Short-term rapamycin persistently improves cardiac function after cessation of treatment in aged male and female mice.

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Abstract

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Cardiac aging is an intrinsic process that results in impaired cardiac function and dysregulation of cellular and molecular quality control mechanisms. These effects are evident in the decline of diastolic function, increase in left ventricular hypertrophy, metabolic substrate shifts, and alterations to the cardiac proteome. This thesis covers the quality control mechanisms that are associated with cardiac aging, results from an anti-aging intervention in aged mice, and a review of mitochondrial dysfunction in the heart. Chapter one is a review of the quality control mechanisms in aging myocardium. Chapter two consists of the results of several mouse experiments that compare the cardiac function, proteomes, and metabolomes of aged and young controls, along with rapamycin treated aged mice. The novelty of this study comes from the inclusion of a group of animals treated only transiently with the drug, then followed for eight weeks post-drug-removal. This persistence cohort may hold clues to deriving long-lasting benefits of rapamycin with only transient treatment. Chapter three includes more results from the cohorts used in chapter two, from work done by our collaborators in two laboratories at the University of Washington. Finally, chapter four is a review of the mechanisms and phenotypes of mitochondrial dysfunction in the aging heart. The goal of my thesis work is to test the persistence of the improvement of cardiac function by rapamycin treatment, and use the correlating changes in the cardiac proteome and metabolome to discover a novel mechanism of functional improvement of the heart in aged animals.
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DEDICATION

For Chris.
1.1 Abstract

This chapter is adapted from (Quarles et al. 2015).

Cardiac aging is an intrinsic process that results in impaired cardiac function, along with cellular and molecular changes. These degenerative changes are intimately associated with quality control mechanisms. This review provides a general overview of the clinical and cellular changes which manifest in cardiac aging, and the quality control mechanisms involved in maintaining homeostasis and retarding aging. These mechanisms include autophagy, ubiquitin-mediated turnover, apoptosis, mitochondrial quality control and cardiac matrix homeostasis. Finally, we discuss aging interventions that have been observed to impact cardiac health outcomes. These include caloric restriction, rapamycin, resveratrol, GDF11, mitochondrial antioxidants and cardiolipin-targeted therapeutics. A greater understanding of the quality control mechanisms that promote cardiac homeostasis will help to understand the benefits of these interventions, and hopefully lead to further improved therapeutic modalities.

1.2 Introduction

Cardiac aging is an intrinsic process that results in impaired cardiac function, along with cellular and molecular changes. These degenerative changes are intimately associated with quality control mechanisms. This review provides a general overview of the clinical and cellular changes which manifest in cardiac aging, and the quality control mechanisms involved in maintaining homeostasis and retarding aging. Finally, we discuss aging interventions that have been observed to impact cardiac health outcomes.

1.3 Overview of Cardiac Aging

1.3.1 Human cardiac aging

A growing body of studies examining human aging and centenarians are beginning to address what healthy aging means for the CV system (Galioto et al. 2008). Centenarians have lower prevalence of CV diseases, hypertension, myocardial infarction, angina, and diabetes than younger persons (ages 70–99 years) (Selim et al. 2005; Galioto et al. 2008). This trend toward protection from CV-related causes of death (hypertension, heart disease, diabetes) is also present in their descendants, pointing to a genetic or epigenetic healthy aging profile (Perls & Terry 2003). Multiple studies have followed CV risk factors and CV health in long-lived populations and while some aspects of disease incidence and primary risk factors differ between groups, the recurring conclusion is that a boost to cardiac health occurring early in life (either through genetics or lifestyle) and maintained through life (also by some combination of genetics and lifestyle) is a common piece of the longevity puzzle (Curb et al. 1990; Yashin et al. 2006). As more studies (and the cohorts within them) mature, there will be more data available on why some humans succumb to aging-related disease early, while others last into their 10th decade.
The Framingham Heart Study and the Baltimore Longitudinal Study on Aging demonstrated that in apparently healthy adults, aging is associated with increase in left ventricular wall thickness measured by echocardiography. The Doppler measurement of the $E/A$ ratio, the ratio between early ($E$) and late ($A$) diastolic LV filling, declines dramatically with age in both mice and humans (Dai & Rabinovitch 2009; Dai et al. 2009). This decline in the $E/A$ ratio suggests that a greater portion of blood filling in the LV results from late diastolic filling as opposed to early diastolic filling, which is clinically defined as diastolic dysfunction or heart failure with preserved ejection fraction (HFpEF). The prevalence of LV hypertrophy and diastolic dysfunction significantly increased in the elderly (Bursi et al. 2006), even in an apparently healthy elderly population without hypertension, suggesting that intrinsic cardiac aging may manifest as the above changes.

Although systolic function determined from ejection fraction is relatively preserved at rest in the elderly, exercise capacity and cardiovascular reserve after prolonged exercise significantly declines with age (Correia et al. 2002). Aging also contributes to the decline of the maximal heart rate during strenuous exercise, but does not affect the resting heart rate when lying face up (Fleg et al. 1995). The decrease in exercise capacity in the elderly is attributed to a modest decrease in ejection fraction after maximal exercise and a prominent decline in maximal heart rate at peak exercise. Likewise, there is age-dependent decline in maximal cardiac index, another measure of systolic function calculated as the cardiac output normalized to the body surface area, which is mostly due to a decline in maximal heart rate after strenuous exercise.

The increased fraction of LV filling performed by atrial contraction in diastolic dysfunction also increases atrial pressure, adversely contributing to atrial hypertrophy and dilatation and subsequently increasing the risk of atrial fibrillation, consistent with the significant age-dependent increase in the prevalence of atrial fibrillation (Lakatta 2003; Lakatta & Levy 2003a; Lakatta & Levy 2003b). Atrial fibrillation adversely affects exercise capacity in the geriatric population. It also predisposes to the development of HFpEF. Indeed, HFpEF accounts for more than half of all heart failure cases in patients older than 75 years old, especially in those without structural or ischemic heart diseases.

Valvular changes in old age include myxomatous degeneration, deposition of collagen and calcium leading to sclerosis of the valves. Aortic valve sclerosis is present in 30–80% of the elderly (Stewart et al. 1997; Nassimiha et al. 2001; Karavidas et al. 2010), which is detected by echocardiography as calcification of aortic valve leaflets and aortic annulus (Otto et al. 1999; Freeman & Otto 2005). Age-related aortic valve sclerosis predisposes to the development of aortic stenosis and increased leaflet calcification and decreased leaflet mobility may predict the progression to aortic stenosis. Hypertension, LV hypertrophy, hyperlipidemia, smoking, end-stage renal disease and congenital bicuspid aortic valves are important risk factors for the progression to aortic valve stenosis (Olsen et al. 2005).

In the elderly, fibrosis and valvular calcification are the most common factors contributing to the development of aortic stenosis, which occurs when the aortic valve opening narrows due to the stiffening and calcification of the aortic valve leaflets (Olsen et al. 2005). This narrowing prevents effective blood pumping through aortic valve, generating a pressure gradient between the aorta and the left ventricle. To compensate for this obstruction, the walls of the left ventricle
thicken with myocardial hypertrophy to maintain sufficient systolic function. Later in the progression, increased wall stress due to pressure overload causes the left ventricle to dilate, leading to deterioration of systolic function. In addition, aortic regurgitation, also related to the calcification of the aortic cusps and annulus, increases with age, and is present in 13–16% of the elderly population (Nassimiha et al. 2001). The presence of aortic regurgitation results in ineffective work of the left ventricle and volume overload that may lead to LV dilatation and systolic heart failure.

The above ventricular and valvular changes in cardiac aging compromise the cardiac functional reserve capacity as well as lower the threshold for development of heart failure (Correia et al. 2002). This makes the aged heart more susceptible to stress and disease-related challenges, leading to increased prevalence of heart failure and CV mortality in the geriatric population.

1.3.2 Large mammal models of cardiac aging

Canine hearts develop several aging changes, including myocardial hypertrophy, accumulation of lipofuscin and amyloid which cause increased myocardial stiffness. Degenerative valvular heart diseases are also common in dogs older than 16 years, the prevalence of which approaches 75% in some breeds (Kwart & Haggstrom 2000). The dog model has been widely used for electrophysiological studies since the distribution of cardiac conduction system and activation sequence (electrophysiological properties) in dogs closely resembles that of the human heart (Hamlin & Smith 1960). Aged dog hearts demonstrated prolonged action potential duration and decreased responsiveness to adrenergic stimulation as well as increased risk of developing sick sinus syndrome and atrial fibrillation (Anyukhovsky et al. 2005).

Aged rhesus monkey demonstrate several age-related cardiac pathologies, including aortic and mitral valve degenerative calcifications, loss or degeneration of myocardial fibers with hypertrophy of remaining cardiomyocytes, lipofuscin accumulation and variable degrees of myocarditis, multifocal interstitial fibrosis, myocardial infarction, and congestive heart failure (Lane et al. 1999; Mattison et al. 2003; Roth et al. 2004). As shown by the National Institute of Aging's longitudinal study of aging in rhesus monkeys (Macaca mulatta), monkeys fed with normal diets develop many of the above cardiac pathologies but did not develop spontaneous atherosclerotic plaques unless they were fed high fat diets.

1.3.3 Rodent models of cardiac aging

Cardiac aging in the mouse model closely recapitulates the age-related changes found in human hearts (Dai et al. 2009; Boyle et al. 2011). Using echocardiography to examine the age-related changes in cardiac structure and function in a mouse longevity cohort, we found a significant age-dependent increase in LV mass index (LVMI) and left atrial dimension (Dai et al. 2009). Systolic function measured by fractional shortening showed only a modest decline with age. Diastolic function, measured by tissue Doppler imaging of $E_d/A_a$, significantly declined with age, with substantial fraction of mice older than 24 months with diastolic dysfunction (defined by $E_d/A_a < 1$). Morphometric analysis indicated an increased myocardial fiber size, increased fibrosis and amyloid deposition with age, especially in the subendocardial areas (Dai et al. 2009). Myocardial performance index (MPI), an indicator of global systolic and diastolic
function, was also significantly impaired with age. All of the above aging phenotypes are also found in middle age mitochondrial mutator (Polg<sup>D257A/D257A</sup>) mice, a model of “premature aging” (Dai <i>et al. 2010</i>).

Previous studies in Fischer rat heart aging using a pressure–volume catheter and echocardiography consistently revealed age-dependent left ventricular hypertrophy, impairment of systolic and diastolic function, as well as increased prevalence of mitral regurgitation (Anversa <i>et al. 1989</i>; Forman <i>et al. 1997</i>; Boluyt <i>et al. 2004</i>). Histopathology of aged rat hearts demonstrated cardiomyocyte hypertrophy and increased LV fibrosis (Forman <i>et al. 1997</i>), which reduced LV elasticity and led to diastolic dysfunction. Aging rat hearts also showed decreased responsiveness to sympathetic and dobutamine stimulation (Ahmet <i>et al. 2011</i>).

### 1.4 MECHANISMS OF CARDIAC AGING

The mechanism of age-dependent LV hypertrophy in mice includes activation of the calcineurin-NFAT pathway, which is well known to mediate pathological hypertrophy (Dai <i>et al. 2009</i>). Calcineurin is a phosphatase that dephosphorylates and activates the transcription factor NFAT, which then translocates into nucleus and interacts with several other transcription factors (e.g., GATA4) to initiate transcription of hypertrophic genes, such as atrial natriuretic peptides and brain natriuretic peptides. The mechanisms of diastolic dysfunction in aged heart include fibrosis and subsequent reduced elasticity of the ventricles. In addition to increased interstitial collagen, there is increased matrix metalloproteinase (MMP) and decreased tissue inhibitor of metalloproteinase (TIMP) abundance in fibrotic aged heart (Lindsey <i>et al. 2005</i>). Delay in active ventricular relaxation in aged heart is attributable to a reduced abundance of sarco(endo)plasmic reticulum calcium ATP-ase (SERCA2), and to an oxidative modification of SERCA2, both of which affect the rate of diastolic calcium reuptake (Adachi <i>et al. 2004</i>; Dai <i>et al. 2009</i>).

#### 1.4.1 Metabolic changes in cardiac aging

Metabolic dysfunction is associated with aging and many age-related diseases (Hu & Liu 2014). In the heart, aging changes in metabolism include reduced fatty acid (FA) metabolism and oxidative phosphorylation (OSPHOS), and compensatory increases in glucose uptake and glycolysis in mice (Stanley <i>et al. 2005</i>). These changes in FA metabolism and OXPHOS recapitulated those seen in humans (Kates <i>et al. 2003</i>). Despite intense research in this area, there is little consensus on the causes of this “metabolic substrate shift” or whether the shift in substrate utilization in failing and aging myocardium is detrimental or compensatory (van Bilsen <i>et al. 2009</i>). The possibility of the latter is raised by observations that increasing glucose as a substrate, rather than FAs, increased contractility in the LV in humans, pigs, and dogs (Stanley <i>et al. 2005</i>). One explanation for this is that FA oxidation both leads to excessive loss of ATP through UCP3, and that it carbohydrate utilization is more efficient than FA oxidation in terms of ATP synthesis (Lardy & Pressman 1956; Himms-Hagen & Harper 2001; Stanley & Chandler 2002). Consequently, the power of the LV contraction seems to be improved when the primary metabolic substrate is glucose rather than fatty acids in adult Sprague-Dawley rats (Burkhoff <i>et al. 1991</i>). It could be that the substrate shift is therefore a compensatory mechanism to improve contractility in the aging heart.
Sirtuins may play an important role in cardiovascular aging. Sirtuins are NAD⁺-dependent class III histone deacetylases involved in the post-translational modification (PTM) of numerous targets (Longo & Kennedy 2006). They can catalyze succinylation, malonylation, and lysine deacetylation (Du et al. 2011; Rardin et al. 2013) and reviewed in (Nakagawa & Guarente 2011). Sirtuins are highly conserved, being found in organisms from bacteria to humans (Brachmann et al. 1995). The yeast Sir2 (silent information regular 2) was the first to be well characterized and shown to affect lifespan and stress response (Kaeberlein et al. 1999). Seven mammalian sirtuins (Sirt1–Sirt7) have been described (Dali-Youcef et al. 2007), and Sirt3, -4, and -5 are present in the mitochondria. Of these, Sirt3 has the strongest activity in the heart. Sirt1, -6, and -7 are largely nuclear, and Sirt2 is located in the cytoplasm (Houtkooper et al. 2012).

There are many examples in the literature suggesting connections between sirtuin enzyme activity, metabolism, and aging (for recent review of this topic see (Pillai et al. 2014; Rehan et al. 2014). It is thought that sirtuins contribute to the regulation of metabolism by modifying mitochondrial enzymes and by acting as a sensor of energy status through their dependence on NAD⁺ concentration (Wu et al. 2014b). Due to this important connection to energy metabolism and the broad range of downstream targets, sirtuins have been proposed to function as “watchdogs” for energy dysregulation (Choudhary et al. 2009; Ozden et al. 2011). SIRT3 in particular may act as a regulator of mitochondrial metabolism and fatty-acid oxidation (Hirschey et al. 2010).

1.4.2 Age-related contractility changes

There are many potential causes of the contractility changes in aging noted above, including alterations in autophagy, proteostasis, inter-/intracellular signaling, mitochondrial lipid composition, and circulating factors.

In a study of 20-week calorie restriction (CR) treatment on aged C57BL/6 mice, changes in indicators of autophagy (LC3B-II to LC3B-I ratio, Beclin-1 expression) following CR were associated with preservation of cardiac geometry and contractile function, as determined by echocardiography (Han et al. 2012). Cardiomyocyte cell area was reduced by CR as was phosphorylation of mTOR. Interestingly, a decrease of phosphorylation of Akt/glycogen synthase kinase-3β (GSK–3β) due to CR treatment aligns this study with others that suggest that Akt regulation of autophagy in the heart is disrupted by aging. Hua and colleagues investigated the role of Akt on cardiac aging through the use of Akt over activation transgenic mice (Hua et al. 2011). They found that both wild-type Akt and especially overactive Akt were associated with decreased autophagic flux, Ca²⁺ dysregulation, and cardiac hypertrophy in aged (24–26 month old) mice. The authors suggest that autophagic dysregulation may play an important role in cardiac aging phenotypes such as contractile defects and hypertrophy (Hua et al. 2011).

The insulin/insulin-like growth factor (IGF) signaling cascade contains a multitude of participating enzymes and co-factors and affects many other signaling cascades (Corpas et al. 1993; Abbas et al. 2008). Perturbations in the IGF signaling have been implicated in alterations in body composition and neuroendocrine signaling along with cardiac functional declines (Yakar et al. 2005). IGF plays a significant role in somatic growth and regulation of apoptosis. Due to the varied functions of IGF, combined with its reduced secretion with aging, researchers have
looked to IGF signaling as a critical link in the biology of aging (Corpas et al. 1993). Cardiomyocyte mechanical function may be detrimentally affected by circulating insulin-like growth factor-1 (IGF-1) levels in aging mammals. A 2008 study found that liver IGF-1 deficient mice had improved cardiomyocyte function compared to aged controls. It also discussed that down-regulation of Akt, Klotho and phosphorylated-AMPK (adenosine monophosphate-activated protein kinase) due to aging was abrogated by IGF-1 deficiency, and this might play a role in protection from aging-induced cardiac functional decline (Li et al. 2008).

Regulation of endothelin-1 (ET-1) appears to be associated with contractile function. ET-1, which is secreted by endothelium, binds to membrane-bound receptor ETA on cardiomyocytes (Takayanagi et al. 1991; Yamamoto et al. 2005). It is involved in cell hypertrophy and vasoconstriction and it may be up-regulated with aging (Ito et al. 1993; Pieske et al. 1999). Ceylan-Isik and colleagues found that short-term treatment of 26–28 month old C57BL/6 mice with ETA receptor antagonist, and knock-out of the ETA receptor, partially abrogated aging-associated decline in contractile function and cardiac hypertrophy (Ceylan-Isik et al. 2013). This effect was dependent on autophagy and also resulted in a reduction of ROS generation and protection from protein damage (Ceylan-Isik et al. 2013).

