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PRECLINICAL RESEARCH

Targeting Calpain for Heart Failure Therapy

Implications From Multiple Murine Models

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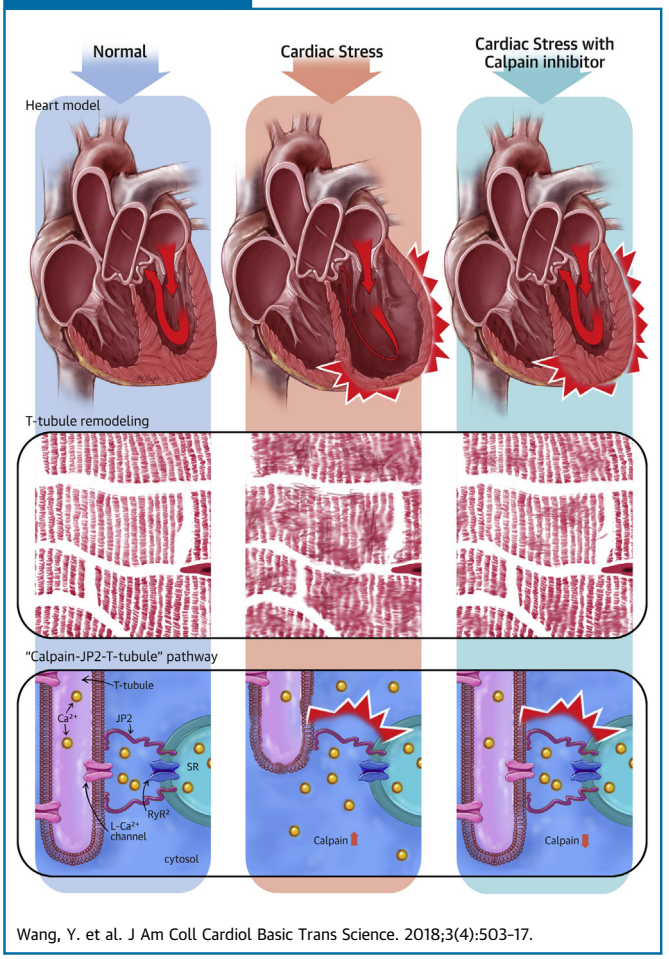
VISUAL ABSTRACT



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HIGHLIGHTS

- **Calpain is hyperactivated in human failing hearts and rodent heart failure models of different etiologies.**
- **Inhibition of calpain activity with MDL-28170 protects against cardiac dysfunction by preserving JP2 expression and T-tubule ultrastructural integrity in murine models of heart failure.**
- **Overexpression of JP2 delays the onset of early cardiac sudden death and heart failure, induced by calpain overactivation.**

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ABBREVIATIONS AND ACRONYMS

CAPN1-OE = calpain-1 overexpressing

E-C coupling = excitation-contraction coupling

EF = ejection fraction

IP = intraperitoneally

ISO = isoproterenol

JP2 = junctophilin-2

JP2-OE = junctophilin-2 overexpressing

LV = left ventricle/ventricular

MI = myocardial infarction

RV = right ventricular

SR = sarcoplasmic reticulum

TAB = transverse aortic banding

T-tubule = transverse tubule

TT_{power} = strength of regularity of the T-tubule system

WT = wild-type

SUMMARY

Heart failure remains a major cause of morbidity and mortality in developed countries. There is still a strong need to devise new mechanism-based treatments for heart failure. Numerous studies have suggested the importance of the Ca^{2+}-dependent protease calpain in cardiac physiology and pathology. However, no drugs are currently under development or testing in human patients to target calpain for heart failure treatment. Herein the data demonstrate that inhibition of calpain activity protects against deleterious ultrastructural remodeling and cardiac dysfunction in multiple rodent models of heart failure, providing compelling evidence that calpain inhibition is a promising therapeutic strategy for heart failure treatment. (J Am Coll Cardiol Basic Trans Science 2018;3:503–17)

Heart failure affects nearly 6 million Americans and is the most common cause of hospitalization in patients over 65 years of age. Classical heart failure treatments, in particular angiotensin-converting enzyme inhibitors, angiotensin II receptor blockers, and beta-blockers, have been shown to improve heart function, prolong life, and reduce heart failure-associated sudden cardiac death. A recent PARADIGM-HF (Prospective comparison of ARNI with ACEI to Determine Impact on Global Mortality and morbidity in Heart Failure) trial demonstrated superior cardiovascular mortality benefit by combining an angiotensin receptor inhibitor with an inhibitor of neprilysin, a neutral endopeptidase that degrades vasoactive peptides and contributes to excessive neurohormonal activation and thereby vasoconstriction and maladaptive cardiac remodeling (1). However, despite a clear improvement in survival, it is important to note that a high percentage of patients (21.8%) still experienced death from cardiovascular causes or disease progression during the clinical trial phase (1). Thus, additional strategies that address the complex mechanisms of heart failure and new therapeutic approaches for heart failure treatment are still urgently needed.

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Calpains are a family of Ca^{2+}-dependent intracellular cysteine proteases that are ubiquitously expressed. At least 16 calpain isoforms have been identified to date (2). Calpain 1, the predominant isoform expressed in cardiomyocytes, is activated by micromolar concentrations of Ca^{2+} and proteolytically cleaves multiple target proteins that are critical for normal cardiac functions, such as protein kinase C, calcineurin, caspases, spectrin, sarcoplasmic reticulum (SR) Ca^{2+} ATPase, L-type Ca^{2+} channel, and junctophilin-2 (JP2) (3). Calpain activity is increased in a variety of pathological conditions such as pressure overload, myocardial infarction (MI), ischemia reperfusion, and isoproterenol (ISO)-induced cardiac disease (4,5). Calpain activation has been implicated in pathogenesis of myocardial remodeling and heart failure (6). Using an inducible cardiac-specific overexpression mouse model system, the Dorn group demonstrated that exogenous overexpression of calpain-1, but not calpain-2, in vivo results in heart failure and early mortality (7). Conversely, genetic inhibition of calpain activity reduces myocardial injury and improves cardiac function in mice post-MI (8). However, limited studies have explored use of pharmacological calpain inhibitors as a therapeutic strategy for antagonizing heart failure development or progression.

In the present study, we tested the therapeutic efficacy of pharmacological calpain inhibition in multiple animal models of heart failure. Due to the diversity of calpain substrates and the known function of calpain-mediated JP2 proteolysis, we then examined the specific contribution of JP2 cleavage in

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calpain-mediated cardiac remodeling and dysfunction using calpain-1 and JP2 double-transgenic mice. Our study provides proof-of-concept evidence that inhibition of calpain activity is a potential strategy for the treatment of heart failure.

METHODS

HUMAN HEART SAMPLES. Left ventricular (LV) samples from patients with ischemic or dilated cardiomyopathies were obtained from explanted hearts at the University of Pennsylvania. Nonfailing donor hearts without evidence of overt cardiac dysfunction were obtained through organ donor networks/organ procurement agencies. All human heart tissue samples were obtained under an organ research donation protocol approved by the institutional review boards at the University of Iowa.

ANIMALS. Animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication 85-23, revised 1996) and were approved by the Institutional Animal Care and Use Committee at the University of Iowa. Chronic heart failure models were established using male C57BL/6N mice (9 to 10 weeks of age). Cardiac-specific, inducible calpain-1 overexpressing mice (CAPN1-OE) (7), (obtained from the Jackson Laboratory, Bar Harbor, Maine) and JP2 overexpressing mice (JP2-OE) (both on a C57BL/6N background) (9) were crossed to generate double-transgenic mice (CAPN1-OExJP2-OE mice). Briefly, both overexpression mouse models were made using modified αMHC promoter which contains the tet-operon (tet-off) (see **Figure 1A** of Guo et al. [9]). Each transgenic mouse strain was crossed first with αMHC-tTA to generate tTA-CAPN1 mice or tTA-JP2 mice. These 2 strains of mice were then crossed to generate CAPN1-OExJP2-OE double-transgenic mice (controlled by αMHC-tTA). The expression of transgenes (CAPN1 and/or JP2) can be turned off by adding doxycycline to chow (625 mg/kg) and turned on by ceasing doxycycline feeding. Here, in the present study, the mice were studied in the absence of doxycycline. In other words, calpain and/or JP2 overexpression occurs since embryonic stage. Either sex of the transgenic mice and wild-type littermates was used for experiments.

