COP9 Signalosome Regulates Autophagosome Maturation

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Background—Autophagy is essential to intracellular homeostasis and is involved in the pathophysiology of a variety of diseases. Mechanisms regulating selective autophagy remain poorly understood. The COP9 signalosome (CSN) is a conserved protein complex consisting of 8 subunits (CSN1 through CSN8), and is known to regulate the ubiquitin-proteasome system. However, it is unknown whether CSN plays a role in autophagy.

Methods and Results—Marked increases in the LC3-II and p62 proteins were observed on Csn8 depletion in the cardiomyocytes of mouse hearts with cardiomyocyte-restricted knockout of the gene encoding CSN subunit 8 (CR-Csn8KO). The increases in autophagosomes were confirmed by probing with green fluorescent protein–LC3 and electron microscopy. Autophagic flux assessments revealed that defective autophagosome removal was the cause of autophagosome accumulation and occurred before a global ubiquitin-proteasome system impairment in Csn8-deficient hearts. Analyzing the prevalence of different stages of autophagic vacuoles revealed defective autophagosome maturation. Downregulation of Rab7 was found to colocalize strikingly with the autophagosome accumulation at the individual cardiomyocyte level. A significantly higher percent of cardiomyocytes with autophagosome accumulation underwent necrosis in CR-Csn8KO hearts. Long-term lysosomal inhibition with chloroquine induced cardiomyocyte necrosis in mice. Rab7 knockdown impaired autophagosome maturation of nonselective and selective autophagy and exacerbated cell death induced by proteasome inhibition in cultured cardiomyocytes.

Conclusions—Csn8/CSN is a central regulator in not only the proteasomal proteolytic pathway, but also selective autophagy. Likely through regulating the expression of Rab7, Csn8/CSN plays a critical role in autophagosome maturation. Impaired autophagosome maturation causes cardiomyocytes to undergo necrosis. (Circulation. 2011;124:2117-2128.)

Key Words: autophagy • COP9 signalosome complex • lysosomes • necrosis • rab7 protein

Macroautophagy (commonly known as autophagy) sequesters a portion of the cytoplasm into a double-membrane vesicle (known as an autophagosome) for fusion with and degradation by lysosomes. The fusion of autophagosomes with lysosomes, also known as autophagosome maturation, is critical to the removal of autophagosomes. Autophagy is essential to maintaining homeostasis in the cell. Nonselective autophagy during starvation helps the cell to survive an energy crisis temporarily by self-eating a portion of its cytoplasm. Selective autophagy, on the other hand, serves as a major executor of quality control by removing aged/damaged organelles and aggregated proteins. Alterations in autophagy were observed in many diseases and are emerging as important pathogenic factors and therapeutic targets. However, our understanding of the regulation of autophagy, especially selective autophagy, remains rudimentary.

Clinical Perspective on p 2128

The COP9 signalosome (CSN) is an evolutionarily conserved protein complex consisting of 8 unique subunits (CSN1 through CSN8). All 8 subunits are required for CSN holo-complex assembly and functioning. The bona fide biochemical activity of CSN is cullin deneddylation, which regulates the dynamics of cullin-based RING ligases, a large family of ubiquitin E3 ligases. Hence, CSN research has so far focused on the regulation of CSN on ubiquitin-proteasome system (UPS)–mediated proteolysis.11,12 We have recently demonstrated that perinatal cardiomyocyte-restricted Csn8 knockout (CR-Csn8KO) causes dilated cardiomyopathy and premature death in mice. Impaired UPS-mediated degradation of misfolded proteins and massive cardiomyocyte necrosis were among the likely causes. Ubiquitinated proteins are accumulated in an aggregated form in CR-Csn8KO mouse hearts.12 Protein aggregation and proteasome inhibition were shown to activate autophagy in cardiomyocytes.13 Increased autophagosomes are often found to colocalize with cell death in failing hearts, although the significance of this phenomenon remains mysterious.14 Hence, we sought to investigate the impact of Csn8 defi-
ciency on cardiac autophagy and tested a link between autophagy impairment and cardiomyocyte necrosis. We have discovered that Csn8/CSN is required for autophagosome maturation and autophagosome flux in the heart and that Csn8/CSN regulates the autophagic-lysosomal pathway through supporting Rab7 expression. Impaired autophagosome maturation can cause cardiomyocyte necrosis.

Methods

Animal Models
CR-Csn8KO was achieved with the use of the Cre-loxP system as described,12 in which transgenic Cre expression is driven by the mouse α-mysin heavy chain (Mhc6) promoter. The Csn8flox/flox::Mhc6-CreNTG littermates of CR-Csn8KO (Csn8flox/flox::Mhc6-CreTG) mice were used as control (CTL). Transgenic mice expressing green fluorescence protein (GFP)–fused LC3 (GFP-LC3) were described and generously donated by Dr Noboru Mizushima.15 The care and use of animals in this study conformed to institutional guidelines.

Transmission Electron Microscopy
Transmission electron microscopy was performed as we recently described.3 See the online-only Data Supplement for details.

Assessing Autophagic Flux in the Heart
This assessment was performed as previously described, with minor modifications.3 Briefly, mice were injected intraperitoneally with bafilomycin-A1 (BFA; 3 μg/kg, Sigma). Tissues were collected 1 hour after the injection for assessment of LC3-II protein levels and measured by the Image-Pro Plus software (LEEDS).

Quantification of Autophagic and Lysosomal Vesicles in Myocardium
Three mouse hearts per group, 3 sections per heart, and 3 fields per section were assessed. An identical threshold was used to subtract the background fluorescence of both CTL and CR-Csn8KO myocardial sections. The colocalization between GFP-LC3 direct fluorescence (autophagosomes) and LAMP1 immunofluorescence (lysosomal vesicles) was assessed by the MetaMorph software, as described.16 The cardiomyocyte compartment area in an image was measured by the Image-Pro Plus software (LEEDS).

Cell Culture and siRNA Transfection
The isolation and culture of NRVMs were performed as previously described.15 For glucose deprivation (GD), myocytes were washed twice with PBS and incubated with glucose-free and serum-free Dulbecco modified Eagle medium (Gibco). The small interference RNA (siRNA) specifically against rat Rab7 and luciferase were purchased from Qiagen.

