solution was preheated to 37°C and the chamber was heated by channel temperature controller (TC-324B, Warner).

### $Ca^{2+}$ spark and transient measurements

Intact ventricular myocytes were loaded with Fluo-4 AM dye (5 μmol/L) for 30 min, transients and sparks were recorded as previously described.[64,65] $Ca^{2+}$ transients were obtained by field stimulation at 0.5 Hz in normal Tyrode's buffer, and treatments including Hi-Glu/Thm-G was perfused and cells were pre-pacing with 0.5Hz for 3min before recording. SR $Ca^{2+}$ load was evaluated by the $Ca^{2+}$ transient amplitude induced upon rapid caffeine application (10 mmol/L). Images were acquired with confocal microscopy (Nikon, ×40 objective) using line scan mode with excitation at 488 nm, emission at >505 nm. Image analysis used ImageJ software and Sparkmaster.[66]

### Redox sensor measurements and $E_{GSH}$ calculation

Isolated cardiac myocytes plated onto laminin-coated glass coverslips were incubated for at least 30 min before imaging at room temperature. Then, the chamber and solution are pre-heated to

35°C for more physiological measurement. The redox measurements were performed using a confocal microscope (Nikon). The roGFP2 sensor was excited at 405 and 488 nm, and the emitted light from the sample was detected at >530 nm. Images were acquired in every 20 secs. Sensor calibration has been done according to a previous study with the parent sensors.[67] Oxidation difference (OxD) and $E_{GSH}$ calculations were performed as described therein. Determining the $E_{GSH}$ values in basal conditions requires analyses of fluorescence intensities at 405 and 488 nm after stimulation with 100μmol/L $H_2O_2$ (maximum oxidation) and 1mmol/L dithiothreitol (maximum reduction). The OxD is the ratio of the number of oxidized molecules to the total number of molecules (OxD roGFP2=[roGFP2ox]/([roGFP2red+roGFP2ox]).[11]

$$\mathrm{OxD}_{\mathrm{roGFP2}} = \frac{I405 \times I488red - I405red \times I488}{I405 \times I488red - I405 \times I488ox + I405ox \times I488 - I405red \times I488}$$

$$E_{\mathrm{roGFP2}} = \mathrm{E}^{\circ}_{\mathrm{roGFP2}} - \frac{RT}{2F} \ln\left(\frac{1 - \mathrm{OxD}_{\mathrm{roGFP2}}}{\mathrm{OxD}_{\mathrm{roGFP2}}}\right)$$

**hiPSC-CMs redox and $Ca^{2+}$ transient measurements**

Human induced pluripotent stem cell derived cardiac myocytes (iPSC-CMs) were prepared by Dr. Mercola's lab by methods previously described.[68,69] At day 25, cells were dissociated and plated onto Matrigel-coated coverslips in a 24-well plate.[70] Then, hiPSC-CMs were subjected to adenoviral-mediated gene transfer of mito- and cyto-Grx1-roGFP2 and cultured for 24 hr. Before starting experiments, culture media was replaced by NT buffer with 1.8mmol/L and 5.5mmol/L glucose. For $Ca^{2+}$ transient measurement, cells were loaded with Fluo-4 AM dye (5 μmol/L) for 30 min and imaged with confocal microscopy (Biorad, 40X oil objective).

**Solution and experimental protocols**

Normal Tyrode's solution (NT) contained (mmol/L) 140 NaCl, 6 KCl, 10 HEPES, 1 $MgCl_2$, 1.8 $CaCl_2$ and 5.5 glucose (RT, pH7.4). For Hi-Glu exposures, myocytes were usually paced at 0.5 Hz, and first equilibrated with NT containing 2 mmol/L glucose plus 28 mmol/L mannitol (or 5.5 mmol/L glucose plus 24.5 mmol/L mannitol). Then, an acute switch was made to NT in which the mannitol was replaced by glucose (bringing [glucose] to 30 mmol/L).

