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COP9 Signalosome Controls the Degradation of Cytosolic Misfolded Proteins and Protects Against Cardiac Proteotoxicity

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Abstract

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 (UPS) 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 (CRLs), thereby controlling ubiquitination of a large number of proteins; however, neither CSN nor CRLs are 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 haploinsufficiency ($CSN8^{hypo}$) mice were used. Myocardial neddylated forms of cullins were markedly increased and myocardial capacity of degrading a surrogate misfolded protein was significantly reduced by $CSN8^{hypo}$. When introduced into proteinopathic mice in which a bona fide misfolded protein $CryAB^{R120G}$ is overexpressed in the heart, $CSN8^{hypo}$ 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

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DISCLOSURE

The authors declare there is no conflict of interest to disclose.

proteins, and reduced levels of total ubiquitinated proteins and LC3-II in the heart. In cultured cardiomyocytes, both CSN8 knockdown and CRL 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) CRLs participate in degradation of cytosolic misfolded proteins.

Subject Terms

Cardiomyopathy; Heart Failure; Myocardial Biology; Genetically Altered and Transgenic Models

Keywords

COP9 signalosome; ubiquitin; autophagy; misfolded proteins; Cops8; desmin-related cardiomyopathy; ubiquitin-proteosome system genetics; proteasome; crystalin; proteotoxicity

INTRODUCTION

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;[3–5] 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 pre-amyloid 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;[8–11] conversely, PQC improvement via chaperone overexpression or enhancement of target proteolysis confers cardiac protection against proteotoxicity in experimental animals.[12–15] These experimental demonstrations are corroborated by clinical observations that a significant portion of cancer patients receiving proteasome inhibitors in their chemotherapy develop cardiac dysfunction or even heart failure.[16, 17] A significant role of UPS malfunction in cardiac pathogenesis is further underscored by the identification of dominant negative mutations in *TRIM63*, the gene encodes a Ub ligase (muscle ring finger 1), as a cause of human familial hypertrophic cardiomyopathy.[18] 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 two 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.[19] 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.[20] Hrd1 was recently confirmed to be pivotal to endoplasmic reticulum (ER) associated degradation of ER misfolded proteins in mammalian cardiomyocytes.[21] However, very little is known about the identity and regulation of Ub ligases responsible for ubiquitination and degradation of cytosolic misfolded proteins in mammals.[22]

The COP9 signalosome (CSN) is an evolutionarily conserved protein complex, playing an important role in regulating the catalytic dynamics of cullin-RING Ub ligases (CRLs).[23] By estimate, CRLs are responsible for ~20% of Ub-dependent degradation of cellular proteins.[24] CRLs are activated by covalent conjugation of a Ub-like protein NEDD8 (Neural precursor cell expressed developmentally down-regulated 8) to cullin proteins via a ubiquitination-like process known as neddylation which is catalyzed by the NEDD8 activating enzyme (NAE), conjugating enzyme, and ligases.[25] CSN-mediated cullin deneddylation is essential to CRL catalytic dynamics in vivo, likely by promoting exchange of substrate receptors of CRLs.[26] The deneddylation activity of CSN requires a holo-complex formed by all 8 subunits (CSN1 through CSN8). By regulating CRLs activities, CSN participates in the regulation of many cellular processes including cell cycle control, DNA repair, gene expression, apoptosis and signaling transduction.[27] We have previously reported that conditional knockout of *cops8*, the gene encoding CSN8, in mouse hearts impairs cullin deneddylation and compromises myocardial UPS and ALP functions,[28, 29] which leads to rapidly deteriorated HF and mouse premature death, preventing them from being used for studying the role of CSN8/CSN in a chronic setting. Hence, the *Cops8* hypomorphic ($CSN8^{hypo}$) 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).[30] DRC 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.[30] Similar to other conformational disorders, protein misfolding and aggregation are identified as the proximal pathogenic factors to DRC.[6, 14, 31–33] Human DRM-linked R120G missense mutation of CryAB ($CryAB^{R120G}$) has proven to be a *bona fide* misfolded cytosolic protein.[34] Mice with cardiomyocyte-restricted transgenic (tg) expression of $CryAB^{R120G}$ develop cardiomyopathy and HF, recapitulating human DRC.[35] Hence, the $CryAB^{R120G}$ tg mice represent a highly relevant animal model of HF,[36] especially for studying cardiac PQC and proteotoxicity.[6, 13, 14, 31–33]

Here we report that $CSN8^{hypo}$ mouse hearts display decreased deneddylation and significantly reduced performance to degrade a surrogate misfolded protein. Myocardial CSN8 is significantly upregulated in $CryAB^{R120G}$ tg mice. When introduced into the $CryAB^{R120G}$ tg mice, $CSN8^{hypo}$ aggravated the $CryAB^{R120G}$-based DRC. Further

experimentation reveals that CSN8 deficiency suppresses the ubiquitination and degradation of CryAB^{R120G}, resulting in accumulation of protein aggregates and exacerbation of CryAB^{R120G} cytotoxicity; similarly, inactivation of CRLs via inhibiting neddylation stabilizes CryAB^{R120G} 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 Supplement.

Mouse models

Mice with *Cops8* conditionally targeted alleles were previously described.[37] Briefly, *Cops8*^{neoflox} allele contains a neomycin resistant 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 GFPdgn 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 cardiomyocytes (NRVMs) cultures and adenoviral delivery

Primary NRVMs culture and adenoviral delivery of CryAB^{R120G} were performed as reported.[39]

SiRNA transfection

To knock down the target gene expression, the Lipofectamine™-2000 transfection reagent (Invitrogen) was used for siRNA 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 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.

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 $CSN8^{neoflox/+}$ control (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, 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 to the CTL (Online Table I). Neither increased mortality nor gross abnormality was discerned in $CSN8^{hypo}$ mice by 1-year-of-age, the longest time monitored.

