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Involvement of activated SUMO-2 conjugation in cardiomyopathy

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ABSTRACT

Sumoylation is a posttranslational modification that regulates a wide spectrum of cellular activities. Cardiomyopathy is the leading cause of heart failure. Whether sumoylation, particularly SUMO-2/3 conjugation, is involved in cardiomyopathy has not been investigated. We report here that SUMO-2/3 conjugation was elevated in the human failing hearts, and we investigated the impact of increased SUMO-2 conjugation on heart function by using the gain-of-function approach in mice, in which cardiac specific expression of constitutively active SUMO-2 was governed by alpha myosin heavy chain promoter (MHC-SUMO-2 transgenic, SUMO-2-Tg). Four of five independent SUMO-2-Tg mouse lines exhibited cardiomyopathy with various severities, ranging from acute heart failure leading to early death to chronically developed hypertrophic cardiomyopathy with aging. We further revealed that SUMO-2 directly regulated apoptotic process by at least partially targeting calpain 2 and its natural inhibitor calpastatin. SUMO conjugation to calpain 2 promoted its enzymatic activity, and SUMO attachment to calpastatin mainly promoted its turnover and altered its subcellular distribution. Thus, enhanced SUMO-2 conjugation led to increased apoptosis and played a pathogenic role in the development of cardiomyopathy and heart failure.

1. Introduction

SUMO conjugation, or sumoylation, is the posttranslational modification in which SUMO proteins are covalently and reversibly attached to the substrates via a series of enzymatic reactions [1]. SUMO family contains three conjugatable members named SUMO-1, SUMO-2 and SUMO-3. While SUMO-1 only exhibits ~45% sequence similarity with SUMO-2 and SUMO-3, the latter two share ~95% identify at the level of amino acid sequence, and are believed to have largely overlap functions in vivo [1]. The number of SUMO targets has been increasing rapidly in recent years, and through modifying its targets, SUMO conjugation has been implicated in diverse cellular activities such as DNA repair [2] and chromatin function [3]. In the past several years SUMO conjugation pathway has been receiving a great deal of attention due to its potential implication in the pathogenesis of certain human diseases such as cancer [4] and neurodegeneration [5].

Accumulating evidence suggests that the SUMO conjugation is altered under certain pathological conditions in both vitro (cultured cells) and vivo (tissues/organs). In brain ischemic study, SUMO-2/3 conjugation increased in the harmful ischemic brain model [6–8], but decreased in delayed ischemic tolerance in an in vitro model [9], indicating its potentially harmful role in ischemic-induced injury. However, an increased expression of Ubc9, the sole sumoylation conjugation enzyme identified in mammals, augmented global sumoylation and limited the ischemic damage in mouse brain [10]. Therefore, a neuroprotective effect of increased SUMO conjugation against ischemic-induced injury has also been proposed. In addition, increased SUMO-1 conjugation was also observed in the polyglutamine diseases [11,12], pointing to its pathogenic role in neurodegenerative pathology. In the cardiovascular field, studies suggest that SUMO-1 conjugation plays an important role in early heart development, as evidenced by the congenital heart defects present in the SUMO-1 knockout mice [13], and in the transgenic mouse model with cardiac specific expression of SENP2, an isopeptidase that deconjugates all SUMO proteins [14]. SUMO-1 conjugation was also responsive to hypoxic insult to murine hearts [15], and was proposed to play a protective role in human and mouse heart failure via modulating the activity of SERCA2 [16]. However, the activity of Erk5 (extracellular signal-regulated kinase 5), an important factor against ischemic/reperfused injury and inhibitor of apoptosis, was suppressed by

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sumoylation [17]. Sumoylation of Erk5 was increased in myocardial infarction, in the H_2O_2-induced inflammation and in the diabetic aorta of mice [17,18]. Thus, an increase in the level of SUMO-1 conjugated Erk5 appears to enhance the inflammation, induce cell death, and consequently worsen the injury.

Compared with the aforementioned several lines of research that implicate SUMO-1 conjugation in cardiac development and pathophysiology, little is known about whether and how SUMO-2/3 conjugation play a role in the cardiovascular diseases. Several studies suggest the potential implication of SUMO-2/3 conjugation in cardiomyopathy. First, SENP2 transgenic mice also developed cardiac hypertrophy and dysfunction with aging, and these symptoms were not ameliorated by overexpressed SUMO-1 [14]. Second, a recent study revealed that the levels of SENP5, another SUMO isopeptidase that mainly deconjugated SUMO-2/3, were elevated in the human failing hearts, and the gain-of-function murine model suggests that SENP5 played a pathogenic role in dilated cardiomyopathy and heart failure [19]. However, the direct evidence that implicates the SUMO-2/3 conjugation in cardiovascular disease is still missing, despite the fact that SUMO-2/3 had more free reservoir and rendered stronger responses to external stimuli such as heart shock than SUMO-1 in the cultured cells [20]. Given that many SUMO substrates that possess opposing activities are implicated in cardiovascular pathophysiology [21], the net functional consequence of the altered conjugation of SUMO-2/3 warrants investigation.

The calpain–calpastatin proteolytic system, a calcium-dependent intracellular proteolytic system, is involved in a variety of cellular processes including apoptosis [22], and plays an important role in cardiovascular pathophysiology and heart failure [23–25]. The function of one of the two ubiquitously expressed calpains (Capns), Capn2, has been well studied. Neonatal induction of Capn2 in murine hearts decreased fractional shortening and increased LVESD (left ventricular end-systolic dimension) [26], indicating the harmful impact of increased activity of Capn2 on heart function. However, how the activity of Capn2 is modulated in vivo remains poorly understood. In this regard, posttranslational modifications serve as a device to modulate Capn2's function. For instance, phosphorylation of Capn2 by PKA suppressed, but Erk-induced phosphorylation elevated its activity [27,28]. Also, sumoylation modified Capn2 on lysine 390 promoted Capn2-dependent cell mobility [29]. However, how the association of sumoylated Capn2 with apoptotic pathway has not been investigated. On the other hand, knockout of calpastatin (CAST), a natural endogenous inhibitor for Capns, in mice led to potentiated activity of Capns [30]. However, overexpression of CAST in murine hearts also caused cardiomyopathy [26], indicating the importance of a balanced calpain–calpastatin system to the maintenance of normal cardiac function. Although CAST exhibits high selectivity for Capns' function, how the activity of CAST itself is regulated is largely unknown.

In the present report, we first showed that an increase in SUMO-2/3 conjugation was observed in the human failing hearts. We then used a gain-of-function approach to generate a number of independent transgenic mouse lines that expressed constitutively active form of SUMO-2 in the hearts (SUMO-2-Tg). The majority of these mouse lines showed premature death, increased apoptosis and cardiomyopathy. We further revealed that SUMO targeted at least two components, Capn2 and the natural calpain inhibitor CAST, of the calpain–calpastatin proteolytic system, a critical regulatory pathway of apoptotic process. Thus, our studies demonstrate a direct regulation of the apoptotic pathway by SUMO-2/3 conjugation, and suggest a pathogenic role that SUMO-2/3 conjugation plays in the development of cardiomyopathy and heart failure.

2. Materials and methods

2.1. Experimental animals

The construct alpha-MHC-flag-SUMO-2, which was used to generate SUMO-2 transgenic mouse lines, was generated as detailed previously [14,19,31]. Briefly, the transgene flag-tagged SUMO-2-GG was subcloned into the region between the 5.4 kb mouse α-MHC promoter (provided by Dr. J. Robbins, University of Cincinnati) and the Simian virus 40 polyadenylation sequence via SalI sites. The orientation of the inserted flag-SUMO-2 cDNA was confirmed by sequencing. This construct was microinjected into the fertilized eggs of FBV mouse strain. The founders (F0) of SUMO-2-Tg lines were backcrossed with C57BL/6 mice for over one to five generations. The positive offspring were identified using PCR with the following oligos (5′ to 3′): forward, CGGCACTC TTAGCAAACCTC and reverse, CTAACCTCCCGTCTGCTGT. All animal experimental protocols were approved by the Institute for Animal Studies of the Institute of Biosciences and Technology at Texas A&M Health Sciences Center and the University of Texas Health Science Center Animal Welfare Committee.

2.2. Human samples

Idiopathic myocardial samples from patients with end-stage heart failure were provided from the Department of Cardiac Pathology at Texas Heart Institute, and the donated non-transplantable normal human heart samples were provided by the International Institute for the Advancement of Medicine (IIAM) based on an official agreement with the laboratory of electrophysiology at Texas Heart Institute. Formalin fixed and paraffin embedded tissue blocks from the left ventricular free wall of human hearts, including five non-failing controls without a history of cardiac diseases and five failing hearts with idiopathic dilated cardiomyopathy were selected for immunohistochemical staining. The protocols for the use of human heart samples were approved by the Institutional Review Board at St. Luke's Episcopal Hospital and at the University of Rochester Medical Center.

