Peptidomimetic Targeting of Ca\(_{\gamma}\)\(\beta\)2 Overcomes Dysregulation of the L-Type Calcium Channel Density and Recovers Cardiac Function

**BACKGROUND:** L-type calcium channels (LTCCs) play important roles in regulating cardiomyocyte physiology, which is governed by appropriate LTCC trafficking to and density at the cell surface. Factors influencing the expression, half-life, subcellular trafficking, and gating of LTCCs are therefore critically involved in conditions of cardiac physiology and disease.

**METHODS:** Yeast 2-hybrid screenings, biochemical and molecular evaluations, protein interaction assays, fluorescence microscopy, structural molecular modeling, and functional studies were used to investigate the molecular mechanisms through which the LTCC Ca\(_{\gamma}\)\(\beta\)2 chaperone regulates channel density at the plasma membrane.

**RESULTS:** On the basis of our previous results, we found a direct linear correlation between the total amount of the LTCC pore-forming Ca\(_{\alpha}\)1.2 and the Akt-dependent phosphorylation status of Ca\(_{\gamma}\)\(\beta\)2 both in a mouse model of diabetic cardiac disease and in 6 diabetic and 7 nondiabetic cardiomyopathy patients with aortic stenosis undergoing aortic valve replacement. Mechanistically, we demonstrate that a conformational change in Ca\(_{\gamma}\)\(\beta\)2 triggered by Akt phosphorylation increases LTCC density at the cardiac plasma membrane, and thus the inward calcium current, through a complex pathway involving reduction of Ca\(_{\alpha}\)1.2 retrograde trafficking and protein degradation through the prevention of dynamin-mediated LTCC endocytosis; promotion of Ca\(_{\alpha}\)1.2 anterograde trafficking by blocking Kir/Gem-dependent sequestration of Ca\(_{\beta}\)2, thus facilitating the chaperoning of Ca\(_{\alpha}\)1.2; and promotion of Ca\(_{\alpha}\)1.2 transcription by the prevention of Kir/Gem-mediated shuttling of Ca\(_{\gamma}\)\(\beta\)2 to the nucleus, where it limits the transcription of Ca\(_{\alpha}\)1.2 through recruitment of the heterochromatin protein 1\(\gamma\) epigenetic repressor to the Cacna1c promoter. On the basis of this mechanism, we developed a novel mimetic peptide that, through targeting of Ca\(_{\gamma}\)\(\beta\)2, corrects LTCC life-cycle alterations, facilitating the proper function of cardiac cells. Delivery of mimetic peptide into a mouse model of diabetic cardiac disease associated with LTCC abnormalities restored impaired calcium balance and recovered cardiac function.

**CONCLUSIONS:** We have uncovered novel mechanisms modulating LTCC trafficking and life cycle and provide proof of concept for the use of Ca\(_{\gamma}\)\(\beta\)2 mimetic peptide as a novel therapeutic tool for the improvement of cardiac conditions correlated with alterations in LTCC levels and function.
Clinical Perspective

What Is New?

- A tight control of the Ca\(^{2+}\) cardiac machinery is essential for normal cardiac physiology. Alterations in the voltage-dependent L-type calcium channel (LTCC), the key mediator of intracellular Ca\(^{2+}\) entry, are associated with various life-threatening cardiovascular conditions such as pathologic hypertrophy, atrial fibrillation, post-infarct myocardial hypertrophy, and diabetic cardiomyopathy. The potential use of positive Ca\(^{2+}\) modulators for the treatment of cardiovascular pathologies has received considerable attention during the past decades. However, hitherto, pharmacologic approaches aimed at enhancing Ca\(^{2+}\) current and inotropism in systolic heart failure have frequently been found to favor arrhythmogenesis and diastolic dysfunction, thereby limiting their use in the clinic.

- Here, we identified a novel peptidomimetic therapeutic tool for the treatment of cardiovascular diseases that, via an unconventional mechanism, bypasses the arrhythmogenic limitations of current channel-activator inotropes. In particular, by dissecting new regulatory pathways modulating the LTCC life cycle, we generated a mimetic peptide that, through multiple levels of regulation, specifically targets the LTCC \(\alpha_{1}2\) cytosolic chaperone, thereby controlling LTCC assembly and density at the plasma membrane while preserving its physiologic channel function.

What Are the Clinical Implications?

- We provide proof of concept for the exploitation of a novel therapy based on mimetic peptide technology and demonstrate that cardiac dysfunctions (eg, diabetic cardiomyopathy) associated with LTCC abnormalities can be effectively treated by in vivo administration of the generated mimetic peptide. Thus, mimetic peptide technology associated with nanocarriers for safe and cardiac-specific delivery may provide novel innovative therapeutic tools for the treatment of cardiac diseases.

Maintenance of calcium homeostasis is critical for preserving the physiology of the cell. A variety of complex mechanisms intervene in the regulation of intracellular levels of Ca\(^{2+}\) and its compartmentalization between subcellular compartments. In the heart, Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR), the major intracellular Ca\(^{2+}\) store, to the cytosol is regulated through a process called Ca\(^{2+}\)-induced Ca\(^{2+}\) release.\(^{1,2}\) Ca\(^{2+}\)-induced Ca\(^{2+}\) release is initiated by the entry of Ca\(^{2+}\) into cardiac cells through sarcolemmal voltage-dependent L-type Ca\(^{2+}\) channels (LTCCs), which triggers the release of Ca\(^{2+}\) from the SR to the cytoplasm through ryanodine receptor 2. A close association between LTCC and ryanodine receptor 2 is required for efficient Ca\(^{2+}\)-in-
duced Ca\(^{2+}\) release and is dependent on the density and organization of LTCCs within the T-tubular invagination of the plasma membrane.\(^{2}\) The increase in free intracellular Ca\(^{2+}\) allows Ca\(^{2+}\) to bind to troponin C, initiating muscle contraction. This is terminated subsequently by the removal of cytosolic Ca\(^{2+}\) through its reuptake into the SR via the cardiac SR Ca\(^{2+}\)-ATPase and, to a lesser extent, by other Ca\(^{2+}\) transport systems. This process is a key regulator of cardiac excitation-contraction coupling and a major determinant for intrinsic physiologic properties of the beating heart.

Modifications in the appropriate trafficking of LTCC to and density at the cell surface and changes in its subcellular localization and gating properties can cause functional alterations in the overall inward Ca\(^{2+}\) current ($I_{\text{Ca,L}}$) and consequently the cellular Ca\(^{2+}\) machinery. In line with this, acquired and genetically determined LTCC dysfunction has been found to be associated with various human diseases,\(^{4-14}\) including life-threatening cardiovascular pathologies.\(^{4-14}\)

LTCCs are expressed in all excitable cells (ie, striated, smooth muscle, and neuronal cells).\(^{15}\) The cardiac LTCC is composed of the pore-forming Ca\(_{\alpha1.2}\) and the accessory Ca\(_{\beta2}\) and Ca\(_{\alpha2\delta}\) subunits.\(^{15-17}\) Ca\(_{\beta2}\), the cytoplasmic chaperone of Ca\(_{\alpha1.2}\), is composed of a globular domain (including an Src homology 3 [SH3] and a guanylate kinase-like [GK] domain) and a C-terminal coiled-coil tail (C-tail) and belongs to the membrane-associated GK family, which is involved in intracellular signaling and the establishment/maintenance of cell polarity.\(^{16}\) Ca\(_{\beta2}\) binds to the pore unit at the \(\alpha\)-interaction domain,\(^{18}\) affecting its trafficking to the plasma-membrane,\(^{19-21}\) its internalization,\(^{22}\) and its gating properties.\(^{17,23}\) However, although LTCC channel physiology has been intensively studied during the last decades, more in-depth insights into the mechanisms regulating the trafficking and function of LTCCs are still required. Here, we unraveled new regulatory pathways modulating the LTCC life cycle and generated a mimetic peptide (MP) that, through multiple levels of regulation, specifically targets Ca\(_{\beta2}\), thereby controlling LTCC levels at the plasma membrane. Additionally, we provide proof of concept for in vivo administration of MP as an effective therapeutic strategy for the treatment of cardiac disorders associated with LTCC abnormalities.

