The COP9 Signalosome Is Required for Autophagy, Proteasome-Mediated Proteolysis, and Cardiomyocyte Survival in Adult Mice

Huabo Su, PhD; Jie Li, MD, PhD; Hanna Osinska, PhD; Faqian Li, MD, PhD; Jeffrey Robbins, PhD; Jinbao Liu, MD, PhD; Ning Wei, PhD; Xuejun Wang, MD, PhD

Background—The COP9 signalosome (CSN) is an evolutionarily conserved protein complex composed of 8 unique protein subunits (CSN1 through CSN8). We have recently discovered in perinatal mouse hearts that CSN regulates not only proteasome-mediated proteolysis but also macroautophagy. However, the physiological significance of CSN in a post-mitotic organ of adult vertebrates has not been determined. We sought to study the physiological role of CSN8/CSN in adult mouse hearts.

Methods and Results—Csn8 was conditionally ablated in the cardiomyocytes of adult mice (CSN8CKO) using a temporally controlled Cre-LoxP system. Loss of CSN8 accumulated the neddylated forms of cullins and noncullin proteins, increased ubiquitinated proteins, and stabilized a surrogate substrate of the proteasome in the heart. Autophagic flux was significantly decreased, whereas autophagosomes were markedly increased in CSN8CKO hearts, indicative of impaired autophagosome removal. Furthermore, we observed increased oxidized proteins, massive necrotic cardiomyocytes, and morphological and functional changes characteristic of dilated cardiomyopathy in CSN8CKO mice.

Conclusions—CSN deneddylates substrates more than cullins and is indispensable to cardiomyocyte survival in not only perinatal hearts but also adult hearts. CSN8/CSN regulates both proteasome-mediated proteolysis and the autophagic-lysosomal pathway, critical to the removal of oxidized proteins in the heart. (Circ Heart Fail. 2013;6:1049-1057.)

Key Words: autophagy ■ COP9 signalosome ■ heart ■ NEDD8 ■ proteasome endopeptidase complex

Protein quality control (PQC) is pivotal to proteostasis in the cell. Targeted removal of terminally misfolded/damaged proteins is the last line of defense in PQC and performed by the ubiquitin proteasome system (UPS) and macroautophagy (hereafter referred to as autophagy), 2 main intracellular proteolytic pathways.1 UPS-mediated proteolysis involves ubiquitination of a protein molecule and the subsequent proteasome-mediated degradation of the ubiquitinated protein. Autophagy uses specialized double-membrane structures to sequester a portion of the cytoplasm, proteins aggregates, or damaged organelles into autophagosomes for fusion with and degradation by lysosomes.2,3 UPS and autophagy dysfunctions are implicated in the pathogenesis of many cardiac disorders.1,11 However, molecular mechanisms by which the UPS and autophagy are regulated in the heart remain poorly understood.

Clinical Perspective on p 1057

The COP9 signalosome (CSN), identified first in Arabidopsis thaliana,12 is an evolutionarily conserved multiprotein complex consisting of 8 unique subunits (CSN1 through CSN8).13 The attachment of a ubiquitin-like protein NEDD8 to cullin via a process known as neddylation is critical for the activation of cullin-based RING (really interesting new gene) ligases (CRLs).14 The bona fide biochemical activity of CSN is to remove NEDD8 from cullin (ie, deneddylation);15 therefore, CSN is believed to play an important role in regulating CRLs. Studies from lower organisms and cultured mammalian cells have suggested that CSN participates in a variety of biological processes, including invertebrate development, DNA repair, cell cycle, kinase signaling, nuclear transport, and T cell proliferation.16 These observed roles are more or less tied to the deneddylation activity of CSN; but this does not preclude other unidentified functions that CSN might possess. Despite these observations, the physiological role of the CSN in mammals has just begun...
to be investigated. Germ-line deletion of the genes encoding CSN subunits in mice all resulted in embryonic lethality, at least partially because of defect in cell proliferation, underscoring its essential role in embryonic development.16,17 CSN8 is the smallest and least conserved subunit of CSN.17 By conditional gene targeting at the perinatal stage of murine development, we have recently found that CSN8 regulates both the UPS and the autophagic-lysosomal pathway in perinatal hearts and is essential for both postnatal cardiomyocyte survival and cardiac function.9,18 However, the biochemical function and physiological significance of CSN have never been tested in a postmitotic organ of vertebrate animals. Given that CSN is required for cell division and rodent cardiomyocytes do not stop proliferating until several days after birth,3,19 the phenotypes, including increased cell death which is often intimately linked to cell cycle perturbation, and resultant cardiac failure observed in mice with perinatal cardiomyocyte-restricted Csn8 knockout may be unique to the perinatal stage. In other words, the heart with cardiomyocytes undergoing active proliferation at the perinatal stage may respond to CSN8/CSN deficiency differently from an adult heart in which cardiomyocyte proliferation has ceased. Furthermore, neddylation and CSN are emerging therapeutic targets in adult malignancies.20,21 Understanding the impact of Csn8/CSN deficiency on adult hearts should help unveil the potential adverse impact of these new therapeutic strategies on adult hearts. Hence, this study has determined the impact of cardiomyocyte-restricted ablation of the Csn8 gene initiated in adult mice (CSN8 CKO) on cardiac function.9,18 Hence, this study determined the impact of cardiomyocyte-restricted ablation of the Csn8 gene initiated in adult mice (CSN8 CKO) on cardiac function and heart structure and function. The results demonstrate for the first time in a postmitotic organ of intact adult ventricular animals that CSN8 is required for the dneddylation of cullins and unknown noncullin proteins and regulates both the UPS and autophagy and, thereby, is essential for PQC and the functioning and survival of cardiomyocytes.

