CARDIOMYOCYTE AUTOPHAGY IS INDUCED BY PROTEIN AGGREGATION IN HEART DISEASE

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DEDICATION

If there was one moment after which everything changed, it was a conversation I had with Dr. Sanford M. Markham. He recognized my interest in biomedical science and set in motion a career trajectory I never would have imagined. From Iowa, to England, to Texas, I will always be grateful for his guidance.

To Dr. Joseph A. Hill who has for many years been a mentor, role model, and friend. I did not really know what a physician-scientist was until I met Dr. Hill, but after just three months of working with him I decided to become one. His belief in my ability and dedication to my training went beyond all expectations; he pushed me when I needed a push, picked me up when I fell, and never said a word about the bar tab I charged to our hotel room in Italy. Thanks Joe, I owe you a lot.

To Dr. Beverly A. Rothermel and her open-door policy. Much of this work is the product of the hours I spent talking with Bev, chasing ideas to whatever conclusion they found. I am truly sorry there is not more discussion of MCIP in these pages, but there is always time for that during my post-doc.

Most of all, endless thanks and gratitude goes to my family. My parents, Raymond and Andrée, who cultivated an environment of intellectual curiosity and taught me to embrace all challenges faced. And to my siblings Beatrice, Pierre, and Marc; I do not know where I would be without their support and humor.

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CARDIOMYOCYTE AUTOPHAGY IS INDUCED BY PROTEIN AGGREGATION IN HEART DISEASE

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Autophagy is associated with diverse forms of myocardial stress. When I initiated my studies activators of this pathway had not been identified in the heart, nor was it clear weather autophagy is an adaptive or maladaptive response in the stressed myocardium. My initial research focused on autophagy in hypertension-induced heart failure, the most common cardiovascular disease in Western nations.

Early evidence demonstrated generation of reactive oxygen species, protein damage, and protein aggregation in the acute period of pressure overload. Given the simultaneous presence of autophagosomes

and aggregates, and autophagy's role in bulk degradation, I postulated these events were mechanistically linked. I designed experiments to test the hypotheses that protein aggregates are activators of autophagy in the heart, and that autophagy functions in aggregate clearance.

Here I report novel findings that link pressure overload-induced protein aggregation to increased cardiomyocyte autophagy. Specifically, in the pressure-stressed ventricle 1) generation of reactive oxygen species is an early pathological event, 2) there is extensive protein aggregation with higher-order processing into aggresomes, 3) protein aggregation induces cardiomyocyte autophagy, and 4) in this setting autophagy functions in its role as a mechanism of bulk protein degradation. These findings are the first to demonstrate proteinopathy of non-genetic etiology contributes to hypertension-induced heart failure and that protein aggregates are robust activators of cardiomyocyte autophagy.

To directly address the role of autophagy in cardiomyocyte clearance of toxic protein species, I turned my attention to $CryAB^{R120G}$-induced desmin-related cardiomyopathy (DRCM), an aggregate-associated disease with autosomal dominant inheritance. My studies demonstrated that 1) autophagy is activated by $CryAB^{R120G}$-induced protein aggregation, 2) aggregate formation is inversely proportional to the degree of autophagic activity and 3) blunting autophagy accelerates pathological myocardial remodeling and the onset of heart failure. Extending this work to clinical medicine, we observed increased autophagy in the skeletal muscle from patients with desmin-related skeletal myopathy.

Cumulatively these data are the first to demonstrate autophagy is induced in DRCM and functions as a protective cellular response. These findings suggest autophagy is a pathway amenable to therapeutic

intervention in patients suffering from myofibrillar myopathy, a disease class for which there are limited therapeutic options.

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LIST OF FIGURES AND TABLES

ABBREVIATIONS

3MA: 3-methyladenine
ALS: Amyotrophic lateral sclerosis
BW: Body weight
CryAB: αB-Crystallin
$CryAB^{R120G}$: αB-Crystallin with an R to G missense mutation at amino acid 120
CTL-A: Chymotrypsin-like activity
DHE: Dihydroethidium
dnATG5: dominant-negative ATG5
DRCM: Desmin related cardiomyopathy
FS: Fractional shortening
GFP-LC3: GFP-tagged LC3 (see MAP-LC3)
HIER: High intensity epitope retrival
HW: Heart weight
I/R: Ischemia and reperfusion
IDC: idiopathic dilated cardiomyopathy
IPB: Immunoprecipitation buffer
LungW: Lung weight
LV: Left ventricle
LVIDd: End diastolic diameter (cm)
LVIDs: End systolic diameter (cm)
MAP-LC3, LC3: microtubule-associated protein 1 light chain 3
MTOC: microtubule organizing center
mTOR: mammalian target of Rapamycin
NRVM: Neonatal rat ventricular myocytes
PFA: Paraformaldehyde
PFA: Paraformaldehyde
PI3KIII: Class III phosphoinositide-3-kinase
Rapa: Rapamycin
ROS: Reactive oxygen speices
RV: Right ventrile
sTAB: severe thoracic aortic banding
UPS: Ubiquitin proteasome system

CHAPTER 1

INTRODUCTION

Heart failure- the public health impact

Heart failure is currently the leading cause of morbidity and mortality in the United States[1, 2]. Roughly 5 million people are afflicted by this condition and treatment costs over 28 billion dollars annually. In the past decade there has been a 159 percent increase in the rate of heart failure-related hospitalization and, with our aging population, the economic and social costs will continue to rise[3].

Despite tremendous efforts made towards understanding the cellular and molecular mechanisms that control the development of heart failure, no single pathway has been identified as a master regulator of disease progression. As an indication of the complicated and multi-factorial nature of this disease, optimal therapy currently involves administration of angiotensin-converting-enzyme inhibitors[4, 5], aldosterone antagonists[6], beta-receptor blockers[7, 8], and resynchronization therapy[9, 10]. Even with optimal treatment, symptomatic heart failure has a one-year mortality rate of 45 percent, clearly demonstrating the need for identification of novel therapeutic targets[11, 12].

Myocardial responses to stress

Efforts toward identifying new therapeutic targets have focused on understanding the mechanisms of myocardial remodeling and cell death. These processes are of central importance because cardiomyocytes, the contractile cells of the heart, are a terminally differentiated population. As such, simple division of healthy cardiomyocytes cannot replace tissue that is lost or damaged, and loss of tissue results in a life-long decrease in total functional capacity. In the last decade there has been an dramatic expansion in knowledge on almost every mechanism of myocardial remodeling with detailed molecular analysis of hypertrophic growth and

atrophic regression, deposition of fibrosis, as well as necrotic, apoptotic, and oncotic cell death.

In addition to the study of degenerative pathways and cell death, there is burgeoning interest in the heart's capacity for regeneration. Virtually all somatic tissues contain resident stem cells which function in maintenance and regeneration[13]. In addition to resident stem cells, peripheral populations (usually from the bone marrow) also demonstrate limited capacity to participate in cardiac muscle regeneration after ischemic insult[14]. Likewise, the presence of Y chromosomes-positive cardiomyocytes in postmortem specimens of female to male adult human cardiac transplants demonstrates some capacity for myocyte renewal from non-cardiac sources[15-17]. These studies demonstrate it is possible to generate new cardiomyocytes in an adult, but clearly this regenerative capacity is not sufficient to halt or reverse the vast majority of heart disease.

While the notion of using stem cells to regenerate myocardial tissue holds exciting potential, this field is still in the early stages of basic experimentation and no therapeutic applications are currently viable. As such, there is pressing need for the identification of novel therapeutic targets that will benefit patients suffering from cardiac disease. Toward these ends, macroautophagy, a ubiquitous cellular pathway for protein degradation, is quickly gaining appreciation as an important myocardial stress response. As discussed in the following sections, the mounting evidence suggests autophagy is a pathway amenable to therapeutic intervention in diverse forms of myocardial illness.

The two faces of autophagy: survival and death

Macroautophagy (herein referred to as autophagy), literally meaning "self-eating," is best-characterized as a process of bulk-protein degradation involved in the turnover of mitochondria, peroxisomes, and non-selective degradation of cytoplasmic components during periods of starvation or stress[18, 19]. Autophagy begins with formation of the autophagosome, a double membrane structure of unknown origin, which then engulfs an entire region of cytoplasm without clearly defined substrate specificity[20, 21]. As the autophagosome matures it fuses with a lysosome, forming the autolysosome, resulting in the proteolysis of engulfed materials[21]. This intermediate form of metabolism is an essential starvation response providing the cell otherwise unavailable macromolecular precursors such as amino acids, fatty acids and nucleotides. In a dramatic demonstration of the necessity of autophagy; mice homozygous null for the autophagy-related gene *ATG5* or *ATG7* develop normally *in utero,* but at birth, when faced with severe metabolic stress incurred by loss of the trans-placental blood supply, the inability to induce autophagy results in death within 24 hours[22, 23].

While the protective role of starvation-induced autophagy is indisputable, our understanding of autophagic cell death (so called type II programmed cell death) is much less clear. The most rigorous work has been performed in cancer studies where the significance of autophagy is an extremely complex and context dependent event. Confounding this work is a large degree of overlap between autophagic and apoptotic cellular machinery. Indeed, many genes originally characterized as being exclusively autophagic or apoptotic have now been demonstrated as being regulated by, or as regulators of, the alternate pathway.

For example; Beclin 1, a protein involved in the proximal steps of autophagosome formation, was recently characterized as a binding partner of the anti-apoptotic protein Bcl2. This interaction results in the attenuation of Beclin 1-mediated autophagy, thus demonstrating that Bcl2 is a negative regulator of both apoptosis and autophagy[24]. Clinically, Beclin 1 haploinsufficiency is found in human breast, ovarian, and other tumors, likewise, mice carrying a heterozygous deletion of *Beclin 1* demonstrate a higher incidence of mid-life malignancy[25, 26]. The finding that reduced levels of Beclin 1 enhance tumorgenesis provides strong evidence that autophagy can function in a tumor-suppressor manner.

ATG5 is another classic “autophagy” protein involved in the early stages of autophagosome formation. It was recently reported that calpain-mediated cleavage of ATG5 results in mitochondrial localization where it then functions in a pro-apoptotic manner[27]. Furthermore, some of the most commonly mutated tumor suppressor proteins, such as p53 and PTEN, have recently been described as powerful induces of autophagy[28, 29].

Clinical and experimental data demonstrate autophagy has a relevant tumor suppressor role, suggesting this pathway can function as a mechanism of caspase-independent cell death. Conversely there are examples of autophagy functioning as a tumor survival mechanism. A number of reports demonstrate that in certain tumor lines the induction of autophagy on growth factor withdrawal is necessary for cell survival[30, 31]. Indeed, the growing consensus is that the protective or cell death function of autophagy is context dependent and relies on the nature of the malignancy, the context of growth, and possibly co-mutations that have not yet been identified.

Considering that the physiological / pathophysiological significance of autophagy is highly dependent on the context of pathway activation, caution and skepticism must be used when discussing the role of autophagy in human heart disease. As explained in the following section, the earliest reports implicating cardiomyocyte autophagy as a mechanism of myocardial remodeling were limited in both scope and perspective, and the conclusions from these reports must be interpreted with this in mind.

Clinical evidence of autophagy in heart disease

Since 2001 there have been a number of reports indicating that autophagy is a remodeling mechanism in the diseased human heart[32-35]. These studies identified a robust presence of autophagosomes in patients with idiopathic dilated cardiomyopathy (IDC), severe aortic valve stenosis, and ischemic cardiomyopathy. In each case autophagy was assessed by strictly morphological methods with the authors' stated objective being the quantification and assessment of cardiomyocyte death; cell death scored as being either oncotic, apoptotic (type I programmed cell death), or autophagic (type II programmed cell death).

Three of these studies included patients with end-stage heart failure due to IDC. Evidence of oncosis, apoptosis, and autophagy was found in all IDC patients, but of particular interest, the authors consistently observed that, within a single cell, morphological evidence of apoptosis and autophagy was mutually exclusive[32, 33, 35]. That is, cells undergoing apoptosis do not demonstrate evidence of autophagy; likewise, cells with active autophagy do not demonstrate evidence of apoptosis. Further characterization of engulfed substrates demonstrated that ubiquitinated proteins were concentrated within autophagosomes, suggesting there is

selective autophagic sequestration of ubiquitinated (*i.e.* damaged) proteins in the diseased state.

Patients with aortic valve stenosis, a condition resulting in severe after-load stress on the heart, were stratified to three different levels of functional decline[32]. The authors reported an increase of protein ubiquitination in degenerating myocytes which occurred in direct proportion to the decrease in fractional shortening. The presence of autophagosomes was again reported in this study and cited as a cause of cell death, but limited evidence was shown to support this claim. Furthermore, while the relationship between total ubiquitin levels and declining performance was described, how these findings relate to the prevalence of autophagosomes was not explored.

Cumulatively these reports demonstrate that autophagy is a prominent and under-appreciated myocyte feature in distinct forms of heart disease. That said, before autophagy is cast as a detrimental response to cardiovascular stress it is important to interpret the results in context of the authors' stated bias. These studies were initiated with the notion that the presence of autophagosomes is patho-pneumonic of type II programmed cell death. Neither explored nor discussed in these reports is the concept that active autophagy may be indicative of an attempt at cell survival. To claim *a priori* that the presence of autophagosomes is solely indicative of autophagic cell death fails to take into consideration the well-established salutary roles of autophagy in other stressed states, most notably starvation. Indeed, the finding that apoptosis and autophagy are mutually exclusive events in a sick cardiomyocyte could also be interpreted as evidence that autophagy functions in a protective, potentially anti-apoptotic manner. Likewise, the extensive accumulation of ubiquitinated protein within the autophagosome may be indicative of

enhanced lysosomes-mediated proteolysis of damaged cellular constituents, presumably to the benefit of the cell.

In conclusion, several studies now demonstrate the presence of autophagic activity in the diseased human heart. At this point it is unknown whether autophagy is adaptive or maladaptive in the setting of heart disease. Furthermore, nothing is known regarding the mechanisms that activate stress-induced autophagy. In this context, our group and others have initiated studies designed to elucidate the mechanistic underpinnings of cardiomyocyte autophagy induced by diverse forms of myocardial stress, and more importantly, to determine in what context the net effect of autophagy is adaptive or maladaptive.

Basic science discoveries of cardiomyocyte autophagy

Stress-induced cardiomyocyte autophagy has been most rigorously studied in two paradigms; ischemia / reperfusion (I/R) and severe pressure-overload. In the former setting, ischemic tissue is deprived of oxygen and nutrient delivery, thus the affected areas of the heart must function in what is effectively a starved state. In contrast, severe pressure-overload places increased energetic demands on the heart as well as severe biomechanical stress; the heart is not so much under-fed as it is over-worked. In addition to the mechanistic differences between these two models, it is very important to note the time-scales with which the studies were done. The I/R studies address the role of autophagy in the acute setting; with one exception, each of these studies involves ischemic stress on resting hearts or cultured cells, with outcomes generally measured on a scale of hours. In contrast, pressure overload-induced failure represents a “chronic” condition, where the development of disease progresses and was studied over days and weeks.

Cardiomyocyte autophagy is an adaptive response to ischemia and reperfusion

The cumulative evidence thus far suggests I/R-induced autophagy functions in a salutary manner. Beginning with the *in vitro* data; simulated I/R in atrial derived HL-1 cells was found to increase the incidence of autophagosomes[36]. To test the significance of this activation the authors used both gain and loss-of-function approaches. Cellular autophagy was increased by overexpression of Beclin 1 or decreased by expression of dominant negative ATG5 (dnATG5). Consistent with a protective effect, enhancing autophagy attenuated I/R-induced activation of pro-apoptotic factors and thereby decreased cell death. In the complementary loss-of-function studies, attenuation of autophagy resulted in increased cell death. In summary, these results demonstrate that, *in vitro*, I/R-induced autophagy is a protective cellular response.

In a more clinically relevant *ex vivo* model of ischemic heart disease, rat hearts were excised, attached to a Langendorf apparatus and subjected to 30 or 90 minutes of global ischemia[37]. The results in this study support the initial *in vitro* findings where autophagy is upregulated in response to ischemia. In elegant gain and loss of function studies, enhancing autophagy via forced over-expression of ATG5 significantly reduced cell death, suggesting a salutary effect of this induction. Conversely, inhibition of autophagy via the delivery of dnATG5 resulted in more extensive myocardial damage measured as cell death and mitochondrial illness. In conclusion, these studies support the notion that autophagy is an adaptive cellular response to ischemic stress.

In the model closest to human disease, pigs were subjected to repeated bouts of I/R defined as 90 minutes of coronary stenosis (with 30% occlusion) followed by a 12 hour reperfusion time[38]. Hearts were harvested after 1, 3, or 6 bouts of I/R and processed for morphological and biochemical markers of autophagy and apoptosis. Of note, the authors again describe a mutually exclusive incidence of cardiomyocyte apoptosis and autophagy. Apoptosis was most marked during the first 3 episodes of I/R and progressively declined during each subsequent episode. In contrast, autophagic activity (defined by increased expression of the autophagy-related genes; cathepsin D, cathepsin B, beclin 1, and processing of MAP-LC3 to the autophagosome specific form) was low during the first three episodes, but progressively increased with each subsequent I/R challenge. Of note, the quantification of apoptosis was imaged based and therefore it was possible to specifically study cardiomyocytes rather than neighboring interstitial cells. The measures of autophagy were performed on ventricular lysates, and consequently, it is possible that some of the signal derives from interstitial cells, not the cardiomyocytes.

Cumulatively these data present a consistent model whereby cardiomyocyte autophagy induced by an acute challenge with ischemia and reperfusion functions in a salutary manner. It is particularly interesting that the mutually exclusive incidence of apoptosis and autophagy, as described in the clinical biopsies, was also observed in both *in vitro* and *in vivo* model systems. The experimental evidence suggests that the presence of autophagy in I/R-stressed cardiomyocytes is indicative of an active cellular survival pathway, not autophagic cell death. Given autophagy's canonical role as a starvation-response mechanism, these findings are not at all surprising. Ischemia results in decreased oxygen

and nutrient delivery to downstream tissue. The fact that autophagy is protective under such conditions makes intuitive sense when you consider this pathway is best characterized as a form of intermediate metabolism.

Cardiomyocyte autophagy is a maladaptive response to severe pressure overload

One of every four Americans will suffer from chronic hypertension. This condition places increased after-load stress on the heart with subsequent activation of the hypertrophic program. Initially hypertrophy is adaptive, allowing for preserved myocardial function despite the increased workload. In the chronic setting, however, there is irreversible pathological myocardial remodeling characterized by ventricular dilation, reactive fibrosis, diminished contractile function, and ultimately heart failure. Despite the high prevalence of this disease, mechanisms that govern the transition from compensated hypertrophy to decompensated failure are still poorly understood.

In our studies of pressure overload-induced ventricular remodeling we found that hemodynamic stress induced a robust autophagic response. Electron micrographs from animals 48h after severe thoracic aortic banding (sTAB) revealed extensive accumulation of double-membrane vacuoles, a gold-standard measure of autophagy. Further evidence of autophagy was seen by anti-LC3 western blots in which we observed high levels of autophagosome-specific LC3 in sTAB animals relative to sham operated controls.

As a third measure of autophagy we generated “autophagy reporter” mice in which there is cardiomyocyte restricted expression of GFP-LC3. In this model GFP-LC3 is diffusely cytosolic at rest, but upon induction of autophagy it redistributes to clearly defined puncta

(representing autophagosomes). This is a powerful tool as it allows for monitoring of cardiomyocyte specific autophagy (rather than activation of interstitial cells) and for high-resolution assessment of regional differences in autophagic activity. Using these reporter mice we observed a robust increase in autophagy in response to sTAB. Levels peak in the first 72 hours, and while activity slowly declines, it remains significantly elevated through at least 3 weeks post sTAB.