MOUSE MODELS OF HEART FAILURE. Chronic heart failure was modeled in male C57BL/6N mice at 9 to 10 weeks of age using 3 different methods: 1) MI surgery by suturing the left anterior descending branch of the coronary artery as previously described (10); 2) pressure overload by transverse aortic banding (TAB) (9); and 3) ISO infusion by insertion of an Alzet Micro-Osmotic Pump (Model 1004, Alzet, Cupertino, California) filled with 420 mg/kg body weight of ISO to provide a constant supply of 30 mg/kg (body weight)/day of ISO. For the MI and TAB models, sham-operated surgery served as control; for the ISO model, untreated mice were used as the control. After surgery/ISO minipump insertion, mice were divided into 2 groups and administered MDL-28170 (10 mg/kg/day, intraperitoneally [IP], dissolved in dimethyl sulfoxide [DMSO]) or an equal concentration of DMSO saline solution only daily beginning on day 3 after surgery. Mice were usually euthanized 5 weeks after surgery (TAB/MI) or 2 weeks after minipump implantation. For some experiments, mice were euthanized at other time points (i.e., 1, 2, 3, or 4 weeks post-surgery or birth).

ECHOCARDIOGRAPHY FOR CARDIAC FUNCTION. Transthoracic echocardiograms were performed at the University of Iowa Cardiology Animal Phenotyping Core Laboratory using a Vevo 2100 Imager (VisualSonics, Toronto, Ontario, Canada) as previously described (9,10). After completion of 2-dimensional imaging, the animals were euthanized, and both the heart and lungs were dissected and weighed.

IN SITU TRANSVERSE TUBULE AND CA^{2+} IMAGING. Confocal imaging of transverse tubules (T-tubules) and Ca^{2+} handling in Langendorff-perfused intact hearts were performed as described previously (11,12). The strength of regularity of T-tubules (TT_{power}) was analyzed using AutoTT custom software (13). Analyses of Ca^{2+} transients were performed as previously described (14).

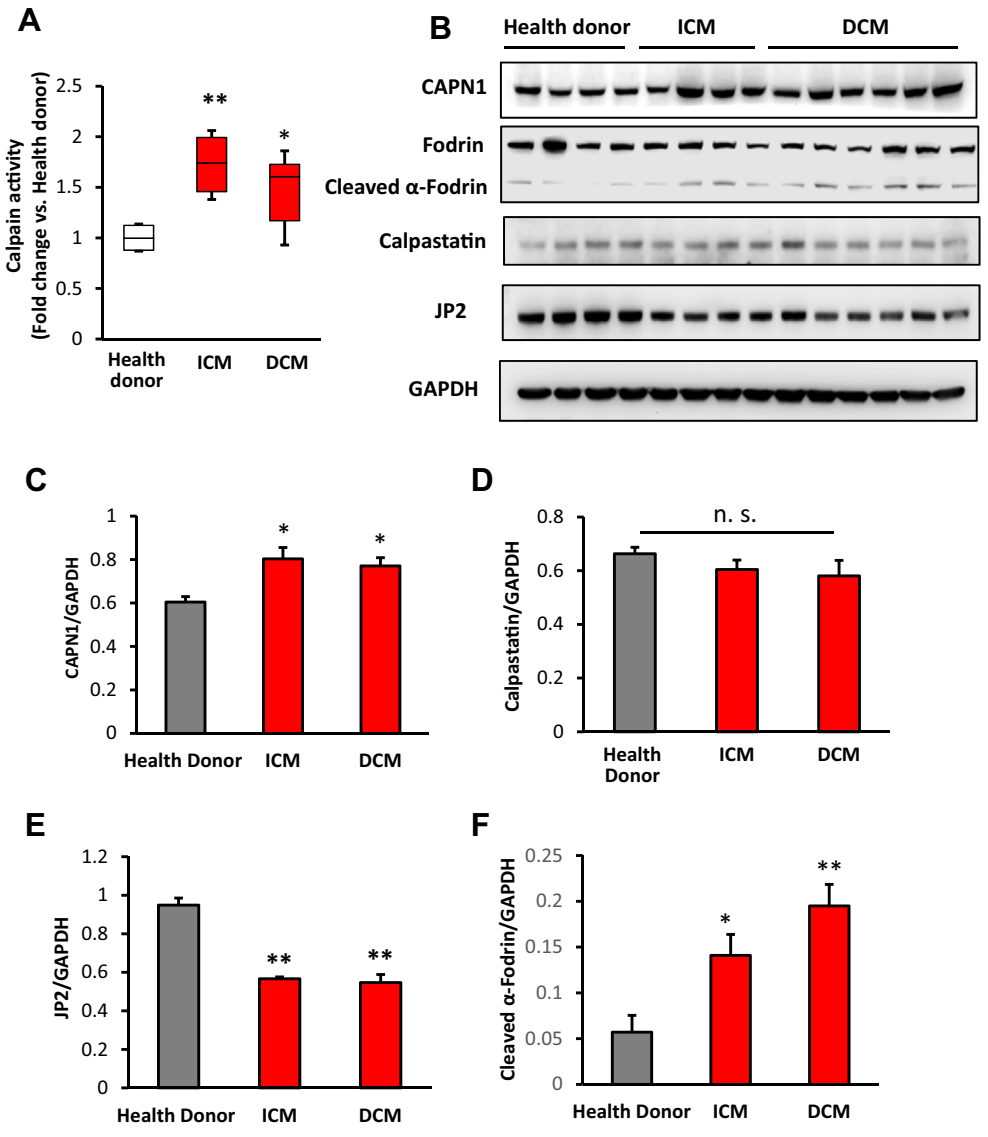
WESTERN BLOT ANALYSIS. Western blotting was performed as described previously (15).

CALPAIN ACTIVITY ASSAY. Calpain activity in heart lysates was determined using a calpain activity assay kit (ab65308, Abcam, Cambridge, Massachusetts), according to the manufacturer's protocol. Heart lysates were obtained from mice treated with MDL-28170 or saline for 5 weeks since day 3 after surgery. Twenty-four h after last injection of MDL-28170, mice were subjected to echocardiography, and then hearts were collected for histology, biochemical, or enzyme activity assays.

HISTOLOGY ASSAY. Masson's trichrome staining was used for the detection of collagen fibers in the heart tissues. ImageJ version 1.8.0 (NIH, Bethesda, Maryland) was used to quantify the relative area of fibrosis (blue staining in the myocardial tissue), by measuring the ratio of blue staining region to total myocardial area in the image.

STATISTICS. Data are expressed as mean ± SEM. Student's *t* test and one-way analysis of variance with

FIGURE 1 Calpain Activity Is Increased in Human Heart Failure



(A) Calpain activity assay in myocardium from healthy donors (n = 4, 45.0 ± 5.3 years of age), ICM patients (n = 4, 47.0 ± 4.7 years of age), and DCM patients (n = 6, 44.0 ± 7.5 years of age). **(B–E)** Representative images of Western blots **(B)** and quantitation of calpain-1 **(C)**, calpastatin **(D)**, JP2 **(E)**, and cleaved form of fodrin **(F)** in left ventricular lysates from samples of **A**. GAPDH was used as the loading control. *p < 0.05, **p < 0.01 versus healthy donor. DCM = dilated cardiomyopathy; ICM = ischemic cardiomyopathy; n.s. = not significant.

