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## CaMKII Serine 280 O-GlcNAcylation Links Diabetic Hyperglycemia to Proarrhythmia

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### Abstract

**Rationale:** Diabetic hyperglycemia is associated with cardiac dysfunction and increased arrhythmia risk, and calcium/calmodulin-dependent protein kinase II (CaMKII) function has been implicated. CaMKII activity is promoted by both oxidation and *O*-linked  $\beta$ -N-acetylglucosamine (*O*-GlcNAc) of known CaMKII sites.

**Objective:** To investigate which post-translational modifications occur in human diabetic hearts and how they alter electrophysiological and Ca<sup>2+</sup> handling properties in hyperglycemia.

**Methods and Results:** We assessed echocardiography, electrophysiology, Ca<sup>2+</sup>-handling, and protein expression in site-specific CaMKII mutant mice (*O*-GlcNAc-resistant S280A and oxidation-resistant MM281/2VV knock-ins, and global and cardiac-specific knockouts), in myocytes subjected to acute hyperglycemia and angiotensin II (Ang-II) and mice after streptozotocin injections (to induce diabetes). Human patients with diabetes exhibit elevated CaMKII *O*-GlcNAcylation but not oxidation. In mice, acute hyperglycemia increased spontaneous diastolic Ca<sup>2+</sup> sparks and waves and arrhythmogenic action potential changes (prolongation, alternans and delayed afterdepolarizations), all of which required CaMKII-S280 *O*-GlcNAcylation. Ang-II effects were dependent on NADPH oxidase 2 (NOX2)-mediated CaMKII MM281/2 oxidation. Diabetes led to much greater Ca<sup>2+</sup> leak, RyR2 S2814 phosphorylation, electrophysiological remodeling, and increased susceptibility to *in vivo* arrhythmias, requiring CaMKII activation, predominantly via S280 *O*-GlcNAcylation and less via MM281/2 oxidation. These effects were present in myocytes at normal glucose, but were exacerbated with the in-vivo high circulating glucose. Phospholamban (PLB) *O*-GlcNAcylation was increased and coincided with reduced PLB S16 phosphorylation in diabetes. Dantrolene, that reverses CaMKII-dependent

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DISCLOSURES

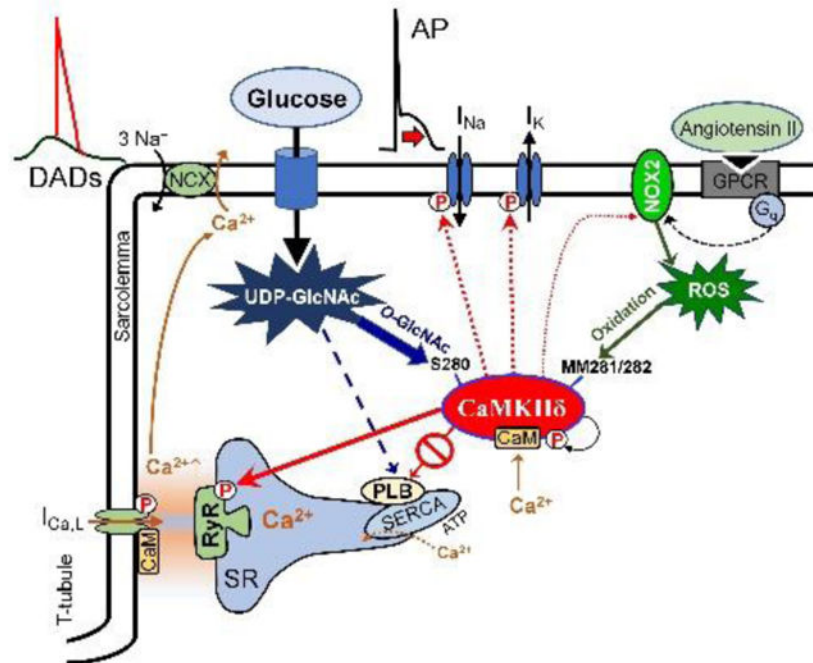
None.

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proarrhythmic RyR-mediated  $\text{Ca}^{2+}$  leak, also prevented hyperglycemia-induced APD prolongation and delayed afterdepolarizations.

**Conclusions:** We found that CaMKII-S280 O-GlcNAcylation is required for increased arrhythmia susceptibility in diabetic hyperglycemia, which can be worsened by an additional angiotensin II-NOX2-CaMKII MM281/2 oxidation pathway. CaMKII-dependent RyR2 S2814 phosphorylation markedly increases proarrhythmic  $\text{Ca}^{2+}$  leak and PLB O-GlcNAcylation may limit SR  $\text{Ca}^{2+}$  reuptake, leading to impaired excitation-contraction coupling and arrhythmogenesis in diabetic hyperglycemia.

### Graphical Abstract



### Keywords

Post-translational modifications; diabetic cardiomyopathy; excitation-contraction coupling; electrophysiology; action potential; diabetes mellitus; calcium signaling; calcium/calmodulin-dependent protein kinase II; Arrhythmias; Basic Science Research; Calcium Cycling/Excitation-Contraction Coupling; Cardiomyopathy; Cell Signaling/Signal Transduction

### INTRODUCTION

Cardiovascular complications including cardiomyopathy and arrhythmias are common sequelae of diabetes mellitus (DM) and the leading causes of mortality representing a major burden on human health.<sup>1, 2</sup> As the incidences of diabetes, prediabetes and metabolic syndrome steadily increase worldwide while effective therapies for diabetic cardiomyopathy remain lacking, there is a pressing need for a better understanding of the exact molecular mechanisms by which diabetes promotes cardiac disease, to uncover novel therapeutic targets in precision medicine. Diabetic hyperglycemia and oxidative stress are major

contributors to detrimental cardiac remodeling<sup>3</sup> in which impaired intracellular Ca<sup>2+</sup> handling and enhanced activity of Ca<sup>2+</sup>/calmodulin-dependent protein kinase II (CaMKII) have been implicated.<sup>4</sup> CaMKII delicately senses and translates the periodic intracellular Ca<sup>2+</sup> concentration rises in cardiomyocytes during excitation-contraction coupling to kinase activity.<sup>5</sup> Besides the Ca<sup>2+</sup>/calmodulin-dependent activation of CaMKII that may lead to autophosphorylation (pT287),<sup>6</sup> additional post-translational modifications (PTMs) by oxidation<sup>7</sup> and *O*-linked  $\beta$ -N-acetylglucosamine (*O*-GlcNAc)<sup>4, 8</sup> can also be enhanced in diabetes. Oxidation of MM281/282 pair<sup>9</sup> and *O*-GlcNAcylation of S280<sup>4</sup> have been identified in the regulatory hub region of CaMKII, and both promote autonomous, pathologically sustained kinase activity (molecular memory) leading to cardiac remodeling, heart failure (HF) and arrhythmias.<sup>10</sup>

Preventing the consequences of hyperglycemia and oxidative stress, including those by PTMs, is the focus of current research and clinical therapies in diabetes. Glycemic control is the basis of diabetes treatment and reduces cardiovascular complications and mortality.<sup>1</sup> Postprandial hyperglycemia and glucose-variability may further increase cardiovascular risk,<sup>11</sup> which may point to the memory function of CaMKII. *O*-GlcNAcylation of proteins, added by *O*-GlcNAc transferase (OGT) and removed by *O*-GlcNAcase (OGA),<sup>12</sup> is generally enhanced in the diabetic heart; however, reduction of either OGT<sup>13</sup> or OGA<sup>14</sup> expression leads to deleterious effects on the diseased heart suggesting a complex regulation of differential downstream targets including CaMKII,<sup>4</sup> phospholamban (PLB),<sup>15</sup> STIM1,<sup>16</sup> and histone deacetylase 4 (HDAC4)<sup>8</sup> among >4000 potentially *O*-GlcNAcylated proteins.<sup>17</sup> In line with this, intense glucose-lowering treatment may paradoxically increase the risk of HF,<sup>18</sup> which again raises the need for a detailed understanding of the exact molecular mechanisms in this signaling network. Antioxidant therapies have also been proposed based on a large body of preclinical evidence,<sup>19</sup> but to date, no clinical trial has shown clear benefit of antioxidant administration in patients with diabetes. Yet, the reduction of angiotensin-induced oxidative stress and better glycemic control can contribute to the benefit of neprilysin-angiotensin inhibition (sacubitril/valsartan) therapy to treat HF in patients with diabetes (PARADIGM-HF trial).<sup>20</sup> This evidence and unresolved questions prompted us to investigate the contribution of PTMs in CaMKII to the diabetic cardiac phenotype. A mechanistic understanding of this nuanced regulation may have important therapeutic implications by pointing to specific downstream targets that may explain ambiguous clinical results of glucose-lowering and antioxidant therapies in DM. Using PTM-resistant CaMKII mutant animals (MM281/2VV, S280A), we mechanistically investigated the contribution of each of these neighboring PTM sites in CaMKII to disease phenotype during acute and chronic diabetic hyperglycemia.

Here, we identified CaMKII S280 *O*-GlcNAcylation as the predominant regulator of increased arrhythmia risk in diabetic hearts. CaMKII MM281/2 oxidation was independent of hyperglycemia, was mediated by an angiotensin II - NADPH oxidase 2 (NOX2) pathway and played a secondary, yet synergistic role to promote autonomous kinase activity. The upregulated CaMKII induced sarcoplasmic reticulum (SR) Ca<sup>2+</sup> leak drove the arrhythmogenic electrophysiological remodeling in diabetic hyperglycemia.

## METHODS

### Data availability.

The authors declare that all data and methods supporting the findings of this study are available in the Data Supplement or from the corresponding authors on reasonable request. Please see expanded methods and the Major Resources Table in the Data Supplement.

