Autophagy and p62 in Cardiac Proteinopathy

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Rationale: Recent studies suggest an important role of autophagy in protection against αB-crystallin-based (CryABR120G) desmin-related cardiomyopathies (DRC), but this has not been demonstrated in a different model of cardiac proteinopathy. Mechanisms underlying the response of cardiomyocytes to proteotoxic stress remain incompletely understood.

Objective: Our first objective was to determine whether and how the autophagic activity is changed in a mouse model of desminopathy. We also investigated the role of p62 in the protein quality control of cardiomyocytes.

Methods and Results: Using an autophagosome reporter and determining changes in LC3-II protein levels in response to lysosomal inhibition, we found significantly increased autophagic flux in mouse hearts with transgenic overexpression of a DRC-linked mutant desmin. Similarly, autophagic flux was increased in cultured neonatal rat ventricular myocytes (NRVMs) expressing a mutant desmin. Suppression of autophagy by 3-methyladenine increased, whereas enhancement of autophagy by rapamycin reduced the ability of a comparable level of mutant desmin overexpression to accumulate ubiquitinated proteins in NRVMs. Furthermore, p62 mRNA and protein expression was significantly up-regulated in cardiomyocytes by transgenic overexpression of the mutant desmin or CryABR120G both in intact mice and in vitro. The p62 depletion impaired aggresome and autophagosome formation, exacerbated cell injury, and decreased cell viability in cultured NRVMs expressing the misfolded proteins.

Conclusions: Autophagic flux is increased in desminopathic hearts, and as previously suggested in CryABR120G-based DRC, this increased autophagic flux serves as an adaptive response to overexpression of misfolded proteins. The p62 is up-regulated in mouse proteinopathic hearts. The p62 promotes aggresome formation and autophagy activation and protects cardiomyocytes against proteotoxic stress. (Circ Res. 2011;109:296-308.)

Key Words: p62/SQSTM1 ■ autophagy ■ aggresome ■ ubiquitin ■ desmin-related cardiomyopathy

Protein quality control (PQC) functions to support protein folding and to segregate and remove terminally misfolded proteins, thereby preventing abnormal proteins from damaging the cell.1 The removal of abnormal proteins is primarily done by the ubiquitin–proteasome system (UPS), the major protein degradation pathway in the cell.1 Macropathogenesis (commonly referred to as autophagy) engulfs a portion of cytoplasm, often including organelles, in a membrane-bound compartment for degradation by lysosomes.2 Through self-digestion of portions of cytoplasm, nonselective autophagy provides fuel for energy supply during starvation, whereas the selective autophagy disposes of damaged organelles and perhaps aberrant protein aggregates for intracellular quality control.3,4 The 2 degradation systems appear to collaborate with each other in PQC.5 It has been shown that increased expression of misfolded proteins in cardiomyocytes causes proteasome functional insufficiency,6,7 aggresome formation,8 and perhaps autophagic activation.9 However, very little is known about the molecular underpinnings of these responses in cardiomyocytes during proteotoxic stress, a condition that is often seen in common forms of heart disease.8,10

Proteinopathies are caused by increased expression of misfolded proteins and featured by the presence of aberrant protein aggregates in the affected cells. Desmin-related cardiomyopathy (DRC) is a family of cardiomyopathy caused by genetic mutations of desmin, αB-crystallin (CryAB), and other desmin partner proteins.11 The most prominent pathological feature of DRC is the presence of aberrant desmin-positive aggregates in muscle cells, which qualifies DRC as a bona fide cardiac proteinopathy. Several transgenic (tg) mouse models of DRC were created by cardiomyocyte-restricted tg overexpression of human DRC-linked mutant genes, including a 7 amino acid (R172~E178) deletion mutation of the desmin gene (D7-des) and a missense mutation (R120G) of CryAB (CryABR120G).8,12,13 These transgenic mice provide excellent intact animal models for studying cardiomyocyte responses to proteotoxic stress.6–8 Most, if not all, of the neurodegenerative disease belongs to

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proteinopathies. Autophagy is up-regulated and appears to facilitate the removal of aberrant aggregates in neurodegenerative diseases.14,15 Studies on autophagy in cardiac proteinopathy have begun to emerge. Hill and colleagues reported a robust increase of autophagosomes in CryABR120G mouse hearts.9 However, an increase in autophagosomes does not always represent increased autophagic activity. On the basis of ultrastructural studies, Robbins’ laboratory suspected that the autophagy activity was decreased in the heart of a slightly different CryABR120G tg mouse model.16,17 Hence, the change of autophagic activities in mouse hearts with CryABR120G-based DRC remains controversial. Nevertheless, blunting autophagy via beclin1 haploinsufficiency was shown to accelerate disease progression in the CryABR120G-based DRC mice.9 However, it remains unclear whether autophagy enhancement would attenuate cardiac proteinopathy, although an increase in autophagy appeared to associate with the Bcl2 overexpression induced delay in the premature-death of CryABR120G tg mouse.16 Moreover, although mutations in either CryAB or desmin genes can cause DRC, the pathogenic pathways are not necessarily identical. Despite sharing DRC characteristics in terms of the presence of desmin aggregates in cardiomyocytes, D7-des-based and CryABR120G-based DRC mice do display diverse phenotypes.12,13 Therefore, in the first part of this study, we sought to investigate changes in the autophagic activity in the heart of a mouse model of cardiac desminopathy and the impact of altered autophagic activities on the removal of misfolded proteins in cardiomyocytes.

Recently, it has been reported that protein aggregates can trigger cardiomyocyte autophagy;10 however, the molecular events that mediate the activation of autophagy by aberrant protein aggregates in cardiomyocytes remain undefined. The finding of the late-onset neurodegeneration in p62 null mice suggests the involvement of p62 in PQC.18 Moreover, several p62 knock-down and knockout experiments further reveal that p62 is required for aggresome formation in metabolically stressed conditions.19,20 Aggresome formation is an important cytoprotective process in proteinopathies. Notably, several lines of evidence from noncardiac cells suggest that p62 may mediate the activation of autophagy by protein aggregates. The p62 has also been identified as a common component in protein aggregates in neurodegenerative diseases.21 The p62 has also been identified as a common component in protein aggregates in neurodegenerative diseases.21 Mean-

### Methods

A full description of Methods can be found in the Online Supplement available at http://circres.ahajournals.org.

#### Transgenic Mice

The protocol for the care and use of animals in this study was approved by the University of South Dakota Institutional Animal Care and Use Committee. The GFP-LC3 transgenic (tg) mouse model was generously donated by Dr. N. Mizushima.24 FVB/N tg mice with cardiac-specific overexpression of D7-des or CryABR120G have been described.12,13

#### Neonatal Rat Ventricular Myocytes (NRVMs) Culture and Adenoviral Gene Delivery

NRVMs culture was performed as previously described.25 The generation of recombinant adenoviruses encoding a 469 amino acid, 53kDa mutated form of the mouse desmin gene with human DRC-associated A360P/N393I compound missense mutations (Ad-MT-des).26 The mouse wild-type desmin gene (Ad-WT-des), a missense (R120G) mutant form of the mouse CryAB gene (Ad-CryABR120G).7 or the LacZ gene (Ad-β-gal) were previously described.27 Adenoviral infection of cultured NRVMs for gene delivery was done as previously described.28

Transmission electron microscopy (TEM),12 Western blot analyses,29 and semiquantitative reverse transcription (RT)–PCR were performed as previously described.30

#### siRNA Transfection

To knock down the target gene expression, we plated 2×10⁶ NRVMs in each 60-mm dish. The Lipofectamine™-2000 transfection reagent (Invitrogen, Carlsbad, CA) was used for siRNA transfection following the manufacturer’s protocol. The siRNA transfec-
tion 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. To achieve sustained knock-down, we required a second round of siRNA transfection, and this was performed 3 days after the first transfection.

**LDH Assay**
LDH activity in the collected medium was measured using a cytotoxicity detection kit (Roche, Indianapolis, IN) following the manufacturer’s protocols. LDH release from NRVMs to culture media of an experimental group was evaluated as a percentage of the mean LDH activity of the corresponding control group.