METHODS

Detailed procedures are provided in the online-only Data Supplement.

Plasmids

Yeast 2-hybrid bait constructs were generated in the pGBK7 vector (Clontech), and the mouse adult heart cDNA library was generated with the Make Your Own Mate & Plate Library System (Clontech). For the nanoluciferase and bioluminescence


Mimetic peptide controls the LTCC life cycle
resonance energy transfer (BRET) assays, cDNAs were cloned into the pNLF1-N and pNL1-F1-N and the HaloTag- pFN21A vector (Promega), respectively.

**Western and Dot Blot Analyses**

Ca$_{\alpha}1.2$ Ca$_{\beta}2$ (S8b-1), HA, and transferrin receptor were from Abcam; c-myc 9E10 was from Santa Cruz; HP-1$_{\gamma}$ 42S2 came from Millipore; GAPDH 14C10 was purchased from Cell Signaling Technology; Polyaginine 9R was from Cell Applications; and goat anti-mouse and anti-rabbit horseradish peroxidase was purchased from Thermo Fisher Scientific. A rabbit polyclonal antibody against the phosphorylated Akt consensus site of Ca$_{\beta}2$ was generated by GenScript.

**Nanoluciferase Assay**

In NanoLuc Luciferase (Promega), HEK293 cells pretreated for 30 minutes with 20 μmol/L cycloheximide were transfected as described in the Results section. Time-course analysis was performed with a Synergy 4 instrument (BioTek).

**NanoBRET Assay**

In the NanoBRET Assay (Promega), HEK293-transfected cells were treated with 100 nmol/L NanoBRET 618 ligand, and signals were detected 5 hours after treatment. For the peptide-protein binding assay, tetramethylrhodamine peptides were used. Signals were detected with a Synergy 4 instrument (BioTek).

**Human Patients**

Patients with aortic stenosis who underwent aortic valve replacement were recruited retrospectively from the biobank available at the University Hospital of Verona, Division of Cardiac Surgery, Verona, Italy. Approval for studies on human tissue samples was obtained from the ethics committee of the University Hospital of Verona. All patients or their relatives gave informed consent before surgery.

**Animals**

All procedures on mice were performed according to institutional guidelines in compliance with national (D.L. N.26, 04/03/2014) and international laws and policies (new directive 2010/63/EU). The protocol was approved by the Italian Ministry of Health. Special attention was paid to animal welfare to minimize the number of animals used and their suffering. All experiments were performed on 12-week-old male mice with at least 8 animals per group. Diabetes mellitus was induced in adult C57B6/J male mice by intraperitoneal injection of streptozotocin (50 mg·kg$^{-1}$·d$^{-1}$) for 5 days. At 2 weeks after the last injection of streptozotocin, whole blood was obtained and glucose levels were measured with the Accu-Chek active blood glucose monitoring system (Roche). Mice with hyperglycemia (>300 mg/dL) were used for the study. Citrate buffer–treated mice were used as nondiabetic controls. After streptozotocin treatment, mice were treated with peptides (2.5 mg·kg$^{-1}$·d$^{-1}$ IP) as described in the Results section.

**Statistical Analysis**

Data are presented as mean±SD. The normality of the data was assessed with the Kolmogorov-Smirnov test. Statistical comparison was performed in at least 3 independent experiments with the Mann-Whitney test, and comparisons between groups were analyzed by repeated-measures ANOVA combined with the Tukey multicomparison. The Pearson correlation coefficient test was used for the analysis of human data. Prism 6.0 software (GraphPad Software) was used to assess the normality of the data and for statistical calculation. A value of $P<0.05$ was considered significant.

**RESULTS**

**Ca$_{\beta}2$ Molecular Reorganization Stabilizes Ca$_{\alpha}1$**

We previously demonstrated that Ca$_{\alpha}1.2$ total protein levels are affected by the phosphorylation status of Ca$_{\beta}2$ at the Akt consensus site in its C-tail. To determine the mechanism by which phosphorylated Ca$_{\beta}2$ controls the Ca$_{\alpha}1.2$ level, we performed yeast 2-hybrid screenings of human and mouse heart cDNA libraries using phosphomimetic Ca$_{\beta}2$ (Ca$_{\beta}2$-SE$^{24}$) as bait. Unexpectedly, several positive clones corresponded to the SH3 region in the globular domain of Ca$_{\beta}2$ (Figure 1A), and cotransformation assays confirmed the binding of Ca$_{\beta}2$-SE, but not wild-type Ca$_{\beta}2$, to the globular region of Ca$_{\beta}2$ (Figure 1B). The specificity of the interaction was further confirmed by coimmunoprecipitation assays (Figure 1C). Analysis of the Ca$_{\beta}2$ structure revealed a solvent accessible sequence within the minimal common region of the identified Ca$_{\beta}2$ clones (Figure 1A), which we predicted to be responsible for the binding to the Ca$_{\beta}2$ C-tail on Akt phosphorylation. Taken together, these results suggest that the Akt-dependent protective effect on Ca$_{\alpha}1.2$ stability is not mediated through interaction with other proteins but relies entirely on Ca$_{\beta}2$ structural rearrangements, triggered by Akt-mediated phosphorylation of its C-terminal interacting domain (TID).

To determine whether the TID directly affects Ca$_{\alpha}1.2$ protein stability, we used site-specific mutagenesis to replace positively charged lysines (K) at positions 141, 149, and 161 in the TID sequence with glutamines (Q) to destroy any potential ionic interaction between the TID and the C-tail. In cells cotransfected with Ca$_{\alpha}1.2$ and Ca$_{\beta}2$-SE, similar protein levels of Ca$_{\alpha}1.2$ total protein were observed in the presence and absence of Akt activation (ie, with and without serum, corresponding to phosphorylated and nonphosphorylated Ca$_{\beta}2$, respectively; Figure 1F, inset), whereas significantly reduced Ca$_{\alpha}1.2$ levels were found in cells cotransfected with K161Q-mutant Ca$_{\beta}2$-SE under serum free conditions (Figure 1D). Consistently, the K161Q mutation prevented the interaction with Ca$_{\beta}2$-SE in the yeast 2-hybrid...
system (Figure 1E), suggesting a direct role of the TID in the protective effect of Ca\(\beta\)2-SE on Ca\(\alpha\)1.2 stability. Similarly, the intracellular Ca\(^{2+}\) concentration was significantly higher in cells cotransfected with Ca\(\alpha\)1.2 and Ca\(\beta\)2-SE compared with wild-type Ca\(\beta\)2 after serum starvation, whereas cotransfection with K161Q-mutant Ca\(\beta\)2-SE ablated this effect (Figure 1F). In addition, I\(_{\text{Ca,L}}\) was significantly reduced in cells transfected with K161Q-mutant Ca\(\beta\)2-SE compared with Ca\(\beta\)2-SE (Figure 1G). Taken together, these results provide evidence that the TID within the Ca\(\beta\)2-SH3 domain plays a direct role in stabilizing Ca\(\alpha\)1.2.
MP Affects LTCC Protein Stability and Function In Vitro

We next designed an array of partially overlapping peptides covering the C-tail and tested their effect on Ca_\text{\textalpha}1.2 stability through binding to the Ca_\text{\textbeta}2-TID. Cotransfection of cells with Ca_\text{\textalpha}1.2, Ca_\text{\textbeta}2, and individual peptides, followed by Western blot analyses (Figure 2A) and intracellular Ca^{2+} concentration measurements (Figure 2B), identified several peptides that efficiently regulated Ca_\text{\textalpha}1.2 protein amounts and increased intracellular Ca^{2+} concentration without inducing apoptosis (Figure 2A). For our subsequent studies, we selected peptide 11 designated as MP, the effect of which was most similar to that of Ca_\text{\textbeta}2-SE. Computational docking simulation of the interaction between the MP and the Ca_\text{\textbeta}2-TID (Figure 2C) and additional site-specific mutagenesis (Figure I in the online-only Data Supplement) further defined the TID-MP binding pocket.

