No role for core Ca\textsubscript{v}1.2 phosphorylation

We developed a transgenic approach that enables doxycycline-inducible expression of Flag-tagged, dihydropyridine (DHP)-resistant Ca\textsubscript{v}1.2 channels in mice (Fig. 1b). The transgenic and endogenous Ca\textsubscript{v}1.2 currents are distinguishable by application of nisoldipine, a Ca\textsuperscript{2+}-channel DHP antagonist\textsuperscript{16}. We mutated all 51 conserved and nonconserved serine and threonine residues within the 35 intracellular PKA consensus phosphorylation sites of rabbit α\textsubscript{1C} to alanine ('35-mutant α\textsubscript{1C}'; Extended Data Fig. 1a). In cardiomyocytes, the nisoldipine-insensitive 35-mutant Ca\textsuperscript{2+} currents were both activated at more negative potentials and increased in response to isoproterenol (a β-adrenoreceptor agonist) or forskolin (which stimulates adenylyl cyclase to produce cyclic AMP, thereby activating PKA), to the same extent as were nisoldipine-insensitive wild-type channels ('pseudo-wild-type (pWT) α\textsubscript{1C} channels') (Fig. 1c, d and Extended Data Fig. 1b, c).

Similarly, we mutated to alanine all 37 conserved and nonconserved serine and threonine residues within 28 PKA-consensus phosphorylation sites of human β\textsubscript{2B} ('28-mutant β\textsubscript{2B}'; Extended Data Fig. 1d). Cardiomyocytes expressing green fluorescent protein (GFP)-tagged 28-mutant β\textsubscript{2B} (Extended Data Fig. 1e, f) displayed isoproterenol- or forskolin-induced stimulation of Ca\textsubscript{v}1.2 current amplitude (Fig. 1e, g) and a hyperpolarizing shift in the voltage dependence of activation (Extended Data Fig. 1b), similar to cardiomyocytes isolated from transgenic mice expressing GFP-tagged wild-type β\textsubscript{2B} (ref. 17).

Finally, we crossed 35-mutant α\textsubscript{1C} with 28-mutant β\textsubscript{2B} transgenic mice. Immunoprecipitation with anti-Flag antibody indicated that...
Identifying the CaV1.2 subdomain proteome

Given the foregoing results, we adapted for application to cardiomyocytes an enzyme-catalysed proximity labelling method5,6 in order to comprehensively identify components of the CaV1.2 macromolecular complex. We generated transgenic mice with doxycycline-inducible, cardiomyocyte-specific expression of DHP-resistant α1C or β2B proteins (Extended Data Fig. 3a). These were primarily classified as being membrane, cytoskeletal or sarcomeric proteins (Extended Data Fig. 3b, c and Supplementary Table 2). Some of these proteins, however, were associated with other compartments, probably reflecting the labelling of proteins encountered during CaV1.2–APEX2 synthesis, maturation and trafficking.

Adrenergic modulation of CaV1.2 neighbourhood

It seemed likely that PKA-dependent stimulation of Ca2+ currents in the heart involves recruitment of a distinct activator protein to, or loss of an inhibitory protein from, the CaV1.2 macromolecular complex. Recently, APEX2 labelling, combined with either multiplexed quantitative mass spectrometry7 or quantitative proteomics using a system of spatial references and bystander ratio calculations8, was used to analyse ligand-induced changes in the local environment of G-protein-coupled receptors. Notably, we found that conjugation of peroxidase to either the α1C or β2B subunits did not interfere with β-adrenergic stimulation of CaV1.2 subcellular localization and function in cardiomyocytes, as assessed using TMT SPS MS 3. The relative summed peptide TMT signal-to-noise ratio—indicating relative protein quantification—changed for 150 proteins with the highest peptide counts were remarkably similar in α1C–APEX2 and β2B–APEX2 transgenic mice (Extended Data Fig. 3a). These were primarily classified as being membrane, cytoskeletal or sarcomeric proteins (Extended Data Fig. 3b, c and Supplementary Table 2). Some of these proteins, however, were associated with other compartments, probably reflecting the labelling of proteins encountered during CaV1.2–APEX2 synthesis, maturation and trafficking.

Fig. 1 | Phosphorylation of α1C and β subunits by PKA is not required for β-adrenergic regulation of CaV1.2. a. Diagram showing rabbit cardiac α1C (top) and β (bottom) subunits. Red dots indicate putative sites of phosphorylation by PKA. G, guanylate kinase domain; SH3, Src homology 3 domain. b. Diagrams showing the binary transgene system that permits robust expression of Flag-DHP-resistant (DHP*) α1C or GFP-tagged β2B only in the presence of both a reverse tetracycline-controlled transactivator (rtTA) and doxycycline (Tet-ON). The top diagram shows expression of the rtTA driven by the cardiac-specific α-myosin heavy chain (α-MHC) promoter. The three non-coding exons that make up the 5′ untranslated region of the α-MHC gene are depicted as black boxes, and the introns as lines. The lower two diagrams show coding exons that make up the 5′-untranslated region of the α-MHC gene are depicted as black boxes, and the introns as lines. The lower two diagrams show coding exons that make up the 5′-untranslated region of the α-MHC gene are depicted as black boxes, and the introns as lines. The lower two diagrams show coding exons that make up the 5′-untranslated region of the α-MHC gene are depicted as black boxes, and the introns as lines. The lower two diagrams show coding exons that make up the 5′-untranslated region of the α-MHC gene are depicted as black boxes, and the introns as lines.

cDNAs for Flag–DHP–α1C or GFP–β2B ligated behind seven tandem tetO sequences, which impart tetracycline inducibility. c. Exemplar whole-cell CaV1.2 currents of 35-mutant α1C cardiomyocytes (from transgenic mice) in nisoldipine before (black trace) and after (blue trace) treatment with isoproterenol. Representative of 25 experiments; pAPf–1, picamperes per picofarad. d. Fold change in peak DHP-resistant Ca2+ current at 0 mV caused by isoproterenol or forskolin. Dots show data points. Data are mean ± s.e.m.; P = 0.39 by unpaired two-tailed t-test; n = 45 cardiomyocytes from 5 mice (isoproterenol); n = 25 cardiomyocytes from 5 mice (forskolin). e, f. Exemplar whole-cell CaV1.2 currents of cardiomyocytes from GFP-tagged 28-mutant β2B transgenic mice (e), and from 35-mutant α1C crossed with 28-mutant β2B transgenic mice (f). Representative of 8 and 22 independent experiments respectively. g. Fold change in peak Ca2+ current caused by isoproterenol or forskolin for cardiomyocytes isolated from transgenic mice expressing GFP-tagged wild-type (WT) β2B subunit7, GFP-tagged 28-mutant β2B, or both 35-mutant α1C and GFP-tagged 28-mutant β2B. Data are mean ± s.e.m.; P = 0.27 by one way ANOVA; n = 19, 8 and 21 cardiomyocytes from 4, 4 and 3 mice, from left to right.
on the Ca\textsubscript{1.2} proteome signature in Langendorff-perfused whole hearts (Fig. 2g, h). We detected isoproterenol-induced recruitment of the PKA catalytic subunit (PKA<sub>cat</sub>) to Ca\textsubscript{1.2} channels in cardiomyocytes isolated from both \(\alpha_{c}\)-APEX2 and \(\beta_{2}\)-APEX2 mice (Fig. 2e, f). All three approaches (using isolated cardiomyocytes from \(\alpha_{c}\)-APEX2 and \(\beta_{2}\)-APEX2 mice, and \(\alpha_{c}\)-APEX2 whole hearts) indicated a 30–50% decrease in the amount of the small Ras-like G protein Rad in the neighbourhood of Ca\textsubscript{1.2} following application of isoproterenol (Fig. 2e, f, h, Extended Data Figs. 4a–c, 5a, b and Supplementary Tables 3–5). By analysing the overlap of proteins displaying isoproterenol-induced changes in \(\alpha_{c}\)-APEX2 and \(\beta_{2}\)-APEX2 experiments, we found Rad to be the only candidate protein displaying this behaviour (Extended Data Fig. 5b, c). By contrast, a 10-minute exposure of cardiomyocytes isolated from nontransgenic mice to isoproterenol (Fig. 2i) had a minimal effect on proteins relatively quantified by TMT SPS MS\textsuperscript{5}, and specifically no substantial effect on the amount of Rad by comparison with untreated paired cardiomyocytes (Fig. 2j).

**PKA regulates Ca\textsubscript{1.2} via Rad phosphorylation**

The robust heterologous reconstitution of PKA regulation of Ca\textsubscript{1.2} currents has long been pursued as a crucial step in identifying and
 validating the mechanism. We now find that Rad was the missing ingredient. To prove this experimentally, we coexpressed Rad with α1C and β2B subunits in HEK293T cells, limiting Rad expression by using a 1/3 to 1/6 ratio of Rad/α1C,β2B complementary DNA to avoid complete inhibition of the Ca2+ current. We used a perforated, whole-cell patch clamp technique to preserve the normal intracellular milieu and signalling cascades, and to minimize current run-down. Ba2+ was used as a charge carrier to eliminate Ca2+-dependent inactivation. We found that Rad-transfected cells with only α1C plus β2B-superfusion of forskolin over 1–3 minutes had no impact on a Ba2+ current (Fig. 3a, b, g, h). By contrast, applying forskolin to cells expressing α1C, β2B and Rad increased the maximal conductance (Gmax) by as much as 4.5-fold and by a mean of 1.5-fold, and shifted the V50 for activation (Fig. 3c, d, g, h and Extended Data Fig. 6a, b). The forskolin-induced increase in current was inversely proportional to the basal current density, as observed in cardiomyocytes (Extended Data Figs. 1c, 6c). In HEK293T cells expressing 35-mutant α1C plus 28-mutant β2B plus Rad, applying forskolin increased Gmax by as much as 3.1-fold and by a mean of 1.9-fold, and caused a hyperpolarizing shift in the V50 for activation (Fig. 3i and Extended Data Fig. 6d, e). For both wild-type and phosphorylation-site-mutant α1C and β2B subunits, the forskolin-induced enhancement of Ba2+ current in Rad-transfected cells was greatest at hyperpolarized potentials and fell as the test potential approached the reversal potential (Extended Data Fig. 6f, g), consistent with observations in cardiomyocytes.

