# Supporting Information

## Subramanyam et al. 10.1073/pnas.1308161110

### SI Materials and Methods

**Molecular Biology: Cloning Strategies for All Expression Plasmids Used.** *Generation of intein-flanked $\alpha_{1C}$ constructs in pcDNA3.1.* Plasmids containing the DnaE split inteins (DnaE-n and DnaE-c) were a generous gift from Tom W. Muir (Princeton University, Princeton, NJ). The split inteins were originally cloned from the cyanobacteria *Nostoc punctiforme* PCC-73102 (http://tools.neb.com/inbase/) (1–3). Amino acid sequence of DnaE-n and DnaE-c are shown in Fig. S1.

To create $_{CFP}[I–II]_{N\text{-intein}}$ we first used overlap extension PCR to fuse a 450 bp fragment (Table S3, primers 1 and 2,) of $\alpha_{1C}$[bungarotoxin-binding site, BBS]–YFP[21] (stretching from a unique EcoRI site to residue Pro871 in the middle of the II–III loop) upstream of a 342 bp fragment (primers 3 and 4) comprising the *N. punctiforme* DnaE-n sequence and a unique C-terminal XbaI site. The resulting 760 bp final fragment (containing 435 and 325 bp from $\alpha_{1C}$ and DnaE-n plasmids, respectively) was cloned into $\alpha_{1C}$[BBS]–YFP in the plasmid construct pcDNA3 using EcoRI and XbaI to yield $[I–II]_{N\text{-intein}}$. A second overlap extension PCR was used to fuse a 748 bp fragment (primers 5 and 6) of CFP with a unique N terminus HindIII site upstream of the first 449 bp of $\alpha_{1C}[I–II]_{N\text{-intein}}$ (primers 7 and 8) including a unique C terminus ClaI site. The resulting 1,167 bp fragment (containing 734 and 433 bp from CFP and $\alpha_{1C}[I–II]_{N\text{-intein}}$, respectively) was cloned into $\alpha_{1C}[I–II]_{N\text{-intein}}$ in pcDNA3 using HindIII and ClaI sites, yielding $_{CFP}[I–II]_{N\text{-intein}}$ in pcDNA3.

To create $_{C\text{-intein}}[III–IV]_{YFP}$, we used overlap extension PCR to fuse a 141 bp fragment (primers 9 and 10) comprising the *N. punctiforme* DnaE-c cDNA sequence with a unique N-terminal KpnI upstream of a 947 bp fragment (primers 11 and 12) from $\alpha_{1C}$[BBS]–YFP starting at Glu873 in the II–III loop to a unique C-terminal Tth111I site. The resulting 1,062 bp fragment (containing 130 and 932 bp from DnaE-c and $\alpha_{1C}$, respectively) was cloned into $\alpha_{1C}$[BBS]–YFP in pcDNA3 using KpnI and Tth111I sites yielding $_{C\text{-intein}}[III–IV]_{YFP}$ in pcDNA3. We used overlap extension PCR (primers 13–16) to introduce T1066Y and Q1070M mutations into $_{C\text{-intein}}[III–IV]_{YFP}$ to generate $_{C\text{-intein}}[III–IV^{TQ/YM}]_{YFP}$.

***Generation of intein-flanked $\alpha_{1C}$ constructs in adenoviral vector plasmids.*** Sequences for $_{CFP}[I–II]_{N\text{-intein}}$ (primers 17 and 18), $_{C\text{-intein}}[III–IV]_{YFP}$ (primers 19 and 20), and $_{C\text{-intein}}[III–IV^{TQ/YM}]_{YFP}$ (primers 19 and 20) were amplified by PCR and cloned into pShuttle-CMV vector (Stratagene) using KpnI/XhoI sites. Shuttle vectors were linearized with PmeI and electroporated into BJ5183-AD-1 electrocompetent cells (pretransformed with the pAdEasy-1 viral plasmid; Stratagene). Transformants with successful recombination were confirmed by restriction digestion using PacI. Positive recombinants were amplified using recombination-deficient XL-10-Gold bacterial strain. Purified recombinant adenoviral plasmid DNA is then digested with PacI to expose the inverted terminal repeats (ITRs) before transfection into AD-293 cells using calcium phosphate DNA precipitation.

To generate split-intein $\alpha_{1C}$ III–IV domain fragments that did not contain fused YFP, we PCR-amplified $_{C\text{-intein}}[III–IV]$ and $_{C\text{-intein}}[III–IV^{TQ/YM}]$ (primers 21 and 22) and included a stop codon after the coding sequence. The PCR products were cloned into pAdEasy dsRed Express using In-Fusion reaction according to the manufacturer's instructions (Clontech). The completed In-Fusion reaction was used to transform competent Stellar *Escherichia coli*, and recombinant clones identified by restriction digestion with XhoI. Plasmid DNA from positive clones were digested with PacI to expose viral ITRs and transfected into HEK293 cells to generate replication-deficient adenoviruses.