These studies demonstrated that autophagy is a robust response to severe pressure overload, yet they did not tell us if autophagy functions in an adaptive or maladaptive manner. To determine this we employed mouse models of enhanced and attenuated autophagy, achieved through genetic manipulation of *beclin 1.* As a model of reduced autophagy we performed experiments on animals carrying a heterozygous deletion of beclin 1 (beclin $1^{+/-}$). Conversely, to enhance autophagic activity we generated animals with cardiomyocyte restricted over-expression of beclin 1 (*αMHC-Beclin 1*).

Beclin $1^{+/-}$ animals subjected to sTAB have a hypertrophic response identical to wildtype controls, indicating that Beclin 1 is not necessary for activation of the growth program. While hypertrophic growth was identical, blunting autophagy resulted in improved cardiac performance when measured 3 weeks after sTAB. These findings, then, are consistent with the hypothesis that autophagy contributes to pressure overload-induced heart failure.

Pushing the system in an opposite direction, we next subjected *αMHC-Beclin 1* mice to pressure overload. As predicted, over-expression of Beclin 1 resulted in enhanced levels of cardiomyocyte autophagy. Consistent with autophagy acting as a detrimental response to hemodynamic stress, a number of pathological hallmarks were enhanced

in *αMHC-Beclin 1* animals; relative to controls, *αMHC-Beclin 1* mice demonstrated an exaggerated autophagic response accompanied by increased fibrosis and a dramatic decline in cardiac performance. Thus, these findings demonstrate that stress-induced autophagy, amplified by Beclin 1 over-expression, promotes a deterioration of cardiac performance and enhances pathological remodeling.

Summary and conclusions

Autophagy is increasingly being appreciated as an important cellular response to diverse forms of myocardial stress. Cardiomyocyte autophagy is clinically associated with idiopathic dilated cardiomyopathy, aortic valve stenosis, and ischemic heart disease. Our group and others have used experimental models to demonstrate that cardiomyocyte autophagy is activated by pressure-overload and ischemia / reperfusion. While autophagy is induced by these diverse forms of cardiovascular stress, whether the net effect of this activity is adaptive or maladaptive appears to be context dependent. Hypertension-induced autophagy is maladaptive, whereas ischemia / reperfusion-induced autophagy functions in a protective manner. This context dependent nature of adaptive versus maladaptive cardiomyocyte autophagy adds a layer of complexity to all studies, but this complexity is entirely consistent the cancer literature.

Toward understanding and possibly predicting the contexts in which autophagy is adaptive or maladaptive, I designed studies to determine the proximal activators of stress-induced cardiomyocyte autophagy. The rational behind this approach was that the proximal signals are likely of primary relevance to the final function of this pathway. As such, I began these studies keeping in mind the two most well-established facts; 1)

autophagy, first and foremost, is a mechanism of lysosome-mediated bulk protein degradation, and 2) in failing hearts there is significant accumulation of ubiquitinated proteins within the autophagosome. Given that extensive protein ubiquitination is a marker of protein damage, and that highly-ubiquitinated proteins tend to form intracellular aggregates, I hypothesized that stress-induced protein aggregation is a proximal trigger of cardiomyocyte autophagy, with autophagy then functioning as a mechanism of aggregate clearance.

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CHAPTER 2

INTRACELLULAR PROTEIN AGGREGATION IS A PROXIMAL TRIGGER OF CARDIOMYOCYTE AUTOPHAGY

Introduction

Under conditions of stress, the heart undergoes a compensatory hypertrophic growth response which serves to normalize wall stress and diminish myocardial oxygen demand[1]. In the chronic state, cardiac hypertrophy is an independent risk factor for heart failure and lethal arrhythmia, leading causes of morbidity and mortality in Western society[2]. Numerous pathways have been causally implicated in the transition from myocyte hypertrophy to failure[3,4], including programmed cell death, cellular atrophy, and very recently, autophagy[5]. However, mechanisms governing the transition from compensated hypertrophy to heart failure are incompletely characterized.

Autophagy is a highly conserved process of protein degradation involved in turnover of mitochondria, peroxisomes, and non-selective degradation of cytoplasmic components during periods of starvation or stress[6,7]. The autophagic reaction commences with formation of the autophagosome, a double membrane structure of unknown origin, that engulfs an entire region of cytoplasm without clearly defined substrate specificity[8,9]. As the autophagosome matures, it fuses with a lysosome to form an autolysosome, leading to proteolysis of engulfed materials[9].

Autophagic activity is associated with the pathogenesis of various diseases[8], including neurodegenerative disorders[10-12], skeletal myopathy, cancer[13,14], and microbial infection. Recent reports demonstrate that multiple forms of cardiovascular stress, including pressure overload, chronic ischemia, and diphtheria toxin-induced injury, provoke an increase in autophagic activity in cardiomyocytes[5,15,16]. Our lab demonstrated recently that in pressure-overload heart failure, a common form of heart failure in patients, induction of autophagic activity is maladaptive[5].

A prominent feature among neurodegenerative diseases associated with autophagy – including Huntington's disease, amyotrophic lateral sclerosis, Parkinsonism, and Alzheimer's disease – is deposition of proteins within intracellular aggregates[17]. The prevailing notion is that autophagic pathways serve a salutary function by facilitating removal of aggregates too large for efficient proteasome-mediated clearance[18]. In fact, in several neurodegenerative diseases, including Huntington's disease[19] and amyotrophic lateral sclerosis[20], a strong association exists between induction of autophagy and the presence of protein aggregates. In the absence of basal levels of autophagic activity in brain, abnormal aggregates of intracellular proteins develop[11,12,21], and pharmacologic or genetic induction of autophagy is sufficient to reduce polyglutamine-induced cytotoxicity in animal models of Huntington's disease[22]. Taken together, these data suggest that intracellular protein aggregates are capable of stimulating autophagic activity which serves, in turn, to facilitate clearance of the aggregates.

Here, we report that hemodynamic stress is sufficient to induce a robust accumulation of intracellular protein aggregates. We also report that these aggregates organize into peri-nuclear aggresomes. To test for a causal link between aggresome formation and cardiomyocyte autophagy, we induced accumulation of ubiquitinated proteins in cultured myocytes by inhibition of proteasome activity. In these studies, protein aggregates/aggresomes accumulated rapidly and correlated with robust induction of autophagy. Further, pharmacologic inhibition of autophagy dramatically enhanced aggresome size and abundance, suggesting that aggresomes are cleared through an autophagic mechanism. Together, these data point to intracellular protein aggregation as a contributor to

pathogenesis in load-induced heart disease, and they establish that protein aggregation and aggresome formation are proximal steps in the induction of cardiomyocyte autophagy.

Methods

Pressure-Overload Hypertrophy

Male C57BL6 mice (6-8 weeks old) were subjected to pressure overload by severe thoracic aortic banding (sTAB), a model of pressure-overload heart failure[23]. At time of sacrifice the integrity of aortic banding was confirmed by inspection of the surgical constriction and by visualization of marked differences in caliber of the right and left carotid arteries.

Primary Culture of Neonatal Rat Ventricular Myocytes

Cardiomyocytes were isolated from the ventricles of 1-2-day-old Sprague Dawley rat pups and plated as described[24] at a density of 1250 cells/mm in medium containing 10% fetal calf serum with 100 μM BrdU. Myocyte cultures obtained using this differential plating method contained less than 10% non-cardiomyocytes as determined microscopically using a myocyte-specific α-actinin antibody (data not shown). 48 hours post-plating, cells were transferred to medium supplemented with 1% FBS, 1μM BrdU, at which point treatment began.

Immunohistochemistry

Animals were euthanized with 5% sodium-pentobarbital in PBS, pH 7.4 (n=5, all groups). Hearts were fixed by sequential perfusion with 30mL heparinized PBS, 30mL 4% paraformaldehyde (PFA), followed by overnight incubation in 4% PFA at 4°C with agitation. All immunostaining was done on 5μm sections cut with a 4-chamber view. We performed antigen retrieval by microwave HIER (heat-induced epitope retrieval) using 1X Biogenex Citra (10min at 95°C). When primary antibodies were of

mouse origin, sections were blocked and immunostained using commercially available Mouse On Mouse® immunodetection reagents (Vector BMK-2202). When not using mouse antibodies, sections were blocked in 3% goat serum in PBS. Primary antibody dilutions were as follows: 1:30 CryAB (Vector, VP-A103), 1:50 anti-MAP-LC3 (Santa Cruz, sc-16756), 1:50 anti-vimentin (Santa Cruz, sc-5565), 1:50 anti-ubiquitin (Santa Cruz, sc-9133), 1:1000 anti-ubiquitin (Abcam, ab7254), or 1:50 gamma-tubulin (Santa Cruz, sc-10732).

Immunocytochemistry

Cultured cardiomyocytes were washed 3 times in PBS supplemented with calcium ($CaCl_2$, 0.1g/L) and magnesium ($MgCl_2$-$6H_2O$, 0.1g/L). Cells were then fixed in 4% PFA, permeabilized for 2 minutes in 0.1% Triton-X 100, followed by 15 minutes blocking in PBS with 3% normal goat serum and 1% bovine serum albumin. Primary and secondary antibody dilutions were identical to those used for immunohistochemistry studies. Immunostained sections were imaged using a Zeiss LSM510 META Laser Scanning Confocal Microscope, running LSM-510 Version 3.2 SP2 data acquisition software. All adjustments of brightness, contrast, or color balance were applied to the entire image to avoid misrepresentation of information present in the original.

In Situ ROS Detection

Cardiac tissue was prepared for *in situ* imaging of superoxide levels using the oxidant-sensitive fluorescent dye dihydroethidium (DHE, Molecular Probes, Inc., Eugene OR) as previously described[25]. Briefly,

unfixed flash frozen myocardial segments were cut into 30 μm sections and incubated (30 min, 37ºC humidified chamber) with topically applied DHE (2 μM). Images were obtained with a BioRad laser scanning confocal microscope. Sections from sham and sTAB hearts were processed and imaged in parallel (excitation at 488 nm, detection of fluorescence at 585 nm) using identical laser settings.

Transient Transfection of Cultured Cardiomyocytes

24 hours after plating, NRVM were transfected with a GFP-tagged LC3 construct, as previously described[5]. Briefly, 0.5μg of plasmid DNA was incubated for 15 minutes at room temperature with 50μL Opti-MEM® (Gibco 31985-070) and 3μL of Plus® reagent (Invitrogen 11514-015). This was then combined with pre-incubated Opti-MEM® supplemented with 2μL Lipofectamine® (Invitrogen 18324-012). Cells were washed 2 times with warm PBS, 2 times with pre-warmed Opti-MEM®, and then incubated for 4 hours with the DNA/Lipofectamine mixture. Cells were then washed and treated as indicated.

Subcellular Fractionation

NRVM were harvested (100mm plates) in 100μL immunoprecipitation buffer (IPB) (10mM Tris-HCl, 5mM EDTA, 1% NP-40, 0.5% deoxycholate, 150mM NaCl) and sheared by repeated passage through a 27G needle. Lysates were incubated on ice for 30min, and then centrifuged at 16,000xg (15min). The supernatant was collected and used as the soluble fraction. The insoluble pellet was re-suspended in 10mM Tris-HCl, 100μL of 1% SDS, and incubated at RT for 10 min. 100μL of

IPB was then added to the insoluble fraction, followed by 15 seconds of sonication.

Chymotrypsin-Like Activity

Cells were harvested in buffer H (20mM Tris-HCl, 20mM NaCl, 1mM EDTA, 5mM BME, pH 7.6 at 4ºC), sheared by repeated passage through a 27G needle, and the supernatant collected after sedimentation of insoluble material (centrifugation at 16,000xg, 10 min). 10μg of supernatant protein was added to 300μL activity assay buffer (50mM Tris-HCl, 5mM BME, 50μM Suc-LLVY-AMC, pH 8.0 at 37ºC). Released fluorogenic peptide was measured over a 20 minute period using a BMG FLUOstar OPTIMA plate reader (excitation 360nm, emission 460nm). Signal was normalized to protein loaded.

Immunoblot Analysis

Protein lysates were fractionated by SDS polyacrylamide gel electrophoresis, transferred to Hybond-C Nitrocellulose membrane (HYBOND-ECL®), and subjected to immunoblot analysis. The ratio of antigen:antibody, detergent concentration, and duration and temperature of the reactions were optimized for each antibody.

Statistical Methods

Averaged data are reported as mean ± SEM. Statistical significance was analyzed using a Student's unpaired, 2-tailed *t* test, or one-way ANOVA followed by Bonferroni's method for *post hoc* pair-wise multiple comparisons.

Results

Autophagic activity increases in pressure-stressed ventricle

We have shown previously that cardiomyocyte autophagy contributes to pathological ventricular remodeling induced by severe afterload stress[5]. To define proximal events that trigger autophagy, we subjected mice to severe thoracic aortic banding (sTAB), a procedure which induces pressure-overload heart failure[23]. Autophagic activity was monitored by both morphologic and histological means. In these hearts, we detected increased abundance of multilamellar autophagosomes, indicative of autophagic activity, by 48h post-surgery (**Fig 1A**). Smaller increases were detected at 24h. As before[5], spatial heterogeneity of autophagosome abundance was detected, with greater numbers of these organelles detected in the basal interventricular septum (data not shown).

During the autophagic response, LC3 (microtubule-associated protein 1 light chain 3), an 18kD homologue of Atg8 in yeast, is processed and lipid-conjugated[26]. The resulting 16kD active isoform migrates from the cytoplasm to isolation membranes and autophagosomes. Recently, intracellular migration of LC3 to vesicular membranes has emerged as a reliable marker of autophagic activity[5,27]. Consistent with increased autophagic activity, we detected robust increases in autophagosome-localized LC3 in pressure-stressed cardiomyocytes (**Fig 1B**) and LC3 processing in protein lysates prepared from pressure-stressed ventricle (**Fig 1C)**.

Severe pressure overload induces robust accumulation of ROS

Accumulation of reactive oxygen species (ROS) has been reported at 3 weeks in hearts subjected to increased workload[28]. To test for ROS

accumulation at earlier time points, we measured dihydroethidium (DHE) fluorescence in cryosections from sham and sTAB hearts 24 hours after surgery. Robust DHE staining was observed in sTAB hearts relative to control (**Fig 2A**), suggesting that ROS accumulation is an early event in pressure-overload heart failure.

To test this further, we quantified the cumulative effect of oxidative stress during the chronic stage of disease by staining for tyrosine nitration, a post-translational modification that accumulates during prolonged ROS stress. Anti-nitrotyrosine immunofluorescence was probed in hearts 7 and 21 days post-surgery. Sham-operated animals demonstrated very low levels of baseline nitrotyrosine (**Fig 2B**). In contrast, 7 days post-sTAB, high levels of nitrotyrosine staining were detected in cardiomyocytes. Similarly high levels were seen 21 days post-surgery (**Fig 2B**). Together, these data point to early and prolonged accumulation of ROS in pressure-overload heart failure.

Ubiquitinated proteins accumulate in the pressure-stressed ventricle

Oxidative stress can cause protein damage and consequent protein aggregation and amyloid deposition[29,30]. This concept, combined with the fact that elevated afterload imposes biomechanical and neurohumoral stress on the heart, led us to search for accumulation of damaged proteins and to assess the activation of clearance mechanisms. Damaged proteins are covalently coupled to ubiquitin, which targets the tagged substrate for proteolysis by the proteasome. If accumulation of ubiquitinated proteins outpaces proteasomal degradation, buildup of intracellular ubiquitinated proteins occurs, resulting in formation of protein aggregates, large

heterogeneous complexes that are poor substrates for proteasome-mediated proteolysis[31,32].

To test for increases in ubiquitinated proteins and protein aggregates, we immunostained sections of pressure-stressed heart for ubiquitin. Here, we detected dramatic accumulation of ubiquitin-positive inclusion bodies distributed throughout the left ventricle 72 hours following sTAB (**Fig 3A**). No significant ubiquitin-like immunoreactivity was detected in regions not subjected to biomechanical stress, such as right ventricle or either atria. Of interest, we detected a preponderance of ubiquitin staining in the basal septum, a region previously reported to be a "hot spot" for load-induced autophagic activity[5].

High molecular weight bands have been reported previously to be a marker of protein aggregation in skeletal muscle[33]. These bands represent proteins which are only partially soluble in SDS detergent and which emerge as a smear on a denaturing gel or remain trapped in the stacking gel. Western blot analysis confirmed the dramatic induction of ubiquitinated protein – detected as diffuse, high molecular weight bands on immunoblot – in pressure-stressed LV (**Fig 3B**).

Accumulation of ubiquitinated proteins is generally believed to occur as a consequence of one of three mechanisms: an increase in protein ubiquitination, a decrease in deubiquitination, or a decrease in proteasome-mediated clearance. To determine whether the accumulation of ubiquitinated proteins in pressure-stressed LV was due to a decrease in proteasome activity, we assayed ventricular lysates for chymotrypsin-like activity (CTL-A). One week following sTAB, basal septum CTL-A was increased 35% (± 2) compared to sham-operated controls ($p<0.05$, $n=4$, run in triplicate) (**Fig 3C**). To test for a component of ATP-dependent

proteasome activity, CTL-A was measured in lysates (n=3) supplemented with 2μM ATP. In these experiments, CTL-A was not significantly different than measured without addition of exogenous ATP (data not shown). Together, these data suggest that accumulation of ubiquitinated protein is not a consequence of diminished proteasome activity, but rather is due to increased flux that exceeds proteasome capacity.

In the context of cellular stress, abundance of chaperone proteins – molecules which play a critical role in protein targeting and stability – increase as an adaptive response[34]. Consistent with this notion, we detected significant increases in the abundance of multiple heat shock proteins in pressure-stressed myocardium 7 days following surgery (**Fig 3D**). Steady-state abundances of CryAB, HSP25, and GADD45 were modestly increased and that of BIP was dramatically increased. Interestingly, the increased levels of these heat shock proteins was greatest in basal septum, the region where we detect a preponderance of autophagosomes[5]. Together, these findings are consistent with a model where the intracellular milieu within load-stressed cardiomyocytes is conducive to protein damage and misfolding.

Induction of aggresomes in pressure-stressed ventricle

In certain contexts, protein aggregates coalesce as peri-nuclear structures termed aggresomes[17,35]. Aggresomes are cytoplasmic inclusion bodies located in the perinuclear region near the microtubule-organizing center (MTOC) which result from active, dynein-dependent transport of small aggregates from other parts of the cell. Given our observations of robust increases in chaperone protein levels in the setting of accumulation of ubiquitin-positive high molecular weight proteins, we

hypothesized that aggresome formation was occurring. To test for their presence, we co-stained pressure-stressed LV tissues for CryAB and vimentin, a structural component of the aggresome[36]. After 7 days of severe pressure overload stress, we detected juxta-nuclear organization of CryAB-associated proteins with an adjacent vimentin-containing shell (**Fig 4A, arrow**), a finding consistent with aggresome formation. Further evidence of aggresome formation was found when we co-immunostained for CryAB and ubiquitin. Again, after 7 days of pressure stress, we observed an increase in both ubiquitin and CryAB staining, with co-localization of signal in the peri-nuclear region (**Fig 4B, arrow**). Thus, ubiquitin-like immunoreactivity in pressure-stressed cardiac myocytes accumulated as large peri-nuclear aggregates which co-localize with CryAB and vimentin (additional images are presented in the **Online Data Supplement**). Based on these findings, we conclude that severe pressure overload triggers protein damage, protein ubiquitination, and formation of peri-nuclear aggresomes.