We used single-channel recordings to determine the mechanism of PKA/Rad modulation of CaV1.2. In the absence of Rad, sweeps with no openings or blank sweeps are rare, while most sweeps exhibit either intermediate or high levels of openings (Fig. 3j and Extended Data Fig. 6h, k). In HEK293T cells transfected with α1C plus β2B, cointreatment with forskolin and PKA failed to increase Gmax and hyperpolarizing shift in the current–voltage curve (Fig. 3i). We identified 14 consensus PKA phosphorylation sites in Rad (Extended Data Fig. 7a), and we mutated these sites to alanine. The mutant Rad effectively inhibited CaV1.2 currents; however, the cAMP/PKA-mediated upregulation of CaV1.2 current was lost (Fig. 3g). In lysates from forskolin-stimulated HEK293T cells transfected with GFP–Rad, we observed phosphorylation of Ser25, Ser38 and Ser300 by mass spectrometry (Extended Data Fig. 7b). These residues were previously identified in mouse hearts as phosphorylation targets (Extended Data Fig. 7c). We were unable to detect either unphosphorylated or phosphorylated peptides containing Ser272, notwithstanding prior biochemical studies identifying this residue as a PKA target. Alanine substitutions of Ser25, Ser38, Ser272 and Ser300 in Rad (4-SA mutant Rad) and/or coexpressed with Rad, the fraction of blank sweeps is reduced, the low-activity mode predominates, and the P0 is reduced (Fig. 3k, Extended Data Fig. 6i). By comparison, if the PKA cat subunit is coexpressed with Rad, the fraction of blank sweeps is reduced, the high-activity mode resurges, and the P0 is increased by 10.6 ± 2.9-fold compared with transfection without PKA (Fig. 3k and Extended Data Fig. 6j, l). These results suggest that Rad potently damps the CaV1.2 current, while phosphorylation of Rad allows channels to operate as though they were devoid of Rad.  

A C-terminal polybasic region of 32 amino acids in Rad is involved in plasma-membrane targeting via binding to negatively charged phospholipids such as phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2, also known as PIP2). Deletion of the C terminus of RGK GTPases prevents their inhibition of Ca2+-channel function. We found that alanine substitutions at Ser272 and Ser300 (2-SA mutant) within the C-terminal polybasic membrane region prevented both the forskolin-induced increase in Gmax and hyperpolarizing shift in the current–voltage curve (Fig. 3e, g, h). By contrast with transfection with wild-type Rad, cotransfection of PKA with 4-SA mutant Rad failed to increase P0, (PKA to no PKA, L15 ± 0.56-fold; Fig. 3i).

Role of Rad binding to CaV1.2 β subunits

Rad can inhibit CaV1.2 via β-dependent and β-independent (α1C-dependent) mechanisms. Substituting Rad residues Arg208 and Leu235, or β2B residues Asp244, Asp320 and Asp322, with alanine (Extended Data Fig. 8a, b) attenuates Rad binding to β subunits. These mutations prevented the forskolin-induced increase in Gmax, and the hyperpolarizing shift in the current–voltage curve (Fig. 4a and Extended Data Fig. 8c, d). Thus, the interaction between Rad and β subunits is essential for cAMP–PKA regulation of CaV1.2.
We used a flow-cytometry Förster resonance energy transfer (FRET) two-hybrid assay to probe potential PKA-mediated changes in the binding of \( \beta_{2n} \) subunits and wild-type Rad. At baseline, we detected robust binding between cerulean-tagged \( \beta_{2n} \) subunit and venus-tagged wildcard-type Rad, consistent with previous studies (with an effective dissociation constant \( K_{d,_{eff}} \) of 7,957 ± 418; Fig. 4b and Extended Data Fig. 9c). Coexpression of the PKA\( \alpha_{ct} \) subunit, however, markedly weakened this interaction (\( K_{d,_{eff}} = 4,349 ± 138 \) versus \( K_{d,_{eff}} = 4,346 ± 197 \)). By contrast, coexpression of the PKA\( \alpha_{ct} \) subunit in cells expressing fluorophore-tagged \( \beta_{2n} \) and 4-SA mutant Rad had no effect on FRET binding (\( K_{d,_{eff}} = 4,349 ± 138 \) versus \( K_{d,_{eff}} = 4,346 ± 197 \)). These results suggest that phosphorylation of Rad is required for dissociation of the Rad–\( \beta_{2n} \) interaction. In a similar manner, PKA phosphorylation of Rad also reduced FRET binding to both \( \beta_{i} \) and \( \beta_{p} \) (Extended Data Fig. 9a–c).

**Unified mechanism for PKA regulation**

In adrenal chromaffin cells and the sinus node cells of the heart, L-type Ca\( V_{1.2} \) channels are robustly stimulated by PKA\( ^{32,33} \). We found that, in HEK293T cells transfected with only Ca\( V_{1.3} \) or \( \alpha_{1D} \) plus \( \beta_{2B} \), superfusion of forskolin over 1–3 minutes had no impact on the \( \mathrm{Ba}^{2+} \) current (Fig. 4d–f). By contrast, in cells expressing \( \alpha_{1C}, \beta_{2n} \) and Rad, applying forskolin increased \( G_{\text{max}} \) by as much as 2.3-fold and by a mean of 1.9-fold, and shifted the \( V_{50} \) for activation (Fig. 4d–f). We also expressed the N-type Ca\( V_{2.2} \) \( \alpha_{2B} \) subunit, which is widely expressed in neurons, with \( \beta_{2B} \) and Rad in HEK293T cells. Forskolin increased \( G_{\text{max}} \) through Ca\( V_{2.2} \) when coexpressed with Rad by a mean of 2.2-fold and shifted the \( V_{50} \) for activation (Fig. 4g–i). For both Ca\( V_{1.3} \) and Ca\( V_{2.2} \), attenuating binding of Rad to \( \beta_{n} \) subunits prevented the forskolin-induced modulation of Ca\( V_{2.2} \) current (Extended Data Fig. 8e, f). We also expressed in HEK293T cells the Ca\( V_{2.2} \) \( \alpha_{2B} \) subunit with \( \beta_{2B} \) and Rem, another member of the RGK GTPase family. Forskolin increased \( G_{\text{max}} \) through Ca\( V_{2.2} \) when coexpressed with Rem by 1.6-fold and shifted the \( V_{50} \) for activation (Fig. 4g–i). Thus, PKA-mediated modulation of Ca\( V_{2.2} \) channels is not idiosyncratic, as currently believed; rather, it is emerging to be a universal mechanism transferable to all Ca\( V_{n} \) channels that bind \( \beta_{n} \) subunits.

**Discussion**

The core \( \alpha_{ct}, \beta_{2n} \) subunits, previously hypothesized to contain the PKA target sites required for \( \beta_{2n} \)-adrenergic agonist-induced stimulation of Ca\( V_{1.2} \), do not. Rather, successful reconstitution of regulation in a heterologous expression system requires an additional component, which we now identify as Rad. The cAMP–PKA-mediated regulation of Ca\( V_{1.2} \) requires both phosphorylation by PKA on the C terminus of Rad and the interaction of Rad with the \( \beta_{n} \) subunit. Multiple-alignment analysis of Rad from mice and other species shows that the four phosphorylation sites are conserved (Extended Data Fig. 10a). The required interaction with the \( \beta_{n} \) subunit is consistent with our recent finding that disrupting the \( \alpha_{ct}–\beta_{n} \) interaction prevents the regulation of Ca\( V_{1.2} \) by PKA in the heart\(^{11} \).

Analysis of Rad and other members of the RGK GTPase family indicates that their C-terminal phosphorylation sites are highly similar (Extended Data Fig. 10b). Short stretches of basic and hydrophobic amino acids are known to interact with the membrane\(^{23} \) and phosphorylation of residues within these stretches alters their electrostatic character, thereby reducing membrane affinity\(^{24} \). We found that phosphorylation of two serine residues within the C-terminal polybasic region of Rad releases Ca\( V_{n} \) channels from Rad-mediated inhibition, probably by means of reducing the affinity of Rad with the membrane and with the Ca\( V_{n} \) \( \beta_{n} \) subunit (Fig. 4j–k). This mechanism of regulation is modular and transferable, as Ca\( V_{1.3} \) channels and neuronal Ca\( V_{2.2} \) channels are also imparted with forskolin/PKA-mediated upregulation via Rad and Rem. The activation of Ca\( V_{n} \) channels via release of inhibition...
by PKA phosphorylation is reminiscent of PKA phosphorylation of phospholamban, an inhibitor of the sarcoplasmic reticulum Ca²⁺-ATPase.