Bonferroni post hoc test were applied for paired comparisons when appropriate, and only parametric analyses have been used. Values of $p < 0.05$ were considered statistically significant.

RESULTS

CALPAIN ACTIVITY IS INCREASED IN END-STAGE HEART FAILURE PATIENTS. To determine the relationship between calpain activity and cardiac structure and function in human heart failure, we examined calpain activity and protein expression levels in LV lysates from end-stage heart failure patients with ischemic cardiomyopathy or dilated cardiomyopathy. As compared with healthy donor hearts, calpain activity and calpain-1 expression were significantly increased in failing hearts of both etiologies (Figures 1A to 1C). By contrast, the expression level of calpastatin, an endogenous calpain inhibitor, was not altered in diseased heart samples (Figure 1D).

Calpain proteolytically cleaves a number of cardiac proteins with known roles in cardiac homeostasis. For example, our group demonstrated that JP2, a membrane-binding protein that provides a structural bridge between the plasmalemma and SR, is a direct substrate of calpain in cardiomyocytes (16). JP2 down-regulation is involved in the progression of heart failure in multiple animal models of cardiac stress (16,17). Analysis of JP2 protein levels in failing human hearts revealed a marked ~45% reduction in both ischemic cardiomyopathy and dilated cardiomyopathy samples as compared with healthy myocardium (**Figures 1B and 1E**). As a control, we also observed a significant increase of cleaved α-Fodrin, a typical calpain substrate, in human failing hearts (**Figure 1F**).

CALPAIN INHIBITOR PRESERVES CARDIAC FUNCTION AFTER CARDIAC STRESS. To investigate the impact of calpain inhibition on cardiac structure and function in chronic heart failure, we utilized 3 common animal models of heart failure: MI, pressure overload via TAB, and ISO minipump infusion. To assess the efficacy of calpain inhibition as a therapeutic modality, mice were treated with the calpain inhibitor MDL-28170 (10 mg/kg/day, IP), a cell-penetrating di-peptidyl aldehyde inhibitor, beginning at 3 days after surgery or implantation of the ISO minipump.

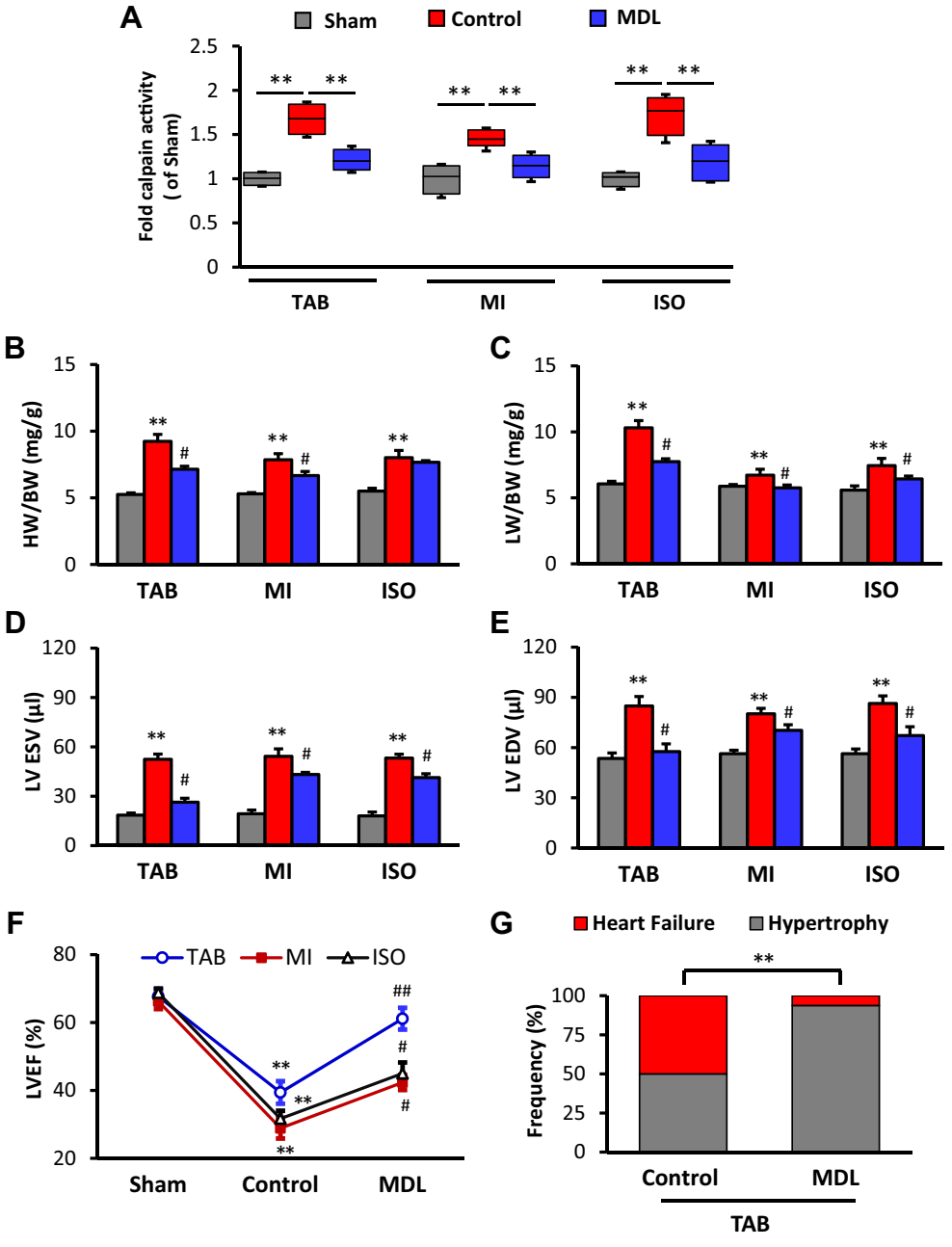
We first established that, as expected, calpain activity is increased in all models of heart failure as compared with sham control, and treatment of mice with MDL-28170 normalized calpain activity close to sham levels (**Figure 2A,** Supplemental Figure S1). The heart weight/body weight ratio and lung weight/body weight ratio were both markedly attenuated in response to MDL-28170 treatment (**Figures 2B and 2C**). Echocardiography was used to examine how MDL-28170 affects LV function following TAB or MI surgery after 5 weeks or ISO minipump after 2 weeks. MDL-28170 significantly prevented LV dilation and attenuated LV systolic dysfunction compared with age-matched TAB, MI, and ISO mice (**Figures 2D and 2E**). Similarly, mice treated with MDL-28170 had significantly better LV contractile function compared with control mice, as shown by increased LV ejection fraction (EF) (**Figure 2F**). We also examined whether MDL-28170 could prevent development of cardiac fibrosis in the TAB model. We found 5-week TAB stress-induced dramatic increase in fibrosis of myocardium, which was significantly inhibited by MDL-28170 treatment (Supplemental Figure S2) Together, these data suggest that elevated calpain activity is a general mechanism for cardiac dysfunction and pathological remodeling induced by cardiac stress, and inhibition of calpain after injury is protective.

In another set of experiments, we further tested whether inhibiting calpain activity will be still effective in TAB mice with established cardiac dysfunction. MDL-28170 was administered beginning at 3 weeks following TAB and continued for 3 weeks (10 mg/kg/day, IP). The EF of TAB mice without MDL-28170 treatment were decreased by 44% during this period, whereas the EF of the MDL-28170 treatment group was reduced by only 18% (Supplemental Figure S3). Taken together, these data suggest that inhibiting calpain activity represents an effective strategy in mitigating the development and progression of heart failure.