Methods

Animal Models and Experimental Protocols

Csn8−/− mice were, respectively, used for measuring chymotrypsin-like and caspase-like activities in crude protein extracts from ventricular myocardium.18

Immunofluorescence Confocal Microscopy

The fixation, embedding, immunostaining, and image acquisition were performed as previously described.18

Western Blot Analyses

Protein extraction from myocardium, protein concentration determination with bicinchoninic acid reagents (Pierce), SDS-PAGE, western blot analyses, and densitometry was performed as previously described.9

RNA Analyses

Total RNA was extracted from ventricular tissue using the Tri-Reagent (Molecular Research Center). Northern blot analysis for Csn8 mRNA and RNA dot blot analyses for GFPdgn and genes of the fetal gene program were performed as previously described.9,25

Proteasome Peptidase Activity Assays

The synthetic fluorogenic substrate Suc-LLVY-minomethycoumarin (25 μmol/L) and Z-LLE-β-naphthamide (25 μmol/L, BIOMOL) were, respectively, used for measuring chymotrypsin-like and caspase-like activities in crude protein extracts from ventricular myocardium.18

Transmission Electron Microscopy

Transmission electron microscopy (TEM) of perfusion-fixed myocardium was performed as previously described.25

Assessing Autophagic Flux in the Heart

Mice were intraperitoneally injected with 2 doses of bafilomycin-A1 (BFA; 3 μmol/kg, Sigma) or vehicle control with 1 hour in between. At 2 hours after the first dose, the mice were euthanized and the ventricular myocardium was sampled for western blot analyses of LC3-I & II levels. BFA induced increases of LC3-II reflect autophagic flux.8

Echocardiography

Mice were anesthetized by inhalation of isoflurane (2.5% for induction and 1.5% for maintenance) via a nose cone. The adequacy of anesthesia was monitored by toe pinch. Transthoracic echocardiography was recorded using the Vevo770™ echocardiography system and a 30-MHz transducer with a focal length of 12.7 mm (Visual Sonics). The LV morphometric and functional parameters were analyzed offline as previously described.18

Left Ventricle Hemodynamics

Mice anesthetized by inhalation of 2.5% isoflurane were intubated through the mouth and mechanically ventilated. The left ventricular (LV) catheterization and hemodynamic assessments were performed as previously described.9

Statistical Analysis

All quantitative data are presented as dot plots with mean±SD superimposed. Differences between 2 experimental groups were evaluated for statistical significance using the Wilcoxon rank-sum test. Differences among multiple groups were evaluated using the Kruskal–Wallis test followed by the Dunn test. The P value <0.05 were considered statistically significant.

Results

Targeted Ablation of the Csn8 Gene in Cardiomyocytes of Adult Mice

We targeted the Csn8 gene in adult mouse hearts using the MarCreMer-mediated inducible Cre-LoxP system (Figure S1A

Institutes of Health (NIH Publication No. 85-23, revised 1996).

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Kruskal–Wallis test followed by the Dunn test. The value <0.05 test. Differences among multiple groups were evaluated using the test. Differences among multiple groups were evaluated using the Kruskal–Wallis test followed by the Dunn test. The P value <0.05 were considered statistically significant.

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in the online-only Data Supplement). Deletion of the Csn8 gene in adult hearts was induced by administration of tamoxifen to 8- to 10-week-old mice. Tamoxifen can bind to MerCreMer and moves the MerCreMer from cytoplasm to the nucleus where the MerCreMer performs the recombination of the floxed alleles.22 The recombination was verified by PCR analyses using genomic DNA extracted from myocardium of the CTL and CSN8CKO mice at 3 days after the first tamoxifen injection. The recombined CSN8lox allele was detected only in the CSN8CKO hearts but not in the CTL hearts (Figure S1B in the online-only Data Supplement). At 3 days after the first tamoxifen injection, the CSN8 mRNA level in the ventricle of CSN8CKO hearts fell nearly below the limit of northern blot analysis (Figure S1C in the online-only Data Supplement), indicative of efficient excision of CSN8lox alleles. Approximately 70% of CSN8 proteins were lost in the CSN8CKO hearts by 5 days after the first tamoxifen injection (Figure 1A). Consistent with the findings from western blot analyses, CSN8 immunostaining was significantly diminished in both the nucleus and cytoplasm of cardiomyocytes in CSN8CKO hearts (Figure S1D in the online-only Data Supplement). Together, these data indicate that highly efficient deletion of the Csn8 gene was achieved in the cardiomyocytes of CSN8CKO adult mice within 5 days after the first tamoxifen injection.