These findings led us to hypothesize that excessive accumulation of ubiquitinated proteins is a proximal step in protein aggregation that subsequently stimulates autophagic clearance. To test this, we treated neonatal rat ventricular myocytes (NRVM) with a proteasome inhibitor (MG-132, 5μM), isolated protein from the insoluble fraction, and probed for poly-ubiquitin. In these studies, we found that insoluble, ubiquitin-tagged protein accumulation was detectable as early as 4 hours following proteasome inhibition (**Fig 5A**). At subsequent time points, accumulation of ubiquitin-tagged protein within the insoluble fraction was progressive and robust. No evidence of cytotoxicity was detected out to 16 hours, although a modest degree of cell death was detected on exposure to MG-

132 for 24 hours or more (data not shown). These data suggest that the cytosol has a limited capacity to retain ubiquitinated protein in the soluble fraction, with excessive accumulation resulting in protein precipitation as an insoluble matrix.

To determine the subcellular localization of ubiquitinated proteins that accumulate in response to proteasome inhibition – and to test further for aggresome formation – we studied NRVM exposed to proteasome inhibition for 16 hours. Here, we detected peri-nuclear coalescence of ubiquitin-conjugated proteins (**Fig 5B, Panel 2**) similar to that seen in pressure-stressed LV *in vivo*. To test whether autophagic activity can reduce the abundance of aggregated proteins, we cultured cells in medium supplemented with 3-methyladenine (3MA, 5mM). 3MA inhibits autophagy at the stage of sequestration, where a double-membrane structure forms around a portion of the cytosol and sequesters it from the rest of the cytoplasm to form an autophagosome. As expected, we detected no change in ubiquitin levels or organization in control cells treated with a short course of 3MA alone (**Fig 5B, Panel 3**). However, in cells where proteasome activity was inhibited (MG-132) and autophagy was blocked (3MA), we observed a dramatic increase in the size of peri-nuclear aggresomes (**Fig 5B, Panel 4**). These data suggest that autophagy antagonizes aggresome formation by providing an alternative clearance mechanism.

Parallel activation of proteasomal and autophagic clearance pathways

In neurons, protein aggregates – similar to those reported here – are capable of triggering autophagy[37]. Thus, we tested whether the

presence of aggregates in cardiomyocytes is sufficient to trigger autophagy. To do this, we monitored autophagic activity *in vitro* by tracking the localization of GFP-tagged LC3 (GFP-LC3) in transiently transfected cardiac myocytes. Using this approach, autophagic activity can be detected as punctate, autophagosome-localized GFP signal, as opposed to the diffuse, cytosolic distribution seen under resting conditions[5,27]. In cells treated with a proteasome inhibitor (MG-132, 5μM, 16 hours), we detected an abundant increase in autophagosome-localized LC3 (**Fig 6A**). In these experiments, autophagic activity, quantified as the number of cells with autophagic vacuoles divided by the total number of transfected cells, was increased 1.8-fold ($p<0.05$) (**Fig 6B**). Of note, induction of cardiomyocyte autophagy in response to proteasome inhibition was similar in magnitude to that triggered by rapamycin (10nM), an established activator of autophagy[38] (**Fig 6B**). Together, these data are consistent with induction of autophagy in response to an accumulation of protein aggregates and argue strongly against nonspecific effects due to MG-132 toxicity.

Given our *in vivo* findings demonstrating that both proteasomal and autophagic clearance pathways are activated in response to pressure overload stress, combined with our *in vitro* data demonstrating that inhibition of the proteasome leads to an increase in autophagic activity, we set out to determine whether the converse pertains, i.e. whether inhibition of autophagy results in an increase in proteasome activity. NRVM were cultured for 7 days in the presence of 3MA or vehicle, with proteasome activity determined by measuring CTL-A. In these experiments, we observed a 73% increase in CTL-A after 7 days of 3MA treatment relative to control (1.73 ± 0.17, $n=3$, run in triplicate, $p<0.05$) (**Fig 6C**). These

results lend support to a model where autophagic and proteasomal clearance mechanisms function in parallel and is consistent with our finding of increases in both proteasomal and autophagic mechanisms in pressure-stressed heart.

Discussion

Recent reports reveal that cardiomyocyte autophagy is activated in response to multiple forms of cardiac injury[5,15,16]. However, proximal triggers of autophagic activity in the heart are unknown. The major findings of this study are 1) pressure-overload hemodynamic stress is associated with oxidative stress, early accumulation of ROS, and changes indicative of protein degradation, including accumulation of ubiquitinated proteins and increases in chaperone protein levels, 2) protein aggregation takes place with consequent formation of intracellular aggresomes, 3) both autophagic and proteasomal clearance pathways are activated in response, 4) protein aggregate and aggresome formation are sufficient to trigger cardiomyocyte autophagy, and 5) autophagic activity in the cardiomyocyte participates in clearance of aggregated proteins. Further, findings reported here are the first to demonstrate that protein aggregation occurs in response to hemodynamic stress, situating pressure-overload heart disease in the category of proteinopathies.

Stress-induced cardiomyocyte proteinopathy

Proteinopathy – toxic aggregations of misfolded proteins – is a growing family of human disorders, which includes Alzheimer's disease, Parkinsonism, amyotrophic lateral sclerosis, and both polyglutamine and polyalanine expansion disorders[39]. In heart disease, abnormal protein aggregation and accumulation of ubiquitinated proteins have been detected in human hearts with idiopathic or ischemic cardiomyopathies[40-43]. In both brain and heart, however, little is known regarding whether these intracellular inclusions are toxic themselves, or whether they

represent a compensatory mechanism that sequesters harmful, soluble proteins within the cytoplasm.

Insoluble protein aggregates are processed by pathways that are just now being deciphered. First, misfolded proteins are delivered to the microtubule organizing center (MTOC) by dynein-dependent retrograde transport along microtubules. When the degradative capacity of the proteasome is exceeded, protein aggregates accumulate in perinuclear inclusions called aggresomes[35], organized structures surrounded by vimentin filaments and which recruit chaperones, ubiquitin, proteasomes, and mitochondria. In pressure-stressed myocardium, we have detected an abundance of large perinuclear inclusions marked by ubiquitin which co-localize with chaperone proteins and vimentin, fulfilling the definition of aggresomes. In liver, aggresomes (Mallory bodies) form in response to a variety of stresses, including proteasome inhibition as seen here[44]. Aggresomes have also been detected in a model of desmin-related cardiomyopathy, a disorder characterized by abnormal amyloid deposition owing to dysfunctional chaperone protein function[43]. Findings reported here, however, are the first to describe aggresome formation in a very common cardiovascular disorder, *viz.* load-induced heart disease.

Cardiomyocyte autophagy as a clearance mechanism

Autophagy occurs in a wide range of eukaryotic cells, and its basic mechanisms are largely conserved from yeast to mammals[45]. Autophagy can be induced by diverse stimuli, including starvation, hypoxia, intracellular stress, and developmental signals[8]. It accomplishes a host of functions critical to normal homeostasis and development. In the context of nutrient deprivation, autophagic activity is adaptive in that degradation

of cytosolic components releases substrates for intermediary metabolism. Autophagy is also a mechanism for eliminating damaged proteins and organelles that might otherwise be toxic or trigger apoptotic death. For example, targeted inhibition of *Atg7*, a gene required for autophagic activity, leads to accumulation of ubiquitin-positive aggregates[21]. During development, autophagy is a means of remodeling tissues or removing unneeded cells[6]. In other settings, however, autophagic activity is associated with the pathogenesis of disease[8], and unrestrained autophagic activity can cause cell death[46-48].

In this study, we establish a link between protein aggregation and induction of cardiomyocyte autophagy. *In vivo,* we describe the accumulation of ubiquitinated proteins in a spatial and temporal pattern consistent with our recent report of pressure overload-induced autophagy[5]. We further characterize the organization of damaged proteins into vimentin associated, peri-nuclear, aggresome-like structures. As aggresome formation is a general response of cells which occurs when proteasome capacity is exceeded by the production of aggregation-prone misfolded proteins[35], we inhibited the proteasome in cultured cardiomyocytes to test directly for a causal link between aggregate formation and cardiomyocyte autophagy. In these experiments, we find that accumulation and aggregation of ubiquitinated protein is a powerful inducer of autophagy, capable of robust activation of autophagy to levels comparable to pharmacologic induction. Together, these findings are consistent with a model where autophagic activity is a general response to protein aggregation in the heart and point to a potential role for autophagy in cardiomyopathies of diverse etiology.

Interestingly, we and others do not detect inclusions that are membrane-bound. Also, the inclusions we detect are much larger than mammalian autophagosomes[49]. Together, these findings suggest that autophagy serves to clear monomeric and oligomeric precursors of aggregates, rather than the large inclusions themselves.

To test involvement of proteasomal activity, we measured chymotryptic activity acting on a small fluorogenic peptide substrate Suc-LLVY-AMC. Owing to its small size, this peptide diffuses easily into the proteolytic chamber of the 20S proteasome[50]; hence, it provides a readout of 20S proteasome activity. Consistent with this, addition of exogenous ATP to stimulate 26S proteasome activity had no significant effect on chymotryptic-like activity (<10%). In these experiments, we find that total proteasome function is increased in heart failure, suggesting increased flux through proteasomal degradation pathways and consistent with similar findings in a model of desmin-related cardiomyopathy[51]. Our data demonstrate that proteasomal and autophagic activities increase in parallel.

Alpha-1-antitrypsin deficiency is another proteinopathy in which autophagy is activated as a clearance mechanism. In this disease, proteolytic destruction of elastic connection tissue matrix occurs in lung due to unrestrained proteolysis. In liver, however, toxicity due to accumulation of misfolded mutant alpha-1-antitrypsin leads to chronic liver inflammation and hepatocellular carcinoma. Recent studies have revealed dramatic increases in autophagic activity in liver, which serves to prevent toxic cytoplasmic accumulation of mutant alpha-1-antitrypsin by selectively targeting insoluble aggregates[52].

Thus, a growing body of evidence implicates autophagy as a protective response in genetic diseases associated with cytoplasmic aggregation-prone proteins[49]. Here, we extend this to disease triggered by environmental stress. Importantly, recent studies demonstrating that pharmacological up-regulation of autophagy is protective in a wide variety of disease models associated with intracellular protein aggregation raise the exciting prospect of autophagic activation as a novel therapeutic strategy[19,53,54]. Our findings here extend this prospect to the myocardium.

Role in heart failure progression

The role of autophagy in heart failure pathogenesis remains unclear. Indeed, it is possible that autophagic activity carries out different functions depending on disease stage and severity. We have shown previously that genetic amplification of the autophagic response to acute-onset pressure stress leads to increased hypertrophic growth of the heart[5]. Here, we extend these findings with evidence that autophagy is activated to eliminate damaged proteins that accumulate within the cardiomyocyte. Several reports point to functional defects in the ubiquitin-proteasome system (UPS) in heart failure resulting in accumulation of misfolded proteins[55]. Consistent with this, we have observed increased ubiquitin staining in pressure-stressed ventricle, which is most prevalent in the basal septum. Further, pathological accumulation of reactive oxygen species plays a role in heart failure[56], which may both damage proteins and inhibit the UPS. Therefore, activation of autophagy at early stages of the disease may be a protective mechanism to scavenge and eliminate misfolded, poly-ubiquitinated protein aggregates that have overwhelmed the degradative capacity of the proteasomal system.

The presence of increased autophagic activity in animal models of heart failure and in samples from human failing hearts provides no insight as to whether cardiomyocyte autophagy is beneficial or pathological. Indeed, the contribution of autophagy to disease pathogenesis may depend upon the nature of the components being degraded and the extent of autophagic activity. We recently demonstrated that in pressure-overload heart failure, a common form of heart failure in patients, induction of autophagic activity is maladaptive[5]. On the other hand, if the protein aggregates themselves are toxic, increased autophagic removal could be beneficial. Further, recent evidence strengthens the assertion that dysregulated autophagic activity induces a caspase-independent programmed cell death program (Type II Programmed Cell Death[46-48]). In order to exploit autophagy as a therapeutic target, it will be essential to identify when it is protective and when it contributes to the pathogenesis of disease.

Perspective

Cardiac hypertrophy is a major predictor of heart failure – a prevalent disorder with high mortality – and there is urgent need for novel therapies to prevent or reverse pathological cardiac remodeling. Our data point to a progression of protein damage, aggregation, and coalescence into aggresomes as a previously unrecognized mechanism of disease. Further, our findings suggest that autophagic activity in cardiomyocytes is a general response to diverse stressors and contributes to disease pathogenesis in multiple contexts. As new advances emerge in deciphering molecular regulation of autophagy, it may soon be possible to enhance or inhibit autophagic activity selectively to impact heart disease meaningfully. Also, given that there are drugs already in clinical use that

alter the process of autophagy, and organ systems where increased autophagic activity is associated with disease, advances in this field are all the more urgent.

Figure 1

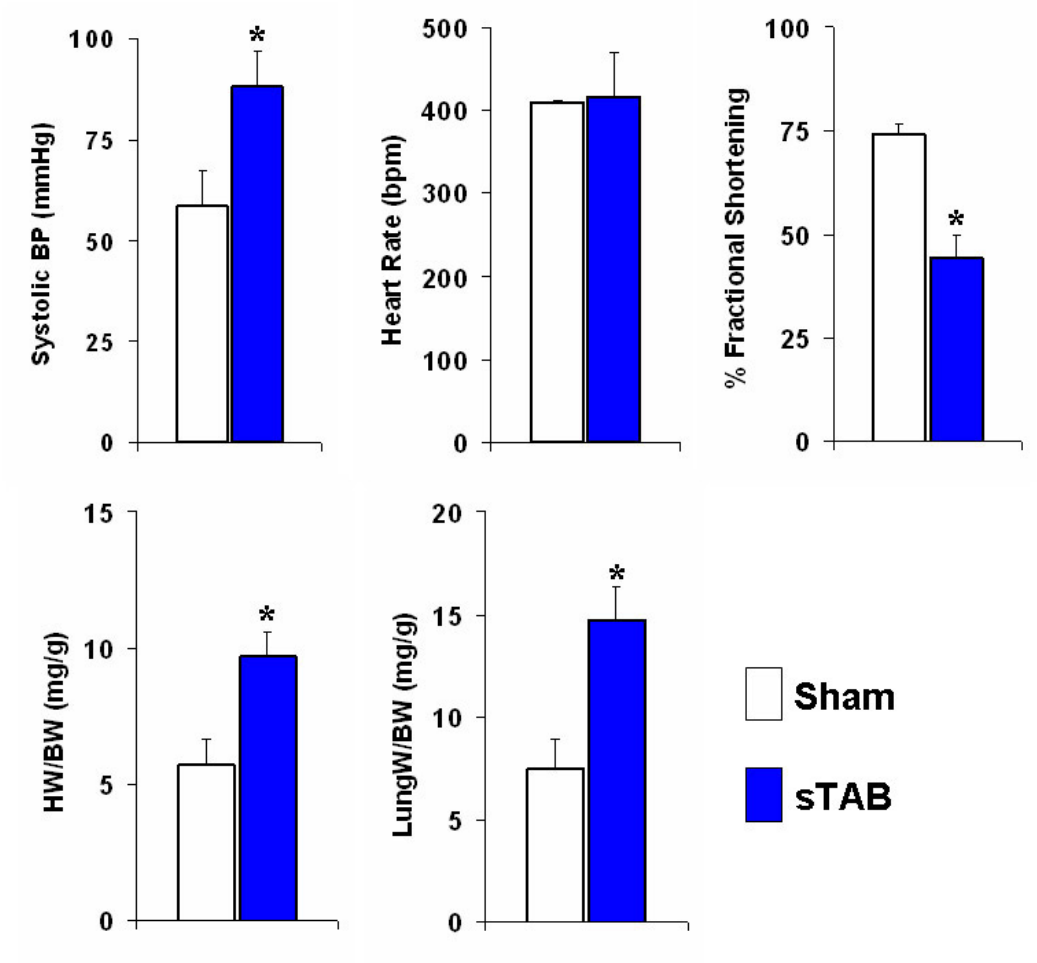

Figure 1: sTAB induces clinical heart failure. Panel A: Seven days after sTAB, animals demonstrated signs of heart failure with significant increases in heart mass, lung mass (indicative of pulmonary edema), and impairment in systolic performance. Mean data from sham (n=5) and sTAB (n=5) mice subjected to surgery as listed. HW/BW indicates heart weight/body weight; LWeight, lung weight. *P<0.05.

Figure 2

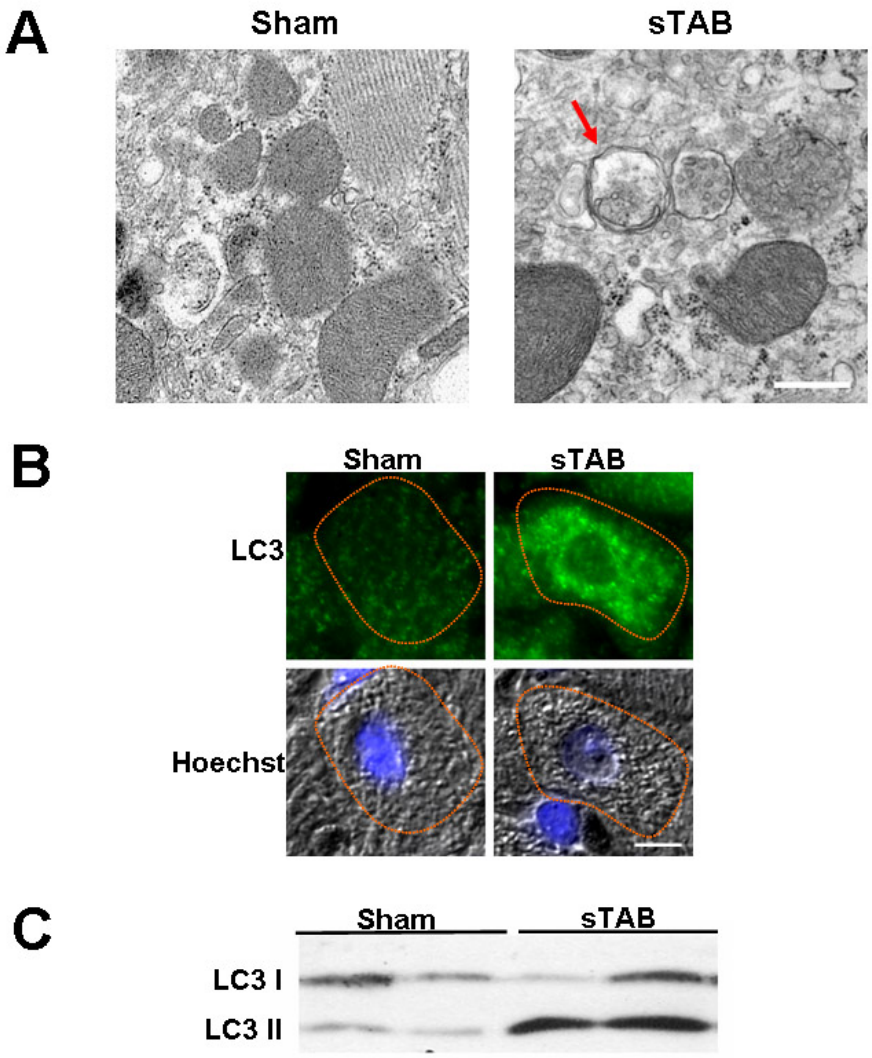

Figure 2: Severe pressure stress induces cardiomyocyte autophagy. Panel A: Representative electron micrograph revealing a multi-lamellar autophagosome in basal septum of LV 48h post-sTAB (scale bar 500ηm). **Panel B:** Anti-LC3 immunofluorescence reveals re-distribution of LC3 into punctate dots, indicative of autophagosome accumulation (scale bar 10μm). **Panel C:** Representative immunoblot demonstrating that severe pressure stress triggers LC3 processing from LC3 I to autophagosome-specific LC3 II.