**MTT Assay**
The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay, an index of cell viability and cell growth, is based on the ability of viable cells to reduce MTT from a yellow water-soluble dye to a dark blue insoluble formazan product. Briefly, $1 \times 10^6$ NRVMs were plated in the 6-well plate. After the experimental treatments were performed, MTT dye (Sigma, 500 $\mu$g/mL) was added to each well of the plate, and the plates were incubated at 37°C for 2 hours. At the end of the incubation, the dye solution was completely removed, 400 $\mu$L 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. Cell viability of the experimental group was determined as a percentage of the reading of the control group.

**Statistical Analysis**
All quantitative data were presented as mean±SD. Differences between groups were evaluated for significance using 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**

**Autophagic Flux Is Increased in Desminopathic Mouse Hearts**
Alterations in the autophagic activity in desminopathic hearts have not been reported. Hence, we investigated autophagy activities in the heart of D7-des tg mice, a well-characterized model of cardiac desminopathy. LC3 is a mammalian homologue of yeast autophagy-related gene (Atg) 8. Native LC3 (LC3-I) is diffusely distributed in the cytoplasm. On autophagic activation, LC3-I is lipidated to form LC3-II. LC3-II integrates to the autophagosomal membrane, thereby displaying a punctate distribution that marks the presence of autophagic vacuoles. Therefore, LC3-II is considered a marker of autophagosomes. Accordingly, GFP-LC3 tg mice have been successfully used in a number of studies to detect in vivo autophagic flux. The probability value <0.05 is considered statistically significant.

For the increased autophagic flux. Taken together. These data indicate that autophagic vacuoles are increased in D7-des hearts. Further accumulation of LC3-II in D7-des mouse hearts on lysosomal inhibition by BFA was observed (Figure 1C and 1D), indicating a significant increase in autophagy in D7-des mouse hearts. Consistent with an increased autophagic activity, the protein level and enzyme activity of cathepsin D, a bona fide lysosomal protease, were significantly increased in D7-des tg hearts (Supplementary Figure I). In the D7-des::GFP-LC3 double tg hearts, the majority of GFP-LC3 puncta stain positive for cathepsin D (Supplementary Figure II), identifying them as autolysosomes. The increase in autophagy activities in D7-des mice is restricted to the heart, where D7-des was overexpressed as both LC3-II abundance and the autophagic flux in the liver were comparable between D7-des tg and Ntg littermate mice (Supplementary Figure III).

According to a recent report, LC3 may also be incorporated into protein aggregates independently of autophagy. To determine whether autophagic structures are increased in D7-des tg mouse hearts, we performed transmission electron microscopy (TEM). At the baseline, autophagic vacuoles were not observed in the cardiomyocytes of the Ntg hearts (Figure 2A and 2C) but were observed, albeit rarely, in the cardiomyocytes of D7-des tg hearts (Figure 2B and 2D, arrow). After 2 hours of BFA treatment to inhibit the lysosome, the frequency with which autophagic structures were observed in the Ntg hearts was markedly increased (Figure 2E), but the frequency for autophagic structures of all stages (the early and the late stages) to be observed in the D7-des tg hearts was significantly greater (Figure 2F). The autophagosome observed in the Ntg heart often engulf a mitochondrion (Figure 2E, open arrow), whereas the autophagic vacuoles (Figure 2F, arrows) in D7-des tg hearts are frequently found in the neighborhood of electron dense protein aggregates, are usually smaller, and usually do not contain mitochondria. Our TEM morphological data further confirm that autophagic flux is increased in D7-des tg mouse hearts.

To further determine whether the autophagic activation is a direct consequence of expression of a misfolded desmin or a secondary response to protein overexpression, we also evaluated autophagic flux in cultured NRVMs expressing DRC-linked MT-des or WT-des. Cultured NRVMs were infected with Ad-MT-des, Ad-WT-des, or Ad-β-gal as a control. Six days after the infection, cells were treated with BFA or vehicle control for 3 hours. LC3-II abundance under basal conditions as well as with lysosomal inhibition was increased in NRVMs expressing MT-des in a dose-dependent manner, in comparison with controls (Figure 1E and 1F). However, at a level comparable to MT-des overexpression, WT-des overexpression did not increase autophagic flux in cultured NRVMs (Supplementary Figure IV), suggesting that the mutation/misfolding of the transgenic protein is responsible for the increased autophagic flux. Taken together, these data...
indicate that autophagy can be up-regulated in cardiomyocytes by the expression of mutant desmin in both intact mice and in vitro cultures.

Manipulating Autophagic Activities Mitigates the Accumulation of Ubiquitinated Proteins in MT-des Expressing NRVMs

Accumulation of ubiquitinated proteins in the cell is a major feature of PQC inadequacy seen in proteinopathy. To determine the role of autophagy in the removal of misfolded proteins in cardiomyocytes, we tested the effects of reducing and enhancing autophagy on MT-des overexpression induced accumulation of ubiquitinated proteins in cultured NRVMs. Inhibition of autophagy was achieved by administration of 3-methyladenine (3-MA), a specific inhibitor of the class III phosphatidylinositol 3-kinase (PI3K) pathway, which is involved in the formation of autophagosomes and initiation of autophagy. Rapamycin, an mTOR inhibitor, was used to enhance autophagy. By monitoring changes in GFP-LC3 distribution and the abundance of endogenous LC3-II, the expected autophagy inhibition and enhancement effects, respectively, by 3-MA and rapamycin were confirmed in cultured NRVMs (Supplementary Figure V). Consistent with previous reports, the accumulation of high molecular weight ubiquitinated proteins were observed in NRVMs expressing MT-des. In comparison with vehicle control, the treatment of 3-MA significantly increased the accumulation of total ubiquitinated proteins (Figure 3A and 3B). The MT-des transcript levels were comparable between the 3-MA and the control treatment groups (Figure 3C and 3D), but the high molecular weight desmin species (HMW-Des), which are likely the ubiquitinated forms of desmin, were markedly increased by 3-MA (Figure 3A). By contrast, rapamycin treatment significantly decreased the accumulation of total ubiquitinated proteins (Figure 4A and 4B) caused by comparable MT-des expression at the mRNA level (Supplementary Figure VI). Moreover, cycloheximide chase experiments revealed that rapamycin treatment substantially shortened the half-life of the MT-des proteins expressed in NRVMs (Figure 4C and 4D).

Figure 1. Expression of a DRC-linked mutant desmin increases autophagic flux in mouse hearts and NRVMs. A and B, Confocal microscopic analysis of GFP-LC3 distribution in ventricular myocardium. D7-des tg mice were cross-bred with GFP-LC3 mice. The resultant GFP-LC3::D7-des double tg (DTG) mice and their littermate GFP-LC3 single tg mice were analyzed at 2 months of age. The representative images (A) and the quantitative analysis of the number of GFP-LC3 puncta (B) are presented. The GFP dot data (B) were quantified from the 3 randomly selected fields per section, 3 representative sections per heart, and 3 hearts per group. C and D, Increases in autophagic flux in the D7-des tg mouse heart. D7-des tg and NTG littermate mice at 2 months were intravenously injected with 1 dose of bafilomycin A1 (BFA, 6 µmol/kg) or vehicle control (CTL) at 3 hours before the hearts were harvested for Western blot analyses of LC3. Representative images (C) and a summary of LC3-II densitometry data (D) are presented. *P<0.05 versus CTL. E and F, Autophagic flux was increased in NRVMs expressing DRC-linked mutant desmin (MT-des). Cultured NRVMs were infected with Ad-MT-des or Ad-β-gal (as control). Six days after infection, the cells were treated with BFA (100 nmol/L) or DMSO for 3 hours before being harvested for Western blot analysis for the indicated proteins. Representative images (E) and a summary of densitometry data (F) are presented. *P<0.05 versus CTL; #P<0.05 versus Ad-β-gal/CTL; n=3 per group in all cases. AU, arbitrary units.
These data are consistent with the hypothesis that the activation of autophagy facilitates the removal of MT-des in cardiomyocytes and enhancing autophagy improves PQC in cardiomyocytes.

