Online Supplement
to

The COP9 signalosome controls the degradation of cytosolic misfolded proteins and protects against cardiac proteotoxicity

Su, The COP9 promotes misfolded protein degradation

Huabo Su, Jie Li, Hanming Zhang, Wenxia Ma, Ning Wei, Jinbao Liu*, Xuejun Wang*

From: State Key Laboratory of Respiratory Disease and Department of Pathophysiology, Guangzhou Medical University, Guangzhou, Guangdong, China (H. S., J. Liu, X. W.); Division of Basic Biomedical Sciences, Sanford School of Medicine of the University of South Dakota, Vermillion, South Dakota 57069, USA (H. S., J. Li, H. Z., J. Liu, X. W.); Vascular Biology Center (H. S., J. Li, W. M.) and Department of Pharmacology and Toxicology (H. S.), Medical College of Georgia, Georgia Regents University, Augusta, Georgia 30912, USA; Department of Molecular, Cellular and Developmental Biology, Yale University, New Haven, Connecticut 06520, USA (N. W.).

*Correspondence: Dr. Xuejun Wang, Division of Basic Biomedical Sciences, Sanford School of Medicine of the University of South Dakota, 414 East Clark Street, Lee Medical building, Vermillion, SD 57069, USA, Tel: (605) 658-6345; Fax: (605)677-6381; e-Mail: xuejun.wang@usd.edu; or Dr. Jinbao Liu, State Key Laboratory of Respiratory Diseases and Department of Pathophysiology, Guangzhou Medical University, Guangzhou, Guangdong, China, Tel: +86 20 81340720; Fax: +86 20 81340542, e-mail: jliu@gzhmu.edu.cn

SUPPLEMENTAL METHODS

Mouse models

Mice with *Cops8* conditionally targeted alleles were previously described.[1] Briefly, *Cops8*^{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 *Cops8*$^{neoflox/+}$ and *Cops8*$^{+/-}$ mice were backcrossed into FVB/N background for at least six generations. The homozygous *Cops8*$^{neoflox/neoflox}$ mice were then mated with *Cops8*$^{+/-}$ mice to produce *Cops8*$^{neoflox/-}$ and *Cops8*$^{neoflox/+}$ mice, which were used as CSN8 hypomorphic (CSN8^{hypo}) mice and control mice (CTL), respectively. The FVB/N CryAB^{R120G} transgenic (tg) mice and FVB/N GFPdgn tg mice were described previously.[2, 3] 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.[4] 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.[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 $MgCl_2$) (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 $2^{-\Delta\Delta ct}$ 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^2 plates at a density of 1.5×10^6 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-$CryAB^{R120G}$ 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 2×10^6 NRVMs in each 60-mm dish. The Lipofectamine™-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'-AACGTACGCGGAATACTTCGA-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 wlel 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

