SUPPLEMENTAL MATERIAL

Expanded Materials & Methods

CaMKIIδ S280A knock-in mouse model

CaMKIIδ S280A knock-in animals were newly generated here to study *O*-GlcNAc post-translational modification effects on CaMKII function in health and disease. For the vector construction, the sgRNA was designed using the Zhang laboratory website (http://crispr.mit.edu). The oligo DNAs CAMK-CRISPR-F (5'-caccgctgttgcctccatgatgcac-3') and CAMK-CRISPR-R (5'-aaacgtgcatcatggaggcaacagc-3') were synthesized by IDT. After phosphorylation by T4 PNK (NEB), the oligo pair was annealed in a thermocycler using the following parameters: 95°C for 5 minutes and then ramp down to 25°C at 5°C/5 minutes. After 1:100 dilution, the oligo DNA was inserted into the Bbsl restriction site in the pX330-U6-Chimeric_BB-CBh-hSpCas9 vector (addgene #42230). This plasmid was designated PX330-CAMK. Female C57BL/6J mice were injected with pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) with a 48-hour interval, and mated with male C57BL/6N mice. The fertilized one-cell embryos were collected from the oviducts. Then, 5 ng/μL PX330-CAMK DNA vector (circular) and 20 ng/μL ssDNA donor (5'-atgttttaaatatcaagtcacccttctcctcttcttttagcaacgctctactgttgccgccatg-atgcacaggcaggagactgtagactgcttgaagaaatttaatgctagacggaaactgaag-3') were injected into the pronuclei of these one-cell-stage embryos. The injected one-cell embryos were then transferred into pseudopregnant ICR mice. The screening of founder mice and examination of off-target effects were performed by PCR and direct sequencing using DNA obtained from the tail. PCR was performed with the following primers: CAMK-WT-F genotyping: 5'-tggctgcagtagtgtgactg-3', CAMK-WT-R genotyping: 5'-ctgcctgtgcatcatgga-3', CAMK-KI-R genotyping: 5'-ctgcctgtgcatcatggc-3'. The PCR products were subcloned into the pCR-Blunt II-TOPO and sequenced using the T7 primer according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). We crossed the positive founder mice with C57BL/6N mice, then sequenced the offspring from the founder and confirmed germline transmission. Mice were subsequently crossbred for >8 generations with C57BL/6J mice. The CaMKIIδ mRNA was sequenced to confirm the amino acid substitution in the S280A knock-in. Subsequent genotyping of the mice was performed with rhPCR and primers WTforward 5'-gcaacgctctactgttgccruccatt/3SpC3/-3', mutforward 5'-gcaacgctctactgtt-gccrgccatc/3SpC3/-3' and reverse 5'-ggagagaaagcccagcacat-3'.

Murine echocardiography

Systolic and diastolic ventricular functions of diabetic mice were assessed by transthoracic echocardiography using the Vevo 2100 echocardiography system (FUJIFILM VisualSonics, Toronto, ON, Canada) equipped with a 40 MHz linear probe. Mice were shaved the day before echocardiography recordings. During recordings, mice were anesthetized with isoflurane inhalation (1.5%), which was later individually adjusted (between 0.5 to 2%) to achieve a stable heart rate of 400-500 beats/minute to avoid fusion of the waves. ECG monitoring was obtained using limb electrodes, and core temperature was carefully monitored and maintained at 37°C during the entire procedure. Left ventricular (LV) B-mode and M-mode two-dimensional echocardiography in parasternal short- and long-axis views were performed for assessment of LV dimensions and systolic function. Pulsed wave Doppler and tissue Doppler images were acquired from the apical four-chamber view to assess diastolic function. At least five consecutive cardiac cycles were sampled for each measurement taken, and evaluations have been performed off-line in two separate blinded analyses. At the end of the procedures all mice recovered from anesthesia without difficulties.

Enzymatic isolation of left ventricular cardiomyocytes

Mice injected with heparin (5000 U/kg body weight) and anesthetized with isoflurane (5%). Hearts were excised and retrograde perfused on constant flow Langendorff apparatus (4 min, 37ºC) with Ca^{2+}-free normal Tyrode's solution, gassed with 100% O_2. Then, the heart was perfused for 8-12 minutes with

0.8 mg Liberase TM (Roche, Cat#: 5401127001) in 50 mL Tyrode's solution to enzymatically isolate cardiomyocytes. Following digestion, the myocytes were gently triturated with a pipette, then filtered through a nylon mesh and allowed to sediment for ~10 minutes. The sedimentation was repeated three times using increasing [Ca^{2+}] from 0.125 to 0.25 then 0.5 mmol/L. Finally, ventricular myocytes were kept in Tyrode's solution (0.5 mmol/L [Ca^{2+}]) at room temperature until use.

Cellular electrophysiology

Isolated single murine ventricular cardiomyocytes were placed in a temperature-controlled perfusion chamber (Warner Instruments, Hamden, CT, USA) mounted on a Leica DMI3000 B inverted microscope (Leica Microsystems, Buffalo Grove, IL, USA). Cells were bathed at 37°C (for 10 minutes before starting the experiments) and continuously perfused (2 mL/min) with Tyrode's solution containing (in mmol/L): NaCl 140, KCl 4, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 5, Na-HEPES 5, glucose 5.5 and mannitol 24.5; pH=7.40 and osmolality=320±2 mOsm/L. High-glucose effects were assessed by switching the bathing medium to a Tyrode solution containing 30 mmol/L glucose and 0 mannitol (osmolality and pH matched). Electrodes were fabricated from borosilicate glass (World Precision Instruments Inc., Sarasota, FL, USA) having tip resistances of 2 to 2.5 MΩ when filled with internal solution containing (in mmol/L): K-aspartate 100, KCl 30, NaCl 8, Mg-ATP 5, phosphocreatine dipotassium salt 10, HEPES 10, EGTA 0.01, cAMP 0.002, and calmodulin 0.0001; pH=7.20 (with KOH). Using this internal solution, the intracellular Ca^{2+} transient and contraction of the cardiomyocyte are preserved.[58] Axopatch 200B amplifier (Axon Instruments Inc., Union City, CA, USA) was used for recordings and the signals were digitized at 50 kHz by a Digidata 1322A A/D converter (Axon Instruments) under software control (pClamp10.4). Series resistance was typically 3 to 5 MΩ and it was compensated by ≥90%. Experiments were discarded when the series resistance was high or increased by ≥20% during the recordings. Reported voltages are already corrected for liquid junction potential. Experiments were conducted at 37±0.1°C.

APs were recorded in whole-cell I-clamp conditions where cells were stimulated using supra-threshold depolarizing pulses (2 ms duration) delivered via the patch pipette at various pacing frequencies from 1 Hz to 10 Hz. 50 consecutive APs were recorded at each pacing frequency to examine the average behavior. AP duration at 90% repolarization (APD_{90}) was used to characterize AP repolarization. APD_{90} alternans magnitude was calculated as the difference between the average APD_{90} of odd and even numbered beats during 50 consecutive APs recorded. Diastolic arrhythmogenic activities were elicited by cessation of 1-min burst pacing (10 Hz), and membrane potential was recorded for additional 3 min. Delayed afterdepolarizations (DADs) were defined as an increase in resting membrane potential exceeding 1 mV in amplitude within 0.5 seconds. Spontaneous APs (sAPs) were defined as depolarizations showing overshoot with a fast upstroke phase.

Chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), if not specified otherwise.

Calcium imaging

Intracellular Ca^{2+} transients and diastolic Ca^{2+} events (sparks and waves) were measured in freshly isolated ventricular cardiomyocytes loaded with Fluo-4 AM (10 μmol/L, Invitrogen) and Pluronic F-127 (0.02%, Invitrogen). The dye was loaded for 30 minutes at room temperature followed by wash and de-esterification for 30 minutes. Fluo-4 was excited at 488 nm using an Argon laser, and emission was collected using a 500-530 nm bandpass filter. Images were recorded using confocal microscopy in line scan mode (Bio-Rad Radiance 2100, 40x objective, 6 ms/line). Intact cardiomyocytes were plated on laminin-coated coverslips and paced at 1 Hz in a field stimulation chamber (Warner Instruments). Paired experiments were performed at room temperature in Tyrode solution containing normal glucose (100 mg/dL), then high glucose (540 mg/dL). ImageJ was used for image processing and analysis, and Ca^{2+} sparks were analysed using the SparkMaster plugin.

Fluorescence recovery after photobleaching (FRAP)

Rabbit cardiomyocytes were isolated from young adult (male, 3-4 month-old) New Zealand White rabbits as previously described.[27] Freshly isolated rabbit myocytes were plated on sterile, laminin-coated glass coverslips in PC-1 media (Lonza). Non-attached myocytes were washed away after an hour, and attached myocytes were then infected with recombinant adenoviruses containing GFP-tagged CaMKIIδ_C WT or S280A, with subsequent culture for 18 hours. GFP-CaMKIIδ_C constructs were generated as previously described.[59] Cultured myocytes were used for FRAP experiments within 18-22 hours of adenovirus infection. All FRAP measurements were performed at room temperature, using an Olympus FV-1000 confocal microscope and 60x objective. Under stimulus setting in the FV-1000 software, a bleaching region (BR) with a diameter of 7.3 μm was selected in each myocyte. Software was set to activate in series: acquiring three images prior to bleaching and several post-bleach images, with image acquisition occurring every 2.71 seconds. The BR fluorescence signal was bleached down to 30-40% of the initial value using 50% 488 nm and 22% 405 nm laser for 1 s. The cells were bathed in normal glucose (5.5 mmol/L, with 24.5 mmol/L mannitol) or high-glucose (30 mmol/L) containing Tyrode's solution and were paced for 5 minutes with electric field stimulation of 25 V at 0.5 Hz before FRAP. ImageJ was used to analyze FRAP experiments. A circular region of interest (ROI, half the size of the BR) was used to measure the fluorescence of the BR over time. Background measurements were obtained using the same ROI. Time points were background subtracted and then normalized for analysis with GraphPad Prism. A region on the opposite end of the cell from the BR was also measured in a subset of cells to verify repeated imaging did not result in a decrease in overall fluorescence over the entire time course.

Western blotting and coimmunoprecipitation in murine heart samples

Hearts from different diabetic and healthy mice were collected and rinsed three times in ice-cold 0 Ca^{2+} normal Tyrode's solution, then cut in 6-12 pieces, flash-frozen, and stored at -80°C until processing. Flash-frozen hearts were thawed and lysed in ice-cold buffer containing (in mmol/L): NaCl 150, HEPES (pH=7.5) 10, NaF 50, sodium pyrophosphate 1, $MgCl_2$ 1, EGTA 1, EDTA 1, 1% Triton X-100, and protease and phosphatase inhibitors (EMD Millipore, set III and V, respectively). Heart lysates were flash-frozen and stored at -80°C. Protein content in heart lysate was determined using a BCA assay (Thermo Fisher Scientific, Cat#: 23225). Sample proteins were then separated on Tris-HCl SDS-PAGE (4-15% for CaMKII, 4-20% for PLB) before transferring to a 0.2 μm nitrocellulose membrane. Immunoblots were blocked with 8% milk in Tris-buffered saline (TBS). The blots were then incubated overnight at 4°C with primary antibodies: CaMKIIδ (custom, ZYMED Inc., San Francisco, CA, USA, 1:15,000, rabbit), CaMKII pT286 (Thermo Fisher Scientific, Cat#: MA1-047, 1:1,000, mouse), PLB (Badrilla, PLN, mAB A1, Cat#: A010-14, 1:5,000, rabbit), PLB pS16 (Badrilla, Cat#: A010-12AP, 1:5,000, rabbit), PLB pT17 (Badrilla, Cat#: A010-13, 1:5,000, rabbit) and GAPDH (Millipore, Cat#: AB2302, 1:5,000, chicken). Following five TBS-Triton (TBST) washes, specific secondary antibodies (goat anti-mouse IRDye800CW, LI-COR, P/N: 926-32210; goat anti-rabbit IRDye680RD, LI-COR, P/N: 926-68071; goat anti-rabbit IRDye 800CW, LI-COR, P/N: 926-32211, all used in 1:10,000; and goat anti-chicken Alexa Fluor 488, Thermo Fisher Scientific, Cat#: A-11039, 1:5,000) were applied and left for 2 hours at room temperature. The blots were again washed in TBST before imaging on the Sapphire Biomolecular Imager (Azure Biosystems).

To detect RyR pS2814 in diabetes, ventricular homogenate proteins were separated on a 7.5% TGX Criterion gel (Bio-Rad) before transfer to 0.2 μm nitrocellulose and blocking with 8% milk in TBS. Blots were then probed for pS2814 (custom antibody, 1:1,000 dilution in 1xTBST, gift from Dr. Xander H.T. Wehrens) followed by anti-rabbit horseradish peroxidase-conjugated secondary antibody (Thermo Fisher Scientific, Cat#: 31460, 1:5,000). Detection was with Supersignal West Dura (Thermo Fisher Scientific). The blots were then washed and reprobed for total RyR antibody (C3-33, 1:1000 dilution in 1xTBST, Thermo Fisher Scientific, Cat#: MA3-916, mouse) followed by goat anti-mouse secondary antibody IRDye800CW (1:10,000 dilution in 1xTBST, LI-COR, P/N: 926-32210).