CALPAIN INHIBITION ATTENUATES T-TUBULE REMODELING AFTER CARDIAC STRESS VIA MAINTENANCE OF JP2 EXPRESSION. Because T-tubule integrity is a major determinant of excitation-contraction coupling (E-C coupling) function and cardiac contractility (18), we hypothesized that excessive calpain activity contributes to T-tubule remodeling after cardiac stress. As shown in the representative in situ confocal T-tubule images and quantitated TT_{power} data, an index of T-tubule regularity, LV cardiomyocytes from the TAB+MDL-28170 group had improved T-tubule organization compared with the TAB group (**Figures 3A and 3B**). Similarly, calpain inhibition protected against ISO-induced severe T-tubule disorganization and subcellular T-tubule loss in cardiomyocytes (**Figures 3C and 3D**). In the MI model, we examined T-tubule structure in the infarct zone, the region adjacent to the infarct (border zone), and LV myocytes in the posterior wall (remote zone). As expected, MI resulted in a complete loss of the T-tubule structure in the infarct zone, a marked disruption of T-tubule integrity in the border zone, and a less pronounced, but substantial, remodeling in the remote zone. Treatment with MDL-28170 attenuated the loss of T-tubule integrity in the border and remote zones (**Figures 3E and 3F**). Together, these results demonstrate treatment with a calpain inhibition after cardiac stress minimizes deleterious T-tubule remodeling, thus preserving cardiac function.

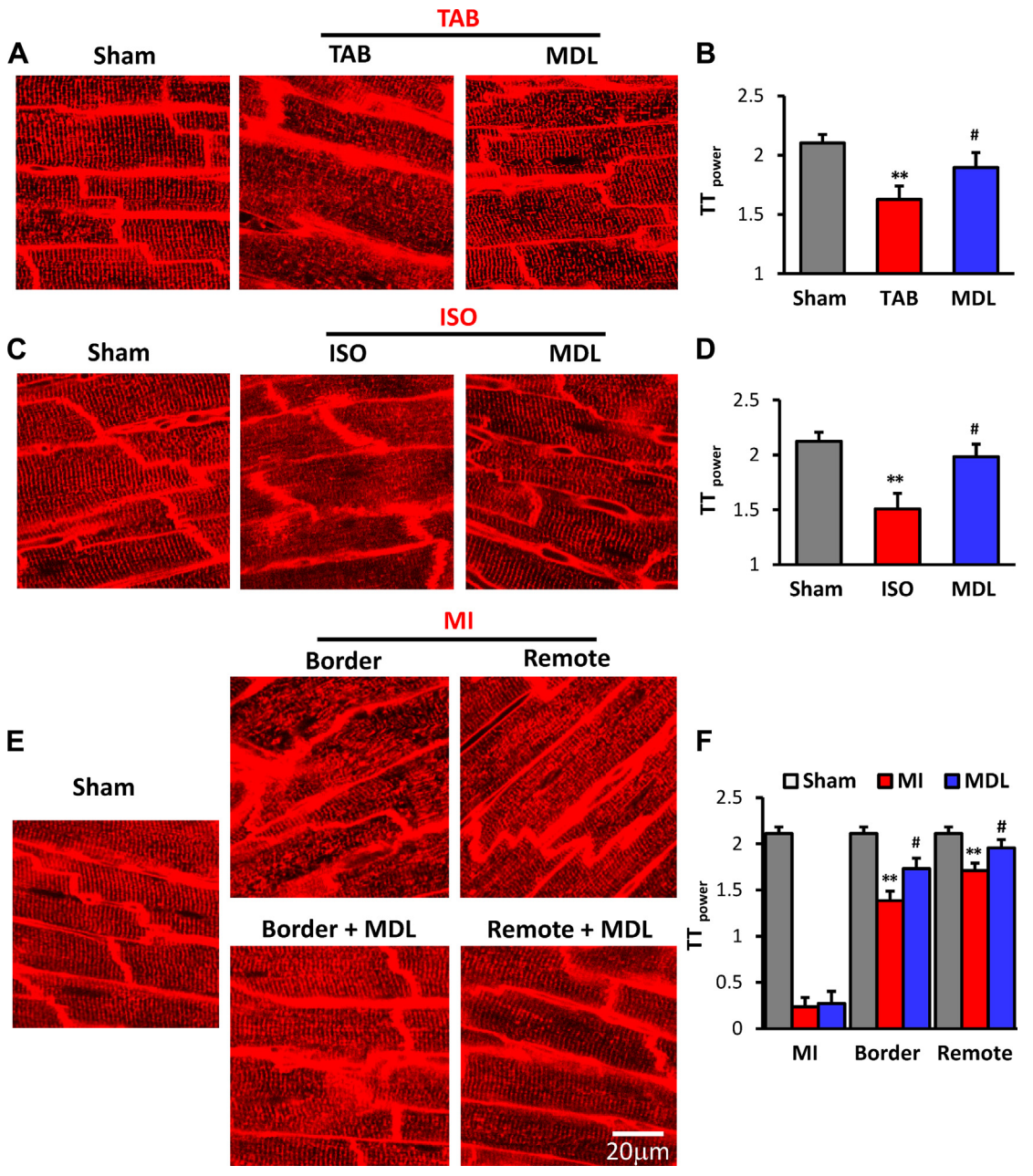
Work from our group and others implicate JP2 as an important stabilizer of the T-tubule network and the integrity of E-C coupling ultrastructure (9,19-24). We next assessed whether calpain inhibition preserves JP2 expression following TAB, MI, or ISO. In all 3 models, JP2 was significantly down-regulated in response to cardiac stress (**Figure 4,** Supplemental Figure S4), in line with findings in human failing hearts. However, treatment with MDL-28170 protected against JP2 down-regulation, suggesting that inhibition of calpain activity prevents loss of cardiac

FIGURE 2 MDL-28170 Protects Against Cardiac Stress-Induced Heart Failure



(A) Calpain activity was assessed in LV lysates after TAB, MI, or ISO minipump infusion. **(B and C)** Average heart weight/body weight **(B)** and lung weight/body weight **(C)** ratios at 5 weeks after surgery or 2 weeks after ISO minipump implantation. **(D to F)** End-systolic volume (ESV) **(D)**, end-diastolic volume (EDV) **(E)**, and ejection fraction (EF) **(F)** were assessed by echocardiography. n = 7 to 11 mice per group. For **A**, **p < 0.01 as indicated. For **B to F**, *p < 0.05, **p < 0.01 versus sham; †p < 0.05, ††p < 0.01 versus saline control. Control = the saline control beginning 3 days after surgery; HW/BW = heart weight/body weight; ISO = isoproterenol; LV = left ventricular; LW/BW = lung weight/body weight; MDL = MDL-28170 beginning 3 days after surgery; MI = myocardial infarction; Sham = sham surgery; TAB = transverse aortic banding.

FIGURE 3 Calpain Inhibitor MDL-28170 Ameliorates T-Tubule Remodeling in Response to Cardiac Stress