### Human Heart Samples.

Human right atrial appendage (RAA) and left ventricular (LV) tissue samples were collected from patients undergoing on-pump coronary artery bypass graft surgery. Our informed consent practice, which required both verbal and written consent by each patient, conformed to the principles outlined in the Declaration of Helsinki. All human tissue work was carried out as outlined in an Ethics Agreement approved by the Human and Disability Ethics Committee of New Zealand (LRS/12/01/001). For each of the 12 patients in the diabetic and non-diabetic cohorts, RAA samples were removed under normothermic conditions before cross clamping for cardiopulmonary bypass. For a subset of 6 patients in each cohort, a small wedge (1.5×1.5×10 mm) was surgically excised from the epicardium of the LV anterior wall. Immediately after removal, tissue samples were placed in a sealed vial containing modified, low Ca<sup>2+</sup> (0.5 mmol/L) Krebs-Henseleit buffer containing (in mmol/L): NaCl 118.5, KCl 4.5, NaH<sub>2</sub>PO<sub>4</sub> 0.3, MgCl<sub>2</sub> 1, NaHCO<sub>3</sub> 25 and glucose 11. Within 5–10 minutes after removal, all tissues were flash-frozen and stored at –80°C.

### Mouse Models and Animal Procedures.

Several types of adult (10–12-wk-old) C57BL/6J mice were used, including wild-type (WT, Jackson Laboratory, Stock No. 000664), NOX2 knockout (Jackson Laboratory, Stock No. 002365), CaMKII $\delta$  cardiac-specific and global KOs,<sup>21, 22</sup> CaMKII $\delta$ -S280A-KI (that ablates the key O-GlcNAcylation site) created for this study,<sup>23</sup> but also used in two parallel studies,<sup>24, 25</sup> and MMVV-KI (that ablates key oxidation sites)<sup>7</sup>.

Diabetes mellitus was induced in 6 to 8-wk-old male mice by intraperitoneal injections with low-dose streptozotocin (STZ, 50 mg/kg body weight in 40 mmol/L sodium citrate, pH=4.0) for 5 consecutive days and littermates received sodium citrate as vehicle control. Blood glucose levels were measured in fresh blood samples collected from the middle tail vein using OneTouch UltraMini blood glucose monitoring system and test strips (LifeScan; measurement range: 20 to 600 mg/dL). Only mice exhibiting >300 mg/dL blood glucose levels following STZ injections (~70% success rate) were included in the study.

Enzymatic isolation of left ventricular cardiomyocytes was performed as previously described.<sup>25</sup> All animal handling and laboratory procedures were in accordance with the approved protocols (#19721 and #21064) of the Institutional Animal Care and Use Committee at University of California, Davis conforming to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (8<sup>th</sup> edition, 2011).

### Calcium Imaging.

Intracellular  $\text{Ca}^{2+}$  transients and diastolic  $\text{Ca}^{2+}$  events (sparks and waves) were measured using confocal microscopy in freshly isolated ventricular cardiomyocytes loaded with Fluo-4 AM. Paired experiments were performed at room temperature in Tyrode's solution containing normal glucose (100 mg/dL), then high glucose (540 mg/dL).

### Cellular Electrophysiology.

Recordings were performed in isolated ventricular cardiomyocytes using whole-cell patch-clamp with physiological solutions at 37°C (for ionic composition see Data Supplement).<sup>26</sup> APs were evoked with short suprathreshold depolarizing pulses at 1–10 Hz pacing frequencies in current-clamp experiments. Arrhythmogenic diastolic activities were assessed during a 3-minute period following 1-minute tachypacing at 10 Hz.

### In vivo ECG and Pharmacological Stress Test.

*In vivo* experiments were performed in anesthetized (isoflurane, 1–2%) diabetic (STZ-treated) and control (vehicle-treated) mice. For pharmacological stress test, mice were injected with isoproterenol (IP, 2 mg/kg) and caffeine (IP, 120 mg/kg). Premature ventricular complexes (PVCs) were counted during the next 60-minute period.

### Murine Echocardiography.

Transthoracic echocardiography was performed in anesthetized (isoflurane, 1.5%) animals before and after 4-wk STZ (or vehicle) treatment. Left ventricular B-mode, M-mode and Doppler images were acquired using a Vevo 2100 echocardiography system (FUJIFILM VisualSonics) equipped with a 40 MHz transducer. Body temperature was carefully monitored, and anesthesia was adjusted to achieve 400–500 beats/minutes heart rate in each animal.

### Fluorescence Recovery after Photobleaching (FRAP).

FRAP experiments were performed in intact rabbit ventricular cardiomyocytes expressing GFP-tagged WT or S280A CaMKII $\delta_C$  using an Olympus FluoView-1000 confocal microscope as previously described.<sup>27</sup> Isolated myocytes were plated on laminin-coated coverslips, cultured, and used for experiments within 18–22 hours of adenoviral infection. Myocytes were electrically field stimulated at 0.5 Hz for 5 minutes at room temperature before initiating FRAP by bleaching a circular region with a diameter of 7.3  $\mu\text{m}$  to 30–40% of the initial fluorescence value. Images were subsequently collected every 2.71 seconds to measure fluorescence recovery.

### Statistical Analysis.

Data are presented as Mean $\pm$ SEM. Normality of the data was assessed by Shapiro-Wilk test. Statistical significance of differences for normally distributed data was determined using two-tailed Student's *t*-test (paired or unpaired) or ANOVA, when applicable. If the data was not normally distributed, we used non-parametric tests. Categorical outcomes were evaluated using Fisher's exact test. In cellular experiments we performed hierarchical statistical analyses (nested tests) to account for inter-subject variability and non-independent sampling

(as multiple cells may come from one animal). GraphPad Prism 9 was used for data analysis.  $P < 0.05$  was considered statistically significant.

## RESULTS

### CaMKII O-GlcNAcylation is Enhanced in the Diabetic Human Heart.

Figure 1A illustrates five known PTM sites in the highly conserved regulatory region of CaMKII, immediately adjacent to the calmodulin (CaM) binding site. These include oxidation (MM281/282) and O-GlcNAcylation (S280), autophosphorylation (pT287) and S-nitrosylation (C290), each of which promotes autonomous kinase activity after  $\text{Ca}^{2+}$ /CaM dissociation (memory).

To directly test whether CaMKII $\delta$  S280 O-GlcNAcylation activates CaMKII autonomous activity, Figure 1B shows measurements of CaMKII-dependent incorporation of  $^{32}\text{P}$  from  $^{32}\text{P}$ -ATP into an artificial substrate, syntide-2 (as previously described<sup>28</sup> and in Data Supplement). Lysates of HEK293 cells expressing WT, S280A and T287A CaMKII $\delta$  were used. Maximal kinase activation was achieved by treating the CaMKII samples for 10 min with  $\text{Ca}^{2+}$  (200  $\mu\text{mol/L}$ ) and CaM (10  $\mu\text{mol/L}$ ; white bars), showing similar activation of all three CaMKII $\delta$  forms by  $\text{Ca}^{2+}$ -CaM. That activation is acutely reversed when  $\text{Ca}^{2+}$  is chelated with EGTA (10 mmol/L; grey bars). Robust autonomous kinase activity in WT CaMKII $\delta$  was achieved by inclusion of either ATP (100  $\mu\text{mol/L}$ ) or a combination of UDP-GlcNAc (100  $\mu\text{mol/L}$ ) and recombinant human OGT (0.5  $\mu\text{g}$ ) during the  $\text{Ca}^{2+}$ -CaM exposure and  $\text{Ca}^{2+}$ -quench with EGTA (pink and red bars, respectively). In CaMKII-S280A, only ATP induced autophosphorylation induced autonomous activity, whereas in T287A only O-GlcNAcylation induced autonomous CaMKII activation. Notably, the direct substrate for O-GlcNAcylation by itself (UDP-GlcNAc) was insufficient to activate autonomy in any case (teal bars) without inclusion of the endogenous enzyme (OGT) that mediates O-GlcNAcylation (red bars). These data demonstrate that S280 and T287 are independently required for O-GlcNAcylation-dependent and autophosphorylation-dependent CaMKII $\delta$  autonomy, respectively.

In human atrial and ventricular samples from patients with type 2 DM, we measured increased O-GlcNAcylation, but not oxidation, of CaMKII versus non-diabetic human samples (Figure 1C–D). Patients in the diabetic cohort exhibited higher fasting blood glucose and hemoglobin A1c values, were obese and slightly hypertensive, but did not exhibit chronic kidney disease, atrial fibrillation, or myocardial infarction, compared to age- and sex-matched non-diabetic (nDM) patients (Supplemental Table I). Importantly, the cardiac ejection fraction was preserved in both cohorts (EF >50%), although diabetic hearts exhibited diastolic dysfunction (60% increase in  $E/e'$ ). Thus, cardiac CaMKII O-GlcNAcylation is elevated and might contribute to cardiac dysfunction in human DM.

To study the functional consequences of CaMKII O-GlcNAcylation in the heart, we used a knock-in mouse model in which a previously identified serine residue<sup>4</sup> was mutated to alanine (S280A-KI) in CaMKII $\delta$  conferring resistance to O-GlcNAcylation (Supplemental Figure IA). CaMKII-S280A-KI mice exhibited unaltered baseline CaMKII $\delta$  expression, and its functional readouts including CaMKII autophosphorylation (pT287) and phospholamban

T17 phosphorylation (PLB pT17, a characteristic CaMKII target) at 12-week of age (Supplemental Figure IB–C). Likewise, the S280A-KI mice exhibited unaltered baseline cardiac contractile function on echocardiography, overall morphological parameters, and blood glucose levels (Supplemental Table II). Thus, the new *O*-GlcNAc-resistant mutant mice exhibit a normal baseline phenotype.