To detect PLB *O*-GlcNAcylation levels in diabetes, PLB was immunoprecipitated from 100 μg of homogenate with 2 μg of anti-PLB antibody (clone A1, Badrilla, Cat#: A010-14). Prior to addition of the

antibody the homogenate was diluted to 0.5 µg/µL with immunoprecipitation buffer (IP buffer, containing in mmol/L: 150 NaCl, 10 HEPES pH=7.5, 1 Na pyrophosphate, 1 $MgCl_2$, 0.5 EGTA, 0.5 EDTA, 0.1 PUGNAc, 1% Triton X100, 1x protease and phosphatase inhibitor cocktail (Millipore Sigma)). After 2 hours of incubation with end-over-end rotation, 50 µL of Dynabeads protein G was added (beads washed and resuspended in IP buffer) and incubation resumed for another 2 hours. After removal of the supernatant, beads were washed with 1 mL IP buffer 6 times and final resuspension in 100 µL sample buffer. Immunoprecipitate was removed after heating beads to 95°C for 5 min, flash-frozen and stored at -80°C. Immunoprecipitate was analyzed by separation on 10-20% TGX criterion gels and transfer to nitrocellulose. Blots were probed with anti-PLB antibody (1:1,000 in 1xTBST, Thermo Fisher Scientific, Cat#: PA5-82945, rabbit) and anti-*O*-linked N-acetylglucosamine antibody (1:1,000 in 1xTBST, Abcam, Cat#: ab2739, mouse) and visualized with goat anti-mouse IRDye800CW (LI-COR, P/N: 926-32210, 1:10,000) and anti-rabbit IRDye680RD (LI-COR, P/N: 926-68071, 1:10,000). Two technical replicates have been made for each blot. Western blot images were analyzed in ImageJ.

Transcript analysis

Total RNA from the hearts of 12-week-old CaMKIIδ S280A and WT littermate mice was extracted using RNeasy Mini Kit (Qiagen). Conversion to cDNA was performed using QuantiTect Reverse Transcription Kit (Qiagen). Transcript analysis of genes encoding CaMKIIδ (CAMK2D), CaMKIIγ (CAMK2G), β-myosin heavy chain (MYH7), natriuretic peptide A (NPPA) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was carried out using real-time quantitative polymerase chain reaction (RT-qPCR) on an Applied Biosystems 7900HT Fast Real-Time PCR System and Primer-Probe detection. The following thermocycle steps have been used: (1) 50°C for 2 minutes, (2) 95°C for 10 minutes, (3) 40 cycles of 95°C for 15 seconds, and (4) 58°C for 1 minute. Three technical replicates were performed for each biological sample. The primers were from Eurofins Genomics with the following sequences: CAMK2D (forward, gtgacacctgaagccaaaga; reverse, catcatggaggcaacagtagag), CAMK2G (forward, aagctggagcctacgatttc; reverse, gcgctttgcagggtttatg), MYH7 (forward, agatggctggtttggatgag; reverse, ttggccttggtcagagtattg), NPPA (forward, caggccatattggagcaaatc; reverse, gggcatgacctcatcttctac), GAPDH (forward, aacagcaactcccactcttc; reverse, cctgttgctgtagccgtatt). Transcript data were normalized to GAPDH, and analysed using the threshold cycle (C_T) relative quantification method, then linearized ($2^{-\Delta CT}$) to make comparison.

Human echocardiography

Functional parameters for each patient were collected using echocardiography (Vivid E9 ultrasound system, GE Medical Systems, Milwaukee, WI, USA). All images were obtained by a trained sonographer using conventional echocardiographic patient positioning. Left ventricular volumes at end-diastole (LVEDV) and end-systole (LVESV) were obtained in the apical four- and two-chamber views. Ejection fraction (EF) was derived using two-dimensional echocardiography. Peak early diastolic filling velocity (E), diastolic relaxation velocity (e'), and late diastolic filling velocity (A) were obtained in the apical four-chamber view using pulsed wave Doppler with the sample volume placed between the mitral valve leaflets. Volumes were visually traced with papillary muscles excluded and calculated using the modified Simpson's biplane method in accordance with the guidelines of American Society of Echocardiography.

Protein analysis in human heart samples

Tissue from human patients was homogenized in buffer containing: 50 mmol/L Tris-HCl, pH=7.5, 3% SDS, phenyl methyl sulfonyl fluoride and phosphatase inhibitor (Roche). Immunoprecipitation was carried out using a Classic IP Kit (Pierce). Beads were conjugated with an anti-CaMKIIδ antibody (Badrilla, Cat#: A010-56AP, 5 µg antibody/50 µg protein homogenate, rabbit). The IgG heavy chain band was removed using Clean-Blot IP Detection Reagent (Thermo Fisher Scientific), which allowed better visualization of the CaMKII bands. Subsequent immunoblot was performed using antibodies against either total CaMKIIδ (Badrilla, Cat#: A010-56AP) or *O*-GlcNAc (Millipore, Cat#: MABS1254, 1:10,000, mouse), followed by secondary treatment with horseradish peroxidase-conjugated antibodies (Thermo

Fisher Scientific, Cat#: 31430 and 31460, respectively, both in 1:5,000) were used subsequently. Oxidized CaMKII was detected using a primary antibody specific to the oxidized MM281/282 sites (Millipore, Cat#: 07-1387, 1:2,500, rabbit). GAPDH was detected using a specific primary antibody (GeneTex, GT239, Cat#: GTX627408, 1:5,000, mouse). Chemiluminescent detection was performed with SuperSignal West Pico (Millipore) and blots were imaged using a Syngene gel doc system.

CaMKII activity assay

CaMKII activity was measured via the incorporation of ^{32}P from ^{32}P-ATP into an artificial substrate, syntide-2, as previously described.[9] HEK293 cells expressing wild type, S280A, or T287A CaMKII were lysed by sonication. Maximal activation was achieved by treating the CaMKII samples for 10 minutes with Ca^{2+} (200 µmol/L $CaCl_2$) and CaM (10 µmol/L), followed by incubation with syntide-2 (a CaMKII substrate) and ^{32}P-ATP for an additional 10 minutes. Samples were blotted on Whatman filter paper (Sigma) to quench the reaction and washed three times to remove the remaining ^{32}P-ATP, followed by scintillation counting to determine ^{32}P incorporation into syntide-2. Control samples were not exposed to Ca^{2+}/CaM to measure basal activity. To measure the effects of *O*-GlcNAcylation or phosphorylation on each CaMKII mutant, we followed the procedure outlined above to activate with Ca^{2+}/CaM, followed by exposure to either a combination of UDP-GlcNAc (100 µmol/L) and recombinant human *O*-GlcNAc transferase (OGT, 0.5 µg) or to ATP (100 µmol/L). After a 10-minute exposure, Ca^{2+} was chelated with EGTA (10 mmol/L). Any remaining detected activity was associated with autonomous activation of CaMKII. Control experiments were performed with only EGTA (to show reversal of Ca^{2+}/CaM dependent activation) and in the presence of UDP-GlcNAc but without OGT (to eliminate the possibility of metabolic effects independent of *O*-GlcNAcylation).

Statistical analysis

Pooled data are presented as Mean±SEM. The number of biological and technical replicates in each experimental group is reported in the figures and figure legends. Normality of the data was assessed by Shapiro-Wilk test and the equality of group variance was tested using Brown-Forsythe test. Statistical significance of differences for normally distributed data and $N \geq 6$ was determined using two-tailed Student's *t*-test (paired on unpaired) and ANOVA with Dunnett's multiple comparisons test, when applicable. If the data was not normally distributed or $N < 6$, we used non-parametric tests, including Wilcoxon matched-pairs signed rank test, Mann-Whitney *U* test, and Kruskal-Wallis one-way ANOVA followed by Dunn's multiple comparisons test. Categorical outcomes were evaluated using Fisher's exact test. GraphPad Prism 9 software was used for data analysis.

