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Supplementary Materials for

An improved reporter identifies ruxolitinib as a potent and cardioprotective CaMKII inhibitor

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The PDF file includes:

Other Supplementary Material for this manuscript includes the following:

SUPPLEMENTARY METHODS

Plasmids and Molecular Biology

To create CaMKAR, we amplified cpGFP and FHA1 domains from pcDNA3.1(+)-ExRai-AKAR2 (gift from Jin Zhang, Addgene plasmid # 161753) into pcDNA3.1 and pET-6xHis/TEV plasmid backbones. To screen for peptide sequences that rendered our reporter sensitive to CaMKII, human codon-optimized CaMKII substrate sequences (amino acid sequences for Syntide-2: MPLARTLSVAGLPGKK, FRESCA: MPLARALTVAGLPGKK, Autocamtide-2: MKKALRRQETVDAL, AC3: MKKALHRQETVDAL, and hCaMKIIδ: MHRQETVDCLK) were added to the 5' end of cpGFP by site-directed mutagenesis (KDL Enzyme Mix, NEB)(complete DNA sequence in supplemental datafile 3). deadCaMKAR^{T6A} was generated by site-directed mutagenesis of pcDNA3.1-CMV-CaMKAR. For lentiviral delivery of CaMKAR and CaMKIIδ, CaMKAR was cloned into pLV-hEF1a plasmid backbone to create pLV:hEF1a-CaMKAR-P2A-BlastR (VectorBuilder); CaMKIIδ and its variants were cloned into the doxycycline-inducible pLVX:TetONE-Puro-hAXL construct (gift from Kenneth Pienta, Addgene plasmid # 124797) along with a non-fused mCherry reporter. For neuronal expression, CaMKAR was subcloned into pCAGGS. PKC reporter pcDNA3.1-ExRai-CKAR was a gift from Jin Zhang (Addgene plasmid # 118409). Constitutively active CaMKI^{T177D} was synthesized by Twist Bioscience. Constitutively active CaMKIV-dCT was a gift from Douglas Black (Addgene plasmid #126422). pcDNA3-Camui-NR3 was a gift from Dr. Michael Lin.

Cell Culture

HEK293T/17 cells (ATCC CRL-11268), K562 cells (ATCC CCL-243), and NRVMs were maintained in DMEM (L-glutamine, Sodium Pyruvate, Non-essential amino acids; Gibco) supplemented with 10% FBS (Gibco) and Pen/Strep (Gibco). Cells were maintained between 10%-95% confluence (HEK293T) or 100k-1M cells/mL (K562). NRVMs were isolated from Sprague-Dawley rats as previously described *(92)*. Rat hippocampal neurons were cultured as previously described *(38)*. All cells were maintained in a humidified incubator at 37C with 5% CO_2. To determine viability, cells were lysed in CellTiter-Glo 2.0 (Promega) reagent and read with a Synergy MX microplate reader.

Recombinant CaMKAR: Purified CaMKAR was generated by transforming NEBExpress Competent E. coli (NEB) with pET-6Xhis/TEV-CaMKAR. After addition of Isopropyl Thiogalactoside (IPTG, 0.4 mM) and incubation at 15°C for 24 hours, cells were lysed and sonicated. CaMKAR was isolated from soluble lysate by nickel chromatography NEBExpress columns (NEB). Soluble protein was quantified by Pierce BCA assay (ThermoFisher) and stored in 50% glycerol at -80 C.

Gene transfer to cultured cells

Lentivirus production and infection: HEK293T/17 cells under passage #6 were cultured in 10cm dishes at 400k cells/mL. These cells were transfected using TransIT-Lenti (Mirus Bio) using a ratio of 5:3.75:1.25 of packaging plasmid:psPAX2:pMD2.G for a total of 10 µg per dish. After 48 hours, supernatant was collected, clarified, and concentrated 10-fold using Lenti-X concentrator (Takara). Lentivirus aliquots were stored at -80 °C until functional titration and use. Infection occurred at indicated multiplicity of infection in the presence of 10 µg/mL polybrene reagent (Sigma).

Plasmid transfection: HEK293T/17 cells were plated into well plates at 400k cells/mL unto PDL coated 24 well plates. Each well was transfected with 500 ng of DNA complexed with 1 µL of JetPrime reagent. Cells were examined 24-48 hours post transfection. For cells receiving doxycycline-inducible constructs (CaMKIIδ and variants in pLVX:TetONE-Puro plasmid), doxycycline was added to the culture media at 1 µg/mL 24 hours prior to terminal assay. During imaging, Tyrode's solution was supplemented with doxycycline 1 µg/mL. Successful dox-on induction was ascertained by fluorescence imaging verification of mCherry reporter included in the plasmid. Rat neurons were transfected with the indicated plasmids using Lipofectamine 2000 (Invitrogen) at 13–18 days in vitro.

Adenovirus and siRNA in NRVMs: CMV-CaMKAR-encoding Ad5 adenovirus was synthesized by Vector Biolabs. NRVMs were infected 24 hours post isolation at multiplicity of infection 100. Anti-CaMKIIδ siRNA (s127546, Thermo Fisher) and scrambled siRNA (AM4611, Thermo Fisher) were transfected using 10pmol complexed with Lipofectamine RNAiMAX (Thermo Fisher).

Microscopy

Timelapse microscopy was performed using an Olympus IX-83 inverted widefield microscope equipped with an ORCA Flash 4.0 and Lumencor SOLA light source. CaMKAR signal was captured at 200 ms exposure using the following channels: excitation filters ET402/15x and ET490/20x and emission filter ET525/35m (Chroma Technology). CaMKAR Signal (R) is defined as the ~488-nm-excited intensity divided by the ~400-nm-excited intensity. Calcium imaging was collected in the TRITC channel ex 555/em. Confocal imaging was performed using a Zeiss LSM880 Airyscan FAST. Using excitation lasers 405 nm and 488 nm and collecting emission window at 520 ± 10 nm.

Otsu segmentation was used to track individual cells and their intensity in 488 nm and 405 nm channels at every timepoint in CellProfiler.

Rat neurons were imaged 3–7 days after transfection on a Zeiss spinning-disk confocal microscope. Dual excitation-ratio imaging was performed using the 488- and 405-nm lasers and a 525/50 emission filter. Image analysis was performed using custom scripts in Fiji/ImageJ.

Induced pluripotent stem cells (iPSCs) generation, iPSC-cardiomyocyte differentiation and imaging.

A patient with a pathogenic *RYR2* mutation (p.S404R) manifesting catecholaminergic polymorphic ventricular tachycardia (CPVT) provided informed consent to participate in the study under protocols approved by the Boston Children's Hospital Institutional Review Board. Peripheral blood mononuclear cells (PBMCs) from this patient were reprogrammed to pluripotency with the CytoTune Sendai reprogramming kit (ThermoFisher). iPSCs were differentiated to iPSC-CM and their purity was assessed by flow cytometry *(13)*. Only clones with greater than 90% positivity of the pluripotency marker SSEA4 were used for differentiation and functional studies.

Kinetic Image Cytometry

For adult mouse cardiomyocytes: The isolation procedure for adult murine cardiomyocytes is described in the "Experimental mouse procedures" section. After isolation, adult cardiomyocytes were stained with 8 μM Rhod-2AM for 15 minutes at 37C, washed with Tyrode solution and incubated with DMSO (1:1000), experimental

compounds (2 μM), isoproterenol (100 nM), or isoproterenol (100 nM) plus autocamtide-2-related inhibitory peptide (AIP) (3 μM) for calcium transient measurements after electrical stimulation (30 pulses) in a CyteSeer Scanner (Vala Sciences) at 8 Volts.

For iPSC-CMs: iPSC-CMs were stained with 5 μM Fluo-4 for 15 minutes at 37C, washed with RPMI and incubated with DMSO (1:1000), experimental compound (2 μM), isoproterenol (1 μM), or isoproterenol (1 μM) plus autocamtide-2-related inhibitory peptide (AIP) (3 μM) for calcium transient measurements after electrical stimulation (10 pulses) in a CyteSeer Scanner (Vala Sciences) at 15 Volts. After CyteSeer data acquisition, offline data analysis was performed, where ImageJ was used for manual cell segmentation, and a custom MATLAB (Mathworks) script was used for calcium transient data filtration, parameter calculation, and post-pacing abnormal calcium release event analysis. Post-pacing abnormal calcium release events were defined by a peak amplitude >5% of the median pacing calcium transient amplitude for a given cell. Microsoft Excel, and GraphPad Prism 9.3 software were then used for data compilation, statistical analysis, and graphical representation. Imaging data obtained from adult cardiomyocytes or iPSC-CMs were analyzed using mixed ANOVA followed by Wald's chi-squared test *(93)*.

CaMKAR-based high throughput screening

We created a polyclonal K562$^{\text{CaMKAR-CaMKII}}$ line by infecting 3M cells with lentivirus containing pLVX:EF1a-CaMKAR-BlastR (multiplicity of infection=5) and pLVX:TetONE-CaMKII^{T287D}-P2A-mCherry (multiplicity of infection=1). These cells were expanded to 600M and incubated with 1 μg/mL doxycycline 24 hours prior to screening. Cells were

resuspended in Live Imaging Cell Solution (Gibco) supplemented with doxycycline and 4.5 g/L glucose. These cells were then distributed across 15 clear-bottom 384 well plates at 50k cells per well. 5 µL of 10x compound from the Johns Hopkins Drug Library v3.0 was added at a final concentration of 5 µM per compound. The Johns Hopkins Drug Library v3 was assembled by combining the Selleckchem FDA-Approved Drug Library (3,174 compounds, Catalog No: L1300) with non-duplicate compounds in APExBIO DiscoveryProbe FDA-approved Library (483 compounds, Catalog No. L1021) and the MicroSource US Drug Collection (817 compounds). The library was formatted for use in 384-well plates and stored in DMSO at -80 °C. Each plate contained 2 columns of untreated cells, and 1 column of cells treated with AS100397 10 µM. Cells were incubated for 12 hours before reading by high content imager (MolDev IXM High Content Imager). Images were automatically segmented and quantified using CellProfiler as above. CaMKII inhibitory % was calculated by min-max normalizing the data between untreated and AS100397-treated samples. For secondary screen, see supplementary methods.

Secondary drug screen

118 candidates from the primary in cellulo screen were then subjected in vitro CaMKAR screen. The in vitro CaMKAR reaction was carried in 384-well plates with 25 µL total volume, 12.5 µL 2X buffered PBS, 1.25 µL 10mM CaCl, 0.5uL 50 µM CaM, 0.11 µL CaMKAR (25 pmol), 0.0625 µL CaMKII (125 fmol), completed with nuclease-free water. Plate was read at baseline via Tecan Safire microplate reader (Channel 1: 400 nm ± 15 nm excitation, 525 ± 20 nm emission; Channel 2: 480 ± 15 nm excitation, 525 ± 20 nm emission), then 5 µL of 5x drug was added (5 µM final), plate read again, then 5 µL ATP was added (100 µM final) and kinetic assay was read for 60 mins. Slope in the change of

CaMKAR ratio was used as metric for CaMKII activity. Data was min-max normalized between untreated and AS100397-treated wells. Hits for both screens were defined by significant deviation from untreated distribution by the z-Statistic p-Value. This statistical approach was reviewed by the Johns Hopkins Biostatistics, Epidemiology, and Data Management Core.