Probing Autophagosome Maturation With a Tandem-Fluorescence LC3
To differentiate autolysosomes from autophagosomes, we infected cultured NRVMs with a recombinant adenoviral vector expressing a tandem-fluorescence LC3 (Ad-tf-LC3; a gift from J. Sadoshima of the University of Medicine and Dentistry of New Jersey, Newark).10 The tf-LC3 is a modified LC3 (microtubule-associated protein 1 light chain 3) with N-terminal fusion of an enhanced GFP and a monomeric red fluorescence protein (mRFP) in tandem.18 During live cell fluorescence imaging, the tf-LC3–containing autophagosomes show as yellow puncta because both GFP and mRFP fused with LC3 fluoresce; however, the tf-LC3–labeled autolysosomes emit only red fluorescence from mRFP because the acidic environment of autolysosomes quenches GFP but not mRFP.19 In our experiments, direct fluorescence images of live cultured NRVMs were captured with an epifluorescent microscope (Zeiss Axiovert 200M). The number of green, red, and yellow fluorescent puncta in each cell was counted manually from at least 3 different myocyte preparations. At least 50 cells per group were scored in each experiment.

Lactate Dehydrogenase Activity Assay
This assay was performed with a Cytotoxicity Detection Kit (lactate dehydrogenase; Roche) as previously described.3

Propidium Iodide Uptake Assay
Cultured NRVMs were incubated with propidium iodide–containing medium (5 μg/mL) for 10 minutes. The cells were then washed with cold PBS 3 times, fixed with 4% paraformaldehyde, counterstained with DAPI (Sigma-Aldrich), and imaged by an epifluorescent microscope (Zeiss Axiovert 200M). The numbers of propidium iodide–positive cells were normalized by the nuclei numbers revealed by DAPI. A total of >3000 cells from 10 random fields in each group were scored.

Statistical Analysis
All continuous variables are presented as mean±SD unless otherwise indicated. Differences between 2 groups were evaluated for statistical significance with the Student t test when the sample size was appropriate and the population was distributed normally; otherwise, the Mann-Whitney U test was used, and the data were summarized with the use of standard box plots. When differences among ≥3 groups were evaluated, 1-way ANOVA or, when appropriate, 2-way ANOVA followed by the Holm-Sidak test for pairwise comparisons was performed. A value of P<0.05 was considered statistically significant, but a Bonferroni correction was applied to the comparisons of the same parameter at multiple ages.

Results

Csn8 Deficiency Increases Autophagosomes in the Heart
Conversion of LC3-I to LC3-II and the incorporation of LC3-II into autophagic membranes mark important events in autophagic activation and autophagosome formation. Therefore, the protein abundance of LC3-II is commonly used as a biochemical parameter of the steady level of autophagosomes.1 As previously reported,12 the depletion of Csn8 protein in CR-Csn8KO hearts occurred between postnatal days 1 and 7. Concurrently, significant increases in LC3-II proteins were observed in CR-Csn8KO hearts by 1 week of age, and the increase maintained thereafter (Figure 1A and 1B). Interestingly, Western blot analyses revealed no significant change in beclin1, autophagy-related protein 5 (Atg5), and Atg7 in CR-Csn8KO hearts compared with their littermate CTLs (Figure 1C). To track autophagosomes, transgenic GFP-LC3 whose punctate distribution marks autophagosomes,15 was cross-bred into the CR-Csn8KO background. GFP-positive puncta (Figure 1D; see Figure I in the online-only Data Supplement for puncta quantification) and GFP-LC3-II protein levels (Figure 1E and 1F) were significantly increased in CR-Csn8KO hearts. These data indicate that autophagosomes were significantly increased in CR-Csn8KO hearts. We then performed transmission electron microscopy to detect autophagic vacuoles in the cardiomyocytes of CR-Csn8KO and CTL hearts. We found massive increases in autophagosomes in CR-Csn8KO hearts. These autophagic vacuoles frequently enclose mitochondria, and most do not show...
signs of lysis of their contents, suggesting that they are early-stage autophagosomes (Figure 1G).

**Autophagosome Removal Is Impaired Before UPS Proteolytic Function Impairment in Csn8-Deficient Hearts**

p62/SQSTM1, a substrate of selective autophagy, accumulates when the autophagic-lysosomal pathway is disrupted. Our Western blot analyses revealed that p62 protein levels in Csn8-deficient hearts were increased significantly at 1 week of age, and the increase became more pronounced at 3 weeks (Figure 2A and 2B). Marked increases in p62-positive foci were also observed by immunofluorescence (186/11006/11006 dots per 104/9262 m2 for CTLs; P<0.0001). The p62-positive dots were largely colocalized with GFP-LC3 puncta (Figure I in the online-only Data Supplement), suggesting that the accumulated p62 proteins are mostly associated with autophagosomes. These findings are consistent with a decrease in autophagosome removal in CR-Csn8KO hearts. This is indeed confirmed by a direct comparison of autophagic flux in CR-Csn8KO and CTL hearts. CR-Csn8KO and CTL mice at 2 and 3 weeks of age were injected intraperitoneally with saline or BFA (3/11006 mol/kg), a widely used lysosomal inhibitor.1 At 2 weeks of age, BFA treatment increased LC3-II significantly in both the CTL and the CR-Csn8KO hearts compared with their corresponding saline-treated CTLs, but the increase is much less in the CR-Csn8KO mice than in the CTL mice (25% versus 270%). At 3 weeks of age, BFA treatment caused a 2.8-fold increase in LC3-II over the saline treatment in the CTL mouse hearts. However in the CR-Csn8KO hearts in which LC3-II was already high at the baseline, BFA failed to further elevate the LC3-II level (Figure 2C and 2D). As a control, the BFA treatment induced similar degrees of LC3-II accumulation in the livers of CR-Csn8KO and CTL mice at both 2 and 3 weeks of age (Figure II in the online-only Data Supplement).