Next, to facilitate intracellular uptake, MP was fused to an oligoarginine (R7W) cell-penetrating peptide. The R7W was fused to the N-terminus of the MP, which, on the basis of the Ca_\text{\textbeta}2-MP 3D model (Figure 2C), was not predicted to create steric hindrance or to affect MP function. To monitor the extent and specificity by which R7W-MP binds to Ca_\text{\textbeta}2, we performed a BRET protein interaction assay between a Ca_\text{\textbeta}2-NanoLuc donor and increasing doses of R7W-MP or R7W-scramble peptide conjugated to the tetramethylrhodamine fluorophore (acceptor). This revealed binding of R7W-MP to Ca_\text{\textbeta}2 in a dose-dependent manner, a finding specific for the Ca_\text{\textbeta}2-SH3 domain and not the GK domain (Figure 2D). Dot blot analyses after Ca_\text{\textbeta}2 immunoprecipitation on lysates from cells transfected with Ca_\text{\textbeta}2 constructs and treated with R7W-peptides supported the above results (Figure II A in the online-only Data Supplement). Furthermore,
R7W-MP was found to colocalize with Ca\(\beta\)2 in adult cardiomyocytes (Figure 1B in the online-only Data Supplement). As expected, R7W-MP was shown to increase the half-life (Figure 2E) and total protein levels (Figure IIC in the online-only Data Supplement) of Ca\(\alpha\)1.2 in a dose-dependent manner. Replacement of either K161 in the TID sequence or its direct interacting residue in MP (E7) with glutamines (Q) ablated the protective effect of R7W-MP on Ca\(\alpha\)1.2 protein levels (Figure 2F) and half-life (Figure IID in the online-only Data Supplement), confirming the specificity of the TID-MP interaction. R7W-MP, but not R7W-scramble, was found to efficiently preserve \(I_{\text{Ca,L}}\) (Figure 2G) without affecting the steady-state voltage dependency of the channel under serum free conditions (data not shown).

**R7W-MP Prevents Dynamin From Binding to Ca\(\beta\)2 and Protects Ca\(\alpha\)1.2 From Protein Degradation**

The Ca\(\beta\)2-SH3 domain was previously shown to downregulate LTCC density at the plasma membrane through interaction with dynamin, a GTPase involved in endocytosis and vesiculation.\(^{22}\) Furthermore, a dynamin-specific inhibitor was found to protect Ca\(\alpha\)1.2 from protein degradation\(^{22}\) and to extend its half-life (data not shown). This led us to question whether R7W-MP might compete with dynamin for binding to the Ca\(\beta\)2-SH3 domain, thereby enhancing the Ca\(\alpha\)1.2 half-life. To test this hypothesis, we performed a BRET assay that revealed that R7W-MP indeed prevents dynamin from binding to Ca\(\beta\)2 (Figure 3A). In addition, we demonstrated that Ca\(\beta\)2 dissociates from Ca\(\alpha\)1.2 when bound to dynamin, whereas R7W-MP recovered the interaction of Ca\(\alpha\)1.2 with its chaperone (Figure 3B). Finally, in a cell surface protein biotinylation assay, a significant reduction in biotinylated Ca\(\alpha\)1.2 (membrane fraction) was found in cells cotransfected with dynamin, an effect that was fully counteracted by R7W-MP (Figure 3C).

**R7W-MP Facilitates Ca\(\alpha\)1.2 Chaperoning to the Plasma Membrane by Preventing Kir/Gem Binding to Ca\(\beta\)2**

Kir/Gem, together with Rad, Rem, and Rem2, is a member of the RGK small GTP-binding protein family\(^{26}\) known to negatively affect the amount of LTCCs at the cell surface.\(^{27,28}\) In particular, overexpression of Kir/Gem in Xenopus oocytes was reported to compromise the association of Ca\(\beta\)3 with Ca\(\alpha\)1.2 and to prevent the trafficking of the channel to the plasma membrane by sequestering Ca\(\beta\)3 in intracellular compartments.\(^{27}\) From this finding and evidence that Kir/Gem binds to Ca\(\beta\)3 through a region within its SH3 domain,\(^{29}\) we hypothesized that R7W-MP promotes anterograde trafficking of Ca\(\alpha\)1.2 by preventing Kir/Gem from complexing with Ca\(\beta\)2. A BRET interaction assay confirmed the binding of Kir/Gem to Ca\(\beta\)2, which was compromised by R7W-MP (Figure 4A). In addition, the association of Ca\(\beta\)2 with Ca\(\alpha\)1.2, which was drastically affected by the presence of Kir/Gem, was gradually recovered by R7W-MP (Figure 4B). The above results were supported by a surface protein biotinylation assay showing that R7W-MP treatment is sufficient to prevent the Kir/Gem-mediated reduction of Ca\(\alpha\)1.2 at the plasma membrane (Figure 4C).
that, when bound to Kir/Gem, cytoplasmic Caβ2 relocates to the nucleus, where it affects the transcription of a specific set of genes through recruitment of HP1γ. As predicted, overexpression of Kir/Gem in HL-1 cardiac cells resulted in the translocation of Caβ2 from the cytosol/plasma membrane to the nucleus, where it was found to colocalize with HP1γ (Figure 5A). The specific interaction between Caβ2 and HP1γ was confirmed by BRET (Figure 5B) and coimmunoprecipitation (Figure 5C) assays, whereas R7W-MP prevented the interaction (Figure 5B and 5C). In contrast, Caβ2 did not interact with bromodomain 4, an epigenetic factor that facilitates transcriptional activation (Figure III in the online-only Data Supplement). Taken together, these data point to a specific interaction between the nucleus-relocalized Caβ2 and HP1γ that is potentially required for the regulation of specific genes.

In muscle progenitor cells, Caβ1 was recently shown to translocate to the nucleus and to regulate gene expression by recognizing noncanonical heptameric sites at the promoter region of a number of genes. To determine whether nuclear Caβ2 might play a similar role and be part of a negative feedback loop in cardiac cells by regulating genes encoding LTCC subunits, we performed promoter analysis that identified 3 and 1 noncanonical sequences in the promoter region of Cacna1c (encoding Caα1.2) and Cacnb2 (encoding Caβ2), respectively (Figure 5F and data not shown). Consistently, Caα1.2 RNA levels were found to be reduced in Kir/Gem-transfected HL-1 cells (causing translocation of Caβ2 to the nucleus), whereas Caα1.2 expression was recovered by R7W-MP treatment (Figure 5D). In contrast, Caβ2 expression was unaltered under all analyzed conditions (data not shown). Cotransfection with HP1γ-specific RNAi (Figure IV in the online-only Data Supplement) prevented the reduction in Caα1.2 RNA levels (Figure 5E), demonstrating that the ability of nuclear Caβ2 to repress LTCC pore unit expression is dependent on its interaction with HP1γ. Consistent with a direct role of nuclear Caβ2 in regulating Cacna1c gene expression, chromatin immunoprecipitation with Caβ2 antibody, followed by quantitative polymerase chain reaction analysis for the above promoter sequences revealed enrichment for the NC1 site at the Cacna1c promoter, which was prevented by R7W-MP treatment (Figure 5F). Furthermore, a luciferase-based promoter assay confirmed the negative regulatory effect of nuclear Caβ2 on Cacna1 gene expression (Figure 5G), supporting the idea that relocated Caβ2 contributes to adjusting channel density in cells via a negative feedback loop.

