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Direct Therapeutic Modulation of RYR2 Activity by CMYA5

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Ryanodine receptor 2 (RYR2), the main cardiomyocyte intracellular Ca^{2+} release channel, is central to excitation-contraction coupling. Aberrant RYR2 regulation causes cardiac dysfunction and arrhythmias, making relief of pathological RYR2 activation an important therapeutic strategy for heart disease. A Rosetta stone for understanding the pathophysiological significance of RYR2 dysregulation has been catecholaminergic polymorphic ventricular tachycardia (CPVT), a potentially lethal arrhythmia caused by pathogenic RYR2 missense variants.

RYR2 interaction with the protein CMYA5 (cardiomyopathy-associated 5) is important for its localization and the assembly of dyads, the nanoscopic structures responsible for cardiomyocyte Ca^{2+} release.[1–3] CMYA5 knockout caused aberrant RYR2 Ca^{2+} release, which was ascribed to dyad disorganization.

All experiments followed protocols approved by the relevant institutional committees. Data, methods, and materials are provided in the article or will be provided by the corresponding author on reasonable request.

Whether CMYA5 directly regulates RYR2 activity was not known. Prior work showed that CMYA5 residues 2731 through 3739 (called MD9; Figure [A]) bound RYR2 and CMYA5[3039–3739] did not.[1–3] We used adeno-associated virus serotype 9 (AAV9) to express CMYA5[2731–3041], dubbed the RYR2 interaction domain ($CMYA5^{RID}$), or MD9 along with GFP (green fluorescent protein) in cardiomyocytes in vivo and measured their binding to RYR2. In cardiomyocytes, RYR2 and MD9 or $CMYA5^{RID}$ colocalized within ≈40 nm based on the proximity ligation assay (Figure [B and C]) and coimmunoprecipitated (Figure [D]).

To test whether $CMYA5^{RID}$ directly regulates RYR2 activity, we recorded RYR2 single-channel activity by incorporating endoplasmic reticulum vesicles from RYR2-expressing HEK293T cells into lipid bilayers. Bacterially produced $CMYA5^{RID}$ but not GFP sharply decreased RYR2 open probability (P_O; Figure [E–G]), demonstrating that $CMYA5^{RID}$ directly inhibits RYR2 activity. In single-channel recordings, RYR2-R176Q, a well-studied CPVT variant, had 3-fold higher P_O than wild type (WT; Figure [E–G]). $CMYA5^{RID}$ strongly inhibited RYR2-R176Q P_O.

We tested the effect of $CMYA5^{RID}$ on RYR2 activity in cardiomyocytes by treating WT and $Ryr2^{R176Q/+}$ CPVT mice with AAV9 expressing $CMYA5^{RID}$ or GFP from the cardiomyocyte-selective *Tnnt2* promoter. We then dissociated cardiomyocytes and recorded Ca^{2+} sparks (Figure [H]). Under basal conditions, there was no significant difference in spark frequency. During isoproterenol stimulation, Ca^{2+} spark frequency was higher in $Ryr2^{R176Q/+}$ cardiomyocytes, and this was suppressed by $CMYA5^{RID}$, suggesting that $CMYA5^{RID}$ might be useful to therapeutically inhibit disease-causing RYR2 variants.

To further investigate the efficacy of $CMYA5^{RID}$ gene therapy for CPVT, we treated neonatal WT or $Ryr2^{R176Q/+}$ mice with AAV9-GFP, AAV9-MD9, or AAV9-$CMYA5^{RID}$

Key Words: catecholaminergic polymorphic ventricular tachycardia ■ gene therapy ■ ryanodine receptor calcium release channel

