Atg7 Induces Basal Autophagy and Rescues Autophagic Deficiency in CryABR120G Cardiomyocytes

J. Scott Pattison, Hanna Osinska, Jeffrey Robbins

Rationale: Increasing evidence suggests that misfolded proteins and intracellular aggregates contribute to cardiac disease and heart failure. Several cardiomyopathies, including the cB-crystallin R120G mutation (CryABR120G) model of desmin-related cardiomyopathy, accumulate cytotoxic misfolded proteins in the form of preamyloid oligomers and aggresomes. Impaired autophagic function is a potential cause of misfolded protein accumulations, cytoplasmic aggregate loads, and cardiac disease. Atg7, a mediator of autophagosomal biogenesis, is a putative regulator of autophagic function.

Objective: To determine whether autophagic induction by Atg7 is sufficient to reduce misfolded protein and aggregate content in protein misfolding-stressed cardiomyocytes.

Methods and Results: To define the gain and loss of function effects of Atg7 expression on CryABR120G protein misfolding and aggregates, neonatal rat cardiomyocytes were infected with adenoviruses expressing either wild-type CryAB or CryABR120G and coinfected with Atg7 adenovirus or with Atg7 silencing siRNAs to produce gain-of or loss-of Atg7 function. Atg7 overexpression effectively induced basal autophagy with no detrimental effects on cell survival, suggesting that Atg7 can activate autophagy with no apparent cytotoxic effects. Autophagic flux assays on CryABR120G-expressing cardiomyocytes revealed reduced autophagic function, which probably contributed to the failure of misfolded proteins and aggregates to be cleared. Coexpression of Atg7 and CryABR120G significantly reduced preamyloid oligomer staining, aggregate content, and cardiomyocyte cytotoxicity. Conversely, Atg7 silencing in the CryABR120G background significantly inhibited the already reduced rate of autophagy and increased CryABR120G aggregate content and cytotoxicity.

Conclusions: Atg7 induces basal autophagy, rescues the CryABR120G autophagic deficiency, and attenuates the accumulation of misfolded proteins and aggregates in cardiomyocytes. (Circ Res. 2011;109:151-160.)

Key Words: autophagy ■ Atg7 ■ aggregate ■ amyloid

Cellular homeostasis is necessary for cell survival. To maintain homeostasis, a cell must balance synthetic with catabolic processes. Eukaryotic cells have 2 major protein degradation pathways: the ubiquitin-proteasome system and the autophagy-lysosomal pathway. The proteasome specializes in the selective degradation of short-lived proteins.1,2 Macroautophagy, hereafter referred to as autophagy, is a process of bulk protein degradation, which functions to engulf and degrade damaged or long-lived proteins and organelles.3,4

Autophagy is initiated by elongation of an initial isolation membrane, the phagophore, which surrounds cytoplasmic material for sequestration.5 As the membrane expands, it forms a double-membrane vesicle referred to as an autophagosome. Autophagosomes completely surround the cytoplasmic products and act as a delivery vehicle for cargo degradation. Autophagosomes often fuse with other vacuolar structures such as endocytic vesicles to form amphisomes. Finally, the outer membrane of an amphisome fuses with the lysosome. The hydrolytic enzymes within the autolysosome degrade the inner membrane and subsequently, the cytoplasmic contents. The resulting macromolecules are released from the lysosome and recycled for use by the cell. This process is associated with both normal homeostasis and for countering cell stress.

Autophagy occurs at a basal level in most tissues, including the heart, contributing to the routine turnover of cellular trash. Under conditions of stress, such as nutrient starvation, autophagy is upregulated as an energy salvage process. In addition to energetic homeostasis, autophagy is involved in tissue development, differentiation, and remodeling.6 Autophagy is also associated with several human diseases and models of cell death.7 and data indicate that autophagy can be either beneficial or detrimental, depending on the cellular context.8,9

Gene ablation of critical components in the autophagic pathway has shown that autophagy is clearly necessary for mammalian survival. Beclin 1−/− mice die in early embry-
genetics, and Atg5−/− and Atg7−/− mice do not survive the neonatal starvation period. Conditional models of autophagic impairment cause accumulation of ubiquitinated protein aggregates in cells. Although the effects of autophagic deficiency differ from tissue to tissue, a consensus phenotype is the formation of intracellular protein aggregates. Presumably, these protein aggregates represent undigested protein byproducts that accumulate due to the loss of autophagic function. Numerous human diseases accumulate protein aggregates, leading to the general hypothesis that at least some of the pathology associated with aggregation-based diseases is due to insufficient autophagic function.