**R7W-MP Restores Cardiac Function In Vivo Through Modulation of LTCC**

Our next objective was to explore the use of R7W-MP for potential cardiac treatments in vivo. After dot blot...
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analysis confirming the delivery of administered R7W-MP to the heart, the effect of R7W-MP on cardiac function was monitored (Figure VA in the online-only Data Supplement). In vivo echocardiographic analyses revealed no differences between groups (Table I in the online-only Data Supplement), and Cavα1.2 protein levels were unchanged (Figure VB in the online-only Data Supplement). Furthermore, electrophysiologic properties of isolated adult cardiomyocytes were unaffected (Figure VC–VF in the online-only Data Supplement), and in vivo epicardial multiple lead recording showed no significant changes in cardiac excitability and refractoriness, pointing toward a low index of proarrhythmogenicity (Figure VG in the online-only Data Supplement).

To investigate the potential therapeutic application of R7W-MP, we assessed its effect in a mouse model of streptozotocin-induced diabetes mellitus in which alteration of cardiac contractility and reduced LTCC density are secondary consequences of the disease10,35–39 (Figure 6A and 6B and Tables II and III in the online-only Data Supplement). Notably, R7W-MP treatment normalized ventricular systolic function and restored Cavα1.2 protein levels in diabetic mice (Figure 6A and 6B and Table III in the online-only Data Supplement). In addition, functional analyses
of cardiomyocytes from the same mice revealed restoration of cell contractility, systolic Ca²⁺ amplitude, and \( I_{Ca,L} \) (Figure 6C and 6D), as well as normalization of \( I_{Ca,L} \) inactivation kinetics (Figure VI in the online-only Data Supplement), in R7W-MP treated mice. These results show the ability of R7W-MP to reduce LTCC turnover and to correct myocardial dysfunction associated with diabetes mellitus, a secondary complication of diabetes mellitus.

On the basis of these outcomes, we explored the potential translatability of our results and retrospectively analyzed left ventricular biopsies from diabetic and nondiabetic cardiomyopathy patients with aortic stenosis undergoing aortic valve replacement. Interestingly, we found a direct linear correlation between the total amount of Cavα₁.2 and the Akt-dependent phosphorylation status of Cavβ₂ (Figure 7).

**DISCUSSION**

The Cavβ₂ LTCC subunit is known to dictate the appropriate chaperoning of Cavα₁.2 to and density at the...
cell surface, which is necessary for initiating Ca\textsuperscript{2+} influx and thus proper excitation-contraction coupling in the heart. Under stress conditions, the SH3-dependent scaffolding properties of Ca\textsubscript{\beta}2 are altered, thus affecting the assembly, intracellular mobility, and function of the LTCC multiprotein complex. Here, we provide new insights into Ca\textsuperscript{2+} channel physiology and demonstrate that the density of the pore unit is regulated by molecular reorganization of Ca\textsubscript{\beta}2, which is initiated by Akt-mediated phosphorylation of its C-terminal tail, allowing it to bind to the central SH3 domain. From this finding, we generated an MP that, by targeting the binding region (TID) within the SH3 domain of Ca\textsubscript{\beta}2, increases Ca\textsubscript{\alpha}1.2 density at the plasma membrane. Mechanistically, our results point toward a complex regulatory system that involves multiple levels of regulation (Figure 8): reduces Ca\textsubscript{\alpha}1.2 retrograde trafficking and protein degradation by preventing dynamin-mediated LTCC endocytosis; promotes Ca\textsubscript{\alpha}1.2 anterograde trafficking by blocking Kir/Gem-dependent sequestration of Ca\textsubscript{\beta}2, thus facilitating the chaperoning of Ca\textsubscript{\alpha}1.2; and promotes Ca\textsubscript{\alpha}1.2 transcription by preventing Kir/Gem-mediated shuttling of Ca\textsubscript{\beta}2 to the nucleus, where it limits the transcription of Ca\textsubscript{\alpha}1.2 through recruitment of the HP1\textgamma epigenetic repressor to the Cacna1c promoter. This last mechanism shows that Ca\textsubscript{\beta}2 plays a critical role in a previously unknown negative feedback pathway controlling the highly regulated expression of LTCC. Future work should address whether nuclear Ca\textsubscript{\beta}2 might be involved in the modulation of a family of genes encoding proteins associated with the regulation of Ca\textsuperscript{2+} handling, sarcomeric assembly, and Ca\textsuperscript{2+} sensitivity of myofilaments, thereby affecting contractility.\textsuperscript{34,40–42}

Our results provide the basis for the development of a potential novel therapy for the treatment of car-

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**Figure 7.** Phosphorylation status of Ca\textsubscript{\beta}2 at the Akt consensus site correlates with Ca\textsubscript{\alpha}1.2 levels in the myocardium of patients with diabetic and nondiabetic cardiomyopathy associated with aortic stenosis. Densitometry (top) and Western blot analysis (bottom) on total protein lysates from left ventricular biopsies. A significant correlation was observed (Pearson correlation coefficient). Protein levels were normalized to GAPDH. AU indicates arbitrary units.

**Figure 8.** Working model. Mimetic peptide binds to the Src homology 3 (SH3) domain of Ca\textsubscript{\beta}2 subunit and facilitates L-type Ca\textsuperscript{2+} channel density at the plasma membrane by promoting forward trafficking, reducing reverse trafficking, and inducing gene expression of Ca\textsubscript{\alpha}1.2 subunit. GK indicates guanylate kinase-like; and HP1\textgamma, heterochromatin protein 1\textgamma.
diabetic disorders associated with reduced LTCC density. The MP generated in this study falls in the class of positive Ca\(^{2+}\) modulators, which has received considerable attention in the past decades. However, although remarkably efficient at enhancing Ca\(^{2+}\) current and inotropism in systolic heart failure, this class of compounds was also found to favor arrhythmogene-
sis, thereby limiting their clinical use. For instance, the deleterious effects of the LTCC-activator BAYK8644 was shown to be due at least partly to unwanted modification of gating kinetics of the LTCC pore and Ca\(^{2+}\)-independent effects on ryanodine receptor gating, increasing resting SR Ca\(^{2+}\) release.\(^{43}\) In contrast, the MP described here acts unconventionally by direct chaperone-mediated modulation of LTCC density and, via its direct action on the life cycle of the channel, restores physiologic levels of LTCC only when its density at the plasma membrane is reduced. Additionally, we found that P-Ca\(_{\beta}2\) levels, which, as expected, were reduced in diabetic mice, were normalized to control levels after MP treatment. This leads to the intriguing hypothesis that, by binding to Ca\(_{\beta}2\), MP might facilitate the proximity or availability of the LTCC chaperone for the active Akt at the plasma membrane. If this is the case, in addition to its effect on Ca\(_{\alpha1.2}\) density at the plasma membrane, MP stimulates the "physiologic" Akt-dependent stabilizing effect on LTCC density and function,\(^{24}\) thereby initiating a possible forward mechanism. More dedicated and in-depth studies are required to address this possibility. Although an MP-based therapy might be envisioned, as also supported by the striking linear correlation between phosphorylated Ca\(_{\beta2}\) and Ca\(_{\alpha1.2}\) density in our preliminary retrospective studies of human patient biopsies, further compound optimization, toxicity studies, and largerscale prospective analyses are required. For instance, the detrimental effects previously observed with supraphysiologic levels of Ca\(_{\beta2}\) and Ca\(_{\alpha1.2}\)\(^{44-48}\) raise the question of whether MP administration may cause similar issues. However, although further and more exhaustive studies are required (ie, short- to long-term pharmacokinetic analysis), it is promising that MP administration did not result in any abnormal increase in Ca\(_{\alpha1.2}\) density or I\(_{\text{CaL}}\). Additionally, MP did not affect Ca\(_{\beta2}\) protein levels in either stressed or unstressed conditions. Another issue is the broad tissue distribution of Ca\(_{\beta2}\), necessitating careful re-evaluation of the nature and type of carrier to use because the current R7W carrier is not specific to the heart and thus may also have potential effects at other locations such as the vasculature or the sympathetic nervous system. In this regard, the recent development of novel drug carriers (ie, cell-specific aptamers\(^{49}\) or functionalized nanoparticles\(^{50}\)) might allow specific delivery to the heart and thus minimization of short- or long-term secondary side effects.