Blinded data acquisition and analysis have been performed for all *in vivo* experiments (echocardiography and electrocardiography). Animals were grouped with no blinding but randomized in cellular experiments. Fully blinded analysis was not performed in cellular studies because the same person carried out the experiments and analysis. Male and female animals were used in equal numbers for experiments in healthy animals, whereas STZ (and vehicle) injections have been performed in all male mice. No samples or animals were excluded from analysis, except for animals not exhibiting high blood glucose levels (>300 mg/dL) following STZ injections (limit was set up *a priori* in approved protocols of the Institutional Animal Care and Use Committee at University of California, Davis). Group sizes were determined by an *a priori* power analysis for a two-tailed Student's *t*-test with an α of 0.05 and power of 0.8, in order to detect a 20% difference signal at the endpoint. Representative traces/images reflected the average level of each experiment. Origin 2016 software was used for plotting the data.

	Non-diabetic	Diabetic	P value
Age (year)	64.2±2.5 [43 – 73]	62.9±2.5 [46 – 73]	*P=0.558*
Gender (Female/Male)	1/11	1/11	
Medications • metformin • beta-blocker • ACE inhibitor	 0 (0%) 3 (25%) 1 (8.3%)	 8 (66.7%) 5 (41.7%) 1 (8.3%)	
BMI (kg/m^2)	27.8±1.5 [20.4 – 39.0]	33.4±2.2 [26.3 – 50.0]	*P=0.033*
MAP (mmHg)	98.0±3.7 [83 – 128]	108.3±5.9 [73 – 140]	*P=0.173*
Glucose (mg/dL)	112.4±6.6 [81.1 – 158.5]	158.1±19.3 [91.9 – 297.3]	*P=0.117*
HbA1c (mmol/mol)	36.0±1.2 [29 – 45]	60.5±4.8 [44 – 97]	$P=2x10^{-6}$
DIA (hour)	N/A	7.7±1.2	
LVEDV (mL)	89.2±8.0	92.7±7.8	*P=0.811*
LVESV (mL)	38.7±4.2	43.8±6.0	*P=0.580*
EF (%)	56.1±2.0	54.4±2.7	*P=0.722*
E (cm/s)	60.7±4.1	68.7±4.4	*P=0.235*
A (cm/s)	70.8±4.9	83.4±7.6	*P=0.131*
E/A	0.90±0.09	0.92±0.14	*P=0.702*
e' (cm/s)	6.8±0.5	5.3±0.5	*P=0.045*
E/e'	9.3±0.6	14.7±2.0	*P=0.040*

Supplemental Table I. Demographic and clinical data of patients with and without diabetes included in the study of expressional analysis of CaMKII posttranslational modifications in the heart.

Human heart tissue samples were collected from patients undergoing on-pump coronary artery bypass graft surgery. None of the patients had history of myocardial infarction, atrial fibrillation, or severe chronic kidney disease requiring dialysis. Patients in the diabetic cohort were obese, slightly hypertensive, had elevated fasting blood glucose and glycated hemoglobin levels compared to the age- and sex-matched nondiabetic patients. Systolic heart function was preserved, and ventricular diameters were unchanged in M-mode echocardiography; however, patients with diabetes exhibited diastolic dysfunction in Doppler echocardiography.

Mean±SEM and minimum-maximum ranges are shown. Mann-Whitney test.

(ACE, angiotensin-converting enzyme; BMI, body mass index; HbA1C, glycated hemoglobin A1c; DIA, duration of insulin action; MAP, mean arterial pressure; LVEDV, left ventricular end-diastolic volume; LVESV, left ventricular end-systolic volume; EF, ejection fraction, calculated as EF=[LVEDV−LVESV]/LVEDV×100; E, early diastolic transmitral flow velocity; A, late diastolic transmitral flow velocity; e', diastolic relaxation velocity.)

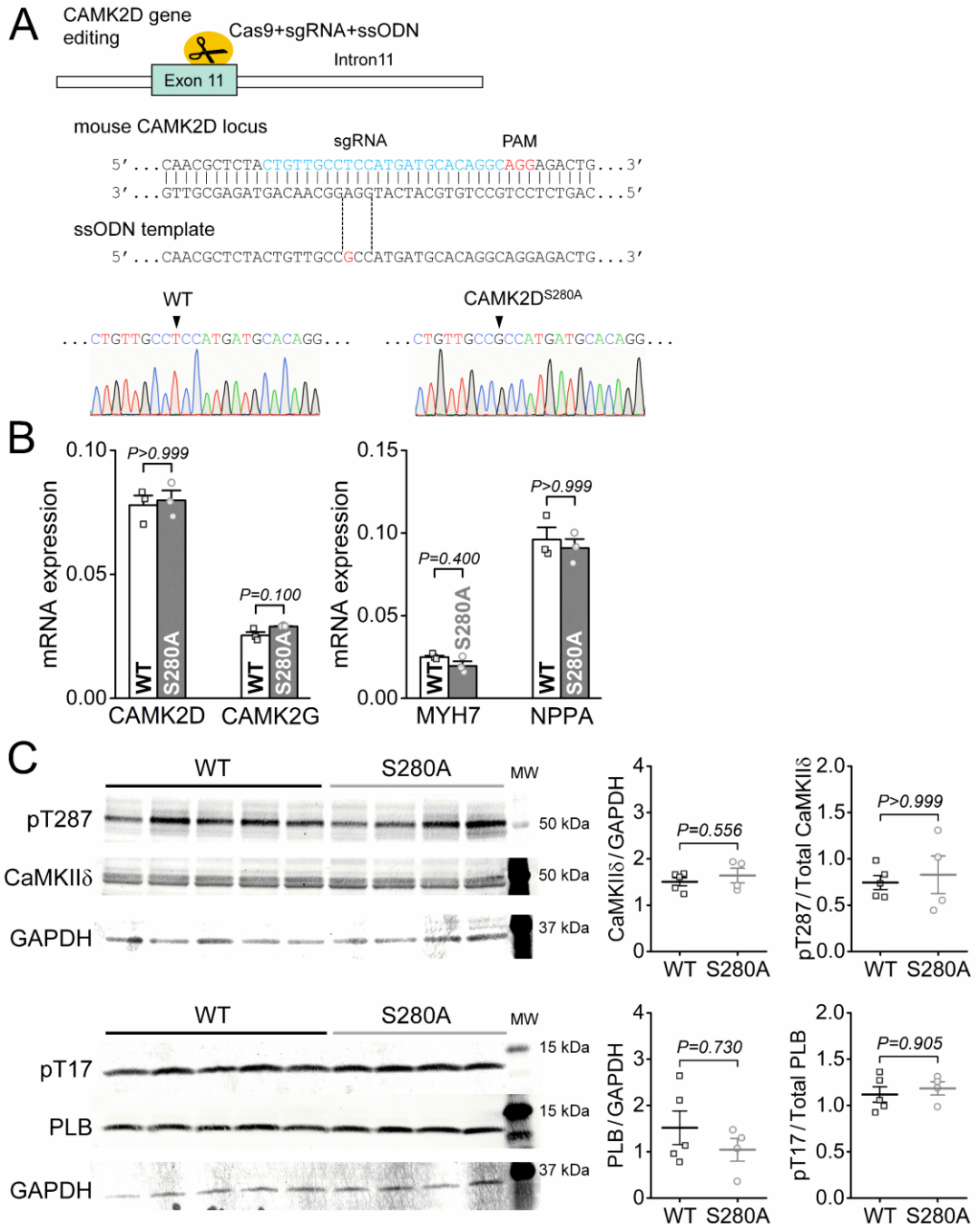
	CaMKIIδ WT	CaMKIIδ S280A	P value
Body weight (g)	23.0±1.4	23.3±0.8	*P=0.839*
Heart weight (mg)	205±18	196±11	*P=0.647*
HW/BW (mg/g)	9.0±0.9	8.6±0.7	*P=0.700*
Lung weight (mg)	148±7	148±7	*P=0.980*
Liver weight (mg)	1109±90	1106±91	*P=0.986*
Body length (mm)	95.5±1.6	95.3±0.6	*P=0.872*
Tibial length (mm)	20.7±0.2	20.9±0.1	*P=0.386*
Blood glucose (mg/dL)	165±7	173±9	*P=0.423*
Heart rate (1/min)	472±17	478±18	*P=0.826*
LVIDd (mm)	3.86±0.09	4.15±0.15	*P=0.087*
LVIDs (mm)	2.93±0.11	3.23±0.15	*P=0.103*
FS (%)	24.4±1.3	23.6±1.6	*P=0.697*
LVPWs	1.19±0.05	1.24±0.10	*P=0.667*
IVSs	1.19±0.05	1.26±0.08	*P=0.469*

