

UNIVERSITY OF CALIFORNIA SAN DIEGO

Secretion of the Mutant Protein AlphaB-crystallin R120G in Extracellular Vesicles

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by

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University of California San Diego

2024

DEDICATION

I dedicate this work to my family, without whom none of this would have been possible.

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LIST OF ABBREVIATIONS

CryAB	α B-Crystallin
CVD	Cardiovascular Disease
DRM	Desmin-Related Myopathy
UPS	Ubiquitin-Proteasome System
EVs	Extracellular Vesicles
KI	Knock-In
BTZ	Bortezomib

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ABSTRACT OF THE THESIS

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by

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Master of Science in Biology

University of California San Diego, 2024

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α B-crystallin (CryAB) is a ~20-kD small heat shock protein commonly expressed in heart tissue. However, the CryAB R120G missense mutation causes misfolding of the protein, leading to the formation of insoluble CryAB aggregates in cardiomyocytes linked to the development of desmin-related cardiomyopathy. Typically, cells utilize mechanisms such as the ubiquitin-proteasome system (UPS) and autophagic-lysosome pathway to maintain proteostasis, while growing evidence shows that secretion of extracellular vesicles (EVs) may also play a role in protein quality control. Here, we analyze how these mechanisms function in the context of CryAB R120G. After confirming presence of insoluble CryAB protein in mouse hearts from a

CryAB R120G KI mutant line, we observed that UPS activity seemed to decrease in mice aged 3-4 months. We identified CryAB in secreted extracellular vesicles from R120G KI heart tissue and observed the presence of autophagic markers, indicating a relationship between EV secretion and autophagy mechanisms. We also found that inhibition of the UPS decreases EV secretion. Further, secreted EVs carrying R120G CryAB do not appear to cause a pro-inflammatory response in macrophages. Our findings suggest insoluble CryAB R120G aggregates impact quality control in cardiomyocytes, and that EVs assist in clearing misfolded CryAB from the cell.

INTRODUCTION

Background & Significance

Around the world, cardiovascular disease (CVD) is the leading cause of death and the largest healthcare expense (Tsao et al, 2023). Individuals with a history of CVD often must limit physical exertion to prevent additional strain on their heart, and can sometimes face debilitating symptoms, such as chronic fatigue, edema, insomnia, and pain, narrowing their scope of activities both day-to-day and long-term, as well as their life expectancy (Komalasari et al, 2019). Frequent rehospitalization is often a reality for these patients as CVD increases the risks associated with other conditions, such as kidney and liver disease (Harjola et al, 2017). Further, only approximately 40% of individuals survive longer than 5 years after being diagnosed with heart failure (Moser & Mann, 2002). Management of heart disease has improved significantly in recent years with more pharmacological interventions and a better understanding of preventive and monitoring practices (Arjmand et al, 2021; Kayani & Ballantyne, 2018). However, while most common approaches are fairly effective in managing cases of CVD originating from lifestyle choices or the environment, treatments for CVD due to genetic mutation are still limited. It is not always possible to manage genetic CVDs with lifestyle changes and while many treatments may address symptoms, none are a cure (Arjmand et al, 2021). Understanding the mechanisms underlying genetic CVD may help identify new effective treatments and therapies for this patient population.

Cardiomyopathy, which is defined as disease of the cardiac muscle, or myocardium, causing negative impact to cardiac function, accounted for approximately 6 million patients suffering from a cardiovascular disease worldwide in 2020 (Richard et al, 2006; Tsao et al, 2023). Although cardiomyopathy accounts for a smaller subset of cases under the larger CVD

classification, it is disproportionally responsible for many instances of heart transplantation, sudden cardiac death (SCD) and heart failure, especially in adolescents (Tsao et al, 2023; Kim et al, 2021). In a study performed in France in 2020, cardiomyopathy patients accounted for 51% of heart transplantation procedures, increasing to 71% when only patients under the age of 40 were considered (Lannou et al, 2020). In short, while the afflicted population of those suffering from cardiomyopathy is small, the burden and lethality of the condition is very impactful, leading the condition to account for a greater proportion of hospitalizations and deaths.

A main reason that many cardiomyopathies are difficult to treat is their basis in genetic causes (Kim et al, 2021). There is no current way to “cure” genetic cardiomyopathy, and all patients will inevitably require a heart transplant, should they develop heart failure. Current treatment options only seek to manage symptoms and slow disease progression. (Torres & Perez-Villa, 2018). If patients with genetic cardiomyopathy do not adhere to regimented treatments, monitoring, and lifestyle changes to manage their condition, their disease will only continue to worsen (Tun, 2021). It is not possible to reverse existing tissue damage as cardiac myocytes are post-mitotic and have limited regenerative potential, incapable of replenishing in numbers to replace damaged structures (Carvalho & de Carvalho, 2010). Loss of cardiomyocytes leads to remodeling and reduced ability to sustain contraction, leading to dysfunction (Orogo & Gustafsson, 2013).

The key to identifying more effective treatments for inherited cardiomyopathy and other heart conditions may lie in better understanding of these diseases and how they affect cardiomyocytes at the molecular level. Further, internal quality control mechanisms are already vital to preserving long-term integrity in cardiac myocytes (Carvalho & de Carvalho, 2010). Such pathways may be able to be targeted for better therapeutics in the future; however, it must

first be understood what these regulatory mechanisms are and how they are impacted in the context of disease.

α B-crystallin (CryAB) R120G

There are many identified genetic causes of cardiomyopathy. One such related mutation is known as α B-crystallin R120G (CryAB R120G), an autosomal dominant missense mutation that replaces arginine with glycine at the 140th amino acid in the peptide chain, causing misfolding of the highly expressed α B-crystallin protein in cardiomyocytes (Bova et al, 1999). The misfolded proteins form aggregates, which negatively interact and bind with desmin-filaments in the cytoskeleton to create what is called a desmin-related myopathy (Wang et al, 2001). Desmin-related myopathy (DRM) describes the build up of abnormal desmin aggregates in skeletal and cardiac muscle, known to cause multiple subtypes of cardiomyopathy (Abraham et al, 1998). Although familial DRM appears very rare, identified patients encompass a wide variety of genetic backgrounds and origins, suggesting many individuals may go undiagnosed. Further, other desmin and aggregation-based disorders follow similar patterns of development and presentation to R120G desmin-related myopathy, meaning better understanding of the rare mutation may have other disease applications as well (McLendon & Robbins, 2011; Goldfarb et al, 2004).

α B-crystallin (CryAB) is a 20-kD small heat shock protein commonly expressed in the lens, skeletal and cardiac muscles (Vicart et al, 1998). Small heat shock proteins (sHSPs) are known to protect against misfolded protein aggregation in the cell, as well as help mediate the cell cycle, inflammatory responses, signal transduction, apoptosis, and cell differentiation (Bakthisaran et al, 2014). CryAB specifically plays a pivotal role in protecting the heart against cellular stress and damage, functioning as a molecular chaperone and stabilizing the cytoskeleton

via binding to desmin and cytoplasmic actin (Yin et al, 2019; Wang et al, 2001). CryAB is upregulated in cardiac tissue in response to stress in order to help preserve cellular function, comprising somewhere between 3-5% of soluble protein found in cardiac tissue (Yin et al, 2019; Dimauro et al. 2017). Interestingly, unlike other molecular chaperones, CryAB does not renature misfolded proteins after binding, nor does it release them. Instead, CryAB remains attached to its target, forming a soluble aggregate that can be degraded by the cell (Yamamoto et al, 2014).

Whereas functional CryAB protects other proteins from cellular stress in the heart, the R120G autosomal dominant mutation causes misfolding, leading to the formation of insoluble aggregates within cardiomyocytes (Vicart et al, 1998). Large electron-dense aggregates have been observed in cardiac tissue, containing misfolded CryAB at the center with bound desmin at the perimeter. Misfolded CryAB still binds with desmin as it does in its functional form; however, instead of stabilizing it, CryAB R120G forms protein aggregates containing desmin (Wang et al 2001). In patients, this aggregate build-up is linked to contractile dysfunction and myofibrillar degeneration, as well as the diagnosis of desmin-related myopathy (DRM) (Alam et al, 2020; Vicart et al, 1998). The association between CryAB R120G aggregate formation and the development of desmin-related cardiomyopathy is fairly well known, yet the course of disease and proteotoxic effects within cardiomyocytes are less characterized.

Proteostasis and Proteotoxicity

An important part of cellular quality control and homeostasis is the maintenance of proteostasis. Proteostasis is the balance of protein synthesis, folding, transport, and degradation in the cell, crucial for maintaining health and function (Labbadia & Morimoto, 2015). Degradation is an important component of this process, crucial to preventing the buildup of

misfolded proteins and allowing for the recycling of components into new, functional proteins (Pohl & Dikic, 2019). In some cases, chaperone proteins may refold misfolded proteins or the ubiquitin-proteasome system (UPS) will be employed to degrade them, but in other instances, these mechanisms are simply not sufficient to clear an overload or aggregation of misfolded proteins (Li et al, 2022). When this occurs, the autophagy-lysosome pathway is activated– a process in which large vesicles engulf and degrade severe aggregates, recycling the amino acids for later utilization in the cell (Pohl & Dikic, 2019). All three aforementioned mechanisms– chaperone proteins, the ubiquitin-proteasome system, and autophagy– are effective for preventing accumulation of protein aggregates; however, they are often compromised with age or disease (Henning & Brundel, 2017).

Proteotoxicity refers to the impact of misfolded or damaged protein build-up in the cell, eliciting negative effects on organelles or even cellular death. In organs such as the brain or heart, which have limited regenerative potential due to their post-mitotic cellular composition, proteotoxicity is a much bigger cause for concern (Sandri & Robbins, 2014). Parkinson's disease, Huntington's disease, and other amyloid-based neurological conditions are marked by a buildup of protein aggregates, exhibiting a negative effect on neural cells. However, studies suggest that a similar mechanism is present in cardiomyocytes and is associated with heart failure, such as in the case of desmin-related myopathy caused by the CryAB R120G mutation (McLendon & Robbins, 2015; Sandri & Robbins, 2014). In both cases, unchecked formation of aggregates at the cellular level aligns with poor health consequences overall, though the exact mechanism of disease and proteotoxic effects of aggregates are still not fully understood.

