

Molecular Determinants of Calpain-dependent Cleavage of Junctophilin-2 Protein in Cardiomyocytes*

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Background: Down-regulation of junctophilin-2 is associated with a variety of cardiac diseases.

Results: Junctophilin-2 is cleaved by calpain at multiple sites, resulting in dysfunctional junctophilin-2 truncations.

Conclusion: Calpain-mediated proteolysis contributes to posttranslational down-regulation of junctophilin-2.

Significance: These data reveal, for the first time, the detailed molecular determinants responsible for calpain proteolysis of junctophilin-2.

Junctophilin-2 (JP2), a membrane-binding protein that provides a structural bridge between the plasmalemma and sarcoplasmic reticulum, is essential for precise Ca²⁺-induced Ca²⁺ release during excitation-contraction coupling in cardiomyocytes. In animal and human failing hearts, expression of JP2 is decreased markedly, but the molecular mechanisms underlying JP2 down-regulation remain incompletely defined. In mouse hearts, ischemia/reperfusion injury resulted in acute JP2 down-regulation, which was attenuated by pretreatment with the calpain inhibitor MDL-28170 or by transgenic overexpression of calpastatin, an endogenous calpain inhibitor. Using a combination of computational analysis to predict calpain cleavage sites and *in vitro* calpain proteolysis reactions, we identified four putative calpain cleavage sites within JP2 with three N-terminal and one C-terminal cleavage sites. Mutagenesis defined the C-terminal region of JP2 as the predominant calpain cleavage site. Exogenous expression of putative JP2 cleavage fragments was not sufficient to rescue Ca²⁺ handling in JP2-deficient cardiomyocytes, indicating that cleaved JP2 is non-functional for normal Ca²⁺-induced Ca²⁺ release. These data provide new molecular insights into the posttranslational regulatory mechanisms of JP2 in cardiac diseases.

Myocardial infarction, one of the most common causes of heart failure, is characterized by defects in cardiac excitation-contraction (E-C)² coupling (1, 2). In a normal cardiomyocyte,

E-C coupling depends on Ca²⁺-induced Ca²⁺ release, in which L-type Ca²⁺ channel-mediated Ca²⁺ influx triggers Ca²⁺ release from the sarcoplasmic reticulum (SR) via type 2 ryanodine receptors (RyR2) (3, 4). L-type Ca²⁺ channels and RyR2s are physically and functionally organized into a tightly regulated structure known as the Ca²⁺ release unit, which provides the structural basis for Ca²⁺-induced Ca²⁺ release (4, 5). The integrity of the Ca²⁺ release unit is maintained by the structural protein junctophilin-2 (JP2) that bridges the T-tubule membrane and the SR (6–8). Disruption of the fine architecture of the E-C coupling machinery impairs Ca²⁺-induced Ca²⁺ release, thereby leading to loss of contractility and heart failure (9).

JP2, the major junctophilin isoform expressed in the heart, contains eight N-terminal membrane occupation and recognition nexus (MORN) domains that mediate interactions with the plasmalemma, a space-spanning α helix, and a C-terminal transmembrane (TM) domain that anchors JP2 to the SR membrane (6). Consistent with a critical role for JP2 in E-C coupling, conditional silencing of JP2 in cardiomyocytes results in contractile dysfunction, abnormal Ca²⁺ handling, and acute heart failure (7, 10, 11). On the contrary, cardiac-specific overexpression of JP2 attenuated the development of heart failure induced by pressure overload (12). Moreover, mutations in the JP2 coding region have been discovered in patients with hypertrophic cardiomyopathy (13, 14). The pathological relevance of JP2 has been revealed by observations that dysregulation of JP2 protein is associated with pathological progression in multiple models of heart failure, including pressure overload-induced hypertrophy/heart failure and myocardial infarction (15–21). Toward understanding the mechanism of JP2 down-regulation, a recent report has demonstrated that JP2 is targeted by the microRNA miR-24, which may be responsible for the down-regulation of JP2 during long-term pressure overload-induced hypertrophy

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² The abbreviations used are: E-C, excitation-contraction; SR, sarcoplasmic reticulum; RyR2, type 2 ryanodine receptor; MORN, membrane occupation and recognition nexus; MORN, membrane occupation and recognition

nexus; KD, knockdown; TM, transmembrane; I/R, ischemia/reperfusion; T-tubule, transverse tubule; Z-LLY-FMK, benzyloxycarbonyl-LLY-fluoromethyl ketone; HF, heart failure; CAST, calpastatin; JP2, junctophilin-2.

and heart failure (19, 22). Recently, a Ca^{2+} -dependent mechanism of junctophilin proteolysis has been reported (23). More specifically, the activity of calpain, a family of Ca^{2+} -dependent proteases has been found to be related to the degradation of JP2 (21). However, it remains unclear whether JP2 is a direct substrate of calpain and the specific molecular site for JP2 proteolysis.

In this study, our objective was to determine whether and how JP2 expression is regulated posttranslationally in response to cardiac injury. We first demonstrated that JP2 expression is down-regulated in failing hearts from patients with ischemic heart disease. Using a murine model of ischemia/reperfusion (I/R) injury, we identified the Ca^{2+} -dependent protease calpain as a key mediator of JP2 down-regulation, including a comprehensive determination of the molecular sites for calpain cleavage. We also found that JP2 fragments corresponding to the primary cleavage site fail to rescue E-C coupling in JP2-deficient cardiomyocytes. These data provide important insights into the mechanism by which JP2 expression is lost in failing hearts.

Experimental Procedures

Human Heart Samples and Animal Studies—Samples from patients with ischemic heart disease were obtained from the University of Iowa Heart Failure Transplant Program. Rejected healthy donor hearts were obtained through the Donor Network. All human heart tissue samples were obtained under an organ research donation protocol that was approved by the Institutional Review Board of the University of Iowa. Animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication no. 85-23, revised 1996) and were approved by the Institutional Animal Care and Use Committee at the University of Iowa.

Cardiac-specific JP2 Knockdown (JP2-KD) was achieved with the use of transgenic mice conditionally expressing a JP2 shRNA (shJP2). Specifically, conditional shJP2 mice (provided by Dr. Wehrens) (7) were crossed with mice carrying the cardiac-specific α -myosin heavy chain promoter upstream of Cre (α MHC-Cre, also FVB-Tg(Myh6-cre)2182Mds/J, The Jackson Laboratory). The shJP2 mice were maintained on a C57BL/6 background for more than 10 generations. The α MHC-Cre mice were maintained on an FVB background. PCR was used to genotype the offspring. The near complete depletion of JP2 protein in JP2-KD (α MHC-Cre X shJP2) hearts has been reported recently (11). JP2-KD mice were sacrificed at 2 months of age for isolation and culture of ventricular cardiomyocytes as described below.