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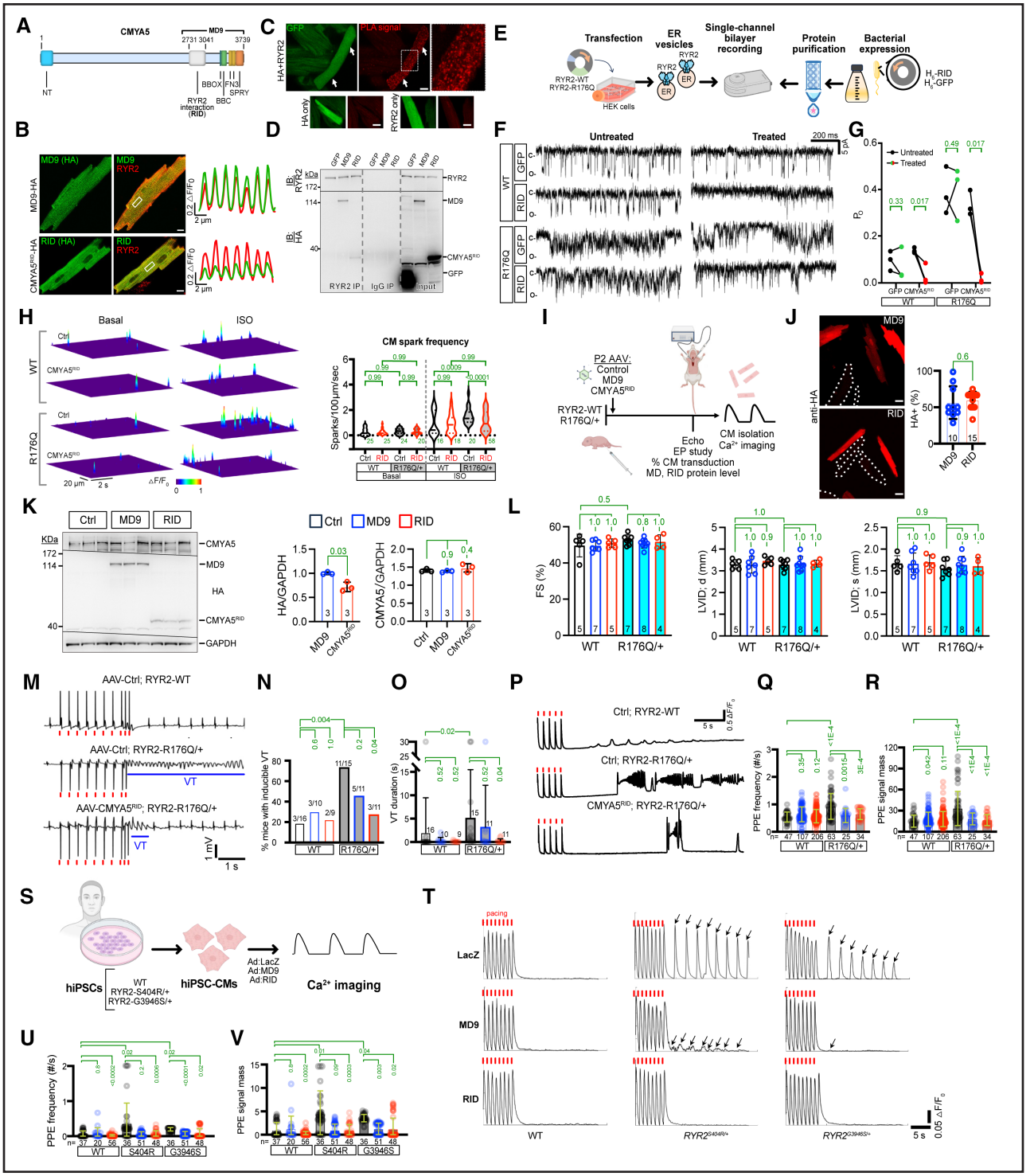

Figure. CMYA5^{RID} directly regulates RYR2 channel activity.

A, CMYA5 (cardiomyopathy-associated 5) protein domain structure. The ryanodine receptor 2 (RYR2) interaction domain (RID) and MD9 region boundaries are shown. **B**, Colocalization of adeno-associated virus 9 (AAV9)–expressed, HA-tagged MD9 or RID of CMYA5 (CMYA5^{RID}) with RYR2 in mature murine cardiomyocytes (CMs). Intensity plots within the boxed areas are shown on the **right**. **C**, Proximity ligation assay (PLA) of HA-tagged RID and RYR2. Mice were transduced with AAV9-HA-CMYA5^{RID}-P2A-GFP (green fluorescent protein). PLA staining was performed with both HA and RYR2 antibodies (**top**) or only HA or RYR2 antibody (**bottom**). GFP (green) identified transduced CMs (arrows). A region of the PLA signal (red; dashed box) is enlarged on the **right**. Nontransduced CMs are negative controls. **D**, HA-tagged CMYA5-derived proteins co-immunoprecipitated with RYR2. AAV9-transduced heart lysates were immunoprecipitated with RYR2 antibody and probed with RYR2 (**top**) or HA (**bottom**) antibodies. **E** through **G**, Single-channel recordings of wild-type (WT) or R176Q mutant RYR2 in lipid bilayers. **E**, Schematic workflow. RYR2-WT or RYR2-R176Q was ectopically expressed in HEK293T cells. Endoplasmic reticulum (ER) vesicles were extracted and incorporated into lipid bilayers. Recombinant His_6 -GST-CMYA5^{RID} or His_6-GST-GFP was expressed in bacteria and purified with nickel (*Continued*)