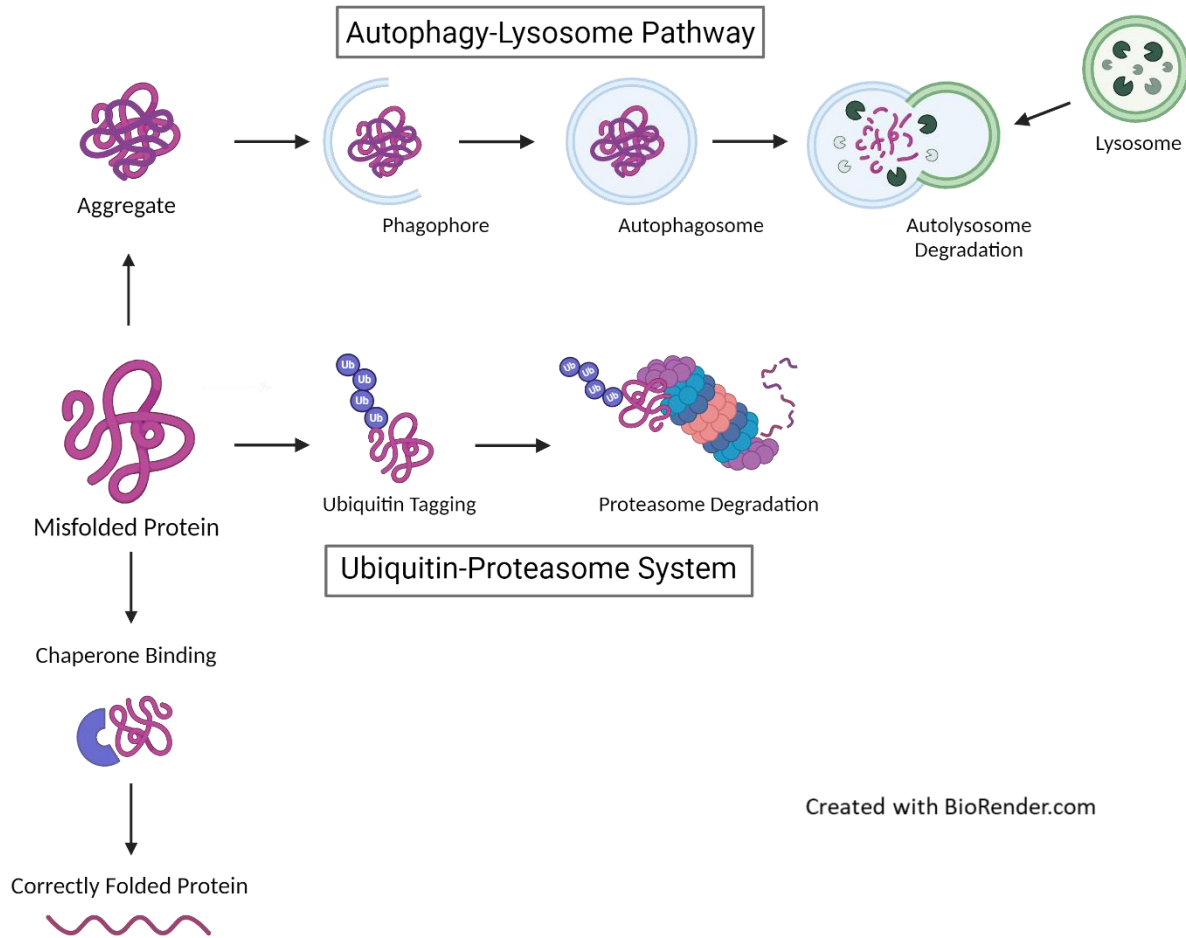


Figure 1: Overview of cellular quality control. Misfolded proteins can either be refolded by bound chaperone proteins or are degraded via the ubiquitin-proteasome system. When misfolded proteins become insoluble aggregates, the autophagy-lysosome pathway must be employed. In the ubiquitin-proteasome system, aggregates are tagged with ubiquitin, which is recognized by the 26S proteasome. The proteasome cleaves the protein into its amino acid components. In the autophagy-lysosome pathway, aggregates are enveloped in autophagosomes, which fuse with lysosomes to degrade the contents inside.

Ubiquitin-Proteasome System

The Ubiquitin-Proteasome System (UPS) is the primary pathway for degrading misfolded or unwanted cellular proteins and is crucial in maintaining proteostasis. When a defective protein is detected by the cell, it is first labeled for degradation through a series of ubiquitinating reactions utilizing E1, E2, and E3 enzymes. After the target protein has been ubiquitinated, the 26S proteasome recognizes and binds, utilizing a multi-ubiquitin chain (Kitajima et al, 2020). The 26S proteasome is a large protein complex found in the cytosol, as well as the site of protein cleavage in the UPS mechanism. The proteasome mainly consists of a 20S core with multiple sites for catalytic activity, and one or two 19S subunits meant to assist in regulating the transfer of proteins to the core (Park et al, 2020).

Autophagy-Lysosome Mediated Degradation

Another important pathway for cellular quality control and proteostasis is the autophagy-lysosome pathway (ALP). When chaperone proteins or the ubiquitin-proteasome system cannot clear insoluble misfolded protein, autophagy is often responsible for degrading the aggregates and clearing them from the cytosol (Finkbeiner, 2020). Autophagy is activated in cells in response to various stressors such as nutrient deprivation, energy deficiency, infection, or damage to organelles (Burman & Ktistakis, 2010). Activation of the autophagy-lysosome pathway begins with the *de novo* formation of autophagosomes, which form around targeted molecules for degradation in the cell. After the target has been engulfed, the autophagosome fuses with the lysosome where the contents are then degraded. At this stage, components of the degraded molecules are either released back into the cytosol for recycled use, or they are secreted via exocytosis (Karanasios et al, 2016; Finkbeiner, 2020).

Extracellular Vesicles

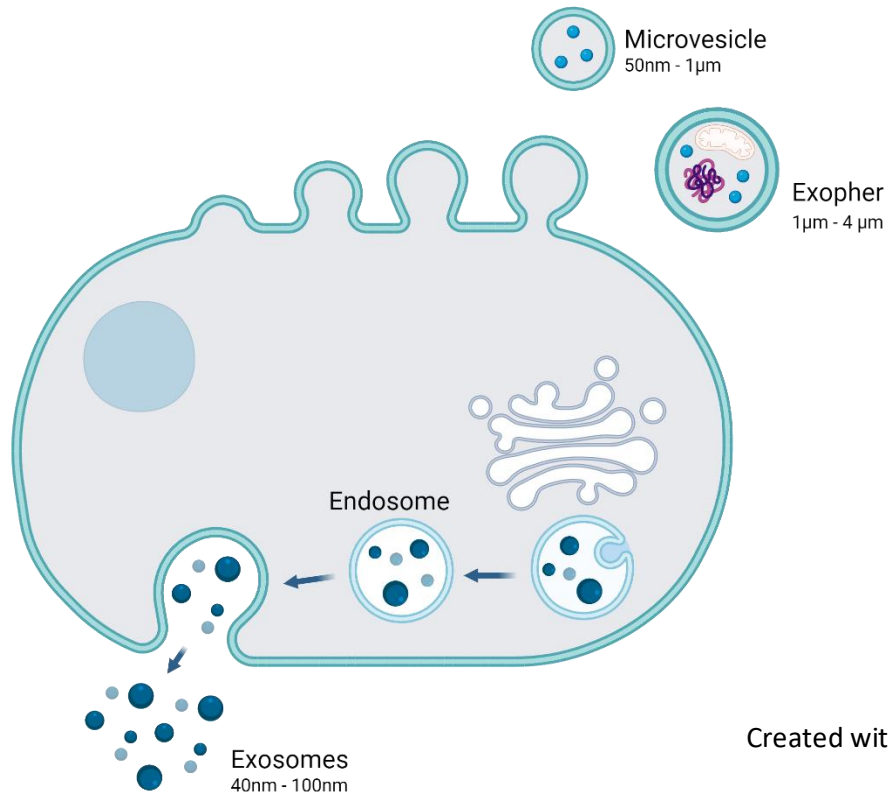
Extracellular vesicles (EVs) are a class of cargo vesicles tasked with transporting bioactive molecules between cells. These lipid membrane-bound carriers are secreted into extracellular space and once released can either act locally or travel within plasma to reach distant tissues (Zaborowski et al, 2015). Common cargo of EVs include lipids, proteins, DNA, and RNA, delivered between cells as a form of communication. The recipient cell will take up the vesicle either through endocytosis, membrane fusion, or phagocytosis, though it is still not fully clear how the contained cargo is released within the recipient cell. In this way, EVs also play a significant role in the immune system, mediating the transportation of immunoreactive cargo between cells. In instances such as systemic inflammation, infection, and disease, EV secretion is shown to be increased (Zaborowski et al, 2015; Yanez-Mo et al, 2015).

There are two broad classifications of EVs based on their origin in the cell: microvesicles and exosomes. Microvesicles, which are formed via direct budding of the cellular plasma membrane, are typically between 50 nm-1 μ m. Exosomes, on the other hand, are formed via intraluminal budding of endosomes and released when the late endosome fuses with the plasma membrane. They are typically between 40 - 100 nm in size. These vesicle types can also be classified into “small” and “large” EVs based on relative size. (Vader et al, 2016; Saludas et al, 2022).

Evidence also suggests that extracellular vesicles (EVs) may play a role in cellular housekeeping, positively contributing to the maintenance of proteostasis in cells (Sheta et al, 2023). We recently reported that large EVs are integral in mitochondrial quality control in the presence of limited lysosomal function in cardiac tissue. Characterization of the vesicles revealed intact mitochondria as cargo, increasing in secretion when lysosomal mediated degradation was

compromised (Liang et al, 2023). If extracellular vesicles appear to play a role in the transport and degradation of intact organelles, the same may be true for protein aggregates. Investigating CryAB in optical tissue discovered that the protein is in secreted exosomes (Kore & Abraham, 2016). This further raises the question whether extracellular vesicles also play a role in secretion of protein aggregates in the heart.

A different kind of extracellular vesicle known as exophers have been shown to exhibit function in protein quality control in the presence of neurotoxic protein aggregation (Cooper et al, 2021). Like microvesicles, exophers bud from the plasma membrane of the cell (Arnold et al, 2020). Exophers are typically larger than microvesicles or exosomes, averaging around 3µm in size, and as a result can carry bigger cargo. Although exophers were first characterized in *C. elegans* studies, more recent findings have isolated similar vesicles released from cardiomyocytes in mice (Nicolás-Ávila et al, 2022; Nicolás-Ávila et al, 2020). In short, exophers do appear to contribute to the maintenance of proteostasis in the cell and may play a key role in eliminating protein aggregation in the context of the CryAB R120G mutation.