***Generation of adenoviruses.*** HEK cells cultured in 60 mm diameter dishes at 70–80% confluency were transfected with *Pac*I-digested linearized adenoviral DNA. Transfected plates were monitored for cytopathic effects (CPEs) and adenoviral plaques. Cells were harvested and subjected to three consecutive freeze-thaw cycles, followed by centrifugation (3,900 × *g*) to remove cellular debris. The supernatant (2 mL) was used to infect a 10 cm dish of 90% confluent HEK293 cells. Following observation of CPEs after 2–3 d, cell supernatants were used to reinfect a new plate of HEK293 cells. Viral expansion and purification was carried out as previously described (4). Briefly, confluent HEK293 cells grown on 15 cm culture dishes were infected with viral supernatant (1 mL) obtained as described above. We used 8–10 plates per preparation. After 48 h, cells from all of the plates were harvested, pelleted by centrifugation, and resuspended in 8 mL of buffer containing (in mM) Tris·HCl 20, $CaCl_2$ 1, and $MgCl_2$ 1 (pH 8.0). Cells were lysed by four consecutive freeze-thaw cycles and cellular debris pelleted by centrifugation. The virus-laden supernatant was purified on a cesium chloride (CsCl) discontinuous gradient by layering three densities of CsCl (1.25, 1.33, and 1.45 g/mL). After centrifugation (50,000 rpm; SW41Ti Rotor, Beckman-Coulter Optima L-100K ultracentrifuge; 1 h, 4 °C), a band of virus at the interface between the 1.33 and 1.45 g/mL layers was removed and dialyzed against PBS (12 h, 4 °C).

**Transfection of HEK293 Cells Using Calcium Phosphate–DNA Precipitation.** Briefly, plasmid DNA was mixed with 62 μL of 2.5M $CaCl_2$ and sterile deionized water (to a final volume of 500 μL). The mixture was added dropwise, with constant tapping to 500 μL of 2× Hepes buffered saline (HBS) (in mM: Hepes 50, NaCl 280, $Na_2HPO4$ 1.5, pH 7.09). The resulting DNA–calcium phosphate mixture was allowed to sit for 20 min, followed by dropwise addition to confluent HEK cells. Cells were washed with serum-free DMEM after 8 h and maintained in supplemented DMEM.

**Culture of Primary Adult Rat Ventricular Myocytes.** Primary cultures of adult rat heart ventricular cells were prepared as previously described (4, 5). Adult male Sprague–Dawley rats were killed with an overdose of halothane in accordance with the guidelines of Columbia University Animal Care and Use Committee. Hearts were excised and ventricular myocytes isolated by enzymatic digestion with 1.7 mg Liberase–TM enzyme mix (Roche) using a Langendorff perfusion apparatus. Healthy rod-shaped myocytes were cultured in Medium 199 (Life Technologies) supplemented with (in mM) carnitine (5), creatine (5), taurine (5) penicillin-streptomycin-glutamine (0.5%, Life technologies), and 5% (vol/vol) FBS (Life Technologies) to promote attachment to dishes. After 5 h, the culture medium was switched to Medium 199 with 1% (vol/vol) serum, but otherwise supplemented as described above. Cultures were maintained in humidified incubators at 37 °C and 5% $CO_2$.

**Quantum Dot Labeling of Cell Surface BBS-tagged L-Type Calcium Channels.** Transfected HEK293 cells or fixed cardiomyocytes were used for this assay. At 48 h posttransfection or postinfection, cells on 35 mm MatTek dishes (MatTek Corporation) were gently washed with PBS with $Ca^{2+}$ and $Mg^{2+}$ (in mM: 0.9 $CaCl_2$, 0.49 $MgCl_2$, pH 7.4) and then incubated for 30 min in blocking medium [DMEM with 3% (wt/vol) BSA]. Cells were then incubated with 1 μM biotinylated α-bungarotoxin (BTX; Life Technologies) in DMEM/3% BSA at room temperature (RT) for 1 h followed by washing twice with DMEM/3% BSA to

remove unbound biotinylated BTX. Cells were then incubated with 10 nM streptavidin-conjugated quantum dot $(QD)_{655}$ (Life Technologies) for 1 h at 4 °C in the dark.

**Immunofluorescence Staining.** Primary cultured myocytes infected with viruses encoding appropriate constructs were used in this assay. At 48 h postinfection, myocytes were fixed with a 4% (wt/vol) solution of paraformadehyde in PBS for 20 min at RT. Cells were washed twice with PBS for 10 min each, followed by incubation in PBS with 0.1 M glycine (10 min, RT) to block free aldehyde groups. Fixed cells were permeabilized using PBS supplemented with 0.2% Triton X-100 (20 min). Nonspecific binding was blocked by incubation of the cells in PBS with 0.1% Triton, 3% (vol/vol) normal goat serum (NGS), and 1% (wt/vol) BSA (1 h, RT). Cells were subsequently incubated with primary antibodies in PBS with 0.1% Triton, 1% NGS, and 1% BSA (4 h, RT). Cells were washed three times for 10 min each with PBS with 0.1% Triton followed by staining with fluorescent secondary antibodies (1 h, RT). Antibody dilutions were prepared in PBS with 0.1% Triton, 1% NGS, and 1%BSA. The cells were then washed with PBS/0.1% Triton. Cells were imaged immediately in the same buffer.