NT with 2 mmol/L glucose was chosen to minimize activation of *O*-GlcNAcylation at baseline, while still nourishing myocytes. To confirm that the central observations regarding HiGlu-induced ROS production occur in physiological NT ([glucose] =5.5 mM) we measured the increases in ROS production from either 2 or 5.5 mmol/L upon increase to HiGlu (30 mmol/L glucose; Online Figure 1C). The results were similar, except that when starting from 5.5 mmol/L glucose the mean ROS increases were slightly smaller. This is consistent with prior observations on the monotonic rise in CaMKII activation in going from 5 .5 to 33 mM (100-500 mg/dl).[8]

Protein samples of hearts were harvested after 20 min Langendorff perfusion at 37°C with various treatments and frozen down immediately.[71] Cardioplegia solution contained (mmol/L) 110 NaCl, 1.2 $CaCl_2$, 16 KCl, 16 $MgCl_2$ and 10 $NaHCO_3$. Perfusing Tyrode's solution contained (mmol/L): 128.2 NaCl, 1.3 $CaCl_2$, 4.7 KCl, 1.05 $MgCl_2$, 1.19 $NaH_2PO_4$, 20 $NaH_2CO_3$. Western blot and immunoprecipitation were performed using a customized rabbit derived anti-CaMKII$\delta$ antibody (made against the C-terminal sequence by ZYMED Inc, San Francisco, CA). For O-GlcNAcylation levels of CaMKII, monoclonal antibody (Sigma, MABS125, Lot#3083560) was used. For protein extraction, ice cold Lysis buffer (in mmol/L: 20 Tris-HCl, pH 7.4, 150 NaCl, 1 EDTA, 1 DTT, 40 GlcNAc, and 1% Non-idet P-40) with protease inhibitor cocktail set III (Calbiochem, 539134) and phosphatase inhibitor cocktail set III (Calbiochem, 524627) were used. GentleMACS dissociators from Miltenyi Biotec was used to homogenize hearts. Negative control with alkaline-induced β-elimination was performed with 1% triethylamine and 0.1% NaOH pH12.5

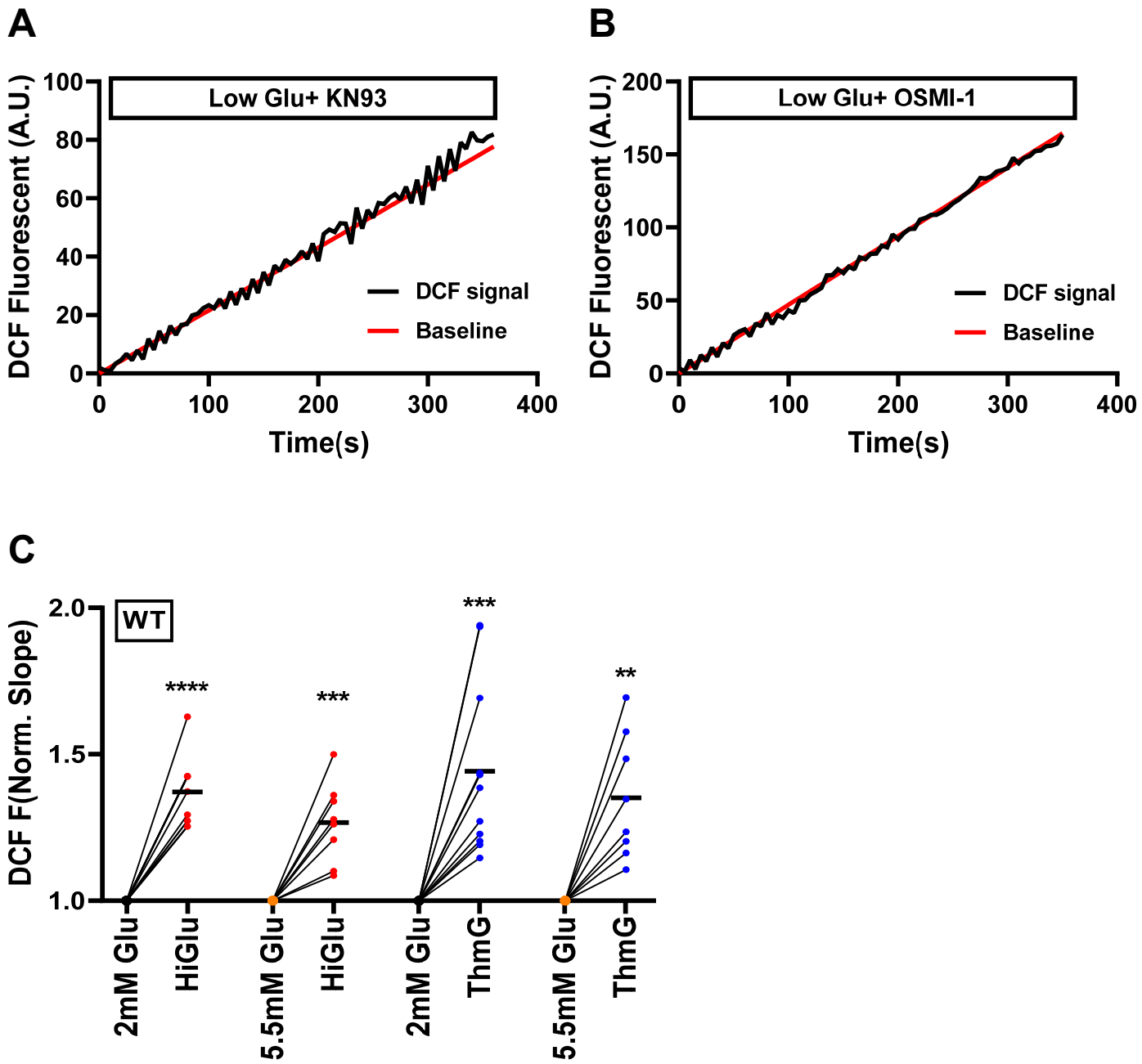
at 4°C for over 18 hrs, and the reaction was quenched by neutralizing to 2% trifluoroacetic acid. Positive control was prepared with 1μmol/L Thm-G perfused heart.[72,73]