Impaired deneddylation activity in $CSN8^{hypo}$ mice

In $CSN8^{hypo}$ 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 $CSN8^{hypo}$ increased neddylated forms of cullin (Cul) 1, 2, 3, and 4a (Figure 1D and 1E). Notably, $CSN8^{hyop}$ also increased the neddylated forms of many other non-cullin proteins (Figure 1F and 1G), indicating that CSN has a broad range of deneddylation substrates. Together, these data demonstrate that $CSN8^{hypo}$ 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 green fluorescence protein (GFP) with degron CL1, a 17-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;[25] hence, GFPdgn is considered a surrogate misfolded protein.[40] To assess whether $CSN8^{hypo}$ affects UPS-mediated degradation of misfolded proteins in vivo, we employed GFPdgn tg mice, in which GFPdgn is ubiquitously expressed.[38] By cross-breeding tg GFPdgn into the CTL or $CSN8^{hypo}$ mice, we found myocardial GFPdgn protein levels were significantly accumulated in $CSN8^{hypo}$ 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 appear to arise from alterations in proteasome activities because all three proteasome peptidase activities were comparable between CTL and $CSN8^{hypo}$ 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^{R120G} (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 line 11 is greater than in line 134.[35] To determine the role of CSN8/CSN upregulation in DRC mice, we crossbred the CSN8^{hypo} mice with CryAB^{R120G} tg mice, a *bona fide* model of cardiac proteinopathy with defined disease progression.[14, 32, 35] We obtained a cohort of mice with a genotype of CTL, CSN8^{hypo}, CTL::CryAB^{R120G}, or CSN8^{hypo}::CryAB^{R120G} and performed a Kaplan-Meier survival analysis which revealed that CSN8^{hypo} 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^{R120G} mice displayed a cardiac functional phenotype characteristic of compensatory restrictive cardiomyopathy, as evidenced by marked decreases in left ventricular (LV) internal diameters and volumes at the end of diastole. These abnormalities were further augmented in CSN8^{hypo}::CryAB^{R120G} mice (Table 1).

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^{hypo}, western blot analyses showed that myocardial total NEDD8 conjugate levels were increased by over 60% ($p<0.05$) in CSN8^{hypo}::CryAB^{R120G} mice, compared with the CTL::CryAB^{R120G} mice (Figure 3D and 3E). Since protein aggregation is a causative pathogenic factor of DRC, we then sought to determine if the exacerbation of DRC in CSN8^{hypo} mice was associated with altered protein aggregation. Immunofluorescence confocal microscopy revealed that CryAB-positive protein aggregates were significantly increased by CSN8^{hypo} in DRC mouse hearts (Figure 4A and 4B). Consistently, filter-trap assays also showed a substantially increase in detergent-resistant CryAB-positive aggregates in the myocardium from CSN8^{hypo}::CryAB^{R120G} 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 CSN8^{hypo}::CryAB^{R120G} 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 CryAB^{R120G} in cultured cardiomyocytes

To further test if CSN8^{hypo}-induced accumulation of protein aggregates in DRC mouse hearts is due to impaired degradation of misfolded CryAB^{R120G}, we assessed the impact of CSN8 down-regulation on the protein stability of HA-tagged CryAB^{R120G} overexpressed in cultured cardiomyocytes. In NRVMs, CSN8 knockdown (CSN8KD) by small interfering RNA (siRNA) substantially increased the steady-state protein levels of CryAB^{R120G} 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

CryAB^{R120G}-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 CryAB^{R120G}, we further performed a cycloheximide chase experiment. We found that CSN8KD substantially prolonged the half-life of CryAB^{R120G} (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 CryAB^{R120G}, a *bona fide* cytosolic misfolded protein, and promotes protein aggregation in cardiomyocytes.

CSN8/CSN and CRLs control CryAB^{R120G} ubiquitination and degradation

CSN is known to control the stability of a number of native proteins by regulating their respective CRLs' activity.[27] We next tested if CSN could regulate the ubiquitination of a *bona fide* misfolded protein. Immunoprecipitation of HA-CryAB^{R120G} followed by western blot analyses showed that proteasome inhibition by MG132 largely increased high-molecular-weight species of CryAB^{R120G}. Probing the immunoprecipitates with anti-Ub antibodies identified these high-molecular-weight species as ubiquitinated forms of CryAB^{R120G}. 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 CryAB^{R120G} and that CSN8/CSN is required for CryAB^{R120G} ubiquitination. Since 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 hypothesize that CRLs are responsible for the ubiquitination of CryAB^{R120G}. To examine this hypothesis, we assessed the effect of CRLs inactivation via inhibiting their neddylation using MLN4924, a specific NAE inhibitor.[24] We found that NAE inhibition significantly elongated the half-life of CryAB^{R120G} expressed in cultured NRVMs (Figure 6B and 6C). These results demonstrate that the activation of CRLs is required for the degradation of CryAB^{R120G}, suggesting that CSN controls CryAB^{R120G} 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.[29, 41] Hence, we examined these parameters in CSN8^{hypo} hearts but we found none of them were discernibly altered at baseline (Online Figure IV), suggesting that autophagic activity is not perturbed in CSN8^{hypo} mouse hearts under basal condition. Myocardial LC3-II, p62, and autophagic activity are known to increase in DRC mice.[33, 39] When coupled with the CryAB^{R120G}-based DRC, CSN8^{hypo} significantly suppressed the increase of LC3-II, but not that of p62, in the heart (Figure 7A and 7B), suggesting that CSN8^{hypo} 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 (MEFs) in which autophagic flux was monitored using the tandem fluorescence protein-fused LC3 (tf-LC3) as a reporter.[42] During autophagic activation triggered by simulated starvation, autophagosome-lysosome fusion impairment was detected in CSN8^{hypo} MEFs (Online Figure VI). Moreover, CSN8KD significantly decreased LC3-II flux in NRVMs overexpressing CryAB^{R120G} (Online Figure VII). Taken together, these results indicate that impairment of autophagic flux by CSN8^{hypo} becomes discernible only when the demand for autophagy is elevated. This also implicates that UPS impairment in Csn8^{hypo} 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 heart failure in DRC.[31, 32, 39] Given the critical role of CSN8/CSN in the removal of misfolded protein CryAB^{R120G}, we then tested if CSN8/CSN protects cardiomyocytes from proteotoxic stress-induced cytotoxicity. The proteotoxic stress was imposed to cultured cardiomyocytes by overexpression of CryAB^{R120G}. Cell death and cell viability were respectively assessed with the lactate dehydrogenase (LDH) leakage assay and the MTT assay. CSN8KD did not affect the survival of cardiomyocytes in basal condition. However, expression of CryAB^{R120G} significantly increased cytotoxicity, as evidenced by a significant increase of LDH activity in media and a decrease of cell viability. CSN8 depletion further aggravated the CryAB^{R120G}–induced cytotoxicity, as revealed by a ~70% increase of LDH release and a ~54% decrease of MTT reading compared to CryAB^{R120G}-expressed cardiomyocytes (Figure 7C and 7D). These data indicate that CSN8-deficient cardiomyocytes are more susceptible to proteotoxicity.

