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Functional Calsequestrin-1 Is Expressed in the Heart and Its Deficiency Is Causally Related to Malignant Hyperthermia-Like Arrhythmia

Running Title: *Sun & Wang, et al.; Casq1 Regulates Cardiomyocyte Ca^{2+} Release*

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Abstract

Background: Calsequestrins (Casqs), comprising the Casq1 and Casq2 isoforms, buffer Ca^{2+} and regulate its release in the sarcoplasmic reticulum (SR) of skeletal and cardiac muscle, respectively. Human inherited diseases associated with mutations in *CASQ1* or *CASQ2* include malignant hyperthermia/environmental heat stroke (MH/EHS) and catecholaminergic polymorphic ventricular tachycardia. However, patients with an MH/EHS event often suffer from arrhythmia for which the underlying mechanism remains unknown.
Methods: Working hearts from conventional (*Casq1*-KO) and cardiac-specific (*Casq1*-CKO) *Casq1* knockout mice were monitored *in vivo* and *ex vivo* by electrocardiogram and electrical mapping, respectively. MH was induced by 2% isoflurane and treated intraperitoneally with dantrolene. Time-lapse imaging was used to monitor intracellular Ca^{2+} activity in isolated mouse cardiomyocytes or neonatal rat ventricular myocytes (NRVMs) with knockdown, over-expression or truncation of the *Casq1* gene. Conformational change in both Casqs was determined by crosslinking Western blot analysis.
Results: Like MH/EHS patients, *Casq1*-KO and *Casq1*-CKO mice had faster basal heart rate, and ventricular tachycardia upon exposure to 2% isoflurane, which could be relieved by dantrolene. Basal sinus tachycardia and ventricular ectopic electrical triggering also occurred in *Casq1*-KO hearts *ex vivo*. Accordingly, the ventricular cardiomyocytes from *Casq1*-CKO mice displayed dantrolene-sensitive increased Ca^{2+} waves and diastole premature Ca^{2+} transients/oscillations upon isoflurane. NRVMs with Casq1-knockdown had enhanced spontaneous Ca^{2+} sparks/transients upon isoflurane, while cells over-expressing Casq1 exhibited decreased Ca^{2+} sparks/transients that were absent in cells with truncation of 9 amino acids at the C-terminus of Casq1. Structural evaluation showed that most of the Casq1 protein was present as a polymer and physically interacted with RyR2 in the ventricular SR. The Casq1 isoform was also expressed in human myocardium. Mechanistically, exposure to 2% isoflurane or heating at 41ºC induced Casq1 oligomerization in mouse ventricular and skeletal muscle tissues, leading to a reduced Casq1/RyR2 interaction and increased RyR2 activity in the ventricle.
Conclusions: Casq1 is expressed in the heart, where it regulates SR Ca^{2+} release and heart rate. Casq1 deficiency independently causes MH/EHS-like ventricular arrhythmia by trigger-induced Casq1 oligomerization and a relief of its inhibitory effect on RyR2-mediated Ca^{2+} release, thus revealing a new inherited arrhythmia and a novel mechanism for MH/EHS arrhythmogenesis.

Key Words: Calsequestrin-1; oligomerization; arrhythmia; malignant hyperthermia; volatile anesthetics

Non-standard Abbreviation and Acronym

AMVMs	adult mouse ventricular myocytes
AnkB	ankyrin-B
Casqs	calsequestrins
Casq1	calsequestrin-1
Casq1-CKO	cardiac specific *Casq1*-knockout mice
Casq1-KO	conventional *Casq1*-knockout mice
Casq1-Δ9	truncation of 9 amino acids at the C-terminus of Casq1
Casq1-wt	wild-type Casq1 gene
CO	Ca^{2+} oscillations

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HET	heterozygous
Isof	isoflurane
JPH2	juncophilin-2
MCM	mhy6-mer-Cre-mer
NRVMs	neonatal rat ventricular myocytes
PCT	premature Ca^{2+} transients
PLN	phospholamban
VPB	ventricular premature beats
VT	occasional ventricular tachycardia

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Clinical Perspective

What is new?

- Calsequestrin-1 (Casq1), the skeletal isoform, is indeed expressed in cardiomyocyte sarcoplasmic reticulum (SR) from murine and human hearts, mostly presenting as a polymeric form and interacting with ryanodine-2 receptor (RyR2) in ventricles.
- Casq1 deficiency causes sinus tachycardia in basal condition, a novel finding which may be associated with sinus beat regulation, and ventricular arrhythmia as an independent arrhythmogenesis if a high concentration of volatile anesthetics is used.
- Volatile anesthetics and heating (41ºC) can directly induce Casq1 oligomerization, thereby causing enhancement of diastolic Ca^{2+} leak and premature Ca^{2+} transients through a reduced regulatory effect of Casq1 on RyR2 activity.

What are the clinical implications?

- This novel mechanism underlying the arrhythmia occurring in patients with a malignant hyperthermia/environmental heat stroke (MH/EHS) episode may provide different strategies for heart disorders as an independent profile in MH/EHS syndrome.
- The finding of Casq1 conformational change induced by triggers of MH/EHS could lead to novel therapeutic approaches to prevent MH/EHS episodes, which may also be useful to treat heatstroke.
- Understanding the fundamental function of Casq1 in the heart, which is likely different from that of Casq2, can improve the knowledge on SR homeostasis and pathogenesis of its disturbance, in particular in the regulation of sinus beats.

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Introduction

Dysfunction of Ca^{2+} handling proteins in the sarcoplasmic reticulum (SR) is one of the key pathological features of acquired arrhythmia in patients with various heart diseases and inherited arrhythmia in patients with genetic defects.[1] Mutations in genes encoding ryanodine receptor-2 (RyR2), a component of a Ca^{2+} releasing channel, and calsequestrin-2 (CASQ2), a Ca^{2+} buffering protein, in myocardial SR are dominantly responsible for human catecholaminergic polymorphic ventricular tachycardia (CPVT), an inherited arrhythmogenic disorder characterized by exercise- or stress-induced ventricular tachycardia with a high lethal rate in untreated patients.[1–3] Premature spontaneous SR Ca^{2+} release during diastole, activation of Na^{+}/Ca^{2+} exchange and the generation of delayed after depolarizations (DADs) are considered as high tachycardia risks for CPVT.[4–7] Similarly, the excessive increase of free intracellular Ca^{2+} resulting from gain-of-function mutations in *RyR1* or loss-of-function mutations in *CASQ1*, both encoding skeletal muscle isoforms, has been suggested to induce a hypermetabolic syndrome known as malignant hyperthermia (MH) and environmental heat stroke (EHS).[8–11] MH/EHS is a hereditary subclinical myopathy with tachycardia, hypercapnia, muscle rigidity, hyperthermia, acidosis and rhabdomyolysis triggered by volatile anesthetics, depolarizing muscle relaxants and strenuous exercise or heat. This syndrome is fatal in up to 80% of cases if left untreated. Dantrolene, a RyR antagonist, is the drug specifically used to treat MH/EHS, with a survival rate of ~90% for patients suffering MH/EHS episodes.[12, 13]

Casqs are SR Ca^{2+} storing proteins that maintain sufficient Ca^{2+} release upon membrane depolarization in striated muscle cells. In addition, Casqs are physically tethered to RyR via triadin and junctin, forming a quaternary complex to regulate the Ca^{2+} release channel function.[5, 6, 14–16] Deficiency in Casq1 and Casq2 abundance or function results in disturbed SR Ca^{2+}

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buffering and disrupted RyR activity, thus generating an uncontrolled SR Ca^{2+} release, activation of the contractile apparatus and a massive increase in cellular energy production in skeletal muscle (MH/EHS)[11, 12, 14, 15] and CPVT in the heart,[6, 16, 17] respectively. In addition, another autosomal dominant hereditary disease called tubular aggregate myopathy (TAM) has been found to be linked to a dramatic CASQ1 polymerization and SR morphological changes due to *CASQ1* mutation in patients who usually have a disturbed SR storage with abnormal Ca^{2+} release/entry and consequent diffuse myalgia, muscle weakness and post-exercise fatigue.[14, 18–20] Moreover, inheritable *CASQ1* mutants of D244G and M87T are found to be associated with TAM and MH patients, respectively. They display a totally different CASQ1 polymerization behavior.[20, 21] These unexplained associations of CASQ1 abundance and conformation changes with multiple hereditary skeletal diseases reflect its importance and complexity in the regulation of SR integrity and Ca^{2+} homeostasis.

It is still puzzling why most inherited myopathies result in obvious cardiac disorders.[21–23] Also, more than 60% of patients suffering an MH/EHS episode have a first sign of tachycardia under either normal or abnormal condition of body temperature and blood gas indexes.[12, 21–24] In addition, some studies have shown ~40% incidence of ventricular arrhythmia and unexplained cardiac pathological alterations, including myocardial necrosis, in MH susceptible individuals.[12, 21, 24–26] Although CASQ1 protein expression has not yet been detected in the heart, clues for *CASQ1* gene expression in human heart (www.proteinatlas.org) and rat ventricle[27] have been revealed, and the variants in *CASQ1* intron 2 were found to be associated with faster resting heart rate in Caucasian population.[28] Therefore, we hypothesize that CASQ1, the skeletal isoform, is also expressed in the heart. As an independent pathogenic factor its change induces abnormal Ca^{2+} signal and arrhythmia in MH/EHS subjects upon trigger exposure.

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In the present study, we generated conventional (*Casq1*-KO) and cardiac-specific *Casq1* knockout (*Casq1*-CKO) mice. Like MH/EHS patients, *Casq1*-KO and *Casq1*-CKO mice demonstrated basal sinus tachycardia, and ventricular tachycardia under exposure to 2% isoflurane *in vivo* and basal ventricular ectopic electric triggering *ex vivo*. *Casq1*-CKO ventricular cardiomyocytes had significant intracellular Ca^{2+} disorders including increased Ca^{2+} waves and diastole premature Ca^{2+} transients upon isoflurane exposure *in vitro*. Furthermore, isoflurane treatment induced Casq1 oligomerization in mouse ventricle, resulting in a reduced interaction between Casq1 and RyR2. To investigate the possible mechanism involved *in vivo*, we used neonatal rat ventricular myocytes (NRVMs) with Casq1-knock down, or over-expression of full-length Casq1 or Ca^{2+} binding domain-truncated Casq1. These results support the findings that the inducer of MH/EHS, isoflurane or heating, can cause Casq1 depolymerization and a reduced inhibitory effect on RyR2-mediated Ca^{2+} release, resulting in a high risk for MH/EHS arrhythmogenesis.

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Methods

The data that support the findings of this study within the article are available from the corresponding author on reasonable request.

Animal experiments complied with the “Guide for the Care and Use of Laboratory Animals” adopted by the Beijing Municipal People’s Government and in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 8523, revised 2011). All animals were housed in the Center of Experimental Animals (Grade SPF), Capital Medical University (Beijing, China), and kept in a 12-h light-dark cycle, in a temperature-controlled room. To avoid sex effects, male mice (wide

type (WT): 87, *Casq1*-KO: 52 and *Casq1*-CKO: 62) were used and counted for evaluation and statistical analysis in this study, excluding mice that died of sudden death of unknown reason. Experimental project, protocol, and human sample collection were approved by Capital Medical University (AEEI-2016-072 and Z2020SY144).

Statistical analysis

All experimentation and data analysis were performed blinded to genotype and intervention. All data are expressed as the Mean±SD, and were examined for normality of distribution before statistical analysis using Shapiro-Wilk normality test. For unpaired observations, Student's *t* test was performed, and ANOVA followed by Tukey's multiple comparison test (normally distributed) or Kruskal-Wallis followed by Dunn's multiple comparison's test (non-normally distributed) were used for multiple groups. Hierarchical analysis was performed for data obtained from isolated cells in separate experiments.[29] Analysis of arrhythmia occurrence percentage was performed using Fisher's exact test. Statistically significant differences between groups were defined as $P<0.05$, and the P values <0.05 are shown in the Figures.

The detail experimental methods and materials can be found in the Data Supplement online.

Results

1. Expression of Casq1 isoform in murine and human hearts

We investigated whether Casq1 is expressed in the heart by analyzing its expression in rat and mouse hearts, NRVMs and a rat embryonic cardiomyocyte cell line (H9C2). We found lower expression of Casq1 mRNA and protein than that of Casq2 in whole tissue and cell lysates (Figure 1A and 1B). However, in the murine ventricle and atrium SR fractions, Casq1 was abundantly expressed as a low molecular weight monomer (63 kDa) and a high molecular weight

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Casq1-like protein, which is presumed to be a polymeric form of Casq1 present in skeletal muscle (Figure 1C, Figure IA in the Data Supplement).[30, 31] We further verified the conformation of Casq1 and Casq2 in the SR fraction from freshly isolated ventricular myocytes that had been crosslinked by treatment with chemical crosslinking reagents. Casq1 appeared mostly as a polymer that disappeared in the non-crosslinking lysate treated with 10 mM egtazic acid (EGTA) before adding the loading buffer, while Casq2 was present in its monomeric, dimeric, and trimeric conformations (Figure 1D) as reported previously.[14, 30, 31] In reference to the expression ratio (0.47:1) of Casq2:Casq1 evaluated in rat soleus,[32, 33] we calculated the relative ratio of Casq isoforms in mouse ventricular myocytes as 3.11:1 (Figure IB in the Data Supplement). The Casq1 protein was also found expressed in human auricle and ventricle from the patients with mitral regurgitation and cardiac hypertrophy, respectively. The ventricle Casq1 appeared to be modified to a heavier molecular weight (Figure 1E, Table I in the Data Supplement), due likely to this proliferative disease as Casq1 monomers were found with a similar molecular weight in human auricle and mouse ventricle. Immunostaining analysis of murine ventricles and cardiomyocytes demonstrated that Casq1 and Casq2 were respectively distributed along the transverse striations with a periodicity of about 2 μm at the terminal cisternae. A non-specific labeling in the intercellular space was observed in anti-Casq1 stained ventricles, but not in isolated myocytes (Figure 1F).

2. MH-like tachycardia induced by isoflurane in cardiac-specific and conventional *Casq1* knockout mice

To determine the function of Casq1 in the heart, we generated *Casq1*-CKO and *Casq1*-KO mice (Figure II in the Data Supplement). Unlike *Casq1*-CKO mice, *Casq1*-KO offspring was not consistent with Mendel's law of heredity (Figure IIIA in the Data Supplement). No statistical

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difference was found between WT and *Casq1*-KO mice with respect to the evaluation of cardiac morphology (Figure IIIB through IIID in the Data Supplement), and function (Table II in the Data Supplement). However, ultrastructural analysis by electron microscopy revealed a wide and empty space with intermittent longitudinal SR in ventricular myocytes of 10-week-old *Casq1*-KO mice compared to the continuous longitudinal SR observed in WT mice (Figure IIIE in the Data Supplement). This SR ultrastructural alteration is different from that previously reported in *Casq2*-KO mice,[17, 34] and thus further studies are needed in this regard.

Casq1 knockout was confirmed by Western blot analysis in ventricular and skeletal muscles (Figure IVA in the Data Supplement), and the abundant expression of Casq1 in *Casq1*-CKO mouse skeletal muscle demonstrated the successful development of *Casq1*-CKO (Figure IVB in the Data Supplement). Like for *Casq2*-null ventricle and *Casq1*-KO skeletal muscle,[3, 15, 34] both *Casq1*-KO and *Casq1*-CKO mouse ventricles had a down-regulated expression of junctin compared to WT mice, an important protein linking Casqs to RyR1 and RyR2, respectively. In addition, the expression of RyR2, phospholamban (PLN), juncophilin-2 (JPH2) and ankyrin-B (AnkB), as well as RyR2 phosphorylation at Ser2808 and Ser2814 and PLN phosphorylation at Ser16/Thr17 were not significantly changed in both ventricles of the *Casq1* knockout mice compared to those in the WT controls, except for a ~25% enhancement at RyR2-pSer2814 (Figure IVC in the Data Supplement), suggesting a hyperactivity state of RyR2 in the ventricles of *Casq1* deficient mice.