### High-Glucose Induced Diastolic SR Ca<sup>2+</sup> Leak and CaMKII Mobility Require CaMKII S280 *O*-GlcNAcylation.

CaMKII $\delta$  phosphorylation of the cardiac ryanodine receptor (RyR2) is a major contributor to pathological intracellular Ca<sup>2+</sup> mishandling. We tested whether diabetic hyperglycemia might alter intracellular Ca<sup>2+</sup> transients (CaTs) and SR Ca<sup>2+</sup> release events (Ca<sup>2+</sup> sparks and waves) in intact ventricular cardiomyocytes. We exposed freshly isolated cardiomyocytes to levels of high-glucose (540 mg/dL; 30 mmol/L) corresponding to what is observed in severe diabetes versus osmotically-matched (glucose substituted with equimolar mannitol) low-glucose (100 mg/dL; 5.5 mmol/L) conditions. Acute high-glucose treatment (6 min) did not alter the pacing-induced CaT amplitude or kinetics, but slightly reduced SR Ca<sup>2+</sup> content in WT, cardiac-specific CaMKII $\delta$ -KO (cKO) or S280A-KI mice (Figure 2A–B). However, high glucose induced a major increase in Ca<sup>2+</sup> spark frequency, whether normalized to SR Ca<sup>2+</sup> load or not (Figure 2C–D), but this effect was completely prevented in both the CaMKII $\delta$  cKO and S280A-KI mice. Likewise, there were more arrhythmogenic Ca<sup>2+</sup> waves induced by high-glucose in WT than cKO or S280A-KI mice (Supplemental Figure II). We conclude that the glucose-induced increase in diastolic SR Ca<sup>2+</sup> leak requires *O*-GlcNAcylation of CaMKII $\delta$  at S280, that may contribute to arrhythmogenesis.

It was recently shown that CaMKII $\delta$  activation promotes its subcellular mobility, which may facilitate phosphorylation of downstream targets outside the dyadic cleft to regulate contractile function, electrophysiology and gene transcription.<sup>27</sup> Figure 2E shows fluorescence recovery after photobleaching (FRAP) experiments in cardiomyocytes expressing GFP-tagged WT or S280A CaMKII $\delta$ . High-glucose treatment increased the FRAP kinetics for WT, but not CaMKII $\delta$ -S280A (Figure 2E), indicating that this increased CaMKII $\delta$  mobility is also dependent upon S280 *O*-GlcNAcylation.

### High-Glucose Induces Arrhythmogenic Action Potentials by CaMKII S280 *O*-GlcNAcylation

Enhanced CaMKII $\delta$  activity is associated with cardiac arrhythmias. We tested whether acute diabetic hyperglycemia alters ventricular action potential duration (APD) and arrhythmogenic activities. High-glucose treatment prolonged APD at lower pacing rates (1–3 Hz) and induced significant APD alternans in subsequent beats at high pacing rates (9–10 Hz; Figure 3A). Both of these arrhythmogenic AP changes were prevented in CaMKII $\delta$ -cKO and S280A but not in the oxidation-resistant CaMKII $\delta$ -MMVV (Figure 3B–D). Similar arrhythmogenic AP responses were observed in males and females (Supplemental Figure III). Acutely blocking *O*-GlcNAcylation using the specific OGT inhibitor OSMI-1 (50  $\mu$ mol/L) or chelating intracellular Ca<sup>2+</sup> using EGTA in the pipette (10 mmol/L) both prevented the arrhythmogenic AP alterations by high-glucose (Figure 3E–F). Conversely, blocking *O*-GlcNAcase using the specific OGA inhibitor Thiamet-G (Thm-G, 100 nmol/L) induced these same APD changes even at normal [glucose], and further enhanced the

hyperglycemia effects. These results indicate that CaMKII *O*-GlcNAcylation, but not oxidation mediate the acute hyperglycemic arrhythmogenic effects on APD.

Angiotensin-II (Ang-II) promotes both CaMKII activation and ROS production, and could contribute to the DM disease phenotype. When we added Ang-II (100 nmol/L) on top of high glucose it exacerbated the high-glucose effects on APD in WT myocytes (Figure 3E–F), but not in MMVV and NOX2-KO mice (NOX2<sup>-/-</sup>). Notably, both the high-glucose and Ang-II effects on APD were completely absent in CaMKIIδ-cKO myocytes. Taken together, these data indicate that NOX2-mediated CaMKIIδ oxidation at MM281/2 as a separate and additive effect of Ang-II to that caused by high glucose alone.

Diastolic arrhythmogenic activities such as delayed afterdepolarizations (DADs) and spontaneous APs (sAPs) were also enhanced by acute hyperglycemia and angiotensin II (Figure 4A–D and Supplemental Figure IV). Indeed, the changes closely paralleled the glucose and Ang-II effects on APD with respect to the five different mouse lines studied. That is, high glucose induction of DADs and sAPs required CaMKIIδ-S280 and *O*-GlcNAcylation, but not NOX2 or MM281/2 on CaMKIIδ, whereas Ang-II required NOX2 and CaMKIIδ-MM281/2. CaMKIIδ was essential to both high glucose and Ang-II effects, and those were only additive in the WT mice. As for APD effects above, this suggests two distinct signaling pathways that activate CaMKIIδ at neighboring regulatory sites. Dantrolene, which shifts back to normal the pathological and leaky RyR2 conformation that is induced by CaMKII phosphorylation,<sup>29</sup> prevented hyperglycemia-induced arrhythmogenic AP changes and diastolic DADs (Supplemental Figure V).

### Diabetes Increases Arrhythmia Susceptibility by CaMKII Activation.

Next, we tested the contribution of these CaMKIIδ PTM sites to disease phenotype in chronic DM. On repeated low-dose streptozotocin (STZ) injections, mice developed severe hyperglycemia and were subject to morphometric and functional evaluation following 4 weeks of diabetes (Figure 5A). This time point was chosen based on literature data showing that 4-wk following STZ injections mice already exhibit cardiac remodeling<sup>8</sup> without chronic kidney disease.<sup>30</sup> This allowed us to focus primarily on the hyperglycemia effects on the heart rather than the secondary renal complications. Similar levels of hyperglycemia were seen in WT, S280A and MMVV diabetic animals (Figure 5B) and similar weight loss with unaltered heart weight to body weight ratio were observed in all three mouse lines (Figure 5C). The ejection fraction and ventricular wall thickness in echocardiography were unchanged following STZ in all experimental groups, indicating preserved systolic function at this early diabetic stage (Figure 5B, D). However, STZ induced some changes in diastolic function (enlarged left atria, 21% decrease in E/A, 26% increase in E/e') in WT, which were attenuated in CaMKIIδ-S280A and slightly also in MMVV (Figure 5E). STZ-treated WT mice also exhibited higher incidence of premature ventricular complexes (PVCs) on *in vivo* ECG recordings following caffeine+isoproterenol stress test, whereas PVC incidence was decreased in STZ-treated CaMKIIδ-KO (Figure 5F), indicating a CaMKIIδ-dependent marked increase in arrhythmia susceptibility in diabetes. In line with this, RyR2 S2814 phosphorylation in diabetic WT hearts was found to be 1.93 times higher than in non-diabetic WT hearts, while phosphorylation of RyR S2814 was absent in CaMKIIδ-KO



(Figure 5G). Moreover, there was a 26% increase in PLB O-GlcNAcylation in STZ-treated WT and 6% increase in STZ-treated CaMKII $\delta$ -KO (Figure 5G), which was reported to occur at Ser16.<sup>15</sup> There was an increasing tendency for PLB expression (by 26%) in diabetic hearts, whereas PLB phosphorylation on S16 site was decreased by 22% and phosphorylation on T17 site was unchanged in diabetes (Supplemental Figure VI).

### Diabetes Promotes Diastolic SR Ca<sup>2+</sup> Leak via CaMKII S280 O-GlcNAcylation.

We assessed myocyte Ca<sup>2+</sup> handling in ventricular cardiomyocytes freshly isolated from diabetic hearts. STZ induced a slight reduction in intracellular CaT amplitude and rate of [Ca<sup>2+</sup>]<sub>i</sub> decay in all three genotypes (Figure 6A–B). SR Ca<sup>2+</sup> load was preserved in normal glucose and slightly reduced in high-glucose conditions (Figure 6B), independent of CaMKII $\delta$  PTMs. However, diabetes increased Ca<sup>2+</sup> sparks and waves in WT in normal glucose and even further in high-glucose (as occur *in vivo* in STZ-treated diabetic animals, Figure 6C–D and Supplemental Figure IIC). Focusing on the Ca<sup>2+</sup> spark frequency normalized to SR Ca<sup>2+</sup> load (Figure 6D, right) STZ induced an elevation of baseline Ca<sup>2+</sup> sparks in WT and CaMKII $\delta$ -MMVV-KI, but not in S280-KI mice. Moreover, restoring [glucose] to the levels seen *in vivo* in the STZ mice (540 mg/dL) further increased Ca<sup>2+</sup> sparks in all but the S280A myocytes. This has two important implications: 1) in the *in vivo* situation the WT diabetic mice have nearly 4 times the rate of arrhythmogenic SR Ca<sup>2+</sup> leak as in normal WT mice, and 2) that the reversal of high glucose during cell isolation and incubation at normal 100 mg/dL glucose only partially reverses the chronic activation (i.e., there is both an acutely reversible and a chronic component). Notably, these effects were completely prevented in CaMKII $\delta$ -S280A and slightly attenuated in CaMKII $\delta$ -MMVV.