CONCLUSION
This study identifies new regulatory mechanisms controlling the LTCC life cycle and provides proof of concept for a novel therapy for the treatment of cardiac conditions associated with reduced LTCC density.

ACKNOWLEDGMENTS
We thank A. Rodano and C. Ronchi for their expertise with animal models and electrophysiologic analysis, respectively; M.V.G. Latronico for graphic preparation; and Dr Emilio Macchi for the helpful discussion about the in vivo electrophysiologic data. HL-1 cells were kindly provided by Dr W. Claycomb; Kir/Gem cDNA by Drs P. Bèguin and S. Seino; and dynamin cDNA by Drs R. Bonecchi and M. Locati.

SOURCES OF FUNDING
This work was supported by the Cariplo Foundation (2008.2504) and the Italian Ministry of Health (GR-2011-02352546) (DC). Dr Rusconi is supported by the Fondazione Umberto Veronesi Young Investigator Program 2011 to 2012.

DISCLOSURES
None.

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FOOTNOTES
Received January 19, 2016; accepted June 27, 2016.

The online-only Data Supplement is available with this article at http://circ.ahajournals.org/lookup/suppl/doi:10.1161/CIRCULATIONAHA.116.021347/-/DC1.

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Peptidomimetic Targeting of CaVβ2 Overcomes Dysregulation of the L-Type Calcium Channel Density and Recovers Cardiac Function
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_Circulation_. 2016;134:534-546; originally published online August 2, 2016; doi: 10.1161/CIRCULATIONAHA.116.021347

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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SUPPLEMENTAL MATERIAL

Peptidomimetic Targeting of Caβ2 Overcomes Dysregulation of the L-Type Calcium Channel Density and Recovers Cardiac Function

Rusconi, Mimetic peptide controls the LTCC life cycle

Francesca Rusconi, PhD* 1,2; Paola Ceriotti, BsC* 1; Michele Miragoli, PhD 1,5; Pierluigi Carullo, BsC 1,2; Nicolò Salvarani, PhD 1,2; Marcella Rocchetti, PhD 3; Elisa Di Pasquale, PhD 1,2; Stefano Rossi, PhD 4; Maddalena Tessari, MSc, PhD 6; Silvia Caprari, PhD 7; Magali Cazade, PhD 8; Paolo Kunderfranco, PhD 1; Jean Chemin, PhD 8; Marie-Louise Bang, PhD 1,2; Fabio Polticelli, PhD 7,9; Antonio Zaza, PhD 3; Giuseppe Faggian, MD 6; Gianluigi Condorelli, MD, PhD 1; and Daniele Catalucci, PhD 1,2.

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**DNA constructs and peptides**

Yeast two-hybrid bait constructs were generated in the pGBKT7 vector (Clontech), whereas the mouse adult heart cDNA library was generated using the “Make Your Own “Mate & Plate Library System, (Clontech). For the NanoLuciferase assay, Ca\(\alpha\)1.2 cDNAs were cloned into the pNLF1-N vector. For the BRET Assay, Ca\(\alpha\)1.2, Ca\(\alpha\)\(\beta\)2, Ca\(\alpha\)\(\beta\)2-SH3, Ca\(\alpha\)\(\beta\)2-GK, Dynamin, and Kir/Gem cDNAs were cloned into the pNLF1-N vector, while Ca\(\alpha\)\(\beta\)2cDNA was cloned into the HaloTag- pFN21A vector. All vectors for NanoLuciferase and BRET assays were obtained from Promega. The Ca\(\alpha\)\(\beta\)2-DsRed overexpression plasmid was constructed by inserting the Ca\(\alpha\)\(\beta\)2cDNA into the pDsRed-Express Vector (Clontech). To generate the cacna1c luciferase promoter plasmid, the region corresponding to -1 to -2456 from chromosome 6 was synthetized by Genscript (USA) and cloned into the pGL3-Enhancer vector (Promega). Deletion of the E-box 1 was performed. All cloning steps were performed using the In-fusion HD Cloning Plus kit (Clontech). Site-directed mutagenesis or deletions were performed using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene) following the manufacturer’s instructions and confirmed by DNA sequencing. Peptides were synthetized by GenScript (USA).

**Yeast two-hybrid assay**

Full-length Ca\(\alpha\)\(\beta\)2-WT and Ca\(\alpha\)\(\beta\)2-SE cDNA cloned into the pGBKT7 vector were used as baits for screening of a Mate & Plate human adult heart cDNA library and a custom made Mate & Plate mouse adult heart cDNA library (using the Matchmaker Gold Yeast Two-Hybrid System (Clontech) according to the manufacturer’s instructions.
Cell culture conditions and transfection

HEK293 cells were cultured in DMEM (Sigma) with 10% HI-FBS (Life Technologies), 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Euroclone). HL-1 cells were cultured in Claycomb medium (Sigma) with 10% FBS (Sigma), 100 U/ml penicillin, 0.1 mg/ml streptomycin (Euroclone), 1% Ultraglutamine 1 (Lonza), and 0.1M Norepinephrine (Sigma) in a gelatin/fibronectin pre-coated flask. Transfection of plasmids was performed in serum free medium (Opti-MEM I reduced-serum medium, Life Technologies) using Lipofectamine 2000 (Life Technologies). Viafect transfection reagent (Promega) was used for all Nanoluciferase and BRET assays. Peptides (without the R7W cell penetrating sequence) were transfected with the DeliverX Peptide transfection kit (Panomics) according to the manufacturer’s instructions. 48 h after transfection, cells were collected and analyzed. Adult cardiomyocytes were isolated as previously described.1-3

siRNA

A pool of four different HP1γ siRNAs and negative control siRNA (Qiagen) were transfected in HL1 cells using Lipofectamine 2000 (Life Technologies) according to the manufacturer’s instructions. 48 h after transfection, gene expression was evaluated in total RNA.

Immunoprecipitation

HEK293 and HL1 transfected cells were lysed in buffer containing 10 mM Tris-HCl pH 7.2, 150 mM NaCl, 30 mM Na4P2O7, 50 mM NaF, 0.1 mM Na3VO4, 1% Triton-X, 5 μM ZnCl2, 0.1 mM PMSF, and Protease Inhibitors 1X (Thermo Fisher Scientific). Extracts were subjected to immunoprecipitation using appropriate antibodies (2-4 ug antibodies) and
immune-complexes were captured with Protein A sepharose beads (GE-Healthcare) or Protein G Dynabeads (Life technologies). After extensive washing, proteins were resolved with SDS-PAGE and subjected to Western blot analysis.

**Western and dot blot analyses**

Protein expression was evaluated in total lysates by Western and dot blot analyses according to standard procedures. Samples obtained from transfected cells or mouse left ventricle were homogenized in RIPA buffer (150 mM NaCl, 10 mM Tris pH 7.2, 0.1 % SDS, 1% Triton-X100, 5 mM EDTA, 100 μM Na3VO4, 10 mM NaF, and Protease inhibitor 1X (Thermo Fisher Scientific)), loaded onto a 4-12% or 10-20% NuPAGE Tris-Glycine Gel, or 3-8% NuPAGE Tris-Acetate Gel (Life Technologies), separated by electrophoresis, and transferred to a PVDF membrane (Millipore). Nitrocellulose membrane was used for the dot blot assay. Antibodies against the following proteins were used: CaVα1.2 CACNA1, CaVβ2 CACNB2 (S8b-1), HA, and Transferrin Receptor from Abcam; c-myc (9E10) from Santa Cruz; Heterochromatin Protein-1 gamma (42S2) from Millipore; GAPDH (14C10) from Cell Signaling Technology; Polyarginine 9R from Cell Applications; Goat anti-mouse-HRP and Goat anti-rabbit-HRP from Thermo Fisher Scientific. A rabbit polyclonal antibody against the phosphorylated Akt consensus site of CaVβ2 was generated by GenScript (USA). ECL (Millipore) was used for protein detection using a GBox iChemi System (Syngene) or Chemidoc MP Imaging System (Biorad). Image J software (National Institutes of Health) was used for densitometry analysis.