Whereas phosphorylation of Ser1928 is definitively not required for β-adrenergic regulation of Ca₉.2 in the heart, substitution of this residue with alanine prevents β-adrenergic stimulation of Ca²⁺ channels in hippocampal neurons and hyperglycemia-induced stimulation of Ca²⁺ currents in arterial smooth muscle cells.4,15 Perhaps there are tissue-specific differences in Ca₉.2 regulation. Phosphorylation of the α₁C subunit may also affect the trafficking and clustering of channels in neurons and cardiomyocytes.4,16,17

Our results demonstrate that proximity labelling using APEX2 is feasible in the heart and, combined with multiplexed TMT proteomics, can identify a dynamically evolving network of interactions induced by β-adrenergic stimulation. This study establishes the utility of proximity labelling in animals and provides an important foundation for future studies that will investigate how diseases, such as heart failure, change the proteomic subdomain of the excitation–contraction coupling machinery.

Augmented Ca²⁺ entry enhances the opening of ryanodine receptors via Ca²⁺-induced Ca²⁺ release and increases cardiac contractility. Dysregulation of Ca₉.2 activity can result in cardiac arrhythmias, heart failure and sudden death. Supporting an important role of RGK GTPases in humans, a Rem2 variant has been identified as a genetic modifier in long QT syndrome 2 (ref. 8), and a Rad variant was recently linked to Brugada syndrome.11 Our results identify potential targets and interaction sites for the therapeutic modulation of β-adrenergic regulation of Ca²⁺ currents in the heart and other tissues. For instance, disrupting the interaction between Rad and β subunits can be inotropic by increasing Ca²⁺ entry in the heart. Conversely, interfering with the α₁C–β interaction, blocking PKA phosphorylation of Rad or potentially enhancing the interaction of Rad with the plasma membrane could attenuate the sympathetic nervous system activation of cardiac Ca²⁺ entry and inotropy more specifically than β-blockers.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41586-020-1947-2.
Methods

Clone construction and cell culture
All mouse N-terminally GFP-tagged Rad (GenBank accession number XM_006531206) constructs in a pEGFP-C1 vector were generated by gene synthesis (Gene Universal). Human Ca,2,2 (pSAD442-1) was a gift from D. Lipscombe (Addgene plasmid 62,574; http://n2t.net/addgene:62574; Resource Identification (RRID) number Addgene_62574). All cDNA clones were authenticated by sequencing.

HEK293T cells (American Type Culture Collection (ATCC), CRL-3216) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, and were transfected with 3 μg rabbit α, (accession number X15539), human α, (for Ca,2,2 experiments) or rat α, (for Ca,1,3 experiments, accession number AF3070009), 3 μg human β (NM_201590.3) and 0.5 μg N-terminal-GFP-tagged mouse Rad using Lipofectamine 2000 (Thermo Fisher Scientific). For the FRET experiments, rat β, (accession number NM_012828) and rat β, (accession number L02315) were used. The media was changed 4–6 h after transfection. The cells were split onto coverslips coated with attachment factor protein (Gibco). Electrophysiological recordings were carried out at room temperature 24–48 h after transfection. The cells were authenticated by ATCC and tested for mycoplasma contamination.

Generation of transgenic mice
The Institutional Animal Care and Use Committee at Columbia University approved all animal experiments. We used male and female mixed-strain mice at 6 weeks to 4 months of age. Sample sizes exceeded the number of samples determined by power calculations, which were based on effect sizes reported previously. The number of mice was always greater than three per genotype. The investigators were blinded to group allocation during data acquisition and analysis.

Transgenic constructs were generated by fusing rabbit α, cDNA or human β, cDNA to the modified murine α-myosin heavy chain (MHC) tetracycline-inducible-promoter vector (a gift from J. Robbins and J. Molkentin, University of Cincinnati, Cincinnati, OH)44,45. A 3′ Flg epigote was ligated in-frame to the N terminus of α, C. The α, subunit was engineered to be dihydropyridine (DHP)-insensitive with the substitutions T1066Y and Q1070M (refs. 46,47). GFP was ligated to the N terminus of β, C. The creation of GFP–WT β, transgenic mouse was described previously. The V5 epitope and APEX2 cDNA48,49, created by gene synthesis, were conjugated to the N terminus of DHP-resistant α, and WT β,.

The 35-α mutant and the 28-β mutant cDNA were generated by site-directed mutagenesis. The optimal PKA phosphorylation motif is a tetrapeptide with arginine at the second and third positions (termed −2 and −3) before the phosphorylated serine or threonine, and a large hydrophobic residue immediately thereafter (R-R-X-S/T-Φ)46,47. The requirement for a positive charge is highest for residues at −2 and −3, but can be found for residues as far as position 6 in PKA target sites. Sites with arginine in positions between 4 and −1 are strongly preferred, and histidine or lysine to a lesser extent. We identified all potential intracellular PKA phosphorylation sites (Extended Data Fig. 1a, d) in rabbit α, and human β, using both manual sequence analysis and several web-based PKA phosphorylation prediction tools, including pKaP50, DISPHOS51, GPS52, NETPHOS53 and SCANSITE54. Each predicted serine or threonine was mutated to alanine. We also mutated additional serines and threonines within several amino-acid residues C-terminal to the arginine or lysine, in order to ensure that we fully captured each phosphorylation regulatory site. We replaced 51 residues in rabbit α, with alanine at 35 potential phospho-regulatory domains in the 35-mutant α, construct, and replaced 37 residues with alanine at 28 putative phospho-regulatory domains of β, C. We excluded those sites that were predicted to be extracellular or within the plasma membrane.

Transgenic mice with non-targeted insertion of these tetracycline-regulated cDNAs (Fig. 1b) were bred with cardiac-specific (α-MHC) doxycycline-regulated, codon-optimized reverse transcriptional transactivator (rtTA) mice (obtained via the Mutant Mouse Resource and Research Center) to generate double-transgenic mice. For the α,–APEX2 and β,–APEX2 mice, transgene expression did not require doxycycline owing to a low basal binding of rtTA protein to the tet operator sequences (so called ‘leak’). These expression levels result in Ca2+ current levels similar to those found under native conditions in the heart. The results presented here were consistent across all founder lines and gender, and therefore were pooled.

Isolation of adult cardiac myocytes
Mice ventricular myocytes were isolated by enzymatic digestion using a Langendorff perfusion apparatus as described. Cardiomyocytes were isolated from 8–12-week-old non-transgenic and transgenic mice. Only non-contracting rod-shaped cells with clear striations were used for functional studies (electrophysiology and excitation–contraction coupling).

Proximity labelling biotinylation
Proximity labelling was performed as described with minor modifications. Isolated ventricular cardiomyocytes were incubated in labelling solution with 0.5 mM biotin-phenol (Iris-biotech) for 30 min. For some experiments, during the final 10 min of incubation, 1 μM isoproterenol (Sigma I5627) was added. To initiate labelling, H2O2 (Sigma H1009) was added to a final concentration of 1 mM for 1 min. Exactly 1 min after H2O2 treatment, the cells were washed three times with cold quenching solution containing 10 mM sodium ascorbate (VWR 95035-692), 5 mM Trolox (Sigma 238813) and 10 mM sodium azide (Sigma S2502). After cells were harvested by centrifugation, the quenching solution was aspirated and the pellet was flash-frozen and stored at −80 °C until streptavidin pull-down.

For biotinylation in Langendorff-perfused hearts, mice were injected with 5 mg kg−1 propranolol (Sigma PHR1308) in order to suppress adrenergic stimulation during isoflurane anaesthesia and cardiectomy. Hearts were retrograde perfused with Krebs solution for 10 min before addition of biotin-phenol for 15 min. During the final 5 min, 1 μM isoproterenol or vehicle was added to the perfusate. Electrocardiograms were monitored throughout the experiment to ensure viability of the preparation and an isoproterenol-induced increase in heart rate.

The cells or whole heart tissue were lysed with a hand-held tip homogenizer in a solution containing 50 mM Tris (tris(hydroxymethyl)aminomethane), 150 mM NaCl, 10 mM EGTA, 10 mM EDTA, 1% Triton X-100 (v/v), 0.1% SDS (w/v), 10 mM sodium azide, phosphatase inhibitors (Sigma 4906845001), protease inhibitors (Sigma 469319001), calpain inhibitor I (Sigma A6185) and calpain inhibitor II (Sigma A6060). Biotin labelling of the samples was confirmed after size fractionation of proteins on SDS–polyacrylamide gel electrophoresis (PAGE), transfer to nitrocellulose membranes, and probing with streptavidin–conjugated horseradish peroxidase (HRP) (Thermofisher, S911, lot number 1718956, 0.6 mg ml−1). The response to isoproterenol was assessed by immunoblotting with an anti-phosphopholamban (Ser6/Thr7) antibody (Cell Signalling, lot number 8496, lot number 1:1,000 dilution).

Biotinylated proteins were bound to streptavidin magnetic beads (Thermo Fisher Scientific 88817), and the beads were washed three times with a solution containing 4 M urea, 0.5% SDS (w/v) and 100 mM sodium phosphate pH 8. Proteins were size-separated on SDS–PAGE, transferred to nitrocellulose membranes, and probed with anti-V5 antibody (Thermofisher, R960-25; 1/5,000 dilution), a custom-made polyclonal anti-α, antibody (Yenzym, 1/1,000 dilution)50,51, a custom-made polyclonal anti-β, antibody (epitope: mouse residues 120–138, DSYTSPRSDSVLEEDRE; Yenzym, 1/1,000 dilution), an anti-JPH2 antibody (Pierce, PA-20642, lot number NG1583142; 1/1,000 dilution),...
an anti-calmodulin antibody (Millipore Sigma 05-173, 1/1,000 dilution), a custom-made anti-RyR2 antibody (1/5,000 dilution)46, and an anti-Kv1.5 antibody (Alomone, APC-150, lot number APC004AN0850; 1/1,000 dilution).