Surface electrocardiogram recordings showed an increased basal heart rate, which was sensitive to dantrolene treatment, in both *Casq1*-KO and *Casq1*-CKO mice under 1% isoflurane or pentobarbital anesthesia. Their heart rate was faster upon sympathetic stimulation with isoproterenol (Figure 2A through 2C, Table III in the Data Supplement), but did not show any

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CPVT sign as found in *Casq2*-KO mice. In Langendorff-perfused hearts, electrocardiogram recordings showed an increased sinus heart rate (Figure 2D) as found *in vivo*. Additionally, epicardial multielectrode array mapping exhibited ectopic spontaneous discharge and frequent ventricular premature beats (VPB) or occasional ventricular tachycardia (VT) in the same *Casq1*-KO or *Casq1*-CKO mice (Figure 2E, Figure VA and VD in the Data Supplement), suggesting that the abnormal discharges and tachycardia are independent of somatic neuromodulation. Notably, when the isoflurane dosage was increased to 2%, a concentration can induce MH event in patient and animal model,[8, 11, 12] both *Casq1*-KO and *Casq1*-CKO mice exhibited dantrolene-sensitive VPB and unidirectional VT (Figure 2B and 2C, Figure VA through VE in the Data Supplement), unlike the CPVT in *Casq2*-deficient mice and patients that mostly are di- or poly-morphic VT upon exposure to an inducer.[1–5]

3. Increased Ca^{2+} waves and premature Ca^{2+} transients are responsible for MH-like arrhythmia

Disorders in SR Ca^{2+} content and recycling may lead to disturbances in cellular electrical homeostasis and consequently induce afterdepolarizations, including DADs[4–7] and early afterdepolarizations (EADs).[35, 36] Accordingly, the intracellular Ca^{2+} activity in *Casq1*-CKO freshly isolated and field-stimulated cardiomyocytes was examined by confocal microscopy. Compared to WT myocytes (Figure 3A), *Casq1*-CKO myocytes exhibited higher frequency of diastolic premature Ca^{2+} transients (PCT) and Ca^{2+} oscillations (CO) in basal condition (PCT: 18% *vs*. 3%, *P*=0.0093; CO: 8% *vs*. 2%, *P*=0.106) and robustly increased PCT and CO upon exposure to 2% isoflurane (PCT: 18 to 39% *vs*. 3 to 7%, *P*=0.0099; CO: 8 to 24% *vs*. 2 to 3%, *P*=0.0109, n=34 (CKO) *vs*. 43 (WT) cells analyzed using Fisher's exact test). Application of dantrolene was effective (PCT: 8% *vs*. 39%, *P*=0.0073, and CO: 0% *vs*. 24%, *P*=0.0151). PCT

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may occur at any time during diastolic phase, and thus is called early after-PCT or delayed after-PCT in *Casq2*-null mice.[35] In this study, local or global premature Ca^{2+} release was usually a continuous process that affected Ca^{2+} baseline to be reached (Figure 3A1, Figure VI in the Data Supplement), thus the isoflurane-induced abnormal Ca^{2+} signals were classified into two types: namely PCT and CO. Detailed incidence rates also showed much higher PCT occurrence in a single cell and during Ca^{2+} transient intervals in *Casq1*-CKO myocytes (Figure 3A3). The baseline and amplitude of Ca^{2+} transients were not altered, but the time to 50% peak in the absence or presence of isoflurane was significantly decreased compared to those in control cells (Figure 3B). Whereas the time of Ca^{2+} decay remained unchanged, the SR Ca^{2+} content and the time to 50% peak of Ca^{2+} release were also reduced, as evaluated by caffeine in the absence or presence of isoflurane in *Casq1*-CKO myocytes (Figure VII in the Data Supplement). In addition, *Casq1*-CKO myocytes showed increases in basal and isoflurane-triggered Ca^{2+} sparks and waves (across the whole cell), which particularly significant upon isoflurane exposure (Figure 3C). All the above abnormal Ca^{2+} releases were corrected by dantrolene treatment *in vitro*, consistent with its effect *in vivo*.

In addition, using a method described previously,[16, 37] Ca^{2+} leak was analyzed by measuring the steady state Ca^{2+} level in the absence or presence of tetracaine, an inhibitor of RyR. We found that the SR Ca^{2+} leak (the tetracaine-induced Ca^{2+}-shift) was not different in baseline and isoflurane-treated *Casq1*-CKO myocytes compared with WT myocytes, and the relationship between the exponentially increased Ca^{2+} leak and increased SR Ca^{2+} content was not different between WT and *Casq1*-CKO myocytes (Figure 3D).

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4. Up-regulation of RyR activity is responsible for ventricular arrhythmia in *Casq1*-KO mice

Previous studies showed an interaction of Casq1 with RyR1 via the scaffolding proteins, triadin and junctin, which span the SR membrane and bind through their luminal domains to both Casq1 and RyR1 in skeletal muscle.[5, 9, 12, 14] To investigate the association of Casq1 with cardiac SR proteins, we pulled down Casq1 with specific antibodies to RyR2, junctin and triadin, respectively, and observed detectable Casq1 bands in the three blots (Figure 4A) similar to those found in skeletal SR.[11, 12, 14] Notably, the interactions of Casq1 monomer/dimer with RyR2 were significantly reduced, but the interactions of polymers with RyR2 and their co-localization remained unchanged in *Casq*1-CKO ventricle compared to WT ventricle (Figure 4B and 4C). Similarly, concerning their interaction, the Casq1 polymer/RyR2 complex detected as >800 kDa by the crosslinking analysis was also unaltered in freshly isolated *Casq1*-CKO cardiomyocytes (Figure VIII in the Data Supplement). Thus, these findings likely explain the largely unchanged ventricular rhythm in *Casq1*-CKO mice without trigger exposure *in vivo* (Figures 2 and 3).

To further investigate the Casq1/RyR2 relationship in the heart, we analyzed the relationship between expression alteration and functional abnormality in Casq1 using *Casq1*-siRNA, or over-expressed rat *Casq1* (Casq1-wt) or a truncated *Casq1* gene encoding the Ca^{2+} binding domain of Casq1 (C-terminal 9 amino acids (Casq1-Δ9)), by adenovirus transfection in cultured NRVMs. Dramatic decreases in the expression of both Casq1 mRNA and protein were observed. Junctin expression was also reduced in Casq1-knockdown cells (Figure 5A and 5B). Remarkably, large amounts of the Casq1 monomer but less polymer were found in Casq1-Δ9 cells compared to Casq1-wt cells (Figure 5C), and RyR2 expression was unexpectedly decreased in Casq1-wt, but not in Casq1-Δ9 cells. In addition, Casq1-Δ9 cells showed reduced triadin and junctin

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expression, which is likely due to the interference of its interaction with triadin/junctin by the truncation (Figure 5D).

Functional evaluation revealed increased basal spontaneous Ca^{2+} transients, Ca^{2+} oscillation upon exposure to 2% isoflurane, and decreased SR content and time to 50% peak detected by caffeine-induced Ca^{2+} release in Casq1-knockdown cells (Figure 6A, Figure IX in the Data Supplement). In contrast, Casq1-wt transfected cells, but not Casq1-Δ9 cells, showed decreased Ca^{2+} transient rate and amplitude with higher SR Ca^{2+} content compared to vector-transfected cells (Figure 6B). Ca^{2+} sparks were also increased upon exposure to 2% isoflurane in Casq1-knockdown cells (Figure 6C), whereas basal sparks were decreased in Casq1-overexpressing cells but not in Casq1-Δ9 transfected cells (Figure 6D). Importantly, co-immunoprecipitation analysis revealed enhanced interaction between Casq1-polymers and RyR2 in Casq1-wt cells, but not in Casq1-Δ9 cells compared to vector control (Figure X in the Data Supplement).

5. Casq1 depolymerization induced by isoflurane or environmental heat is responsible for MH/EHS-like arrhythmia

Finally, we investigated why MH/EHS-like arrhythmias are induced by specific triggers such as isoflurane and heating. Since the conformation of Casq1 is so important for performing its function, we tested whether the conformation of Casq1 was changed upon exposure to 2% isoflurane in crosslinked WT ventricle. As shown in Figure 7A, normal ventricles treated with isoflurane, but not those treated with isoproterenol, had less Casq1 in polymer form and more in monomer form, and showed a decrease in the high molecular weight (>800 kDa) RyR2 complex compared to controls, while the conformation of Casq2 remained unchanged. *Casq1*-CKO ventricles underwent even more depolymerization upon exposure to isoflurane (Figure 7B).

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Although RyR2 abundance (~500 kDa) remained unaltered in regular Western blot analysis, a dramatic decrease in Casq1 and Casq1/RyR2 complex (>800 kDa) was found after exposure to isoflurane using crosslinking Western blot analysis (Figure 7C), suggesting a disrupted Casq1 binding to RyR2.

Strong Casq1 oligomerization and even degeneration was also found in WT ventricles upon heating at 41ºC (Figure 7D), an inducer of EHS in susceptible patients. To avoid possible tissue response to heat, we directly treated SR lysates from rat ventricle and gastrocnemius with heating at 41ºC and observed significant Casq1 oligomerizations in both types of muscles from rat (Figure 8A) as observed in mice (Figure 7D). Taken together, these results suggest that Casq1 conformational change is sensitive to volatile anesthetics and high temperature.

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Discussion

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Sinus tachycardia and ventricular arrhythmia are high-rate signs in subjects with an MH/EHS event after exposure to triggers through an as yet unclear mechanism, since, theoretically, mutations in the *RyR1* or *CASQ1* gene, the causes of MH/EHS, only impair the skeletal-specific isoform. In this study, we have demonstrated for the first time that Casq1 is expressed in the heart and regulates the basal heart rates. In addition, it contributes to a MH/EHS-like ventricular tachycardia due to the induction of Casq1 depolymerization by triggers, resulting in a diastolic spontaneous Ca^{2+} transient from the SR and premature heartbeat.

Casq1 is expressed in the heart, where it regulates the heart rate, and its deficiency induces ventricular arrhythmia upon exposure to trigger

Casq1 is predominantly expressed in skeletal muscle and it is still obscure whether it is expressed in myocardium where Casq2 is abundantly present. This study revealed that in murine

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and human myocardium SR Casq1 is clearly expressed, mostly in polymer form, and colocalized with RyR2 in mouse heart (Figures 1 and 4, Figures IV, VIII and X in the Data Supplement), resembling the Casq1 conformation and interaction with RyR1 in skeletal muscle.[12, 14, 15] The detection of Casq1 mRNA expression in murine ventricles in this study further supports its presence in the heart, consistent with observations in human (www.proteinatlas.org) and rat heart.[27] Noteworthy, *Casq1* knockout mice had increased sinus heart rates, which could be inhibited by dantrolene (Figure 2), simulating the sinus tachycardia before and during the MH/EHS event originally described in patients.[12, 21–24] In addition, non-sustained ventricular tachycardia was induced in *Casq1* knockout mice upon exposure to 2% isoflurane, but not to catecholamine. Dantrolene suppressed this ventricular tachycardia in ~85% of the animals (Figure 2), which is comparable to the ~90% clinical cure rate of ventricular arrhythmia in MH/EHS patients.[12, 13, 38] Remarkably, the ventricular tachycardia was unidirectional (Figure V in the Data Supplement), unlike the CPTV in *Casq2*- KO mice and mutated patients.[1–3, 6]

Casq1 deficiency induces ventricular arrhythmia *via* down-regulation of RyR activity and induction of premature spontaneous Ca^{2+} transients

RyR2 is the only known SR Ca^{2+} release channel coupling electrical excitation to contraction at the myocardial triads, where Casq2 plays a regulatory role in RyR2 activity. Here, we have shown that Casq1 also interacts with RyR2 and exerts an inhibitory effect on RyR2 function in the heart based on the following findings: compared with counterpart controls, (1) less and more interaction between Casq1 monomer and RyR2 was observed in *Casq1*-CKO mice and Casq1-overexpressing NRVMs, respectively (Figure 4B, Figure X in the Data Supplement), and although it remained stable under normal condition, Casq1 polymer/RyR2 interaction could be dramatically down-regulated by 2% isoflurane treatment (Figure 7); (2) the in *vivo* basal sinus

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tachycardia and ventricular arrhythmia, and the *in vitro* dramatic increase in the diastolic Ca^{2+} leak upon exposure to isoflurane were all sensitive to the inhibition of RyR in *Casq1*-CKO mice (Figures 2 and 3), and the slight increase of RyR2 phosphorylation in *Casq1*-CKO mice (Figure IVC in the Data Supplement), suggesting a RyR2 hyperactivity in *Casq1*-deficient cardiomyocytes; (3) in contrast, Casq1 over-expressing NRVMs exhibited increased Casq1/RyR2 interaction and Ca^{2+} load, but reduced spontaneous Ca^{2+} transients and Ca^{2+} sparks, while the expression of Casq1 with truncated C-terminus Ca^{2+} binding site and knockdown of Casq1 reversed these effects of Casq1-wt (Figure 6); (4) reduced junctin expression was found in *Casq1*-KO heart and *Casq1*-knockdown NRVMs, analogous to that found in the myocardium of *Casq2*-null mice and the skeletal muscle of *Casq1*-null mice; (5) faster Ca^{2+} release induced by pacing or caffeine was observed in *Casq1*-CKO myocytes and *Casq1*-knockdown NRVMs (Figure 3B, Figures VII and IXB in the Data Supplement), suggesting an intrinsic functional relationship between Casq1 and RyR2 in the heart.

Compared with frequent diastolic DADs, the proposed arrhythmogenesis in CPVT patients and *Casq2*-KO mice,[6, 12, 16, 17] premature spontaneous Ca^{2+} transients and Ca^{2+} oscillations in *Casq1*-CKO mice upon exposure to triggers seem to be the major arrhythmogenic component for the premature ventricular beats. It is well established that under pathological conditions aberrant SR Ca^{2+} activity is prone to generate DADs and EADs, and causes whole heart premature ventricular contractions through the increased Na/Ca exchange (DADs and EADs) or the activation of L-type Ca^{2+} channels (EAD) during the diastolic phase.[39, 40] In this study, β-adrenergic agonist that can activate L-type channels failed to elicit abnormal Ca^{2+} release and ventricular arrhythmia in *Casq1*-CKO mice *in vivo* (Figure 2) and freshly isolated cardiomyocytes *in vitro* (data not shown). However, isoflurane-induced Ca^{2+} waves, diastolic

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spontaneous Ca^{2+} transients and premature ventricular beats in Casq1-deficient cardiomyocytes were all suppressed by the inhibitory effect of dantrolene *in vivo* and *in vitro*, suggesting a potentiated RyR2-associated aberrant Ca^{2+} handling as a candidate molecular mechanism for the cause of MH-like ventricular arrhythmogenesis.

It is still unclear why the ventricular disorder requires specific triggers. Furthermore, based on clinical studies, it remains a puzzle why the sinus and ventricular tachycardia are seen in ~60 and ~40% MH/EHS patients, most of whom are identified as carriers of *RyR1* mutations rather than *CASQ1* mutations.[8–11, 21] We thus speculate that RyR function remains normal in *CASQ1*- or *RyR1*- mutated MH/EHS patients due to a normal or compensated CASQ1 inhibitory mechanism. In the exposure to an MH or EHS trigger, an unknown change occurs in the conformation or function of CASQ1, which impairs the CASQ1 normal or compensatory regulatory effects on RyR1 and RyR2 functions and causes skeletal syndrome and sinus tachycardia. The hyperthermia in patients with a MH attack provides a further trigger to exaggerate the cardiac CASQ1 change, resulting ventricular tachycardia (see below).

Isoflurane and environmental heat induce Casq1 depolymerization, which is responsible for MH/EHS-like arrhythmia

Casq function is closely associated with its molecular conformation. Casq2 is found mostly as a monomer, while Casq1 exists in a polymeric form in the SR at Ca^{2+} concentration below 1 mM.[20, 30, 31, 41] As the SR Ca^{2+} concentration increases, more protein molecules of Casqs polymerize, thereby providing more Ca^{2+} binding site as a buffer.[30, 31, 41, 42] In addition, it has been found that Casq polymers, including Casq2 monomers, interact with and affect RyR1- and RyR2- mediated Ca^{2+} release in skeletal and cardiac muscles, respectively.[5, 6, 12, 43] In this study, we have shown for the first time that a high fraction of Casq1 is present as a polymer and forms a

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Casq1/RyR2 complex in the heart. This complex (interaction) was not changed in *Casq1*-KO ventricles compared with WT heart in basal state (Figure 4B, Figure VIII in the Data Supplement), providing a relatively normal regulation in *Casq1*-KO ventricular rhythm, even though *ex vivo* ventricular ectopic discharge was seen with their reduced monomer/dimer interactions with RyR2 (Figures 2E and 4B). However, upon exposure to isoflurane or heating a fraction of Casq1 depolymerized to form monomers/dimers, while Casq2 remained unaltered (Figures 7 and 8). Such conformational change in Casq1 perhaps quickly impairs Casq1 binding and interacting with RyR2 as well as the SR Ca^{2+} buffering ability, leading to a dysfunctional SR recycling and more diastolic Ca^{2+} release, predisposing MH/EHS patients to high risks for premature Ca^{2+} transients and ventricular arrhythmia (Figure XI in the Data Supplement). This Casq1 oligomerization-based pathogenesis mechanism is strongly supported by the clinical requirement of specific triggers for an MH or EHS episode. In addition, Casq1-Δ9 truncated NRVMs' failures in the enhancements of Casq1 polymerization and interaction with RyR2, and the inhibition of spontaneous Ca^{2+} transients with an increased SR Ca^{2+} content found in Casq1-overexpressing cells further support this notion (Figures 5 and 6, Figure X in the Data Supplement). Moreover, an exaggerated Casq1 polymerization in TAM patients with an opposite syndrome including dominant myalgia, muscle weakness and post-exercise fatigue,[18–20] and the M87T mutation, a *CASQ1* defect found in MH patients, with a weakness in Casq1 tetramerization and a destabilization of Casq1 lattice and abnormal Ca^{2+} leak/SR Ca^{2+} oscillations[20, 21, 42, 44] provide more evidence supporting this mechanism and illustrate the importance of the Casq1 polymer in the regulation of RyR activity.[42, 45]

Differences between Casq2 and Casq1 in the regulation of SR Ca^{2+} release in the heart

Our results identify a novel profile of MH-like arrhythmia originated from the heart that may

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differ from that of CPVT reported previously in mouse model. Specifically, we have observed (1) faster sinus heart rates in *CASQ1* mutated patients[9–12] and *Casq1*-KO mice in this study, whereas sinus bradycardia was reported in *CASQ2* mutated patients[3] and *Casq2*-KO mice;[2, 3, 46] (2) increased dantrolene-sensitive Ca^{2+} waves, premature transients/oscillations and unidirectional ventricular arrhythmia upon exposure to isoflurane were found in *Casq1*-CKO mice in this study, while Ca^{2+} waves/DADs (occasionally EADs and oscillations[6, 17, 35]) and bidirectional/polymorphic ventricular arrhythmia upon exposure to catecholamine have been reported in *Casq2*-KO mice,[1–4, 11] suggesting different electrophysiological bases for the two arrhythmogenic conditions; (3) upon exposure to volatile anesthetics or heating, a dramatic Casq1 depolymerization is responsible for the MH/EHS-like arrhythmia in this study, while unchanged Casq2 level with increased SR content and Ca^{2+} leak due to catecholamine stimulation rather than a relief of Casq2 inhibition on RyR2 may underlie the aberrant SR recycling for CPVT.[1, 16, 46]

In conclusion, this study demonstrates for the first time that Casq1 is expressed in cardiomyocytes, functionally buffering SR Ca^{2+} and regulating the heart rate. Casq1 deficiency causes basal sinus tachycardia, increased diastolic spontaneous Ca^{2+} release and ventricular tachycardia upon exposure to the trigger, recapitulating the arrhythmia that occurs in patients with an MH/EHS episode. Isoflurane and heating, the specific MH/EHS triggers, can induce Casq1 oligomerization and reduce the regulatory effect of Casq1 on RyR, accounting for the ventricular tachycardia and likely the skeletal syndrome in MH/EHS patients.