**p62 Is Up-Regulated in Proteinopathic Mouse Hearts**

On the basis of the findings from studying noncardiac cells, p62 was purported to play a critical role in mediating the activation of autophagy by ubiquitinated proteins, but this has not been tested in cardiac proteinopathy. Therefore, we first examined p62 transcript and protein expression and protein distribution in the hearts of D7-des and CryABR120G tg mice, 2 well-established mouse models of cardiac proteinopathy. In comparison with their respective littermate Ntg controls, both CryABR120G and D7-des tg mouse hearts showed marked increases in both the transcript and the protein levels of p62 (Figure 5). Immunofluorescence staining revealed that the up-regulation of p62 was limited in cardiomyocytes (Supplementary Figure VII). Furthermore, Western blot analyses showed that p62 protein levels were also markedly increased in cultured NRVMs expressing CryABR120G or MT-des (Supplementary Figure VIII), suggesting that up-regulation of p62 in proteinopathic hearts is specifically induced by expression of misfolded proteins rather than a secondary response to cardiac dysfunction.

We next examined the distribution of p62 in relation to autophagic vacuoles and ubiquitinated protein aggregates in D7-des tg hearts using immunofluorescence confocal microscopy. The p62 formed small speckles in NTG mouse hearts, whereas in D7 mouse hearts, p62 formed large punctate structures with dimensions consistent with autophagosomes. To monitor autophagosomes in D7-des tg hearts, we crossbred D7-des mice with GFP-LC3 mice. The colocalization among p62, ubiquitin, and GFP-LC3 was observed in D7-des mouse hearts (Supplementary Figure VII). These findings suggest a potential functional relationship between p62 and ubiquitin-positive aggregates as well as between p62 and autophagosomes in cardiomyocytes.

**p62 Depletion Decreases LC3-II in Cardiomyocytes**

To test the functional role of p62 in the cell models of cardiac proteinopathy, we performed p62 knock-down experiments in NRVMs to test the impact of p62 depletion on LC3 lipidation, which renders LC3-I to LC3-II and represents a critical event in autophagosome formation. We achieved a very efficient p62 knock-down in cultured NRVMs via 2 consecutive
transfections of p62 siRNA at a dose of 160 pmol for 2×10^6 cells with an interval of 72 hours. Three days after the second p62 siRNA transfection, p62 proteins in the cells were reduced to a level undetectable by Western blot analysis (Figure 6). The protein abundance of LC3 was assessed in NRVMs with p62 depletion. We found that p62 knock-down reduced LC3-II levels under basal conditions. Expression of MT-des or CryABR120G significantly increased LC3-II abundance in NRVMs. This increase was blunted by p62 knockdown (Figure 6). These results suggest that p62 is essential to the formation of at least a significant portion of autophagosomes in cardiomyocytes both under basal conditions and during overexpression of misfolded proteins.

p62 Is Required for Efficient Formation of Aggresomes in NRVMs Expressing MT-des or CryABR120G

Aggresome formation is considered a protective response to increased expression of misfolded proteins in the cell. Transgenic expression of CryABR120G has been shown to cause aggresome formation in cardiomyocytes in vivo and in vitro. Aggresomes always contain elevated levels of ubiquitin and SEC61α. SEC61α is a normal constituent of endoplasmic reticulum (ER). To determine a potential obligatory role of p62 in the formation of aggresomes in cardiomyocytes, we assessed the prevalence of aggresomes resulting from overexpression of misfolded proteins in
cultured NRVMs. Immunofluorescence images showed that p62 formed small speckles in the perinuclear region in the control cardiomyocytes. Consistent with previous reports, NRVMs expressing HA-CryABR120G and treated with control siRNA showed numerous large perinuclear HA-positive aggregates (Figure 7A). These aggregates showed elevated levels of ubiquitin (Supplementary Figure IX) and SEC61, indicative of aggresomes. Interestingly, both p62 and SEC61 were concentrated in these aggresomes. However, after p62 knock-down, Ad-CryABR120G infection at the same multiplicity of infection (MOI) induced substantially fewer and smaller aggresomes in NRVMs, and aggresomes were formed in fewer CryABR120G cells (Figure 7 and Supplementary Figure IX). Similar results were also obtained from NRVMs expressing Myc-MT-des (Supplementary Figure X–XII). These data reveal the necessity of p62 in the formation of aggresomes in cardiomyocytes expressing misfolded proteins.

Given that aggresomes are insoluble, its chief components, including misfolded proteins and ubiquitin conjugates, are present in the insoluble fraction of the cell lysates. To further test the necessity of p62 in aggresome formation in a more...
quantitative manner, we assessed the effects of p62 depletion on the abundance of the overexpressed mutant proteins and the total ubiquitin conjugates in the Triton-X100 soluble and insoluble fractions of cardiomyocytes. Western blot analyses revealed that expression of CryABR120G (Figure 8) or MT-desmin (Supplementary Figure XIII) in NRVMs both accumulated ubiquitinated proteins in the soluble and insoluble fractions. Overexpression of CryABR120G (Figure 8A and 8B) or MT-des- (Supplementary Figure XIIIA and XIIIB) induced accumulation of ubiquitinated proteins in the insoluble fraction was significantly attenuated by p62 knockdown. Moreover, HA-CryABR120G and Myc-Mt-des protein levels in the insoluble fraction were both reduced with p62 depletion (Figure 8A and 8D, Supplementary Figure XIIIA and XIIID). However, the mutant protein levels in the Triton-X100 soluble fraction responded differently with p62 depletion. With p62 depletion, soluble HA-CryABR120G proteins were not altered, whereas Myc-Mt-des protein levels in Triton-X100 soluble fractions were decreased (Figure 8A and 8C, Supplementary Figure XIIIA and XIIIC). The difference might be due to the different functions and distributions of these 2 proteins in the cell.42 Taken together, these data show that p62 plays an important role in the recruitment of soluble species of misfolded proteins into the insoluble aggresomes and, therefore, was critical in aggresome formation.

**p62 Deficiency Sensitizes Cell Injury Induced by Expressing MT-des and CryABR120G:**

Our findings demonstrate that p62 regulates both aggresome formation and autophagy activation in cardiomyocytes. Given that both aggresome formation and autophagy activation may protect the cell from toxic proteins in cardiomyocytes,8,9 we sought to determine whether p62 plays a protective role in cells expressing mutant proteins. Cell injury and cell viability were assessed respectively by LDH leakage and the MTT assay. Knocking down p62 significantly increased cytotoxicity under basal conditions, as evidenced by a significant increase of LDH activities in the culture medium and decreased cell viability in the NRVM cultures transfected by p62-siRNA, in comparison with those transfected by the control siRNA (Luc-siRNA). LDH activities in cultured media were also elevated by expression of CryABR120G in NRVMs. Strikingly, after p62 depletion, the elevation of LDH activity induced by CryABR120G expression was more than doubled (from a 43% increase to a 92% increase; Figure 8E). In agreement with the LDH data, the MTT assays revealed that p62 knock-down reduced cell viability under basal conditions. CryABR120G expression caused a significant loss of cell viability. Notably, after p62 knock-down, the same extent of CryABR120G overexpression caused a twice greater loss of cell viability (from a 25% loss to a 46% loss; Figure 8F). Moreover, LDH activities in the medium were normalized by the number of viable cells of the same dish as

**Figure 7. The p62 is required for efficient formation of aggresomes in NRVMs expressing CryABR120G.** NRVMs were treated as described in Figure 6. On day 7 after Ad-HA-CryABR120G infection, cultured NRVMs were fixed in 2% paraformaldehyde and used for indirect double immunofluorescence labeling for the HA-tag of HA-CryABR120G (green) and for p62 (red, A) or SEC61α (red, B). Nuclei were stained blue with DAPI. HA-labeled aggregates, induced by HA-CryABR120G expression, colocalize with p62 (A) and SEC61α (B). To show the normal distribution of SEC61α, we obtained the images of the groups infected with Ad-β-gal by a longer exposure (1.5 s) in comparison with the Ad-CryABR120G groups (400 ms).
measured by the MTT assay. The normalized LDH activities (ie, LDH/MTT ratio) further confirmed that p62 knock-down enhanced cell injury and decreased cell viability in NRVMs (Figure 8G). Similar results were also obtained from NRVMs expressing MT-des (Figure 8E–8G). The significantly decreased cell viability and increased cell injury caused by p62 deficiency indicate a protective role of p62 in the PQC of cardiomyocytes.