**Figure 1.** Increased abundance of autophagosomes in CR-Csn8KO (indicated by both Csn8KO and KO) hearts. A, Representative Western blot images for the indicated proteins in myocardium from mice of the indicated age. GAPDH was probed as loading control. B, A summary of the densitometric data of LC3-II from the experiments as illustrated in A. *P<0.05, †P<0.01 vs control (CTL), Student t test. C, Western blot analyses for the indicated proteins in the heart at 3 weeks. D, Confocal micrographs of myocardial green fluorescent protein (GFP)–LC3 direct fluorescence from CTL/GFP-LC3 and CR-Csn8KO/GFP-LC3 mice at 3 weeks. Bar=10 μm. E and F, Western blot analyses of myocardial GFP-LC3 in mice as described in D. Representative images (E) and a summary of the densitometric quantification (F) are shown. n=4 for each group; †P<0.01 vs CTL. G, Electron micrographs of ventricular myocardium from 3-week-old mice. Representative images from CTL (a) and CR-Csn8KO (b–e) are shown. c through e, Magnified images from the indicated areas of b illustrating examples of autophagosomes (arrows). Bar=1 μm.
Consistently, BFA treatment significantly accumulated GFP-LC3 puncta in CTL hearts but failed to do so in the hearts of CR-Csn8KO mice at 3 weeks (Figure III in the online-only Data Supplement). These findings indicate that Csn8 deficiency impairs autophagosome removal, leading to accumulation of autophagosomes in cardiomyocytes. To delineate the temporal relationship between defective autophagy and the impaired UPS-mediated protein degradation in CR-Csn8KO hearts, we further determined the time course of UPS mal-function by introducing GFPdgn, a validated reverse reporter of UPS function, into CR-Csn8KO and CTL littermate mice by cross-breeding as previously described. GFPdgn proteins were significantly increased in the hearts of CR-Csn8KO mice at 3 weeks but not at 2 weeks of age compared with their littermate CTLs (Figure 2E and 2F). These data indicate that impaired degradation of UPS substrates in the CR-Csn8KO heart is not discernible until after 2 weeks of age, which is after impaired autophagosome removal has occurred.

Changes in Lysosomal Genesis in Csn8-Deficient Hearts
The decreased removal of autophagosomes can be caused by a decrease in lysosomes. Hence, we investigated the impact of Csn8 deficiency on lysosomal genesis in the heart. Compared with CTL, the levels of 2 major lysosomal membrane sialglycoproteins (LAMP-1 and LAMP-2) were markedly increased in CR-Csn8KO hearts, as were the intermediate and mature forms of cathepsin D but not the cathepsin D precursor (Figure 3A and Figure IV in the online-only Data Supplement). Cathepsin D activity was also increased (Figure 3B). Consistently, double immunostaining of LAMP1 and cathepsin D showed that both LAMP1-positive vesicles and cathepsin D–positive vesicles were significantly increased (Figure 3C and 3D); however, the density of vesicles positive for both LAMP1 and cathepsin D, which is indicative of lysosomes, remained unchanged in CR-Csn8KO cardiomyocytes (Figure 3D). Interestingly, the relative abundance of

Figure 2. Autophagic flux assessments. A and B, Time course of changes in p62 protein expression in CR-Csn8KO (KO) mouse hearts. Representative images (A) and a summary of densitometry data (B) of Western blot analyses of p62 are shown. *P<0.05, #P<0.01 vs control (CTL), Student's t test. C and D, Autophagic flux assays based on bafilomycin-A1 (BFA)–induced changes in the endogenous LC3-II protein level. Three-week-old CTL and CR-Csn8KO mice were treated with BFA (3 μmol/kg IP) or vehicles and euthanized 1 hour after the BFA injection. LC3 protein levels in ventricular myocardium and liver tissue were quantified with Western blot analysis. Representative Western blot images (C) and densitometric quantification (D) of LC3 proteins are presented. n=4 mice for each group. *P<0.05, #P<0.01, 2-way ANOVA followed by Holm-Sidak test. E and F, Probing myocardial ubiquitin-proteasome system (UPS) proteolytic function in CR-Csn8KO mice using a surrogate UPS substrate (GFPdgn). Through cross-breeding, transgenic GFPdgn was introduced into CR-Csn8KO and CTL mice. The resultant CTL/GFPdgn and CR-Csn8KO/GFPdgn mice at 2 and 3 weeks of age were examined for myocardial protein levels of GFPdgn. Representative images of Western blot analyses for GFPdgn (E) and a summary of changes in GFPdgn protein levels (F) are shown. LC indicates a nonspecific band used as loading control. *P<0.05 vs CTL.
cathepsin D–positive/LAMP1–positive dots to either cathepsin D–positive or LAMP1-positive dots was significantly reduced in CR-Csn8KO hearts (Figure 3E and 3F). A plausible interpretation of these results is that Csn8-deficient cardiomyocytes attempt to increase their lysosomal synthesis by synthesizing more cathepsin D and LAMPs, but these lysosomal components fail to productively assemble a greater number of lysosomes. Nonetheless, it appears that a normal number of lysosomes are available for fusion with autophagosomes because the total number of lysosomes was not decreased in the CR-Csn8KO cardiomyocytes.

**Fusion Between Autophagosomes and Lysosomes Is Impaired in Csn8-Deficient Hearts**

The removal of autophagosomes by lysosomes occurs after the autophagosome fuses with the lysosome to form autolysosomes. Therefore, the accumulation of autophagic vacuoles could result from a blockage in the fusion between autophagosomes with lysosomes or from a defect in lysosomal proteolysis in the autolysosomes. A defect in the fusion is characterized by a reduced amount of autolysosomes relative to autophagosomes or lysosomes, whereas a defect in lysosomal proteolysis after fusion would increase the relative prevalence of autolysosomes. We measured the density of autophagic vacuoles, lysosomes, and autolysosomes through quantification of GFP-LC3 puncta (autophagosomes and autolysosomes), LAMP1-positive dots (lysosomes and autolysosomes), and GFP-LC3::LAMP1 double-positive dots (autolysosomes only), respectively. Confocal microscopy was performed with myocardial cryosections from the CR-Csn8KO and CTL mice (Figure 4A). To better understand the changes in membrane dynamics caused by Csn8 deficiency, we sought to compare the changes in the relative abundance of the different stages of autophagic vesicles in CR-Csn8KO hearts with those observed in the hearts of GFP-LC3 transgenic mice that had undergone 12 hours of starvation (the starved group) or had received an intravenous injection of the proteasomal inhibitor MG262 (the MG262 group; see Figure 3).
V in the online-only Data Supplement for representative confocal micrographs). Both starvation and proteasome inhibition are known to activate autophagy and to increase autophagic flux.

