The COP9 signalosome is required for autophagy, proteasome-mediated proteolysis, and cardiomyocyte survival in adult mice

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Supplemental Materials

I. **Supplemental Methods**

II. **Supplemental Figures (Figures S1 through S5)**

I. Supplemental Methods

Immunostaining and fluorescence confocal microscopy

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

Western blot analyses

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

RNA analyses

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

Transmission electron microscopy (TEM)

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

Left ventricle (LV) hemodynamics

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

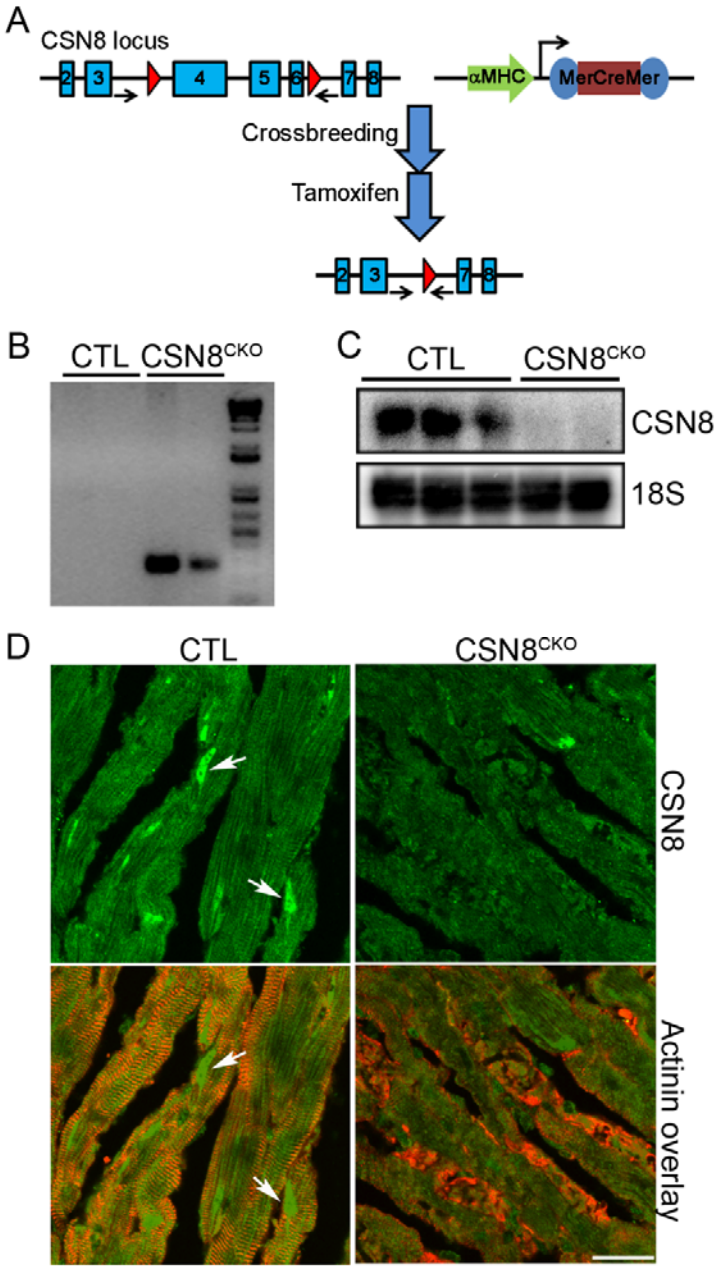
pressure (LVEDP) were measured directly from the waveforms. All the other parameters were derived from the PowerLab software as described.[2]

References of Supplemental Methods

1. Li J, Horak KM, Su H, Sanbe A, Robbins J, Wang X. Enhancement of proteasomal function protects against cardiac proteinopathy and ischemia/reperfusion injury in mice. *J Clin Invest*. 2011;121:3689-3700
2. Kumarapeli AR, Su H, Huang W, Tang M, Zheng H, Horak KM, Li M, Wang X. Alpha b-crystallin suppresses pressure overload cardiac hypertrophy. *Circ Res*. 2008;103:1473-1482