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Figure 2: Extracellular vesicles. There are three broad types of extracellular vesicles (EVs): exosomes, microvesicles, and exophers. Exosomes are formed in the endosome, budding off its intraluminal membrane. Microvesicles and exophers are both formed by budding of the cellular plasma membrane. Of these, exophers are typically much larger and able to carry bigger cargo.

Macrophages

Macrophages are an important part of the innate immune system in humans, functioning as a line of defense against foreign pathogens, as well as assisting in wound healing and tissue regeneration. If a signal of infection, injury, or inflammation is received, macrophages will travel to the site and engage in a process called phagocytosis to engulf the pathogens and possible debris for internal digestion (Lendeckel et al, 2022). Macrophages are also found within all tissues of the body and display a great diversity of functionality depending on the environment they occupy (Wynn et al, 2013). Even after the cells have matured from monocytes to macrophages, they can differentiate further via a process called polarization. In macrophage polarization, cells can either become type M1, also referred to as “classically activated”, or type M2, referred to as “alternatively activated,” M1 macrophages (Lendeckel et al, 2022). M1 macrophages display pro-inflammatory qualities, which are important for mounting a defense against infection, but can damage tissue over time if inflammatory signals persist. In contrast, M2 macrophages display anti-inflammatory qualities, playing a more reparative role and initiating tissue healing (Perez & Rius-Perez, 2022).

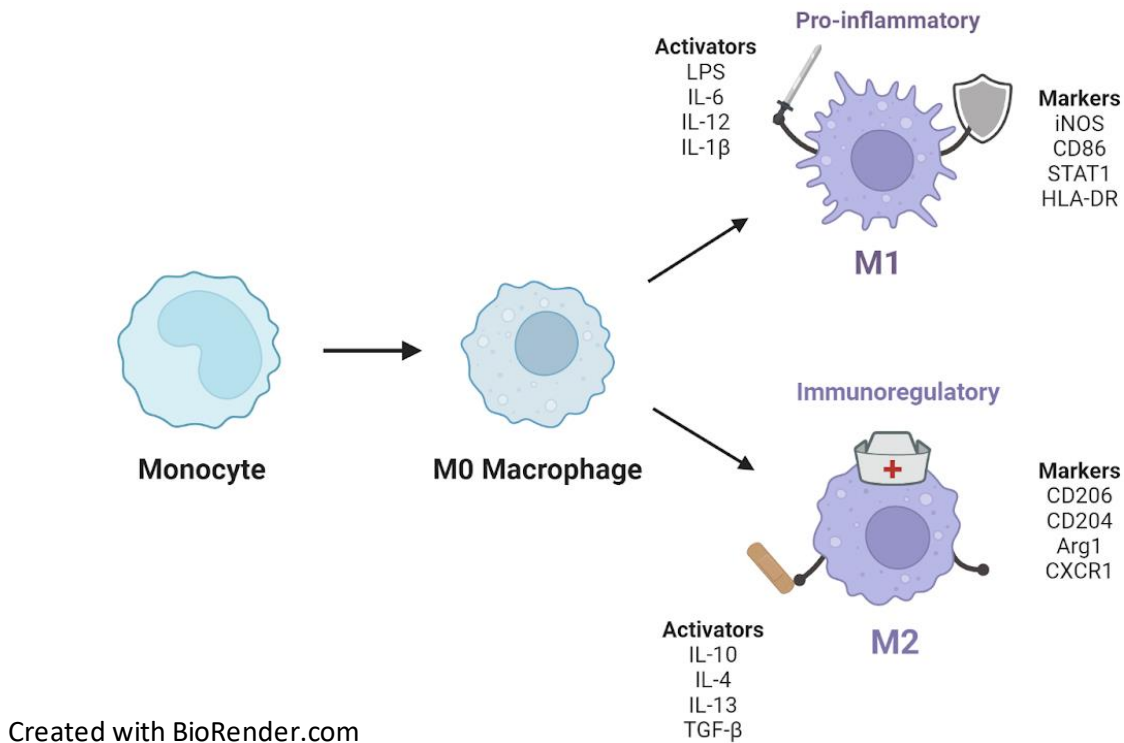


Figure 3: Macrophage differentiation. After developing from a monocyte, M0 macrophages can differentiate into M1 or M2 macrophages. M1 macrophages are activated by cytokines such as TNF α , eliciting a pro-inflammatory response typically used to combat foreign pathogens. M2 macrophages are activated by cytokines such as IL-4, generating a reparative response typically used to begin wound healing.

Rationale

CryAB R120G is linked to desmin-related cardiomyopathy in humans, leading to contractile dysfunction and heart failure as the disease progresses. Whereas the larger physiological implications are understood, the cellular mechanisms impacted by the mutant protein are still unclear. Multiple processes in the cell contribute to the maintenance of proteostasis, such as the ubiquitin-proteasome system and autophagy. Investigating the effect of CryAB R120G protein on quality control systems may reveal potential paths for better understanding of the disease progression, as well as identify potential therapeutic targets for future treatments.

In my research I have used both *in vivo* and *in vitro* models to examine the CryAB R120G mutation and its proteotoxic impact on cardiomyocyte structure and function. I have examined the following questions to test the hypothesis that cells secrete CryAB R120G protein aggregates in EVs to limit accumulation of the mutant protein in cells and that these EVs are consequently taken up by macrophages.

1. How does CryAB R120G affect cardiac structure with age?
2. Does CryAB R120G aggregation impact ubiquitin-proteasome system activity?
3. Are CryAB R120G aggregates secreted in extracellular vesicles?
4. Does CryAB R120G aggregate presence in secreted extracellular vesicles confer an inflammatory response in macrophages?

MATERIALS AND METHODS

Mouse Models

All mice were housed under standard conditions, given free access to chow and water, and subjected to a 12/12-hr-dark-light cycle. CryAB R120G knock-in mice were generated at the Department of Ophthalmology and Visual Sciences at Washington University School of Medicine via stem cell-based techniques on a C57Bl/6J background. Further information on generation of this line has been described previously (Andley et al. 2011). All R120G KI mice were homozygous for the mutant gene. C57BL/6J mice were used as age-matched wild-type controls. Both male and female mice were used in experiments.

For tissue collection, mice were anesthetized using a lethal dose of sodium pentobarbital via IP injection. Hearts were removed, rinsed in cold phosphate-buffered saline (PBS) and then flash-frozen in liquid nitrogen. After freezing, samples were stored at -80°C until use. For samples used for EV isolation, hearts were weighed before freezing to ensure an equal amount of each sample.

For Bortezomib injections, Bortezomib powder (Selleckchem) was resuspended in saline and retro-orbitally injected into mice at a dose of 1mg/kg at 0 and 24 hours. At 48 hours after initial injection, mice were anesthetized using sodium pentobarbital and hearts were collected and frozen.

Histology

Hearts were fixed in 10% neutral buffered formalin for 24 hours and then transferred to 70% dehydrant alcohol for at least another 24 hours. Dehydrated hearts were embedded in paraffin and cut into 5-µm thick sections using a microtome (Leica Biosystems). Hearts were cut at the transverse plane, allowing for visualization of the two ventricular chambers. Sections were

stained with H&E to visualize structure and Masson's trichrome to visualize collagen (MilliporeSigma). Sections were imaged via a Nikon Eclipse Ti2 microscope. Images were captured at 4x and 10x magnification.

EV Isolation

For EV isolation from mouse cardiac tissue, frozen tissue samples were placed in Hanks' Balanced Salt solution (HBSS) containing 200ug/ml Liberase HD (Roche) and 20U/ml DNase I (Roche). While in solution on ice, tissue was cut into small pieces (2x2x2 mm) and then incubated for 45 minutes at 37°C with mild agitation (50rpm). After enzymatic digestion, the resulting solution and debris was subjected to serial centrifugation at 50 x g for 5 min and 300 x g for 10 min. The resulting supernatant was centrifuged at 1000 x g for 10 minutes and separated from the formed pellet containing exophers. The supernatant was spun at 16,500 x g to pellet large EVs and again at 118,000 x g to pellet small EVs. The exopher, large, and small EV pellets were resuspended with RIPA buffer (Cell Signaling) containing PhosSTOP (Roche) and cOmplete Protease Inhibitor Cocktail (Roche) for Western blot analysis. EV protein concentration was analyzed via Bradford assay.

Macrophage EV Uptake Assay

Raw 267.4 macrophages were cultured in RPMI 1640 containing 10% fetal bovine serum (FBS, Gibco) and 1% antibiotic-antimycotic (Gibco). Cells were incubated at 37°C in a 5% CO₂ atmosphere. Cells were routinely tested for presence of mycoplasma.

Raw 267.4 cells were plated on 35mm dishes at a density of 1x10⁵ cells per dish. Before treatment with EVs, cells were rinsed with PBS and changed to RPMI 1640 containing 10% exosome-depleted FBS and 1% antibiotic-antimycotic. After 24 hours in exosome-depleted media, EVs were introduced to the macrophages. Positive control plates were introduced with

lipopolysaccharide (LPS; Sigma-Aldrich) at 100ng/ml or IL-4 (PeproTech) at 30ng/ml. Negative control plates were introduced with PBS. After cell collection, pellets were resuspended with RIPA buffer (Cell Signaling) containing PhosSTOP (Roche) and cOmplete Protease Inhibitor Cocktail (Roche) for Western blot analysis.

Western Blot Analysis

For isolation of soluble and insoluble fractions, frozen tissue samples were homogenized in detergent-free lysis buffer (1M Tris-HCl pH 7.4, 5M NaCl, 100mM EDTA, 100mM EGTA), mincing with scissors and mixing via Polytron for 3 seconds at 22,000rpm. Triton-X was then added to the lysates at a final concentration of 1%. After incubation on ice for 45 minutes, samples were centrifuged at 20,000 x g for 20 minutes at 4°C and supernatant was then separated from the pellet. The supernatant was analyzed as the “soluble” fraction of proteins present in the sample. To isolate the “insoluble” fraction, the pellet was washed with PBS and homogenized in buffer containing 5mM Tris-HCl (pH 6.8), 2% SDS, 100mM DTT, and 10% glycerol. Samples were then sonicated 5 times at 20% amplitude for 5 seconds, then boiled at 95°C for 5 minutes. After centrifugation at 20,000 x g for 20 minutes, the supernatant was separated and analyzed as the “insoluble” fraction.