**Confocal Laser Scanning Microscopy: Laser Lines and Detector Settings.** The following laser lines and detector settings were used: CFP, 405 nm diode laser, 465–502 nm detection; Alexa 488, 488 nm Ar ion, 500–540 nm detection; YFP, 514nm Ar ion, 520–560 nm detection; Alexa 555, 543 nm HeNe, 550–600 nm detection; and $QD_{655}$, 405 nm diode laser, 620–700 nm detection. Laser power was set at 30% and detector gain at 550–700 V to reduce noise and prevent saturation of image pixels. Pinhole size was set at 1 Airy unit.

**Intracellular Calcium Transient Measurements.** Primary myocytes in culture were loaded with rhod2–AM (5 μM with 0.05% Pluronic F127 detergent) in Tyrode solution (containing in mM: NaCl 138, KCl 4, CaCl2 2, MgCl2 1, $NaH_2PO_4$ 0.33, and Hepes 10) for 20 min at RT. Cells were then washed and maintained in Tyrode. The Leica SP2 microscope equipped with a 63×, oil immersion, N.A. 1.4 objective was used for confocal linescan imaging. The electronic zoom was adjusted to fit the cells, and the scan field was rotated to be along the long axis of the cells. Static bright-field and fluorescent images were taken before measuring intracellular $Ca^{2+}$ release. Rhod-2 was excited at 543 nm and emission detected between 550–600 nm. Cells were paced at 1 Hz using a Myopacer EP (Ion Optix). Confocal linescan frequency was set at 400 Hz and cells were scanned for 20 s.

**Electrophysiology.** Whole-cell recordings were conducted at RT on transfected HEK293 cells 48 h posttransfection using an EPC-8 patch clamp amplifier (HEKA Electronics) controlled by PULSE software (HEKA). Micropipettes were prepared from 1.5 mm thin-walled glass (World Precision Instruments) using a P97 microelectrode puller (Sutter Instruments). The internal solution contained (in mM) cesium methanesulfonate (135), cesium chloride (5), EGTA (5), $MgCl_2$ (1), MgATP (4, added fresh), and Hepes (10, pH 7.4). When filled with internal solution, the resistance of the pipette was typically 1.7–2.5 MΩ. External solution contained (in mM) tetraethylammonium-methanesulfonate (140), $BaCl_2$ (5), and Hepes (10, pH 7.4). There was no electronic series resistance compensation. Currents were sampled at 25 KHz and filtered at 10 KHz. Traces were acquired at a repetition interval of 6 s. Leak and capacitive currents were subtracted using a P/8 protocol.

For whole-cell recordings of cultured rat ventricular myocytes, microelectrodes typically had a resistance of 1–2 MΩ when filled with an internal solution containing (in mM) cesium-methanesulfonate (150), EGTA (10), CsCl (5), $MgCl_2$ (1), MgATP (4, added fresh), and Hepes (10). For formation of gigaohm seals and initial break-in to the whole-cell configuration, cells were perfused with normal tyrode solution containing (in mM) NaCl (138), KCl (4), $CaCl_2$ (2), $MgCl_2$ (1), $NaH_2PO4$ (0.33), and Hepes (10, pH 7.4). Following successful break-in, the perfusing medium was switched to an external recording solution containing (mM) *N*-methy-D-glucamine-aspartate (155), 4-aminopyridine (10), $MgCl_2$ (1), $BaCl_2$ (5), and Hepes (10, pH 7.4 adjusted with *N*-methyl-D-glucamine). Signals were filtered at 2 KHz and sampled at 25 KHz. Data traces were acquired at a repetition interval of 45 s. Leak and capacitive currents were subtracted using a P/8 protocol.

**Western Blotting.** HEK293 cells and adult rat ventricular myocytes were harvested in lysis buffer containing (in mM) Tris (20, pH 7.4), EDTA (1), NaCl (150), 1% (wt/vol) SDS, 0.1% Triton X-100, and supplemented with protease inhibitor mixture (10 μL/mL, Sigma-Aldrich). Cells were lysed with three pulses of sonication for 10 s each. Total protein concentration in cell lysates was determined using the bis-cinchonic acid protein estimation kit (Pierce Technologies). Lysates were boiled (95 °C, 10 min) after addition of sample buffer [50 mM Tris, 10% (vol/vol) glycerol, 2% SDS, 100 mM DTT, and 0.2 mg/mL bromophenol blue]. Proteins were resolved on a 4–12% Bis·Tris gradient precast gel (Life Technologies) in Mops-SDS running buffer (Life Technologies) at 200 V constant for 1 h. We loaded 15 μL of the Precision Plus All Blue molecular weight standard (10–250 kDa, BioRad) alongside the samples. Protein bands were transferred by tank transfer onto a nitrocellulose membrane (2.5 h, 4 °C, 30 V constant) in transfer buffer (25 mM Tris pH 8.3, 192 mM glycine, 15% (vol/vol) methanol, and 0.1% SDS). The membranes were blocked with a solution of 5% nonfat milk (BioRad) in tris-buffered saline-tween (TBS-T) (25 mM Tris pH 7.4, 150 mM NaCl, and 0.1% Tween-20) for 1 h at RT and then incubated overnight at 4 °C with primary antibodies in blocking solution. The blots were washed with TBS-T three times for 10 min each and then incubated with secondary horseradish peroxidase-conjugated antibody for 1 h at RT. After washing in TBS-T, the blots were developed with a chemiluminiscent detection kit (Pierce Technologies) and then visualized on a gel imager.