Many studies have pointed to the regulation of autophagy in aging hearts as a lynchpin for the preservation or loss of cardiac function/geometry with interventions or aging, respectively. Promotion of autophagy by various mechanisms is associated with decreased hypertrophy, improved contractile function, reduced protein damage, and intracellular Ca²⁺ regulation (Goswami & Das 2006; Gurusamy & Das 2009; Taneike et al. 2010; Hua et al. 2011; Han et al. 2012; Kobayashi & Liang 2014; Mei et al. 2014). See Section 1.5.4 below.

Alterations in mitochondrial cardiolipin (CL) composition in aging heart mitochondria have been associated with cardiac functional impairment and mitochondrial respiratory dysfunction (Lee et al. 2006; Chicco & Sparagna 2007). Interestingly, a recent study by Mulligan and colleagues found that inhibition of certain types of CL remodeling could improve cardiac contractile function, along with hypertrophy and dilation, in 25 month old mice without altering age-associated disruption of mitochondrial function and ROS production (Mulligan et al. 2014). Delta-6 desaturase inhibition was used to prevent the age-related reallocation of poly-unsaturated fatty acids (PUFAs) on CL, in particular to prevent the switch between of linoleic acid (in young animals) to long-chain PUFAs (found in older animals). Contractility was much improved in the treated cohort, despite an apparent lack of change in mitochondrial function or measures of ROS production such as H₂O₂ production or lipid peroxidation (Mulligan et al. 2014). Studies of a novel CL-targeted therapeutic, SS-31, are discussed in Section 1.6.3 below.

Nitric oxide (NO), produced by three nitric oxide synthases (NOS) in myocardium, effects many aspects of cardiac maintenance and function including endothelial vasorelaxation, gene expression, contractility, oxygen consumption, apoptosis, remodeling during hypertrophy, and regeneration (Massion et al. 2005; Sverdlov et al. 2014). Negative CV outcomes associated with aging also appear to be associated with a proportionate decrease in measurable NO availability in the myocardium either by decreased NO production or increased scavenging (Paulus 2001; Massion et al. 2005). An exception to this is neuronal NO synthase (nNOS). By measuring RNA and protein levels, nNOS has been shown to be up-regulated, in humans with congestive heart
failure (Damy et al. 2004). Whether the increased expression of nNOS with heart failure is beneficial or detrimental is as yet unknown.

1.5 QUALITY CONTROL MECHANISMS IN CARDIAC AGING

1.5.1 mTOR Signaling in regulation of protein homeostasis

The TOR (and in mammals, mTOR) signaling pathway is a mechanism of transmitting a wide variety of extracellular environmental cues (nutrient availability, amino acids, hormonal signals, mitogens) and producing adaptive responses within the cell. These adaptive responses are important throughout the body, and the heart is no exception. By regulating apoptosis, mitochondrial biogenesis, transcription, translation, lipid metabolism, glycolysis and inflammation, the mTOR pathway plays a critical role in cardiomyocyte growth, function, and structure in aging. Numerous reviews (Balasubramanian et al. 2009; Evans et al. 2011; C 2013; O'Neill 2013; Johnson et al. 2015) are available regarding this pathway and its implications in growth, disease, and aging. mTOR, the mechanistic target of rapamycin, is a serine/threonine kinase in the PI3K family. It is the catalytic subunit of two distinct complexes – mTORC1 and mTORC2. mTORC1 is downstream of the AKT and PI3K pathways, and mTORC2 is activated by the RAS and RAF signaling cascade (Dobashi et al. 2011). mTORC1 has been the more thoroughly studied complex in mammalian aging, largely due to its inhibition by rapamycin. This pharmacological inhibition is mediated through the rapamycin–FKBP12 complex (Evans et al. 2011). Important pathways downstream of TORC1 include regulation of cap-dependent initiation of translation via 4EBP1, control of ribosomal protein biosynthesis via S6K, and regulation of autophagy via Ulk1 (Johnson et al. 2013). However, chronic treatment with rapamycin can also inhibit mTORC2 in a cell-specific manner (Lamming et al. 2012). mTOR complex 2 (mTORC2) includes Rictor. This complex may also regulate some aspects of cardiac homeostasis in aging through stimulating autophagy and the clearance of pro-apoptotic factors, and removal of Akt from FOXO3 (Gurusamy & Das 2009; Jung et al. 2010; Kurdi & Booz 2011).

1.5.2 mTORC1

mTORC1 is an important regulator of cell growth and size, and signaling of mTORC1 is depressed in stress conditions such as low ATP concentration and low nutrient availability (Dobashi et al. 2011). Modulation of mTORC1 has been shown to improve cardiac geometry and function (Balasubramanian et al. 2009). It has many downstream functions critical to proteostasis including stimulating protein synthesis, inhibiting autophagy, ribosomal biogenesis, and translation initiation. 4E-BP1, and eukaryotic elongation factor 2 (eEF2), and ribosomal protein S6 kinase (S6K) are downstream effectors of mTORC1 and are largely responsible for mTORC1's control over protein synthesis. A study by Wessells and colleagues provided evidence that in Drosophila melanogaster, upregulation of d4eBP was sufficient to mitigate the age-related decline in fly cardiac function (Wessells et al. 2009). 4eBP binds eIF4E, inhibiting cap-dependent initiation of translation (Sonnenberg & Hinnebusch 2009).
1.5.3 Proteostasis

Protein homeostasis (proteostasis) is the equilibrium between protein synthesis, maintenance, and degradation. Maintenance of proper protein homeostasis is essential to cellular and organismal health – as illustrated by many studies indicating that age-related diseases and conditions are associated with the inability of the cell to maintain healthy proteins or get rid of defective proteins (Bedford et al. 2008). These conditions include neurodegenerative disease (Douglas & Dillin 2010), cardiac dysfunction (Hedhli et al. 2005; Christians & Benjamin 2012), cataracts (Surguchev & Surguchov 2010), and sarcopenia (Vinciguerra et al. 2010; Marzetti et al. 2012). Similar dysfunctions in proteostasis have been observed in “normally” aging cells which are free of disease (de Magalhães 2004), indicating a potentially important role for protein regulation in both health and aging.

Aside from the correlative association between aging, health, and protein quality control, direct interventions to modulate quality control mechanisms may potentially increase lifespan and improve health. Several such examples can be seen among interventions that inhibit mTOR, including rapamycin and calorie restriction, discussed further below. A number of other protein quality control interventions have been shown to improve health and aging in both invertebrate and mammalian models as well (Morimoto & Cuervo 2009; Douglas & Dillin 2010; Madeo et al. 2010; Koga et al. 2011).

Collectively, these studies may suggest that dysfunctional proteostasis has some causative role in aging or, alternatively, restoration of protein homeostasis machinery is protective against some other driving force in aging and age-related disease. In either scenario, the major mechanistic question of how these processes extend lifespan and healthspan remains as yet poorly answered, as an incomplete understanding of the various interactions, specificity, and targets of quality control pathways currently limits the ability of researchers to close this gap. Fortunately, several quality control pathways, such as autophagy and ubiquitin-mediated degradation, are receiving increased attention from several areas of biomedical research as their roles are recognized in a number of diseases (Madeo et al. 2010). In addition, the emergence of sophisticated tools in genomics and proteomics has provided powerful resources in cellular biology, allowing researchers to acquire and analyze an unprecedented depth and volume of data.

The aging cardiac proteome recapitulates most hallmarks of the aged cellular proteome including the appearance of protein aggregates and lipofuscin, increased protein oxidation and damage, increased ubiquitination, and declines in autophagy and the ubiquitin proteasome system (Morimoto & Cuervo 2009; Johnson et al. 2013). All of these changes have an impact on global levels of proteostasis to some degree, consistent with a notion of proteome remodeling during aging. It is unlikely, however, that all protein changes are equally or significantly contributing to the aging phenotype – presenting a challenge for researchers to identify the most phenotypically relevant downstream targets and their changes during aging.

The majority of studies in mice have reported a decline in the efficiency of protein degradation machinery with advanced age, contributing to a popular notion that aging is associated with a decrease in overall protein turnover. However, using a sensitive method of heavy label proteomics, our group has consistently observed that proteome turnover is either unchanged or
modestly increased in the various mouse tissues examined to date, including mouse heart (Dai et al. 2014a) as well as skeletal muscle (Kruse et al. 2016), brain (unpublished), and liver (Karunadharma et al. 2015b). Unlike earlier studies, these finding were based on direct measurements of individual protein turnover rates in vivo (Hsieh et al. 2012), rather than using bulk protein synthesis measurements or cellular markers of degradation as a proxy. Additionally, other recent studies utilizing a similar metabolic labeling-based MS approach to assess in vivo protein turnover have observed turnover rates consistent with our observations in aging mice (Price et al. 2010; Miller et al. 2012).

In mice, we have shown that the age related functional declines discussed previously are accompanied by proteomic remodeling of both energetic and structural pathways (Dai et al. 2014a). Levels of mitochondrial respiratory proteins, key for the production of most of the cardiac ATP, declined in the old heart, with concurrent reductions in metabolic proteins involved in fatty acid beta oxidation, amino acid metabolism, ketogenesis, and the TCA cycle, together likely contributing to an overall energy deficiency in aging hearts (Dai et al. 2014a). Conversely, glycolytic metabolic pathways as well as extracellular structural proteins were significantly increased in protein abundance with age (Dai et al. 2014a). The metabolic shift from TCA to glycolysis/gluconeogenesis was confirmed by metabolomics. This remodeling of the cardiac proteome with age is consistent with a number of proteomic studies focused on cardiac aging and disease. These changes may be the result of an underlying decline in protein quality control systems, which in turn leads to accumulation of damaged proteins. Two processes are known to turn over the majority of cellular proteins: autophagy and the ubiquitin proteasome system.

1.5.4 Autophagy

Autophagy is one of two primary cellular systems which degrade the vast majority of proteins in the cell (its counterpart, the ubiquitin proteasome, is discussed below). Any cellular degradation involving lysosomes, single membrane vesicles containing various enzymes for the digestion of macromolecules, is generally categorized under the umbrella term “autophagy” (Morimoto & Cuervo 2009). There are three major ways by which proteins can be delivered to a lysosome for degradation, which define three primary categories of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy. For brevity these will not be covered in detail, however, readers are referred to a number of comprehensive reviews on each topic (Morimoto & Cuervo 2009; Madeo et al. 2010; Koga et al. 2011).

There are four general physiological roles of autophagic degradation: cell and protein quality control, conserving cellular resources, cellular remodeling, and cell defense (Madeo et al. 2010). In context of quality control, autophagy is responsible for the clearance of damaged proteins, insoluble protein inclusions, and abnormal organelles, all of which are hallmarks of aged and dysfunctional tissues. Knocking down components of autophagy leaves cells unable to remove damaged organelles and proteins (Ravikumar et al. 2002; Madeo et al. 2010; Wong & Cuervo 2010), demonstrating that autophagy plays a key role in protein homeostasis.

Numerous lines of evidence suggest that autophagy is likely to have an important role in organismal aging, however, there has as yet been no “smoking gun”. Many studies, mostly in Caenorhabditis elegans, have demonstrated that autophagy components are required for lifespan
extension by CR, mTOR inhibition, IGF-1 inhibition, and a few other longevity pathways (Ryazanov & Nefsky 2002; Douglas & Dillin 2010; Koga et al. 2011), although these have not yet been confirmed in other model systems. Unfortunately, there is no genetic or pharmacological intervention known to specifically increase autophagy without targeting other processes.

A recent report has found that genetic over-expression of ATG5, a vital autophagy protein involved in autophagosome formation, extends lifespan in mice (Pyo et al. 2013). ATG5 has an important pro-apoptotic function, which cannot be excluded as the longevity-promoting factor. In particular clearance of defective mitochondria by autophagy (mitophagy) is known to be essential in cardiac development and response to stress or injury. Thus, cardiac-specific knockdown of ATG5 in mice has been shown to accelerate aspects of aging in the heart, including accelerated left ventricular hypertrophy, decreased fractional shortening, and premature death (Taneike et al. 2010). In addition, the accumulation of ubiquitinated proteins and p62 in ATG5 mutant mice suggests that removal of damaged or aggregating proteins is a protective mechanism (Taneike et al. 2010; Wohlgemuth et al. 2014b). In agreement with this, a study performed using cardiomyocyte cell lines found that induction of autophagy was protective against oxidative stress-induced protein aggregation and reduced levels of protein ubiquitination (Dutta et al. 2013). Further, this study found that induction of autophagy improved mitochondrial function and reduced cell death, confirming that autophagy has an important role in maintaining mitochondrial quality.

Sirt1 expression stimulates basal levels of autophagy, likely through deacetylation of autophagy genes (Atg)5, -7, and -8, and Sirt1 overexpression increases autophagic flux in cultured mouse embryonic fibroblasts (Lee et al. 2008). The reduction in Sirt1 expression with age may contribute to susceptibility of the heart to injury, as it is no longer able to promote autophagy through deacetylation of FoxO1, leaving cardiomyocytes with a lessened ability to respond to ischemia (Hariharan et al. 2010). The induction of autophagy mediated by CR and nutrient deprivation, but not autophagy stimulated by rapamycin treatment, appear to be dependent on functioning Sirt1 (Morselli et al. 2010).

The mTOR pathway, when inhibited, is well known to increase autophagy and extend lifespan. In fact rapamycin (discussed below), by inhibiting mTOR, is one of the few drugs available which can be used to pharmacologically increase autophagy. Longevity studies with rapamycin, and other forms of mTOR inhibition, have reported increased autophagy in animals across many studies (Harrison et al. 2009; Morimoto & Cuervo 2009; Stanfel et al. 2009; Johnson et al. 2013), and offer further evidence that autophagy may play a central role in aging. Even so, due to the inability to specifically over-express autophagy components without targeting non-specific processes, it is still not certain whether augmenting autophagy can in itself extend lifespan or slow aging.

In the future, to better understand the mechanisms underlying improved cardiac function following induction of autophagy, it will be critical to better understand the detailed roles of autophagic pathways with oxidative stress, mitochondrial quality control, and clearance of unwanted proteins in the heart.
1.5.5 Ubiquitin-mediated turnover

The ubiquitin-proteasome system (UPS) is the primary non-lysosomal protein degradation pathway. In contrast to autophagy, its action is limited specifically to individual proteins, and cannot degrade other macromolecules, organelles, or groups of proteins. Where autophagy often degrades its targets in bulk, the UPS very specifically targets thousands of proteins and utilizes a sophisticated array of mechanisms to do so with spatial and temporal precision. The UPS is also active in all regions of the cell, and targets proteins localized in organelles. For most proteins, degradation through this pathway is characterized by 2 major steps: first the recognition and “tagging” of a protein for elimination with a poly-ubiquitin modification, followed by translocation to the proteasome for degradation (Morimoto & Cuervo 2009; Wong & Cuervo 2010; Koga et al. 2011). This extraordinarily complex process has been extensively studied and described in very great detail in literature, and readers are referred to several detailed reviews of UPS functions (Ryazanov & Nefsky 2002; Morimoto & Cuervo 2009; Douglas & Dillin 2010; Wong & Cuervo 2010; Koga et al. 2011; Jana 2012).

Similar to autophagy, the UPS is essential for maintaining overall cellular homeostasis. Inhibiting or deleting its components often leads to severe cellular phenotypes, toxicity, and cellular death. Almost immediately after inhibition, an accumulation of protein inclusions can be observed in cells. Interestingly these resemble the inclusions described in a number of neurodegenerative diseases (Ryazanov & Nefsky 2002; Keck et al. 2003; Bedford et al. 2008; Robinson 2008). Genetic depletion of proteasome subunits in the brains of mice has been shown to induce a neurodegenerative phenotype, suggesting a role in neurodegenerative diseases characterized by protein inclusions (Bedford et al. 2008). In the heart, the role of the UPS is less well known. Some evidence exists of proteasomal degradation of various cardiac proteins including myofibrillar proteins, connexins, actin, and myosin (Pagan et al. 2013). These mechanisms, for the most part, have not been well-characterized in heart. Pharmacological and genetic intervention of the UPS with proteasome inhibitors has, however, made it evident that the proteasome can have powerful effects on the heart. In models of ischemia–reperfusion injury, for instance, proteasome inhibitors decrease infarct size – sometimes by over 50% (Pagan et al. 2013). Little has been reported in the literature about the role of the ubiquitin proteasome in the context of cardiac aging specifically.

Most of the evidence linking the UPS-intervention to longevity comes from C. elegans studies (Li et al. 2007; Koga et al. 2011; Jana 2012). Generally, these can be explained by the specific action of UPS on longevity pathways, rather than a global change in the proteolytic system. The ubiquitin ligase RLE-1, for example, selectively targets and poly-ubiquitinates a key component in the homologous insulin/IGF pathway in worms, daf-16, and leads to its degradation by the proteasome (Li et al. 2007). As a result, inhibition of RLE-1 extends lifespan in C. elegans. In flies it has been shown that overexpression of parkin-1, a ubiquitin ligase involved in familial Parkinson's disease, extends lifespan (Rana et al. 2013).

On a broad scale, it is not known if general protein maintenance by the UPS is intimately involved in aging. Correlatively, proteasomal activity becomes less functional with age and is restored in long-lived animals under CR (Koga et al. 2011; Jana 2012). It is also important to note that autophagy and the UPS must work in synchrony to direct protein homeostasis and an
intervention in either process is likely to cause changes in both. Interestingly, it has been shown that poly-ubiquitination can promote the clearance of proteins through autophagy (Tan et al. 2008).