**Immunoprecipitations**
Cardiomyocytes were lysed with a hand-held tip homogenizer in a 1% (v/v) Triton X-100 buffer containing 50 mM Tris-HCl (pH 7.4) 150 mM NaCl, 10 mM EDTA, 10 mM EGTA and 0.01 mM calpain inhibitor I, 0.01 mM calpain inhibitor II, and complete protease inhibitors (1 per 7 ml, Roche). The lysates were incubated on ice for 30 min and centrifuged at 14,000 r.p.m. at 4 °C for 10 min; supernatants were then collected. Anti-Flag antibody (Sigma, F7425, lot number 078M4886V) immunoprecipitations were performed, as described47, overnight in a lysis buffer consisting of 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.25% 1% Triton X-100 (v/v), 10 mM EDTA, 10 mM EGTA, 0.01 mM calpain inhibitor I, 0.01 mM calpain inhibitor II, and complete protease inhibitors (1 per 7 ml). Antibody–protein complexes were collected using protein-A-conjugated agarose (Amersham) for 2 h, followed by three washes in lysis buffer. Proteins were size-separated by SDS, transferred to nitrocellulose membranes and probed with HRP-conjugated anti-Flag antibody (Sigma, A8592), a custom-made anti-β-antibody and HRP-conjugated secondary goat anti-rabbit antibody. Detection of luminescence was performed, as described47, with a luminometer.

**Immunofluorescence**
Isolated cardiomyocytes were first exposed to biotin-phenol and H2O2 (Sigma, A8592), a custom-made anti-β-antibody and HRP-conjugated secondary goat anti-rabbit antibody. Detection of luminescence was performed with a charge-coupled-device (CCD) camera (Carestream Imaging). The uncult gels are shown in Supplementary Fig. 1.

**Processing biotinylated proteins for mass spectrometry**
Proteins were prepared as described48 with some modifications. Proteins were precipitated with trichloroacetic acid (TCA; Sigma T9159) and then centrifuged at 21,130g at 4 °C for 10 min. The pellet was washed with –20 °C cold acetone (Sigma 650501), vortexed, and centrifuged at 21,130g at 4 °C for 10 min. Following centrifugation, acetone was aspirated and the pellet was washed with acetone three more times. After the last washing step, the pellet was resuspended in 8 M urea, 100 mM sodium phosphate pH 8, 100 mM ammonium bicarbonate and 1% SDS (w/v), and then rotated at room temperature until fully dissolved. Resuspended proteins were centrifuged at 21,130g at room temperature for 10 min and the cleared supernatant was transferred to a new microcentrifuge tube. To reduce disulfides, we added 10 mM Tris (2-carboxyethyl)phosphine hydrochloride (TCEP-HCl; Thermo Fisher Scientific PG82089) in Milli-Q water titrated to pH 7.5 with sodium hydroxide. To alkylate free cysteine, we added freshly prepared 400 mM iodoacetamide (Thermo Fisher Scientific 90034) stock solution in 50 mM ammonium bicarbonate to the supernatant to a final concentration of 20 mM, then immediately vortexed and incubated the solution in the dark for 25 min at room temperature. After alkylation, freshly prepared dithiothreitol (DTT) stock solution was added to a final concentration of 50 mM in order to quench alkylation. Water was added to each sample to reach a final concentration of 4 M urea and 0.5% (w/v) SDS.

A 100 μl suspension equivalent per sample of streptavidin magnetic beads was washed twice with a solution containing 4 M urea, 0.5% SDS (w/v) and 100 mM sodium phosphate pH 8, and was added to each 1 mg of protein sample, diluting each sample with an equal amount of water to reach a final concentration of 2 M urea, 0.25% SDS (w/v), 50 mM sodium phosphate pH 8 during pull-down. The tubes were rotated overnight at 4 °C. Following streptavidin pull-down, the magnetic beads were washed three times with a solution containing 4 M urea, 0.5% SDS (w/v), 100 mM sodium phosphate pH 8, and three times with the same buffer without SDS. The beads were transferred to new tubes for the last wash step. Before final pulldown of the beads for mass-spectrometry analysis, streptavidin–HRP blotting was performed on 5% of the resuspended beads.

**On-bead digestion and TMT labelling**
Samples were prepared as described47. Liquid reagents used were of high-performance liquid chromatography (HPLC) quality grade. Washed beads were resuspended in 50 μl of 200 mM 3-(4-(2-hydroxyethyl)-piperazin-1-yl)propane-1-sulfonic acid (EPPS) buffer pH 8.5, 2% acetonitrile (v/v) with 1 μl of LysC stock solution (2 mg ml−1, Wako), vortexed briefly and incubated at 37 °C for 3 h. Then, 50 μl of trypsin stock (Promega V5111) diluted 1/100 (v/v) in 200 mM EPPS pH 8.5 was added. After mixing, digests were incubated at 37 °C overnight and beads were magnetically removed. Peptides were directly labelled after digest. For this, acetonitrile was added to a concentration of 30% (v/v) and peptides were labelled with TMT 10-plex or 11-plex reagents (Thermo Fisher Scientific 90406 and A34807) for 1 h. Reactions were quenched with hydroxylamine at a final concentration of 0.3% (v/v) for 15 min, and 1% of labelled peptides were analysed for efficiency of label incorporation and relative ratios by mass spectrometry. After quenching, peptide solutions were acidified with formic acid, trifluoroacetic acid (TFA) was added to a concentration of 0.1%, and peptides were desalted and fractionated by high pH reversed phase chromatography (Thermo Fisher Scientific 84868). After loading of labelled peptides onto preconditioned columns and a single wash with water, excess unincorporated TMT label was removed by washing reversed phase columns once with 0.1% trimethylamine (TEA) buffer containing 5% acetonitrile. Samples were fractionated under alkaline conditions into 12 fractions with increasing concentrations of acetonitrile: 10%, 12.5%, 15%, 17.5%, 20%, 25%, 30%, 35%, 40%, 50%, 65% and 80%. Fractions 1 and 7, 2 and 8, 3 and 9, 4 and 10, 5 and 11, and 6 and 12 were pooled to obtain 6 final pooled fractions for subsequent analysis. Pooled fractions were dried to completion and further purified and desalted by acidic C18 solid phase extraction (StageTip). Labelled peptides were finally resuspended in 1% formic acid (v/v) and 3% acetonitrile (v/v).

**Whole-cell proteomics**
Isolated nontransgenic cardiomyocytes were lysed by homogenization (QiA shredder cartridges, Qiagen) in lysis buffer (2% SDS, 150 mM NaCl, 50 mM Tris pH 7.4). Lysates were reduced with 5 mM DTT and alkylated with 15 mM iodoacetamide for 30 min in the dark; alkylation reactions were quenched with freshly prepared DTT added to a concentration of 50 mM, and proteins were precipitated by methanol/chloroform precipitation. Digests were carried out in 1 M urea freshly prepared in 200 mM EPPS pH 8.5 in the presence of 2% acetonitrile (v/v) with LysC (Wako, 2 mg ml−1, 1/75 w/w protease/substrates during digest) for 3 h at room temperature and after subsequent addition of trypsin (Promega V5111, stock 1/100 w/w protease/substrates) overnight at 37 °C. The missed cleavage rate was assessed from a small aliquot by mass spectrometry. For whole-proteome analysis, digests containing approximately 60 μg of peptide material were directly labelled with TMT reagents (Thermo Fisher Scientific). Labelling efficiency and TMT ratios were assayed by mass spectrometry, while labelling reactions were stored at –80 °C. After quenching of TMT labelling reactions with hydroxylamine, TMT labelling reactions were mixed, solvent evaporated to near completion, and TMT-labelled peptides purified and desalted by acidic reversed phase C18 chromatography. Peptides
were then fractionated by alkaline reversed phase chromatography into 96 fractions and combined into 24 samples.

**Mass-spectrometry analysis**

Data collection followed a MultiNotch MS³ TMT method by using an Orbitrap Lumos mass spectrometer coupled to a Proxeon EASY-nLC 1200 liquid chromatography system (both Thermo Fisher Scientific). The capillary column used was packed with C₄ resin (length 35 cm, inner diameter 75 μm, matrix 2.6 μm Accucore (Thermo Fisher Scientific)). Peptides of each fraction were separated for 4 h over acidic acetonitrile gradients by liquid chromatography before mass-spectrometry analysis. The scan sequence started with an MS³ scan (Orbitrap analysis; resolution 120,000; mass range 400–1,400 Th). MS² analysis followed collision-induced dissociation (CID; CID energy = 35) with a maximum ion-injection time of 150–300 ms and an isolation window of 0.4 m/z. In order to obtain quantitative information, we fragmented MS³ precursors by high-energy collision-induced dissociation (HCD) and analysed the fragments in the Orbitrap at a resolution of 50,000 at 200 Th. Further details on liquid chromatography and mass-spectrometry parameters and settings were described recently.