**Cell fractionation**
Protein lysates from nuclear and cytosolic fractions were obtained using the Protein Isolation System (Life Technologies), according to the manufacturer’s instructions.

**Cell Surface Biotinylation**

The assay was performed as described elsewhere. Briefly, transfected HEK293 cells were cooled to 4°C, washed in ice-cold PBS, and biotinylated for 20 minutes with 0.5 mg/mL EZ Link Sulfo-NHS-LC-Biotin (Thermo Scientific) at 4°C. After extensive washing, cells were lysed in RIPA buffer and incubated with 50 µL High Capacity NeutrAvidin agarose beads (Thermo Scientific) for 2 hours at 4°C. NeutrAvidin beads were washed, after which Western blot analysis was performed.

**Nano Luciferase Assay**

The NanoLuc Luciferase assay was performed as described by the manufacturer (Promega). Briefly, HEK293 cells pretreated for 30 min with 20 µM cycloheximide were transfected as described in the result section. Time-course analysis (NanoLuc Luciferase activity from 0.5 to 8 hours post treatment) was performed using a Synergy 4 instrument (BioTek) as described by the manufacturer (Promega). Results were analyzed using Prism 6.0 software (GraphPad Software, CA).

**Nano-BRET Assay**

The NanoBRET Assay was performed as described by the manufacturer (Promega). Briefly, for protein-protein interaction assays, HEK293 transfected cells were treated with 100 nM NanoBRET 618 Ligand (Promega) and signals were detected five hours after treatment. For
the peptide-protein binding assay (Promega), HEK293 transfected cells were treated with different concentrations of tetramethylrhodamine-peptides and signals were detected five hours post treatment. Signals were detected using a Synergy 4 instrument (BioTek) and results were analyzed using Prism 6.0 software (GraphPad Software, CA).

**Promoter Assay**

Consensus sequences for the noncanonical heptameric E-box (CANNNTG)\(^5\) were computationally identified within the \textit{cacna1c} and \textit{cacnab2} promoters (-4Kb + 1Kb). HEK293 cells were transfected as described in the result section and the luciferase signal was detected 24 hours later with a Synergy 4 instrument (BioTek) as described by manufacturer (Promega). Results were analyzed using Prism 6.0 software (GraphPad Software, CA).

**Chromatin immunoprecipitation (ChIP)**

ChIP was performed as described elsewhere.\(^6\) Briefly, 5x10\(^6\) transfected/treated cells were cross-linked for 10 minutes at room temperature using 1% formaldehyde. Crosslinking was quenched by addition of glycine to a final concentration of 0.125 M. Cells were then collected, resuspended in lysis buffer (5 mM PIPES pH 8, 85 mM KCl, 0.5% NP40 and protease inhibitors), and incubated on ice for 15 min. Samples were sonicated to generate 200–400 bp fragments and the efficiency of sonication was assessed by agarose gel electrophoresis. Chromatin samples were pre-cleared for 1 hour with protein-G beads (Life technologies) and then immunoprecipitated overnight at 4°C with the following specific antibodies: anti-CACNB2 (S8b-1) and rabbit IgG (Millipore-Upstate). After incubation, the immunocomplexes were bound to protein-G beads for 2 hours and subsequently washed with low-salt wash buffer (0.1% SDS, 2 mM EDTA, 20 mM Tris HCl pH8, 1% Triton X-100, 150
mM NaCl, and protease inhibitors), high-salt wash buffer (0.1% SDS, 2 mM EDTA, 20 mM Tris HCl pH8, 1% Triton x-100, 500 mM NaCl, and protease inhibitors), and TE buffer. Immunocomplexes were eluted in elution buffer (1% SDS, 100 mM NaHCO3) and cross-linking reverted overnight at 65°C. Samples were then treated with proteinase K, extracted with phenol/chloroform, and precipitated with ethanol. Purified DNA was evaluated by quantitative PCR (qPCR) using SYBR green PCR master mix (Applied Biosystem). The following primers inside the Cacna promoter were used: control Fw (GCTCCCTCTACATCCTGCTCT), control Rev (GCATAACCACGTTGGCAAAA); E-Box1 Fw (CCTGCTTCCCTGCTCTAC), E-Box1 Rev (GGTTCAGGCGTGAGARGAG); E-Box2 Fw (GCAATCCCAGCTGAGCCATTAC); E-Box3 Fw (GGTCAGAGCTATGCGGAGAC), E-Box3 Rev (AGGCCTATCACCCTCCAC). Values obtained were normalized to the input.

RNA isolation, qPCR and gene expression analysis

Total RNA was isolated using the RNeasy Mini Kit (Qiagen) and treated with DNase I according to the manufacturer’s protocol. cDNA was generated using the Super Script VILO cDNA Synthesis Kit (Life Technologies) and amplified by quantitative real-time PCR (qRT-PCR) using SYBR Green PCR master mix (Applied Biosystems). Relative expression analysis was performed using the ∆∆Ct method by normalization to Gapdh. The following primers were used: Gapdh Fw (GGTCACCAGGGCTGCCATTTG); Gapdh Rev (TTCCAGAGGGCCATCCACAG); Cacna Fw (GCAGCAGCTTGTCTCTCTC), Cacna Rev (TGATCCAGCCCTTTGCTCTTC); Cbx3 (HP1γ) Fw (GAGAGATGCTGCTGACAAACC); Cbx3 (HP1γ) Rev (GGTCAGAGCGTGAGCTTAC); Cacna (HP1γ) Fw (GCTCCTCGTAGAAGGCAATG).
Modeling and docking

Structural models of the mimetic peptide were constructed using both the threading program I-TASSER\(^7\) and the *ab initio* modeling program Rosetta.\(^8\) Docking of the structural model of the mimetic peptide onto the three-dimensional structure of the Ca\(_{\beta2}\) functional core [PDB code: 1T3S\(^9\)] was performed using RosettaDock.\(^10\)

Ca\(^{2+}\) assays

Electrophysiological recordings in tsA-201, HEK293 cells, and adult CMCs were performed as previously described.\(^11\) Briefly, adult mouse ventricular myocytes were isolated and used within 6 h from isolation. The myocyte suspension was placed in a 30 mm Petri dish on the stage of an inverted microscope (Nikon Eclipse TE200, Tokyo, Japan). A thermostated manifold, allowing for fast (electronically timed) solution switch, was used for cell perfusion. Experiments were performed at 35.0 ± 0.5°C and the temperature was monitored at the pipette tip with a fast-response digital thermometer (Physitemp Instruments, Clifton, NJ). During measurements, transfected cells or myocytes were superfused at 2 ml/min with Tyrode solution containing 140 mM NaCl, 4 mM KCl, 2 mM CaCl\(_2\), 1 mM MgCl\(_2\), 5 mM HEPES-NaOH, and 10 mM D-glucose, pH 7.4. To measure L-type Ca\(^{2+}\) current (I\(_{\text{Ca,L}}\)), extracellular Na\(^+\) was replaced with equimolar TEA and K\(^+\) was omitted from the solution. The internal pipette solution contained 115 mM CsCl, 20 mM TEACl, 0.5 mM MgCl\(_2\), 10 mM EGTA, 5 mM HEPES, 0.4 mM GTP-tris salt, 5 mM ATP-Mg\(^{2+}\) salt, and 5 mM creatine phosphate-tris salt, pH 7.2. Cells were voltage-clamped in the whole-cell configuration (Multiclamp 700B, Axon Instruments). Membrane capacitance (C\(_m\)) and series resistance were measured in every cell and compensated to 90% before I\(_{\text{Ca,L}}\) measurements and the
estimated voltage error was <5 mV in all cases. Trace acquisition and analysis was controlled by dedicated software (Axon pClamp 10). \( I_{Ca,L} \) I/V relations were obtained by measuring activated current at different test potentials (300 ms; -50 mV to 80 mV) from a holding potential of -80 mV; steady state activation curves were derived from each I/V relation. Steady-state inactivation curves were obtained by measuring the current activated at 0 mV after preconditioning voltage steps (2 sec) from -50 to 40 mV and normalizing it to the maximal current. Activation and inactivation curves were described by fitting experimental points with the Boltzmann equation to estimate the voltages of half-maximal activation and inactivation (\( V_{0.5} \)) and each slope\(^1 \) factor (k). \( I_{Ca,L} \) inactivation kinetics were obtained by fitting the current activated at 0 mV with a biexponential function. \( V_m \) of single CMCs were determined in current clamp mode and membrane resistances of cells (\( R_m \)) were calculated from voltage changes in response to hyperpolarizing current steps (5 pA, 500 ms). Threshold action potentials (APs) were measured in CMCs by application of 3 ms long depolarizing current pulses. To take into account the variations in cell size, current amplitude were normalized to \( C_m \) in all measurements. Cell membrane capacitance was not affected in diabetic mice either in untreated mice or in the presence of MP or scramble.