2.3. Plasmid constructs

For adenoviral expression, flag-epitobed SUMO-2-GG or SUMO-2-ΔGG was PCR amplified, and ligated into pShuttle-IRES-hrGFP-1 vector via EcoRV and XhoI sites. Capn2 wild type (wt) and the sumoylation-deficient K390R mutant (the conversion of lysine 390 to arginine) were PCR amplified and subcloned into pShuttle-IRES-hrGFP-1 on SpeI and SalI sites. For SUMO-2 fused Capn2 construct, Capn2 cDNA was first subcloned into pcDNA4A vector on XhoI and XbaI sites (pcDNA-Capn2), followed by SUMO-2 cDNA subcloning in frame into pcDNA-Capn2 before Capn cDNA on NheI and XhoI sites (SUMO-Capn2). The SUMO-Capn2 was then PCR amplified and ligated in frame into pShuttle-IRES-hrGFP-1 on SpeI and SalI sites to generate the adenoviral expression vector. Mouse CAST cDNA was purchased from Openbiosystem and subcloned into pcDNA4A-V5/His vector on EcoR and XbaI sites (CAST-V5). To generate SUMO fused CAST expression vector, SUMO-1 or -2 cDNA was subcloned in frame before CAST on HindIII and EcoRV sites, generating SUMO-CAST-V5 expression vector. HA- and His_6-tagged SUMO-1-wt (HA-His_6-SUMO-1-wt) was obtained by ligating PCR-amplified His_6-tagged SUMO-1 provided by Dr. Ron T. Hay in frame into PCGN vector via XbaI and KpnI sites.

2.4. Antibodies and chemicals

Anti-V5-HRP, Annexin-V Alexa Fluor 594, Alexa Fluoro® 488 anti-goat antibody, goat serum, Histostain-SP Kit (Invitrogen), anti-V5 antibody (Bethyl Laboratories, Inc.), anti-HA-HRP (Genscript), anti-GAPDH-HRP, anti-SUMO-1, anti-SUMO-2/3 and anti-Calpastatin antibodies (Santa Cruz), anti-Calpain 2 antibody (Cell Signaling Technology), anti-V5 agarose affinity gel (Sigma-Aldrich), bead-conjugated anti-SUMO-2/3 antibody (Santa Cruz), anti-mouse IgG agarose (Sigma-Aldrich), t-BOC-Leu-Met-chloromethylaminocoumarin (t-BOC-LM-CMAC, Invitrogen), Ni-NTA agarose (Qiagen), Restore Western blot stripping buffer (Thermo Scientific Pierce), inhibitors for calpains 1 and 2, cathepsin, and caspases (pan caspase inhibitor Z-VAD(OM3)-FMK) (Santa Cruz).

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2.5. Cell culture and transfection

Regular Hela cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) plus 10% fetal bovine serum (FBS). Transient transfection was performed using Lipofectamine 2000 on either 6 cm plates for Western blot analysis or 10 cm plates for *nickel-nitrilotriacetic acid* (Ni-NTA) pulldown assays (see below). Neonatal rat cardiomyocyte culture was described previously [32]. For CAST-V5 and SUMO-CAST-V5 turnover and protease inhibition analysis, 24 h posttransfection, cycloheximide (10 μg/ml) was added into the cell culture medium for 12 or 16 h in the absence or presence of various protease inhibitors with two dosages per inhibitor as indicated in the legend of Fig. 7, followed by Western blots to detect the expression levels of CAST-V5 and SUMO-CAST-V5. GAPDH was used as a loading control in the Western blot analysis.

2.6. Western blot, sumoylation assays and Ni-NTA pulldown assays

For Western blot analysis, the lysis buffer (50 mM Tris, 150 mM NaCl, 1% NP-40) was used to lyse cells or homogenize hearts. 40 μg proteins purified from Hela cell lysates containing overexpressed proteins of interest, or 100 μg of protein lysates extracted from mouse left ventricles, were boiled, subjected to 4–12% NuPAGE, and transferred to the polyvinylidene difluoride (PVDF) membrane. The blot was probed with the antibodies of interest as indicated in each figure legend. The protein bands were visualized using HyGlo Quick Spray (Denville Scientific) or ECL plus (GE Healthcare). The procedure for in vivo sumoylation assays was similar to that for the Western blot, except that the isopeptidase inhibitor N-ethylmaleimide (NEM) was added to the cell lysates (final concentration 25 mM) to prevent desumoylation. Ni-NTA affinity chromatography was detailed previously [33].

2.7. Co-immunoprecipitation

Protein lysates purified from frozen human hearts were prepared. 400 μg of total protein was diluted to a final concentration of 0.4 mg/ml in the lysis buffer. 4 μg of bead-conjugated anti-SUMO-2/3 antibody was applied to the lysis buffer (as a negative control) or the protein sample and incubated for 2 h at 4 °C on a rotary platform. The beads were subsequently pelleted by centrifugation, washed five times with binding buffer. These protein lysates were subsequently subjected to 4–12% NuPAGE, transferred to PVDF membrane, detected by the desired antibodies, which was then visualized with chemiluminescence.

2.8. Adenovirus-mediated expression

Recombinant adenoviruses were generated using the AdEasy XL Adenoviral Vector System (Stratagene). Briefly, all cDNAs of interest in adenoviral-mediated expression were subcloned into the pShuttle-IRES-hrGFP1 vector as described above. The entire expression cassettes from the resulting vectors were recombined in BJ5183 bacterial strain with serotype 5 first-generation adenoviral backbone, AdEasy-1. These recombinant adenoviral backbones were transfected into AD293 cells to generate infectious viral particles. Viral titer was determined by the tissue culture infectious dose method [34]. Cardiomyocytes were infected with recombinant adenoviruses for 2 h at a multiplicity of infection (MOI) of 100 particles/cell and incubated for additional 24–48 h to ensure transgene expression.

2.9. Cardiomyocyte size measurement

WGA-TRITC staining was used on heart sections of P5 or P80 of wt and SUMO-2-Tg mice to distinguish sarcolemmal membrane. 10–20 randomly selected fields from each individual heart sample were used to measure surface areas of cardiomyocytes using Software ImageJ (http://rsbweb.nih.gov).

2.10. Immunofluorescence

Hela cells were plated into two well Lab-Tek II chamber slide (Nunc) and were transiently transfected with CAST-V5 and SUMO-CAST-V5 using Lipofectamine 2000 (Invitrogene) according to the manufacturer's protocol. Hela cells were then fixed in 3.5% paraformaldehyde (PFA) and permeabilized with 0.5% Triton X-100. Staining was conducted using anti-V5 antibody (1:200). Cells were sequentially incubated with secondary antibodies, Alexa Fluoro® 488 anti-goat antibody and then mounted with Vectashield with DAPI (Vector Laboratory). Annexin-V staining was performed using Annexin-V Alexa Fluor 594 on neonatal rat cardiomyocytes cultured on cover slides. Cardiomyocytes were incubated with 5 μl of Annexin-V Alexa Fluor 594 for 15 min at room temperature. Thereafter the cells were washed and mounted with Vectashield (Vector Laboratory).

2.11. Immunobiochemistry

4 μm sections of human hearts were deparaffinized and antigen retrieval was performed in sodium citrate buffer (pH = 6.0) by heating to 99 °C for 20 min with PT Link system by Dako (Carpinteria, CA). Endogenous peroxidase activity was quenched with 3% H_2O_2 and non-specific binding was blocked with 10% non-immune goat serum and then incubated with primary antibody at 4 °C overnight. The signals were amplified with the Histostain-SP Kit and detected with DAB substrate (Dako). Special attention was paid to avoid color overdevelopment. Hematoxylin was used as a counterstain. Negative controls were incubated with appropriate serum instead of primary antibody under the same conditions.

2.12. In vivo Calpain 2 activity assay

In vivo Calpain 2 activity was analyzed using synthetic calpain substrate t-BOC-LM-CMAC [35]. Hela cells were plated at 70–80% confluence in 22 mm square coverglass (Corning) and grown in the complete media for 24 h. Cells were thereafter transfected with the pShuttle-IRES-hrGFP-1 alone (as a control) or the individual expression vectors encoding Capn2, SUMO-2-Capn2 or Capn2-K390R, respectively. After 48 h incubation, cells were incubated for 30 min in the presence of 10 μM t-BOC-LM-CMAC. The cells were then washed with 1× PBS and covered with a glass cover slide. Both GFP and t-BOC-LM-CMAC positive cells were scored for analysis. Fluorescence was visualized using a fluorescence microscope (Olympus fluorescence microscope). Intensity of fluorescence caused by cleavage of synthetic substrate was measured using ImageJ. Because the backgrounds among the slides prepared slightly varied, background measurements were also taken and subtracted. Capn2 activity was expressed as CTCF (corrected total cell fluorescence), which is calculated as follows: integrated density − (area of selected cell × mean fluorescence of background readings).

2.13. Echocardiography

Mice of interest were anesthetized by inhalation of 1% isofluorane and rested on a warm pad during transthoracic measurements of cardiac function using two dimensional M-mode of a Vevo 770 in vivo micro-imaging system (Visual Sonics, Toronto, Canada). The probe contacted the hair-removed chest to record cardiac function indices. The investigator that performed and analyzed cardiac functions was blind to animal genotypes.