DISCUSSION

Aberrant protein aggregation is best exemplified in DRC but it is also implicated in the heart of humans with CHF of common causes,[6, 7] suggesting that cardiac proteotoxicity is pathogenic in a large subset of cardiac disease. Indeed, improving UPS performance was recently shown to protect against not only DRC but also myocardial ischemia/reperfusion injury.[14] 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 non-cardiac disease. Here we have shown that CSN8^{hypo} impairs the ubiquitination and degradation of a surrogate misfolded protein (GFPdgn) as well as a *bona fide* misfolded protein CryAB^{R120G}, 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.

CSN8 is an indispensable subunit of the CSN holoenzyme for cullin deneddylation;[23] 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^{hypo} mice do not display abnormal phenotypes for at least the first 6 months of age. Nevertheless, CSN8^{hypo} exacerbates cardiac proteinopathy and reduced ubiquitination

emerges as a primary defect caused by CSN8^{hypo} during proteotoxic stress. This is supported by (1) myocardial UPS performance in CSN8^{hypo} mice was decreased without altering proteasomal activities; (2) Ub conjugates were markedly decreased in both protein aggregates and total myocardial protein extracts from CryAB^{R120G}::CSN8^{hypo} mice; and (3) CSN8KD reduced ubiquitinated forms of CryAB^{R120G} in cultured NRVMs.

Ubiquitination not only is essential to proteasomal degradation but also can indirectly promote ALP-mediated degradation.[19, 20] Based on the prevalent model, when escaped form 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^{hypo}-derived defect in ubiquitination reduces primarily UPS performance but does not affect ALP activity at baseline; however, CSN8^{hypo} discernibly limits autophagic activity under a stress condition (e.g., 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^{hypo} significantly decreased LC3-II protein levels in CryAB^{R120G} 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 (e.g., reduced autophagic flux) and destabilizing factors (e.g., reduction of Ub chains in the aggregates) counter each other, resulting in unaltered p62 protein levels in CSN8^{hypo}::CryAB^{R120G} hearts, compared with CTL::CryAB^{R120G} hearts.

In cardiomyocytes, the degradation of overexpressed misfolded proteins such as CryAB^{R120G} depends on both UPS and ALP.[13, 14, 19, 20] CSN8^{hypo} 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^{R120G} contribute to the exacerbation of protein aggregation and disease progression in DRC mouse hearts by CSN8^{hypo}.

The specificity of ubiquitination is determined principally by Ub ligases. In mammals, HRD1, Parkin, and CHIP (C-terminus of Hsp70-interacting protein) have been shown to serve as the Ub ligases of the endoplasmic reticulum (ER) associated degradation (ERAD), responsible for ubiquitination of misfolded proteins retro-translocated from the ER 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 so far 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^{hypo} 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 lastly, 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.[43] 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^{hypo} 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^{R120G}, resulting in accumulation of protein aggregates and exacerbation of CryAB^{R120G} 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 due to genetic mutations or acquired causes are highly conceivable and, in some cases, well-demonstrated in cardiac remodeling and heart failure.[1, 2, 6] However, presently there is no treatment specifically aiming at enhancing degradation of misfolded proteins in the heart. Meanwhile, the firs-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]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms

ALP	the autophagic-lysosomal pathway
CHX	cycloheximide

CRLs	cullin-based RING ligases
CryAB	αB-crystallin
CSN	the COP9 signalosome
CSN8^{hypo}	CSN8 hypomorphism
DRC	desmin-related cardiomyopathy
GFP	green fluorescent protein
GFPdgn	GFP modified by carboxyl fusion of degron CL1
HF	heart failure
LDH	lactate dehydrogenase
LV	left ventricle
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
NEDD8	neural precursor cell expressed, developmentally down-regulated 8
NAE	NEDD8 activating enzyme
NEO	neomycin phosphotransferase II gene
PQC	protein quality control
siRNA	small interfering RNA
Ub	ubiquitin
UPS	the ubiquitin proteasome system

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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 *Cops8* impairs myocardial protein degradation by the ubiquitin-proteasome system (UPS) and autophagic-lysosomal pathway (ALP), 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 down-regulation 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.

UPS- and ALP- mediated protein degradation constitutes the last line of defense in protein quality control (PQC) 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 PQC 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 PQC 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 NEDD8 activating enzyme (NAE) 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 ALP. These findings suggest that both CSN8/CSN and CRLs may represent key targets for improving cardiac PQC and caution that NAE inhibition may yield cardiac toxicity.

