Protein quality control (PQC) functions to minimize the level and toxicity of misfolded proteins in the cell, pivotal to intracellular proteostasis and cell survival.\(^1\)\(^,\)\(^2\) PQC is accomplished by intricate collaboration between molecular chaperones and targeted proteolysis. The latter is done primarily by the ubiquitin (Ub)-proteasome system (UPS) and, sometimes, the autophagic-lysosomal pathway (ALP). PQC inadequacy allows misfolded proteins to undergo aberrant aggregation which can further impair PQC via mechanisms, including suppressing UPS function;\(^3\)\(^,\)\(^4\)\(^,\)\(^5\) hence aberrant protein aggregation is both a consequence and a further cause of PQC inadequacy. Striking aberrant protein aggregation in cardiomyocytes, as evidenced by the presence of intracellular preamyloid oligomers and congophilic fibrils,\(^6\)\(^,\)\(^7\) occurs in a large subset of human heart failure (HF) resulting from idiopathic cardiomyopathies. This links PQC inadequacy to the pathogenesis of common forms of heart diseases. PQC suppression via either ablating a chaperone gene or inhibiting targeted proteolysis is sufficient to cause cardiomyopathy and HF or to facilitate maladaptive cardiac remodeling;\(^8\)\(^,\)\(^9\)\(^,\)\(^10\)\(^,\)\(^11\) conversely, PQC improvement via chaperone overexpression or enhancement of target proteolysis confers cardiac protection against proteotoxicity in experimental animals.\(^12\)\(^,\)\(^13\)\(^,\)\(^14\)\(^,\)\(^15\) These experimental demonstrations are corroborated by clinical observations that a significant portion of cancer patients receiving proteasome inhibitors demonstrate cardiotoxic effects.\(^16\)\(^,\)\(^17\)\(^,\)\(^18\)\(^,\)\(^19\) We sought to investigate the role of CSN8/CSN in misfolded protein degradation and cardiac proteinopathy.

**Rationale:** Impaired degradation of misfolded proteins is associated with a large subset of heart diseases. Misfolded proteins are degraded primarily by the ubiquitin-proteasome system, but the ubiquitin ligases responsible for the degradation remain largely unidentified. The cullin deneddylation activity of the COP9 signalosome (CSN) requires all 8 CSN subunits (CSN1 through CSN8) and regulates cullin-RING ligases, thereby controlling ubiquitination of a large number of proteins; however, neither CSN nor cullin-RING ligases is known to regulate the degradation of cytosolic misfolded proteins.

**Objective:** We sought to investigate the role of CSN8/CSN in misfolded protein degradation and cardiac proteinopathy.

**Methods and Results:** Cardiac CSN8 knockout causes mouse premature death; hence, CSN8 hypomorphism (CSN8\(^{\text{hypo}}\)) mice were used. Myocardial neddylated forms of cullins were markedly increased, and myocardial capacity of degrading a surrogate misfolded protein was significantly reduced by CSN8 hypomorphism. When introduced into proteinopathic mice in which a bona fide misfolded protein R120G missense mutation of αβ-crystallin (CryAB\(^{R120G}\)) is overexpressed in the heart, CSN8 hypomorphism aggravated CryAB\(^{R120G}\)-induced restrictive cardiomyopathy and shortened the lifespan of CryAB\(^{R120G}\) mice, which was associated with augmented accumulation of protein aggregates, increased neddylated proteins, and reduced levels of total ubiquitinated proteins and LC3-II in the heart. In cultured cardiomyocytes, both CSN8 knockdown and cullin-RING ligase inactivation suppressed the ubiquitination and degradation of CryAB\(^{R120G}\) but not native CryAB, resulting in accumulation of protein aggregates and exacerbation of CryAB\(^{R120G}\) cytotoxicity.

**Conclusions:** (1) CSN8/CSN promotes the ubiquitination and degradation of misfolded proteins and protects against cardiac proteotoxicity, and (2) cullin-RING ligases participate in degradation of cytosolic misfolded proteins. (Circ Res. 2015;117:956-966. DOI: 10.1161/CIRCRESAHA.115.306783.)

**Key Words:** autophagy ■ COP9 signalosome ■ Cops8 ■ desmin-related cardiomyopathy ■ proteasome ■ ubiquitin

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Editorial, see p 914
Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ALP</td>
<td>the autophagic-lysosomal pathway</td>
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<tr>
<td>CRLs</td>
<td>cullin-RING ligases</td>
</tr>
<tr>
<td>CryAB</td>
<td>αB-crystallin</td>
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<tr>
<td>CSN</td>
<td>the COP9 signalosome</td>
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<tr>
<td>CSN8&lt;sup&gt;hypo&lt;/sup&gt;</td>
<td>CSN8 hypomorphism</td>
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<tr>
<td>CSN8KD</td>
<td>CSN8 knockdown</td>
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<tr>
<td>CTL</td>
<td>control mice</td>
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<td>DRC</td>
<td>desmin-related cardiomyopathy</td>
</tr>
<tr>
<td>DRM</td>
<td>desmin-related myopathy</td>
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<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GFP&lt;sub&gt;dgn&lt;/sub&gt;</td>
<td>GFP modified by carboxyl fusion of degron CL1</td>
</tr>
<tr>
<td>HF</td>
<td>heart failure</td>
</tr>
<tr>
<td>NAE</td>
<td>NEDD8-activating enzyme</td>
</tr>
<tr>
<td>NEDD8</td>
<td>neural precursor cell expressed, developmentally downregulated 8</td>
</tr>
<tr>
<td>NRVM</td>
<td>neonatal rat ventricular cardiomyocytes</td>
</tr>
<tr>
<td>POC</td>
<td>protein quality control</td>
</tr>
<tr>
<td>tg</td>
<td>transgenic</td>
</tr>
<tr>
<td>Ub</td>
<td>ubiquitin</td>
</tr>
<tr>
<td>UPS</td>
<td>the ubiquitin proteasome system</td>
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</table>

The proteasome and lysosomes can degrade 2 distinct repertoires of misfolded proteins, with the former degrading individual protein molecules and the latter removing protein aggregates; however, both often require the misfolded proteins to be covalently modified by Ub via a process known as ubiquitination. The specificity of ubiquitination is conferred by Ub ligases, which recognize and bind a mature degron on the substrate proteins. In yeast, several Ub ligases, such as Hrd1, San1, Ubr1, and Hul5, were identified to ubiquitinate misfolded proteins. Hrd1 was recently confirmed to be pivotal to endoplasmic reticulum–associated degradation of endoplasmic reticulum misfolded proteins in mammalian cardiomyocytes. However, little is known about the identity and regulation of Ub ligases responsible for ubiquitination and degradation of cytosolic misfolded proteins in mammals.

The COP9 signalosome (CSN) is an evolutionarily conserved protein complex playing an important role in regulating the catalytic dynamics of cullin-RING ligases (CRLs). By estimate, CRLs are responsible for ≈20% of Ub-dependent degradation of cellular proteins. CRLs are activated by covalent conjugation of a Ub-like protein neural precursor cell expressed, developmentally downregulated 8 (NEDD8) to cullin proteins via a ubiquitination-like process known as neddylation, which is catalyzed by the NEDD8-activating enzyme (NAE), conjugating enzyme, and ligases. CSN-mediated cullin deneddylation is essential to CRL catalytic dynamics in vivo, likely by promoting exchange of substrate receptors of CRLs. The deneddylation activity of CSN requires a holo-complex formed by all 8 subunits (CSN1 through CSN8). By regulating CRL activities, CSN participates in the regulation of many cellular processes, including cell cycle control, DNA repair, gene expression, apoptosis, and signaling transduction. We have previously reported that conditional knockout of <i>cops8</i>, the gene encoding CSN8, in mouse hearts impairs cullin deneddylation and compromises myocardial UPS and ALP functions, which leads to rapidly deteriorated HF and mouse premature death, preventing them from being used for studying the role of CSN8 in a chronic setting. Hence, the <i>Cops8</i> hypomorphic (CSN8<sup>hypo</sup>) mice were used here to address an unanswered question: does CSN8/CSN regulate the ubiquitination and degradation of misfolded proteins?