Compared with the corresponding control groups, the density of autophagic vacuoles (ie, GFP-LC3 puncta) was significantly increased in the cardiomyocytes of all 3 groups (CR-Csn8KO, starved, and MG262-treated groups; Figure 4B). However, the density of lysosomes (ie, LAMP1-positive puncta) was increased only in the CR-Csn8KO group, not in the starved or the MG262 group (Figure 4C). Notably, the density of autolysosomes (ie, GFP-LC3/LAMP1 double-positive puncta) was significantly increased in the starved and MG262 groups but not in the CR-Csn8KO group (Figure 4D). Consequently, the abundance of autolysosomes in CR-Csn8KO cardiomyocytes is remarkably decreased relative to either autophagic vacuoles or lysosomal vacuoles (Figure 4E) and unchanged relative to autophagic vacuoles (Figure 4E and 4F) compared with their respective control groups. These analyses reveal that the nature of the increased autophagic vacuoles in CR-Csn8KO hearts differs sharply from that caused by starvation or proteasome inhibition in that Csn8 deficiency impairs autophagosome maturation whereas starvation and proteasome inhibition enhance autophagosome maturation. Furthermore, these data strongly suggest that a reduced ability of autophagosomes to fuse with lysosomes contributes, at least in part, to the defective removal of autophagosomes in the CR-Csn8KO mouse hearts.

Severe Impairment of Autophagosome Maturation May Be a Cause of Cardiomyocyte Necrosis in CR-Csn8KO Mice

As we previously reported,12 a marked increase in cardiomyocyte necrosis, as detected by Evans blue dye (EBD) uptake and increased leukocyte infiltration, is present in CR-Csn8KO mouse hearts before elevated apoptosis becomes discernible. The necrotic nature of the EBD-positive cardiomyocytes is confirmed by intracellular positive staining for mouse endogenous immunoglobulin (Figure VI) and the increased uptake of intravenously injected rabbit anti-desmin antibodies (data not shown). From the findings described so
far, we reasoned that a greater autophagosome accumulation in a cardiomyocyte of the CR-Csn8KO heart is indicative of more severe impairment in autophagosome maturation in the cell. The heterogeneity of GFP-LC3 puncta accumulation among cardiomyocytes in CR-Csn8KO hearts (Figures 1D and 4A) allows us to determine whether impaired autophagosome maturation correlates to the increased necrosis at the individual cardiomyocyte level. Quantification of GFP-LC3 puncta in the EBD-negative cardiomyocytes of CR-Csn8KO hearts showed that the 95% confidence range of the GFP-LC3 puncta number density is from 1 to 16 per 100 μm². Accordingly, all cardiomyocytes (EBD positive and EBD negative) in CR-Csn8KO hearts were divided into 2 groups: group A containing ≤16 GFP-LC3 puncta per 100 μm² and those showing >16 puncta per 100 μm² (B) are presented. Scale bar—20 μm. **P<0.0001, Fisher exact test.

Rab7 Downregulation Colocalizes With and Correlates to Autophagosome Accumulation in the Cardiomyocytes of CR-Csn8KO Mouse Hearts

The mechanisms underlying autophagosome maturation remain poorly understood. Nevertheless, a small G protein, Rab7, has been shown to play an indispensable role in

Figure 5. Impaired autophagosome maturation causes cardiomyocyte necrosis in mice. A and B, Cardiomyocytes with severe accumulation of autophagosomes more likely undergo necrosis (arrowhead). Green fluorescent protein (GFP)-LC3::CR-Csn8KO and GFP-LC3::CTL mice at 3 weeks were administered Evan blue dye (EBD; 100 mg/kg IP) 18 hours before the heart was flushed with saline and fixed with 4% paraformaldehyde via retrograde perfusion through the abdominal aorta. Cryosections of ventricular myocardium were stained with Alexa-568–conjugated phalloidin to identify cardiomyocytes. The sections were imaged with fluorescence confocal microscopy for GFP-LC3 (green), EBD (red), and F-actin (blue). Representative confocal micrographs (A) and a comparison of the prevalence of EBD-positive cells in CR-Csn8KO hearts between the cardiomyocytes containing ≤16 GFP-LC3 puncta per 100 μm² and those showing >16 puncta per 100 μm² (B) are presented. Scale bar—20 μm. **P<0.0001, Fisher exact test. C and D, Long-term blockade of autophagic flux causes cardiomyocytes necrosis (arrows) in wild-type mice. Adult mice were injected with chloroquine (CQ; 10 mg/kg daily) or saline for 3 weeks. At the end of the experiment, mice were injected with EBD, and ventricular myocardium samples were processed to detect cardiomyocyte EBD uptake (C) as described in A. The sections were counterstained with wheat germ agglutinin (WGA; green) and DAPI (blue) to reveal cell membrane and the nuclei. An adjacent set of sections were immunostained for CD45 (red), and the nuclei were stained with DAPI (blue) for quantification of the percentage of CD45-positive nuclei among all nuclei in the field (D). Bar=20 μm; n=3 mice per group. *P<0.05.
It was recently shown that increased Rab7 expression is likely a nexus through which Csn8/CSN regulates autophagosome maturation and lysosomal genesis in cultured cardiomyocytes. To explore the molecular mechanisms underlying the defective selective autophagy in Csn8-deficient cardiomyocytes, we measured Rab5, Rab7, and Rab11 protein expression and found a significant upregulation of both Rab5 and Rab11 but a remarkable downregulation of Rab7 in CR-Csn8KO mouse hearts (Figure 6A and 6B). Furthermore, a potential causal relationship between Rab7 downregulation and the impaired autophagosome removal is suggested by our examination of Rab7 cellular distribution in CTL/GFP-LC3 and CR-Csn8KO/GFP-LC3 mouse hearts. In the CTL, Rab7 is readily detectable with immunofluorescence and, as expected, is located in the cytoplasm with a comparable expression level among different cardiomyocytes. In CR-Csn8KO hearts, the overall immunofluorescence of Rab7 was decreased, which is consistent with results of the Western blot analysis. Rab7 expression appeared uneven among different cardiomyocytes in the same section. More interestingly, the accumulation of GFP-LC3 puncta (ie, autophagosomes) was most intense in the cardiomyocytes with the lowest Rab7 protein expression (Figure 6C). This remarkable colocalization between the downregulation of Rab7 and autophagosome accumulation in individual cells strongly suggests that Rab7 is likely a nexus through which Csn8/CSN regulates autophagosome maturation.

Figure 6. Downregulation of Rab7 in Csn8-deficient mouse hearts. A and B, Representative images (A) and a summary of densitometry data (B) of Western blot analysis of Rab5, Rab7, and Rab11 in mouse hearts at 3 weeks. n = 4 mice per group; *P < 0.05 vs control (CTL). C, Representative confocal micrographs illustrating the colocalization between Rab7 downregulation and autophagosome accumulation at the individual cardiomyocyte level in CR-Csn8KO hearts. Scale bar = 10 μm.