1. Perler FB, et al. (1994) Protein splicing elements: Inteins and exteins—a definition of terms and recommended nomenclature. *Nucleic Acids Res* 22(7):1125–1127.
2. Iwai H, Züger S, Jin J, Tam PH (2006) Highly efficient protein trans-splicing by a naturally split DnaE intein from Nostoc punctiforme. *FEBS Lett* 580(7):1853–1858.
3. Zettler J, Schütz V, Mootz HD (2009) The naturally split Npu DnaE intein exhibits an extraordinarily high rate in the protein trans-splicing reaction. *FEBS Lett* 583(5): 909–914.
4. Colecraft HM, et al. (2002) Novel functional properties of Ca(2+) channel beta subunits revealed by their expression in adult rat heart cells. *J Physiol* 541(Pt 2):435–452.
5. Xu X, Colecraft HM (2009) Primary culture of adult rat heart myocytes. *J Vis Exp*, 10.3791/1308.

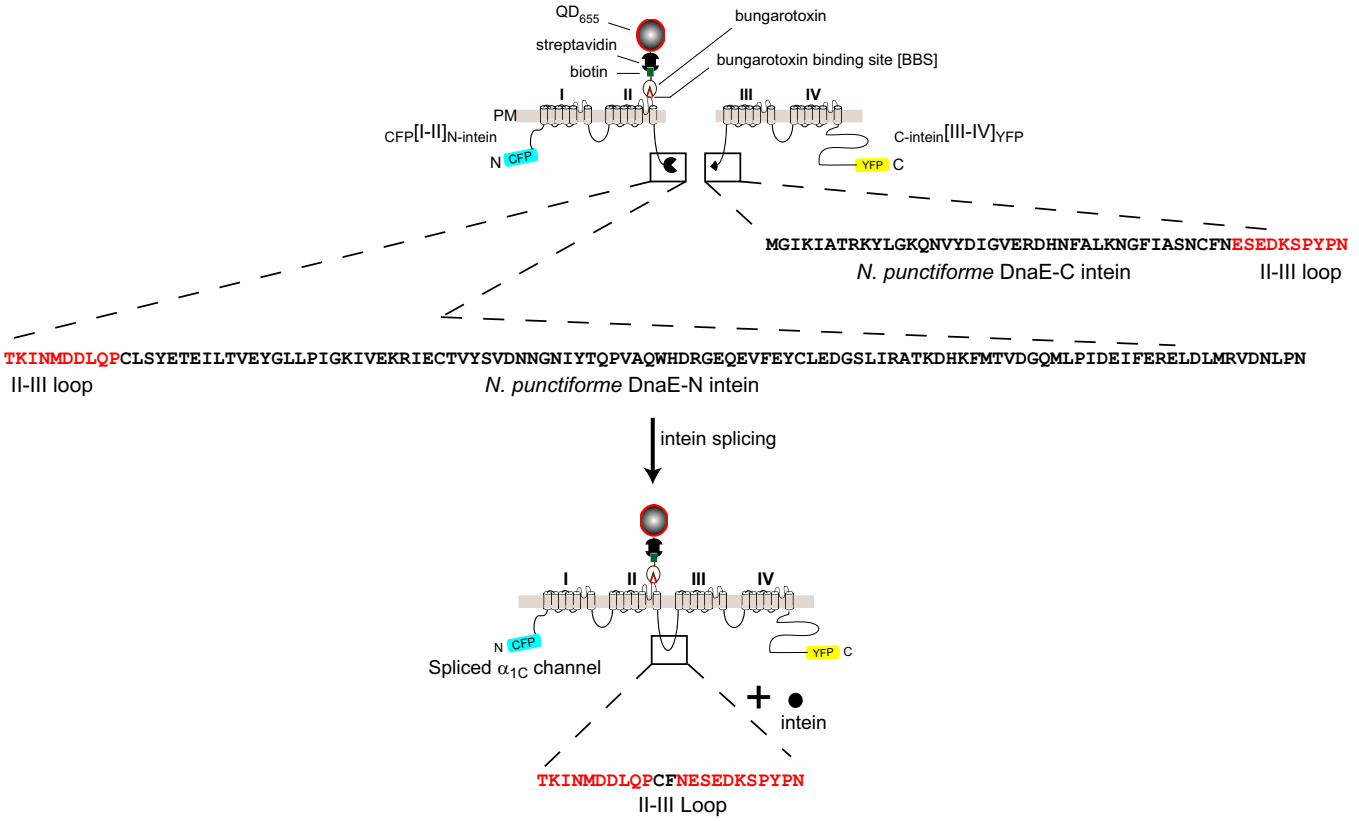


**Fig. S1.** Design of the intein-flanked $\alpha_{1C}$ moieties. (*Upper*) Depiction of the CFP- and YFP-tagged $\alpha_{1C}$ halves with location of the extracellular BBS tag and precise intein splice site within the II–III loop. Intein amino acid sequence (black) and II–III loop at splice site (red) is given. (*Lower*) Postsplicing inteins facilitate joining of $\alpha_{1C}$ halves, leaving three intein residues—C,F, and N—in the spliced product. Taking advantage of an $\alpha_{1C}$ asparagine residue at the split site, construct design ensures only two foreign residues are left behind: C and F.

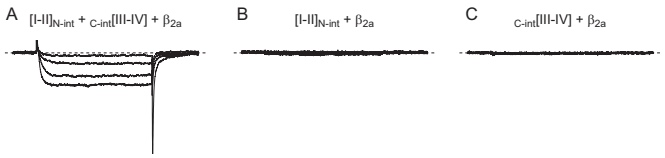