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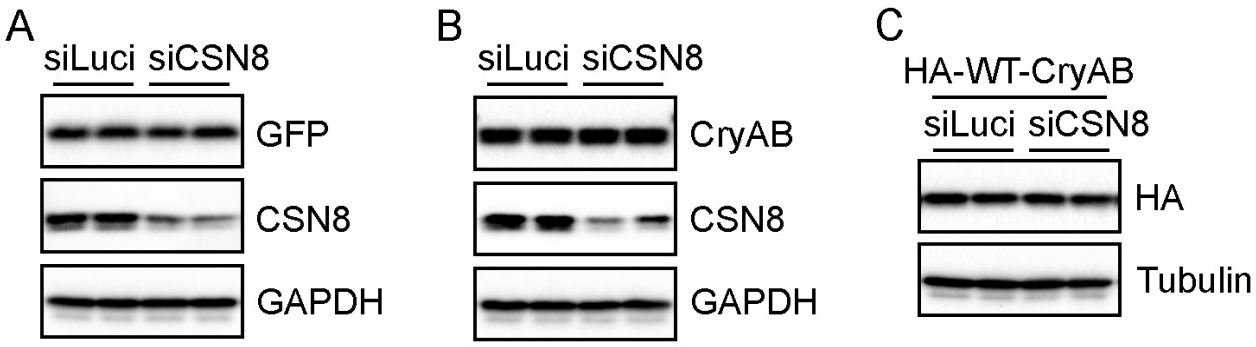
2. Wang X, Osinska H, Klevitsky R, Gerdes AM, Nieman M, Lorenz J, Hewett T, Robbins J. Expression of r120g-alphab-crystallin causes aberrant desmin and alphab-crystallin aggregation and cardiomyopathy in mice. *Circ Res*. 2001;89:84-91.
3. Kumarapeli AR, Horak KM, Glasford JW, Li J, Chen Q, Liu J, Zheng H, Wang X. A novel transgenic mouse model reveals deregulation of the ubiquitin-proteasome system in the heart by doxorubicin. *FASEB J*. 2005;19:2051-2053.
4. Zheng Q, Su H, Ranek MJ, Wang X. Autophagy and p62 in cardiac proteinopathy. *Circ Res*. 2011;109:296-308.
5. Su H, Li J, Menon S, Liu J, Kumarapeli AR, Wei N, Wang X. Perturbation of cullin deneddylation via conditional csn8 ablation impairs the ubiquitin-proteasome system and causes cardiomyocyte necrosis and dilated cardiomyopathy in mice. *Circ Res*. 2011;108:40-50.

Supplementary Data (1 Online Table and 8 Online Figures)

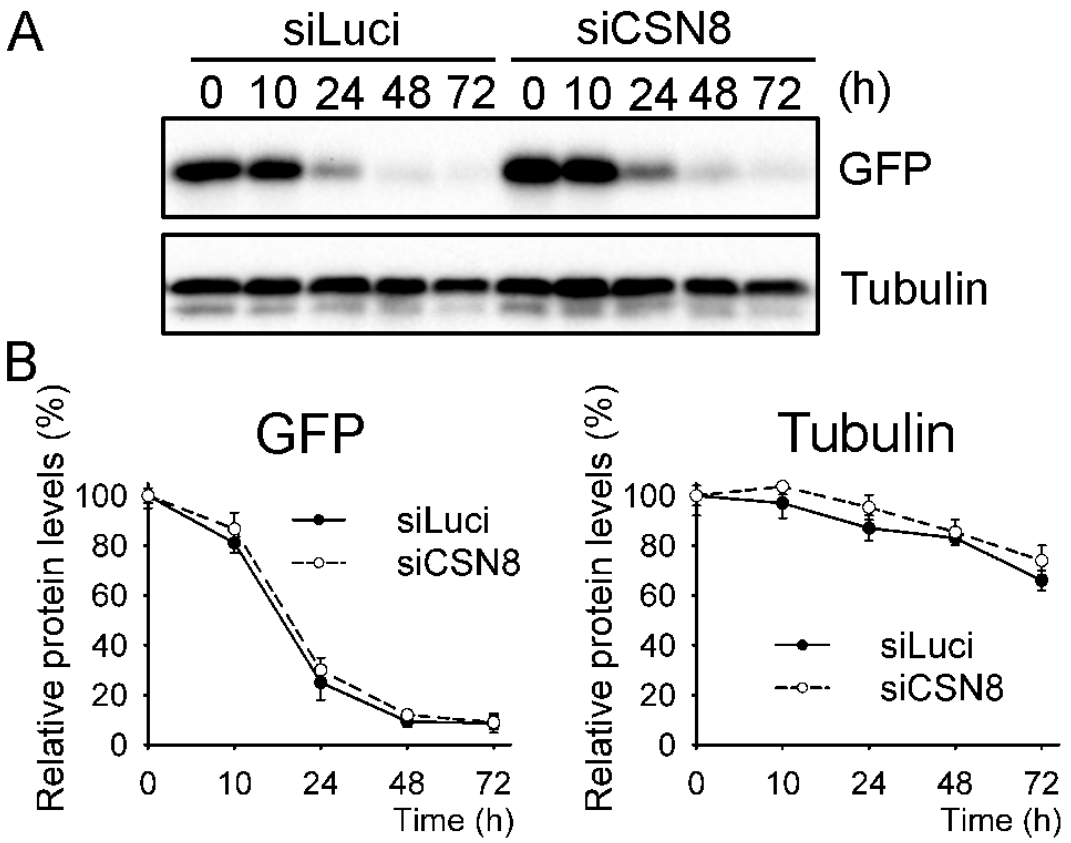
Online Table I. Echocardiographic Parameters at 6 months of Age