**Downregulation of Rab7 Impairs Autophagosome Maturation and Exacerbates the Induction of Cell Death by Proteasome Inhibition in Cultured Cardiomyocytes**

To begin testing a causal relationship between the Rab7 downregulation and the impaired autophagosome maturation in Csn8-deficient cardiomyocytes, we next investigated the impact of siRNA-mediated Rab7 knockdown (Rab7KD) on autophagosome maturation under the baseline condition and during starvation or proteasome inhibition in cultured NRVMs. We measured autophagic flux by assessing the changes in biochemical markers (LC3-II, p62) in the presence and absence of BFA-mediated lysosomal inhibition. Under basal condition in the absence of BFA treatment, Rab7KD significantly increased both LC3-II and p62 compared with the control siRNA (siLuci) group (Figure 7A and 7B). BFA-induced lysosome inhibition led to a marked increase in LC3-II and p62 levels in the control siRNA group, but this increase was significantly less in the Rab7KD group (Figure 7C and 7D). These findings indicate that Rab7 downregulation is sufficient to impair basal autophagic flux.

Nutrition starvation by GD activates nonselective autophagy and increases autophagic flux. GD-induced increase of autophagic flux was measured by the difference in LC3-II protein levels between the BFA-treated and vehicle-treated cells. GD-activated autophagic flux was significantly less in Rab7KD cells than in the siLuci-transfected cells (Figure 7E and 7F). Furthermore, we tested the effect of Rab7KD on proteasome inhibition–activated selective autophagy. As expected, proteasome inhibition by bortezomib increased autophagic flux. The bortezomib-induced autophagic flux was also significantly attenuated by Rab7KD (Figure 7E and 7H). These data indicate that Rab7 is required for autophagic flux activated by either GD or proteasome inhibition in cardiomyocytes.

To further demonstrate that Rab7 downregulation impairs autophagosome maturation during not only nonselective autophagy, but also proteasome inhibition–activated selective autophagy, we used a tf-LC3 (GFP-mRFP-LC3). After incorporation into autophagosomes, the tf-LC3 can effectively differentiate autophagosomes from autolysosomes because the acidic environment in autolysosomes quenches GFP but not mRFP fluorescence. The tf-LC3 was expressed in cultured NRVMs via Ad-tf-LC3 infection. We found a modest but statistically significant increase in the yellow puncta but not red puncta in Rab7KD cardiomyocytes at baseline (Figure 8B and Figure VII in the online-only Data Supplement), suggesting that Rab7 is required for basal autophagy in the cultured NRVMs. Importantly, GD or bortezomib treatment markedly increased both yellow and red-only puncta. However, Rab7KD exacerbated the GD- or bortezomib-induced increases in the yellow puncta but significantly attenuated the GD- or bortezomib-induced increase in the red-only puncta compared with the control siRNA-treated cells (Figure 8A and 8B and Figure VIII in the online-only Data Supplement). These results demonstrate compellingly that Rab7 downregulation is sufficient to impair autophagosome maturation in both nonselective and selective autophagy in cardiomyocytes.
Finally, we determined the effect of Rab7 downregulation on proteasome inhibition–induced cell death. Cardiomyocytes undergoing necrosis or late apoptosis lose their cell membrane integrity, which can be assessed by measuring the leakage of lactate dehydrogenase into the culture medium and the uptake of a cell membrane–impermeable dye, propidium iodide, which will then bind to double-stranded DNA and fluoresce. A low dose of bortezomib (10 nmol/L) significantly increased lactate dehydrogenase activities in the medium and propidium iodide uptake in a time-dependent manner, which was significantly augmented by Rab7KD (Figure 8C–8E). These data indicate that Rab7 downregulation disrupts autophagosome maturation and sensitizes cardiomyocytes to proteasome inhibition–induced cell death.

Discussion

The deletion of the Csn8 gene in cardiomyocytes destabilizes other CSN subunits; therefore, the observed phenotypes in CR-Csn8KO mice may not be attributed exclusively to the Csn8 deficiency but also to the CSN holo-complex. CSN is known to regulate UPS proteolytic function by modulating the functioning of cullin-based ubiquitin E3 ligases. In the present study, we have expanded for the first time the regulatory role of CSN to macroautophagy, another critical catabolic and quality control pathway in the cell. We have demonstrated here that (1) Csn8/CSN is indispensable for macroautophagy in cardiomyocytes of intact mice; (2) Csn8/CSN is required for autophagosome maturation or the fusion between autophagosomes and lysosomes; (3) Csn8/CSN regulates autophagosome maturation likely by regulating Rab7 expression; and (4) impaired autophagosome removal in mouse hearts appears to promote cardiomyocytes necrosis.
tured K562 cells; however, they did not investigate whether the increase in autophagosome was caused by increased formation or decreased removal.

Csn8/CSN Regulates Autophagosome Maturation Likely Through Modulating Rab7 Expression

The regulation of autophagosome maturation is poorly understood. In cell culture studies, Rab7 was shown to be indispensable for autophagosome-lysosome fusion and lysosomal genesis, but this remains to be tested in intact animals. Hariharan et al recently demonstrated that upregulation of Rab7 plays an important role in mediating starvation-induced increases in autophagic flux in cardiomyocytes. The present study suggests that downregulation of Rab7 likely mediates the Csn8 deficiency–induced impairment of autophagosome maturation. This contention is supported by multiple lines of evidence. First, the protein expression of Rab7, but not Rab5 or Rab11, was significantly decreased in CR-Csn8KO mouse hearts. Second, perhaps because of differential onset of Csn8KO in various cardiomyocytes of a heart, the degree of Rab7 protein downregulation varied among cardiomyocytes in the heart; more importantly, the downregulation of Rab7 strikingly colocalized with and correlated to GFP-LC3 accumulation. The highest GFP-LC3 puncta increase is found in the cardiomyocytes with the lowest Rab7 protein expression. Third, we failed to decrease Rab7 protein levels in cultured NRVMs by overexpressing GFP-LC3 (data not shown). Finally, Rab7KD in cultured NRVMs recapitulates the autophagosome maturation impairment phenotype observed in CR-Csn8KO mouse hearts. Rab7KD impairs the autophagosome maturation not only at baseline but also during GD and proteasome inhibition, both known to increase autophagic flux. GD activates nonselective autophagy, whereas, likely through upregulation