**Discussion**

Two reported studies have investigated autophagy in cardiac proteinopathy, and both used mouse models of CryAB$^{R120G}$, based DRC. However, the 2 reports do not appear to agree on whether autophagic activity is increased in the DRC mouse hearts. Moreover, DRC can be caused by expression of other misfolded proteins such as mutations in the desmin gene; but no study has been reported on autophagy in the heart with DRC caused by a mutant gene other than CryAB$^{R120G}$. More important, the mechanism underlying autophagic activation in cardiac proteinopathy is unknown. In an attempt to address some of these unanswered questions, we have found in the present study that (1) autophagic flux is increased in the heart of a mouse model of desminopathy, (2) lysosomal function is also up-regulated in desminopathic mouse hearts, (3) autophagy helps to clear ubiquitinated proteins in cells expressing human DRC-linked misfolded proteins, and (4) p62 plays a crucial role in the formation of aggresomes as well as the activation of autophagy in cardiomyocytes expressing misfolded proteins. These new findings provide strong evidence that autophagic flux is increased in desminopathic hearts and establish an important role of p62 in the PQC in cardiomyocytes. Our study suggests that p62 can potentially be a therapeutic target for improving PQC in the heart.

**Autophagy Is Up-Regulated in Cardiac Proteinopathies**

A recent study on cultured SH-SY5Y cells, a cell line derived from human neuroblastoma, suggests that activation of autophagy is not a universal response to all misfolded proteins.
It shows that aggresomes formed by ectopic expression of a DRC-linked mutant desmin do not trigger autophagy and are not cleared by autophagy.43 In the present study, however, we have collected multiple lines of compelling evidence supporting the notion that autophagy is up-regulated in mutant desmin-based proteinopathy, both in cultured cardiomyocytes and in the heart of intact mice. Both endogenous LC3 and overexpressed GFP-LC3, commonly used autophagosome markers, are markedly increased in D7-des mouse hearts. Cathepsin D expression levels and enzyme activities are up-regulated in D7-des mouse hearts (Supplementary Figure I). The superimposing immunolabeling of cathepsin D and GFP-LC3 puncta indicates the formation of autolysosome (Supplementary Figure II). Furthermore, the additional increases in both LC3-II protein levels and TEM detected autophagic vacuoles in the heart after lysosomal inhibition demonstrate that autophagic flux is significantly increased in D7-des mouse hearts. These data indicate that the increase in autophagosomes in D7-des heart is not due to diminished lysosomal function but is rather caused by increased autophagosome formation. The increased autophagic flux is unlikely a secondary response to heart malfunction because our results also show an increased autophagic flux in cultured cardiomyocytes expressing MT-des.

Using TEM, Maloyan et al failed to detect any increase in autophagic vacuoles in the heart of CryABR120G tg mice under basal conditions.16 Indeed, we also found in our TEM study that autophagic vacuoles can be found but are very rare in the D7-des mouse hearts at baseline. However, the situation is dramatically changed by a short-term lysosomal inhibition. At 2 hours after intraperitoneal injection of BFA, autophagic vacuoles become detectable in the Ntg control mouse hearts and are markedly more prevalent in D7-des tg mouse hearts (Figure 2). These data suggest that the entire process from the formation of an autophagosome to the complete removal of the autophagosome by lysosomes is extremely fast in cardiomyocytes both under the normal condition and during desminopathy. Notably, there is an apparent discrepancy between the 4-fold increase of LC3-II proteins and the very modest increase in autophagic vacuoles detected in the hearts of D7-des mice under basal conditions. This discrepancy suggests that a portion of the up-regulated LC3-II proteins in D7-des cardiomyocytes is not associated with typical autophagosomes, consistent with a recent report.37 Nevertheless, paralleling increases in both LC3-II protein levels and autophagic vacuoles were observed in both D7-des tg and Ntg hearts after lysosomal inhibition. Our TEM study observed all stages of autophagic vacuoles in the D7-des hearts. These vacuoles tend to be smaller than those observed in the control hearts. As previously reported,13 the large and electron-dense protein aggregates in D7-des tg cardiomyocytes are always surrounded by and continued with amorphous less-dense materials. The latter appear to be an intermediate species of small protein aggregates that are at a stage either before merging with the aggresomes or immediately after breaking off the dense aggresomes. Another interesting and perhaps important TEM feature of the autophagic vacuoles detected in the cardiomyocytes of D7-des tg mice is that they often appear in the vicinity of the electron-dense aggresomes but show no intent of engulfing the large and electron-dense aggregates (or aggresomes). Rather, the content engulfed in the early-stage autophagosome appears to be those intermediate species of amorphous aggregates (Figure 2F). This observation suggests that autophagy removes the misfolded proteins in the vicinity of aggresomes rather than directly removing the aggresome per se. The aggresome constituent may need to be broken down to smaller and less-dense materials before being engulfed and removed by autophagosomes. It is also possible that the loose and small protein aggregates have the opportunity of being removed by autophagy before merging with the aggresome.

The soluble misfolded proteins are mainly degraded by the proteasome, whereas the aggregated forms resist proteasomal degradation.44 This resistance to UPS degradation is probably caused by the difficulty of aggregated proteins to enter the narrow opening of the 20S proteasome barrel.7,44 Moreover, aberrant protein aggregation, in turn, overwhelms and impairs proteasome function,7,26 potentially disrupting other important cellular processes.45 In contrast, autophagy has a more remarkable capability of degrading aggregated proteins.46 We have previously reported that cardiac proteasome function is insufficient in both D7-des-based and CryABR120G-based DRC mice.6,7 Therefore, the up-regulated autophagic flux observed in cardiac proteinopathy suggests a likely collaborative relationship between the UPS and autophagy in handling misfolded proteins in the heart.

**Autophagy Removes Ubiquitinated Proteins in Cardiomyocytes Expressing MT-des**

Detrimental and protective roles of an increased autophagic activity in cardiac dysfunction have both been reported.33,34 The role of autophagy in a disease may depend on disease stages and the nature of the stress that causes the disease.

Here we have tested, and obtained results consistent with, the hypothesis that autophagy helps remove the DRC-linked MT-des proteins in cardiomyocytes, thereby protecting against desminopathy. Terminally misfolded proteins in the cell, when escaped from the surveillance from chaperones and the UPS, trigger aberrant protein aggregation that can damage the cell by a number of mechanisms, including impairing the proteasome.1,26 Accumulation of ubiquitinated proteins in the form of protein aggregates is a common feature of cells under proteotoxic stress. Under comparable levels of MT-des overexpression, suppression of autophagy by administration of 3-MA significantly reduces—whereas enhancement of autophagy by rapamycin accelerates—the ability of the cultured cardiomyocytes to eliminate ubiquitinated proteins and MT-des proteins. Activation of autophagy by rapamycin significantly decreased the half-life of MT-des, whereas suppression of autophagy increased the HMW species of MT-des expressed in cultured cardiomyocytes (Figures 3 and 4). These data suggest that autophagy functions to remove ubiquitinated proteins and the MT-des proteins in cardiomyocytes overexpressing MT-des. Consistent with our findings, Tannous et al demonstrated a protective effect of autophagy on the pathogenesis of CryABR120G in mouse hearts by a loss-of-function approach.8 Hence, in DRC caused by either a mutant CryAB or a mutant desmin, it appears that...
autophagy is activated and plays an essential role in maintaining protein homeostasis in the cardiomyocytes.

p62 Is Up-Regulated in Cardiac Proteinopathy and Facilitates Aggresome Formation and Autophagy Activation

Recently, p62 is purported to act as an adaptor molecule linking ubiquitinated proteins to the autophagic machinery. It was shown in Hela cells that p62 directly interacts with LC3 and is degraded by autophagy. Here we report that both the transcript and protein levels of p62 are significantly up-regulated in the heart of 2 mouse models of cardiac proteinopathy. The up-regulation was recapitulated in cultured NRVMs. The p62 has also been identified as a common component in the inclusion bodies seen in a number of neurodegenerative diseases. Our study has extended this prospect to cardiac proteinopathy. We found that p62 is enriched in the ubiquitin-positive aggregates in D7-des mouse hearts and colocalizes with autophagosome marker GFP-LC3, prompting us to hypothesize that p62 provides a key link between protein aggregation and selective autophagy in cardiomyocytes. To the best of our knowledge, this is the first study on the functional role of p62 in cardiac disease.