To date, few models of autophagic induction have been developed, and most are limited to starvation-induced autophagy. Transgenic overexpression of Beclin1-GFP had no effect on basal autophagy. Cardiomyocyte autonomous overexpression of Beclin1 also failed to induce basal autophagy but did significantly increase starvation-induced autophagy. FoxO1, FoxO3, and Sirt1 studies in cardiomyocytes have also been limited to starvation-induced autophagy. We hypothesized that Atg7 might be a viable target through which to activate basal autophagic function. Atg7 has dual functions in autophagosomal biogenesis. First, Atg7 acts as an E1-like ligase, which conjugates Atg5 to Atg12, a necessary step for formation of a functional autophagosome. Second, Atg7 converts Atg8 (LC3) from an immobile, cytosolic form to a mature autophagosomal membrane protein by adding a phosphatidylethanolamine group. Genetic deletion of Atg7 causes an overt loss of autophagy, proving that Atg7 is necessary for autophagic function.

In this report, we test the hypothesis that Atg7 overexpression is sufficient to induce increased basal levels of autophagic function. We hypothesized that if Atg7 did induce autophagy, we could then use this to evaluate whether autophagic upregulation is beneficial or detrimental to a cardiomyocyte under different, stressed conditions or in the unstressed cell. We tested this hypothesis by expressing Atg7 in neonatal rat cardiomyocytes (RNCs) and by determining the ability of increased levels of Atg7 to clear protein aggregates formed by αβ-crystallin R120G mutation (CryABR120G) expression.

Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Primary Cultures and Adenoviral Infection

Primary RNCs were isolated from the ventricles of 1- to 2-day old Sprague-Dawley pups and plated on 10-cm² plates at a density of 1.5×10⁶ cells in 10% FBS in DMEM. Twenty-four hours after plating, cells were infected with adenoviral constructs (10 MOI, unless otherwise noted) for 2 hours in DMEM media. Adenoviral constructs expressing LacZ, Atg7, CryAB, CryABR120G, and GFP-LC3 were used. Postinfusion cells were maintained in 2% FBS, 1% penicillin/streptomycin in high-glucose DMEM until fixed or harvested.

Atg7 Adenovirus Construction

The mouse coding sequence for Atg7 (BC058597) was subcloned into a Blunt TOPO vector (Invitrogen), and a COOH-terminal FLAG tag added. The Flag-tagged Atg7 was then subcloned into the pCMV-Shuttle vector (Stratagene) for adenovirus synthesis. The adenovirus was expanded in HEK cells and CsCl-purified before use.

siRNA Knockdown of Atg7

To specifically silence Atg7 expression, a pool of siRNAs (Invitrogen) was tested for their capacity to reduce Atg7 mRNA and protein levels in RNC transfections. The most potent silencing siRNA was used for all subsequent experiments. A nonspecific siRNA was used for a negative control siRNA in all silencing experiments. Twenty-four hours after plating, cells were transfected with 100 μmol/L siRNA, with Lipofectamine 2000 (Invitrogen), in OptiMem (Invitrogen) media overnight. In experiments using both siRNAs and adenoviruses, cells were transfected first, returned to growth media for 6 hours, and then infected for 2 hours.

Autophagic Flux Assays

To measure autophagic synthesis versus degradation, LC3-II protein levels were quantified with and without lysosomal inhibition for each genetic condition. To inhibit lysosomal function, media containing 50 nmol/L Bafilomycin (Baf)A1 (Sigma) was added to the cells for 4 hours. An equal volume of DMSO was used as a vehicle control. Adenovirus (Ad)GFP-LC3 (provided by J. Sadoshima, University of New Jersey Medical School) was used to quantify GFP-LC3-positive puncta under different experimental conditions as a reporter of autophagy levels. Fluorescent images were captured at ×60 magnification on a BX60 microscope (Olympus), using NIS elements software (Nikon). The number of GFP-LC3-positive puncta per cell and treatment were quantified with Image J software (NIH). Images from 30 to 50 different cells were captured per well of a chamber slide, and 4 different wells per genetic condition were quantified. GFP-LC3 analyses were not done on cells expressing CryABR120G because GFP-LC3 became trapped in the aggregate structures, preventing puncta-specific quantification.