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Disclosures

None

Circulation

Supplemental Materials

Supplementary material and data are available with this article at Circulation online.

Expanded Materials

Supplemental Figures I–XI

Supplemental Tables I – III

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Figure Legends

Figure 1. Casq1 expression in murine and human hearts

Samples of rat and mouse ventricular and skeletal muscles, neonatal rat ventricular myocytes (NRVMs), rat embryonic cardiomyocyte line (H9C2 cells), and human auricle and ventricle were prepared. **A:** Mean±SD Casq1 and Casq2 mRNAs in the murine ventricular tissues and cardiomyocyte samples were determined by quantitative RT-PCR and normalized with GAPDH mRNA gene. **B:** Representative Western blots of whole tissue and cell lysates for Casq1 and Casq2 detection in murine samples, n=3 animals for each sample. **C:** Representative Western blots of ventricular and skeletal muscle SR and cytosol fractions from one mouse. Calnexin, a SR protein, indicates a successful SR isolation. Casq1 monomer at 63 kDa and dimer/polymer around 120/260 kDa (Casq1-like protein) were clearly detected in ventricular SR, paralleled with those observed in skeletal muscle. Casq2 monomer around 50 kDa in both SR and cytosol fractions and dimer/trimer around 100/150 kDa in SR fraction were detected, n=5 animals for each sample. **D:** Representative Western blots of SR lysate from freshly isolated ventricular cardiomyocytes that had been crosslinked by treatment with chemical crosslinking reagents for 5 min. Most aggregated Casq1 remained in the well and as a polymer form around 260 kDa that disappeared in the lysate with the presence of 10 mM EGTA, while Casq2 monomer at 50 kDa and polymer bellow 260 kDa were observed, n=4 animals for each sample. **E:** Representative Western blots of SR lysates from human auricle and ventricular tissues, and mouse skeletal muscle tissue. Here, Casq1 protein weight in patients with hypertrophic left ventricle was heavier than that detected in human auricle and mouse skeletal muscle, implying a post-transcription modification of Casq1 in these ventricles, n=4 patients for each sample. **F**: Casq1 localizes along

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with the transverse striations in murine ventricles. **F1:** Representative immunostaining images of Casq1 and Casq2 distribution in ventricles. Yellow arrows indicate a non-specific labeling in the intercellular space. **F2:** Representative immunostaining images of Casq1 and Casq2 in freshly isolated adult mouse ventricular myocytes (AMVMs) and NRVMs. Squares indicate the areas enlarged in the right images, n=5 animals and 3−4 distinctive cell experiments for each panel. Bars: 10 and 20 μm as indicated.

Figure 2. Electrocardiographic changes in cardiac-specific (*Casq1*-CKO) and conventional *Casq1* knockout (*Casq1*-KO) mice

DII peripheral lead ECG was performed on anesthetized mice. **A:** Representative ECG curves (A1) and Mean±SD of basal heart rate (A2) in WT and *Casq1*-CKO mice under anesthesia by 1% isoflurane or pentobarbital (70 μg/g). Isoproterenol (2 mg/kg, i.p.) was used for sympathetic stimulation. **B:** Representative ECG curves (B1) and Mean±SD basal heart rate under 1% isoflurane anesthesia, and ventricular arrhythmia upon 2% isoflurane exposure in *Casq1*-CKO mice (B2). **C:** Representative ECG curves (C1) and Mean±SD of basal heart rate, and ventricular arrhythmia under 2% isoflurane exposure in *Casq1*-KO mice (C2). Treatment with dantrolene (Dan, 27.3 mg/kg, i.p.) was effective for the increased heart rate and ventricular tachycardia in both *Casq1*-CKO and *Casq1*-KO mice. **D:** Representative ECG curves (D1) and Mean±SD sinus tachycardia (D2) in working hearts from WT, *Casq1*-KO and *Casq1*-CKO mice. **E:** Representative activation electrical mapping (E1) and quantification of the abnormal triggering (E2), and accordant ECG curves (E3) and quantification of the ventricular tachycardia (E4) under sinus rhythm of working hearts from the same WT, *Casq1*-KO and *Casq1*-CKO mice. The mouse numbers were indicated as arrhythmia/total in each panel. The *P* values given were

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compared between two groups as indicated with a line and obtained with Student's *t* test performance for unpaired observations of heart rates (A2 right panel), two-way ANOVA with Tukey analysis (A2−C2 left panels), one-way ANOVA with Tukey analysis (D2), and Fisher's exact test analysis for arrhythmic occurrence percentage in B2 and C2, right panels.

Figure 3. Intracellular Ca^{2+} disturbance in freshly isolated ventricular myocytes from *Casq1*-CKO mice

Freshly isolated ventricular myocytes from adult mice were loaded with 10 μM Fluo4/AM for 30 min, washed and then mounted on laser confocal microscopy in Tyrode's solution. Ca^{2+} transients were induced by field-stimulated pacing at 1 Hz. **A**: Representative premature spontaneous Ca^{2+} transients (PCT) and Ca^{2+} oscillations (CO) induced by 2% isoflurane and suppressed by dantrolene (10 μM) (A1). Pie charts of the incidence percentages of PCT and CO in total tested cells (A2), and mean PCT incidence per cell during six pacing period (left) or during the interval between two transients (trans, right) in *Casq1*-CKO and WT myocytes (A3). **B**: Mean±SD values in basal intracellular Ca^{2+}, and time to 50% peak and peak of Ca^{2+} transients in the absence or presences of 2% isoflurane. Decay 50 could not be correctly obtained because continuous PCT occurred upon isoflurane. **C**: Representative images of spontaneous Ca^{2+} sparks, Ca^{2+} wavelets and Ca^{2+} waves prior to or after isoflurane exposure followed by dantrolene treatment (C1), and their respective Mean±SD percentages in total tested cells (C2) and incidence ratios per cell (C3). **D**: Determination of the Ca^{2+} leak. Experimental protocol for the Ca^{2+}-leak detection (D1). Myocytes were field stimulated at 1 Hz and then the bath solution was changed to $0Na^{+}/0Ca^{2+}$ solution. Tetracaine (1 mM) was added to block RyR, and the shift drop in basal intracellular Ca^{2+} is proportional to SR Ca^{2+}-leak. The caffeine-induced Ca^{2+} transient

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was used as a measure of total SR Ca^{2+} content. Mean±SD tetracaine-dependent SR Ca^{2+}-leak (D2) and SR Ca^{2+}-content (D3) in the absence or presence of isoflurane, and their relationship (D4) in WT and *Casq1*-CKO myocytes were then obtained. The *P* values given were compared between groups as indicated with a line and obtained with hierarchical analysis in panels B, C2 (1st panel) and D, and Fisher' exact test analysis in panel C2, 5−6 mice were used in each panel.

Figure 4. Interaction between Casq1 and RyR2, junctin or triadin in mouse ventricles

A: Representative immunoprecipitating Western blots of ventricular SR lysates from WT and *Casq1*-CKO ventricles using antibody specific for RyR2, triadin or junctin, and then blotted by antibody for Casq1, n=3 mice for triadin and junction blots. **B:** Mean±SD Casq1/RyR2 interaction normalized to WT control for 9 mice. The *P* values given were compared between groups as indicated with a line and obtained with Student's *t* test performance for unpaired observations. **C:** Representative ventricle images of double immunostaining with antibodies against Casq1 (red) and RyR2 (green) (C1), and plots scanned at the white lines (C2). Bar: 5 μm, 5 mice were used for each group.

Figure 5. Expression changes in SR associated proteins in NRVMs with manipulated Casq1 expression

NRVMs were cultured for 24 h, and then incubated with Casq1-siRNA or vector, or with adenovirus-vector or adenovirus carrying Casq1-wt or Casq1-Δ9 gene for 48 h. **A:** Identification of Casq1 knockdown in NRVMs. Representative Western blot of Casq1 expression (A1) and Mean±SD Casq1 mRNA and protein expression normalized to vector controls (A2). **B:** Mean±SD expression levels of SR associated proteins due to Casq1 knockdown. Representative

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Western blots of SR associated proteins including RyR2, SERCA2a, Casq2, triadin, junctin and phospholamban (PLN) (B1) and Mean±SD expression levels of these proteins normalized to vector controls (B2) in NRVMs with Casq1 knockdown. **C:** Identification of Casq1 over-expression in NRVMs. Representative Western blots of Casq1 in NRVMs transfected with Casq1-wt or Casq1-Δ9 gene (C1) and Mean±SD Casq1 expression normalized to vector control (C2). **D:** Representative Western blots of SR associated protein expression in NRVMs transfected with Casq1-wt or Casq1-Δ9 gene (D1) and Mean±SD protein expression levels normalized to vector control (D2). The *P* values given were compared between groups as indicated with a line and obtained with Student's *t* test performance for unpaired observations (A2 and B2), and one-way ANOVA followed by Tukey's or Kruskal-Wallis followed by Dunn's multiple comparison's test (C2 and D2), n=independent experiments for each treatment, and 40 animal hearts were used for each experiment.

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Figure 6. Intracellular Ca^{2+} signaling change in NRVMs with manipulated Casq1 expression

NRVMs were cultured for 24 h, and then incubated with Casq1-siRNA or vector, or with adenovirus vector or adenovirus carrying Casq1-wt or Casq1-Δ9 gene for 48 h. NRVMs were loaded with 2 μM Fluo4/AM for 30 min, washed and mounted on confocal microscopy for measurement of intracellular Ca^{2+} signals. **A:** Ca^{2+} signaling change due to Casq1 knockdown. Representative curves of spontaneous Ca^{2+} transients in basal state, tonic Ca^{2+} oscillations upon 2% isoflurane and Ca^{2+} release induced by caffeine (10 mM) (A1), and Mean±SD values of the rate and amplitude of spontaneous Ca^{2+} transients, and the percentage of oscillated cells upon isoflurane in Casq1-siRNA and vector treated NRVMs (A2). **B:** Ca^{2+} signaling changes due to

Casq1 over-expression. Representative curves of basal spontaneous Ca^{2+} transients and caffeine-induced Ca^{2+} release (B1), and their Mean±SD values (B2) in NRVMs transfected with vector, Casq1-wt gene or Casq1-Δ9 gene. **C:** Local Ca^{2+} signal changes due to Casq1 knockdown. Representative images of Ca^{2+} sparks (C1) and Mean±SD incidence (C2) in control and Casq1-knockdown NRVMs. **D:** Local Ca^{2+} signal changes due to Casq1 over-expression. Representative images of Ca^{2+} sparks (D1) and Mean±SD spark incidence (D2) in adenovirus-vector, Casq1-wt and Casq1-Δ9 NRVMs. The *P* values given were compared between groups as indicated with a line and obtained with Student's *t* test performance for unpaired observations in panel A2, Fisher's exact test in panel A2 (right), one-way ANOVA followed by Tukey's post-hoc test in panel B2 (middles), Kruskal-Wallis followed by Dunn's multiple comparison test in panels B2 and D2, and two-way ANOVA with Tukey's multiple comparison test in panel C2, cells in each panel were isolated from 40 neonatal rat hearts in at least 3 independent experiments.

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Figure 7. Casq1 depolymerization induced by isoflurane or heat

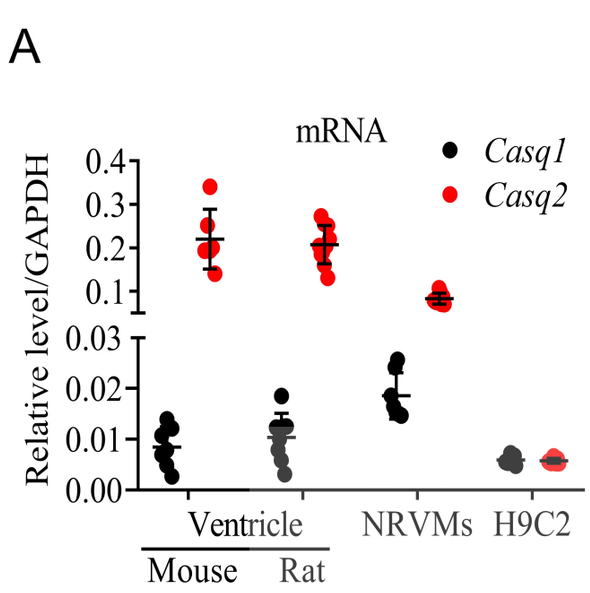
Mouse ventricles were immersed in Tyrode's solution containing 2% isoflurane (Isof) or 1 μM isoproterenol (Iso) when needed. **A:** Representative Western blots of a half ventricle treated with Isof and another half treated with Iso for 2 min from 3 mice for each panel. **B:** Identification of Casq1 conformational change due to the presence of Isof in both WT and *Casq1*-CKO ventricles. Representative Western blots of ventricles treated with Isof for 2 min (B1), and Mean±SD Casq1 oligomer/polymer ratio for 6−8 mice (B2). The *P* values given were compared between groups as indicated with a line and obtained with Student's *t* test performance for unpaired observations. **C:** Identification of changes in Casq1 binding with RyR2 due to Isof exposure. Representative

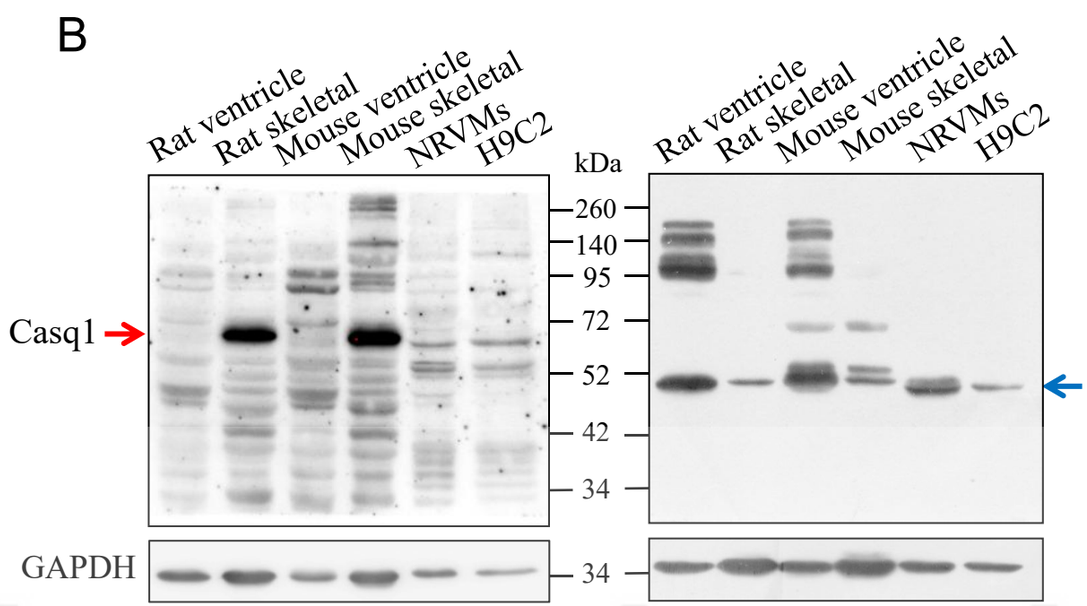
Western blots of ventricles treated with Isof, and their lysates were incubated in the absence (C1) or presence of crosslinking (C2) reagents. The overlay (yellow) of Casq1 (green) and RyR2 (red), both one clear band, was dramatically reduced at molecular weight >800 kDa because of Casq1 oligomerization upon Isof (C3), n=5 mice for each panel. **D:** Representative Western blots of Casq1 depolymerization in ventricles treated with heat at 41ºC for 2 or 5 min for 6 mice, respectively.

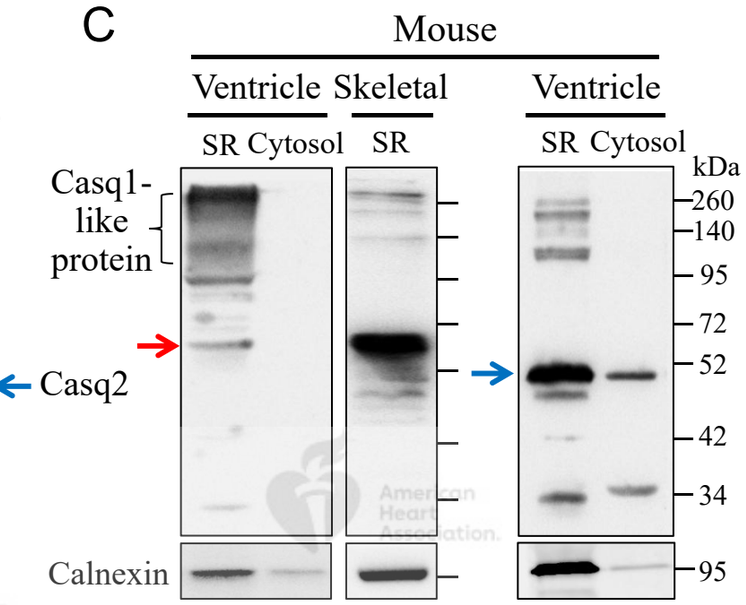
Figure 8. Casq1 depolymerization by heat in rat ventricular and skeletal muscles

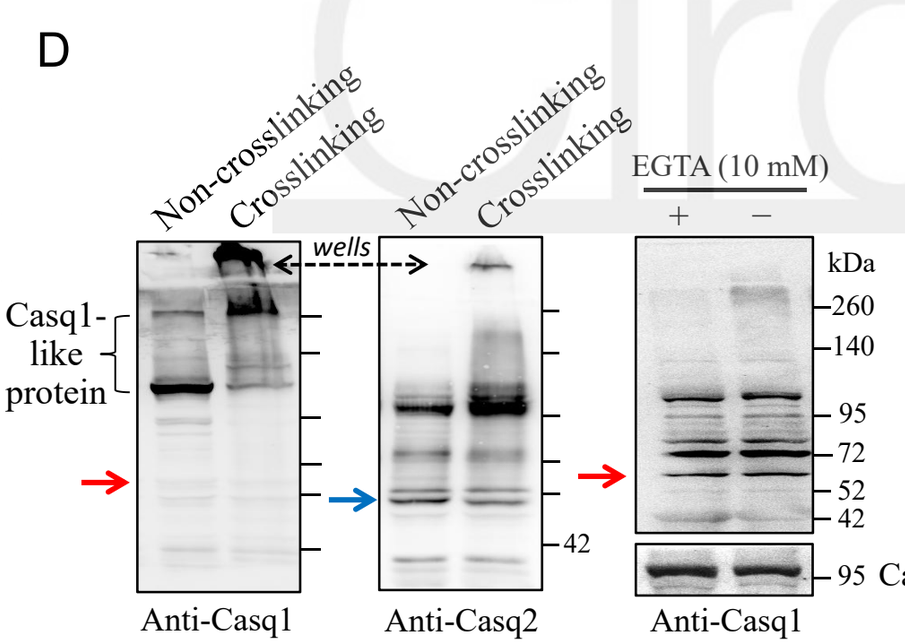
SR lysates from rat ventricular and skeletal muscles were directly treated with ice (0ºC), room temperature (22±1ºC) and 41ºC for 2 or 8 min, respectively, and detected by native Western blot using specific antibody against Casq1 or Casq2. **A:** Identification of Casq1 conformational change in SR lysates upon heating. Representative Western blots of Casq1 and Casq2 conformational change (A1) and Mean±SD Casq1 dimer/polymer fraction ratios (A2) in ventricular and skeletal muscle SR lysates treated with heat. Arrows indicate the bands with abundance changed. Casq2 conformation was not changed because of heating. The *P* values given were compared between groups as indicated with a line and obtained with one-way ANOVA followed by Tukey's multiple comparison test performance.