Dynabeads (Invitrogen,10003D) were used for immunoprecipitation. For each IP sample, 50μl Dynabeads, 300 μg protein and 5 μl of anti-CaMKII antibody were used. IP buffer was used containing (in mmol/L): 100 NaCl, 20 HEPES, 10 $MgCl_2$, 0.2 EGTA, protease inhibitor and phosphatase inhibitor. Washing buffer was made with 0.05% Tween 20 in PBS. Magnetic bead-Ab-Ag complex was resuspended in Loading buffer (6X laemmli buffer, Biorad). CoIP proteins were heated at 70°C for 10 min and separated by SDS-PAGE. And electroblotted to nitrocellulose membrane. Then block the membranes with 5% blocking buffer in TBST. CTD110.6 antibody diluted 1/500 in Antibody dilution buffer (5% BSA in TBST). IRDye800CW Goat anti-Mouse IgM (LI-COR, Lot#C70808-03) was used at 1/10000. The membrane images were acquired with Azure Sapphire Biomolecular Imager. ImageJ software was used to analyze the blots.

**Statistics**

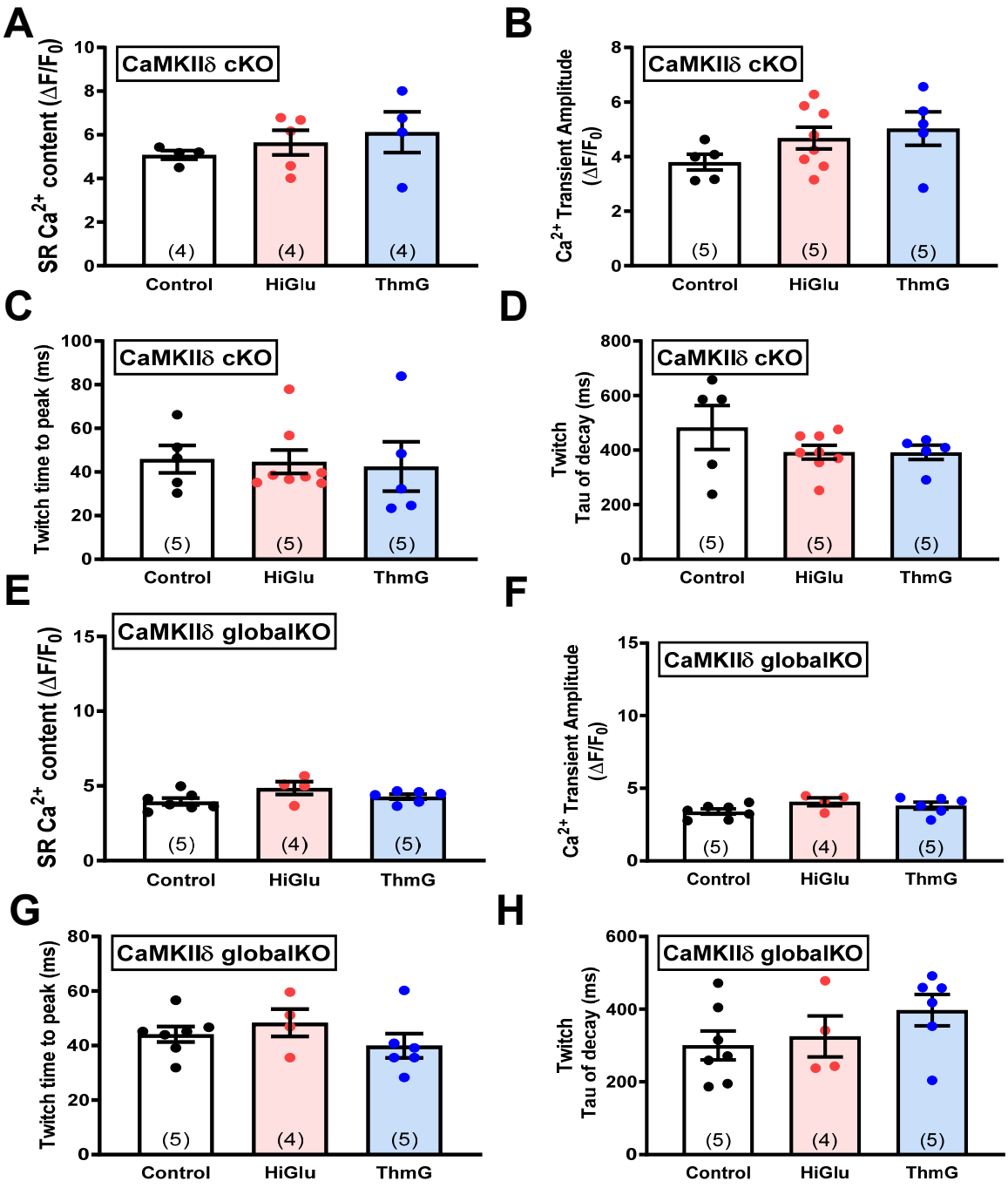
Pooled data are represented as the mean ± SEM. Group sizes were determined by an a priori power analysis for a two-tailed, two-sample t-test with an α of 0.05 and power of 0.8, in order to detect a 10% difference signal at the endpoint. Animal were grouped with no blinding but randomized during the experiments. Fully blinded analysis was not performed because the same person carried out the experiments and analysis. Male and female animals were used in equal numbers for all experiments. No samples or animals were excluded from analysis. Representative figures/images reflected the average level of each experiment. Normality of the data was done by D'Agostino-Pearson test and the equality of group variance (using Brown-Forsythe test) were performed. Comparisons between two groups were performed by one-sample t test (for all the DCF and contractility experiments), unpair (for all others), two tailed t-test, between more than two groups by oneway ANOVA followed by Bonferroni's post-hoc using Prism 8.0 software (GraphPad). Western blot analysis was done using two-way ANOVA. DCF signal was fitted linearly with RSQ >0.9. Redox sensor calibration data was fitted with sigmoidal curve in GraphPad. A value of two-tailed $P < 0.05$ was considered statistically significant.