2.14. Histopathology

Mouse hearts were dissected and fixed overnight in 4% paraformaldehyde (PFA). Hematoxylin and eosin (H&E) or Masson's trichrome staining was performed on heart sections (10 μm) according to standard protocols.

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2.15. Transmission electron microscopy (TEM)

Left ventricle tissues were fixed in 2% paraformaldehyde (PFA) and 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3 and prepared according to the standard protocol. Electron microscopy was examined in a JEM 1010 transmission electron microscope (JEOL, USA, Inc.) at an accelerating voltage of 80 kV. Digital images were obtained using AMT Imaging System (Advanced Microscopy Techniques Corp.). This work was performed in MD Anderson HREM facility.

2.16. Statistical analysis

The number of mice for each group used was indicated in the figure legends. Two-tailed Student's *t* test or one way ANOVA followed by Bonferroni correction was used to determine statistical significance between groups when applicable and data were represented as mean ± SEM. $p < 0.05$ was considered statistically significant and $p < 0.01$ highly significant.

3. Results

3.1. Increased SUMO-2/3 conjugation in human failing hearts

As an initial step to explore if SUMO-2/3 conjugation is implicated in human cardiac muscle disorders, we performed Western blots on the protein lysates purified from five untransplantable healthy human hearts (control) and seven failing hearts caused by idiopathic cardiomyopathy, the diagnosis of which was confirmed by clinical symptoms and cardiac functional analysis, to evaluate the changes in SUMO-2/3 conjugation in these samples. As shown in Fig. 1A and SF1, increased levels of SUMO-2/3 conjugates (high molecular weight conjugates, HMW) were observed in the human failing heart muscles, while they were barely detected in the control heart samples. Consistent with this finding, immunobiochemistry also revealed enhanced SUMO-2/3 staining in human cardiomyopathic heart samples compared with the control ones (Fig. 1B). Thus, SUMO-2/3 conjugation was altered under pathological conditions in the human failing hearts, although its role in the development of cardiac muscle disorders was not clear.

3.2. Elevation of SUMO-2 conjugation in murine hearts leads to cardiomyopathy

To investigate whether increased SUMO-2/3 conjugation in the heart was just an irrelevant event, or had any beneficial/detrimental impacts on cardiac function, we generated six independent transgenic mouse lines that expressed constitutively active SUMO-2-GG in the hearts under the control of murine cardiac α-myosin heavy chain (MHC) promoter. The mice from four lines (#9606, #9610, #9592, #9608) were confirmed to develop cardiomyopathy with various severities and mortality rate (Fig. 2A). The expression of SUMO-2 transgene in these SUMO-2-Tg hearts was also confirmed (Fig. 2B). The levels of increased SUMO-2/3 conjugation in the heart were correlated to the phenotypic severity/mortality rate of SUMO-2-Tg line. For instance, line #9606, which showed the highest mortality rate, exhibited the highest levels of SUMO-2/3 conjugation, followed by line #9610. Line #9614 mice, which showed the least cardiac phenotypic severity, exhibited the least increase in SUMO-2/3 conjugation in the heart. The SUMO-2-Tg mice from line #9601 also had premature death, but due to the fact that we lost this line without obtaining sufficient number of animals (see below), this line was not further analyzed. The brief summary of phenotypic manifestation of each of these SUMO-2-Tg mouse lines was presented below:

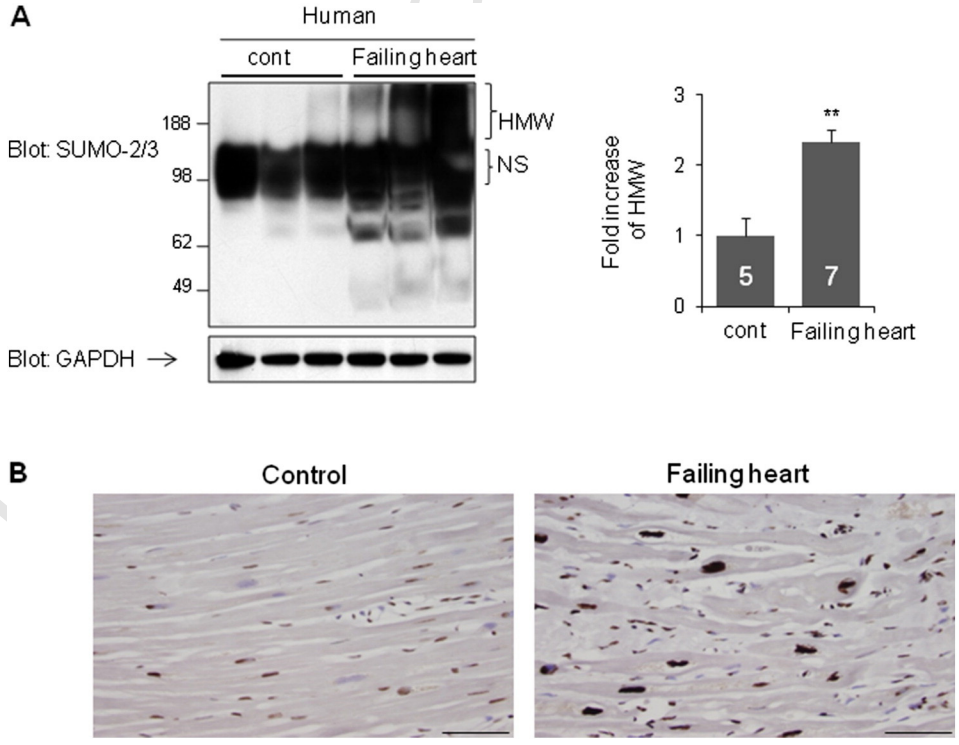

Fig. 1. Increased SUMO-2/3 conjugation in human failing hearts. A. Western blots were performed on protein lysates purified from the left ventricles (LV) of human control and failing hearts caused by idiopathic cardiomyopathy. Left panels: blotted with anti-SUMO-2/3 (up) and anti-GAPDH (down) antibodies, respectively. HMW, high molecular weight conjugates. Right panel is the statistical analysis of the right panel and Supplemental Fig. 1 (SF1). **$p < 0.005$. n, the number of samples in each experimental group was indicated within each bar. B. Immunohistochemistry showed enhanced SUMO-2/3 staining in human control and failing heart tissues. Experiments were performed on five human control and failing hearts and nuclei were count-stained with hematoxylin. Representative data were shown. Magnification, ×400.

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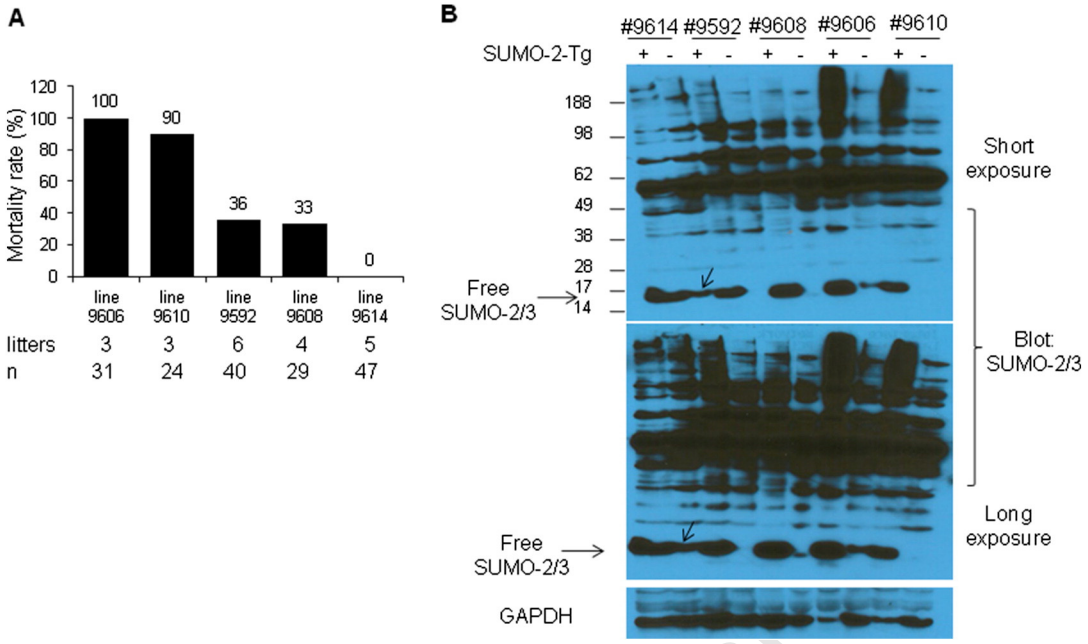

Fig. 2. Mortality rate analysis in five independent SUMO-2-Tg mouse lines. A. The mortality rate was recorded within one year after birth. The number of litters and animals from each line was indicated under each bar. B. SUMO-2/3 conjugation was increased in the SUMO-2-Tg mouse hearts. Western blot was performed on heart protein lysates purified from mice from five independent mouse lines as indicated. Upper panel, anti-SUMO-2-/3; lower panel, anti-GAPDH. HMW, high molecular weight conjugates. Arrows indicate protein bands caused by the overflow from the neighboring lane during sample loading.