	CTL (n=7)	$CSN8^{hypo}$ (n=7)
Body Weight (BW, g)	29.3±7.1	31.8±7.3
Heart Rate (bpm)	460±68	489±76
LVID; d (mm)	4.14±0.19	4.11±0.23
LVPW; d (mm)	0.73±0.14	0.78±0.06
LVID; s (mm)	2.97±0.24	2.86±0.44
LVPW; s (mm)	0.95±0.19	1.18±0.22
IVS; d (mm)	0.75±0.15	0.80±0.09
IVS; s (mm)	1.11±0.25	1.18±0.16
LV %FS	30.81±6.10	30.22±10.58
LV %EF	59.88±7.66	56.64±15.30
LVVd (mm^3)	76.04±8.11	74.75±9.65
LVVs (mm^3)	35.01±6.96	32.21±11.29
SV (μl)	41.0±7.6	42.6±12.8
CO (ml/min)	19.1±5.5	21.3±9.1
LV Mass (mg)	109.9±27.5	121.4±17.1
LV Mass/BW (mg/g)	3.81±0.95	3.92±0.49

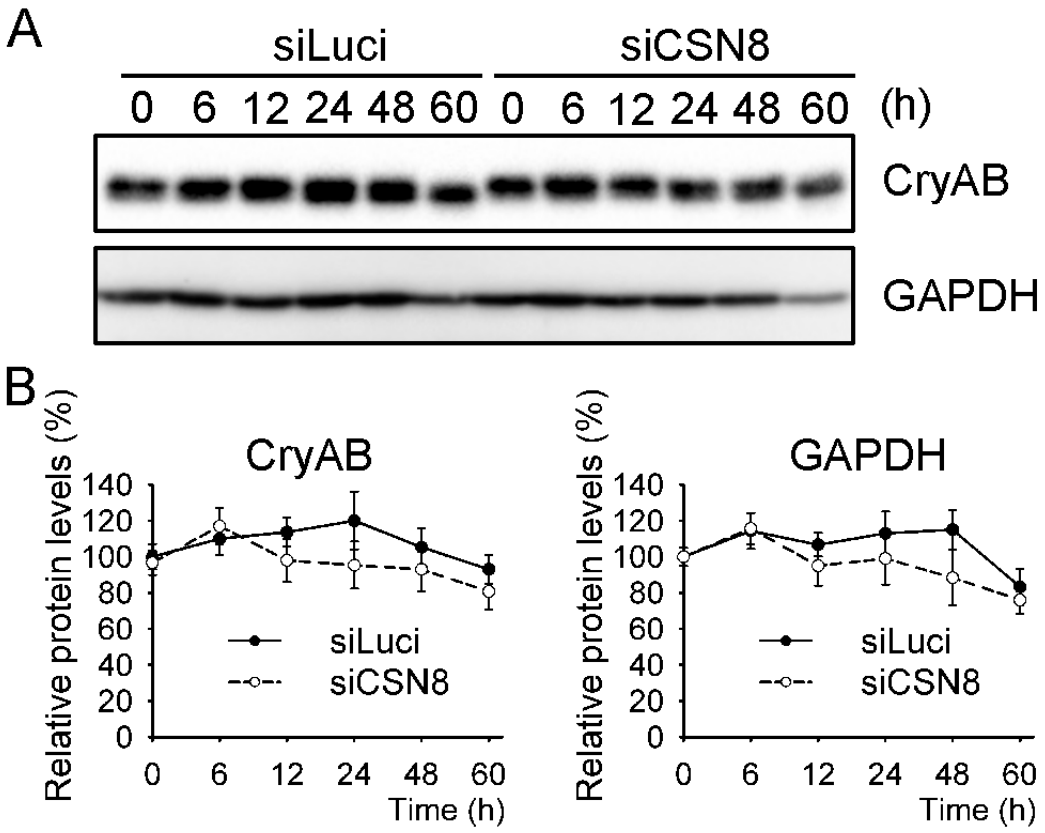
Student's *t*-tests show that the difference in each of the parameters between the two groups is not statistically significant ($p>0.05$). Mean ± STD.