Figure 1. Cardiomyocyte-restricted Csn8 knockout initiated in adult mice impairs deneddyllylation activity in the heart. At 5 days after the first tamoxifen injection, ventricular myocardium was sampled from Csn8flox/flox (control [CTL]) or Csn8flox/::MerCreMer littermate mice for western blot analyses. Representative images are shown with each lane representing a mouse. α-Tubulin or GAPDH was probed for loading control. A, Changes in CSN8, CSN1, and CSN5. B, Increases in the deneddyllylated form (arrow) and decreases in the native form (*) of cullin1 (Cul1), Cul2, Cul3, and Cul4A in CSN8CKO hearts. To verify the identity of the deneddyllylated cullins, cultured neonatal rat cardiomyocytes were treated with a specific NEDD8 E1 inhibitor MLN4924 (MLN, 1 μmol/L; Active Biochem, Maplewood, NJ) or vehicle (−) for 24 hours. MLN4924, which inhibits neddylation, abolished the slow-migrating band of all cullins confirming their identity as deneddyllylated cullins. C, Increased Nedd8 conjugates in CSN8CKO hearts. AU indicates arbitrary unit; CSN, COP9 signalosome; and GADPH, glyceraldehyde 3-phosphate dehydrogenase.

Loss of CSN Deneddyllylation Activity in CSN8CKO Hearts

The bona fide cullin deneddyllylation activity of CSN resides in CSN5.13 Neither CSN5 nor CSN1 protein abundance were altered in CSN8CKO hearts (Figure 1A), but the deneddyllylated form of cullin (Cul) proteins, including Cul1, Cul2, Cul3, and Cul4A was markedly increased in the CSN8CKO hearts (Figure 1B). A number of noncullin proteins can also be deneddyllylated.27 Interestingly, increases in the deneddyllylated form of unknown proteins were also detected in CSN8CKO hearts (Figure 1C). These data demonstrate that deletion of the Csn8 gene suffices to compromise CSN deneddyllylation activity in adult cardiomyocytes.

Impairment of UPS Proteolytic Function in CSN8CKO Hearts

We recently showed that perinatal loss of CSN8 in cardiomyocytes caused cardiac PFI but this has not been tested in adult hearts.18 To monitor UPS proteolytic function in vivo, a reporter mouse model was previously generated and validated.21 In this tg mouse, a UPS surrogate substrate (GFPdgn) was ubiquitously and constitutively expressed and GFPdgn protein levels inversely reflect UPS proteolytic function. Tg GFPdgn was introduced into CSN8CKO and littermate CTL mice through cross-breeding. GFPdgn protein levels in CSN8CKO::GFPdgn mouse hearts were significantly increased at 3 days after the first tamoxifen injection, compared with the littermate CTL::GFPdgn mice (Figure 2A). Consistently, fluorescent confocal microscopy showed that GFPdgn fluorescence in cardiomyocytes was brighter in the CSN8CKO::GFPdgn mice than in CTL::GFPdgn mice (Figure 2B). RNA dot blot analysis revealed that the GFPdgn transcripts were not significantly different between the CTL::GFPdgn mice and the CSN8CKO::GFPdgn mice (Figure 2C), indicating that the increased GFPdgn protein levels likely result from decreased UPS-mediated degradation. These results reveal that cardiac UPS proteolytic function is severely impaired by CSN8CKO. This impairment does not appear to be intrinsic to the proteasome because the proteasomal chymotrypsin-like and caspase-like activities both in presence or absence of ATP were significantly increased in the CSN8CKO mice compared with the CTL (Figure 2D).

