Published in final edited form as:
Circ Res. 2011 January 7; 108(1): 40–50. doi:10.1161/CIRCRESAHA.110.230607.

Perturbation of cullin deneddylation via conditional Csn8 ablation impairs the ubiquitin-proteasome system and causes cardiomyocyte necrosis and dilated cardiomyopathy in mice

Huabo Su, PhD[1,2], **Jie Li, MD, PhD**[1,2], **Suchithra Menon, PhD**[3], **Jinbao Liu, MD, PhD**[2,4], **Asangi R. Kumarapeli, MD, PhD**[1,2], **Ning Wei, PhD**[3], and **Xuejun Wang, MD, PhD**[1,2]

[1]Division of Basic Biomedical Sciences, Sanford School of Medicine of the University of South Dakota, Vermillion, South Dakota 57069, USA.

[2]Cardiovascular Research Institute, Sanford School of Medicine of the University of South Dakota, Vermillion, South Dakota 57069, USA.

Address correspondence to: Dr. Xuejun Wang, Division of Basic Biomedical Sciences, Sanford School of Medicine of the University of South Dakota, 414 East Clark Street, Lee Medical building, SD 57069, USA, Tel. 605 677-5132; Fax. 605 677-6381; xuejun.wang@usd.edu.

Current address: Dr. Menon, Department of Genetics and Complex Diseases, Harvard School of Public Health, Boston, Massachusetts; Dr. Kumarapeli, Department of Pathology, Buffalo General Hospital, Buffalo, New York.

Disclosure: None.

Novelty and Significance

What Is Known?

- The bona fide biochemical activity of the COP9 signalosome (CSN) is cullin deneddylation by which the CSN regulates cullin-RING E3 ligases and thereby the ubiquitination of a large sub-proteome.
- The necessity and function of the CSN in a post-mitotic organ has not been reported.
- Ubiquitin-proteasome system (UPS) dysfunction has been observed in cardiac remodeling and failure but its pathophysiological significance remains obscure.

What New Information Does This Article Contribute?

- The first compelling evidence that Csn8 deficiency/CSN malfunction impairs UPS proteolytic function in mouse hearts.
- The first evidence that Csn8 deficiency primarily causes cardiomyocyte necrosis and that the mode of cell death caused by Csn8 deficiency is cell-type specific.
- The first demonstration that Csn8/CSN is essential to postnatal cardiac development and function.
- The first demonstration that cardiomyocyte-restricted UPS impairment is sufficient to cause cardiac hypertrophy and failure.

The importance of UPS-mediated proteolysis in cardiac pathogenesis and experimental therapeutics is increasingly recognized. Delineating how cardiac UPS function is regulated should enable a better assessment of its the pathophysiological significance to the development of new therapeutic strategies. Previous studies have shown that the CSN regulates the UPS via removing NEDD8 from NEDD8-conjugated Cullins: the Cullins constitute a large family of E3 ligases. However, this has not been tested in a critical vertebrate organ. Moreover, the role of the CSN in a post-mitotic organ has not been determined. Through cardiomyocyte-restricted genetic deletion of Csn8, a subunit of the CSN, we demonstrate for the first time that Csn8/CSN is required for postnatal cardiac development and heart function. Csn8 deficiency impaired UPS proteolytic function and caused, primarily, cardiomyocyte necrosis rather than apoptosis. This study establishes a new model of cardiomyocyte-restricted UPS impairment and shows for the first time that cardiomyocyte-restricted UPS malfunction is sufficient to cause cardiac hypertrophy and heart failure. The findings of this study also indicate that additional caution should be exercised in the use of drugs that interfere with neddylation. Such drugs are currently being tested in clinical trials to treat cancer and our data indicate that the potential cardiotoxicity of these compounds should be monitored closely.

[3]Department of Molecular, Cellular and Developmental Biology, Yale University, New Haven, Connecticut 06520, USA.

[4]Department of Pathophysiology, Guangzhou Medical College, Guangzhou, Guangdong, China.

Abstract

Rationale—Ubiquitin-proteasome system (UPS) dysfunction has been implicated in cardiac pathogenesis. Understanding how cardiac UPS function is regulated will facilitate delineating the pathophysiological significance of UPS dysfunction and developing new therapeutic strategies. The COP9 signalosome (CSN) may regulate the UPS but this has not been tested in a critical vertebrate organ. Moreover, the role of CSN in a post-mitotic organ and the impact of cardiomyocyte-restricted UPS dysfunction on the heart have not been reported.

Objective—We sought to determine the role of CSN-mediated deneddylation in UPS function and postnatal cardiac development and function.

Methods and Results—Cardiomyocyte-restricted Csn8 gene knockout (CR-Csn8KO) in mice was achieved using a Cre-LoxP system. CR-Csn8KO impaired CSN holocomplex formation and cullin deneddylation and resulted in decreases in F-box proteins. Probing with a surrogate misfolded protein revealed severe impairment of UPS function in CR-Csn8KO hearts. Consequently, CR-Csn8KO mice developed cardiac hypertrophy, which rapidly progressed to heart failure and premature death. Massive cardiomyocyte necrosis rather than apoptosis appears to be the primary cause of the heart failure. This is because (1) massive necrotic cell death and increased infiltration of leukocytes were observed prior to increased apoptosis; (2) increased apoptosis was not detectable until overt heart failure was observed; and (3) cardiac overexpression of Bcl2 failed to ameliorate CR-Csn8KO mouse premature death.

Conclusions—Csn8/CSN plays an essential role in cullin deneddylation, UPS-mediated degradation of a subset of proteins, and the survival of cardiomyocytes; therefore is indispensible in postnatal development and function of the heart. Cardiomyocyte-restricted UPS malfunction can cause heart failure.

Keywords

COP9 signalosome; ubiquitin E3 ligases; proteasome; cell death; heart failure

INTRODUCTION

The ubiquitin-proteasome system (UPS) mediates the main proteolytic pathway of targeted protein degradation in the cell. UPS dysfunction has been observed in animal models of a variety of cardiac disorders,[1-4] including ischemic heart disease, pressure-overload cardiac hypertrophy and failure, diabetic cardiomyopathy, familial hypertrophic and dilated cardiomyopathy,[5, 6] desmin-related cardiomyopathy, and doxorubicin cardiotoxicity.[7, 8] UPS dysfunction has also been implicated in failing human hearts.[6, 9, 10] Both clinical observation and some animal experiments have suggested that proteasome functional insufficiency may play an important role in the genesis of congestive heart failure (CHF).[11-14] Therefore, a better understanding of the molecular mechanisms by which the UPS function is regulated in the heart may provide critical information for developing novel therapeutic strategies to treat the related cardiac disorders or to protect against cardiotoxicity from chemotherapies. To this end, a few recent reports have excitingly begun to decipher the regulation of proteasome activities in the heart under the baseline and certain pathological conditions by factors including posttranslational modifications.[14-20] Meanwhile, currently, proteasome inhibition in intact animals can only be achieved in a non-selective manner using pharmacological agents. An animal model of cardiomyocyte-restricted UPS functional

impairment is currently lacking but would remarkably benefit the elucidation of pathophysiological significance of cardiac UPS dysfunction in cardiac pathogenesis.

Ubiquitination, the initial and essential step to target a protein molecule for degradation by the UPS, is achieved by a cascade of enzymatic reactions that covalently attaches ubiquitin to the target protein molecule or the preceding ubiquitin. The ubiquitin E3 ligase plays the rate-limiting and specificity determination roles in ubiquitination. The largest family of ubiquitin E3 ligases is cullin-RING ligases (CRLs). In a CRL, cullin serves as a scaffold for other components of the E3 ligase to assemble and function. A classic example of CRLs is the SCF (Skp1-Cullin1-F-box) subfamily of E3's. Neddylation, a process analogous to ubiquitination, covalently attaches an ubiquitin-like small protein NEDD8 to the side chain of lysine residues of a target protein molecule such as cullin. Cullin neddylation is essential to the assembly and activation of the CRL whereas cullin deneddylation appears to be critical for the disassembly and thereby the dynamics of CRLs.[21, 22]