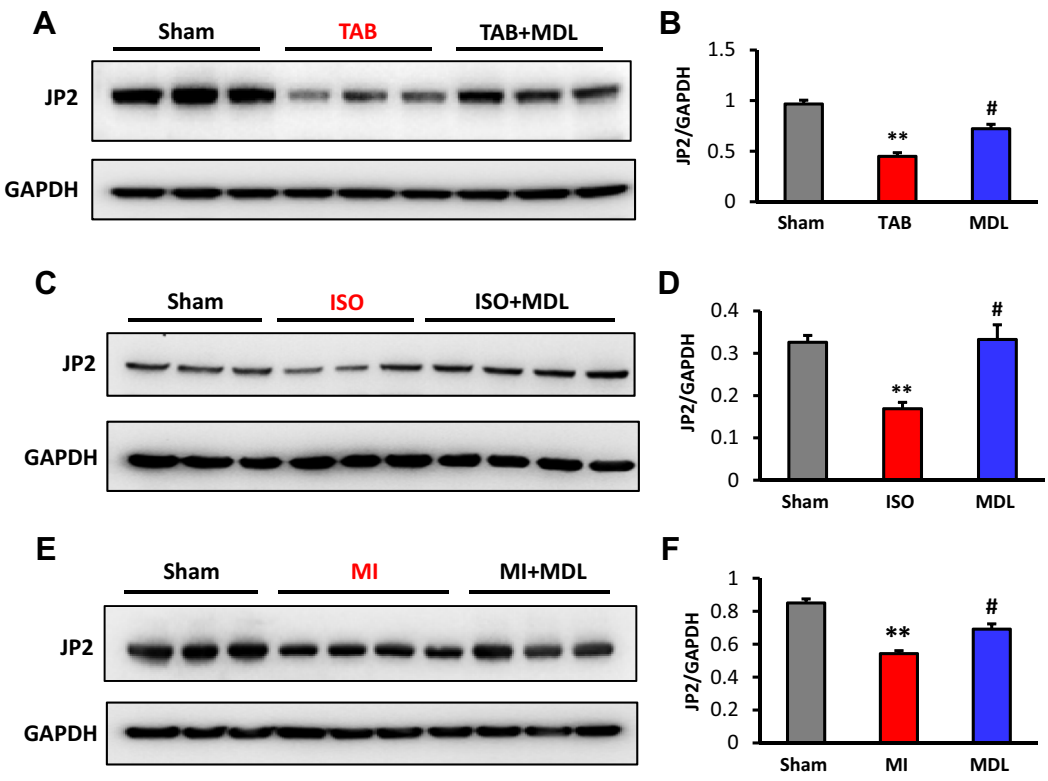


(A, C, E) Representative in situ LV T-tubule confocal images after staining with lipophilic marker MM 4-64. **(A, C)** LV cardiomyocytes after TAB **(A)** or ISO infusion **(C)**. **(E)** Representative images of the border and remote zones relative to the infarct zone in the MI model. **(B, D, F)** Mean values of TT_{power}. n = 4 to 5 mice per group. **p < 0.01 versus sham; †p < 0.05 versus saline control. T-tubule = transverse tubule; TT_{power} = strength of regularity of the T-tubule system; other abbreviations as in Figure 2.

function, at least partially, by preserving JP2 expression and T-tubule structural integrity.

JP2 OVEREXPRESSION INITIALLY PROTECTS AGAINST CARDIAC DYSFUNCTION IN MICE WITH CALPAIN OVEREXPRESSION. In addition to JP2, many other calpain substrates, such as dystrophin, utrophin, spectrin, calcineurin, Ca^{2+}/calmodulin-protein kinase and actin, may play important roles in maintaining cardiac structure and function (3). For example, cardiac hypertrophy induced by ISO administration leads to calpain-mediated breakdown of sarcomeres and heart failure (25). We therefore examined

FIGURE 4 Calpain Inhibition Blunts Stress-Induced JP2 Dysregulation



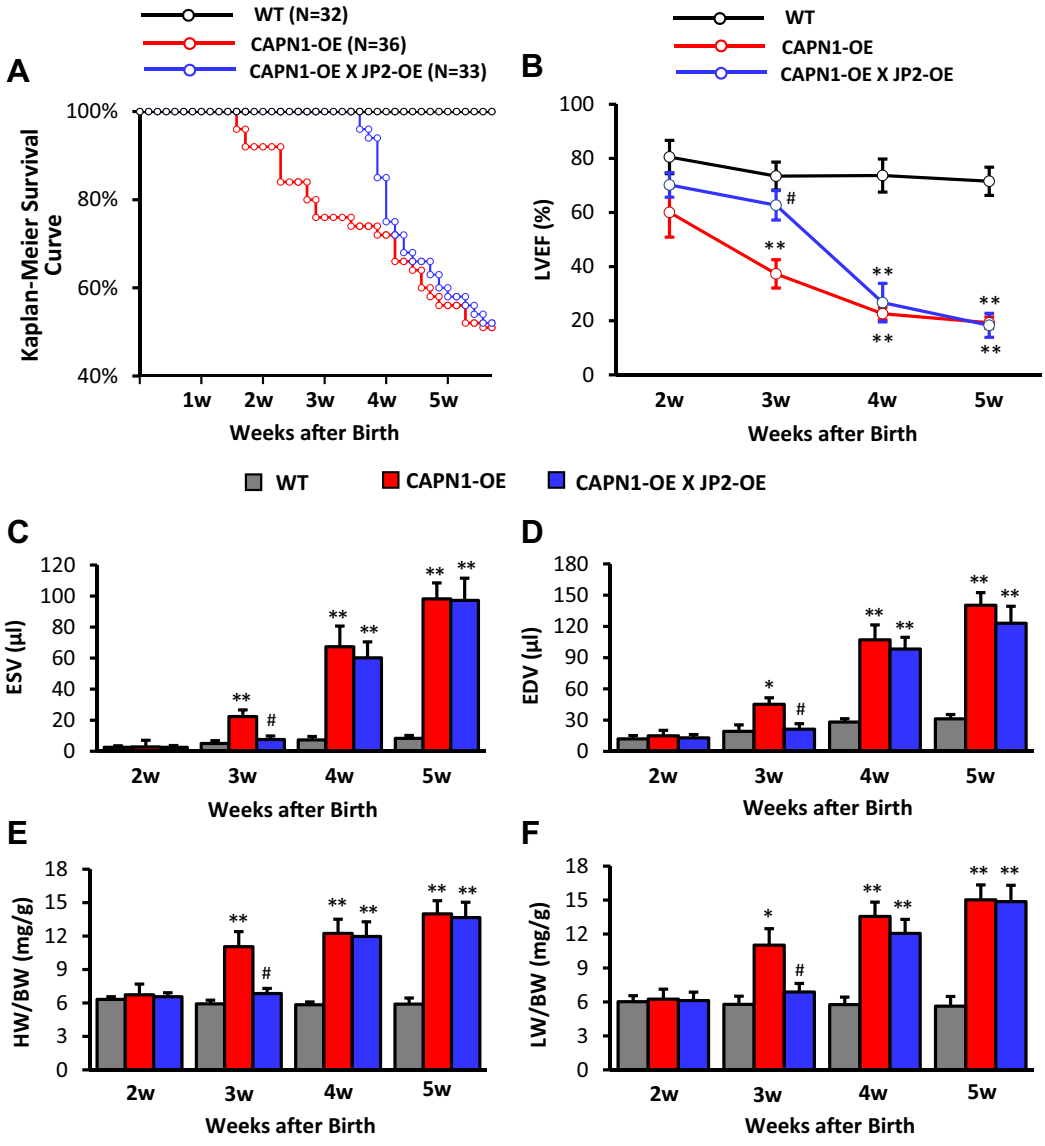
(A to F) Western blots **(A, C, E)** and quantitation **(B, D, F)** of JP2 expression in heart lysates after TAB or MI at 5 weeks or ISO infusion at 2 weeks. GAPDH was used as the loading control. **p < 0.01 versus sham; †p < 0.05 versus saline control. n = 3 to 4 mice per group. JP2 = junctophilin-2; other abbreviations as in Figure 2.

the relative role of calpain-dependent cleavage of JP2 in heart failure mediated by excessive calpain activation. To test this, we examined transgenic mice with cardiac-specific overexpression of calpain-1 alone (CAPN1-OE mice) or with dual overexpression of calpain-1 and JP2 (CAPN1-OExJP2-OE mice), which were generated by crossing CAPN1-OE mice with JP2-OE mice. Expression of the transgene was increased by ~3-fold in both CAPN1-OE mice and JP2-OE mice (Supplemental Figures S5 and S6).

We first asked whether JP2 overexpression can protect against the deleterious effects of calpain-1 overexpression. As compared with wild-type (WT) mice, the overall 2- and 3-week relative survival rates of CAPN1-OE mice were 92% and 76%, respectively, whereas the corresponding survival rates for CAPN1-OExJP2-OE mice were higher, with ~100% at 2 and 3 weeks. The double-transgenic mice started to die at day 25. However, beginning at 4 weeks of age, the relative survival rates were nearly identical between the CAPN1-OE and CAPN1-OExJP2-OE groups (72% to 75%). The Kaplan-Meier survival curves continued to overlap between groups at 5 weeks (~58%) (Figure 5A). These data indicate that JP2 overexpression delayed the onset of cardiac sudden death and thus initially prolonged survival, but was not sufficient to protect against the adverse effects of sustained calpain-1 overactivation.