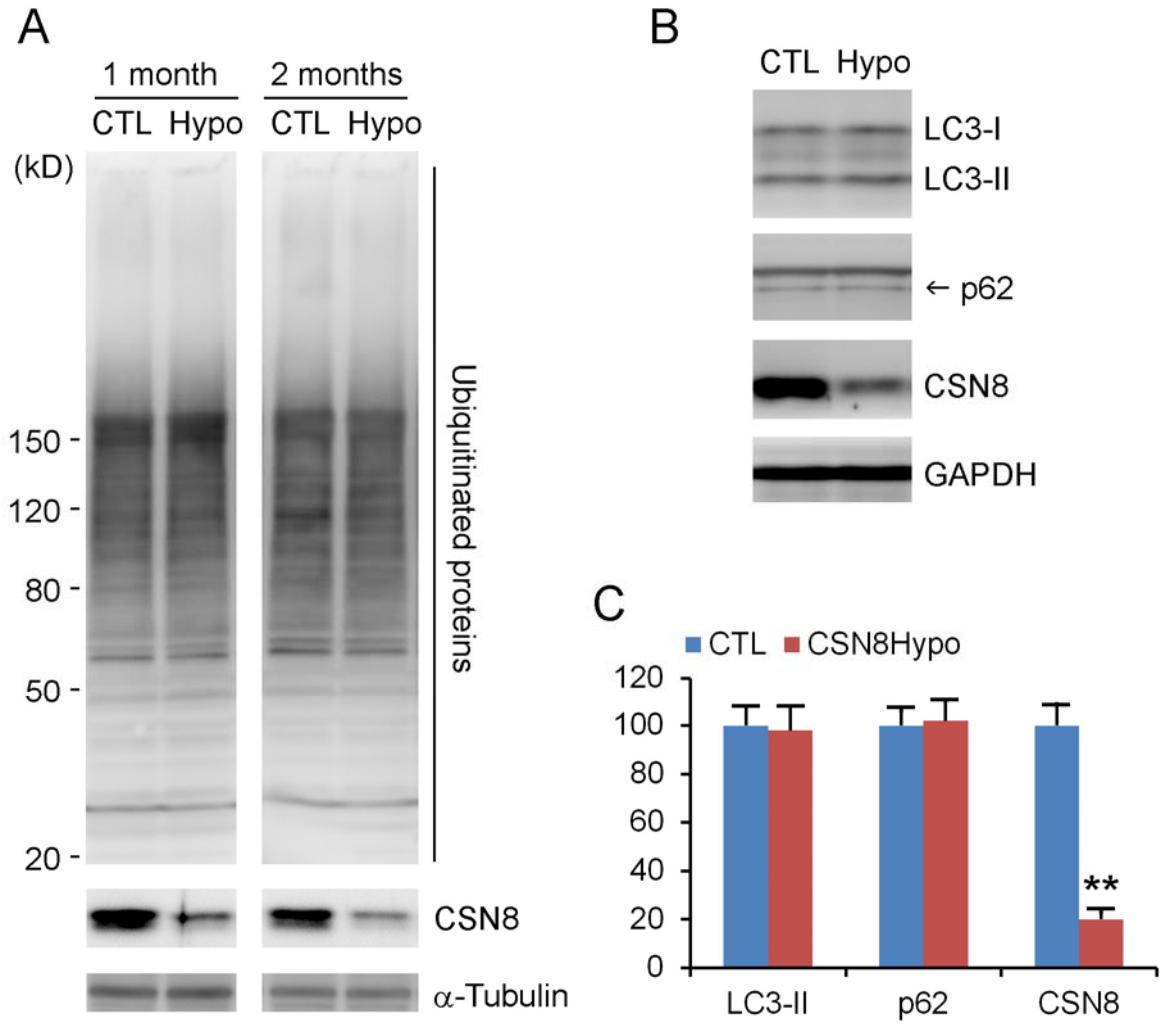
Online Figure I. CSN8 knockdown does not alter the steady state protein levels of endogenous CryAB or overexpressed wild type (WT-) CryAB or conventional GFP in cultured NRVMs.