### Diabetes Induces Arrhythmogenic APDs Predominantly by CaMKII S280 O-GlcNAcylation.

Finally, in Figures 7–8 we subjected WT and diabetic myocytes to similar electrophysiological analysis as was done for acute hyperglycemia in Figures 3–4, informed by the *in vivo* proarrhythmic tendencies observed at the whole animal level in Figure 5F. Diabetes induction by STZ led to pronounced AP remodeling even when measured at 100 mg/dL glucose, with APD prolonged versus vehicle controls and alternans already at physiological pacing rates (8 Hz) versus negligible alternans in vehicle controls even at 10 Hz (Figure 7A, 7E–F). Raising glucose in STZ myocytes to 540 mg/dL further increased APD and induced APD alternans already at 6 Hz. These arrhythmogenic AP changes were prevented by acute cell pretreatment with a selective CaMKII inhibitor peptide AIP (1  $\mu$ mol/L; Figure 7B). Importantly, high glucose also failed to increase APD or alternans in diabetic CaMKII $\delta$ -S280A myocytes (Figure 7C), but in MMVV myocytes the effects were nearly as large as in WT+STZ (Figure 7D). Conversely, Ang-II did not induce additional APD prolongation or alternans in MMVV myocytes, although it did in the other three myocyte groups studied (Figure 7E–F). The conclusions here parallel the above for SR Ca<sup>2+</sup> leak, in that CaMKII $\delta$ -S280 appears essential for the high-glucose induced effects on APD and alternans, whereas the Ang-II effect requires CaMKII $\delta$ -MM281/2. Furthermore, the 4-week STZ treatment alone produces effects that are incompletely reversed by simply lowering extracellular glucose from the high *in vivo* levels to those in the standard perfusate during cell isolation and incubation (leftmost 2 white bars in Figure 7E–F). However, even

the acute restoration of the diabetic extracellular glucose level can further promote these APD effects, and may more closely resemble the *in vivo* situation in these diabetic mice.

Spontaneous diastolic DADs and sAPs were also measured in vehicle and STZ-treated mice (Figure 8A–D and Supplemental Figure IVC) and the effects qualitatively parallel and extend those for control animals (in Figure 4A–D) and for APD (Figure 7A–F). That is, STZ promoted DADs and sAPs substantially, even at baseline glucose levels, in WT and CaMKII $\delta$ -MMVV mice and this was exacerbated at the diabetic glucose level, especially in WT mice. However, those effects were completely prevented in CaMKII $\delta$ -S280A mice. An interesting note here is that Ang-II recruited a further increase in DADs (versus high glucose) only in the S280A mice (which had the lowest value in high glucose). But that may reflect a sort of ceiling effect for DADs in the WT cells, because Ang-II still promoted sAPs in all but the MMVV mice (Figure 8C–D).

## DISCUSSION

CaMKII upregulation has been linked to various heart diseases including cardiac hypertrophy, HF and arrhythmias.<sup>31</sup> Altered intracellular Ca<sup>2+</sup> signaling can be a cause but also a consequence of pathological CaMKII activation that leads to impairment of fundamental cellular processes such as excitation-contraction coupling, transcriptional regulation, cell mechanics and energetics.<sup>10, 31, 32</sup> PTMs of CaMKII may lead to sustained kinase activation when the intracellular Ca<sup>2+</sup> transient has not yet been changed, thus promoting disease. CaMKII upregulation has been shown in diabetic cardiomyopathy, and CaMKII inhibition provided substantial benefit in various diabetic animal models.<sup>10</sup> Increased *O*-GlcNAcylation of CaMKII has previously been identified in diabetes;<sup>4, 8</sup> however, its contribution to disease remained elusive. Here we show that a site-specific mutation of CaMKII that prevents *O*-GlcNAcylation (S280A knock-in) largely attenuated the cellular arrhythmogenic consequences of diabetic hyperglycemia without affecting the baseline phenotype.

Increased production of reactive oxygen species (ROS), previously shown in diabetes,<sup>33</sup> may further enhance CaMKII activation by oxidation of two neighboring methionines (281/2) in the regulatory domain of CaMKII $\delta$ .<sup>9</sup> Interestingly, it was previously shown that the increased ROS production and upregulation of p47phox and p67phox (NOX2 associated proteins) were attenuated following CaMKII inhibition in diabetic cardiomyocytes.<sup>34</sup> However, we found that acute hyperglycemia led to CaMKII activation and its cellular consequences (RyR leak-mediated arrhythmogenic AP changes) exclusively via *O*-GlcNAcylation of S280 and not via MM281/2 oxidation. Even in chronic diabetes, CaMKII $\delta$ -S280A was found to be largely protective, whereas CaMKII $\delta$ -MMVV had only a minor role. These data suggest that CaMKII *O*-GlcNAcylation can be an early step in hyperglycemia-induced signaling, which then triggers kinase activation and may lead to further detrimental cellular remodeling, including increased ROS production.<sup>24</sup> Moreover, a stimulated renin-angiotensin-aldosterone system, characteristic of type 2 diabetes and metabolic syndrome,<sup>35</sup> can induce NOX-2-dependent ROS production and CaMKII oxidation.<sup>4, 36</sup> In contrast with our data here, Mesubi *et al.* recently reported a predominant role for CaMKII oxidation in atrial fibrillation susceptibility in diabetes induced by one-time

high-dose STZ or repeated low-dose STZ+high-fat diet.<sup>37</sup> Therefore, the contribution of *O*-GlcNAcylation and oxidation of CaMKII may differ in different diabetes types, cardiac regions, and stages of disease. Overall, our results highlight the importance of tight blood glucose control in patients with diabetes and suggest that even significant temporary rises in blood glucose levels (impaired glucose tolerance) may lead to significant CaMKII activation by *O*-GlcNAcylation.

A dynamic cross-talk between *O*-GlcNAcylation and phosphorylation at the same or neighboring serine/threonine residues may occur in many nucleocytoplasmic proteins.<sup>38</sup> In acute hyperglycemia, both S280 *O*-GlcNAcylation and T287 autophosphorylation of CaMKII $\delta$  were previously found to be enhanced,<sup>4</sup> suggesting a synergy between the two PTMs, creating molecular memory and prolonged active open state of the kinase. On the contrary, *O*-GlcNAcylation of PLB at S16 was shown to inhibit the phosphorylation of the same site by protein kinase A (PKA) and inhibit SERCA2, where only one of these modifications is possible at a given serine.<sup>15</sup> This mechanism may also affect the CaMKII-dependent phosphorylation of the neighboring T17 in PLB because the Stokes radius of an *O*-GlcNAc moiety is ~5 times larger than a phosphate. In line with this, PLB *O*-GlcNAcylation was increased, pS16 was decreased, and pT17 levels were unchanged in diabetes (Figure 5G and Supplemental Figure VI), and the intracellular Ca<sup>2+</sup> transient decay was prolonged (Figure 6) indicating slowed SR Ca<sup>2+</sup> ATPase (SERCA) and higher inhibition by PLB. These results agree with literature data reporting increased PLB expression and reduced pPLB levels in diabetes.<sup>39</sup> Similarly, *O*-GlcNAcylation of HDAC4 at S642 was shown to prevent CaMKII-dependent phosphorylation at S632, which can be a protective mechanism against remodeling and systolic HF.<sup>8</sup> However, the exact spatiotemporal details of these signaling networks (e.g., which events may occur first) is yet to be determined. According to the current dogma, CaMKII gets activated predominantly in the dyadic cleft where local intracellular [Ca<sup>2+</sup>] may rise to 50  $\mu$ mol/L during systolic SR Ca<sup>2+</sup> release. From here, CaMKII may translocate to phosphorylate cytoplasmic and nuclear targets. As for Ca<sup>2+</sup>-dependent CaMKII activation,<sup>27</sup> we found CaMKII-S280 *O*-GlcNAcylation in acute hyperglycemia further enhances CaMKII $\delta$  mobility in the cytosol.

Remodeling in several ion channels, including voltage-gated Na<sup>+</sup>, Ca<sup>2+</sup> and K<sup>+</sup> channels have been shown in diabetes resembling the one that occurs via CaMKII signaling.<sup>10, 31</sup> We have recently shown that K<sup>+</sup> channel downregulation in diabetes was markedly attenuated in CaMKII-S280 mice.<sup>25</sup> Diabetes is also frequently associated with HF, which is known to induce complex ion channel remodeling<sup>26, 40–42</sup> and intracellular Ca<sup>2+</sup> mishandling<sup>32, 43, 44</sup> leading to impaired AP repolarization and SR Ca<sup>2+</sup> leak (via RyR pS2814), and subsequent increase in the susceptibility to triggered activities and arrhythmias. This is in line with a recently demonstrated two-hit arrhythmia model in diabetic hyperglycemia.<sup>45</sup> CaMKII inhibition has been shown to prevent these arrhythmogenic events in various preclinical models of diabetes and HF.<sup>10, 31</sup> However, global CaMKII inhibition may have some detrimental effects in specific conditions relevant to diabetes, such as acidosis.<sup>46</sup> Preventing just the *O*-GlcNAcylation of CaMKII and repressing its pathological autonomous activation may serve as a precision medicine approach to prevent arrhythmias in diabetic hyperglycemia.

Diastolic dysfunction is an early manifestation of diabetic cardiomyopathy and frequently reported in patients.<sup>47</sup> Here, we show that CaMKII -S280 *O*-GlcNAcylation may contribute to diastolic dysfunction in diabetes. This effect could be mediated by the increased diastolic SR Ca<sup>2+</sup> leak and also the phosphorylation of titin which increases diastolic stiffness in HF.<sup>48</sup> However, assessing the contribution of CaMKII to diastolic dysfunction requires further investigation in additional animal models. Another limitation of our study is that we did not broadly assess possible phosphorylation differences attributed to CaMKII-S280 *O*-GlcNAcylation among the many CaMKII substrates in the diabetic hearts. HF with preserved ejection fraction (HFpEF) is a major clinical challenge in diabetes, with very limited treatment options currently available.<sup>49</sup> Empagliflozin, a sodium-glucose cotransporter-2 (SGLT2) inhibitor was demonstrated to significantly reduce cardiovascular mortality and hospitalization for HF in patients with diabetes (EMPA-REG OUTCOME trial).<sup>50</sup> Importantly, empagliflozin was shown to reduce CaMKII activation and its cellular consequences in failing murine and human ventricular myocytes.<sup>51</sup> Along those lines, the related drug exenatide induced of Ca/CaM dependent serine protein kinase (CASK) that inhibit CaMKII, and could partially ameliorate hyperglycemic effects that we described here.<sup>52</sup> Moreover, empagliflozin blunted postprandial glucose excursions and reduced glucose-variability in patients with diabetes, which may attenuate *O*-GlcNAc-dependent CaMKII activation.<sup>53</sup> In line with this, dapagliflozin, another SGLT2 inhibitor was shown to reduce *O*-GlcNAcylated protein levels in a type 2 diabetic murine model.<sup>54</sup>