Aggregate–Filter Trap Assays

To quantify changes in aggregate content, RIPA-insoluble proteins were treated with DNase (1 mg/mL in 10 mmol/L Tris, 15 mmol/L MgCl₂) (Roche) for 1 hour and protein quantified with a modified Bradford assay. The insoluble protein was then diluted to 0.015 μg/μL with 2% SDS, 20 mmol/L EDTA, and 50 mmol/L DTT dissolved in TBS. Five micrograms of resuspended insoluble protein was dotted onto a nitrocellulose membrane (BioRad), which was blocked and immunoblotted with appropriate antibodies, as outlined.

Statistical Analyses

Data are expressed as mean ± SEM. All statistical tests were done with SigmaPlot 9.0 software. Comparisons between 2 groups were analyzed with the Student t test (P<0.05). Comparisons between multiple groups were analyzed with 1-, 2-, or 3-way ANOVAs followed by Tukey post hoc correction (P<0.05).

The authors had full access to and take full responsibility for the integrity of the data. The authors have read and agree to the manuscript as written.

Results

To test the hypothesis that increased levels of Atg7 are sufficient to induce autophagy, we generated an adenovirus in which the CMV promoter was used to drive Atg7 expression
in RNCs (Figure 1). AdAtg7 induced Atg7 protein levels by 7.8-fold while leaving other autophagic markers such as Beclin1, Atg5–12, p62, and cathepsin D unchanged (Figure 1A). These data suggest that any effects of Atg7 overexpression subsequently detected would not be due to changes in lysosomal content or the upregulation of Beclin1. Localization of Atg7 was examined by immunocytochemistry, which showed a diffuse cytoplasmic staining pattern with stronger perinuclear staining on Atg7 overexpression (Online Figure I, A). To determine whether Atg7 overexpression induces the intracellular structures characteristic of autophagy, we evaluated the ultrastructure of the infected RNCs (Figure 1B) and observed a marked upregulation in the number of autophagic structures. Most notably, there was a large number of amphisomes, an intermediate structure that develops after an autophagosome engulfs its cargo and fuses with other vesicular structures such as multivesicular endosomes, which have yet to fuse with and be degraded by lysosomes. To evaluate the extent of autophagy, autophagic flux assays were performed using a GFP-LC3 reporter. The active form of LC3 (LC3-II) localizes in autophagosomes, which appear as punctate structures, whereas inactive LC3 (LC3-I) stains diffuse in the cytoplasm. Increased levels of LC3-II could be due to either increased LC3 synthesis or impaired LC3 degradation, and the current gold standard for evaluating autophagic function is the autophagic flux assay, which uses the lysosomal inhibitor BafA1 to block lysosomal function and thus LC3 degradation. If LC3-II levels increase as a result of Atg7 expression and in the presence of BafA1 relative to the LacZ controls, then the increase in LC3-II levels must be due to an increase in LC3 synthesis (increased autophagy). Conversely, if gene expression with BafA1 blockade results in less LC3-II accumulation, the decrease would be due to impaired LC3 synthesis (decreased autophagy). Atg7 overexpression significantly increased the number of GFP-LC3 puncta per cell with Bafilomycin treatment over LacZ-expressing controls (Figure 1C and 1D).

Flux assays were performed on mock-infected versus AdLacZ-infected cells to show that the infection process does not alter autophagy (Online Figure I, B). Alterations in autophagic flux were further confirmed by immunoblotting. AdAtg7- and AdLacZ-infected cells exhibit similar LC3-II levels under control (+ vehicle) conditions, whereas lysosomal inhibition with BafA1 differentially increased LC3-II levels 1.7-fold as compared with AdLacZ control-infected RNCs (Figure 1E and 1F). Thus, Atg7 significantly induces autophagic flux (LC3-II synthesis) above control levels. Because these data were performed on cardiomyocytes grown in media with high glucose and 2% serum and not in starved cells, the data confirm that Atg7 overexpression is sufficient to induce basal autophagy and that Atg7-induced autophagy is not limited to starvation-induced autophagy.
Because autophagy has been associated with both beneficial and detrimental outcomes, we directly tested the hypothesis that Atg7-induced autophagy is not detrimental by carrying out cytotoxicity assays. At 5 days after infection, Atg7 expression did not lead to any increased cytotoxicity as measured by both the adenylate kinase and lactate dehydrogenase release assays relative to LacZ expressing or mock-infected controls (Online Figure I, C).

