



# Nuclear localization of a novel calpain-2 mediated junctophilin-2 C-terminal cleavage peptide promotes cardiomyocyte remodeling

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## Abstract

Heart failure (HF) is a leading cause of morbidity and mortality worldwide. Patients with HF exhibit a loss of junctophilin-2 (JPH2), a structural protein critical in forming junctional membrane complexes in which excitation–contraction takes place. Several mechanisms have been proposed to mediate the loss of JPH2, one being cleavage by the calcium-dependent protease calpain. The downstream mechanisms underlying HF progression after JPH2 cleavage are presently poorly understood. In this study, we used Labcas to bioinformatically predict putative calpain cleavage sites on JPH2. We identified a cleavage site that produces a novel C-terminal JPH2 peptide (JPH2-CTP) using several domain-specific antibodies. Western blotting revealed elevated JPH2-CTP levels in hearts of patients and mice with HF, corresponding to increased levels of calpain-2. Moreover, immunocytochemistry demonstrated nuclear localization of JPH2-CTP within ventricular myocytes isolated from a murine model of pressure overload-induced HF as well as rat ventricular myocytes treated with isoproterenol. Nuclear localization of JPH2-CTP and cellular remodeling were abrogated by a genetic mutation of the nuclear localization sequence within JPH2-CTP. Taken together, our studies identified a novel C-terminal fragment of JPH2 (JPH2-CTP) generated by calpain-2 mediated cleavage which localizes within the cardiomyocyte nucleus during HF. Blocking nuclear localization of JPH2-CTP protects cardiomyocytes from isoproterenol-induced hypertrophy in vitro. Future in vivo studies of the nuclear role of JPH2-CTP may reveal a causal association with adverse remodeling during HF and establish CTP as a therapeutic target.

**Keyword** Calpain · Heart failure · Junctophilin-2 · Nuclear translocation

## Abbreviations

Ca<sup>2+</sup> Calcium  
CAPN Calpain  
CTP C-terminal peptide

HF Heart failure  
ISO Isoproterenol  
JPH2 Junctophilin-2  
NLS Nuclear localization signal  
RyR2 Ryanodine receptor type 2  
SR Sarcoplasmic reticulum  
TAC Transverse aortic constriction

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## Introduction

Heart failure (HF) results from inefficient cardiac contraction and relaxation, which fails to match the body's metabolic demands. HF is a major public health concern, affecting over 23 million people worldwide with a median mortality rate of approximately 5 years [5]. HF disproportionately affects minorities and the elderly and is the leading cause of hospitalization of those over 65 in the United States. Moreover, HF poses a substantial financial burden on the health-care system [5]. Currently available therapeutic approaches to treat HF patients are limited due to unfavorable side effects and limited ability to rescue cardiac contractility in HF with reduced ejection fraction [2]. Understanding the underlying mechanism of HF will be required to achieve the long-term goal of reducing mortality and improving patients' quality of life.

HF leads to adverse remodeling in the myocardium at multiple levels [13]. At the molecular level, HF progression is marked by disturbances in intracellular calcium ( $\text{Ca}^{2+}$ ) handling within cardiomyocytes [6, 9, 20]. Cardiomyocyte  $\text{Ca}^{2+}$  handling is coordinated by the dyad-spanning protein junctophilin-2 (JPH2), the cardiac isoform of the junctophilin family of proteins, which facilitates excitation–contraction coupling in excitable cells. JPH2 maintains junctional membrane complexes by approximating plasmalemmal and intracellular ion channels within cardiomyocyte dyads [17, 18]. Additionally, JPH2 directly binds to and regulates several  $\text{Ca}^{2+}$  channels including the ryanodine receptor type 2 (RyR2) and L-type  $\text{Ca}^{2+}$  channel [3]. Previous studies have demonstrated that loss of JPH2 promotes increased  $\text{Ca}^{2+}$  release through “leaky” RyR2 channels, indicating that JPH2 maintains normal  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$ -release by directly stabilizing the RyR2 channel. In addition, JPH2 helps maintain the appropriate dyadic distance between the plasma membrane invaginations known as transverse tubules and the sarcoplasmic reticulum (SR) [22, 27]. Several studies have revealed a loss of transverse tubule structures and junctional membrane complexes during HF, which has been attributed to a loss of JPH2 levels in HF [27, 31, 38]. Moreover, over-expression of JPH2 in transgenic mice and JPH2 re-expression via adeno-associated virus in a murine model of pressure overload-induced HF prevented decompensated HF by restoring transverse tubule regularity and stabilizing RyR2, establishing JPH2 as a potential HF therapy [28].

The mechanisms by which JPH2 levels are reduced in HF are incompletely understood. Three overarching hypotheses have been examined as a mechanism underlying JPH2 loss including reduced translation mediated by micro RNAs [34], mechanical redistribution by

microtubules [37], and cleavage by proteases such as  $\text{Ca}^{2+}$ -sensitive proteolytic enzyme calpain (CAPN) [11, 12, 33] and matrix metalloproteinase-2 (MMP-2) [8]. Recent studies revealed that overexpression of CAPN1 can cause cleavage of JPH2 into multiple N- and C-terminal fragments [11, 33]. Moreover, calpastatin, a physiological inhibitor of CAPN, was shown to have a protective role in murine models of ischemic HF [36] whereas calpain inhibition by pharmacological inhibitor MDL-28170 protects cardiac function in pressure-overload-induced HF, ischemic HF and stress-induced HF in mice [30]. Another recent study revealed JPH2 degradation in ischemia due to MMP-2 mediated cleavage [8]. These studies are important because they suggest that proteases such as CAPN and MMP-2 could potentially cleave recombinant JPH2 overexpressed by means of gene therapy, thus limiting the therapeutic effects of such an approach [27]. Recently, Guo et al. [12] reported that a large N-terminal JPH2 peptide is formed as a result of CAPN1 activation. This peptide can translocate into the nucleus where it exerts a protective effect on cardiomyocytes through *Mef2* gene repression. However, the role of CAPN2 mediated proteolysis of JPH2 in HF remains unknown. Given that CAPN2 is activated under higher concentrations of intracellular  $\text{Ca}^{2+}$ , which are often seen during HF progression, we hypothesize that JPH2 is also cleaved by CAPN2 during adverse remodeling associated with heart failure.

## Methods

For full details refer to the methods in Online Resource 1.

### Cloning

N-terminal or C-terminally HA-tagged JPH2 was amplified with KOD Xtreme Hot Start polymerase and cloned into a pcDNA3.1 + vector as previously described [25]. See Online Resource 1, Supplementary Table 1 for details.

### Cell culture and transfection

HEK293T, H9C2, and neonatal rat ventricular myocytes (NRVMs) were cultured and transfected according to established protocols [25].

### Human samples

Left ventricular samples were obtained from patients undergoing heart transplantation for end-stage heart failure (HF) after obtaining informed consent. Non-failing (NF) human hearts (which could not be used for transplantation) were obtained from the ‘International Institute

for the Advancement of Medicine' (IIAM) or the Gift of Hope. Human samples used in this study are listed in Online Resource 1, Supplementary Table 2. All studies were performed with IRB approval from Baylor College of Medicine and Texas Heart Institute (H31918), or the human studies committees at the University of Alabama, Birmingham [26].

## Animal studies

Studies including transverse aortic constriction (TAC) and echocardiography were performed on 2-month old male and female C57Bl6/J mice (Online Resource 1, Supplementary Table 3) as described [28], according to protocols approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine conforming to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996).

## Western blot

Western blots were performed according to previously described protocols [25, 28]. See Online Resource 1 for details.

## Immunocytochemistry

JPH2 localization in TAC isolated cardiomyocytes and H9C2, HEK293T, and NRVM cell cultures were visualized by immunostaining and confocal microscopy according to

previously established protocols with minor adjustments [28]. Refer to Online Resource 1 for more detail.