Q15 Line #9606: Mice of this Tg line had the most severe phenotypes, and the mortality rate analysis from three litters of totally 31 animals showed 100% premature death of those Tg+ pups before P10 with enlarged hearts (Figs. 2A and 3A). WGA staining showed slightly but significantly enlarged sizes of cardiomyocytes in Tg+ hearts compared with those in littermate controls at P5 (Fig. 3B and C). Apoptosis was significantly increased prior to a change in heart weight (HW) to body weight (BW) ratio (Fig. 3D), indicating a pathogenic role of apoptosis in SUMO-2-induced cardiomyopathy. At a later stage P7, although a more substantial increase in apoptosis was observed in the Tg+ heart, TEM examination revealed no significant changes such as swelling in the mitochondrial structure of the Tg+ heart (SF2). The increased levels of SUMO-2 conjugates in Tg+ hearts from this Tg line were equivalent to those observed in the human cardiomyopathic hearts (Fig. 3E).

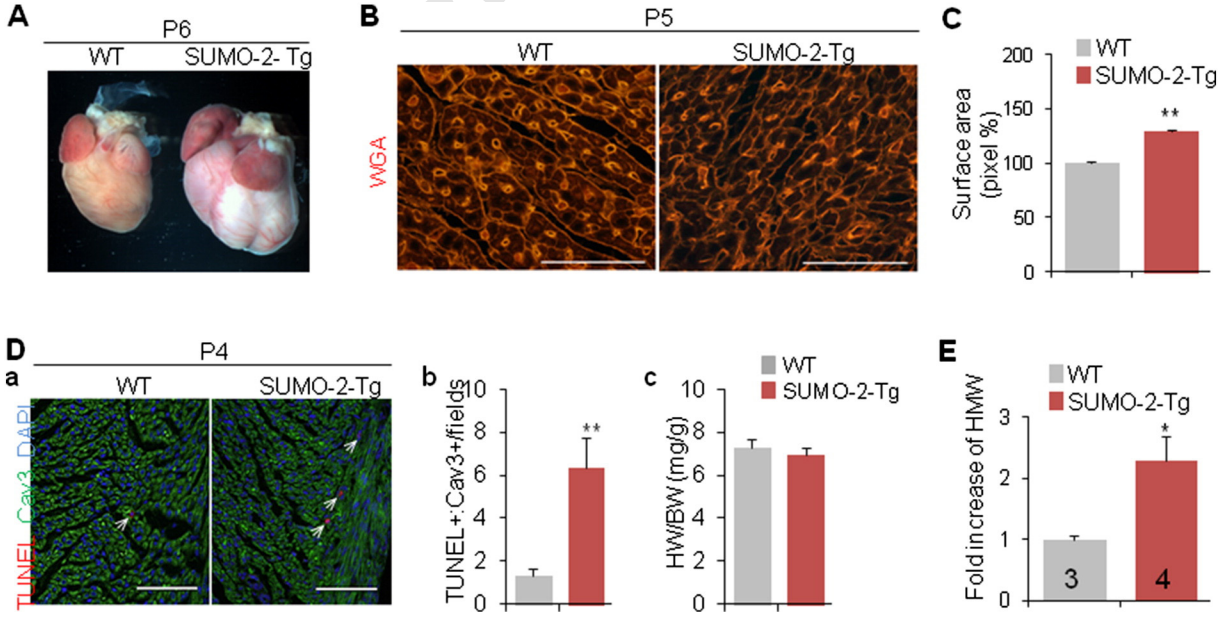

Fig. 3. Line #9606 mice had a severe cardiac phenotype leading to early postnatal death. A. SUMO-2-Tg heart was significantly larger than the littermate control heart at P6. B and C. WGA staining showed slightly but significantly enlarged size of cardiomyocytes of SUMO-2-Tg hearts compared with the control hearts at P5. Bar, 50 μm. C is the statistical analysis of B. **$p < 0.001$. D. Increased TUNEL positive cells preceded the significant change in heart weight (HW)/body weight (BW) ratio. TUNEL staining was performed on heart sections from SUMO-2-Tg and littermate controls at P4 (a and b), at which time HW/BW ratio of SUMO-2-Tg mice was comparable to that of littermate control (c). Bar, 200 μm. E. The level of HMW of SUMO-2-Tg hearts was equivalent to that observed in the human cardiomyopathic hearts. Quantitative analysis of those HMW conjugates was measured as in Fig. 1A. The number inside each bar represents the number of samples used for the analysis of each group.

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Line #9610: Mice of this Tg line exhibited the second most severe phenotypes. Over 90% of Tg+ mice analyzed from three litters of totally 24 animals died before P90. Non-invasive cardiac functional analysis performed at ~P60 showed that these Tg mice exhibited left ventricular (LV) mass/BW ratio and significantly decreased %EF and FS%. Consistent with the finding of increased LV mass, Tg+ cardiomyocytes were enlarged compared with control cardiomyocytes (Fig. 4B), suggesting cardiac hypertrophy. Masson's trichrome staining revealed extensive interstitial fibrosis in Tg+ mouse hearts compared with that in control hearts (Fig. 4C).

Line #9592: The mortality rate analysis from 6 litters of totally 40 animals collected from this Tg line showed that 36% Tg mice died within one year after birth (Fig. 2A). The surviving Tg+ mice gradually developed cardiac hypertrophy over duration of one year, with increased LV mass/BW ratio and LVPW/d (SF3. A and B), although no significant impairment in cardiac contractility in Tg+ mice was observed in comparison with that in control mice (data not shown), indicating a compensatory stage of cardiac hypertrophy at this age. The transcription levels of a number of cardiac disease markers such as ANF, BNP, β-MHC, and skeletal alpha actin were significantly elevated, while α-MHC transcripts were decreased, as revealed by the real time PCR (SF3. C), in agreement with the development of cardiac muscle disorders.

Line #9608: The mortality rate analysis from four litters of totally 29 animals from this Tg line showed that 33% of Tg+ mice died within one year after birth. The surviving Tg+ mice gradually developed cardiomyopathy with compromised cardiac functions analyzed by echocardiography (Supplement Table 1). The time course of developing cardiomyopathy of this Tg line was similar to that of the line #9592.

Line #9614: Analysis from five litters of totally 47 animals of this line showed no premature death of Tg mice within one year after birth, and the cardiac functions of Tg+ mice measured at ~P404 showed at equivalent levels compared with those of littermate controls (data not shown).

Line #9601: The founder 9601 (male) had very low fertility, and gave birth to totally three litters of 11 animals, including only two positive pups. The first Tg+ pup was used for transgene expression analysis, which showed expression of flag-SUMO-2-GG and increased SUMO-2 conjugation in the heart (data not shown). The second Tg+ pup died at P2. This line was excluded from the study due to insufficient number of Tg+ animals.

3.3. SUMO-2 promotes apoptotic process in cultured cardiomyocytes

Since increased apoptosis preceded the heart enlargement (Fig. 3D), we wondered if SUMO-2 conjugation could directly regulate apoptosis in cardiomyocytes. SUMO-2-GG or SUMO-2-ΔGG (unconjugatable mutant of SUMO-2) was introduced into cultured cardiomyocytes via adenovirus (Ad) infection. Ad-mediated GFP expression alone was used as a control. As expected, SUMO-2-GG, but not SUMO-2-ΔGG, significantly increased SUMO-2 conjugates (Fig. 5A). The immunostaining against Annexin-V, which marks the early stage of apoptotic process, was performed on those cardiomyocytes 36 h after infection. As shown in Fig. 5B and C, the number of double positive (Annexin-V+/GFP+) cardiomyocytes in the Ad-SUMO-2-GG group was significantly higher than that of either Ad-GFP control group or Ad-SUMO-2-ΔGG group. Also, we compared the SUMO-2/3 conjugation level in Ad-SUMO-2-GG-infected cardiomyocytes with that in line #9606 mouse heart, which showed increased apoptosis (Fig. 3D). While the similar amount of proteins was loaded for both samples, it seems that transgenic heart showed more robust modification than the cardiomyocytes overexpressing SUMO-2, although the free SUMO-2 levels in these two samples were comparably expressed (Fig. 5D). These findings suggest that increased SUMO-2 conjugation, but not free SUMO-2 itself, promotes or initiates apoptotic process. It is also very possible that SUMO-2/3 conjugation in the heart is mediated by the microenvironment such as local neurohormonal activity.