Under normal conditions, p62 distributes as cytosolic speckles, but under conditions of metabolic stress, p62 redistributes into aggregates. Here we found that p62 colocalizes with the mutant proteins in the large aggregates in cultured cardiomyocytes expressing CryABR120G or MT-des. The aggregates also contain ubiquitin and SEC61β and most of them are perinuclear, identifying them as aggresomes. Notably, consistent with what we showed previously in mouse hearts, here we observed that overexpression of CryABR20G or MT-desmin in cultured NRVMs also led to formation of large protein aggregates that are not perinuclear. By definition, these nonperinuclear aggregates may not be considered as aggresomes. Importantly, our study shows that p62 depletion impairs the formation of both aggresomal and nonaggresomal large aggregates from the overexpressed misfolded proteins in cardiomyocytes. Accordingly, the insoluble fractions of ubiquitin conjugates are reduced by p62 depletion. This is in agreement with the previous observations in noncardiac cells and tissues. Depletion of p62 diminishes the formation of ubiquitin-positive inclusions following puromycin treatment in Hela cells or in Atg7-deficient mouse livers and brains. These lines of evidence suggest an important role of p62 in the cells under proteotoxic stress through promoting the formation of aggresomes. It is generally believed that aggresomes restrict the intracellular distribution of misfolded proteins. Aggresome formation sequesters toxic soluble oligomers into the less harmful insoluble aggregates, thereby protecting the cell. Given the similarity of their composition to aggresomes, nonperinuclear large aggregates, which also help to sequester the misfolded proteins, may conceivably make misfolded proteins not only less toxic but also more accessible to selective autophagy. This is because autophagy machinery is not limited to the perinuclear location.

An important way by which aggresome formation protects the cell is to facilitate the delivery of dispersed small protein aggregates to the autophagic machinery for bulk degradation. The p62 is a proven substrate of, and constitutively degraded by, selective autophagy. Therefore, it is likely that by interacting with LC3-II, p62 incorporation into aggresomes recruits the phagophore and thereby triggers autophagic degradation of the aggresome. Supporting this hypothesis, our study shows that p62 knock-down significantly decreased LC3-II formation in cardiomyocytes both under basal conditions and during overexpression of misfolded proteins. The p62 is not required for the nonselective autophagy because starvation-induced autophagy is not affected in p62-deficient mice. Hence, our study shows an important functional role of p62 in the selective autophagy in cardiomyocytes.

As a further demonstration of a protective role for p62-mediated aggresome formation and autophagic activation in cardiomyocytes in response to increased proteotoxic stress, we have observed that p62 depletion increases cell injury and decreases cell viability in NRVMs under basal conditions and during overexpression of misfolded proteins, as evidenced by the increased leak of LDH to the culture media and the decreased number of viable cells (Figure 8).

Collectively, our study shows that p62 is important for aggresome formation as well as the activation of selective autophagy in cardiomyocytes in response to proteotoxic stress. On the basis of our data and previous reports, the following model is proposed for the role of p62 in the response of cardiomyocytes to the increase in misfolded proteins in the cytosol: (1) ubiquitinated misfolded proteins escape from proteasome-mediated degradation and form the relatively small and soluble oligomeric aggregates that are active and toxic to the cell or alternatively, the misfolded proteins that have escaped from the UPS surveillance form the oligomers first and are then ubiquitinated; (2) the toxic oligomers are packed into the less-harmful aggresomes in a p62-dependent manner; and (3) aggresome-associated p62 recruits autophagic machinery to the aggresome and triggers the selective autophagy to remove the aggregated misfolded proteins.

Acknowledgments
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Disclosures
None.
References

What Is Known?

- Increased autophagosomes were observed in CryAB<sup>R120G</sup>-based desmin-related cardiomyopathy (DRC) mouse hearts, but autophagic flux has not been determined in a model of cardiac proteinopathy.
- The p62 is purported to mediate the formation of inclusion bodies and activate selective autophagy, but this remains to be demonstrated in cardiomyocytes.
- The role of p62 in protein quality control in the cell appears to be cell type dependent.

What New Information Does This Article Contribute?

- The first report of an increased autophagic flux in the heart of a mouse model of proteinopathy and the first evidence that enhancing autophagy facilitates the removal of misfolded proteins in cardiomyocytes.
- The p62 expression is up-regulated in the heart of DRC mouse models, representing the first study of p62 in the heart.
- The first demonstration that p62 is required for aggresome formation and activation of selective autophagy in cardiomyocytes under the proteotoxic stress and protects cardiomyocytes from proteotoxic stress.

Understanding the mechanism by which cardiomyocytes handle increased expression of misfolded proteins, an inevitable consequence and cause of increased cardiac stress, is essential for delineating the pathogenesis of a large subset of heart disease. This is because aberrant aggregation and accumulation of misfolded proteins in cardiomyocytes have been observed in failing human hearts resulting from common forms of heart disease. However, the molecular mechanisms of protein quality control, in cardiomyocytes in particular, remain incompletely delineated. Using intact mice or cultured cardiomyocytes, this study reveals for the first time that (1) autophagic flux is adaptively increased in a mouse model of cardiac proteinopathy; (2) p62 is transcriptionally up-regulated in cardiomyocytes and the heart by overexpression of human DRC-linked misfolded proteins; and (3) p62 appears to play a pivotal and protective role in cardiomyocytes in mediating 2 major defending mechanisms against increased misfolded proteins: aggresome formation and the activation of selective autophagy. These mechanistic findings suggest that autophagy and p62 can potentially be key targets for developing therapeutic strategies to improve cardiac protein quality control.
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Autophagy and p62 in Cardiac Proteinopathy

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ONLINE SUPPLEMENTARY MATERIALS

I. A Full Description of Methods
II. Supplementary Figures and Legends
I. A Full Description of Methods

Reagents

E64D, pepstatin A methyl ester, and MG132 were purchased from EMD Chemicals Inc. (Gibbstown, NJ). Trichostatin A ( TSA), rapamycin, Ethylenediaminetetraacetic Acid (EDTA), Phenylmethylsulfonyl Fluoride (PMSF), and 5-Bromo-2'-Deoxyuridine (BrdU) were purchased from Sigma-Aldrich (St. Louis, MO). 3-MA and nonidet P-40 detergent (NP 40) were purchased from ThermoFisher Scientific (Waltham, MA). Bafilomycin A1 (BFA) and bortezomib were from LC laboratory (Woburn, MA). MG262 was purchased from BIOMOL (Plymouth Meeting, PA). DNase I and complete protease inhibitor cocktail was from Roche Applied Science (Indianapolis, IN)

Transgenic mice

The protocol for the care and use of animals in this study was approved by University of South Dakota Institutional Animal Care and Use Committee. The GFP-LC3 tg mouse model was generously donated by Dr. N. Mizushima from National Institute for Basic Biology in Japan and maintained in FVB/N inbred background.1 FVB/N tg mice with cardiac-specific overexpression of D7-des (line 641) or CryABR120G (line 134) have been described.2, 3 Genotyping was carried out by PCR analysis. The primers used for PCR genotyping are as follows: GFP-LC3: Forward 5'-ATAACTTGCTGGCCTTTCCACT-3'; intermediate 5'-GCAGCTCATTGCTGTTTCTCAAA-3'; reverse 5'-CGGGCCATTTCACCCTGTAAGTT-AT-3'. CryABR120G: Forward 5'-GAGTCTGACCTCTTCTCAACAGCC-3'; reverse 5'-CTGGCGTTCGTGGTGCTTGCGGGCTGGT-3'; D7-des: Forward 5'-CAGCTTCGGAACAGCAGCGGCTCC; reverse 5'-CATCAATCTCGAGGCTGTTAGGACT-3'.