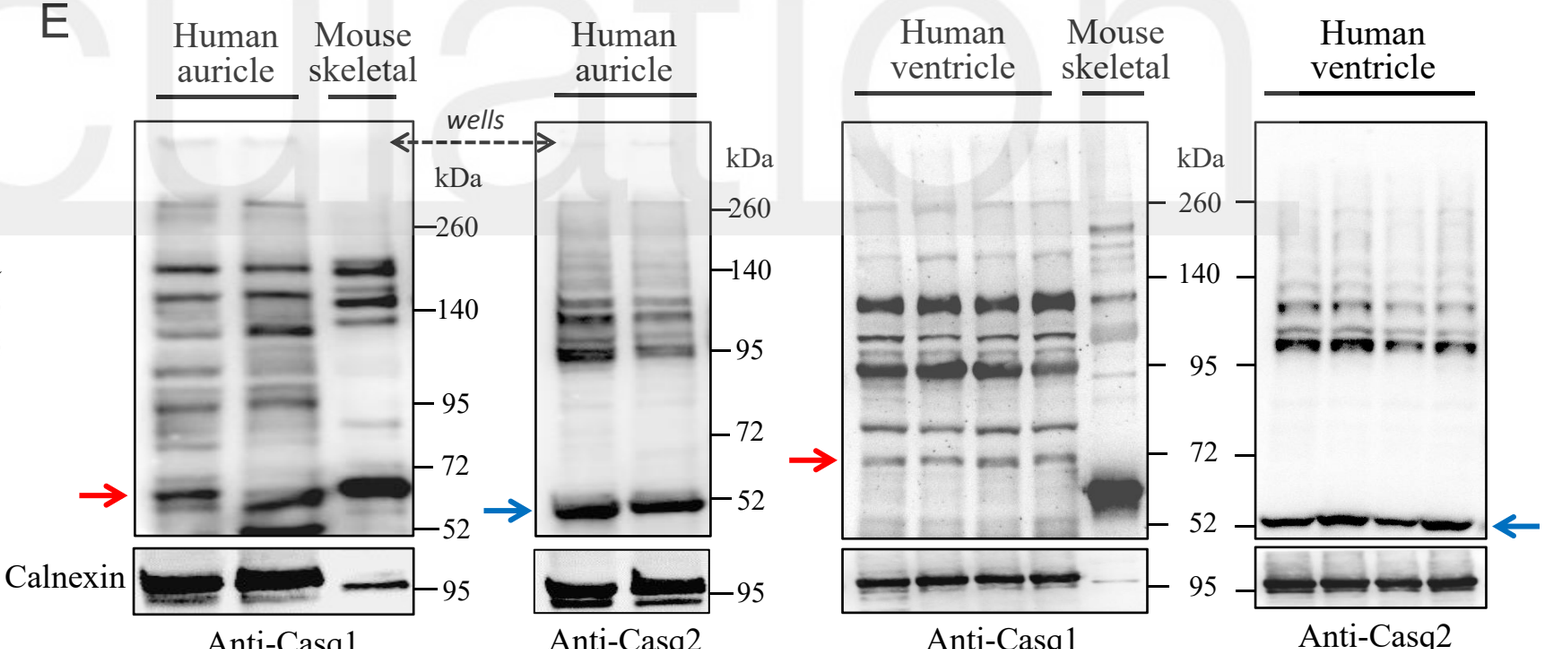
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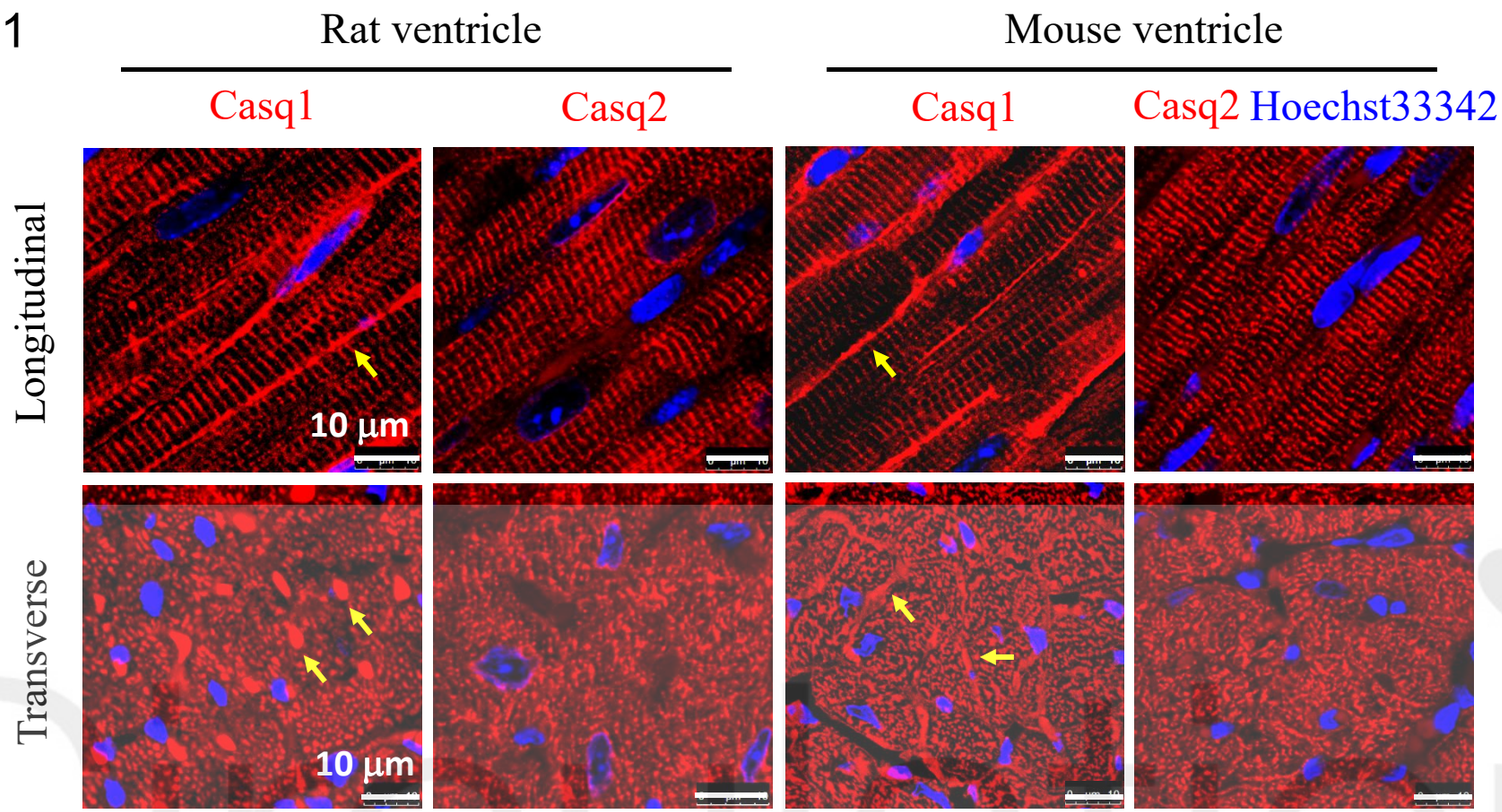




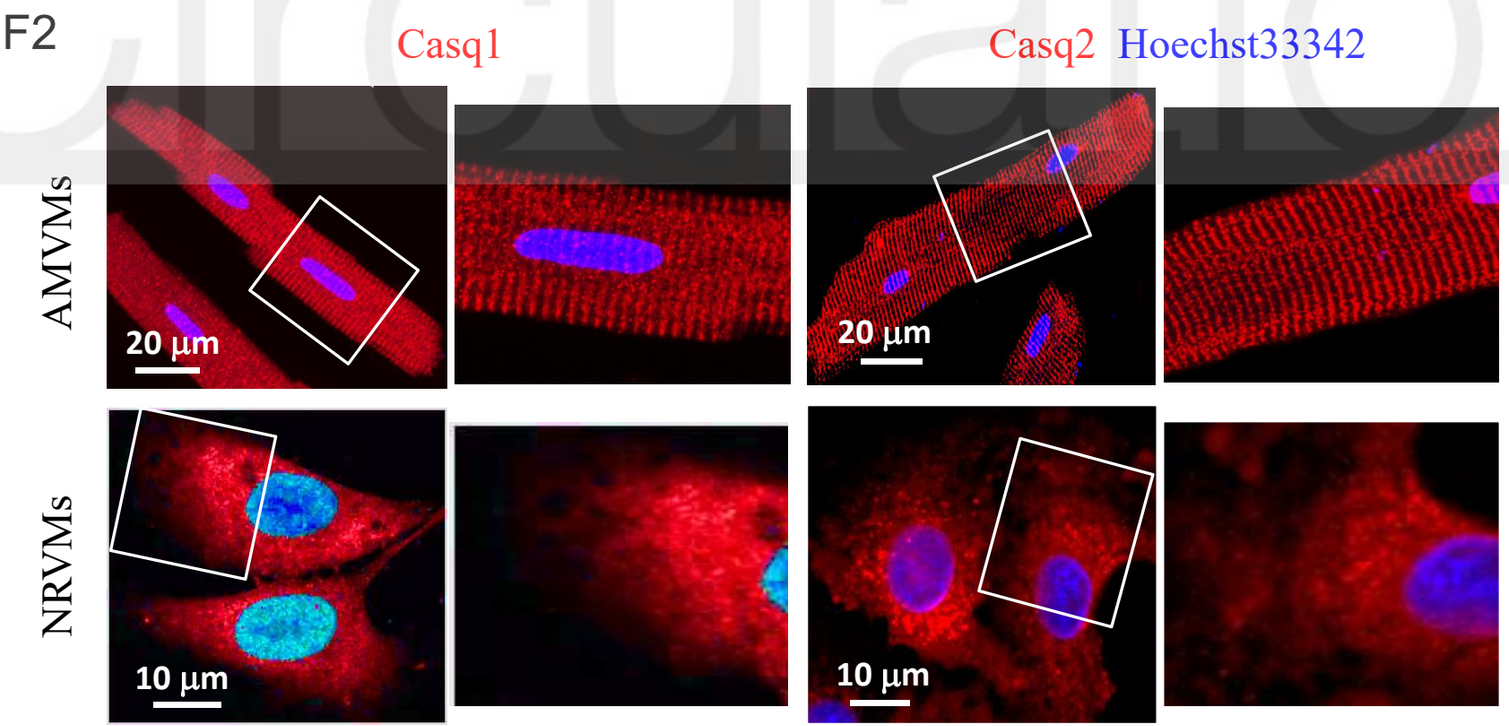


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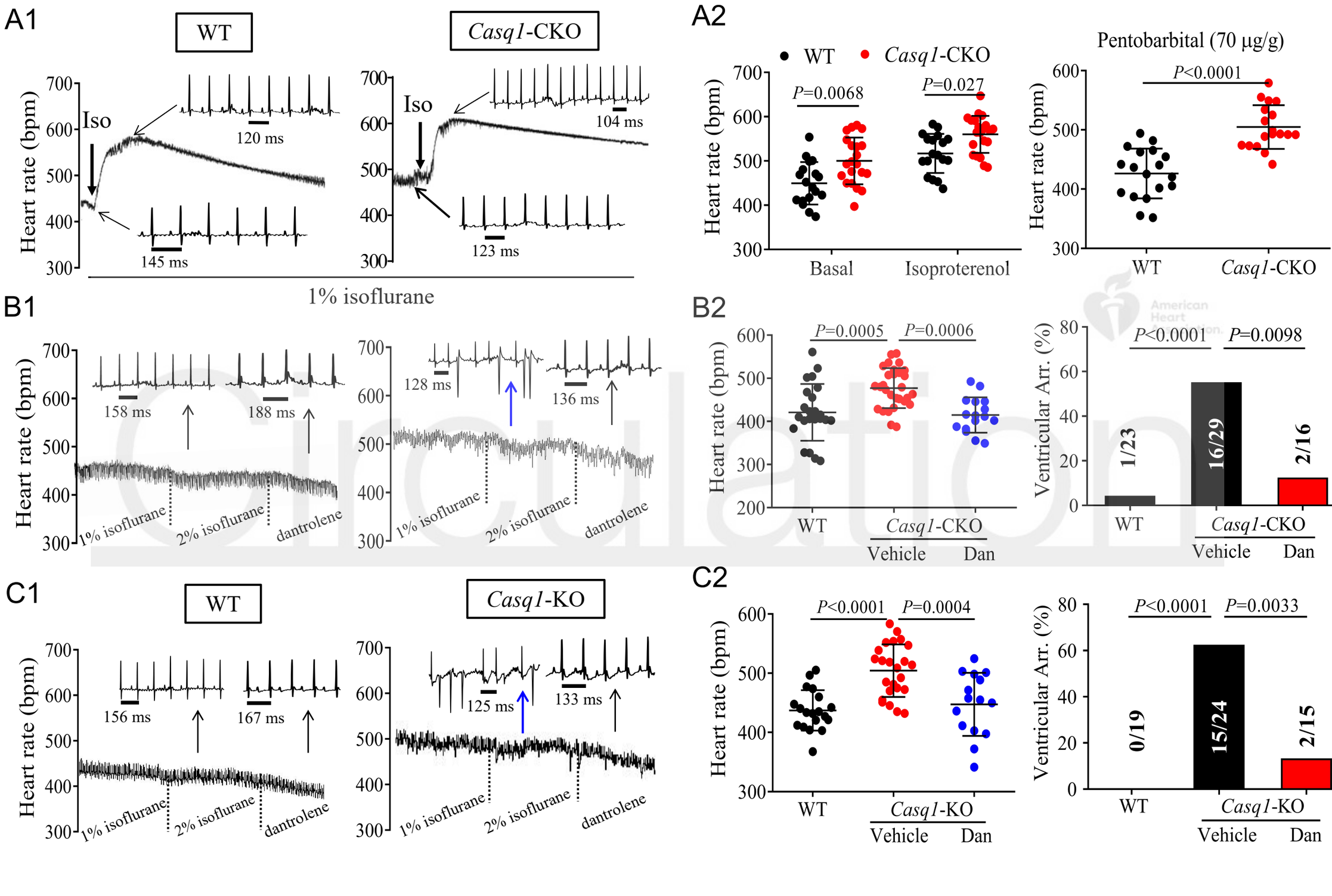


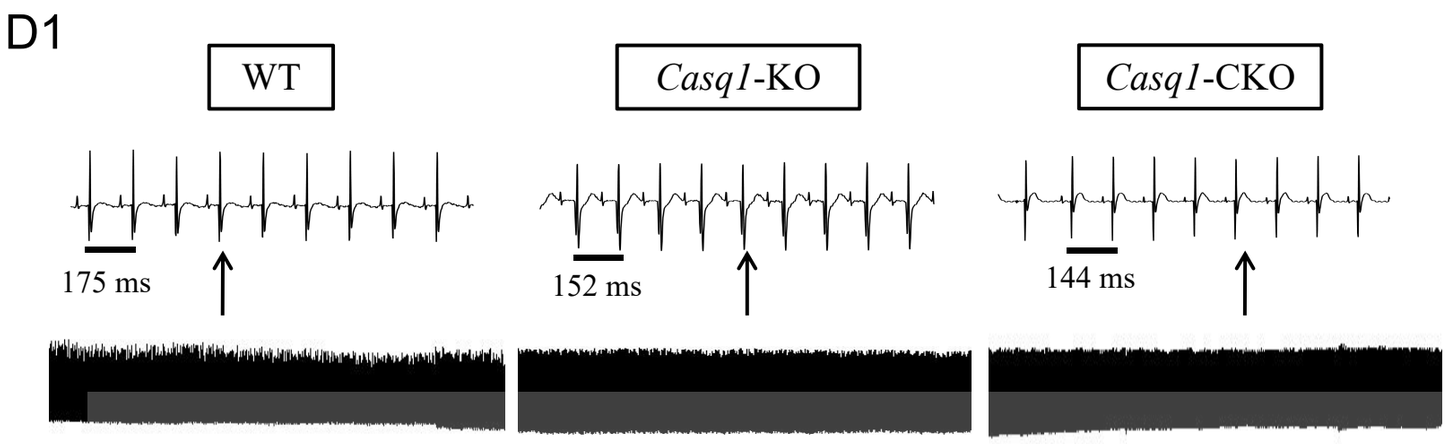
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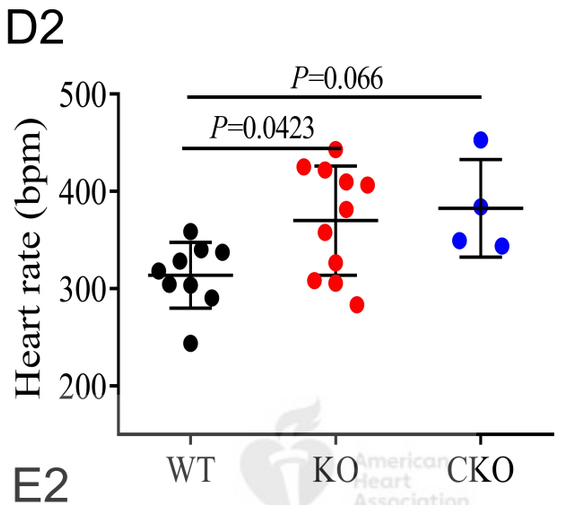