The removal of Nedd8 from cullin through a process known as deneddylation is carried out by the COP9 (**CO**nstitutive **P**hotomorphogenesis mutant **9**) signalosome (CSN),[23] which is an evolutionarily highly conserved multi-protein complex consisting of 8 unique subunits (CSN1 through CSN8).[21] Molecular and genetic characterization of the CSN in several organisms has revealed its involvement in a wide variety of signaling and developmental processes, including cell cycle progression, DNA repair, transcriptional regulation, nuclear export, angiogenesis, circadian rhythms, embryogenesis, and the immune response.[22] Although the exact mechanisms underlying the cellular functions of the CSN remain unclear, most of these functions can be tied to its capabilities of regulating the activity of CRLs and the subsequent UPS-mediated proteolysis of their substrates. The regulation of CRLs by the CSN relies on its CSN5-resided isopeptidase activity, which removes an ubiquitin-like protein Nedd8 from cullin family proteins. It seems that cycling of neddylation and deneddylation are required to sustain CRLs' ubiquitination activity, as loss of CSN function led to destabilization of many substrate-recognizing adaptors in CRLs and thereby accumulating their substrates.[24-27] Additionally, the CSN recruits deubiquitination enzymes, thereby helping remove ubiquitin from mono- or poly- ubiquitinated substrates.[21]

The CSN is structurally homologous to the lid of 19S proteasomes, therefore is proposed as an alternative lid of the 26S proteasome.[28] Although the isopeptidase catalytic center resides in CSN5, an intact holocomplex of all 8 subunits appears to be required for the deneddylation activity. Typically, loss of one CSN subunit destabilizes other CSN subunits to various degrees and leads to impaired deneddylation activity in mammalian cells.[26, 29-31]

The physiological significance of the CSN has begun to be understood. Loss of CSN function led to growth arrest in the early stages of the development in plants, fungi, flies, and mice.[21, 31-34] Defective cell-cycle progression and gene expression are attributable to the observed phenotypes. Conditional deletion of Csn8 during late T-cell development has been carried out, which revealed the role of the Csn8/CSN in T-cell activation. The study showed that Csn8 is essential for quiescent T-cells to re-enter the rigorous cell divisions in response to immune stimulation.[31] The lack of T-cell clonal expansion in the Csn8 T-cell knockout is associated with inadequate gene expression of important cell cycle regulators.[31] We have recently shown that conditional deletion of Csn8 in hepatocytes causes massive hepatocyte apoptosis and impaired liver regeneration.[35]

Here we generated perinatal cardiomyocytes-restricted Csn8 knockout mice (CR-Csn8KO). Unlike the T-cell and hepatocyte knockout models, CR-Csn8KO does not involve robust proliferation of the parenchymal cells. Rather, this knockout model allowed the physiological role of the CSN in a critical post-mitotic vertebrate organ to be investigated

for the first time. We report that depletion of Csn8 caused cardiac hypertrophy and subsequent CHF and premature death in mice. Csn8 deficiency compromises UPS-mediated proteolysis and causes primarily necrotic cell death in cardiomyocytes, indicating an essential role of the CSN in regulating UPS-mediated proteolysis and cardiomyocyte survival in the heart.

METHODS

(A full description of Methods can be found in online supplement.)

Animal models

The Csn8-floxed mouse model ($Csn8^{flox/+}$) was originally created in the 129/Sv background.[31] For the present study, it was converted to the C57BL6 inbred background through >10 generations of backcross. In the Csn8-floxed allele, exons 4 through 6 of the *Csn8* gene are flanked by two loxP sites.[31] The transgenic (tg) mouse model with expression of *Cre* driven by the mouse α myosin heavy chain (Mhc6) promoter ($\alpha MHC\text{-}Cre^{+}$) was created and maintained in the C57BL6 background.[14] From the cross-breeding scheme depicted in Fig. 1A, $CSN8^{flox/flox}/\alpha MHC\text{-}Cre^{+}$ mice (CR-Csn8KO) and littermate $CSN8^{flox/flox}/\alpha MHC\text{-}Cre^{-}$ mice (CTL) were obtained and used in this study. Notably, we did not observe any phenotypic difference between the $CSN8^{flox/flox}/\alpha MHC\text{-}Cre^{-}$ and the $CSN8^{+/+}/\alpha MHC\text{-}Cre^{+}$ mice within the time frame studied here.

The creation and validation of the FVB/N GFPdgn tg mice as a reverse reporter of UPS proteolytic function have been previously described.[7] The Bcl2 tg mice were previously described.[36] The breeding strategies to introduce the GFPdgn or the Bcl2 transgene into the CR-Csn8KO background were similar (Supplementary Fig. I). The care and use of animals in this study conform to institutional guidelines for the use of animals in research.

Western blot analyses, gel filtration, RNA dot blot analysis, echocardiography, terminal dUTP nick end-labeling (TUNEL) assays, and immunofluorescence and confocal microscopy were performed as we previously described.[29, 35, 37]

Determining cell membrane permeability to Evan's blue dye (EBD)

This was done as previously described.[38]

Statistical Analysis

All quantitative data are presented as mean±SD unless otherwise indicated. Differences between experimental groups were evaluated for statistical significance using Student's *t*-test for unpaired two group comparison or one-way ANOVA where appropriate. The *P* value <0.05 was considered statistically significant.

RESULTS

Establishing CR-Csn8KO in mice

The creation and initial characterization of the *Csn8* floxed mouse were recently reported.[31] To study the physiological role of Csn8/CSN in the heart, we used the transgenic (tg) *Cre* driven by the mouse *Mhc*6 gene promoter to conditionally ablate the *Csn8* gene (CR-Csn8KO, Fig. 1A).[14] Although germ line deletion of *Csn8* in mice resulted in early embryonic lethality,[31] our *Mhc*6-dependent cardiomyocyte-restricted deletion of *Csn8* is viable, as CR-Csn8KO mice were born with the expected Mendelian frequency (Fig. 1A). Csn8 protein level was significantly decreased at postnatal day 1 in CR-Csn8KO hearts and largely depleted by day 7 (Fig. 1B), indicating that the depletion of Csn8 in cardiomyocytes

is achieved between postnatal day 1 and day 7. The specificity of *Csn8* gene deletion was confirmed by the loss of nuclear-enriched Csn8 staining in cardiomyocytes, but not in non-cardiomyocytes, of the CR-Csn8KO hearts (Fig. 1C), and by unaltered Csn8 protein levels in other major organs (Fig. 1D).

Csn8 is essential to CSN complex formation and CSN *bona fide* activities in the heart

The CSN holo-complex consists of 8 subunits and each subunit appears to be essential for CSN complex formation and the *bona fide* deneddylation activity in the cell.[22] In cardiomyocytes, loss of Csn8 led to reduced protein levels of other tested CSN subunits (Fig. 2A). We investigated further into the CSN complex formation by separating the holo-complex and the mini-complex via gel filtration followed by western blot analyses for Csn1, Csn2, and Csn6. Loss of Csn8 disrupted the CSN holo-complex formation as evidenced by reduced amount of 450 kD CSN holo-complex and substantially increased levels of 150-300 kD CSN mini-complexes in CR-Csn8KO hearts (Fig. 2B). Moreover, CR-Csn8KO heart lysate displayed a marked increase of neddylated cullin 1 (Cul1), Cul2, Cul3, and Cul4A, which showed a slower migration rate than the corresponding native forms (Fig. 2C, D), indicating that the cullin deneddylation activity was compromised in Csn8-deficient hearts.

Csn8 deficiency in cardiomyocytes causes cardiac hypertrophy

CR-Csn8KO mice were grossly indistinguishable from their littermate CTLs at birth. The body weight of CR-Csn8KO mice did not differ from that of their littermate CTLs during the first 3 weeks. However, CR-Csn8KO mice failed to gain further body weight between weeks 3 and 4 while their littermates grew rapidly (Fig. 3A). Csn8 ablation by the employed strategy did not appear to cause early cardiac developmental defects, as the CR-Csn8KO hearts were morphologically indistinguishable from the CTLs at birth (data not shown). However, starting from postnatal 2 weeks, the CR-Csn8KO hearts were significantly enlarged (Fig. 3B), which was confirmed by increased heart weight (Hw) to tibial length (TL) ratio as well as increased ventricle weight (Vw) to TL ratio (Fig. 3C). We found that the cross-sectional area of CR-Csn8KO cardiomyocyte was significantly increased at 2 weeks (Fig. 3D, E), which indicates cardiomyocyte hypertrophy and is consistent with increased Hw/TL ratios. In addition, two representative fetal genes, α-skeletal actin (Sk. actin) and atrial natriuretic factor (ANF), were up-regulated in CR-Csn8KO hearts at 2 weeks of age (Fig. 3F, G). These results indicate that loss of Csn8 in cardiomyocytes causes cardiac hypertrophy in mice.