Figure Continued. affinity resin. Single-channel recordings were performed with the Nanion Orbit Mini system before and after the addition of GFP or CMYA5^{RID} to 130 nmol/L. **F**, Representative recordings before (untreated) and after (treated) protein addition. **G**, Quantification of RYR2 open probability (P_O) before or after protein addition. P_O values were measured from 1-minute recordings before and after protein addition. Paired *t* test with Benjamini, Krieger, and Yekutieli false discovery rate. **H**, CMYA5^{RID} reduced spontaneous RYR2 opening in CMs. Adult CMs from mice treated with AAV9-CMYA5^{RID}-P2A-GFP or AAV9-GFP control were loaded with Rhod-2 AM and analyzed by confocal line scan imaging before and after the addition of isoproterenol (ISO; 100 nmol/L). Quantification of Ca^{2+} spark frequency is shown on the **right**. Generalized estimating equation that accounted for clustering of cell measurements within each mouse with Holm-Bonferroni correction for multiple testing. **I** through **O**, Murine electrophysiological (EP) study. **I**, Schematic. WT or CPVT (*Ryr2*$^{R176Q/+}$) mice of both sexes were randomized to treatment with 1E14 vg/kg AAV9-GFP (Ctrl), AAV9-CMYA5^{RID}-P2A-GFP, or AAV9-MD9-P2A-GFP. Ventricular tachycardia (VT) vulnerability was assessed by EP study and heart function by echocardiography. **J**, HA immunostaining to measure transduction efficiency. Quantification is shown on the **right**. *t* Test. Bar, 20 µm. **K**, Immunoblot to measure the expression of endogenous CMYA5, HA-MD9, or HA-CMYA5^{RID} in transduced hearts. Quantification is shown on the **right**. **Left**, Welch *t* test. **Right**, ANOVA with Dunnett multiple-comparison test. **L**, Echocardiographic analysis of CMYA5^{RID} effect on cardiac systolic function. Two-way ANOVA with Sidak multiple-comparison test. **M**, Programmed stimulation to assess VT vulnerability. The stimulation protocol used right ventricular pacing at 10% above the unpaced rate, followed by 1 and then 2 extra stimuli, with sequential treatment with ISO (2 mg/kg) and then epinephrine (3 mg/kg).[4] Red and blue lines indicate pacing and VT, respectively. **N**, Percent of mice with inducible VT. Fisher exact test. **O**, Duration of induced VT. Bootstrapped zero-inflated ANOVA with Holm-Bonferroni multiple-testing correction. **P** through **R**, Single-cell Ca^{2+} imaging of AAV9-transduced CMs. After the EP study, dissociated CMs were loaded with Rhod-2 AM and electrically paced for 30 s at 1 Hz. Spontaneous postpacing Ca^{2+} release events (PPEs) were quantified over the subsequent 30 seconds. **P**, Representative Ca^{2+} signal. Red lines indicate pacing. **Q** and **R**, Quantification of PPE frequency (**Q**) and signal mass (product of amplitude and duration; **R**). Generalized estimating equation that accounted for clustering of cell measurements within each mouse with Holm-Bonferroni correction for multiple testing. **S** through **V**, Effect of CMYA5^{RID} on Ca^{2+} handling of human induced pluripotent cell–derived cardiomyocytes (hiPSC-CMs) with *RYR2*$^{+/+}$ (WT), *RYR2*$^{S404R/+}$, and *RYR2*$^{G3946S/+}$ genotypes. **S**, Experimental outline. Isogenic adenovirus-transduced hiPSC-CMs were incubated with 5 µmol/L Fluo-4 AM for 10 minutes at 37°C and 5% CO_2, washed twice in Tyrode solution, and electrically paced at 1 Hz for 30 seconds at 37°C and 5% CO_2. **T**, Representative traces. A ×20 field was imaged at 99 ms/frame. The change in Fluo-4 intensity in individual cells was measured over time. Red lines show electrical field stimulation. Arrows indicate PPEs. **U** and **V**, Quantification of spontaneous PPE frequency (**U**) and signal mass (**V**). Bootstrapped zero-inflated ANOVA with clustering by well and Holm-Bonferroni multiple-testing correction. Green numbers within graphs indicate sample sizes and *P* values. Graphs show mean±SD. Statistical testing with the zero-inflated *t* test and general estimating equation was performed with SAS. Other statistical tests were performed with GraphPad Prism. Antibodies: CMYA5, custom[3]; RYR2, Sigma R128; GAPDH, Proteintech 60004-1-Ig; HA, Cell Signaling Technologies 3724S. c indicates closed; FS, fractional shortening; LVID;d, left ventricular end-diastolic internal diameter at end diastole; LVID;s, left ventricular end-diastolic internal diameter at end systole; and o, open. Figure [E] and [S] were created using Biorender.com.