Online Figure I



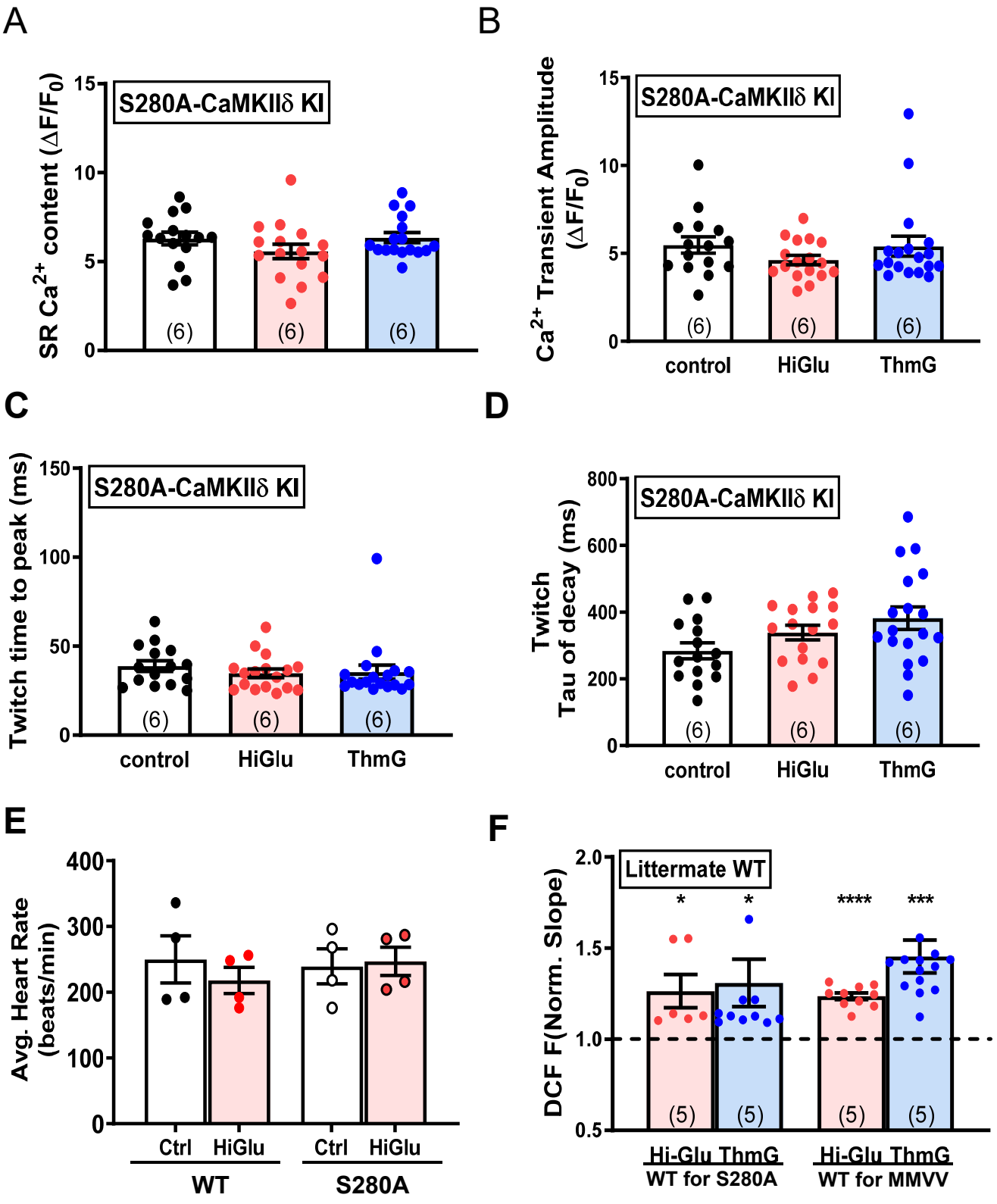

**Online Figure I Acute perfusion of KN93 and OSMI-1 do not change the ROS generation rate** (A) ROS generation rate remains linear with perfusion of KN93. (B) Short period of OSMI-1 perfusion do not perturb the ROS generation at low Glu. (C) Initial glucose with 2mmol/L and 5.5mmol/L perfusion in WT. (**P<0.01, ***P<0.001, ****P<0.0001)