Cell-free kinase activity assays

In vitro CaMKIIδ assays were performed by Reaction Biology Corporation as follows: Substrate was prepared in freshly prepared Reaction Buffer (20 mM Hepes (pH 7.5), 10 mM MgCl2, 1 mM EGTA, 0.01% Brij35, 0.02 mg/ml BSA, 0.1 mM Na3VO4, 2 mM DTT, 1% DMSO) along with CaMKII-specific co-factors. Kinase was delivered into the substrate solution. Compounds were delivered in 100% DMSO into the kinase reaction mixture by Acoustic technology (Echo550; nanoliter range) followed by incubation for 20 min at room temp. 33P-ATP was delivered into the reaction mixture to initiate the reaction and incubated for 2 hours at room temperature. Kinase activity was detected by P81 filter-binding. In vitro assays for confirmation of secondary chemical screening (Datafile S2) were performed by Eurofins DiscoverX Corporation using the scanELECT assay.

Mouse model maintenance

All mouse studies were carried out in accordance with guidelines and approval of Animal Care and Use Committees of JHU (#MO20M274), Boston Children's Hospital (#20-05-4139), and Baylor College of Medicine (#AN-4044).

At Johns Hopkins University, 8-12 week old C57BL/6J mice (The Jackson Laboratory, ME, USA) and 16-18 week old O-GlcNAc transferase overexpressing (OGT-

TG, *62*) mice were housed in a facility with 12 hour light/12 hour dark cycle at 22 ± 1 °C and 40 ± 10% humidity. C57BL/6J mice were fed Teklad global 18% protein rodent diet and tap water ad libitum, whereas OGT-TG mice were fed normal chow diet (NCD) (7913 irradiated NIH-31 modified 6% mouse/rat diet – 15 kg, Envigo, Indianapolis, IN) and tap water ad libitum.

At Boston Children's Hospital, animals are housed in a facility with automated light/dark cycles of 12 hours in an temperature and humidity controlled environment. Animals are provided a standard diet with Prolab® IsoPro® RMH 3000 and distilled water through an automated dispensing system. RyR2-R176Q animals were backcrossed with wild type C57BL/6J mice for more than 6 generations and genotyping confirmed by sequencing prior to breeding. 8-12 week-old animals were used for all experiments with wild type litter-matched mice as controls.

At Baylor College of Medicine, 8-months-old CREM-IbΔC-X mice (provided by Dr. Frank U. Müller) were housed in a facility with 12 hour light/12 hour dark cycle in accordance with the Guide for the Care and Use of Laboratory Animals. Mice were provided an irradiated, soy-protein–free, extruded rodent diet (19.4% protein, no. 2920X, Harlan Teklad Global, Madison, WI) free choice and received acidified, autoclaved municipal water ad libitum. Mice were housed in autoclaved, shoebox-size, microisolator cages on 1/4-in. corncob bedding with positive-pressure air flow. Cages were changed weekly under a laminar-flow hood by the same caretaker using microisolator technique.

Experimental mouse procedures

Isoproterenol challenge: Ruxolitinib phosphate (MedChemExpress) was suspended in sterile saline with 10% DMSO. Drug was administered via intraperitoneal injection. 10

minutes later, 5 mg/kg isoproterenol (Sigma-Aldrich) in saline was injected via intraperitoneal injection. Mice were euthanized 10 minutes after the isoproterenol challenge.

Cardiomyocyte Isolation: Adult ventricular cardiomyocytes were isolated from wildtype or transgenic *Ryr2*$^{R176Q/+}$ CPVT mice by retrograde perfusion of the aorta with enzymatic digestion (collagenase type II, Worthington) in a Langendorff apparatus *(13)*, and plated on laminin-coated 96-well glass-bottomed plate for calcium transient measurements initiated by electrical stimulation (8Hz). Kinetic Image Cytometry (KIC) measurements were performed to the attached cardiomyocytes after staining with Rhod-2AM (15 minutes at 37°C). The cells were constantly maintained in Tyrode solution containing 137 mM NaCl, 20 mM HEPES, 10 mM D-glucose, 5.4 mM KCl, 1.2 mM $MgCl_2$ x $6H_2O$, 1.2 mM NaH_2PO_4 x $2H_2O$, 10 mM Taurine, 10 mM BDM, 1 mM $CaCl_2$ x $2H_2O$, pH 7.5.

Diabetic Mouse Model: Type-1 diabetes (T1D) was induced as previously described *(59)* in adult male C57BL/6J mice (The Jackson Laboratory, ME, USA) by a single intraperitoneal injection of streptozotocin (STZ) (185 mg/kg, Sigma-Aldrich) dissolved in a citrate buffer (citric acid and sodium citrate, pH 4.0) after a six hour fast. Mice were maintained for at least 2 weeks on normal chow diet (NCD) (7913 irradiated NIH-31 modified 6% mouse/rat diet – 15 kg, Envigo, Indianapolis, IN) prior to subsequent electrophysiological studies. Mice were considered diabetic if blood glucose was ≥ 300mg/dl via tail vein blood checked with a glucometer - OneTouch Ultra 2 (LifeScan, Zug, Switzerland).

Electrophysiological studies in CPVT mice: Adult mice (aged 8-12 weeks old) were anesthetized and instrumented to a 2-lead (leads I and II) electrocardiogram (ECG) with a 1.1F octapolar catheter (ADInstruments) being inserted into the right ventricle via a right jugular vein. Dimethyl sulfoxide (DMSO) (1:1000), ruxolitinib (75mg/Kg), isoproterenol (2 mg/kg) and epinephrine (3 mg/kg) were administered intraperitoneally, while surface and intracardiac ECGs were recorded for 3 to 15 minutes post-injection. More than 3 premature ventricular contractions (PVCs) or a single ventricular couplet during this recording period was considered a positive response. Programmed ventricular stimulation was performed using a digital stimulator (ADInstruments) that delivered x8 stimuli, designated as stimulus-1 (S1 x 8 impulses, followed by 2 early extra-stimuli [S2 and S3]). Ventricular arrhythmias were defined at ≥3 induced ventricular beats. The duration of arrhythmias was measured from the last paced beat *(13)*. QT dispersion was measured by comparing the absolute difference in the corrected QT interval between ECG leads I and II, while the heart rate–corrected QT (QTc) interval was calculated with an automated algorithm in LabChart *(13)*.

Atrial fibrillation induction: Anesthetized mice were injected via intraperitoneal route with either ruxolitinib phosphate (MedChemExpress) – 75 mg/kg dissolved in sterile saline with 10% dimethylsulfoxide (DMSO) and 2.5% tween-20 – or vehicle 10 minutes prior to rapid atrial burst pacing to assess atrial fibrillation (AF) inducibility. In vivo electrophysiology (EP) studies were performed as previously reported *(59)* in mice anesthetized with isoflurane (2% for induction and 1.5% for maintenance of anesthesia;

Isotec 100 Series Isoflurane Vaporizer; Harvard Apparatus). AF was defined as the occurrence of rapid and fragmented atrial electrograms with irregular AV-nodal conduction and ventricular rhythm for at least 1 second. If 1 or more atrial bursts (out of 5) were an AF episode, the mouse was considered to have inducible AF. Type 1 diabetic mice underwent a single round of AF induction 10 minutes after either vehicle or ruxolitinib intraperitoneal administration. O-GlcNAc transferase overexpressing mice (OGT-TG, *62*) underwent 2 sequential inductions: first induction was 10 minutes after intraperitoneal vehicle administration; mice were then allowed to return to sinus rhythm still under anesthesia and were given a 2-minute resting period; then, intraperitoneal ruxolitinib 75 mg/kg was administered followed by a 10 minute incubation followed by a second round AF induction and recording. All mice were euthanized immediately following the procedure and atrial tissue from both right and left atria were obtained and flash frozen in liquid nitrogen and then stored at -80°C.

Spontaneous AF: 8-month-old CREM-IbΔC-X mice were monitored via surface electrocardiogram (ECG) for 15 minutes to establish the baseline for time spent in atrial fibrillation (AF). AF was defined as loss of p-waves and irregularly irregular R-R intervals for > 10 seconds. Each mouse was monitored at least twice with more than 24 hours interval between two recordings. After establishing the baseline, the mice were injected with vehicle (10% DMSO, 2.5% Tween-20 in saline; i.p. injections) and a 15-minute ECG was recorded between 10-25 minutes post injection. In these same mice, 24 hours after the aforementioned vehicle injection and ECG measurement, ruxolitinib was injected (75

mg/kg in 10% DMSO, 2.5% Tween-20 in saline; i.p. injections) and another 15-minute ECG was recorded between 10-25 minutes post ruxolitinib injection.

Behavioral paradigms

Memory tests were performed at the JHU Behavioral Core by a blinded experimentalist on male mice. Short term memory was assessed using the novel object recognition test. The test consisted of three phases over two days. On day 1 mice were habituated to the arena for 10 minutes. On day 2, mice were allowed to explore the arena consisting of two identical objects, referred to as the familiar objects, for 10 minutes and were then placed back in the home cage for 30 minutes. Following the delay, the mice were placed back into the arena with one “familiar” object and one “novel” object and allowed to explore the objects for 5 minutes. Distance travelled and time spent investigating each object was automatically recorded using Anymaze tracking software (Stoelting Co., Wood Dale, IL, USA).

Spatial memory was tested via the Y-maze spatial recognition test. The Y-maze consists of three 38 cm-long arms (San Diego Instruments). During the training phase, one arm of the Y-maze was blocked. The mouse was placed at the end of one of the two open arms and allowed to explore for 5 min. After a 30-min inter-trial interval, the test phase began: the blockade was removed, and the mouse was allowed to explore all three arms of the maze for 5 min. Distance traveled and time spent in each arm was automatically recorded using Anymaze tracking software. Data from the first 2 min of the test phase were used to evaluate percent time spent in the novel arm.

For the Barnes maze: A brightly lit (1100 lux) Barnes maze with 20 evenly spaced holes and an escape box placed under one of the holes was used (Maze Engineers). During

training, each mouse was placed in the center of the maze and allowed to explore the maze for 3 minutes per trial. During the trial, the number of head dips and the latency to find and then enter the escape box were recorded. Mice were given two trials per day for four days. 24 hours following final training, mice were given a probe trial, with the number of head dips and latency to find and then enter the escape box recorded. Two mice (one from each treatment group) were excluded from analysis due to failure to explore the maze during training.