CR-Csn8KO mice develop heart failure and die prematurely

To determine the impact of Csn8 deficiency on cardiac function, echocardiography was performed at both 2 and 3 weeks. Despite cardiac hypertrophy, no functional abnormality in CR-Csn8KO hearts was discernible at 2 weeks (data not shown). However at 3 weeks, CR-Csn8KO mice showed increased end-systolic and end-diastolic left ventricular (LV) diameters and reduced fractional shortening, indicating impaired LV function (Fig. 4A, B; Supplementary Table I). The LV function of CR-Csn8KO mice was further deteriorated at 4 weeks, as indicated by a sudden drastic increase in relative lung weight compared to a normal kidney weight (Fig. 4C). CR-Csn8KO eventually led to 100% postnatal lethality within 52 days; the majority of mice died between 4 and 5 weeks (Fig. 4D).

Csn8 deficiency impairs UPS proteolytic function in the heart

The CSN has been proposed as a UPS regulator by virtue of its capability to regulate CRL activities and its association with de-ubiquitinating enzymes and the 19S proteasome.[28, 39] To assist the investigation of UPS proteolytic function in intact animals, we previously created a tg mouse model that ubiquitously overexpresses a modified green fluorescence

protein (GFP) with its carboxyl terminus fused to degron CL1. The modified GFP is known as GFPdgn and is a surrogate substrate of the UPS.[7] In absence of changes in synthesis, GFPdgn protein levels inversely correlate to UPS proteolytic function in the cell.[40, 41] Degron CL1 signals for ubiquitination by surface exposure of a stretch of hydrophobic amino acid residues,[42] a signature conformation of misfolded proteins.[1] Therefore, GFPdgn is considered a surrogate of misfolded proteins although its solubility remains very good when it is stably expressed in mice.[43] Here, to explore the in vivo impact of Csn8 deficiency on UPS-mediated proteolysis, tg GFPdgn was introduced into CTL and CR-Csn8KO mice by cross-breeding. CR-Csn8KO significantly increased GFPdgn protein levels (~ 3 fold relative to that of CTLs, $p<0.01$) (Fig. 5A) but not GFPdgn transcript levels at 3 weeks (Supplementary Fig. II). The accumulation of GFPdgn was further confirmed by the increased GFP immunofluorescence intensity in CR-Csn8KO mouse hearts (Fig. 5B). These experiments compellingly demonstrate that UPS-mediated proteolysis, at least the removal of a surrogate misfolded protein in the heart, is severely compromised by Csn8 deficiency. Consistent with this important finding, our quantitative western blot analyses showed a significant increase in the level of total ubiquitinated proteins in CR-Csn8KO hearts and immunostaining of myocardial sections further revealed that the increase was in the form of ubiquitin-positive protein aggregates in cardiomyocytes rather than a global increase throughout the cell (Fig. 5C, D). In CTL hearts, ubiquitin immunostaining distributes rather evenly and no ubiquitin-positive protein aggregates are discernible (Fig. 5D).

To pinpoint the defective step in the UPS, we assessed the proteasome proteolytic function using the in vitro peptidase activity assays with synthetic fluorogenic substrates. Interestingly, compared with the CTL group, the 3 proteasomal peptidase activities: chymotrypsin-like, caspase-like, and trypsin-like peptidase activities in either presence or absence of ATP were all significantly increased in the CR-Csn8KO hearts (Fig. 6A), suggesting that the impaired UPS proteolytic function is not due to defective proteasome peptidase activities. Supporting this conclusion, the protein abundance of representative subunits of the 19S proteasome: Rpt5, Rpn11 and Rpn12 and the representative subunit of 20S proteasomes: β5 (PSMB5), were all up-regulated in CR-Csn8KO hearts (Fig. 6B). Furthermore, as illustrated by gel filtration analyses of PSMB5 and Rpn2 (Fig. 6C), the 26S and 20S proteasome complexes were increased in CR-Csn8KO hearts.

It has been reported that loss of CSN function in cultured cells or lower species destabilizes some substrate-recognizing adaptors in CRLs and thereby accumulating their substrates.[24-27] Here, we found that representative F-box proteins: Atrogin-1, VHL (von Hippel-Lindau), and β-TrCP (β-transducin repeats containing protein), which are a critical component of the SCF-type of CRLs, were decreased in CR-Csn8KO hearts whereas examples of single-unit RING E3 ligases (e.g., MuRF1 and MDM2) which do not interact with cullin for their ligase activation,[44, 45] were not (Fig. 6D). Interestingly, an accumulation of calcineurin-A, HIF1α, and β-catenin, which are the representative substrates respectively for Atrogin-1, VHL, and β-TrCP, was not observed in CR-Csn8KO hearts (Supplementary Fig. III).

Csn8 deletion does not cause apoptosis but enhances susceptibility to apoptotic agent in cardiomyocytes

Given that deficiency in certain CSN subunits may elicit apoptosis,[29, 35, 46] we tested whether apoptosis is a primary cause of the cardiac phenotype in CR-Csn8KO mice. Surprisingly, neither the number of TUNEL positive cardiomyocytes (Fig. 7A, B) nor the cleaved form caspase-3 (Fig. 7C) was increased in CR-Csn8KO hearts at 3 weeks, indicating no increase in apoptosis. Interestingly, anti-apoptotic protein Bcl2 was increased in the CR-Csn8KO mouse hearts. Moreover, tg overexpression of Bcl-2, an anti-apoptotic protein, in

cardiomyocytes failed to improve the lifespan of CR-Csn8KO mice (Fig. 7D). These results strongly indicate that CR-Csn8KO did not directly induce apoptosis.

Nevertheless, acute infusion of isoproterenol (i.p., 15 mg/kg), an elicitor of apoptosis, resulted in a more pronounced increase of TUNEL-positive cardiomyocytes in 3-week-old CR-Csn8KO mice, compared with CTL mice (Fig. 7B), suggesting that Csn8 deficiency increases the susceptibility of cardiomyocytes to cell death induced by pathological insults. Indeed, 4-week-old CR-Csn8KO mice, which are in the late stage of heart failure, showed more TUNEL-positive cardiomyocytes than the CTL (Fig. 7B).

Loss of Csn8 in cardiomyocytes causes massive necrosis

Since apoptosis is ruled out as a primary cause of cardiomyocytes loss in CR-Csn8KO hearts, we examined the hearts for the prevalence of necrosis. A characteristic early event of necrotic cell death is the loss of plasma membrane integrity. This can be tested by using Evans Blue Dye (EBD), which is taken up by cardiomyocytes with leaky sarcolemma but is excluded by cells with intact membranes. A large number of EBD-positive cardiomyocytes were detected in the CR-Csn8KO hearts at 18 hours after intraperitoneal injection of EBD, whereas few were found in the CTL hearts (180 ± 50 vs. 4 ± 1 per 10^4 cardiomyocytes, $p<0.01$; Fig. 8A, C). Necrosis is often accompanied by inflammatory responses, including neutrophils infiltration. Immunohistopathological analysis using an antibody against CD45, a marker of leukocytes, revealed that significantly more leukocytes were present in the CR-Csn8KO heart than in the CTL (Fig. 8B, D). Collectively, these data show that loss of Csn8 in cardiomyocytes induces necrosis.

DISCUSSION

Germ-line deletion of any CSN subunits including Csn8 all led to early embryonic lethality in mice,[31, 34, 46, 47] preventing its application in study of mechanisms of CSN functions in any organs. By conditionally targeting the *Csn8* gene in mouse hearts, we demonstrate for the first time that Csn8 plays an essential role in cullin deneddylation, UPS-mediated misfolded protein removal, and the survival of cardiomyocytes. Therefore, Csn8/CSN is indispensible in the postnatal development and function of the heart. From the point of view of cardiac pathogenesis, we demonstrate here for the first time in intact animals that genetically induced cardiomyocyte-restricted perturbation of UPS function is sufficient to cause CHF. In terms of CSN biology, this study is the first in-depth elucidation of the physiological significance of Csn8/CSN in a critical post-mitotic vertebrate organ and reveals that Csn8 deficiency/CSN malfunction causes primarily necrotic cell death in the heart.

Csn8 is essential to CSN holocomplex formation and cullin deneddylation in cardiomyocytes

CSN8 is the smallest and least conserved subunit of the CSN in *Arabidopsis Thaliana* from which CSN was initially discovered.[23] The homologues of all CSN subunits except CSN8 have been identified in fission yeast *S. pombe*.[48] Hence, it was unclear whether CSN8 is an essential subunit of the CSN holocomplex in all higher eukaryotic cells. Previous studies with cultured HEK-293 cells or the T-cells and hepatocytes in intact mice suggested that Csn8 is an indispensible subunit of CSN holo-enzyme.[29, 31, 35] The present study comes to the same conclusion in cardiomyocytes by analyzing CSN complex distribution, protein abundance of other CSN subunits, and the change in cullin neddylation status in CR-Csn8KO mouse hearts (Fig. 2). The deletion of the *Csn8* gene in cardiomyocytes destabilized other CSN subunits, disrupted the formation of CSN holo-complexes, and accumulated neddylated proteins, including the neddylated form of all 4 cullins examined

(Fig. 2). These findings also suggest that the observed phenotypes in CR-Csn8KO mice are attributable to not only Csn8 deficiency but also the loss of CSN holo-complex and resultant perturbation of cullin deneddylation.