Primary cell culture of neonatal rat ventricular myocytes (NRVMs)

NRVMs were isolated using the Cellutron Neomyocytes isolation system (Cellutron Life Technology, Baltimore, MD, Cat. No. nc-6031) following the manufacturer’s instructions.4, 5 Ventricles were collected from 0- to 2-day-old rat neonates and then digested at 37°C in a beaker with stir bar stirring at a speed of 150rpm for 12 minutes. Cells were collected using 1200rpm centrifugation for 2 minutes and then resuspended in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 μM BrdU, and 100 U/ml penicillin/streptomycin ( Invitrogen, Carlsbad, CA). Several repeats of the digestion process were performed until most of the tissues were digested. To selectively enrich cardiomyocytes, the cells were preplated in 100mm non-coated dishes for 1 hour. The resulting suspended cells were counted with a hemocytometer and then plated evenly on 1% gelatin-coated plates in appropriate cell densities. The plated cells were then cultured in a 5% CO2 incubator at 37°C for at least 24 hours before the medium was changed to meet the needs of the follow-up experiments.

Recombinant adenoviruses infection of NRVMs

Creation and validation of recombinant adenoviruses harboring the β-galactosidase (Ad-β-Gal) expression cassette were previously reported.6 As previously reported, adenoviruses harboring Myc-tagged MT-des expression cassette (Ad-MT-des) and HA-tagged CryABR120G (Ad-CryABR120G) were generated using the AdEasy Adenoviral Vector System (Stratagene, Santa Clara, CA) and accompanying protocol. A c-myc tag to the amino-terminus of MT-des and an HA- tag to the carboxyl terminus of CryAB differentiate them from their endogenous counterparts as previously reported.6 GFP-LC3 expressing adenoviruses (Ad-GFP-LC3) were generously provided by Dr. R. Gottlieb of San Diego State University.7 Adenoviruses harboring HDAC6 expression cassette (Ad-HDAC6) were purchased from Vector Biolabs. Infection of
cultured cardiac myocytes with any of these recombinant adenoviruses was generally started at 48–72 hours after myocytes were plated. Adenoviral infection of NRVMs was performed in serum-free DMEM in a 5% CO₂ incubator at 37°C for 3 hours. Control cells were infected with Ad-β-gal at comparable multiplicity of infection (MOI) with the adenovirus applied to the experimental cells. Three hours after infection, the cultured mediums containing the adenovirus were replaced with fresh mediums containing 2% FBS and the cells were incubated for at least 48 hours to allow the transgene expression.

RNA interference
The small interference RNA (siRNA) specific for rat p62 (p62 siRNA: 5’-CATGTCTATGTGAAAGATGA-3’) and the siRNA targeting luciferase serving as a control siRNA (luc siRNA: 5’-AACGTACGCGGAATACTTCGA-3’) were purchased from Qiagen (Cat.#: SI03074113; Valencia, CA). For the transient knock down of target genes, 2X10⁶ cells were plated in 60mm dishes, and transfection of cultured NRVMs with siRNA was generally started at 48-72 hours after myocytes were plated. Lipofectamine™ 2000 transfection reagent (Invitrogen) was used for siRNA transfection following the manufacturer’s protocol. In knock down experiments, the same amounts of luciferase siRNA and p62 siRNA were applied to the control and experimental groups, respectively. Six hours after the transfection, the siRNA-containing medium was replaced with the fresh medium containing 2% FBS. Two consecutive rounds of transfection of p62 siRNA at a dose of 160pmol for 2X10⁶ cells with an interval of 72 hours were performed. Three days after the second p62 siRNA transfection, the follow-up assessments were performed.

Protein extraction and western blot analysis
To prepare total proteins, ventricular myocardium 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 and boiled for 5 minutes. After cell/tissue homogenates were centrifuged at 10,000×g for 10 minutes at 4°C, the supernatants were obtained as total proteins.

To prepare soluble and insoluble fractions, cells were harvested 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 mixture and then incubated on ice for 30 minutes, vortexed 30 seconds every 10 minutes. The cell extracts 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 being sonicated 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.

The protein concentration was determined using Bicinchoninic acid (BCA) reagents (Pierce biotechnology, Rockford, IL). Equal amounts of samples were subjected to sodium dodecyl sulfate -polyacrylamide gel electrophoresis (SDS-PAGE), transferred to PVDF membrane using a Trans-blot apparatus (Bio-Rad, Hercules, CA). The membranes were blocked with 5% non-fat-dry milk in PBS containing 0.1% Tween-20 (PBS-T) for 1 hour at room temperature and then probed with appropriate primary antibodies overnight at 4°C. The following primary antibodies were used: anti-ubiquitin (U5379, Sigma-Aldrich; 1:100), anti-desmin (D1033, Sigma-Aldrich; 1:20000), anti-α-tubulin (T6199, Sigma-Aldrich; 1: 2000), anti-acetylated α-tubulin (T7451, Sigma-Aldrich; 1:20000), anti-α-actinin (A5044, Sigma-Aldrich; 1:5000), anti-GAPDH (G8795, Sigma-Aldrich; 1:1000), anti-cathepsin D (IM16, EMD Chemicals Inc.; 1:500), anti-LC3 (M115-3, Medical & Biological Laboratories Co., MBL, Nagoya, Japan; 1:1000), anti-HA (sc-7392, Santa Cruz Biotechnology, Santa Cruz, CA; 1:20000), anti-p62 (03-GP62-C, American Research products, Belmont, MA; 1:10000), anti- CryAB (SPA 227, Stressgen; 1:20000), anti-proteasome
subunit β5 (customized antibody, 1:5000), and anti-c-myc (MB600-336, Novus Biological, Littleton, CO; 1:10000), GFP B2 (sc-9996, Santa Cruz Biotechnology; 1:2000). The corresponding horseradish peroxidase-conjugated goat anti-mouse, goat anti-rabbit or goat anti-guinea secondary antibodies (Santa Cruz Biotechnology) were used respectively for chemiluminescence-based western blot analysis. The signal was detected using either enhanced chemiluminescence (ECL-Plus) reagents (GE Healthcare, Piscataway, NJ) or, for weak signals, ECL Advance Western Blotting Kit (GE Healthcare) and visualized with a VersaDoc3000 imaging system (model 3000, Bio-Rad). The signal was quantified with the Quantity One software (Bio-Rad).

Cathepsin D activity assay

Cathepsin D activity was measured using Cathepsin D assay kit (Sigma-Aldrich) and following the manufacturer’s protocol. The internally quenched fluorescent substrate, MCA-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu- Lys (DNP)-D-Arg-NH2 trifluoroacetate salt, was used. Snap-frozen myocardium tissues were homogenized on ice in lysis buffer (10 mM HEPES buffer at pH 7.9 containing 10 mM NaCl, 1 mM DTT, 10% glycerol, 15 mM MgCl2, 0.2 mM EDTA, 0.1%NP40, protease inhibitor cocktail), and then centrifuged at 10,000×g for 10 minutes at 4 °C. The resultant supernatants were obtained as soluble proteins for the assay. Reactions were performed in the absence or presence of the aspartic protease inhibitor pepstatin A to inhibit the Cathepsin D activity. In each well of a 96-well plate, the 98 µl reactions include 10 µg of soluble protein and 20 µl assay buffer (0.1 M sodium acetate, 5 mM DDT, 1 mM EDTA, pH 5.5) with and without pepstatin A (final concentration: 0.2 mg/ml). The 96-well plate was pre-incubated at 37°C for 10 minutes to allow for inhibition of the enzyme. After 2 µl of the Cathepsin D substrate solution was added to each well of the enzymatic assay, fluorescence was measured using the Tecan plate reader with the excitation/emission wavelength of 328/393nm. The activity inhibited by Pepstatin A was equivalent to the cathepsin D activity. The presented relative cathepsin D activity of D7-des mouse hearts was determined as a percentage of the activity of Ntg control hearts.