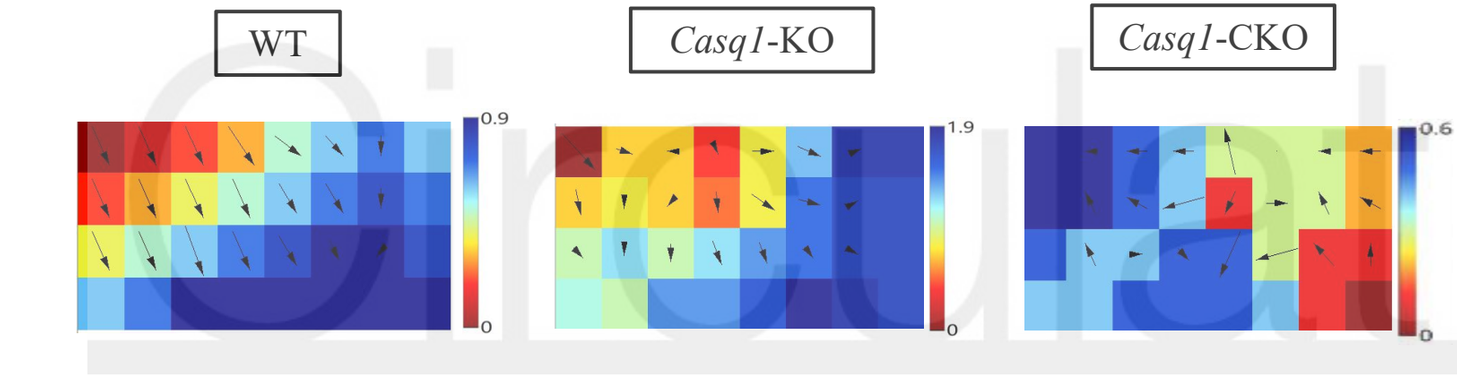
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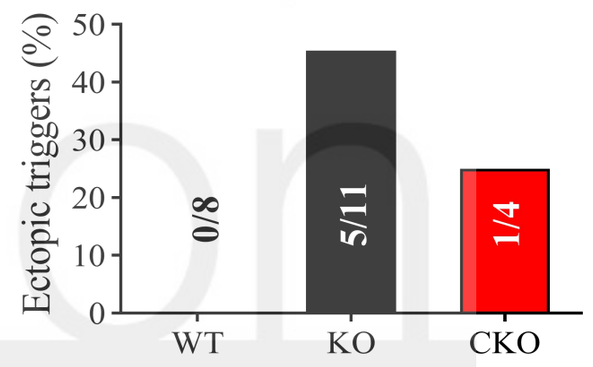
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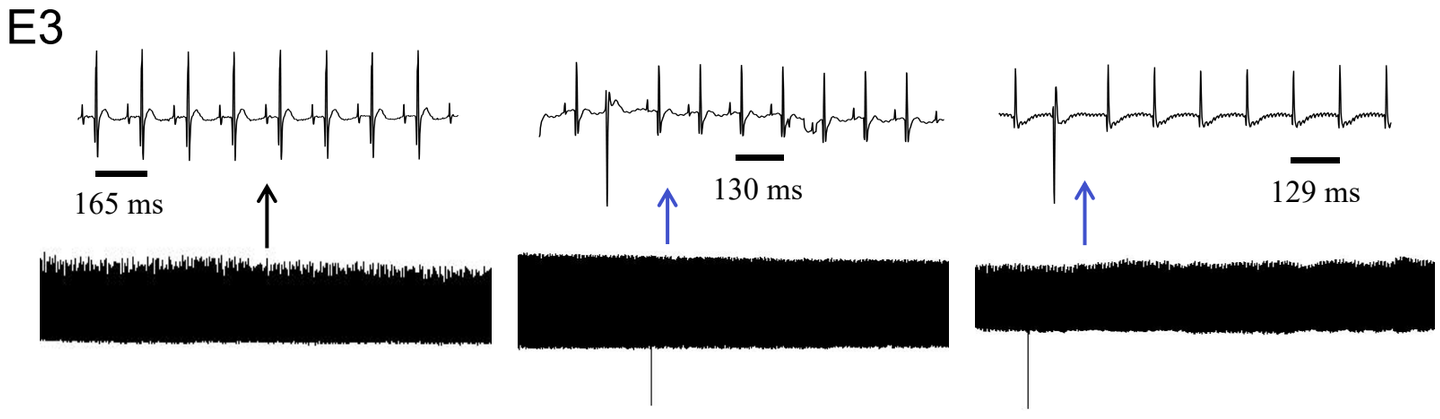


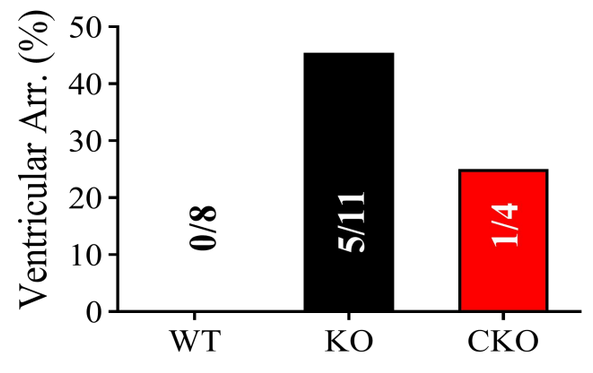






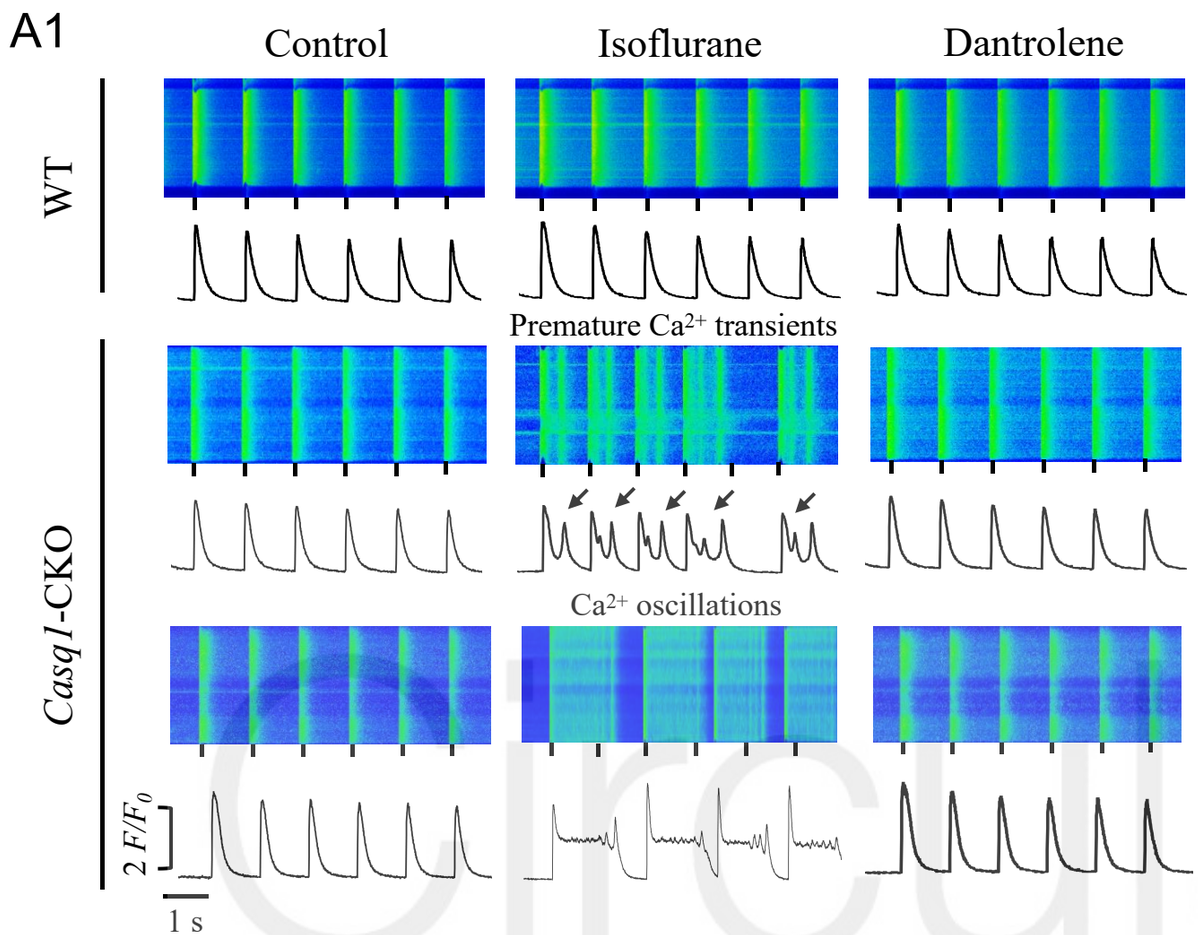


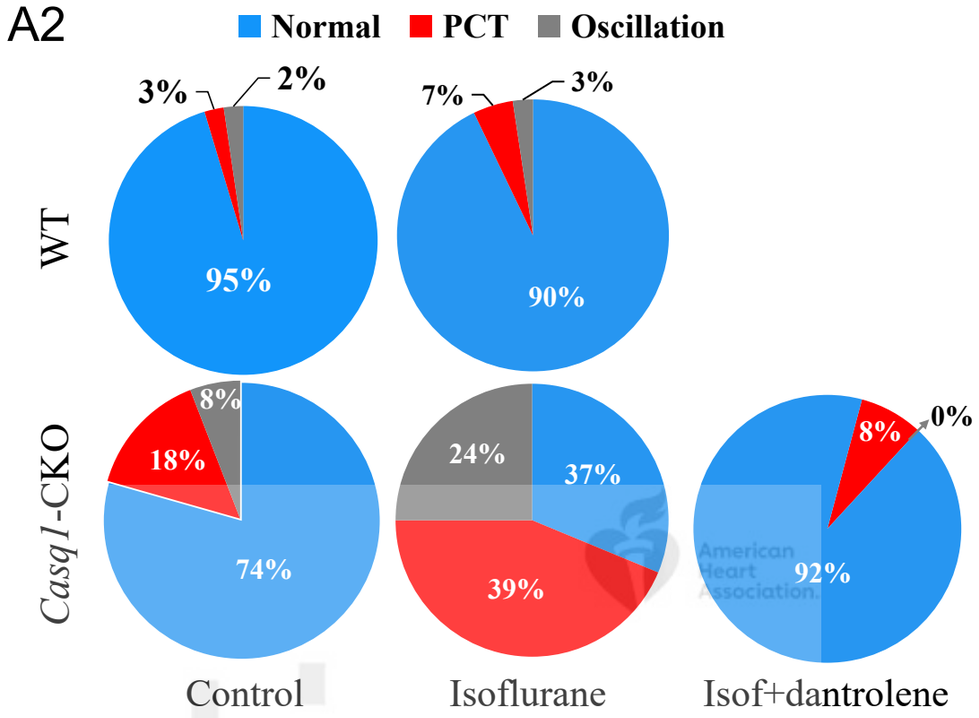


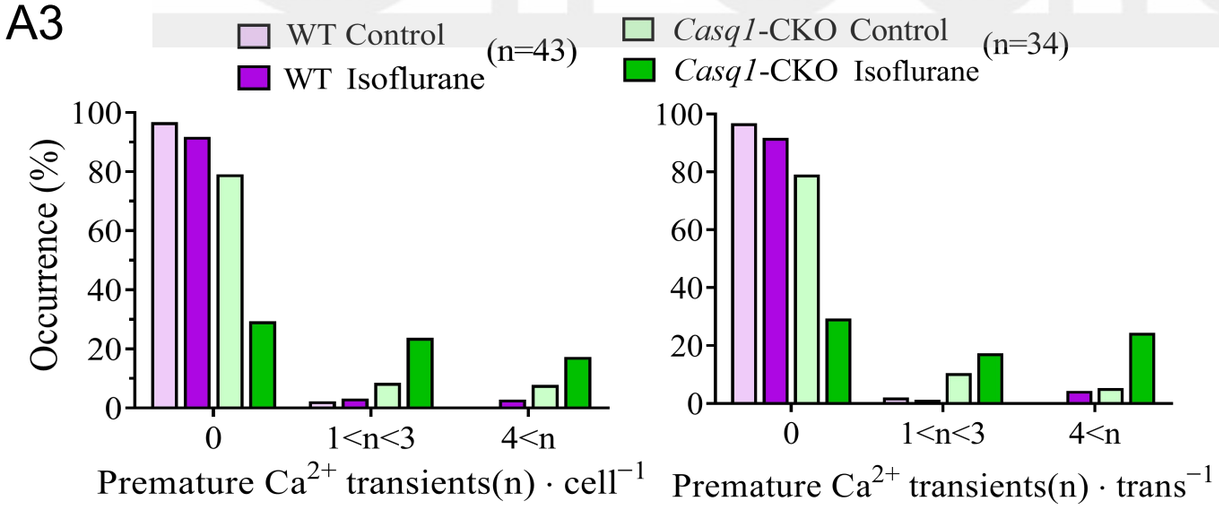


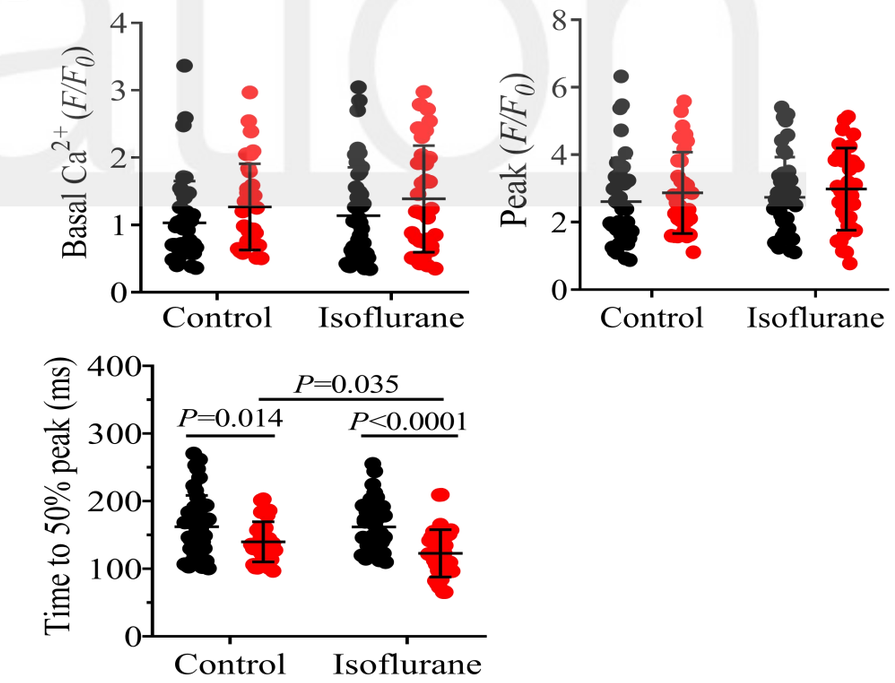
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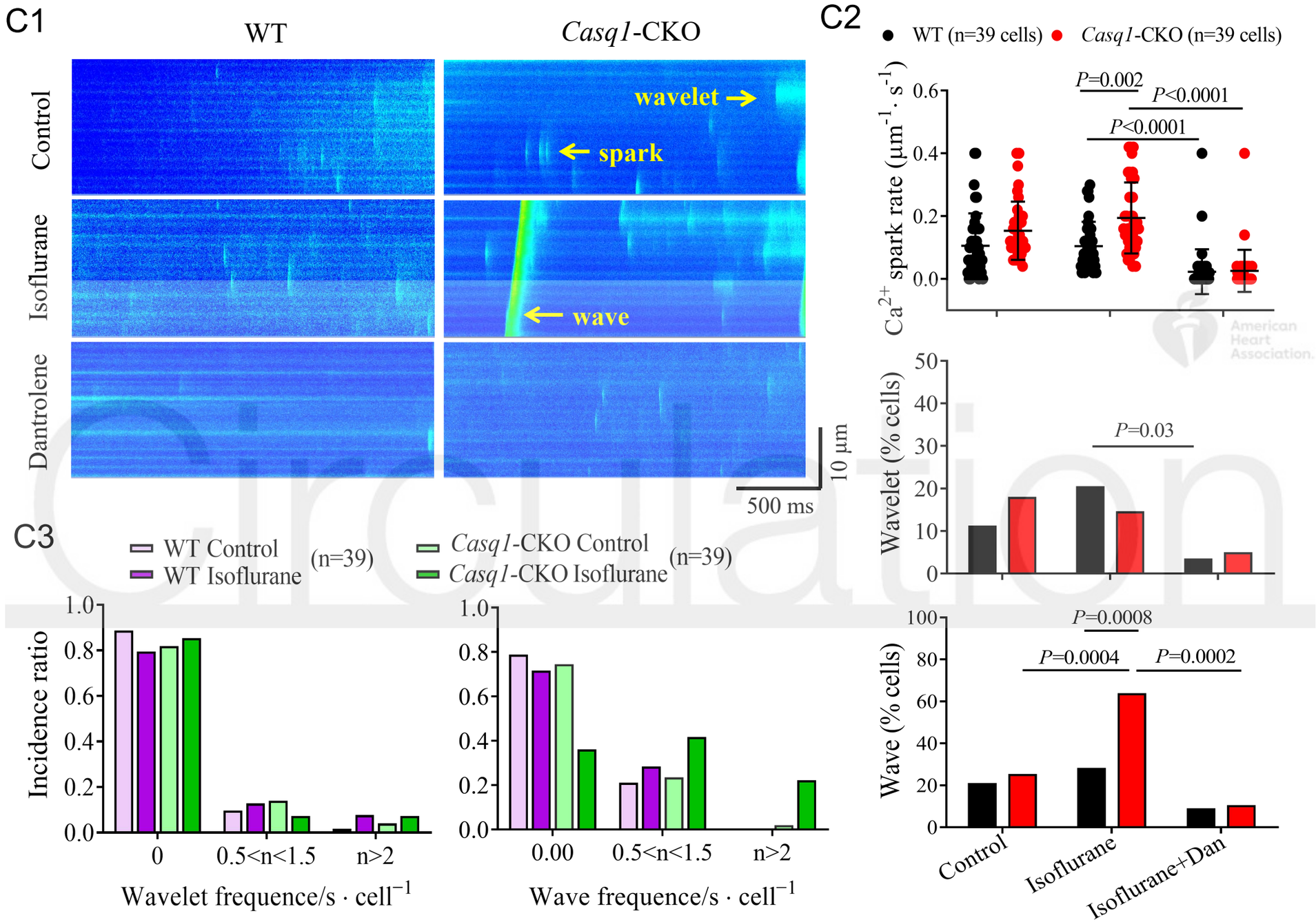
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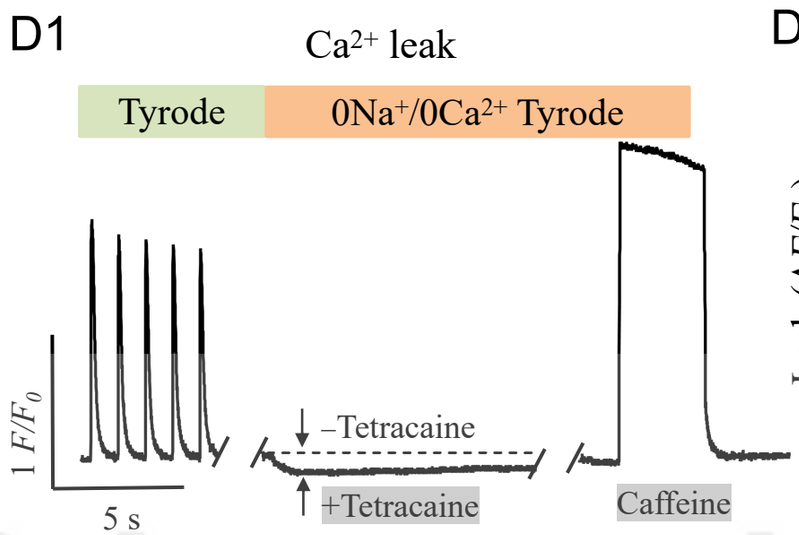