Accumulation of Autophagosomes in CSN8CKO Hearts

Targeted protein degradation in the cell is performed mainly by the UPS and the autophagic-lysosomal pathway. Proteasome functional insufficiency (PFI) can activate autophagy in cardiomyocytes;23,28 but chronic inhibition of autophagy has been shown to hinder the degradation of UPS substrates.29 Because we have observed severe impairment in UPS-mediated protein degradation in CSN8CKO hearts, we sought to investigate whether autophagy was altered. The conversion of LC3 from the cytosolic form (LC3-I) to the lipidated form (LC3-II) targets LC3-II to autophagosome membrane during autophagosome formation. Therefore, LC3-II protein levels are extensively used as a marker for autophagic vesicles. We found LC3-II protein levels were markedly increased in the
CSN8<sup>CKO</sup> hearts (Figure 3A and 3B), suggesting that Csn8 deficiency increases autophagosomes in adult mouse hearts. This was confirmed using the GFP-LC3 reporter and TEM. We cross-bred the GFP-LC3 tg mice with the CSN8CKO mice. Direct fluorescent confocal microscopy revealed more GFP puncta in LV myocardium from the CSN8CKO mice compared with the CTL mice (Figure 3C). Quantification showed that GFP-LC3 puncta increased by a factor of >8 in CSN8 CKO compared with the littermate GFP-LC3 control mice (Figure 3D). Morphological detection of autophagic vacuoles via TEM remains the gold standard to visualize autophagosomes in the cell. Our TEM analyses further confirmed the markedly increase in autophagic vacuoles, especially the early stage ones (ie, autophagosomes) in the cardiomyocytes of CSN8CKO hearts (Figure 3E and 3F). These autophagosomes often contain organelles, such as degenerative mitochondria.

**Diminished Autophagy Flux in CSN8<sup>CKO</sup> Mouse Hearts**

The increase in autophagosomes could result from elevated formation or defective removal. Interestingly, the protein levels of p62, an autophagy substrate, were also significantly increased in CSN8<sup>CKO</sup> hearts. This finding prompted us to test whether autophagic flux is compromised. We used intraperitoneal injection of BFA to inhibit lysosomes. As expected, BFA treatment substantially accumulated LC3-II in the CTL mouse hearts. However, the BFA treatment failed to induce a significant increase in LC3-II in the CSN8<sup>CKO</sup> hearts (Figure 4). These data indicate that the increase of autophagosomes in CSN8<sup>CKO</sup> hearts is because of decreased lysosomal removal.

**Accumulation of Abnormal Proteins in CSN8<sup>CKO</sup> Hearts**

Terminally damaged/misfolded proteins are degraded by the UPS and autophagy. Consistent with PFI and the impaired autophagic-lysosomal pathway, the total ubiquitinated proteins (Figure 5A) in the form of aggregates (Figure 5B) and the level of protein carbonyls, a common feature of oxidized proteins (Figure 5C), were greatly increased in CSN8<sup>CKO</sup> hearts. These ubiquitin-positive protein aggregates are frequently colocalized with GFP-LC3 puncta and enriched in p62 in CSN8<sup>CKO</sup> hearts (Figure S2 in the online-only Data Supplement).

**Massive Cardiomyocyte Necrosis in CSN8<sup>CKO</sup> Hearts**

A hallmark of necrotic cells is the loss of cell membrane integrity. To assess the prevalence of cardiomyocyte necrosis, we first tested the uptake of intraperitoneally injected Evan’s blue dye by the cardiomyocytes in intact mice. Nearly none of the cardiomyocytes of CTL mice showed Evan’s blue dye uptake but over 10% of cardiomyocytes in the CSN8<sup>CKO</sup> mice did (Figure 5D and 5E), suggesting that Csn8 deficiency causes cardiomyocytes to die in the necrotic form in adult mice. Consistently, TEM also revealed evidence of necrotic cardiomyocytes in the CSN8<sup>CKO</sup> mice, including markedly cell swollen and mitochondrial swollen, decreased overall electron density, and myofibril fragmentation (Figure S3 in the online-only Data Supplement).

**CSN8<sup>CKO</sup> Mice Developed Dilated Cardiomyopathy**

Five days after the first tamoxifen injection, the heart of CSN8<sup>CKO</sup> mice seemed to be enlarged, compared with the CTL (Figure S4A in the online-only Data Supplement).
Gravimetric analyses showed that CSN8 CKO mice had modest but statistically significant increases in the heart weight to body weight ratio, the ventricle weight to body weight ratio, and the lung weight to body weight ratio (Figure 6A) but not the kidney weight to body weight ratio (data not shown), compared with the CTL mice. The cross-sectional area of LV cardiomyocytes increased by 30% compared with the CTL mice (Figure S4B and S4C in the online-only Data Supplement). A hallmark of cardiac hypertrophy/pathology is reactivation of the fetal gene program. The steady-state transcript levels of atrial natriuretic factor and skeletal α-actin in CSN8 CKO hearts were upregulated by a factor of 8 and 9, respectively, whereas the SERCA2a mRNA levels were downregulated by a factor of 4 (Figure 6B). These results show that Csn8 ablation in adult cardiomyocytes triggers hypertrophic responses.