It has been hypothesized that models accumulating misfolded proteins and protein aggregates may have impaired autophagy.22–24 Previously, we studied a model of desmin-related cardiomyopathy that occurred as result of cardiomyocyte-specific expression of a CryAB carrying a mutation causative for the disease.25–28 CryABR120G expression results in the accumulation of cytotoxic preamyloid oligomers (PAOs) and aggresomes, which cause cardiomyocyte toxicity in vitro and heart failure in vivo.27,28 To test the hypothesis that CryABR120G expression also results in impaired autophagy, we measured autophagic flux in RNCs transfected with adenoviruses overexpressing either wild-type CryAB (AdCryAB) or CryABR120G (AdCryABR120G). CryABR120G expression resulted in reduced LC3-II levels under basal and lysosome-inhibited conditions (Figure 2A). CryABR120G expression causes a significant reduction in LC3 synthesis (-37%) relative to CryABWT-expressing cells (Figure 2B). Thus, CryABR120G expression leads to decreased autophagic function. CryABWT flux did not differ from flux levels in mock-infected cells (Online Figure II, A). Next, we tested the hypothesis that AdAtg7 overexpression could rescue the autophagic deficiency present in the CryABR120G-expressing cardiomyocytes. Autophagic flux assays showed that enhanced levels of Atg7 rescued the autophagic deficiency observed in CryABR120G cardiomyocytes (Figure 2C and 2D). As with Atg7 expression alone, when Atg7 is coexpressed with CryABR120G no alterations in the protein levels of lysosomal markers or Beclin 1 were observed (Figure 2E). Induction of autophagy by Atg7 was further confirmed by electron microscopy, which showed an increase in autophagic structures in AdAtg7- and AdCryABR120G-coexpressing cardiomyocytes (Online Figure II, B).

To test the hypothesis that aggregate accumulation was a result of decreased autophagy, we coexpressed CryABR120G with Atg7 to rescue autophagic function and determine if aggregate accumulation correspondingly decreased. Aggresomal accumulation was quantified by CryAB staining (Figure 3A).25 Atg7 expression resulted in a significant reduction in aggregate content (Figure 3B). To quantify aggregate content, we measured the area occupied by CryAB-positive aggregate staining (green) relative to Troponin I cardiomyocyte staining (red). We found a significant, 48% reduction in aggregate area when Atg7 was coexpressed with CryABR120G. A second method of quantifying the reduction in aggregate content was through use of filter trap assays (Figure 3C), with significant reductions of 44% and 36% in insoluble CryAB and ubiquitin, respectively, when Atg7 was coexpressed with CryABR120G (Figure 3D).

To better define the effective dose of Atg7, we tested the effects of increasing MOI of AdAtg7 relative to AdLacZ.
Increasing MOI of AdAtg7 results in a dose-dependent increase in Atg7 protein levels (Figure 4A). Increasing MOI of Atg7 has no effect on cytotoxicity (Online Figure IV). Increasing the MOI of AdAtg7 with a constant MOI of AdCryABR120G resulted in a dose-dependent reduction in CryABR120G aggregate content (Figure 4B and 4C). To determine whether increasing doses of AdAtg7 resulted in increasing levels of autophagy, autophagic flux assays with GFP-LC3 were performed (Figure 4D). Autophagic flux was significantly increased in a dose-dependent manner from 100 MOI AdLacZ to 10, 30, and 100 MOI of AdAtg7 (Figure 4E).

Previous data from the CryABR120G model showed that PAO accumulation is associated with cytotoxicity and heart failure.25,27,28 To test the hypothesis that Atg7 induction of autophagy could reduce PAO content, we immunostained transfected RNCs with a conformation-specific antibody29–31 that uniquely stains PAOs and, under these culture conditions, nuclei (Figure 5A). The nonnuclear staining represents toxic oligomer accumulation.25,27,28 We observed a significant, 4.2-fold reduction in nonnuclear PAO staining when AdAtg7 was coexpressed with AdCryABR120G (Figure 5B).

To quantify nonnuclear PAO reduction, the area of cytoplasmic PAO staining (green) was measured relative to cardiomyocyte area as visualized using Troponin I staining (red). Consistent with PAO cytotoxicity, we observed that Atg7 expression also significantly reduced CryABR120G cytotoxicity as measured by adenylate kinase and lactate dehydrogenase release assays (Figure 5C).