Figure 3

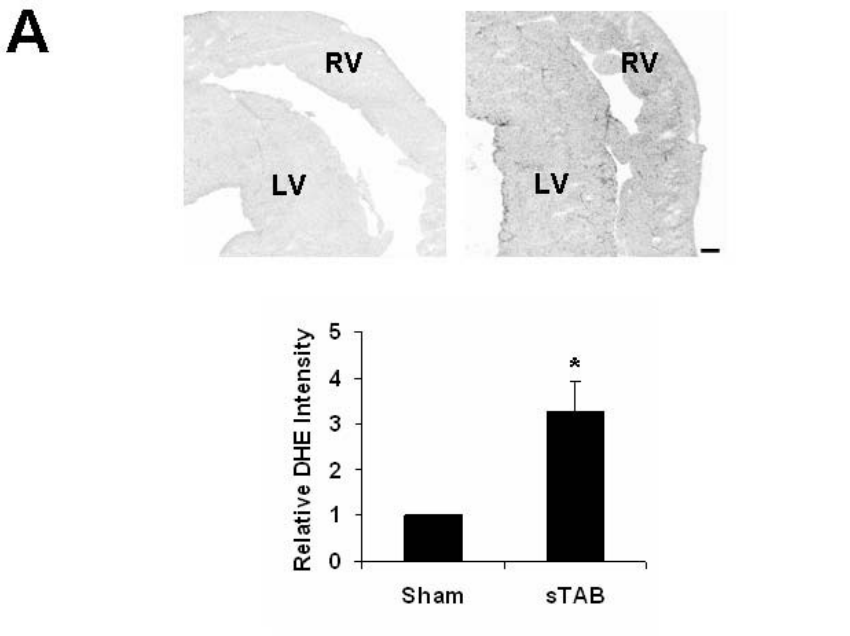
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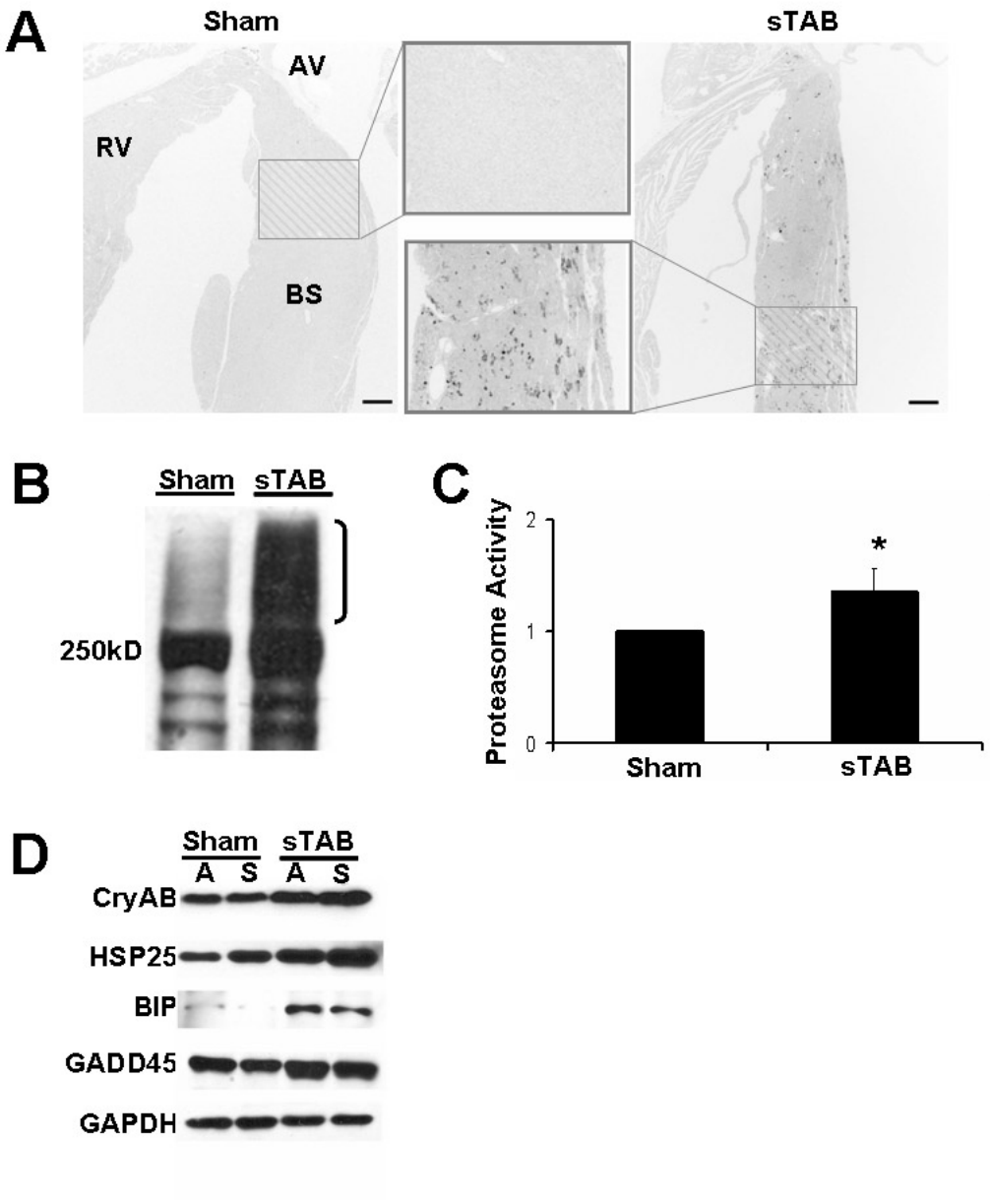
Figure 3: Severe pressure stress induces robust accumulation of reactive oxygen species. Panel A: DHE staining in hearts harvested 24 hours after sham or sTAB operation demonstrates early sTAB-induced increases in ROS (scale bar 500μm). Mean data from 10 fields taken from 2 independent experiments, * denotes $p<0.01$ relative to Sham. **Panel B:** Anti-nitrotyrosine staining shows evidence of cumulative oxidative stress 7 days post-operation with only modest decreases in signal at 3 weeks (scale bar 50μm).

Figure 4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GADD45
GAPDH

Figure 4: Proteotoxic stress in load-induced heart failure. **Panel A:** Representative immunohistochemical staining for ubiquitin revealing accumulation of ubiquitin-conjugated proteins in basal interventricular septum 72h post sTAB**.** RV= right ventricle, AV= aortic valve, BS= basal septum (scale bar 200μm). **Panel B:** Representative immunoblot of LV lysates 72h post-sTAB or Sham operation and probed for ubiquitin. **Panel C:** Proteasome activity assayed 1 week post-sTAB is increased (1.35±0.2, n=4) relative to sham-operated controls (1.0±0.3, n=4, $p<0.05$). **Panel D:** Representative immunoblots demonstrating that abundance of multiple protein chaperones is increased in pressure-stressed ventricle. Consistent with prior studies, stress markers are generally higher in basal septum (S) relative to apex (A).

Figure 5

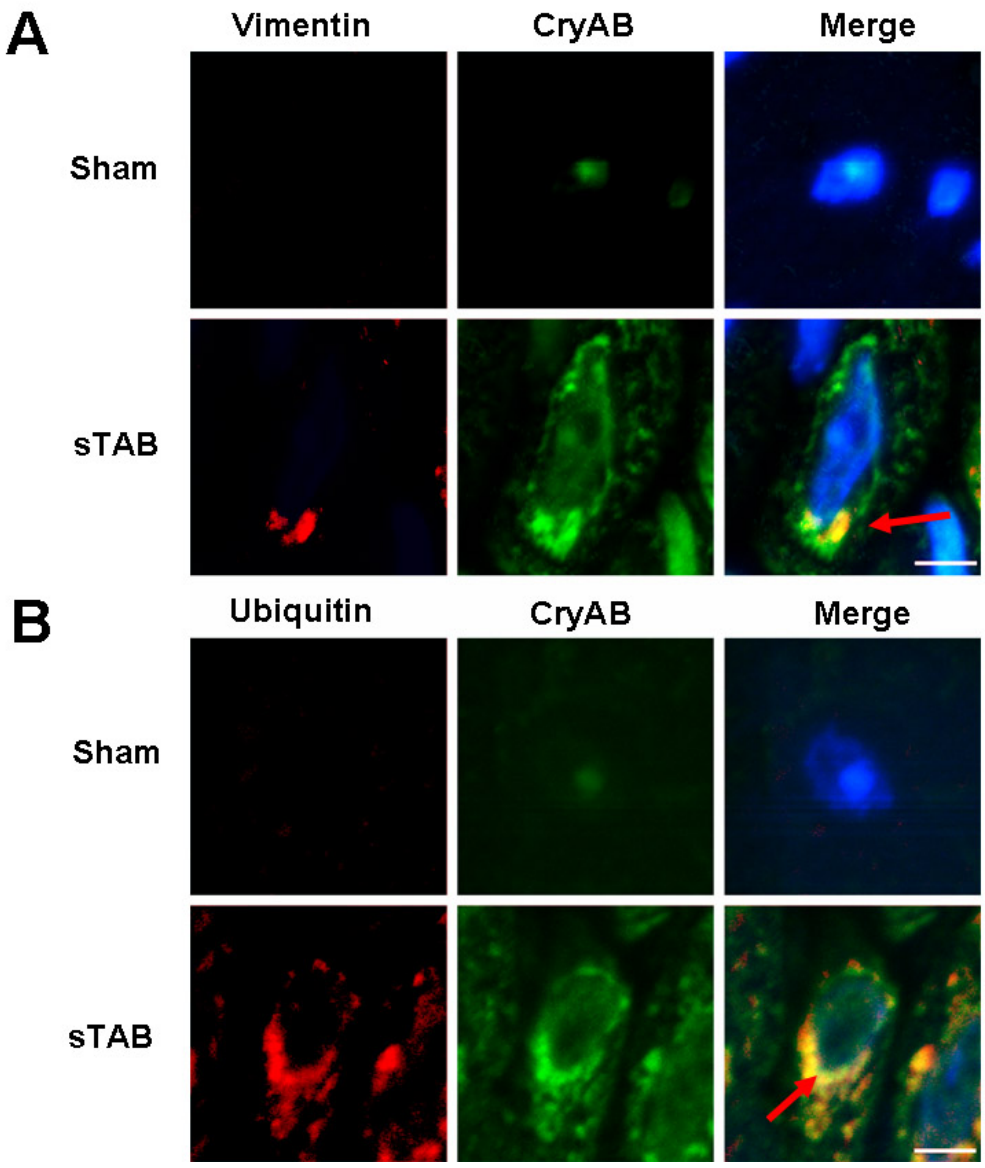

Figure 5: Aggresome formation in pressure-stressed LV. Panel A: Representative immunohistochemical sections 7 days post-sTAB immunostained for vimentin and CryAB (**Panel A**) or ubiquitin and CryAB (**Panel B**). (scale bar 25μm, n=4 in each group). Confocal imaging revealed peri-nuclear co-localization of these proteins, consistent with aggresome formation (arrow).

Figure 6

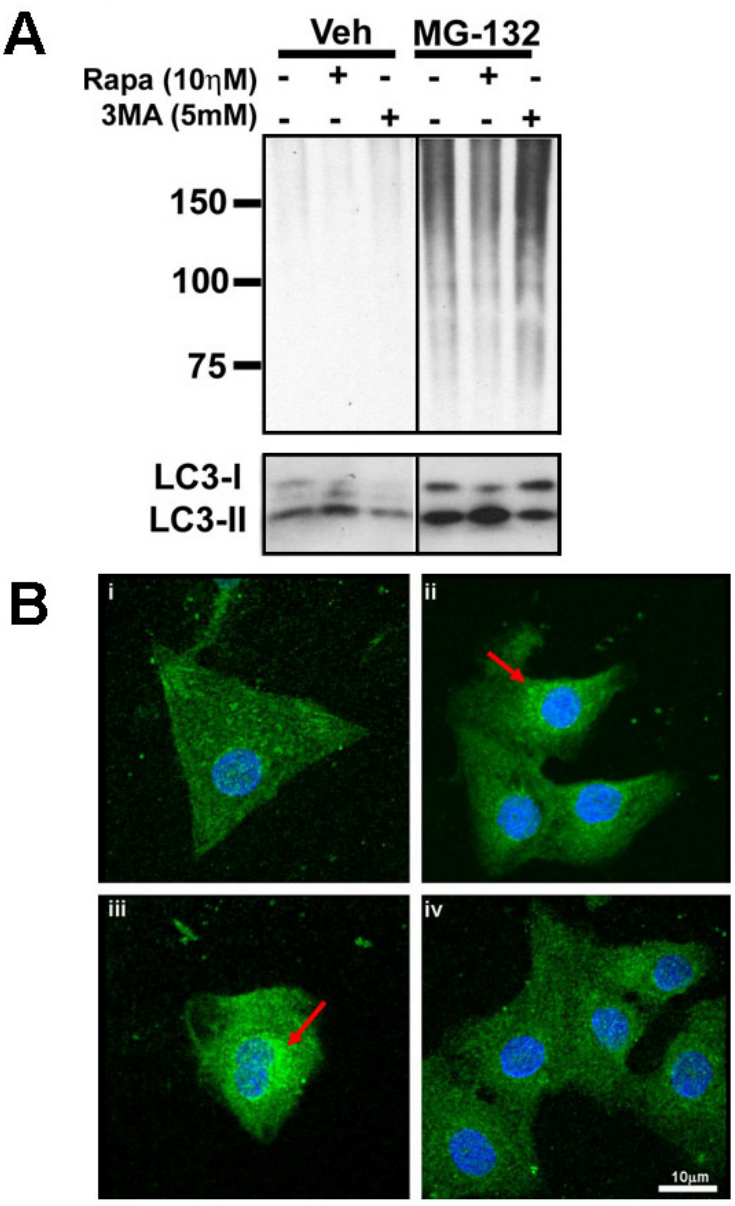
A
Veh
MG-132
Rapa (10ηM) - + - - + -
3MA (5mM) - - + - - +
150
100
75
LC3-I
LC3-II
B
i
ii
iii
iv
10μm

Figure 6: *In vitro* aggresome formation is secondary to accumulation of insoluble ubiquitinated protein. Panel A: NRVM were loaded with poly-ubiquitinated protein by selective proteasome inhibition (MG-132, 5μM). Representative immunoblot demonstrating that ubiquitinated protein accumulates in the setting of 16h of proteasome inhibition. Blunting or enhancing autophagy (with 3MA, 5mM or Rapamycin, 10ηM, respectively) alters this process. **Panel B:** Anti-ubiquitin immunocytochemistry reveals an ordered processing of the accumulating ubiquitinated proteins. Representative immunocytochemical staining after 16h of proteasome inhibition revealing peri-nuclear coalescence of ubiquitin tagged protein (**ii**) which are absent in vehicle treated cells (**i**). Consistent with a role of autophagy as a bulk degradation process, inhibition of autophagy by 3MA (5mM) amplified aggregate formation (**iii**). In contrast, Rapamycin treated cells demonstrate attenuated aggregate formation (**iv**).

Figure 7

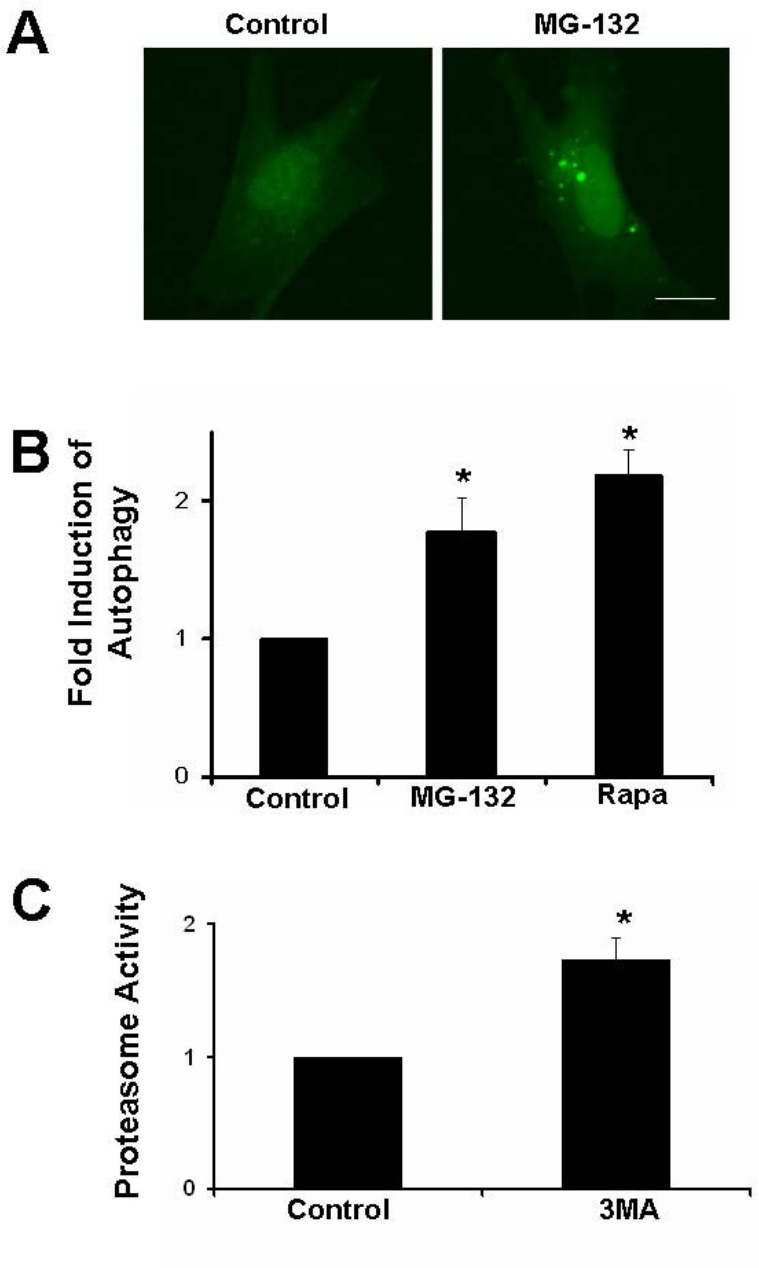
A
Control
MG-132
B
Fold Induction of Autophagy
0
1
2
*
*
Control
MG-132
Rapa
C
Proteasome Activity
0
1
2
*
Control
3MA

Figure 7: Aggresomes are a proximal trigger of cardiomyocyte autophagy. Panel A: NRVM were transfected with GFP-LC3 and subjected to proteasome inhibition (16h) resulting in redistribution of LC3 in an autophagosome-specific punctate pattern. Images of representative cells are depicted (scale bar 10μm). **Panel B.** Quantification of the numbers of cells manifesting autophagic activity (* denotes $p<0.05$ relative to control). Greater than 300 cells were evaluated in each treatment condition in 2 independent experiments. **Panel C.** Cells treated with 3MA for 7 days were assayed for proteasome activity, revealing a robust increase in activity (* denotes $p<0.05$).

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CHAPTER 3

AUTOPHAGY IS AN ADAPTIVE RESOPNSE IN DESMIN-RELATED CARDIOMYOPATHY

Introduction

Protein conformation disease – toxic aggregations of misfolded proteins – is a growing family of human disorders, which includes Alzheimer's disease, Parkinsonism, amyotrophic lateral sclerosis, and both polyglutamine and polyalanine expansion disorders[1]. A common feature of these diseases is the formation of intracellular aggregates of toxic proteins. In muscle, where myofibrillar architecture is maintained by desmin and other intermediate filaments, intracellular protein aggregates contain desmin and perturbation of the desmin cytoarchitecture is a major feature of disease (leading to the designation desmin-related myopathy). [With the discovery that a number of other proteins are present in these intracellular inclusions, the more generic term myofibrillar myopathy is often used[2].] In all these muscle disorders, lower extremity weakness and gait disturbances are prominent features and cardiomyopathy is a major cause of mortality.