**Fig. S2.** (*A*) Exemplar whole-cell current traces and gating charge movement in cells expressing intein-spliced $\alpha_{1C}$. (*B* and *C*) Cells expressing either $_{CFP}[I–II]_{N\text{-intein}}$ or $_{C\text{-intein}}[III–IV]_{YFP}$ separately do not exhibit either currents or gating charge movement. $n = 3$ for each condition.

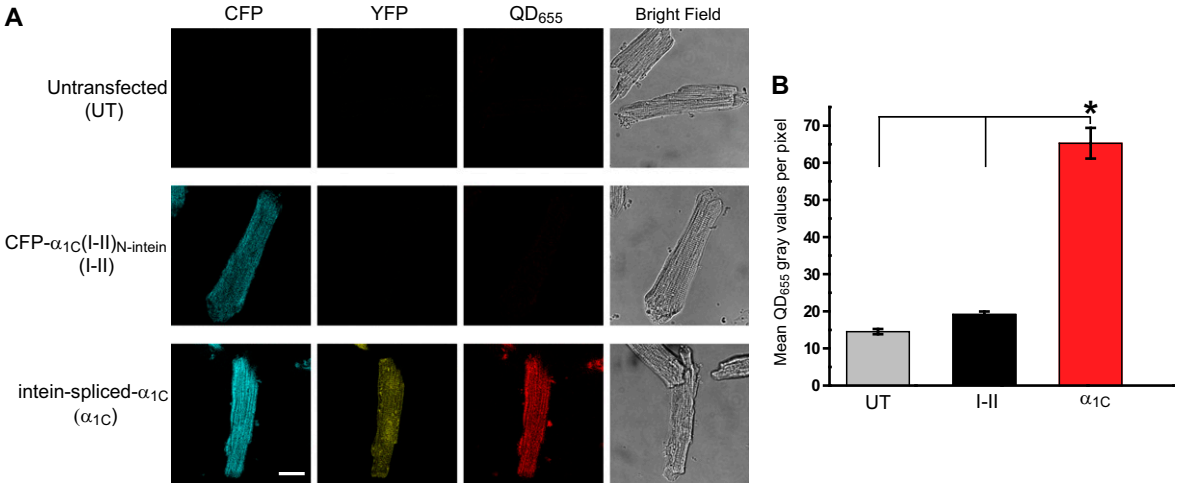


**Fig. S3.** Surface trafficking of intein-spliced $\alpha_{1C}$ subunits in cardiomyocytes. (*A*) Confocal images of myocytes depicting bright-field images, CFP/YFP fluorescence, and $QD_{655}$ surface staining of extracellular BBS tag present in intein-spliced $\alpha_{1C}$ channel moieties. Uninfected control myocytes (*Top*, UT) and myocytes expressing either $_{CFP}[\alpha_{1C}$ I–II$]_{N\text{-intein}}$ (*Middle*, I–II) or intein-spliced $\alpha_{1C}$ (*Bottom*, $\alpha_{1C}$) are shown. $QD_{655}$ signal is negligible in uninfected myocytes and those expressing only the N-terminal moiety but is very robust in myocytes expressing intein-spliced $\alpha_{1C}$. (Scale bar, 20 μm.) (*B*) Quantitation of $QD_{655}$ signal shown in *A*. Mean gray values per pixel were calculated for each image acquired in $QD_{655}$ channel for a population of 15 cells from each condition. Myocytes expressing intein-spliced $\alpha_{1C}$ showed significantly higher $QD_{655}$ signal. Error bars, SEM; * significantly different from UT and I–II using one-way ANOVA [$F(2, 50) = 108.9$, $P = 1.02E\text{-}16$] and Bonferonni pairwise comparisons. $n = 15$ cells for each condition.