Enhanced *O*-GlcNAcylation was found in HF also in non-diabetic patients<sup>4, 55</sup> and animals<sup>13</sup> as a result of altered cardiac metabolism. CaMKII *O*-GlcNAcylation was also enhanced in human failing hearts, and even higher in HF patients with diabetes.<sup>4</sup> Therefore, it may represent a therapeutic target in a broader range of heart diseases. Moreover, CaMKII *O*-GlcNAcylation is not restricted to the heart, but it has also been shown in brain samples of patients with diabetes and may occur in other organs as well.<sup>4</sup> Impaired *O*-GlcNAcylation has been implicated in the pathogenesis of Alzheimer's disease,<sup>56</sup> neurodegeneration<sup>57</sup> and cancer.<sup>57</sup> The potential involvement of CaMKII *O*-GlcNAcylation in those diseases is yet to be tested. Indeed, the ability of hyperglycemia to promote autonomous activation of the widespread and multifunctional CaMKII (already implicated in pathological heart disease) suggest value in further exploration of this pathway in other diseases.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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### Nonstandard Abbreviations and Acronyms:

<b>AIP</b>	autocamtide-2-related inhibitory peptide
<b>AP</b>	action potential
<b>APD</b>	action potential duration
<b>Ang-II</b>	angiotensin II
<b>CaM</b>	calmodulin
<b>CaMKII</b>	Ca <sup>2+</sup> /calmodulin-dependent protein kinase II
<b>CaT</b>	Ca <sup>2+</sup> transient
<b>cKO</b>	cardiac-specific knockout
<b>DAD</b>	delayed afterdepolarization
<b>DM</b>	diabetes mellitus
<b>EF</b>	ejection fraction
<b>EGTA</b>	ethylene glycol-bis( $\beta$ -aminoethyl ether)-N,N,N',N'-tetraacetic acid
<b>FRAP</b>	fluorescence recovery after photobleaching
<b>HDAC4</b>	histone deacetylase 4
<b>HF</b>	heart failure
<b>HFpEF</b>	HF with preserved ejection fraction
<b>LV</b>	left ventricular
<b>MMVV</b>	CaMKII with mutated Met281Val and Met282Val
<b>NOX2</b>	NADPH oxidase 2
<b>O-GlcNAc</b>	O-linked $\beta$ -N-acetylglucosamine
<b>OGA</b>	O-GlcNAcase
<b>OGT</b>	O-GlcNAc transferase
<b>OSMI-1</b>	$\alpha$ R-[[[1,2-dihydro-2-oxo-6-quinoliny]sulfonyl]amino]-N-(2-furanylmethyl)-2-methoxy-N-(2-thienylmethyl)-benzeneacetamide
<b>PLB</b>	phospholamban
<b>PKA</b>	protein kinase A

<b>PTM</b>	posttranslational modification
<b>ROS</b>	reactive oxygen species
<b>RyR</b>	ryanodine receptor
<b>S280A</b>	CaMKII with mutated Ser280Ala
<b>sAP</b>	spontaneous AP
<b>SERCA</b>	sarcoplasmic reticulum Ca <sup>2+</sup> ATPase
<b>SGLT2</b>	sodium-glucose cotransporter-2
<b>SR</b>	sarcoplasmic reticulum
<b>STZ</b>	streptozotocin
<b>Thm-G</b>	Thiamet-G
<b>WT</b>	wild type

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## NOVELTY AND SIGNIFICANCE

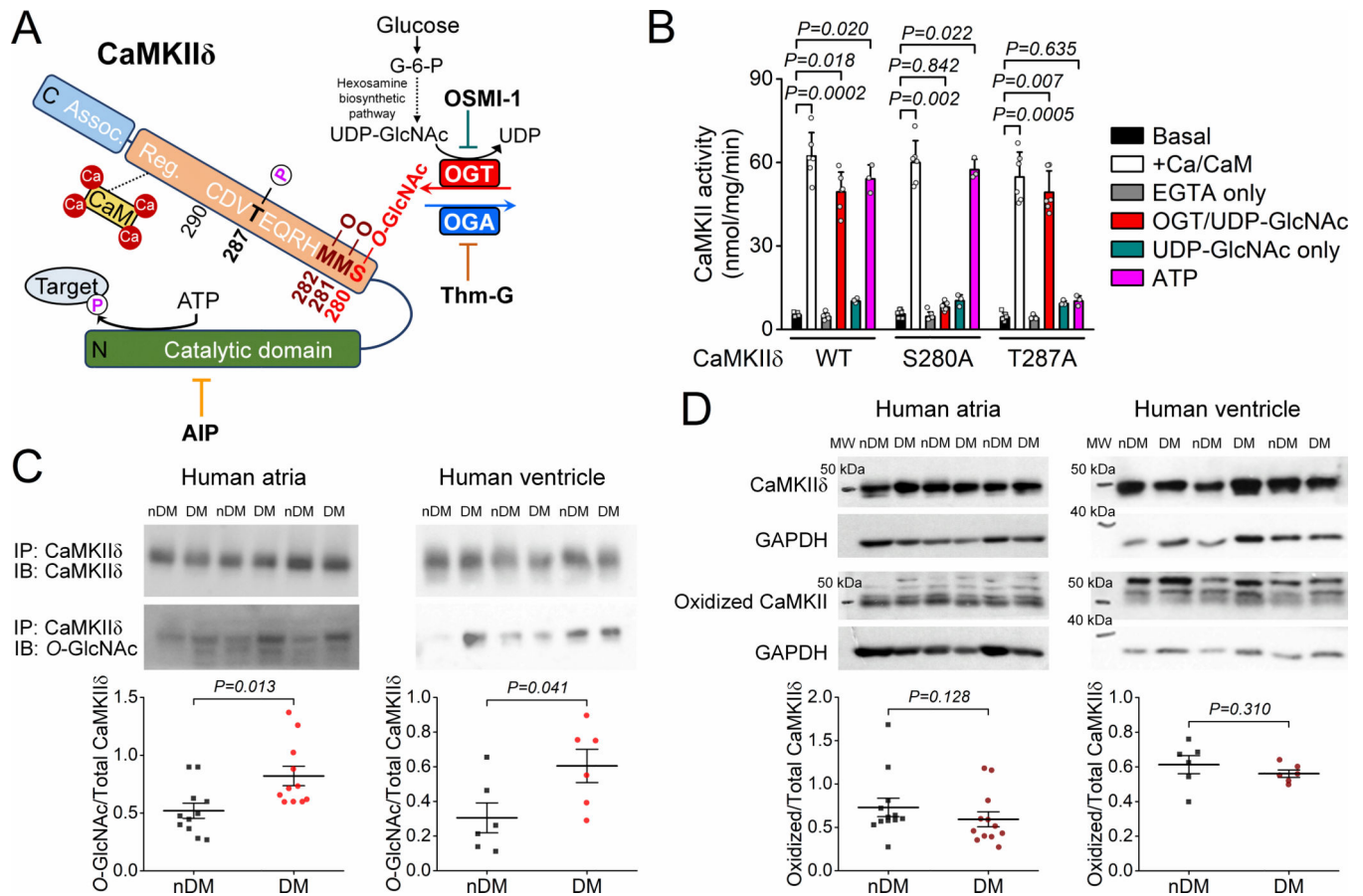
### What Is Known?

- In diabetes, excessive intracellular *O*-GlcNAcylation and reactive oxygen species (ROS) have been reported.
- Both *O*-GlcNAcylation and ROS induce autonomous CaMKII activation.
- Chronic CaMKII activation plays detrimental roles in the pathophysiology of impaired excitation-contraction coupling, cardiac remodeling, arrhythmias, and heart failure.

### What New Information Does This Article Contribute?

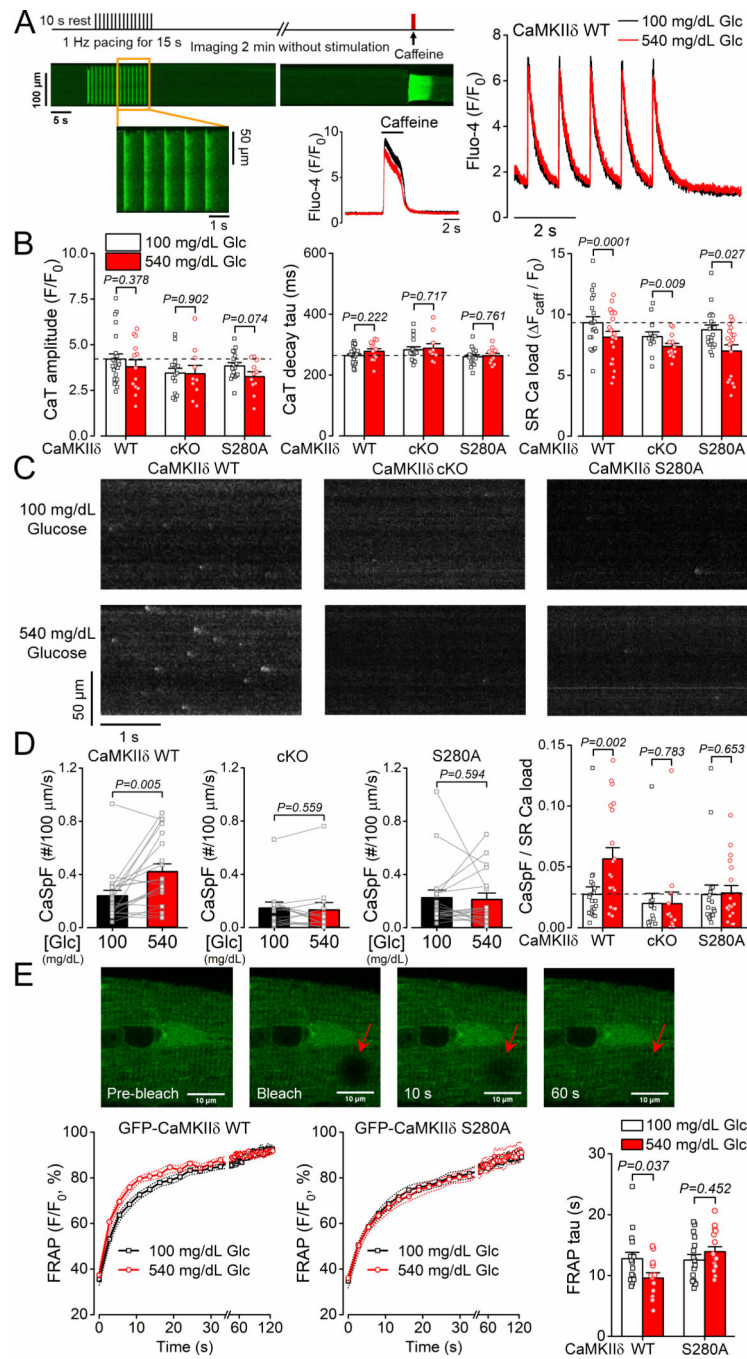
- We show that *O*-GlcNAcylation of CaMKII at serine 280 is the predominant mechanism for autonomous CaMKII activation in diabetic hyperglycemia. Oxidation of CaMKII at two neighboring methionine residues (281/2) is independent of hyperglycemia but is mediated by an angiotensin II - NADPH oxidase 2 (NOX2) pathway, and it plays a secondary, yet synergistic role to promote autonomous kinase activity.
- CaMKII-dependent phosphorylation of the main sarcoplasmic Ca<sup>2+</sup> release channel (ryanodine receptor 2, RyR2) at serine 2814 markedly increases proarrhythmic Ca<sup>2+</sup> leak and drives proarrhythmic electrophysiological changes in diabetic hyperglycemia.
- We found an increased *O*-GlcNAcylation of phospholamban (PLB) that increases its inhibition of sarcoplasmic reticulum Ca<sup>2+</sup> ATPase (SERCA) thus limiting SR Ca<sup>2+</sup> reuptake and further impairing excitation-contraction coupling in diabetic hyperglycemia.