In Vitro and in Vivo I/R Protocols in Mouse Hearts—For *in vitro* I/R injury, hearts were excised rapidly from anesthetized mice and perfused with Krebs-Henseleit solution at 37 °C using a Langendorff apparatus at a constant pressure of 80 mm Hg with 95% O_2 , 5% CO_2 . After 20 min of equilibration, hearts were subjected to 35 min of global no-flow ischemia followed by 50 min of reperfusion. Some hearts were treated with MDL-28170 (10 μM) via perfusion 10 min before ischemia and after the onset of reperfusion. For *in vivo* I/R injury in mouse hearts, myocardial ischemia was produced by ligation of the left ante-

rior descending coronary artery for 20 min, followed by reperfusion for 30 min (longer periods of ischemia/reperfusion lead to a high death rate under our conditions). Left ventricles were dissected for analysis by Western blotting.

Molecular Cloning, Mutagenesis, and Adenovirus Construction—Wild-type JP2 cDNA was provided by Dr. Takeshima (6). FLAG and HA epitope tags were added to 5' and 3' ends, respectively, in the same reading frame of the coding sequence of JP2 cDNA by PCR. The dual epitope-tagged cDNA was cloned into the pCMV-XL5 plasmid under the control of a CMV promoter. cDNAs of JP2 truncations were cloned using PCR. QuikChange II (Agilent) was used for site-directed mutagenesis. Adenoviruses containing full-length JP2 (Ad-JP2), truncated JP2(1–565) (Ad-JP2(1–565)), or JP2(566-end) (Ad-JP2(566-end)) or empty Ad5 virus (Ad-empty) were produced by the University of Iowa Gene Transfer Vector Core. In the adenovirus construct, the full-length JP2 and truncation JP2(1–565) were tagged with FLAG at the N terminus and HA at the C terminus. The truncation JP2(566-end) was tagged with HA at the C terminus.

Adult Cardiomyocyte Culture, Cell Transfection, and Adenovirus Infection—Genecarrier-1 (Epoch Lab) was used to transfect plasmids into HEK293T cells. Isolation and culture of cardiomyocytes were performed as described previously (24). Adenoviruses were applied at a multiplicity of infection of 100, which resulted in transfection of almost all cells, as confirmed by immunostaining of epitope tags. Ca^{2+} imaging experiments were performed 40 h after adenoviral infection.

In Vitro Calpain-mediated Proteolysis Reaction—Mouse and human heart tissues as well as HEK 293T cells (obtained from the ATCC and cultured according to the protocol of the manufacturer) overexpressing tagged JP2 were washed with PBS and homogenized in lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, and 1% Triton X-100), followed by sonication. Homogenates were centrifuged for 10 min at 14,000 rpm at 4 °C. Protein content in the supernatant was determined using the BCA assay (Thermo Scientific). Cell extracts were diluted in ice-cold calpain reaction buffer (135 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , 10 mM glucose, 10 mM Hepes (pH 7.25), 2 μM lactacystin, and 10 mM 2-mercaptoethanol) to a concentration of 4 mg protein/ml. Just prior to starting the protease reaction, CaCl_2 was added to achieve a final concentration of 3 mM free Ca^{2+} (in the presence of EDTA), as calculated by winmaxc32 (Stanford University). For some experiments, the free Ca^{2+} concentration was adjusted to 500 μM , as calculated by winmaxc32. Purified human calpain I (Calbiochem) was added at a concentration of 1 mg of total protein/1 μg of calpain I. Reactions without Ca^{2+} or without purified calpain I were used as controls. Reactions were incubated at 30 °C for the indicated time and stopped by adding EDTA to a final concentration of 10 mM and incubating for 5 min at 95 °C. The reaction products were subjected to Western blotting.

Western Blot Analysis and Immunostaining—Western blotting and immunostaining of cardiomyocytes were performed as described previously (24) using antibodies to JP2 (catalog no. sc-51313, Santa Cruz Biotechnology) and epitope tags (anti-FLAG, catalog no. A00187, Genescript; anti-HA, catalog no. sc-805, Santa Cruz Biotechnology). Immunofluorescence was

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imaged by using a confocal microscope (Carl Zeiss MicroImaging Inc.).

Confocal Ca^{2+} Imaging—Cells were loaded with Rhod-2 AM at 37 °C for 20 min, followed by washing with Tyrode's solution at room temperature for 15 min before Ca^{2+} imaging. Confocal images were acquired using a $\times 63$, 1.3 numerical aperture oil immersion objective mounted on a Zeiss LSM 510 confocal microscope. Confocal line scanning was used to record Ca^{2+} signals. Steady-state Ca^{2+} transients were recorded in Tyrode's solution containing 1.8 mM Ca^{2+} under field stimulation of 1 Hz. At least five steady-state transients for each cell were analyzed and averaged to represent Ca^{2+} signals of the cell. Ca^{2+} imaging data were analyzed by using a home-complied software, CaTeasy, which is coded in MATLAB 2013a (Mathworks). Briefly, the program normalizes Ca^{2+} transient images on a column-by-column basis (512 columns/image). The characteristic parameters of normalized transients were detected automatically. The firing time of every scanning pixel was detected column by column on the basis of the maximum local variability of pixel intensity. The profile of the point-to-point firing time was indicated by a red line overlapping on the Ca^{2+} image. Index of dyssynchronization, defined by the mean absolute deviation of firing time of each scanning pixel (with every 8 pixels binned), was used to evaluate the dyssynchrony of Ca^{2+} transients. This automated software for calcium transient analysis is available upon request.

Statistics—Data are presented as mean \pm S.E. Analysis of variance and Student's *t* test were applied when appropriate. A *p* value of <0.05 was considered statistically significant.

Results

Calpain Mediates Down-regulation of JP2 in Response to Cardiac Ischemia/Reperfusion Injury—A Western blotting assay showed that JP2 protein was reduced by 60% in left ventricular lysates from end-stage heart failure patients with ischemic heart disease (Fig. 1A), similar to previous reports on human failing hearts of different etiologies, such as dilated and hypertrophied cardiomyopathies (10, 25). To dissect the molecular mechanisms responsible for JP2 down-regulation in disease, we used a murine model of acute cardiac stress. Following global cardiac I/R injury in Langendorff-perfused WT mouse hearts, we observed a significant down-regulation of JP2 protein in the left ventricle (Fig. 1B). JP2 down-regulation was also observed in an *in vivo* I/R model induced by *in situ* left anterior descending coronary artery ligation/reperfusion in mouse hearts (Fig. 1D). These data suggest that JP2 down-regulation is not solely accomplished by miRNA-mediated gene silencing (19), given that loss of JP2 expression was evident within the short time period of I/R injury in our study.

JP2 down-regulation was attenuated by perfusion with the calpain inhibitor MDL-28170 before and after ischemia (Fig. 1B). These data are in line with the observed increase in calpain activity under a variety of pathological conditions, such as pressure overload, myocardial infarction, I/R injury, and isoproterenol-induced cardiac disease (26–37). In the post-ischemic heart, calpain degrades a myriad of structural and myofibrillar proteins, resulting in cardiac dysfunction (29, 38–41). We next performed studies in transgenic mice overexpressing the

endogenous calpain inhibitor protein calpastatin (CAST) (42, 43). JP2 levels were statistically unchanged in hearts from CAST mice after both *in vitro* and *in vivo* I/R injury (Fig. 1, C and E), further suggesting that calpain contributes to the reduction in JP2 protein expression.