The pathogenic role of cardiac proteotoxicity is best illustrated by desmin-related cardiomyopathy (DRC), which is the cardiac manifestation of desmin-related myopathy (DRM). DRM is a heterogeneous group of myopathies caused by mutations in desmin or its partner proteins, such as αB-crystallin (CryAB). DRM eventually progresses to HF and is the main cause of death in DRM. At cellular level, DRM is characterized by intrasarcoplasmic desmin-positive aberrant protein aggregates and disruption of the cytoskeletal network. Similar to other conformational disorders, protein misfolding and aggregation are identified as the proximal pathogenic factors to DRC. Human DRM-linked R120G missense mutation of CryAB (CryAB<sup>R120G</sup>) has proven to be a bona fide misfolded cytosolic protein. Mice with cardiomyocyte-restricted transgenic (tg) expression of CryAB<sup>R120G</sup> develop cardiomyopathy and HF, recapitulating human DRC. Hence, the CryAB<sup>R120G</sup> tg mice represent a highly relevant animal model of HF, especially for studying cardiac PQC and proteotoxicity.

Here we report that CSN8<sup>hypo</sup> mouse hearts display decreased deneddylation and significantly reduced performance to degrade a surrogate misfolded protein. Myocardial CSN8 is significantly upregulated in CryAB<sup>R120G</sup> tg mice. When introduced into the CryAB<sup>R120G</sup> tg mice, CSN8<sup>hypo</sup> aggravated the CryAB<sup>R120G</sup>-based DRC. Further experimentation reveals that CSN8 deficiency suppresses the ubiquitination and degradation of CryAB<sup>R120G</sup>, resulting in accumulation of protein aggregates and exacerbation of CryAB<sup>R120G</sup> cytopotoxicity; similarly, inactivation of CRLs via inhibiting neddylation stabilizes CryAB<sup>R120G</sup> proteins in cardiomyocytes. Our results demonstrate that CSN8/CSN promotes the ubiquitination and degradation of misfolded proteins and protects against cardiac proteotoxicity and that CRLs participate in degradation of cytosolic misfolded proteins.

**Methods**

A detailed Methods section is provided in the Online Data Supplement.

**Mouse Models**

Mice with <i>cops8</i> conditionally targeted alleles were previously described. Briefly, <i>cops8<sup>neoflox</sup></i> allele contains a neomycin-resistant inhibitors in their chemotherapy develop cardiac dysfunction or even HF. A significant role of UPS malfunction in cardiac pathogenesis is further underscored by the identification of dominant negative mutations in <i>TRIM63</i>, the gene encodes a Ub ligase (muscle ring finger 1), as a cause of human familial hypertrophic cardiomyopathy. Hence, a better understanding of the molecular underpinnings of cardiac PQC is of paramount significance to developing strategies to improve cardiac PQC and thereby more effectively to treat a large subset of heart diseases.

The proteasome and lysosomes can degrade 2 distinct repertoires of misfolded proteins, with the former degrading individual protein molecules and the latter removing protein aggregates; however, both often require the misfolded proteins to be covalently modified by Ub via a process known as ubiquitination. The specificity of ubiquitination is conferred by Ub ligases, which recognize and bind a mature degron on the substrate proteins. In yeast, several Ub ligases, such as Hrd1, San1, Ubr1, and Hul5, were identified to ubiquitinate misfolded proteins. Hrd1 was recently confirmed to be pivotal to endoplasmic reticulum–associated degradation of endoplasmic reticulum misfolded proteins in mammalian cardiomyocytes. However, little is known about the identity and regulation of Ub ligases responsible for ubiquitination and degradation of cytosolic misfolded proteins in mammals. The COP9 signalosome (CSN) is an evolutionarily conserved protein complex playing an important role in regulating the catalytic dynamics of cullin-RING ligases (CRLs). By estimate, CRLs are responsible for ≈20% of Ub-dependent degradation of cellular proteins. CRLs are activated by covalent conjugation of a Ub-like protein neural precursor cell expressed, developmentally downregulated 8 (NEDD8) to cullin proteins via a ubiquitination-like process known as neddylation, which is catalyzed by the NEDD8-activating enzyme (NAE), conjugating enzyme, and ligases. CSN-mediated cullin deneddylation is essential to CRL catalytic dynamics in vivo, likely by promoting exchange of substrate receptors of CRLs. The deneddylation activity of CSN requires a holo-complex formed by all 8 subunits (CSN1 through CSN8). By regulating CRL activities, CSN participates in the regulation of many cellular processes, including cell cycle control, DNA repair, gene expression, apoptosis, and signaling transduction. We have previously reported that conditional knockout of <i>cops8</i>, the gene encoding CSN8, in mouse hearts impairs cullin deneddylation and compromises myocardial UPS and ALP functions, which leads to rapidly deteriorated HF and mouse premature death, preventing them from being used for studying the role of CSN8 in a chronic setting. Hence, the <i>Cops8</i> hypomorphic (CSN8<sup>hypo</sup>) mice were used here to address an unanswered question: does CSN8/CSN regulate the ubiquitination and degradation of misfolded proteins?

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cassette in intron between exon 3 and 4; the CSN8 knockout allele (CSN8\(^{-}\)) has a deletion of exon 4 to 6. The homozygous Cops8\(^{neoflox/neoflox}\) mice were then mated with Cops8\(^{+/−}\) mice to produce Cops8\(^{neoflox/−}\) and Cops8\(^{neoflox/+}\) mice, which were in the FVB/N inbred background and used as CSN8 hypo mice and control mice (CTL), respectively. The CryAB, CryAB R120G, and green fluorescent protein (GFP) modified by carboxyl fusion of degron CL1 (GFP\(^{dgn}\)) tg mice were described previously.\(^{35,38}\)

Echocardiography

Trans-thoracic echocardiography was performed on mice using the VisualSonics Vevo 770 system and a 30-MHz probe as previously described.\(^{28}\)

Neonatal Rat Ventricular Cardiomyocyte Cultures and Adenoviral Delivery

Primary neonatal rat ventricular cardiomyocyte (NRVM) culture and adenoviral delivery of CryAB\(^{R120G}\) were performed as reported.\(^{39}\)

Small Interfering RNA Transfection

To knock down the target gene expression, the Lipofectamine-2000 transfection reagent (Invitrogen) was used for small interfering RNA transfection following the manufacturer’s protocol.\(^{39}\)

Immunostaining and Aggregate Quantification

Immunofluorescence staining of mouse myocardial sections or cultured NRVMs were performed as described.\(^{29}\) Immunofluorescence images of CryAB-positive aggregates were quantified using Image-Pro Plus as described.\(^{14}\)

Statistical Analyses

All continuous variables are expressed as mean±SD. Differences between groups were evaluated for significance using 2-tailed Student’s t test for unpaired 2-group comparison or 1-way or 2-way analysis of variance followed by the Scheffé test when appropriate. The probability value <0.05 is considered statistically significant.