Figure 8. Rab7 downregulation disrupts autophagosome maturation and increases proteasome inhibition–induced cell death in cultured neonatal rat ventricular myocytes (NRVMs). A and B, Changes in autolysosome formation as probed with tandem fluorescence LC3 (tf-LC3). Cultured NRVMs were infected with Ad-tf-LC3 24 hours after transfection of siRab7 or siLuci (as control); 24 hours later, the cells were treated with bortezomib (BZM; 20 nmol/L) for 12 hours or subjected to glucose deprivation (GD) for 4 hours before the cells were imaged live for the green fluorescence protein (GFP; green) and monomeric red fluorescence protein (mRFP; red) signals from tf-LC3. Representative epifluorescent images (A) and a summary (B) of the changes of autophagic vacuole abundance with indicated treatments. Insets are the enlarged images of the indicated area. Arrowheads indicate red-only puncta (autolysosome); arrows, green and red superimposed puncta (autophagosomes); scale bar=50 μm; *P<0.05; #P<0.01. C through E, Rab7 knockdown exacerbates BZM-induced cell death. BZM (10 nmol/L) treatment was initiated 48 hours after the siRNA transfection. At the indicated time points of BZM treatment, culture media were sampled for measuring lactate dehydrogenase (LDH) activities (C). At 24 hours of BZM treatment, propidium iodide (PI) was applied to the cell culture. Ten minutes later, the unbound PI was removed completely, and the cells were then fixed in 4% paraformaldehyde, counterstained with DAPI, and imaged for PI and DAPI staining. Representative epifluorescent micrographs are shown in D. The percent of PI-positive (red) nuclei among all DAPI-stained nuclei (blue) was determined from 3 independent repeats (E). Scale bar=100 μm; *P<0.01 vs siLuci; #P<0.01 vs siLuci+BZM.
of p62, proteasome inhibition activates selective autophagy. The critical role of Rab7 in starvation-induced autophagy in cardiomyocytes was recently delineated by Hariharan and colleagues.10 Our data demonstrate for the first time in cardiomyocytes that Rab7 is essential to autophagosome maturation in selective autophagy. To fully prove that Rab7 downregulation mediates the autophagic defect in CR-Csn8KO hearts, it is important to test whether cardiomyocyte-restricted Rab7 overexpression rescues the defect in CR-Csn8KO mice. At this point, we could not rule out that an Rab7-independent mechanism may also contribute to impaired autophagosome maturation in Csn8-deficient hearts.

The mechanism by which Csn8/CSN regulates Rab7 expression is currently unknown, but a potential link has been implicated in the existing literature. First, we have shown that the F-Box protein atrogin1 is downregulated in Csn8KO hearts,12 which is consistent with the prevalent theory that CSN-mediated deneddylation stabilizes F-Box proteins.11 Second, atrogin1, which is a target gene of FoxO1/FoxO3, promotes FoxO1/FoxO3 transactivation via a feed-forward mechanism that depends on K63-linked polyubiquitination of the FoxO proteins by atrogin1.23 Finally, Rab7 is a target gene of the FoxO transcription factors, at least during autophagy activation.10 Indeed, we found that Rab7 mRNA levels are significantly decreased in CR-Csn8KO mouse hearts (data not shown). These data are consistent with the proposition that CSN may regulate the transcription of Rab7 via atrogin1 and FoxO proteins.

Failure of Autophagosome Maturation Promotes Cardiomyocyte Necrosis

As we described previously, massive cardiomyocyte necrosis was observed in CR-Csn8KO mouse hearts as early as 3 weeks of age when increased apoptosis and congestive heart failure are not discernible. Here we found impaired autophagosome removal in CR-Csn8KO hearts as early as 1 week of age or 2 weeks before a global impairment of UPS proteolytic function was evident or increases in cardiomyocyte necrosis were detected.12 This temporal relationship represents the first line of evidence for a causal relationship between autophagy impairment and cardiomyocyte necrosis. Second, a significantly higher percent of cardiomyocytes with severe accumulation of autophagosomes showed increased EBD uptake compared with those with a relatively lower autophagosome density in CR-Csn8KO hearts. Third, blocking autophagosome maturation by Rab7KD exacerbates proteasome inhibition–induced lactate dehydrogenase leakage and propidium iodide uptake in cultured NRVMs. Finally, long-term inhibition of autophagic flux by chloroquine significantly increased EBD uptake and CD45 infiltration in mouse hearts with wild-type Csn8.

Notably, mice with similarly achieved cardiomyocyte-restricted Atg5 knockout show impaired autophagosome formation,19 but they do not display the severe abnormal phenotypes we observed in CR-Csn8KO mice, which have impaired autophagosome removal. This discrepancy suggests that impaired autophagosome removal may be more detrimental than decreased autophagosome formation, as recently hypothesized by Gottlieb and Mentzer.9 One potential mechanism by which impaired autophagosome maturation causes cardiomyocyte necrosis is that, when cardiomyocytes cannot self-digest the defective organelles or protein aggregates sequestered by autophagosomes, they may alternatively dump these highly inflammatory autophagosome contents to the extracellular space.9 This can conceivably have at least 2 consequences: The cell loses the opportunity to recycle the ingredients, and the dumped content may act as autocrinal and/or paracrine factors to trigger inflammation and even activate the programmed necrosis pathway.

Taken together, our results show that we have uncovered a novel function of Csn8 as an important player in the autophagosome removal stage of selective autophagy. The autophagy defect can be detected in CR-Csn8KO hearts when Csn8 protein is just depleted (1 week) or 2 weeks before the onset of UPS functional deficiency and discernible cardiomyopathy.12 Long-term inhibition of autophagy was shown to compromise the degradation of UPS substrates in noncardiac cells.24 Hence, it is very possible that the accumulation of a surrogate UPS substrate (GFPdgn) observed in the later stage of CR-Csn8KO mouse hearts may also be attributable to impairment of the autophagic pathway. A working model for the role of Csn8/CSN in the cardiomyocytes of postnatal hearts can be summarized as follow: Csn8/CSN suppresses the necrotic pathway by enhancing autophagosome maturation via supporting Rab7 expression. Given that autophagy dysfunction and cardiomyocyte necrosis have been observed in congestive heart failure of various types,14 it is important to decipher how Csn8/CSN regulates Rab7 expression and how impaired autophagosome maturation causes necrosis. Considering that CSN has already been established as an important player in the UPS, our study, which places CSN in the autophagy-mediated degradation pathway, further underscores the prominent role of CSN in catabolic processes in the cell.