1.5.6 **Apoptosis**

There is increasing evidence of significant age-related loss of cardiac myocytes (Kajstura et al. 1996a; Kajstura et al. 1996b; Liu et al. 1998; Lee et al. 1999; Li et al. 2007) which contributes to the increased susceptibility of the aged heart in models of myocardial infarction (MI), ischemic heart attack, and congestive heart failure (Liu et al. 1998; Azhar et al. 1999; Narula et al. 1999; Crow et al. 2004; Lehrke et al. 2006). Studies on the interrelation between age and apoptotic cell loss have been contradictory, with some apoptotic markers decreasing while others increase with age and in pathologies generally associated with aging (Maury & Teppo 1989; Levine et al. 1990; Lane et al. 1993; Torre-Amione et al. 1996; Kavathia et al. 2009). However, there is consensus that apoptosis plays a significant role in deteriorating function of senescent hearts. Several cellular processes have been hypothesized to contribute to this. A significant increase in oxidative stress may precede cardiomyocyte apoptosis (Kajstura et al. 1996b; Nitahara et al. 1998; Mather & Rottenberg 2000; Phaneuf & Leeuwenburgh 2002; Crow et al. 2004). Similarly, the reduction of SIRT1 deacetylase activity of and the increased acetylation of the Foxo1 transcription factor in senescent hearts has been shown to lead to activation of pro-apoptotic Bim signaling (Sin et al. 2014). Sirt1 and Sirt7, both localized to the nucleus, modulate p53 activity to act in a protective manner against apoptosis (Alcendor et al. 2004; Vakhrusheva et al. 2008b). Sirt3 can also influence the path to autophagy by targeting the mitochondrial permeability transition pore (MPTP). Cyclophilin D is a component of the MPTP and Sirt3 maintains cyclophilin D in its deacetylated form, preventing the release of pro-apoptotic factors (Hafner et al. 2010). Age-related decline in the Bcl2 anti-apoptotic marker and significant elevation of cytosolic cytochrome c in aged hearts can also trigger apoptosis (Narula et al. 1999; Phaneuf & Leeuwenburgh 2002). Furthermore, cytochrome c-dependent activation of cysteine proteases and caspase 3 is known to mediate myopathic apoptosis in human cardiomyopathy (Beltrami et al. 1994; Narula et al. 1999).

1.5.7 **Mitochondrial quality control**

Mitochondrial dysfunction and dysregulation are well documented in old age. Mitochondrial dysfunction in old age is associated with abnormal mitochondrial ROS production and detoxification (reviewed in (Terzioglu & Larsson 2007; Trifunovic & Larsson 2008; Mammucari & Rizzuto 2010)). Mitochondrial oxidative phosphorylation declined with age, as evident by the decline in mitochondrial state 3 respiration (maximal stimulated respiration), related to diminished activity of electron transport complexes I and IV in old age. The function of complexes II, III and V are relatively unaffected in old age (see review (Navarro & Boveris 2007)).

As the heart is a highly metabolic active organ and rich in mitochondria, it is particularly susceptible to mitochondrial oxidative damage. Several studies have demonstrated the deficiency of mitochondrial energetics in human and experimental animals with heart failure (Ventura-Clapier et al. 2008). The mechanisms by which mitochondrial dysfunction lead to heart failure
may include mitochondrial biogenesis that does not keep up with the increasing demand in cardiac hypertrophy (see review (Goffart et al. 2004)), mitochondrial uncoupling and decreased substrate availability (Murray et al. 2004), and increased mitochondrial DNA deletions (Dai et al. 2011b) and altered energetics (see Section 1.4.1, above).

We have previously shown that mitochondrial protein carbonylation, indicative of oxidative damage to mitochondrial proteins, significantly increased in aged mouse hearts (Dai et al. 2009; Dai et al. 2010). This suggests that damaged mitochondria in aged mouse hearts produce more ROS than healthy mitochondria in young hearts. Furthermore, aged mouse hearts had a 3–4-fold increase in mitochondrial DNA point mutations and deletions (Dai et al. 2009). Defective mtDNA produce defective subunits of mitochondrial electron transfer complexes, especially complexes I and IV, leading to increased ROS production. This may lead to vicious cycle of ROS amplification within mitochondria (Dai et al. 2012a; Dai et al. 2012c). The most direct evidence for the critical role of mitochondrial ROS in cardiac aging was shown by mice overexpressing catalase targeted to the mitochondria (mCAT). The mCAT mice were significantly protected from the age-dependent increase in LVMI, decline in diastolic function and impairment of myocardial performance through better preservation of SERCA2, as well as amelioration of cardiac fibrosis and cardiomyocytes hypertrophy (Dai et al. 2009). Consistent with this, mCAT attenuates mitochondrial oxidative damage, as displayed by significant reductions of mitochondrial protein carbonyls and mtDNA deletion frequencies in aged mCAT hearts (Dai et al. 2009).

Another line of evidence for the critical role of mitochondria in aging is demonstrated by mice with proofreading-deficient homozygous mutation of mitochondrial polymerase gamma (PolgD257A/D257A designated as Polg\textsuperscript{m/m}), which induces a substantial increase in mtDNA point mutations and deletions (Kujoh et al. 2005) (Trifunovic et al. 2004). The accumulation of mitochondrial DNA mutations has been shown to increase apoptosis (Kujoh et al. 2005). These mice were shown to have shortened lifespan and an “accelerated aging-like” phenotypes, such as kyphosis, graying and loss of hair, anemia, osteoporosis and age-dependent cardiomyopathy (Trifunovic et al. 2004; Dai et al. 2010), which include marked LV hypertrophy, systolic and diastolic dysfunction, impairment of myocardial performance, increased cardiac fibrosis, apoptosis and hypertrophy of remaining cardiomyocytes. The observations that mitochondrial damage and cardiomyopathy in these mice can be partially rescued by mCAT suggests that mitochondrial ROS and mitochondrial DNA damage are part of a vicious cycle of ROS-induced ROS release (Dai et al. 2010).

A recent paper reports the striking result that endurance exercise in Polg\textsuperscript{m/m} mutant mice can prevent progeroid phenotypes in both skeletal and cardiac muscles (Safdar et al. 2011). It is proposed that the augmented mitochondrial biogenesis induced by endurance exercise in these mice is a critical factor in maintaining mitochondrial function in these muscles. Age-associated accumulation of mtDNA deletions have been documented in human hearts (Corral-Debrinski et al. 1991; Zhang et al. 1997). The beneficial effects of endurance exercise seen in Polg\textsuperscript{m/m} mutant mice reinforce the well-known benefit of regular aerobic exercise for human hearts.

Sirtuins are believed to influence cardiac aging through modulation of mitochondrial stress responses. Sirt3 has been shown to contribute to this by up-regulating mitochondrial antioxidant
defenses, leading to lower levels of ROS (Wu et al. 2013). Indeed, mice without Sirt3 have a compromised ability to benefit from caloric restriction in the face of oxidative stress (Tao et al. 2010) and develop cardiac hypertrophy and fibrosis very early in life (Sundaresan et al. 2009). Age-related loss of Sirt3 has been associated with cardiac aging phenotypes, including hypertrophy (Pillai et al. 2010; Pillai et al. 2014). Sirt3 modulates the Foxo3a and catalase to reduce ROS in vitro, along with decreasing signaling from Ras to the MAP/ERK and PI3K/Akt pathways, preventing cardiomyocyte hypertrophy (Sundaresan et al. 2009). Sirt3 also deacetylates the mitochondrial antioxidant MnSOD in vitro, contributing to its improved antioxidant effects (Qiu et al. 2010).

1.5.8 Mitochondria fusion and fission

Mitochondria fusion/fission is a highly conserved quality control process in which a balance between fusion and fission is vital for normal functioning of the mitochondria and overall cellular homeostasis. These processes regulate mitochondria number, morphology, and function (Bereiter-Hahn & Voth 1994) and their role in maintenance of mitochondrial quality control is likely necessary to retard the detrimental effects of aging. As noted above, mitochondrial dysfunction is a hallmark of aging and this is manifested by impairment of OXPHOS bioenergetics, and accumulation of ROS. Furthermore, cardiomyocyte mitochondrial morphological changes have been reported in aging and heart disease (Hom & Sheu 2009; Ong & Hausenloy 2010). This suggests that there are diminished mitochondrial quality control mechanisms with age and a greater understanding may offer some insight into the function decline in cardiac function with aging.

In recent years, it has become clear that mitochondria exist as a dynamic network within cells, in which the active processes of mitochondrial fission and fusion are a balanced process by which mitochondrial quality control is maintained (Hom & Sheu 2009). A key mechanism by which this is accomplished is that mitochondrial fission fragments that have low membrane potential (indicative of poor OXPHOS activity) are targeted for degradation by ubiquitination via the activity of Pink and Parkin (Matsuda et al. 2010). Key proteins that regulate mitochondrial fusion and fission are mitofusin1/2 and OPA1, and their dysregulation impairs mitochondrial structure and function, with loss of efficiency of cellular respiration in many tissues (Frieden et al. 2004; Szabadkai et al. 2004; Chen et al. 2005; Westermann 2010), including the aging heart (Bossy-Wetzel et al. 2003; Papanicolaou et al. 2011). Complete genetic ablation of mfn1/2 is embryonic lethal. Deletion of mfn1 results in mitochondrial fragmentation, although mfn1-KO murine hearts have normal left ventricular function and their mitochondria exhibited normal respiratory function (Papanicolaou et al. 2012). However, other studies have found that Mfn1 partial deletion resulted in mild respiration deficiency, cardiac hypertrophy, and impaired contractile reserve (Papanicolaou et al. 2011).

Under normal conditions mitofusins 2 (Mfn2) is highly expressed in adult hearts and its deficiency in cardiomyocytes is associated with disruption of cell cycle progression, cardiac hypertrophy, reduced oxidative metabolism and altered mitochondrial permeability transition and systolic dysfunction (Papanicolaou et al. 2011). Piquereau et al. also found that partial down-regulation of Mfn2 and optic atrophy-1 (OPA1) in cardiac tissue resulted in altered mitochondrial morphology in which large pleomorphic mitochondria with disorganized cristae...
were arranged in irregular patterns (Papanicolaou et al. 2011; Piquereau et al. 2012).
Suppression of mfn2 expression has also been reported in cardiac diseases such as SHR, murine pressure-overload hypertrophy and in $\beta_2$-TG mice with cardiomyopathy (Fang et al. 2007). Partial knockout of both Mfn1 and 2 in murine models results in mitochondrial fragmentation, impaired mitochondrial respiration, and fatal cardiomyopathy (Chen et al. 2011). Overexpression of Mfn2 may also promote apoptosis, however (Shen et al. 2007; Ikeda et al. 2014).

It is thus clear that mitochondrial dynamics, including fission and fusion, is a necessary component of cardiomyocyte homeostasis and maintenance of cardiac function. The decline in efficiency of these functions is likely implicated in cardiac aging, however, this is clearly a subject that warrants further study.

1.5.9 Cardiac matrix homeostasis: matrix metalloproteases

Matrix metalloproteases (MMPs) are known to degrade the extracellular matrix (ECM) and play a role in tissue homeostasis (Parks et al. 2004). As organisms age, this homeostatic role can be thrown off balance with overexpression of MMPs. With increased age, MMP2, MMP9, and MMP28 have all been shown to play a role in the cardiac tissue (Chiao et al. 2012; Horn et al. 2012; Ma et al. 2013; Yabluchanskiy et al. 2014).

MMP2 is a gelatinase that is up-regulated in multiple aged organisms (Horn et al. 2012). A study using sheep demonstrated that MMP2 expression is increased in old animals, and this expression shows the same trend in old mice (Horn et al. 2012). When angiotensin-converting enzyme 2 (ACE2) is knocked out in mice, there is a spike in MMP2 expression that is not seen in wild type controls, suggesting an increase in matrix degradation when angiotensin cannot be properly regulated (Patel et al. 2014).

MMP9, a collagenase, is the most commonly upregulated MMP in aging heart (Parks et al. 2004; Chiao et al. 2012). Chiao et al. showed an increase in MMP9 expression and protein levels in both the left ventricle and plasma in senescent mice, which was tied to decreased collagen deposits and TGF$\beta$ activation. In the same study, when MMP9 was knocked out, MMP8, which has a high affinity for collagen I and III, was upregulated. Similar to MMP2, when ACE2 was knocked out, MMP9 was increased (Chiao et al. 2012). Despite MMP9 being a collagenase, with its increased expression collagen accumulates and there is increased angiogenic signaling (Yabluchanskiy et al. 2014). However, there is no increase in vessel numbers or prevention of vascular leakage unless MMP9 expression is attenuated (Yabluchanskiy et al. 2014).

MMP28, an epilysin and the most recently cloned MMP, has been shown to be dysregulated in cardiac aging (Ma et al. 2013). When MMP28 is knocked out, the aging heart has an increased inflammatory response (Ma et al. 2013). MMP28 will be a protease to investigate in the future, since many of its interactions and roles are still being elucidated, especially in cardiac aging.

Besides the MMPs, the role of collagen and fibrinogen in the ECM composition is important. If there is too much extracellular matrix there is an increase in cardiac stiffness and diastolic dysfunction in aged organisms (Ma et al. 2013). Cardiac stiffness increased with age due to the
increased amount of collagen content and fibrinogen deposit in old animals (Horn et al. 2012; Rodriguez-Menocal et al. 2014; Yabluchanskiy et al. 2014).

1.6 CARDIAC AGING INTERVENTIONS

Interventions to prevent or delay CV disease have been widely publicized and often successful, however these have focused on cardiac-extrinsic risk factors, such as hypertension, cholesterol, smoking, diabetes and exercise (http://www.cdc.gov.offcampus.lib.washington.edu/heartdisease/risk_factors.htm). Age-related factors (see Section 1) are generally considered immutable, even though aging is by far the largest risk factor for heart disease and failure. There are however, several recognized methods of delaying the negative outcomes of aging, the best characterized of these being restriction of caloric intake. More recently, there has been some progress in defining caloric restriction (CR) mimetics that may recapitulate positive changes due to CR, without the necessary reduction of food intake. These largely function by modulating the activity of mTOR. Candidates for CR mimetics presently including rapamycin and resveratrol (Wohlgemuth et al. 2014b). Other interventions include antioxidants targeted to the mitochondria, circulating factors, and cardiolipin-targeted pharmaceutics. Below are summaries of some of the important cardiac aging interventions that have been pursued to date.

1.6.1 Caloric/dietary restriction and mimetics

1.6.1.1 CR

CR, also called dietary restriction (DR), is a powerful and reproducible technique to improve both healthspan and lifespan in many model organisms from yeast to mice (reviewed in (Speakman & Mitchell 2011). CR is sustained calorie restriction without restriction of vitamins and micronutrients. CR's cardioprotective activity includes reduced cardiac hypertrophy as measured by left ventricular mass and wall thickness, improved cardiomyocyte contractile function and reduced cardiomyocyte size (Han et al. 2012). While both CR and rapamycin affect metabolism through decreased mechanistic target of rapamycin (mTOR) activity, there is evidence that DR works though other pathways as well in a complex net of changes resulting in a whole-body stress response and adjustment in tissue maintenance (reviewed extensively in (Speakman & Mitchell 2011)). CR can delay the onset of cardiac aging and ameliorate the effects of cardiovascular disease. In humans, rodents and monkeys, chronic dietary restriction reduces the aging-associated decline in cardiac function, ameliorates cardiac hypertrophy, and reduces signs of cardiomyopathy (Maeda et al. 1985; Taffet et al. 1997; Colman et al. 2009; Niemann et al. 2010; Shinmura et al. 2011; Dai et al. 2014a).

CR protects against cardiomyopathy, in part by reducing age-associated apoptosis through protection from DNA damage, enhanced DNA repair, and alterations in apoptosis-related gene expression (Maeda et al. 1985; Dhahbi et al. 2006). It can also modulate expression of genes involved in fibrosis, extracellular matrix maintenance, inflammation, and fatty acid metabolism (Dhahbi et al. 2006). Many other processes associated with cardiac aging are modulated by DR, including reduced perivascular collagen deposition, reduced left ventricular cardiac hypertrophy,
protective effects against ischemia, and a reduction of chronic vascular inflammation (Spaulding 
et al. 1997; Broderick et al. 2001; Dhabhi et al. 2006).

The mechanisms by which CR modulates cardiac aging are still a matter of intense research. One hypothesis is that limited nutrient/energy availability drives tissues from a proliferative and energetic state to a somatic maintenance state to allow the best use of limited resources. Indeed, Drake and colleagues found that DNA synthesis, a measure of proliferation, was reduced while measures of mitochondrial biogenesis were maintained during life-long CR (Miller et al. 2012; Drake et al. 2013). Many of these effects may be mediated through the mTOR pathway, particularly mTORC1, which regulates protein synthesis and autophagy (Dobashi et al. 2011). Aged rats that are subjected to CR over their lifespan show increased autophagy in conjunction with improved LV diastolic function (Shinmura et al. 2011). Conversely, when autophagy is reduced in the heart in Atg5 knockout mice, after three months they exhibit a reduced lifespan, LV hypertrophy, decreased fractional shortening, and defective structure and function of cardiac mitochondria compared to controls (Taneike et al. 2010). Cardiovascular disease and cardiac aging have long thought to be influenced by oxidative stress. Since CR has been shown to reduce mitochondrial reactive oxygen species (ROS) production in the heart and other tissues, and decrease NAD(P)H oxidase activity, it may abrogate the effects of aging through a modulation of the redox environment (Gredilla et al. 2001; Csiszar et al. 2010; Csiszar & Ungvari 2010). The most direct evidence of this is derived from study of mice overexpressing catalase targeted to the mitochondria. These mice have longer mean and maximal lifespans (Schriner et al. 2005), and moreover, show functional and biochemical evidence of reduced cardiac aging (Dai et al. 2009). Notably, diastolic failure (HFpEF), which is common in aged mice, as well as in man, was substantially attenuated in mCAT mice.

As life-long CR is unlikely to be translatable as a human therapeutic regime, there has recently been greater attention to the potential benefits of short term CR initiated later in life. We have examined cardiac function and molecular alterations following 10 week CR given to mice at 24 months of age (Dai et al. 2014a). We found that both CR and rapamycin reversed age-related diastolic dysfunction and cardiac hypertrophy. Also, by using deuterated-leucine protein labeling, we observed protein turnover differences between CR-treated and old control animals.

1.6.1.2 Rapamycin

CR in humans is an unappealing regimen, and thus, the search continues for CR mimetics that reproduce beneficial effects of CR without necessitating drastic changes in diet.

Rapamycin is by far the best documented agent that is believed to function as a CR mimetic. By inhibiting the target of rapamycin, mTOR, several important growth and cellular quality control mechanisms are modulated including ribosomal biogenesis, autophagy, lipid synthesis, and translation (reviewed in (Johnson et al. 2013)). These effects have been long- and well-documented in invertebrate models of aging, including flies (Kapahi et al. 2004), nematodes (Vellai et al. 2003; Jia et al. 2004), and yeast (Kaeberlein et al. 2005; Powers et al. 2006). Interest in rapamycin in mammalian systems greatly increased following reports from the National Institute on Aging Intervention Testing Program that long-term treatment of mice with rapamycin improves healthspan measures and extends lifespan (Harrison et al. 2009; Anisimov
et al. 2010; Miller et al. 2011). Aging tissues throughout the body are affected by rapamycin, including the liver, adrenal glands, tendons, bone marrow, and heart (Chen et al. 2009; Wilkinson et al. 2012). Benefits of rapamycin on the aging heart included a reduced incidence of nuclear atypia in cardiomyocytes (Wilkinson et al. 2012).