3.4. SUMO directly targets calpain–calpastatin proteolytic system

Since increased SUMO-2 conjugation led to elevated apoptosis, we hypothesized that SUMO targeted one or more factors that are implicated in the apoptosis pathway. It is well documented that the Capns, a family of calcium-dependent cysteine proteases that are widely distributed among tissues, along with their natural and highly specific endogenous inhibitor CAST, are involved in apoptotic processes [22]. We therefore asked if any of the components of the calpain–calpastatin proteolytic system was a SUMO substrate. We first identified CAST as a novel

A

P63	Control (n=3) average	SEM	SUMO-2-Tg (n=3) average	SEM	P value
LVID; s (mm)	1.90	0.23	4.34	0.38	0.006
LVPW; d (mm)	1.10	0.07	1.35	0.11	0.139
LVPW; s (mm)	1.25	0.13	1.37	0.30	0.739
Diameters; s (mm)	1.77	0.09	4.01	0.52	0.013
Diameters; d (mm)	3.20	0.20	5.02	0.69	0.065
Volume; s	9.43	1.21	74.07	20.00	0.032
Volume; d	41.46	6.33	125.62	35.04	0.077
stroke volume (ul)	32.03	5.16	51.55	15.15	0.290
%EF	77.08	0.91	40.43	1.40	0.000
%FS	44.59	0.96	19.84	1.00	0.000
cardiac output (ml/min)	13.77	1.77	21.70	6.89	0.328
LV mass (mg)	123.00	13.26	295.76	61.24	0.051
BW (g)	27.53	0.24	22.73	0.13	0.000
LV mass/BW	4.46	0.46	13.04	2.75	0.037

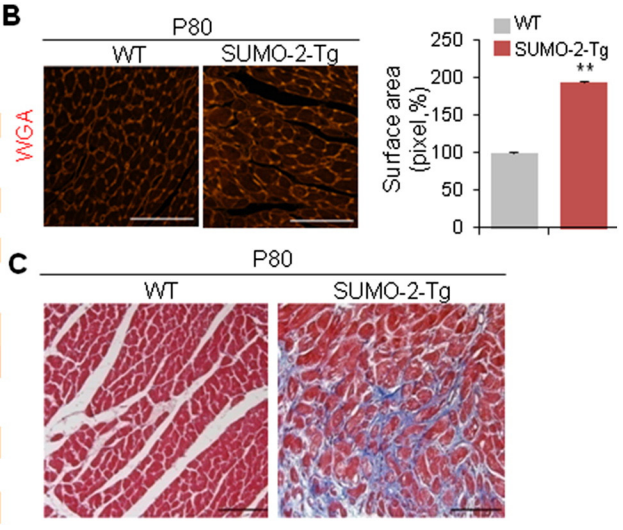

Fig. 4. Line #9610 mice exhibited cardiac hypertrophy with compromised cardiac function. A. Non-invasive echocardiography was performed on age-matched control and SUMO-2-Tg mice with p value shown on each cardiac function index. Note that compared with control mice, SUMO-2-Tg mice exhibited elevated LV mass/BW ratio and decreased %EF and %FS. $p < 0.05$ was considered significant. B. WGA staining showed a significant increase in the size of cardiomyocytes of SUMO-2-Tg hearts compared with its littermate controls at P80. $^{**}p < 0.001$. Bar, 100 μm. C. Masson's trichrome staining revealed massive fibrosis in the SUMO-2-Tg hearts. Bar, 200 μm.

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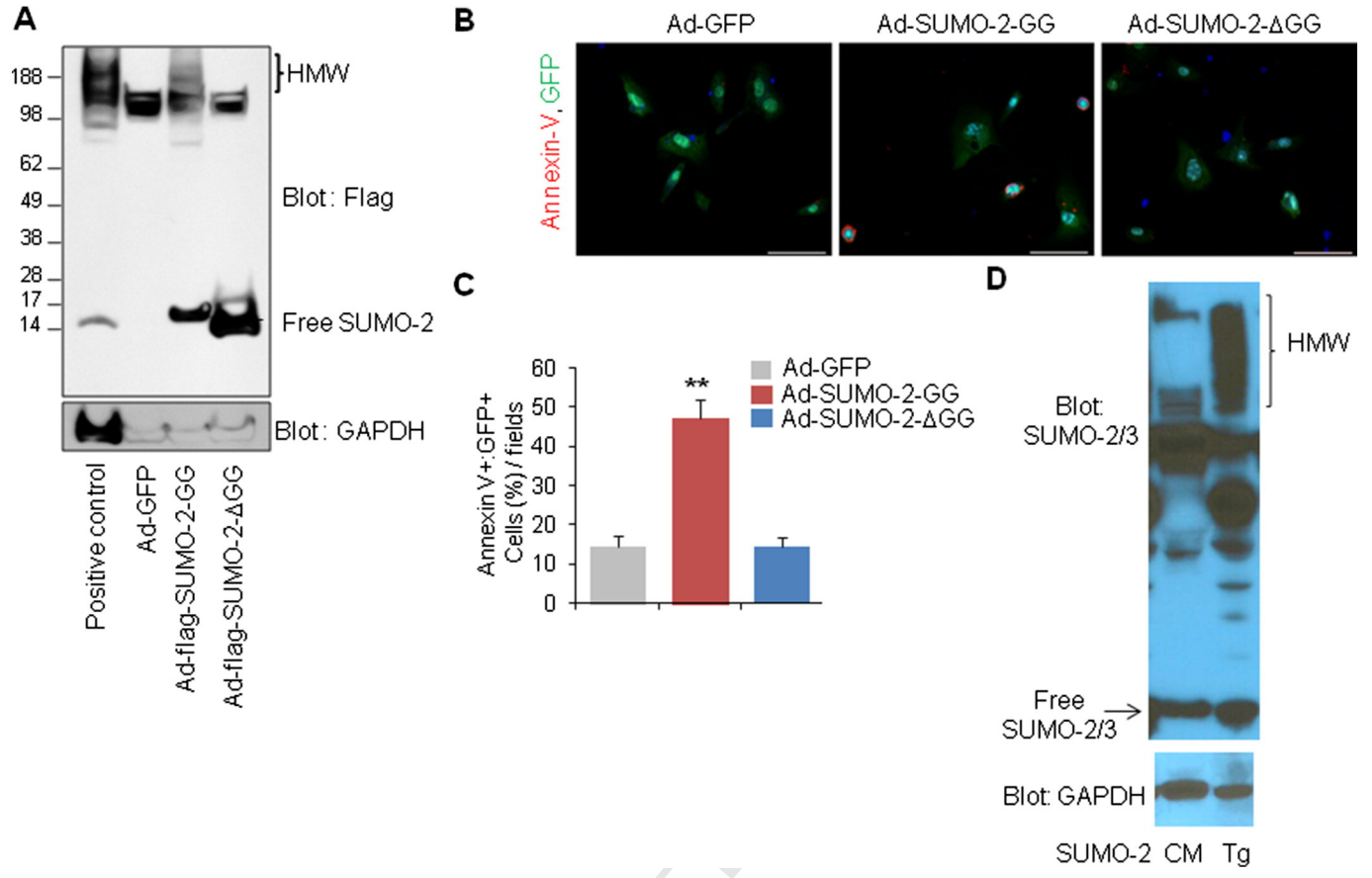

Fig. 5. SUMO-2 directly regulated apoptotic process in cardiomyocytes. A. Overexpression of SUMO-2-GG in cardiomyocytes led to increased SUMO-2 conjugation. Western blot was performed on cell lysates purified from cardiomyocytes with adenovirus-mediated expression of GFP alone, flag-tagged SUMO-2-GG or SUMO-2-ΔGG, respectively. Positive control: Hela cells transfected with SUMO-2 expression vector. Upper panel: blotted with anti-flag antibody; lower panel: blotted with anti-GAPDH-HRP as a control. B. Overexpression of SUMO-2-GG in cardiomyocytes led to increased Annexin-V staining. Immunofluorescence staining against Annexin-V was performed on cardiomyocytes expressing GFP alone, SUMO-2-GG or SUMO-2-ΔGG, respectively. Annexin-V, red; GFP, green. Bar, 50 μm. C. Statistical analysis of Annexin-V staining shown in B. n = 3 for each group. **p < 0.001. The number of double positive (Annexin-V+/GFP+) cells was chosen from at least 100 cells on randomly selected fields. All adenoviral constructs were delivered at MOI = 100. D. Comparison of the levels of SUMO-2/3 conjugation in SUMO-2-Tg hearts (#9606 line) and cultured cardiomyocytes expressing Ad-flag-SUMO-2-GG. It appears that a more robust SUMO-2/3 conjugation was seen in Tg heart than in cultured cardiomyocytes (CM), given a even less loading in Tg group than in cardiomyocyte group based on GAPDH.