II. Supplemental Figures

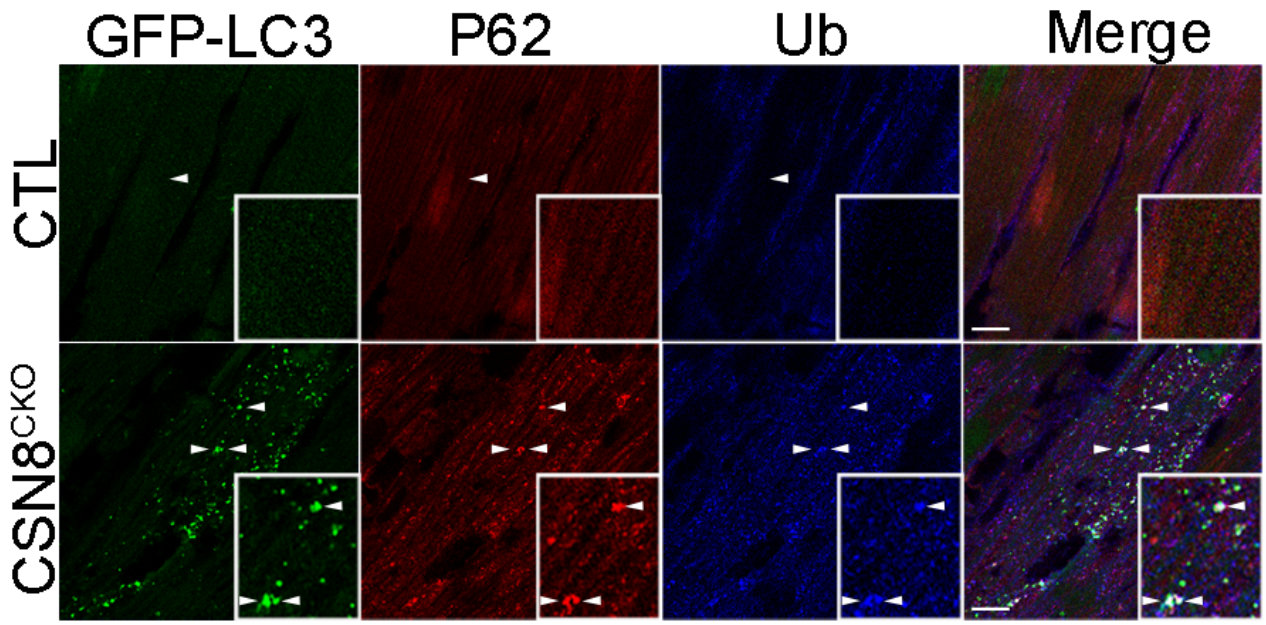
Supplemental Figure 1



Supplemental figure 1. Cardiomyocyte-restricted Csn8 gene ablation in adult mice. (**A**) Schematic representation of the tamoxifen (Tam)-induced excision of exons 4, 5 and 6 of the csn8 gene. The primers (arrows) for detection of the excision by PCR and the loxP sites (red arrowheads) are indicated. (**B**) PCR analysis of myocardial genomic DNA showing a 270bp band characteristic of recombination in $CSN8^{CKO}$ hearts after Tam injections. Sense primer I3F (5'-AACAGCTCAGCTGATAAGAG TGG - 3') and antisense primer I6R (5'-GTAGGT GACCTTCAATGTCAC-3') were used for the PCR analyses. (**C**) Northern blot analysis of *csn8*