Results

Characterization of CSN8 Hypomorphic Mice

The early postnatal lethality of cardiomyocyte-restricted knockout of Cops8 in mice prevents the use of these mice to study the role of CSN in the degradation of misfolded proteins in adult hearts. To circumvent this problem, we used mice with different Cops8\(^{targeted}\) alleles (Figure 1A) to generate CSN8\(^{neoflox/−}\) (CSN8\(^{hypo}\)) and littermate CSN8\(^{neoflox/+}\) CTL mice. CSN8\(^{hypo}\) mice are viable, fertile, and morphologically indistinguishable from wild-type mice and the CTL mice. Western blot analyses revealed that the CSN8\(^{hypo}\) mice displayed \(≈ 80\%\) reduction of CSN8 proteins in the heart (Figure 1B and 1C). In contrast, the CTL mice showed comparable CSN8 expression to wild-type mice (data not shown). Under the baseline condition, echocardiography showed no alteration in cardiac function, morphology, or growth during the first 6 months of life of these CSN8\(^{hypo}\) mice, compared with the CTL (Online Table I). Neither increased mortality nor gross abnormality
was discerned in CSN8<sup>ypo</sup> mice by 1 year of age, the longest time monitored.

**Impaired Deneddylation Activity in CSN8<sup>ypo</sup> Mice**

In CSN8<sup>ypo</sup> hearts, the reduction of CSN8 proteins was sufficient to discernibly reduce the protein levels of several other CSN subunits tested, including CSN1, CSN2, CSN3, and CSN5 (Figure 1B and 1C), confirming the essential role of CSN8 in the integrity and stability of the CSN holocomplex in cardiomyocytes. Consistent with the notion that the intact CSN complex is required for CSN deneddylation activity, we found that CSN8<sup>ypo</sup> increased neddylated forms of cullin 1, 2, 3, and 4a (Figure 1D and 1E). Notably, CSN8<sup>ypo</sup> also increased the neddylated forms of many other noncullin proteins (Figure 1F and 1G), indicating that CSN has a broad range of deneddylation substrates. Together, these data demonstrate that CSN8<sup>ypo</sup> impairs CSN deneddylation activity in the heart.

**CSN8 Hypomorphism Impairs Degradation of a Surrogate Misfolded Protein in the Heart**

A proven UPS substrate protein GFPdgn was created by carboxyl fusion of a GFP with degron CL1, a 16-amino acid sequence with surface exposure of a stretch of hydrophobic residues that mimics the signature conformation of misfolded proteins and is capable of triggering ubiquitination of its fusion protein by a pathway known to target misfolded proteins; hence, GFPdgn is considered a surrogate misfolded protein. To assess whether CSN8<sup>ypo</sup> affects UPS-mediated degradation of misfolded proteins in vivo, we used GFPdgn tg mice, in which GFPdgn is ubiquitously expressed. By cross-breeding tg GFPdgn into the CTL or CSN8<sup>ypo</sup> mice, we found myocardial GFPdgn protein levels were significantly accumulated in CSN8<sup>ypo</sup> mouse hearts (Figure 2A and 2B) in absence of changes in GFPdgn mRNA levels (Figure 2C), suggesting that CSN8 haploinsufficiency impairs UPS degradation of GFPdgn. The defect does not seem to arise from alterations in proteasome activities because all 3 proteasome peptidase activities were comparable between CTL and CSN8<sup>ypo</sup> hearts (Figure 2D).

**CSN8 Hypomorphism Exacerbates DRC in Mice**

Our examination of protein expression of representative CSN subunits (CSN1, CSN2, CSN8) revealed that CSN abundance was significantly increased in CryAB<sup>R120G</sup> (line 134), but not wild-type CryAB (line 11), tg mouse hearts (Figure 3A and 3B), although previous studies have shown that CryAB mRNA and protein overexpression in 11 is greater than in line 134. To determine the role of CSN8/CSN upregulation in DRC mice, we crossed the CSN8<sup>ypo</sup> mice with CryAB<sup>R120G</sup> tg mice, a bona fide model of cardiac proteinopathy with defined disease progression. We obtained a cohort of mice with a genotype of CTL::CryAB<sup>R120G</sup>, CTL::CryAB<sup>R120G</sup>, or CSN8<sup>ypo</sup>::CryAB<sup>R120G</sup> and performed a Kaplan–Meier survival analysis, which revealed that CSN8<sup>ypo</sup> significantly accelerated the premature death of the DRC mice (Figure 3C). Transthoracic echocardiography was performed on these animals at 12 weeks of age. Compared with CTL mice, CTL::CryAB<sup>R120G</sup> mice displayed a cardiac functional phenotype characteristic of compensatory restrictive cardiomyopathy, as evidenced by marked decreases in left ventricular internal diameters and volumes at the end of diastole. These abnormalities were further augmented in CSN8<sup>ypo</sup>::CryAB<sup>R120G</sup> mice (Table).

**CSN8 Hypomorphism Increases NEDD8 Conjugates and Aberrant Protein Aggregation in DRC Mouse Hearts**

Confirming that the deneddylation activity in DRC hearts was decreased by CSN8<sup>ypo</sup>, Western blot analyses showed that myocardial total NEDD8 conjugate levels were increased by over 60% (P<0.05) in CSN8<sup>ypo</sup>::CryAB<sup>R120G</sup> mice compared with the CTL::CryAB<sup>R120G</sup> mice (Figure 3D and 3E). Because protein aggregation is a causative pathogenic factor of DRC, we then sought to determine whether the exacerbation of DRC in CSN8<sup>ypo</sup> mice was associated with altered protein aggregation. Immunofluorescence confocal microscopy revealed that CryAB-positive protein aggregates were significantly increased by CSN8<sup>ypo</sup> in DRC mouse hearts (Figure 4A and
4B). Consistently, filter-trap assays also showed a substantial increase in detergent-resistant CryAB-positive aggregates in the myocardium from CSN8hypo::CryABR120G mice compared with those from CTL::CryAB R120G mice (Figure 4C). These data compellingly demonstrate that CSN8 hypo accumulates protein aggregates in DRC hearts. Surprisingly, the increased protein aggregates in CSN8hypo::CryABR120G hearts were accompanied by significantly reduced Ub conjugates in both detergent-resistant myocardial fraction (Figure 4C) and total myocardial protein extract (Figure 4D), suggesting that CSN8 is required for the ubiquitination of misfolded proteins in DRC hearts.

CSN8/CSN Regulates the Stability of CryABR120G in Cultured Cardiomyocytes

To further test whether CSN8hypo-induced accumulation of protein aggregates in DRC mouse hearts is caused by impaired degradation of misfolded CryABR120G, we assessed the impact of CSN8 knockdown (CSN8KD) by small interfering RNA substantially increased the steady-state protein levels of CryABR120G in the soluble fraction of cardiomyocyte lysate, and the increase was even more pronounced in the insoluble fraction (Figure 5A). We also assessed the prevalence of protein aggregates in CryABR120G-overexpressed cardiomyocytes by immunostaining for HA-CryAB R120G and SEC61α, the latter serving as a marker of protein aggregates.39 Immunofluorescence images showed that CSN8KD increased both the abundance and the size of protein aggregates in cardiomyocytes (Figure 5B). To dynamically assess the degradation of CryABR120G, we further performed a cycloheximide chase experiment. We found that CSN8KD substantially prolonged the half-life of CryABR120G (Figure 5C and 5D). By contrast, CSN8KD did not discernibly increase the steady state protein levels of conventional GFP and endogenous or overexpressed wild-type CryAB (Online Figure I) nor did it elongate the half-life of GFP and CryAB (Online Figures II and III). Taken together, these results demonstrate that CSN8 depletion impairs degradation of CryABR120G, a bona fide cytosolic misfolded protein, and promotes protein aggregation in cardiomyocytes.