Western Blotting

Mouse hearts were disrupted using a tissue blender in 1% Triton X-100 containing protease and phosphatase inhibitors. To detect phospho- and total phospholamban, samples were left unboiled to preserve pentameric form. Samples were run on 4-12% bis-tris acrylamide gels and transferred onto nitrocellulose membranes. Total and phosphorylated-T17 phospholamban were assayed by incubation with respective antibodies (Phospholamban pT17 pAb (Badrilla A010-13), 1:5000; Phospholamban (Thermofisher/Pierce MA3-922), 1:2000) followed by secondary labeling (Goat Anti-Rabbit IgG Antibody 680LT (Licor 926-68021) 1:10,000; Goat Anti Mouse IgG Antibody 800CW (Licor 926-32210) 1:10,000) and quantified using a LICOR Odyssey imager and ImageStudio. CaMKIIδ was assayed via Anti-CaMKII delta antibody (Abcam ab181052). Total protein was assayed via Revert reagent (Licor).

For RyR2$^{R176Q/WT}$ animals

Heart tissues were shock frozen and homogenized in a buffer (~500µL) containing: 120mM NaCl, 1mM EDTA, 10mM Glycerophosphate, 40mM HEPES, 40mM NaF, 0.3% CHAPS, 1% Triton X-100 and 1% HALT, pH 7.5. Proteins were quantified using Pierce

BCA protein assay (Thermo Fischer Scientific). Samples were boiled at 95°C for 5 min and 10-15 µg of total protein were loaded per lane and subjected to 15% SDS-PAGE and immunoblot analysis. For uncalibrated optical density, all scanned blots were analyzed by ImageJ software.

Schematics

Schematics and drawings made with Biorender.com. CaMKAR Ratio pseudocoloring for fluorescence microscopy performed with ImageJ using the Ratio Plus plugin. Kinase dendrogram illustration made with KinMap *(94)* and reproduced courtesy of Cell Signaling Technology, Inc. (www.cellsignal.com).

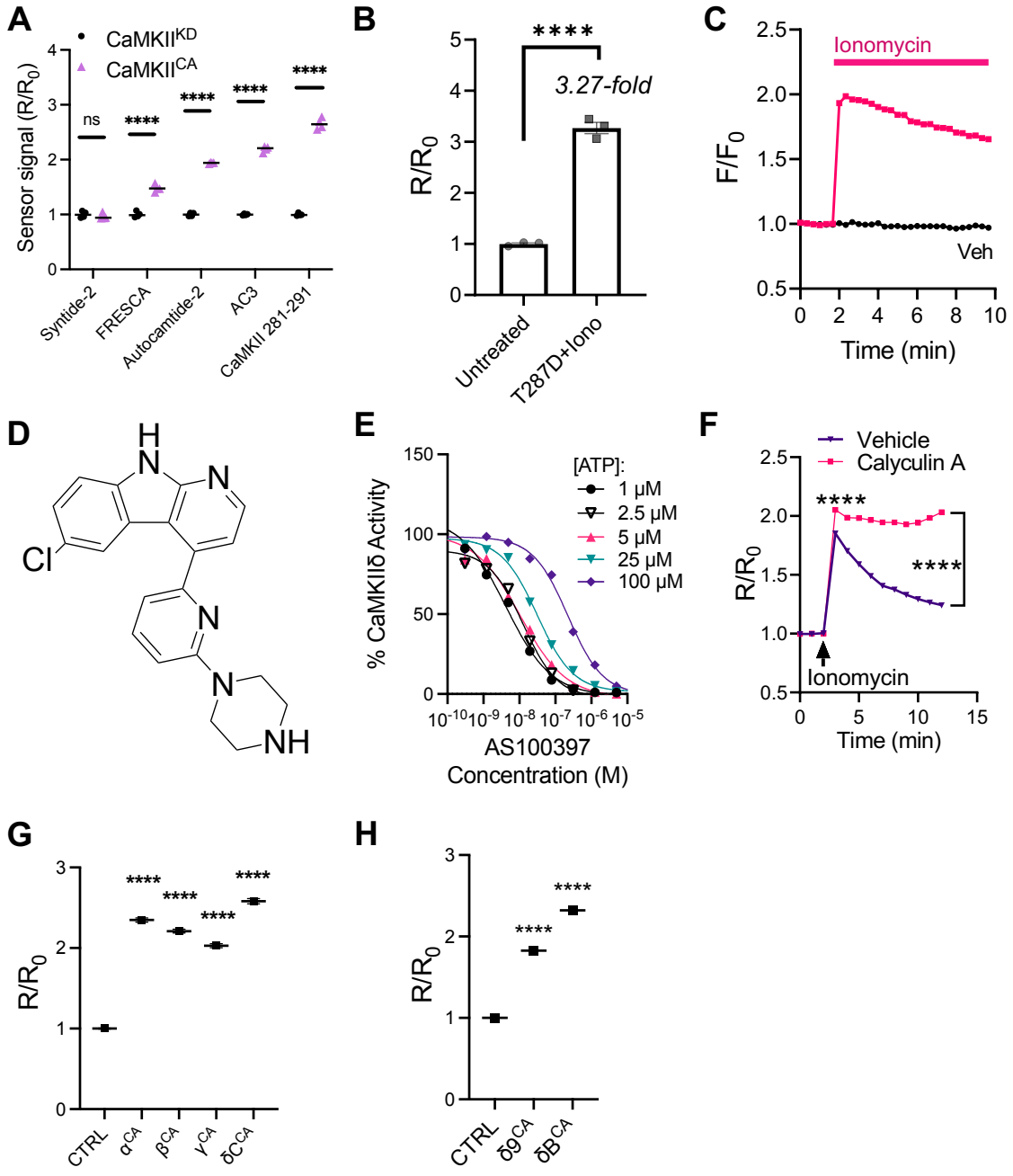
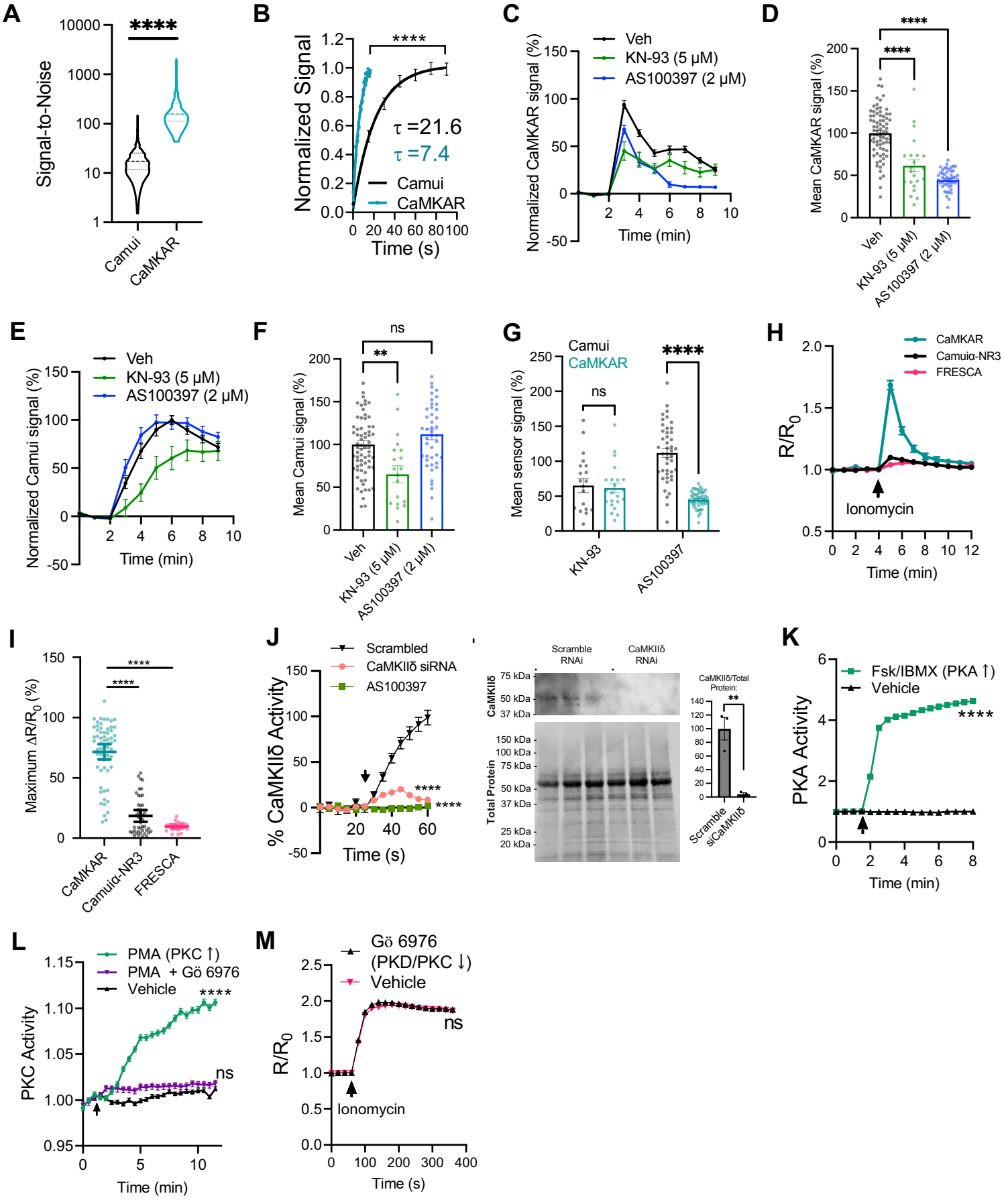


Figure S1. CaMKAR development and validation. (A) Screening to identify peptide sequence that renders sensor sensitive to CaMKIIδ: plate reader fluorometry of 293T cells transfected with CaMKIIδ (CA=constitutively active, T287D mutation; KD=kinase dead, K43M mutation) and prototype sensors. Each peptide was fused to the 5' end of kinase-sensing cpGFP-FHA1. Sensor signal (R) defined as ratio of 488-nm-excited intensity divided by 405-nm-excited intensity; R_0 = R in control condition or baseline. N=3 wells per observation. **(B)** Maximal stimulation of CaMKAR in 293T cells by expression of $CaMKII^{T287D}$ plus ionomycin treatment (5 μM; n=3 wells) or untreated (n=3 wells). **(C)** Intensiometric CaMKAR signal (Ex. 488 nm, Em. 520 nm) after vehicle (Veh, n=2,488-2,615 cells) or ionomycin stimulation (n=1,733-2,220 cells), from same dataset as Fig. 1C. F/F_0= Fluorescence intensity normalized to baseline fluorescence intensity. **(D)** Structure of CaMKII inhibitor AS100397. **(E)** In vitro CaMKII kinase assay (HotSpot substrate phosphorylation) with varying concentrations of AS100397 and ATP. % activity defined as relative to DMSO control (set to 100%). **(F)** CaMKAR signal in 293T cells pre-treated with vehicle (n=1,746-1,930 cells) or Calyculin A (10 nM; n=1,051-1,595), then stimulated with ionomycin. **(G)** CaMKAR response in control (CTRL; n=151) cells or after transfection of constitutively active CaMKIIα (n=194 cells), β (n=229), δ (n=201), or γ (n=194) and **(H)** control (CTRL; n=772) vs constitutively active CaMKIIδ splice variants 9 (n=1,136) and B (n=1,140). Data shown as mean ± SEM (error not displayed whenever it is smaller than the data point). All observations done in biological triplicate. ns=p>0.05, ****p<0.0001; significance was determined via one-way ANOVA and Sidak's multiple comparisons test (*A*) or Dunnett's multiple comparisons test (*G and H*); two-way ANOVA and Sidak's multiple comparison test (*F*); unpaired Student's T-test (*B).*