Clinically, diabetes is associated with increased risk of arrhythmias and heart failure. Excessive *O*-GlcNAcylation and ROS production, and upregulated CaMKII have been implicated in the pathophysiology of diabetic cardiomyopathy. However, the molecular details of this signaling were unclear. Here, we identified CaMKII S280 *O*-GlcNAcylation and subsequent SR Ca<sup>2+</sup> leak as the predominant regulator of increased arrhythmia risk in diabetic hearts. This finding provides mechanistic insights into disease pathophysiology and may lead to better targeted therapies in diabetes.



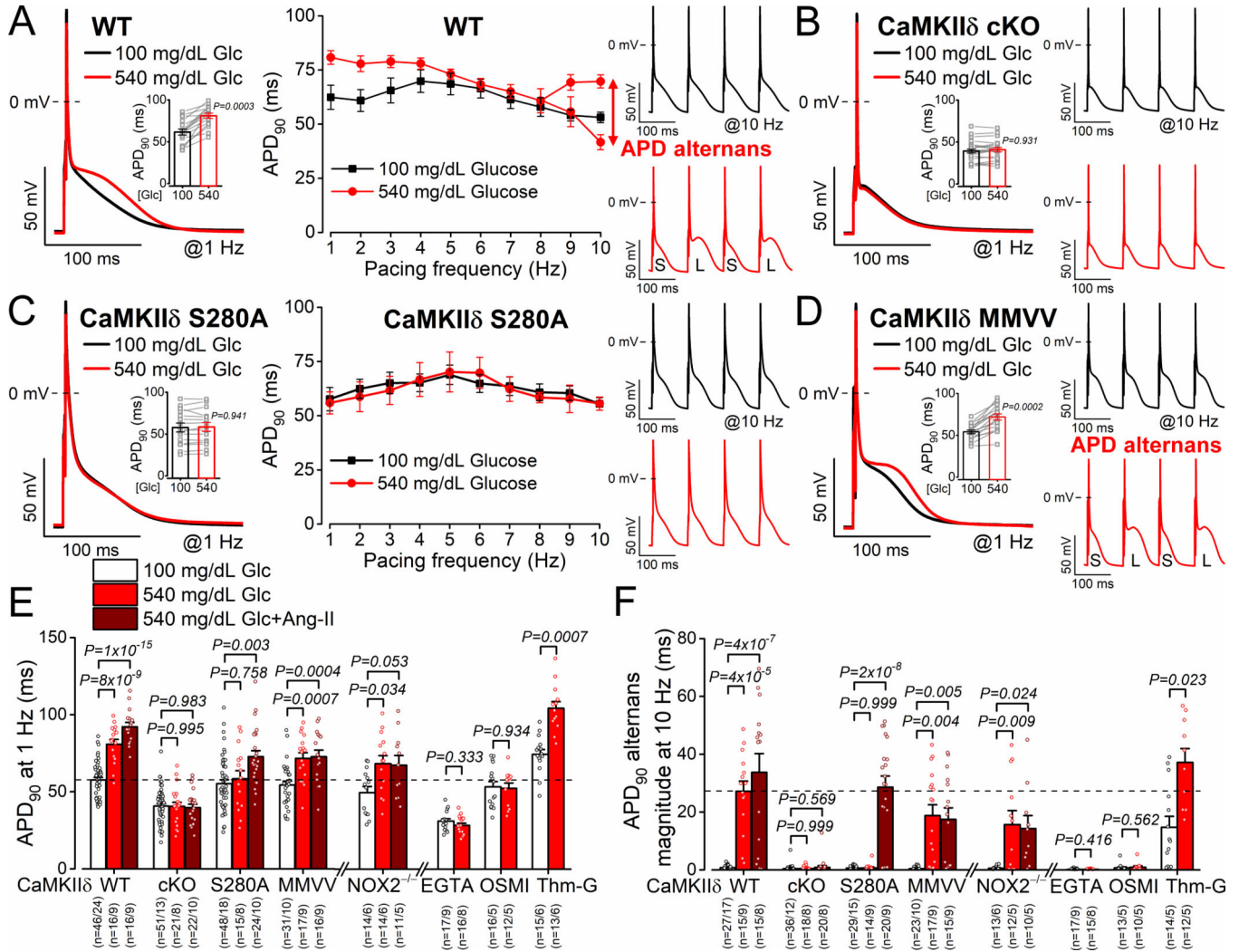
**Figure 1. CaMKII $\delta$  O-GlcNAcylation is increased in human diabetic hearts.**

**A**, Schematic of CaMKII $\delta$  structure and sequence of the regulatory hub domain and the O-GlcNAcylation pathway. **B**, CaMKII activity measured as <sup>32</sup>P incorporation in HEK293 cell lysates expressing WT, S280A, and T287A CaMKII $\delta$  (n, basal=6, Ca/CaM=6, EGTA=6, OGT/UDP-GlcNAc=6, UDP-GlcNAc=3, ATP=3 in all three CaMKII $\delta$  forms). Kruskal-Wallis one-way ANOVA, followed by Dunn's multiple comparisons test. **C**, Western blot data showing enhanced CaMKII O-GlcNAcylation in human atrial and ventricular samples from diabetic (DM) versus non-diabetic (nDM) patients (n, atrial-DM=11, atrial-nDM=11, ventricular-DM=6, ventricular-nDM=6). Mann-Whitney test. **D**, CaMKII oxidation was not increased in atrial and ventricular samples from patients with diabetes (n, atrial-DM=12, atrial-nDM=12, ventricular-DM=6, ventricular-nDM=6). Mann-Whitney test. MW indicates molecular weight.



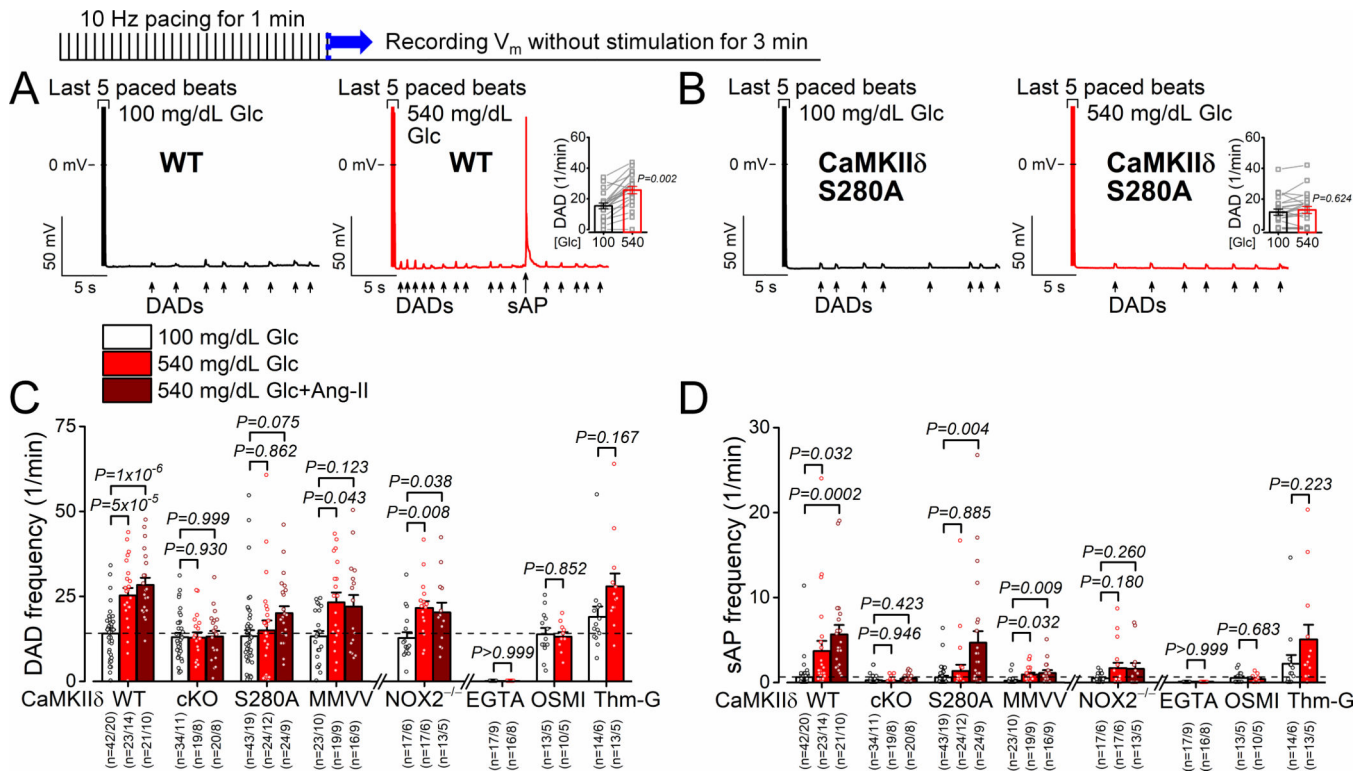
**Figure 2. Glucose-induced diastolic SR Ca<sup>2+</sup> leak is CaMKII $\delta$ -S280 O-GlcNAcylation dependent.** **A**, Experimental protocol and representative intracellular Ca<sup>2+</sup> signals (quantified as changes in Fluo-4 fluorescence). **B**, High-glucose treatment (540 mg/dL, 6 minutes) did not change the amplitude and decay of intracellular Ca<sup>2+</sup> transient (CaT), but reduced SR Ca<sup>2+</sup> load similarly in CaMKII $\delta$  wild-type (WT), cardiac-specific knockout (cKO), and O-GlcNAc-resistant CaMKII $\delta$ -S280A knock-in (n[CaT]=total number of cells/animals, WT/normal glucose=24/8, WT/high-glucose=13/8, cKO/normal glucose=15/7, cKO/high-glucose=10/7, S280A/normal glucose=17/6, S280A/high-glucose=11/6; n[SR load]= total number of cells/