Prior to loading on gel, cell and tissue samples were analyzed with a Bradford assay to determine protein concentration for normalization during loading. Isolated EVs were not normalized before loading. For cell, tissue, and EV analysis 4X NuPAGE LDS Sample Buffer (Novex) and 50μM dithiothreitol (DTT) was added to samples. Proteins were separated on 10% NuPAGE Bis-Tris gel (Life Technologies) and then transferred to nitrocellulose membranes for antibody staining. Proteins were detected using antibodies against α B-crystallin (Enzo), flotillin-1 (Cell Signaling), GAPDH (GeneTex), actin (Cell Signaling), ubiquitin (brand), LC3 (Cell

Signaling), p62 (Cell Signaling), CD81 (Cell Signaling), CD206 (Cell Signaling), and iNOS (Cell Signaling). Proteins were visualized with a BioRad ChemiDoc XRS+, and bands were quantified with ImageLab.

UPS Assay

Hearts were first homogenized in assay buffer (50mM HEPES, 10mM NaCl, 1.5mM MgCl₂, 1 mM EDTA, 1mM EGTA, 250 mM sucrose, 2mM ATP, 5mM DTT, pH 7.8) and centrifuged at 16,000g for 10 minutes. Supernatant was separated from the formed pellet and analyzed for protein concentration via Bradford assay. In a clear-bottomed/black walled 96-well plate (MilliporeSigma) 20ug of protein was added per well. Assay buffer was added until a volume of 195ul was reached per well. 5ul of 5mM fluorescent substrate (chymotrypsin-like, trypsin-like or caspase-like, Enzo) was then added per well. Plates were incubated for 60 minutes at 37°C, 5% CO₂. Fluorescence was measured at A360ex/A460em on a fluorescent plate reader. Each sample was measured in triplicate for each substrate. Additional samples were boiled at 95°C for 30 minutes, heat-inactivating contained proteins and serving as a negative control.

Statistical Analysis

Data are mean +/- S.E.M. Student's t-test was used to evaluate differences between 2 groups using GraphPad Prism 7 software. A p-value of less than 0.05 was considered the threshold for statistical significance.

Chapter 1

aB-Crystallin R120G causes insoluble protein aggregation in cardiomyocytes

CryAB is highly expressed in cardiac tissue and the R120G mutation causes misfolding of the protein and formation of protein aggregates (Wang et al, 2001). These aggregates are insoluble, requiring mechanisms such as autophagy to be cleared from the cell. Without elimination of these aggregates, they can exert negative effects on organelles and cell function. Here, we investigated the levels of soluble and insoluble protein in wild-type and CryAB R120G KI mouse hearts to determine if there was a significant accumulation of CryAB in the insoluble protein fraction in hearts with the R120G mutation.

To quantify the levels of soluble and insoluble CryAB protein levels, we examined soluble and insoluble protein fraction prepared from wild-type and R120G heart tissue by Western blot experiments. Correctly folded CryAB is soluble whereas the misfolded protein is expected to form insoluble aggregates. Thus, under normal conditions, it would be anticipated for the majority of CryAB to be present in the soluble protein fraction of a tissue sample. We found that much of the wild-type protein was found in the soluble fraction (Fig. 4A & 4B), whereas a significant portion of CryAB R120G was present in the insoluble fraction (Fig. 4A & 4C). Overall, these results demonstrate that CryAB in wild-type hearts is a soluble protein whereas the majority of the mutant CryAB in R120G KI hearts is an insoluble protein.

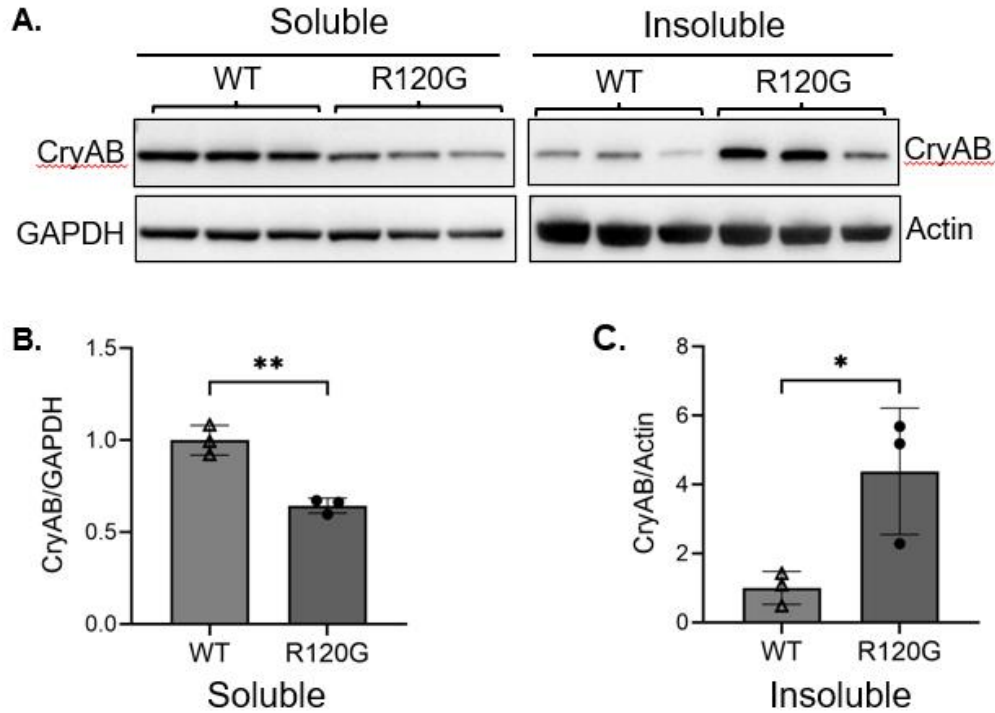


Figure 4: Levels of insoluble CryAB increase in R120G KI cardiomyocytes. **A)** Hearts from wild-type (WT) and CryAB R120G KI mice at 8-10 weeks of age were harvested and whole heart homogenates prepared. Representative blot of CryAB levels in Triton X-100 soluble and insoluble fractions. GAPDH and actin were used as loading controls. **B)** Quantification of soluble CryAB in WT and R120G samples (n=3; **p<0.01). **C)** Quantification of insoluble CryAB in WT and R120G samples (n=3; *p<0.05). Analysis was performed using Student's T-test.

Histological Evaluation of Wild type and CryAB R120G KI hearts

While the CryAB R120G mutation results in cardiomyopathy within afflicted individuals, how the mutant protein affects cellular processes in cardiomyocytes is still not well understood. Cardiomyocytes are non-regenerative, meaning they do not undergo mitosis. When these cells die, they are replaced by collagen, allowing the myocardium to still retain its shape and structure. However, the loss of too many cardiomyocytes will lead to contractile dysfunction as increasing amounts of the myocardium are replaced with collagen rather than functioning myocytes. This is the basis of many types of heart disease (Hinderer & Layland, 2019). Thus, cardiomyocyte death due to CryAB R120G protein aggregation will lead to deposits of collagen resulting in a stiffer heart. To assess changes to structure and levels of collagen in cardiac tissue, both hematoxylin and eosin (H&E) and Masson's trichrome staining were performed on heart sections from wild-type and R120G KI mice. Both young (3-4 month) and old (1 year) mice from each group were evaluated for structural changes to the myocardium, as well as levels of fibrosis.

Overall, we observed no substantial differences in H&E and Masson's Trichrome staining between wild type and R120G KI hearts (Figure 5A & 5B). While young versus old mice displayed some changes to structure in line with the aging process, there wasn't an apparent difference between the wild-type and the mutant at either age point. Collagen levels appear similar in both wild-type and R120G hearts.

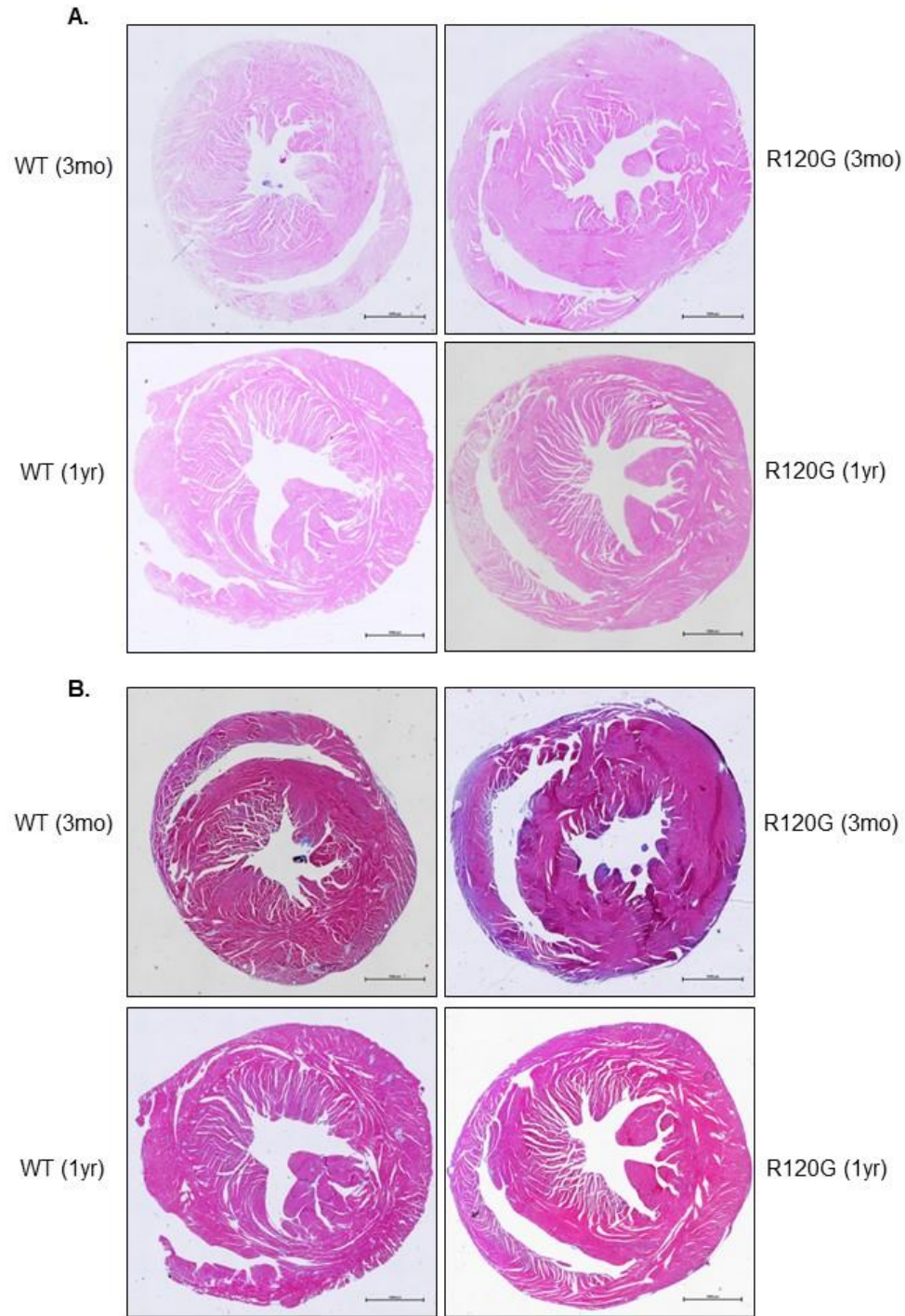


Figure 5: No observable difference between WT and R120G cardiomyocyte structure and collagen levels. **A)** Hearts from WT and CryAB R120G KI mice were fixed in formalin, sectioned and stained with hematoxylin and eosin. Images were captured at 4x magnification. Scale bar = 1mm. **B)** Heart sections were stained with Masson's trichrome and images captured at 4x magnification. Scale bar = 1mm.