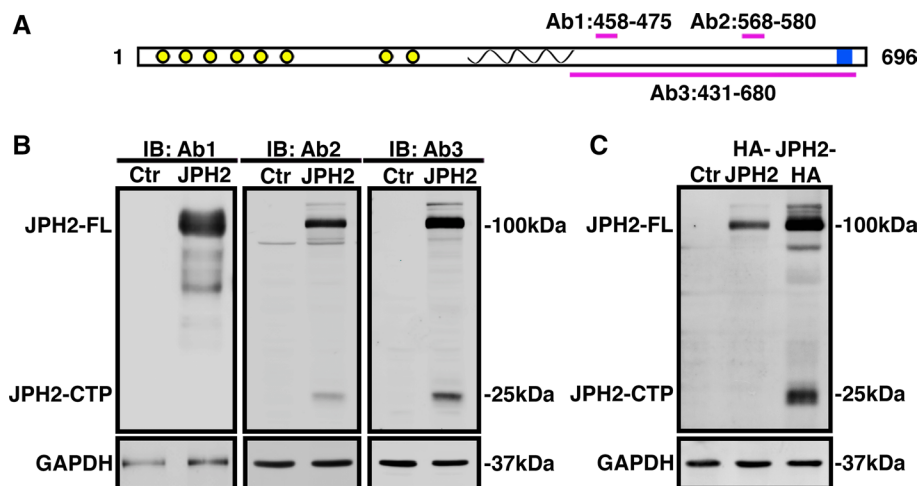
## Statistics

Results are expressed as mean  $\pm$  standard error of the mean (SEM). The Shapiro–Wilk test was performed to determine whether data were normally distributed. The unpaired *T*-test, Mann–Whitney test, one-way ANOVA, or nonparametric Kruskal Wallis test were used for comparisons between groups, as specified in figure legends. A *p* value of less than 0.05 was considered statistically significant.

## Results

### JPH2 is cleaved at its C-terminus

Full-length JPH2 is a 696-bp protein consisting of six 'membrane occupation and recognition nexus' (MORN) domains at its N-terminus which mediates attachment to the plasma membrane, followed by a dyad-spanning alpha-helical region, a divergent region, and a transmembrane domain that inserts into the SR at its C-terminus (Fig. 1a). While previous studies focused on N-terminal peptides cleaved from JPH2 following calpain-mediated proteolysis [11, 12], we set out to identify potential C-terminal cleavage peptides. First, we identified three different JPH2 antibodies that recognize epitopes consisting of amino acids (aa) 458–475 (Ab1), 568–580 (Ab2), and 431–680 (Ab3), respectively (Fig. 1a; Online Resource 1, Supplementary Table 4). Lysates from



**Fig. 1** JPH2 is cleaved at its C-terminus. **a** Cartoon of JPH2 protein with N-terminal MORN domains (yellow),  $\alpha$ -helix (spiral), and C-terminal transmembrane domain (blue). Antibody epitopes are labeled Ab1, Ab2 and Ab3. **b** Western blots using three different JPH2 antibodies revealing expression of full-length (FL) JPH2 and a 25-kDa C-terminal peptide in lysates of HEK293 cells expressing

either JPH2 or pcDNA3.1 vector control (Ctr). **c** Western blots using HA antibody revealing expression of JPH2-FL and/or JPH2-CTP in lysates of HEK293 cells that were not transfected (Ctr; lane 1), expressing HA-JPH2 (lane 2), or JPH2-HA (lane 3), respectively. All the experiments were performed at least 3 times to validate reproducibility

HEK293 cells expressing mouse JPH2 cDNA or empty pcDNA3.1 + vector control were exposed to  $\text{CaCl}_2$  (2 mM) for 2 h at 37 °C to activate endogenous calpains. Immunoblotting revealed that all 3 antibodies identified full-length JPH2 (JPH2-FL) (Fig. 1b). In contrast, only Ab2 and Ab3 detected a distinct 25-kDa C-terminal fragment (JPH2-CTP). To confirm that JPH2-CTP is comprised of the C-terminus of JPH2, the JPH2 sequence was fused with an N-terminal (HA-JPH2) or C-terminal (JPH2-HA) HA-tag for identification on immunoblots. Following expression of HA-JPH2, JPH2-HA, and empty vector control in HEK293 cells, lysates were exposed to 2 mM  $\text{Ca}^{2+}$  as before. Immunoblotting using HA antibody detected the 25-kDa cleavage peptide only when the HA-tag was fused to the C-terminus, thus confirming that JPH2-CTP is comprised of the C-terminus of JPH2 (Fig. 1c). Taken together these results indicate that JPH2 is most likely cleaved near its C-terminus resulting a novel 25-kDa peptide that is different from the C terminal peptide shown in previous calpain/JPH2 studies [11].

### JPH2 amino acids G482 and T483 are required for the formation of JPH2-CTP

Previous studies identified three CAPN cleavage sites in JPH2 (V<sup>155</sup>, L<sup>201</sup>, R<sup>565</sup>) with R<sup>565</sup> being the most predominant among them [11]. We ran the Labcas algorithm to identify unbiased potential calpain cleavage sites on JPH2. Our initial prediction indicated 65 possible calpain cleavage sites. To narrow the list of putative calpain cleavage sites, we filtered these initial predictions based on antibody detection (Ab2 positive, Ab1 negative), since results from Fig. 1b demonstrate that JPH2 cleaved product was detected by Ab2 but not Ab1. Therefore, any predicted calpain sites N-terminal of amino acid 464 were excluded. HA antibody also detected the C-terminally HA-tagged JPH2 ~ 25 kDa band (Fig. 1c), suggesting that the JPH2 fragment includes the JPH2 C-terminal end. Therefore, ExPASy was used to predict the molecular weights of fragments from the cleavage site to the C-terminal end of JPH2 and predicted molecular weights under 20 kDa were excluded based on expected size (~ 25 kDa). These filters resulted in a list of 13 cleavage sites, which we then ordered by cleavage probability rank (Online Resource 1, Supplementary Table 5 and Supplementary Fig. 1a, b). The top four ranked sites include G<sup>482</sup>, L<sup>500</sup>, A<sup>481</sup> and E<sup>467</sup> (Fig. 2a; Online Resource 1, Supplementary Table 5). Based on these result and predictions, the top predicted cleavage site is G<sup>482</sup>, which would result in the production of a C-terminal peptide including amino acids T<sup>483</sup>-T<sup>696</sup> with a molecular weight of ~ 24 kDa. The C terminal R<sup>565</sup> described in a previous study [11] is detected by the algorithm. The probability score of R<sup>565</sup> (0.008908) is almost half of G<sup>482</sup> (0.017001). Taken together, we inferred