SUMO substrate as shown in Fig. 6A. Western blot was performed on Hela cell lysates transfected with V5-tagged CAST alone, or together with SUMO-1-wt or SUMO-1-ΔGG (defective in conjugation). The presence of SUMO-1-wt, but not SUMO-1-ΔGG, promoted the appearance of a retarded migratory band. SUMO conjugation to CAST was further confirmed by Ni-NTA assays (SF4). In the cultured Hela cells, CAST was a weak SUMO-2 substrate compared with SUMO-1 (Fig. 6B). We also found that SUMO conjugated CAST exhibited a shorter halflife than free CAST as shown in Fig. 6C, in which 12 h treatment of cycloheximide (CHX), a protein synthesis inhibitor, promoted turnover of SUMO-conjugated CAST but not free CAST. To further confirm this observation, we tested turnover of SUMO-ligated V5-tagged-CAST (SUMO-CAST-V5) in the absence or presence of CHX for 12 h. Indeed, CHX treatment decreased the level of SUMO-CAST-V5, but not the level of free CAST-V5 (Fig. 6D). Since CAST can be cleaved by a number of proteases, we next sought to find which protease(s) was responsible for the cleavage of SUMO-attached CAST. Specific inhibitors for calpain 1, calpain 2, cathepsins and pan-caspase with two dosages each were tested in the presence of CHX treatment for 16 h, and we found that only pan-caspase inhibitor significantly slowed turnover of SUMO-1-CAST-V5 (Fig. 6E, compare lanes 9 and 10 with lane 2). SUMO-2 fused CAST exhibited a similar turnover pattern caused by CHX treatment (data not shown). Collectively, these data indicated that SUMO conjugation promoted cleavage of CAST by caspases. Also, it appears that most of caspases contributed more or less to this degradation, because only pan-caspase inhibitor, but not any other individual caspase inhibitors tested substantially stabilized SUMO-CAST-V5 (data not shown). In addition to the increased turnover of SUMO-CAST, we also found that SUMO fusion to CAST altered subcellular distribution of CAST. As shown in Fig. 7A and B, free CAST mainly exhibited diffused distribution pattern in the cytoplasm, however, the majority of SUMO-CAST showed aggregated (dotted) pattern of cytoplasmic distribution. Taken together, SUMO conjugation alters cytoplasmic distribution pattern of CAST, and promotes its turnover.

Capn2, one of the two major Capns that were extensively studied, was previously identified as a SUMO substrate, and SUMO conjugation enhanced its ability to promote cell mobility [29]. We confirmed that SUMO targeted Capn2 on lysine 390 (data not shown). To probe if SUMO conjugation to Capn2 could positively mediate apoptotic pathway, Ad-GFP, Ad-Capn2, Ad-SUMO-2 fused Capn2 (Ad-SUMO-Capn2), or Ad-Capn2-K390R (unsumoylatable mutant) were introduced into the cultured cardiomyocytes and up to 36 h after infection, Annexin-V staining was performed. In agreement with the previous report [29], K390R mutant showed less activity than Capn2 wt in promoting Annexin-V staining in cardiomyocytes (Fig. 8A and B), and SUMO-Capn2 possessed the most potent capability to activate the apoptotic pathway. To further evaluate the changes in the enzymatic activity of Capn2 once SUMO-conjugated, cultured cardiomyocytes were infected with Ad-GFP, Ad-Capn2, Ad-SUMO-Capn2, or Ad-Capn2-K390R, in the presence of the synthetic substrate, t-BOC-LM-CMAC for 30 min, which is visualized by calpain via a cleavage-dependent manner [35]. As shown in Fig. 8C, Capn2 increased fluorescence from

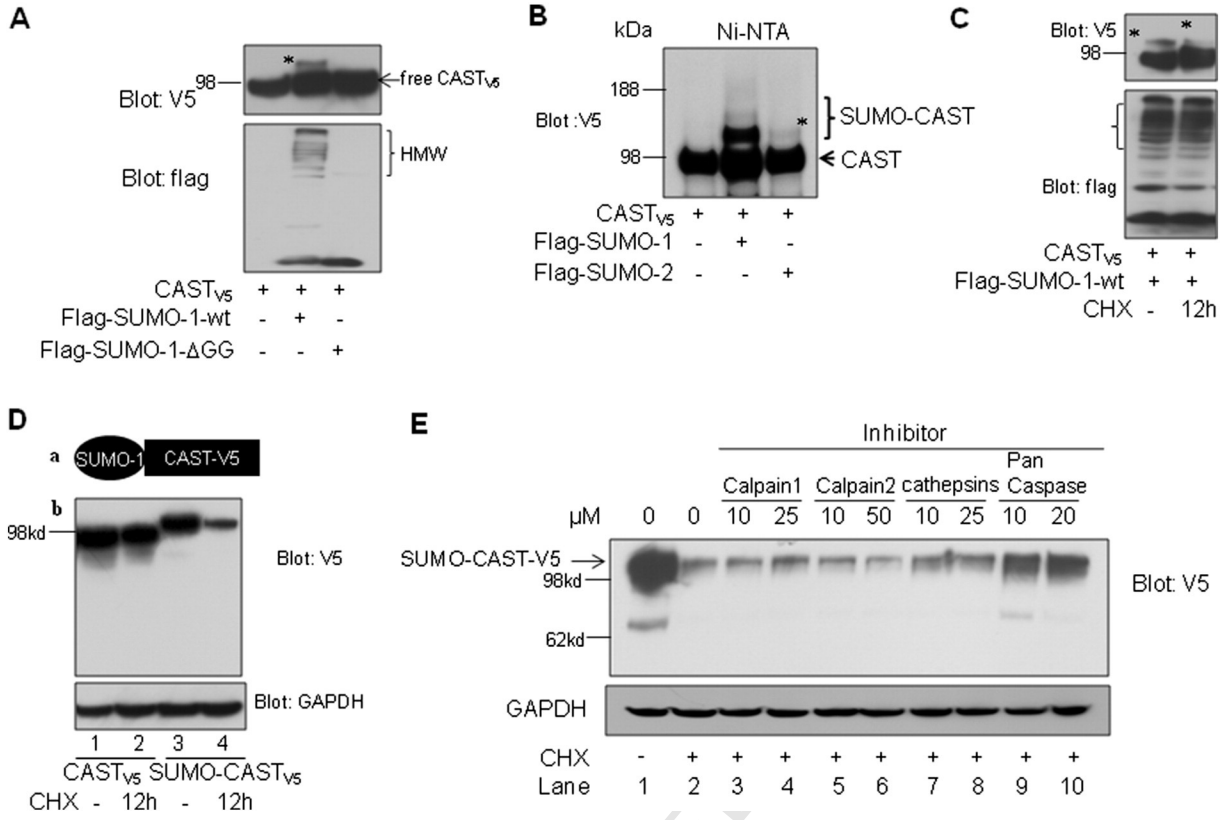

Fig. 6. SUMO targeted CAST for turnover. A. CAST was modified by SUMO. Western blot was performed on Hela cell lysates overexpressing V5-tagged CAST (CAST-V5) alone, or in the presence of either flag-epitoped SUMO-1-wt or SUMO-1-ΔGG, the latter which is the unconjugatable form of SUMO-1. Note that that a retarded species (SUMO-1 modified CAST, asterisk) was only detected in the presence of both CAST-V5 and SUMO-1-wt, but not in the presence of both CAST-V5 and SUMO-1-ΔGG. Upper panel: anti-V5 blot; lower panel: anti-flag blot. HMW, high molecular weight conjugates. B. CAST was a weak SUMO-2 target in Hela cells. Ni-NTA pulldown assay was performed on Hela cells transfected with 6× His tagged CAST-V5 alone, or in the presence of either SUMO-1 or SUMO-2, as indicated. The blot was probed with anti-V5 antibody. C. SUMO conjugation promoted CAST turnover. Western blot was performed on Hela cells expressing both CAST-V5 and SUMO-1-wt with or without cycloheximide (CHX) treatment (10 μg/ml) for 12 h as indicated. Note that the level of SUMO-conjugated CAST was significantly decreased after 12 h CHX treatment, while no obvious changes in the level of free CAST in these two groups. Asterisks indicate the sumoylated CAST. D. SUMO-fused CAST (SUMO-CAST) exhibited quick turnover. a, Schematic representation of SUMO-fused CAST-V5. b, Western blot was performed on Hela cell lysates expressing either CAST-V5 or SUMO-CAST-V5 in the presence or absence of CHX for 12 h, as indicated. Note that while CAST-V5 showed no significant changes in the protein levels after 12 h CHX treatment (compare lane 1 with lane 2, upper panel), SUMO-CAST-V5 showed a significant decrease at protein levels after CHX treatment (compare lane 4 with lane 3, upper panel). GAPDH served as a control. E. SUMO-CAST was cleaved by caspases. Hela cells were transfected with SUMO-CAST-V5 in the absence (lane 1) or the presence of CHX for 16 h. Various protease inhibitors with two dosages were added into the culture medium together with CHX from lane 3 to lane 10 as indicated. Note that only pan-caspase inhibitor was able to delay the turnover of SUMO-CAST-V5 (compare lanes 9 and 10 with lane 2 treated with vehicle). Upper panel: anti-V5; lower panel: anti-GAPDH-HRP as a control.

t-BOC-LM-CMAC as expected, and mutation of lysine 390 to arginine partially repressed its enzymatic activity. SUMO-Capn2 exhibited highest enzymatic function among these three tested. Thus, we conclude that SUMO attachment to Capn2 substantiates its enzymatic activity, thus leading to increased capacity to activate the apoptotic pathway.