In line with the survival data, CAPN1-OE mice had significantly decreased LVEF beginning at 3 weeks of age as compared with WT mice (Figure 5B). Dual expression of calpain and JP2 attenuated this decrease at 3 weeks, but the protection was lost beginning at 4 weeks. Similarly, end-diastolic volume and end-systolic volume were significantly improved and heart weight/body weight and lung weight/body weight ratios were decreased in the CAPN1-OExJP2-OE mice at 3 weeks, but not at later ages, as compared with CAPN1-OE mice (Figures 5C to 5F). These results indicate that JP2 overexpression in the setting of sustained calpain-1 overexpression is not sufficient to mitigate spontaneous heart failure.

FIGURE 5 Effects of JP2 Overexpression on Survival and Cardiac Function in CAPN1-OE Mice

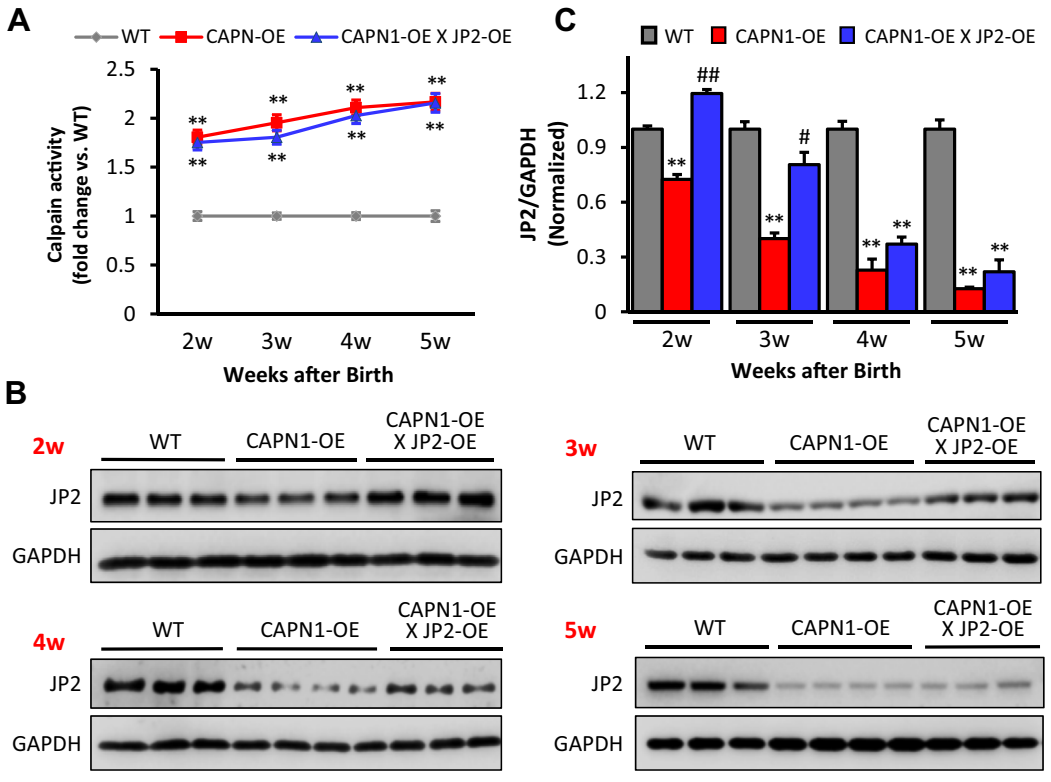


(A) Kaplan-Meier survival curve for WT, CAPN1-OE, and CAPN1-OExJP2-OE mice (n = 32, 36, and 33, respectively). **(B to D)** Ejection fraction **(B)**, ESV **(C)**, and EDV **(D)** were assessed by echocardiography. **(E, F)** Average HW/BW **(E)** and LW/BW **(F)** ratios. n = 7 to 11 per group. *$p < 0.05$, **$p < 0.01$ versus age-matched WT; †$p < 0.05$ versus age-matched CAPN1-OE. CAPN1-OE = calpain-1 overexpressing; CAPN1-OExJP2-OE = calpain-1 overexpressing junctophilin-2 overexpressing double-transgenic cross; WT = wild-type; other abbreviations as in Figures 1, 2, and 4.

JP2 EXPRESSION PROGRESSIVELY DECLINES IN CAPN1-OExJP2-OE MICE. To understand the underlying mechanism of the aforementioned findings, we first examined the impact of JP2 overexpression on calpain activity. CAPN1-OE mice had a 1.8-fold increase in calpain activity in the heart at 2 weeks after birth as compared with WT mice (Figure 6A). Calpain activity progressively increased between 2 and 5 weeks after birth, with a 2.2-fold increase in calpain activity at 5 weeks. In CAPN1-OExJP2-OE mice, calpain activity was nearly identical to that observed in CAPN1-OE mice at all time points examined (Figure 6A), demonstrating that co-overexpression of JP2 does not alter calpain function.

We next investigated whether JP2 expression is sustained or down-regulated in CAPN1-OExJP2-OE mice. As expected, JP2 levels in CAPN1-OE mice

FIGURE 6 JP2 Expression Progressively Declines in CAPN1-OExJP2-OE Mice



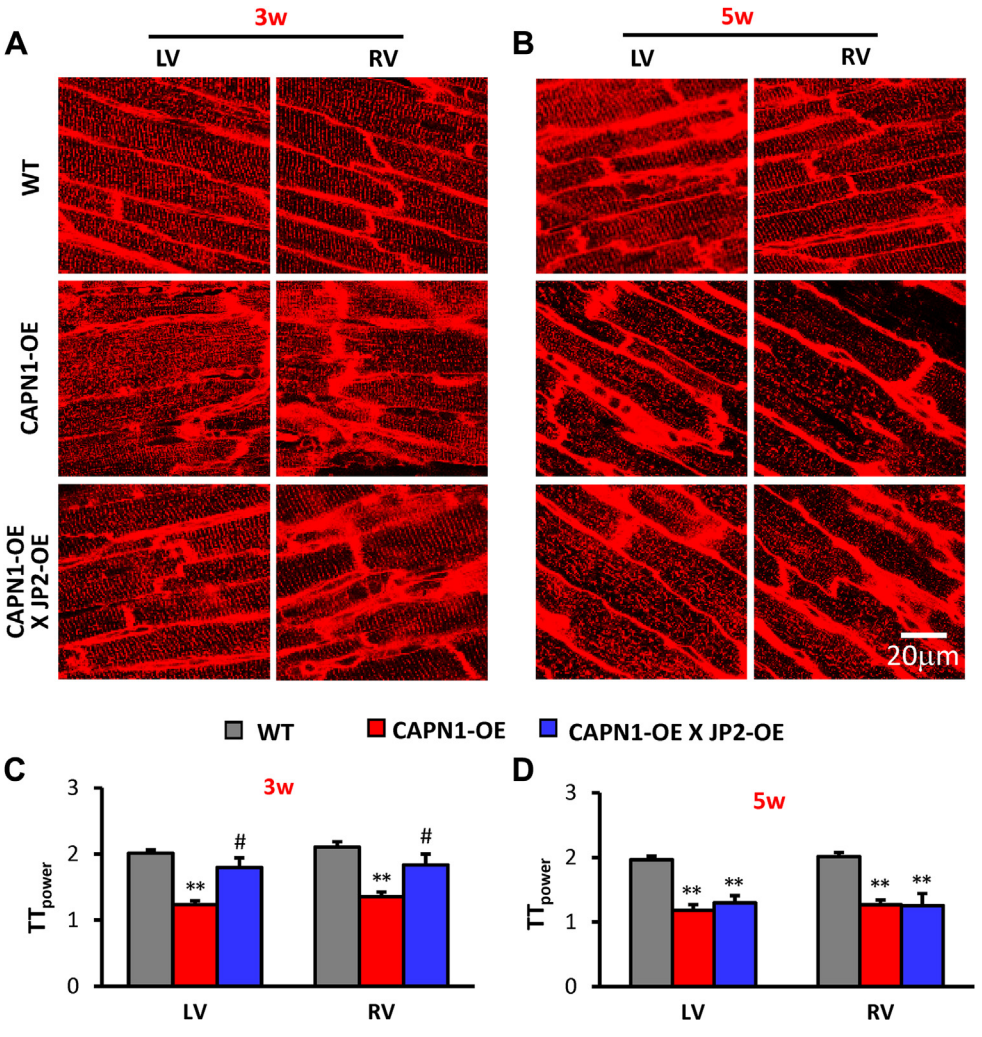
(A) Calpain activity assay in heart lysates from WT, CAPN1-OE, and CAPN1-OExJP2-OE mice at different ages. **(B, C)** Representative Western blot **(B)** and summary data **(C)** of JP2 expression in heart lysates WT, CAPN1-OE, and CAPN1-OExJP2-OE mice. n = 6 to 11 per group. *p < 0.05, **p < 0.01 versus age-matched WT; †p < 0.05, ††p < 0.01 versus age-matched CAPN1-OE. Abbreviations as in Figure 5.