Since the longevity effect of rapamycin was very similar after the drug was administered from 9 months of age or beginning at 20 months of age, it has been suggested that the cardiac benefits of rapamycin might be delivered to even older mice after briefer treatment. Flynn and colleagues (Flynn et al. 2013) demonstrated an improvement in the age-related loss of contractile function and a reduction in evidence of age-related sterile inflammation after rapamycin was administered to 24 month old female mice for 3 months. In our laboratory, we observed that 10 week rapamycin treatment of 25 month-old mice conferred a substantial reversal of diastolic dysfunction and cardiac hypertrophy, as well as an attenuation of age-related cardiac proteomic changes (Dai et al. 2014a). While the total proteome turnover of the aging mouse heart was not significantly different from young controls, the cardiac proteome had a significantly increased half-life after both CR and rapamycin treatment, concurrent with a reduction of detectable protein oxidation and ubiquitination. These results may point to proteome remodeling as a mechanism behind the cardiac functional benefits granted by rapamycin. We also found an age-dependent abundance decrease in proteins associated with young mitochondrial functional profile (electron transport chain, TCA cycle, fatty acid metabolism) and an increase in proteins that transition the mitochondria to a more glycolytic program. Short-term rapamycin and CR both reversed this phenotypic age-related change (Dai et al. 2014a).

1.6.1.3 Resveratrol

Resveratrol, a potential CR mimetic initially studied for its anticancer benefits, has enjoyed some popularity as an aging intervention. Resveratrol (3,4′,5-trihydroxy-trans-stilbene) is a phytoalexin that is produced by plants as a reaction to stresses such as infection or injury (Baur 2010). Many studies point to resveratrol as an activator of sirtuins, modulating protein acetylation states (Wohlgemuth et al. 2014b). Sirt1 and Sirt3 and cause the nuclear translocation of phosphorylated FoxOs (Mukherjee et al. 2010), and this mechanism may underlie resveratrol's hormetic action in preconditioning that increases stress response pathway activation and autophagy (Petrovski et al. 2011). Other possible mechanisms of action include activation of AMPK and inhibition of cAMP phosphodiesterases (PDEs) (Chung 2012). In an ischemia/reperfusion model of Sprague-Dawley rats, a formulation of resveratrol with 5% quercetin plus 5% rice bran phytate was shown to protect cardiac performance and minimize infarct area, while also increasing autophagy. It has also been suggested that resveratrol can be combined with short term CR to potentiate autophagy in the hearts of 26-month-old rats and provide protection against doxorubicin-mediated cardiac toxicity (Dutta et al. 2013). However, a recent meta-study found that the published work on resveratrol has sufficient study variability, including dose ranges and methodological variability, to reduce the confidence in conclusions drawn from the clinical literature (Pollack & Crandall 2013).

1.6.1.4 GDF11

There is recent evidence that the circulating growth differentiation factor 11 (GDF11, also known as bone morphogenetic protein 11, BMP11) may contribute to myocardial aging
(Loffredo et al. 2013). Using heterochronic parabiosis, a technique that joins the circulatory systems of two mice, in this case old to young, Loffredo and colleagues determined that age-related cardiac hypertrophy was abrogated in old mice due to a circulating factor originating from the young mice. They concluded that GDF11 was the factor responsible for the age-related hypertrophy reversal. Indeed, old mice given GDF11 had similar cardiac benefits (Loffredo et al. 2013). However, the mechanism by which GDF11 may be working remains unclear. GDF11 shares much homology with myostatin, and myostatin is a negative regulator of muscle mass (McPherron 2010). Also the homologous gene myoglianin has been shown to preserve muscle function in fly aging models, together suggesting that GDF11 may preserve cardiac homeostasis and other tissues (Patel & Demontis 2014).

1.6.2 Mitochondrial antioxidants

Mitochondria are critical for maintaining protein, lipid, and overall cellular quality control, and mitochondrial dysfunction is associated with aging tissue dysfunction. Moreover, as noted above, mCAT mice show considerable protection from the functional and biochemical effects of aging (Dai et al. 2009). Consequently several pharmaceutical therapies have been proposed to target and improve mitochondrial function.

Triphenylphosphonium ion (TPP+) conjugation is an effective method of targeting the mitochondria by using the potential gradient across the inner membrane to trap the molecules there at up to 100- to 1000-fold higher concentration than in the cytosol (Murphy & Smith 2007). An example is TPP+ conjugated to coenzyme Q, a compound termed MitoQ (Smith et al. 2012). MitoQ and other TPP+ conjugates have been shown to reduce systolic blood pressure and cardiac hypertrophy in rats (Graham et al. 2009; Dikalova et al. 2010), prolong lifespan in SOD−/− flies (Magwere et al. 2006), and be protective against AD and PD in rodent models (Ghosh et al. 2010; Manczak et al. 2010).

Plastoquinone conjugated to TPP+ (SkQ1) (Skulachev et al. 2009), is another strategy employed to reduce intracellular ROS and improve lifespan (Skulachev 2013). Both MitoQ and SkQ1 can reduce IR injury, but they may also inhibit oxidative phosphorylation and ATP production (Szeto 2014).

1.6.3 Cardiolipin-targeted therapeutics

SS-31 (Szeto-Schiller compound 31, H-d-Arg-Dmt-Lys-Phe-NH2) is a tetrapeptide which preferentially targets and concentrates in the inner mitochondrial membrane (Szeto 2014). SS-31 has been the focus of several recent Phase I and Phase II clinical trials under the name Bendavia (Chakrabarti et al. 2013; Szeto 2014). Though it can scavenge free radicals such as \( H_2O_2 \), hydroxyl radical and peroxynitrite, the in vivo effects seem to be primarily due to its interaction with cardiolipin. Cardiolipin is an inner mitochondrial membrane phospholipid critical for maintenance of cristae structure and the formation of electron transport chain super complexes (Zhang et al. 2002; Pfeiffer et al. 2003), as well as anchoring cytochrome c at the inner membrane (Rytomaa & Kinnunen 1994; Rytomaa & Kinnunen 1995). This interaction is important for cytochrome c function, but cardiolipin also causes cytochrome c to unfold through hydrophobic interactions. Peroxidase activity of cytochrome c is then greatly increased, which
can lead to cardiolipin peroxidation and subsequent loss of proper cristae structure and super complex stability (Basova et al. 2007; Wiswedel et al. 2010). Quality control mechanisms of the mitochondria are dependent on homeostatic ROS, and this disruption of electron transport function and organization leads to increased ROS formation by complex 1 (Maranzana et al. 2013). SS-31 binding to cardiolipin blocks the peroxidase activity of cytochrome c (Birk et al. 2014).

After a coronary artery ligation method of myocardial infarction, rats chronically receiving Bendavia were found to have reduced LV volume, scar area, and ROS production, and improved LV fractional shortening and ejection fraction (Dai et al. 2014b). Interestingly, this was accompanied by reduced apoptosis in the infarct border zone and maintenance of mitochondrial function and gene expression.

Our laboratory has shown that SS-31 attenuates Goq overexpression-induced heart failure and reduced angiotensin-II induced LV hypertrophy and diastolic dysfunction (Dai et al. 2011b). It may also protect cardiac mitochondrial ultrastructure in a pressure-overload model of transverse aortic constriction by preserving most of the mitochondrial and non-mitochondrial cardiac proteome in the pre-overload state (Dai et al. 2013).

1.7 CONCLUSION

Quality control of genetic material, proteins, and cellular processes degrades with aging. In the heart, this progressive loss of maintenance mechanisms leads to clinically relevant cardiac dysfunction and a susceptibility to age-associated diseases. Many aspects of cardiac dysfunction manifest similarly in humans and other mammals, allowing the use of genetically altered and pharmacologically treated model organisms to dissect mechanisms of cardiac aging. We now know that molecular pathways that affect longevity also tend to affect cardiac healthspan. Pathways that respond to and modulate proteostasis, nutrient signaling, autophagy and mitochondrial maintenance are clearly important for CV health, and interventions that directly interact with these pathways are promising avenues for preserving optimal cardiac function. Through powerful new methods of investigating quality control mechanisms and cardiovascular dysfunction, in tandem with progress in interventions that modulate them, we can look forward to more therapies that directly influence cellular maintenance to improve cardiovascular health.
Chapter 2. SHORT TERM RAPAMYCIN PERSISTENTLY IMPROVES CARDIAC FUNCTION AFTER CESSATION OF TREATMENT IN AGED MALE AND FEMALE MICE.

2.1 ABSTRACT

Even in the context of healthy aging, cardiac morbidity and mortality increase with age in both mice and humans. These effects are evident in the decline of diastolic function, increase in left ventricular hypertrophy, metabolic substrate shifts, and alterations to the cardiac proteome. Previous work from our lab indicated that short-term (10-week) treatment with rapamycin, an mTORC1 inhibitor, improved measures of these age-related changes. In this report we demonstrate that the improvement of diastolic function is highly persistent 8 weeks after cessation of an 8-week treatment of rapamycin in both male and female 24+-month-old C57BL/6NIA mice. The proteomic and metabolomic abundance changes that occur after 8 weeks of rapamycin treatment have varying persistence after two further months without the drug. However, rapamycin treatment did lead to a persistent increase in abundance of electron transport chain (ETC) complex components, most of which belonged to Complex I. Although ETC protein abundance and Complex I activity were each differentially affected in males and females, the ratio of Complex I activity to Complex I protein abundance was equally reduced in both sexes and this change was highly persistent in both sexes. Thus rapamycin treatment in the aged mice persistently improved diastolic function, persistently alters the cardiac proteome in the absence of persistent metabolic changes, and leads to persistent alterations in mitochondrial respiratory chain activity. These observations suggest that an optimal translational regimen for rapamycin therapy or other treatments that promote proteostasis for enhancement of healthspan may involve intermittent short term treatment.

2.2 INTRODUCTION

It is estimated that by 2030, the prevalence of heart failure (HF) will be over 8 million people in the US alone (Heidenreich et al. 2013). In North America, Europe, Latin America, Oceania, and Central Asia, HF is most responsible for poor healthspan in males (age-standardized years lived with disability, (Moran et al. 2014). HF is not only a pervasive problem, but also a costly one: Estimates project the cost of HF in the US at $69.7 billion USD by 2030 (Heidenreich et al. 2013). Historically most attention has been focused on heart failure with reduced ejection fraction (HFrEF), such as may result after myocardial infarction; however, the Atherosclerosis Risk in Communities (ARIC) study recently reported that 47% of US hospitalizations due to HF were due to heart failure with preserved ejection fraction (HFpEF) (Chang et al. 2014). HFpEF is generally defined clinically by a signs/symptoms of HF combined with preserved left ventricular (LV) ejection fraction (EF). In this setting, impaired cardiac output is related to impaired diastolic LV filling, resulting in exercise intolerance and contributing to frailty. Although LV diastolic dysfunction can provide important evidence of HFpEF, it is no longer considered the definitive marker for diagnosis in humans, as other signs of detrimental CV remodeling may be observed that contribute to HFpEF (Hummel & Kitzman 2013). Diastolic dysfunction is still the primary diagnostic criterion for HFpEF in rodent models. Cardiac aging
Phenotypes are similar between humans and rodents, including a linear decrease in diastolic function with age and increased LV hypertrophy with age. These similarities make rodents a good model for diastolic function research using pharmacotherapy (Dai & Rabinovitch 2009). While in recent decades pharmacotherapy has enjoyed substantial success in improving health and survival after HFrEF in humans, effective treatment for HFpEF has been elusive. Despite the efforts of several large randomized clinical trials designed to improve quality of life in patients with HFpEF, results have thus far been largely disappointing (Hummel & Kitzman 2013).

Rapamycin is an FDA approved drug which directly inhibits the mechanistic target of rapamycin (mTOR) complex I. Inhibition of mTORC1 has wide ranging effects in vivo, including altering protein synthesis, inhibiting cell growth, and stimulating stress response mechanisms and autophagy (Li et al. 2014). Transient or life-long treatment extends lifespan and healthspan in many organisms, ranging from nematodes to primates (Bitto et al. 2015). Rapamycin extends murine lifespan in both sexes, even when administered at 9 or 20 months of age in genetically heterogeneous mice (Harrison et al. 2009; Miller et al. 2011), and at 19 (Zhang et al. 2014a) or 20-21 months of age (Bitto et al. 2016) in C57BL/6 mice. The lifespan and healthspan extension due to rapamycin is dose- and sex-dependent (Miller et al. 2014). Clinically, rapamycin and rapalogs are used to prevent rejection in de-novo organ transplantation (Tang et al. 2015; Fine & Kushwaha 2016) and for the prevention of restenosis after insertion of cardiac stents (Park et al. 2013a). Major concerns in considering potential clinical translation of rapamycin treatment are the detrimental on- or off-target effects including immunomodulation, gonadal atrophy, and stomatitis (Boers-Doets et al. 2013; Pallet & Legendre 2013; Verhave et al. 2014). However, these adverse effects are generally reversible, leading to the question of whether the more desirable healthspan effects of rapamycin might persist for long durations after the undesirable effects are resolved.

Work from our lab and others has shown that rapamycin improves cardiac function, most specifically diastolic function, when administered to middle or late aged mice (Flynn et al. 2013; Dai et al. 2014a), and it can improve cardiac structure and function in the context of various genetic/experimental cardiac defects (Marin et al. 2011; Das et al. 2014; Paul et al. 2014).

In this study, we analyzed functional and molecular outcomes from continuous and transient rapamycin treatment in aged, male and female C57BL/6 mice. In both sexes rapamycin treatment replicated our previous results showing a significant improvement in cardiac diastolic function, and this effect was persistent for 2 months after rapamycin was eliminated from the diet. By focusing on molecular changes due to rapamycin treatment that persist after drug removal, we hoped to shed light on the specific mechanisms of cardiac functional rejuvenation during rapamycin treatment.

2.3 RESULTS

2.3.1 Rapamycin persistently improves diastolic function.

In both humans and mice, diastolic function is measured by comparing the relative proportion of left ventricular filling that takes place in early diastole by LV relaxation (Ea) or in late diastole secondary to atrial contraction (Aa). In healthy hearts the early component is greater than the
late, and diastolic dysfunction is conventionally ascribed to a reversal of this ratio, i.e., an early to late filling ratio below 1.0. The 24-month old mice in this study demonstrated an Ea/Aa ratio averaging close to one at the beginning of the study (approximately half the mice above and half below 1.0), which is typical of this age of C57BL/6NIA mice. In the control group, this ratio stayed steady, but animals exposed to rapamycin improved their diastolic function significantly (by one way ANOVA with repeated measures – essentially a “paired” ANOVA to control for each individual) (Figure 2.1A). After 8 weeks treatment, rapamycin-induced improvement persisted for 8 weeks after removal of the drug, with cardiac performance maintained at levels near those of mice receiving 16 week continuous rapamycin treatment (diastolic function 82% persistent in females at 16 weeks, 78% in males at 16 weeks).

Our previous work in 24-26 month old female C57BL/6NIA mice showed no significant change in systolic functional measures (fractional shortening, FS and ejection fraction, EF) during 10 weeks of rapamycin treatment (Dai et al. 2014a). Concordantly, there were no measurable differences in FS or EF in the female mice at 16 weeks in this study (Figure 2.1B). However, males showed a small, but statistically significant reduced FS and EF in Old compared to Young Control mice (Tukey post-hoc test performed after a significant ANOVA). While rapamycin treated old mouse EF and FS were intermediate between young and old values, these differences did not reach significance.

We measured cardiac hypertrophy by measuring cardiac weight normalized to tibial length at necropsy (Figure 2.1C). Female mice at 16 weeks showed a decrease in cardiac hypertrophy with rapamycin and this effect trended towards persistent (p = 0.086) by T-test. Males also showed a reduction in hypertrophy with rapamycin treatment (p = 0.012) and again this difference approached significance in the persistence group (p = 0.081) by T-test. Combining both sexes, the persistence was significant (p = 0.013, see Figure 2.2). This reduction in cardiac hypertrophy cannot be explained by reduction in overall body size, as the body weight over time in all groups were similar and relatively stable (Figure 2.1D). Young animals were smaller for both sexes (mean ± sem F: 21.01 ± 0.60, M: 29.57 ± 0.39).
Figure 2.1 Rapamycin persistently improves diastolic function and reverses cardiac hypertrophy.
A) Ea/Aa ratios of female and male mice over the course of treatment (average +/- SEM). Continuous rapamycin treatment (rapa, dotted line), persistence (pers, dashed line), aged control (control, solid line). Both rapa and persistence groups are statistically significantly higher than controls for weeks 8, 12, and 16 by one-way ANOVA followed by Tukey posthoc for all groups at each time point per sex (R vs C 10^{-8} < p-value < 10^{-11}, P vs C 10^{-5} < p-value < 10^{-16}). Rapa and persistence groups’ Ea/Aa increased significantly by one-way ANOVA with repeated measures for each group over time. B) Systolic function parameters measured by echocardiography at 16 weeks. EF – ejection fraction, FS – fractional shortening. Black bars, %EF; white bars, %FS. C= Old Control, P= Persistence, R= Rapa, Y= Young. *significant by T-test between Old Control and Young groups. C) Heart mass in grams normalized to tibia length (mm) for all groups at 16 weeks. P-values from Tukey post-hoc tests when sex specific one-way ANOVA was significant. * vs Young, # vs Old Control, & vs Rapa. D) Body mass in grams for all groups over time. *p<0.05, **p<0.01, ***p<0.001.
2.2 Heart mass:tibia length is significantly persistent in combined data.

The heart mass:tibia length data from Figure 1.1 were normalized to the old control groups for each sex, then combined for the bar chart and T-tests. Asterisks (C vs Y) *p<0.05, **p<0.01, ***p<0.001. # = Y vs P, p < 0.01. C – old control, P – persistence, R – continuous rapamycin, Y – young control.