\( Ca^{2+} \) fluorescent analyses were performed using the FLIPR Calcium 5 Assay kit (Molecular Devices) following the manufacturer’s instructions. Briefly, transfected HEK293 cells were seeded overnight in a 96 well plate at a density of 50.000 cells/well and incubated with Loading Buffer for 1 hour. Bay K8644 (Sigma Aldrich) at a final concentration of 1 \( \mu \)M was added as LTCC agonist during detection on a Synergy 2 instrument (BioTek) with the following fluorescence parameters: excitation wavelength: 485/20 nm, emission wavelength: 528/20 nm, and automatic emission cut-off: 510 nm. Results are expressed as intracellular \( Ca^{2+} \) and analyzed using Prism Software (GraphPad Software). Each experiment (n=8) is representative of a total of four independent FLIPR experiments.
Cardiomyocyte contractility and intracellular Ca\textsuperscript{2+} transient measurements

Measurements of cardiomyocyte contractility and Ca\textsuperscript{2+} transients were carried out as previously described\textsuperscript{11}. Briefly, cardiomyocytes, previously loaded with 1 μM of the Ca\textsuperscript{2+} probe Fura-2 AM (Life technologies), were placed in a perfusion system and continuously perfused with a standard Tyrode solution containing 1.2 mM Ca\textsuperscript{2+}. Loaded cells were field stimulated at increasing frequency as described in the result section and sarcomere length and Fura-2 ratio (measured at 512 nm upon excitation at 340 and 380 nm) were simultaneously recorded. Data were analyzed using Ion Wizard software (IonOptix Corp., Milton, USA).

Confocal Microscopy

Transfected HEK293 cells, HL1 cells, and isolated adult cardiomyocytes were blocked and permeabilized with 3% normal goat serum, 0.1% Triton X-100, 50 mM glycine, and 1% cold water fish gelatin in 1 x PBS for 1 hour. After incubation with primary and secondary antibodies, cells were scanned with an Olympus FluoView FV1000 confocal laser scanning microscope. Images were analyzed using Fiji Image J software (National Institutes of Health). Measurements of the relative increase in plasma membrane localization were performed as described elsewhere.\textsuperscript{12} Internalization was detected by calculation of cytoplasm fluorescence (Fc)/membrane fluorescence (Fm). The following antibodies were used: Ca\textsubscript{\alpha}1.2 CACNA1, Ca\textsubscript{\beta}2 CACNB2 (S8b-1), Heterochromatin Protein-1 gamma (42S2), and Polyaginine 9R (Cell Applications).

Human patients
Patients with aortic stenosis that underwent aortic valve replacement were recruited retrospectively from the biobank available at the University Hospital of Verona, Division of Cardiac Surgery, Verona, Italy. Approval for studies on human tissue samples was obtained from the Ethical Committee of the University Hospital of Verona. All patients or their relatives gave informed consent before operation. Non-diabetic cardiomyopathy patients with aortic stenosis: age = 74.4 ± 2.4 years; ejection fraction = 63.60 ± 7.70 (%); glycemia = normal. Diabetic cardiomyopathy patients with aortic stenosis: age = 78.0 ± 6.3; ejection fraction = 52.67 ± 11.37 (%); glycemia = 6.79 ± 1.45 (nmol/L).

Animals

All procedures on mice were performed according to institutional guidelines in compliance with national (D.L. N.26, 04/03/2014) and international law and policies (new directive 2010/63/EU). The protocol was approved by the Italian Ministry of Health. Special attention was paid to animal welfare and to minimize the number of animals used and their suffering. All experiments were performed on 12-week-old male mice with at least 8 animals per group. Diabetes was induced in adult C57B6/J male mice by intraperitoneal injection of streptozotocin (Stz, 50 mg/kg per day) for 5 days. 7 days after the last injection of Stz, whole blood was obtained from the mouse tail vein and glucose levels were measured using the Accu-check Active blood glucose monitoring system (Roche). Mice were considered diabetic and used for the study only if they had hyperglycemia. Citrate buffer–treated mice were used as non diabetic controls. After Stz treatment, mice were treated with peptides (2.5 mg/kg/day injected intraperitoneally) as described in the Result section.

Echocardiography
A Vevo 2100 high-resolution *in vivo* imaging system (VisualSonics Fujifilm) with a MS550S probe "high frame" scanhead was used for echocardiographic analysis. Mice were anesthetized with 1.0% isoflurane and imaged in the M-mode.

**In vivo epicardial potential mapping**

*In vivo* epicardial potential mapping was performed as previously described.13 Briefly, 8-week-old C57BL6/J male mice (Charles River, IT) were anesthetized by intraperitoneal injection of a mixture of ketamine (50 mg/kg) and xylazine (10 mg/kg), and ventilated at 100 cycles per minute. Subsequently, the heart was exposed through longitudinal sternotomy. To determine cardiac excitability and refractoriness as measures of susceptibility to arrhythmias, an epicardial electrode (EE) array (5×5 with a 0.6 mm resolution square mesh) was used to record unipolar epicardial electrograms during sinus rhythm and ventricular pacing. As a measure of cardiac excitability, the threshold for activation at 1 ms duration was obtained using 15 selected electrodes of the EE array; refractoriness was obtained by delivery of ten baseline stimuli (S1; 1 ms width and twice diastolic threshold intensity) with a frequency slightly higher than the basal cycle length using 5 selected electrodes of the EE array. The S1 pacing sequence was followed by an extra-stimulus (S2, four-fold the S1 intensity), whose delay from the previous S1 was first progressively decremented by 10 ms steps until capture was lost and then progressively augmented by 2 ms steps till capture was resumed. The effective refractory period (ERP), which was defined as the shortest S1–S2 time interval at which excitation from S2 failed, and the spatial dispersion of the ERP, measured as the maximum difference (range) and the standard deviation (SD) of the mean, were considered.

**Supplemental tables**
Table I. Echocardiographic analysis of 10-weeks-old male WT mice treated with R7W-scr and R7W-MP.