Csn8/CSN is critical for UPS-mediated degradation of misfolded proteins in the heart

Although Csn8 has been conditionally ablated in T-cells and hepatocytes the impact of Csn8 deficiency on UPS-mediated degradation of misfolded proteins in intact organs has not been reported. By tg expression of GFPdgn in CR-Csn8KO and CTL littermate mice, we were able to detect a significant increase in GFPdgn protein but not transcript levels in CR-Csn8KO hearts prior to the manifestation of CHF (Fig. 5A, B; Supplementary Fig. II), suggesting that Csn8 deficiency impairs UPS-mediated degradation of a surrogate misfolded protein and may contribute to the development of heart failure. Currently, a reliable method to directly detect changes in the amount of endogenous misfolded proteins in the heart is lacking but the accumulation of misfolded proteins activates characteristic cellular responses, including the up-regulation of chaperone proteins and the formation of protein aggregates. Indeed, the protein expression of several important heat shock proteins (Hsp), αB-crystallin, Hsp25, and Hsp90 were markedly increased in CR-Csn8KO hearts (Supplementary Fig. IV). Increases in total ubiquitinated proteins in the form of ubiquitin-positive protein aggregates were also observed in Cr-Csn8KO hearts (Fig. 5C, D).

Neddylation of cullin activates CRL's but the impact of deneddylation of cullin by CSN on CRLs remains elusive. Earlier in vitro biochemistry studies suggested that cullin deneddylation negatively regulates CRLs' activities but genetic studies with cultured cells showed the opposite.[21] A currently prevalent hypothesis is that cullin deneddylation is essential to the disassembly of the CRL complex when the complex has completed ubiquitinating a target protein molecule. In absence of cullin deneddylation, the active CRL complex may ubiquitinate and destabilizes its own components, such as the F-box protein in the SCF complex, by self-ubiquitination.[25] Hence, deneddylation-triggered CRL disassembly allows the components of the CRL to be preserved and to move onto next target protein molecule to continue doing their job. This hypothesis has gained some support from studies using cultured cells or lower model species but remains to be tested in intact vertebrates.[24-27] We examined this hypothesis in the heart with the CR-Csn8KO mice. Muscle-specific RING finger proteins (MuRF's) and Atrogin-1/MAFbx are among the best known muscle-specific E3's in myocytes. VHL (von Hippel-Lindau) and MDM2 (mouse double minute 2) are ubiquitously expressed. Both Atrogin-1 and VHL are F-Box proteins and can assemble complexes with cullins to form SCF-type of E3's.[44, 49] As predicted by the current prevalent model for the role of CSN-mediated cullin deneddylation, Csn8 deficiency destabilized Atrogin-1 and VHL, leading to their decreases in CR-Csn8KO hearts (Fig. 6D). Atrogin-1 is a striated muscle-specific F-box protein and teams up with Skp1 and cullin1 to ubiquitinate myofibrillar proteins, myogenic factors, and hypertrophy signaling molecules for degradation or for a non-proteolytic regulation.[50-52] Hence, Atrogin-1 down-regulation may conceivably have participated in the development of CHF in CR-Csn8KO mice. However, a known substrate (calcineurin A) of Atrogin-1, along with HIF1α, β-catenin, cyclin E, and IκBα whose ubiquitination are normally mediated by E3's of the CRL family, [53-55] was surprisingly not accumulated in CR-Csn8KO hearts under basal condition. These findings indicate that CRLs-mediated ubiquitination was not globally impaired in CR-Csn8KO hearts. This is also supported by the findings that ubiquitinated GFPdgn was not significantly decreased by CR-Csn8KO (Supplementary Fig. V).

Since our data do not support a global impairment of CRLs, we propose that CR-Csn8KO induced impairment of UPS proteolytic function may occur as a result of uncoupling between ubiquitination and proteasome-mediated degradation steps. This is because: (1) in vitro assays showed that proteasome peptidase activities in CR-Csn8KO myocardial extracts

were significantly increased rather than decreased (Fig. 6A); (2) the abundance of the 19S, 20S, and 26S proteasomes were significantly increased (Fig. 6B, C); and (3) the total ubiquitinated proteins were significantly increased in CR-Csn8KO hearts (Fig. 5). The CSN is structurally homologous to the lid of 19 proteasomes and can associate with both CRLs and the 26S proteasome.[56, 57] Therefore, it is very possible that Csn8/CSN plays a previously unrecognized role in coupling the ubiquitination with the subsequent proteolytic removal of misfolded proteins in post-mitotic cells.

It should also be noted that the increase in total ubiquitin conjugates in CR-Csn8KO hearts is consistent with CSN's deubiquitination activity,[21] whereas the accumulation of ubiquitin positive protein aggregates suggests that Csn8/CSN may be critical to the removal of aggregated proteins by a mechanism such as macroautophagy. It will be interesting and important to determine if Csn8/CSN plays a role in the autophagic-lysosomal pathway.

The mode of cell death caused by Csn8 deficiency is organ-specific

Necrosis instead of apoptosis appears to be the primary mode of cell death in CR-Csn8KO hearts. This is strongly supported by the remarkable increase in the cardiomyocyte membrane permeability to EBD and significantly elevated leukocyte infiltration (Fig. 8) before the increase of apoptosis became discernible in CR-Csn8KO muse hearts (Fig. 7B). Both TUNEL assays and analysis of caspase 3 activation did not show an increase in apoptosis in CR-Csn8KO hearts until after 3 weeks (Fig. 7A, B). Cardiac overexpression of Bcl2 has previously been shown to protect against cardiomyocyte apoptosis,[36] but failed to attenuate premature death of CR-Csn8KO mice (Fig. 7D). By contrast, we have recently found that postnatal hepatocyte-restricted Csn8 deletion using the same Csn8-floxed mice induced massive hepatocyte apoptosis. EBD staining did not show an increase in necrotic cell death in the Csn8-deficient liver.[35] Taken together, these results indicate that the mode of cell death caused by Csn8 deficiency appears to be organ-specific. The mechanisms underlying the specificity are unknown at this time but will be important and interesting to be investigated.[35] Differences in functional obligations and in proliferation property may contribute to the phenotypic difference.

Significance and clinical implications

The pathophysiological significance of cardiomyocyte UPS dysfunction observed in a variety of cardiac disorders has not been well understood. A major barrier is the inability to inhibit UPS function specifically in cardiomyocytes without affecting UPS function in the non-cardiomyocyte compartment, with a pharmacological method. Pharmacologically-induced systemic proteasome inhibition or global knockout of a specific E3 ligase, such as MuRF1, MuRF2, MuRF3, and CHIP, has been shown to impair cardiac function or make the heart more vulnerable to ischemic injury.[3, 13, 43] The approaches used so far, however, could not differentiate whether the cardiac impairment resulted from the UPS malfunction of the cardiomyocyte compartment or non-cardiomyocyte compartments. The present study represents the first report of a genetic model of cardiomyocyte-restricted UPS malfunction. We have unequivocally demonstrated that UPS function impairment caused by Csn8 deficiency/CSN malfunction in cardiomyocytes has severe consequences on cardiomyocyte fate and cardiac function during postnatal development.

Because the activity of CRLs is critical to tumor cell division and survival, a new drug (e.g. MLN4924) has been developed employing a strategy to block cullin neddylation and is currently under phase 1 clinical trials for treating both solid tumor and hematological malignancies.[58] Hence, an immediate clinical implication of this study is that patients receiving agents inhibiting cullin neddylation should be closely watched for potential cardiac toxicity because both inhibition of neddylation by MLN4924 and inhibiting

deneddylation by Csn8 deficiency disrupt the catalytic dynamics of CRLs and thereby yielding the same adverse consequence to the heart.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Frances Day for assistance in confocal microscopy analyses, Ms. Andrea Jahn for outstanding technical assistance in maintaining mouse colonies and genotype determination. Dr. X. Wang is a recipient of the Established Investigator Award of the American Heart Association.

Sources of Founding

This work was in part supported by NIH grants R01HL085629, and R01HL072166, American Heart Association grants 0740025N (to X. W.) and 0625738Z (to H.S.), and the Physician Scientist Program of the University of South Dakota.