2.3.2 Rapamycin dramatically alters proteome abundances in both sexes, however the persistence of these changes varies by sex.

In our previous work, we applied proteomics to detect many differences in protein abundances due to 10 weeks of rapamycin treatment beginning at 24 months of age in female C57BL/6NIA mice. Thus, an important question was whether the proteome abundance changes due to rapamycin treatment were persistent after drug removal. Figure 2.3A shows heatmaps of all proteins in each sex that had a significant difference between control and continuous rapamycin treatment at the 16 week time point (by Students T-test, adjusted for multiple comparisons as described in methods, using q < 0.05). Overall, the females had close similarities between the rapamycin and persistence groups, while the males showed protein abundances that were intermediate between old control and rapamycin groups. Qiagen Ingenuity Pathway Analysis (IPA) software was used to identify significantly changed canonical pathways. This revealed that 8 of 10 top pathways were conserved between sexes. The 5 most significantly changed pathways in each sex are shown in the heatmap of Figure 2.3B, four of which are conserved between sexes; again it is apparent that the rapamycin and persistence groups are similar in the females but in the male cohort, the persistence group is more intermediate. The distribution of percent persistence of the proteins within each IPA category is plotted in Figure 2.3C. It can be seen that for all top 6 pathways but Mitochondrial Dysfunction, the median protein abundance in the female persistence groups is actually a slightly larger change (120-125% effect in the same direction) than in the continuous rapamycin treatment group, whereas the median Mitochondrial Dysfunction pathway persistence is “only” ~86% persistent in females (Table 2.2). The Mitochondrial Dysfunction pathway is a larger set of proteins (females n=27, males n=55) compared to the other top 5 pathways (females 6<n<18, males 19<n<29), and was more heterogeneous. When ETC proteins alone were examined, persistence was varied between the sexes and the individual complexes of the ETC (Table 2.1), with the females again generally showing greater persistence per complex than the males. Interestingly, the mean persistence of proteins in Complex V of the respiratory chain (ATP synthase) were very high in both sexes. Many of the proteins found to be altered significantly in both sexes for the Mitochondrial Dysfunction category were associated with Complex I (NADH:ubiquinone oxidoreductase) of the electron transport chain; persistence of proteins in this complex was 76.90% in females and
26.34% in males. As predicted by the overall proteomics, persistence within IPA pathways was lower in males than females and differences between pathways were less apparent.

One explanation for variation in the level of persistence of these proteins might be a relationship with half-life of the proteins; however, we did not find any significant correlation between persistence and our previously measured half-lives of the same proteins in the heart (Dai et al. 2014a); data not shown).

### Table 2.1. Average percent persistence per individual complex of the Respiratory Chain per sex.

The four complexes of the ETC, along with ATP synthase are shown along with the average percent persistence of all proteins found in each category. The standard deviation (SD) and the number of proteins in each complex (n) are also shown. The data were limited to eliminate outliers (Q1-IQR*1.5 < protein percent persistence < Q3 + IQR*1.5).

<table>
<thead>
<tr>
<th>Complex</th>
<th>females %</th>
<th>SD</th>
<th>n</th>
<th>males %</th>
<th>SD</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>CI</td>
<td>76.90</td>
<td>41.55</td>
<td>41</td>
<td>26.34</td>
<td>38.27</td>
<td>39</td>
</tr>
<tr>
<td>CII</td>
<td>67.61</td>
<td>22.01</td>
<td>3</td>
<td>19.00</td>
<td>49.78</td>
<td>4</td>
</tr>
<tr>
<td>CIII</td>
<td>94.25</td>
<td>78.93</td>
<td>9</td>
<td>-6.54</td>
<td>68.64</td>
<td>9</td>
</tr>
<tr>
<td>CIV</td>
<td>92.70</td>
<td>85.36</td>
<td>14</td>
<td>32.24</td>
<td>54.82</td>
<td>16</td>
</tr>
<tr>
<td>CV / ATP Synthase</td>
<td>145.75</td>
<td>108.29</td>
<td>8</td>
<td>143.24</td>
<td>238.64</td>
<td>7</td>
</tr>
</tbody>
</table>

### Table 2.2. Average percent persistence per IPA category per sex.

The top 5 IPA categories per sex, 6 altogether with two different between the sexes and four the same, are listed along with the average percent persistence of all proteins found in each category. The standard deviation (SD) and the number of proteins in each category (n) are also shown. The data were limited to proteins with percent persistence between -200% and 200% to eliminate outliers.

<table>
<thead>
<tr>
<th>IPA Category</th>
<th>females %</th>
<th>SD</th>
<th>n</th>
<th>males %</th>
<th>SD</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>actin cytoskeleton signaling</td>
<td>116.6</td>
<td>54.1</td>
<td>11</td>
<td>39.1</td>
<td>31.1</td>
<td>24</td>
</tr>
<tr>
<td>calcium signaling</td>
<td>132.8</td>
<td>55.5</td>
<td>15</td>
<td>56.1</td>
<td>15.2</td>
<td>19</td>
</tr>
<tr>
<td>ILK signaling</td>
<td>119.6</td>
<td>56.4</td>
<td>12</td>
<td>42.5</td>
<td>37</td>
<td>26</td>
</tr>
<tr>
<td>mito dysfunction &amp; oxphos</td>
<td>86.1</td>
<td>43.6</td>
<td>27</td>
<td>21.3</td>
<td>41.5</td>
<td>55</td>
</tr>
<tr>
<td>protein kinase A signaling</td>
<td>113.3</td>
<td>37.3</td>
<td>18</td>
<td>39.7</td>
<td>39.9</td>
<td>29</td>
</tr>
<tr>
<td>protein ubiquitination pathway</td>
<td>121.7</td>
<td>34.4</td>
<td>6</td>
<td>40.9</td>
<td>46.7</td>
<td>23</td>
</tr>
</tbody>
</table>
Figure 2.3 Persistence of abundance changes of proteins in top IPA pathways differs by sex. A) Dendrograms and heatmaps showing all significantly altered protein abundances due to rapamycin for each sex. Dendrograms show the spearman’s distance as a measure of relatedness. Color show z-scores of protein abundance differences by protein, with red indicating greater abundance and blue meaning less abundant. B) Z-score heatmaps of protein abundance, organized into the five most significantly altered pathways (by Ingenuity Pathway Analysis – IPA) for each sex – females on the left, males on the right. C) Asymmetrical beanplots show the range of the percent persistence for proteins in each IPA category (y-axis), with females (light gray) on the left side of each bean, and males (dark gray) on the right. Black bars denote the median of the range for each sex/category. The data range was limited to 0% to 200% for easier visualization. All data shown are from tissue collected at the 16-week time point. C – control, R – rapamycin, P – persistence, all at 16 weeks.
2.3.3 Metabolome differences seen at 8 weeks of rapamycin treatment are mainly not persistent after a further eight weeks with or without the drug.

Previous studies by our laboratory and others showed evidence of a metabolic substrate switch from dependence on fatty acid oxidation (FAO) to glycolysis with aging (Chiao et al. 2016; Wende et al. 2017). Concordant with a more youthful function, we previously found evidence of a reversal of this aging substrate shift in mice treated for 10 weeks with rapamycin (Dai et al. 2014a). This was confirmed by $^{13}$C glucose labelling and NMR in Langendorff perfused hearts (Chiao et al. 2016). In the present study we found that this reversal of shift largely disappears after a further eight weeks of treatment with rapamycin (Table 2.3). Categories shown in Table 2.3 were related to the metabolic substrate switch previously seen at 10 weeks of treatment with rapamycin. (See Figures 2.4 and 2.5 for more detailed information on the metabolite and enzyme abundances in the TCA cycle and glycolysis, respectively.) This does not appear to be simply a survivorship effect because A) the hearts continue to show poor diastolic function at this later timepoint, and B) the 16 week control old mice still remain different from young animals at this time. It thus appears more likely that the rapamycin effects on cardiac metabolism are transient in nature. Thus, the metabolic shift may contribute to persistent cardiac remodeling, but it appears unlikely to be one of the major causes of the persistent functional changes that we observe.

There is a growing evidence that rapa-induced metabolic shifts in mice and marmosets (Fang et al. 2013; Flynn et al. 2013; Ross et al. 2015), and humans (Blum 2002), change over the duration of treatment and can be reversible (Liu et al. 2014). Even though these studies have focused on insulin sensitivity and glucose tolerance, the idea that rapamycin can affect metabolism differently at different times during longer-term treatment has precedent – it adds to the already complex story of how rapamycin, and mTORC1/2 inhibition in general, can modulate tissue function and organismal health. mTORC2 inhibition by long-term rapamycin treatment is often used as a partial explanation for the phasic effects of rapamycin on metabolism (Lamming et al. 2012; Ye et al. 2012). Studying these effects seems important to future work on rapamycin treatment in mammals.
Table 2.3. Metabolome changes due to aging are not persistently reversed by rapamycin.
Left column shows the proteins and metabolites in either glycolysis or TCA cycle pathways. For both sexes, each protein/metabolite shows an up/down arrow (increased or decreased abundance compared to old control, respectively). Significance was determined by one-way ANOVA (metabolites) or two-way ANOVA (proteins) and those with p-values under 0.05 were further subjected to a Tukey post-hoc test for group comparisons, the significance level of which is displayed by the asterisks (*p<0.05, **p<0.01, ***p<0.001). R/C – rapa/control, P/C – persistence/control, Y/C – young/control. Grey arrows – not significantly different by ANOVA.

Table 2.4 Metabolomic and proteomic abundance changes in the TCA cycle.
This diagram shows the log2-fold-change from old control mice, in both sexes, in the enzymes (blue) and metabolites (orange) present in the TCA cycle. nd – no data. Sdh(a/b/c/d) – succinate dehydrogenase a/b/c/d respectively. Statistical significance by one-way ANOVA followed by Tukey post-hoc test: group was significantly different compared to old control (a) or group was significantly different from young control (b) of the same sex.
Figure 2.5 Metabolomic and proteomic abundance changes in glycolysis.
This diagram shows the log2fold-change from old control mice, in both sexes, in the enzymes (blue) and metabolites (orange) present in glycolysis. nd – no data. Statistical significance by one-way ANOVA followed by Tukey post-hoc test: group was significantly different compared to old control (a) or group was significantly different
from young control (b) of the same sex. G6P and F6P were indistinguishable by metabolomics.

2.3.4 Passive stiffness of the left ventricle

To examine whether the change in diastolic function was due to passive rather than active relaxation of the left ventricle, we formed a collaboration with the Regnier Lab at the University of Washington. The Regnier lab extracted left ventricular trabeculae from our old control, old rapamycin, and young animals (n = 5–8 per group) and tested how much force it took to passively stretch the muscle (Figure 2.6). This stiffness generally increases with age in mice, rats, dogs and humans (Asif et al. 2000; Alwardt et al. 2006; Rozenberg et al. 2006; Campbell & Sorrell 2015). We found that rapamycin treatment substantially and significantly reversed the age-related increase in passive stiffness of the fibers. In a separate experiment, we repeated the test with old control mice and old persistence mice and found a large and significant difference, with the previously rapamycin-treated mice exhibiting reduced muscle passive stiffness. These data together suggests that passive stiffness is a significant contributor to the diastolic dysfunction seen with aging, is substantially reversed by 8 weeks rapamycin treatment, and remains persistently decreased 8 weeks after rapamycin withdrawal.

Figure 2.6 Passive stiffness increases with age, and is persistently decreased with rapamycin in female mice.

These graphs show the force required to stretch LV trabeculae to varying percent length changes. Left graph: Female old control (blue), old rapa (red), and young mice. Right graph: Female old control (blue) and old persistence mice (green). P-values shown on the graphs are from one-way ANOVAs for each graph, followed by Tukey post hoc tests. n = 13~15 for left graph, and 4~5 for right graph. Error bars are SEM.
2.3.5 Sarcomeric protein phosphorylation may contribute to reduced passive stiffness and improved diastolic function after rapamycin.

Aging, rapamycin treatment, and changes in the redox status of the myocardium lead to reversible changes to phosphorylation status of sarcomeric proteins that can directly contribute to the stiffness of the myocardium. The altered cellular environment due to aging, rapamycin, or redox status can also affect the activation/inhibition of kinases and phosphatases that alter sarcomeric proteins and participate in cell signaling. Therefore, we endeavored to find evidence of aging and rapamycin-dependent changes to the phosphorylation status of several sarcomeric proteins, as well as proteins downstream and upstream of mTORC1/2. Together, the changes (or similarities) found could partially explain the differences in LV stiffness that persist for 8 weeks after removal of rapamycin.

To test whether rapamycin treatment does indeed lead to persistence reductions in post-translational modifications (PTMs) considered detrimental to the sarcomere, and diastolic function in general, we focused on phosphorylation on targets of interest in a set of sarcomeric proteins.

We focused on targets that have been shown to have changes in phosphorylation in the context of more oxidized cellular environment leading to aberrant kinase activity (CamK2d, PKA, PKC) that are known to modify sarcomeric proteins to increase myocardial stiffness (Hamdani et al. 2013). The list of sites to examine was also limited by the availability of appropriate mass spectrometry targets: these must be peptides that map to a single protein, contain the phosphosites of interest without other likely phosphosites, and be of an acceptable length for adequate detection and identification by LC-MS/MS. We therefore chose the following targets: Mybpc3, Ryr2, Pln, Camk2d, Sgk1, Tnni3, Eif4ebp1, Rps6, Ampka1, and Ampka2. Eif4ebp1 (eukaryotic translation initiation factor 4E-binding protein 1) and Rps6 (40S ribosomal protein S6) are downstream targets of mTORC1 and their phosphorylation levels are expected to be low during inhibition of mTORC1 by rapamycin. These serve as a positive control of the targeted MS/MS. Sgk1 (serine/threonine-protein kinase 1) is a downstream target of mTORC2, and changes from old control in its phosphosites may be indicative of inhibition of mTORC2 due to long-term rapamycin treatment. Of the remaining targets, some are sarcomeric proteins (Mybpc3 – Myosin-binding protein C cardiac-type, Ryr2 – ryanodine receptor 2, Pln – cardiac phospholamban, Tnni3 – cardiac troponin I) that have all been shown to alter cardiac function when their phosphorylation status is changed (Hamdani et al. 2013; Bovo et al. 2017; Li et al. 2017; Rajtik et al. 2017; Wu et al. 2017). Ampka1 and Ampka2 are important targets upstream of mTORC1/2, and their phosphorylation status may be indicative of a changed REDOX environment (Shirwany & Zou 2014). Camk2d (Ca++/calmodulin dependent kinase 2d) is important for regulating calcium ion signaling and phosphorylating Ryr2 (ryanodine receptor 2), a protein that regulates sarcoplasmic reticulum Ca++ release (Currie 2009). All of these targets but those immediately downstream of mTORC1 have been identified as important for diastolic function or are redox sensitive and may play at least an indirect role in diastolic function. Ideally, we would also have included PKC-alpha and PKA, due to their redox sensitivity and their complex interactions with both mTORC1/2 and many proteins of the sarcomere, but suitable target peptides were not available. Targeted LC-MS/MS to follow-up on these potential changes remains ongoing, and we are hopeful that it may shed further light on possible contributions of
passive stiffness within the myocardium to the persistent improvement of diastolic function after rapamycin treatment. Work completed to date focuses on the important protein Titin.

The largest protein in the sarcomere, indeed the largest protein known (Zile et al. 2015), is titin. Titin spans half the distance of the sarcomere, is involved in assembly of the sarcomere, serves as a scaffold for many other proteins and enzymes, and contributes to the passive stiffness of the muscle. This contribution to stiffness is due to long sections of titin that behave as a spring, and phosphorylation of these regions alters the spring’s ability to bounce back after contraction of the sarcomere. Type-switching of titin, the proportional abundance shift between N2BA and N2B sequences of titin, is one mechanism by which stiffness can be modulated in the LV. No evidence of titin type switching had previously been observed in hearts from mice fed for 10 weeks with rapamycin (Henk Granzier, personal communication). Titin isoform type switching in 16-week rapamycin treated or persistence hearts was independently examined in LC/MS-MS data by comparing the abundance of peptides found only on one isotype versus the other; this data confirmed the absence of any significant differences from old control groups or the young cohort (data not shown). Post-translational phosphorylation is another mechanism of titin stiffness modulation; when phosphorylated, the PEVK region alters titin into a more stiff conformation, and the N2Bus region allows a more loose configuration (Hamdani et al. 2017). Thus, targeted phosphoproteomics was used to examine 9 sites of interest on titin which are well accepted as either increasing or decreasing stiffness when phosphorylated (Figure 2.7). Several significant differences between groups emerged, but only in females. The persistence group in the female dataset was significantly different from every other treatment/age group at one N2Bus phosphosite and two PEVK phosphosites. Typically, when a phosphosite in the N2Bus region of titin is phosphorylated, the molecule becomes looser, whereas phosphorylation at PEVK phosphosites leads to a stiffer protein. In this fashion, the N2Bus phosphosite found to be significantly reduced in phosphorylation in the persistence group is well known to increase stiffness when phosphorylated. There are exceptions, however, and both of the PEVK peptides that showed an increase in phosphorylation in the persistence group for females have been shown to lead to a decrease in stiffness of titin when phosphorylated (Roe et al. 2017). Thus, all three phosphosites that are significantly altered in the female persistence group are known to be associated with reduced stiffness of titin, consistent with observations, at least in this sex. Overall, the lack of differences between the young controls and old controls in either sex were surprising, and potentially confound the interpretation of the persistence group results. The continuous rapamycin groups of both sexes also showed no significant differences to old control by repeated measures ANOVA with a Tukey post hoc test. Since only females, and only the persistence group, showed significant changes in the phosphorylation status of titin, these changes may not fully explain the rapamycin-dependent improvement in diastolic function.
Figure 2.7 Titin is differentially phosphorylated by sex, after rapamycin treatment.