Acknowledgments

We thank the Imaging Core of the Division of Basic Biomedical Sciences for assistance in confocal microscopy, Andrea Jahn for outstanding technical assistance in maintaining mouse colonies and genotype determination, Suleman Said for technical support on transmission electron microscopy sample preparation, and Dr Evelyn Schlenker for statistical assistance. Dr Wang is a recipient of the Established Investigator Award of the American Heart Association.

Sources of Funding

This work was in part supported by National Institutes of Health (NIH) grants R01HL085629 and R01HL072166, American Heart Association grants 0740025N (to Dr Wang), 0625738Z (to Dr Su), and 11PRE5730009 (to M.J. Ranek). The Imaging Core is supported by NIH grant (5P20RR015567).

Disclosures

None.

References


**CLINICAL PERSPECTIVE**

Macroautophagy sequesters portions of the cytoplasm into double-membrane vesicles (known as autophagosomes) for fusion with and degradation by lysosomes. Autophagy is essential to intracellular homeostasis and is involved in the pathophysiology of a variety of diseases. Targeting autophagy can potentially become a therapeutic strategy to treat heart disease; this is being intensively explored. Thus, it is critical to better understand the mechanisms that regulate autophagy, especially the selective autophagy that removes defective organelles and protein aggregates. The COP9 signalosome (CSN), a conserved protein complex consisting of 8 subunits (CSN1 through CSN8), is known to regulate the ubiquitin-proteasome system, another major intracellular proteolytic pathway. We report that mice with perinatal cardiomyocyte-restricted ablation of the Csn8 gene (CR-Csn8KO) display massive cardiomyocyte necrosis, develop dilated cardiomyopathy, and die prematurely. However, it is unknown whether CSN plays a role in autophagy. Here, we report that the fusion between autophagosomes and lysosomes is impaired in the cardiomyocytes of CR-Csn8KO mice; as a result, the removal of autophagosomes is impaired. This appears to be caused by a downregulation of Rab7, a small G protein known to be indispensable to the fusion process. Impaired autophagosome removal is likely responsible for the massive cardiomyocyte necrosis in CR-Csn8KO mice because cardiomyocytes with more severe autophagosome accumulation undergo necrosis more often in CR-Csn8KO mice and long-term lysosomal inhibition with chloroquine is sufficient to cause cardiomyocyte necrosis in mice. Therefore, we conclude that Csn8/CSN regulates both the ubiquitin-proteasome system and autophagy in the heart.
COP9 Signalosome Regulates Autophagosome Maturation
Huabo Su, Faqian Li, Mark J. Ranek, Ning Wei and Xuejun Wang

_Circulation_. 2011;124:2117-2128; originally published online October 10, 2011; doi: 10.1161/CIRCULATIONAHA.111.048934

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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SUPPLEMENTAL MATERIAL

For

The COP9 Signalosome Regulates Autophagosome Maturation

Huabo Su, PhD\textsuperscript{1,2}; Faqian Li, MD, PhD\textsuperscript{3}; Mark J. Ranek, BS\textsuperscript{1,2}; Ning Wei, PhD\textsuperscript{4}; Xuejun Wang, MD, PhD\textsuperscript{1,2}

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Online Supplementary Materials

The online supplements contain:
- Supplementary Methods
- 1 supplementary table;
- 5 supplementary figures and legends.
Supplementary Methods

Western blot analyses. Protein extraction from either myocardial tissues or cultured neonatal rat ventricular cardiomyocytes (NRVMs), protein concentration determination with BCA reagents (Pierce), SDS-PAGE, immunoblotting analysis, and densitometry were performed as previously described. Antibodies include: CSN8 (BIOMOL), Rab5 and Rab11 (Cell Signaling), LC3 (MBL), p62 (ARP), cathepsin D (Calbiochem), Rab7, α-actinin and GAPDH (Sigma). The rat anti-LAMP1 and anti-LAMP2 antibodies developed by Thomas August (John Hopkins University) were obtained from the Developmental Studies Hybridoma Bank (DSHB) developed under the auspices of the NICHD and maintained by The University of Iowa.

Transmission electron microscopy (TEM). TEM was performed as described. Mice were anesthetized with isoflurane and the hearts fixed by gravity-fed (600 mmH2O) 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. At least 3 tissue samples from each area of the heart were chosen randomly for ultrastructural analysis. Two mice for each genotype were examined. Ultrathin sections were counterstained with uranyl acetate and lead citrate and viewed/imaged with a JEOL model 1210 electron microscope at 100 kV.

Immunostaining and fluorescence confocal microscopy. These were performed as previously described. The primary antibodies used for immunostaining include: guinea pig anti-p62 (ARP), rat anti-LAMP1 (DSHB, University of Iowa), rabbit anti-cathepsin D (Calbiochem), and goat anti-CD45 (R&D system). The secondary antibodies used are the Alexa-Fluor 488 donkey anti-rabbit Ig, the Alexa-Fluor 568 goat anti-guinea pig Ig, and the Alexa-Fluor 568 donkey anti-mouse Ig (Molecular Probes). The immunostaining was visualized using a fluorescence confocal microscope (Olympus Fluoview 500) and the images were captured and digitalized using the associated software.

Cell culture and siRNA transfection. The isolation and culture of neonatal rat ventricular cardiomyocytes (NRVMs) were performed as previously described. For glucose deprivation (GD), myocytes were washed twice with phosphate buffered saline (PBS) and incubated with glucose-free and serum-free DMEM (Gibco). The small interference RNA (siRNA) specifically against rat Rab7a (siRab7-1, Cat. #: SI03021725; siRab7-2, Cat. #: SI03037748) and luciferase (siLuci, Cat. #: SI03074113) were purchased from Qiagen. For transient knockdown of target genes, 100 pmol of siRNA were transfected to 2X10^6 NVRMs in 60-mm dishes with Lipofectamine™ 2000 (Invitrogen) by following the manufacturer’s instruction. SiLuci served as the control siRNA. siRab7-1 showed better knockdown effect than siRab7-2 in the initial tests and hence was used in all subsequent experiments.