progressively decreased with age as compared with WT controls (Figures 6B and 6C). At 2 weeks after birth, JP2 levels in CAPN1-OExJP2-OE mice were 1.2-fold higher than in WT mice. By 3 weeks of age, however, JP2 levels were trending toward a decrease in CAPN1-OExJP2-OE mice as compared with WT mice. JP2 levels continued to decline with age in CAPN1-OExJP2-OE mice, with levels nearly identical to CAPN1-OE mice at 5 weeks (Figures 6B and 6C). These results indicate that JP2 overexpression is not sufficient to antagonize the deleterious effects of calpain overexpression.

JP2-OE INITIALLY PRESERVES T-TUBULE ORGANIZATION AND Ca^{2+} HANDLING IN THE SETTING OF EXCESS CALPAIN ACTIVATION. To determine whether the progressive decline in JP2 expression mediates T-tubule remodeling in CAPN1-OExJP2-OE mice, we evaluated T-tubule integrity in 3- and 5-week-old mice using in situ T-tubule confocal imaging. As compared with myocytes from WT hearts, calpain overexpression resulted in a severely disorganized T-tubule pattern in LV and right ventricular (RV) myocytes (Figures 7A and 7B), with a statistically significant decrease in TT_{power} (Figures 7C and 7D). The T-tubule network in LV and RV myocytes from 3-week-old CAPN1-OExJP2-OE mice was much better organized as compared with myocytes from age-matched CAPN1-OE mice, consistent with a significantly higher level of JP2 protein in CAPN1-OExJP2-OE mice, compared with CAPN1-OE alone mice. However, at 5 weeks, there was a marked loss in T-tubule integrity in both LV and RV myocytes from CAPN1-OExJP2-OE mice, which corresponds with the decline in JP2 expression and cardiac function.

Because a fundamental effect of T-tubule remodeling in cardiomyocytes is defective E-C coupling, we analyzed Ca^{2+} transients in ventricular myocytes by in situ imaging of perfused intact hearts. Autonomously beating cardiomyocytes from CAPN1-OE

FIGURE 7 JP2 Overexpression Initially Preserves T-Tubule Integrity in CAPN1-OE Mice



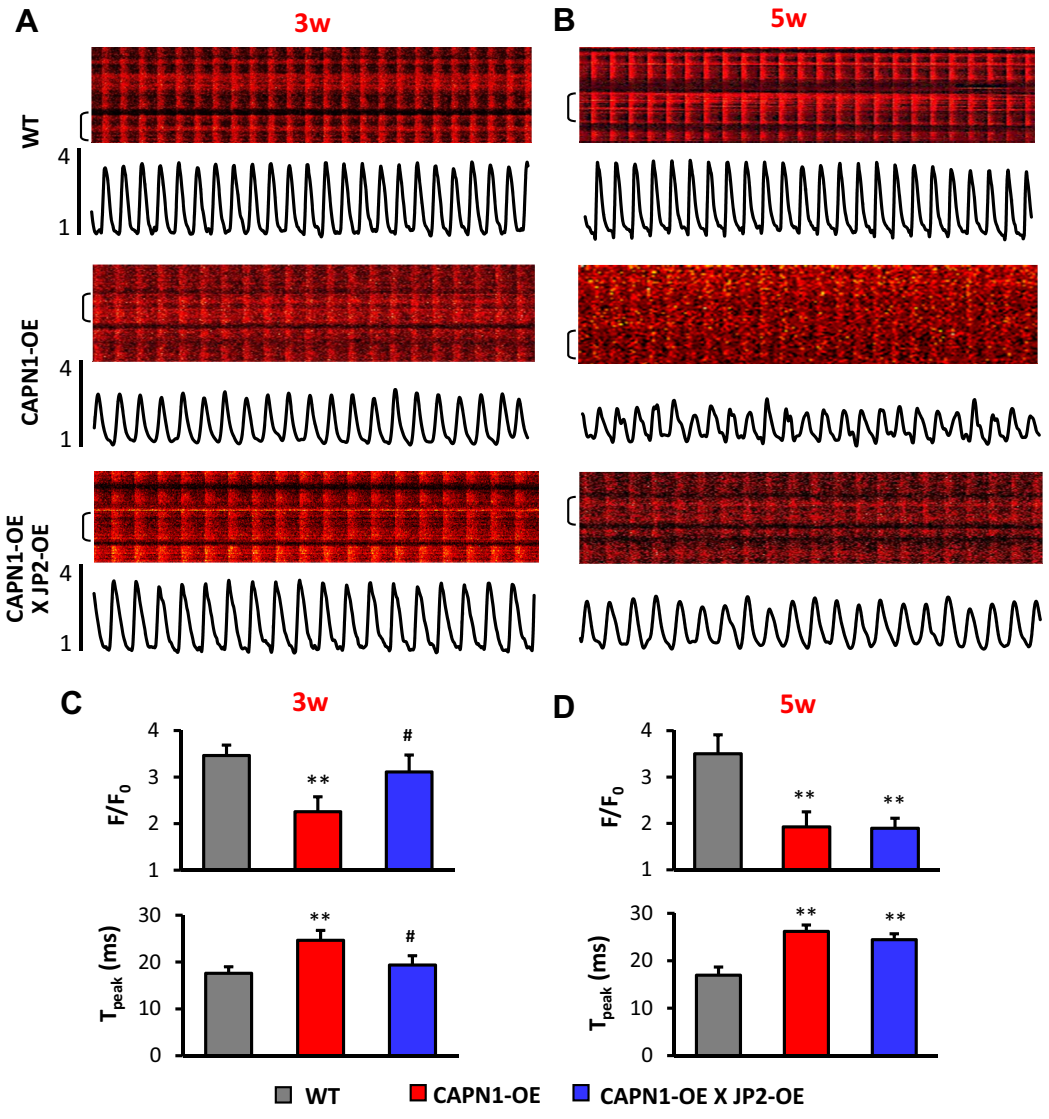
(A and B) Representative in situ confocal images of T-tubules in LV and RV myocytes from WT, CAPN1-OE, and CAPN1-OExJP2-OE mice at 3 weeks **(A)** and 5 weeks **(B)** of age. **(C, D)** Average TT_{power} in LV and RV myocytes from WT, CAPN1-OE and CAPN1-OExJP2-OE mice at 3 weeks **(C)** and 5 weeks **(D)** of age. n = 4 to 6 hearts for each group. **p < 0.01 versus age-matched WT; †p < 0.05 versus age-matched CAPN1-OE. RV = right ventricle; other abbreviations as in Figures 4 and 5.

mice displayed a depressed amplitude of Ca^{2+} transients and prolonged time to peak as compared with age-matched WT myocytes (Figure 8). In accordance with the observation of T-tubule structure, we observed a significantly improved Ca^{2+} amplitude and shortened time to peak in CAPN1-OExJP2-OE relative to CAPN1-OE mice at 3 weeks, but not 5 weeks. These data suggest that calpain-dependent JP2 cleavage and loss of T-tubule integrity play an important role in the mechanism by which calpain hyperactivity promotes myocyte Ca^{2+} handling dysfunction.