Chapter 2

Activity of the 26S proteasome is decreased in α B-Crystallin KI R120G hearts

As previously described, CryAB misfolding increases in cardiac tissue with the R120G mutation (Bova et al, 1999). The activity of the proteasome, which is primarily responsible for degrading misfolded proteins within cells, is therefore expected to increase in hearts with the R120G mutation. However, in instances of stress or age, the UPS pathway can become compromised, leading to a decrease in protein degradation and increased buildup of misfolded proteins and aggregate formation. If UPS activity is reduced, it could account for the increased presence of insoluble CryAB aggregates in R120G KI hearts. Further, it is important to characterize how the function of the UPS is affected over time, to determine if it may be an underlying factor of age-related heart disease. Therefore, we evaluated proteasomal activity in young and aged wild type and R120G KI hearts.

To identify potential differences in proteasomal activity levels between wild-type (WT) and CryAB R120G KI mice, as well as between young (3-4 month & 6 month) and aged (1 year & 26 month) mice, an assay was performed on heart tissue isolated from each genotype using proteasome-specific substrates with fluorescent tags. After incubating samples with substrates for 1 h, changes in fluorescence were assessed using a plate reader. The proteasome employs three main categories of biochemical processes— caspase-like, chymotrypsin-like, and trypsin-like activity—to carry out degradation (Britton et al., 2009). By evaluating these three proteolytic activities, the overall activity of the proteasome can be assessed.

In hearts from young mice (3-4mo), we observed a significant decrease in two types of proteasomal activity (chymotrypsin-like and trypsin-like) in R120G KI hearts compared to the wild-type. Further, although the difference wasn't statistically significant, caspase-like activity

followed the same trend of decreased activity in R120G KI hearts. However, in the aged mice (1yr), we found no significant differences across any activity type between WT and R120G KI heart samples (n=3; Figure 6A). In comparing wild-type mice at 6mo and 26mo of age, there was an apparent increase of proteasomal activity with age, but only the difference in caspase-like activity was statistically significant. This was likely due to the small number of repeats in the experiment (n=3; Figure 6B). Overall, the findings suggest that proteasomal activity is decreased in hearts of young mice with the R120G KI mutation. However, activities are restored to levels observed in wild type hearts by 12 months of age. Also, proteasomal activity seems to be retained in hearts with age.

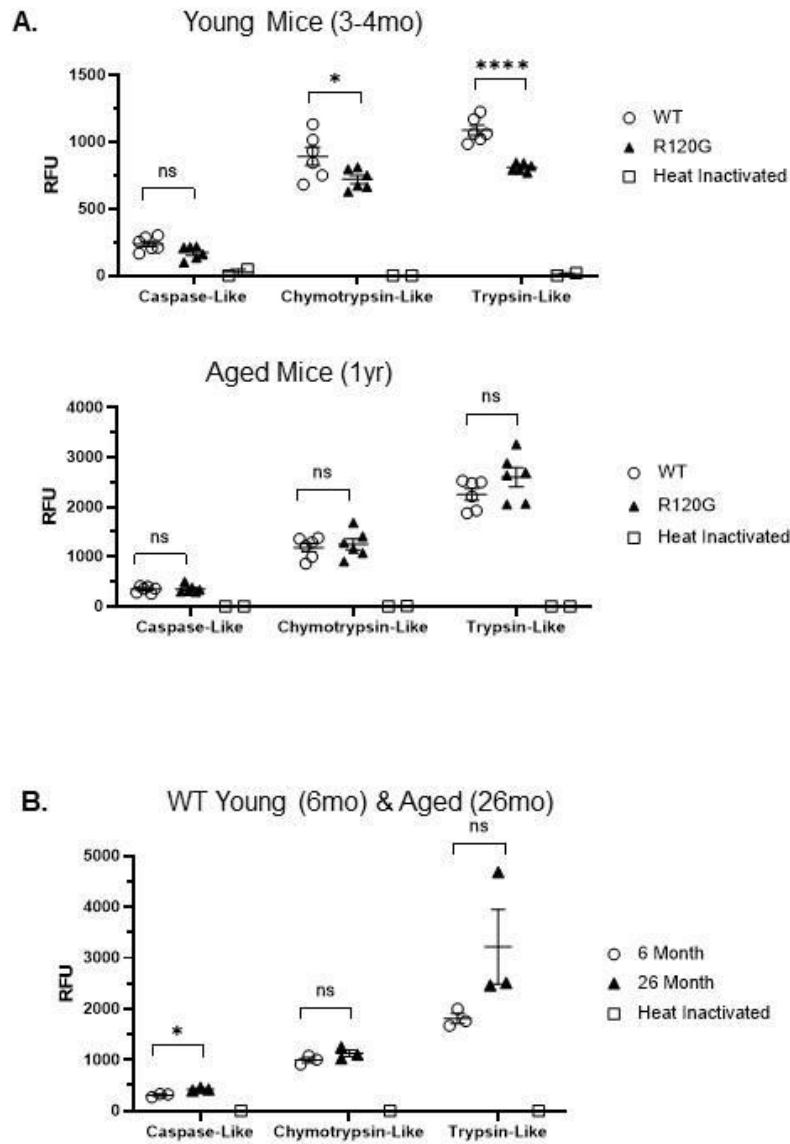


Figure 6: Proteasomal activity is decreased in young R120G KI hearts. A) WT and CryAB R120G KI mouse hearts were lysed and analyzed via fluorescent assay for three types of proteasomal activity: caspase-like, chymotrypsin-like, and trypsin-like. Relative fluorescence Units (RFU) was quantified after an hour of incubation at 37°C. Activity in hearts from mice at 3-4 months and 1 year of age were compared (n=6; ns=not significant, *p<0.05, ****p<0.0001). **B)** Comparison of activities in hearts of WT mice at 26 months and 6 months of age for proteasomal activity (n=3; ns=not significant, *p<0.05). Analysis was performed using Student's T-test.

Extracellular vesicles from R120G KI mice contain aB-Crystallin

Extracellular vesicles (EVs) are secreted by cells and have been shown to function as a quality control function within cells by secreting damaged organelles and misfolded proteins from the cell. They can be divided into three categories— large EVs, small EVs, and exophers. Exophers were first shown to expel neurotoxic protein aggregation in *C. elegans* but have also been reported to play a similar role in mouse cardiomyocyte tissue (Nicolás-Ávila et al, 2022). Large and small EVs have been shown to participate in eliminating damaged organelles from cells, but may also function similarly to exophers in protein quality control. Further, there is evidence of a link between EV secretion and autophagy. Previously, the regulation of EV secretion has not been well understood, but recent findings point to components of autophagy controlling the process (Leidal & Debnath, 2021). In the context of the R120G mutation, clearance of protein aggregates is vital to cardiomyocyte survival and maintenance of proteostasis. With reduced activity of the UPS in R120G KI hearts, and the predominant fraction of insoluble CryAB in the cell as examined previously, other mechanisms for quality control may be employed, such as the utilization of extracellular vesicles. To investigate if EVs participate in quality control, we conducted experiments to determine if the mutant CryAB protein was secreted from cells in R120G KI hearts.

First, we isolated EVs from both wild-type and R120G KI mouse hearts using a differential centrifugation protocol (Figure 7). After isolation of small EVs, large EVs, and exopher fractions, the proteins were separated on SDS-PAGE and analyzed by Western blotting. The membranes were stained with anti-CryAB and EV markers CD81 and flotillin (Zaborowski et al, 2015). To examine possible crosstalk between EV secretion and autophagic activity,

common autophagy-lysosome pathway markers ubiquitin, LC3, and p62 were also measured (Leidal & Debnath, 2021). We also determined total vesicle yield from each sample by Bradford assay. We found that CryAB levels significantly increased in exophers and small EV fractions prepared from R120G KI mice as compared to wild-type. In contrast, CryAB was not secreted in EVs in wild-type hearts (Figure 8A & 8C). Further, ubiquitin, LC3II and p62 levels all increased in R120G KI EVs compared to the wild-type EVs, though only the ubiquitin and p62 levels in exophers were significantly different (Figure 8B & 8C). This is likely due to high variation within the data. Total vesicle yield was similar between wild-type and R120G KI mice (Figure 8C). The data indicates that cells are secreting the mutant R120G proteins in exophers and EVs, suggesting that this might function as an alternative control mechanism to remove CryAB R120G aggregates from the cell. Further, increased levels of p62, LC3II and ubiquitin in EVs suggest potential cross talk between the autophagy and secretion pathways in the heart.