# Online Figure II



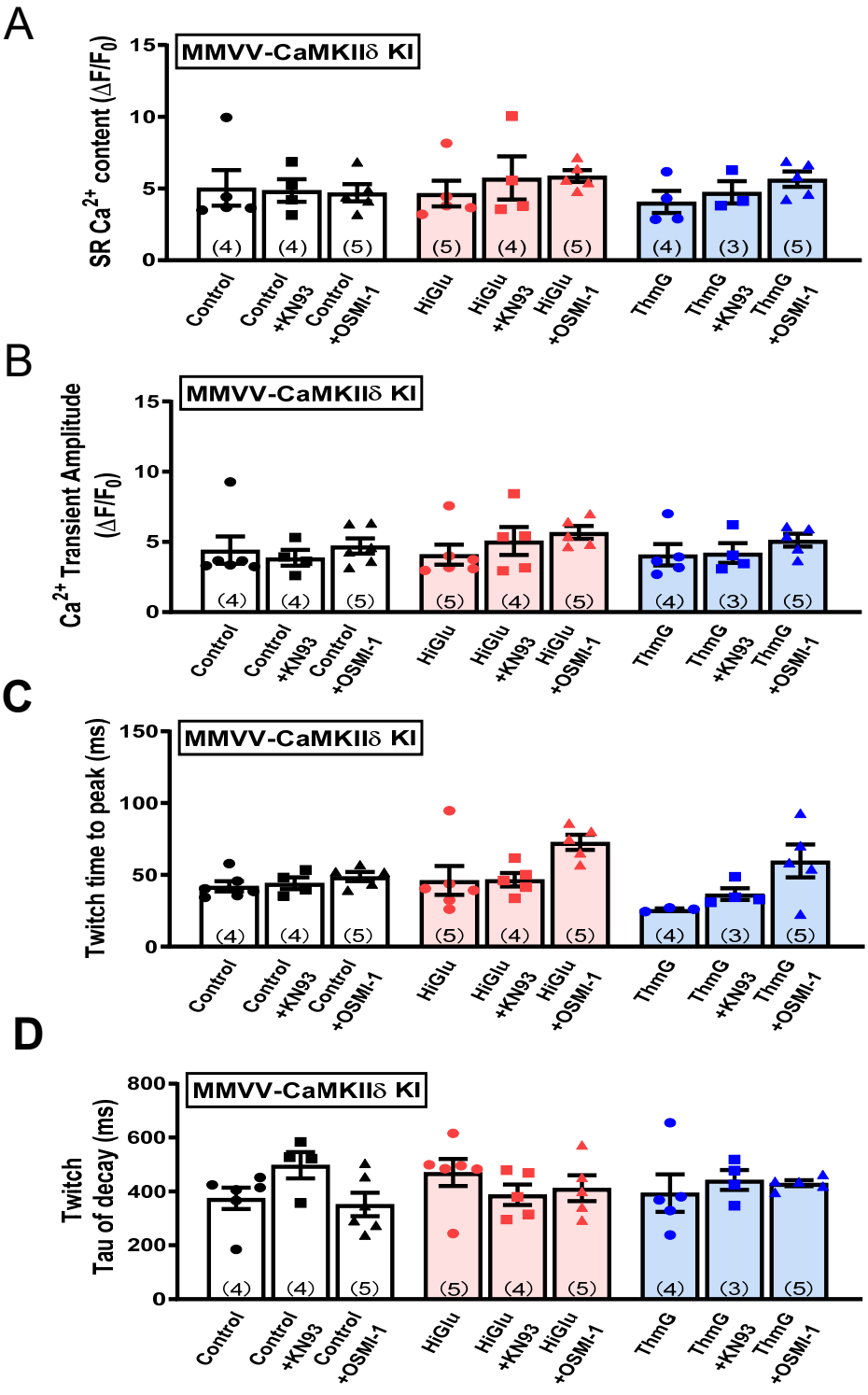

**Online Figure II SR $Ca^{2+}$ handling in different strains** (A-D) Hi-Glu/ ThmG has no effects on SR $Ca^{2+}$ handling in CaMKIIδ cKO. (E-H) Hi-Glu/ ThmG has no effects on SR $Ca^{2+}$ handling in CaMKIIδ globalKO.