Silencing Atg7 can reduce autophagy.12,32 However, the question of whether autophagic deficiency leads directly to increased aggregate content is relatively unexplored. To test this hypothesis, we used an Atg7-silencing siRNA (Online Figure III, A, through C) with AdCryABR120G-infected RNCs to see if aggregate content increased as a result of autophagic silencing. To confirm that Atg7 siRNA successfully inhibited autophagy, we measured autophagic flux (Figure 6A). The data showed that much like CryABR120G expression, decreased Atg7 levels significantly impaired LC3 synthesis (Figure 6B). Silencing Atg7 expression while overexpressing CryABR120G resulted in a marked 47% increase in aggregate content per cardiomyocyte (Figure 6C and 6D).

Impaired autophagic function has not previously been associated with PAO content. We therefore tested whether Atg7 silencing with CryABR120G expression could increase PAO content in cardiomyocytes. Immunocytochemistry showed that knockdown of Atg7 exacerbated the degree of PAO staining in CryABR120G expressing cardiomyocytes (Figure 7A). By quantifying PAO area (green) relative to cardiomyocyte area (red), we found that Atg7 silencing significantly increased PAO/cardiomyocyte area (Figure 7B). Atg7 siRNA doubled the magnitude increase in PAO staining between CryABWT and CryABR120G. Similarly, knocking
down Atg7 in CryABR120G-expressing cardiomyocytes resulted in increased cytotoxicity, as measured by adenylate kinase and lactate dehydrogenase release assays (Figure 7C and 7D).

**Discussion**

Our data demonstrate that enhanced levels of Atg7 are sufficient to induce autophagy. Furthermore, the ability of Atg7 to affect autophagy did not require preconditioning by cell starvation, meaning that Atg7 can, unlike Beclin1, increase basal autophagy, not just starvation-induced autophagy.14 The capacity to induce autophagy without starvation makes Atg7 a unique tool for autophagic studies. Numerous studies have concluded that elevated autophagy is detrimental, after observing an increase in autophagic markers in association with a deleterious context.14,33 Our work demonstrates that Atg7 overexpression clearly increases autophagic flux (autophagic function) without increasing cytotoxicity. In fact, when Atg7 was coexpressed with cytotoxic CryABR120G expression, it reduced overall cytotoxicity. These data show that autophagy upregulation is not universally detrimental but can be beneficial. Whether Atg7 induction represents a widely used pathway for beneficial autophagy remains to be determined.

Because of the necessary roles of Atg7 in processing LC3-I to active LC3-II and in the conjugation of Atg5 to Atg12, we hypothesized that upregulating what might be a rate-limiting enzyme, Atg7, would result in more available LC3-II and increase autophagosomal content. However, we observed no depletion in LC3-I with Atg7 overexpression, suggesting that Atg7 upregulates the process of autophagy overall rather than simply affecting a single, rate-limiting step.

Increasing evidence suggests that misfolded proteins, PAOs, and aggregates can all cause or functionally participate in the development of cardiotoxicity and heart failure,27,28,34,35 and pharmacological inhibition of autophagy has been associated with CryABR120G aggregate accumulation.36 Although our laboratory has studied the CryABR120G model...
Extensively, we have not defined a single mechanism that is necessary and sufficient for PAO and aggregate accumulation. The autophagic flux assays for CryABR120G expressing cardiomyocytes clearly demonstrate that CryABR120G expression causes an autophagic deficiency. Atg7 silencing causes a similar autophagic deficiency, such that when we combined the autophagic deficiency of CryABR120G with Atg7 knockdown, we observed increased accumulation of aggregates.

Figure 5. Atg7 reduces CryABR120G PAO content and cytotoxicity. A, Immunofluorescent staining shows decreased preamyloid oligomer PAO content when Atg7 is coexpressed with CryABR120G. Cardiomyocytes counterstained by a TnI antibody. B, PAO area relative to the cardiomyocyte area (n=4 wells/treatment). *P<0.001, significant difference between CryABR120G versus CryABWT. †P<0.05, significant difference between CryABR120G + Atg7 versus CryABR120G + LacZ (P<0.001). C, CryABR120G adenylate kinase and lactate dehydrogenase release are reduced by Atg7 coexpression at 5 days after infection (n=4/treatment). *P<0.001, significant difference between CryABR120G versus CryABWT. †P<0.001, significant difference between CryABR120G + Atg7 versus CryABR120G + LacZ.