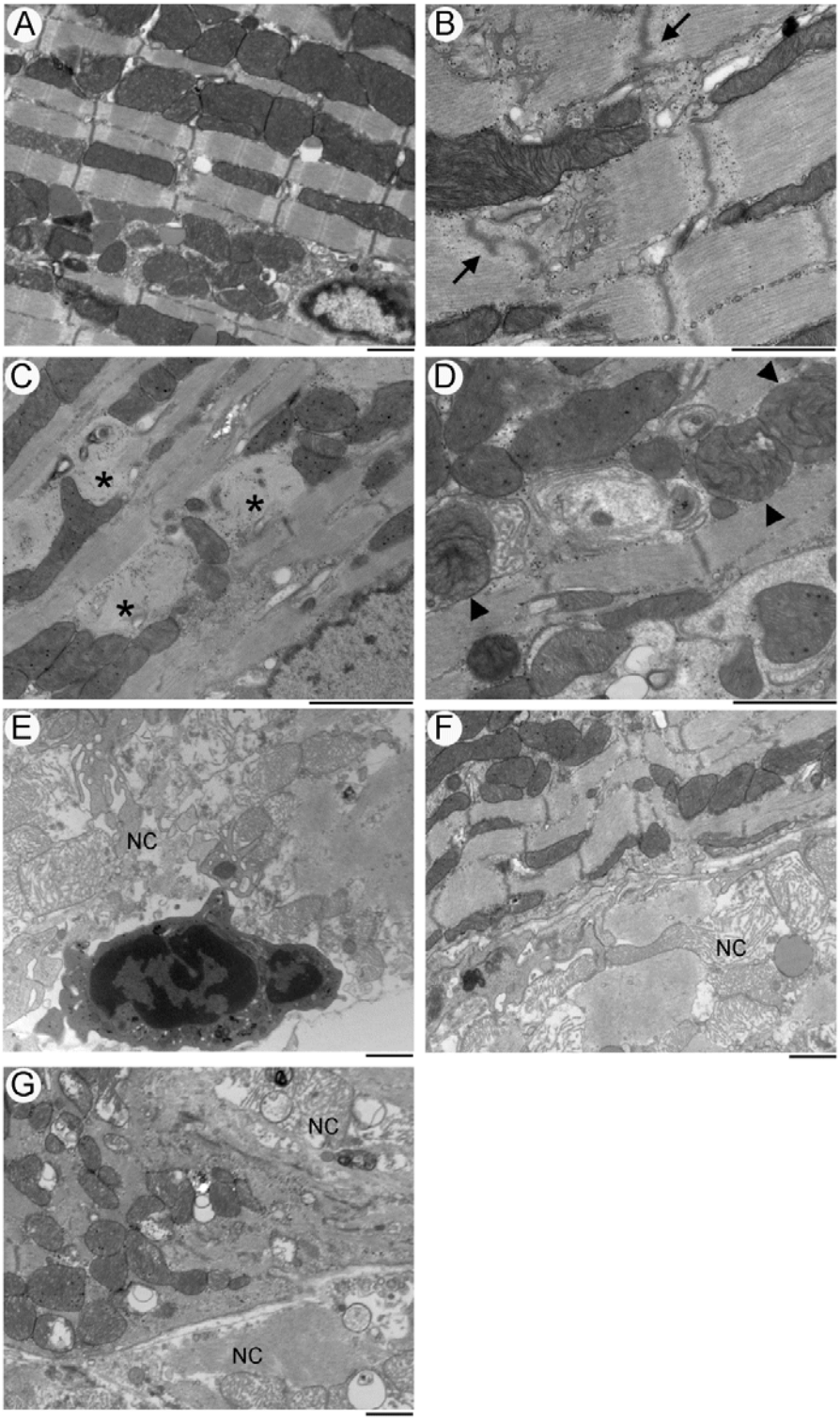
transcripts in the hearts 3 days after the first Tam injection. The 18S rRNA was probed as loading control. (**D**) Confocal micrographs of immunofluorescence labeled CSN8 (green) and α-actinin (red) in myocardium. The nuclear-enriched CSN8 staining in the cardiomyocytes of CTL hearts (arrows) was lost in the CSN8^{CKO} heart. Scale bar = 50 μm.