Morphometric and cardiac functional assessments further revealed that Csn8 deficiency initiated in adult hearts caused dilated cardiomyopathy (DCM). Echocardiography at 5 days after the first tamoxifen injection revealed significant increases in the LV end-diastolic dimension and end-systolic dimension and significant decreases in the posterior wall thickness at the end of diastole and LV fractional shortening in CSN8 CKO mice (Figure 7A; Figure S5 in the online-only Data Supplement). Hemodynamic analysis via monitoring LV pressure (Figure 7B) revealed a marked reduction of LV systolic pressure and the maximum dP/dt in CSN8 CKO mice, indicative of reduced cardiac contractility. The accompanying marked increase of LV end-diastolic pressure and a significant reduction of the minimum dP/dt in CSN8 CKO hearts indicate that LV relaxation is also impaired. Taken together, these findings demonstrate that loss of CSN8 in the cardiomyocytes of adult mice causes rapidly severe congestive heart failure.

Discussion

The physiological significance of CSN has not been demonstrated in a postmitotic organ of intact adult vertebral animals. Here, we have shown that CSN8 deficiency initiated in adult mouse hearts caused severe duo-impairment of both the UPS and autophagy, resulted in cardiomyocyte necrosis, and led to rapid left heart failure. Along with the similar findings from perinatal mice, it is now comprehensively demonstrated

Figure 3. Marked increases of autophagic vacuoles in CSN8 CKO hearts. A and B, Representative images of western blot analyses of LC3 and p62 (A) and a summary of LC3-II densitometry data (B) in CTL and CSN8 CKO hearts at 5 days after the first tamoxifen injection. C and D, Probing autophagy in CSN8 CKO hearts using GFP-LC3. GFP-LC3 was introduced into the CTL and CSN8 CKO mice via cross-breeding. Perfusion-fixed ventricular myocardium from CTL::GFP-LC3 mice and CSN8KO::GFP-LC3 littermate mice at 5 days after the first injection of tamoxifen was subjected to GFP-LC3 direct fluorescence confocal microscopy. Images from a 2.1-μm thick slide of tissue were projected (C, representative images) and analyzed for the GFP-LC3 puncta density (D). The inset in C shows the area indicated by the arrow in a higher magnification. Bar=10 μm. E and F, Electron micrographs of myocardium from CTL (E) and CSN8 CKO (F) hearts. Abundant autophagosomes (Av) containing degrading mitochondria and other cytoplasmic contents are evident in CSN8 CKO hearts. CSN indicates COP9 signalosome; CTL, control; and GADPH, glycer-aldehyde 3-phosphate dehydrogenase.

Figure 4. Impaired autophagic flux in CSN8 CKO hearts. Three days after the first tamoxifen injection, CTL and CSN8 CKO littermate mice were treated with 2 doses of BFA (3 μmol/kg, IP) or vehicles with 1 hour in between and euthanized 2 hour after the first dose. LC3-II protein levels in ventricular myocardium were quantified using western blot analysis. Representative western blot images (A) and densitometric quantification (B) of LC3 proteins are presented. AU indicates arbitrary unit; BFA, bafilomycin A1; CSN, COP9 signalosome; and CTL, control.
that CSN8/CSN regulates protein degradation by not only the UPS but also lysosomes in the heart during both the early development and adulthood. Moreover, we show here for the first time in intact animals that CSN8/CSN may modulate the redox state in the heart.

CSN8 Is Required for CSN-Mediated Deneddylation of a Variety of Substrates

Using the same Csn8-floxed allele but temporally less controllable conditional gene targeting, we and others have previously showed that CSN-mediated cullin deneddylation in mouse hepatocytes, neonatal cardiomyocytes, and T cells was compromised by CSN8 deficiency.\(^1^7,18,32\) In those previous studies, the accompanied downregulation of other CSN subunits, such as CSN5 which harbors the deneddylase, could not be dissociated from the impaired deneddylation. Because the strategy used for the inducible Csn8 targeting in this study allows the timing of gene ablation to be controlled and pinpointed, we were able to observe severe impairment of CSN deneddylation in CSN8-ablated hearts before reduction of other key CSN subunits becomes evident, providing compelling evidence that CSN8 is required for CSN5-mediated deneddylation. Cullins are the first and best studied proteins that undergo neddylation but the list of the latter is growing.\(^27,33\) It is unknown whether CSN plays a role in the deneddylation of proteins other than cullins. Besides neddylated cullins, a large number of neddylated proteins of unknown identities were also accumulated in CSN8CKO hearts. This is in agreement with our recent findings in postnatal CSN8-deficient heart.\(^18\) Taken together, it is now strongly demonstrated that CSN-mediated deneddylation in the heart has an array of substrates besides neddylated cullins. It will be interesting to identify these noncullin substrates using the CSN8-deficient hearts.
serving as an alternate lid of the 19S proteasome, or stabilizing the ubiquitin conjugation enzyme. Our previous study showed impaired UPS function in postnatal CSN8-deficient hearts, which may be caused by uncoupling of ubiquitination from proteasome-mediated degradation. Consistent with this notion, we observed that loss of CSN8 in adult hearts rapidly accumulated the proteasome surrogate substrate GFPdgn but proteasomal peptidase activities were increased. Taken together, it is revealed that CSN8 is crucial to the functioning of the UPS in both neonatal and adult cardiomyocytes. Because DCM occurred immediately after the induction of CSN8 CKO and cardiomyopathy can result in cardiac PFI, it cannot be ruled out that the concomitant DCM may contribute to the UPS malfunction.