Figure 6. Atg7 silencing inhibits autophagy and increases aggregate content of CryABR120G. A, Representative blot showing autophagic flux assay with reduced LC3-II levels from Atg7 knockdown with BafA1 (n=4/treatment). B, Decreased LC3-II protein levels with Atg7 siRNA. *P<0.05, significant difference between vehicle versus BafA1. †P<0.05, significant difference Atg7 siRNA versus Ctrl siRNA. C, Increased CryAB aggregate content (green) when Atg7 siRNA is coupled with CryABR120G. Cardiomyocytes are counterstained with a TnI antibody. D, Aggregate area relative to the cardiomyocyte area (n=4/treatment). *P<0.001, significant difference CryABWT versus CryABR120G. †P<0.001, significant difference CryABR120G + Atg7 siRNA versus CryABR120G + Ctrl siRNA.
PAOs and aggregates. Additional PAO and aggregate accumulation led to increased cytotoxicity. These data show that conditions of autophagic deficiency exacerbate the accumulation of misfolded proteins and aggregates, contributing to the proteotoxicity phenotype in the cardiomyocyte.

If autophagic insufficiency plays a role in the CryAB\textsuperscript{R120G} phenotype of PAO and aggregate accumulation, then overexpression of Atg7 to induce autophagy should attenuate the pathology. Indeed, Atg7 expression decreased CryAB\textsuperscript{R120G} aggregate and PAO content in cardiomyocytes. Consistent with the hypothesis that impaired autophagy with PAO and aggregate accumulation induces a proteotoxic stress, Atg7 stimulation of autophagy reduced CryAB\textsuperscript{R120G}-induced cardiomyocyte toxicity. Additionally, increasing levels of Atg7 expression led to a dose-dependent reduction in CryAB\textsuperscript{R120G} aggregate content, suggesting that further elevating autophagic function increased clearance of misfolded protein structures.

Although it is logical to think that upregulation of autophagy, which functions to degrade misfolded proteins and aggregates, enhances their clearance, few if any models have shown the sufficiency of autophagy for this process.\textsuperscript{25} Our data clearly show that inducing autophagy by Atg7 overexpression is sufficient to reduce CryAB\textsuperscript{R120G} PAO and aggregate content, with a concomitant decrease in cytotoxicity. Thus, Atg7 rescues the autophagic deficiency caused by CryAB\textsuperscript{R120G} expression. Future studies will be aimed at testing the effects of Atg7 overexpression in vivo and its capacity to prevent aggregate formation and heart disease as well as its potential for rescuing preexisting disease.

Our understanding of the role of autophagy is still in its infancy. It is currently unclear how prevalent autophagic deficiency is in models of heart disease or neurodegeneration because most publications report associative studies with autophagic markers and disease. Several genetic models of heart disease, including Tg-Mst1, truncated MYBPC3, Des\textsuperscript{-}/H90047, PQ83, and CryAB\textsuperscript{R120G}, result in the accumulation of intracellular aggregates.\textsuperscript{27,28,34,38,39} However, recent evidence suggests that autophagic induction may not be generally effective for all aggregate diseases because, in an expanded polyglutamine model of aggregate formation, autophagosomes failed to recognize and engulf the polyglutamine aggregates.\textsuperscript{40} It remains to be seen whether upregulation of autophagy is beneficial in other models of protein aggregation, PAO accumulation, and heart disease. However, if other aggregate-inducing models of heart failure are the result of insufficient autophagy, inducing autophagy may provide a mechanistic rescue for those models of heart disease.

A limitation in many studies of autophagy is the exclusive reliance on measuring levels of autophagic markers such as LC3 either by blotting or by puncta accumulation. Upregulation of LC3 levels can be observed under both conditions of increased autophagic synthesis and decreased lysosomal function.\textsuperscript{20} Actual, autophagic function, determined with autophagic flux assays, which compares LC3 synthesis versus degradation through the use of a lysosomal inhibitor,\textsuperscript{21} is relatively rare in the literature. As assays of autophagic function come into widespread use, the relationship between autophagic function and disease will be better defined.

One of the biggest hurdles to our understanding of the processes’ implications is the lack of genetic tools by which autophagy can be regulated. Our data describe a new genetic
target for the upregulation of autophagy, Atg7. Additional genetic reagents will also need to be developed to better define the pathways that induce and repress autophagic function to more fully understand the role of autophagy in cardiac disease.

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Disclosures

None.

References


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**Novelty and Significance**

**What Is Known?**

- **Atg7** is necessary for autophagic function.
- Impaired autophagy results in aggregate accumulation.
- Mutant β-crystallin expression causes the accumulation of misfolded preamyloid oligomers and aggregates.