To determine whether calpain directly cleaves JP2, we next performed *in vitro* cleavage reactions by incubating protein lysates from WT mouse hearts with purified human calpain I. Blotting with a JP2 C-terminal antibody revealed a significant reduction in full-length JP2 upon addition of calpain I (Fig. 2A). The Ca^{2+} chelator EDTA or the calpain inhibitor Z-LLY-FMK inhibited JP2 degradation. *In vitro* calpain cleavage assays using human heart lysates also showed a time-dependent and Ca^{2+} /calpain-dependent JP2 down-regulation, identifying JP2 as a direct substrate for calpain (Fig. 2B). These data provide direct evidence that, in cardiac tissue, JP2 is a substrate of the Ca^{2+} -dependent protease calpain.

Identification of Calpain Cleavage Sites on JP2—We overexpressed murine JP2 in 293T cells and subjected lysates to a calpain proteolysis assay. Using an antibody against the JP2 C-terminal epitope, we detected a dramatic decrease in the amount of full-length JP2 but could not detect the faster-migrating species (Fig. 2C), indicating that calpain cleavage may have destroyed the antibody epitope.

To determine the extent of JP2 proteolysis by calpain, we next generated a JP2 construct with an N-terminal FLAG tag and a C-terminal HA tag (Fig. 3A). After cotransfection of the dually tagged JP2 and human calpain I into 293T cells, we used the Ca^{2+} ionophore ionomycin to induce Ca^{2+} -dependent cleavage of JP2. Blotting with an antibody against the N-terminal FLAG tag demonstrated the calpain-dependent appearance of four N-terminal cleavage products, denoted N-I, II, III, and IV (Fig. 3B, a). The antibody against the HA tag detected one C-terminal cleavage product (denoted C-I, Fig. 3B, b). Treatment with the calpain inhibitor MDL-28170 resulted in a substantial reduction in the presence of both the N- and C-terminal cleavage products and restored JP2 full-length levels to those observed in untreated cells (Fig. 3B, a and b). Cleavage products of a similar molecular weight were also observed in the *in vitro* calpain cleavage assays at 3 mM Ca^{2+} (Fig. 3C) and 500 μ M Ca^{2+} (Fig. 3D). Removing Ca^{2+} from the reaction or inhibition of calpain with Z-LLY-FMK resulted in a substantial reduction in cleavage products and preservation of full-length JP2 (Fig. 3, C and D). These data suggest that JP2 contains at least four calpain cleavage sites.

Using the computational tools CaMPDB (44) and GPS-CCD 1.0 (45), we identified several putative calpain cleavage sites in JP2. We first generated a panel of tagged JP2 truncations on the basis of the predicted N- and C-terminal calpain cleavage sites (Fig. 4A). When expressed exogenously in 293T cells, JP2(1–155), JP2(1–201), and JP2(1–565) have a similar molecular weight as the cleavage products N-I, N-II, and N-IV, respectively (Fig. 4B, a). JP2(1–155), which corresponds to N-I, was only detectable in 293T cells treated with the proteasomal inhibitor lactacystin (Fig. 4B, a), suggesting that this cleavage product is not stable and undergoes further degradation by the proteasome (Fig. 4B, a). Note that JP2(566-end), which is the C-terminal counterpart of JP2(1–565), has a similar molecular