Desmin-related cardiomyopathies (DRCM) are severe and progressive forms of heart disease for which there are currently no effective treatments. This class of disease arises from mutations in several different proteins, including desmin, myotilin, and dystrophin. Indeed, to date, over 30 distinct mutations have been causally implicated in DRCM[3]. In a subset, disease is due to a failed interaction between desmin and αB-crystallin (CryAB), a small heat shock protein[4,5]. In healthy striated muscle, desmin provides structural support for the myofibrils and acts as a cytoskeletal protein linking Z bands to the plasma membrane. CryAB associates with desmin and functions as a molecular chaperone, preventing aggregation of desmin and thus maintaining myofibrillar structure[6]. Mutations that disrupt the interaction between

desmin and CryAB produce a phenotype of protein aggregation, myofibrillar disarray, cardiac dysfunction, and sudden cardiac death[3-5].

DRCM due to a missense mutation of αB-crystallin ($CryAB^{R120G}$) is the most extensively studied form of this disease. Clinical presentation of the index family was characterized by multi-generational early-onset cataracts, proximal and distal muscle weakness, and severe cardiomyopathy[5]. Many groups have studied $CryAB^{R120G}$-associated DRCM which has now been replicated in two independently-derived transgenic mouse models[7,8]. Based on these two animal models, insights have been gleaned regarding pathogenic mechanisms in this disease. The $CryAB^{R120G}$ mutation results in protein aggregation[5], aggresome formation[9], mitochondrial toxicity[10], disruption of proteasome function[11,12], and induces a state of "reductive stress"[8]. However, whereas $CryAB^{R120G}$-induced pathogenesis has been well characterized, we have limited understanding of the adaptive cellular pathways that function to protect cardiomyocytes from $CryAB^{R120G}$-induced proteotoxicity.

Autophagy is increasingly appreciated as an important cellular response to diverse forms of myocardial stress. Best characterized as a mechanism of lysosome-mediated proteolysis, work by our group and others has demonstrated that cardiomyocyte autophagy is activated by severe pressure-overload[13,14], ischemia/reperfusion[15-18], and diphtheria toxin-induced cell death[19]. Given that excessive protein aggregation is central to $CryAB^{R120G}$ pathology, we postulated that autophagy, a process of bulk-protein degradation, could be a mechanism through which the heart protects itself in the setting of DRCM.

A number of characteristics associated with $CryAB^{R120G}$–induced cardiomyopathy led us to postulate that, in this setting, autophagy

functions in a protective manner. CryAB^{R120G}-associated DRCM disease results from the chronic expression of an aggregate-inducing protein in terminally differentiated, non-dividing cells. This paradigm is very similar to neurodegenerative diseases such as Huntington's disease, Parkinson's disease, or amyotrophic lateral sclerosis (ALS), where the abnormal deposition of proteins within intracellular aggregates is a prominent pathological feature[20]. The prevailing theory in this field is that autophagic pathways facilitate removal of aggregates too large for efficient proteasome-mediated clearance – thus acting in a salutary fashion[21-23]. Similarly, we previously reported that pressure overload or pharmacologically-induced protein aggregation are sufficient to induce robust cardiomyocyte autophagy which then functions to attenuate the accumulation of protein aggregates and aggresome formation[24]. Based on the prominent role of protein aggregation in these diseases, we hypothesized that autophagy is up-regulated in DRCM, decreases the accumulation of toxic protein aggregates, and thereby attenuates disease progression.

Methods

Primary Culture of Neonatal Rat Ventricular Myocytes

Cardiomyocytes were isolated from the ventricles of 1-2-day-old Sprague Dawley rat pups and plated as described[25] at a density of 1250 cells/mm in medium containing 10% fetal calf serum with 100 μM BrdU. Myocyte cultures obtained using this differential plating method contained less than 10% non-cardiomyocytes as determined microscopically using a myocyte-specific α-actinin antibody (data not shown). 48 hours post-plating, cells were transferred to medium supplemented with 1% FBS, at which point treatment would begin.

Transient Transfection of Cultured Cardiomyocytes

24 hours after plating, NRVM were transfected with a GFP-tagged LC3 construct, as previously described[13] Briefly, 0.5μg of plasmid DNA was incubated for 15 minutes at room temperature with 50 μL Opti-MEM (Gibco 31985-070) and 3μL of Plus reagent (Invitrogen 11514-015). This was then combined with pre-incubated Opti-MEM supplemented with 2 μL Lipofectamine (Invitrogen 18324-012). Cells were washed 2X with warm PBS, 2X with pre-warmed Opti-MEM, then incubated for 4 hours with the DNA/Lipofectamine mixture. After this incubation period cells were washed and treated as indicated.

Immunocytochemistry

Cultured cardiomyocytes were washed 3X in PBS supplemented with calcium and magnesium. Cells were then fixed in 4% PFA, permeabilized for 2 minutes in 0.1% Triton-X 100, followed by 15 minutes blocking in PBS with 3% normal goat serum and 1% bovine serum

albumin. Primary and secondary antibody dilutions were identical to those used for our immunohistochemistry studies, described above. Immunostained sections were imaged using a Zeiss LSM510 META Laser Scanning Confocal Microscope.

Adenoviral Infection of Cultured Cardiomyocytes

Experiments in which adenovirus expressing green fluorescent protein (AdGFP)-infected cells were simultaneously labeled with a fluorescent-tagged antibody recognizing α-actinin demonstrated that adenoviral particles at a multiplicity of infection (MOI) of 50 or higher infected greater than 90% of cells, as previously described[26]. For aggresome studies, NRVM were infected 48 hours post-plating with adenovirus containing wild-type or mutant human CryAB (Ad-CryAB^{WT}, Ad-CryAB^{R120G}, respectively). Experiments were performed at MOI = 100, yielding a ~95% infection rate. Cells were subsequently grown for 7 days in 2% FBS media supplemented with either Rapamycin (10ηM), or 3MA (5mM).

Cell Viability Measurements

NRVM were cultured and infected as described above. Culture medium was changed every 24 hours, and cell viability was measured after 5 days by the addition of 20μL CellTiter 96® AQ_{ueous} Solution Reagent (Promega, G3580) to 100μL of fresh culture medium. Cells were incubated for 2 hours at 37ºC in a humidified 5% CO_2 atmosphere, with absorbance then recorded at 490 nm using a 96-well plate reader.

Subcellular Fractionation

NRVM were harvested from 100mm tissue culture plates in 100µL of immunoprecipitation buffer (IPB) (10mM Tris-HCl, 5mM EDTA, 1% NP-40, 0.5% deoxycholate, 150mM NaCl) and sheared by repeated passage through a 27 gauge needle. Lysates were incubated on ice for 30min, and then centrifuged at 16,000 X g for 15min. The supernatant was collected and used as the soluble fraction. The insoluble pellet was re-suspended in 10mM Tris-HCl, 100µL of 1% SDS and incubated at room temperature for 10 min. 100µL of IPB was then added to the insoluble fraction, followed by 15 seconds of sonication.

CryAB transgenic lines

Transgenic mice expressing mutant human CryAB (*CryAB*^{R120G}) were engineered as described previously[8]. Only *CryAB*^{R120G}*High* animals was used in our study of mutants, herein referred to simply as *CryAB*^{R120G}.

Beclin 1 knockout animals

beclin 1 knockout animals were generously provided by Dr. Beth Levine[27].

Immunohistochemistry

Animals were euthanized with 5% sodium-pentobarbitol in PBS, pH 7.4 (n=5, all groups). Hearts were fixed by sequential perfusion with 30 mL heparinized PBS, 30 mL 4% paraformaldehyde (PFA), followed by overnight incubation in 4% PFA at 4°C with agitation. All immunostaining was done on 5 µm sections cut with a 4-chamber view. We performed antigen retrieval by microwave high intensity epitope retrieval (HIER)

using 1X Biogenex Citra (10min at 95C). When primary antibodies were of mouse origin sections were blocked and immunostained using commercially available Mouse On Mouse immunodetection reagents (Vector BMK-2202). When not using mouse antibodies sections were blocked in 3% normal goat serum in PBS. Primary antibodies dilutions were; 1:30 CryAB (Vector, VP-A103), 1:50 anti-MAP-LC3 (Santa Cruz, sc-16756), 1:50 anti-vimentin (Santa Cruz, sc-5565), 1:50 anti-ubiquitin (Santa Crux, sc-9133), 1:1000 anti-ubiquitin (Abcam, ab7254), or 1:50 gamma-tubulin (Santa Cruz, sc-10732).

Electron Microscopy

Hearts were retrograde perfused using PBS and 2% glutaraldehydye in 0.1 M cacodylate buffer. Post-fixation occurred in 2% osmium tetroxide in 0.1 M cacodylate buffer and 1% aqueous uranyl acetate each for one hour. An ascending series of ethanol washes (50%, 70%, 90%, 100%) was used to dehydrate the tissue followed by transitioning to propylene oxide and then a 1:1 mixture of propylene oxide and EMBED 812. The tissue was allowed to sit in EMBED for one hour then placed in a 70^{o} oven to polymerize the plastic. Sections were cut approximately 75-80 nm thickness using a Leica ultramicrotome and a Diatome diamond knife, collected on 200-mesh copper grids and post stained in 5% uranyl acetate in ethanol (for 10 min) and Reynold's lead citrate (for five min). A JEOL 1200 EX transmission electron microscope, operating at 40 to 120 V and equipped with a digital camera, was used to photograph the sections.

Immunoblot Analysis

Protein lysates were fractionated by SDS polyacrylamide gel electrophoresis, transferred to Hybond-C Nitrocellulose membrane (HYBOND-ECL Nitrocellulose), and subjected to immunoblot analysis. The ratio of antigen:antibody, detergent concentration, and duration and temperature of the reactions were optimized for each antibody.

Echocardiography

Transthoracic echocardiograms were recorded in conscious sedated mice as described previously[28]. Left ventricular internal diameters and wall thicknesses were measured (at least three cardiac cycles) at end-systole and end-diastole from two-dimensionally targeted M-mode cross-sectional views at the level of the chordae tendineae. Heart rate was determined from mitral inflow Doppler envelopes.

Statistical Methods

Averaged data are reported as mean ± SEM. Statistical significance was analyzed using a Student's unpaired, 2-tailed *t* test, or one-way ANOVA followed by Bonferroni's method for *post hoc* pair-wise multiple comparisons. Comparison of rates of aggregate accumulation was tested with logistic regression. The logit of the fraction of affected cells was modeled with main factors of cell line and time and a cell line × time interaction. The cell line difference between data points was estimated as a linear contrast within this model. Comparison of rates of decline in fractional shortening was tested with the log-rank statistic in a time-to-event context with events defined at the level of individual mice as attainment of 40% fractional shortening. SAS/STAT® software, version 9.13, was used for all analyses

Results

Mutant CryAB^{R120G} increases the abundance of autophagosomes in cardiomyocytes

We hypothesized that the presence of aggregate-prone protein would induce autophagic activity in cardiac myocytes. To test this, NRVM were infected with virus expressing wild-type human CryAB (Ad-CryAB^{WT}), virus expressing mutant human CryAB^{R120G} (Ad-CryAB^{R120G}), or empty virus (control). Infected cells were cultured for 5 days and then processed for analysis by transmission electron microscopy (EM) to evaluate for double membrane-bound vacuoles consistent with autophagosomes.

As expected, and consistent with the known "housekeeping" function of constitutive autophagy in many cells, modest numbers of autophagosomes were detected in both control and Ad-CryAB^{WT}-expressing cells (**Figure 1A**). These autophagosomes were relatively uniform in morphology, appearing as clearly discernable double-membrane vacuoles, >0.5μm in diameter, and containing heterogeneous proteinaceous material. In striking contrast, 5 days of Ad-CryAB^{R120G} expression triggered the appearance of large, perinuclear structures (**Figure 1A, asterisks**). The appearance of these structures was suggestive of aggresomes, intermediate filament-encaged collections of damaged proteins localized in a microtubule-dependent manner at the microtubule organizing center (MTOC)[29], consistent with previous reports[9]. Interestingly, we detected increased numbers of autophagosomes in the perinuclear region of aggresome-positive cells, with morphology distinct from the autophagosomes seen in healthy control cells. CryAB^{R120G}-induced autophagosomes were heterogeneous in morphology, multi-lamellar (in contrast to just one double membrane), contained high density

proteinacious material, and showed evidence of mitochondrial sequestration (**Figure 1A, arrows)**. Despite the presence of large perinuclear aggresomes and accumulated autophagosomes, cell viability was not altered through the 5 day experimental period **(Figure 1B)**.

To quantify the increase in autophagic activity in response to Ad-CryAB^{R120G} expression, NRVM were transfected with a GFP-LC3 autophagy-reporter construct, followed by infection with control adenovirus, Ad-CryAB^{WT}, or Ad-CryAB^{R120G}. LC3 is an intermembrane component of the early autophagosome, and its redistribution from a diffuse cytosolic signal to punctate dots is a sensitive and specific indicator of autophagy[30]. After 24 hours of infection, the abundance of autophagic vesicles was measured in live cells, quantified as the number of GFP-LC3 punctate-positive cells divided by the total number of GFP-positive cells. In these experiments, autophagic activity was increased >2-fold ($p<0.05$) in NRVM expressing Ad-CryAB^{R120G}, whereas infection with Ad-CryAB^{WT} had no effect relative to control (**Figure 1C**).

Aggresomes invariably recruit cytoplasmic components including chaperones and elements of the ubiquitin and proteasome pathways. In certain polyglutamine-expansion disorders, intracellular protein aggregates sequester mTOR (mammalian target of rapamycin), a well-established inhibitor of autophagy, reducing levels of the soluble protein with a consequent increase in autophagy[31]. Given our findings of autophagic activation in association with protein aggregate accumulation, we evaluated mTOR localization in NRVMs expressing CryAB^{R120G}. Consistent with findings reported in Huntington's disease, we detected a perinuclear coalescence of mTOR in cells expressing mutant crystallin (**Figure 1D**). In contrast, mTOR remained freely distributed through the

cytoplasm of cell expressing wild-type CryAB (**Figure 1D**). These data, then, lend credence to the notion that protein aggregates induced by mutant CryAB trigger an autophagic response in cardiac myocytes.

Inhibiting autophagy increases the abundance and size of CryAB^{R120G}-induced aggregates

To test the hypothesis that autophagosomes help clear CryAB^{R120G}–induced protein aggregates, NRVM were infected with either Ad-CryAB^{WT} or mutant Ad-CryAB^{R120G}. After infection, the cells were treated daily with 5mM 3-methyladenine (3MA), an inhibitor of Class III phosphoinositide-3-kinase (PI3K), an enzyme required for initiation of autophagosome formation[32]. After five days in culture, immunocytochemical analysis revealed that wild-type CryAB protein was distributed diffusely throughout the cytoplasm of vehicle-treated NRVM infected with Ad-CryAB^{WT} (**Figure 2A**). Inhibition of autophagy with 3MA increased the intensity of the CryAB-like immunoreactivity signal, but the immunoreactivity remained diffusely distributed throughout the cytoplasm with no evidence of protein aggregation or formation of aggresomes (**Figure 2A**).

Consistent with our EM studies (**Figure 1A**), CryAB-associated perinuclear aggregates were detected in the Ad-CryAB^{R120G}-infected NRVM (**Figure 2A**). Gamma-tubulin, a structural component of the MTOC was found to co-localize with the structures, supporting the idea that they are aggresomes (**Figure 1, Online Supplement**). In addition, disruption of microtubules with nocodazole (10μM) was sufficient to prevent perinuclear coalescence (**Figure 1, Online Supplement**). Together, these findings confirm that these intracellular structures are aggresomes.

As autophagic mechanisms are activated in neurodegenerative disease to clear protein aggregates, we tested the role of autophagy in aggresome formation in cardiac myocytes by blunting autophagy with 3MA. Here, we observed a dramatic increase in aggresome size (**Figure 2A**). To evaluate for changes in the distribution of CryAB proteins, NP-40 soluble and insoluble proteins were fractionated from CryAB-over-expressing cells. In CryAB^{WT}-expressing cells, CryAB was detected only in the detergent-soluble fraction, regardless of the addition of 3MA to block autophagy **(Figure 2B)**. In contrast, CryAB was found in both the soluble and insoluble fractions of Ad-CryAB^{R120G}-infected cells, and blunting of autophagy with 3MA elicited a significant increase in the levels of insoluble CryAB **(Figure 2B)**. No increases in soluble CryAB were detected in Ad-CryAB^{R120G}-infected cells treated with 3MA, suggesting that mutant CryAB^{R120G} proteins which are not eliminated via autophagic pathways go on to form insoluble aggregates. These results, then, suggest that autophagy plays a role in the clearance of CryAB^{R120G} protein, which may serve to protect myocytes from the accumulation of misfolded aggregates and subsequent incorporation into aggresomes.

Inhibition of autophagy increased the size of protein aggregates in CryAB^{R120G}-expressing cells (**Figure 2A**). In addition, the prevalence of CryAB-positive aggregates was increased in response to 3MA. To examine the kinetics of aggresome formation, we quantified the number of NRVM with detectable aggresomes at 3 and 5 days post-infection. Three days after Ad-CryAB^{R120G} infection, 23.1% (±7.1) of all NRVM (n=479) were positive for aggresomes. Treatment with 3MA significantly increased the number of aggresome-positive cells to 33.5% (±9.3, n=455, $p<0.05$). Five days after infection, 48.8% (±14.4) of vehicle-treated cells (n=274)

were aggresome-positive, whereas in cells exposed to 3MA (n=317), the prevalence increased to 66.3% (±10.1, $p<0.05$) (**Figure 2C)**. Logistic regression analysis revealed that the rate of aggresome accumulation in 3MA-treated cells was statistically significantly accelerated ($p=0.03$). Collectively, these findings demonstrate that expression of CryAB^{R120G} protein is sufficient to trigger robust autophagic activity in cardiac myocytes. Further, these data suggest that autophagic activity functions as a clearance pathway for these potentially toxic protein aggregates.

Hearts expressing mutant CryAB^{R120G} display increased autophagic activity

To evaluate the relationship between autophagy and DRCM, we studied transgenic mice expressing cardiomyocyte-restricted hCryAB^{R120G} (*αMHC-CryAB^{R120G}*), a model of DRCM where disease progression is remarkably similar to that of humans carrying a mutant allele[8]. The *αMHC-CryAB^{R120G}* transgene triggers a late-onset cardiomyopathy with severe pathological remodeling, protein aggregation, and death due to heart failure at 10-12 months of age[8]. Initial analysis was performed on 2 month-old animals, a time point which precedes ventricular remodeling or detectable reductions in cardiac function.

Consistent with our *in vitro* studies, *αMHC-CryAB^{R120G}*–expressing cardiomyocytes contained perinuclear aggregates detectable by anti-CryAB immunohistochemistry (**Figure 3A**), even at this early, preclinical stage of disease (see below). Ultrastructural analysis also revealed numerous protein aggregates (**Figure 3B, asterisk**). By contrast, normal cellular architecture, with tightly packed arrangements of sarcomeres and

mitochondria, was seen in wild-type littermates (**Figure 2B**). Again, we detected an abundance of autophagosomes immediately adjacent to the aggregates in α*MHC-CryAB*^{R120G}–expressing cardiomyocytes, suggesting a functional relationship between aggregated proteins and autophagy (**Figure 3B, arrows**).

Because metabolic stress and starvation are capable of activating autophagy, we tested whether increases in autophagic activity were the result of CryAB^{R120G} expressed within myocytes or a secondary consequence of systemic illness. To do this, we studied skeletal muscle from each animal in tandem with the heart samples. Ultrastructural analysis of skeletal muscle (soleus) from α*MHC-CryAB*^{R120G} mice revealed an absence of aggregates, no sarcomeric disarray, and no increase in autophagic activity (**Figure 3B**). Indeed, skeletal muscle from these transgenic mice was identical on EM to wild-type littermates, suggesting that systemic factors do not account for the observed activation of autophagic pathways. Together, these findings demonstrate that both accumulation of protein aggregates and induction of autophagic activity occur in cardiac myocytes from α*MHC-CryAB*^{R120G} mice prior to any clinical manifestations of disease.