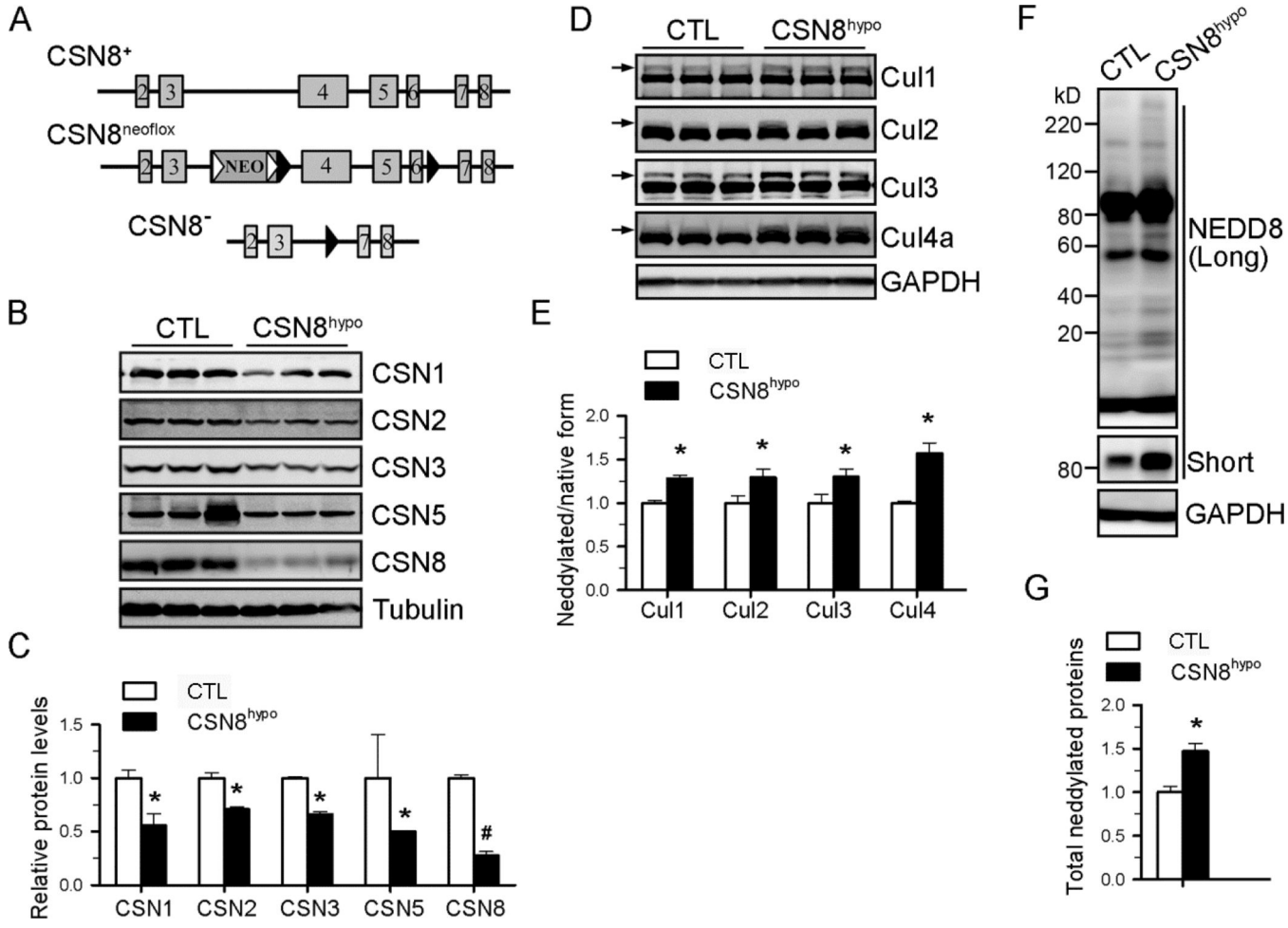

Figure 1. Decreased deneddylation activity in CSN8$^{neoflox/-}$ (CSN8^{hypo}) mouse hearts

Age-matched littermate CSN8$^{neoflox/+}$ mice were used as controls (CTL). (**A**) *Csn8*-targeted alleles described in this study. The numbered light grey rectangles denote the exons. In the *neoflox* allele (CSN8^{neoflox}), a neomycin phosphotransferase II gene (NEO) flanked by *Frt* sites (empty triangles) are inserted between exon 3 and 4; and exons 4 through 6 are flanked by *LoxP* sites (solid triangles). In CSN8 knockout allele (CSN8^{-}), the NEO cassette and exons 4 through 6 are deleted by FLP- and Cre- mediated recombination. (**B, C**) Western blot analyses of indicated proteins. Representative images (B) and pooled densitometry data (C) are shown. GAPDH serves as a loading control. (**D, E**) Representative images (D) and pooled densitometry data (E) from western blot analyses of indicated cullin proteins in mouse hearts. Arrows mark the neddylated form of cullins. (**F, G**) Representative images (F) and pooled densitometry data (G) from western blot analyses of neddylated proteins in mouse hearts. Total neddylated proteins (revealed by long-time exposure) and neddylated cullin (revealed by short-time exposure) are increased in CSN8^{hypo} hearts. N=3 mice/group; **p*<0.05, #*p*<0.01 vs. CTL; *t*-test.

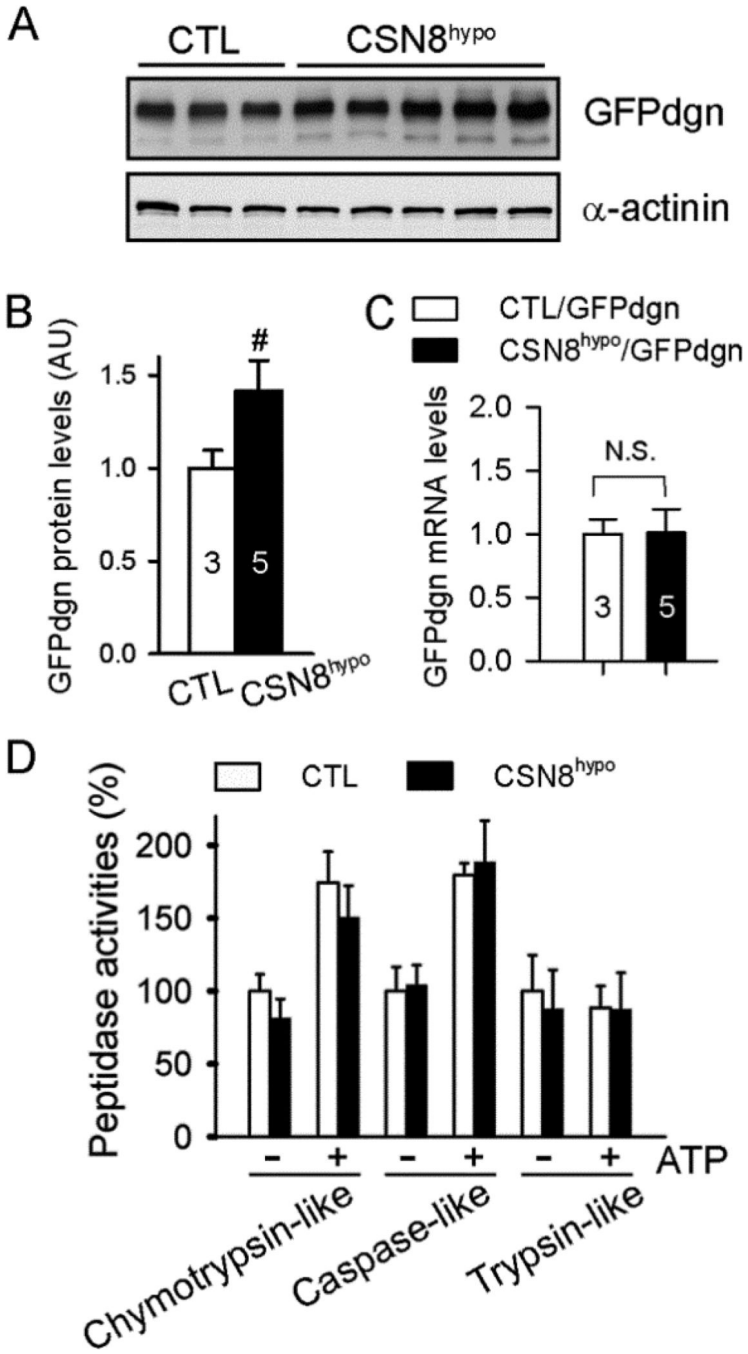

Figure 2. Effect of CSN8^{hypo} on myocardial UPS function

Mice were created via cross-breeding between CSN8$^{neoflox/neoflox}$ and CSN8$^{+/-}$::GFPdgn mice. The resultant 8-week-old CTL::GFPdgn mice and CSN8^{hypo}::GFPdgn mice were used. (**A, B**) Images (A) and pooled densitometry data (B) from western blot analyses for myocardial GFPdgn. Alpha-actinin was probed as a loading control. n= 3 or 5 mice per group, #$p < 0.01$ vs. CTL. (**C**) GFPdgn mRNA levels are assessed by real time RT-PCR using total RNA extracted from mouse ventricular myocardium. (**D**) Proteasomal peptidase activity assays of ventricular myocardium. n=4~6 per group, *t*-test.