A
Signal-to-Noise
****
Camui
CaMKAR
B
Normalized Signal
****
τ =21.6
τ =7.4
Camui
CaMKAR
Time (s)
C
Normalized CaMKAR signal (%)
Veh
KN-93 (5 μM)
AS100397 (2 μM)
Time (min)
D
Mean CaMKAR signal (%)
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Veh
KN-93 (5 μM)
AS100397 (2 μM)
E
Normalized Camui signal (%)
Veh
KN-93 (5 μM)
AS100397 (2 μM)
Time (min)
F
Mean Camui signal (%)
ns
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Veh
KN-93 (5 μM)
AS100397 (2 μM)
G
Mean sensor signal (%)
Camui
CaMKAR
ns
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KN-93
AS100397
H
R/R0
CaMKAR
Camuiα-NR3
FRESCA
Ionomycin
Time (min)
I
Maximum ΔR/R0 (%)
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CaMKAR
Camuiα-NR3
FRESCA
J
% CaMKIIδ Activity
Scrambled
CaMKIIδ siRNA
AS100397
****
****
Time (s)
Scramble RNAi
CaMKIIδ RNAi
CaMKIIδ
75 kDa
50 kDa
37 kDa
Total Protein
150 kDa
100 kDa
75 kDa
50 kDa
37 kDa
25 kDa
20 kDa
CaMKIIδ/Total Protein:
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Scramble
siCaMKIIδ
K
PKA Activity
Fsk/IBMX (PKA ↑)
Vehicle
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PKC Activity
PMA (PKC ↑)
PMA + Gö 6976
Vehicle
****
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Time (min)
M
R/R0
Gö 6976 (PKD/PKC ↓)
Vehicle
ns
Ionomycin
Time (s)

Figure S2. CaMKAR benchmarking and specificity compared to other CaMKII sensors. (A) CaMKAR and Camui-NR3 signal response to ionomycin in 293T cells: assayed for signal-to-noise ratio (CaMKAR n=447 cells; Camui n=469 cells), and **(B)** activation kinetics (CaMKAR n=73 cells; Camui n=3,576-4,147 cells). Normalized signal: Each sensor was min-max normalized to their respective pre-stimulation baseline (equal to 0) and their respective post stimulation maxima (equal to 1). **(C)** CaMKAR activity tracking in rat hippocampal neurons pre-treated with CaMKII inhibitors KN-93 or AS100397 and then stimulated with ionomycin (added at 2 minute mark). **(D)** Normalized mean sensor signal across all timepoints post-stimulation in *C*. Three baseline values were captured prior to ionomycin stimulation; datapoints represent individual cells (Vehicle n=78, KN-93 n=22, AS100397 n=49). **(E)** Camui activity tracking in rat hippocampal neurons pre-treated with CaMKII inhibitors and then stimulated with ionomycin (added at 2 minute mark). **(F)** Normalized mean sensor signal across all timepoints post-stimulation in *E*. Three baseline values were captured prior to ionomycin stimulation; datapoints represent individual cells (Vehicle n=69, KN-93 n=20, AS100397 n=44). **(G)** Effect size comparison of KN-93 (5 μM) and AS100397 (2 μM) treatment for each sensor (same data as in *D, F*). **(H)** Sensor-expressing rat hippocampal neurons stimulated with ionomycin and tracked for sensor activity over time. **(I)** Maximal signal change from *H* (CaMKAR n=61, Camui n=43, FRESCA n=21). **(J)** *Left:* CaMKAR-expressing neonatal ventricular cardiomyocytes (NRVMs) were pretreated with AS100397 (10 μM, n=722-749 cells), anti-CAMKIIδ siRNA (n=665-700) or scrambled siRNA (n=571-594) and stimulated with 2 Hz pacing (arrow). *Right:* validation of anti-CaMKIIδ siRNA (immunoblot and densitometry against Total Protein) in technical triplicate from the same cell preparation as *left.* **(K)** 293T cells expressing PKA sensor ExRai-AKAR2 treated with vehicle (n=1,066-1,092 cells) or Fsk (50 μM)/IBMX (100 μM)(n=908-1,135 cells). Addition of Fsk/IBMX or vehicle at arrow. **(L)** 293T cells expressing PKC sensor ExRai-CKAR treated with vehicle (n=1,490-1,591 cells), PMA (100 ng/mL; n=932-970), or PMA + Gö 6976 (500 nM; n=770-823). Addition of PMA, PMA+Gö 6976, or vehicle at arrow. **(M)** Effect of pre-treatment with vehicle (n=722-792) or Gö 6976 (500 nM; n=509-587) on ionomycin induced CaMKAR response in 293T cells. Data shown as mean ± SEM (error not displayed whenever it is smaller than the data point). All observations done in biological triplicate. ns=p>0.05,**p<0.01, ****p<0.0001; significance was determined via unpaired Student's T-test (*A, J*); nonlinear regression (*B*); one-way ANOVA and Dunnet's multiple comparison test (*D, F, and I*); two-way ANOVA and Sidak's multiple comparisons test (*G, K, and M*) and Tukey's multiple comparisons test (*J and L*).

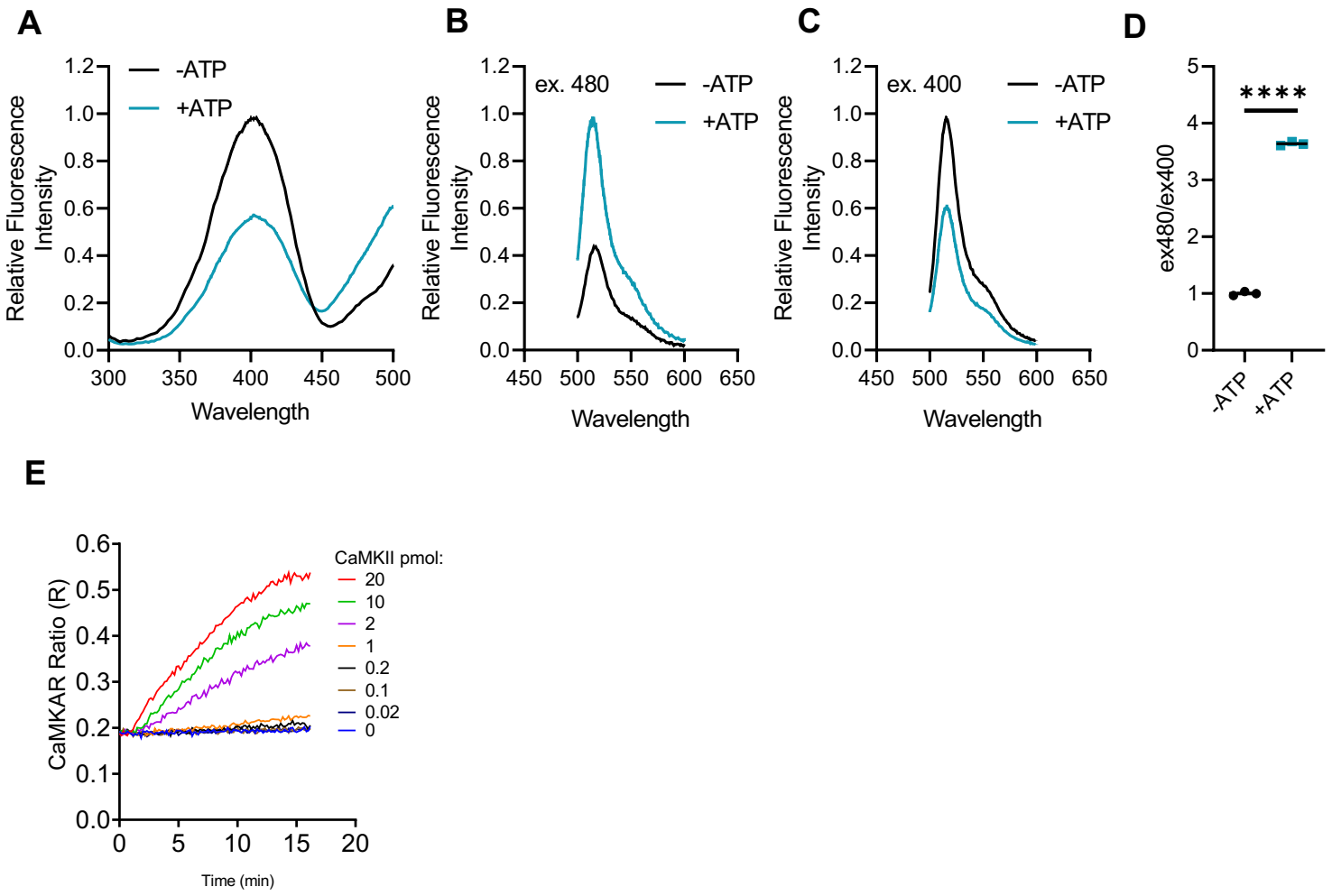


Figure S3. CaMKAR is functional in vitro. Purified recombinant CaMKAR was co-incubated with CaMKIIδ_C, Ca^{2+}/CaM ± ATP and assayed via fluorescence plate reader for excitation spectrum at 520 nm emission **(A)**, emission spectrum at 400 nm excitation **(B)**, emission spectrum at 488 nm excitation **(C)**, and maximal CaMKAR ratio **(D)** (see methods). **(E)** Purified CaMKAR co-incubated with increasing concentrations of purified CaMKIIδ_C (see methods). Data shown as mean ± SEM (error not displayed whenever it is smaller than the data point). Observations done in technical triplicate (*A-D*) or single replicate (*E*).****=p<0.0001; significance determined via unpaired two-tailed t-test.