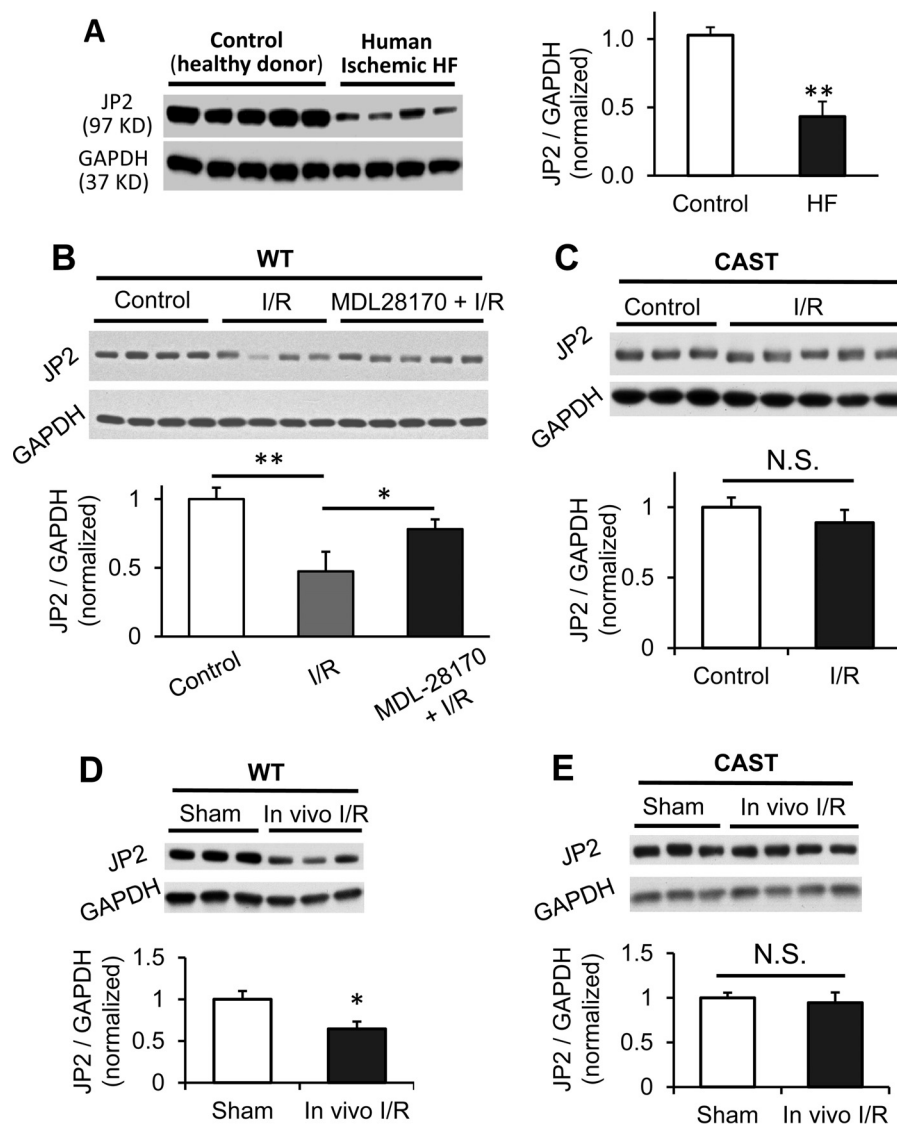


FIGURE 1. JP2 is down-regulated by calpain in response to *in vitro* and *in vivo* I/R injury. A, Western blot of JP2 expression in left ventricular lysates from patients with ischemic heart failure (HF) ($n = 4$) or rejected donor hearts (Control) ($n = 5$). B, representative Western blot and summary data of JP2 expression in WT murine left ventricles after *in vitro* I/R injury under Langendorff perfusion (at least four hearts for each group). MDL-28170 ($10 \mu\text{M}$) perfusion after the onset of reperfusion and 10 min before ischemia attenuated JP2 down-regulation. C, representative Western blot and summary data of JP2 expression in left ventricles from calpastatin (CAST) transgenic mice after *in vitro* I/R injury under Langendorff perfusion (at least three hearts for each group). D and E, JP2 protein level in left ventricles from WT mice (D) and calpastatin transgenic mice (E) after *in vivo* coronary artery occlusion-induced I/R injury (at least three hearts for each group). Data are normalized to GAPDH and expressed relative to the values of control or sham-operated hearts for each genotype. *, $p < 0.05$; **, $p < 0.01$; N.S., not significant.

weight as C-terminal cleavage product C-I (Fig. 4B, b), indicating N-IV and C-I may be generated by a single cleavage of JP2 at Arg-565/Thr-566. We also examined the susceptibility of JP2(1–565) to calpain-mediated proteolysis and found that this fragment can be further processed by calpain to generate N-I, N-II, and N-III fragments (Fig. 4C). These data provide evidence that deletion of the extreme C-terminal tail of JP2 does not attenuate the calpain recognition of other cleavage sites.

We next verified the predicted calpain cleavage at Val-155/Arg-156, Leu-201/Leu-202, and Arg-565/Thr-566 by creating site-directed deletions in the surrounding area. A six-residue deletion around the putative cleavage site Val-155/Arg-156 ($\Delta(153-158)$) completely abolished the generation of N-I in the *in vitro* calpain cleavage assay (Fig. 4D, a), whereas cleavage at the extreme C terminus remained largely intact compared with WT JP2 (Fig. 4D, a and b). Next, an eight residue deletion

around the putative cleavage site Leu-201/Leu-202 ($\Delta(198-205)$) resulted in complete loss of the JP2 cleavage product N-II but had no effect on the generation of N-I (Fig. 4D, c), suggesting that cleavage at Val-155/Arg-156 is not dependent on prior cleavage at Leu-201/Leu-202. Finally, a six-amino acid deletion around the putative cleavage site Arg-565/Thr-566 ($\Delta(563-568)$) led to complete loss of N-IV (Fig. 4D, d) and a severe reduction in C-I (Fig. 4D, e). Several C-terminal cleavage products (arrows, Fig. 4D, e), which may be counterparts of N-terminal cleavage products detected by anti-FLAG antibody, were detected by the anti-HA antibody in the *in vitro* calpain cleavage reaction of $\Delta(563-568)$ but not WT JP2 (Fig. 4D, e). Unlike loss of the N-terminal cleavage sites, loss of the C-terminal Arg-565/Thr-566 cleavage site preserved full-length JP2 (Fig. 4D, d and e), suggesting that the C-terminal Arg-565/Thr-566 cleavage site is more sensitive to calpain than the N-terminal

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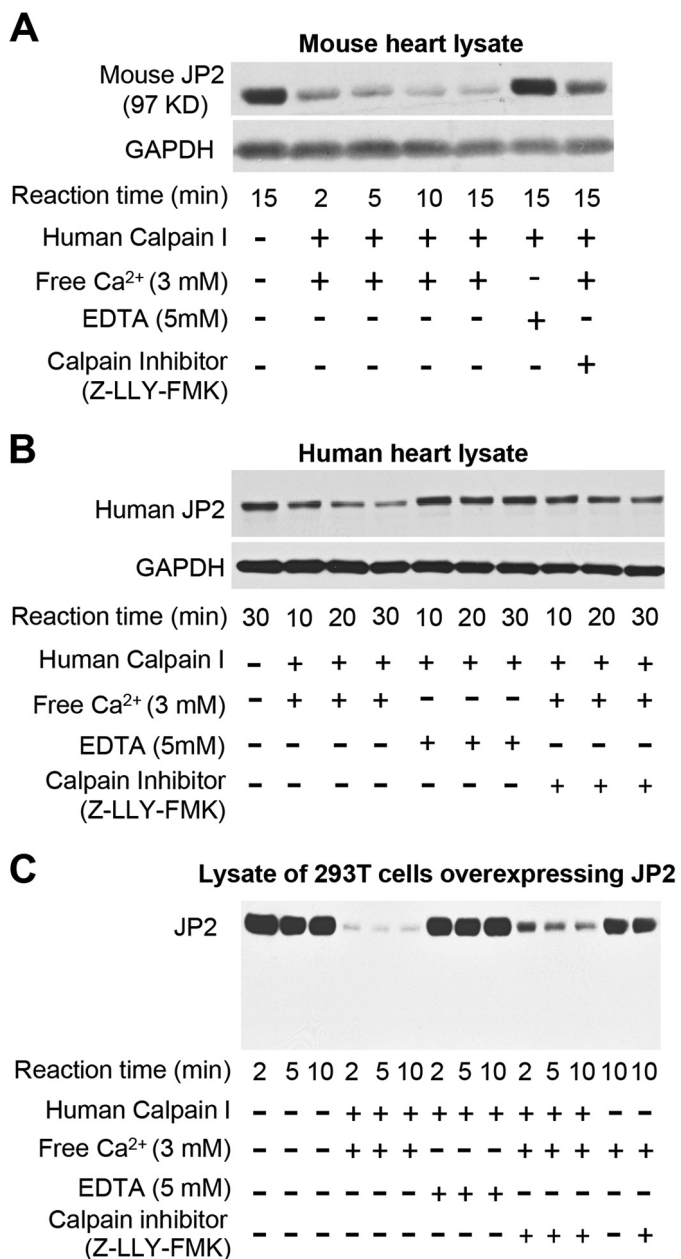


FIGURE 2. *In vitro* calpain cleavage reactions identify calpain as the JP2 protease in mouse and human heart. *A* and *B*, *in vitro* proteolysis of JP2 in mouse (*A*) and human (*B*) heart lysates (1 mg total protein) in the presence of human calpain I (1 μ g of calpain I) and free Ca²⁺ (3 mM) with or without EDTA (5 mM) or the calpain inhibitor Z-LLY-FMK (20 μ M). *C*, protein lysates from 293T cells overexpressing JP2 (untagged) were incubated with purified human calpain I in the presence or absence of Ca²⁺ or calpain inhibitor. Data are representative of at least three independent experiments.

cleavage sites. Taken together, these data identify Val-155/Arg-156, Leu-201/Leu-202, and Arg-565/Thr-566 as calpain cleavage sites that result in the generation of the cleavage products N-I, N-II and N-IV, and C-I, respectively.