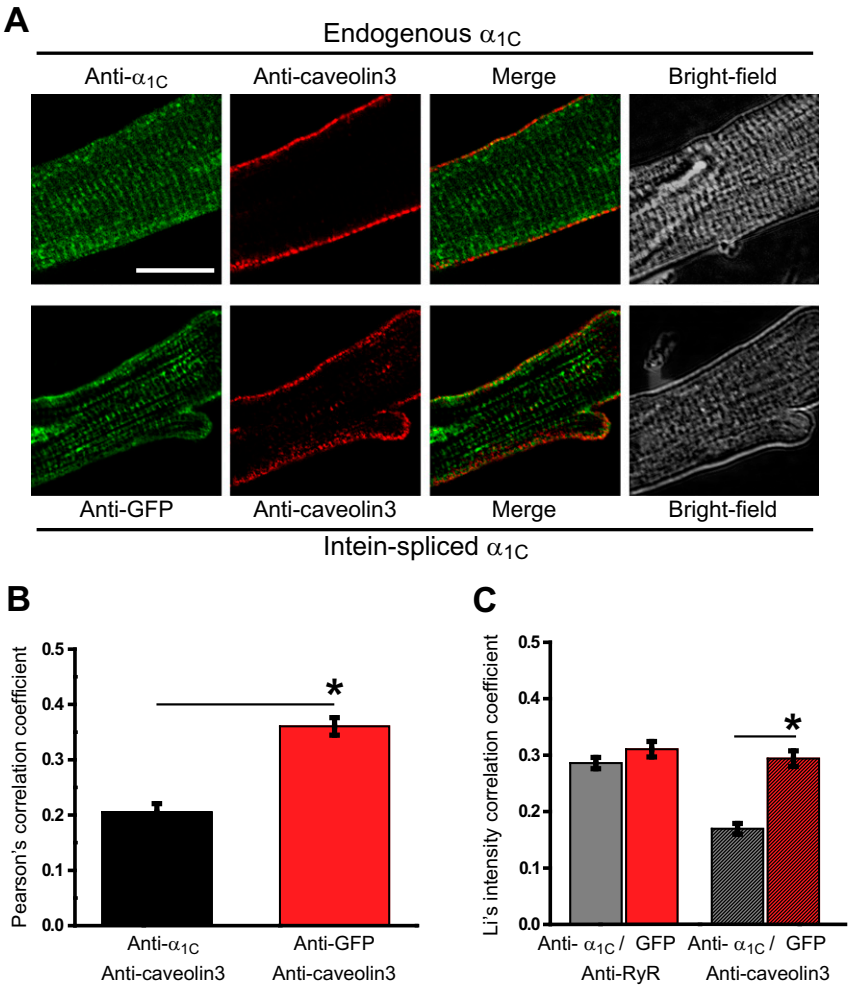


**Fig. S4.** Colocalization of intein-spliced $\alpha_{1C}$ with caveolin-3 in cardiac myocytes. (*A*, *Upper*) Confocal images of cardiac myocytes depicting colocalization of endogenous $\alpha_{1C}$ (anti-$\alpha_{1C}$, green) and endogenous caveolin-3 (anti–caveolin-3, red). (*A*, *Lower*) Significant $\alpha_{1C}$ /caveolin-3 colocalization is also found in myocytes expressing intein-spliced $\alpha_{1C}$ (anti-GFP, green). (Scale bar, 20 μm.) (*B*) Quantitation of colocalization shown in *A*. Colocalization coefficient of pixels in the green ($\alpha_{1C}$) and red (caveolin-3) channels was calculated using the JaCoP plugin in ImageJ from images acquired from 20 individual cells. Pearson's coefficient data depict robust $\alpha_{1C}$/caveolin-3 colocalization between control (uninfected) and recombinant (expressing intein-spliced $\alpha_{1C}$) myocytes. *P6E-11 through unpaired *t* test. (*C*) Quantitation of $\alpha_{1C}$/ryanodine receptor (RyR) (shown in Fig. 4*D*) and $\alpha_{1C}$/caveolin (shown here in *A* and *B*) as measured through Li's intensity correlation coefficient using the JaCoP plugin on the same images analyzed in Fig.4 *D* and *E* and Fig. S3 *A* and *B*). Similar results to that obtained through Pearson's correlation corroborate strong colocalization between $\alpha_{1C}$/RyR and $\alpha_{1C}$/caveolin-3 in both uninfected (control) and adenovirally infected (expressing intein-spliced $\alpha_{1C}$) myocytes. *P1.2E-10 through unpaired *t* test.