Titin has two regions known for stiffness changes due to phosphorylation – N2BUS and PEVK (left side and right side of figure, respectively). All data are ratios of the amount of non-phosphorylated peptides containing the phosphosite indicated (e.g. S3754) to the amount of the same peptides without phosphorylation, as determined by targeted mass spectrometry. Boxplots represent data from 6 animals per group, in the order (left to right) of old control (blue), old persistence (purple), old rapamycin (red), and young (gray). Female data are in the top row, and male data are in the bottom row. ‡ = Persistence group is significantly (0.038 < p-value < 10⁻⁸) different from all other groups for the indicated phosphosite by repeated measures ANOVA followed by Tukey post-hoc test.

2.3.6 Rapamycin effects on age-related fibrosis of the myocardium

An alternate explanation for the changes to passive stiffness is a reversal of age-related increase in fibrosis of the myocardium. Such fibrosis is known in other settings to impair diastolic function (Eghbali et al. 1989; Bradshaw et al. 2010; Chiao et al. 2012; Chen et al. 2015). We therefore stained samples from Old Control, Old Rapa, and Young female mouse hearts with the Trichrome stain. We found evidence of only minor fibrosis (2-4% of myocardial area) in all groups, and no significant differences between groups (data not shown). Follow-up to this would be to use more sophisticated measures of changes in extracellular matrix (ECM), for example, by mass spectrometry.
2.3.7 *Rapamycin differentially alters respiratory chain complex activity by sex.*

Since rapamycin did persistently alter ETC Complex I peptide abundances for both sexes, there may be a connection between individual ETC complex activity and diastolic function. We hypothesized that rapamycin might persistently alter the function of the ETC in both sexes in the same direction and more specifically, that rapamycin changes the ratio of complex activity to complex abundance, thereby reducing the flux through the ETC. Electron flux is largely responsible for determining the mitochondrial membrane potential ($\Delta \Psi_M$), by creating a proton gradient across the inner mitochondrial membrane. An increase in $\Delta \Psi_M$ has been correlated with an increase in the production of ROS in myocardium and in isolated cardiac mitochondria (Aon *et al.* 2008; Chen & Knowlton 2010; Chen & Zweier 2014). Rapamycin has previously been shown to lower $\Delta \Psi_M$, through a mechanism independent of its well-known cellular targets (Schieke *et al.* 2006). Thus, changing the ratio of complex activity to complex abundance might reduce the $\Delta \Psi_M$ from a more pathological level in the aging heart, to a more physiological level. The impact of this could be reduced ROS while maintaining an appropriate ATP pool necessary for cardiac cycling activity.

We therefore compared the amount of Complex proteins in the ETC, and activity level of the individual ETC complexes, thereby obtaining a measure of activity/quantity of ETC protein.

![Diagram](image)

**Figure 2.8** *Rapamycin alters ETC Complex activity differentially by sex.*

A) Boxplots of the activity in nmol min$^{-1}$ mg$^{-1}$ of each of the complexes, normalized to the activity of citrate synthase for each sample, then as a ratio against the control average activity per complex. Each data point is one mouse (average from technical triplicates). N per group is 8 to 16. OC – old control at 16 wks, OP – old persistence, OR – old rapa, Y – young. Stars indicate significance, by Tukey post-hoc after an ANOVA of all groups per sex/complex which had an ANOVA $p < 0.05$, compared to control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

In the female cohorts we were unable to detect significant differences between treatment groups (by ANOVA) in activity of Complexes I, II, and IV (Figure 2.8, top panel). There was, however,
an aging-related increase in CIII activity in females, which was reduced persistently by rapamycin. Males showed a significant increase in Complex I activity with aging, which was partially reversed, again persistently, with rapamycin (Figure 2.8, bottom panel). The males also showed a significant increase in Complex II activity with rapamycin, though this was not persistent eight weeks after drug removal. All complex activity data were normalized to mitochondrial content, as determined by citrate synthase (CS) activity (See Figure 2.9A). We also measured mitochondrial:nuclear DNA ratios in all samples, and determined that mitochondrial content by this metric was similar between all old groups within each sex (Figure 2.9B).

![citrate synthase activity](image)

**Figure 2.9** Measures of mitochondrial content in both sexes.
Panel A) Citrate synthase activity per min per mg tissue. Left: females, right: males. Significance determined by ANOVA followed by Tukey post-hoc test. * p<0.05, *** p<0.001. n per group: 6 ~ 15. Error bars are SEM. Panel B) Aging in females, but not males, increases mitochondrial:nuclear DNA ratio. Bar charts for females (left) and males (right) show the ratio of mitochondrial:nuclear DNA ratio (mt-nd1:CYP1a1). Error bars are SEM. Significance was determined by one-way ANOVA with Tukey post hoc test. C = old control, P = old persistence, R = old rapa, Y = young mice. * p<0.05, *** p<0.001. n per group: 7~8

The activity of the ETC complexes was determined in the context of the mitochondrial content, by normalizing to the citrate synthase (CS) activity of each sample. This is a commonly accepted method of accounting for differences in the mitochondrial content of a sample. There was a slight decrease in CS activity in the control and persistence groups compared to young animals in the males, but this difference did not noticeably alter the degree of significance found in the comparisons of ETC complex assays normalized to the CS activity. I also investigated another measure of mitochondrial content, mitochondrial:nuclear DNA ratio. In females, which generally had higher ratios of mito:nuclear DNA than males, there were significant increases in all old groups with age, but no significant differences between the old groups (control, rapamycin, persistence). In males, there were no detected differences due to aging or rapamycin. The CS
activity data and the mito:nuclear DNA ratio data both show modest to no detectable differences between the old mice groups for either sex, leading to the conclusion that the mitochondrial content was similar between all old groups within each sex.

We used these data to examine potential changes in activity per unit abundance of ETC complexes after rapamycin treatment. Both sexes consistently demonstrated a much greater ratio of activity:abundance in old controls than in young animals (Figure 2.10), in spite of the sexual dimorphism in the abundance and activity of components of ETC CI and CIII. The 16 week rapamycin and the persistence treatment groups both showed a significant decline in the ratio of activity:abundance of CI, bringing levels towards that of the young animals (Figure 2.10A). Inclusion of accessory components in addition to core components of CI did not substantially alter this effect. In CIII, females, but not males showed a reversal of the age-dependent increase in activity:abundance ratio in rapamycin and persistence groups (Figure 2.10B). Consistent changes were not found in CII or CIV.

Figure 2.10  Rapamycin reduces ETC Complex I activity:Complex I protein ratios in both male and female mice, and Complex III activity:protein ratios in females. Calculation of activity to abundance ratio of protein was: (average individual complex activity per group divided by the average individual complex activity level of old control of same sex) / (average individual complex protein fold changes of each group divided by the old control). A) Complex I activity:abundance ratio data, separated by sex, and by inclusion (Core + Acc.) of accessory proteins in the list of protein fold changes or exclusion (Core) of those proteins. B) Complex III activity:abundance ratio data, separated by sex. C) Table of p-values from one-way ANOVAs performed per sex/complex, followed by Tukey post hoc test. Numbers in grey bars are from ANOVA
alone, the rest of the p-values are from the Tukey post hoc. Black bars – old control, white bars – rapamycin, dark grey bars – persistence, and light grey bars – young. All graphs show mean+/− SEM. p<0.05 *, p<0.01 **, p<0.001 ***

2.3.8 Do markers of cellular senescence change after rapamycin treatment and if so, are they persistent?

Senescence is an irreversible arrest of cell proliferation leading to radically altered cellular function and signaling. Selectively removing senescent cells results in longer lifespan and improved tissue function (Wang et al. 2017), including in the heart (Zhu et al. 2015). Rapamycin can inhibit some aspects of cell senescence in vitro (Wang et al. 2017), which lead us to question whether the senescent cell burden was reduced persistently in our rapamycin treated cohorts. Therefore, we investigated whether rapamycin alters the proportion of senescent cells using qPCR and IHC. The p16 and p53 tumor suppressors are key mediators of senescence (Rayess et al. 2012). By qPCR, we found that expression of p16INK4a was increased with age, and reduced persistently with rapamycin in female, but not male, mice (Figure 2.11). Part of the senescence phenotype is the production and secretion of the SASP (senescence associated secretory phenotype) which promotes inflammation. The High Mobility Group Box-1 (HMGB1) protein, which usually resides in the nucleus, is relocated as an Alaramin into the extracellular space around senescent cells (Davalos et al. 2013). To further interrogate whether the proportion of senescent cells was reduced with rapamycin, we will be staining frozen slides with antibodies to p16INK4a, HMGB1, and beta galactosidase. There are other factors to investigate with regard to rapamycin-reduced SASP. For instance, Laberge and colleagues have shown that rapamycin can modulate the SASP, as measured by decreased IL6 secretion, by suppressing NF-kB activity thereby suppressing IL1A translation (Laberge et al. 2015). Future work is needed that directly tests the relationship between reduction of senescence in response to rapamycin, and cardiac function in aging animal models.

Figure 2.11  p16INK4a mRNA quantities are increased with age in both sexes and persistently decline in females after rapamycin treatment.
Bar charts show relative quantities of mRNA (cDNA) for p16INK4a compared to old controls for each sex. N = 7–8 per group. Error bars are SEM. P-values from one-way ANOVA followed by Tukey post-hoc tests: * p<0.05, *** p<0.001. OC – old control, OR – old rapa, OP – old persistence, Y – young.

2.4 Discussion

Age-related diastolic dysfunction is a pervasive problem with no current treatment options. Diastolic dysfunction limits cardiac reserve, which can lead to symptoms of fatigue and dyspnea, and ultimately muscle wasting, loss of independence, and pulmonary venous congestion.
Rapamycin has been shown to reverse age-related diastolic dysfunction in rodents (Chiao et al. 2016; Luck et al. 2017) and dogs (Urfer et al. 2017). However, thus far there have not been studies that include both sexes and test for the persistence of the rapamycin-dependent improvement. Our work indicates that in C57BL/6NIA mice rapamycin can persistently improve diastolic function in aged animals of both sexes, even 8 weeks after cessation of an 8 week treatment. We also explored possible mechanisms for this persistent benefit. This work can help inform further studies of transient rapamycin treatment, and adds insights into possible mechanisms for improvement of cardiac function in old age.

2.4.1 Echocardiography

Both sexes of C57BL/6 mice show age-related diastolic dysfunction which is partially reversed by rapamycin treatment. This occurred in the absence of systolic changes in females, and with only small changes in systolic function in males, and it was persistent for two months post drug removal. This is an exciting finding because it suggests that future treatments for diastolic dysfunction may be transient, potentially reducing cost, side-effects (Pallet & Legendre 2013; Verhave et al. 2014; Salmon 2015), and other negative effects of chronic drug treatment.

2.4.2 Proteomics and ETC activity

We interpreted the large proteomic abundance changes seen after 10 week rapamycin treatment in previous experiments as evidence of improved proteostasis in the myocardium and postulated that these effects might remain persistent in the absence of the drug. Using shotgun proteomics (MS/MS), we found sex-specific differences in protein abundance with rapamycin treatment, and sex-specific differences in the persistence of many of these abundance changes. The IPA pathway most significantly affected by rapamycin treatment in both sexes was Mitochondrial Dysfunction and Oxidative Phosphorylation. Within this category, many proteins found to be significantly altered were related to Complex I of the ETC, either as assembly factors, core proteins, or accessory proteins. This lead us to hypothesize that there are activity level changes to the ETC, or at least to Complex I, with rapamycin treatment.

We found that both sexes had persistent decreases in ETC complexes activity with rapamycin. Complex I activity was significantly reduced with rapamycin in male mice, and Complex III was reduced in females. These changes were not coordinate with the abundance proteomics. However, we reasoned that the ratio of activity to abundance of proteins involved in Complexes I and III might be coordinately altered by rapamycin treatment in both males and females, which proved to be the case. In both sexes, this change (a reduction in activity:abundance ratio) was also persistently reduced in Complex I and a similar effect was seen in CIII in females. While males showed a stronger effect than females in CI, the significantly and persistently reduced ratio in Complex III in females may equalize this effect. Thus, both the males and females may have accomplished a similar functional outcome via modulating ETC activity and abundance.

It is tempting to theorize that changes to the ETC activity/abundance ratio may alter the mitochondrial membrane potential ($\Delta \Psi_m$), by modulating the electron flux through the ETC. Both Complex I and Complex III have been implicated repeatedly as prominent sources of ROS in mitochondria and an increase in $\Delta \Psi_m$ has also been shown to lead to an increase in ROS in myocardial cells, especially at higher $\Delta \Psi_m$ (Chen & Knowlton 2010; Chen & Zweier 2014). This
may be in part due to higher $\Delta \Psi_M$ driving reverse electron flux through the respiratory chain, primarily through Complex I (Batandier et al. 2006; Selivanov et al. 2011). Rapamycin has been previously demonstrated to reduce $\Delta \Psi_M$ (Schieke et al. 2006). This information, especially combined with various clinical and animal model studies that demonstrate an increase in ROS generation in heart failure (Chen & Knowlton 2010), lead us to posit that in old hearts rapamycin may persistently reduce the electron flux through the respiratory chain, leading to a lower $\Delta \Psi_M$ and reduced ROS generation. By reversing some of the burden of deleterious reactive species, and preventing new formation of reactive species, rapamycin may be reducing the need to expend cellular energy on damaged protein/DNA/lipid repair and more efficiently maintain the aged cellular environment. Studies that directly test this hypothesis are needed to more fully understand the connection between ETC complex activity and abundance and rapamycin’s organ-wide functional improvement.

Another intriguing way that rapamycin may alter whole heart function is through the regulation of mitochondrial membrane potential depolarization waves. In isolated cardiac mitochondria and intact cardiomyocytes, regular cycles of mitochondrial membrane potential depolarization create oscillations in important functions that include sarcolemmal potassium ion currents, excitation-contraction coupling, and changes in action potential duration, all of which impact cardiac function (Romashko et al. 1998; Aon et al. 2008). Membrane potential depolarization waves are, however, associated with increased ROS. Aon and colleagues provided evidence that the activity of the electron transport chain is directly responsible for the oscillations in $\Delta \Psi_m$ and the accumulation of ROS that leads to depolarization (Aon et al. 2008). They proposed that physiological mitochondrial oscillators are weakly coupled with low levels of ROS, and under stress (oxidative or metabolic) that ROS production supersedes a threshold that leads to strong coupling. This strong coupling changes the oscillations from high-frequency, low-amplitude to a pattern of low-frequency, high-amplitude. This pathological-state pattern of oscillations may be detrimental to the coordinated function of the mitochondria in a single cell and the entire heart. By reducing electron flux, rapamycin may be indirectly improving mitochondrial efficiency (less ROS production/ATP) and/or regulating the manner of REDOX waves in the myocardium.

2.4.3 Metabolomics

Our lab has previously shown that 10 week rapamycin treatment reverses the age-related metabolic switch from dependence on fatty acid oxidation (beta oxidation, FAO) to glycolysis. Since this change was concurrent with improved diastolic function, we analyzed global metabolomics to see whether that shift was persistent 8 weeks after drug removal. While found some evidence of the switch occurring in the cohorts in this study, those changes did not persist 8 weeks later. Intriguingly, all old groups from both sexes (16 weeks continuous rapamycin treatment and 8 weeks treatment followed by 8 weeks without rapamycin) were indistinguishable from each other, but remained significantly different from young animals of each sex, indicating that the changes to the metabolome due to rapamycin were transient. Thus, the metabolomes of the 16-week rapamycin treated animals (28 mo of age at that time) seemed to revert back to resembling the old control animals. Other studies have indicated that rapamycin’s effects on glucose metabolism and insulin regulation are also phasic and reversible (Liu et al. 2014). While this metabolomic profile change within the first few weeks of rapamycin treatment may be important for diastolic functional improvement, it does not appear to be necessary for the
persistent benefits. These data emphasize the importance at studying the kinetics of phenotypes, rather than single time points, when trying to determine possible mechanisms of drug effects.

2.4.4 Senescence

While we found one measure of senescence increased with aging (p16\textsuperscript{INK4a} expression), and was partially reversed by rapamycin treatment in female mice. This effect was persistent to eight weeks after drug removal. However, we did not detect a significant reduction of age-related expression of p16\textsuperscript{INK4a} in male mice. One measure of senescence is insufficient to draw conclusions of rapamycin’s effect on the burden of senescence in the aging hearts. Our ongoing work includes staining of tissue directly with HMGB1, p16, and beta galactosidase, so we can investigate the possible connection between rapamycin treatment and changes to senescence that correlate with the persistence diastolic function improvement.

2.5 CONCLUSION

We have found that rapamycin treatment in vivo leads to a persistent improvement in diastolic function, possibly through an altered cardiac proteome and changes in mitochondrial electron transport chain flux. There remain many interesting questions stemming from this work: Is ROS production from Complexes I and III reduced in rapamycin treated old hearts, and is this persistent? Is the persistently altered ETC flux seen in rapa hearts essential to the improvement in cardiac function? Do other longevity therapies lead to improved altered ETC flux?

We observed considerable sexual dimorphism in cellular and molecular changes due to rapamycin. Changes that are concordant and persistent in both sexes may best explain the persistence of diastolic improvement and inform the mechanisms of new therapies or more targeted approaches. The goal of any treatment in humans to is maximize the benefits while minimizing the undesirable effects, in as small and infrequent of dosing as possible. To that end, many clinical studies have monitored the side effects of rapamycin treatment (Pallet & Legendre 2013; Verhave et al. 2014) and animal model studies have sought to modulate those by either changing treatment duration or amount (Fang et al. 2013; Miller et al. 2014; Arriola Apelo et al. 2016; Bitto et al. 2016). Most side effects of rapamycin are, however, reversible upon cessation of treatment (Kaplan et al. 2014), although this was not addressed in the present study. These studies present a compelling case for the ability to minimize or eliminate side effects of continuous rapamycin use, primarily by using a transient dosing regimen. Our results complement this body of work by showing that transient treatment may be enough to confer long-term health benefits.