Supplemental Table II. Morphometric and echocardiography data of CaMKIIδ S280A mice.
Morphometric and 2D echocardiography data of 12-wk-old CaMKIIδ S280A knock-in and wild-type (WT) littermate mice. 4 male and 4 female mice were in each group. Mean±SEM are shown. Student's *t*-test, two-tailed.
(HW/BW, heart weight to body weight ratio; LVIDd, left ventricular internal diameter at diastole; LVIDs, left ventricular internal diameter at systole; FS, fractional shortening, calculated as FS=[LVIDd−LVIDs]/LVIDd×100; LVPWs, left ventricular posterior wall thickness at systole; IVSs, interventricular wall thickness at systole.)

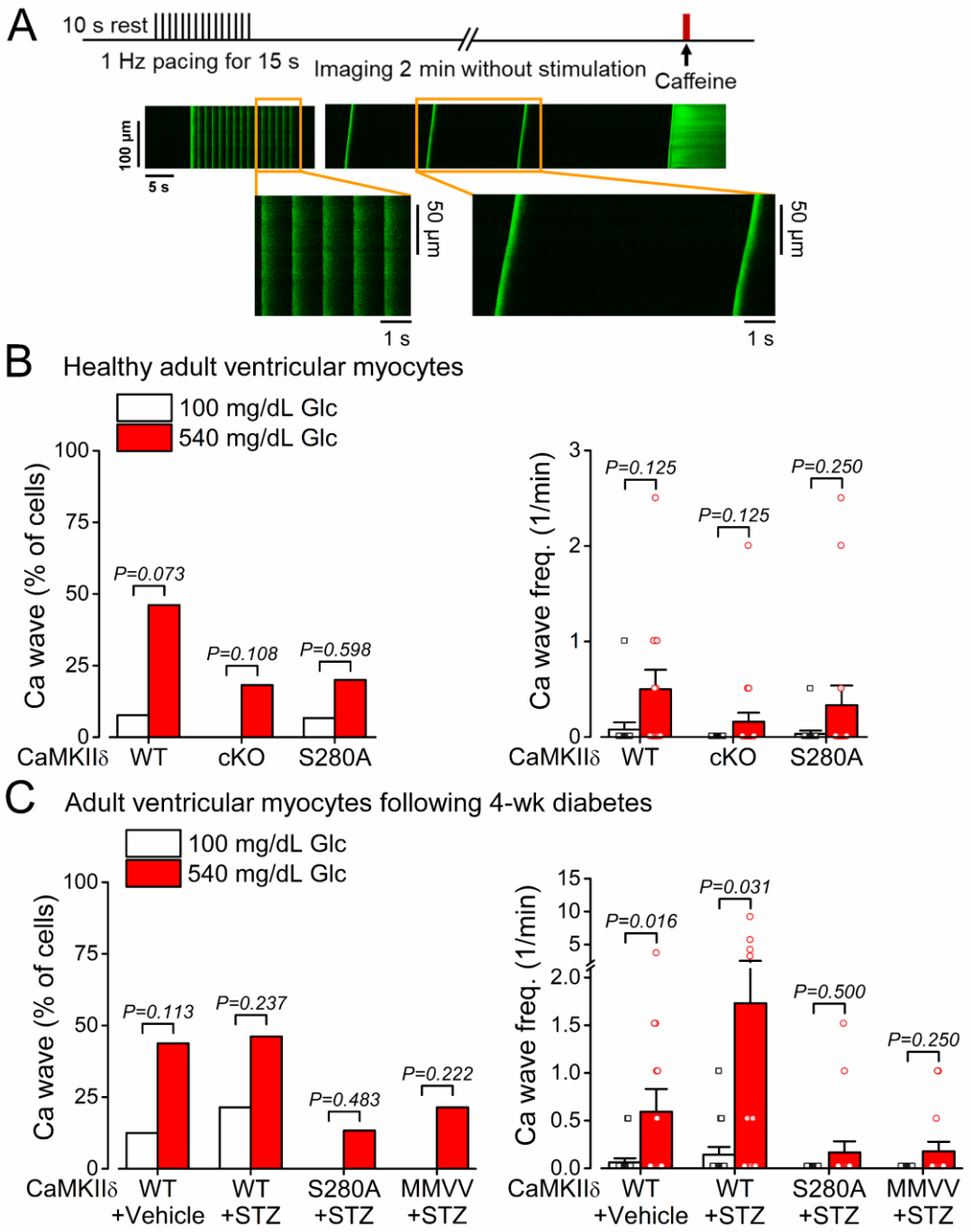
	WT + Vehicle		WT + STZ		S280A + STZ		MMVV + STZ	
	Pre-injection	Post-injection	Pre-injection	Post-injection	Pre-injection	Post-injection	Pre-injection	Post-injection
N (animals)	12		9		6		7	
Ejection fraction (%)	46.5±2.4	45.7±2.5^{NS}	47.8±3.4	44.5±1.8^{NS}	47.7±3.4	48.2±3.4^{NS}	47.6±2.5	47.9±2.3^{NS}
Fractional shortening (%)	22.4±1.5	22.6±1.5^{NS}	23.8±2.0	21.8±1.1^{NS}	24.6±2.4	25.7±2.5^{NS}	23.4±1.5	24.0±1.5^{NS}
LVIDd (mm)	3.73±0.16	4.03±0.09^{NS}	3.86±0.09	4.02±0.13^{NS}	4.11±0.14	4.25±0.20^{NS}	3.57±0.15	3.82±0.07^{NS}
LVIDs (mm)	2.92±0.17	3.12±0.08^{NS}	2.95±0.13	3.14±0.11^{NS}	3.28±0.15	3.14±0.16^{NS}	2.73±0.11	2.90±0.05^{NS}
LVPWd (mm)	0.87±0.06	0.83±0.03^{NS}	0.82±0.06	0.79±0.05^{NS}	0.78±0.08	0.76±0.03^{NS}	0.78±0.06	0.83±0.07^{NS}
LVPWs (mm)	1.24±0.08	1.19±0.06^{NS}	1.15±0.07	1.09±0.07^{NS}	1.14±0.09	1.09±0.15^{NS}	1.03±0.05	1.20±0.10^{NS}
IVSd (mm)	0.85±0.05	0.89±0.3^{NS}	0.83±0.05	0.90±0.06^{NS}	0.86±0.04	0.80±0.05^{NS}	0.94±0.04	0.88±0.04^{NS}
IVSs (mm)	1.16±0.05	1.26±0.05^{NS}	1.21±0.06	1.22±0.08^{NS}	1.19±0.09	1.11±0.06^{NS}	1.25±0.03	1.25±0.06^{NS}
LA area (mm^2)	2.51±0.04	2.61±0.05^{NS}	2.50±0.05	3.21±0.13$^{P=0.0002}$	2.55±0.05	2.90±0.08$^{P=0.01}$	2.55±0.06	2.99±0.08$^{P=0.002}$
E (mm/s)	643±22	706±37^{NS}	612±39	553±52^{NS}	726±49	598±28^{NS}	653±47	640±41^{NS}
A (mm/s)	416±25	480±047^{NS}	401±38	449±35^{NS}	477±46	403±28^{NS}	442±048	446±027^{NS}
E/A	1.58±0.06	1.56±0.11^{NS}	1.57±0.07	1.24±0.09$^{P=0.01}$	1.57±0.13	1.50±0.07^{NS}	1.52±0.08	1.46±0.10^{NS}
e' (mm/s)	17.0±0.6	19.8±1.2^{NS}	17.0±1.7	13.3±2.0^{NS}	19.5±1.0	17.6±1.6^{NS}	18.2±1.9	16.5±1.1^{NS}
E/e'	38.2±1.5	36.2±1.3^{NS}	37.2±1.7	45.8±4.9$^{P=0.11}$	37.3±2.5	35.7±4.2^{NS}	38.2±1.5	39.3±2.6^{NS}
Heart rate (1/min)	441±21	453±14^{NS}	441±21	453±14^{NS}	448±15	422±9^{NS}	465±26	424±23^{NS}