**What New Information Does This Article Contribute?**

- **Atg7** expression is sufficient to induce autophagic function in cardiac myocytes without nutrient deprivation.
- β-Crystallin R120G mutation (CryABR120G) expression inhibits autophagy.
- **Atg7**-induced autophagy rescues the impaired autophagy of CryABR120G expression, as evidenced by dose-dependent reduction in aggregate and preamyloid oligomer content per cardiomyocyte, demonstrating that autophagy upregulation can be beneficial for proteotoxic disease.

Only a few genes have been demonstrated to regulate autophagy. We show that **Atg7** expression can upregulate autophagic flux in cardiac myocytes. A model of desmin-related myopathy, brought about through expression of **CryABR120G** that causes the human disease, forms protein aggregates and preamyloid oligomers in cardiomyocytes. We found that CryABR120G expression resulted in impaired autophagy, which may contribute to the accumulation of aggregates and preamyloid oligomers, characteristic of desmin-related myopathy. To test whether inducing autophagy could reduce CryABR120G pathology, we coexpressed Atg7 with CryABR120G, which resulted in reduced aggregate and preamyloid oligomer content per cardiomyocyte with a corresponding reduction in cytotoxicity. Conversely, silencing Atg7 inhibited autophagy, which exacerbated the aggregate and preamyloid accumulation with CryABR120G expression. Our findings suggest a novel role for Atg7 in inducing a beneficial form of autophagy, which may be a potential therapeutic avenue for the treatment of desminopathy pathology.
Atg7 Induces Basal Autophagy and Rescues Autophagic Deficiency in CryAB$^{R120G}$ Cardiomyocytes

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Atg7 Induces Basal Autophagy and Rescues Autophagic Deficiency in CryAB\textsuperscript{R120G} Cardiomyocytes.

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Running title: Atg7 Induces Autophagy

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**SUPPLEMENTARY METHODS**

**Western Blot Analyses**
To lyse RNCs, growth media was aspirated, plates were washed with 1X PBS, which was then aspirated and the cells were lysed with 250uL Cell Lytic M (Sigma) lysis buffer, supplemented with Complete protease inhibitor cocktail (Roche). After 5 minutes of lysis, cells were scraped and transferred to 1.5 mL tubes and sonicated for 6 seconds at 0.06 watts. The lysates were centrifuged at 14,000 x g for 15 minutes to sediment any insoluble material. The protein content of the soluble lysates was measured using the modified Bradford protocol/ reagent relative to a BSA standard curve (BioRad). Equal protein concentrations of each lysate were diluted in 3X SDS loading dye (New England Biolabs). Samples were boiled for 5 minutes prior to loading on SDS-PAGE gels. Proteins were separated on SDS-PAGE gels (BioRad) and transferred to PVDF membranes (BioRad). Membranes were blocked for 1 hour in 5% non-fat dried milk and exposed to primary antibodies overnight. Membranes were then washed in 1X TBS-T, 2 times for 5 minutes and incubated for 1 hour with alkaline-phosphatase-conjugated secondary antibodies (Santa Cruz Biotechnology). Membranes were washed in 1X TBS-T 2 times for 5 minutes and exposed with ECF reagent (Amersham) for up to 5 minutes. Bands were detected on a STORM 820 fluorescent scanner (Molecular Dynamics). The following primary antibodies were used for immunoblotting: anti-LC3B (2775, Cell Signaling), anti-Atg7 CT (3615, ProSci), anti-Atg7 (2631, Cell Signaling), anti-FLAG (F7425, Sigma), anti-αB crystallin (SAP-223, Assay Designs), anti-p62 COOH-terminal (GP62-C, ProGen), Anti Atg5-12 (CAC-TMD-PH-AT5, Cosmo Bio Co), anti-Beclin1 D-18, anti-cathepsin D, C-20, anti-ubiquitin P4D1 and anti-LAMP1 1D4B (sc-10086, sc-6486, sc-8017, sc-19992, Santa Cruz Biotechnology) and anti-GAPDH (MAB374, Chemicon). Densitometry on scanned membranes was done using ImageQuant version 5.2 (Molecular Dynamics).

**EM analyses**
RNCs were cultured for 5 days on 10 cm dishes coated with gelatin. Cells were washed with cardioplegic buffer and fixed in the dishes with 1% paraformaldehyde, 2% glutaraldehyde, 0.05M cacodylate buffer for 15 minutes on ice. The cells were then scraped and pelleted by centrifugation. The pellets were post-fixed in 1% OsO4 in cacodylate buffer, for 2 hours on ice, then dehydrated in series of acetone washes and embedded in EMbed812 in silicone pyramid tip molds. Ultrathin sections were counterstained with uranium and lead salts and examined with a Hitachi7600 transmission electron microscope. Images were acquired with an AMT digital camera.