2.6 MATERIALS AND METHODS

Animals and Husbandry

C57BL/6J female and male mice (17 to 24 months old) from the National Institute of Aging (NIA) (originating from Charles River) were housed and maintained according to the guidelines of the Institutional Animal Care and Use Committee of the University of Washington. Both sexes were used to account for sex-specific differences in response to rapamycin treatment (Miller et al. 2014). Both sexes began treatment at the 75% survival mark for the National Institutes of Aging colony of C57BL/6 mice (Turturro et al. 1999). Animals were randomized
and divided into three experimental groups per sex. At 24 months of age (male) or 22 months of age (female), animals received encapsulated rapamycin (microencapsulated rapamycin in EUDRAGIT purchased from the University of Texas Health Science Center, San Antonio) at 42 ppm (males) or 14 ppm (females) in standard chow (rapa group), or encapsulation alone in the chow (control group), for 8 or 16 weeks. Another group received rapamycin chow for 8 weeks, followed by control chow for a further 8 weeks (persistence group). Young mice of both sexes were the same genotype, acquired from the NIA at 3 months old, and used for the studies at 4 months old. Body weight remained stable for all groups throughout the experiment. Animals were removed from the study when one of several possible conditions were met A) loss of 20% body weight, B) tumors or masses interfered with daily activity, or C) other illness or unknown cause led to the need to euthanize the mouse early. Diet was prepared in house, by combining powdered standard rodent diet (LabDiet PicoLab Rodent diet 20, #5053) with food coloring, agar agar, water, and either EUDRAGIT or encapsulated rapamycin. This mix was then pressed into patties and frozen until used. Animals had *ad libitum* access to food and water with a 12-hour light/dark cycle.

All mice were used for baseline echocardiography up to one week before treatment diets were introduced. Experimental animals were euthanized by cervical dislocation. Hearts were immediately removed and rinsed in ice-cold PBS, blotted dry on Kim Wipes, and trimmed to remove large vessels and fat. The tissue was weighed, and a donut of tissue was sliced through the middle of the ventricles for fixation in 4% formalin. The remainder of the tissue was minced and flash frozen in liquid nitrogen. The frozen tissues were pulverized using a bead beater (TissueLyser II, Qiagen) and stored in LN2 until use.

**Genomic DNA and cDNA expression**
Genomic DNA and mRNA were both isolated from each sample using a commercially available kit (TRIzol, Invitrogen, cat. # 15596026) according to manufacturer’s instructions. cDNA synthesized from these samples was used in quantitative real-time PCR using SYBER Green (ThermoFisher Scientific), on a Rotor-Gene Q qPCR machine, using the p16INK4a probe from ABI gene expression assays. To quantify mitochondria copy number, we performed quantitative PCR on the genomic DNA using the following primers: Cyp1a1 (Forward 5’ GACACAGTTGATCGAGAT 3’, Reverse 5’ TCTGATCAATGTCAACCGG 3’), ND1 (Forward 5’ GAACGAAAAATCTTAGGCTACATA 3’, Reverse 5’ GCCGTATGGACAAATGTT 3’). Mitochondrial copy number was calculated as the ratio of the amount of the mitochondrial gene NADH dehydrogenase 1 (ND1) to the single copy nuclear gene cytochrome P4501a1 (Cyp1a1).

**Echocardiography**
Echocardiography was used to measure cardiac function longitudinally. At each time point, (0, 8, 12, and 16 weeks after start of treatment), mice were anesthetized with 1-2% isoflurane in an oxygen mix to keep the heart rate between 500-550 bpm. Breathing and heart rates were continuously monitored and body temperature was kept stable using a circulating warm water pad. Images were captured using a 13 MHz probe with a Siemens Acuson CV-70 (Siemens Medical Solution, Mountain View, CA, USA), using M-mode and B-mode views along with LV parasternal long axes view (D-mode and TDI). Images taken when the heart rate was not within the 500 to 550 bpm range were excluded from analysis.
**Trichrome stains**
Cardiac tissue sections were sliced into 2 mm donut sections with a coronal heart slicer (Zivic Labs, product number HSMA001-1) in the largest cross-sectional area of each heart and frozen in O.C.T. medium. 4 µm sections were stained with Masson’s Trichrome stain. All stained slides were imaged with a Nuance spectral deconvolution microscope. Images were subsequently quantified using Nuance software and the data were analyzed using R.

**Proteomics sample processing**
Flash-frozen and pulverized heart tissues were sonicated in ice-cold 50 mM ammonium bicarbonate + 0.1% (wt/vol) RapiGest (Waters Corporation, product number 186001860), then centrifuged at 8000 g for 10 min at 4°C. Concentration of soluble protein in the supernatant was determined by BCA (Company). DTT was added to 20 µg of protein to a final concentration of 5 mM, and incubated at 50°C for 30 minutes. After cooling to room temperature, iodoacetamide (IAA) was added to a final concentration of 15 mM and incubated in the dark for 30 minutes. Trypsin (sequencing grade, Promega, cat #), was added at a ratio of 1:50 enzyme:protein, incubated 2 hrs in dark while shaking at 37°C. Trypsin was neutralized with HCl at 200 mM, incubated with shaking for 45 minutes at 37°C. Samples were centrifuged at 16k g, at 4°C, for 20 minutes. Supernatant was cleaned with preconditioned MCX columns (company, cat #), washing with 0.1% formic acid in water, 90% acetonitrile:10% water. Peptides were eluted with 600 µL 2.8% NH4OH in methanol. Peptides were dried under vacuum until only a few µL were left per sample, after which they were reconstituted with 160 µL 0.1% formic acid in water and stored at 4°C until submission to the MacCoss lab at the University of Washington. LC-MS/MS analysis was performed with a Waters nanoAcquity UPLC and an Orbitrap Fusion Mass Spectrometer.

**Shotgun Proteomic Analysis**
Statistical analyses were performed using R (R Core Team 2016) and Bioconductor (Fred Hutchinson Cancer Research Center, Seattle, WA, USA). Raw data are available on Panorama (https://panoramaweb.org) and R scripts used in the proteomics analysis pipeline are available upon request from the corresponding author.

*Mapping peptides to proteins:* Peptides that mapped to a single UniProt (Apweiler et al. 2004) protein accession for Mus Musculus were used for quantification of protein abundance. 31,791 entries (male dataset) and 27,165 entries (female dataset) from UniProtKB/Swiss-Prot and UniProtKB/TrEMBL were found. Where one protein consisted of multiple peptides, statistical models were modified to appropriately account for these by using the peptides in each protein as a blocking factor. Total number of proteins found were 7,166 (males), 5,870 (females).

*Relative abundance:* Statistically significant abundance changes of proteins between groups were determined by Students T-test, and p-values were corrected for multiple testing by calculating q-values. q-values under 0.05 were considered statistically significant.

**Pathway Analysis and Heatmaps**
We used Ingenuity Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity) to generate lists of pathways that were significantly affected by
rapamycin. Multiple testing corrections, to control for estimated false discovery rate, were performed using the Bioconductor q-value package. Only proteins whose expression was significantly different by Students T-test, (threshold of q-value < 0.05), between Control and Rapamycin (continuous) groups were included in the queries to identify canonical pathways from IPA. IPA uses a Fisher’s exact test to determine the p-value of enrichment into canonical pathways, after deriving the significance of the association between the data set and a pathway (number molecules from dataset fitting into a pathway/total number of molecules present in curated pathway). Z-scores of abundance of proteins in each pathway, and all significantly affected proteins per sex, were visualized on heatmaps created in R using the heatmaps.2 function in the gplots (Warnes et al. 2016) package. Clustering in Dendrograms was performed using Ward’s method.

**Targeted Phosphoproteomics**

*Alterations to MSMS prep protocol:* The samples were processed in the presence of inhibitors of the specific kinases that affect these sites, and inhibitors of the enzymes that remove those modifications at these sites. The kinase inhibitors were K252a (Sigma, cat # K2015) and KN-83 (Enzo, cat # BML-E1268-0001) and the phosphatase inhibitor was HALT phosphatase cocktail (Thermo Scientific, cat # 78426). Each was added at a concentration of 1 mM immediately after thawing the samples.

*Approach to picking targets:* Each phosphosite target was chosen by first identifying in the literature which phosphosites on each protein of interest were affected by mTORC1/2 or by PKC-alpha, Camk2d, PKG, and PKA. Then the protein was digested in silico by ExPasy (http://web.expasy.org/peptide_cutter/) to find the tryptic peptide containing the phosphosite of interest. Each peptide sequence found this way was subjected to three criteria to be considered for use in the targeted proteomics: 1) the peptide must be between 5 and 25 aa long, 2) it must contain the phosphosite of interest in a region more likely to be detected by MS2 (a smaller numbered y- or daughter-ion), and 3) it must contain one or no other potential phosphosites.

**Metabolite Extraction and Analysis**

*Metabolite extraction* - 10-12 mg of flash-frozen and pulverized heart tissue was homogenized in 200 μL water:HPLC grade methanol (1:4, -75°C, on dry ice). To each sample, we added 800 μL water:methanol (1:4) and incubated for 30 minutes on dry ice. Then the samples were centrifuged at 14K rpm for 5 minutes at 4°C. Supernatants were saved on dry ice. Pellets were resuspended in 500 μL water:HPLC grade methanol (1:4) and incubated for 15 minutes on dry ice. These were centrifuged again, 14k rpm, 5 minutes, at 4°C and the soluble extract was combined with the first supernatant. Samples were dried completely under vacuum at 30°C. Metabolic profiling by LC-MS was performed as previously described (Dai et al. 2014a).

*Analysis* - Data were log2 transformed, centered and scaled (by standard deviation), then analyzed using one-way ANOVA per sex and metabolite in R. Sample sizes for each group (old control, old rapa, old persistence, young controls) were (females – 8,8,8,6) and (males – 8,7,8,7).

**Statistical Analysis**

For specific statistical analyses, see each methods subsection. In general, all tests involving one sex with more than two groups were one-way ANOVAs. Two-way ANOVA with repeated
measures was used when one sex with more than two groups was tested over multiple time points (i.e. for Ea/Aa ratios). Following ANOVAs, Tukey post-hoc tests were used to determine group-group differences. Students T-tests were used when comparing two groups. Multiple testing corrections were performed using the Bioconductor package q-value. \( p \)-values and \( q \)-values less than 0.05 were considered statistically significant.
Chapter 3. ADDITIONAL OBSERVATIONS FROM THE RAPAMYCIN-PERSISTENCE ANIMAL COHORTS

3.1 ABSTRACT

I engaged in several collaborations that utilized the animal cohorts described in Chapter 2. Two of those collaborations have led to publications thus far, and the relevant portions of those works are detailed below. The first is a study lead by Johnathan An, in Dr. Matt Kaeberlein’s lab at the University of Washington, investigating the effects of aging and transient rapamycin treatment on periodontal disease in mice (An et al. 2017). The second was an effort lead by Dr. Warren Ladiges, also of the University of Washington, to develop a tool to measure biological aging with and without interventions outside of the traditional lifespan study (Ladiges et al. 2017). The data below is contained in these published reports, in which I am a co-author.

3.2 RAPAMYCIN TREATMENT REVERSES ALVEOLAR BONE LOSS IN AGED MICE

Periodontal disease is an aging-related disorder defined by the loss of alveolar bone (thickened area of bone in the jaws that holds the tooth sockets) and connective tissue (Socransky & Haffajee 1994; Darveau 2010). Dysregulated immune function leading to systemic inflammation may be one cause of periodontal disease. mTOR inhibition has been demonstrated to improve immune function in aged mice and humans (Chen et al. 2009; Mannick et al. 2014). In (An et al. 2017), aging clearly worsens periodontal disease as seen in the comparison of young to old control mice from the animals described in Chapter 2. However, 8-week rapamycin treatment in aged animals was sufficient to dramatically recover the alveolar bone. These results provide the first evidence of an effective treatment for periodontal disease.

In Figure 3.1, the authors quantified alveolar bone loss with aging using 10-11 week old mice and 26 month old mice by high-resolution microCT imaging of the maxilla. They measured the cementoenamel junction (CEJ) to alveolar bone crest (ABC) distance at 14 sites, using 5 independent observers. Quantification of their findings is shown in Figure 3.2.

When the authors compared old controls to animals treated with rapamycin for 8 weeks, they found that the treated animals had restored levels of alveolar bone (Figure 3.3).
Figure 3.1 Aging is associated with alveolar bone loss in C57BL6JNia mice. Representative microCT scan showing significant alveolar bone loss in old mice vs. young mice. Palatal aspect is shown for a representative (A) young 10-11 week-old mouse and a (B) old 24-26 month-old mouse. Buccal aspect is shown for (C) young and (D) old mice. Yellow lines represent distance between the cementoenamel junction (CEJ) to the alveolar bone crest (ABC) as landmarked by an observer who was blinded to the identity of each animal. The larger distance in panel D compared to panel C is indicative of alveolar bone loss in the aged animal compared to the young animal. Figure and figure legend taken directly from (An et al. 2017).

Figure 3.2 Representative microCT scan showing predetermined landmarks for quantifying alveolar bone levels in mice. Distance from the cementoenamel junction (CEJ) to the alveolar bone crest (ABC) was measured at 14 predetermined maxillary sites, bilaterally. The readings were totaled for each mouse. (A) 8 predetermined maxillary buccal sites (B) 6 predetermined maxillary palatal sites. Figure and figure legend taken directly from (An et al. 2017).
Figure 3.3  *A single, transient 8 week treatment with rapamycin attenuates alveolar bone loss in aged C57BL/6JNia mice.*

Female 24-26-month old C57BL/6JNia treated were treated with either a control diet or 14 ppm eRAPA diet for 8 weeks. MicroCT image analysis indicated less alveolar bone loss in the rapamycin treated mice at the end of the treatment period compared to control animals. Representative images of (A) control (n=8) and (B) rapamycin-treated mice (n=8) after 8 weeks (26-28 months of age at time of sacrifice). (C) Boxplot showing total distance measured from CEJ-ABC buccal and palatal aspect only for 26 month old female C57BL/6JNia mice treated with control or 14 ppm rapamycin beginning at 24 months of age. Rapamycin-treated animals had significantly greater alveolar bone levels compared to controls (p<0.005). *Figure and figure legend taken directly from* (An et al. 2017).

3.3  **RAPAMYCIN REDUCES OCCURRENCE OF TISSUE LESIONS IN AGED MICE**

Ladiges and colleagues have sought to develop a set of guidelines for testing therapeutics meant to slow aging (Ladiges et al. 2017). Currently, lifespan studies are conducted in mice to test “anti-aging” drugs, but these are not ideal. They are costly, both in time and money, and they cannot be done with human subjects. The authors have thus derived a new paradigm, based on the detection and determination of severity of histopathological lesions in a variety of tissues (Ladiges et al. 2013). Part of the data used to validate this Geropathology Grading Platform (GGP) were derived from animals used in Chapter 2.
Each tissue used in the GGP is given a score for each of its age-related lesions, which are then summed and averaged over multiple mice to form the composite lesion score (CLS). These CLS scores have been shown to be both sensitive to changes by anti-aging interventions, and correlate with age.

In Figure 3.4, panel C, it can be seen that animals treated for eight weeks with rapamycin have significantly lower CLS than age-matched controls. The authors contend that detection of the reduced CLS with this intervention is evidence that the GGP may be useful to detect longevity benefits in other interventions even with short term or transient treatment, before performing a lifespan study.

**Figure 3.4** Composite lesion scores generated by the Geropathology Grading Platform in mice change in an age- and drug-dependent manner. (A) Composite lesion scores in four age groups of C57BL/6N male mice increase with increasing age and in an organ-dependent manner, \( N = 12/\text{cohort} \). (B) Composite lesion scores in four age groups of CB6F1 male mice increase with increasing age and in an organ-dependent manner, \( N = 12/\text{cohort} \). (C) Composite lesion scores are suppressed in 24-month-old C57BL/6 mice after 2 months of oral rapamycin, 42 ppm, \( N = 6–7/\text{cohort}, p \leq .05 \). (D) Composite lesion scores in the heart increase in alignment with left ventricular mass index (LVMI) and organ weight as measures of the progression of cardiac decline with increasing age in C57BL/6N mice, \( N = 12/\text{cohort} \). Figure and figure legend taken directly from (Ladiges et al. 2017).
Chapter 4. MITOCHONDRIAL DYSFUNCTION IN CARDIAC AGING

4.1 ABSTRACT

This chapter is adapted from (Tocchi et al. 2015).

Cardiovascular diseases are the leading cause of death in most developed nations. While it has received the least public attention, aging is the dominant risk factor for developing cardiovascular diseases, as the prevalence of cardiovascular diseases increases dramatically with increasing age. Mitochondria play a great role in these processes, as cardiac function is an energetically demanding process. In this review, we examine mitochondrial dysfunction in cardiac aging. Recent research has demonstrated that mitochondrial dysfunction can disrupt morphology, signaling pathways, and protein interactions; conversely, mitochondrial homeostasis is maintained by mechanisms that include fission/fusion, autophagy, and unfolded protein responses. Finally, we describe some of the recent findings in mitochondrial targeted treatments to help meet the challenges of mitochondrial dysfunction in aging.

4.2 INTRODUCTION

The heart is a highly metabolic organ that is reliant on the maintenance of cellular-energetic homeostasis, precisely regulated mitochondrial dynamics, and optimal mitochondrial function. Mitochondria are important determinants of cellular homeostasis and longevity since they are the main producers of cellular ATP and play a vital role in regulation of apoptotic death pathways in many tissues. Due to its high energetic demand and high density of mitochondria, the heart is especially vulnerable to mitochondrial dysfunction via structural disruption, energetic fluctuations, and mitochondrial signaling. Cardiac senescence is accompanied by a general decline in mitochondrial function, clonal expansion of dysfunctional mitochondria, increased production of reactive oxygen species (ROS), suppressed mitophagy, and dysregulation of mitochondrial quality control processes such a fusion and fission (Khrapko et al. 1999; Terman et al. 2003; Das & Muniyappa 2013; Dorn 2013). These detrimental alterations in mitochondrial function have been widely correlated with several age-related cardiac diseases, as will be described below. The mechanisms responsible for age-related mitochondrial dysfunctions in cardiac tissue are only partially defined and it is not yet clear the extent to which mitochondrial dysfunction is directly linked to aging (Bratic & Larsson 2013). Nevertheless, the information below will illustrate that there is abundant evidence that mitochondrial function is intimately tied to cardiac health and, likely, largely related to cardiac aging.