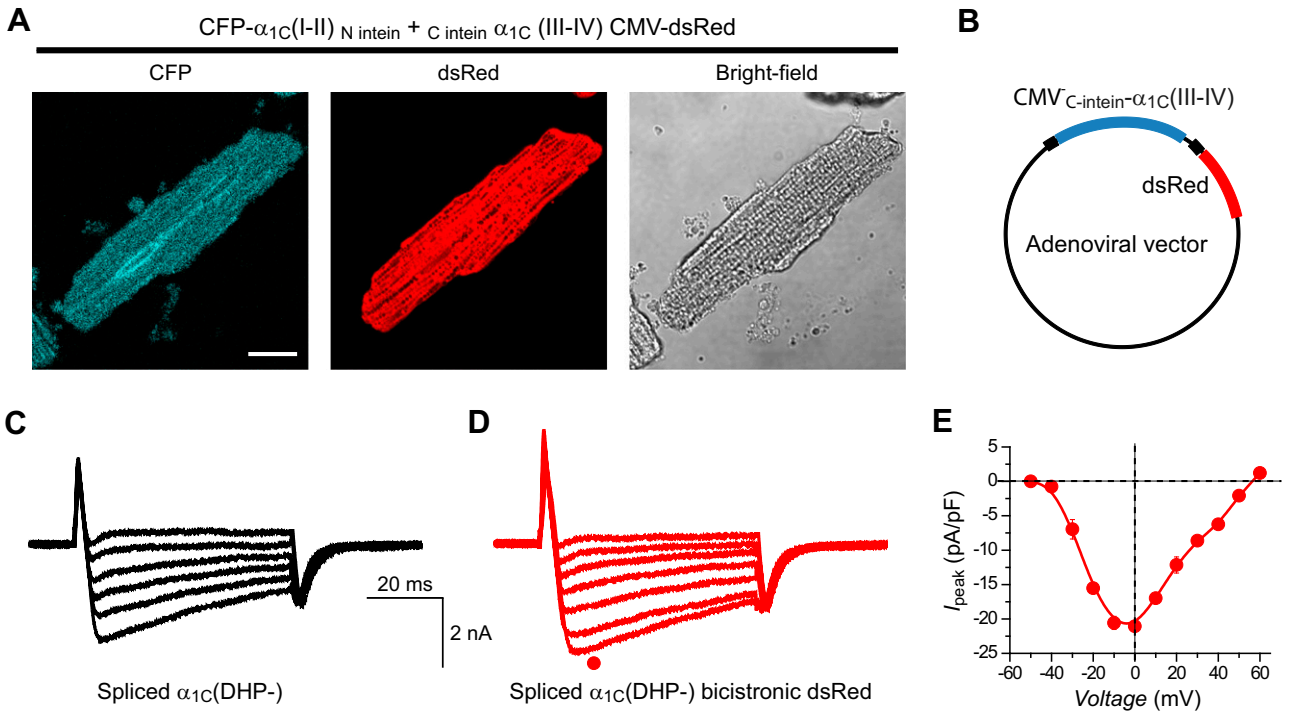


**Fig. S5.** Characterization of intein-spliced dihydropyridine (DHP)-insensitive $\alpha_{1C}$ expressed in bicistronic vectors in cardiac myocytes. (*A*) Confocal images of myocytes expressing intein-spliced CFP-tagged intein-spliced $\alpha_{1C}$ and dsRed from a bicistronic vector. (Scale bar, 20 μm.) (*B*) Construction of the $_{C\text{-intein}}\alpha_{1C}$III–IV_dsRed biscistronic vector. dsRed expression is under the control of a separate CMV promoter. (*C*) Exemplar current traces from cells expressing intein-spliced CFP/YFP-tagged DHP-insensitive $\alpha_{1C}$, $n = 8$. (*D*) Exemplar current traces and (*E*) population current-voltage (*J–V*) relationships from myocytes expressing intein-spliced DHP-insensitive $\alpha_{1C}$ channels ($n = 7$) with bicistronic dsRed. Intein-spliced DHP– $\alpha_{1C}$ derived from bicistronic dsRed constructs exhibit large whole-cell $Ba^{2+}$ current in presence of Nifedipine. Similar current traces, *C* and *D* indicate that YFP fusion on C terminus of the channels does not affect function.

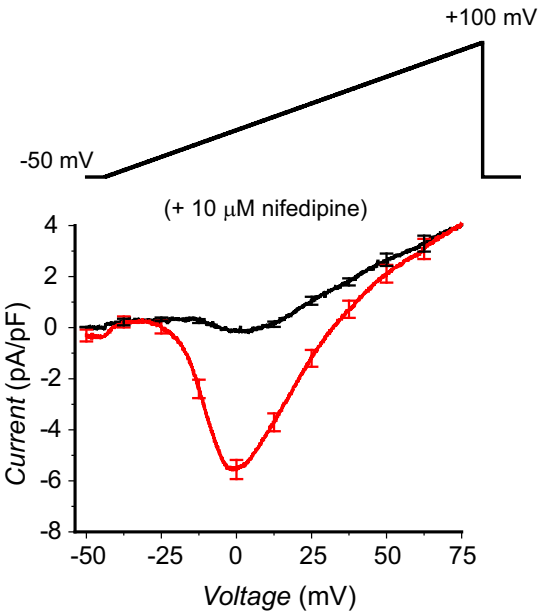


**Fig. S6.** Nifedipine-based separation of WT and DHP-resistant $\alpha_{1C}$ in cardiac myocytes expressing intein-spliced $\alpha_{1C}$ by using a –50 mV holding potential and 2 mM $Ca^{2+}$ as charge carrier. Under these conditions, WT $Ca_V1.2$ whole-cell currents elicited by a ramp protocol were deeply inhibited by 10 μM nifedipine (black trace, $n = 4$), whereas myocytes expressing intein-spliced DHP-resistant $\alpha_{1C}$ (red trace, $n = 3$) displayed a sevenfold larger remnant current.