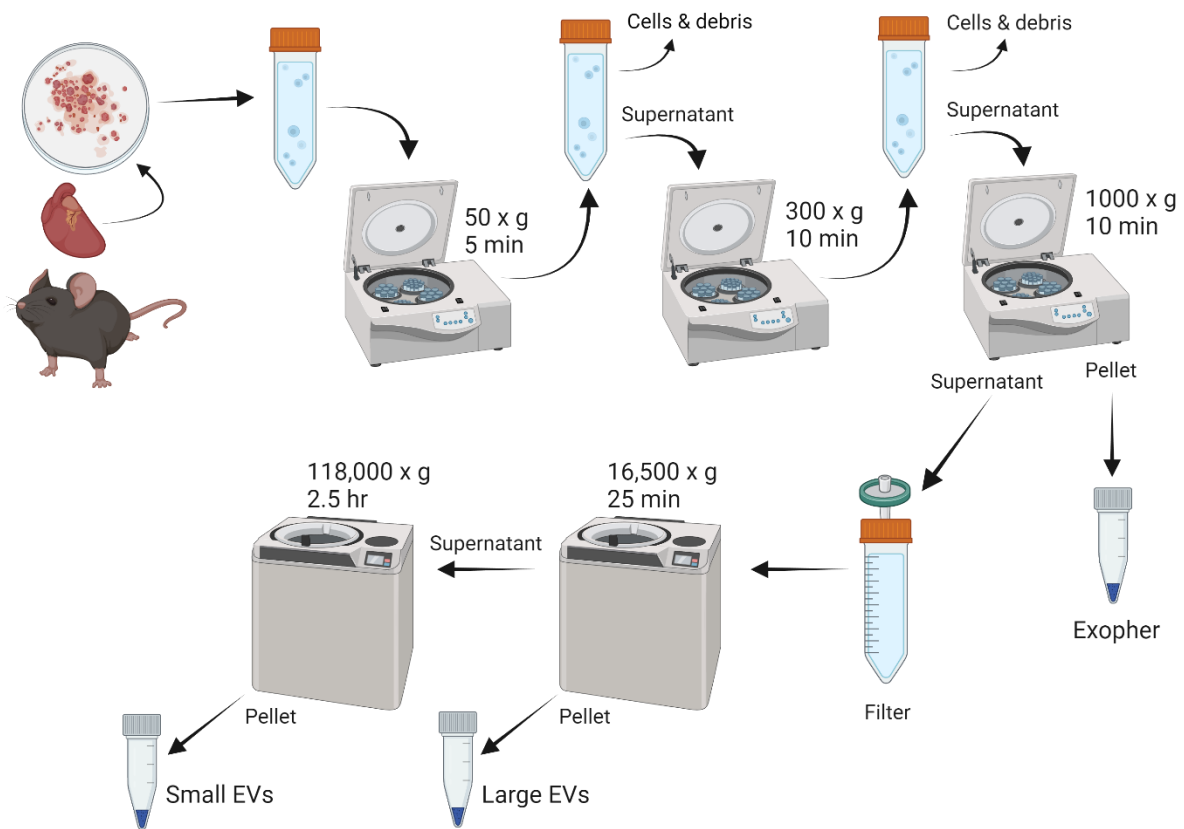


Figure 7: Protocol for isolating exophers, small and large extracellular vesicles using differential centrifugation. After mincing tissue in HBSS containing Liberase HD and DNase I, cellular debris is separated from supernatant by a series of centrifugation spins. Exophers are isolated at 1000 x g. Further centrifugation at 16,500 x g and 118,000 x g isolate large and small EVs respectively.

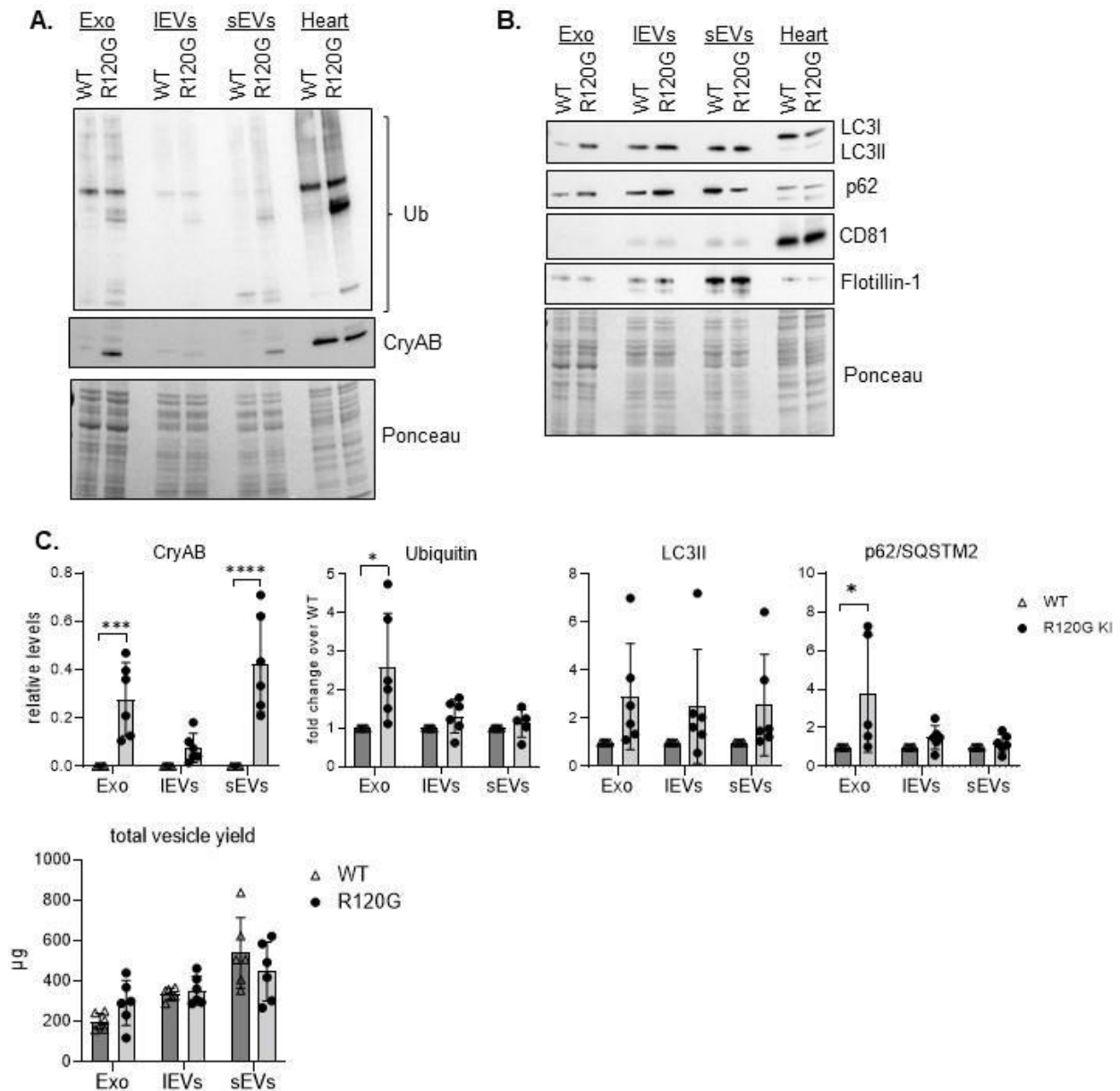


Figure 8: CryAB R120G cardiomyocytes secrete vesicles carrying CryAB, which display higher levels of ubiquitin, LC3II and p62. **A)** Exophers, large and small EVs were isolated from wild-type and R120G KI mouse hearts (aged 3-4mo) and evaluated by western blot. Whole heart lysates were also included for comparison. Representative Western blots of CryAB and ubiquitin protein levels. Ponceau stain is shown to display total protein levels in each sample. **B)** Representative Western blots of LC3I, LC3II, p62, CD81, and flotillin-1 protein levels. **C)** Quantification of CryAB, Ubiquitin, LC3II, p62 and total vesicle levels (n=6; *p<0.05, ***p<0.001, ****p<0.0001). Analysis was performed using Student's T-test.

Inhibition of the UPS decreases EV secretion in CryAB R120G cardiomyocytes

Evaluation of CryAB in extracellular vesicles in R120G hearts suggests that secretion might function as an alternative quality control mechanism due to a decline in proteasomal activity. Therefore, we conducted experiments evaluating the relationship between UPS activity and EV secretion in the heart. Bortezomib is a proteasome inhibitor, typically employed as a cancer treatment drug (Chen et al, 2011). By inhibiting function of the ubiquitin-proteasome system with Bortezomib, we expect protein aggregation to increase, thereby eliciting a greater need for quality control in the cell to preserve proteostasis. If extracellular vesicles participate in this action, more will be secreted in response to accumulating levels of CryAB R120G aggregates and transport them out of the cytosol.

We first injected wild-type and R120G KI mice (age 3-4mo) with either Bortezomib (BTZ) or saline at 1mg/kg. Injections occurred at 0 and 24 hours, with hearts harvested at 48 hours after initial injection. Exophers, large and small EVs were evaluated by Western blot analysis. Results show that treatment with BTZ lowers EV secretion in R120G KI mice, indicated by the ponceau staining and decreased levels of CD81; however, differences between sEV and exopher CD81 levels were not considered statistically significant. This is possibly due to large amounts of variation in the small data pool and more experimental repeats may reveal more definitive trends (Figure 9A & B). Assessing relative levels of CryAB in R120G KI EVs, there was a decrease in BTZ-treated fractions, in line with observed changes in vesicle yield from the ponceau staining. This change was statistically significant in large and small EVs, but not in exophers due to an outlier in the data (Figure 9C & 9D). Finally, levels of LC3I & II increased in BTZ treated R120G KI hearts, suggesting increased formation of autophagosomes (Figure 9E).