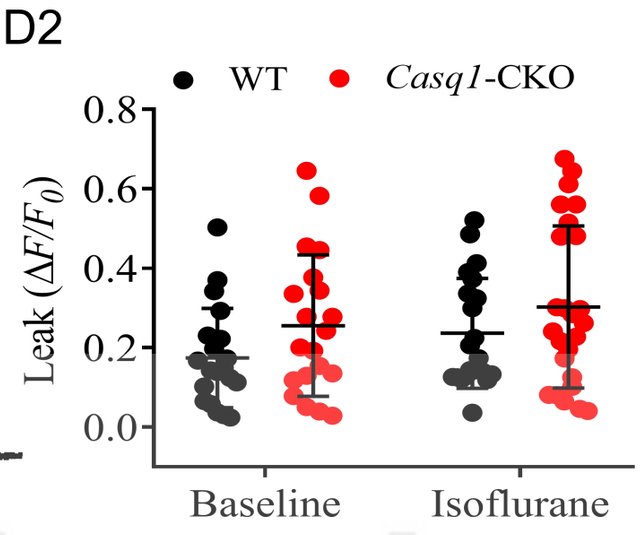


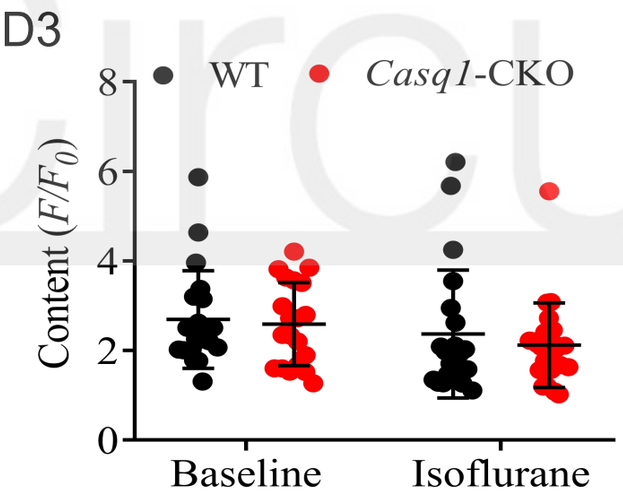


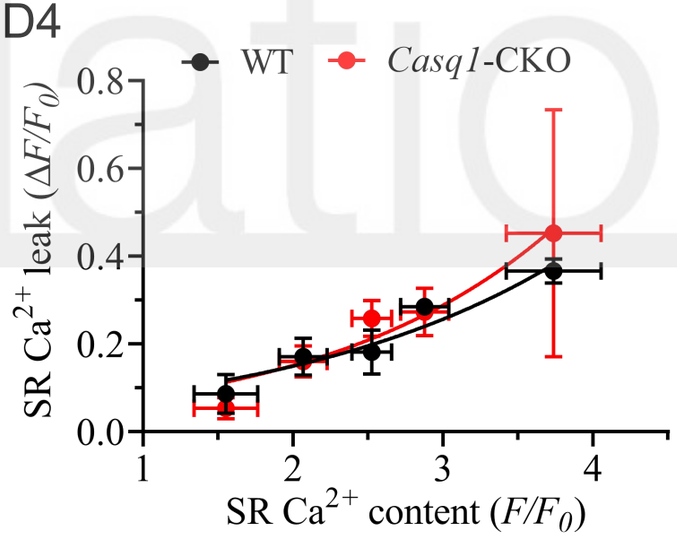


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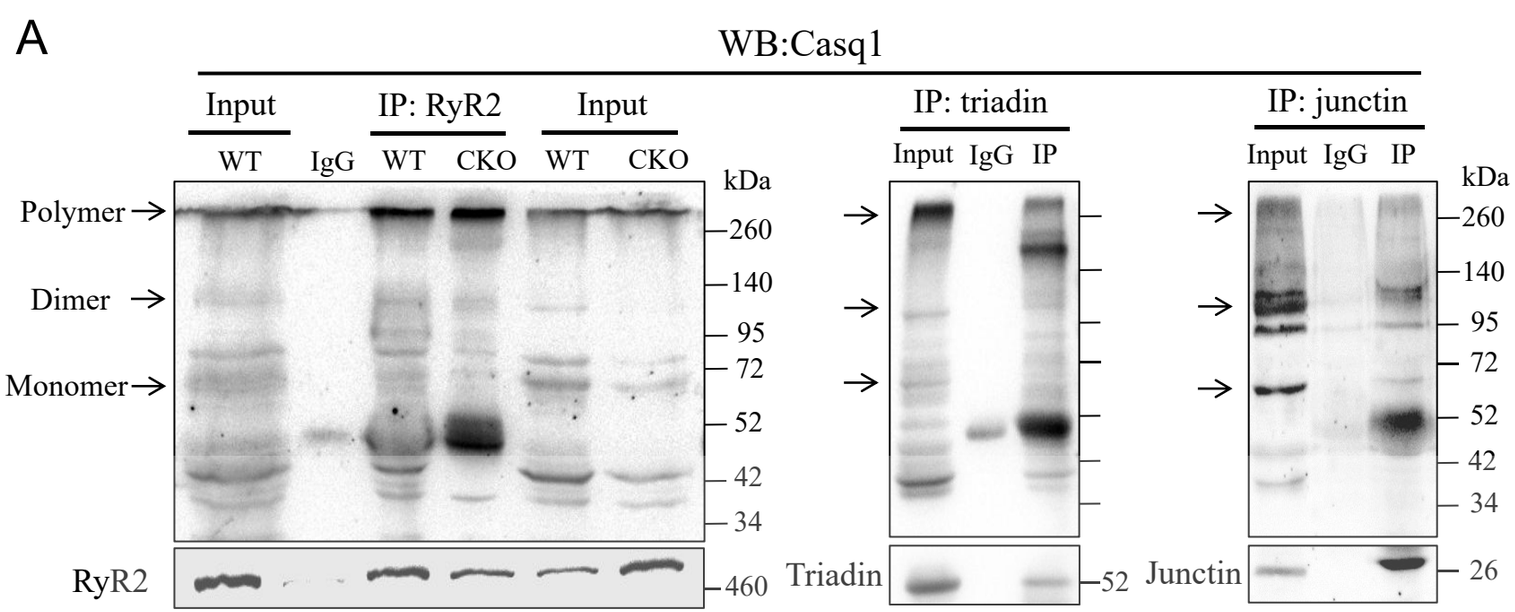


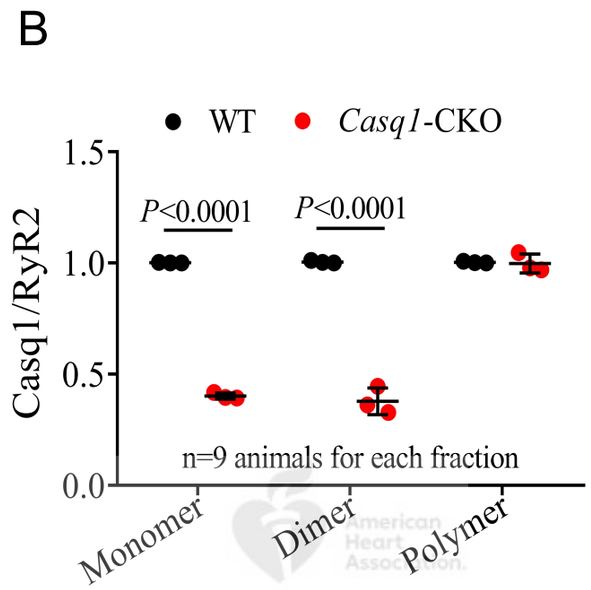


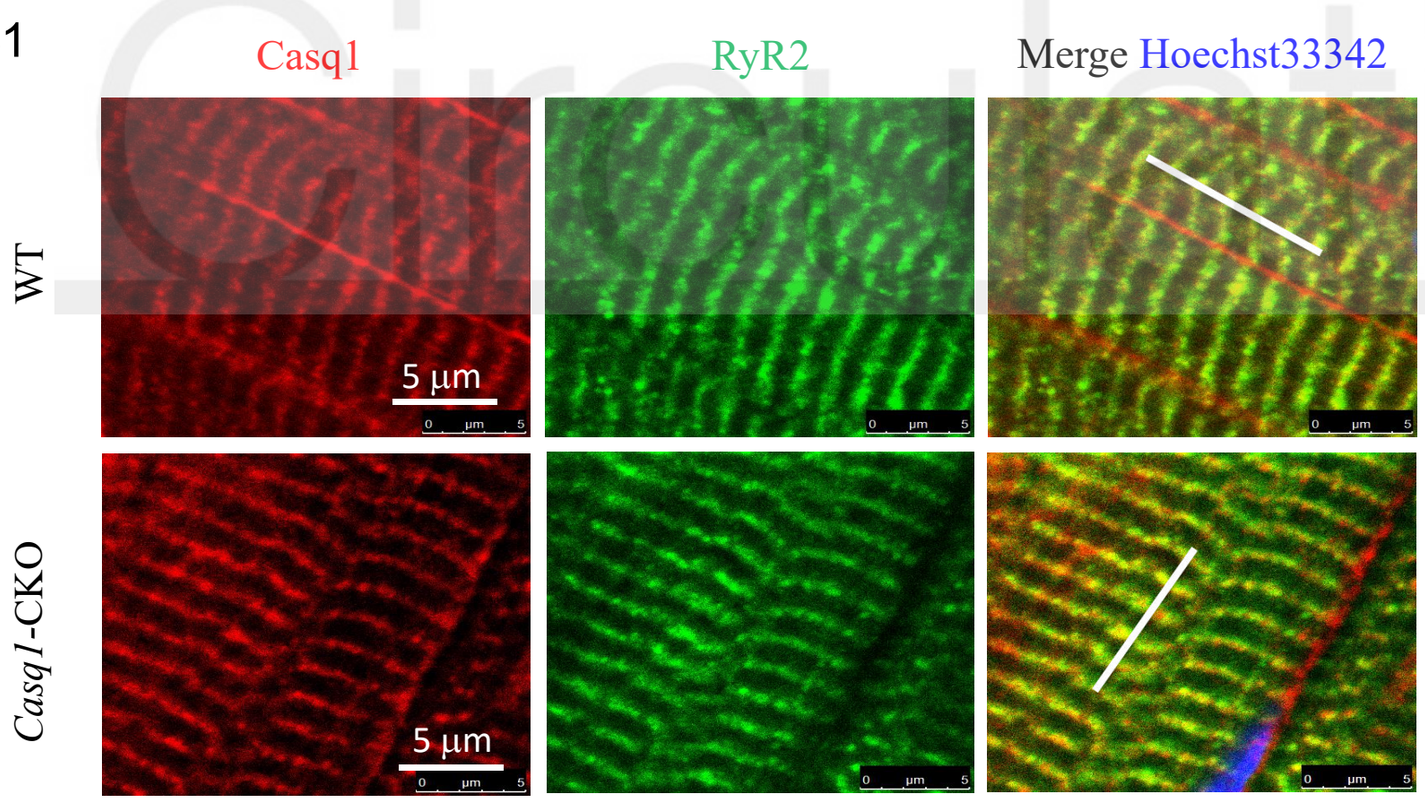


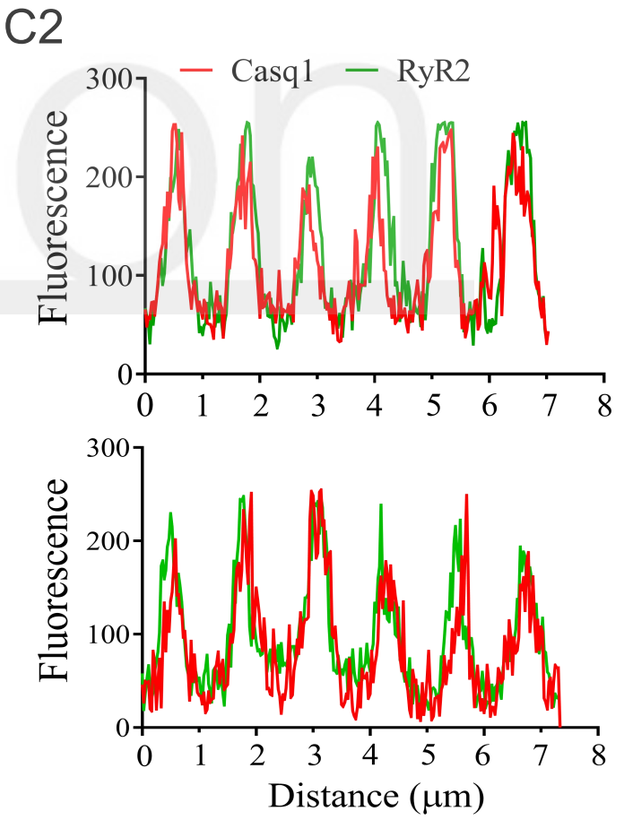
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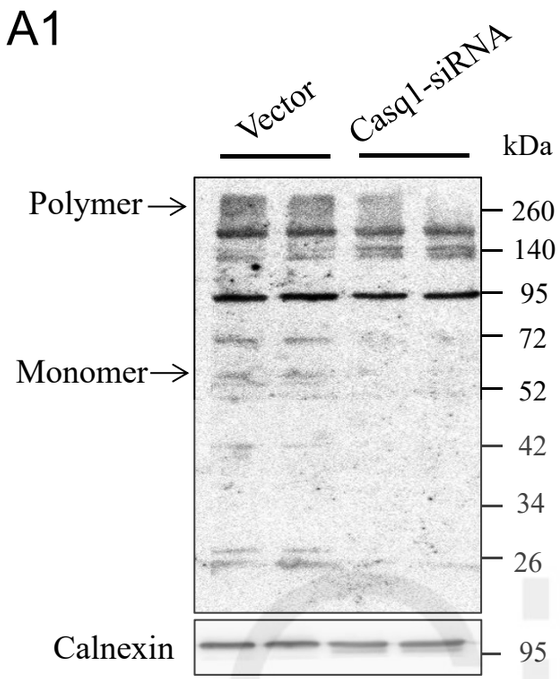