Supplemental Figure 2



Supplemental Figure S2. **Colocalization of GFP-LC3 with p62 and ubiquitin in CSN8^{CKO} hearts**. GFP-LC3direct fluorescence (green) and immunofluorescence detection of p62 (red) and ubiquitin (blue)-positive inclusions in CTL and CSN8^{CKO} myocardium sections. Higher magnification views are shown in insets. Scale Bar=10 μm.

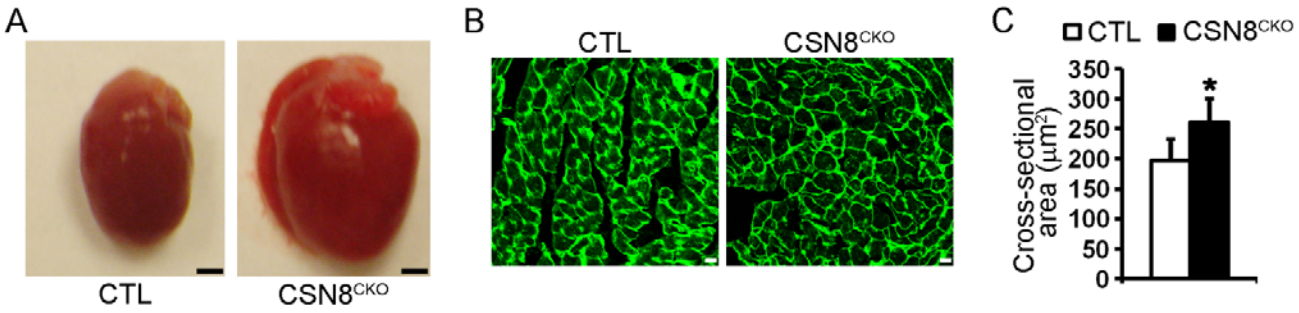
Supplemental Figure 3



Supplemental Figure S3. Electron micrographs of myocardium from CTL and CSN8^{CKO} littermate mice at 5 days after the first Tam injection. TEM analysis of LV myocardium from CTL (**A**) and CSN8^{CKO} (**B-G**) hearts revealed marked myofibril and mitochondrial degeneration as evidenced

by wavy Z-lines (arrows in B), focal myofibrillar lysis (* in C), and swollen as well as lumpy/collapsed mitochondria with disorganized cristae (arrowheads in D) in the cardiomyocytes of CSN8^{CKO} hearts. Necrotic cardiomyocytes with a significantly decreased electron density and overall structural disruption and lysis were also observed in CSN8^{CKO} hearts (E to G). NC: necrotic cardiomyocytes. Scale bar = 1 μm.

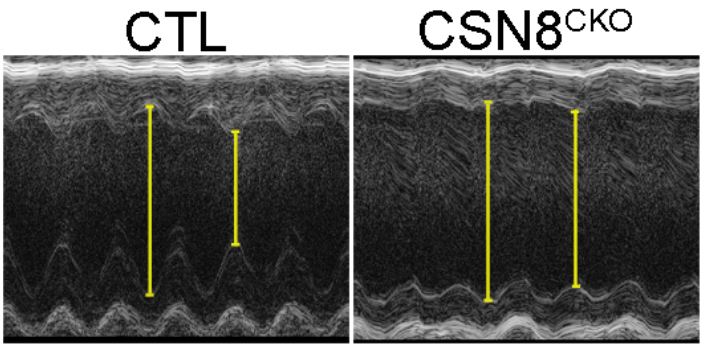
Supplemental Figure 4



Supplemental Figure S4. Cardiac hypertrophy in CSN8^{CKO} mice.

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

Supplemental Figure 5



Supplemental Figure S5. Representative M-mode echocardiographs recorded at 5 days after the first tamoxifen injection.