<table>
<thead>
<tr>
<th>Weight</th>
<th>IVSd</th>
<th>LVIDd</th>
<th>LVPWd</th>
<th>IVSs</th>
<th>LVIDs</th>
<th>LVPWs</th>
<th>FS</th>
<th>EF</th>
<th>HR</th>
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<td>(g)</td>
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**Basal**

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<td>Ctl</td>
<td>24.1±1.7</td>
<td>0.77±0.07</td>
<td>3.29±0.13</td>
<td>0.75±0.07</td>
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<td>1.90±0.10</td>
<td>1.19±0.05</td>
<td>42.17±1.73</td>
<td>74.39±1.93</td>
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<tr>
<td>R7W-scr</td>
<td>21.9±0.8</td>
<td>0.82±0.05</td>
<td>3.13±0.11</td>
<td>0.76±0.06</td>
<td>1.23±0.09</td>
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<td>42.41±1.39</td>
<td>74.83±1.62</td>
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<td>R7W-MP</td>
<td>21.9±1.1</td>
<td>0.77±0.07</td>
<td>3.29±0.13</td>
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<td>1.90±0.10</td>
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</table>

**4 days post treatment**

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<tr>
<td>R7W-scr</td>
<td>23.4±1.5</td>
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<tr>
<td>R7W-MP</td>
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<td>1.19±0.03</td>
<td>42.63±2.13</td>
<td>74.99±2.38</td>
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IVSd, diastolic septal wall thickness; LVIDd, left ventricular end-diastolic diameter; LVPWd, left ventricular posterior wall thickness in diastole; IVSs, systolic septal wall thickness; LVIDs, left ventricular end-systolic diameter; LVPWs, left ventricular posterior wall thickness in systole; FS, left ventricular fractional shortening; HR, heart rate. (n=6-8). No statistical significances were found.
<table>
<thead>
<tr>
<th></th>
<th>LV</th>
<th>RV</th>
<th>Atria</th>
<th>Tibia</th>
<th>Glycemia</th>
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<tr>
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<td>(g)</td>
<td>(g)</td>
<td>(g)</td>
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<td>(mg/dl)</td>
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<tr>
<td>Ctl</td>
<td>0.077±0.012</td>
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<td>0.007±0.001</td>
<td>2.2</td>
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<td>Vehicle</td>
<td>0.061±0.006</td>
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<td>0.012±0.005</td>
<td>2.2</td>
<td>&gt;300*</td>
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<tr>
<td>R7W-scr</td>
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<td>&gt;300*</td>
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<td>R7W-MP</td>
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<td>0.018±0.003</td>
<td>0.006±0.002</td>
<td>2.2</td>
<td>&gt;300*</td>
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LV, left ventricle; RV, right ventricle. (n=10) * P<0.05 vs Ctl.
### Table III. Echocardiographic analysis of control and DCM mice not treated or treated with vehicle, R7W-scr, and R7W-MP.

<table>
<thead>
<tr>
<th></th>
<th>Weight (g)</th>
<th>IVSd (mm)</th>
<th>LVIDd (mm)</th>
<th>LVPWd (mm)</th>
<th>IVSs (mm)</th>
<th>LVIDs (mm)</th>
<th>LVPWs (mm)</th>
<th>FS (%)</th>
<th>EF (%)</th>
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<tbody>
<tr>
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<td>Ctl</td>
<td>25.2±0.5</td>
<td>0.67±0.01</td>
<td>3.59±0.04</td>
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<td>1.21±0.02</td>
<td>2.08±0.08</td>
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<td>42.39±1.74</td>
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<td>3.39±0.07</td>
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<td>1.24±0.02***‡‡</td>
<td>46.57±1.57****‡‡‡</td>
<td>78.66±1.89††***</td>
<td>520±63</td>
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IVSd, diastolic septal wall thickness; LVIDd, left ventricular end-diastolic diameter; LVPWd, left ventricular posterior wall thickness in diastole; IVSs, systolic septal wall thickness; LVIDs, left ventricular end-systolic diameter; LVPWs, left ventricular posterior wall thickness in systole; FS, left ventricular fractional shortening; HR, heart rate. Stz: streptozotocin. (n=10 per each group). * P<0.05, ** P<0.01, *** P<0.005 vs R7W-Scr; † P<0.05, †† P<0.01, ††† P<0.005, †††† P<0.001 vs Ctl; ‡ P<0.05, ‡‡ P<0.01, ‡‡‡ P<0.005 vs vehicle.
Figure I. Site-specific mutagenesis in the Ca,β2 TID binding site affects Ca,α1.2 protein levels. Western blot analysis for Ca,α1.2 in total protein lysates from HEK293 cells transfected as indicated. Representative experiments are shown (n = 4).
Figure II. Targeting of R7W-MP to Ca\(\beta\)2 and effects on Ca\(\alpha\)1.2. (A) Interaction between Ca\(\beta\)2 and R7W-MP or R7W-scr as examined by co-IP with the indicated antibodies, (n = 3). (B) Immunofluorescence stainings for R7W-MP or R7W-scramble (Red) and Ca\(\alpha\)1.2 or Ca\(\beta\)2 (Green) in adult cardiomyocytes. Bar = 10 μm, (n = 15). (C) Western blot analysis for Ca\(\alpha\)1.2 in total protein lysates from serum starved HEK293 cells transfected and treated as indicated. Representative experiments are shown (n = 4). Increasing doses of peptides were 0.16, 0.62, 1.3, 2.6, 5.2, and 10.4 μM. (D) Ca\(\alpha\)1.2 half-life measured by NanoLuc luciferase assay. HEK293 cells were transfected with Ca\(\alpha\)1.2-NanoLuc and either Ca\(\beta\)2-WT or Ca\(\beta\)2-K161Q mutant. (n = 6). No statistical significance was observed (Mann-Whitney test).
Figure III. No interaction between nuclear Ca,β2 and Bromodomain 4. Ca,β2-

Bromodomain 4 protein affinity was measured by BRET analysis in HEK293 cells transfected
with Ca,β2-NanoLuc and Bromodomain 4-Halo, and treated with increasing doses (0.12, 1.3,
and 10.2 μM) of R7W-MP and R7W-scr. (n = 6). No statistical significance was observed.
Figure IV. siRNA interference for HP1γ expression. qRT-PCR analysis for HP1γ expression in HL1 cells transfected as indicated (n = 12). All data are shown as mean ± SD; **, P<0.01, ***, P<0.001 (Mann–Whitney test).
Figure V. *In vivo* administration of R7W-MP in WT mice. (A) Tissue distribution of peptides as examined by dot blot analysis following co-IP with the indicated antibodies. Mice were intraperitoneally injected (2.5 mg/kg) with R7W-MP or R7W-scr once a day for 4 consecutive days (n = 4). (B) Western blot analysis for Ca\(\alpha\)1.2 in left ventricular homogenates from mice treated as in (A). No statistical significance was observed. (C) Single traces (top) and steady-state activation/inactivation curves (bottom) from isolated cardiomyocytes of mice treated as in (A). (n = 15 cells per mouse). No statistical significance was observed. (D) Electrophysiological passive properties of isolated cardiomyocytes from mice treated as in (A). No statistical
significance was observed for any of the electrophysiological parameters (n = 11-13 cells per mouse). (E) Action potential parameters of isolated cardiomyocytes from mice treated as in (A). No statistical significance was observed (n = 14 cells per mouse). (F) Strength-duration relations recorded in isolated cardiomyocytes from mice treated as in (A). No statistical significance was observed for action potential (AP) current thresholds at any duration of the stimulus between control and treated cardiomyocytes (n = 12-13). Rheobase (1.7 ± 0.4, 2.2 ± 1.6, and 1.5 ± 0.9 pA for control, R7W-scr, and R7W-MP, respectively) and Chronaxie (4.2 ± 0.5, 4.3 ± 0.7, and 4.5 ± 1.2 ms for control, R7W-scr, and R7W-MP, respectively) measured from corresponding strength duration curves are not statistically different. (G) Cardiac excitability (left) and refractoriness (right) from mice treated as in (A). No statistical significance was observed for the parameters, which are both indexes of pro-arrhythmogenicity.
Figure VI. Effect of MP on LTCC inactivation kinetics. Upper panels: Effect of Stz and its reversal by MP (but not by its scrambled form, scr) on the fast and slow components of $I_{CaL}$ inactivation. $\tau$ = time constant, $\tau_{0.5}$ = time at 50% inactivation, $A_{fast}/A_{slow}$ = weight ratio of kinetic components. Bottom panel: example of $I_{CaL}$ inactivation (black trace) fitted by the biexponential function (red trace).
Supplemental references