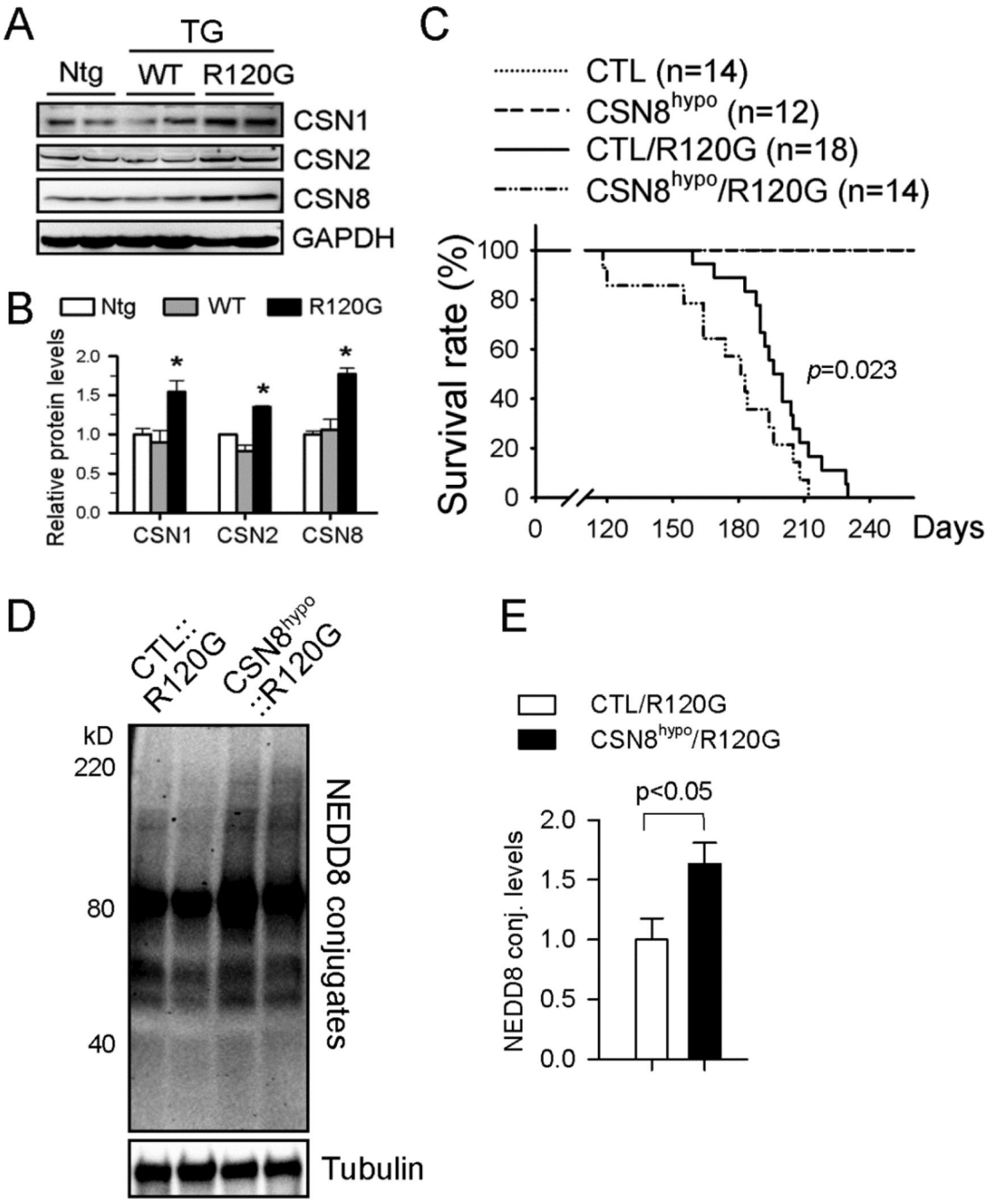

Figure 3. DRC exacerbation by CSN8^{hypo}

(**A, B**) Western blot analysis for the indicated CSN subunits in mouse hearts with tg overexpression of wild type CryAB (WT) or CryAB^{R120G} (R120G). Representative images (A) and pooled densitometry data (B) are shown. n=4 mice/group; *p<0.05 vs. Ntg or WT; ANOVA followed by the Scheffé test. (**C**) Kaplan-Meier survival analysis of a cohort of mixed-sex littermate CTL, CSN8^{hypo}, CTL::CryAB^{R120G}, and CSN8^{hypo}::CryAB^{R120G} mice. Log-rank test. (**D, E**) Representative image (D) and pooled densitometry data (E) of

western blot analysis for myocardial NEDD8 conjugates (conj.) of mice of indicated genotypes at 8 weeks (n=4 mice/group); *t*-test.