Nonstandard Abbreviations and Acronyms

AAV9	adeno-associated virus serotype 9
CMYA5	cardiomyopathy-associated 5
CMYA5^{RID}	RYR2 interaction domain of CMYA5 (residues 2731–3041)
CPVT	catecholaminergic polymorphic ventricular tachycardia
GFP	green fluorescent protein
hiPSC-CM	human induced pluripotent stem cell-derived cardiomyocyte
MD9	C-terminal portion of CMYA5 (residues 2731–3739)
PPE	postpacing spontaneous calcium release event
RYR2	ryanodine receptor 2
WT	wild type

(1E14 vg/kg; Figure [I]), transducing ≈60% of cardiomyocytes (Figure [J]). MD9 expression was slightly greater than CMYA5^{RID} expression (Figure [K, left]). Neither altered CMYA5 levels (Figure [K, right]) or impaired heart function (Figure [L]). We assessed arrhythmia vulnerability using programmed ventricular stimulation.[4] AAV9-GFP–treated *Ryr2*$^{R176Q/+}$ mice had more frequent and longer ventricular tachycardia than WT (Figure [M–O]). AAV9-CMYA5^{RID} but not AAV9-MD9 reduced VT frequency and duration.

After the electrophysiological study, dissociated cardiomyocytes were loaded with Rhod-2. After isoproterenol treatment and pacing at 1 Hz, postpacing spontaneous Ca^{2+} release events (PPEs) were measured for 30 seconds (Figure [P]). Under control treatment, PPEs were infrequent in WT and higher in *Ryr2*$^{R176Q/+}$ (Figure [Q]). PPE signal mass, the product of PPE amplitude and duration, was also markedly elevated in *Ryr2*$^{R176Q/+}$ compared with WT (Figure [R]). Both AAV9-MD9 and AAV9-CMYA5^{RID} strongly suppressed PPE frequency and signal mass in *Ryr2*$^{R176Q/+}$ (Figure [Q and R]). These data indicate that CMYA5^{RID} reduces VT vulnerability and aberrant Ca^{2+} release in the *Ryr2*$^{R176Q/+}$ CPVT model.

To assess CMYA5^{RID} efficacy in human cardiomyocytes and other CPVT-causing RYR2 variants, we tested human induced pluripotent cell–derived cardiomyocytes (hiPSC-CMs) that were Cas9 genome edited to harbor CPVT-causing variants *RYR2*$^{S404R/+}$ and *RYR2*$^{G3946S/+}$ (Figure [S]). Bioreactor-differentiated hiPSC-CMs[5] were transduced with adenovirus expressing LacZ (control), MD9, or CMYA5^{RID}; loaded with Fluo-4; treated with 1 µmol/L isoproterenol; and electrically paced at 1 Hz for 30 seconds. PPEs were measured during the subsequent 30-second postpacing interval (Figure [T]). WT hiPSC-CMs exhibited few PPEs in all 3 treatment groups, whereas control-treated, isogenic

RYR2$^{S404R/+}$ and *RYR2*$^{G3964S/+}$ hiPSC-CMs had elevated PPE frequency and signal mass (Figure [U and V]). CMYA5^{RID} strongly reduced PPE frequency and signal mass in both CPVT genotypes. MD9 reduced both parameters in *RYR2*$^{G3964S/+}$ but not *RYR2*$^{S404R/+}$ hiPSC-CMs. These data indicate that CMYA5^{RID} inhibits aberrant Ca^{2+} handling in human CPVT cardiomyocytes with 2 additional pathogenic RYR2 variants.

In summary, our study showed that CMYA5^{RID} directly inhibits RYR2 channel activity and that this regulatory mechanism may be therapeutically exploited in CPVT. The pathological conditions with aberrant RYR2 regulation such as acquired heart failure or atrial fibrillation that respond to CMYA5^{RID} require further investigation.

ARTICLE INFORMATION

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Disclosures

Drs Pu and Lu have filed for intellectual property on CMYA5^{RID}. The other authors report no conflicts.

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