CSN8/CSN and CRLs Control CryABR120G Ubiquitination and Degradation

CSN is known to control the stability of several native proteins by regulating their respective CRLs' activity.27 We next tested whether CSN could regulate the ubiquitination of a bona fide misfolded protein. Immunoprecipitation of HA-CryABR120G followed by western blot analyses showed that proteasome inhibition by MG132 largely increased high molecular weight species of CryABR120G. Probing the immunoprecipitates with anti-Ub antibodies identified these high molecular weight species as ubiquitinated forms of CryABR120G. Furthermore, CSN8KD significantly reduced the ubiquitinated forms of HA-CryAB R120G in both absence and presence of proteasome inhibition (Figure 6A). These data indicate that the proteasome is responsible for degradation of ubiquitinated CryABR120G and that CSN8/CSN is required for CryABR120G ubiquitination. Because CSN per se does not ubiquitinate any proteins but rather it regulates the activity of CRLs, an important family of Ub ligases,26 these in vivo and in vitro findings led us to

Table. Echocardiographic Measurements at 12 Weeks of Age

<table>
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<tr>
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<th>CTL n=6</th>
<th>CSN8hypo n=8</th>
<th>CTL::CryABR120G n=10</th>
<th>CSN8hypo::CryABR120G n=8</th>
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<tr>
<td>Body weight, g</td>
<td>30.4±2.1</td>
<td>27.2±3.5</td>
<td>28.2±3.7</td>
<td>28.8±4.2</td>
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<td>Heart rate, bpm</td>
<td>471±24</td>
<td>487±42</td>
<td>432±43</td>
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<td>0.88±0.06‡</td>
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<td>2.45±0.22*</td>
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<td>65.19±8.95‡</td>
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<td>47.3±10.3</td>
<td>35.2±6.2</td>
<td>43.9±6.7</td>
<td>38.3±8.6</td>
</tr>
<tr>
<td>CO, mL/min</td>
<td>22.3±4.9</td>
<td>17.2±3.9</td>
<td>19.0±4.3</td>
<td>18.1±4.2</td>
</tr>
</tbody>
</table>

ANOVA indicates analysis of variance; CO, cardiac output; CryABR120G, R120G missense mutation of α- crystallin; CSN8hypo, CSN8 hypomorphism; CTL, control mice; EF, ejection fraction; FS, fractional shortening; LV, left ventricle; LVIDd, end-diastolic left ventricular (LV) internal diameter; LVIDs, end-systolic LVd; LVVPd, end-diastolic LV posterior wall thickness; LVVPWs, end-systolic LVWP; LVWd, end-diastolic LV anterior wall thickness; LVWAs, end-systolic LVW; LVd, end-diastolic LV volume; LVs, end-systolic LV; and SV, stroke volume.

*P<0.01 versus CTL.
†P<0.05 versus CTL::CryABR120G.
‡P<0.05 versus CTL; ANOVA followed by the Scheffé test.
hypothesize that CRLs are responsible for the ubiquitination of CryAB<sup>R120G</sup>. To examine this hypothesis, we assessed the effect of CRLs inactivation via inhibiting their neddylation using MLN4924, a specific NAE inhibitor. We found that NAE inhibition significantly elongated the half-life of CryAB<sup>R120G</sup> expressed in cultured NRVMs (Figure 6B and 6C). These results demonstrate that the activation of CRLs is required for the degradation of CryAB<sup>R120G</sup>, suggesting that CSN controls CryAB<sup>R120G</sup> ubiquitination via its regulation on CRLs.

CSN8 Hypomorphism Blunts Autophagic Responses in Cardiomyocytes Under Stress

We have previously demonstrated that Cops8 loss-of-function impairs autophagosome–lysosome fusion, thereby accumulating Ub conjugates, LC3-II (a marker of autophagosomes), and p62/SQSTM1 (a substrate of autophagy) in the heart. Hence, we examined these parameters in CSN8<sup>hypo</sup> hearts, but we found none of them were discernibly altered at baseline (Online Figure IV), suggesting that autophagic activity is not perturbed in CSN8<sup>hypo</sup> mouse hearts, but we found none of them were discernibly altered at baseline condition. Myocardial LC3-II, p62, and autophagic activity are known to increase in DRC mice. When coupled with the CryAB<sup>R120G</sup>-based DRC, CSN8<sup>hypo</sup> significantly suppressed the increase of LC3-II, but not that of p62, in the heart (Figure 7A and 7B), suggesting that CSN8<sup>hypo</sup> may impair cardiac autophagy under a stress condition. To examine this postulate further, we performed cell culture experiments. CSN8KD in cultured NRVMs did not reduce LC3-II flux or p62 flux at baseline but did so during simulated starvation (Online Figure V). Similar evidence was obtained in mouse embryonic fibroblasts in which autophagic flux was monitored using the tandem fluorescence protein–fused LC3 as a reporter. During autophagic activation triggered by simulated starvation, autophagosome–lysosome fusion impairment was detected in CSN8<sup>hypo</sup> mouse embryonic fibroblasts (Online Figure VI). Moreover, CSN8KD significantly decreased LC3-II flux in NRVMs overexpressing CryAB<sup>R120G</sup> (Online Figure VI). Taken together, these results indicate that impairment of autophagic flux by CSN8<sup>hypo</sup> becomes discernible only when the demand for autophagy is elevated. This also implicates that UPS impairment in Csn8<sup>hypo</sup> mouse hearts, which is discernible at baseline, is not secondary to potential ALP impairment.

CSN8 Deficiency Sensitizes Cardiomyocytes to Proteotoxic Stress

Protein misfolding and aberrant aggregation are associated with cytotoxicity and HF in DRC. Given the critical role of CSN8/CSN in the removal of misfolded protein CryAB<sup>R120G</sup>, we then tested whether CSN8/CSN protects cardiomyocytes from proteotoxic stress–induced cytotoxicity. The proteotoxic stress was imposed to cultured cardiomyocytes by overexpression of CryAB<sup>R120G</sup>. Cell death and cell viability were respectively assessed with the lactate dehydrogenase leakage assay and the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl...
tetrazolium bromide assay. CSN8KD did not affect the survival of cardiomyocytes in basal condition. However, expression of CryAB<sup>R120G</sup> significantly increased cytotoxicity, as evidenced by a significant increase of lactate dehydrogenase activity in media and a decrease of cell viability. CSN8 depletion further aggravated the CryAB<sup>R120G</sup>-induced cytotoxicity, as revealed by an ≈70% increase of lactate dehydrogenase release and an ≈54% decrease of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide reading when compared with CryAB<sup>R120G</sup>-expressed cardiomyocytes (Figure 7C and 7D). These data indicate that CSN8-deficient cardiomyocytes are more susceptible to proteotoxicity.

Figure 4. CSN8<sub>hypo</sub> aggravates CryAB<sup>R120G</sup>-induced aberrant protein aggregation and decreases the ubiquitination in mouse hearts. Littermate mice with the indicated genotypes were used at the age of 8 weeks. A, Representative confocal fluorescent images of myocardial sections immunostained for CryAB (white). Scale bar=20 μm. B, Quantitative analysis of the relative abundance of the CryAB-positive protein aggregates in the heart. n=3 mice/group. *P<0.05 vs CTL::CryAB<sup>R120G</sup>, t test. C, Filter trapping assays for CryAB and ubiquitin (Ub) in ventricular myocardium of mice with indicated genotypes. The proteins retained on the filter were immunostained for CryAB or Ub. The relative density of each dot is shown below the blots. D, Western blot analyses of myocardial total ubiquitinated proteins. β-Tubulin was probed as a loading control. CSN indicates COP9 signalosome; CryAB<sup>R120G</sup>, R120G missense mutation of αβ-crystallin; and CTL, control mice.