DISCUSSION

Abundant evidence demonstrates that calpains target many proteins that are required for cardiac homeostasis, and calpain activity is markedly enhanced in multiple heart failure models (3,6). Despite this resounding evidence, no clinical studies have pursued calpain inhibition as a therapeutic opportunity for heart failure. In this study, we first established that calpain is hyperactive in human failing hearts of different etiologies and in 3 different rodent models of heart failure. Second, we observed

FIGURE 8 JP2 Overexpression Initially Alleviates Impaired Ca^{2+} Handling Dysfunction Induced by Excessive Calpain Activation



(A, B) Representative examples of in situ confocal Ca^{2+} images in WT, CAPN1-OE, and CAPN1-OExJP2-OE hearts at 3 weeks **(A)** and 5 weeks **(B)** of age under autonomous beating. Bottom traces under each image are profiles of Ca^{2+} transients. **(C, D)** Summary data of the amplitude **(left)** and time to peak **(right)** of Ca^{2+} transients under autonomous beating at 3 weeks **(C)** and 5 weeks **(D)** after birth. n = 250 to 300 cells from 4 to 5 hearts per group. **p < 0.01 versus age-matched WT; †p < 0.05, ††P < 0.01 versus age-matched CAPN1-OE. Abbreviations as in Figures 4 and 5.

that JP2, one of the substrates of calpain proteolysis and a key protein in cardiomyocyte E-C coupling, is down-regulated in these samples. Third, we found that inhibiting calpain activity in vivo prevented TAB-, MI-, and ISO-induced structural and functional remodeling, as well as heart failure development. Lastly, we investigated the mechanistic role of JP2 in calpain-cleavage–mediated cardiac remodeling and dysfunction. JP2 overexpression initially improves cardiac function and extends survival in the setting of calpain-1 overexpression, but is not sufficient to mitigate the effects of sustained calpain-1 overexpression.

Our data substantiate previous reports of the protective role of calpain inhibition in cardiomyopathies. For example, transgenic overexpression of the

endogenous calpain inhibitor, calpastatin, protects against myocardial dysfunction in models such as myocardial infarction (26) and endotoxemia (27). The Peng group has also used genetic deletion of calpain-4, a regulatory subunit for both calpain-1 and calpain-2, to demonstrate the protective effect of inhibiting calpain activity on cardiac function, deleterious reactive oxygen species production, endoplasmic reticulum stress, and mitochondrial metabolic function in multiple different experimental models, including diabetes, ischemia (8), high-fat diet (28), and endotoxemia (29). Studies have also explored the use of MDL-28170 in the acute heart failure model of cardiac ischemia/reperfusion injury and found that administration of the calpain inhibition at the time of injury limits ischemia/reperfusion-induced infarction (30). A recent paper from the David Garcia-Dorado group reported that oral administration of SNJ-1945 (a calpain inhibitor) attenuated calpain activation and reduced scar expansion, ventricular dilation and dysfunction, probably through prevention of inflammation pathway in a rat model of transient ischemia (30 min) followed by 21 days of reperfusion (31). Herein, our new data extend these findings by identifying calpain inhibition as a general therapeutic strategy to protect against deleterious ultrastructural remodeling and cardiac dysfunction in multiple common models of chronic LV heart failure.

Recent work from our group established that JP2, a structural protein spanning T-tubules and the SR membrane, is crucial for maintaining normal T-tubule organization (9,11,32). Our previous data on animal models of cardiac stress show that down-regulation of the JP2 is associated with T-tubule disorganization and heart failure development (11), whereas overexpression of JP2 protects against stress-induced heart failure and sudden cardiac death (9). We also found that JP2 down-regulation is driven by activation of the Ca^{2+}-dependent protease, calpain (16,17). Supporting this work, herein we demonstrate in human failing hearts that increased calpain activity correlates with loss of JP2 expression. Similarly, calpain activity was increased and JP2 expression decreased in 3 different animal models of heart failure. In all 3 models, treatment with the calpain inhibitor MDL-28170 preserved JP2 expression as well as T-tubule structure and cardiac function. Interestingly, overexpression of JP2 provided only short-term protection against sudden death and cardiac failure in the dual CAPN1-OExJP2-OE model, compared with CAPN1-OE alone mice. This observation is consistent with the data that JP2 expression level was maintained at high or close to normal control levels at early stage (2 to 3 weeks) (**Figures 6B and 6C**), but fell off quickly afterwards (4 to 5 weeks) in CAPN1-OExJP2-OE hearts. This is not surprising, as JP2 was continuously cleaved in the presence of excessively elevated calpain activities in CAPN1-OExJP2-OE hearts.

A handful of calpain inhibitors have advanced into clinical trials for Alzheimer's disease, amylotrophic lateral sclerosis, multiple sclerosis, muscular dystrophy, and spinal muscular atrophy (33), though to date, none has been explored for cardiovascular indications. Despite the wide use of angiotensin-converting enzyme inhibitors and beta-blockers, heart failure remains a major cause of morbidity and mortality in developed countries. Thus, there remains a strong need to devise new mechanism-based treatments for heart failure. However, no drugs are currently under development or testing in human patients to target calpain for heart failure treatment. Our data reported in the present study provide compelling evidence that calpain inhibitors should be tested in clinical trials for its efficacy in treatment of human chronic heart failure.

STUDY LIMITATIONS. MDL-28170 at the concentration we used may also inhibit cathepsin B (34). The expression levels of cathepsin B are elevated gradually following TAB stress, and deficiency of cathepsin B protects against pathological cardiac remodeling to pressure overload stress (35). Although we consider the pathway of calpain elevation → JP2 down-regulation → T-tubule disorganization → E-C coupling dysfunction as the primary mechanism contributing to heart failure development, we recognize that MDL-28170 may also exert its beneficial effects via inhibition of cathepsin B-mediated signaling.

CONCLUSIONS

Our data demonstrate that hyperactivation of the calpain proteolytic system leads to heart failure in part through JP2 down-regulation and T-tubule remodeling, and these harmful effects can be prevented through inhibition of calpain activity with MDL-28170. JP2 overexpression initially improves cardiac function and increases survival in calpain-overexpressing mice, yet JP2 expression wanes over time in this dual-transgenic model. The progressive decline in JP2 expression is accompanied by T-tubule

remodeling, loss of E-C coupling function, and cardiac dysfunction. Together, these data identify calpain inhibition as a promising therapeutic strategy to mitigate cardiac remodeling and the progression of heart failure.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: Classical heart failure treatments, in particular angiotensin-converting enzyme inhibitors, angiotensin II receptor blockers, and beta-blockers, have been shown to improve heart function, prolong life, and reduce heart failure–associated sudden cardiac death. However, there is still a strong need to devise new mechanism-based treatments for heart failure.

TRANSLATION OUTLOOK: Inhibiting calpain activity is a promising therapeutic strategy for heart failure treatment by mitigating cardiac remodeling and the progression of heart failure. When combined with other heart failure medications, calpain inhibitor may exert synergistic effects on protection of cardiac structure and function, and on prolongation of patient lifespan.

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APPENDIX For supplemental figures, please see the online version of this paper.