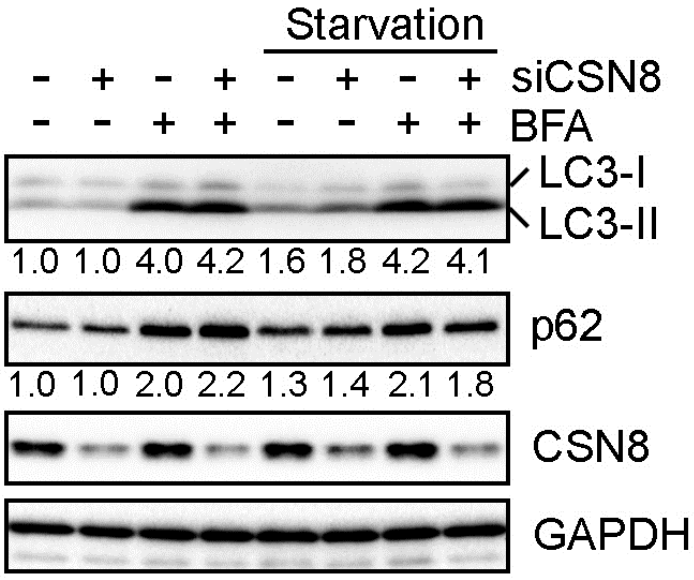
Online Figure II. Cycloheximide (CHX) chase assays showing that CSN8 knockdown does not slow down the degradation of conventional GFP proteins in cultured NRVMs. Represnetative western blot images (**A**) and pooled quantitative densitometry data (**B**) are shown; mean±STD, n=3 repeats, $p>0.05$ for each time point between the two groups, *t*-test.



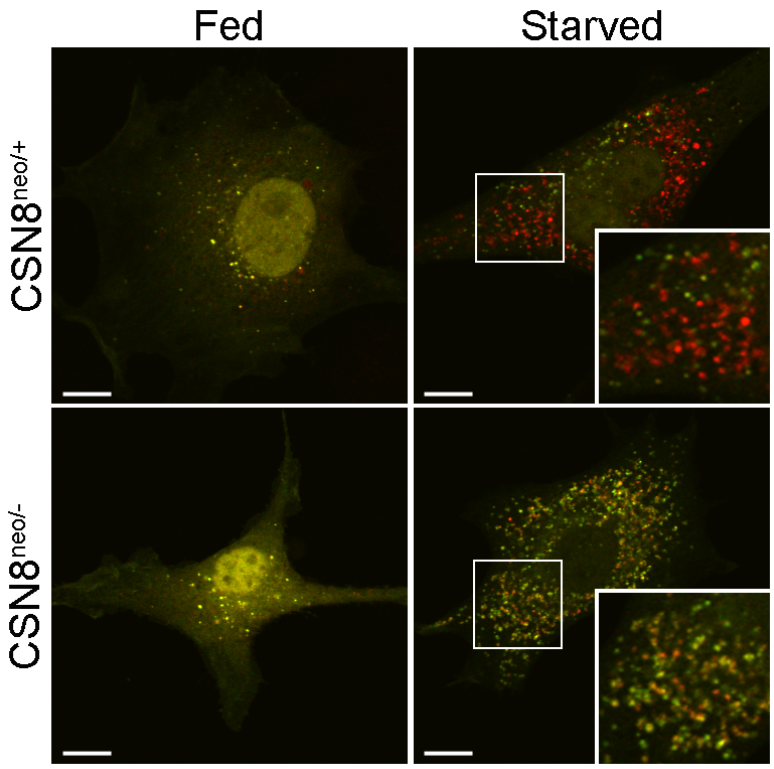
Online Figure III. Cycloheximide (CHX) chase assays showing that CSN8 knockdown does not slow down the degradation of endogenous CryAB proteins in cultured NRVMs. Represnetative western blot images (**A**) and pooled quantitative densitometry data (**B**) are shown; mean±STD, n=3 repeats, $p>0.05$ for each time point between the two groups, *t*-test.