Transmission Electron microscopy (TEM)

TEM was performed essentially as previously described.2 Mice were anesthetized with isoflurane and the hearts 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 (800 mmH2O) into the abdominal aorta via a retrograde cannula with the distal end of the abdominal aorta occluded. The right atrium was cut open during the perfusion. 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 picked up on nickel grids, dried and etched with a saturated solution of sodium m-periodate and 0.1N HCl. Thin sections were counterstained with uranyl acetate and lead citrate. The sections were viewed in a JEOL model 1210 electron microscope at 100 kV.

Semi-quantitative reverse transcription-(RT-) polymerase chain reaction (PCR) analysis

Total RNA was isolated from cultured cardiomyocytes in 60mm dishes or ventricular myocardium tissue using the TRizol Reagent (Molecular Research Center, Inc., Cincinnati, CA) following the manufacturer’s protocol. The concentration of RNA was determined using Agilent RNA 6000 Nano assay (Agilent technologies, Inc. Germany) following the manufacturer’s protocol.

Semi-quantitative RT-PCR is a PCR amplification technique that employs both reverse transcriptase and thermostable Taq DNA polymerase. The first step is reverse transcription which synthesizes complementary DNA (cDNA) from the total mRNA template. The
subsequent step is traditional PCR amplifying the synthesized cDNA to detectable levels. For reverse transcription reaction, 1μg of RNA was used as a template to generate cDNA using the SuperScript III First-Strand Synthesis kit (Invitrogen) and was carried out according to the manufacturer’s instructions. For PCR amplification reaction, 2μl of solution resulting from the reverse transcription reaction and specific primers towards the gene of interest were used. The transcript levels of MT-des and p62 were semi-quantitated by PCR at the minimum of cycles that can detect PCR products. The sequences of the specific primers were as follows, MT-des: forward 5’-GCATCAATGCAGAAGCTGAT -3’ and reverse 5’-CTCTTGCAGCTCCACCTT-CT-3’; p62: forward 5’-CCTTGCCCCTACAGCTGAGTC-3’ and reverse 5’-GTCA-TCGTCTCCTCTCGACGC-3’. Primers corresponding to GAPDH were included as a control. Relative transcript levels were normalized with GAPDH transcript levels.

**Cycloheximide (CHX) chase assay:**

The protein degradation rate of MT-des was tested in the cultured NRVMs with rapamycin treatment and HDAC6 overexpression, respectively. Cells were incubated in serum-free DMEM containing 10μM CHX (Sigma-Aldrich) to block further protein synthesis. To evaluate the effect of rapamycin treatment on the degradation rate of MT-des proteins, starting three days after infection of Ad-MT-des or Ad-β-gal, cells were incubated with rapamycin or DMSO in the presence of CHX. To evaluate the effect of HDAC6 overexpression on the degradation rate of MT-des proteins, cells were infected with Ad-HDAC6 or Ad-β-gal at one day after being infected with Ad-MT-des or Ad-β-gal. Cells were treated with CHX at two days after the second infection. In both experiments, cells were collected at the indicated time points (0, 12, 24, 36, and 48 hours) following CHX treatment and whole-cell lysates were analyzed by western blot analyses using the c-myc antibody. Band densities were normalized to the one at 0 time point which was set at 1.

**Immunolabeling and fluorescence microscopy**

Mouse tissues were fixed with 4% paraformaldehyde (Electron Microscopy Science, Hatfield, PA) by perfusion fixation, saturated with 40% sucrose solution and embedded in Tissue-Tek O.C.T. (Sakura Finetek. USA, Inc, Torrance, CA), and then underwent tissue sectioning at 5 micron 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 specimens were then incubated with primary antibodies overnight at 4°C. The primary antibody dilutions were as follows: anti-p62 (03-GP62-C, ARP American Research products; guinea pig, 1:100), anti-ubiquitin (U5379, Sigma-Aldrich; rabbit, 1:100), anti-cathepsin D (IM16, EMD Chemicals Inc.; rabbit, 1:50), anti-SEC61α (sc-12322, Santa Cruz Biotechnology; goat, 1:50), anti-HA (sc-7392, Sigma-Aldrich; mouse,1:100), and anti-c-myc (MB600-336 and MG600-335, Novus Biological; rabbit and goat, 1:500). Subsequently, the corresponding Alexa-488, -568 and -647 conjugated secondary antibodies (Invitrogen) were used to mark the protein. Alexa Fluor 568-conjugated phalloidin (Invitrogen) was used to stain F-actin and identify cardiomyocytes. DAPI (Sigma-Aldrich) was used to stain nuclei. The immunolabeling was visualized and imaged using a fluorescence confocal microscope (Olympus Fluoview 500, Center Valley, PA) or an epi-fluorescence microscope (Zeiss Axiovert 100, Branson, MO). Unless stated otherwise, representative confocal images shown were taken using maximum projections of Z-stacks.

**MTT assay**

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay, an index of cell viability and cell growth, is based on the ability of viable cells to reduce MTT from a yellow
water soluble dye to a dark blue insoluble formazan product. Briefly, 1X10^6 neonatal cardiomyocytes were plated in 6-well plates. After the treatments had been performed, 500 μg/ml MTT dye (Sigma-Aldrich) was added to cardiomyocytes and the plates were incubated at 37°C for 2 hours. At the end of the incubation period, after the dye solution was discarded completely, 400μl solvent solution (1 volume of 1N HCl in 9 volume of anhydrous isopropanol) was added and the absorbance was determined at 570nm in a Tecan plate reader (Tecan group Ltd., Männedorf, Switzerland). Cell viability of the experimental group was determined as a percentage of the reading of the control group.

**LDH assay**

Cellular injury was determined by measuring the LDH released into the cell culture medium. Culture medium was collected from individual dishes or wells, and cell debris was removed by centrifugation. LDH activity in the collected culture medium was measured using the cytotoxicity detection kit (Roche Applied Science) following the manufacturer's protocols. Briefly, both 100 μl of culture medium and 100 μl of reaction reagent were added to each well of a 96-well plate, and the mixture was incubated for 30 minutes at 25°C. Absorbance of the samples at 490 nm was read on a Tecan plate reader. LDH release of an experimental group was determined as a percentage of the mean LDH activity of the corresponding control group. Moreover, LDH activities in the medium were normalized by the number of viable cells of the same dish as measured by the MTT assay.

**Heart perfusion fixation**

Mice were anesthetized by inhalation of isoflurane in room air supplemented with 100% oxygen. For perfusion fixation, a 25G butterfly cannula was connected to the perfusion apparatus which was placed at 80cm above the working table and generated 80mmHg pressure by height. The chest of a mouse was opened and the butterfly cannula was then introduced into the left ventricle of the mouse. Immediately after the heart started to fill up with the fixative, an incision was made in right atrium to release fluid. The whole system was flushed with PBS for 5 minutes and then fixed with 4% paraformaldehyde at 80 mmHg for 15 minutes. After fixation, the organs of interest were removed and equilibrated overnight in 40% sucrose at 4°C.

**Statistical analysis**

All quantitative data were presented as mean ± S.D. Differences between experimental groups were evaluated for significance using Student’s t-test for unpaired two group comparison, non-parametric statistic if variances were unequal. One-way or two-way ANOVA followed by the post-hoc test was used when appropriate. The p-value <0.05 is considered statistically significant.