Figure 5. CSN8 knockdown stabilizes CryAB<sup>R120G</sup> in cardiomyocytes. NRVMs were infected with adenoviruses expressing HA-CryAB<sup>R120G</sup> (Ad-HA-CryAB<sup>R120G</sup>) or β-Gal as indicated. The cells were also transfected with small interfering RNAs (siRNAs) against either luciferase (siLuci) or CSN8 (siCSN8). At 72 hours after the siRNA transfection, the cells were harvested for the analyses (A and B) or treated with cycloheximide (CHX, 100 μmol/L) for the indicated times (C). A, Representative Western blot images of indicated proteins in the Triton X-100 soluble and insoluble fraction of cell lysate. GAPDH and α-actinin were probed as loading controls. B, Immunofluorescent images showing increased protein aggregates in CSN8 knockdown cells. HA-tag (green) and Sec61α (red) were stained for CryAB<sup>R120G</sup> and aggresomes, respectively. Scale bar=50 μm. C and D, Cycloheximide (CHX) chase assay for HA-CryAB<sup>R120G</sup>. HA-CryAB<sup>R120G</sup> protein levels at the indicated time points were measured using Western blot analyses for HA-tag. A representative image (C) and a summary of the relative levels of HA-CryAB<sup>R120G</sup> (D) are shown; *P<0.05 vs the siLuci+CryAB<sup>R120G</sup> group, n=3 repeats; t test. CSN indicates COP9 signalosome; CryAB<sup>R120G</sup>, R120G missense mutation of αβ-crystallin; and NRVM, neonatal rat ventricular cardiomyocytes.
Aberrant protein aggregation is best exemplified in DRC, but it is also implicated in the heart of humans with CHF of common causes, suggesting that cardiac proteotoxicity is pathogenic in a large subset of cardiac disease. Indeed, improving UPS performance was recently shown to protect not only DRC but also myocardial ischemia/reperfusion injury. Hence, a better understanding of the clearance of misfolded proteins may provide new strategies to treat heart disease or prevent cardiotoxicity of the treatment of noncardiac disease. Here we have shown that CSN8 hypomorphism impairs the ubiquitination and degradation of a surrogate misfolded protein (GFPdgn), as well as a bona fide misfolded protein CryABR120G, leading to accumulation of protein aggregates and exacerbation of DRC in mice. Our data demonstrate that CSN8/CSN is essential to the ubiquitination and degradation of misfolded proteins via UPS and ALP and that CRLs participate in the degradation of cytosolic misfolded proteins.

**Figure 6.** Inhibition of cullin-RING ligases (CRLs) impairs CryABR120G ubiquitination and degradation in cardiomyocytes. **A**, CSN8 knockdown impairs CryABR120G ubiquitination. NVRMs were treated as described in Figure 5. Seventy-two hours after small interfering RNA (siRNA) transfection, MG132 (5 μmol/L) treatment was initiated and lasted for 6 hours before the cells were harvested. Representative images of Western blot analyses (IB) of the indicated proteins in immunoprecipitated (IP) HA-CryABR120G are shown. **B** and **C** MLN4024 stabilizes CryABR120G in cultured NVRMs. MLN4924 (1 μmol/L) or vehicle control (DMSO) treatment was initiated at 48 hours after Ad-HA-CryABR120G infection in cultured NVRMs. Cycloheximide (CHX, 50 μmol/L) was added to the culture media at 30 min after initiating MLN4924 treatment. Cells were harvested at the indicated time points for extraction of total proteins. Representative images (B) and pooled densitometry data (C) of Western blot analyses of HA-CryABR120G are shown. *P<0.05 vs the DMSO group, n=3 repeats; t test. CSN indicates COP9 signalosome; CryABR120G, R120G missense mutation of αβ-crystallin; and NRV, neonatal rat ventricular cardiomyocytes.

**Figure 7.** CSN8 deficiency mitigates autophagic responses in mouse hearts and augments proteotoxicity in cardiomyocytes. **A and B**, Western blot analyses for myocardial LC3 and p62. CSN8 hypomorphism was cross-bred into CryABR120G (R120G) mice. Ventricular myocardial total protein extracts from 8-week-old mice was used. Representative images (A) and pooled densitometry data (B) are shown, n=4–6 mice/group. #P<0.01 vs CTL; ‡P<0.05 vs CTL::R120G. **C and D** CSN8 knockdown augments CryABR120G-induced cardiomyocyte injury. NVRMs were treated as described in Figure 5. The assays were performed 72 hours after small interfering RNA (siRNA) transfection. Lactate dehydrogenase (LDH) activities in the cultured medium (C) were measured to assess cell injury. Cell viability was assessed by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay (D). *P<0.01 vs silLuc; †P<0.01 vs silLuc+R120G; n=4–6. ANOVA followed by the Scheffé test. CSN indicates COP9 signalosome; CryABR120G, R120G missense mutation of αβ-crystallin; and NRV, neonatal rat ventricular cardiomyocytes.
CSN8 is an indispensable subunit of the CSN holoenzyme for cullin deneddylation;21 hence, CSN8 deficiency is expected to compromise CRLs catalytic dynamics, thereby affecting ubiquitination efficiency of a large family of proteins.24,43 Somewhat surprisingly, CSN8<sup>hypo</sup> mice do not display abnormal phenotypes for at least the first 6 months of age. Nevertheless, CSN8<sup>hypo</sup> exacerbates cardiac proteinopathy, and reduced ubiquitination emerges as a primary defect caused by CSN8<sup>hypo</sup> during proteotoxic stress. This is supported by the following: (1) myocardial UPS performance in CSN8<sup>hypo</sup> mice was decreased without altering proteasomal activities; (2) Ub conjugates were markedly decreased in both protein aggregates and total myocardial protein extracts from CryAB<sup>R120G</sup>:CSN8<sup>hypo</sup> mice; and (3) CSN8KD reduced ubiquitinated forms of CryAB<sup>R120G</sup> in cultured NRVMs.

Ubiquitination not only is essential to proteasomal degradation but also can indirectly promote ALP-mediated degradation.18,20 Based on the prevalent model, when escaped from proteasomal degradation, misfolded proteins form aggregates and their Ub chains may bind p62/SQSTM1 which, in turn, recruits LC3-II–positive phagophores to trigger autophagosomal engulfment and degradation of the aggregates (Online Figure VIII). Our results show that the CSN8<sup>hypo</sup>-derived defect in ubiquitination reduces primarily UPS performance but does not affect ALP activity at baseline; however, CSN8<sup>hypo</sup> discernibly limits autophagic activity under a stress condition (eg, nutrient deprivation, misfolded protein overexpression) that normally upregulates autophagy. This expands our prior findings from cardiac-specific CSN8 knockout mice that CSN8/CSN is essential to both UPS and ALP.29,41 This also explains why CSN8<sup>hypo</sup> significantly decreased LC3-II protein levels in CryAB<sup>R120G</sup>tg mouse hearts. Via binding ubiquitinated proteins, p62 facilitates aggresome formation and is often enriched in aberrant aggregates,39 whereas p62 is stabilized by ALP impairment.44 It is likely that p62 stabilizing factors (eg, reduced autophagic flux) and destabilizing factors (eg, reduction of Ub chains in the aggregates) counter each other, resulting in unaltered p62 protein levels in CSN8<sup>hypo</sup>:CryAB<sup>R120G</sup> hearts compared with CTL::CryAB<sup>R120G</sup> hearts.

In cardiomyocytes, the degradation of overexpressed misfolded proteins, such as CryAB<sup>R120G</sup>, depends on both UPS and ALP.13,19,20 CSN8<sup>hypo</sup> reduces baseline myocardial UPS performance and limits stress-induced ALP activity in cardiomyocytes. Hence, we submit that reduced UPS- and ALP-mediated degradation of CryAB<sup>R120G</sup> contribute to the exacerbation of protein aggregation and disease progression in DRC mouse hearts by CSN8<sup>hypo</sup>.