Supplemental Table III. Echocardiography parameters in diabetic mice.

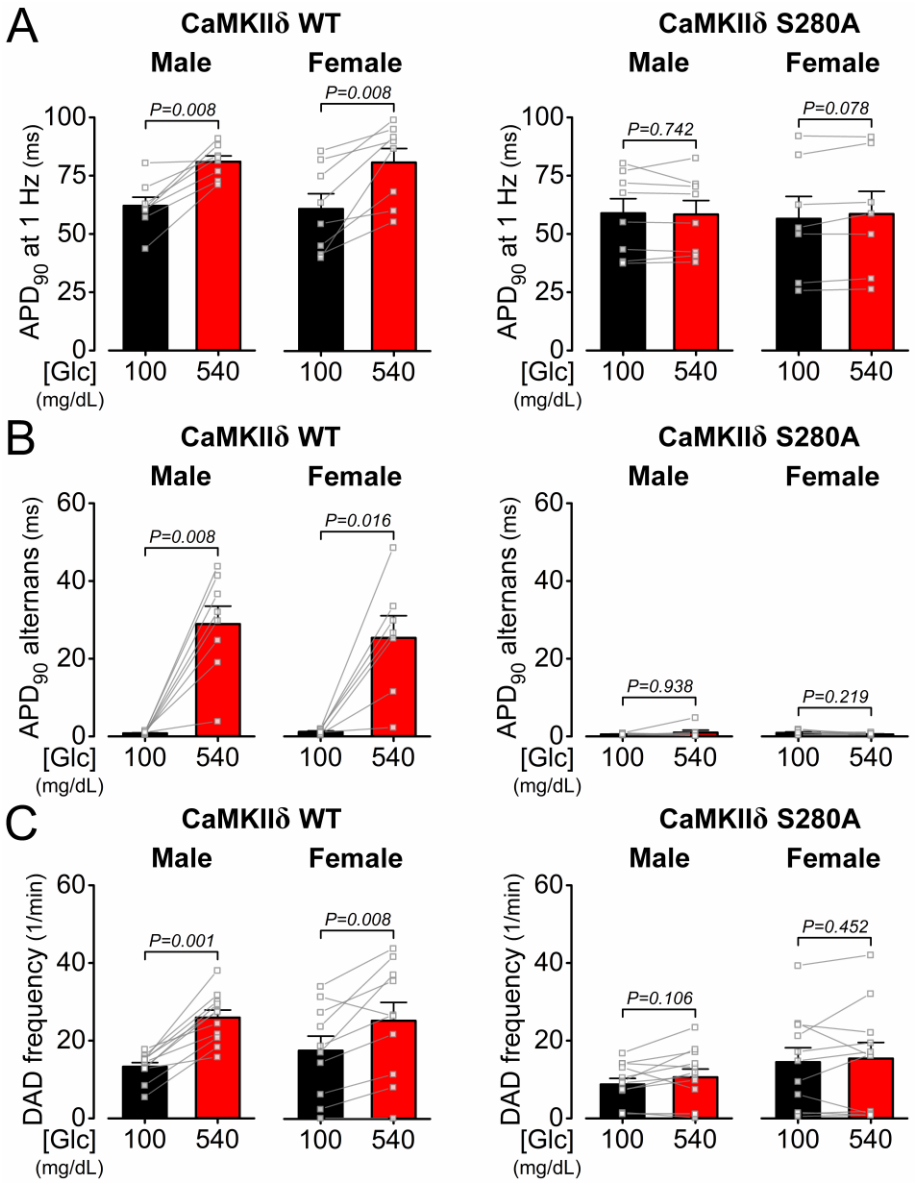
Systolic and diastolic heart functions were assessed by echocardiography before and 4 weeks after injection of low-dose streptozotocin (STZ) [or vehicle for control] to induce diabetes mellitus in CaMKIIδ wild-type (WT), S280A (*O*-GlcNAc resistant) and MMVV (oxidation-resistant) male mice. Comparisons between pre- and post-injection parameters were made in each genotype. Paired, two-tailed Student's *t*-test. *NS* indicates non-significance. (LVIDd and LVIDs are left ventricular end-diastolic and end-systolic diameters, respectively; LVPWd and LVPWs are left ventricular posterior wall thicknesses at diastole and at systole, respectively; IVSd and IVSs are intraventricular septal thicknesses at diastole and at systole, respectively; LA area, left atrial area; E, early diastolic transmitral flow velocity; A, late diastolic transmitral flow velocity; e', diastolic relaxation velocity.)