**Table S1. Gating parameters for WT and intein-spliced $Ca_V1.2$ channels**

| Channel type | $G$ pApF$^{-1}$·mV$^{-1}$ | $V_{0.5}$ mV | $k$ mV | $n$ |
|---|---|---|---|---|
| $\alpha_{1C} + \beta_{2a}$ | 0.58 ± 0.21 | −11.20 ± 1.06 | 5.67 ± 0.3 | 7 |
| Spliced WT $\alpha_{1C} + \beta_{2a}$ | 0.48 ± 0.15 | −11.99 ± 1.10 | 6.99 ± 1.00 | 7 |
| Spliced $\alpha_{1C}$[TQ/YM] + $\beta_{2a}$ | 0.52 ± 0.11 | −9.66 ± 0.82 | 5.5 ± 0.16 | 11 |

I–V relations were fit to the following equation:

$$I = G(V - V_{rev})\frac{1}{1 + exp(\frac{V_{0.5} - V}{k})},$$

where $I$ is the whole-cell current density (pA/pF), $G$ is the specific conductance (pA pF-1 mV-1), $V_{rev}$ is the reversal potential (mV), $V_{0.5}$ is the voltage of half-maximal activation (mV), and $k$ is a slope factor (mV).

**Table S2. Accession numbers of cDNAs**

| GenBank ID | Gene name | Accession no. |
|---|---|---|
| $\alpha_{1C}$($Ca_V1.2$) rabbit cardiac | CACNA1C/CACH2C | X15539.1 |
| β2a rat cardiac/brain | Cacnb2 | M80545.1 |
| *N. punctiforme* PCC73102 DnaE-n intein | Npun_F4872 | ZP_00111398.2 |
| *N. punctiforme* PCC73102 DnaE-c intein | Npun_F5684 | ZP_00108882.1 |

**Table S3. List and sequences of cloning primers**

| Primer no. | Sequence 5′–3′ | Included restriction or in-fusion site |
|---|---|---|
| 1 | ACCCGGAGGAGCACGTTCGAC | |
| 2 | TTCATAGCTTAAACAGGGCTGGAGGTCATCCATGTTGATCTTGGTG | |
| 3 | TGGATGACCTCCAGCCCTGTTTAAGCTATGAAACGGAAATATTG | |
| 4 | AAACGGGCCCTCTAGATTAATTCGGCAAATTATC | XbaI |
| 5 | ATATAAGCTTGCCACCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTG | HindIII |
| 6 | TCGAAGCATTGTCAGCTTGTACAGCTCGTCCATGCCGAGAGTGATC | |
| 7 | GACGAGCTGTACAAGCTGACAATGCTTCGAGCCCTTGTTCAGCCAG | |
| 8 | ATGGGGTTCTTCAGGGTGAGG | |
| 9 | ATATGGTACCATGGGAATCAAAATAGCCACACG | KpnI |
| 10 | CTTATCCTCACTCTCATTGAAACAATTAGAAGCTATGAAGCCATT | |
| 11 | TCTAATTGTTTCAATGAGAGTGAGGATAAGAGTCCCTACCCCAAC | |
| 12 | ACTCGGTAGTTGTAGATAGGG | |
| 13 | ATATGGTACCATGGGAATCAAAATAG | KpnI |
| 14 | CATCAGCAGCGTGTAGACAATCACGATGTTCCCAATGGTCCGGATG | |
| 15 | TACACGCTGCTGATGTTCATGTTCGCCTGCATCGGAGTCCAGCTC | |
| 16 | AGCAGGATGAGGACGAACATCAGG | |
| 17 | ATATGGTACCGCCACCATGGTGAGCAAGGG | KpnI |
| 18 | ATATCTCGAGTTAATTCGGCAAATTATCAACCCG | XhoI |
| 19 | ATATGGTACCGCCACCATGGGAATCAAAATAGCCAC | KpnI |
| 20 | ATATCTCGAGTTACTTGTACAGCTCGTCCATG | XhoI |
| 21 | GTAACTATAACGGTCGCCACCATGGGAATCAAAATAGCCACACG | In-fusion, GTAACTATAACGGTC |
| 22 | ATTACCTCTTTCTCCTTAAGACAGGCTGCTGACGCCGGCCCTGCGGTCC | In-fusion, ATTACCTCTTTCTCC |