JP2 Fragments Corresponding to the Primary Cleavage Site (Arg-565/Thr-566) Have No Effect on Cardiomyocyte Ca²⁺ Handling—Cleavage at the primary calpain proteolysis site (Arg-565/Thr-566) splits apart the C-terminal SR-binding TM domain and N-terminal plasma membrane-binding MORN domains of JP2. It is believed that intact JP2 is required for its

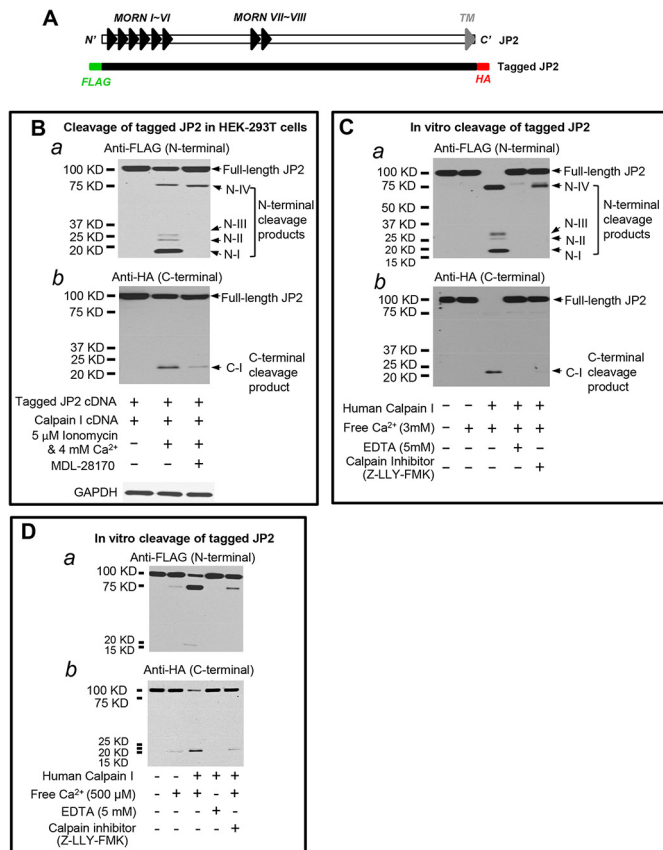


FIGURE 3. Calpain cleaves JP2 at multiple sites in the N- and C-terminal regions. *A*, schematic of the N- and C-terminal epitope-tagged full-length JP2 cDNA. *B*, after cotransfection of 293T cells with calpain I and full-length JP2 containing an N-terminal FLAG tag and a C-terminal HA tag, cells were exposed to 5 μ M ionomycin and 4 mM extracellular Ca²⁺ for 1 h in the absence or presence of 10 μ M MDL-28170. JP2 expression and degradation were assessed by Western blotting with anti-FLAG (*a*) or anti-HA (*b*) to detect N- and C-terminal cleavage fragments, respectively. N-I, N-II, N-III, and N-IV denote the four N-terminal cleavage products detected with anti-FLAG. C-I denotes the major C-terminal cleavage product detected by anti-HA. *C*, *in vitro* calpain-I mediated cleavage assay as in Fig. 2 using lysates from 293T cells overexpressing JP2 with N-terminal FLAG and C-terminal HA epitopes. Cleavage products were detected with anti-FLAG (*a*) or anti-HA (*b*). *D*, *in vitro* calpain proteolysis of tagged JP2 at lower concentrations of Ca²⁺ (500 μ M). Data are representative of at least three independent experiments.

normal function to tether the plasma membrane and SR together, but it is unknown whether truncated JP2 fragments exert any additional functions on myocyte Ca²⁺ handling. To test this, we expressed epitope-tagged (see “Experimental Procedures”) full-length JP2 and JP2 truncations corresponding to the primary calpain cleavage site in cardiomyocytes via adenoviruses. Immunostaining showed the striated pattern of these three versions of JP2 in infected mouse ventricular cardiomyocytes (Fig. 5A), indicating that the two truncations can still be localized to the T-tubule/SR junction via either MORN domains or TM domains, respectively. Coimmunoprecipitation showed that full-length JP2 and JP2(1–565), but not JP2(566–end), interact with RyR2 and Cav1.2 (Fig. 5B), demonstrating that JP2 interacts with complexes of E-C coupling channels via its N terminus.

Next we evaluated the potential role of JP2 truncates on Ca²⁺ handling in cardiomyocytes. We expressed epitope-tagged full-length JP2, N-terminal truncation JP2(1–565) and C-terminal