The specificity of ubiquitination is determined principally by Ub ligases. In mammals, HRD1, Parkin, and C-terminus of Hsp70-interacting protein have been shown to serve as the Ub ligases of the endoplasmic reticulum–associated degradation responsible for ubiquitination of misfolded proteins retro-translocated from the endoplasmic reticulum to the cytosolic side.22 However, the identities of Ub ligases responsible for the degradation of cytosolic misfolded proteins are virtually unknown, although CHIP is implicated in cytosolic PQC.22 Notably, none of these PQC ligases reported to date belongs to CRLs. Here we present multiple lines of strong evidence to support an important role of CRLs in the ubiquitination and degradation of cytosolic misfolded proteins. First, the degradation of a surrogate misfolded protein (GFpDgn) by the UPS is impaired in CSN8<sup>hypo</sup> mouse hearts with unaltered proteasome peptidase activities; second, reduction of CSN8/CSN and its deneddylation function decreased the misfolded proteins–induced protein ubiquitination in the heart of intact animals; third, disruption of CSN-mediated deneddylation activities by CSN8KD inhibited the ubiquitination and increased aberrant aggregation of a bona fide misfolded protein in cultured cardiomyocytes; and finally, inhibition of CRLs by a NAE-specific inhibitor MLN4924 significantly slowed down the degradation of a bona fide misfolded protein in cardiomyocytes. It is unlikely that a single E3 ligase can account for the ubiquitination of all misfolded proteins, given the multitude of conformations that misfolded proteins can assume. There are 7 cullin proteins in the cullin family, each assembled with multiple substrate-recognizing adaptors to regulate the ubiquitination of the substrates.41 Therefore, the diversity and flexibility of CRLs seem well suited to accommodate the multitude of conformations that misfolded proteins may assume. It will be important to identify specific cullins and adaptors responsible for ubiquitination of misfolded proteins.

In conclusion, here we demonstrate that upregulation of CSN8/CSN is adaptive in DRC hearts, and CSN8<sup>hypo</sup> exacerbates cardiac proteinopathy; the exacerbation is associated with augmented accumulation of protein aggregates, increased NEDD8 conjugates, and reduced levels of total Ub conjugates in the heart. Cardiomyocyte culture experiments further show that both CSN8 deficiency and CRLs inhibition suppress the ubiquitination and degradation of CryAB<sup>R120G</sup>, resulting in accumulation of protein aggregates and exacerbation of CryAB<sup>R120G</sup> cytotoxicity. Hence, we have obtained compelling evidence that CSN8/CSN is essential to the ubiquitination and clearance of misfolded cytosolic proteins and protects against proteotoxicity and that CRLs participate in the degradation of cytosolic misfolded proteins.

Increased production and impaired removal of misfolded proteins in the heart because of genetic mutations or acquired causes are highly conceivable and, in some cases, well-demonstrated in cardiac remodeling and HF.1,2,6 However, presently there is no treatment specifically aiming at enhancing degradation of misfolded proteins in the heart. Meanwhile, the first-in-class NAE inhibitor (MLN4924), which inhibits neddylation and activation of CRLs, is in clinical trials for treating cancers.45 Increased NEDD8 conjugates in end-stage failing human hearts were recently reported.46 The present study identifies that CSN8/CSN and CRLs contribute to degradation of cytosolic misfolded proteins. This represents one major step closer to identification of specific Ub ligases for targeted degradation of toxic misfolded proteins; on the other hand, this also cautions that NAE inhibition may potentially exert cardiotoxicity, just like proteasome inhibitors.16,17

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Disclosures

None.

References


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**Novelty and Significance**

**What Is Known?**
- The COP9 signalosome holocomplex (CSN) consisting of 8 unique proteins (CSN1 through CSN8), which via cullin deneddylation, regulates cullin-RING ligases (CRLs), the largest family of ubiquitin ligases.
- Cardiac ablation of Cop8 impairs myocardial protein degradation by the ubiquitin-proteasome system and autophagic-lysosomal pathway, resulting in cardiac hypertrophy, heart failure, and premature death in mice.
- Neither CSN nor CRLs are known to control the ubiquitination of cytosolic misfolded proteins.

**What New Information Does This Article Contribute?**
- Myocardial CSN8/CSN is increased in a mouse model of proteinopathy, whereas downregulation of CSN8/CSN impairs the ubiquitination and degradation of a bona fide misfolded cytosolic protein in cardiomyocytes.
- CRLs participate in ubiquitination and degradation of a bona fide misfolded cytosolic protein.
- CSN8/CSN protects against cardiac proteinopathy.

Ubiquitin-proteasome system- and autophagic-lysosomal pathway-mediated protein degradation constitutes the last line of defense in protein quality control, which acts to minimize the level and the toxicity of misfolded proteins in the cell. Increases of misfolded proteins in cardiomyocytes are an inevitable consequence and cause of increased cardiac stress, whereas protein quality control inadequacy has been implicated in the progression to heart failure from a large subset of heart diseases. However, no specific treatment aimed at improving cardiac protein quality control is currently available. The present study reveals that (1) myocardial CSN8 is upregulated in a classical mouse model of proteinopathy and, when introduced into this mouse, CSN8 hypomorphism hastens disease progression; (2) the degradation of a bona fide misfolded protein in cardiomyocytes is suppressed by CSN8 knockdown or the inhibition of CRLs using a neural precursor cell expressed, developmentally downregulated 8–activating enzyme inhibitor which is in clinical trial to treat cancers; and (3) CSN8 increases ubiquitination of misfolded proteins, thereby promoting their degradation by the proteasome and autophagic-lysosomal pathway. These findings suggest that both CSN8/CSN and CRLs may represent key targets for improving cardiac protein quality control and caution that neural precursor cell expressed, developmentally downregulated 8–activating enzyme inhibition may yield cardiac toxicity.
COP9 Signalosome Controls the Degradation of Cytosolic Misfolded Proteins and Protects Against Cardiac Proteotoxicity
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The COP9 signalosome controls the degradation of cytosolic misfolded proteins and protects against cardiac proteotoxicity

Su, The COP9 promotes misfolded protein degradation

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SUPPLEMENTAL METHODS

Mouse models
Mice with *Caps8* conditionally targeted alleles were previously described. Briefly, *Caps8*\(^{\text{neoflox}}\) allele contains a neomycin resistant cassette in intron between exon 3 and 4 while CSN8 knockout allele (CSN8\(^{-}\)) has a deletion of exon 4 to 6. The C57BL/6J *Caps8*\(^{\text{neoflox}/+}\) and *Caps8*\(^{+/+}\) mice were backcrossed into FVB/N background for at least six generations. The homozygous *Caps8*\(^{\text{neoflox/neoflox}}\) mice were then mated with *Caps8*\(^{+/+}\) mice to produce *Caps8*\(^{\text{neoflox/-}}\) and *Caps8*\(^{\text{neoflox/+}}\) mice, which were used as CSN8 hypomorphic (CSN8\(^{\text{hypo}}\)) mice and control mice (CTL), respectively. The FVB/N CryAB\(^{R120G}\) transgenic (tg) mice and FVB/N GFPdgn tg mice were described previously. Mice of mixed sexes were used in this study.

All protocols involving the use of animals were in compliance with the National Institutes of Health’s and approved by the Institutional Animal Care and Use Committee of the University of South Dakota.

Protein extraction and western blot analysis
To prepare total proteins, ventricular tissues or cultured cells were lysed in 1×SDS sampling buffer (50mM Tris-Cl at pH 6.8 containing 2% SDS and 10% glycerol and a complete protease inhibitor cocktail). The extracts were homogenized on ice, boiled for 5 minutes and centrifuged at 10,000×g for 10 minutes at 4°C. The supernatants were obtained as total proteins.

To prepare soluble and insoluble fractions, myocardial tissue or cells were homogenized in cold phosphate-buffered saline (PBS) at pH 7.4 containing 1% Triton-X100, 2.5mM EDTA, 0.5mM PMSF and a complete protease inhibitor cocktail and incubated on ice for 30 minutes with 30-second vortex every 10 minutes. The homogenates were centrifuged at 12,000×g for 15 minutes and the supernatants were collected as soluble fractions. The pellets were dissolved in 1× SDS sampling buffer by sonication on ice and boiled for 5 minutes, then centrifuged at 10,000×g for 10 minutes at 4°C. The supernatants were obtained as insoluble fractions of proteins.