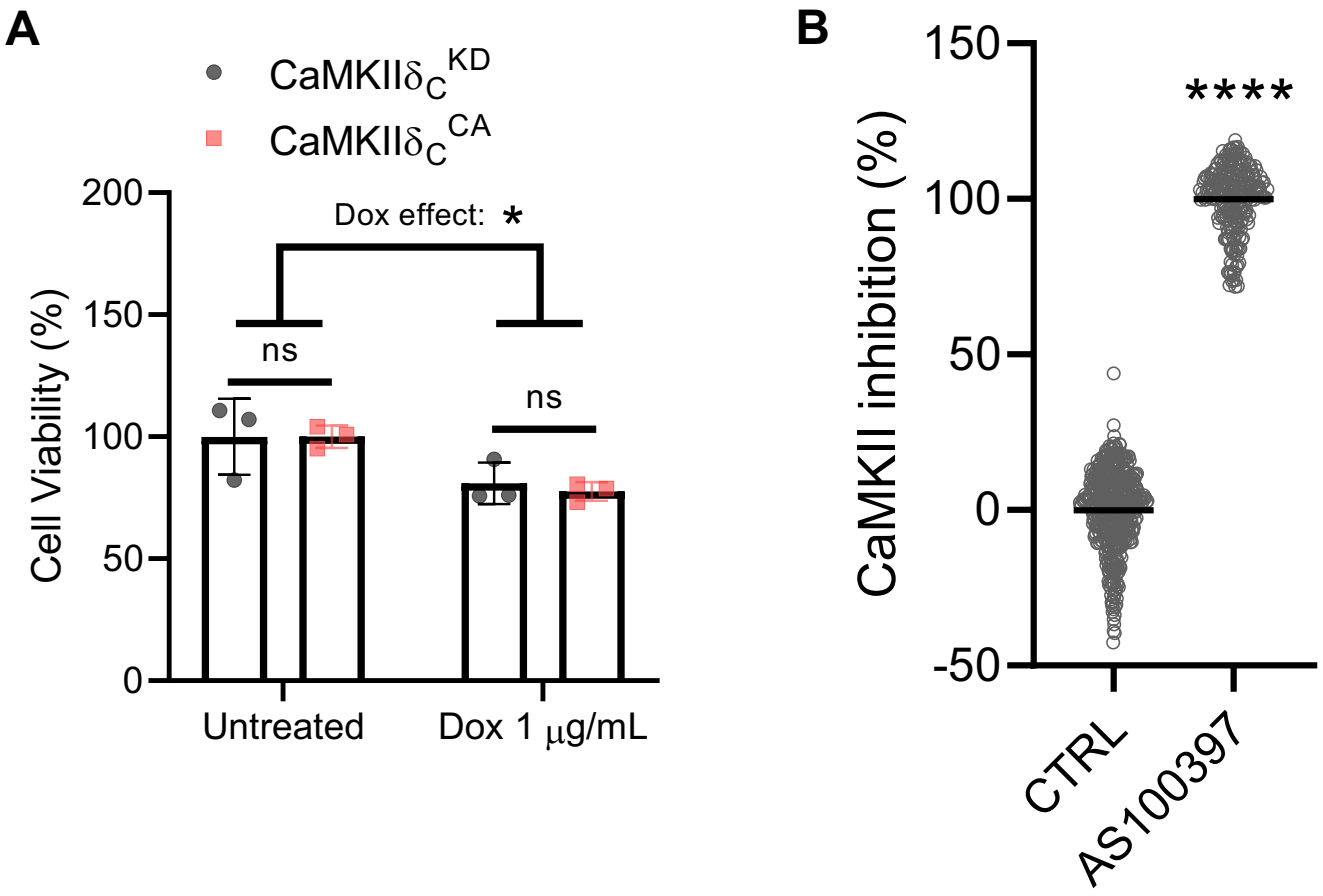


Figure S4. K562 cells tolerate CaMKIIδ^{T287D} overexpression and are suitable for screening. (A) K562 cells infected with lentiviruses encoding CMV-driven CaMKAR and TetON-driven kinase-dead or constitutively active CaMKIIδ$_C$ were treated with doxycycline for 24 hours and assayed for viability via CellTiterGlo 2.0 luminescence. Cells displayed doxycycline-associated toxicity but tolerated hyperactive CaMKII. Observations done in biological triplicate. Data points represent individual wells normalized to untreated condition. ns=p<0.05, *p<0.05; significance determined via two-way ANOVA and Tukey's multiple comparisons test. **(B)** CaMKAR signal in K562$^{CaMKAR/CaMKII}$ cells co-incubated with AS100397 (10 μM, n=240 wells) or vehicle (CTRL, n=504 wells) in 384 well plate format. Data min-max normalized to 0-100% based on means of the two groups. ****p<0.0001; significance determined via unpaired Student's t -test.

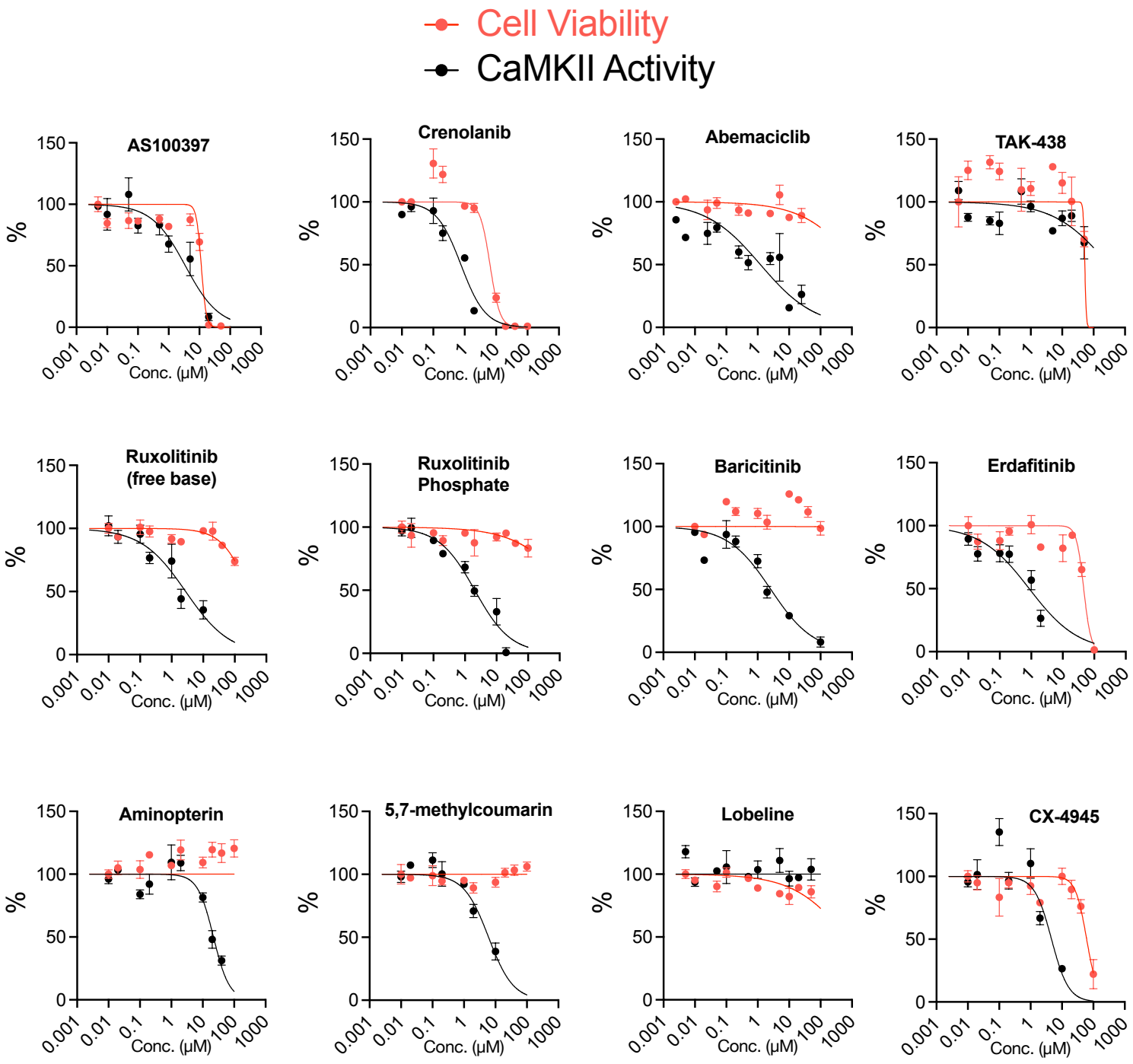


Figure S5. CaMKII inhibition and cell viability in 293T cells. CaMKAR- and CaMKII^{T287D}-expressing 293T cells were treated with hits identified in fig. 2C, AS100397 as positive control. Compounds were incubated for 12 hours and assayed via high content imaging for CaMKII activity (black) and via CellTiter-Glo 2.0 luminescence for cell viability (red). Data represented as mean ± SEM from 3 independent wells, normalized against vehicle-treated cells, with overlayed dose-response IC50 curves (variable slope curve-fitting). TAK-438, erdafitinib, aminopterin, 5,7-methylcoumarin, and lobeline were subsequently confirmed to be false positives (supplementary data file S2).

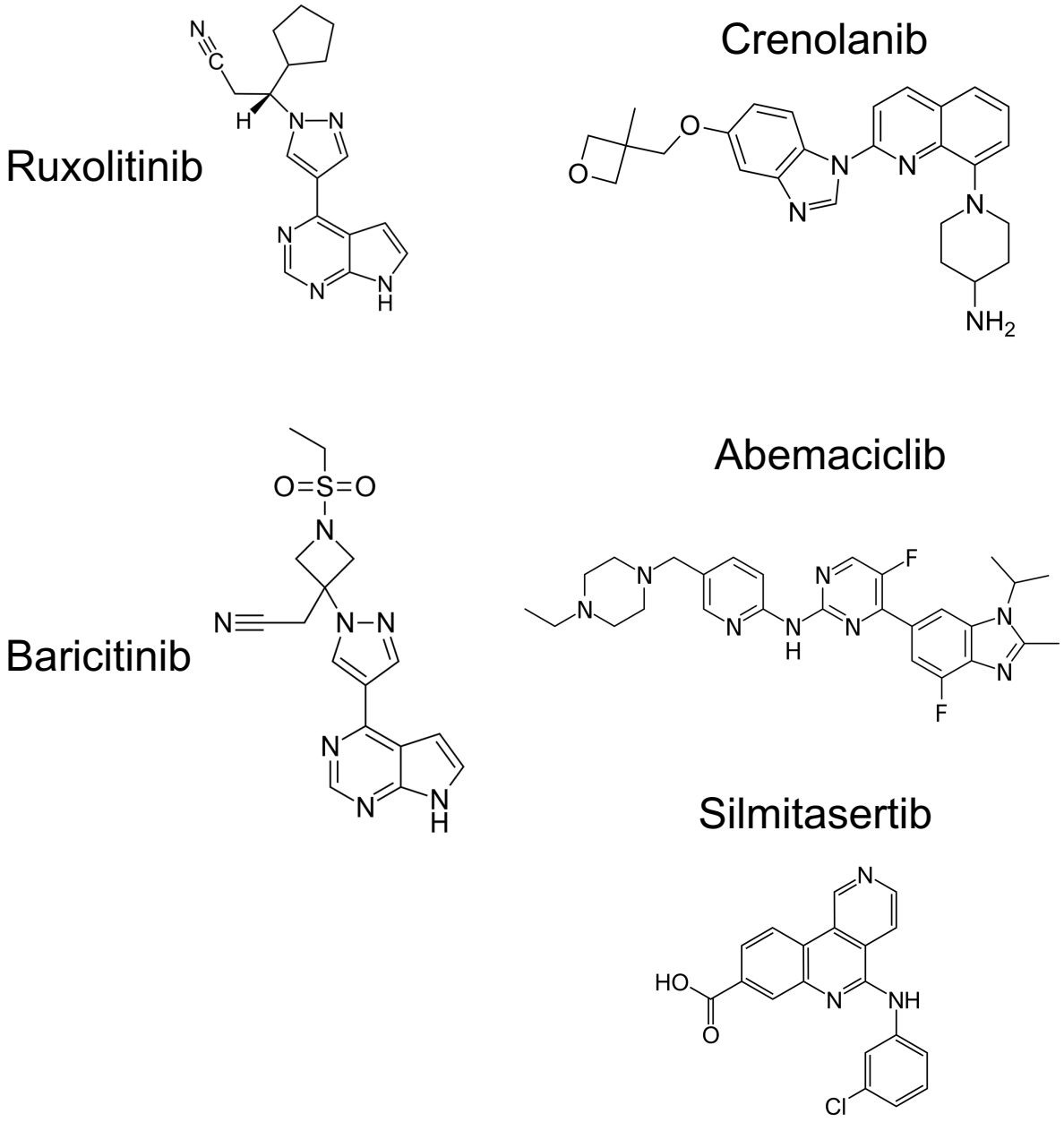


Figure S6. Chemical structures of CaMKII inhibitors identified by drug screening.