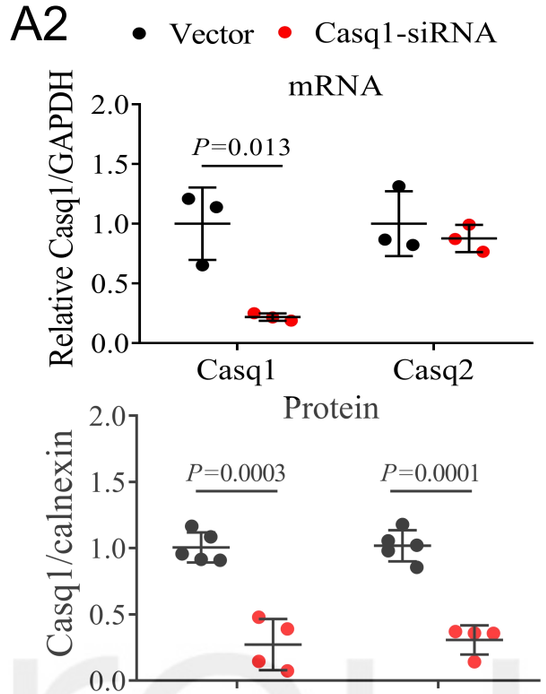


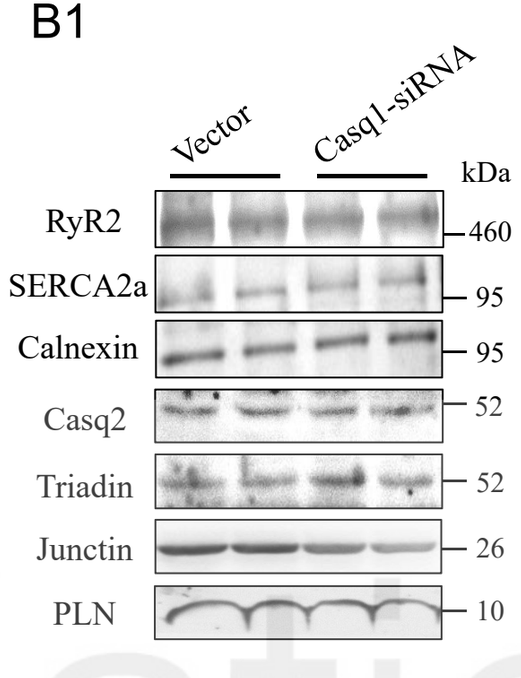


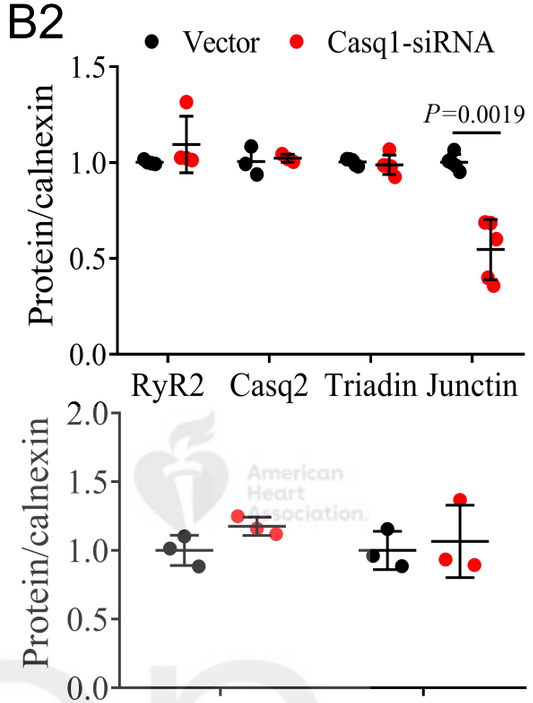


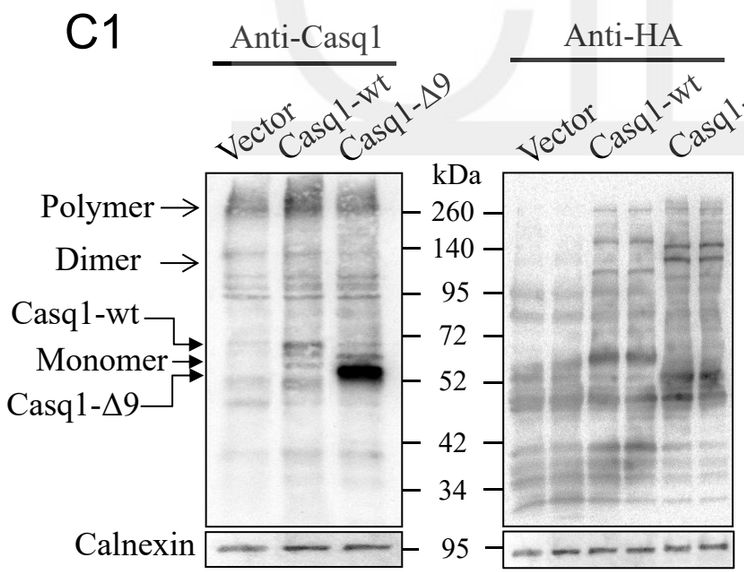
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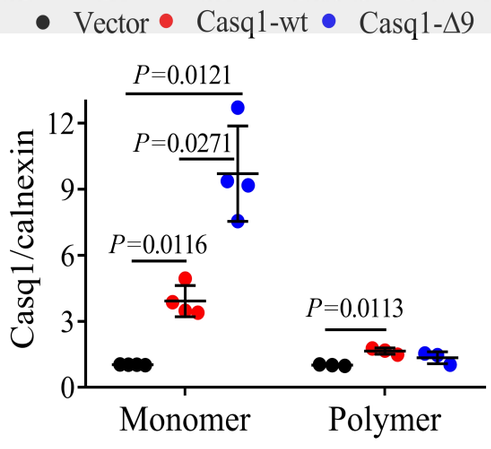