Another exciting contribution of this study is to have confirmed an important role of CSN8/CSN in the autophagic-lysosomal pathway that was initially revealed by our studies on the perinatal Csn8 knockout. Here, we found the coexistence of increased autophagosomes with decreased autophagic flux in the adult mouse hearts with Csn8 ablated in cardiomyocytes confirming that CSN8/CSN is required for autophagosome removal. Impaired autophagosome removal can result from defective fusion between autophagosomes and lysosomes or from lysosome malfunction.

UPS-mediated protein degradation is a highly regulated process. Polyubiquitination of target proteins, the delivery of polyubiquitinated proteins to the proteasome, and proteasome activities can all impact on UPS functioning. Given that the proteasome peptidase activities were increased in Csn8-deficient myocardium, the impairment of UPS proteolytic function as evidenced by GFPdgn accumulation is likely caused by defective ubiquitination and impaired delivery of ubiquitinated proteins to the proteasome. The latter is consistent with the increases of total ubiquitinated proteins in Csn8 CKO hearts. As discussed below, macroautophagy is impaired and p62 increased in Csn8 CKO hearts, which may impair delivery of ubiquitinated proteins to the proteasome. Chronic inhibition of macroautophagy was shown to accumulate p62, which in turn binds ubiquitinated proteins and hinders their degradation by the proteasome.

Selective autophagy contributes to quality control in the cell by targeted removal of defective organelles and protein aggregates. p62 is involved in the recruitment of autophagosome to misfolded proteins. Consistent with PFI and inadequate PQC, both ubiquitinated proteins and oxidized proteins were markedly increased in CSN8 CKO hearts. The accumulation of ubiquitinated proteins in the form of aggregates and the colocalization of p62 and GFP-LC3 with the aggregates suggest that defective removal of autophagosomes at least contributes to, if is not the primary cause of, PQC inadequacy in CSN8 CKO hearts.

The increased level of protein carbonyls in CSN8 CKO hearts suggests that CSN8/CSN may regulate the redox state via supporting the removal of oxidized proteins and suppressing oxidative modifications. Indeed, a recent study on the transcriptome, proteome, and metabolome of the filamentous fungus, Aspergillus nidulans, with CSN5 gene deletion has revealed a critical role of CSN in mediating transcriptional and metabolic responses to oxidative stress. Hence, this study presents the first evidence that CSN8/CSN may regulate the redox state in vertebral animals.

**CSN8 Deficiency Causes Cardiomyocyte Necrosis and DCM**

We have previously reported that CSN8 deficiency causes primarily apoptosis in hepatocytes but predominantly necrosis in neonatal cardiomyocytes. Here, we observed massive cardiomyocyte necrosis in adult mice with CSN8 CKO. These results suggest that CSN8/CSN is essential for cardiomyocyte survival but the type of cell death caused by CSN8 deficiency is tissue/organ-specific. Similarly to Csn8 knockout initiated at the perinatal stage, cardiomyocyte-restricted Csn8 ablation initiated in adult mice causes cardiac structural and functional changes that are characteristic of DCM. Interestingly, it takes a shorter time for cardiac Csn8 ablation to cause DCM in adults than in neonates. This is strikingly reminiscent of the cardiac ablation of Arg5, a protein required for autophagy, implicating that impairment of autophagy might be primarily responsible for the DCM in CSN8-deficient hearts. Massive cardiomyocyte necrosis is associated with, and likely an underlying cause of, the DCM in both perinatal and adult CSN8-deficient hearts. Indeed, inhibition of autophagosome removal is sufficient to cause...
cardiomyocyte necrosis in adult mice. Moreover, accumulation of autophagic vacuoles was also observed in necrotic cardiomyocytes in diseased human and mouse hearts, raising an interesting possibility that impaired autophagosome removal may be a cause of cardiomyocyte necrosis in a diseased heart. It will be important to test this possibility and to investigate how impaired autophagosome removal causes necrotic cell death.