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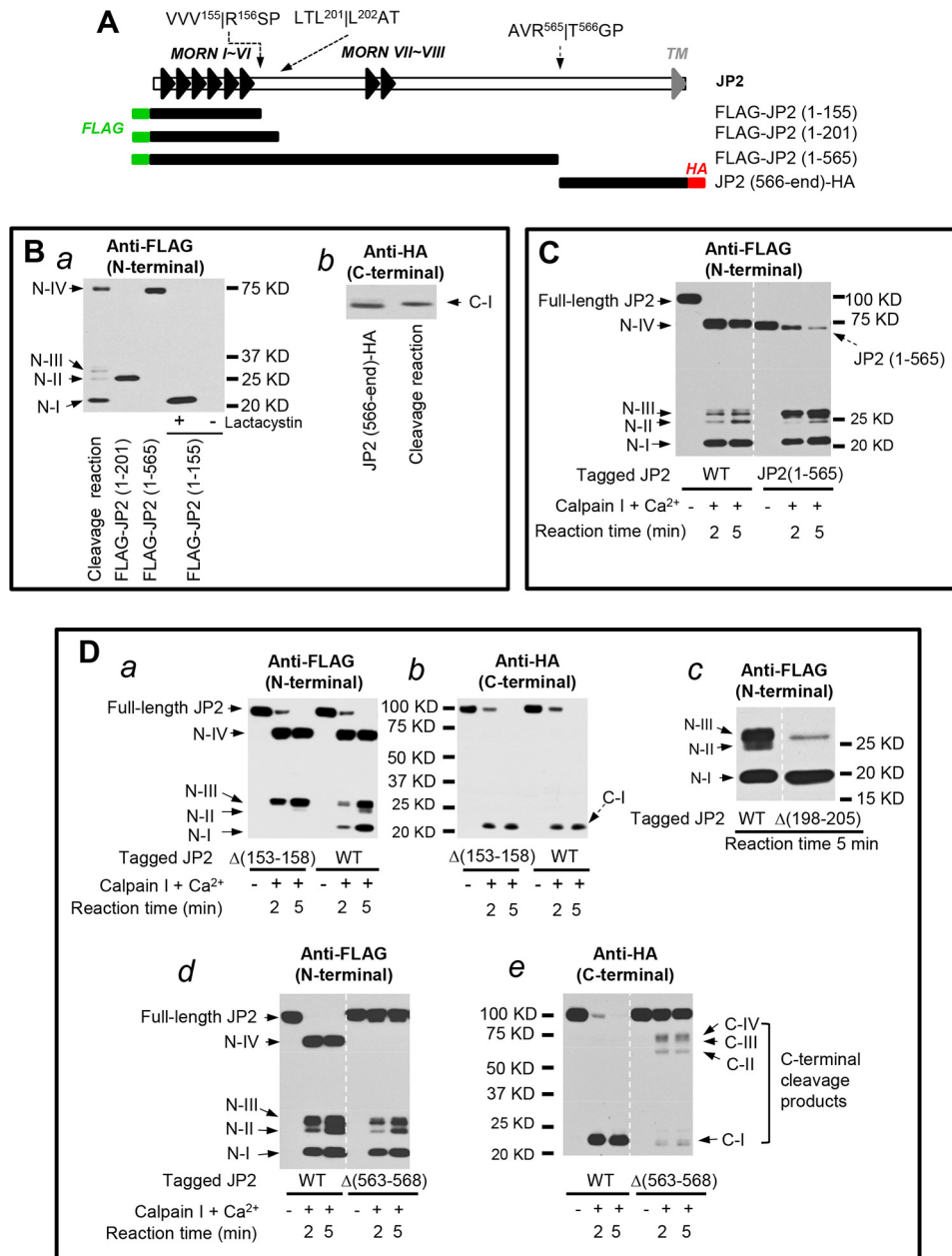
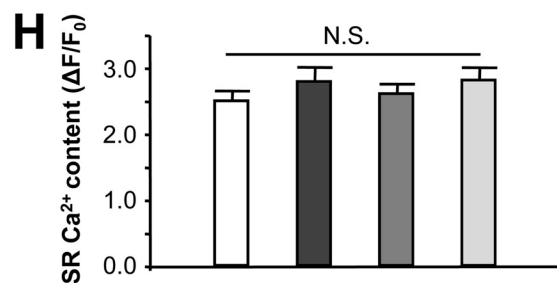
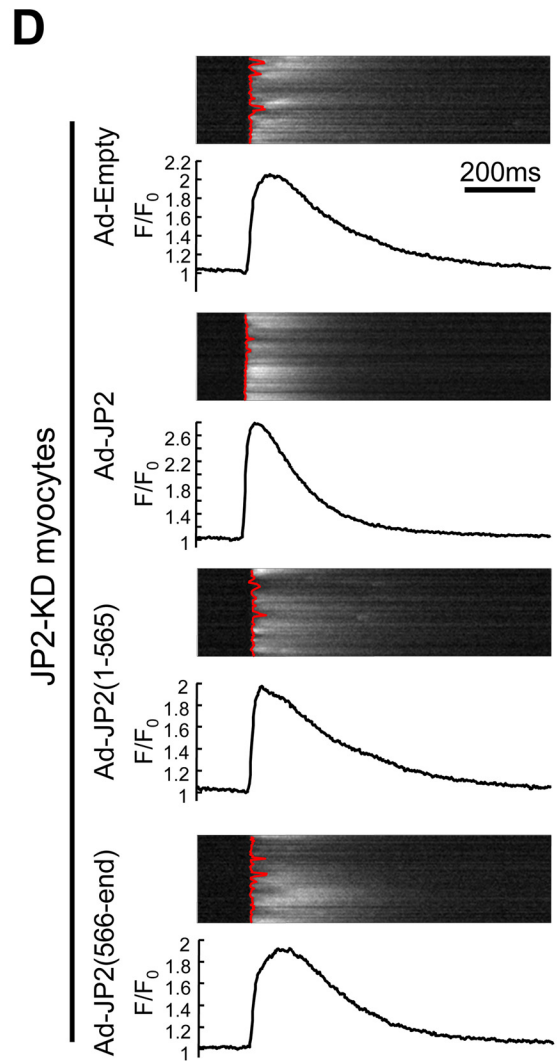
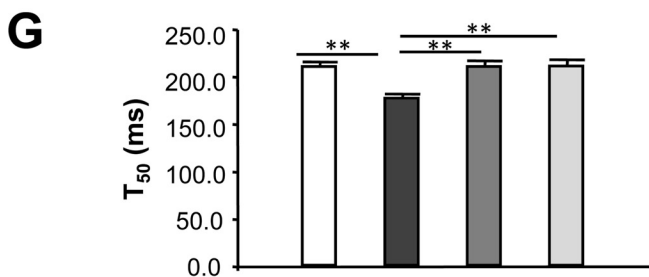
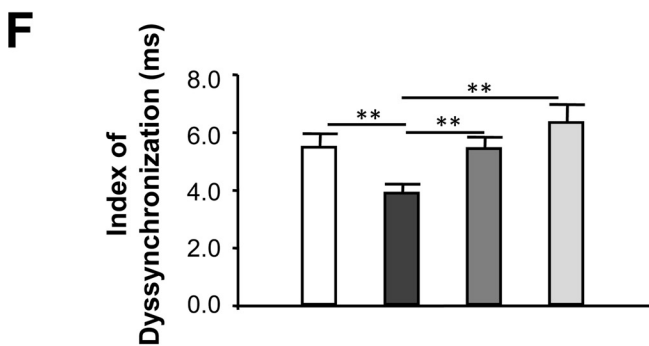
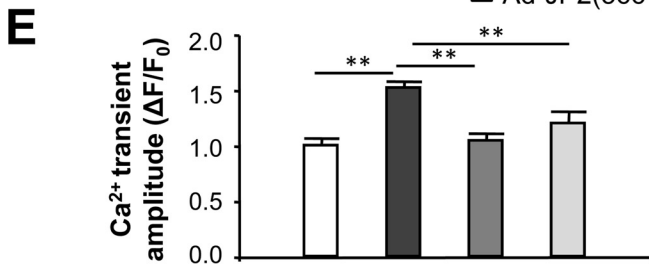
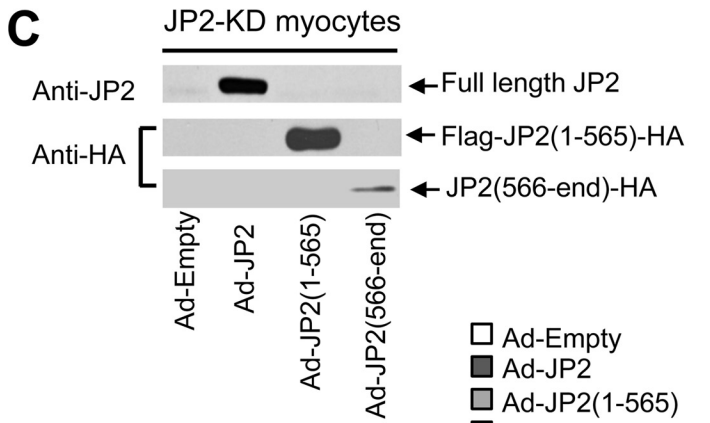
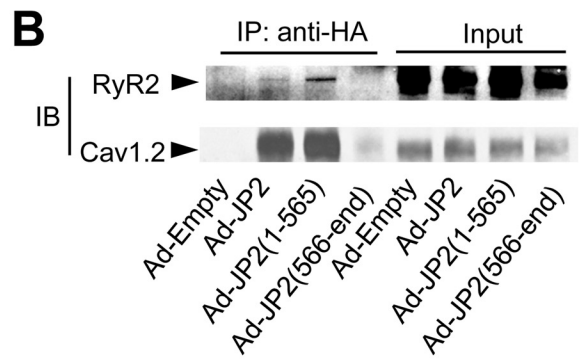
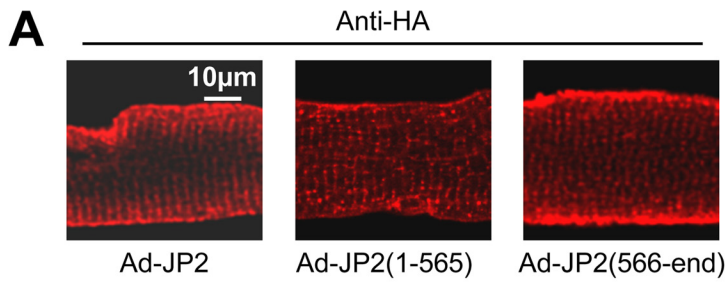