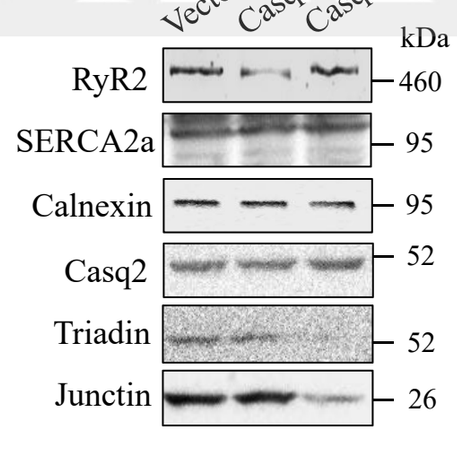


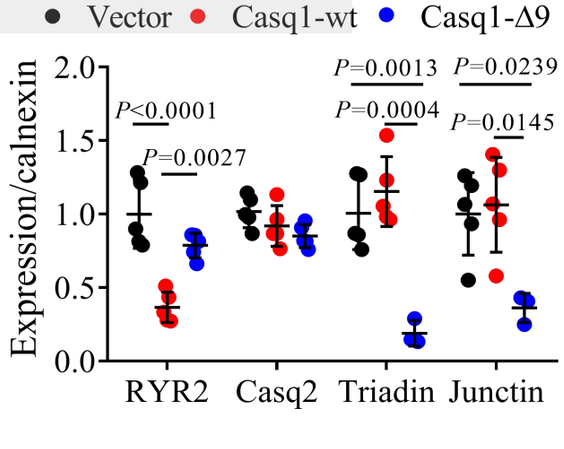




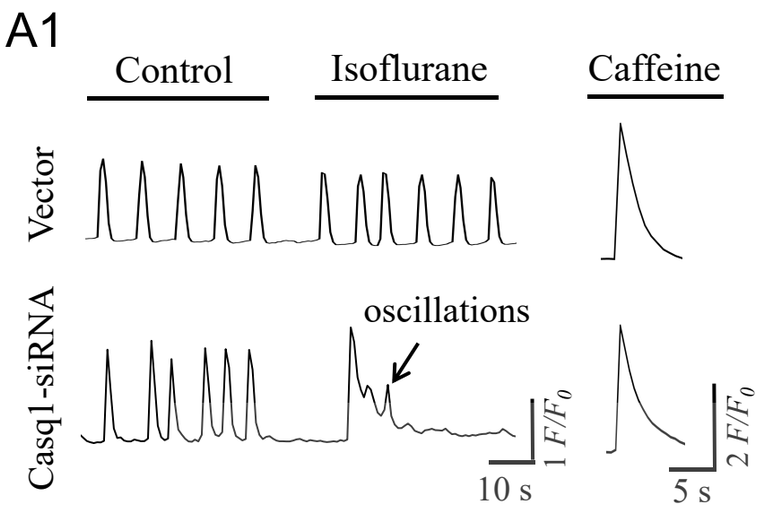


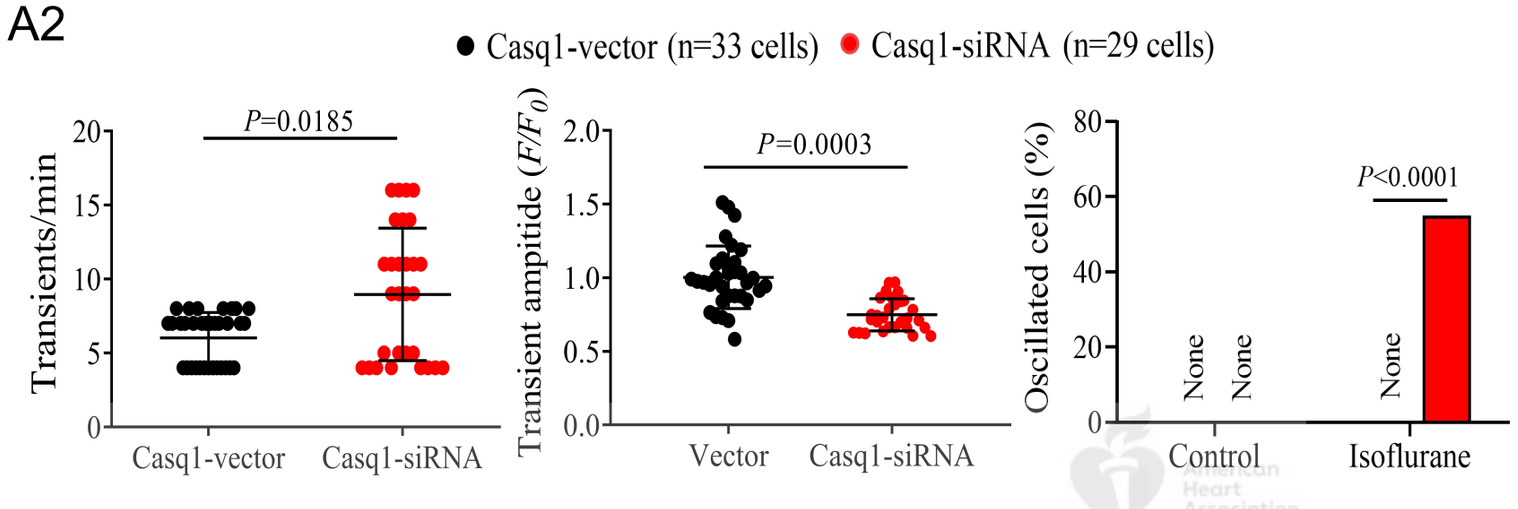


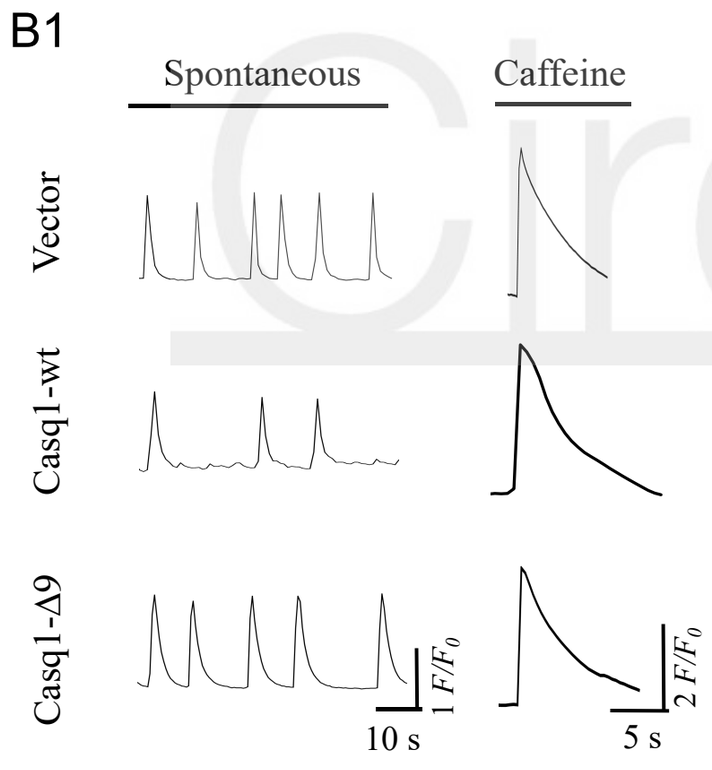


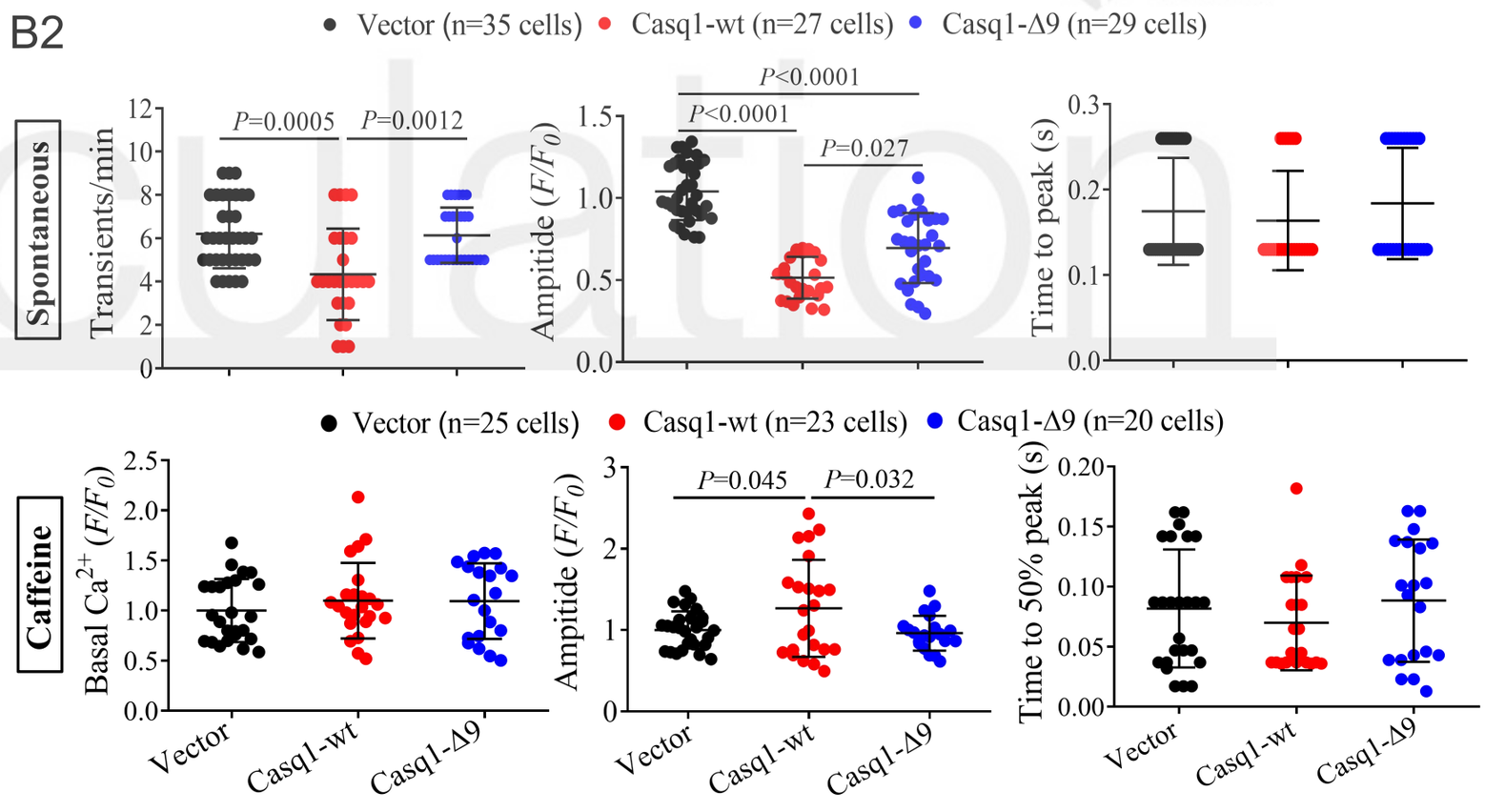


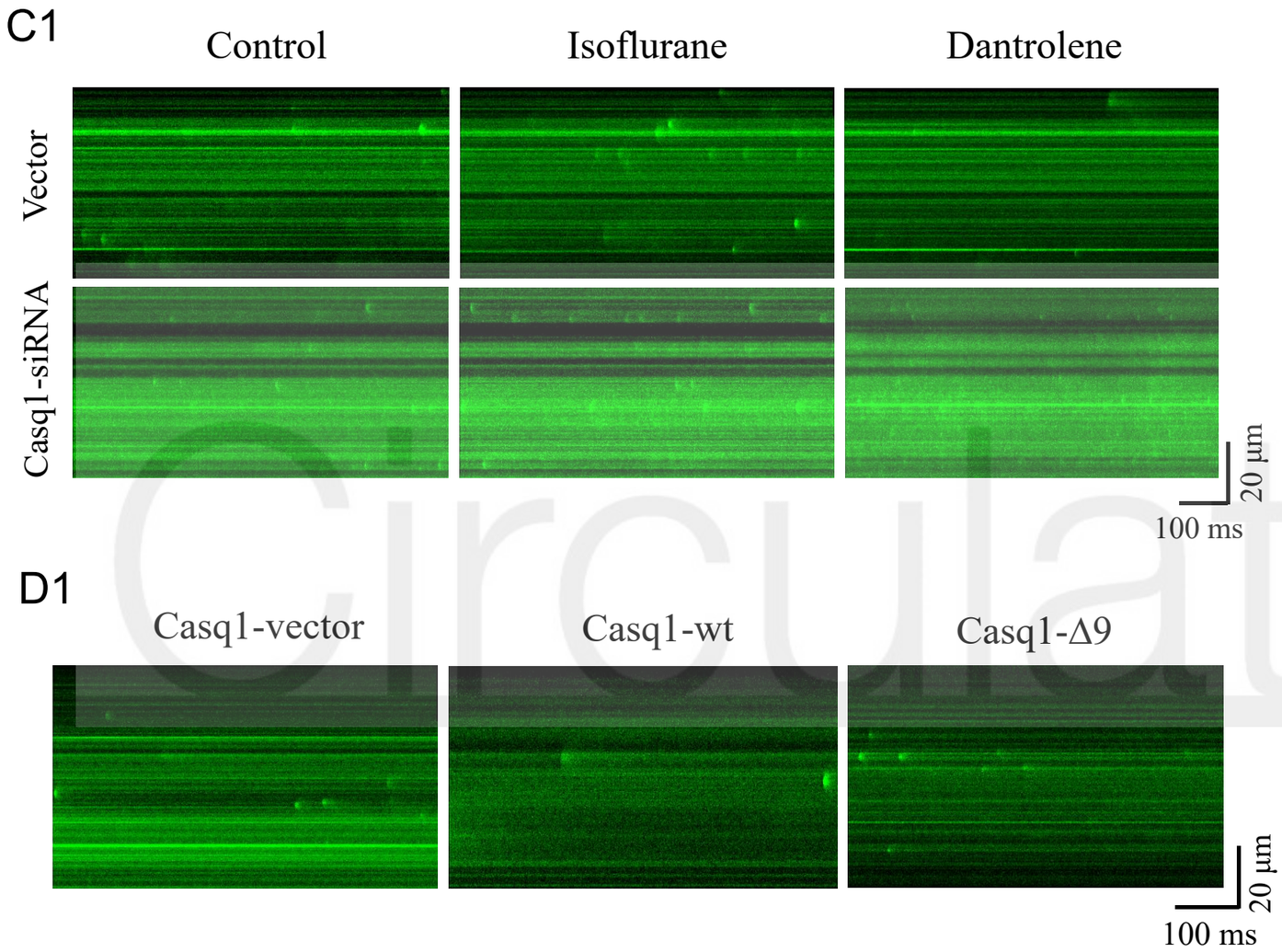
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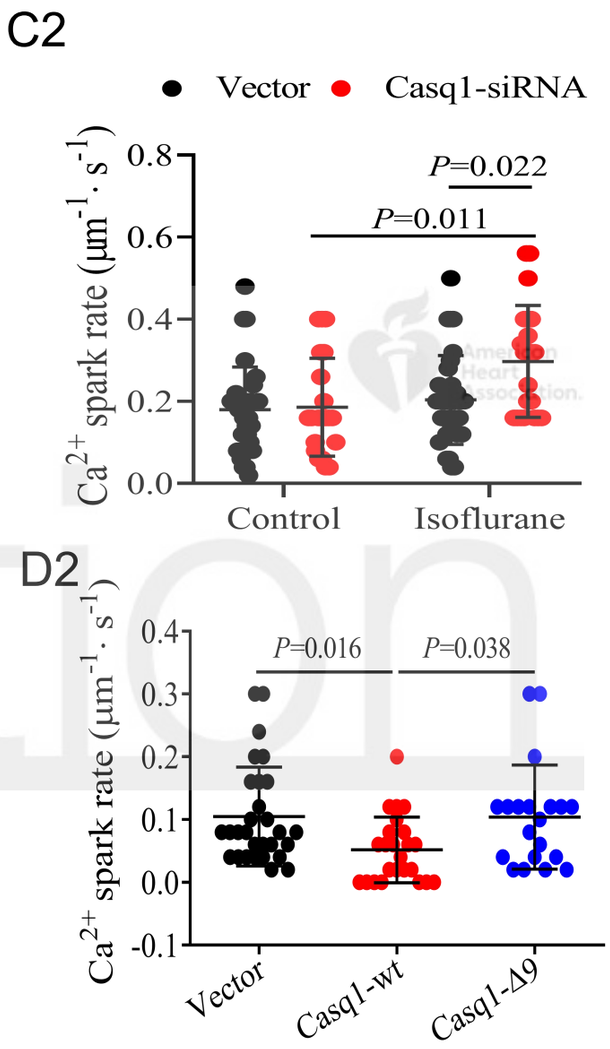




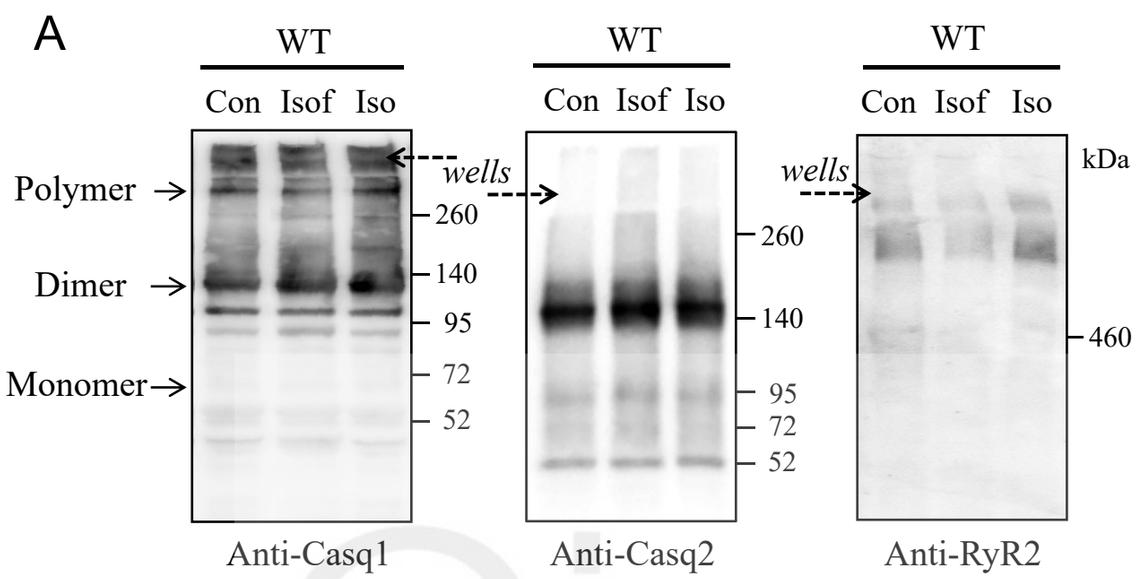


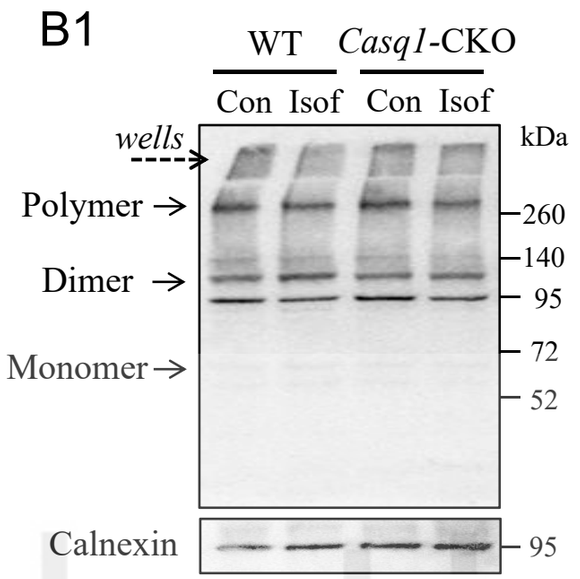


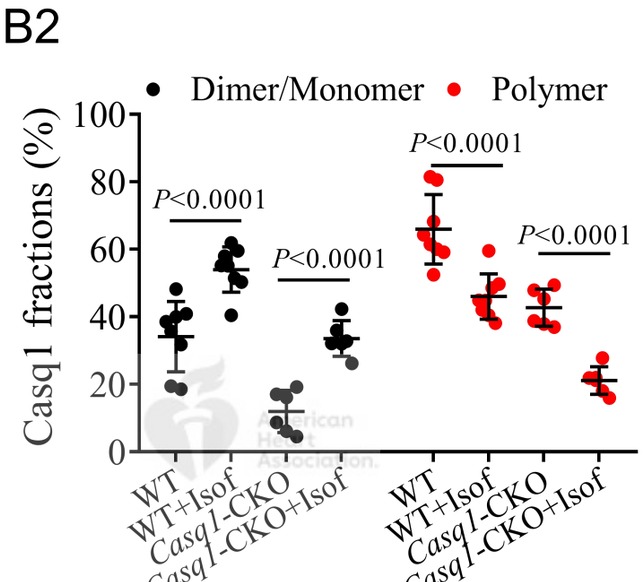




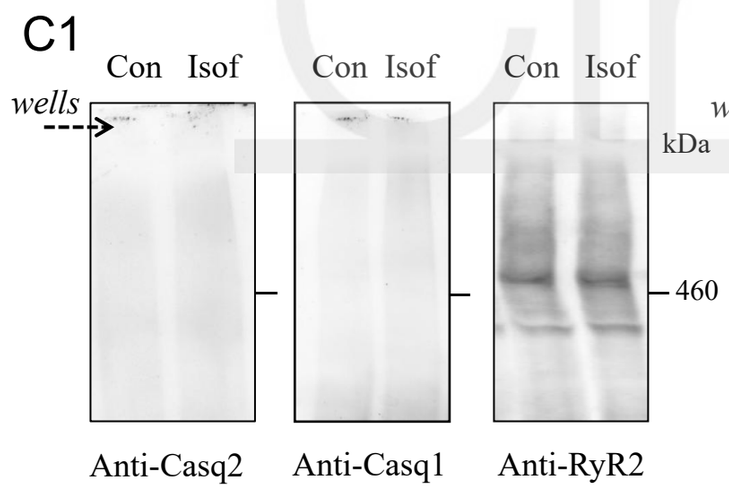
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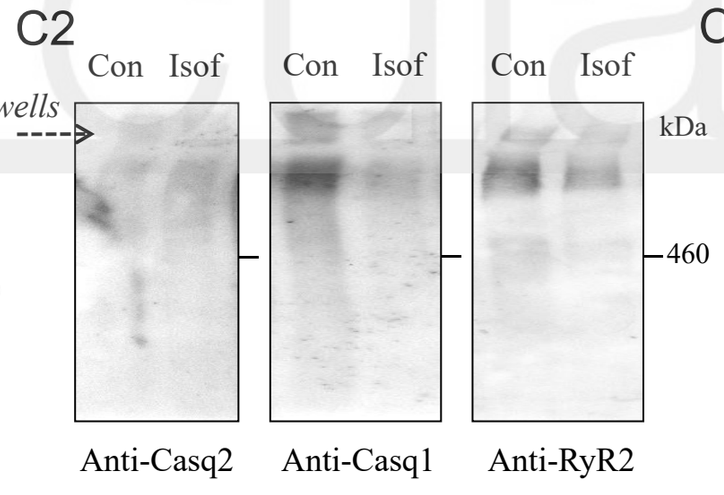


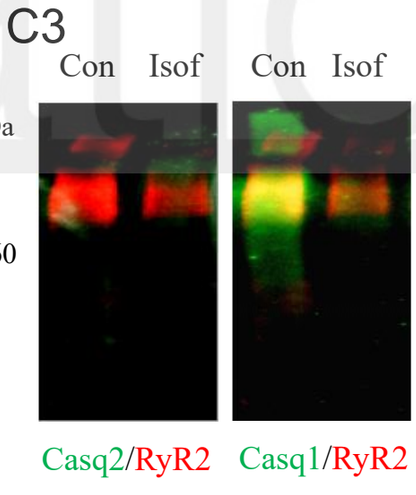


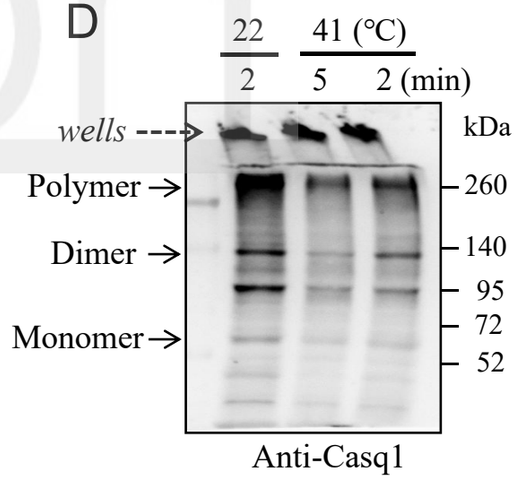


Crosslinking, isoflurane (Isof) or isoproterenol (Iso) (2 min)







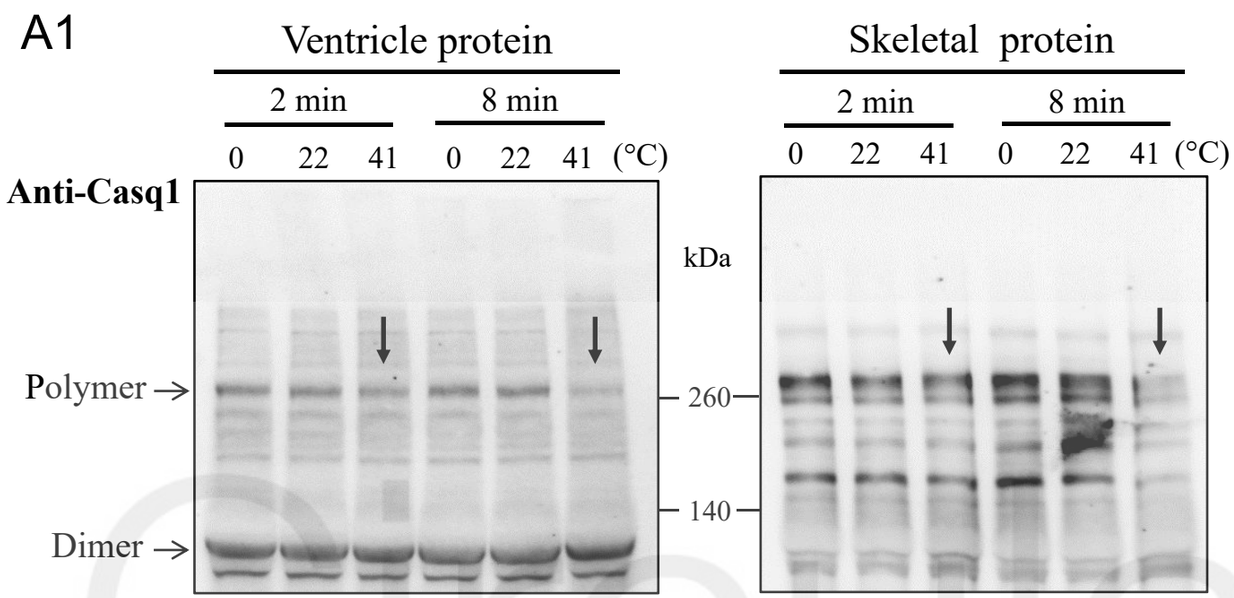


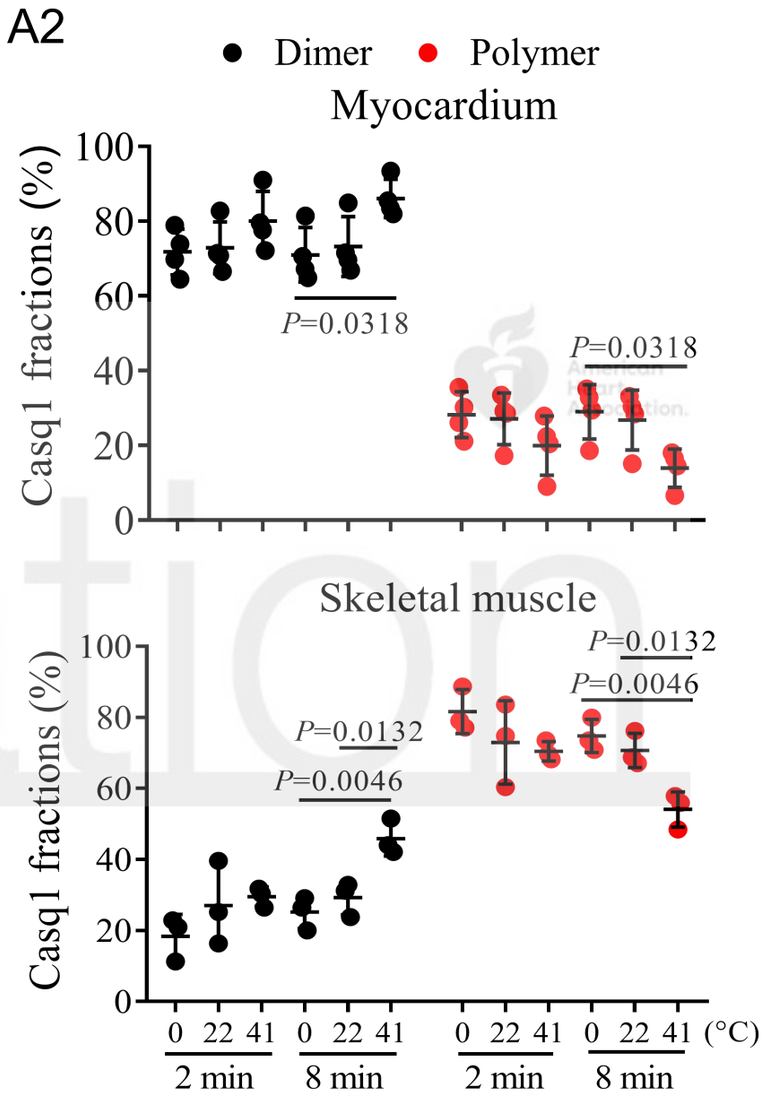
Non-crosslinking, isoflurane (2 min)

Crosslinking, isoflurane (2 min)

Heating

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