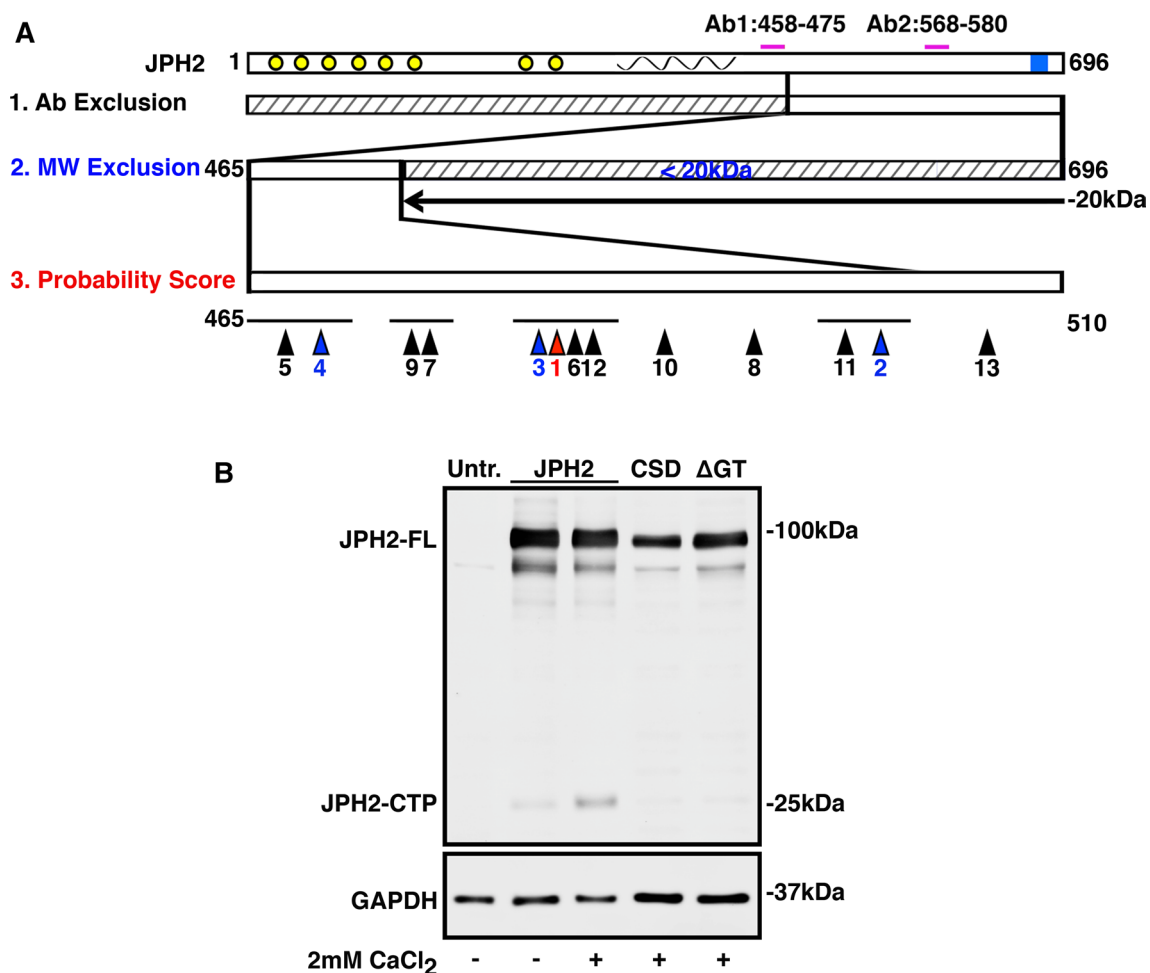
that <sup>479</sup>SPAG/TPPQ<sup>486</sup> is the predicted cleavage motif for CTP formation due to cleavage after G<sup>482</sup>.

To test this hypothesis, we constructed two cleavage site mutants, (1) JPH2-CSD deleting the complete cleavage motif <sup>479</sup>SPAG/TPPQ<sup>486</sup> and (2) JPH2-Δ<sup>482</sup>GT<sup>483</sup> deleting <sup>482</sup>GT<sup>483</sup> only. Deleting the complete motif would prevent JPH2 cleavage at secondary cleavage sites in close proximity such as A<sup>481</sup>, T<sup>483</sup> or P<sup>484</sup> (3rd, 6th and 12th predicted cleavage sites, respectively; Online Resource 1, Supplementary Table 5). We expressed C-terminally HA-tagged JPH2 cleavage site mutants in HEK293 cells and exposed cell lysates to 2 mM  $\text{CaCl}_2$ . Western blotting with anti-JPH2 antibody Ab2 revealed that deletion of either the complete motif or only the 2 amino acids surrounding the predicted cleavage site diminished JPH2-CTP formation. This suggests that <sup>479</sup>SPAG↓TPPQ<sup>486</sup> with <sup>482</sup>GT<sup>483</sup> at its core is the calpain cleavage site that produces the JPH2-CTP (Fig. 2b).

### Activated CAPN2 is required for JPH2-CTP formation

Based on previous reports showing CAPN-mediated JPH2 proteolysis and our observation that  $\text{Ca}^{2+}$  increased JPH2-CTP formation at a predicted CAPN-cleavage site (Fig. 2b), we investigated which CAPN isoform(s) is/are responsible for generating JPH2-CTP. We transfected JPH2 in HEK cells grown on separate wells as biological repeats ( $N=6$ ). We collected cell lysates from separate wells after 48 h and exogenous treatment with 2 mM  $\text{CaCl}_2$  individually resulted in a significant increase in JPH2-CTP formation ( $2.67 \pm 0.47$  arbitrary units (AU) in  $\text{CaCl}_2$  treated compared to  $1.16 \pm 0.11$  AU in untreated;  $p=0.0073$ ) and decreased full length (FL)-JPH2 ( $0.59 \pm 0.05$  in  $\text{CaCl}_2$ -treated compared to  $0.96 \pm 0.04$  AU in untreated;  $p=0.0006$ ). By contrast, 2.5 μM calpastatin treatment blocked this  $\text{Ca}^{2+}$ -induced JPH2 cleavage ( $1.46 \pm 0.14$  AU in  $\text{CaCl}_2$  + calpastatin-treated compared to  $2.67 \pm 0.47$  AU in  $\text{CaCl}_2$ -only group;  $p=0.03$ ). Moreover, calpastatin was able to rescue JPH2-FL protein levels ( $0.95 \pm 0.06$  AU in the  $\text{CaCl}_2$  + calpastatin-treated group compared to  $0.59 \pm 0.05$  AU in  $\text{CaCl}_2$ -treated group;  $p=0.0008$ ) (Online Resource 1, Supplementary Fig. 2a–c), suggesting that calpain is required for the formation of the CTP.

However, since calpastatin is a nonselective inhibitor of CAPNs [32], we also used shRNA-mediated reduction of specific CAPN isoforms to determine which isoform was responsible for JPH2 cleavage at the predicted site. The most active forms of CAPN in cardiac tissue are CAPN1 and CAPN2 while CAPN4 serves as the small subunit for both these CAPNs [23]. Differentiated H9C2 rat ventricular myocytes grown in separate plates ( $N=6$ ) were infected with lentiviral vectors expressing shRNAs against *Capn1* (*shCapn1*), or *Capn2* (*shCapn2*), or scrambled shRNA control (*shScr*). The specificity of these shRNAs was confirmed



**Fig. 2** JPH2 amino acids G482 and T483 are required for the formation of JPH2-CTP. **a** JPH2 cartoon showing full-length protein with the antibody epitopes (top). Since JPH2-CTP is not detected by Ab1, the domain N-terminal of Ab1 is excluded (see 1). Based on western blotting, the molecular weight of JPH2-CTP is estimated to exceed 20 kDa, hence the C-terminal domain is excluded (see 2). The domain spanning amino acids 465–510 (see 3) is predicted to contain the calpain cleavage site. Bioinformatic predictions of possible cleav-