Supplemental Figure I. mRNA and protein expression in CaMKIIδ S280A murine hearts.
A, Generation of CaMKIIδ-S280A knock-in mouse model using CRISPR/Cas9 gene editing. **B,** mRNA expression of CaMKIIδ (CAMK2D), CaMKIIγ (CAMK2G), β-myosin heavy chain (MYH7), and natriuretic peptide A (NPPA) was unchanged CaMKIIδ-S280A knock-in (n=3 hearts) vs. wild-type (WT, n=3 hearts) littermate mouse heart samples. Three technical replicates were performed for each heart sample. Mann-Whitney test. **C,** Unaltered CaMKIIδ expression and autophosphorylation (pT287), and phospholamban (PLB) expression and phosphorylation (pT17, CaMKII site) in the CaMKIIδ-S280A knock-in (n=4 hearts) versus WT (n=5 hearts). Two technical replicates (blots) were performed for each heart sample. Mann-Whitney test.

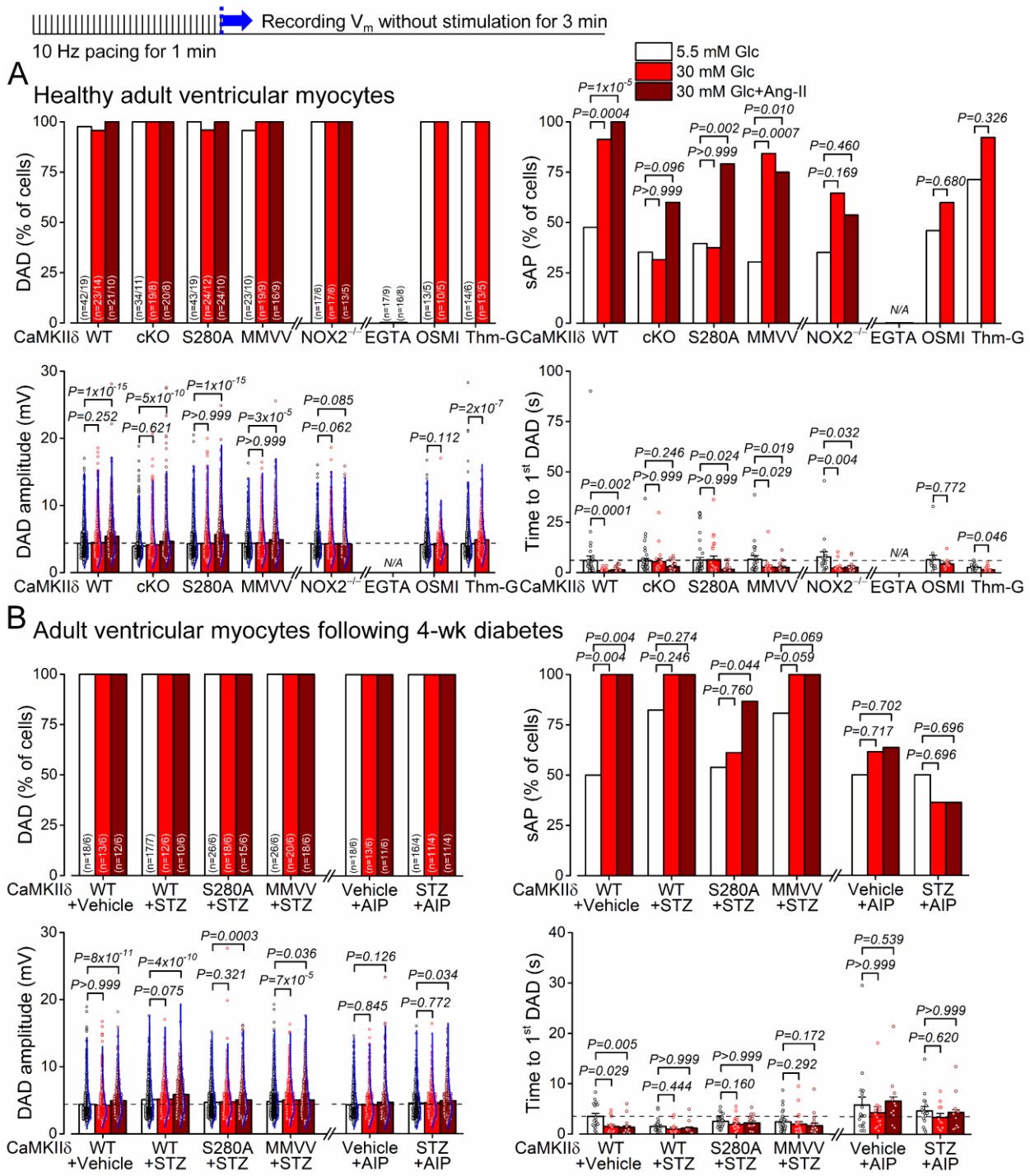


Supplemental Figure II. Glucose-induced spontaneous Ca^{2+} waves.
A, Experimental protocol and representative intracellular Ca^{2+} signals (Fluo-4 fluorescence) showing Ca^{2+} waves following high-glucose treatment (540 mg/dL, 6 minutes) in a wild-type (WT) murine ventricular cardiomyocyte. **B,** High-glucose treatment slightly increased the occurrence and frequency of Ca^{2+} waves in WT, which were prevented in CaMKIIδ cardiac-specific knockout (cKO) and in *O*-GlcNAc-resistant CaMKIIδ-S280A knock-in (n, WT=13 cells from 8 animals, cKO=22 cells from 9 animals, S280A=15 cells from 6 animals). **C,** Diabetes further increased Ca^{2+} waves, which were attenuated in both CaMKIIδ-S280A and oxidation-resistant CaMKIIδ-MMVV (n, WT+Vehicle=16 cells from 7 animals, WT+STZ=14 cells from 6 animals, S280A+STZ=15 cells from 5 animals, MMVV+STZ=14 cells from 5 animals). The number of cells exhibiting Ca waves in high-glucose was tested using Fisher's exact test. The change in Ca wave frequency in high-glucose was tested using Wilcoxon matched-pairs signed rank test.