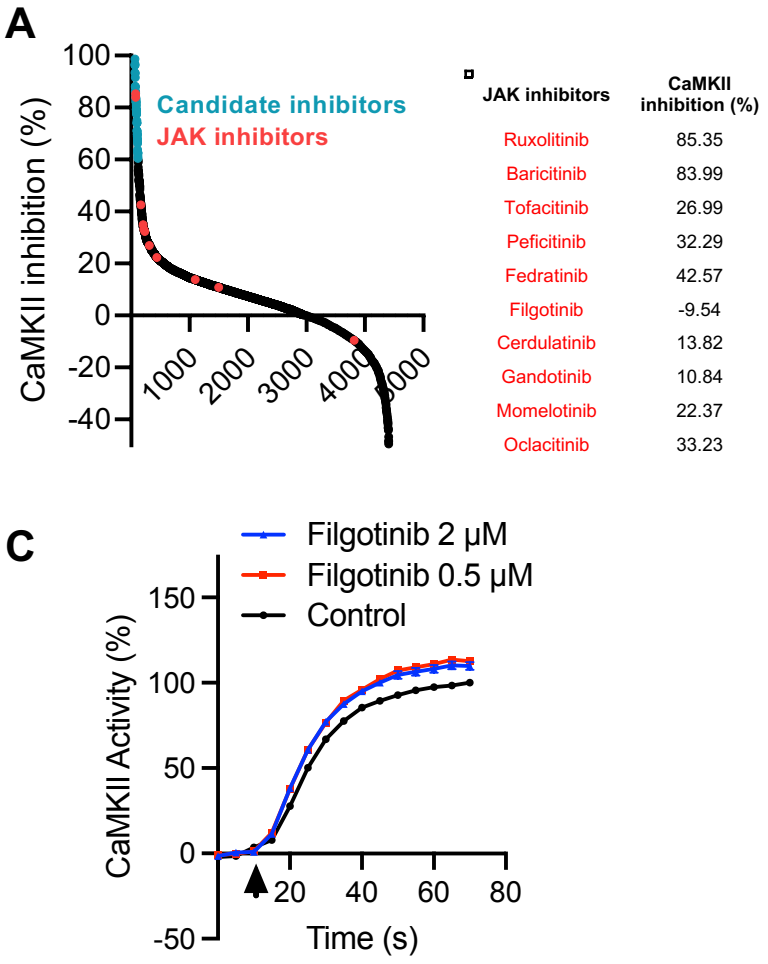


Figure S7. CaMKII inhibition is independent of JAK1/2 inhibition. (A) Known JAK1/2 inhibitors included in the primary screen (from Fig. 2B) are displayed in red (analyzed drugs shown on right with their corresponding % CaMKII inhibition). **(B)** JAK1/2 inhibitors from *A* plotted by their known in vitro IC50 against JAK1 and JAK2 (Ruxolitinib: JAK1=3.3 nM and JAK2=2.8 nM *[50];* Baricitinib: JAK1=5.9 nM and JAK2=5.7 nM *[110];* Tofacitinib: JAK1=112 nM and JAK2=20 nM *[111];* Peficitinib: JAK1=3.9 nM and JAK2=5.0 nM *[112];* Fedratinib: JAK1=105 nM and JAK2=3.0 nM *[113];* Filgotinib: JAK1=10 nM and JAK2=28 nM *[114];* Cerdulatinib: JAK1=12 nM and JAK2=6 nM *[115];* Gandotinib: JAK1=19.8 nM and JAK2=3.0 nM *[116];* Momelotinib: JAK1=11 nM and JAK2=18 nM *[117];* Oclacitinib: JAK1=10 nM and JAK2=18 nM *[118])* versus their in cellulo CaMKII inhibition score identified in this study (same is in panel A). *Insert:* linear regression analysis to determine significance of correlation. **(C)** Pacing-induced CaMKII activity (arrow) in CaMKAR-expressing NRVMs pre-treated with vehicle (n=284-354) or JAK inhibitor filgotinib (0.5 μM; n=291-335; 2 μm n=290-330 cells). **(D)** In vitro kinase activity assay (HotSpot radioactive phosphorous substrate labeling) was performed with varying concentrations of ruxolitinib and ATP. As ATP concentration increases, the apparent IC50 of ruxolitinib also increases, consistent with ATP-competition. Ki=inhibitory constant.

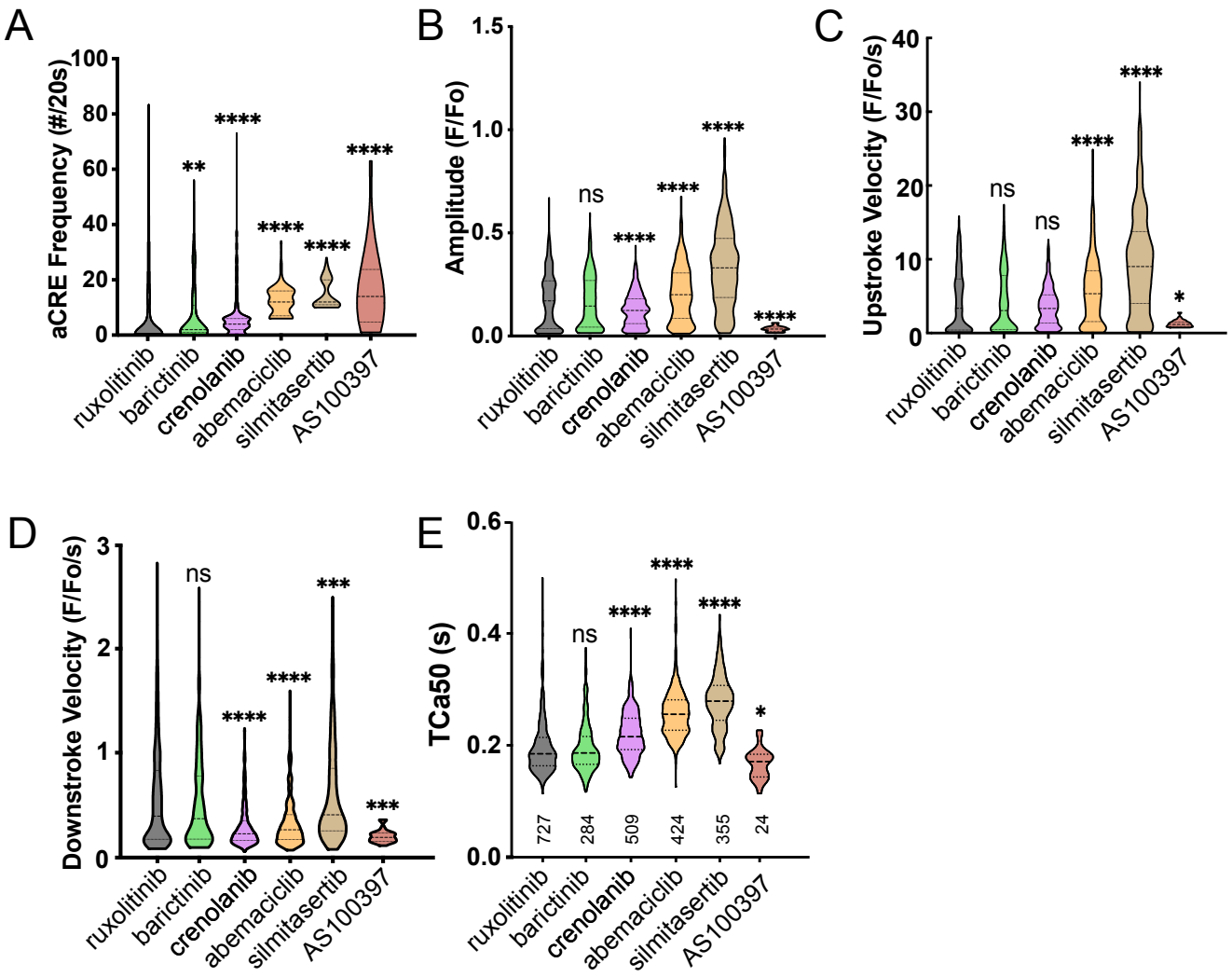


Figure S8. In vitro comparison of calcium transient kinetics among candidate CaMKII inhibitors. Human iPSCs with the pathogenic mutation RyR2$^{S404R/WT}$ were differentiated into cardiomyocytes and loaded with Rhod-2 as a Ca^{2+} indicator. Each test compound was pre-incubated for >10 minutes at a concentration of 2μM. Cells were paced at 1Hz for 10s with continuation of high-speed Ca^{2+} imaging after the cessation of pacing to detect abnormal Ca^{2+} release events **(A)**. Automated analysis of paced Ca^{2+} transients revealed the peak amplitude **(B)**, upstroke velocity **(C)**, Downstroke velocity **(D)**, and Ca^{2+} transient duration measured at 50% of the peak (TCa50) **(E)**. The number of analyzed cells (n) for each parameter is annotated on panel E. Statistics were performed by one-way ANOVA (non-parametric Kruskal-Wallis) against ruxolitinib and Dunn's multiple comparisons test: ns=p>0.1, *p<0.05, ****p<0.0001.