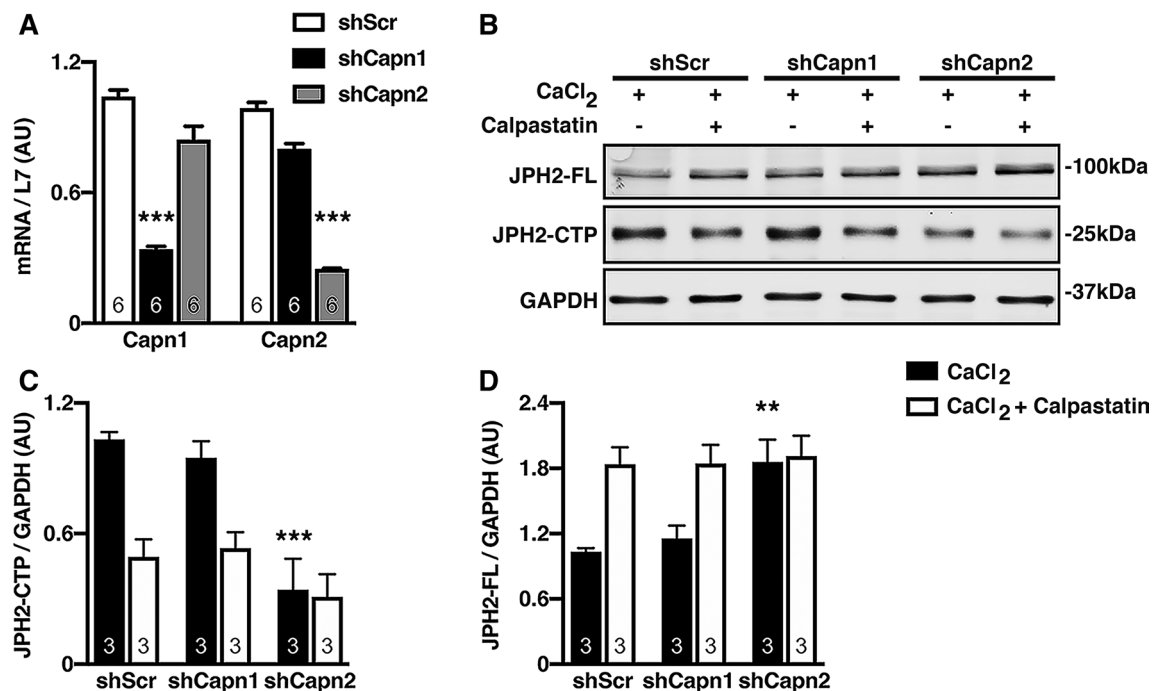
age sites are shown, with the relative rankings below the sites marked by arrow heads. The site with the highest predicted score is colored red, the 2nd–4th highest prediction scores are colored blue. **b** Western blots showing levels of full-length JPH2 (JPH2-FL) and JPH2-CTP in lysates of HEK293 cells expressing wild-type JPH2, JPH2-CSD, and JPH2-ΔGT (in which residues <sup>482</sup>G-<sup>483</sup>T were removed). All the experiments were performed at least 3 times to validate reproducibility

by measuring *Capn* mRNA levels relative to the *L7* house-keeping gene. *shCapn1* transfected cells had decreased levels of *Capn1* ( $0.34 \pm 0.01$  AU) but not *Capn2* ( $0.84 \pm 0.06$  AU) compared to *shScr* controls ( $1.04 \pm 0.03$ ,  $p = 0.0005$ ); whereas, *shCapn2*-transfected cells had decreased *Capn2* ( $0.25 \pm 0.003$  AU) but not *Capn1* transcripts ( $0.81 \pm 0.02$  AU) compared to *shScr* controls ( $0.99 \pm 0.03$  AU,  $p = 0.0002$ , Fig. 3a). These results validated the specificity the isoform-specific *Capn* shRNAs used.

To determine whether CAPN1 or CAPN2 was responsible for the JPH2 C-terminal cleavage, H9C2 cells were treated with *shCapn1*, *shCapn2*, or *shScr* in individual wells ( $N = 3$ ), and these lysates were exposed to 2 mM CaCl<sub>2</sub> with or without calpastatin. *shCapn1* treated cells retained the formation of JPH2-CTP; whereas, *shCapn2*

knockdown and calpastatin inhibited the formation of the JPH2-CTP ( $0.34 \pm 0.14$  AU compared to *shScr*,  $1.03 \pm 0.03$  AU,  $P = 0.0005$ ; and *shCapn1*,  $0.95 \pm 0.08$  AU,  $p = 0.0014$ ; Fig. 3b, c). Moreover, *shCapn2* rescued JPH2-FL protein levels ( $1.86 \pm 0.20$  AU compared to *shScr*,  $1.10 \pm 0.06$  AU,  $p = 0.0126$ ; and *shCapn1*,  $1.15 \pm 0.12$  AU,  $p = 0.0196$  Fig. 3b, d). We did a separate western blot with the Ca<sup>2+</sup> untreated H9C2 lysate alongside Ca<sup>2+</sup> and shRNA treated lysates. Our results (Online Resource 1, Supplementary Fig. 3) further validated CAPN2 mediated generation of CTP as *shCapn2* abrogated CTP to basal level as Ca<sup>2+</sup> untreated controls. These results suggested Ca<sup>2+</sup>-activated CAPN2 is required to generate JPH2-CTP in physiological conditions. Although our data predict CAPN2 to cleave predominantly at G<sup>483</sup> to generate JPH2-CTP and previous





**Fig. 3** Activated calpain-2 is required for JPH2-CTP formation. **a** Quantification of qPCR measurements of *Capn1* and *Capn2* mRNA levels relative to L7 in H9C2 cells infected with lentiviral vectors containing shRNA against *Capn1* (*shCapn1*), *Capn2* (*shCapn2*), or a scrambled control (*shScr*). **b** Representative western blot with JPH2 antibody (Ab2) showing expression of full-length JPH2 (JPH2-FL)

and JPH2-CTP in H9C2 cells following *Capn1* or *Capn2* inhibition by shRNA in presence of 2 mM CaCl<sub>2</sub> plus or minus calpastatin. GAPDH was used as a loading control. Western blot quantification of JPH2-CTP (**c**) and JPH2-FL (**d**) normalized to GAPDH. Data were analyzed using the nonparametric Kruskal–Wallis test (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ )

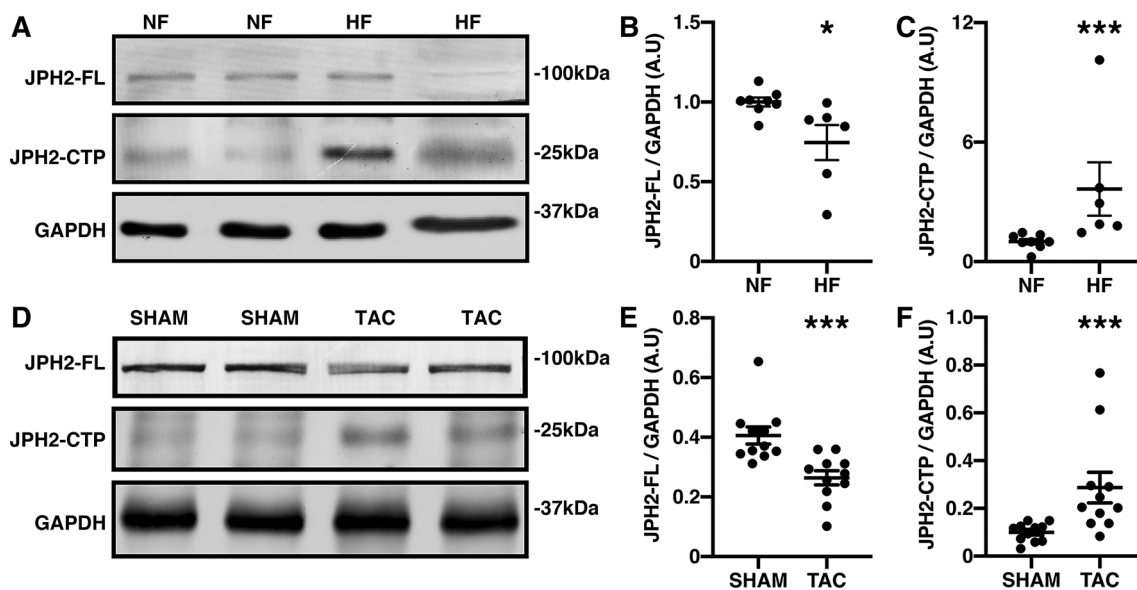
studies showed that CAPN1 cleaves JPH2 to generate the 75 kDa N-terminal fragment (JPH2-NTP), both CAPN1 and CAPN2 could generate NTP and CTP in non-physiological conditions when we incubated recombinant JPH2 with either recombinant CAPN1 or CAPN2 (Online Resource 1, Supplementary Fig. 4). Coomassie staining, JPH2 N term and C term antibody blotting shows that both calpain-1 and calpain-2 cleave JPH2 to generate JPH2-NTP and CTP.