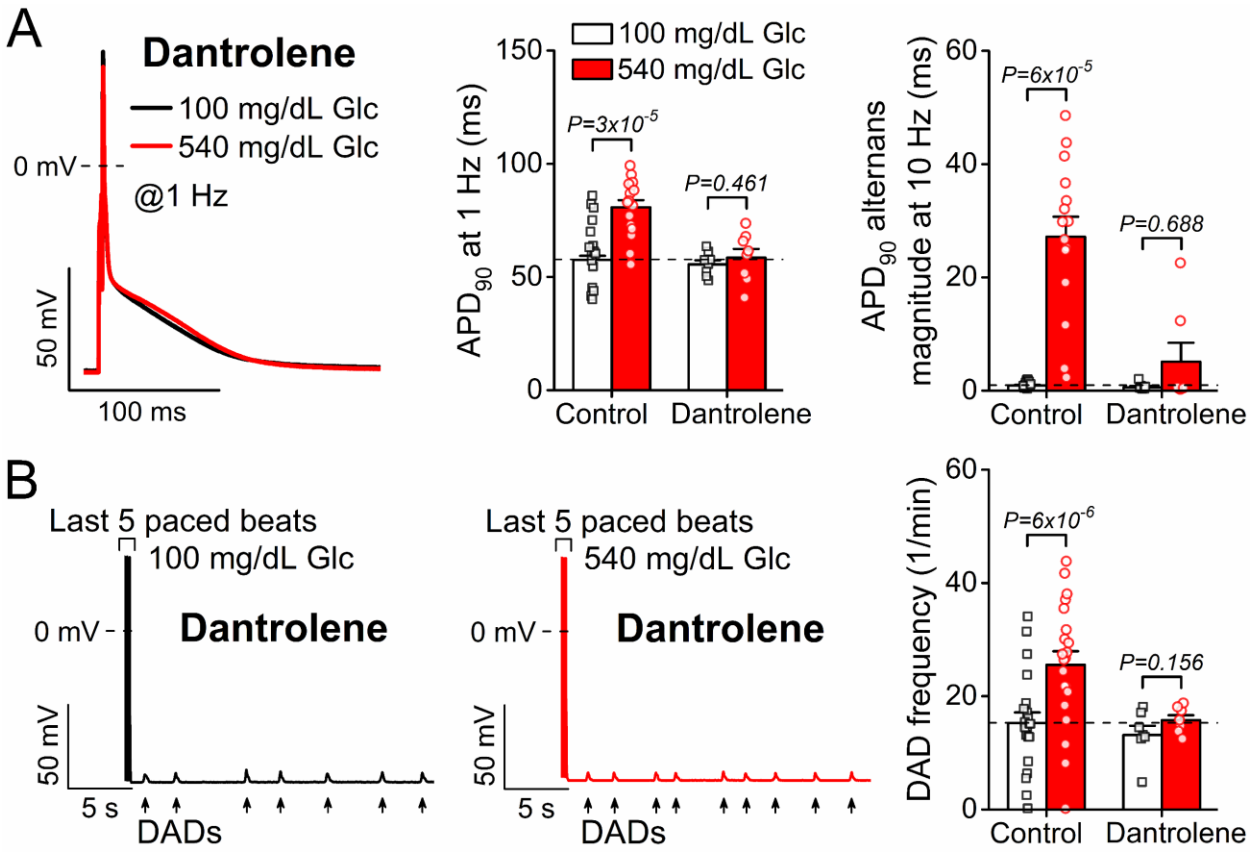
Supplemental Figure III. Similar electrophysiological response to acute hyperglycemia in males and females.

A, High-glucose treatment induced similar prolongation of the action potential duration at 90% repolarization (APD_{90}) at 1 Hz pacing in male (n=8 cells from 5 animals) and female (n=8 cells from 4 animals) wild-type (WT) murine ventricular cardiomyocytes. High-glucose did not prolong APD neither in male (n=8 cells from 4 animals) nor in female (n=7 cells from 4 animals) *O*-GlcNAc-resistant CaMKIIδ-S280A knock-in myocytes. Wilcoxon matched-pairs signed rank test. **B,** High-glucose treatment induced similar APD alternans at 10 Hz pacing in WT males (n=8 cells from 5 animals) and females (n=7 cells from 3 animals) but not in CaMKIIδ-S280A males (n=7 cells from 4 animals) and females (n=7 cells from 4 animals). Wilcoxon matched-pairs signed rank test. **C,** High-glucose treatment induced similar increase in the frequency of delayed afterdepolarizations (DADs) in WT males (n=11 cells from 5 animals) and females (n= 10 cells from 4 animals) but not in CaMKIIδ-S280A males (n=12 cells from 5 animals) and females (n=11 cells from 6 animals). Wilcoxon matched-pairs signed rank test.



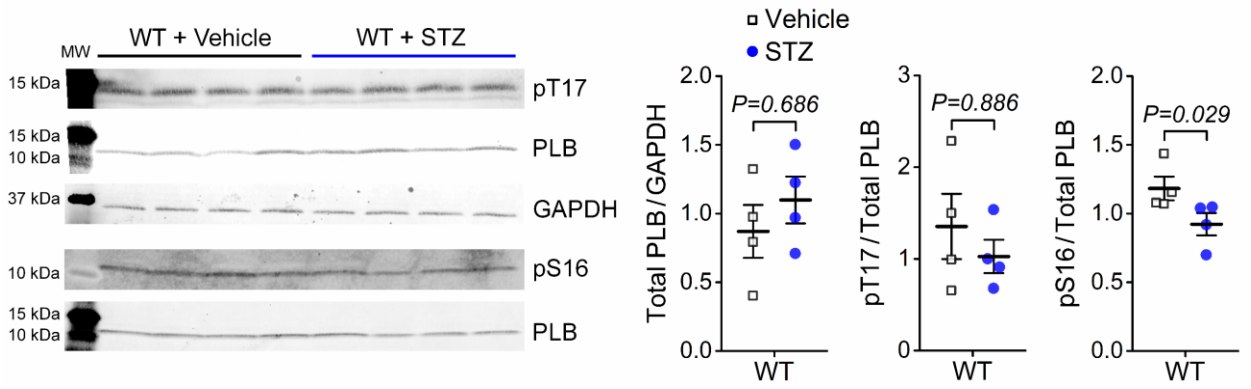
Supplemental Figure IV. Glucose-induced arrhythmogenic diastolic activities.

A, Statistics on arrhythmogenic diastolic activities (delayed afterdepolarizations, DADs; spontaneous action potentials, sAPs) following a tachypacing protocol shown above. **B,** Statistics on arrhythmogenic diastolic activities in diabetic cardiomyocytes 4-week after streptozotocin (STZ)-injection. Kruskal-Wallis one-way ANOVA, followed by Dunn's multiple comparisons test was used to compare 3 groups. Mann-Whitney test was used to compare 2 groups (EGTA, OSMI, Thm-G). The number of cells exhibiting DADs and sAPs was compared using Fisher's exact test. DAD amplitude data were fitted to a log-normal distribution curve (blue lines). The n numbers (total number of cells/animals) are reported in the figure.



Supplemental Figure V. Dantrolene prevents the arrhythmogenic electrophysiological changes in acute hyperglycemia.

A, Dantrolene (10 μM) prevented action potential duration (APD) prolongation and APD alternans induced by acute high-glucose treatment in murine ventricular cardiomyocytes (n, APD_{90}, control=16 cells from 9 animals, dantrolene=8 cells from 4 animals; APD alternans, control=15 cells from 8 animals, dantrolene=7 cells from 4 animals). Wilcoxon matched-pairs signed rank test. **B,** Dantrolene prevented the increase in arrhythmogenic diastolic activities (delayed afterdepolarizations, DADs) in acute high-glucose treatment (n, control=21 cells from 9 animals, dantrolene=7 cells from 4 animals). Wilcoxon matched-pairs signed rank test.



Supplemental Figure VI. Expression levels of total PLB and PLB phosphorylation (pS16, pT17). Western blot data of phospholamban (PLB) and its phosphorylation at S16 (PKA site) and T17 (CaMKII site) in vehicle-treated (n=4 hearts) and streptozotocin (STZ)-treated (n=4 hearts) WT mice. Two technical replicates (blots) were performed. Mann-Whitney test.