Peptides were searched against a SEQUEST (v.28, rev. 12)-based software against a size-sorted forward and reverse database of the Mus musculus proteome (Uniprot Jul 2014) with added common contaminant proteins. For this, spectra were first converted to mzXML. Searches were performed using a mass tolerance of 20 p.p.m. for precursors and a fragment-ion tolerance of 0.9 Da. For the searches, a modified version of the Ascore algorithm to quantify the confidence assignment of phosphorylation sites. Phosphorylation localised to particular residues required Ascore values of more than 13 (P ≤ 0.05) for confident localization. Quantitative information on peptides was derived from MS³ scans. Quant tables were generated requiring an MS³ isolation specificity of more than 70% for each peptide and a sum of TMT signal-to-noise (s/n) ratios of more than 200 over all channels for any given peptide; the tables were exported to Excel and processed further therein. Details of the TMT intensity quantification method and further search parameters were described previously. Proteomics raw data and search results were deposited in the PRIDE archive and can be accessed under ProteomeXchange accession numbers PXD014499, PXD014500 and PXD014501.

The relative summed TMT s/n ratios for proteins between two experimental conditions (referred to as ‘enriched’) were calculated from the sum of TMT s/n ratios for all peptides quantified of a given protein. For enrichment of Gene Ontology (GO) terms, the BINGO package in Cytoscape was used. Scaled quantitation data were subjected to two-way clustering (JMP software package) and changes in enrichment were analysed using Graphpad Prism 8 (Graphpad Software). Statistical significance was determined by multiple t-tests without correction for multiple comparisons and α = 0.05. Data for relative protein quantification can be found in Supplementary Tables I, 3–6.

**Phosphoproteomic analysis of Rad**

HEK293T cells were cultured in DMEM with 10% FBS and 1% penicillin/streptomycin. Cells were transfected with GFP-labelled Rad using Lipofectamine 2000 as above. The medium was changed 4–6 h after transfection. After 24 h, cells were treated with trypsin and spun down for 5 min at 1,000 r.p.m. Cells were then resuspended in PBS with 10 μM forskolin for 5 min. After washing cell pellets three times with PBS, cells were frozen at −80 °C. Cold cell pellets were lysed in PBS with 0.1% triton X-100 (v/v, Sigma) and a phosphatase-inhibitor mixture (Complete and PhosSTOP, both from Roche) by pipetting up and down several times. Lysates were homogenized by passing them through QIAshredder cartridges (QIagen) and incubated with GFP–trap agarose beads (Chromotek, Germany) for 4 h at 4 °C with constant rotation. Beads were washed three times with PBS with 0.1% (v/v) Triton and three times with detergent-free PBS and subjected to on-bead digest with trypsin (Promega V5111). LysC (Wako) or ArgC (Promega V1831). ArgC digestion buffer 50 mM Tris-HCL pH 7.8, 5 mM CaCl₂, 2 mM EDTA, 2% acetonitrile (v/v) separately as described above overnight at 37 °C. After acidification, peptides were purified by reverse phase C₁₈ chromatography and subjected to MS/MS analysis. For this, the same parameters as above for MS³ and MS² scans were used with an isolation window of 1.2 Da and taking a neutral loss of 97.9763 Da into account, with multi-stage activation (MSA) set for MS² scans. Analysis of phospho-site localization was performed as above.

**Fractional shortening**

Freshly isolated myocytes were perfused with a Tyrode’s solution containing 1.8 mM CaCl₂. Myocytes were field stimulated at 1 Hz. Nisoldipine (300 nM) dissolved in Tyrode’s solution was then superfused. Fractional shortening of sarcomere length was measured using the SarcLen module of Ionoptix.

**Whole-cell patch-clamp electrophysiology**

Isolated cardiomyocytes or HEK cells attached on glass 8 × 8 mm coverslips were placed in Bioptechs Delta T dishes filled with solution containing 112 mM NaCl, 5.4 mM KCl, 1.7 mM Na₂HPO₄, 1.6 mM MgCl₂, 20.4 mM HEPES pH 7.2, 30 mM taurine, 2 mM l-carnitine, 2.3 mM creatine and 5.4 mM glucose. The Petri dishes were mounted on the stage of an inverted microscope and served as a perfusion chamber. After establishing a seal and achieving whole-cell configuration, external solutions were changed by the fast local perfusion method.

For cardiomyocytes, pipette resistance was maintained at 1 and 3 MΩ. Membrane currents were measured by the conventional (ruptured) whole-cell patch-clamp method using a MultiClamp 700B or Axopatch200B amplifier and pCLAMP 10.7 software (Molecular Devices). Capacitance transients and series resistance were compensated. Voltage was corrected for local junction potential (~10 mV) during analysis. Leak currents were subtracted by a p/4 protocol. The parameters of voltage-dependent activation were obtained using a modified Boltzmann distribution: \( \Gamma(V) = G_{max} \times \frac{(V - V_{rev})}{1 + \exp((V - V_{0.5})/V_{slope})} \), where \( V \) is the peak current, \( G_{max} \) is the maximal conductance, \( E_{rev} \) is the reversal potential, \( V_{0.5} \) is the midpoint, and \( V_{slope} \) is the slope factor.

The pipette solution contained 40 mM CsCl, 80 mM cesium gluconate, 10 mM 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA), 1 mM MgCl₂, 4 mM Mg-ATP, 2 mM CaCl₂, and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), adjusted to pH 7.2 with CsOH. After the isolated cardiomyocytes were dialysed and adequately buffered with 10 mM BAPTA in the internal solution, cells were superfused with 140 mM tetraethylammonium chloride (TEA-Cl), 1.8 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, adjusted to pH 7.4 with CsOH. To measure peak currents, we held the cell membrane potential at ~50 mV and stepped it to +0 mV for 350 ms every 10 s. To evaluate the current–voltage (I–V) relationship in cardiomyocytes, we repeated the same protocol with steps between ~40 mV and ~60 mV in 10-mV increments. Nisoldipine (Santa Cruz) was stored protected from light at ~20 °C as a 3 mM stock in ethanol. The final dilution of nisoldipine to 300 nM was in the extracellular recording solution immediately before the experiment. Isoproterenol (Sigma I5627) and forskolin (Santa Cruz) were prepared daily and diluted in extracellular solution.
the perforated whole-cell patch-clamp technique in order to minimize current run-down and preserve the intracellular milieu. Amphotericin B (Sigma A9528) was initially dissolved in DMSO (20 mg ml⁻¹) and used in the pipette solution at a final concentration of 200 µg ml⁻¹. The tip of the pipette was filled with amphotericin-free solution containing 80 mM caesium gluconate, 40 mM CsCl, 10 mM Hepes, 10 mM BAPTA, 1 mM MgCl₂, and 1 mM Mg-ATP, pH adjusted to 7.2 with CsOH. The pipette was backfilled with 125 mM CsCl, 10 mM HEPES, 4 mM CaCl₂, 1 mM MgCl₂, pH 7.2 and CsOH containing amphotericin at 200 µg ml⁻¹. CaCl₂ (4 mM) was added to the patch electrode solution to enable the detection of conversion from perforated to ruptured configuration. The external solution contained 130 mM tetraethylammonium methanesulfonate, 10 mM HEPES, 1 mM MgCl₂, 10 mM (with Rad expression) or 2 mM (without Rad expression) BaCl₂, 5 mM glucose. For experiments with HEK293T cells, in addition to step protocols, we used a ramp protocol with a 200-ms voltage ramp from −60 mV to +60 mV (0.6 V s⁻¹) applied every 10 s to monitor the I–V relationship. All experiments were performed at room temperature, 22 ± 1°C. Cells were selected on the basis of the voltage dependency of currents. For each cell, at least 50 sweeps with a repetition interval of 10 s were recorded. The numbers of sweeps are as follows: 388 sweeps; and 4SA mutant Rad plus PKA, 311 sweeps (three one-channel patches). Third, cells expressing the Cer fluorophore alone were used to measure the spectral crosstalk parameter R_C, corresponding to bleed-through of Cer fluorescence into the FRET channel. Third, cells expressing the Cer fluorophore alone were used to measure spectral crosstalk parameter R_C, corresponding to bleed-through of Cer fluorescence into the FRET channel. Third, cells expressing the Cer fluorophore alone were used to measure spectral crosstalk parameter R_C, corresponding to bleed-through of Cer fluorescence into the FRET channel. Third, cells expressing the Cer fluorophore alone were used to measure spectral crosstalk parameter R_C, corresponding to bleed-through of Cer fluorescence into the FRET channel.

Single-channel patch-clamp electrophysiology

Cell-attached single-channel recordings were performed at room temperature as described.70,72 Patch pipettes (5–10 MΩ) were pulled from ultra-thick-walled borosilicate glass (BF200-116-10, Sutter Instruments), and were coated with Sylgard. Currents were filtered at 2 Khz. The pipette solution contained 140 mM tetraethylammonium methanesulfonate; 10 mM Hepes; 40 mM BaCl₂; at 300 mOsm l⁻¹, adjusted with glucose; and pH 7.4, adjusted with sodium hydroxide. To maintain the membrane potential between −50 mV and +40 mV are displayed and analyzed. For each patch, we recorded 80–120 sweeps with a repetition interval of 10 s.

For each experimental run on the flow cytometer, we performed several control experiments. First, the background fluorescence level for each fluorescent channel (Bg_Cer, Bg_Ven and Bg_Cer-Ven) was obtained by measuring fluorescence from cells exposed to PEI without any fluorophore-containing plasmids. Second, cells expressing the Ven fluorophore alone were used to measure the spectral crosstalk parameter R_V, corresponding to bleed-through of Ven fluorescence into the FRET channel. Third, cells expressing the Cer fluorophore alone were used to measure spectral crosstalk parameter R_C, corresponding to bleed-through of Cer fluorescence into the FRET channel.