### JPH2 C-terminal cleavage is increased in HF

Prior studies reported increased JPH2 cleavage of CAPN1 induced JPH2 peptides in mice with HF [33]. To assess the physiological significance of CTP, we investigated its abundance during HF by measuring JPH2-CTP with western blot detected by JPH2 antibody Ab2. Interestingly, CTP was significantly increased almost four-fold in human HF ventricular lysates ( $N=6$ ) compared to non-failing (NF) ( $N=8$ ) controls ( $3.65 \pm 1.34$  AU in HF compared to  $1 \pm 0.14$  AU in NF,  $p=0.0007$ ); whereas, JPH2-FL is decreased significantly in HF samples ( $0.75 \pm 0.11$  AU in HF compared to  $1 \pm 0.03$  AU in NF,  $P=0.0127$ ) (Fig. 4a–c). Further, we subjected 3-month-old B16/J mice to transverse aortic constriction (TAC) to model pressure-overload induced HF. Echocardiography to assess cardiac function 8-weeks post-TAC

revealed reduced EF in TAC mice ( $27.1 \pm 0.7\%$ ) compared to sham controls ( $73.3 \pm 0.8\%$ ,  $p < 0.0001$ ; Online Resource 1, Supplementary Table 3). Western blotting from ventricular lysates with antibody Ab2 revealed significantly decreased levels ( $0.26 \pm 0.02$  AU compared to  $0.41 \pm 0.03$  AU in controls,  $p=0.0004$ ,  $N=11$ ) of full-length (FL) JPH2 but increased levels ( $0.28 \pm 0.06$  AU compared to  $0.1 \pm 0.01$  AU,  $p=0.0006$ ,  $N=11$ ) of JPH2-CTP in TAC mice, compared to sham controls (Fig. 4d–f). GAPDH was used as a loading control. Overall, these results demonstrate elevated JPH2-CTP in both human and murine HF, suggesting that this particular JPH2-CTP plays a potential role in HF progression.

To determine Calpain expressions in these human HF and TAC induced mouse HF samples, we measured both CAPN2 expression at protein level from these samples. Prior studies showed that in HF, CAPN1 promotes JPH2 R<sup>565</sup> cleavage, creating a 75 kDa cleaved N-terminal fragment [12]. So, we also measured CAPN1 protein levels by western blot from these aforementioned samples (Online Resource 1, Supplementary Fig. 5). Western blot with CAPN isoform-specific antibodies (Online Resource 1, Supplementary Fig. 5a–c) revealed a significant increase in protein expression of CAPN2 only in human HF samples ( $2.661 \pm 0.56$  vs.  $1.00 \pm 0.229$  a.u. in HF vs. NF,  $p=0.034$ ,  $N=4$ ) whereas an increase in CAPN1 expression was not



**Fig. 4** JPH2 C-terminal cleavage is increased in HF. **a, b** Representative western blots using JPH2-Ab2 of nonfailing (NF) and heart failure (HF) human heart lysates (**a**) and quantification (**b, c**) showing unaltered full-length (FL) JPH2 but increased C-terminal peptide (CTP) expression normalized to GAPDH loading control. **d-f** Representative western blots and quantification of JPH2-FL (**e**) and

CTP (**f**) in sham and TAC mice relative to GAPDH from mice heart left ventricles after transverse aortic constriction (TAC) compared to sham controls. Data were analyzed using the Mann–Whitney test (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ). All the experiments were performed at least 3 times to validate reproducibility

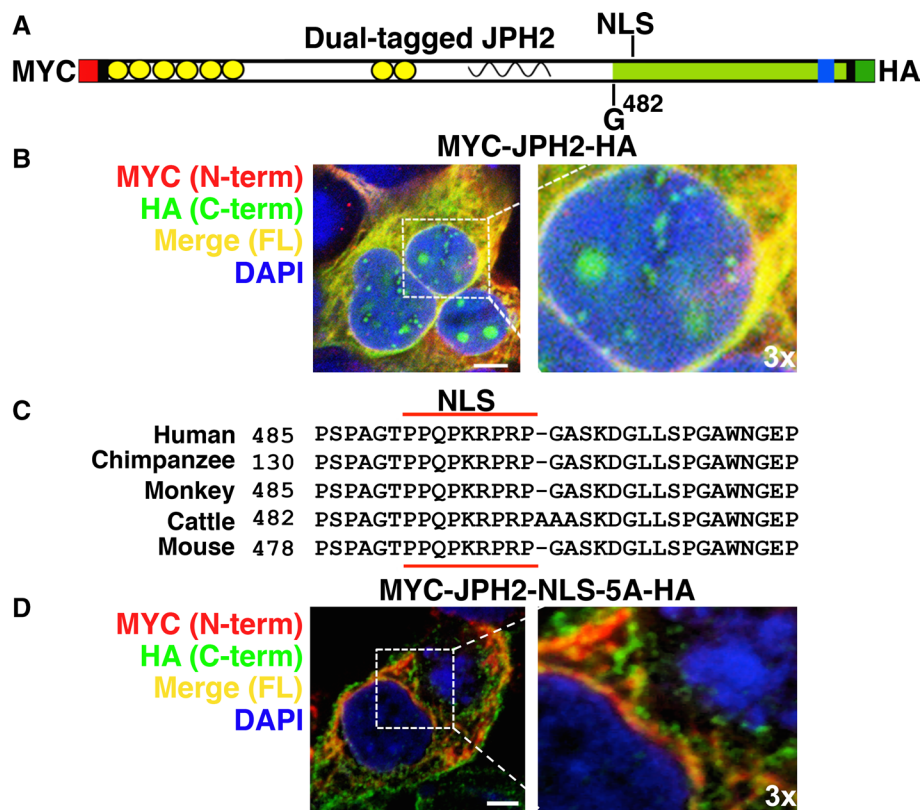
significant ( $1.682 \pm 0.379$  vs.  $1.00 \pm 0.172$  a.u. in HF vs. NF,  $p = 0.11$ ,  $N = 4$ ) in these samples. We further assessed the correlation between calpain isoforms and JPH2-FL and CTP from these human samples [ $N = 8$  (NF + HF)]. Our results (Online Resource 1, Supplementary Fig. 6a–d) interestingly revealed a strong positive correlation between CAPN2 and JPH2-CTP ( $R = 0.8611$ ,  $p = 0.006$ ) whereas the positive correlation between CAPN1 and JPH2-CTP was not significant ( $R = 0.3147$ ,  $p = 0.448$ ) suggesting calpain-2 as responsible to generate CTP. JPH2-FL had a significant negative correlation with both CAPN2 ( $R = -0.8805$ ,  $p = 0.004$ ) and CAPN1 ( $R = -0.8936$ ,  $p = 0.003$ ) further validating the previous calpain-1 mediated JPH2 degradation studies and our current findings with calpain-2. Moreover, western blot from TAC induced HF samples (Online Resource 1, Supplementary Fig. 5d–f) further revealed that CAPN2 protein expression was also significantly increased in TAC-induced HF ( $1.54 \pm 0.15$  vs.  $1.00 \pm 0.10$  a.u. in TAC vs. SHAM,  $p = 0.001$ ,  $N = 8$ ); whereas, CAPN1 was not significantly changed ( $1.23 \pm 0.14$  vs.  $1.00 \pm 0.13$  a.u. in HF vs. NF,  $p = 0.28$ ,  $N = 8$ ). Overall, these results further validate calpain-2 mediated JPH2-CTP formation.