Quantification of protein concentration, SDS-PAGE, immunoblotting analysis, and densitometry were performed as previously described. To probe for total ubiquitinated proteins, 20 μg of total proteins were separated by SDS-PAGE using 4-15% precast gradient Tris-HCl gels (Bio-Rad), transferred to PVDF membrane, and blotted with rabbit anti-ubiquitin antibodies (Sigma). Other used antibodies include: CSN8 (Enzo life Sciences), CSN1, CSN5 and cullin 3 (Novus Biologicals), NEDD8 (Epitomics), cullin 2 (Zymed), β-tubulin, HA-epitope, cullin 1 and GFP (Santa Cruz), CryAB (Stressgen), α-actin and GAPDH (Sigma), CSN2, CSN3 and cullin 4A (custom made).

Proteasome peptidase activity assays
The synthetic fluorogenic substrate Suc-LLVY-AMC (25μM, BIOMOL), Z-LLE-AMC(25μM, BIOMOL) and Ac-RLR-AMC (40 mM, BIOMOL) were respectively used for measuring chymotrypsin-like, caspase-like and trypsin-like activities in crude protein extracts from ventricular myocardium. Assays were performed in either absence or presence of ATP (28μM for chymotrypsin-like activities and 14μM for caspase-like activities). The portion of peptide cleavage inhibited by the proteasome-specific inhibitor MG-132 (20μM, EMD) for chymotrypsin-like and caspase-like activities, or epoxomycin (5μM, CALBIOCHEM) for trypsin-like activity, is attributed to the proteasome.
Echocardiography
Trans-thoracic echocardiography was performed on mice using the VisualSonics Vevo 770 system and a 30-MHz probe as previously described.\textsuperscript{5}

Filter trapping assay
To quantify changes in aggregate content, RIPA-insoluble proteins were treated with DNase I (1 mg/mL in 10 mmol/L Tris, 15 mmol/L MgCl\textsubscript{2}) (Roche) for 1 hour and protein quantified with a modified Bradford assay. The insoluble protein was then diluted with 2% SDS, 20 mmol/L EDTA, and 50 mmol/L DTT dissolved in TBS. Five micrograms of resuspended insoluble protein was dotted onto a nitrocellulose membrane (BioRad), which was blocked and immunoblotted with appropriate antibodies.

Real time reverse transcriptase polymerase chain reaction (qRT-PCR)
Gene expression levels were measured in duplicate per sample by real-time PCR (StepOnePlus Real-Time PCR system, Life Technologies) using the SYBR-Green assay with gene-specific primers at a final concentration of 200 nM. The following primers are used: GFPdgn-forward: GGGCACAAGCTGGAGTACAACT, GFPdgn-reverse: ATGTTGTGGCGGATCTTGAAG. Relative gene expression was calculated using the $2^{-\Delta\Delta C_{t}}$ method against a mouse house-keeping gene hypoxanthine phosphoribosyltransferase (HPRT). Each experiment was repeated at least three times independently.

Neonatal rat ventricular cardiomyocytes (NRVMs) cultures and adenoviral delivery
Primary NRVMs were isolated from the ventricles of 1- to 2-day old Sprague-Dawley rat pups and plated on 10-cm\textsuperscript{2} plates at a density of 1.5×10\textsuperscript{6} cells in 10% FBS in DMEM. Twenty-four hours after plating, cells were infected with recombinant adenoviral vectors (10 MOI, unless otherwise noted) for 3 hours in DMEM media. Adenoviruses expressing HA-CryAB\textsuperscript{R120G} or β-gal were used. Post-infection cells were maintained in 2% FBS, 1% penicillin/streptomycin in high-glucose DMEM until fixed or harvested.

SiRNA Transfection
To knock down the target gene expression, we plated 2×10\textsuperscript{6} NRVMs in each 60-mm dish. The Lipofectamine\textsuperscript{TM}-2000 transfection reagent (Invitrogen) was used for siRNA transfection following the manufacturer's protocol. The siRNA transfection was started 48 to 72 hours after the cells were plated. Six hours after the transfection, the siRNA-containing medium was replaced with the regular medium. SiRNA specific for rat CSN8 (siCSN8, 5’-CAGTCTGCAATGAGAACGCAA-3’) and the siRNA targeting luciferase serving as a control siRNA (siLuci: 5’-AACGTACGCAGAATCTTCCA-3’) were purchased from Qiagen.

Immunostaining
Mouse tissues were perfusion-fixed with 4% paraformaldehyde (Electron Microscopy Science), saturated with 40% sucrose solution and embedded in Tissue-Tek O.C.T. (Sakura Finetek. USA), and then underwent tissue sectioning at 6-µm thickness. NRVMs cultured in dishes were fixed with 2% of paraformaldehyde for 10 minutes. The tissue cryosections or fixed cells were permeabilized with 1% of Triton-X100 in PBS for 1 hour, quenched with 0.1M glycine in PBS for 1 hour, and blocked with 0.5% BSA for 1 hour. The specimen was then incubated with primary antibodies overnight at 4°C. The
primary antibodies against CryAB (Stressgen), SEC61α (Santa Cruz), and HA (Sigma) were used. Subsequently, the corresponding Alexa-488, and -568 conjugated secondary antibodies (Invitrogen) were used to label the protein. The images were captured using a fluorescence confocal microscope (Olympus Fluoview 500) or an epi-fluorescence microscope (Zeiss Axiovert 100).

To quantify the areas of protein aggregates or myocardium tissues, all images were obtained and processed with the identical setting. The areas were quantified by Image-Pro Plus. The area of the protein aggregates were normalized to the area of myocardial tissues. Three mouse hearts per group, 3 representative sections per heart, and 3 representative fields per section were assessed.

**Cycloheximide (CHX) chase assay**

NRVMs in cultures were treated with CHX (100 µmol/L) to block new protein synthesis for various periods of time before being harvested for extraction of total proteins, which were subsequently analyzed by SDS-PAGE followed by western blot for the proteins to be chased, as previously described.4

**Autophagic flux assay in cultured cardiomyocytes**

After subject to designed experimental manipulation and intervention, NRVMs were treated with bafilomycin A1 (BFA; 100 nM) or vehicle control (DMSO) for 2 hours before being harvested for protein extraction. By inhibiting v-type proton-ATPase, BFA block autophagosome and lysosome fusion as wel as lysosomal degradation of autophagosome. Total cell lysates were used for western blot analyses for LC3-II (indicator of autophagosome abundance) and β-tubulin (loading control). The difference of LC3-II protein levels between BFA and DMSO treated cells reflects autophagic flux.

**Lactate dehydrogenase (LDH) activity assay**

The LDH activity in the collected medium was measured using a cytotoxicity detection kit (Roche, Indianapolis, IN) by following the manufacturer's protocols.

**MTT assay**

This was performed as previously described.2 Briefly, MTT dye (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide, 500 µg/mL, Sigma) was added to the cells and the plates were incubated at 37°C for 2 hours. At the end of the incubation, the dye solution was completely removed, 400 µL solvent solution (1 volume of 1N HCl in 9 volume of anhydrous isopropanol) was added, and the absorbance was determined at 570 nm in a Tecan plate reader.