Flow-cytometric FRET two-hybrid assay

For flow-cytometric FRET assays, HEK293 cells (ATCC CRL1573) were cultured in 12-well plates and transfected with pCMV6-XL6-CMV vector (Promega) containing the cytoplasmic domain of the β2-adrenergic receptor (BD Biosciences) flow cytometer, equipped with 405-nm, 488-nm and 633-nm lasers for excitation and 18 different emission channels. Forward- and side-scatter signals were detected and used to gate for single and healthy cells. To determine FRET efficiency, we measured three distinct fluorescence signals: first, S_cer (corresponding to emission from the cerulean tag) is measured through the BV421 channel (excitation, 405 nm; emission, 450/50); second, S_ven (corresponding to emission from the venus tag) is measured via the FITC channel (excitation, 405 nm; dichroic, S05P; emission, 525/50); and third, S_fret (corresponding to FRET emission) is measured via the BV510 channel (excitation, 405 nm; dichroic, S05P; emission, 550/50). These raw fluorescence measurements are subsequently used to obtain Ven_fret (Venus emission due to direct excitation), Cer_fret (Cerulean emission due to direct excitation), and Ven_fret (Venus emission due to FRET excitation). Flow-cytometric signals were collected at a medium flow rate (2,000 to 8,000 events per second). Fluorescence data were exported as FCS 3.0 files for further processing and analysis using custom MATLAB functions (MathWorks).

For each experimental run of the flow cytometer, we performed several control experiments. First, the background fluorescence level for each fluorescent channel (Bg_Cer, Bg_Ven and Bg_Cer-Ven) was obtained by measuring fluorescence from cells exposed to PEI without any fluorophore-containing plasmids. Second, cells expressing the Ven fluorophore alone were used to measure the spectral crosstalk parameter R_V, corresponding to bleed-through of Ven fluorescence into the FRET channel. Third, cells expressing the Cer fluorophore alone were used to measure spectral crosstalk parameter R_C, corresponding to bleed-through of Cer fluorescence into the FRET channel. Fourth, FRET measurements also require determination of instrument-specific calibration parameters g_Cer/Bg_Cer and f_Cer/f_Ven, which are respectively ratios of fluorescence excitation and emission for Ven to Cer fluorophores. These parameters also incorporate fluorophore-dependent aspects, including molar extinction (for g) and quantum yield (for f), as well as instrument-specific parameters, including laser power, attenuation by optical components, and photodetection, amplification and digitization of fluorescence. To determine these parameters, we used Cer–Ven dimers with four different linker lengths (5, 32, 63, and 100 µM) was added to cells 2 h before experimentation to halt synthesis of new fluorophores, in order to allow existing fluorophores to fully mature.
For Cer–Ven dimers, we obtained FRET efficiencies of roughly 0.55, 0.38, and 0.05 for linker lengths 5, 32, and 228 respectively, matching previous work. The relative proportion of Cer and Ven fluorophores in each cell was determined as $N_{Cer} = \frac{\text{Cer}_{\text{area}}}{(1 - E_d)}$ and $N_{Ven} = \frac{\text{Ven}_{\text{area}}}{\text{Cer}_{\text{area}} * \text{Ven}_{\text{area}}}$. To construct FRET two-hybrid binding curves, we imposed a 1:1 binding isotherm as in previous studies. For each FRET pair, we obtained effective dissociation constant ($K_{d,EF}$), $E_{max}$ and 95% confidence intervals by constrained least-squares fit.

For Fig. 4b, c and Extended Data Fig. 9a, b, we show all individual cells from two different transfections. We fit these data with the equation $E_0 = E_{max} \times \frac{[Ven - Rad]_{t0}^\text{Direct} + [Cer]_{t0}^\text{Direct}}{[Ven - Rad]_{t0}^\text{Direct} + [Cer]_{t0}^\text{Direct} + K_{d,EF}}$ using the least-squares algorithm (www.mathworks.com/help/stats/nlinfit.html). The $K_{d,EF}$ fit value based on all of the data points is shown as the bar in Extended Data Fig. 9c. The error is calculated by the fitting algorithm as a 95% confidence interval on the fit parameter (https://www.mathworks.com/help/stats/nlinparci.html).

Statistical analysis
Experiments were not randomized. Results are presented as mean ± s.e.m. For comparisons between two groups, we used Student’s $t$-test. Statistical analyses were performed using Prism 8 (Graphpad Software). For multiple group comparisons, we performed a one-way ANOVA followed by either Dunnett’s or Tukey’s post-hoc test using Prism 8. Differences were considered statistically significant at values of $P < 0.05$.

Data and material availability
All transgenic mice are available from S.O.M. under a material agreement with Columbia University. All data are available in the main text, the Extended Data or the Supplementary Information. Proteomics raw data and search results were deposited in the PRIDE archive and can be accessed via the ProteomeXchange under accession numbers PXD014499, PXD014500 and PXD014501. The FRET software is accessible on github at https://github.com/manubenjohny/FACS_FRET. Source data for Figs 1–4 and Extended Data Figs 1, 2, 6, 8 are provided with the paper.

Acknowledgements
We thank A. Karlin for helpful discussions and editing the manuscript; B. Soda for creating the cell-model illustration; and G. A. Bradshaw for technical assistance. This publication was supported by the National Institutes of Health (NIH; grants RO1 HL113136, RO1 HL12153 and RO1 HL144194) and by the National Center for Advancing Translational Sciences (grant UL1TR000187). These studies used the resources of the Herbert Irving Comprehensive Cancer Center Flow Cytometry Shared Resources, funded in part through Center Grant P30CA13666. Images were collected (and analysed) in the Confocal and Specialized Microscopy Shared Resource of the Herbert Irving Comprehensive Cancer Center at Columbia University, supported by NIH grant P30 CA013666 (National Cancer Institute). A.P. was supported by NIH grant T32 HL120826 and National Science Foundation (NSF) Division of Graduate Education (DGE) grant 1644869. D.R. was supported by grants T32 HL102506 and F31 HL14278. J.K. was supported by grant T32 HL00343 and the Glorney–Raisbeck Fellowship from the New York Academy of Medicine, and J.A.H. was supported by grant T32 HL00385. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

Author contributions
The following authors designed research and analysed data: G.L., A.P., A.N.K., S.I.Z., D.R., J.A.H., and M.B.J. The following authors performed research and analysed data: G.L., A.P., A.N.K., S.I.Z., D.R., J.A.H., and M.B.J. All authors contributed to the writing of the paper with input from all authors: S.O.M., M.K., H.M.C., G.S.P. and M.B.J. All authors declare no competing interests.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41586-020-0847-9.

Correspondence and requests for materials should be addressed to M.K. or S.O.M.

Peer review information
Nature thanks Donald Biers, Alice Ting and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