Disclosures

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References

Targeted removal of terminally misfolded/damaged proteins by the ubiquitin proteasome system (UPS) and the autophagic-lysosomal pathways represents the last line of defense in protein quality control. UPS and autophagy dysfunctions are implicated in cardiac pathogenesis. However, the regulation of the UPS and autophagy is poorly understood. The attachment of a ubiquitin-like protein NEDD8 to cullin via a process known as neddylation activates cullin-based RING ligases, a large family of ubiquitin ligases. The proper functioning of cullin-based RING ligases also requires cullin deneddylation catalyzed by the COP9 signalosome (CSN), a protein complex formed by 8 unique proteins (CSN1 through CSN8). Hence, CSN may regulate cardiac UPS. By conditional gene targeting, we previously discovered that CSN8/CSN regulates both cardiac UPS and autophagy and is essential to cardiomyocyte survival at the perinatal stage when cardiomyocyte proliferation contributes actively to cardiac development. Here, we report that CSN8/CSN also plays indispensable roles in adult hearts. Similarly to perinatal Csn8 knockout, cardiac ablation of the Csn8 gene initiated in adult mice impairs both UPS proteolytic function and autophagosome removal leading to massive cardiomyocyte necrosis and progressive dilated cardiomyopathy and heart failure. This has immediate clinical relevance because neddylation inhibition via NEDD8-activating enzyme inhibitors (eg, MLN4924) is in clinical trial to treat cancer and CSN inhibition is also being tested in the laboratory as a new therapeutic strategy. Similar to CSN8 deficiency, both NEDD8-activating enzyme and CSN inhibitors effect to inhibit cullin-based RING ligases. Our findings caution that the use of these inhibitors may damage the heart.
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The COP9 signalosome is required for autophagy, proteasome-mediated proteolysis, and cardiomyocyte survival in adult mice

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Supplemental Materials

I. Supplemental Methods

II. Supplemental Figures (Figures S1 through S5)
I. Supplemental Methods

Immunostaining and fluorescence confocal microscopy

Ventricular myocardium fixed with 4% of paraformaldehyde was equilibrated with 40% sucrose at 4°C overnight, embedded in O.C.T. (Sakura Finetek U.S.A), and used for preparing cryo-sections. The sections were permeabilized with 1% of Triton X-100 in PBS for 30 minutes, quenched with 0.1M glycine in PBS for 1 hour, and blocked with 0.5% BSA for 1 hour. The sections were then incubated with primary antibodies at 4°C overnight. The unbound antibodies were washed away via rinsing with PBS (pH 7.4) 3 times with 10 minutes per time. Subsequently, the sections were incubated with their respective secondary antibodies at room temperature for 1 hour. The unbound secondary antibodies were rinsed away from the sections by PBS rinsing (3x10min). Antibodies used for immunostaining include: rabbit anti-CSN8 (BIOMOL), mouse anti-α-actinin (Sigma), rabbit anti-GFP (Santa Cruz), guinea pig anti-p62 (ARP), rabbit anti-ubiquitin (Sigma), the Alexa Flour 488 donkey anti-rabbit IgG, the Alexa Flour 568 donkey anti-mouse IgG, Alexa Fluor 568 goat anti-guinea pig, and the Alexa Fluor 647 donkey anti-rabbit IgG (Molecular Probes). FITC-conjugated wheat germ agglutinin (WGA) and Alexa Fluor 568 phalloidin were used for staining cell membrane and F-actin respectively. Immunostaining was visualized using a fluorescence confocal microscope (Olympus Fluoview 500) and the images were captured and digitalized using the associated software.

Western blot analyses

Frozen ventricular myocardial tissues were homogenized in 1X SDS sampling buffer (50mM Tris-Cl at pH 6.8, 2% SDS and 10% glycerol). The crude extracts were further sonicated on ice and boiled for 5 minutes. The supernatant was obtained following a 14,000g centrifugation for 5 minutes at 4°C. The protein concentration was measured using the Bicinchoninic Acid (BCA) method. Equal amount of samples were resolved by SDS-PAGE, transferred to PVDF membrane, probed with appropriate primary and secondary antibodies, and followed by detection with enhanced chemiluminescence (ECL-Plus) reagents (GE Healthcare, Piscataway, NJ) and a VersaDoc3000 imaging system (BioRad, Hercules, CA). The signal was quantified with the Quantity One software (BioRad, Hercules, CA). Antibodies used and their sources include: CSN8 (BIOMOL), CSN1 and CSN5 (Novus Biologicals), cullin 1 and cullin 4A (custom made), cullin 3 (Abcam), NEDD8 (Epitomics), GFP (Santa Cruz), microtubule associated protein 1 light chain 3 (LC3, MBL), p62 (ARP), ubiquitin (Sigma), and DNP (Invitrogen).
RNA analyses

Total RNA was extracted from ventricular tissue using the Tri-Reagent (Molecular Research Center). P$^{32}$-labeled GFPdgn cDNA and mouse CSN8 cDNA probes generated via a nick-translation kit (Roche) and P$^{32}$-labeled synthetic oligonucleotides specific for the transcript of atrial natriuretic factor (ANF), skeletal actin, sarcoplasmic reticulum calcium ATPase 2a (SERCA2a), GAPDH, or 18S rRNA were used as probes for RNA dot blot analyses. The radioactive signals bound to the RNA on the nitrocellulose membrane were exposed to a phosphor screen, detected with a phosphoimager, and qualified with Quantity-One software (Bio-Rad) as previously described.$^1$