**Immunohistochemistry**
RNCs were grown on 2-well chamber slides and plated at a density of 1x10^5 cells/well (Nalgene). Cells were first washed with 1X PBS, and then fixed for 20 minutes in 4% paraformaldehyde, 0.5% Triton-X 100, in 1X PBS. Fixed cells were washed twice with 1X PBS and then exposed to an antigen retrieval solution of 0.1 mol/L glycine (pH=3.5) for 30 minutes. The retrieval solution-treated cells were then twice washed with 1X PBS. The fixed cells were incubated in blocking solution (1% BSA, 0.1% cold water fish gelatin, 0.1% Tween-20, 0.05% sodium azide in 1X PBS) for 1 hour. Primary antibodies were diluted 1:100 in blocking solution and incubated overnight. The following antibodies were used for immunohistochemistry: anti-αB crystallin (SAP-223, Assay Designs) and anti-A-11/PAO antibody (courtesy of C. Glabe, University of California, Irvine). Cells were washed twice with 1X PBS, followed by incubation with a species-specific secondary antibody – conjugated to Alexa488 (Molecular Probes) for 1 hour. Cardiomyocytes were counterstained using an anti-troponin I antibody (MAB3152, Chemicon) at a 1:1000 dilution for 1 hour, followed by washing and incubation with a goat-anti mouse Alexa568 secondary antibody (Molecular Probes) for one hour. Nuclei were
counterstained using TOPRO 1:500 for 45 minutes (Molecular Probes). Following two final washes with 1X PBS, coverslips were mounted using Vectashield Hard Set mounting media (Vector labs) and allowed to dry for 45 minutes. Slides were kept at 4 °C until visualized by confocal microscopy (PCM 2000, Nikon) with images captured by Simple PCv4 software (Compix Inc). To quantify aggregate and PAO content from fluorescent images, images were captured from each experimental group of 4-8 separate chamber slide wells. Each well had 8-12 images from different regions captured per well. The area of aggregate or PAO staining was quantified relative to the area of cardiomyocyte staining using MetaMorph 7 software (Molecular Devices).

Cytotoxicity assays
Adenylate kinase release into cell culture media was measured using the ToxiLight Bioassay kit (Lonza). The assay luminescence was measured on a MonoLight 3010 luminometer (BD Biosciences). Lactate dehydrogenase release was measured by a Cytotoxicity Detection kit (Roche). Color development was measured using a µQuant microplate spectrophotometer with an absorbance of 490nM (BioTek Instruments).
**Supplementary Figure I.**

**A**, Atg7 staining (green) maintains nuclear and cytoplasmic localization with overexpression. Cardiomyocytes counterstained with TnI antibody (red). **B**, Representative blot showing autophagic flux does not differ between AdLacZ and mock-infected cells. *$P<0.05$* significant difference between Veh (vehicle) and BafA1 treated groups. **C**, Adenylate kinase and lactate dehydrogenase release are unchanged by Atg7 expression at five days post-infection (n=4/treatment).
**Supplementary Figure II.**

**A,** Representative blot showing that autophagic flux does not differ between mock-infected and AdCryAB<sup>WT</sup>-infected cells. *P*<0.05; significant difference between Veh and BafA1 treated groups.

**B,** Atg7 increases the number of autophagic structures visible by EM analysis when co-expressed with CryAB<sup>WT</sup> and CryAB<sup>R120G</sup>. >50 fields were randomly scored from both groups and representative fields are shown. Amphisomes denoted by white arrows. Multilamellar bodies denoted by white asterisks.
Supplementary Figure III. A, Real-time PCR analysis shows that one of three siRNAs targeted to Atg7 effectively reduces Atg7 mRNA levels. *P<0.05 significant difference between Ctrl siRNA and Atg7 siRNA#3. B, Representative immunoblot shows Atg7 protein levels are reduced following transfection with Atg7 siRNA. C, Plot shows reduced densitometry of Atg7 protein levels by Atg7 siRNA transfection (n=4/group). *P<0.05 significant difference between Ctrl siRNA and Atg7 siRNA.
Supplementary Figure IV. A, Adenylate kinase and lactate dehydrogenase release are unchanged with increasing Atg7 expression at five days post-infection (n=4/treatment).