Reprints and permissions information is available at http://www.nature.com/reprints.
Extended Data Fig. 1 | Putative PKA phosphorylation sites in α1C and β2B subunits. a, Left column, the 35 putative PKA phosphorylation sites in rabbit α1C. Centre, the 51 residues in red are either predicted phosphorylation sites or within the immediate region of the predicted phosphorylation sites. Right, all 51 residues were replaced with alanine in 35-mutant α1C transgenic mice. b, Combined bar and column scatter plot of Boltzmann function parameters, \( V_{50} \). Data are mean ± s.e.m. **\( P < 0.01; ***P < 0.001; ****P < 0.0001 \) by paired two-tailed t-test. \( pWT \) α, \( n = 19 \); 35-α mutant, \( n = 14 \); 28-β mutant, \( n = 16 \); 35-α mutant × 28-β mutant, \( n = 24 \). Specific P values can be found in the associated Source Data (see Supplementary Information). c, Graph showing isoproterenol- and forskolin-induced increases in nisoldipine-resistant current, stratified by total basal current density before nisoldipine treatment. d, Left, the 28 putative PKA phosphorylation sites in the N-terminal (NT), Hook, GK and C-terminal (CT) domains of β2B. Centre, the 37 residues in red are either predicted phosphorylation sites or within the immediate vicinity of predicted phosphorylation sites, and were mutated to alanine in the 28-mutant GFP-tagged β2B transgenic mice (right). e, Fluorescence imaging of isolated cardiomyocytes expressing the GFP-tagged 28-β mutant. Representative of images from more than five biologically independent mice. f, Anti-β-subunit immunoblot of cleared lysates from doxycycline-fed 35-mutant α1C transgenic (TG) mice or 35-mutant α1C × GFP-tagged 28-β mutant expressing mice hearts. Representative of immunoblots obtained from at least three biologically independent mice. g, Anti-Flag antibody (upper) and anti-β antibody (lower) immunoblots of anti-Flag antibody immunoprecipitations from cleared lysates of hearts from pWT, 35-α and three mice expressing 35-α × GFP-tagged 28-β. Representative images from two independent experiments. For source gel data, see Supplementary Fig. 1.
Extended Data Fig. 2 | Trafficking and function of APEX2-conjugated Ca\(_{\text{v}}\)1.2 subunits in heart. a, Exemplar current–voltage relationship of Ca\(^{2+}\) currents from cardiomyocytes of α\(_{\text{vC}}\)–APEX2 mice, acquired in the absence (black trace) and presence (red trace) of nisoldipine. Insets show series of whole-cell Ca\(_{\text{v}}\)1.2 currents recorded from a series of voltage steps between −40 mV and +60 mV from a holding potential of −50 mV. Scale bars, horizontal 100 ms, vertical 10 pA/pF. Representative of five experiments. b, Time course of changes in sarcomere length after superfusion of nisoldipine-containing solution. Representative of seven experiments. c, Percentage sarcomere shortening in the presence of nisoldipine. Data are mean ± s.e.m. ****P < 0.0001 by unpaired two-tailed t-test. n = 12 and 7 cardiomyocytes from left to right. NTG, nontransgenic. d, Immunofluorescence of cardiomyocytes isolated from mice expressing α\(_{\text{vC}}\)–APEX2 and β\(_{2\text{B}}\)–APEX2, exposed to biotin–phenol and H\(_2\)O\(_2\) or no H\(_2\)O\(_2\). Staining is with anti-V5 and Alexa594-conjugated secondary antibodies and streptavidin-conjugated Alexa488, and nuclear labelling is with DAPI. Scale bar, 5 μm. Representative of 13 and 8 cardiomyocytes from 2 and 3 mice respectively. e, Streptavidin–HRP blot of lysates from isolated ventricular cardiomyocytes, representative of five similar experiments. f, Exemplar whole-cell Ca\(_{\text{v}}\)1.2 currents recorded from cardiomyocytes of α\(_{\text{vC}}\)–APEX2 transgenic mice. Black trace, 300 nM nisoldipine; blue trace, 200 nM isoproterenol plus nisoldipine. Representative of nine cells from two biologically independent mice. g, As in f, except from β\(_{2\text{B}}\)–APEX2 mice. Black trace, control; blue trace, 200 nM isoproterenol. Representative of seven experiments from two biologically independent mice. h, i, Anti-phospho-phospholamban immunoblot of proteins from cardiomyocytes isolated from α\(_{\text{vC}}\)–APEX2 and β\(_{2\text{B}}\)–APEX2 mice. Cardiomyocytes were exposed to either vehicle or 1 μM isoproterenol after incubation with biotin–phenol. Blots are representative of three independent experiments from at least five biologically independent mice for each genotype. j, As in h and i, except that cardiomyocytes were isolated from non-transgenic mice without incubation with biotin–phenol. Blot is representative of three independent experiments from three biologically independent mice. k, As in h and i, except that whole heart was exposed to 1 μM isoproterenol for 5 min after infusion of biotin–phenol. This blot is representative of at least five biologically independent mice for no isoproterenol and at least five biologically independent mice for isoproterenol. For source gel data, see Supplementary Fig. 1.
Extended Data Fig. 3 | Analysis of proteins quantified by mass spectrometry in cardiomyocytes isolated from α1C-APEX2 and β2B-APEX2 mice. a, Proteins with a ratio of more than 2 (measured by normalized TMT signal/noise) in the indicated experimental conditions, compared with a no-labelling control (no H2O2), were sorted by spectral counts. The 150 proteins with the highest peptide counts are displayed in this colour-coded table. α1C–APEX2 and β2B–APEX2 data were collected in biological duplicate experiments. Supplementary Table 1 shows all 3,883 proteins quantified by multiplexed SPS MS3 TMT mass spectrometry. b, Prefuse force-directed map of proteins from a. Peptide counts were used as weight. Proteins mapping to the GO term ‘Z disc’ are in green, to ‘membrane’ in yellow, and to both are in purple. α1C–APEX2 and β2B–APEX2 are in blue. c, GO term (cellular localization) enrichment for proteins in a. See Supplementary Table 2 for the full table.

Supplementary Table 1 shows all 3,883 proteins quantified by multiplexed SPS MS3 TMT mass spectrometry. b, Prefuse force-directed map of proteins from a. Peptide counts were used as weight. Proteins mapping to the GO term ‘Z disc’ are in green, to ‘membrane’ in yellow, and to both are in purple. α1C–APEX2 and β2B–APEX2 are in blue. c, GO term (cellular localization) enrichment for proteins in a. See Supplementary Table 2 for the full table.
Extended Data Fig. 4 | See next page for caption.
Extended Data Fig. 4 | Two-way hierarchical clustering of scaled data from Fig. 2. a, Dendrogram showing two-way hierarchical clustering of scaled TMT s/n data for streptavidin-purified proteins from α1C–APEX2 cardiomyocytes after stimulation with vehicle or isoproterenol. Shown are scaled relative TMT protein quantification data for 1,951 proteins from biological quintuplicate α1C–APEX2 mice. Clustering used Ward’s minimum-variance method. b, Dendrogram showing two-way hierarchical clustering of scaled relative quantification data for 1,936 proteins from biological triplicate β2B–APEX2 experiments. Heterogeneity between cardiomyocyte preparations from different mice is apparent. c, Dendrogram showing two-way hierarchical clustering of scaled relative quantification data for 2,610 proteins from whole-organ α1C–APEX without or with perfusion of isoproterenol. Prominent heterogeneity in relative protein quantification between hearts is apparent. The position of Rad is indicated by a red line. In this experiment, the individual hearts were not paired. d, Dendrogram showing two-way hierarchical clustering of scaled TMT s/n data from non-transgenic mouse cardiomyocytes stimulated with isoproterenol or with vehicle. Scaled data for 4,622 quantified proteins from a biological quadruplicate experiment are displayed. Pairing of samples is apparent.
Extended Data Fig. 5 | See next page for caption.
Extended Data Fig. 5 | Isoproterenol-induced change in Rad detected by mass spectrometry. 

**a.** Right, the MS² spectrum (top) and TMT quantification parameters (bottom) for a Rad peptide changed upon treatment of murine hearts with isoproterenol. Shown is the MS² spectrum that was used to identify the Rad peptide IFGGIEDGPEAEAAGHTYDR. Left, m/z ratios for b and y ions identified in the spectrum and their deviation from theoretical m/z ratios. We measured the precursor mass as 778.71 Da with a charge of +3. Peptide modifications were +229.16 Da for TMT on the peptide N terminus and lysine residues, +57.02 Da for cysteine alkylation and +15.99 for methionine oxidation. Shown are ion injection times, isolation specificity, sum of signal-to-noise (SN) over all TMT channels, TMT raw intensities, adjusted intensities and final SN intensities used for relative quantification, as well as synchronous precursor selection (SPS) ion m/z ratios (isolated in the ion trap with Notch filtering).

**b.** Table showing gene names of proteins with P < 0.05 for the three approaches: cardiomyocytes isolated from α1C–APEX and β2B–APEX mice, and α1C–APEX hearts. Genes in yellow are common to all groups, but note that for Mast2, the fold change is not consistent. Data are mean fold changes for five pairs of biologically independent pairs of α1C–APEX2 cardiomyocyte samples, three pairs of biologically independent pairs of β2B–APEX cardiomyocyte samples, and ten α1C–APEX2 hearts, five without isoproterenol and five with isoproterenol. Non-adjusted unpaired two-tailed t-test.

**c.** Venn diagram showing the data from b. Rad, Rad; Prkaca, PKA catalytic subunit; Acss1, acyl-CoA synthetase short-chain family member 1. Rad is the only protein that is consistently changed amongst the three approaches.

‘Notch mz’ denotes the ion m/z ratio of individual isolated SPS ions prior to HCD fragmentation and MS².
Extended Data Fig. 6 | Rad is required for forskolin-induced activation of heterologously expressed CaV1.2 channels. 

a, Exemplar whole-cell CaV1.2 currents elicited from step depolarizations recorded from HEK293T cells expressing Rad. Voltage command steps were applied every 10 s before (black traces) and during (blue traces) forskolin treatment. Representative of at least ten cells. 

b, Methodology used for generating G/V curves. i, Upper, a 200-ms voltage ramp from −60 mV to +60 mV was applied every 10 s. Lower, current traces, each an average of three traces before (black) and three traces after (blue) forskolin treatment. 

ii, Conversion of time scale to applied voltage. 

iii, Conversion to G–V relationship. Fold change was calculated at Gmax. 

c, Graph showing forskolin-induced fold change in current, stratified by basal current density. 

d, Exemplar traces of Ba2+ currents in the absence and presence of Rad elicited by voltage ramp every 10 s. Black traces, before forskolin treatment; blue traces after treatment; no Rad, 7 cells; Rad, 16 cells. 

e, Boltzmann function parameter V0.5. Data are mean ± s.e.m. **P < 0.01 by paired two-tailed t-test. n = 7 and 16, from left to right. 

f–g, Ratio of Ba2+ current after forskolin treatment to Ba2+ current before treatment for cells transfected without and with Rad. Representative of analyses for three cells for each condition. 

h–i, Distribution of sweep-by-sweep average Po (single-trial Po) for different conditions. 

h, In the absence of Rad, sweeps with no openings or blank sweeps are rare (10%); most sweeps exhibit either intermediate or high levels of openings. 

i, If the PKA catalytic domain is also coexpressed with Rad, the fraction of blank sweeps is reduced and there is a resurgence of the high-activity mode. 

j, Pale blue lines show conditional Po–voltage relationships obtained for sweeps exhibiting high activity in the absence of Rad and PKA. The dark blue line is the Boltzmann fit. 

k, As in i, but with Rad and PKA expression.
Extended Data Fig. 7 | PKA phosphorylation sites in mouse Rad. **a**, Serine/threonine residues (in purple) that are mutated to alanine in the 14-SA mutant.