Online Figure III



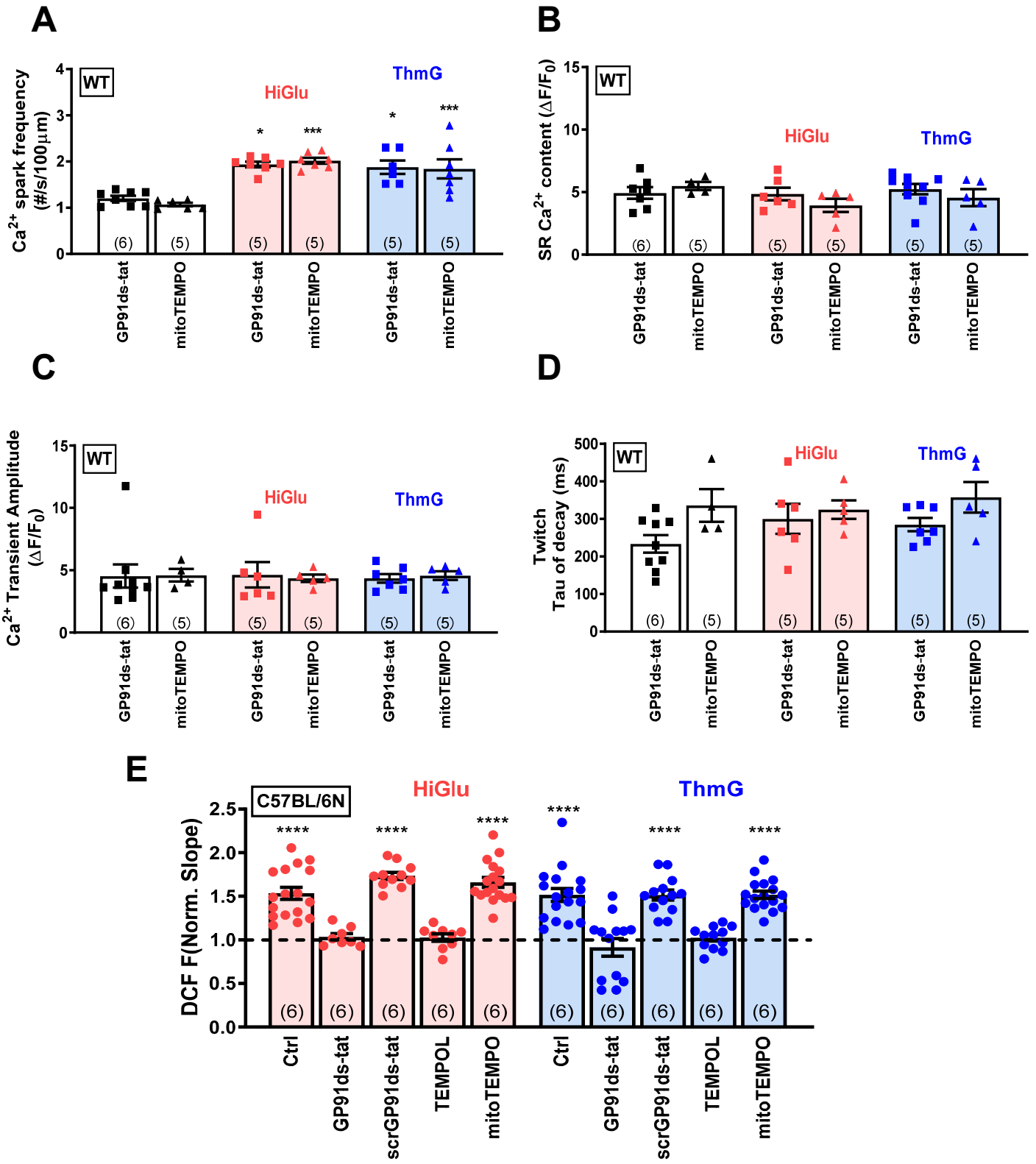

**Online Figure III** (A-D) SR $Ca^{2+}$ handling remains normal in S280A. (E) Average heart rate is stable during the Langendorf Hi-Glu perfusion. (F) Littermate controls of S280A KI and MMVV KI show similar response to stimuli as WT. (*P<0.05, ***P<0.001, ****P<0.0001)