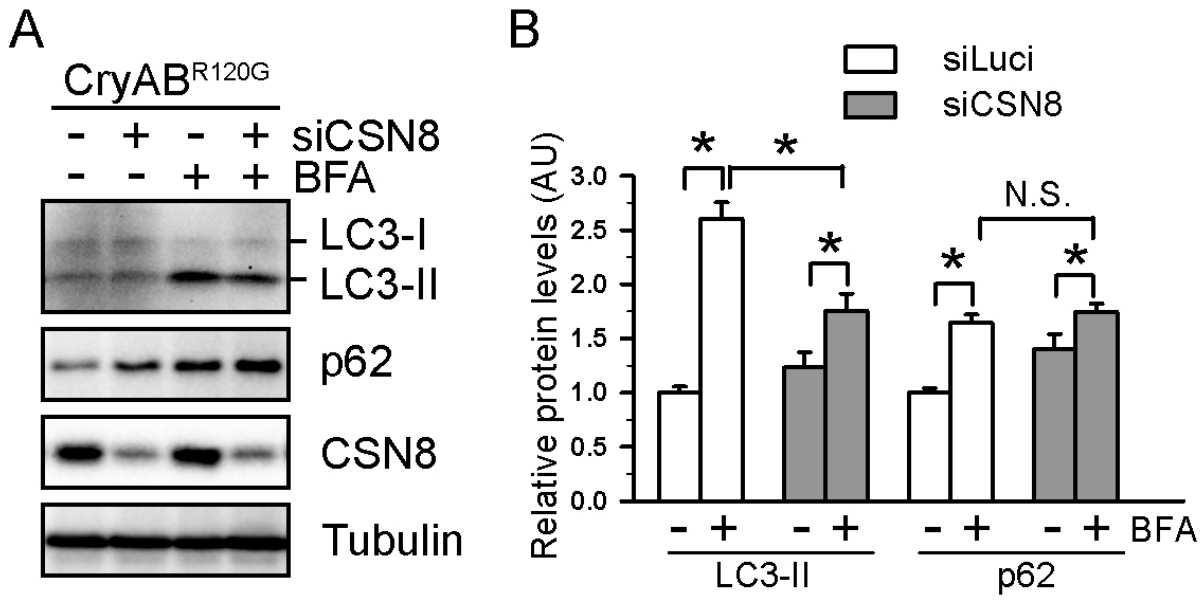
Online Figure IV. Characterization of baseline autophagy in CSN8 hypomorphic mouse hearts. Total protein extracts of ventricular myocardial tissues collected from CSN8$^{neoflox/-}$ (CSN8 hypomorphic, Hypo) and CSN8$^{neoflox/+}$ (control, CTL) littermate mice at 1 or 2 months of age were subject to SDS-PAGE and western blot analyses for the indidated 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.