**b**, Mass-spectrometry identification of phosphorylated residues on Rad enriched with an anti-GFP nanobody matrix, from HEK cells expressing GFP–Rad and treated with forskolin. The number of spectral counts is plotted against the position of the phosphorylated amino acids in Rad. We detected 534 aggregated phosphopeptides in two independent experiments.

**c**, Database entry for phosphorylation sites identified previously in Rad (https://phosphomouse.hms.harvard.edu/site_view.php?ref=IPI00133102). The highest level of Rad phosphorylation was detected in the heart (left panel). The lower right panel shows peptides detected with phosphorylated serine residues on positions 25, 38 and 300 (in bold red; mapped to the Rad expression constructs used here; blue highlighting indicates sequence covered by peptides).

**d**, Serine residues mutated to alanine in the 4-SA mutant (arrows).
Extended Data Fig. 8 | Binding of Rad and β2B is required for regulation of forskolin-induced stimulation of voltage-gated Ca2+ channels. a, The Rad protein sequence shown here indicates the residues Arg208 and Leu235 that were substituted with alanine (yellow). b, The β2B protein sequence indicates the residues Asp244, Asp320 and Asp322 that were substituted with alanine (yellow), resulting in attenuation of Rad binding to the β subunit, as described previously28,29. c, Ba2+ currents from CaV1.2 channels, elicited by voltage ramp every 10 s from −60 mV to +60 mV over 200 ms, before (black) and after (blue) treatment with forskolin. Representative of 20 (top) and 15 (bottom) cells. d, Boltzmann function parameter V50. Data are mean ± s.e.m. ***P < 0.001 by paired two-tailed t test. The data for wild-type Rad are the same as in Fig. 3h. Specific P values can be found in the associated Source Data. n = 16, 19 and 13, from left to right. e, Fold change in Gmax in Cav1.3 channels. Data are mean ± s.e.m. P < 0.0001 by one-way ANOVA; ****P < 0.0001 by Dunnett’s test. The data for wild-type Rad and wild-type β2B are the same as in Fig. 4e. n = 7, 7 and 9 cells, from left to right. f, Fold change in Gmax in Cav2.2 channels. Data are mean ± s.e.m. P < 0.001 by one-way ANOVA; ***P < 0.001 by Dunnett’s test. Data for wild-type Rad and wild-type β2B are as in Fig. 4h. n = 11, 7 and 8 cells, from left to right.
Extended Data Fig. 9 | Phosphorylation-dependent dissociation of Rad, β2, and β4 subunits. (a, b) FRET two-hybrid binding isotherms were determined for Cer-tagged β3 (a) and β4 (b) subunits, and N-terminal Ven-tagged wild-type (left) or 4-SA mutant (right) Rad. FRET efficiency ($E_D$) is plotted against the free concentration Ven–WT or Ven–4SA-mutant Rad. The solid line fits a 1/1 binding isotherm. Coexpression of the PKA catalytic subunit weakened binding in cells expressing wild-type Rad, but not in cells expressing 4-SA mutant Rad. (c) Bar graph summarizing mean $K_{d,EFF}$ for β2, β3, and β4, and wild-type and 4-SA mutant Rad, expressed without and with the PKA catalytic subunit. Data are mean ± 95% confidence intervals; error bars show 95% confidence intervals for the pooled nonlinear fits based on the Jacobians computed. The sample size for each condition is 1,580–10,364 cells, acquired via two independent transfections and then pooled. The distribution of data in this graph is reflected in Fig. 4b, c, and in a, b.
Extended Data Fig. 10 | ClustalW alignment of Rad sequences and RGK GTPases. 

**a.** Conservation of phosphorylation sites from mouse Rad (Ser25, Ser38, Ser272 and Ser300) in other species. Blue highlights basic amino acids (arginine, lysine and histidine), and red highlights serine and threonine.

**b.** C-terminal phosphorylation sites are conserved in other species. The equivalent of the Ser25 phosphorylation site is conserved in Rem1, and the equivalent of Ser38 is probably conserved in Gem. Phosphorylation sites in mouse Rad (Ser25, Ser38, Ser272 and Ser300) are indicated with arrows. Blue highlights basic amino acids (arginine, lysine and histidine), and red highlights serine and threonine.
Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see Authors & Referees and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- The statistical test(s) used AND whether they are one- or two-sided
- Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- A full description of the statistical parameters including central tendency (e.g. mean) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
- Give P values as exact values wherever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection | pClamp 10.7 software (Molecular Devices), Sequest (V.28, rev. 12)

Data analysis | Graphpad Prism 8, Origin 7.5, JMP 14 Pro, The FRET software is on github: https://github.com/manubenjoj/hny/FACS_FRET

For manuscripts utilizing custom algorithms or software that are not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. Github). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The authors declare that the data supporting the findings of this study are available within the paper, its supplemental figures and tables, and all raw mass spectrometry data have been deposited.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.
Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | Sample sizes exceeded the number of samples determined by power calculations, which were based on effect sizes previously reported in the literature. Number of animals was always greater than 3 per genotype. Sample size was determined to be adequate based on the magnitude and consistency of measurable differences between groups and low observed variability between samples. |
| Data exclusions | No data were excluded from analyses. For electrophysiological studies, cells were not studied if baseline electrophysiological determinations demonstrated current run-down. These cells were excluded before any experiment was initiated (a predetermined criteria). Once nisoldipine, isoproterenol or forskolin was added, the cells were included in all analyses. See Methods for details. |
| Replication | All experiments were replicated and all attempts at replication were successful and consistent. |
| Randomization | No randomization of mice. Mice were litter-mates when possible. |
| Blinding | The investigators were blinded to group allocation during data acquisition and analysis. |

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

| Materials & experimental systems | Methods |
|---------------------------------|---------|
| n/a | Involved in the study | n/a | Involved in the study |
| ☑ Antibodies | ☑ ChiP-seq |
| ☑ Eukaryotic cell lines | ☑ Flow cytometry |
| ☑ Palaeontology | ☑ MRI-based neuroimaging |
| ☑ Animals and other organisms | ☑ Clinical data |
| ☑ Human research participants | ☑ Clinical data |
| ☑ Clinical data | ☑ Clinical data |

Antibodies

Additional information for all antibodies are provided in the Methods section.

- Anti-V5 Tag Monoclonal Antibody (Thermofisher, R960-25) 1:5000 dilution
- Anti-iph2 Antibody Pierce, PAS-20642, lot# NG1583142 1:1000 dilution
- Anti-CaMulin Antibody (Millipore Sigma, 05-173) 1:1000 dilution
- Anti-RyR2 Antibody (5029) dilute 1:5000
- Anti-Kv1.5 Antibody (Alomone Labs, APC-004, Lot# APC-004AN0850) 1:10000 dilution
- Anti-phospho-phospholamban [Ser 16/Thr 17] Antibody [Cell Signaling, #8495, Lot# 1] 1:10000 dilution
- Goat anti-Mouse IgG [H+L] Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 594 (Thermofisher, A11032, Lot#2069816) 1:200 dilution
- Streptavidin, Alexa Fluor® 488 Conjugate (Thermofisher, S32354, Lot# 17196556) 1:800 dilution
- Streptavidin, horseradish peroxidase conjugate (Thermofisher, s911, Lot# 1711896) 0.6mg/ml
- Anti-Alpha1C antibody [custom-made by YenZym] 1:1000 dilution
- Anti-CaVbeta antibody [custom-made by YenZym] 1:500 dilution
- Anti-FLAG antibody [Sigma, F7425, Lot#078M4886V] 1:10000 dilution

Validation

These antibodies were used for both heterologous expression studies and mouse cardiomyocytes.

The alpha1C antibody was custom-made by YenZym and has been used for more than a decade in the laboratory. It has been validated using heterologous expression studies.

The anti-beta antibody was also custom-made by YenZym and has been used for more than a decade in my laboratory. We have validated this antibody using heterologous expression studies. Antibodies were also validated in our previous work (Yang et al 2019).

The anti-j-tropin antibody and anti-FLAG antibody were validated using heterologous expression studies, by their manufacturer and in the case of anti-FLAG antibody using transgenic mice created in the laboratory.

- Anti-V5 Tag Monoclonal Antibody - validated by 310 published papers for W8 and 70 published papers for Immunofluorescence
- Anti-iph2 Antibody - validated by Invitrogen and published paper
- Anti-CaMulin Antibody - validated by Millipore Sigma and also multiple published papers
- Anti-RyR2 Antibody - validated by multiple published papers and heterologous expression studies
- Anti-Kv1.5 Antibody - validated by Alomone Laboratory and multiple published paper
Eukaryotic cell lines

Policy information about cell lines

**Cell line source(s)**

HEK293T cells (human, ATCC, CRL-3216) for electrophysiology; HEK293 (human, ATCC, CRL-1573) for FRET were purchased directly from ATCC.

**Authentication**

Cell lines are authenticated by ATCC.

**Mycoplasma contamination**

All cell lines were tested negative for mycoplasma.

**Commonly misidentified lines**

No commonly misidentified cell lines were used.

Animals and other organisms

Policy information about studies involving animals: ARRIVE guidelines recommended for reporting animal research

**Laboratory animals**

Mice, mixed strain, ages 5 weeks - 4 months, male and female. Description of research mice used for experiments can be found in the relevant figure legends and Methods.

**Wild animals**

The study did not involve wild animals

**Field-collected samples**

The study did not involve samples collected from the field

**Ethics oversight**

Columbia University IACUC

Note that full information on the approval of the study protocol must also be provided in the manuscript.