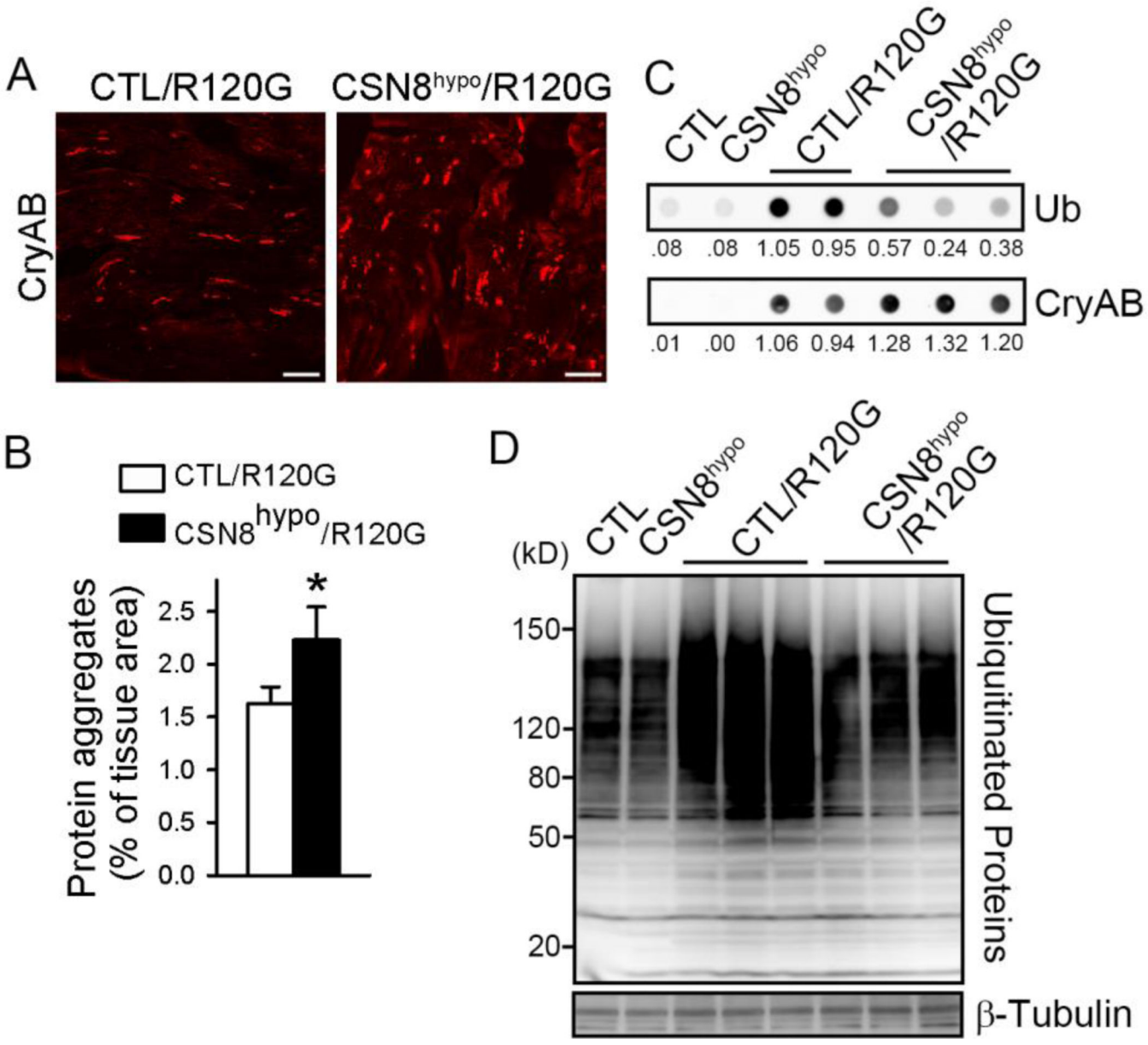

Figure 4. CSN8^{hypo} aggravates CryAB^{R120G}-induced aberrant protein aggregation and decreases the ubiquitination in mouse hearts

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^{R120G}, 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.