**Statistical Analyses**

All continuous variables are expressed as mean±SD. Differences between groups were evaluated for significance using two-tailed Student’s t test for unpaired 2-group comparison or 1-way or 2-way analysis of variance (ANOVA) followed by the Scheffé test when appropriate. The probability value <0.05 is considered statistically significant.
References


Supplementary Data (1 Online Table and 8 Online Figures)

Online Table I. Echocardiographic Parameters at 6 months of Age

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CTL (n=7)</th>
<th>CSN8^hypo (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight (BW, g)</td>
<td>29.3±7.1</td>
<td>31.8±7.3</td>
</tr>
<tr>
<td>Heart Rate (bpm)</td>
<td>460±68</td>
<td>489±76</td>
</tr>
<tr>
<td>LVID; d (mm)</td>
<td>4.14±0.19</td>
<td>4.11±0.23</td>
</tr>
<tr>
<td>LVPW; d (mm)</td>
<td>0.73±0.14</td>
<td>0.78±0.06</td>
</tr>
<tr>
<td>LVID; s (mm)</td>
<td>2.97±0.24</td>
<td>2.86±0.44</td>
</tr>
<tr>
<td>LVPW; s (mm)</td>
<td>0.95±0.19</td>
<td>1.18±0.22</td>
</tr>
<tr>
<td>IVS; d (mm)</td>
<td>0.75±0.15</td>
<td>0.80±0.09</td>
</tr>
<tr>
<td>IVS; s (mm)</td>
<td>1.11±0.25</td>
<td>1.18±0.16</td>
</tr>
<tr>
<td>LV %FS</td>
<td>30.81±6.10</td>
<td>30.22±10.58</td>
</tr>
<tr>
<td>LV %EF</td>
<td>59.88±7.66</td>
<td>56.64±15.30</td>
</tr>
<tr>
<td>LVVd (mm³)</td>
<td>76.04±8.11</td>
<td>74.75±9.65</td>
</tr>
<tr>
<td>LVVs (mm³)</td>
<td>35.01±6.96</td>
<td>32.21±11.29</td>
</tr>
<tr>
<td>SV (μl)</td>
<td>41.0±7.6</td>
<td>42.6±12.8</td>
</tr>
<tr>
<td>CO (ml/min)</td>
<td>19.1±5.5</td>
<td>21.3±9.1</td>
</tr>
<tr>
<td>LV Mass (mg)</td>
<td>109.9±27.5</td>
<td>121.4±17.1</td>
</tr>
<tr>
<td>LV Mass/BW (mg/g)</td>
<td>3.81±0.95</td>
<td>3.92±0.49</td>
</tr>
</tbody>
</table>

Student’s t-tests show that the difference in each of the parameters between the two groups is not statistically significant (p>0.05). Mean ± STD.
Online Figure I. CSN8 knockdown does not alter the steady state protein levels of endogenous CryAB or overexpressed wild type (WT-) CryAB or conventional GFP in cultured NRVMs.
Online Figure II. Cycloheximide (CHX) chase assays showing that CSN8 knockdown does not slow down the degradation of conventional GFP proteins in cultured NRVMs. Representative western blot images (A) and pooled quantitative densitometry data (B) are shown; mean±STD, n=3 repeats, p>0.05 for each time point between the two groups, t-test.
Online Figure III. Cycloheximide (CHX) chase assays showing that CSN8 knockdown does not slow down the degradation of endogenous CryAB proteins in cultured NRVMs. Representative western blot images (A) and pooled quantitative densitometry data (B) are shown; mean±STD, n=3 repeats, p>0.05 for each time point between the two groups, t-test.
Online Figure IV. Characterization of baseline autophagy in CSN8 hypomorphic mouse hearts. Total protein extracts of ventricular myocardial tissues collected from CSN8<sup>neoflox<sup>−/−</sup></sup> (CSN8 hypomorphic, Hypo) and CSN8<sup>neoflox<sup>+/−</sup></sup> (control, CTL) littermate mice at 1 or 2 months of age were subject to SDS-PAGE and western blot analyses for the indicated proteins. Representative images are shown. Under baseline condition, myocardial total ubiquitinated proteins (A), LC3-II, and p62 (B, C) were not discernibly altered in the CSN8 hypomorphic mice, compared with the CTL mice. **p<0.01 vs. CTL, t-test, n=4 mice/group.
Online Figure V. Representative images of western blot analyses for the autophagic flux in NRVMs. NRVMs in cultures were transfected with either control siRNAs (-) or siRNA targeting CSN8 (siCSN8) for 48 hours before subsequent treatment. To activate non-selective autophagy, the cells were subjected to glucose deprivation (GD) for 4 hours to mimic starvation. To assess autophagic flux, cells were treated with bafilomycin A1 (BFA; 0.1 µmol/L) or vehicle (DMSO) at 2 hours before the cells were harvested for western blot analyses of the indicated proteins. The relative abundance of LC3-II/GAPDH and p62/GAPDH of each lane is presented below the respective images. Under the baseline condition, CSN8 knockdown did not decrease LC3-II or p62 flux; however, during glucose deprivation both LC3-II flux and p62 flux were reduced by CSN8 knockdown.
Online Figure VI. Autophagic flux is impaired in CSN8 hypomorphic mouse embryonic fibroblasts (MEFs) during starvation. Shown are direct fluorescence confocal images of cultured MEFs transfected with the tandem GFP-mRFP fused LC3 (tf-LC3) plasmids (#21074, Addgene, Cambridge, MA) under the basal culture condition (Fed) or after 1hr of amino acid deprivation (Starved). Note that after starvation, autophagic vacuoles were markedly increased in both types of cells; they are mainly autolysosomes (red puncta) in the control cell but autophagosomes (yellow puncta) in the Csn8 hypomorphic cell. In cells expressing tf-LC3, autophagosomes show both green and red fluorescence (merged as yellow) whereas autolysosomes display only red fluorescence because of the quench of GFP fluorescence in the acidic lysosomal lumen. Hence, these results corroborate nicely the findings from the CSN8 knockdown experiments using cultured NRVMs (Supplementary Figure S3), illustrating that CSN8 depletion reduces autophagic flux by impairing autophagosome removal.
Online Figure VII. Autophagic flux assays in cultured NRVMs overexpressing CryAB<sup>R120G</sup>
NRVMs were infected with Ad-HA-CryAB<sup>R120G</sup> for 24 hrs, followed by 2 rounds of transfection of the siRNA specifically against CSN8 (siCSN8) or luciferase (siLuci) for 96 hrs, and then treated with bafilomycin A1 (BFA; 100 nM) for another 2 hrs. Total cell lysates were subjected to western blot analyses for the indicated proteins. Representative images (A) and pooled quantitative data (B) are shown. *p<0.05; N.S., not significant.
Online Figure VIII. A schematic illustration of the main findings. Normally, terminally misfolded proteins are polyubiquitinated by cullin-RING ligases (CRLs) and subsequently degraded by the proteasome. When polyubiquitinated misfolded proteins are not timely degraded by the proteasome, they form aggregates which bind p62 via the poly-ubiquitin (Ub) chains. The bound p62 can recruit phagophores through interaction with LC3-II on the phagophores, which triggers autophagosome engulfment of the aggregates. Aggregates-loaded autophagosomes fuse with lysosomes to form autolysosomes whereby the aggregates are degraded by lysosomal enzymes. When CRLs are compromised by CSN8 hypomorphism (hypo) or MLN4924 treatment, terminally misfolded proteins are not timely ubiquitinated and degraded by the proteasome but rather undergo aberrant aggregation, which generates highly unstable and active intermediate oligomers that are toxic to the cell including inhibition of the proteasome, and result in increased formation of insoluble aggregates. These insoluble aggregates are less ubiquitinated than they would be when CRLs are not impaired; therefore they are less effective in binding p62 and in triggering autophagosome formation, thereby decreasing autophagic flux at the autophagosome formation end. Meanwhile, CSN8 deficiency impairs autophagosome-lysosome fusion and thereby decreases autophagic flux at the autophagosome removal end. CSN8 hypo, CSN8 hypomorphism.