Detection of necrotic cardiomyocytes in intact mice. To label cardiomyocytes undergoing necrosis, CR-Csn8KO mice at 3 weeks of age were injected with Evan’s blue dye (EBD, 100mg/kg, i.p.) 18 hrs before the mice were anesthetized and the heart was flushed with saline and fixed with 3.8% paraformaldehyde by retrograde perfusion via the abdominal aorta. Ventricular myocardium was sampled and processed to obtain cryosections as described previously. The cryosections were immunostained with Alexa 488 conjugated anti-mouse...
immunoglobulin (Ig) or anti-rabbit Ig and anti-goat Ig as control. These sections were imaged for EBD (red) and Alexa-488 (green) fluorescence using a confocal microscope.

Reference for Supplementary Methods


Supplementary Table 1. The prevalence of increased EBD uptake among cardiomyocytes with different degrees of autophagosome accumulation in CR-Csn8KO mouse hearts

<table>
<thead>
<tr>
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<th>GFP-LC3 puncta number per 100 μm²</th>
<th>Total</th>
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<tbody>
<tr>
<td></td>
<td>0–16</td>
<td>&gt;16</td>
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<tr>
<td>EBD-</td>
<td>393</td>
<td>12</td>
</tr>
<tr>
<td>EBD+</td>
<td>75</td>
<td>13</td>
</tr>
<tr>
<td>Total</td>
<td>468</td>
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</tr>
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Fisher’s exact test, $p=0.00006765$.
$X^2=20.95$, $p<0.0001$, Chi-square test.
Supplementary figure S1. Increased p62 proteins were mostly co-localized with autophagosomes in CR-Csn8KO hearts. Cryosections of ventricular myocardium from 3-week-old CR-Csn8KO::GFP-LC3 mice and their littermate CTL::GFP-LC3 mice were immunostained for p62 as described in the Methods of main text. (A) Representative confocal micrographs showing increase of p62+ puncta (red) and its co-localization with GFP-LC3 (green). The insets are the enlarged image of the arrowhead-pointed area. Bar=10 μm. (B) Quantification of p62+ dots in myocardial sections from CTL and CR-Csn8KO mice. The number of the p62+ dots was normalized by tissue area. #: p<0.01 vs. CTL; Student’s t-test.
Supplementary Figure S2. Autophagic flux in the liver is comparable between CR-Csn8KO and CTL mice at both 2 and 3 weeks of age. The mice treatment and other related methods are decreased in Figure 2 of main text. *p<0.05, #p<0.01; N.S., not significant; 2-way ANOVA followed by Holm-Sidak test for pair-wise comparisons.
Supplementary Figure S3. Autophagic flux assays based on BFA induced changes in the number of GFP-LC3 puncta. CTL/GFP-LC3 and CR-Csn8KO/GFP-LC3 mice of 3-week-old were treated with BFA or vehicle as described in the panel C Figure 2 in the main text. Cryosections of fixed ventricular myocardium were used for GFP direct fluorescence confocal microscopy. Representative confocal micrographs (A) and a summary of changes in the number density of GFP-LC3 puncta (B) are shown. The number of GFP-LC3 puncta was normalized by tissue area. *p<0.05; N.S., not significant; Student’s t-test.
Supplementary figure S4. A summary of the densitometric data of western blot analyses for the indicated proteins as illustrated in Figure 3A of the main text. p, i, and m denote the precursor, intermediate and matured form of cathepsin D (CathD) respectively. n=4 for each group. *: p<0.05 vs. CTL; Student’s t-test.
Supplementary figure S5. Confocal micrographs of myocardium sections from GFP-LC3 transgenic mice with indicated treatments. GFP-LC3 direct fluorescence (green) and immunostained LAMP1 (red) were shown. For the starvation group (Starved), mice were deprived of food for 12 hours with free access to water. For the MG262 treated group, mice were intravenously injected with MG262 (5μmol/kg) 12 hours before sample collection. The insets are the enlarged images of the asterisk-pointed areas. Arrowheads point to the GFP-LC3+/ LAMP1+ dots.
Supplementary figure S6. The loss of membrane integrity of EBD+ cardiomyocytes in CR-Csn8KO hearts is confirmed by immunostaining for mouse endogenous IgG.

To label cardiomyocytes undergoing necrosis, CR-Csn8KO mice at 3 weeks of age were injected with Evan’s blue dye (EBD, 100mg/kg, i.p.) 18 hrs before the mice were anesthetized and the heart was flushed with saline and fixed with 3.8% paraformaldehyde by retrograde perfusion via the abdominal aorta. Ventricular myocardium was sampled and processed to obtain cryosections as described in the main text. The cryosections were immunostained with Alexa 488 conjugated anti-mouse immunoglobulin (Ig) or anti-rabbit Ig and anti-goat Ig as control. These sections were imaged for EBD (red) and Alexa-488 (green) fluorescence using a confocal microscope. The representative micrographs are shown. As expected, EBD positive cardiomyocytes display distinct red fluorescence which clearly distinguishes these cells from their neighboring EBD negative cells in each section. Note that anti-mouse Ig but not anti-rabbit or anti-goat Ig antibodies were able to positively stain the interior of all EBD-positive cells, confirming that the EBD positive cardiomyocytes had lost their membrane integrity so that not only injected EBD but also endogenous mouse Ig were allowed to enter the cell.
Supplementary Figure S7. Representative epi-fluorescence micrographs showing changes in autophagic vacuole abundance by Rab7 knockdown (Rab7KD) as probed with tf-LC3. Cultured NRVMs were infected with Ad-tf-LC3 24hrs after transfection of siRNA against rat Rab7 (siRab7) or against luciferase (siLuci, as control); 36hrs later, the cells were imaged live for the GFP (green) and mRFP (red) signals from tf-LC3. Autophagosomes (yellow puncta) are increased in Rab7KD cells. Scale bar=50μm.


**Supplementary figure S8. Rab7 knockdown blocks autophagosome maturation in glucose deprivation (GD) activated autophagic flux.** NRVMs were first transfected with siRab7 or siLuci for 24 hours, infected with Ad-tf-LC3 for another 24 hours, and then subject to GD for 4 hours before being imaged live for GFP and mRFP fluorescence. Representative epi-fluorescence images of Ad-tf-LC3 infected cells were shown. Insets are the enlarged images. Arrows and arrowheads point to red-only puncta (autolysosome) and green-and-red puncta (autophagosome) respectively. Scale bar=50 μm.