Online Figure IV



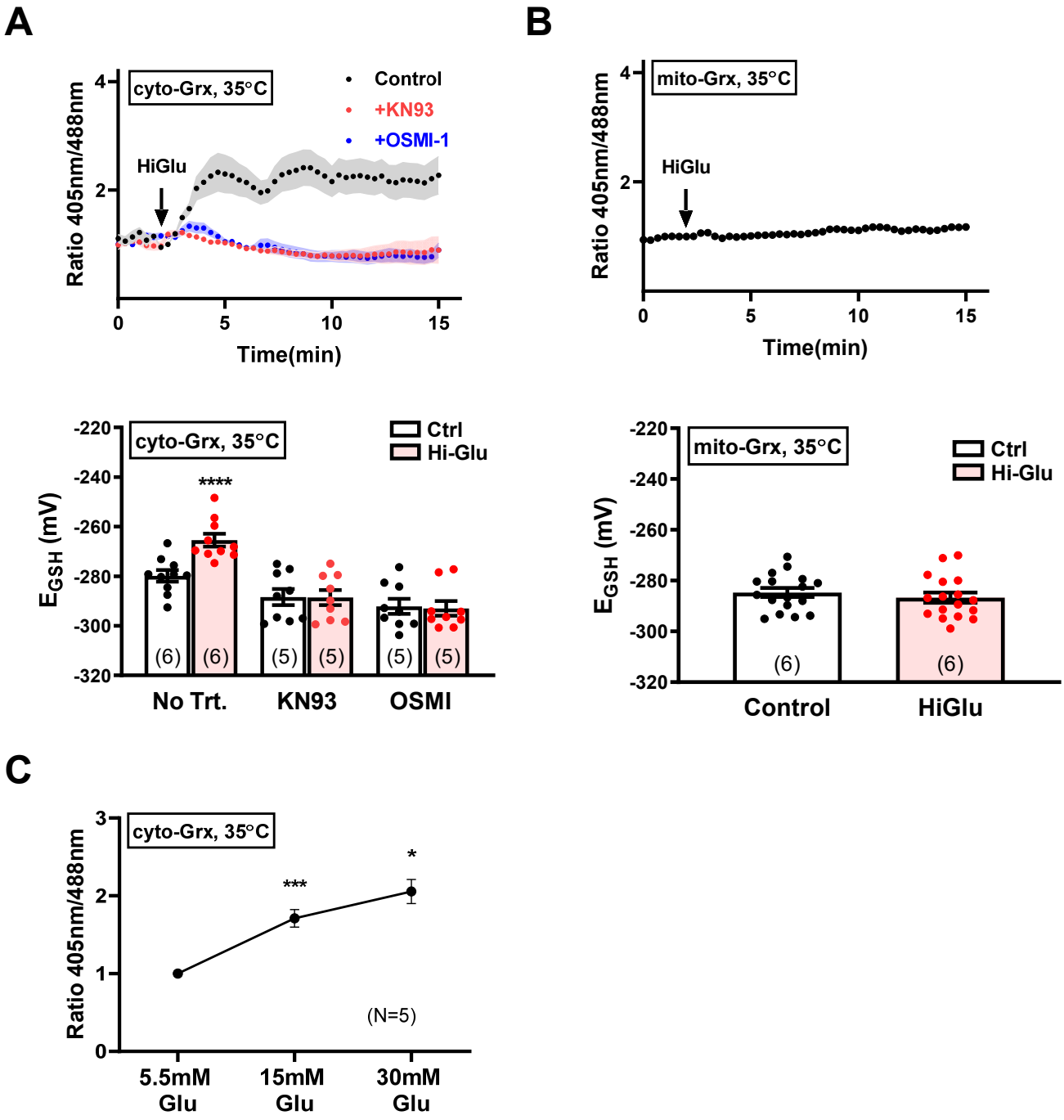

**Online Figure IV.** No obvious SR $Ca^{2+}$ handling was observed with different treatments in MMVV-CaMKIIδ KI.

# Online Figure V




**Online Figure V** (A) $Ca^{2+}$ spark frequency increases in Hi-Glu/ThmG despite of GP91-ds-tat or mitoTEMPO pretreatments. (B-D) SR $Ca^{2+}$ handling was not different in pretreat myocytes. (E) ROS production was increased in Hi-Glu/Thm-G and inhibited by GP91ds-tat and TEMPOL on C57BL/6N (*P<0.05, ***P<0.001, ****P<0.0001)

# Online Figure VI




**Online Figure VI** (A) Representative traces of cyto-Grx ratio metric signal and converted $E_{GSH}$ in response to Hi-Glu/ThmG at 35°C. (B) Representative traces of mito-Grx ratio metric signal and converted $E_{GSH}$ in response to Hi-Glu/ThmG at 35°C. (C) Various glucose levels induced cytosolic ROS generation. (**P<0.01, ****P<0.0001)