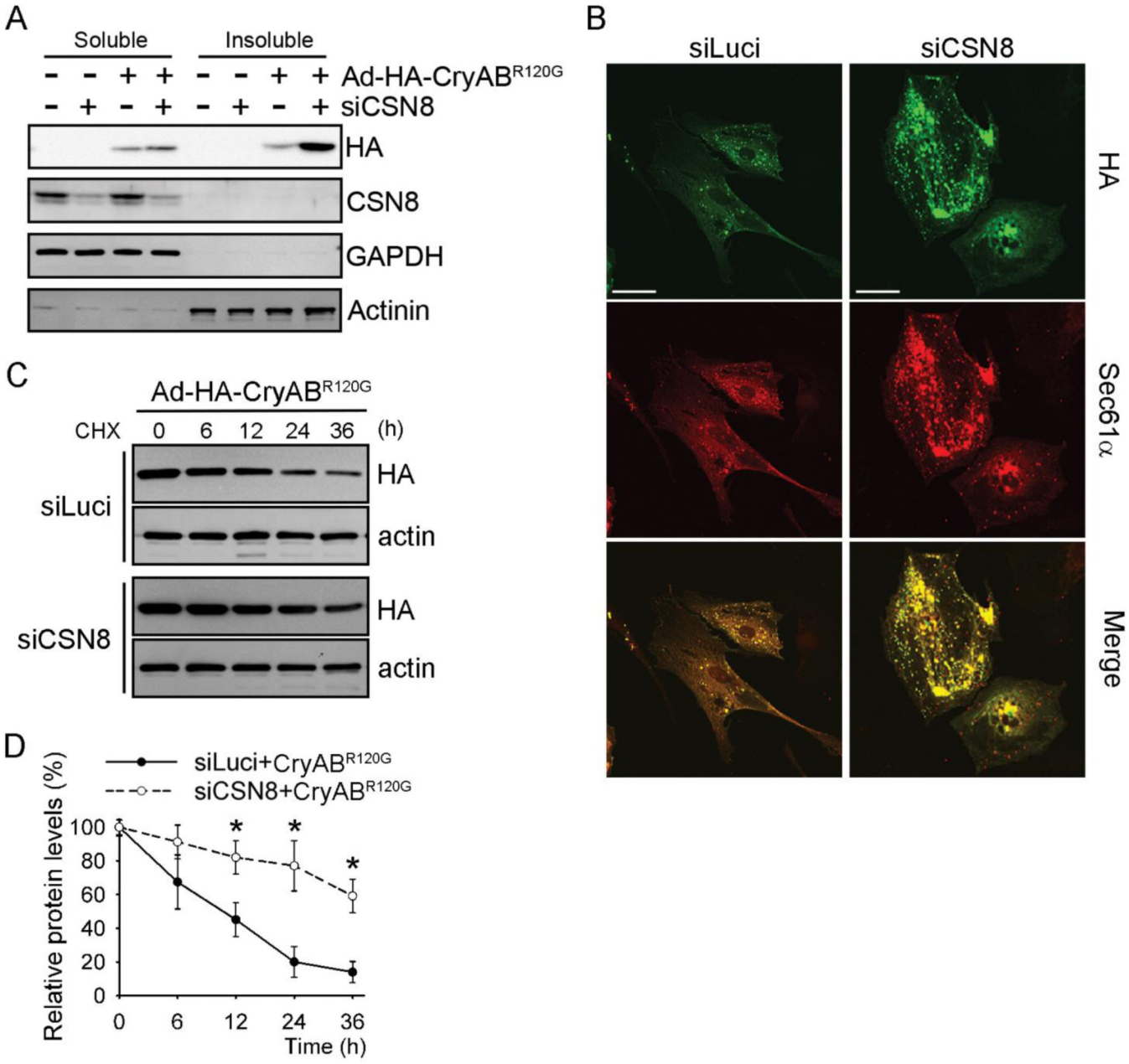

Figure 5. CSN8 knockdown stabilizes CryAB^{R120G} in cardiomyocytes

NRVMs were infected with adenoviruses expressing HA-CryAB^{R120G} (Ad-HA-CryAB^{R120G}) or β-Gal as indicated. The cells were also transfected with siRNAs against either luciferase (siLuci) or CSN8 (siCSN8). At 72 hours after the siRNA transfection, the cells were harvested for the analyses (A, 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^{R120G} and aggresomes, respectively. Scale bar=50 μm. (**C, D**) Cycloheximide

(CHX) chase assay for HA-CryAB^{R120G}. HA-CryAB^{R120G} 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^{R120G} (D) are shown; *p<0.05 vs. the siLuci+CryAB^{R120G} group, n=3 repeats; t-test.

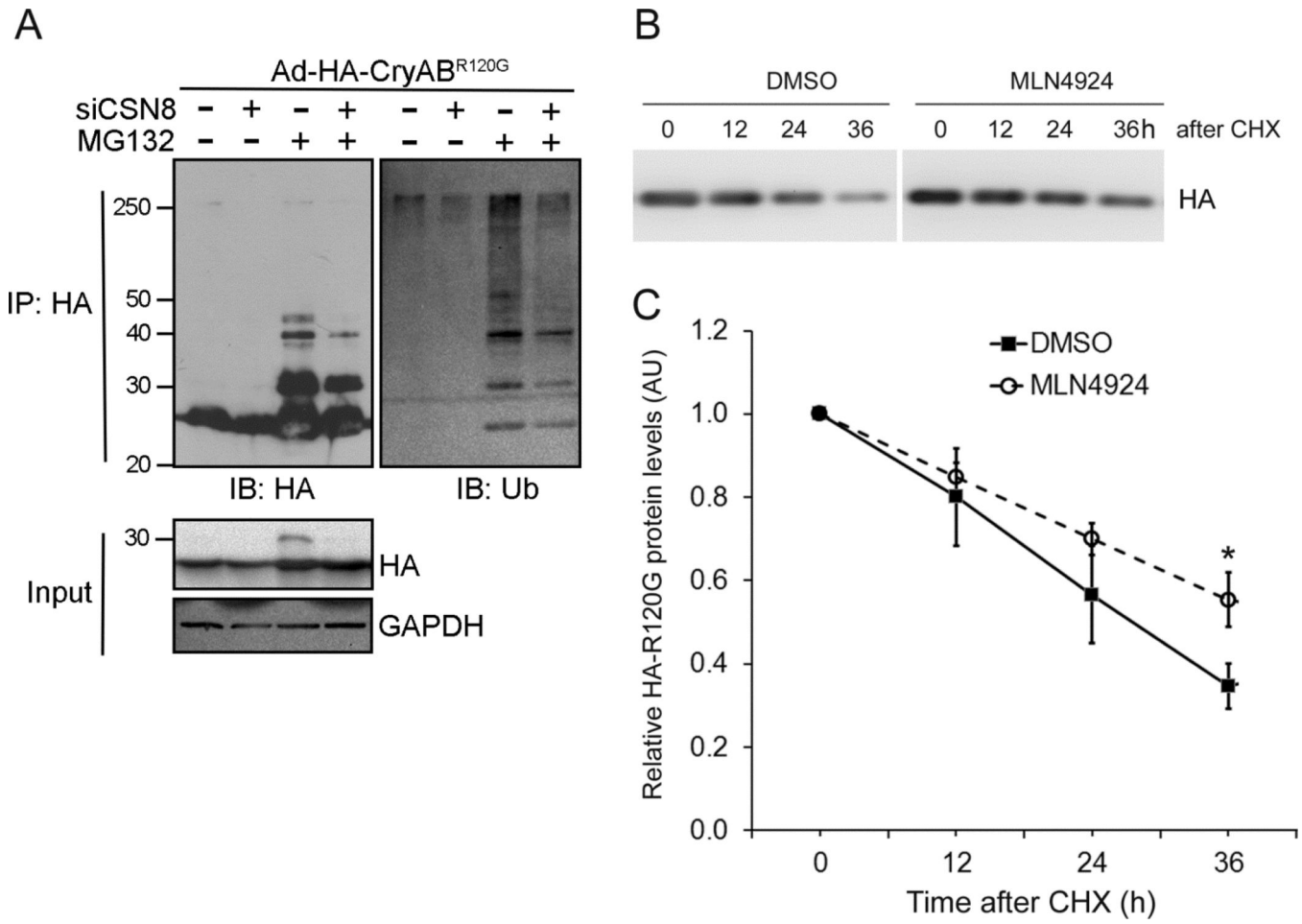

Figure 6. Inhibition of CRLs impairs CryAB^{R120G} ubiquitination and degradation in cardiomyocytes

(**A**) CSN8 knockdown impairs CryAB^{R120G} ubiquitination. NVRMs were treated as described in Figure 5. Seventy-two hours after 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-CryAB^{R120G} are shown. (**B, C**) MLN4024 stabilizes CryAB^{R120G} in cultured NRVMs. MLN4924 (1μmol/L) or vehicle control (DMSO) treatment was initiated at 48 hours after Ad-HA-CryAB^{R120G} infection in cultured NRVMs. Cycloheximide (CHX, 50μmol/L) was added to the culture media at 30min 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-CryAB^{R120G} are shown. *p<0.05 vs. the DMSO group, n=3 repeats; t-test.