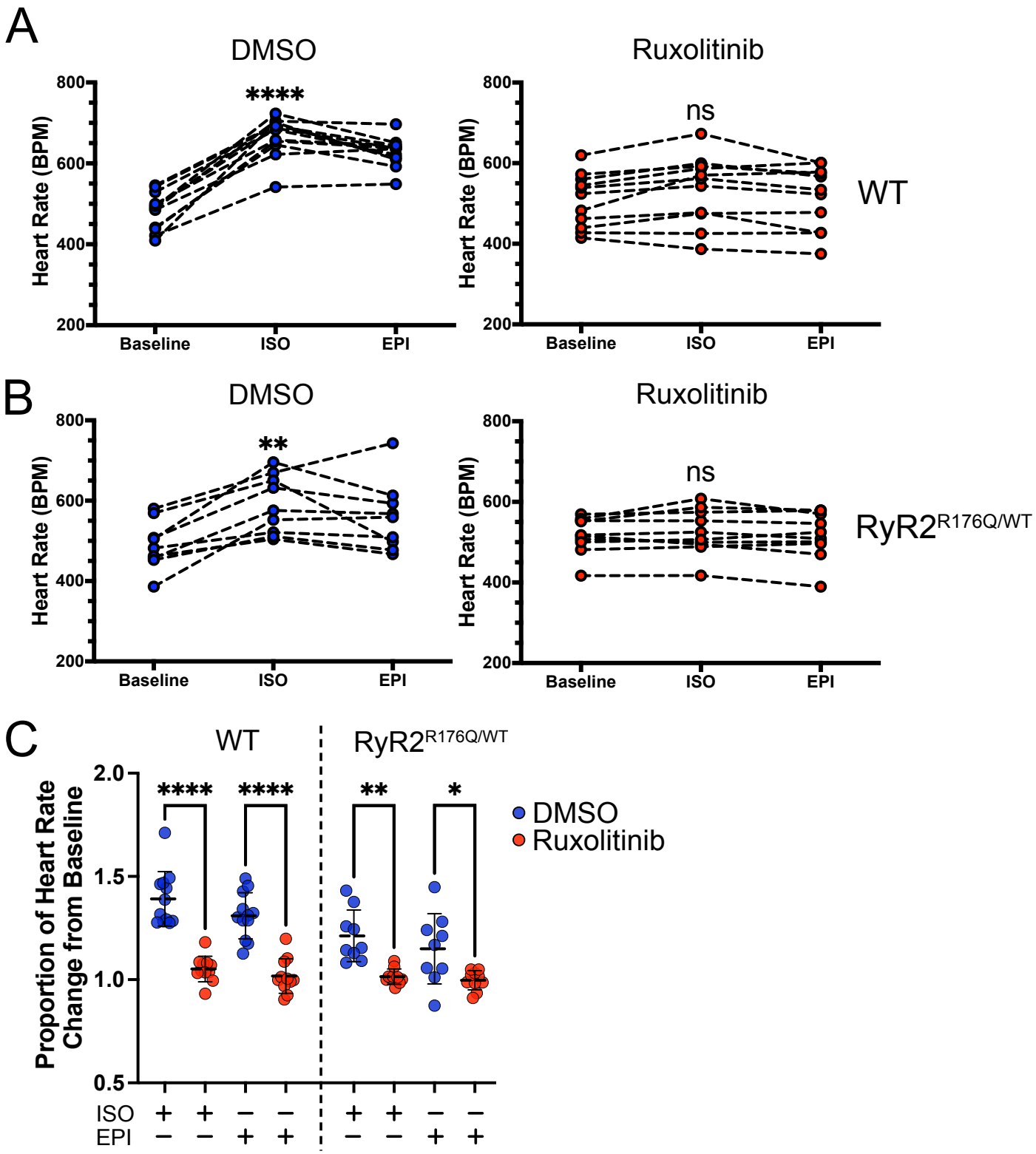


Figure S9. Ruxolitinib inhibits adrenergically induced increases in heart rate in mice. Wild type (WT) and RyR2$^{R176Q/WT}$ animals were instrumented with a 2 lead ECG to monitor heart rate. Prior to stimulation, animals were treated with ruxolitinib (75 mg/kg) or vehicle only (DMSO) by intraperitoneal injection followed by Isoproterenol (ISO; 4 mg/kg) or epinephrine (EPI; 2 mg/kg). Continuous monitoring of the heart rate in WT **(A)** or RyR2$^{R176Q/WT}$ animals **(B)**, demonstrated the inhibitory effects of ruxolitinib. **(C)** The fractional change from baseline was calculated for each animal and compared for each condition. Number of animals in each group (N): WT + DMSO = 12, WT + ruxolitinib = 11, RyR2R176Q/WT + DMSO = 9, and RyR2R176Q/WT + ruxolitinib = 10. Statistics were performed by one-way ANOVA and Tukey's multiple comparisons test: ns=p>0.1, *p<0.05, **p<0.01, ****p<0.0001.

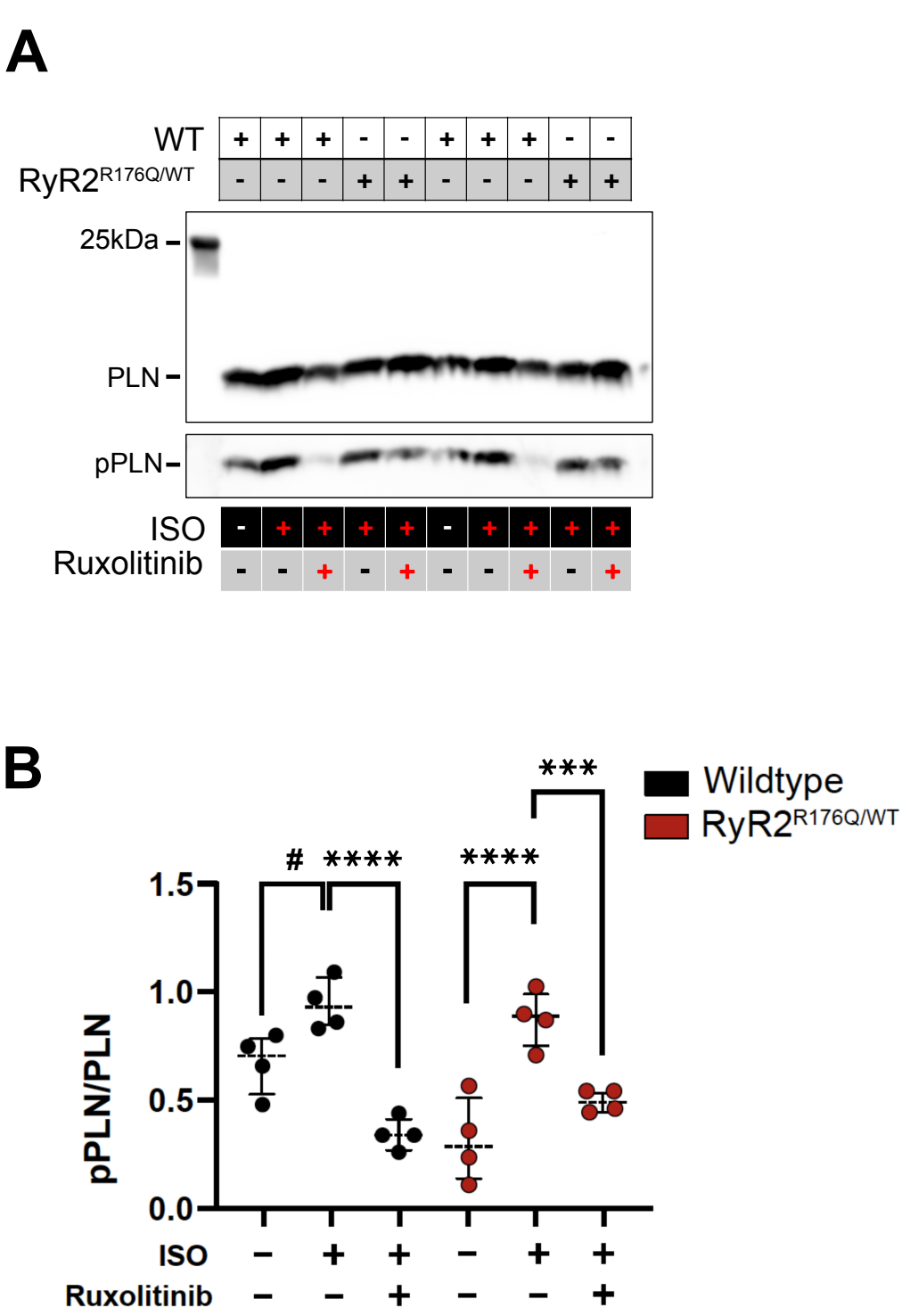


Figure S10. Ruxolitinib inhibits CaMKII in CPVT mice. (A) Representative western blot of whole heart lysates prepared from wild type (WT) and RyR2$^{R176Q/WT}$ animals treated with ruxolitinib and/or isoproterenol (ISO) were separated by SDS-page electrophoresis and probed with specific antibodies to monomeric phospholamban (PLN) and phosphorylated phospholamban (pPLN). **(B)** Quantification of immunoblot from A. Data points represent individual mice, with n=4 mice in each group. Statistics were performed by one-way ANOVA and Tukey's multiple comparisons test: #=p=0.0531, ***p<0.005, ****p<0.0001.