3.5. Increased SUMO-2/3 conjugation to CAST and Capn2 in human cardiomyopathic heart muscles

We next investigated if SUMO-2/3 conjugation to CAST and Capn2 altered in the muscle-diseased human hearts. Co-IP was performed on two control and two diseased human hearts with SUMO-2/3 antibody-conjugated beads, and the blot was subsequently probed with anti-Capn2 antibody or anti-CAST antibody, respectively. Clearly, two specific bands above 98 kDa were detected by anti-Capn2 antibody in both diseased heart samples but not in the either negative control (only containing binding buffer and SUMO-2/3-conjugated beads) or control heart samples (Fig. 9A), indicating increased SUMO-2/3 conjugation to Capn2. Similarly, SUMO-2/3 conjugated CAST was detected in both diseased heart samples but not in the control samples (Fig. 9B). Thus, we argue that in the cardiomyopathic hearts, SUMO-2/3 conjugation to Capn2 and CAST is elevated, which may play a role in disease development/progression.

4. Discussion

4.1. SUMO-2/3 conjugation and cardiomyopathy

Cardiomyopathy represents a major health threat and is a leading cause of heart failure [36]. Two main types of cardiomyopathy are dilated and hypertrophic cardiomyopathy. The exact mechanism underlying these cardiomyopathies is poorly understood, although it is believed that multiple factors, including genetic factors and environmental cues, play important roles in disease initiation and development. In the present study, we reported an increase in SUMO-2/3 conjugation in human failing hearts and recapitulated cardiomyopathy phenotype in the murine model by using the gain-of-function approach, i.e., increasing the levels of SUMO-2 conjugation in cardiomyocytes. Furthermore, the severity of cardiac phenotypes was shown to be associated with the levels of SUMO-2 conjugation in the heart. Thus, we provided the direct evidence that links SUMO-2 conjugation to the development/progression of cardiomyopathy and heart failure. In contrast to this finding, an increase in SUMO-2/3 conjugation was observed in the transient ischemic brain [6–8] and was proposed to be protective [10,37]. While a short

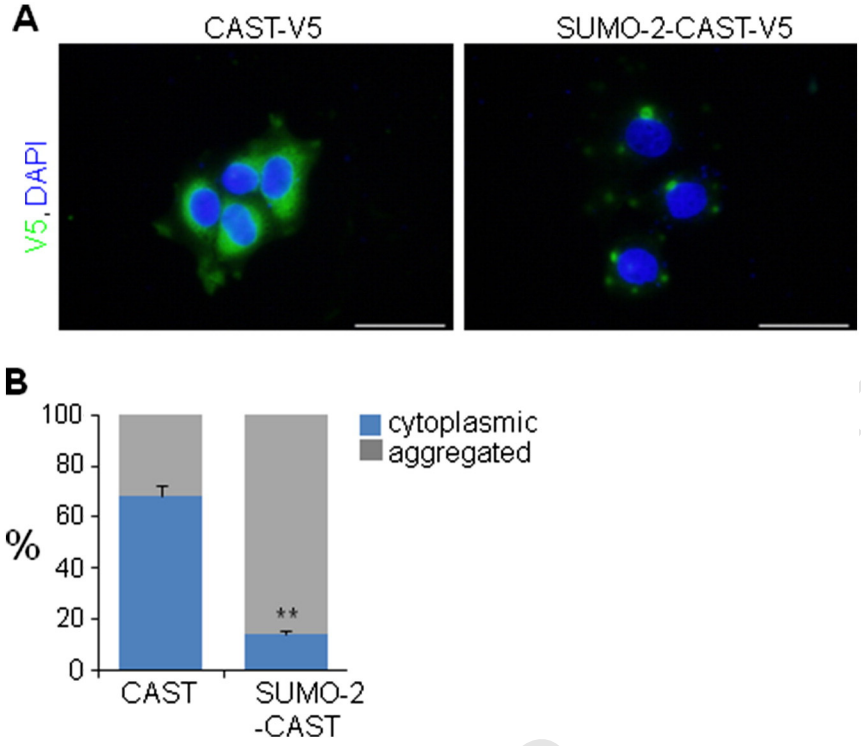

Fig. 7. SUMO fusion altered intracellular localization pattern of CAST. A, CAST-V5 or SUMO-fused CAST-V5 in Hela cells were visualized by immunostaining using anti-V5 antibody (green fluorescence). DAPI (blue fluorescence) was used for nuclear staining. Note the diffused pattern of CAST (left panel) and the aggregated pattern of SUMO-CAST (right panel) in cytoplasm. Bar indicates 50 μm. B, Bar graph indicates the percentage of cells with diffused (CAST-V5) or aggregated (SUMO-CAST-V5) localization, respectively. Data were collected from four independent experiments. $^{**}p < 0.001$.

term or transient increase in pan-SUMO conjugation might be beneficial against certain particular insults, the consequence of a long term increase in SUMO-2/3 conjugation alone had never been investigated. Our data indicated that the increase in SUMO-2 conjugation in the long run may be detrimental to heart function. We noted that cardiac specific overexpression of SUMO-1 did not cause any discernible phenotype(s) at baseline activity [14,16]. One possible explanation for this is that SUMO-1 and SUMO-2 may modify different substrates, or modify the same targets with different affinity in vivo under certain circumstances, subsequently leading to various functional outcomes. We also noted that the SUMO-2-Tg mice (line #9606) with the most severe heart phenotype exhibited an equivalent increase in SUMO-2 conjugation compared with that observed in the human failing hearts. Given the long term nature of development of human heart failure and the acute disease manifestation of line 9606, it is possible that the sensitivity to increased SUMO-2/3 conjugation is species-dependent. In human, it is currently not clear whether the elevated SUMO-2/3 conjugation was an intrinsic nature down the road from cardiomyopathy to heart failure, or it was just related to any particular clinical treatments the patients received. However, since an increase in SUMO-2 conjugation is harmful to cardiac function as shown in our SUMO-2 gain-of-function models, our findings collectively raise the possibility that SUMO-2/3 conjugation may be a potential therapeutic target.

Since overexpression of SUMO-2 did not significantly alter the transcripts of endogenous SUMO-1 and SUMO-3 (data not shown), we believed that cardiomyopathy observed in the SUMO-2-Tg mice was solely attributable to SUMO-2 conjugation. In human and mouse hearts, little SUMO-2/3 conjugation was observed during basal activity, but was substantially potentiated under certain pathological circumstances. This observation is consistent with the previous report showing that SUMO-2/3 responded well to the external stimuli in vitro [20]. It is tempting to further interrogate whether the increase in SUMO-2/3 conjugation is a common phenomenon in other cardiac myopathic models, and the importance of this increase in the initiation/development of cardiac muscle disorders warrants further investigation using loss-of-function mouse models.

We observed an increase in apoptosis in SUMO-2-Tg hearts and in cultured cardiomyocytes overexpressing SUMO-2, indicating the potential implication of cell death in the progression of cardiomyopathy associated with SUMO-2 overexpression. Since the overexpressed SUMO-2-GG, but not the mutant SUMO-2-ΔGG, in cultured cardiomyocytes promoted apoptotic process, we concluded that it was the SUMO-2 conjugation, but not SUMO-2 itself, that was critical in the cell death process and cardiomyopathy development. Thus, it is highly likely that the functional consequence of a globally elevated SUMO-2 conjugation may depend on the primary targets, and any pathological stimuli that shift the substrates for conjugation even without significantly changing the global levels of SUMO-2 conjugation may result in different functional outcomes. In addition, we also observed cardiac hypertrophy induced by increased SUMO-2, suggesting a potential implication of SUMO-2 in cardiac remodeling. We are currently investigating this issue.

4.2. Modulation of calpain–calpastatin proteolytic system by SUMO-2

Calpain–calpastatin system plays an important role in mediating the apoptotic process. Our findings suggested that SUMO-2 targeted at least two components of this system. CAST, the natural inhibitor of Capns, was a novel SUMO substrate identified by our study, and SUMO attachment promoted its turnover and altered its nature of subcellular distribution, subsequently decreasing its inhibitory impact on Capns. CAST had a relatively stable half-life [38], although it can be cleaved/degraded by a number of proteases including calpains, cathepsin and caspases [39–41]. Interestingly, SUMO-linked CAST was mainly cleaved by caspases, but not by other proteases tested, indicating that SUMO conjugation to CAST increased its sensitivity to caspases and/or decreased its