### JPH2-CTP localizes to the nucleus via an NLS

Normally, JPH2 is a junctional membrane complex protein, which tethers the plasma membranes and the SR at cardiomyocyte dyads through its affinity for the plasma

membrane due to its N-terminal membrane occupation and recognition nexus (MORN) domains and C-terminal transmembrane domain insertion in the SR. However, previous studies have shown that during HF a protective N-terminal peptide translocates to the nucleus diffusely [12]. To understand the more about the role of the new JPH2-CTP, we constructed a dual-tagged Myc-JPH2-HA plasmid to observe its subcellular localization after the activation of calpain with  $\text{CaCl}_2$  (Fig. 5a). Anti-Myc and -HA antibodies demonstrated a punctate nuclear localization of the JPH2 C-terminus (HA) but not the N-terminus (Myc) after  $\text{CaCl}_2$  addition (Fig. 5b). Nuclear JPH2 was only detected with HA antibody suggesting this might be the JPH2-CTP, while JPH2-FL (merged HA and Myc staining) was observed in the extranuclear space.

Since JPH2-CTP localized to the nucleus, we analyzed the JPH2 sequence to determine whether the JPH2-CTP contained a nuclear localization sequence (NLS). NLS-mapper identified a consensus NLS downstream of the CAPN2 cleavage site in the JPH2 sequence ( $\text{T}^{484}\text{-PPQKRPR-P}^{492}$ ), which was conserved among different species (Fig. 5c). To validate the predicted JPH2 monopartite NLS, we developed a dual-tagged JPH2 NLS mutant construct (Myc-JPH2-NLS-5A-HA) by alanine mutagenesis of the proline-arginine rich domain of the NLS ( $\text{K}^{488}\text{-RPR-P}^{492}$  to  $\text{A}^{488}\text{-AAA-A}^{492}$ ). While HEK293 cells transfected with wildtype dual-tagged JPH2 demonstrated HA-stained punctate nuclear staining after  $\text{CaCl}_2$  addition, mutagenesis of the NLS abolished



**Fig. 5** Nuclear localization of JPH2-CTP requires the nuclear localization signal (NLS). **a** Cartoon of JPH2 showing the location of the NLS, the G482 cleavage site, and the N-terminal MYC and C-terminal HA tags. **b** Confocal images of HEK293 cells expressing dual-tagged JPH2 stained with MYC (red), HA (green) antibodies, and DAPI (blue). Green staining shows that JPH2-CTP translocated into the nucleus. **c** Alignment of a putative NLS in JPH2 across species.

**d** Confocal images of HEK293 cells expressing dual-tagged JPH2-NLS-5A (mutated NLS peptide) stained with MYC (N-terminus, red) HA (C-terminus, green), and DAPI (nucleus, blue) antibodies. Lack of green staining shows that the NLS is required for nuclear translocation of JPH2-CTP. Dotted white box indicates a magnified region (3× zoom); scale bar is 10 μm. All the experiments were performed at least 3 times to validate reproducibility

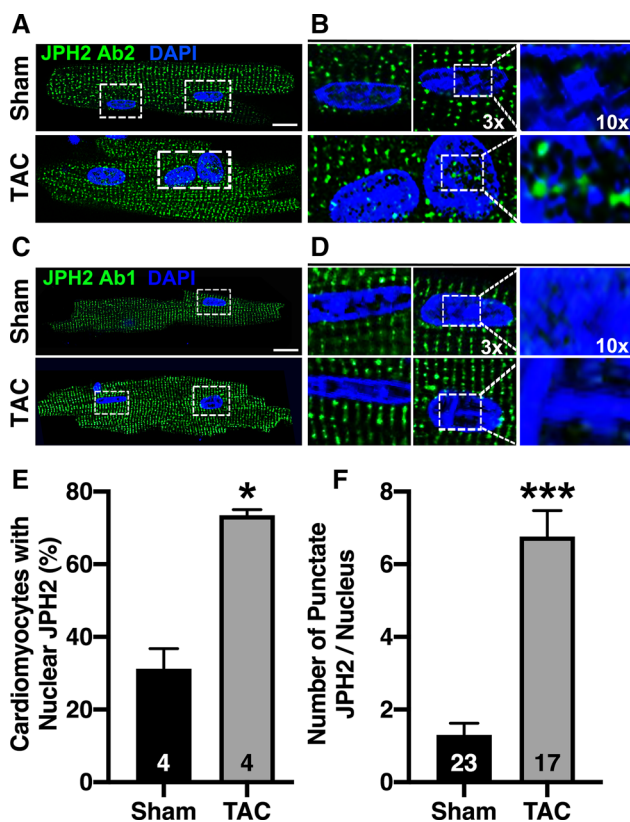
nuclear localization of JPH2 (Fig. 5d; Online Resource 1, Supplementary Fig. 7a). These results suggest that this monopartite NLS is required for nuclear localization of the JPH2-CTP. NLS-dependent punctate nuclear localization of C-terminal JPH2 occurs in other cell types like C2C12 myocytes (Online Resource 1, Supplementary Fig. 7b). To track full-length JPH2 and its cleaved fragments localization in live cells, we cloned JPH2 and NLS mutant tagged with eGFP at its C terminal. HEK cells transfected with JPH2-eGFP showed punctate nuclear GFP staining following exposure to 2 mM  $\text{CaCl}_2$ , while eGFP controls did not localize to the nucleus (Online Resource 1, Supplementary Fig. 8). Overall, these results demonstrate that CTP translocation to nucleus after  $\text{Ca}^{2+}$ -induced calpain cleavage depends on the NLS.

### Nuclear localization of JPH2-CTP is increased in HF

Since we observed elevated JPH2-CTP formation during HF (Fig. 4), we investigated whether nuclear localization

of CTP is also increased. To assess the subcellular localization of JPH2 in mouse hearts, we immune-stained isolated cardiomyocytes from both TAC-induced HF mice and sham controls with JPH2 Ab2, which recognizes both FL-JPH2 and JPH2-CTP, or Ab1 which recognizes FL-JPH2 but not JPH2-CTP. Confocal microscopy revealed increased nuclear JPH2 in TAC compared to sham controls (Fig. 6a). In alignment with our prior findings, Ab2 detected nuclear and non-nuclear JPH2 while Ab1 detected only JPH2 localized to dyads and the periphery of the nucleus (Fig. 6b). To better quantify the nuclear localization of JPH2-CTP in HF, the number of cardiomyocytes with a minimum of one nuclear puncta staining positive for JPH2 were compared between sham and TAC mice. Assessment of cardiomyocytes with JPH2-positive nuclei indicated an increase in the percent of cardiomyocytes positive for nuclear puncta after TAC-induced HF ( $73.50 \pm 1.55\%$  in TAC compared to  $31.25 \pm 5.54\%$  in sham controls;  $p = 0.029$ ,  $N = 4$ ; Fig. 6e). Moreover, the number of puncta within each JPH2-positive nucleus was significantly increased in TAC cardiomyocytes





**Fig. 6** JPH2-CTP translocates into the nucleus in myocytes from mice subjected to TAC. Immunofluorescence of fixed ventricular cardiomyocytes isolated from mice subjected to TAC or a sham procedure following staining with **a** JPH2 C-terminal antibody (Ab2); 3 $\times$  and 10 $\times$  zoom images are shown on the right (**b**). **c** Staining with JPH2 Ab1 which is N-terminal to the CTP; 3 $\times$  and 10 $\times$  zoom images on the right (**d**). White dotted box indicates zoom region. Scale bar = 10  $\mu$ m. **e** Quantification of the percent of cardiomyocytes with positive nuclear staining after TAC,  $N$  = number of mice. **f** Quantification of an average number of JPH2 puncta per nuclei after TAC-induced HF,  $N$  = number of nuclei. Data were analyzed using Mann–Whitney test (\* $p$  < 0.05; \*\* $p$  < 0.01; \*\*\* $p$  < 0.001). All the experiments were performed at least 3 times to validate reproducibility

relative to sham controls. ( $6.77 \pm 0.72$  puncta in TAC cardiomyocyte nuclei compared to  $1.30 \pm 0.32$  puncta in sham control nuclei,  $N$  = number of nuclei, 23 in Sham and 17 in TAC,  $p$  < 0.0001; Fig. 6f). Zoomed images revealed nuclear JPH2 in areas lacking heterochromatin (stained with DAPI; Fig. 6c, d). Taken together our findings indicate that nuclear localization of CTP is increased in a mouse model of HF.