FIGURE 4. C-terminal JP2 proteolysis at R565T is the primary site for calpain-mediated cleavage. *A*, schematic of the N- or C-terminal epitope-tagged truncated JP2 constructs on the basis of putative calpain cleavage sites. These cleavage sites were predicted by using GPS-CCD 1.0 and CaMPDB. Arrows indicate these predicted cleavage sites relative to the MORN and TM domains. *B*, expression of JP2 truncations in 293T cells. Where indicated, cells were pretreated with lactacystin (10 μ M) after transfection. *C*, Western blot with anti-FLAG (N-terminal tag) following *in vitro* cleavage reaction with JP2(1-565) truncation. *D*, six to eight Amino acid deletions surrounding the predicted cleavage sites (V155R, $\Delta(153-158)$; L201L, $\Delta(198-205)$; R565T, $\Delta(563-568)$) were introduced into the full-length JP2 construct with N-terminal FLAG and C-terminal HA epitope tags. Following expression of mutants in 293T cells, lysates were subjected to *in vitro* cleavage reactions. WT JP2 with tags was used as a control.

truncation JP2(566-end) back into ventricular cardiomyocytes from mice with silenced endogenous JP2 (JP2-KD) (Fig. 5C). Expression of full-length JP2 in JP2-KD cardiomyocytes resulted in a significantly increased amplitude (by ~50%) (Fig. 5, *D* and *E*), increased synchronization (quantified by the index of dyssynchronization, Fig. 5, *D* and *F*), as well as a shortened decay phase (T_{50}) of Ca^{2+} transients (Fig. 5G). In contrast, expression of JP2(1-565) or JP2(566-end) failed to restore Ca^{2+} transients in JP2-KD cardiomyocytes (Fig. 5, *E-G*). SR Ca^{2+} content of JP2-KD cardiomyocytes was not influenced by expression of full-length JP2 or JP2 truncations (Fig. 5H).

Because JP2(1-565) and JP2(566-end) can still be localized to the striated T-tubule/SR junction, and because JP2(1-565) even interacts with RyR2 and Cav1.2, we next tested whether these truncations have a dominant negative effect on Ca^{2+} handling in WT cardiomyocytes. Our data showed that overexpression of full-length JP2 or truncations from either terminus did not alter the kinetics of steady-state Ca^{2+} transients under 1-Hz field stimulation and SR Ca^{2+} loading in WT cardiomyocytes (Fig. 6). These data indicate that proteolysis of JP2 at the C terminus results in E-C coupling dysfunction.