Transmission electron microscopy (TEM)

Mice were anesthetized with inhalant isoflurane (2.5% for induction and 1.5% for maintenance). The adequacy of anesthesia was monitored by toe pinch. The hearts were fixed by perfusion with 3.5% glutaraldehyde in cardioplegic buffer for 2 minutes, followed by 3.5% glutaraldehyde in 100 mM cacodylate buffer (pH 7.3) for 2 minutes. The fixatives were gravity fed (600 mm) into the hearts through the apex and right ventricle. Immediately after the hearts started to fill with the fixative, the right atrium was cut open to allow the output of liquids. At least 3 tissue samples from each area of the ventricles were chosen for extensive ultrastructural analysis. Two mice for each genotype were examined. Ultrathin sections were counterstained with uranyl acetate and lead citrate. The sections were viewed in a Zeiss Omega 912 electron microscope at 100 kV.

Left ventricle (LV) hemodynamics

Mice anesthetized by inhalation of 2.5% isoflurane were intubated through the mouth and mechanically ventilated. Mice were kept in light anesthesia with inhalation of isoflurane in room air supplemented with 100% oxygen. The right common carotid artery was isolated in the anterior triangle of the neck under a dissecting microscope. A 1.4F Millar Mikro-Tip catheter transducer (model SPR-835, Millar Instruments) was inserted into the right common carotid artery and advanced to the LV chamber. After stabilizing for 30 min, LV pressure (LVP) and its first derivatives (dP/dt) were recorded using a Powerlab data acquisition system (AD Instruments). Systolic and diastolic LVP and LV end-diastolic
pressure (LVEDP) were measured directly from the waveforms. All the other parameters were derived from the PowerLab software as described.²

References of Supplemental Methods
II. Supplemental Figures

Supplemental Figure 1

**Supplemental figure 1. Cardiomyocyte-restricted Csn8 gene ablation in adult mice.** (A) Schematic representation of the tamoxifen (Tam)-induced excision of exons 4, 5 and 6 of the csn8 gene. The primers (arrows) for detection of the excision by PCR and the loxP sites (red arrowheads) are indicated. (B) PCR analysis of myocardial genomic DNA showing a 270bp band characteristic of recombination in CSN8<sup>CKO</sup> hearts after Tam injections. Sense primer I3F (5’-AACAGCTCAGCTGATAAGAGTGG - 3’) and antisense primer I6R (5’-GTAGGTGACCTTCAATGTCAC-3’) were used for the PCR analyses. (C) Northern blot analysis of csn8
transcripts in the hearts 3 days after the first Tam injection. The 18S rRNA was probed as loading control. (D) Confocal micrographs of immunofluorescence labeled CSN8 (green) and α-actinin (red) in myocardium. The nuclear-enriched CSN8 staining in the cardiomyocytes of CTL hearts (arrows) was lost in the CSN8CKO heart. Scale bar = 50 μm. 

Supplemental Figure 2

Supplemental Figure S2. Colocalization of GFP-LC3 with p62 and ubiquitin in CSN8CKO hearts. GFP-LC3 direct fluorescence (green) and immunofluorescence detection of p62 (red) and ubiquitin (blue)-positive inclusions in CTL and CSN8CKO myocardium sections. Higher magnification views are shown in insets. Scale Bar=10 μm.
Supplemental Figure S3. Electron micrographs of myocardium from CTL and CSN8\textsuperscript{CKO} littermate mice at 5 days after the first Tam injection. TEM analysis of LV myocardium from CTL (A) and CSN8\textsuperscript{CKO} (B-G) hearts revealed marked myofibril and mitochondrial degeneration as evidenced
by wavy Z-lines (arrows in B), focal myofibrillar lysis (*) in C, and swollen as well as lumpy/collapsed mitochondria with disorganized cristae (arrowheads in D) in the cardiomyocytes of CSN8\textsuperscript{CKO} hearts. Necrotic cardiomyocytes with a significantly decreased electron density and overall structural disruption and lysis were also observed in CSN8\textsuperscript{CKO} hearts (E to G). NC: necrotic cardiomyocytes. Scale bar = 1 \mu m.

Supplemental Figure S4

(A) Representative gross morphology of CTL and CSN8\textsuperscript{CKO} hearts at 5 days after the 1\textsuperscript{st} tamoxifen injection. Scale bar = 1 mm. (B) Representative images of the cross-sectional area of cardiomyocytes. LV sections were stained with FITC-conjugated wheat germ agglutinin (green) to outline the boundaries of the cardiomyocytes. Scale bar = 10 \mu m. (C) Quantification of the cross-sectional area of cardiomyocytes (n = 250 cardiomyocytes evenly from 5 hearts for each genotypes). *: p < 0.05 vs. CTL.
Supplemental Figure S5. Representative M-mode echocardiographs recorded at 5 days after the first tamoxifen injection.