Increased autophagic activity in human myofibrillar myopathy

To test for evidence of increased autophagic activity in humans, we studied skeletal muscle biopsies from 5 patients diagnosed with myofibrillar myopathy. In 4 of the 5 patients, we detected histochemical evidence of intracellular protein aggregation and increased lysosomal activity, suggestive of increased flux through autophagosomal/lysosomal

pathways. Specifically, we observed irregular, eosinophilic proteinaceous inclusions within the sarcoplasm of affected fibers on both hematoxylin/eosin (**Figure 4A, arrow**) and Gomori trichrome stain (**Figure 4B, arrow**). Anti-desmin immunohistochemistry revealed that the aggregates are desmin-positive inclusions, even though none of the patients had known mutations in the gene coding for CryAB (**Figure 4D, arrow**). Consistent with our animal studies where autophagosomes accumulate in the peri-aggregate region, enzyme histochemical stain for acid phosphatase activity (a marker of lysosomal activity) demonstrated a dramatic increase in some fibers (**Figure 4C, arrow**). A more modest increase was also observed in the immediate peri-aggregate region (**Figure 4C, arrowheads**). Together, these data strongly suggest that autophagosomal clearance pathways are activated in patients with myofibrillar myopathies.

Blunting autophagy accelerates pathological remodeling in αMHC-CryAB^{R120G} hearts

To evaluate the effects of autophagic activity on the structural remodeling of DRCM, we studied hearts from *αMHC-CryAB^{R120G}* mice crossed into a *beclin 1* haploinsufficient background. *Beclin 1*, the mammalian homologue of the pro-autophagic gene *Atg6* in yeast, is required for recruitment of Atg12-Atg5 conjugates to pre-autophagosomal membranes[33], and hence is required for autophagic vesicle formation. Although *beclin 1$^{-/-}$* mice are not viable, it has been demonstrated that heterozygous *beclin 1$^{+/-}$* animals have an approximate 50% reduction in autophagic capacity[27]. Hearts were studied at 9 months of age, a time point where severe structural abnormalities exist in the *αMHC-CryAB^{R120G}*

model (**Table**), and yet mortality was not yet excessive. At this age, %FS averages ~40%, and values below this seen at later time points are indicative of terminal heart failure with high risk of sudden cardiac death[34]. Echocardiographic determination of posterior wall thickness and necropsy evaluation of heart mass both revealed similar degrees of hypertrophic growth in *αMHC-CryAB^{R120G}* and *αMHC-CryAB^{R120G};beclin 1$^{+/-}$* mice (**Table**).

Hearts were stained with trichrome stain (**Figure 5A**) or picrosirius red (data not shown) for interstitial fibrosis, a hallmark of pathological myocardial remodeling and an indirect marker of cell death. In wild-type and *beclin 1$^{+/-}$* mice, we detected no signs of interstitial fibrosis at 9 months. In contrast, hearts from 9 month-old *αMHC-CryAB^{R120G}* animals manifested extensive intracellular aggregates, but only minor increases in fibrosis were detected. Similar to *αMHC-CryAB^{R120G}* animals and consistent with our *in vitro* findings, numerous intracellular protein aggregates were detected in hearts from *αMHC-CryAB^{R120G};beclin 1$^{+/-}$* mice. However, in contrast with the other 3 genotypes, hearts from *αMHC-CryAB^{R120G};beclin 1$^{+/-}$* mice manifested signs of severe pathological remodeling characterized by extensive – 3-fold increased – deposition of interstitial fibrosis (**Figure 5A**).

Additional qualitative differences were detected on ultrastructural analysis in *CryAB^{R120G}* hearts where autophagy was blunted by *beclin 1* haploinsufficiency. Although both *αMHC-CryAB^{R120G}* and *αMHC-CryAB^{R120G};beclin 1$^{+/-}$* hearts contained protein aggregates, the aggregates were both more prevalent and larger in *αMHC-CryAB^{R120G};beclin 1$^{+/-}$* mice (**Figure 5B**). Additional evidence for protein aggregate accumulation was obtained on immunoblot by evaluating for

high molecular weight poly-ubiquitinated proteins in ventricular lysates (**Figure 5C**). The abundance of these high molecular weight proteins was similar in wild-type and *beclin 1*$^{+/-}$ animals (**Figure 5C**). Consistent with the induction of aggregates containing degraded protein, *αMHC-CryAB*^{R120G} hearts contained increased levels of high-molecular weight poly-ubiquitinated proteins. And consistent with a role for autophagic pathways in the elimination of these proteins, their abundance was even greater in *αMHC-CryAB*^{R120G}*;beclin 1*$^{+/-}$ animals (**Figure 5C**).

A reduction in autophagy does not increase apoptosis

Overlap between apoptotic and autophagic death has been studied extensively during neuronal cell death[35,36]. Indeed, recent studies have uncovered significant interactions between autophagic and apoptotic signaling pathways[37,38]. For example, similar types of stress can induce either apoptosis or autophagy depending on cellular context, and when cells are induced to undergo apoptosis while the activation of caspases is prevented, the cells die via caspase-independent mechanisms[39]. In a different transgenic model of cardiac-restricted expression of mouse CryAB^{R120G}, a model where animals die very early (around 5 months), increases in apoptosis have been reported[10]. To test for apoptosis as a contributor to heart failure in our model of moderately over-expressed (≈6-fold) hCryAB^{R120G}, paraffin-fixed sections of hearts from five and nine month-old animals were processed for TUNEL staining. Cardiac size and performance in *αMHC-CryAB*^{R120G} mice are both normal at five months, whereas signs of heart failure are emerging at 9 months (see below). Hearts from wild type and *beclin 1*$^{+/-}$ mice manifested very low levels of TUNEL-positive cells at both time points **(Figure 6A)**. At five months, the

prevalence of TUNEL-positive cells was only modestly increased in both α*MHC-CryAB*^{R120G} and α*MHC-CryAB*^{R120G}*;beclin 1*$^{+/-}$ hearts (**Figure 6B**), and the increases were similar (p=NS) in both genotypes. Similar findings were seen at nine months of age, a time point where significant pathological remodeling is evident. Indeed, we detected a decrease in apoptotic signal in α*MHC-CryAB*^{R120G}*;beclin 1*$^{+/-}$ heart at nine months as compared with α*MHC-CryAB*^{R120G} (**Figure 6B**). Additional evidence for a lack of up-regulated apoptotic activity was obtained on Western blot, where no changes caspase-3 cleavage (**Figure 6C**) or Bcl-2 phosphorylation (data not shown) were detected in all 4 genotypes.

Attenuation of autophagy accelerates heart failure progression in α*MHC-CryAB*^{R120G} mice

Findings on necropsy at 9 months were consistent with the notion that down-regulated autophagy promotes accumulation of poly-ubiquitinated proteins, aggresome formation, and pathological remodeling (**Figure 5**). To determine whether these increases in autophagic activity are adaptive or maladaptive, α*MHC-CryAB*^{R120G} mice were crossed with *beclin 1* haploinsufficient mice. We took advantage of the diminished autophagic response in these mice to titrate the cellular response to *CryAB*^{R120G}, and we evaluated cardiac structure and function by serial echocardiography for up to one year, followed by necropsy analysis. Thus, we analyzed the cardiac phenotype in four genetic backgrounds; wild type, *beclin 1*$^{+/-}$, α*MHC-CryAB*^{R120G}, and α*MHC-CryAB*^{R120G} in a *beclin 1*$^{+/-}$ background. Monthly echocardiograms were performed on age-matched male siblings.

Wild-type and *beclin 1*$^{+/-}$ mice manifested no signs of compromise of cardiac performance over the 12-month period of study (**Figure 7A**). Percent fractional shortening (%FS), a measure of ventricular systolic function, was constant throughout at 70-80%, consistent with a previous report[13]. As expected, α*MHC-CryAB*^{R120G} mice developed a progressive decline in %FS that reached statistical significance compared to wild type at 9 months of age **(Figure 7A)**. By 12 months of age, these mice manifested overt heart failure (%FS<40%).

To test the role of autophagic activity in α*MHC-CryAB*^{R120G} mice, we studied α*MHC-CryAB*^{R120G}*;beclin 1*$^{+/-}$ mice. Intriguingly, declines in cardiac performance were greatly accelerated in these mice (**Figure 7A**). Indeed, these animals developed significant declines in cardiac function by 6 months with severe heart failure by 9 months of age, a disease progression which was statistically significantly accelerated relative to α*MHC-CryAB*^{R120G} (p=0.007 by log-rank statistic) (**Figure 7A, 7B**). Mortality was significantly increased (p<0.05) in α*MHC-CryAB*^{R120G}*;beclin 1*$^{+/-}$ mice, as well: CryAB^{R120G}*;b*eclin 1$^{+/-}$ mice manifested 25% mortality (2 of 8 animals) by 9 months of age, whereas no mortality was seen in WT (0 of 7), *beclin 1*$^{+/-}$ (0 of 9), or α*-MHC-CryAB*^{R120G} (0 of 8) animals at that time point. Echocardiographic determination of posterior wall thickness and necropsy evaluation of heart mass both revealed similar degrees of hypertrophic growth in α*MHC-CryAB*^{R120G} *and* α*MHC-CryAB*^{R120G}*;beclin 1*$^{+/-}$ *mice* (**Figure 7C**). Interestingly, in both α*-MHC-CryAB*^{R120G} and *CryAB*^{R120G}*;beclin 1*$^{+/-}$ mice, heart failure was systolic in nature, with significant increases developing in end-systolic diameter but with no significant changes in end-diastolic dimensions (**Figure 7D**).

Discussion

Misfolding and aggregation of mutant or damaged proteins underlies the pathogenesis of multiple neurodegenerative diseases, skeletal myopathies, and heart failure. In these disorders, cellular mechanisms responsible for recognizing and disposing of aggregating proteins are overwhelmed, leading to accumulation of soluble and insoluble isoforms of toxic protein. In the case of DRCM, desmin/CryAB^{R120G} aggregates accumulate and impair proteasome function[11,12]. As a result, clearance of defective proteins via this pathway is hindered. The present study is the first to implicate autophagic activity in DRCM and the first to demonstrate that autophagic activity has a protective role by serving to clear toxic aggregates. The major findings reported here are: 1) autophagic activity increases in cardiac myocytes when mutant CryAB^{R120G} protein is expressed; 2) increases in autophagic activity are detected in patients with myofibrillar myopathy; 3) increases in autophagic activity serve to clear the protein aggregates both *in vitro* and *in vivo*; and 4) in an animal model of DRCM that recapitulates human disease, attenuation of autophagy accelerates pathological remodeling and hastens the onset of heart failure. Together, these findings suggest that targeting autophagy may be a promising strategy for therapeutic intervention in DRCM and related disorders.

Proteinopathy

Protein conformational disorders (proteinopathies) are a diverse and expanding class of disease. Among the proteinopathies are conditions in which the aggregated proteins are predominantly cytosolic

(e.g. Parkinson's disease or adult-onset Huntington's disease), predominantly intranuclear (e.g. spinocerebellar ataxia type 1), localized to the endoplasmic reticulum (e.g. neuroserpin mutations that cause familial encephalopathy with neuroserpin inclusion bodies), or secreted extracellularly (e.g. amyloid-β in Alzheimer's disease). In heart, abnormal protein aggregation and accumulation of ubiquitinated proteins in the cytosol have been detected in human hearts with idiopathic or ischemic cardiomyopathies[40-42]. Recent work from our group has demonstrated accumulation of protein aggregates and aggresomes in a very common form of acquired heart disease, *viz.* load-induced heart failure[24].

In the case of the desmin-related myopathies, severe cardiomyopathy and early death are thought to be due, at least in part, to disruption of the desmin architecture within the cell, leading to contractile dysfunction and cell damage. In other cases of proteinopathy, the mechanism whereby a mutated protein is toxic to the cell is less clear. While some mechanisms are likely disease-specific -- related to loss of function of the mutated or misfolded protein -- there is general agreement that early, still-soluble aggregates are the most damaging species[43]. Indeed, in many instances, the mutations responsible for proteinopathies cause disease by conferring a toxic gain of function to the relevant protein. Molecular chaperones, such as HSP70, monitor protein quality and either facilitate refolding of the misfolded protein or promote degradation via the proteasome. Excess misfolded proteins that escape this quality control mechanism begin to aggregate. The presence of protein aggregates, in turn, overwhelms and inhibits proteasome activity, potentially disrupting other important proteasome functions[12]. Autophagy can relieve proteasome inhibition by removing aggregates that have escaped

proteasome clearance. In parallel, unprocessed protein aggregates are directed toward sequestration in the perinuclear aggresome.

It is likely that the low level of apoptotic activity that we observe contributes in part to the cardiac pathology seen in α*MHC-CryAB*^{R120G} mice, but it is unlikely to be the sole cause of cardiac failure. Further, we did not detect increased levels of myocyte apoptosis in α*MHC-CryAB*^{R120G};*beclin 1*$^{+/-}$ mice as compared to α*MHC-CryAB*^{R120G} animals, despite the marked acceleration of heart failure progression in the former. This contrasts with prior studies in another model of DRCM where disease progression is markedly accelerated relative to our model[44]. This suggests that differences exist between cellular responses stemming from increases in the expression of aggregating protein and the processing of those aggregates via autophagic clearance. Interestingly, blunting autophagy with 3MA increased the abundance of insoluble CryAB^{R120G} aggregates but had little effect on the soluble species (**Figure 2**). Whereas evidence suggests that it is the soluble pre-amyloid aggregates that are the most toxic in neurons[45], the dramatically adaptive effects we observe from CryAB^{R120G}-induced autophagic activity suggests this may not be the case in DRCM.

It is interesting to note that our line of α*MHC-CryAB*^{R120G} mice develop systolic failure with no evidence of chamber dilation, whereas the α*MHC-CryAB*^{R120G} mice developed by Robbins and colleagues, which manifest high levels of apoptosis, develop systolic dysfunction with substantial ventricular dilation[44]. This raises the intriguing possibility that these different transgenic lines may highlight distinct, stage-specific features of DRCM.

Autophagy and myofibrillar myopathy

Autophagy occurs in a wide range of eukaryotic cells, and its basic mechanisms are largely conserved from yeast to mammals[46]. Autophagy can be induced by diverse stimuli, including starvation, hypoxia, intracellular stress, and developmental signals[47]. It accomplishes a host of functions critical to normal homeostasis and development. In the context of nutrient deprivation, autophagic activity is adaptive in that degradation of cytosolic components releases substrates for intermediary metabolism. Autophagy is also a mechanism for eliminating damaged proteins and organelles that might otherwise be toxic or trigger apoptotic death. For example, targeted inhibition of *Atg7*, a gene required for autophagic activity, leads to accumulation of ubiquitin-positive aggregates[48]. During development, autophagy is a means of remodeling tissues or removing unneeded cells[49,50]. In other settings, however, autophagic activity is associated with the pathogenesis of disease[47], and unrestrained autophagic activity can cause cell death[51-53].

Some evidence suggests that autophagy can efficiently target species that are not in aggregates large enough to be seen on light microscopy[54]. The observation of inclusion formation in the neurons of mice with neuron-restricted inactivation of autophagy genes is consistent with the ability of autophagy to clear soluble and oligomeric aggregate precursors[55,56]. In general, it seems that the capacity to aggregate – rather than the protein aggregates themselves – is correlated with toxicity. In both brain and heart, however, little is known regarding whether these intracellular inclusions are toxic themselves, or whether they represent a compensatory mechanism that sequesters harmful, soluble proteins within

the cytoplasm. However, a model has been proposed in which increased autophagic activity in neurodegenerative disorders does not directly clear aggregates themselves but clears aggregate precursors, shifting the equilibrium away from aggregate formation[43].

Although the importance of protein degradation via autophagy has long been appreciated in neurodegenerative disorders and in tumor growth, the potential contribution of autophagy to cardiac remodeling has only recently been recognized. Polyglutamine-expansion mutations, such as those seen in Huntington's disease, Parkinson's disease, and different forms of tau are strongly dependent on macroautophagy pathways for their clearance (reviewed[43]). Each disease is caused by the expression of a dominant-negative, aggregate-prone protein in terminally differentiated post-mitotic cells. In that context, mechanisms capable of removing damaged proteins are particularly important, due to limited capacity for replacement of defective cells. Here, we describe an increase in the abundance of autophagic vesicles in cardiac myocytes in response to expression of a mutant protein causing DRCM. Whereas autophagic activity has been shown to be protective in cell and fly models of aggregate-prone diseases[31], to our knowledge this study is the first to demonstrate such in a mammalian model of myopathy. Taken together, these studies suggest that the presence of protein aggregates stimulates an increase in autophagic activity which facilitates their removal.

Suppression of autophagic activity triggered increases in the size and abundance of aggresomes both *in vitro* and *in vivo*. Although studies in neurons suggest that this species is less toxic than the soluble aggregates, we postulate that the presence of aggresomes in muscle may interfere with contractile properties unique to muscle and could contribute

to ventricular stiffness and systolic dysfunction. Further studies will be required to explore mechanisms of heart failure development in DRCM.

Autophagy in heart disease

Very recent studies have uncovered a role for autophagic activity in ischemia/reperfusion injury and heart failure. Depending on the stressor and disease context, these studies point to either adaptive[14,16] or maladaptive[13,18] roles of this protein clearance pathway. Here, we report that cardiomyocyte autophagy triggered by abnormal aggregation of intracellular proteins is beneficial, which is consistent with observations made in neurodegenerative diseases. Indeed, a growing body of evidence implicates autophagy as a protective response in genetic diseases associated with cytoplasmic aggregation-prone proteins[57], which we extend here to heart disease triggered by defective chaperone function. Indeed, it has been suggested that autophagy may have two distinct beneficial effects in protein conformation disease. First, this pathway functions to clear the primary toxin causing these diseases. Second, enhanced autophagy attenuates apoptotic responses to various insults, rendering the cell resistant to programmed cell death. Importantly, recent studies demonstrating that pharmacological up-regulation of autophagy is protective in a wide variety of disease models associated with intracellular protein aggregation raise the exciting prospect of autophagic activation as a novel therapeutic strategy[54,58,59].

It is clear from multiple studies that autophagy can either trigger a type of programmed cell death or carry out functions essential to cell survival (reviewed[60]). Consistent with this, whereas cardiac autophagy is induced by diverse forms of cardiovascular stress, the net effect of this

activity – adaptive versus maladaptive – appears to be context dependent. Indeed, it is possible that autophagic activity carries out different functions depending on disease stage and severity, the nature of the components being degraded, and the extent of autophagic activity. For example, in two different models, autophagy in the setting of severe hypertension can be adaptive[14] or maladaptive[13]. Autophagy appears to be protective in response to ischemia/reperfusion of the heart[15-17], and a recent study demonstrated that activation of autophagy is beneficial during ischemia but damaging during reperfusion[18].

There is some evidence to suggest that activation of autophagy involving an increase in Beclin 1 expression is indicative of damaging autophagic activity[18]. In reperfusion following ischemic injury[18] and in pressure-overload stress[13], Beclin 1 levels increase in concert with activation of autophagy; in *beclin 1*$^{+/-}$ mice subjected to either stress, autophagy has been shown to be maladaptive. Significantly, we see no elevation in Beclin 1 levels in the α*MHC-CryAB*^{R120G} mice (data not shown) consistent with autophagy being beneficial in this setting.