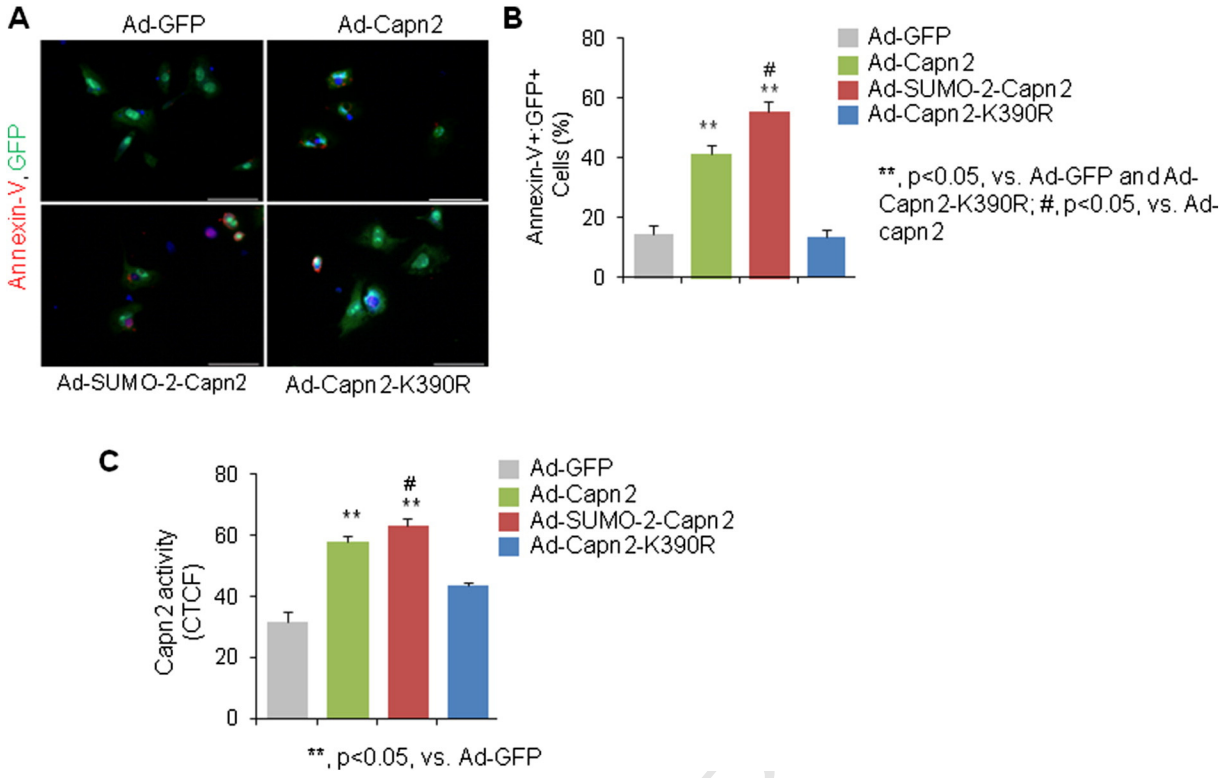

Fig. 8. SUMO-fused Capn2 promoted its enzymatic activity and apoptotic process. A. Overexpression of SUMO-fused Capn2 (SUMO-Capn2) promoted apoptotic process. Annexin-V immunofluorescence staining was performed on cardiomyocytes 36 h after adenovirus-mediated expression of GFP alone, Capn2, SUMO-Capn2 or Capn2-K390R (unsumoylatable form of Capn2), respectively. Red, Annexin-V; green, GFP. Bar, 50 μm. B. Statistical analysis of A. **$p < 0.001$ vs. Ad-GFP; #$p < 0.005$ vs. Ad-Capn2. C. SUMO-Capn2 exhibited higher enzymatic activity than Capn2. Cultured cardiomyocytes were infected with adenovirus expressing empty vector, Capn2, SUMO-Capn2 or Capn2-K390R, respectively, as shown in A. Synthetic substrate, t-BOC-LM-CMAC, was visualized in a cleavage-dependent manner by calpain. Randomly selected cells (at least over 100 cells/field) were scored by double positive signals of GFP and t-BOC-LM-CMAC. Note that SUMO-2 fusion enhanced proteolytic activity of Capn2, while SUMO site mutation attenuated the enzymatic activity of Capn2. Data were collected from three independent experiments. **$p < 0.005$ vs GFP, #$p < 0.005$ vs Capn2.

sensitivity to other proteases. The mechanisms underlying this sensitivity change merit further investigation. MG132, the proteasome-associated degradation inhibitor, also slightly but significantly inhibited the turnover of SUMO-fused CAST (data not shown), indicating that the proteasome-associated protein degradation was also involved in its facilitated turnover to some degree. Thus, it appears that the stability of SUMO conjugated CAST was regulated via at least two means: caspase cleavage and proteasome-associated degradation.

A previous study reported Capn2 as a SUMO target on lysine 390 [29], and SUMO conjugation to Capn2 potentiated, but SUMO site mutation (K390R) repressed, its ability to promote cell mobility [29]. In agreement with the above observations, SUMO-2-fused form of Capn2 exhibited elevated enzymatic activity and promoted apoptotic process in cultured cardiomyocytes, as evidenced by more significantly increased Annexin-V staining in SUMO-2-Capn2 group compared with the free Capn2 group. Interestingly, Capn2 was the only SUMO substrate among the Capn family members we tested; Capn1, Capn3 and Capn4 were not SUMO targeted (data not shown), indicating a unique regulation of Capn2 activity by the SUMO conjugation pathway. Since the SUMO conjugation pathway is governed by a number of enzymes, it will be interesting to probe whether there is any crosstalk between any of those enzymes involved in the SUMO conjugation pathway such as SUMO E3 ligases and/or SENPs and calpain–calpastatin proteolytic system.

In the present study, we used SUMO fused form to study the functions of SUMO modified CAST and Capn2. Although SUMO fused substrates are not identical to the native SUMO conjugated substrates, it has been widely used in the SUMO field [42,43], mainly due to the technical difficulty with purifying a sufficient amount of functionally active SUMO conjugated targets for functional analysis.

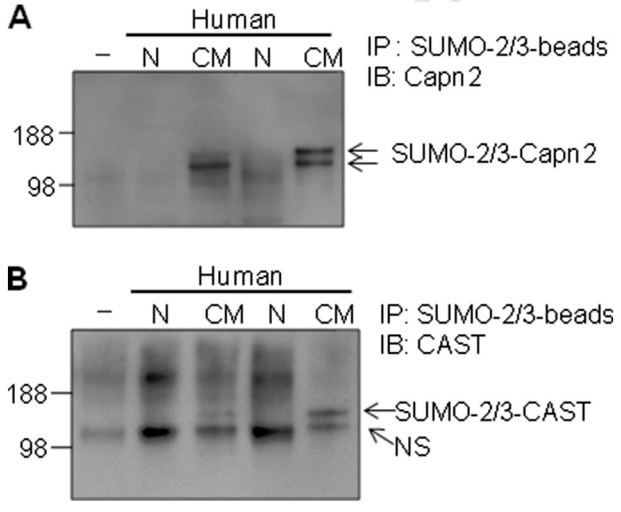

Fig. 9. Increased SUMO conjugation of Capn2 and CAST in the human cardiomyopathic heart muscles. Co-IP was performed on protein lysates extracted from two control and two diseased human left ventricles using SUMO-2/3 conjugated beads, followed by Western blot probed with anti-Capn2 antibody (A) or anti-CAST antibody (B). —, negative control group, which only contained binding buffer and SUMO-2/3 conjugated beads. N, normal heart; CM, cardiomyopathy; NS, non-specific.

4.3. SUMO conjugation and apoptosis

The crosstalk between the SUMO conjugation pathway and the apoptosis pathway has been previously explored in a number of studies. For

instance, an elevated SUMO conjugation was reported to protect cells from oxidative stress-induced apoptosis [44,45]. However, some other reports favored the notion that enhanced SUMO conjugation promoted apoptosis. For example, the increased SUMO-1 conjugation was linked to cell death induced by external stimuli such as H_2O_2 [46] or by treatment with progesterone receptor antagonists [47]. A more recent report suggested that knockout of SENP1, an isopeptidase that performs deconjugation functions, led to elevated SUMO conjugation and increased ER stress-induced apoptosis [48]. Our findings provided the evidence that the enhanced SUMO-2 conjugation promoted cell death. Although we revealed that SUMO-2 conjugation altered the activity of calpain–calpastatin system, we cannot rule out the possibility that SUMO-2 linkage to other substrates, such as caspases [49,50], may also play a role in the apoptotic process. Surprisingly, either increase or decrease in the levels of SENP2, a potent desumoylation enzyme, failed to significantly alter the cardiomyocyte survival status [14,51]. The exact mechanisms underlying this observation are not known. Possibly, under basal activity, little SUMO conjugation to the targets such as Capn2 and CAST was present, as shown in the control hearts; SUMO conjugation to these targets only increased under pathophysiological condition. Thus, altering the SENP2 levels under baseline conditions would not exert any significant effects on apoptosis. It is also possible that the subcellular localization of SENP2 may prevent it from efficiently "contacting" these SUMO modified targets to perform its desumoylation duties. It is highly likely that the functional interplay between the SUMO conjugation pathway and the cell death pathway is context/substrate dependent.

In conclusion, our studies demonstrate that SUMO-2/3 conjugation is involved in regulation of apoptosis at least partially via targeting calpain–calpastatin proteolytic system. Two major components of this system, calpain 2 and calpastatin, are SUMO substrates. SUMO-2 represses the function of calpastatin via inducing its turnover, while it potentiates the enzymatic activity of calpain 2, therefore subsequently increasing the proteolytic activity of this system. We further uncovered the elevated global SUMO-2/3 conjugation accompanied by increased levels of SUMO-2/3-attached calpain 2 and calpastatin in the human cardiomyopathic heart muscles. Collectively, our current findings suggest that the SUMO-2/3 conjugation pathway plays a pathogenic role in the development of cardiomyopathy and heart failure, and this pathway may serve as a potential novel target for therapeutic intervention in clinic.

Disclosures

None declared

Transparency document

The Transparency document associated with this article can be found, in the online version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2015.03.013.

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