animals, WT=20/11, cKO=13/9, S280A=18/9). Nested *t*-test. **C**, Representative diastolic Ca<sup>2+</sup> sparks were increased by high-glucose treatment in WT which was prevented in cKO and S280A. **D**, Increased Ca<sup>2+</sup> spark frequency normalized to SR load indicates sensitized, leaky ryanodine receptors (n=total number of cells/animals, WT=20/11, cKO=13/9, S280A=18/9). Nested *t*-test. **E**, Enhanced fluorescence recovery after photobleaching (FRAP) by high-glucose treatment in cardiomyocytes expressing GFP-tagged WT-CaMKII $\delta$  (n=16 cells from 6 animals in normal glucose and n=13 cells from 6 animals in high-glucose) but not in GFP-CaMKII $\delta$ -S280A (n=16 cells from 6 animals in both normal and high-glucose) indicates increased activation-dependent mobility of CaMKII $\delta$ . Nested *t*-test.



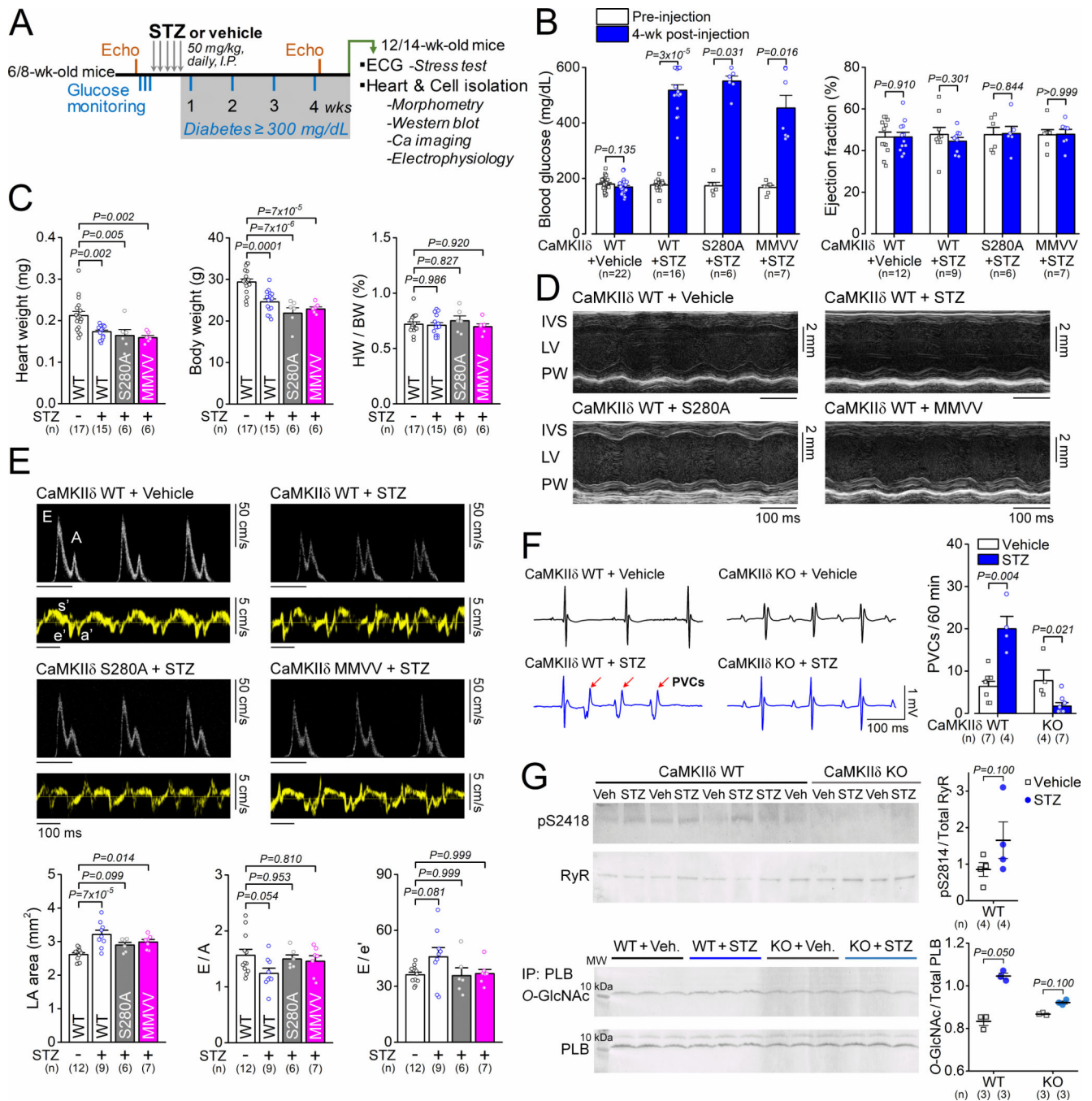
**Figure 3. Glucose-induced arrhythmic action potentials are CaMKIIδ-S280 O-GlcNAcylation dependent.**

**A**, Action potential duration (APD) prolongation and alternans (S, short; L, long) were increased by acute high-glucose treatment in WT myocytes (n=16 cells from 9 animals). **B**, Arrhythmic APD responses were prevented by CaMKIIδ-cKO (n=21 cells from 8 animals). **C-D**, CaMKIIδ-S280A knock-in (n=15 cells from 8 animals) but not MMVV (n=17 cells from 9 animals) was resistant to glucose-induced acute APD changes. Nested *t*-test. **E-F**, Glucose-induced APD changes were prevented by CaMKIIδ-cKO, S280A, intracellular Ca<sup>2+</sup> buffering (EGTA) or the O-GlcNAc transferase inhibitor OSMI-1 but enhanced by the O-GlcNAcase inhibitor Thiamet-G (Thm-G) in WT. Angiotensin II (Ang-II) further enhanced arrhythmic APD changes, which was prevented by CaMKIIδ-cKO, MMVV or in NOX2<sup>-/-</sup> (n=total number of cells/animals is reported in the figure). Nested one-way ANOVA, followed by Dunnett’s multiple comparisons test was used to compare 3 groups. Nested *t*-test was used to compare two groups.



**Figure 4. Glucose-induced delayed afterdepolarizations are CaMKIIδ-S280 O-GlcNAcylation dependent.**

**A-B,** Delayed afterdepolarizations (DADs) and spontaneous action potentials (sAPs) were increased by high-glucose treatment in control (n=21 cells from 13 animals), but it was prevented in CaMKIIδ-S280A (n=23 cells from 12 animals). Nested *t*-test. **C-D,** Glucose-induced spontaneous diastolic activities were prevented by EGTA, OSMI-1 and in CaMKIIδ cKO and S280A. Additional Ang-II treatment further enhanced the arrhythmogenic activities, which were prevented in NOX2<sup>-/-</sup> and CaMKIIδ-cKO and MMVV (n=total number of cells/animals is reported in the figure). Nested one-way ANOVA, followed by Dunnett’s multiple comparisons test was used to compare 3 groups. Nested *t*-test was used to compare two groups (EGTA, OSMI, Thm-G).