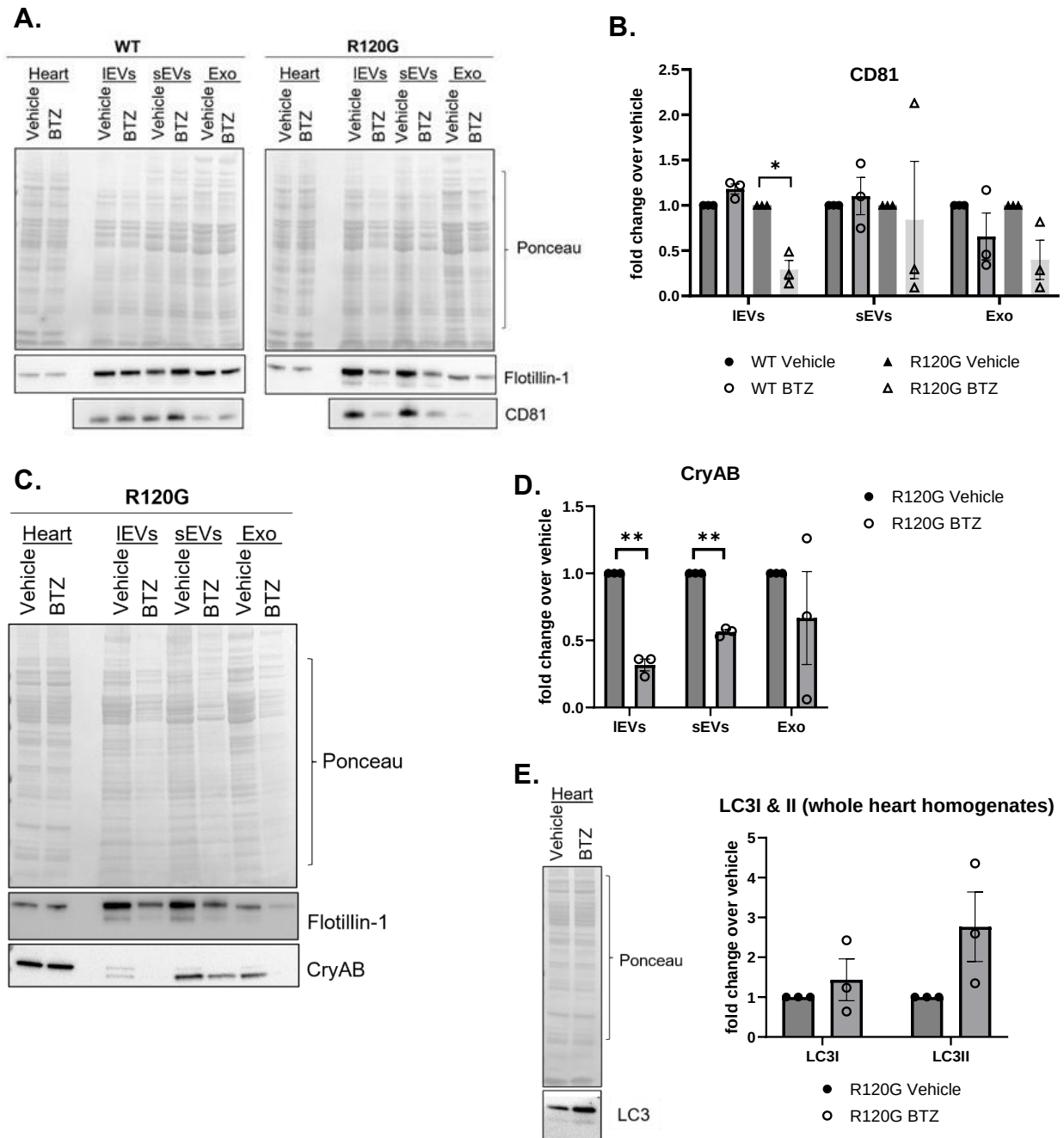


Figure 9: EV secretion decreases with UPS inhibition. **A)** After 2 doses of Bortezomib (BTZ) at 1mg/kg spaced 24 hours apart, EV fractions from WT and R120G KI mice (aged 3-4mo) were evaluated by Western blotting. Representative Western blot of flotillin, CD81, and relative EV levels (Ponceau stain). **B)** Quantification of CD81 levels in vehicle and BTZ mice. Fold change calculated over the vehicle group (n=3, *p<0.05). **C)** Representative Western blot of CryAB, flotillin and LC3, following the same conditions as previous. **D)** Quantification of CryAB and in R120G KI vehicle and BTZ EV fractions (n=3; **p<0.01). **E)** Representative Western blot and quantification of LC3I & II in whole heart homogenates (n=3). Analysis was performed using Student's T-test.

Chapter 3

Macrophages are not activated by exophers and EVs

Macrophages are an important part of the immune system, functioning to clear infections and bolster wound repair. A crucial characteristic of macrophages is their ability to elicit an inflammatory response in tissues, typically responding to foreign pathogens as a mechanism to create a hostile internal environment to prevent the spread of infection. However, prolonged states of inflammation can be damaging to tissue, which is why a countermeasure also exists within macrophages to release out anti-inflammatory signals and initiate tissue repair. Both mechanisms are carried out by two different conformations of macrophages– M1, which are pro-inflammatory, and M2, which are anti-inflammatory. A potential mechanism by which R120G causes tissue damage and myocardial dysfunction may be chronic inflammation.

Extracellular vesicles play a role in cell-cell communication, carrying cargo that is taken up by recipient cells to relay a signal. Thus, it is possible that vesicles secreted in the hearts containing the mutant CryAB protein may be taken up by macrophages, initiating a defensive response and causing inflammation. To test if secreted vesicles containing CryAB R120G trigger activation of macrophages, isolated EVs from hearts of wild-type and R120G KI mice were added to cultured Raw 267.4 macrophages. After 24 h incubation with the vesicles, the cells were analyzed by Western blot to determine whether the vesicles promoted activation of macrophages to M1 or M2 phenotype. iNOS is a protein marker of pro-inflammatory M1 macrophages and CD206 is an indicator of pro-reparative M2 phenotype. Time course experiments of CryAB R120G levels were also performed to evaluate uptake of the mutant protein in macrophages. We discovered that no EV fraction resulted in any expression of iNOS (M1 marker) in macrophages. Increases in CD206 protein levels (M2 marker) were also not

observed after treatment with any EV fraction, remaining at levels matching the untreated macrophages (Figure 10A & 10B). Investigating if we could detect CryAB R120G in macrophages after adding exophers or EVs in time course experiments, we were unable to detect the mutant protein by Western blotting (Figure 10C), suggesting rapid degradation of the protein upon cellular uptake.

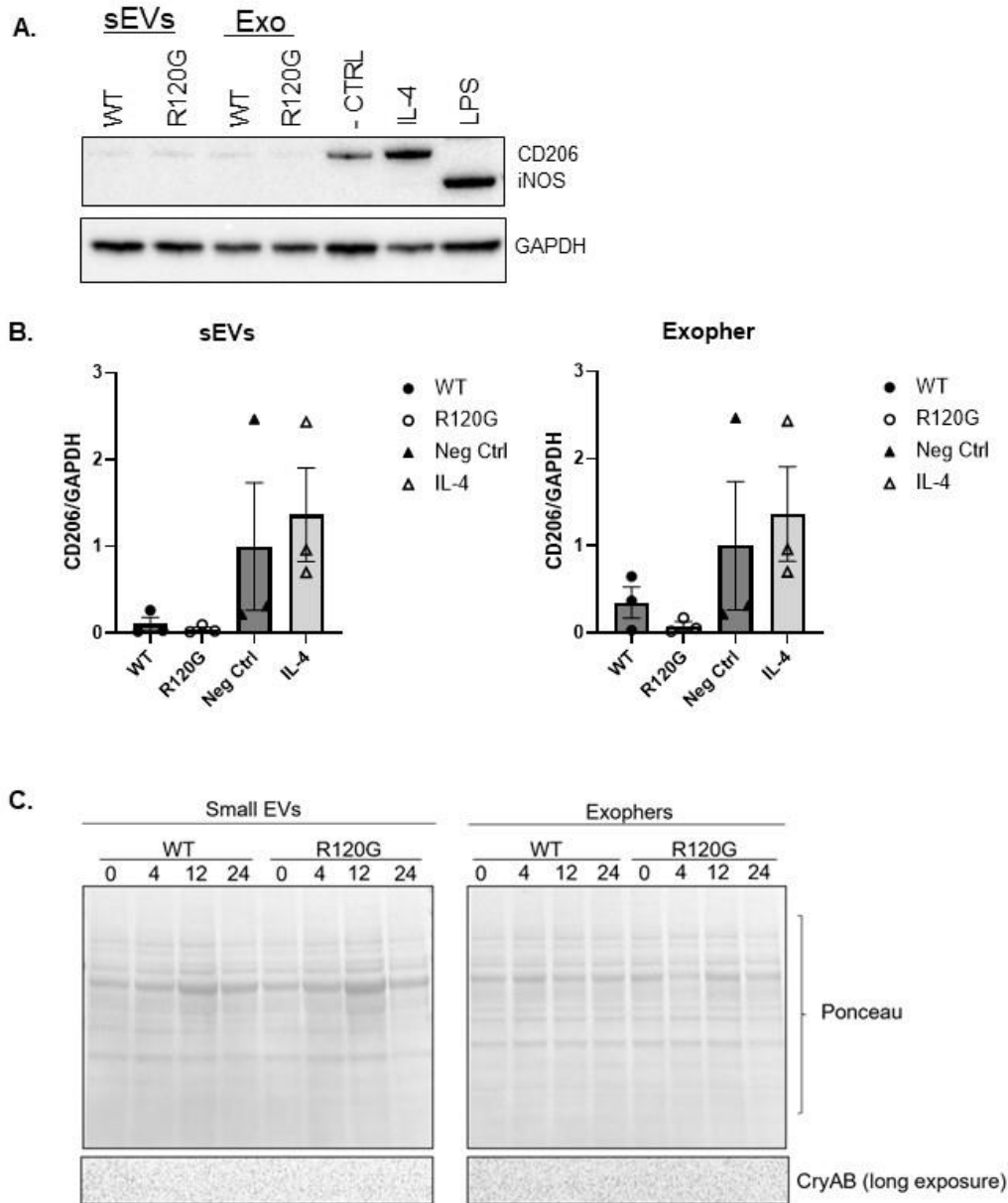


Figure 10: Extracellular vesicles and exophers do not activate macrophages. A) Raw 264.7 macrophage cells were treated with exophers and small EVs isolated from hearts of WT and R120G KI mice (aged 3-4mo). Macrophages were also treated with IL-4 and CD206 to induce differentiation into M2 and M1 conformations respectively, serving as positive controls. Negative control macrophages were untreated. After 24 hours, macrophages were harvested for Western blot analysis. Representative Western blot of CD206, iNOS and GAPDH levels. GAPDH was used as a loading control. **B)** Macrophages were harvested at 0, 4, 12, and 24 hours after treatment with exophers or small EVs isolated from hearts of WT and R120G KI mice. Representative Western blot of CryAB and GAPDH levels. **C)** Quantification of CD206 levels in sEVs and exophers.

SUMMARY

The data shown displays the varying effects of the α B-Crystallin R120G mutation on cardiomyocyte function. We demonstrate that there is a significant presence of insoluble CryAB protein in mouse R120G KI hearts not seen in wild-type. While histological evaluation did not reveal any changes in myocardium structure or collagen levels, multiple experiments do reveal changes in quality control. Activity of the ubiquitin-proteasome system (UPS) decreases while extracellular vesicles secreted from R120G KI hearts contain CryAB. Inhibition of the UPS pathway also appears to reduce EV secretion in R120G KI hearts. Finally, macrophages are not activated when exposed to EVs from R120G KI hearts.

DISCUSSION AND CONCLUSIONS

The results presented gather insight into how proteostasis and quality control are altered in CryAB R120G KI cardiomyocytes. Firstly, histological studies did not reveal significant myocardium restructuring or cell death in R120G KI hearts; however, study of soluble and insoluble protein levels by Western blot did reveal significant presence of insoluble CryAB in R120G KI hearts. We then demonstrated that there is reduced activity of the ubiquitin-proteasome system (UPS) in R120G KI hearts and identified that cells in the heart secrete extracellular vesicles (EVs) containing the mutant CryAB as. Interestingly, inhibition of the UPS system did not increase vesicle secretion but appeared to reduce it in R120G KI hearts. Finally, secreted EVs did not activate macrophages to become pro- or anti-inflammatory cells. The histological sections showed no significant myocardium remodeling or cellular disorganization in R120G KI hearts. Overall, this research suggests the CryAB R120G mutation influences cellular quality control mechanisms, and that extracellular vesicles play a role in clearing mutant CryAB from the cell, possibly linked with autophagic pathways.