Molecular Determinants of Junctophilin-2 Cleavage



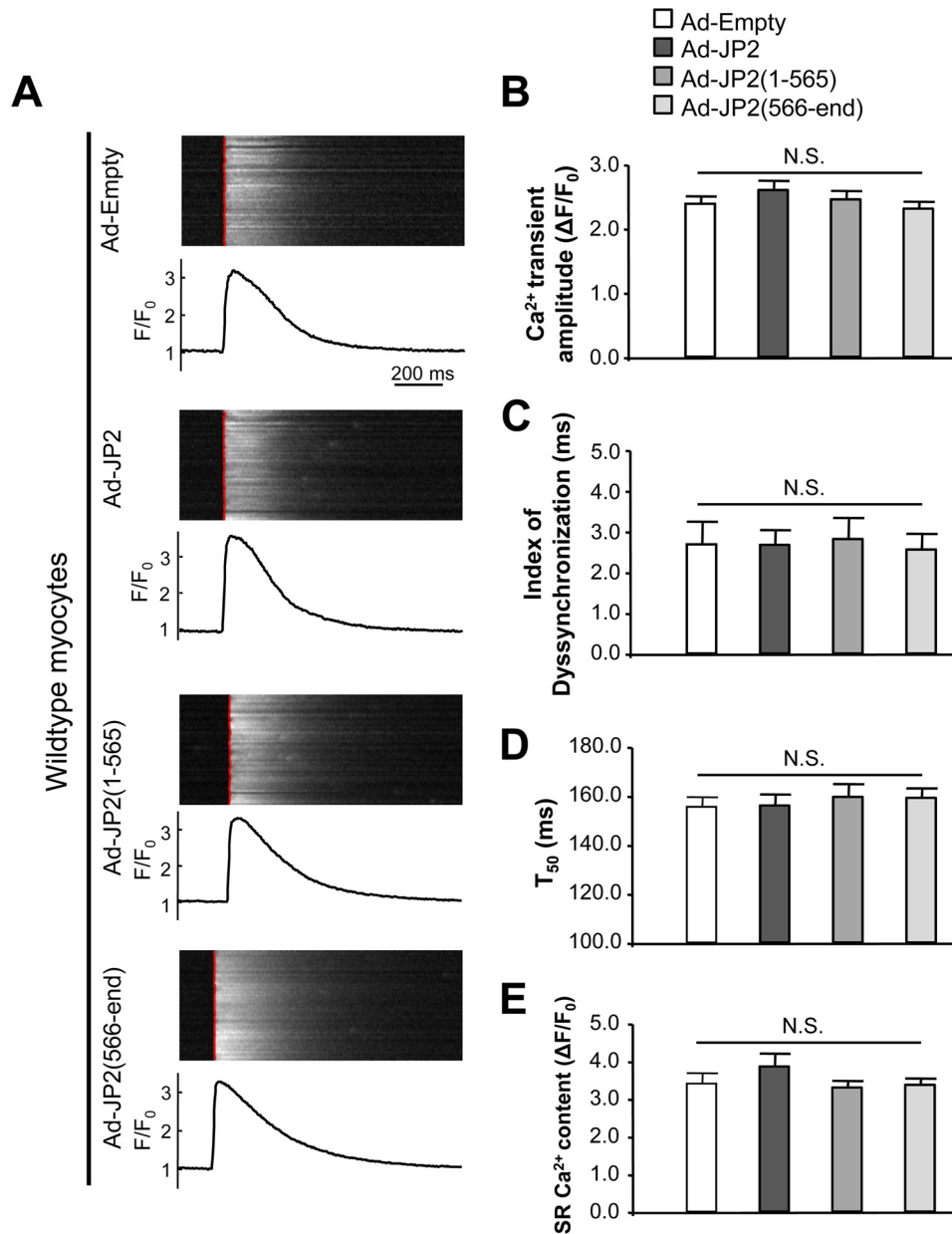


FIGURE 6. JP2 truncations do not have a dominant negative effect on Ca²⁺ transients in wild-type cardiomyocytes. *A*, representative steady-state Ca²⁺ transients under 1-Hz field stimulation. See the legend of Fig. 5 for the definition of the red lines overlapping on Ca²⁺ imaging. *B–D*, summary of Ca²⁺ transient amplitude, index of dyssynchronization, and duration of 50% decay (T_{50}) ($n = 51, 52, 51,$ and 61 for Ad-Empty, Ad-JP2, Ad-JP2(1–565), and Ad-JP2(566-end), respectively). *E*, summary of SR Ca²⁺ content, which was assessed by caffeine-induced SR Ca²⁺ release ($n = 24, 24, 29,$ and 33 for Ad-Empty, Ad-JP2, Ad-JP2(1–565), and Ad-JP2(566-end), respectively).

Discussion

These data reveal, for the first time, the molecular determinants responsible for posttranslational calpain proteolysis of

JP2, providing a molecular mechanism of JP2 down-regulation following cardiac injury. Sequence prediction of calpain cleavage sites identified several putative sites, three of which we con-

FIGURE 5. JP2 truncations are nonfunctional in regulating Ca²⁺ transients. *A*, an antibody against the HA tag was used to reveal the localization of adenoviral expression of tagged full-length JP2 and truncations, as indicated, in adult wild-type cardiomyocytes. All the three version of JP2 can be localized in the striated pattern. *B*, full-length JP2 and JP2(1–565) forms complexes with RyR2 and Cav1.2 *in vivo*. An antibody against HA was used for immunoprecipitation (IP). The RyR2 and Cav1.2 that were pulled down were detected by Western blot analysis. Note that full-length JP2 and JP2(1–565) pulled down both RyR2 and Cav1.2, although JP(566-end) does not form complexes with RyR2 or Cav1.2. *IB*, immunoblot. *C*, adenovirus-mediated expression of full-length JP2, FLAG-JP2(1–565)-HA, and JP2(566-end)-HA in JP2-KD cardiomyocytes. *D*, representative steady-state Ca²⁺ transients under 1-Hz field stimulation. The fluorescence intensity (F) of Ca²⁺ imaging was normalized to the baseline (F_0). The red lines overlapping on Ca²⁺ imaging show the profile of the moment of Ca²⁺ transient firing on the scanning line in a point-by-point way. A straighter line means better synchronization of the Ca²⁺ transients. Note that expression of full-length JP2, but not JP2 truncations, improves the amplitude and synchronization of Ca²⁺ transients. *E–G*, summary of Ca²⁺ transient amplitude, index of dyssynchronization (mean absolute deviation of firing time), and duration of 50% decay (T_{50}) ($n = 68, 70, 70,$ and 52 for Ad-Empty, Ad-JP2, Ad-JP2(1–565), and Ad-JP2(566-end), respectively). Only full-length JP2 improves the amplitude, synchronization, and decay of Ca²⁺ transients. *H*, summary of SR Ca²⁺ content, which was assessed by caffeine-induced SR Ca²⁺ release ($n = 17, 17, 17,$ and 12 for Ad-Empty, Ad-JP2, Ad-JP2(1–565), and Ad-JP2(566-end), respectively). **, $p < 0.01$ versus indicated groups; N.S., not significant.

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firmed by mutagenesis. Our data suggest that C-terminal calpain cleavage of JP2 may be a prerequisite for proteolysis at N-terminal sites. We also found that neither N-terminal nor C-terminal truncations of JP2 are sufficient to restore Ca^{2+} transients in cardiomyocytes in which endogenous JP2 has been knocked down. These data provide important insights into JP2 regulation in cardiomyocyte E-C coupling and may explain, in part, the significant cardiac phenotype associated with loss of JP2 *in vivo* and the protective effect of calpain inhibition (32, 38).

We and others have reported that JP2 is required for maintaining T-tubule structural integrity in adult hearts (7, 18). More recently, we identified JP2 as an essential factor in T-tubule and E-C coupling maturation during development (17). In another study, we found that overexpression of JP2 attenuates the transition from hypertrophy to heart failure, suggesting its significance as a potential therapeutic target (12). JP2 protein down-regulation has been observed in failing human hearts as well as multiple animal models of cardiac disease (10, 15, 17–21, 23, 25, 46). This loss of JP2 is associated with alterations in T-tubule ultrastructure and E-C coupling dysfunction, thereby contributing to the development of heart failure (7, 10, 11, 19). To date, miR-24-mediated translational repression and Ca^{2+} -dependent proteolysis have been implicated mechanistically in JP2 down-regulation (19, 22, 23, 46). Our data extend these latter findings by identifying calpain as the JP2 protease. Moreover, we define the molecular determinants of JP2 proteolysis and provide evidence for this mechanism in an animal model of cardiac stress.

Previous reports have suggested two mechanisms by which JP2 regulates E-C coupling. First, JP2 acts as a physical bridge between the T-tubule and SR membranes to maintain normal Ca^{2+} handling in cardiomyocytes (7, 11). Second, JP2 has been shown to interact with RyR2 and Cav1.2 and regulate their gating function.(7, 47) Our data suggest that down-regulation of JP2 results in E-C coupling dysfunction primarily through disrupting the junctional complex. Specifically, we found that the N-terminal truncation fragment of JP2 interacts with RyR2 and Cav1.2 but is not sufficient to restore Ca^{2+} transients in JP2-KD cardiomyocytes. These data provide compelling evidence that full-length JP2 is required for normal E-C coupling function. Taken together, this study provides novel molecular insights into the structure and function of JP2 in cardiomyocytes. Identification of the precise calpain cleavage sites on JP2 may lead to the development of new targeted therapies that maintain JP2 expression in the setting of cardiac stress.

Author Contributions—A. G. and L. S. S. conceived and designed the research. A. G., D. H., C. Z., and W. K. performed research and analyzed data. J. D. M. and F. J. L. provided human heart samples. T. P. provided reagents. C. E. G. and R. Z. L. supervised the experiments, data analysis, and interpretations. A. G. and L. S. S. wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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