**References**


II. Supplementary Figures and Legends

**Figure I.** Cathepsin D abundance and enzyme activity are increased in D7-des tg mouse hearts. Ventricular myocardium was collected from 2-month-old D7-des tg (D7) and NTG littermates for extraction of total proteins (A and B) or soluble proteins (C). A and B, Western-blot analysis showed that the abundance of cathepsin D intermediates (48KDa) was markedly increased in D7 mouse hearts. The representative images (A) and a summary of densitometry data of cathepsin D intermediates (B) are presented. C, Enzyme activity assay of cathepsin D showed significantly increased cathepsin D activities in D7 mouse hearts. **p<0.01, *** p<0.001 vs. NTG, Student’s t-test; n=4 mice/group.
Figure II. Confocal micrographs of the distribution of GFP-LC3 and cathepsin D in ventricular myocardium. D7-des tg mice were cross-bred with GFP-LC3 mice. The resultant GFP-LC3::D7-des double tg (DTG) mice and their littermate GFP-LC3 single tg mice were analyzed at 2 months of age. Cryosections from perfusion-fixed ventricular myocardium were immunostained for cathepsin D (red). Both direct fluorescence of GFP-LC3 (green) and indirectly immunofluorescence of cathepsin D (red) are shown. The arrows point to the stain of partial co-localization of cathepsin D and GFP-LC3 puncta. Scale bar: 10μm.
Figure III. Autophagic flux was unchanged in the liver tissues of D7-des mice as compared with NTG mice. Liver tissues were collected from the same D7-des tg and NTG littermate mice as used in Figure 1C and 1D in the main text. Representative western blot images (A) and a summary of densitometry data of LC3-II (B) are presented. *p<0.05 vs. NTG/CTL, two-way ANOVA followed by Scheffe test; n=3 mice/group. BFA: bafilomycin 1; AU: arbitrary units.
Figure IV. Autophagic flux is not increased by overexpression of wild type desmin (WT-des) in cultured neonatal rat ventricular myocytes (NRVMs). As described in main text Figure 1E, cultured NRVMs were infected with recombinant replication-deficient adenoviruses encoding either WT-des (Ad-WT-des) or β-galactosidase (Ad-β-gal, as control). Six days after infection, the cells were treated with BFA (100nM) or DMSO for 3 hours before being harvested for western blot analysis for the indicated proteins. Representative images are presented. Overexpression of WT-des at the level comparable to MT-des overexpression (Figure 1E, 1F) did not significantly increase LC3-II levels at the baseline or after BFA induced lysosomal inhibition.
Figure V. Validation of pharmacological interference of autophagy in NRVMs. 
A, Cultured NRVMs were infected with Ad-GFP-LC3. Three days later, NRVMs were incubated with DMSO (control, CTL), rapamycin (Rapa), 3-methyladenine (3-MA), and/or bafilomycin A1 (BFA) as indicated in serum free medium for 6 hours. Cells were then fixed with 2% paraformaldehyde for 20 minutes and imaged using an epifluorescence microscope. Scale bar: 20μM. 
B, After pretreatment with 3-MA (1.5mM) or DMSO for 3 hours, NRVMs were treated with BFA (100nM) or DMSO for another 3 hours. The cells were then harvested for western blot analyses of the indicated proteins. 
C, NRVMs were treated with Rapa (200nM) or DMSO for 6 hours. LC3 protein levels were measured by western blot analysis. GAPDH was probed for loading control.
Figure VI Semi-quantitative analyses of the mRNA levels of transgenic MT-des. NRVMs were treated as in Figure 4 in the main text. Total RNA was then isolated and the transcript levels of MT-des were assessed by semi-quantitative reverse transcription (RT) - PCR analyses. The transcript levels of overexpressed MT-des were comparable between rapamycin and control treatment groups. Representative images (A) and a summary of densitometry data from 3 repeats (B) are shown. GAPDH was analyzed for loading control. NS, not significant (p>0.05) vs. the control treatment; Student’s t-test.
Figure VII. Confocal micrographs of the distribution of p62, GFP-LC3, and ubiquitin in ventricular myocardium. D7-des tg mice were cross-bred with GFP-LC3 mice. The resultant GFP-LC3:D7-des double tg (DTG) mice and their littermate GFP-LC3 single tg mice were analyzed at 2 months of age. Cryosections from perfusion-fixed ventricular myocardium were immunostained for ubiquitin (red) and p62 (blue). Shown are direct fluorescence of GFP-LC3 (green), and indirect immunofluorescence of ubiquitin (red), and p62 (blue). p62 co-localizes with both GFP-LC3 and ubiquitin-positive aggregates in D7-des mouse hearts. The asterisks indicate the magnified frame location. The arrowheads point to the stain of overlapped GFP-LC3, p62 and ubiquitin. Some smaller GFP-LC3/Ub puncta are p62-negative (as denoted by arrows). Scale bar: 10μm.
Figure VIII. p62 protein expression is increased in NRVMs expressing MT-des or CryAB^{R120G}. NRVMs were infected with MT-des (A and B) or CryAB^{R120G} (R120G) (C and D) adenoviruses. Five days after infection, the cells were harvested for western blot analysis of p62. The representative images (A and C) and summaries of densitometry data of p62 from 3 independent repeats (B and D) are presented. *p<0.05, **p<0.001 vs. the β-gal group, Student’s t-test.
Figure IX. Representative micrographs of double immunofluorescence labeling for HA and ubiquitin (Ub) in cultured NRVMs overexpressing HA-CryABR120G. NRVMs were treated as described in Figure 7 in the main text. On day 7 after Ad-HA-CryABR120G infection, cultured NRVMs were fixed in 2% paraformaldehyde and used for indirect double immunofluorescence labeling for the HA-tag of HA-CryABR120G (green) and for Ub (red). Nuclei were stained blue with DAPI. Ub is enriched in the nuclei. HA-labeled aggregates, induced by HA-CryABR120G overexpression, co-localized with Ub. p62 knockdown attenuated aggresome formation.
Figure X. Representative micrographs of double immunofluorescence labeling for c-myc and ubiquitin in cultured NRVMs overexpressing c-myc-MT-des. NRVMs were treated as described in Figure 7 in the main text. On day 7 after Ad-c-myc-MT-des infection, cultured NRVMs were fixed in 2% paraformaldehyde and used for indirect double immunofluorescence labeling for the c-myc-tag of c-myc-MT-des (green) and for ubiquitin (red). Nuclei were stained blue with DAPI. c-myc-labeled aggregates, induced by c-myc-MT-des overexpression, co-localized with ubiquitin. p62 knockdown attenuated aggresome formation.
Figure XI, Representative micrographs of double immunofluorescence labeling for c-myc and SEC61α in cultured NRVMs overexpressing c-myc-MT-des. NRVMs were treated as in Figure 7 of the main text. On day 7 after Ad-c-myc-MT-des infection, cultured NRVMs were fixed in 2% paraformaldehyde and used for indirect double immunofluorescence labeling for the c-myc-tag of c-myc-MT-des (green) and for SEC61α (red). Nuclei were stained blue with DAPI. c-myc-labeled aggregates, induced by c-myc-MT-des overexpression, co-localize with SEC61α. To show the normal distribution of SEC61α, the images of the groups infected with Ad-β-gal were obtained by a longer exposure (1.5s) compared to the Ad-MT-des groups (400ms). p62 knockdown attenuated aggresome formation.
Figure XII, Representative micrographs of double immunofluorescence labeling for c-myc and p62 in cultured NRVMs overexpressing c-myc-MT-des. NRVMs were treated as in Figure 7 of the main text. On day 7 after Ad-c-myc-MT-des infection, cultured NRVMs were fixed in 2% paraformaldehyde and used for indirect double immunofluorescence labeling for the c-myc-tag of c-myc-MT-des (green) and for p62 (red). Nuclei were stained blue with DAPI. c-myc-labeled aggregates, induced by c-myc-MT-des overexpression, co-localize with p62. p62 knockdown attenuated aggresome formation.
Figure XIII. p62 depletion decreases the ubiquitinated (Ub-) proteins and MT-des in both soluble and insoluble fractions in NRVMs overexpressing MT-des. NRVMs were treated as in Figure 6 of the main text. Cells were lysed with a lysis buffer containing 1% Triton X-100. Triton X-100 soluble and insoluble fractions of the cell lysates were used for western blot analysis for the abundance of both ubiquitinated proteins and c-myc-tagged MT-des. GAPDH and α-actinin were probed as loading controls for proteins in the soluble and the insoluble fractions, respectively. Representative images (A) and summaries of quantitative data (B, C and D) from 4 independent repeats are presented. In B and C, *p<0.05 vs. β-gal/luc-siRNA; # p<0.05; two-way ANOVA followed by the Scheffé’s test. In D, # p<0.05; Student’s t-test.