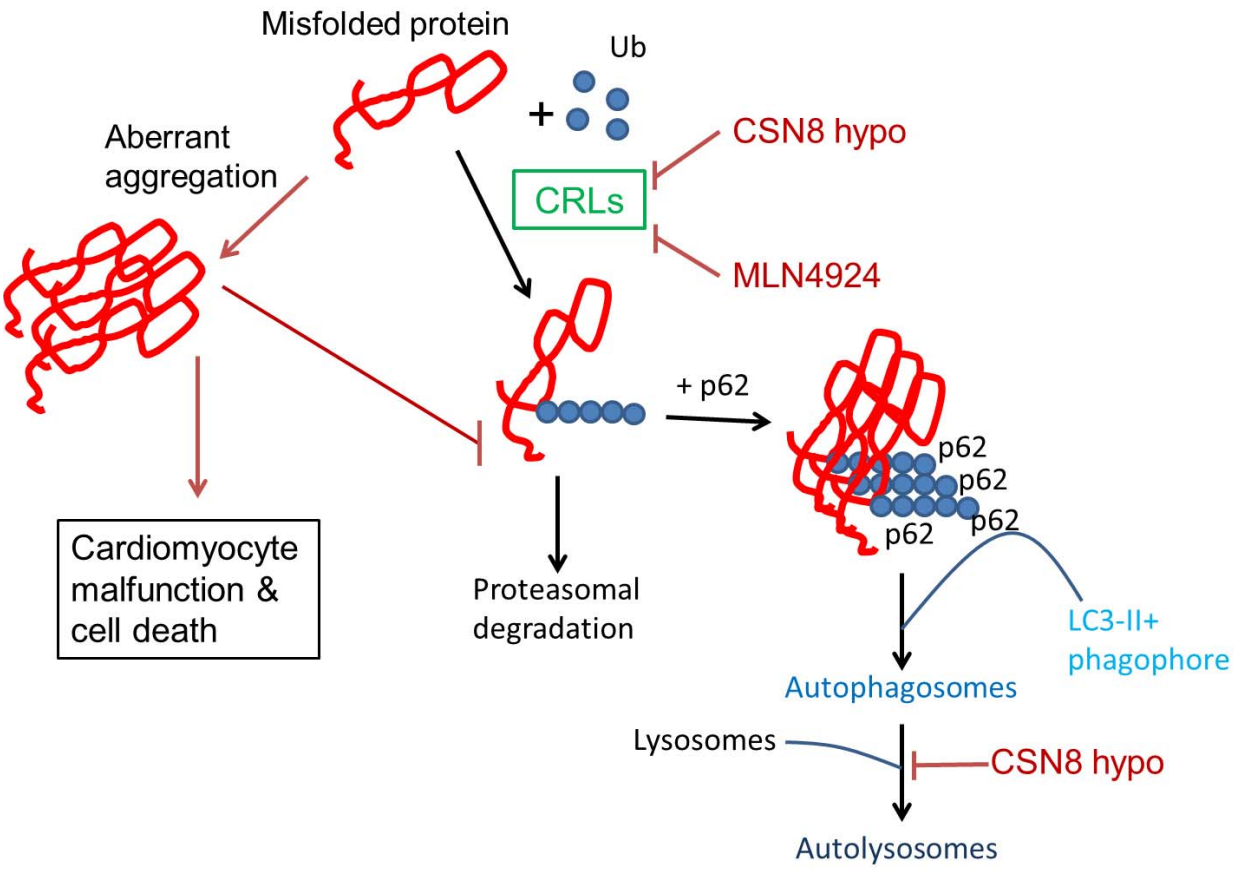
Online Figure V. Representative images of western blot analyses for the autophagic flux in NRVMs. NRVMs in cultures were transfected with either control siRNAs (-) or siRNA targeting CSN8 (siCSN8) for 48 hours before subsequent treatment. To activate non-selective autophagy, the cells were subjected 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^{R120G}
NRVMs were infected with Ad-HA-CryAB^{R120G} 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.