Cardiomyopathy is broadly defined as a disease of the myocardium, which is characterized by either muscular or electrical dysfunction. The disease can be either acquired or genetic— as in the case of CryAB R120G— but ultimately leads to heart failure if severe and left untreated (Wexler et al, 2010). A key component of disease progression in many types of cardiomyopathies is cardiac remodeling. Cardiac remodeling describes a change in morphology of the heart involving fibrosis, often as an attempt to preserve efficiency and function in the presence of cardiac disease or injury, or resulting from genetic mutation (Seidman & Seidman, 2001). Fibrosis is a mechanism in which collagen is secreted by activated fibroblasts to either expand the interstitial space for reinforcement of the myocardium, or to replace dead

cardiomyocytes in order to preserve cardiac shape (replacement fibrosis). In both cases, while these mechanisms can initially preserve function, increased collagen in the heart leads to ventricular wall thickening and stiffness, causing dysfunction and eventual heart failure (Murtha et al, 2017). Desmin-related cardiomyopathy, which is the type of cardiomyopathy linked with CryAB R120G, is characterized by accumulation of insoluble protein aggregates, cell death and subsequent replacement fibrosis (He et al, 2010). We demonstrated aggregation to be present in our investigation of soluble versus insoluble CryAB in the mouse hearts aged 8-10 weeks. We found that CryAB R120G KI hearts contained significantly higher proportions of insoluble CryAB compared to wild-type. This suggests that misfolded CryAB R120G is forming insoluble aggregates in cardiomyocytes, in line with our understanding of how desmin-related myopathies develop.

We then investigated if this insoluble aggregation was causing fibrosis and cardiac remodeling by using histological analysis, staining wild-type and R120G KI mouse heart sections for Masson's trichrome, which can differentiate collagen (blue) and nuclei (black) from muscle fiber (red; de Vlekkert et al, 2020). We expected older R120G KI mice (aged 1 year) to show progression of disease marked by increased collagen levels due to cell death; however, there was not a discernible difference between wild-type and R120G samples in neither young (3-4 months) nor old (1 year) mice. We also used hematoxylin and eosin (H&E) staining on both groups to view possible structural changes to the myocardium, but this also displayed no difference between groups. These results likely suggest the mice studied were not progressed enough in heart disease to display a significant amount of tissue disorganization and remodeling, as it is unlikely these are not factors in CryAB R120G disease progression. In mice, one year is only considered middle-aged, so further studies may need to employ even older mice or mice

artificially progressed further along in the disease to show a difference in cardiac health at the structural level.

The ubiquitin-proteasome system (UPS) is the main quality control mechanism utilized by the cell to degrade misfolded proteins, preventing aggregation and maintaining proteostasis (Li et al, 2022). The R120G mutation in CryAB causes the protein to become misfolded— a change from arginine to glycine causing structural deformity that prevents CryAB from exhibiting its normal chaperone-like functionality (Bova et al, 1999). It would be expected in the case of CryAB R120G that UPS activity would be upregulated, responding to the increased misfolded CryAB present within the cell. However, when we measured and compared proteasomal activity between wild-type and R120G KI hearts, this was not the case. In younger mice (3-4 mo), UPS activity was decreased whereas in older mice (1 year) activity levels were similar. There are a few possible explanations for this. First, protein aggregation from misfolded CryAB may inhibit proteasome function, such as seen with A β aggregates in the progression of Alzheimer's disease (Schmidt & Finley, 2013). We demonstrated that hearts from mice as young as 8-10 weeks contain insoluble CryAB aggregates. However, it may also be that the UPS components are downregulated due to upregulation of autophagy in the presence of the CryAB R120G aggregates. Autophagy, the cell's primary mechanism for degrading protein aggregates, may be employed at a higher level in younger mice with CryAB R120G aggregation, leading to less utilization of the UPS as seen in our results. In following experiments, we measure common autophagic markers to assess any possible changes in activity, but more investigation is needed to determine any causality for these changes in activity.

Recent studies have confirmed that extracellular vesicles (EVs) play a role in cellular quality control, possibly linked with the mechanisms that dictate the autophagy-lysosome

pathway for protein aggregate degradation (Leidal & Debnath, 2021). Studies from our lab reported that large EVs can carry intact mitochondria for degradation and would increase in secretion when lysosomal-mediated degradation was inhibited (Liang et al, 2023). Further, exophers, a sub-type of extracellular vesicle characterized by its large relative size (average 3.5µm), have been shown to mediate extrusion of neurotoxic protein aggregates in *C. elegans* and have been more recently identified to be secreted in mouse cardiac tissue (Nicolás-Ávila et al, 2022). These studies suggest that extracellular vesicles could assist in quality control of CryAB R120G aggregates. To address this question, we compared EVs from wild-type and R120G KI mouse hearts, looking for levels of CryAB cargo within. After conducting the experiments, we found that CryAB was present in small EVs and exophers from hearts of R120G KI mice. CryAB was not detected in vesicles secreted in wild-type hearts. This suggests that in the context of the CryAB R120G mutation, EVs may help export CryAB aggregates out of the cell, participating in quality control and maintenance of proteostasis. Although this result provides strong evidence for this conclusion, a key experiment needed is to determine if inhibiting EV secretion leads to increased CryAB R120G aggregation in hearts.

We also examined levels of p62, ubiquitin, and LCII across EVs from wild-type and R120G KI mouse hearts. Only levels of ubiquitin and p62 were considered statistically significantly increased in R120G KI exophers compared to wild-type, but nearly all EV types showed an increase of these markers in R120G KI. All three markers are indicators of autophagy, suggesting cross talk between the EV secretion pathway and autophagy in the context of CryAB R120G. LC3 and p62 are proteins that facilitate docking of autophagosome membrane to cargo for engulfment by the autophagosome (Liu et al 2016).

Another approach we used to test the utilization of EVs in maintaining cellular quality control was by inhibiting UPS function to determine if EV secretion would increase to compensate loss of internal protein degradation. Bortezomib is an anti-cancer drug, which inhibits the ubiquitin-proteasome system to limit tumorigenesis (Chen et al, 2011). By injecting mice with Bortezomib (BTZ) with 2 doses over 48 h, we sought to inhibit proteasomal activity and assess the downstream effect on EV secretion in heart tissue. We anticipated secretion of EVs to increase with UPS inhibition, accounting for a decrease in protein degradation inside the cell. We specifically expected this in the context of CryAB R120G, where there is an increased amount of misfolded proteins present in cardiomyocytes. Interestingly, we observed the opposite effect from the ponceau and CD81 staining, in which EV secretion decreased in R120G KI hearts with UPS inhibition. This was not observed in wild-type hearts, with inherently no change between vehicle and Bortezomib-treated groups. We then measured levels of LC3I & II in whole heart homogenates to determine if autophagy was upregulated as a possible explanation for this result. Increased LC3II levels indicate more autophagosome formation. In measuring differences between R120G KI hearts, there was an observed increase in both LC3I & II in the R120G KI Bortezomib group, though not considered significant due to variability in samples. Increases in autophagic degradation of R120G may compensate for the inhibition of the UPS, leading to the observed decreased EV secretion. In comparing CryAB in only R120G KI EVs, levels decreased in BTZ-treated groups where vesicle yield was low. The data suggests Bortezomib does not impact existing secretion of CryAB in R120G KI EVs. Further, these experiments do not suggest much about the role of EVs in CryAB R120G quality control but do present more questions for the relationship between EVs, autophagy, and the UPS.

Finally, we examined the possible role of EV secretion in activating macrophages. Data suggests that EVs participate in quality control in the context of CryAB R120G; however, secretion of EVs carrying misfolded CryAB may also induce a pathogenic effect on cardiac tissue. An important characteristic of macrophages is their ability to differentiate between M1 and M2 forms. M1 is a pro-inflammatory phenotype, commonly triggered by the presence of pathogens in the body (Lendeckel et al, 2022). This form can also be triggered by the presence of misfolded protein via the unfolded protein response (UPR)-- a series of signals taking place in the endoplasmic reticulum in response to misfolded protein in the cell, ending with secretion of inflammatory cytokines (Grootjans et al, 2016). Long-term inflammation is damaging to the cell and M2 macrophages, which exhibit a pro-reparative form, are employed to quell inflammation. Macrophages can also rapidly switch between phenotypes in response to changing conditions (Wang et al, 2014). In previous studies, it has also been shown that secreted EVs are taken up by macrophages and can trigger a change in phenotype (Tang et al, 2022). If secreted EVs containing misfolded CryAB are causing consistent inflammation in cardiac tissue, it could lead to the progression of heart failure. Our results suggest that this is not the case. iNOS, a marker for M1 activation, was not measured in macrophages treated with R120G KI EVs. We did measure slight levels of marker CD206 in both wild-type and R120G KI EV-treated macrophages, indicating small amounts of M2 activation, but this is likely non-significant. Levels of CD206 appeared lower in EV-treated macrophages compared to the non-treated negative control, suggesting EVs may affect differentiation, but more research is needed to determine if this is correct. No CryAB R120G is detectable in macrophages introduced to R120G KI EVs. It is possible the amount of CryAB contained in EVs is too low to be detected on Western blot, or that the protein is quickly degraded after uptake.

Overall, data displays that protein quality control mechanisms are altered in the context of the R120G KI mutation and that extracellular vesicles secreted by R120G KI cardiomyocytes contain CryAB, suggesting a role in maintaining proteostasis. Future studies should seek to pharmacologically inhibit extracellular vesicle secretion, observing if misfolded CryAB aggregation increases inside the cells as a result. This would further confirm EV participation in the quality control of aggregates. Additional evidence in this project has continued to point to a possible link between EV secretion and autophagic machinery. More studies should be carried out to better understand the interplay between UPS and autophagy mechanisms, as well as with EV secretion. This could include further repeats of experiments outlined in this project, solidifying trends and statistically significant differences between the wild-type and R120G KI. More investigation of R120G KI extracellular vesicle uptake in macrophages is also needed, likely with different experimental conditions, to better understand how EVs are processed by the macrophage and determine if misfolded CryAB cargo does activate a response not yet measured. Increasing the concentration of EVs in the treatment or investigating if rapid lysosomal degradation is occurring in the macrophages are both potential next steps. Finally, the utilization of older mouse populations (>1yr age) may reveal more comprehensive evidence of disease progression in the R120G KI line.

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