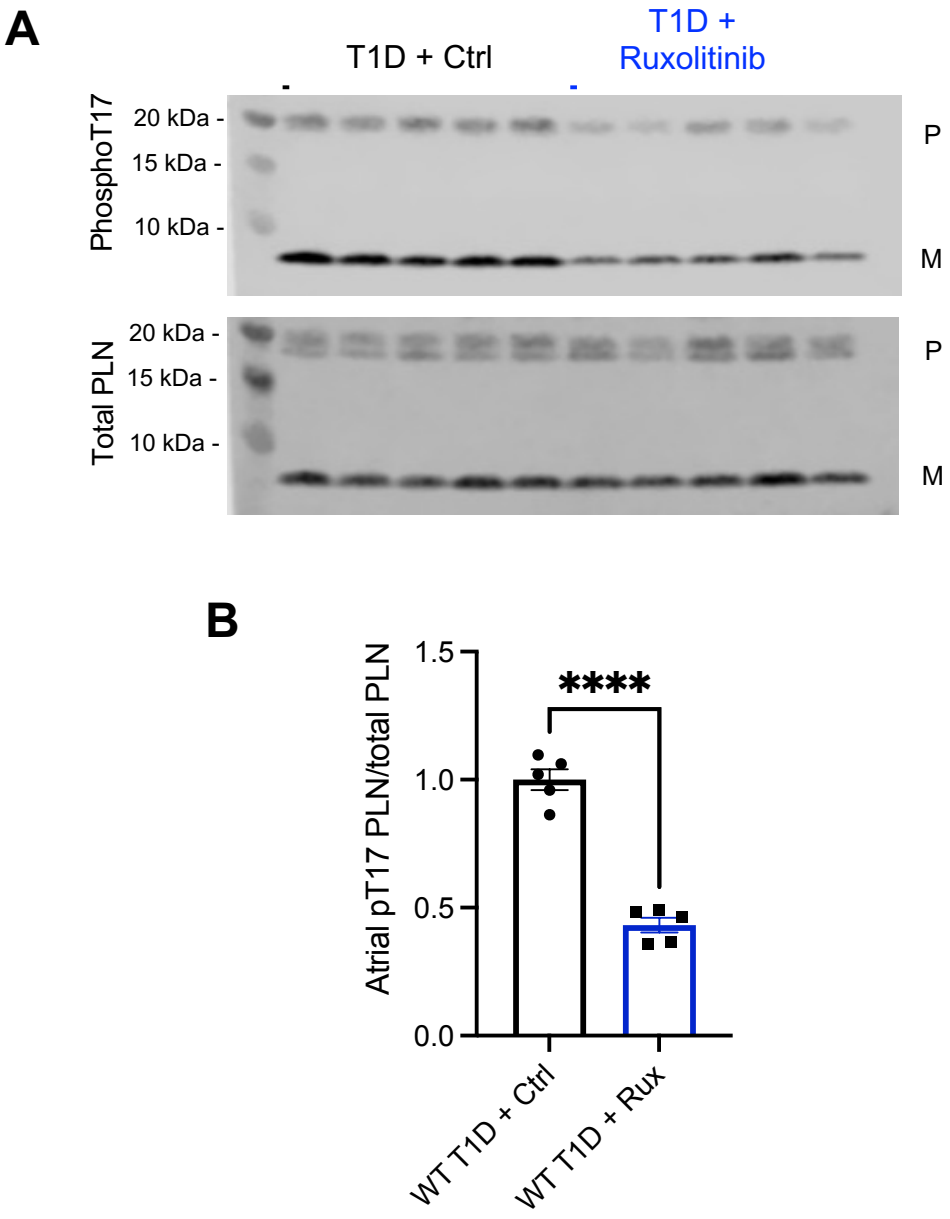
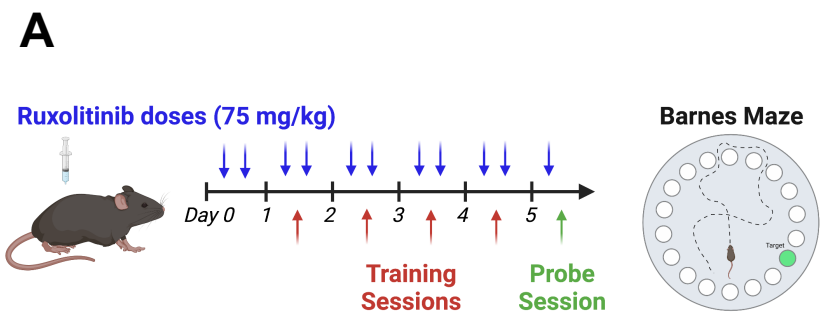
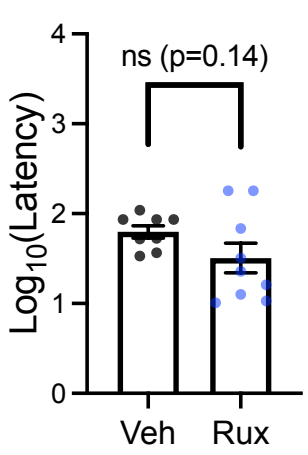
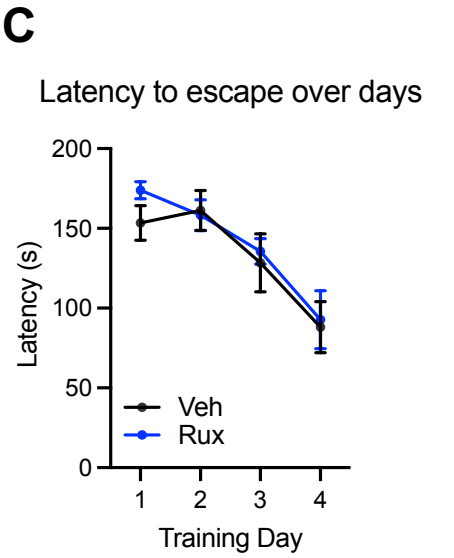
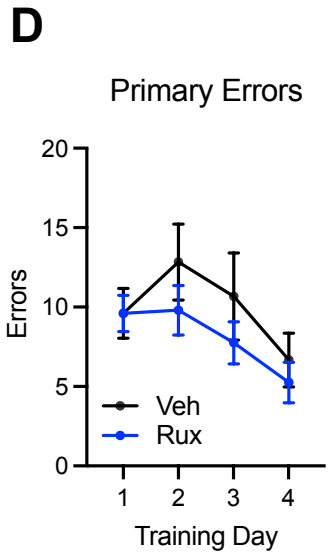


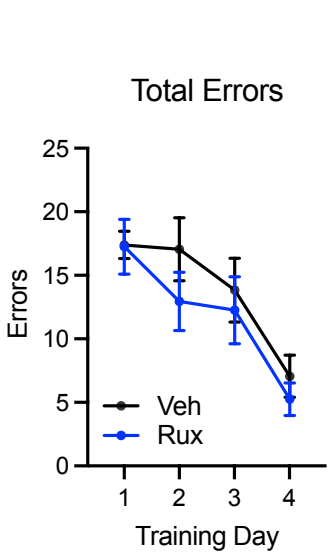
Figure S11. Ruxolitinib inhibits CaMKII in diabetic mouse atria. (A) Atrial heart lysates prepared from type 1 diabetic (T1D) animals treated with ruxolitinib (Rux) or vehicle (Ctrl) were separated by SDS-page electrophoresis and probed with specific antibodies to phospholamban (PLN), phosphorylated phospholamban (pPLN). P = pentameric PLN; M = monomeric PLN. **(B)** Quantification of immunoblot from A. Aggregate of both pentameric and monomeric bands. Data points represent individual mice (n=5 per group). Significance was determined via unpaired T-test; ****p<0.0001.











Source of Variation	% of total variation	P value	
Day	36.71	<0.0001	****
Treatment	0.5729	0.5247	ns
Subject	23.09	0.0555	ns

Source of Variation	% of total variation	P value	
Day	12.11	0.0037	**
Treatment	2.741	0.3365	ns
Subject	47.65	<0.0001	****

Source of Variation	% of total variation	P value	
Day	30.85	<0.0001	****
Treatment	1.617	0.4175	ns
Subject	39.8	<0.0001	****

Figure S12. Ruxolitinib (Rux) does not impair mouse performance in the Barnes maze test compared to vehicle (Veh). **(A)** Wild type C57BL/6J mice were started on intraperitoneal ruxolitinib treatment twice daily prior to longitudinal training in the Barnes maze. Mice received training sessions (days 1-4) and one probe session (day 5). **(B)** Time in seconds (s) for each mouse was measured for latency to escape the maze during the probe session. Values were Log_{10} transformed due to a non-normal distribution and statistically tested using a two-tailed unpaired Student's T-test. There was no significant increase (p=0.14) in latency comparing Rux (n=9) to Veh (n=8) treated mice. Data shown as mean ± SEM. **(C-E)** Latency to escape maze **(C)**, primary errors **(D)**, and total errors **(E)** of mice subjected to Barnes maze during the 4 training trials. Statistical testing via two-way ANOVA found a significant effect by day but not treatment. Data shown as mean ± SEM.

Table S1. Comparison of properties of 3 major CaMKII reporters.

	Camui	**FRESCA**	**CaMKAR**
Maximal amplitude change	~50-80% *(34, 41)*	2.37 ± 0.06% *(35)*	227 ± 11.1%
Kinetics (Time-to-peak)	~1-3 min *(34, 41)*	1.125 min *(35)*	17.6 s
Fluorophores	CFP/YFP, mNeonGreen/mRuby3 *(34, 41)*	CFP/YFP *(35)*	Circularly permuted GFP
Sensing mechanism	Förster resonance energy transfer (FRET) decrease secondary to conformational change of built-in CaMKIIα	Förster resonance energy transfer (FRET) decrease secondary to CaMKII phosphorylation of substrate peptide	Fluorescence intensity increase secondary to CaMKII phosphorylation of substrate peptide
Known Compatible CaMKII isoforms	CaMKIIα *(34, 41)*	CaMKIIγ *(35)*	CaMKIIα/β/δ/γ

Table S2. Comparison of safety profiles of 5 FDA approved compounds that were identified as CaMKII inhibitors. N/A= not available.

	Ruxolitinib	**Baricitinib**	**Abemaciclib**	**Silmitasertib**	**Crenolanib**
Original target(s)	JAK1/2	JAK1/2	CDK4/6	CK2	FLT3, PDGFRα, PDGFRβ, C-Kit
Indications	Atopic dermatitis, Vitiligo, Polycythemia, splenomegaly, myelofibrosis	Rheumatoid Arthritis, Alopecia areata, COVID-19	HR-positive, HER2-negative advanced or metastatic breast cancer	Cholangiocarcinoma, Medulloblastoma	Acute myeloid leukemia, GIST, glioma
Notable adverse effects	Reversible thrombocytopenia and anemia, herpes zoster infection *(83)*	Opportunistic respiratory infections, neutropenia, pulmonary embolism, thrombosis *(95)*	Neutropenia, interstitial lung disease, hepatoxicity, embryo-fetal toxicity, thrombosis *(96, 97)*	Anemia, diarrhea, fatigue, thrombocytopenia *(98)*	Vomiting, nausea, diarrhea, transaminitis *(45, 99, 100)*
Maximum dose used in humans	FDA-approved: 5-25 mg BID *(101)* Healthy volunteers: 200 mg QD *(76)*	FDA-approved: 4 mg QD *(102)* Healthy volunteers: 40 mg *(103)*	200 mg BID *(104)*	1000 mg BID *(98)*	100 mg TID *(105)*
Treatment duration	Median 2.9 years, (COMFORT trials) *(83)*	Median 4.6 years *(95)*	Median 14 months (monarchE trial) *(106)*	21 days *(98)*	9 weeks *(107)*
Year of FDA-approval	2011	2018	2017	FDA orphan drug status: 2017	N/A
Agency approvals	FDA, EMA, PMDA	FDA, EMA, PMDA	FDA, EMA, PMDA	FDA orphan drug status (Phase II trials)	None (Phase II trials)
CNS penetrance	3.5% *(66)*	20% *(108)*	11-16.6% *(109)*	N/A	N/A

Table S3. Electrophysiologic parameters in mice treated with ruxolitinib and either isoproterenol (ISO) or epinephrine (EPI). All parameters are in msec. WT = wild type, PR = P wave R wave interval, QRS = Q wave R wave S wave interval, QTc = corrected Q wave T wave interval.

		DMSO			**Ruxolitinib**		
		Baseline	ISO	EPI	Baseline	ISO	EPI
WT	PR	40.31 ± 2	36.73 ± 1.8	120.7 ± 84	43.4 ± 4	43.8 ± 4	45.9 ± 2.7
	QRS	10.8 ± 0.8	11.31 ± 1	9.9 ± 1	13.2 ± 2	11.5 ± 1	12.5 ± 1
	QTc	39.74 ± 6	41.9 ± 3	46.4 ± 6.2	48.6 ± 6	49.2 ± 4	55.7 ± 8
$RyR^{R176Q/WT}$	PR	41.1 ± 2	36.9 ± 3	40.02 ± 3	39.5 ± 1	43.1 ± 3	42.2 ± 4
	QRS	11.03 ± 1	8.8 ± 0.6	11.1 ± 1	9.3 ± 0.7	11.6 ± 1.2	11.5 ± 0.8
	QTc	38.5 ± 4	46.1 ± 5	50.7 ± 5	36.5 ± 5	51.6 ± 5	49.5 ± 4