### JPH2 nuclear localization is critical for isoproterenol-induced hypertrophy

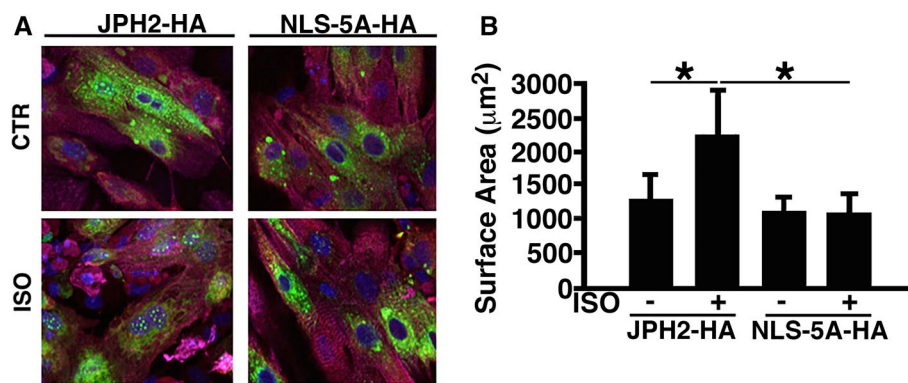
We found that nuclear transport of CAPN-cleaved JPH2-CTP was significantly increased in HF; however, the association of nuclear JPH2-CTP in the context of HF was still

inconclusive. To assess the involvement of JPH2-CTP in cardiomyocyte remodeling, we first developed an in vitro cardiomyocyte hypertrophy model to assess if isoproterenol (ISO), which mimics the excessive  $\beta$ -adrenergic stimulation in HF and increases intracellular  $Ca^{2+}$  concentrations, could induce this CAPN-mediated JPH2 cleavage. Calpains have been shown to mediate isoproterenol mediated hypertrophy [1]. To test our hypothesis, we treated isolated cardiomyocytes from 2-month old mice ( $N=4$ ) with saline or ISO. Immunoblotting with JPH2 Ab2 demonstrated that  $\beta$ -adrenergic stimulation reduced the amount of FL-JPH2 ( $0.25 \pm 0.11$  in ISO vs.  $0.56 \pm 0.04$  in Ctr;  $p=0.029$ ) and increased JPH2-CTP formation ( $0.47 \pm 0.05$  in ISO vs.  $0.13 \pm 0.02$  in Ctr;  $p=0.03$ ) in ISO-treated samples compared to saline-treated controls (Online Resource 1, Supplementary Fig. 9a–c). Our results indicate that our model of cellular stress-activated JPH2-CTP formation.

Next, to test the association of the nuclear localization of JPH2-CTP to cardiomyocyte modeling, we infected neonatal rat ventricular cardiomyocytes (NRVMs) with adenoviral vectors overexpressing JPH2-HA or JPH2-NLS-5A-HA ( $N=3$  NRVM isolations). To assess whether nuclear localization of the CAPN-cleaved CTP plays a role in cardiomyocyte remodeling in our in vitro model, infected NRVM cells were exposed to 10  $\mu$ M of ISO to induce cellular hypertrophy, one of the primary signs of maladaptive cardiac remodeling. After 24 h of ISO exposure, these NRVMs were co-stained with anti- $\alpha$ -actinin and anti-HA antibodies. Anti-HA staining clearly showed increased nuclear JPH2-CTP after ISO exposure in JPH2-HA infected cells, which was abolished in JPH2-NLS-5A-HA infected cardiomyocytes (Fig. 7a). Moreover, ISO treatment promoted cellular hypertrophy quantified by cell surface area in JPH2-HA infected cells ( $2319.4 \pm 666.7 \mu m^2$  in ISO vs.  $1347.2 \pm 361 \mu m^2$  in Ctr;  $p < 0.05$ , Fig. 7b). JPH2-NLS-5A-HA abrogated ISO-induced hypertrophy in NRVMs ( $1152.8 \pm 263.9 \mu m^2$  in ISO vs.  $1166.7 \pm 208.3 \mu m^2$  in Ctr, Fig. 7b) indicating that JPH2-CTP nuclear localization is required for ISO-mediated in vitro cardiomyocyte hypertrophy. Taken together, these data suggest that nuclear CTP is required for cardiomyocyte remodeling. However, further studies with genetically engineered mouse models abrogating JPH2-CTP formation and nuclear localization are warranted to assess whether nuclear CTP-induced remodeling is detrimental or compensatory.

## Discussion

In this study, we identified a calpain-cleaved JPH2 C-terminal fragment (JPH2-CTP), the levels of which are increased in HF. First, we found that JPH2 is cleaved in its C-terminal domain by CAPN2 at the  $^{482}GT^{483}$  site forming a 25 kDa JPH2-CTP. Second, we found that the calpain-2 isoform is



**Fig. 7** JPH2 nuclear localization is critical for isoproterenol-induced hypertrophy. **a** Representative immunofluorescence images of neonatal rat ventricular myocytes infected with adenovirus overexpressing JPH2-HA or JPH2-NLS-5A-HA, following treatment with isoproterenol (ISO) or buffer control (Ctr). Fixed cells were stained with HA

(green, located at JPH2 C-terminus), counterstained with  $\alpha$ -actinin (magenta) and DAPI (blue). **b** Quantification of cell surface area. Scale bar = 10  $\mu$ m. Data were analyzed using one-way analysis of variance (ANOVA) (\* $p < 0.05$ )

responsible for the cleavage of JPH2-CTP. Third, in patients with HF and mice with pressure overload-induced HF, JPH2-CTP levels are elevated. Fourth, we found that JPH2-CTP translocates into discrete areas within the nucleus due to a nuclear localization sequence within this peptide. Nuclear JPH2-CTP was also observed in cardiomyocytes isolated with mice subjected to transverse aortic constriction and myocytes treated with isoproterenol. Finally, mutagenesis of the NLS within JPH2-CTP prevented hypertrophic cardiomyocyte remodeling in ISO induced neonatal rat cardiomyocytes in vitro model, suggesting a potential association of JPH2-CTP in stress-induced cardiomyocyte remodeling.

### Calpains as a therapeutic target in heart failure

CAPNs are  $\text{Ca}^{2+}$ -activated proteolytic enzymes that have been identified as potential therapeutic targets in HF. CAPN1 and CAPN2 are the most active CAPN isoforms within the heart. Prior studies showed that both CAPN1 and CAPN2 are localized to the Z-disk [16], which aligns with the cardiomyocyte dyads where JPH2 is typically found. CAPN1 (activated by  $\mu\text{M}$   $\text{Ca}^{2+}$  concentration) and CAPN2 (activated by  $\text{mM}$   $\text{Ca}^{2+}$  concentration) are referred as  $\mu$ -CAPN and m-CAPN, respectively, based on their  $\text{Ca}^{2+}$  requirements [24]. Prior studies have demonstrated that both TAC and I/R injury-induced  $\text{Ca}^{2+}$  overload in rodent cardiomyocytes leads to CAPN-mediated proteolytic cleavage of several SR proteins promoting contractile dysfunction [19]. A number of studies established the inhibition of CAPN activity as a therapeutic strategy for HF [10, 21]. However, these studies have taken a broad approach of indiscriminate CAPN inhibition to prevent downstream effects of pressure overload. Moreover, they did not look at the long-term effects of CAPN inhibition [21, 23]. Additionally, multiple studies have demonstrated the paradoxical effects of CAPN1 and

CAPN2 in heart disease pathogenesis [29]. For instance, CAPN activation has an anti-hypertrophic response through the eNOS/AKT signaling pathway [4]. Moreover, both CAPN1 and CAPN2 regulate a number of essential cardiac proteins such as spectrin, desmin, PKC $\alpha$ , annexin 1, titin, calcineurin and cardiac troponin [19, 24]. Taken together, these studies suggest that CAPN has an important role in cardiac physiology. Considering the complex nature of CAPN's role in cell signaling, broad CAPN inhibition may have unforeseen side effects. As Calpain has both positive and negative effect on cardiac function, it will be important to distinguish between physiological and deleterious effects of CAPN activation to design Calpain targeted therapeutic approach in HF.