Non-standard Abbreviations and Acronyms

UPS	ubiquitin-proteasome system
CHF	congestive heart failure
CSN	The COP9 Signalosome
RING	really interesting new gene
CRLs	cullin-RING ligases
CTL	the control group
WT	wild type
KO	knockout
CR-Csn8KO	cardiomyocyte-restricted Csn8 KO
Cul	Cullin
TL	tibial length
Hw	heart weight
Vw	ventricular weight
ANF	atrial natriuretic factor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
LVEDD	left ventricle (LV) end-diastolic dimension
LVESD	LV end-systolic dimension
PWTd	posterior wall thickness at the end of diastole
FS	fractional shortening
ISO	isoproterenol
TUNEL	terminal dUTP nick end-labeling
EBD	Evan's blue dye
MuRF	muscle-specific RING finger protein

MDM2	mouse double minute 2
β-TrCP	β-transducin repeats containing protein
VHL	von Hippel-Lindau

References

1. Wang X, Robbins J. Heart failure and protein quality control. Circ Res. 2006; 99:1315–1328. [PubMed: 17158347]
2. Mearini G, Schlossarek S, Willis MS, Carrier L. The ubiquitin-proteasome system in cardiac dysfunction. Biochim Biophys Acta. 2008; 1782:749–763. [PubMed: 18634872]
3. Su H, Wang X. The ubiquitin-proteasome system in cardiac proteinopathy: A quality control perspective. Cardiovasc Res. 2010; 85:253–262. [PubMed: 19696071]
4. Willis MS, Townley-Tilson WH, Kang EY, Homeister JW, Patterson C. Sent to destroy: The ubiquitin proteasome system regulates cell signaling and protein quality control in cardiovascular development and disease. Circ Res. 2010; 106:463–478. [PubMed: 20167943]
5. Carrier L, Schlossarek S, Willis MS, Eschenhagen T. The ubiquitin-proteasome system and nonsense-mediated mrna decay in hypertrophic cardiomyopathy. Cardiovasc Res. 2010; 85:330–338. [PubMed: 19617224]
6. Predmore JM, Wang P, Davis F, Bartolone S, Westfall MV, Dyke DB, Pagani F, Powell SR, Day SM. Ubiquitin proteasome dysfunction in human hypertrophic and dilated cardiomyopathies. Circulation. 2010; 121:997–1004. [PubMed: 20159828]
7. Kumarapeli AR, Horak KM, Glasford JW, Li J, Chen Q, Liu J, Zheng H, Wang X. A novel transgenic mouse model reveals deregulation of the ubiquitin-proteasome system in the heart by doxorubicin. Faseb J. 2005; 19:2051–2053. [PubMed: 16188962]
8. Ranek MJ, Wang X. Activation of the ubiquitin-proteasome system in doxorubicin cardiomyopathy. Curr Hypertens Rep. 2009; 11:389–395. [PubMed: 19895749]
9. Tsukamoto O, Minamino T, Okada K, Shintani Y, Takashima S, Kato H, Liao Y, Okazaki H, Asai M, Hirata A, Fujita M, Asano Y, Yamazaki S, Asanuma H, Hori M, Kitakaze M. Depression of proteasome activities during the progression of cardiac dysfunction in pressure-overloaded heart of mice. Biochem Biophys Res Commun. 2006; 340:1125–1133. [PubMed: 16403436]
10. Kassiotis C, Ballal K, Wellnitz K, Vela D, Gong M, Salazar R, Frazier OH, Taegtmeyer H. Markers of autophagy are downregulated in failing human heart after mechanical unloading. Circulation. 2009; 120:S191–197. [PubMed: 19752367]
11. Voortman J, Giaccone G. Severe reversible cardiac failure after bortezomib treatment combined with chemotherapy in a non-small cell lung cancer patient: A case report. BMC Cancer. 2006; 6:129. [PubMed: 16689991]
12. Hacihanefioglu A, Tarkun P, Gonullu E. Acute severe cardiac failure in a myeloma patient due to proteasome inhibitor bortezomib. Int J Hematol. 2008; 88:219–222. [PubMed: 18633693]
13. Tang M, Li J, Huang W, Su H, Liang Q, Tian Z, Horak KM, Molkentin JD, Wang X. Proteasome functional insufficiency activates the calcineurin-nfat pathway in cardiomyocytes and promotes maladaptive remodelling of stressed mouse hearts. Cardiovasc Res. 2010
14. Drews O, Tsukamoto O, Liem D, Streicher J, Wang Y, Ping P. Differential regulation of proteasome function in isoproterenol-induced cardiac hypertrophy. Circ Res. 2010 (in press):DOI: 10.1161/CIRCRESAHA.1110.222364.
15. Gomes AV, Young GW, Wang Y, Zong C, Eghbali M, Drews O, Lu H, Stefani E, Ping P. Contrasting proteome biology and functional heterogeneity of the 20 s proteasome complexes in mammalian tissues. Mol Cell Proteomics. 2009; 8:302–315. [PubMed: 18931337]
16. Lu H, Zong C, Wang Y, Young GW, Deng N, Souda P, Li X, Whitelegge J, Drews O, Yang PY, Ping P. Revealing the dynamics of the 20 s proteasome phosphoproteome: A combined cid and electron transfer dissociation approach. Mol Cell Proteomics. 2008; 7:2073–2089. [PubMed: 18579562]

17. Drews O, Wildgruber R, Zong C, Sukop U, Nissum M, Weber G, Gomes AV, Ping P. Mammalian proteasome subpopulations with distinct molecular compositions and proteolytic activities. Mol Cell Proteomics. 2007; 6:2021–2031. [PubMed: 17660509]
18. Zong C, Gomes AV, Drews O, Li X, Young GW, Berhane B, Qiao X, French SW, Bardag-Gorce F, Ping P. Regulation of murine cardiac 20s proteasomes: Role of associating partners. Circ Res. 2006; 99:372–380. [PubMed: 16857963]
19. Asai M, Tsukamoto O, Minamino T, Asanuma H, Fujita M, Asano Y, Takahama H, Sasaki H, Higo S, Asakura M, Takashima S, Hori M, Kitakaze M. Pka rapidly enhances proteasome assembly and activity in in vivo canine hearts. J Mol Cell Cardiol. 2009; 46:452–462. [PubMed: 19059265]
20. Divald A, Kivity S, Wang P, Hochhauser E, Roberts B, Teichberg S, Gomes AV, Powell SR. Myocardial ischemic preconditioning preserves postischemic function of the 26s proteasome through diminished oxidative damage to 19s regulatory particle subunits. Circ Res. 2010; 106:1829–1838. [PubMed: 20431057]
21. Wei N, Deng XW. The cop9 signalosome. Annu Rev Cell Dev Biol. 2003; 19:261–286. [PubMed: 14570571]
22. Wei N, Serino G, Deng XW. The cop9 signalosome: More than a protease. Trends Biochem Sci. 2008; 33:592–600. [PubMed: 18926707]
23. Wei N, Chamovitz DA, Deng XW. Arabidopsis cop9 is a component of a novel signaling complex mediating light control of development. Cell. 1994; 78:117–124. [PubMed: 8033203]
24. He Q, Cheng P, Liu Y. The cop9 signalosome regulates the neurospora circadian clock by controlling the stability of the scffwd-1 complex. Genes Dev. 2005; 19:1518–1531. [PubMed: 15961524]
25. Wee S, Geyer RK, Toda T, Wolf DA. Csn facilitates cullin-ring ubiquitin ligase function by counteracting autocatalytic adapter instability. Nat Cell Biol. 2005; 7:387–391. [PubMed: 15793566]
26. Denti S, Fernandez-Sanchez ME, Rogge L, Bianchi E. The cop9 signalosome regulates skp2 levels and proliferation of human cells. J Biol Chem. 2006; 281:32188–32196. [PubMed: 16943200]
27. Luke-Glaser S, Roy M, Larsen B, Le Bihan T, Metalnikov P, Tyers M, Peter M, Pintard L. Cif-1, a shared subunit of the cop9/signalosome and eukaryotic initiation factor 3 complexes, regulates mel-26 levels in the caenorhabditis elegans embryo. Mol. Cell. Biol. 2007; 27:4526–4540. [PubMed: 17403899]
28. Li L, Deng XW. The cop9 signalosome: An alternative lid for the 26s proteasome? Trends Cell Biol. 2003; 13:507–509. [PubMed: 14507477]
29. Su H, Huang W, Wang X. The cop9 signalosome negatively regulates proteasome proteolytic function and is essential to transcription. Int J Biochem Cell Biol. 2009; 41:615–624. [PubMed: 18706515]
30. Cope GA, Deshaies RJ. Targeted silencing of jab1/csn5 in human cells downregulates scf activity through reduction of f-box protein levels. BMC Biochem. 2006; 7:1. [PubMed: 16401342]
31. Menon S, Chi H, Zhang H, Deng XW, Flavell RA, Wei N. Cop9 signalosome subunit 8 is essential for peripheral t cell homeostasis and antigen receptor-induced entry into the cell cycle from quiescence. Nat Immunol. 2007; 8:1236–1245. [PubMed: 17906629]
32. Busch S, Schwier EU, Nahlik K, Bayram O, Helmstaedt K, Draht OW, Krappmann S, Valerius O, Lipscomb WN, Braus GH. An eight-subunit cop9 signalosome with an intact jamm motif is required for fungal fruit body formation. Proc Natl Acad Sci U S A. 2007; 104:8089–8094. [PubMed: 17470786]
33. Oren-Giladi P, Krieger O, Edgar BA, Chamovitz DA, Segal D. Cop9 signalosome subunit 8 (csn8) is essential for drosophila development. Genes Cells. 2008; 13:221–231. [PubMed: 18298797]
34. Lykke-Andersen K, Schaefer L, Menon S, Deng XW, Miller JB, Wei N. Disruption of the cop9 signalosome csn2 subunit in mice causes deficient cell proliferation, accumulation of p53 and cyclin e, and early embryonic death. Mol Cell Biol. 2003; 23:6790–6797. [PubMed: 12972599]
35. Lei D, Li F, Su H, Tian Z, Ye B, Wei N, Wang X. Cop9 signalosome subunit 8 is required for postnatal hepatocyte survival and effective proliferation. Cell Death and Differentiation. 2010 (in press):doi:10.1038/cdd.2010.1098.