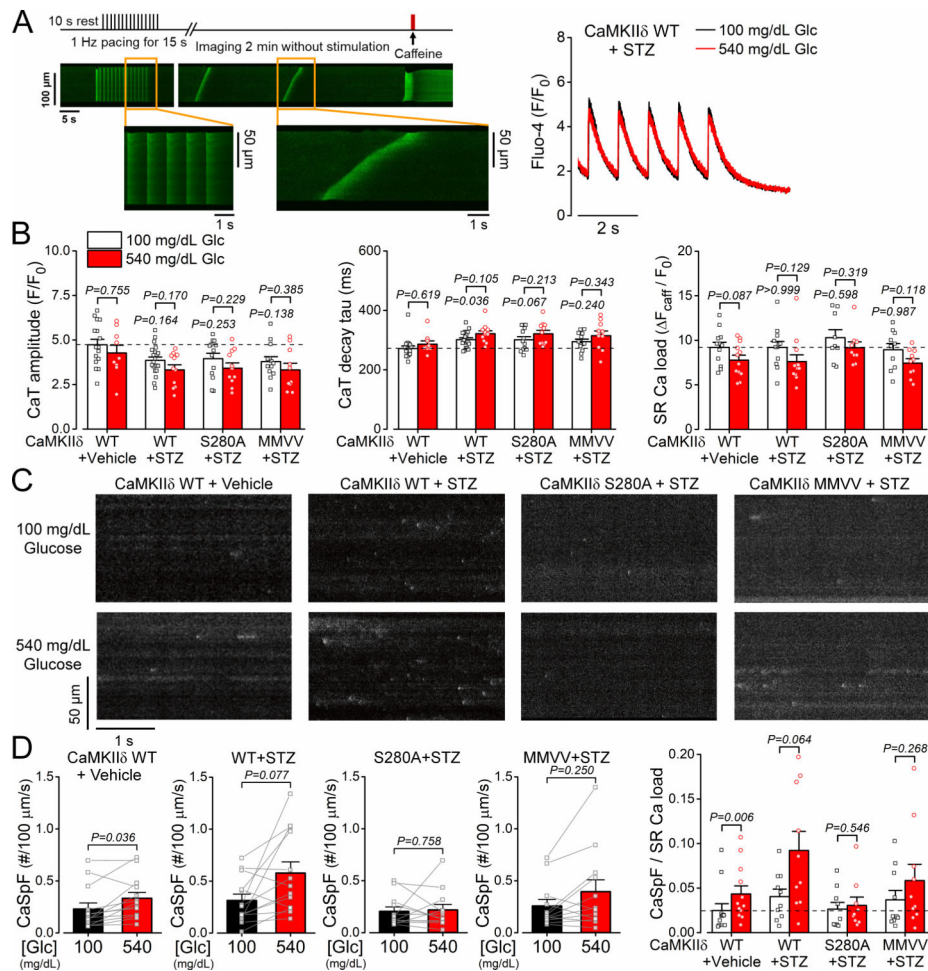


**Figure 5. Diabetes-induced cardiac remodeling and arrhythmias are dependent on CaMKII $\delta$  activation.**

**A**, Study protocol of streptozotocin (STZ)-induced diabetes and assessment of cardiac function. **B**, Blood glucose levels were highly elevated, whereas cardiac ejection fraction was preserved in STZ. Wilcoxon matched-pairs signed rank test. **C**, Morphological parameters in either vehicle-treated or STZ-treated CaMKII $\delta$ -WT, S280A and MMVV mice. One-way ANOVA, followed by Dunnett's multiple comparisons test. **D**, Preserved systolic heart function in STZ. **E**, Diastolic heart function (enlarged left atria, reduced E/A, increased

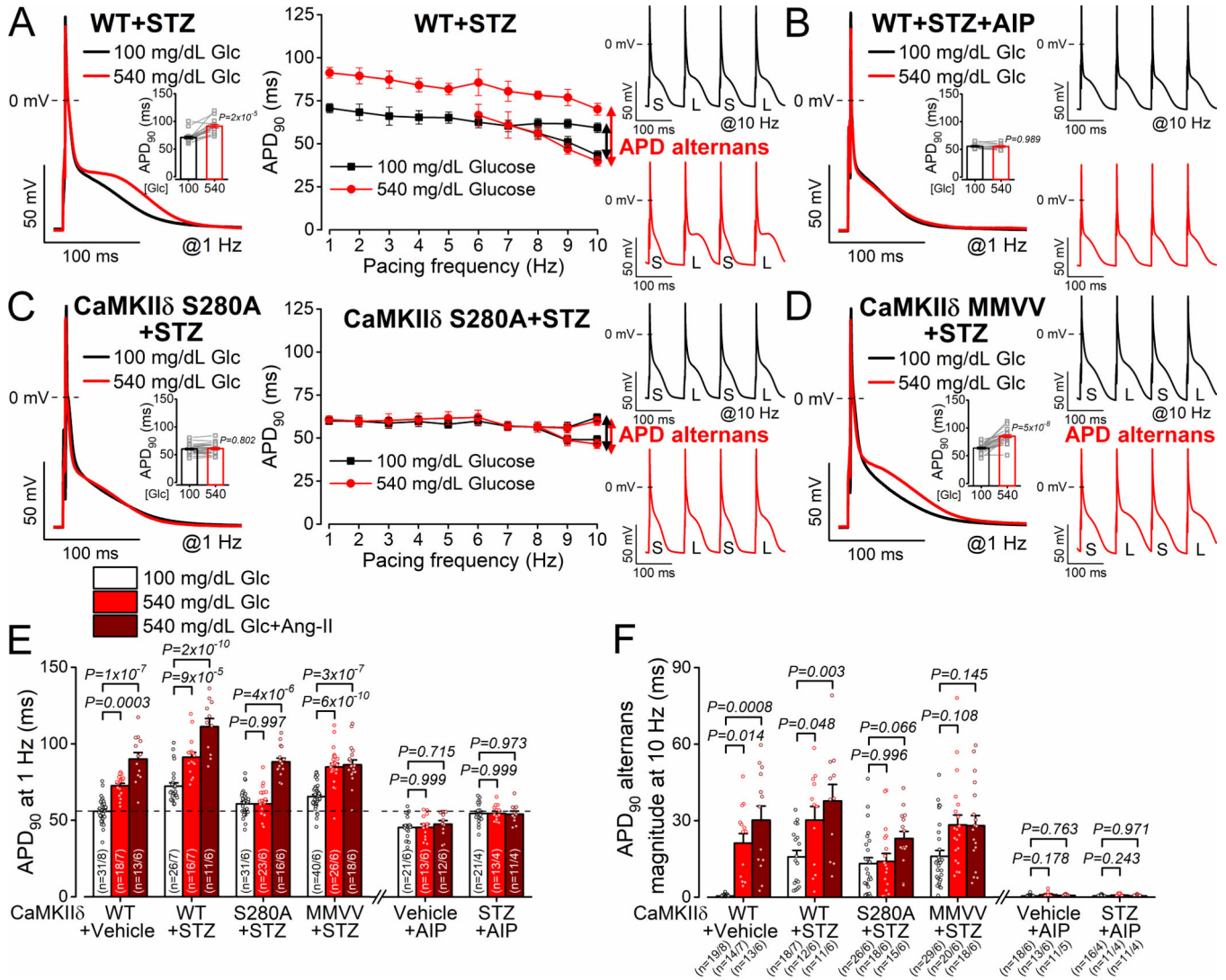


E/e') in 4-week diabetes. One-way ANOVA, followed by Dunnett's multiple comparisons test. **F**, Incidence of premature ventricular complexes (PVCs) following isoproterenol +caffeine stress test was increased in diabetic WT animals, whereas CaMKII $\delta$ -cKO was protective. Mann-Whitney test. **G**, Increased ryanodine receptor (RyR) S2814 phosphorylation and phospholamban (PLB) *O*-GlcNAcylation in STZ. Two technical replicates (blots) were performed for each protein sample. Mann-Whitney test. The number of biological replicates (n) is shown.



**Figure 6. Diabetes-induced diastolic SR Ca<sup>2+</sup> leak is CaMKIIδ-S280 O-GlcNAcylation-dependent.**

**A**, Experimental protocol and representative intracellular Ca<sup>2+</sup> signals (as changes in Fluo-4 fluorescence) showing spontaneous Ca<sup>2+</sup> waves in WT following 4-week of STZ-induced diabetes. **B**, Diabetes slightly reduced the amplitude of the intracellular Ca<sup>2+</sup> transient and prolonged the Ca<sup>2+</sup> transient decay without significantly altering SR Ca<sup>2+</sup> load (n[CaT]=total number of cells/animals, WT+Vehicle/normal glucose=16/7, WT+Vehicle/high-glucose=9/7, WT+STZ/normal glucose=20/6, WT+STZ/high-glucose=11/6, S280A+STZ/normal glucose=13/5, S280A+STZ/high-glucose=11/5, MMVV+STZ/normal glucose=13/5, MMVV+STZ/high-glucose=10/5; n[SR load]=total number of cells/animals, WT+Vehicle=12/6, WT+STZ=12/7, S280A+STZ=9/5, MMVV+STZ=10/5). Acute effect of high-glucose within the same genotype/treatment was tested using nested *t*-test. Effect of STZ-induced diabetes (in normal glucose) versus WT+Vehicle was tested using nested one-way ANOVA, followed by Dunnett’s multiple comparisons test. **C**, Ca<sup>2+</sup> sparks were increased in diabetic WT, and further increased by acute high-glucose treatment. **D**, The increase in Ca<sup>2+</sup> spark rate was prevented in CaMKIIδ-S280A and was attenuated in the CaMKIIδ-MMVV (n=total number of cells/animals, WT+Vehicle=12/6, WT+STZ=10/6, S280A+STZ=9/5, MMVV+STZ=10/5). Nested *t*-test.



**Figure 7. Diabetes-induced arrhythmogenic action potential remodeling is dependent predominantly on CaMKIIδ-S280 O-GlcNAcylation.**

**A**, APD prolongation and alternans in STZ-treated WT murine ventricular myocytes (n=16 cells from 7 animals). **B**, Arrhythmogenic AP remodeling in STZ was abolished by AIP (n=11 cells from 4 animals). **C**, APD prolongation was prevented in STZ-treated CaMKIIδ-S280A (n=22 cells from 6 animals). **D**, APD prolongation and alternans in STZ-treated CaMKIIδ-MMVV (n=17 cells from 6 animals). Nested *t*-test. **E-F**, AP remodeling was CaMKII-dependent and involved both hyperglycemia-dependent S280 O-GlcNAcylation (predominant mechanism) and Ang-II-dependent 281/2MM oxidation (n=total number of cells/animals is reported in the figure). Nested one-way ANOVA, followed by Dunnett's multiple comparisons test.