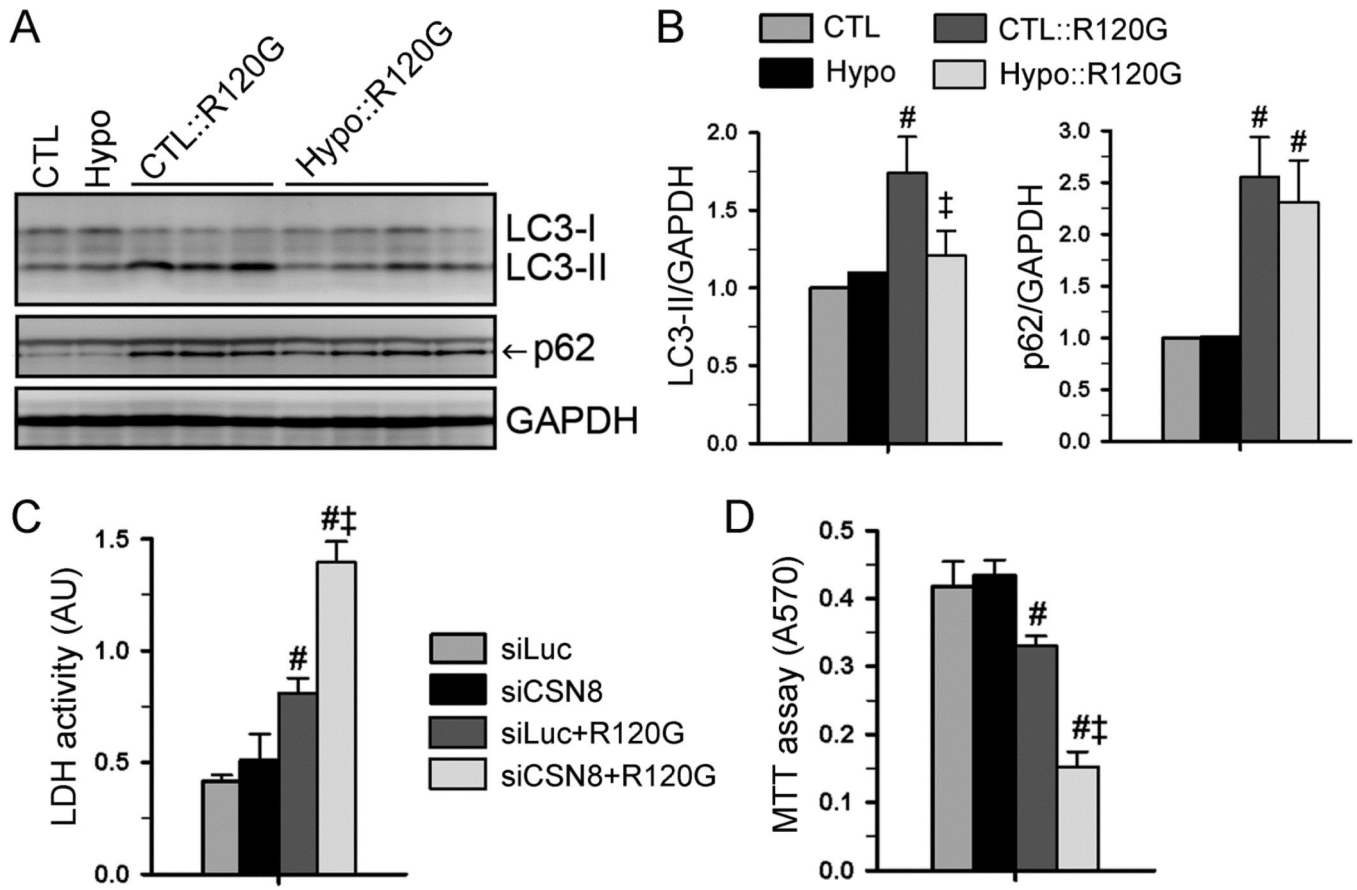

Figure 7. CSN8 deficiency mitigates autophagic responses in mouse hearts and augments proteotoxicity in cardiomyocytes

(**A, B**) Western blot analyses for myocardial LC3 and p62. CSN8 hypomorphism was cross-bred into CryAB^{R120G} (R120G) mice. Ventricular myocardial total protein extracts from 8-week-old mice were 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, D**) CSN8 knock-down augments CryAB^{R120G}-induced cardiomyocyte injury. NRVMs were treated as described in Figure 5. The assays were performed 72 hours after siRNA transfection. LDH activities in the cultured medium (**C**) were measured to assess cell injury. Cell viability was assessed by the MTT assay (**D**). $^{\#}p$<0.01 vs. siLuci; ‡p<0.01 vs. siLuci+R120G; n=4~6. ANOVA followed by the Scheffé test.

Table 1

Echocardiographic Measurements at 12 Weeks of Age

	CTL n=6	CSN8^{hypo} n=8	CTL::CryAB^{R120G} n=10	CSN8^{hypo}::CryAB^{R120G} n=8
Body weight (g)	30.4±2.1	27.2±3.5	28.2±3.7	28.8±4.2
Heart rate (bpm)	471±24	487±42	432±43	472±43
LVIDd (mm)	4.37±0.35	4.09±0.14	3.90±0.26†	3.54±0.28†‡
LVPWd (mm)	0.70±0.13	0.73±0.13	0.93±0.15†	0.88±0.06*
LVIDs (mm)	3.17±0.26	3.12±0.21	2.45±0.22†	1.89±0.22†‡
LVPWs (mm)	1.13±0.12	1.09±0.24	1.45±0.27†	1.44±0.14†
LVAWd (mm)	0.71±0.08	0.67±0.12	1.00±0.20†	0.97±0.19†
LVAWs (mm)	1.15±0.12	1.03±0.27	1.52±0.25†	1.55±0.21†
LV FS (%)	30.02±2.48	28.59±2.00	37.84±5.93*	45.07±4.11†‡
LV EF (%)	53.83±5.68	58.55±5.52	65.19±8.95*	77.25±4.20†‡
LVVd (mm^3)	87.58±15.20	77.33±2.40	66.20±10.92†	49.59±11.02†‡
LVVs (mm^3)	40.28±8.18	41.29±6.18	22.28±9.46†	11.31±3.41†‡
SV (μl)	47.3±10.3	35.2±6.2	43.9±6.7	38.3±8.6
CO (ml/min)	22.3±4.9	17.2±3.9	19.0±4.3	18.1±4.2

LVIDd, end-diastolic left ventricular (LV) internal diameter; LVIDs, end-systolic LVID; LVPWd, end-diastolic LV posterior wall thickness; LVPWs, end-systolic LVPW; LVAWd, end-diastolic LV anterior wall thickness; LVAWs, end-systolic LVAW; FS, fractional shortening; EF, ejection fraction; LVVd, end-diastolic LV volume; LVVs, end-systolic LVV; SV, stroke volume; CO, cardiac output.

* $p<0.05$,

† $p<0.01$ vs. CTL;

‡ $p<0.05$ vs. CTL::CryAB^{R120G}; ANOVA followed by the Scheffé test.