### Differential calpain cleavage of junctophilin-2

One important protein that is cleaved by CAPN is JPH2. In normal cardiomyocytes, JPH2 maintains a critical interdyadic distance through its N-terminal interaction with the plasmalemma via membrane occupation and recognition nexus (MORN) domains, a dyad-spanning alpha helix, and a transmembrane domain that anchors its C-terminus in the SR. Previous studies found that JPH2 is cleaved by CAPN1 to form multiple N-terminal and C-terminal fragments [12]. Our study identifies a novel CAPN-cleaved C-terminal fragment that spans from  $^{483}\text{T}$  to the end of JPH2. In contrast to previously identified CAPN1-cleaved fragments, our data suggest that the CAPN2 isoform cleaves JPH2 at the  $^{482}\text{GT}^{483}$  site since *shCapn2* but not *shCapn1* abolished JPH2-CTP formation. Both recombinant calpain-1 and calpain-2 cleaves JPH2 to generate NTP (amino acids 1–565) and CTP (amino acids 483–696) in vitro. Therefore, although we cannot rule out that the JPH2  $^{482}\text{GT}^{483}$  site may be cleaved by CAPN1 under different physiological

conditions, our results show that the CAPN2 isoform is responsible for cleavage of JPH2-CTP. CAPN1 is activated by  $\mu\text{M}$  amounts of  $\text{Ca}^{2+}$  seen in the dyad during normal physiological  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release, and CAPN2 is activated by  $\text{mM}$   $\text{Ca}^{2+}$  seen in pathological conditions. Therefore, CAPN2 and its cleavage of JPH2 at the  $^{482}\text{GT}^{483}$  site may have a more important role in pathological rather than physiological or compensatory conditions. This difference in how CAPN1 and CAPN2 proteolyze JPH2 could be exploited as a therapeutic strategy to maintain physiological but not pathological calpain activity in HF.

### Junctophilin-2 nuclear localization

This study also complements and expands upon the newly identified role of JPH2 in the nucleus. Guo et al. [12] identified a long N-terminal JPH2 fragment that was cleaved by CAPN at the  $^{565}\text{RT}^{566}$  site, which included a monopartite NLS. The authors of this study found diffuse nuclear localization of this N-terminal peptide, where it was targeted to the chromatin by its alpha helix, where it bound and repressed Mef2 transcription [12]. It was proposed that nuclear localization of this N-terminal peptide acts as a compensatory mechanism opposing the detrimental effects of full-length JPH2 loss in HF. On the other hand, our studies demonstrate that CAPN2-mediated cleavage of JPH2 at the  $^{482}\text{GT}^{483}$  site led to the inclusion of the monopartite NLS with the JPH2-CTP instead of the N-terminal fragment. This led to punctate as opposed to diffuse nuclear localization of the C-terminal fragment within cardiomyocyte nuclei. We investigated if CTP acts as a transcription factor similar to NTP. We utilized the UAS-Gal4 tool to analyze CTP mediated transcriptional regulation. Our results from these experiments suggest that JPH2-CTP does not act as a transcriptional factor as similar to JPH2-NTP (Online Resource 1, Supplementary Fig. 10). In cardiomyocyte nuclei isolated from TAC hearts, JPH2-CTP localized within discrete aggregates in the nuclei, which appear to be separate from the densely stained blue chromatin. It is possible that these aggregates are part of a nuclear subcompartment in the nucleoplasm itself or that they are located within nuclear “tunnels” bound to the nuclear membrane [14].

While further studies are needed to determine the specific role of JPH2-CTP in the nucleus, our present study demonstrates that blocking nuclear localization of the JPH2-CTP prevent ISO-induced cellular hypertrophy in NRVMs. Mutation of the monopartite NLS within JPH2 decreased nuclear localization of JPH2-CTP and abrogated ISO-mediated hypertrophy indicating that JPH2-CTP may play an opposing role to the N-terminal peptide in the nucleus. This finding differs from what was found in mice missing the monopartite NLS in the paper by Guo et al. [12]. In this paper, the authors claim that mice missing

the JPH2 NLS were protected after TAC. However, cardiac function in these mice was only assessed for a very brief time after TAC, which was not sufficient to determine whether loss of the NLS was indeed detrimental. Whether or not the nuclear localization of JPH2 is beneficial or harmful might depend on whether the N-terminal or the C-terminal fragment gains the NLS and translocate to the nucleus. Previous studies with Bid [15] and PARP-1 [7] showed differential cellular functions of cleaved peptides from the same protein, further suggesting that similarly in-depth mechanistic studies with JPH2-NTP and CTP in the cardiomyocyte nucleus may reveal if these fragments cooperate or compete with each other during adverse remodeling associated with HF.

### Therapeutic implications of differential junctophilin-2 cleavage and nuclear localization

The cleavage of JPH2 into different peptides based on the CAPN isoform involved and their subsequent nuclear translocation may be important for designating the timing of JPH2 gene therapy. Adeno-associated virus delivery given prior to acute decompensation in a mouse TAC model halted HF progression in mice [28]. One downside to JPH2 gene therapy is the possibility that CAPNs could theoretically decrease the amount of therapeutic full-length JPH2 over time, although such a decrease was not seen after seven weeks in TAC mice receiving JPH2 gene therapy [28]. Our current study helps to inform the timing of this gene therapy since CAPN2 may be more active in the later stages of HF [35]. Although CAPN1 activation in the early stages of HF could lead JPH2 cleavage at the  $^{565}\text{RT}^{566}$  site leading to N-terminal nuclear localization which may be protective, CAPN2 activation in end-stage HF could enhance JPH2-CTP nuclear localization, which may cause detrimental remodeling. Thus, a  $^{482}\text{GT}^{483}$  site cleavage resistant JPH2 may be beneficial in cases in which CAPN2 activity is increased. While further studies are needed to determine whether nuclear localization of the JPH2-CTP promotes decompensated cardiac remodeling, understanding the differences in JPH2 cleavage by each CAPN isoform will help provide a more specific therapeutic window for JPH2 gene therapy. Moreover, as JPH2 cleavage is in upstream of the signaling cascade, blocking nuclear translocation of CTP wouldn't prevent calpain-2 mediated JPH2 cleavage at  $\text{G}^{482}/\text{T}^{483}$  and it would further accumulate CTP in cytosol which can be potentially harmful. Therefore, it would be more efficient to target JPH2 cleavage site compared to the NLS as it is also shared between different cleaved JPH2 fragments (NTP, CTP) with paradoxical functions.



## Conclusions

Taken together, we discovered a novel C-terminal fragment of JPH2 cleaved by CAPN2, which translocates to the nucleus during HF. Several groups have studied targeting CAPN to prevent the development of HF; however, broad CAPN blockade may be detrimental due to the complex role of CAPN in cellular signaling. In contrast, our work has focused on understanding the pathological effects of CAPN on one of its critical cardiac targets, JPH2. Since our findings indicate that blocking nuclear localization of JPH2-CTP abrogates ISO-induced cellular hypertrophy in our in vitro model, preventing JPH2-CTP formation and its nuclear localization by blocking the CAPN2 cleavage site <sup>482</sup>GT<sup>483</sup> on JPH2 will reveal the nuclear role of JPH2-CTP associated to HF and may represent a more strategic therapeutic approach.

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## Compliance with ethical standards

**Conflict of interest** XHTW is a founding partner of Elex Biotech, a start-up company that develops drug molecules that target ryanodine receptors for the treatment of cardiac arrhythmia disorders.

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