36. Imahashi K, Schneider MD, Steenbergen C, Murphy E. Transgenic expression of bcl-2 modulates energy metabolism, prevents cytosolic acidification during ischemia, and reduces ischemia/reperfusion injury. Circ Res. 2004; 95:734–741. [PubMed: 15345651]
37. Kumarapeli AR, Su H, Huang W, Tang M, Zheng H, Horak KM, Li M, Wang X. Alpha bcrystallin suppresses pressure overload cardiac hypertrophy. Circ Res. 2008; 103:1473–1482. [PubMed: 18974385]
38. Millay DP, Sargent MA, Osinska H, Baines CP, Barton ER, Vuagniaux G, Sweeney HL, Robbins J, Molkentin JD. Genetic and pharmacologic inhibition of mitochondrial-dependent necrosis attenuates muscular dystrophy. Nat Med. 2008; 14:442–447. [PubMed: 18345011]
39. Wolf DA, Zhou C, Wee S. The cop9 signalosome: An assembly and maintenance platform for cullin ubiquitin ligases? Nat Cell Biol. 2003; 5:1029–1033. [PubMed: 14647295]
40. Chen Q, Liu J-B, Horak KM, Zheng H, Kumarapeli ARK, Li J, Li F, Gerdes AM, Wawrousek EF, Wang X. Intrasarcoplasmic amyloidosis impairs proteolytic function of proteasomes in cardiomyocytes by compromising substrate uptake. Circ Res. 2005; 97:1018–1028. [PubMed: 16210548]
41. Liu J, Chen Q, Huang W, Horak KM, Zheng H, Mestril R, Wang X. Impairment of the ubiquitinproteasome system in desminopathy mouse hearts. FASEB J. 2006; 20:362–364. [PubMed: 16371426]
42. Gilon T, Chomsky O, Kulka RG. Degradation signals recognized by the ubc6p-ubc7p ubiquitinconjugating enzyme pair. Mol Cell Biol. 2000; 20:7214–7219. [PubMed: 10982838]
43. Wang X, Su H, Ranek MJ. Protein quality control and degradation in cardiomyocytes. J Mol Cell Cardiol. 2008; 45:11–27. [PubMed: 18495153]
44. Bodine SC, Latres E, Baumhueter S, Lai VK, Nunez L, Clarke BA, Poueymirou WT, Panaro FJ, Na E, Dharmarajan K, Pan ZQ, Valenzuela DM, DeChiara TM, Stitt TN, Yancopoulos GD, Glass DJ. Identification of ubiquitin ligases required for skeletal muscle atrophy. Science. 2001; 294:1704–1708. [PubMed: 11679633]
45. Sun Y. Targeting e3 ubiquitin ligases for cancer therapy. Cancer Biol Ther. 2003; 2:623–629. [PubMed: 14688465]
46. Tomoda K, Yoneda-Kato N, Fukumoto A, Yamanaka S, Kato JY. Multiple functions of jab1 are required for early embryonic development and growth potential in mice. J Biol Chem. 2004; 279:43013–43018. [PubMed: 15299027]
47. Yan J, Walz K, Nakamura H, Carattini-Rivera S, Zhao Q, Vogel H, Wei N, Justice MJ, Bradley A, Lupski JR. Cop9 signalosome subunit 3 is essential for maintenance of cell proliferation in the mouse embryonic epiblast. Mol Cell Biol. 2003; 23:6798–6808. [PubMed: 12972600]
48. Lykke-Andersen K, Wei N. Gene structure and embryonic expression of mouse cop9 signalosome subunit 8 (csn8). Gene. 2003; 321:65–72. [PubMed: 14636993]
49. Stebbins CE, Kaelin WG Jr. Pavletich NP. Structure of the vhl-elonginc-elonginb complex: Implications for vhl tumor suppressor function. Science. 1999; 284:455–461. [PubMed: 10205047]
50. Jogo M, Shiraishi S, Tamura TA. Identification of mafbx as a myogenin-engaged f-box protein in scf ubiquitin ligase. FEBS Lett. 2009; 583:2715–2719. [PubMed: 19631210]
51. Li HH, Willis MS, Lockyer P, Miller N, McDonough H, Glass DJ, Patterson C. Atrogin-1 inhibits akt-dependent cardiac hypertrophy in mice via ubiquitin-dependent coactivation of forkhead proteins. J Clin Invest. 2007; 117:3211–3223. [PubMed: 17965779]
52. Li HH, Kedar V, Zhang C, McDonough H, Arya R, Wang DZ, Patterson C. Atrogin-1/muscle atrophy f-box inhibits calcineurin-dependent cardiac hypertrophy by participating in an scf ubiquitin ligase complex. J Clin Invest. 2004; 114:1058–1071. [PubMed: 15489953]
53. Winston JT, Strack P, Beer-Romero P, Chu CY, Elledge SJ, Harper JW. The scfbeta-trcpubiquitin ligase complex associates specifically with phosphorylated destruction motifs in ikappabalpha and beta-catenin and stimulates ikappabalpha ubiquitination in vitro. Genes Dev. 1999; 13:270–283. [PubMed: 9990852]
54. Read MA, Brownell JE, Gladysheva TB, Hottelet M, Parent LA, Coggins MB, Pierce JW, Podust VN, Luo RS, Chau V, Palombella VJ. Nedd8 modification of cul-1 activates scf(beta(trcp))-

dependent ubiquitination of ikappabalpha. Mol Cell Biol. 2000; 20:2326–2333. [PubMed: 10713156]

55. Koepp DM, Schaefer LK, Ye X, Keyomarsi K, Chu C, Harper JW, Elledge SJ. Phosphorylation-dependent ubiquitination of cyclin e by the scffbw7 ubiquitin ligase. Science. 2001; 294:173–177. [PubMed: 11533444]
56. Enchev RI, Schreiber A, Beuron F, Morris EP. Structural insights into the cop9 signalosome and its common architecture with the 26s proteasome lid and eif3. Structure. 2010; 18:518–527. [PubMed: 20399188]
57. Huang X, Hetfeld BK, Seifert U, Kahne T, Kloetzel PM, Naumann M, Bech-Otschir D, Dubiel W. Consequences of cop9 signalosome and 26s proteasome interaction. FEBS J. 2005; 272:3909–3917. [PubMed: 16045761]
58. Soucy TA, Smith PG, Rolfe M. Targeting nedd8-activated cullin-ring ligases for the treatment of cancer. Clin Cancer Res. 2009; 15:3912–3916. [PubMed: 19509147]

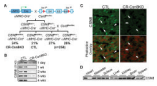

Figure 1.