Although findings presented here suggest that autophagy acts as a protective pathway by removing desmin/CryAB^{R120G} aggregates, it is important to recognize that the increase in autophagic vesicle abundance observed in α*MHC-CryAB*^{R120G} hearts could be caused either by an increase in vesicle formation or by a block in the clearance of vesicles. Our evidence suggests that α*MHC-CryAB*^{R120G} protein stimulates vesicle formation (rather than inhibiting their clearance), because decreasing autophagic capacity, either pharmacologically or genetically, increased the abundance of aggregated proteins. This demonstrates that the protein aggregates caused by CryAB^{R120G} can indeed be removed through

autophagic pathways. That said, it remains possible that CryAB^{R120G}-associated aggregates eventually overwhelm and inhibit autophagy the same way they have been shown to inhibit proteasomal function. In either scenario, our finding that DRCM is more severe and progresses more rapidly in animals with reduced autophagic capacity is important, because it suggests that increasing autophagic capacity may be beneficial in patients with this and related disorders.

Perspective

Desmin-related cardiomyopathy is a severe and progressive disease for which there are limited therapeutic options. In this report, we identify autophagy as a robust cellular response to CryAB^{R120G}-associated proteinopathy, and further demonstrate that this response plays a significant role in attenuating disease progression. These findings are clinically relevant for a number of reasons; 1) they indicate that in DRCM, autophagy is a pathway suitable for consideration as a therapeutic intervention; 2) they suggest that inter-individual variations in autophagic capacity or responsiveness may account for the heterogeneous presentation of disease; and 3) they may serve as a paradigm for myofibrillar myopathy of diverse molecular etiology. Finally, given that drugs that alter the process of autophagy are already in clinical use, advances in this field are all the more urgent.

Table 1: Echocardiographic data recorded at 9 months of age.

Genotype	***FS (%)***	***LVIDd (cm)***	***LVIDs (cm)***	***Post. Wall (cm)***	***HW/TL (mg/mm)***	***HW/LW***
Wild type (n=7)	71 ± 0.9	0.225 ± 0.006	0.067 ± 0.003	0.153 ± 0.016	7.4 ± 0.9	9.5 ± 0.6
beclin $1^{+/-}$ (n=9)	69 ± 2	0.259 ± 0.012	0.085 ± 0.008	0.142 ± 0.001	7.6 ± 0.1	10.5 ± 0.2
R120G (n=8)	59 ± 3*	0.228 ± 0.009	0.093 ± 0.006	0.193 ± 0.015*	10.7 ± 1.2*	10.5 ± 1.0
beclin $1^{+/-}$ x R120G (n=8)	39 ± 6^	0.274 ± 0.04*	0.169 ± 0.041	0.183 ± 0.006*	10.9 ± 0.7*	9.8 ± 0.8

* denotes $p<0.05$ relative to wild type; ^ denotes $p<0.05$ relative to R120G; HW/TL, Heart Weight/Tibia Length; HW/LW, Heart Weight/Lung Weight.

Figure 8

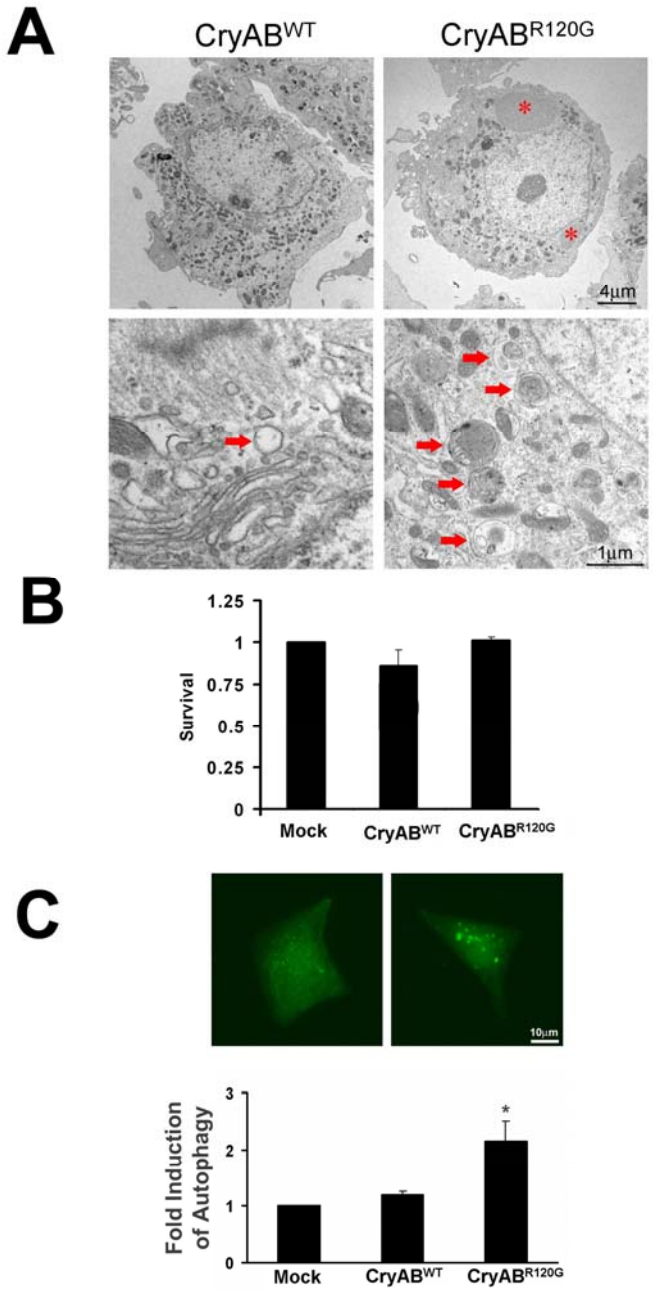

A
CryABWT
CryABR120G
4µm
1µm
B
Survival
1.25
1
0.75
0.5
0.25
0
Mock
CryABWT
CryABR120G
C
10µm
Fold Induction of Autophagy
3
2
1
0
*
Mock
CryABWT
CryABR120G

Figure 8 (continued)

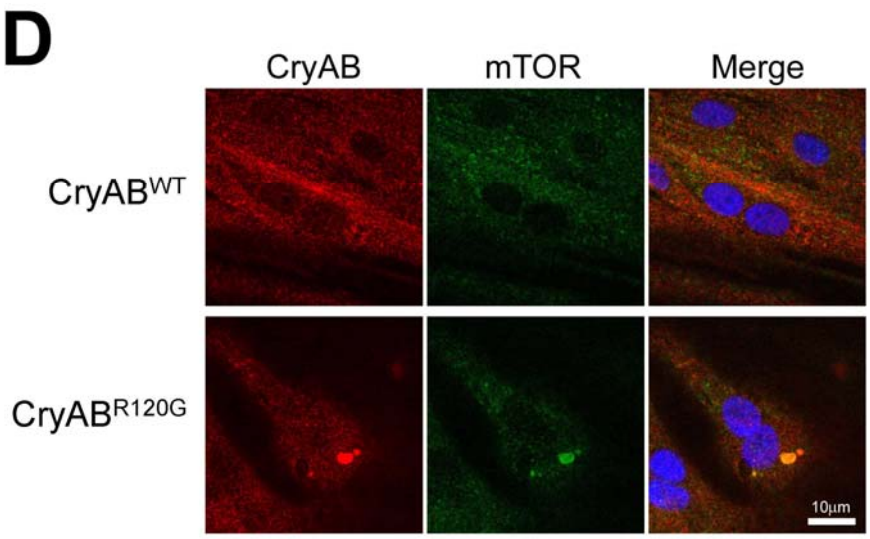


Figure 8: CryAB^{R120G} expression is a potent activator of cardiomyocyte autophagy. A. Representative low (x5,000) and high (x20,000) magnification transmission EM images of NRVMs 5 days after adenoviral-mediated expression of CryAB^{WT} or CryAB^{R120G}. Aggresomes are evident in CryAB^{R120G}-expressing cells (asterisks) as are extensive perinuclear autophagosomes (arrows). Respective genotypes are as follows: **i**, *wild-type*; **ii**, *beclin 1*$^{+/-}$; **iii**, *αMHC-CryAB*^{R120G}; **iv**, *αMHC-CryAB*^{R120G}*;beclin 1*$^{+/-}$*.* **B.** Despite extensive aggresome formation and induction of autophagy, there is no appreciable change in cell viability, detected by MTT assay, 5 days post-infection. Genotypes are as in Panel A. **C.** NRVMs were transiently transfected with a GFP-LC3 construct and then infected with wild-type or mutant CryAB. 24 hours later, cellular autophagy was quantified by determining the number of punctate-positive cells divided by total number of transfected (GFP+) cells. **D.** Representative images of NRVMs (2 examples of each) infected with CryAB and processed for mTOR immunocytochemistry. mTOR is freely distributed throughout the cytoplasm in NRVMs 3 days after adenoviral-mediated expression of wild-type CryAB. In contrast, expression of mutant CryAB resulted in the formation of perinuclear aggregates which stain for both mTOR and crystallin.

Figure 9

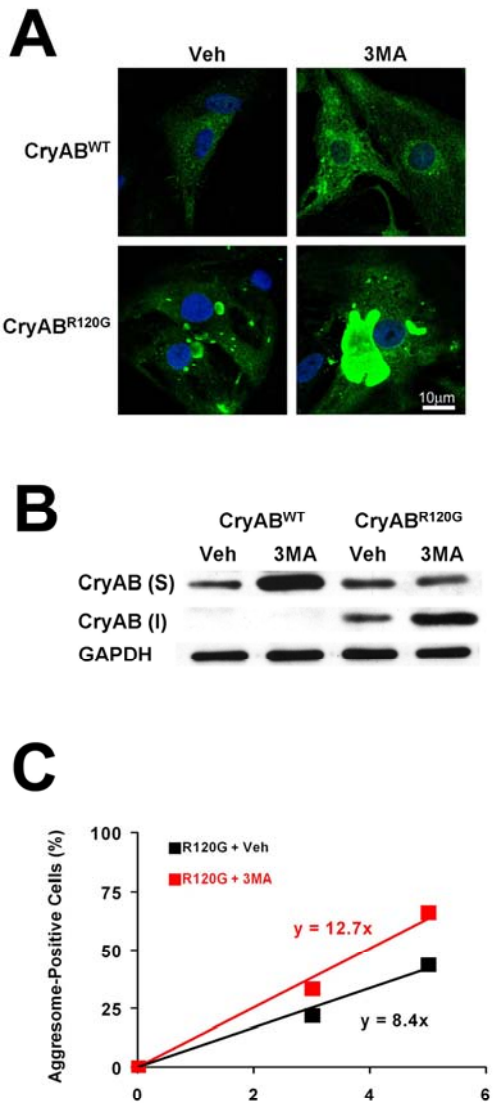

A
Veh
3MA
CryAB^WT
CryAB^R120G
10μm
B
CryAB^WT
CryAB^R120G
Veh
3MA
Veh
3MA
CryAB (S)
CryAB (I)
GAPDH
C
Aggresome-Positive Cells (%)
100
75
50
25
0
0
2
4
6
R120G + Veh
R120G + 3MA
y = 12.7x
y = 8.4x

Figure 9: Autophagic activity serves to clear CryAB^{R120G}-induced protein aggregates. **A.** Representative images depicting NRVMs subjected to 5 days of CryAB^{R120G} expression, demonstrating robust perinuclear aggresome formation. Chronic blunting of autophagy with 3MA (5mM) dramatically increased aggresome size. **B.** Consistent with the immunocytochemical data, blunting autophagy increased the amount of insoluble CryAB in cells expressing mutant, but not wild-type protein. **C.** CryAB^{R120G}-induced aggregate accumulation is accelerated in the setting of blunted autophagy, suggesting that autophagic pathways function to clear the aggregates.

Figure 10

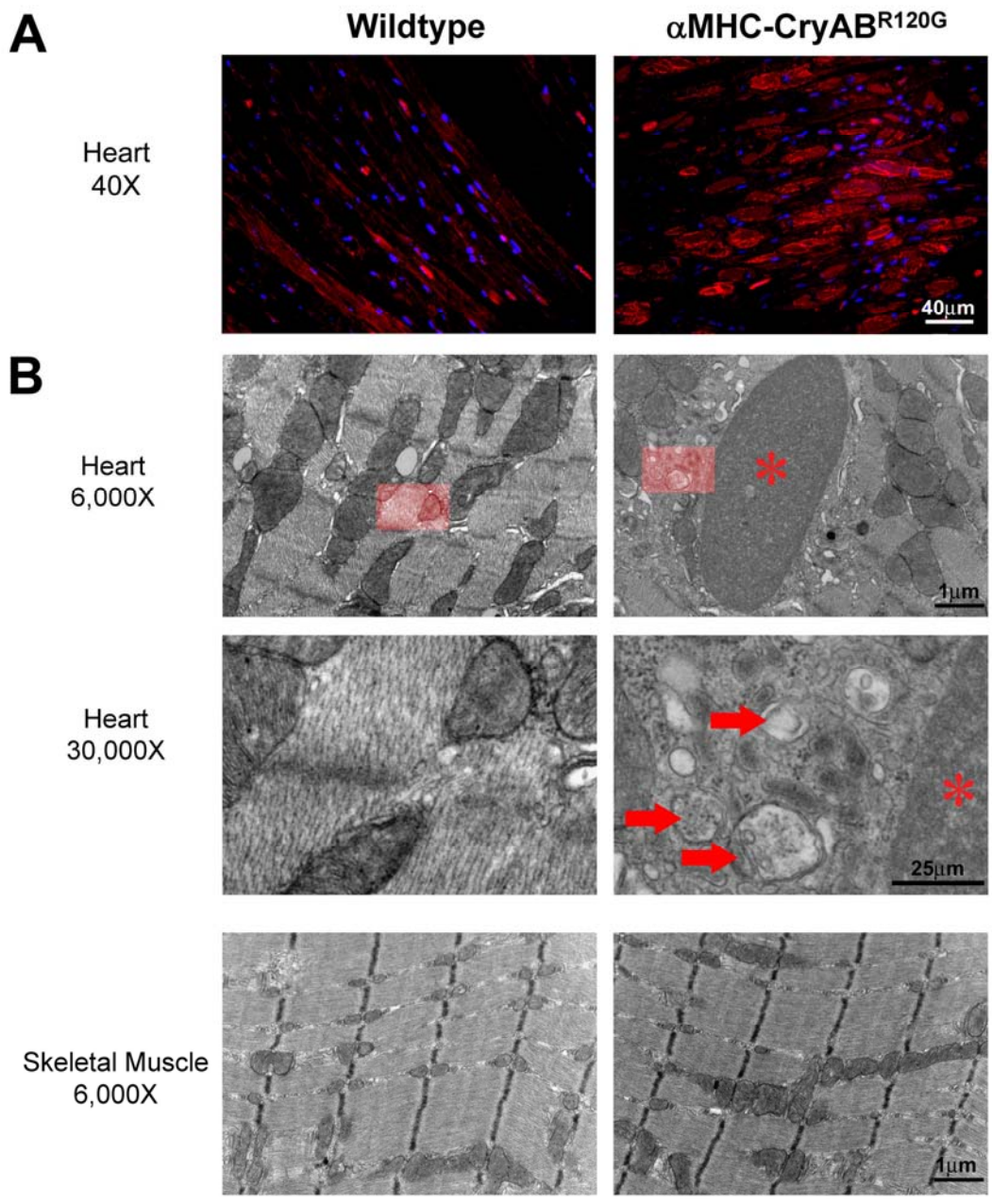

A
Wildtype
αMHC-CryAB^{R120G}
Heart
40X
40μm
B
Heart
6,000X
1μm
Heart
30,000X
25μm
Skeletal Muscle
6,000X
1μm

Figure 10: Mutant CryAB expression induces cardiomyocyte autophagy *in vivo.* **A.** Representative images depicting tissue from a 5 month old α*MHC-CryAB*^{R120G} mouse and a nontransgenic littermate, both of which were processed for anti-CryAB immunohistochemistry. Extensive aggregate formation was detected in animals expressing mutant, but not wild-type CryAB. **B.** Ultrastructural analysis demonstrated morphological changes in the intersarcomeric region in α*MHC-CryAB*^{R120G} animals, including abundant accumulation of autophagosomes (double membrane structures indicated by asterisks) as well as myelin bodies (aged autophagosomes indicated by arrows). No such changes were observed in skeletal muscle from either genotype.

Figure 11

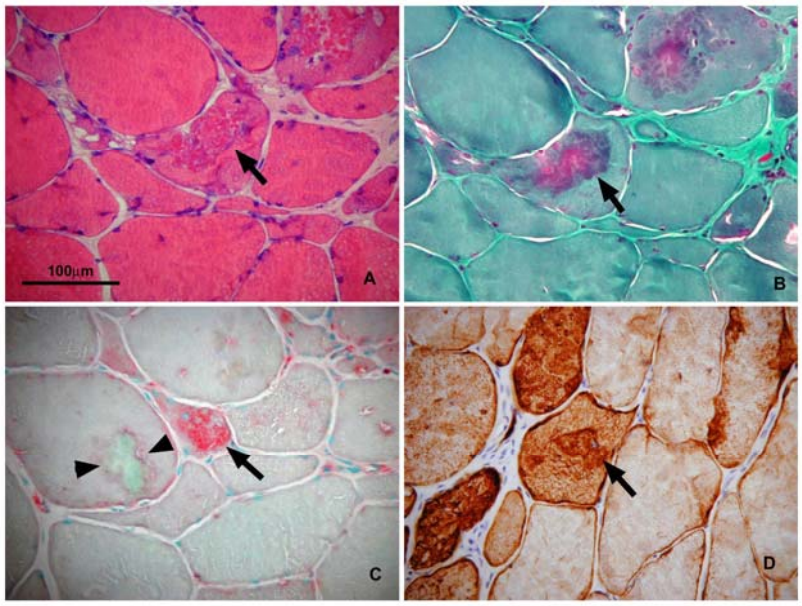
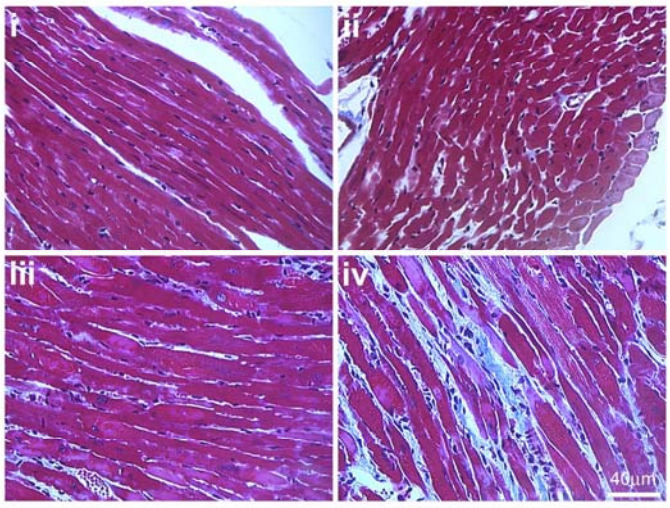


Figure 11: Autophagic activation in skeletal muscle from a patient with myofibrillar myopathy. **A.** Irregular, eosinophilic proteinaceous inclusions within the sarcoplasm of affected fibers (arrow) (H&E, x200). **B.** Gomori trichrome stain demonstrating characteristic blue-green appearance of the protein-rich sarcoplasmic accumulations (arrow) (modified Gomori trichrome, x200). **C.** Enzyme histochemical stain for acid phosphatase, a marker of lysosomal activity. Acid phosphatase activity, localized in this preparation by a red reaction product, is dramatically increased in some fibers (arrow). In other fibers, proteinaceous aggregates (arrowheads) are associated with a more modest increase in enzyme activity, visible in this fiber at the periphery of a hyaline deposit (acid phosphatase, x200). **D.** Immunohistochemical stain demonstrating desmin reactivity associated with some of the sarcoplasmic protein aggregates (x200). Scale bar in lower left corner of panel A = 100μm.