Cardiomyocyte-restricted ablation of the *Csn8* gene (CR-Csn8KO). **A**, The breeding scheme used to obtain CR-Csn8KO and littermate control (CTL) mice. The exons 4 through 6 were floxed in the *Csn8*-floxed allele ($Csn8^{flox}$). The deletor strain, αMHC-Cre^{+}, expresses transgenic Cre under the control of the mouse myosin heavy chain-6 promoter (αMHC). The percent distribution of each genotype among 258 mice at birth is shown. **B**, Western blot analyses of Csn8 in myocardial extracts at the indicated age. **C**, Fluorescence confocal micrographs of myocardial cryosections immunostained for Csn8 (green). F-actin was stained with rhodamine conjugated Phalloidin. Arrows, cardiomyocytes; arrowheads, non-cardiomyocytes. Scale bar = 10 μm. **D**, Western blot analysis of Csn8 in the heart and the indicated non-cardiac organs at 3 weeks (wks). For all organs, the left lane is from CTL and the right from CR-Csn8KO mice.

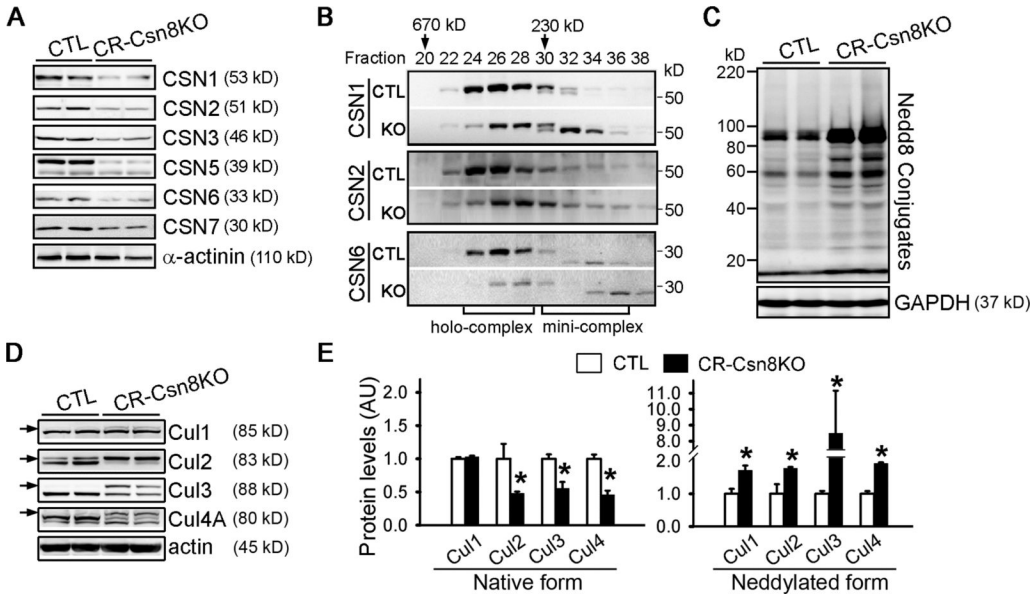

Figure 2.

Effects of Csn8 ablation on the protein abundance of other subunits, the complex distribution, the function of CSN in the heart. **A**, Western blot analysis of other CSN subunits in the heart at 2 weeks of age. **B**, Gel filtration followed by western blot analyses of CSN complex distribution. The results from probing Csn1, Csn2, and Csn6 consistently show significant reduction of CSN holocomplex in the CR-Csn8KO heart (KO). **C**, Western blot analysis of Nedd8 conjugates in total ventricular myocardium. **D** and **E**, Representative images (**D**) and a summary of densitometry data (**E**) of western blot analyses of the native and neddylated (marked by arrows) forms of cullin1 (Cul1), Cul2, Cul3, and Cul4A in ventricular myocardium at 2 weeks of age. Mean±SD, n=4; *:$p<0.05$ vs. CTL, Student's *t*-test.

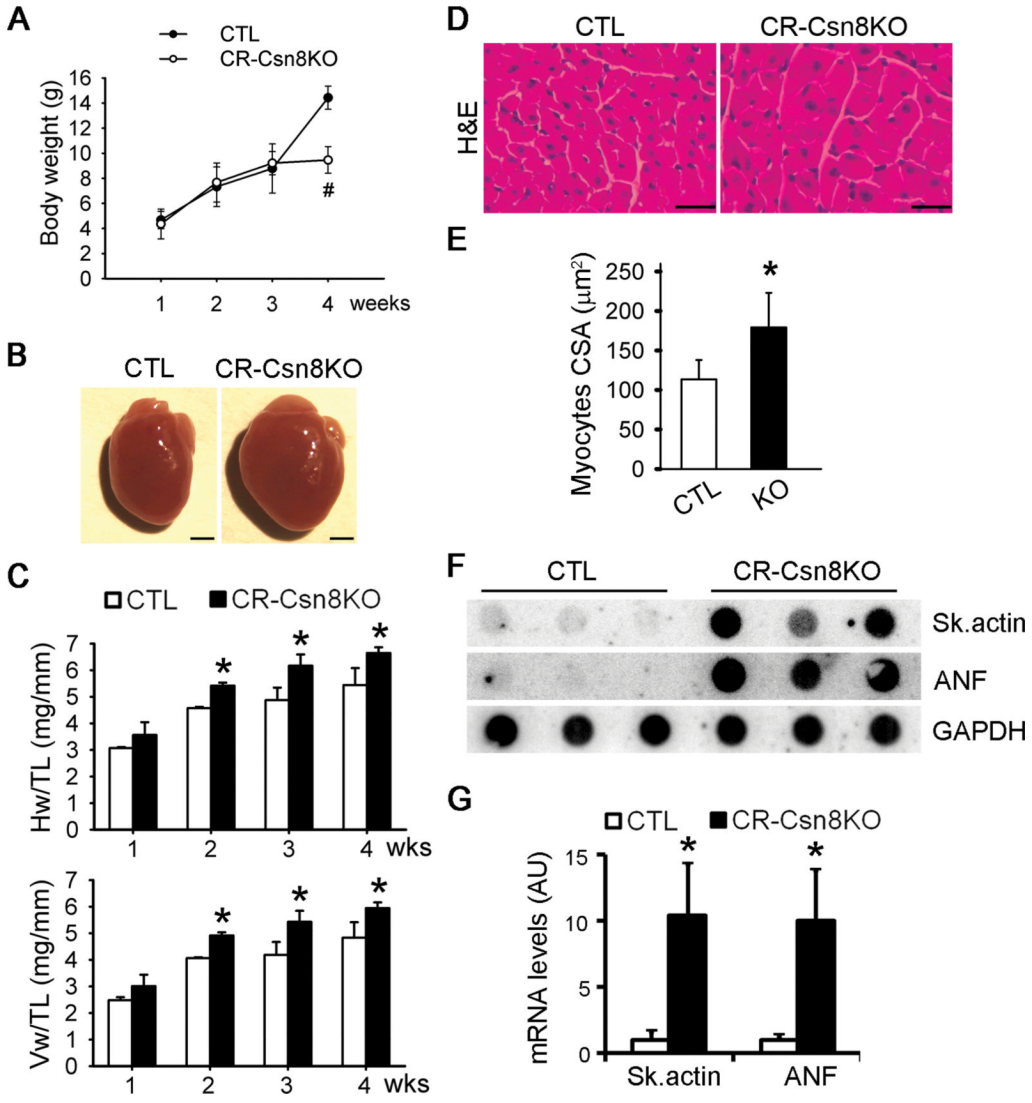

Figure 3.

CR-Csn8KO mice develop cardiac hypertrophy. **A**, The time course of changes in the body weight of CR-Csn8KO mice compared with their littermate CTLs. #: $p<0.01$ vs. CTL, Student's *t*-test. **B**, Representative images of whole hearts from 2-week old mice. Scale Bar=1mm. **C**, Comparison of the heart weight (Hw) to tibial length (TL) ratio and the ventricular weight to TL ratio between CTL and CR-Csn8KO mice at indicated ages. n = 6 ~ 8 for each group. *: $p<0.05$ vs. CTL; the same for other panels. **D** and **E**, Morphometric analysis of cardiomyocyte cross-sectional area (CSA) in 2-week-old mice. Representative images of H&E staining of myocardial sections (**D**) and a bar graph to summarize changes in CSA (**E**) are shown. Sections of 3 areas of each mouse heart and 3 mice per group were analyzed. Scale bar=20μm. **F** and **G**, RNA dot blot analyses of the steady state transcript levels of atrial natriuretic peptide (ANF) and skeletal α-actin (Sk. actin) in the ventricle of 2-week old mice. Total RNA (2 μg) isolated from ventricular myocardium was loaded on and cross-linked to nitrocellulose membrane and hybridized to p^{32}-labelled transcript-specific oligonucleotide probes. The bound probes were exposed to a phosphor-screen and imaged using a BioRad Personal Phosphoimager (**F**) and quantified using the Quantity-One software. GAPDH was probed as loading control. The relative changes are shown in panel (**G**).

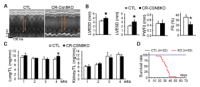

Figure 4.

CR-Csn8KO mice develop dilated cardiomyopathy and die prematurely. **A** and **B**, Representative images (**A**) and key parameters (**B**) of the M-mode echocardiography from 3-week-old mice. LVEDD, left ventricle (LV) end-diastolic dimension; LVESD, LV end-systolic dimension; PWTd, posterior wall thickness at the end of diastole; FS, fractional shortening. **C**, Changes in the Lung weight to TL ratio and the kidney weight to TL ratio during the first 4 weeks (wks) after birth. n = 6 ~ 8 for each group. *: $p<0.05$ vs. CTL; the same for the other panels. **D**, A Kaplan-Meier survival curve. $p<0.0001$, the log rank test.

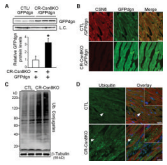

Figure 5.

CSN8 deficiency in cardiomyocytes impairs UPS proteolytic function. **A** and **B**, GFPdgn transgene was introduced into CR-Csn8KO and littermate control mice through cross-breeding. Ventricular myocardium was collected at 3 weeks of age for analyses reported here. **A**, Western blot analysis of myocardial GFPdgn. Representative images are shown in the upper panel and the densitometric data (n=6 for each group) are shown in the graph at the bottom. A non-specific band serves as a loading control (L.C.). *: $p < 0.01$ vs. CTL. Representative fluorescence confocal micrographs of ventricular myocardial sections from CTL/GFPdgn and CR-Csn8KO/GFPdgn mice were shown in panel **B**. Csn8 and GFPdgn were immunostained red and green, respectively. **C** and **D**, Littermate CR-Csn8KO and CTL mice at 3 weeks of age were used for western blot analysis (**C**) and confocal microscopy of immunofluorescence staining (green, **D**) for total ubiquitinated proteins (Ub.) in ventricular myocardium. In panel D, F-actin was stained using Rhodamine-conjugated Phalloidin (red) and the nuclei were stained blue with DAPI. Insets are the enlarged images of the arrowhead-pointed areas. Scale bar = 20 μm.

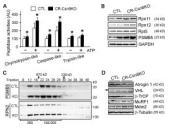

Figure 6.

Effects of Csn8 deficiency on proteasomal abundance and peptidase activities in the heart. **A**, Changes in proteasomal peptidase activities at 3 weeks. Specific synthetic fluorogenic peptide substrates were used to measure the activity of the indicated peptidases in the crude myocardial protein extracts from the CTL (n=4) and the CR-Csn8KO (n=6) mouse ventricles. *: $p < 0.05$ vs. CTL. **B**, Representative images of western blot analyses for the indicated proteasome subunits. **C**, Gel filtration and subsequent western blot analyses for the size distribution of representative proteasome subunits. **D**, Western blot analyses of the indicated representative E3 ligases. GAPDH and β-tubulin were probed for loading control.

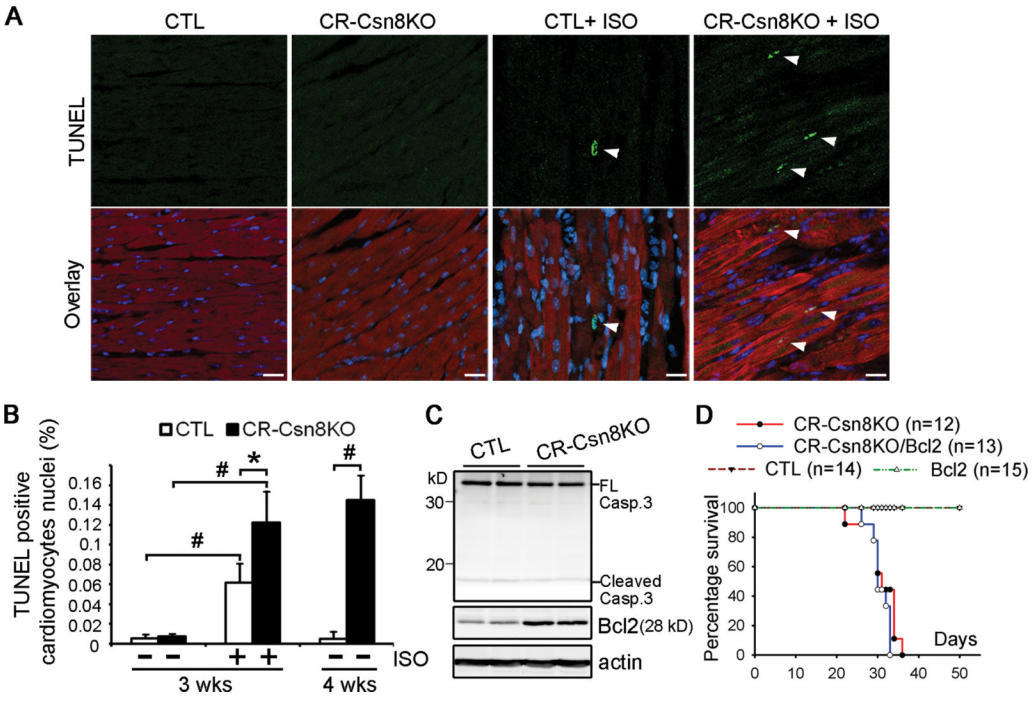

Figure 7.

Effects of Csn8 deficiency on cardiomyocyte apoptosis. **A**, Representative confocal micrographs of TUNEL-stained (green) myocardial sections from 3-week old mice. The sections were counterstained for F-actin (red) with rhodamine phalloidin and for the nucleus (blue) with TO-RPO 3. To challenge the hearts, mice were treated with isoproterenol (ISO, i.p., 15 mg/kg) 24 hours before being sacrificed. Scale bar=20 μm. **B**, Quantification of TUNEL-positive cardiomyocyte nuclei. N = 3 mice/group. *: $p<0.05$, #: $p<0.01$. **C**, Representative images of western blot analysis of full length (FL) and cleaved caspase (Casp.) 3 and Bcl2 in ventricular myocardium from 3-week-old mice. **D**, Kaplan-Meier survival analysis of the impact of cardiac overexpression of Bcl-2 on CR-Csn8KO mice.

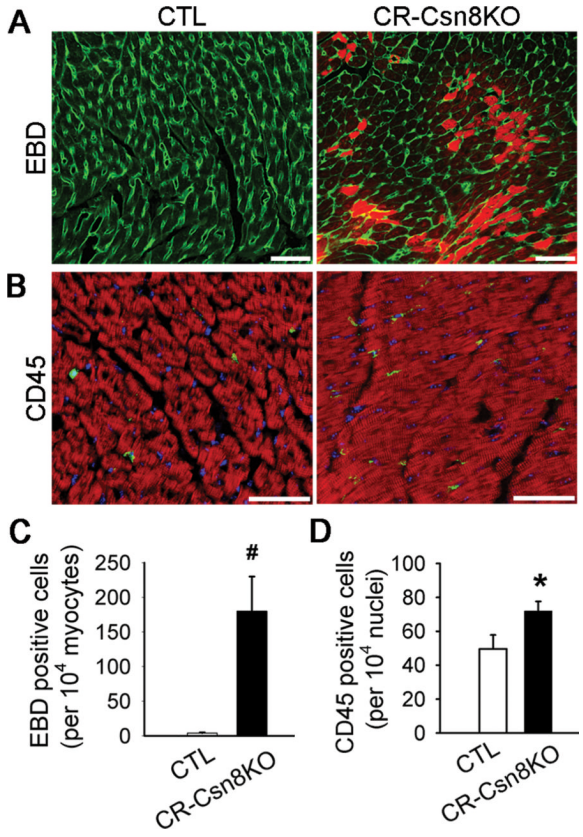

Figure 8.

CR-Csn8KO induces myocytes necrosis. **A** and **B**, Representative confocal micrographs of Evans blue dye (EBD) incorporation (red in **A**) and immunostained $CD45^+$ cells (green in **B**) in myocardium at 3 weeks. EBD was intraperitoneally injected 18 hours before the mice were sacrificed. The cell membrane was stained green in panel **A** with FITC-conjugated wheat germ agglutinin. Cardiomyocytes were stained red in panel **B** with rhodamine phalloidin. **C** and **D**, Quantification of EBD infiltrated cells (**C**) and $CD45^+$ cells (**D**). n = 3 mice/group. Bar=50 μm; *: $p<0.05$, #: $p < 0.01$ vs. CTL.