Figure 12

A


i
ii
iii
iv
40μm

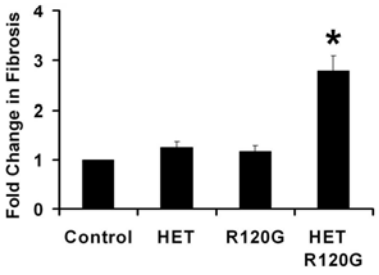

Fold Change in Fibrosis
0
1
2
3
4
*
Control
HET
R120G
HET R120G

Figure 12 (continued)

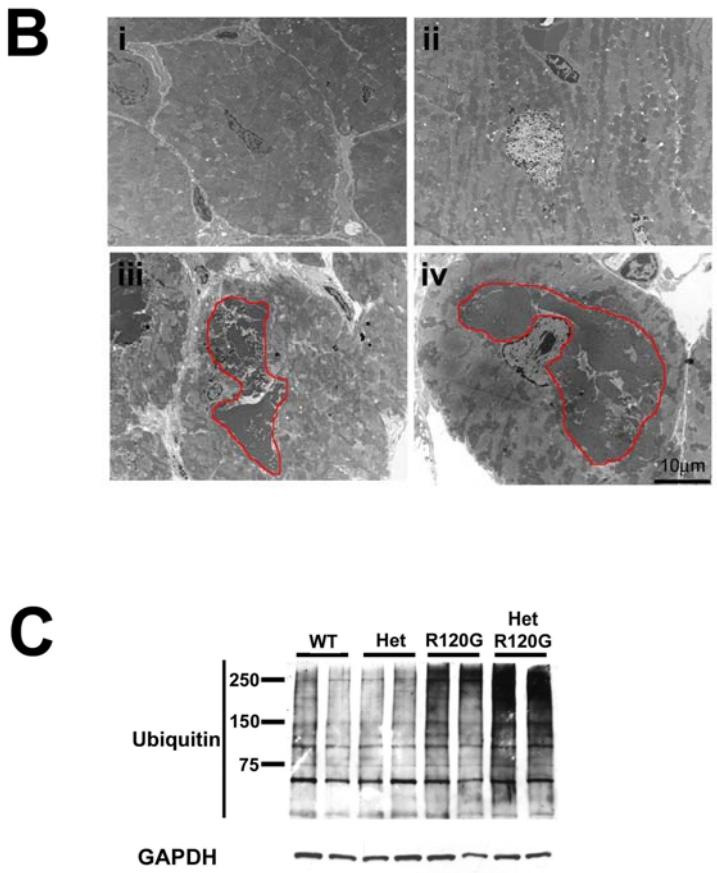


Figure 12: Autophagy attenuates CryAB^{R120G}-induced pathological remodeling of the myocardium. **A.** Representative paraffin-fixed sections from 9 month-old animals were stained with picrosirius red and imaged at 40X magnification. Aggregate accumulation was detected in α*MHC-CryAB*^{R120G} animals, but consistent with the functional data, only minimal increases in total fibrosis were detected. Hearts from α*MHC-CryAB*^{R120G}*;beclin 1*$^{+/-}$ mice, in contrast, contained extensive interstitial fibrosis, a sensitive marker of pathological cardiac remodeling. **B.** Transmission EM analysis revealed similar patterns, where the blunting of autophagy triggered more extensive aggregate accumulation, with more severe pathological remodeling. **C.** Representative immunoblots prepared from cardiac protein lysates demonstrating extensive accumulation of high molecular mass ubiquitin-positive proteins in α*MHC-CryAB*^{R120G}*;beclin 1*$^{+/-}$ mice relative to α*MHC-CryAB*^{R120G}, wild type, or *beclin 1*$^{+/-}$.

Figure 13

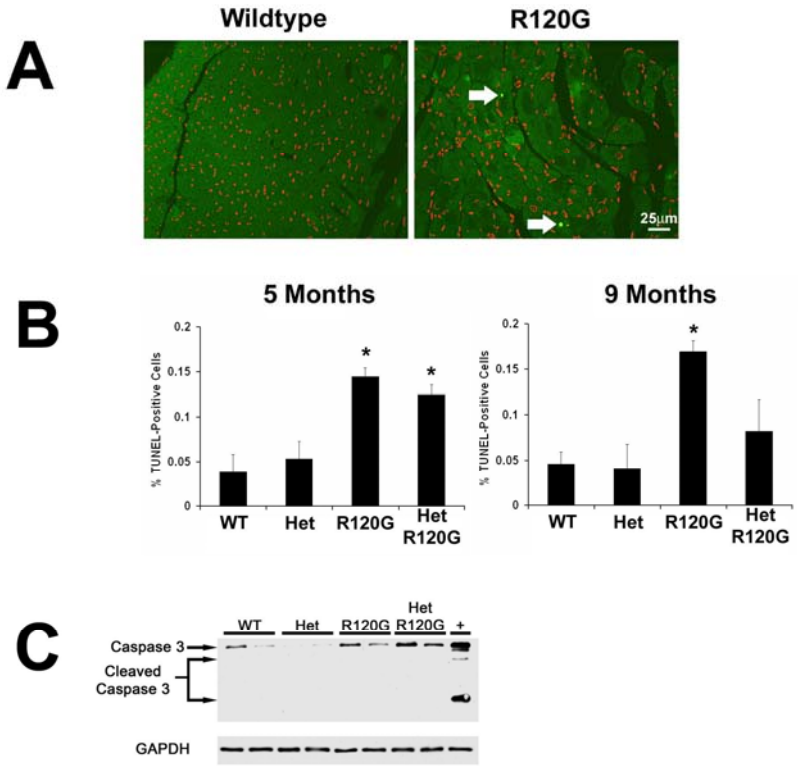


Figure 13: Modest increases in apoptosis in DRCM. A. Representative images of TUNEL staining performed in wild-type and *CryAB*^{R120G}*;beclin 1*$^{+/-}$ hearts. These studies revealed only modest increases in cardiomyocyte apoptosis (arrows depict TUNEL-positive cells). **B.** Apoptosis in 5 and 9 month-old animals was quantified as the proportion of TUNEL-positive cells with >7,000 cells counted per animal (n=3 animals for each genotype). **C.** Consistent with low level induction of apoptosis at 5 months of age, evidence of caspase 3 cleavage was not detectable by immunoblot analysis (+, positive control = staurosporine treated NRVM, 1μM for 2h).

Figure 14

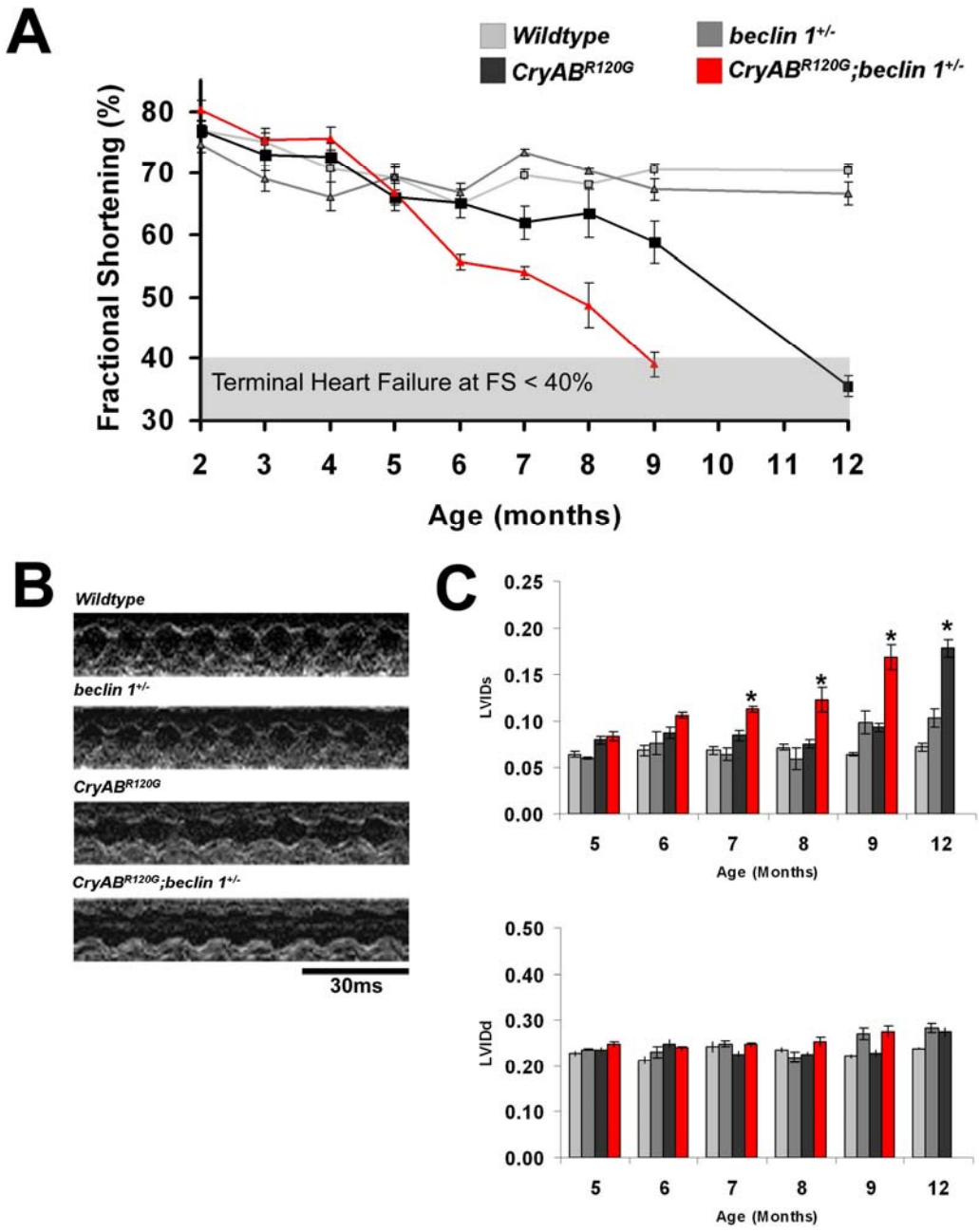

A
Wildtype
beclin 1+/-
CryAB R120G
CryAB R120G;beclin 1+/-
Fractional Shortening (%)
80
70
60
50
40
30
Terminal Heart Failure at FS < 40%
2
3
4
5
6
7
8
9
10
11
12
Age (months)
B
Wildtype
beclin 1+/-
CryAB R120G
CryAB R120G;beclin 1+/-
30ms
C
LVIDs
0.25
0.20
0.15
0.10
0.05
0.00
5
6
7
8
9
12
Age (Months)
LVIDd
0.50
0.40
0.30
0.20
0.10
0.00
5
6
7
8
9
12
Age (Months)

Figure 14: Accelerated heart failure and early mortality in *αMHC-CryAB^{R120G};beclin 1$^{+/-}$* mice. **A.** Cardiac function was monitored by serial echocardiography in non-sedated animals over a 12 month period. *αMHC-CryAB^{R120G}* animals developed late-onset heart failure with the first signs of functional decline appearing at 9 months and development of heart failure by 12 months of age. In contrast, *αMHC-CryAB^{R120G};beclin 1$^{+/-}$ mice* manifested an accelerated disease course, with early signs of functional decline apparent at 6 months and terminal heart failure at 9 months. **B.** Representative M-mode echocardiograms recorded in 9 month-old animals. **C.** Declines in systolic performance are due primarily to progressive increases in left ventricular internal dimension at end systole (LVIDs), with little changes seen in end-diastolic diameter (LVIDd).

Chapter 3 References

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CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

Autophagy in the pressure-stressed heart

Clinical and experimental data clearly demonstrate that autophagy is associated with diverse forms of myocardial stress. At the time I initiated my studies the proximal activators of this pathway had not been identified, nor was it clear weather autophagy functioned as an adaptive or maladaptive response in the stressed myocardium. My initial research focused on autophagy in hypertension-induced heart failure, the most common cardiovascular disease in Western nations. Work by other members of the Hill laboratory had already confirmed that severe thoracic aortic banding (a surgical model of hypertension) induced extensive cardiomyocyte autophagy; my studies were designed to elucidate the functional significance of this activity.

Early evidence demonstrated the generation of reactive oxygen species and protein damage in the acute period of pressure overload, with damaged proteins then coalescing into intracellular aggregates. Given the simultaneous presence of autophagosomes and protein aggregates, and autophagy's best characterized role as a pathway of bulk-protein degradation, I postulated that these two events were mechanistically linked. I generated the hypotheses that protein aggregates are proximal activators of autophagy and that autophagy functions as an aggregate clearance mechanism.

I reported a number of novel findings that link pressure overload-induced protein aggregation to increases in cardiomyocyte autophagy. Specifically, my work demonstrates that in the pressure-stressed ventricle 1) generation of reactive oxygen species is an early pathological event, 2) there is extensive protein aggregation with higher-order processing of damaged substrates into peri-nuclear aggresomes, 3) intracellular protein

aggregation is sufficient to induce robust cardiomyocyte autophagy, and 4) in this setting autophagy functions in its canonical role as a mechanism of bulk protein degradation. These findings, then, are the first to demonstrate that proteinopathy of non-genetic etiology contributes to hypertension-induced heart failure and that protein aggregates are robust activators of cardiomyocyte autophagy.

Future directions in hypertension-induced autophagy

My studies intentionally focused on the interaction between protein aggregation, the induction of autophagy, and subsequent autophagy-mediated protein degradation. In this linear system one would predict that the net effect of pressure overload-induced autophagy is adaptive. The logic being that protein aggregates and their semi-soluble precursors are toxic species and the efficient removal of such substrates should ultimately protect the cell. In contrast to this prediction, a recent publication of ours demonstrates that the net effect of pressure overload-induced autophagy is, in fact, maladaptive.

Understanding this discrepancy is a current focus of our group. One explanation could be the timescale or extent of autophagic induction, where a threshold of activity exists below which autophagy is adaptive, above which it becomes pathologic. Two lines of evidence support this model in pressure overload-induced heart failure. First, time-course analysis of sTAB-induced autophagy demonstrates levels are highest during the first post-operative week (the time during which my studies were performed), and while levels go down over time, autophagy remains elevated after 3 weeks. Second, in a genetic model of enhanced

autophagy animals rapidly progress to overt heart failure when challenged with only a modest degree of pressure-overload.

While it is clear that there is some degree of substrate specificity to autophagy-mediated proteolysis, it is believed that many cellular components are still sequestered in a non-specific manner. As such, chronic autophagic activity may attenuate the accumulation of protein aggregates while also non-specifically depleting the cell of necessary constituents. Continual depletion of proteins involved in house-keeping processes may contribute to the pathologic outcome, thus accounting for the surprising roles our two studies have elucidated for cardiomyocyte autophagy.

The interaction between aggregate formation and induction of cardiomyocyte autophagy demonstrates an interesting biological process, but due to the multi-factorial nature of pressure overload-induced heart failure, this disease may not be the ideal system in which to elucidate the mechanistic machinery and clinical significance of aggregate-induced autophagy. Pressure overload not only induces reactive oxygen species, it also results in abnormal calcium handling, MAPK activation, and places biomechanical and neuro-humoral stress on the heart. Thus, protein aggregation and autophagy are a component of pressure overload-induced heart failure, but they are just two of many pathological events.

Autophagy in Desmin-Related Cardiomyopathy

To directly address the role of autophagy in cardiomyocyte clearance of toxic protein species, I turned my attention to $CryAB^{R120G}$-induced desmin-related cardiomyopathy. As discussed earlier, DRCM is a genetic disease with autosomal dominant inheritance. A missense

mutation in CryAB results in extensive protein aggregation, aggresome formation, and a lethal cardiomyopathy resulting in death during middle-age. Unlike pressure-overload which is associated with protein aggregation amongst a myriad of other pathologic processes, DRCM is defined first by the extensive proteinopathy, though a number of cellular processes are ultimately disrupted as a consequence of $CryAB^{R120G}$ expression.

In my studies on DRCM I found the paradigm elucidated in the pressure-stressed ventricle holds, and was further able to correlate autophagy-mediated proteolysis of aggregates to changes in cardiac remodeling, function, and survival. Specifically, my work demonstrates that 1) autophagy is activated by $CryAB^{R120G}$-induced protein aggregation, 2) the extent of aggregate formation is inversely proportional to the degree of autophagic activity and 3) blunting autophagy dramatically increases pathological myocardial remodeling and 4) autophagy is an adaptive response that significantly attenuates the onset of heart failure. These findings were further extended into clinical medicine where we demonstrate evidence of enhanced autophagy in patients suffering from desmin-related skeletal myopathy. Cumulatively these data are the first to demonstrate autophagy is induced in DRCM and functions as an important protective cellular response to protein aggregate stress. These findings suggest that autophagy is a pathway amenable to therapeutic intervention in patients suffering from diverse forms of myofibrillar myopathy, a disease class for which there are limited therapeutic options.

Future directions in myofibrillar myopathy and autophagy

My work demonstrates that autophagic induction is a robust and clinically important cellular response in desmin-related cardiomyopathy

and the human data suggests this pathway is relevant in diseases that proceed via similar mechanisms, regardless of the specific initiating genetic lesion. With the functional role of autophagy now established many exciting questions remain regarding the molecular pathways that facility this process and the genetic parameters that may alter clinical outcomes.

Recent evidence indicates that specific targeting of protein aggregates to the autophagosome is mediated by the protein p62. As already discussed, damaged proteins are post-translationally modified with ubiquitin moieties which target the protein towards proteasome-mediated proteolysis. When the proteolysis fails and aggregates form, p62 binds ubiquitin and then targets the aggregate towards the forming autophagic vacuole. p62, then, may play a central role in the coupling of CryAB-associated aggregates and the induction of cardiomyocyte autophagy.

If this interaction proves to be true in DRCM a number of mechanistic insights could be gleaned. Specifically, knockdown of p62 *in vitro*, or crossing $CryAB^{R120G}$ and p62 null mice would provide a model where by extensive aggregates form, but do so while uncoupled from the autophagic pathway. It would be interested to see if in this condition autophagy is still induced, if aggregates are cleared, and how this alters the development of heart failure in animals.

From a clinical standpoint my studies indicate that autophagy is a pathway amenable to therapeutic intervention. There are a number of known pharmacologic inducers of autophagy that may be useful. The best characterized small-molecule activator of autophagy is rapamycin, a drug already in clinical use with transplant patients for its immunosuppressive properties. Unfortunately, our data suggests this agent would have

minimal effect in patients with DRCM. Rapamycin induces autophagy via inhibition of mTOR, a repressor of autophagy. As demonstrated in my studies, crystallin aggregates sequester mTOR which one would predict has the same effect as rapamycin. Indeed, the finding that CryABR120G induces autophagy to a similar degree as rapamycin supports this conclusion. Fortunately, work is currently underway to identify novel induces of autophagy, and recent publications report small-molecule inducers of autophagy which function in an mTOR independent manner. While not yet approved for clinical use, these lines of research provide hope that pharmacologic activation of autophagy will soon be a viable option for patients suffering from protein surplus diseases.

Finally, the demonstration that the cell's capacity to induce autophagy can profoundly alter clinical outcomes suggests that inter-individual genetic variations within the population may account for the diverse clinical presentation of patients with this class of disease. For instance, beclin 1 was identified as a tumor suppressor protein only after its deletion was found in multiple forms of human malignancy. From our animals studies one would predict that a patient with beclin haploinsufficieny would present with a much more sever case of DRCM that would a homozygous wildtype individual. Beclin, of course, is only one of many genes involved in the autophagic pathway, likewise there are genes involved in the identification and targeting of toxic proteins to the autophagosome (such as p62). While yet to be explored, my work suggests that an individual's "autophagic potential" can dramatically alter disease progression in patients with myofibrillar myopathy, which carries with it significant prognostic and therapeutic implications.