4.3 MITOCHONDRIAL ENERGETICS IN CARDIAC AGING

Given the high energetic demand of the heart, it is not surprising that age-related defects in mitochondrial bioenergetics have been related to normal cardiac aging (Shigenaga et al. 1994; Tatarkova et al. 2011; Bratic & Larsson 2013). Many factors contribute to the reduced energetic capacity of the cardiac mitochondria including increased ROS, mutation and deletions in the
mitochondrial genome, and dysregulation of proteostasis and mitochondrial biogenesis (Shigenaga et al. 1994; Navarro & Boveris 2007; Lopez-Lluch et al. 2008; Tatarkova et al. 2011; Bratic & Larsson 2013). In rodent models, the total mitochondrial content does not change in liver and brain with age, however, the activity levels of components of the electron transport chain decrease (Navarro & Boveris 2004).

It has been documented that the activity of mitochondrial respiratory chain complexes declines with age in skeletal muscle (Short et al. 2005), brain (Ojaimi et al. 1999), and heart (Tatarkova et al. 2011), particularly in complex I and IV (Lenaz et al. 1997; Tatarkova et al. 2011). Complexes II, III, and V remain less affected by age in cardiomyocytes (Navarro & Boveris 2007; Tatarkova et al. 2011). Differences in the reported activity levels of complex III in aging heart may be due to the inclusion or exclusion of two separate populations of cardiac mitochondria, interfibrillar (IFM) and subsarcolemmal (SSM), a unique aspect of cardiac structure. Of these two populations, complex III activity may only decrease in the IFM with aging (Lesnefsky et al. 2001). The decreased activity of complexes I and V (and possibly III and IV) may be partially compensated for by increased expression of the mitochondrial genes within those complexes in adult mice, but this overexpression was reversed in aged mice (Manczak et al. 2005).

Levels of mitochondrial respiratory proteins and other key proteins involved in mitochondrial metabolism decline in the old heart, including those in fatty acid metabolism. Conversely, glycolytic metabolic pathways as well as extracellular structural proteins increase significantly with age (Dai et al. 2014a). Increased expression of glycolytic proteins, together with a decline in fatty acid oxidation, TCA cycle, and amino acid metabolism, indicates a metabolic remodeling with age that bears some similarity to heart failure in younger individuals (Kolwicz & Tian 2009; Dai et al. 2012b). As described in a subsequent section, treatment of old mice with CR or rapamycin reverses this metabolic substrate shift in the heart, restoring a greater dependence on fatty acid oxidation and mitochondrial function (Dai et al. 2014a). As the heart has uniquely high and continuous energetic requirements, many of the energetic and metabolic changes seen in cardiac aging and failure may be more apparent in the heart than in other organs.

### 4.4 ROS, DNA DAMAGE AND THE AGING HEART

Mitochondria are the main source and target of ROS produced as by-products of cellular respiration (Barja 1999; Judge & Leeuwenburgh 2007). ROS production increases with age and higher ROS content limits proper functioning of macromolecules and signaling pathways (Bartke 2008). The mitochondria free radical theory of aging (MFRTA) hypothesized that age-related increases in mitochondrial ROS resulted in accumulation of mtDNA mutations and oxidized proteins and lipid that impaired mitochondrial respiratory (RC) efficiency, leading to further ROS production in a viscous cycle (Fraga et al. 1990; Stadtman 1992; Chen et al. 2005). Many studies showed data consistent with this theory, including increased ROS production, increases in mitochondrial DNA (mtDNA) mutations, and respiratory chain dysfunction in aging tissues (Pikó et al. 1988; Sato et al. 1989; Trounce et al. 1989; Yen et al. 1989; Corral-Debrinski et al. 1992; Soong et al. 1992; Wanagat et al. 2001; Kujoth et al. 2005; Vermulst et al. 2007; Trifunovic & Larsson 2008; Vermulst et al. 2008; Tatarkova et al. 2011). Decreased mtDNA quantity and deteriorating replication fidelity with age contributes to an accumulation of dysfunctional mitochondria often resulting in pathological outcome (Hayashi et al. 1991; Yoneda et al. 1994; Nakada et al. 2001; Chan 2006; Hom & Sheu 2009). However, there is increasing
recognition that ROS and ROS signaling have beneficial roles, and many studies of mouse models in which cytoplasmic antioxidant enzymes are reduced or increased have failed to support a causal connection to aging (Pérez et al. 2009). The study that is most supportive of the mitochondrial variant of the free radical theory of aging is of transgenic mice that express catalase that is targeted to mitochondria (mCAT); these mice has increased lifespan (Schriner et al. 2005) and numerous reports of resistance to diseases of aging (Wanagat et al. 2010). Most relevant to this review, mCAT mice have a phenotype of delayed cardiac aging that includes both functional and molecular parameters (Hill et al. 1960). Furthermore, mice with mutation in the mitochondrial Polg exonuclease proofreading domain (PolgD181A) have elevated mitochondrial DNA mutations and deletions, exhibit a progeria phenotype and can develop cardiomyopathy leading to congestive heart failure (Kujoth et al. 2007). This cardiomyopathy is attenuated in mCAT mice, indicating that this phenotype is partly mediated by mitochondrial oxidative stress (Dai et al. 2010).

4.5 MITOCHONDRIAL STRUCTURAL CHANGES WITH AGING

In several model systems, and in humans, evidence suggests that the structure of mitochondria in the heart is disrupted by the aging process. Studies in human and hamster hearts show that mitochondria may increase in size with age (Sachs et al. 1977; Fleischer et al. 1978). Electron microscopy has been used to demonstrate a disrupted morphology of mitochondria with age in mice (Dai & Rabinovitch 2009). It has been shown that the mouse cardiac inner mitochondrial membrane displays a loss of cristae with aging (Tate & Herbener 1976), although crista morphology does not appear to change with age in Fischer 344 rats both in situ and in isolated subsarcomal and interfibrillar mitochondria (Riva et al. 2006).

Mitochondrial structure is intimately associated with functional integrity and the cristae provide essential scaffolding for RC complexes; thus it would be no surprise that alterations in mitochondrial structure might be integrally related to the age-related decline in mitochondrial activity. Indeed, reversing the loss of youthful mitochondrial structure with age may result in improved electron transport activity (Hagen et al. 2002).

4.5.1 Cardiolipin in the aging heart

The inner mitochondrial membrane contains cardiolipin (1′-[1,2-diacyl-sn-glycero-3-phosphoryl]-3′-[1″,2″-diacyl-sn-glycero-3″-phosphoryl]-sn-glycerol; 1,3-diphosphatidylglycerol), which is almost entirely absent from the rest of the mammalian cell (Schlame & Haldar 1993; Lee et al. 2006; Chicco & Sparagna 2007). Cardiolipin (CL) was first purified from beef heart in 1942 and has since been classified as a tetra–acyl phospholipid essential for the structural integrity of the mitochondrial membrane (Pangborn 1942) (reviewed in (Chicco & Sparagna 2007)). The acyl chains on CL can vary depending on which kingdom, species and tissue is investigated (Paradies & Ruggiero 1990; Choma & Komaniecka 2003; Guan et al. 2014; Jimenez et al. 2014a) and what diet is being consumed (Yamaoka et al. 1990; Cortie & Else 2012).

CL has an essential role in maintaining optimal mitochondrial structure and function through its ability to maintain curvature of cristae, supporting the assembly and interaction of mitochondrial respiratory chain complexes and supercomplexes, modulating and maintaining the proton
gradient, and preventing apoptosis (reviewed in (Schlame & Ren 2009; Schlame 2013)). Recent studies have begun to help clarify some of these complex interactions between cristae structure, CL, respiratory complexes and the proteins that facilitate their assembly (Cogliati et al. 2013; Harner et al. 2014). A considerable body of evidence (Ames et al. 1995; Paradies et al. 1999; Pepe et al. 1999; Tamburini et al. 2004; Lee et al. 2006), with rare exception (Moghaddas et al. 2002) suggests that CL is selectively lost and/or remodeled in aging mitochondria.

Modification or restoration of CL content has been proposed as a method of reducing age-associated declines in mitochondrial function. Age-dependent loss of CL may be due to oxidative stress (Almaida-Pagan et al. 2014), which can be due to extrinsic ROS or by peroxidase activity of cytochrome C that is closely associated with CL (Aluri et al. 2014). Prevention of peroxidation of CL may attenuate or abrogate mitochondrial dysfunction (Aluri et al. 2014). In the brain, melatonin might help to preserve the structural integrity of cardiolipin by preventing age-related peroxidation of the cardiolipin (Petrosillo et al. 2008). This was observed alongside other improved parameters of mitochondrial aging in the brain and suggests that preservation of intact cardiolipin is an avenue of abrogating age-related declines in mitochondrial function. Similarly, Paradies and colleagues have reported that acyl-carnitine supplementation in aged rats restored CL levels to that of young controls, and that some CL-dependent processes were improved (Paradies et al. 1992; Paradies et al. 1999).

Barth syndrome is an example of CL disease with cardiomyopathies being the most deadly symptom. In Barth syndrome, tafazzin, another protein located in the mitochondria, is mutated or lost. CL is modified into its final 18:2 form from monolysocarlipin (MLCL) by adding and removing acyl chains in two different tafazzin-dependent mechanisms (Ye et al. 2014). The exact mechanism is still unknown for how tafazzin and CL interact, but the disease state suggests an important relationship between the two.

More recently, it has been suggested that the protective effects of mitochondrial targeted SS-31 peptide is due to its affinity for CL and the prevention of cytochrome C peroxidation (see the section on Cardiolipin-Targeted Therapies, below).

4.6 DIETARY INTERVENTION AND THE AGING HEART

CR, the reduction of total calories without nutritional deficits, is the longest studied and most reproducibly successful method of extending lifespan and improving healthspan in model organisms. CR exerts some of its effects through modulating TOR signaling (mTORC1 in particular in mammals), and seems to have a wide range of effects, including modulation of tissue maintenance (reviewed extensively in (Speakman & Mitchell 2011)). In multiple organisms, including humans, rodents, and monkeys, chronic CR delays the onset of cardiac aging. This can be seen as a reduction of aging-associated cardiac functional decline, cardiac hypertrophy, and cardiomyopathy (Maeda et al. 1985; Taffet et al. 1997; Colman et al. 2009; Niemann et al. 2010; Shinmura et al. 2011; Dai et al. 2014a).

CR protects against cardiomyopathy, at least in part, by reducing age-associated apoptosis. This is partially accomplished by a reduced susceptibility to DNA damage, improved DNA repair, and apoptosis-related gene expression alterations (Maeda et al. 1985; Dhahbi et al. 2006). Expression of genes involved in numerous other processes important for mitochondrial function
in aging are also modulated by CR, including extracellular matrix maintenance, inflammation, oxidative phosphorylation, and glucose and fatty acid metabolism (Dhahbi et al. 2006; Dai et al. 2014a). Other protective effects of CR in the myocardium include the reduction of fibrosis and perivascular collagen deposition, reduced vascular inflammation and left ventricular cardiac hypertrophy, along with protective effects against ischemia (Spaulding et al. 1997; Broderick et al. 2001; Dhahbi et al. 2006).

While it is unknown exactly how CR modulates cardiac aging, an attractive hypothesis is that limited nutrient and energy availability allows tissues to switch to a somatic maintenance state that may include optimization of existing cellular resources. For example, Drake and colleagues (2013) found that proliferative rates in heart, while low in controls, were further reduced by life-long CR (measured by DNA synthesis) while measures of mitochondrial biogenesis were maintained (Miller et al. 2012; Drake et al. 2013). Short term (10 weeks) CR has been shown to result in improved cardiac function accompanied by a 30% reduction in protein turnover rates, and remodeling of the cellular and mitochondrial cardiac proteome and metabolome toward an abundance profile more similar to that of young mice, as well as with lower oxidative damage (Dai et al. 2014a).

Oxidative stress increases with age, concurrent with a decreasing ability to prevent or recover from oxidative stress in the heart (Judge et al. 2005). While some evidence suggests that mitochondrial dysfunction may not be due to damage from age-associated ROS alone (Trifunovic & Larsson 2008), modulation of this stress either by direct targeting of catalase to the mitochondria (mCAT) (Schriner et al. 2005; Dai et al. 2009), or by CR (Colom et al. 2007; Niemann et al. 2010; Shinmura et al. 2011; Dai et al. 2014a) results in improvements in cardiac function and in molecular changes indicative of an improved response to oxidative stress. For example, long-term, but not short-term, CR has been shown to dramatically reduce mitochondrial H$_2$O$_2$ production while lowering oxidative damage to mtDNA (Gredilla et al. 2001). A clear understanding of the mechanisms of CR enhancement of cardiac mitochondrial function should provide greater insight into future protective intervention strategies.

### 4.7 SIGNALING PATHWAYS

Modulation of cardiac health and aging, including the effects of CR, is mediated through several signaling pathways, the best characterized of which include mTOR and Insulin-like Growth Factor signaling and downstream of these, regulation of histone acetylation by sirtuins.

#### 4.7.1 mTOR pathway

Rapamycin inhibits mTOR (mechanistic target of rapamycin) and is the best studied CR mimetic. mTOR modulates several important growth and cellular quality control mechanisms including ribosomal biogenesis, autophagy, lipid synthesis, and protein translation (reviewed in (Johnson et al. 2013)). Following the National Institute on Ageing Intervention Testing Program’s (Nadon et al. 2008) demonstration of enhanced longevity after chronic rapamycin treatment of mice (Harrison et al. 2009), several other publications have demonstrated that long-term rapamycin treatment of mice improves healthspan measures and/or extends lifespan (Anismanov et al. 2010; Miller et al. 2011). Inhibitors of TOR (both genetic and pharmacological) also extend lifespan and healthspan in other model organisms including flies (Kapahi et al.)
Rapamycin confers functional benefits to the aging heart. Wilkinson and colleagues found that many measures of healthspan were positively affected by life-long rapamycin treatment in 20–22-month-old genetically heterogeneous mice. In the heart, they found that the incidence of nuclear atypia was reduced in rapamycin treated animals (Wilkinson et al. 2012). Pressure-overload-induced cardiac hypertrophy in young mice is reduced by rapamycin (McMullen et al. 2004). Recently, it has been shown that short-term (10–12 weeks) rapamycin treatment in late-life reversed age-related cardiac functional declines in mice, including improvement in systolic and diastolic dysfunction, and a reversal of cardiac hypertrophy (Flynn et al. 2013; Dai et al. 2014a). Investigators at the Buck Institute reported that this was accompanied by a reduction in age-related sterile inflammation (Flynn et al. 2013), while our laboratory showed that rapamycin recapitulated the CR effect of remodeling the old heart proteome to a more youthful abundance of proteins associated with young mitochondrial function (ETC., TCA cycle, fatty acid metabolism) and decreased abundance of glycolytic pathway proteins (Dai et al. 2014a). These results may point to proteomic and metabolic remodeling as a mechanism behind the cardiac functional benefits granted by rapamycin.

4.7.2 Insulin-like growth factor

The insulin/IGF-1 signaling pathway helps regulate cellular proliferation, survival, and autophagy (Li et al. 2012; Riehle et al. 2013). This pathway is one of the best characterized determinants of lifespan, as deficiency in insulin/IGF-1 signaling is associated with increased lifespan in both invertebrate and vertebrate models of aging and IGF-1 activity is also down regulated in CR (Avogaro et al. 2010; Ziv & Hu 2011). In general, IGF-1 has been shown to be cardio protective, allowing for suppression of ROS and autophagy in the cardiovascular system (Kuo et al. 2005; Sanz et al. 2005; Riehle et al. 2013; Troncoso et al. 2013). Notably, reduction in insulin/IGF-1 signaling improved cardiac performance at advanced age in Drosophila (Wessells et al. 2004). In contrast, an age-dependent decline in serum IGF-1 correlates with an increased risk of heart failure in humans (Khan et al. 2002). It has therefore been proposed that treatments to increase IGF-1 signaling, including growth hormone therapy, may actually be beneficial in some patients with heart failure (Broglio et al. 1999). Thus, much remains to be learned before we understand the full role the IGF-1 pathway plays in cardiac aging.

4.7.3 Sirtuins

Sirtuins (Sirt) are a family of proteins deacetylases. There are seven members of the family, with Sirt3, Sirt4, and Sirt5 being targeted to the mitochondria (Sack 2011; Park et al. 2013b). Sirt3 in particular has been studied in the cardiovascular system and has been shown to prevent apoptosis, interact with nutrient sensing, and post-translationally modify proteins, while also being the only Sirt to be linked to an increase in human lifespan (Pillai et al. 2010; Porter et al. 2014). Sirt3 overexpression leads to a decrease in cardiac hypertrophy via activation of Foxo3a-dependent defenses, while the loss of Sirt3 in cell lines and mice, leads to an increase (Pillai et al. 2010; Giralt & Villarroya 2012). Sirt3 helps prevent apoptosis by inhibiting upstream effectors of Bax, including Ku70 (Sundaresan et al. 2008). Other studies have shown that Sirt3
reduces levels of ROS by regulating antioxidant enzymes such as MnSOD and catalase (Pillai et al. 2010; Park et al. 2013b; Porter et al. 2014). By responding to mitochondrial NAD status, Sirt3 has a key metabolic regulatory role; this is shown mice lacking Sirt3 by reductions in complex I and III of the ETC, decreased in fatty acid oxidation, and a glycogenic state that leads to accelerated cardiac aging (Hafner et al. 2010; Kim et al. 2010; Sack 2011). Sirt3 is thought to be the main deacetylase in the mitochondria, which is supported by the fact that when Sirt3 knockout mice are investigated, there is an increase in acetylation in the ETC, especially complex I (Giralt & Villarroya 2012). Sirt3 is able to maintain mitochondrial integrity by deacetylating cyclophilin D, a protein that helps open the mitochondrial permeability transition pore (Sadoshima 2011). Calcium induced mitochondrial swelling was increased in Sirt3 deficient cells (Hafner et al. 2010). Resveratrol has been shown to activate Sirt3 (Chen et al. 2013; Chen et al. 2015). This interaction has been linked to both the NF-KB signaling and TGF-β/Smad3. With the induction of NF-KB pathway, it is suggested that apoptosis is inhibited by increasing the expression of SOD2 and Bcl2, while decreasing the Bax (Chen et al. 2013). In models of mouse TAC surgery, when resveratrol was given, the mice had less fibrosis, which was linked to the TGF-β/Smad3 pathway preventing the transition of myoblasts to fibroblasts (Chen et al. 2015).

Sirtuins that are not targeted to the mitochondria have also been linked to the heart and aging. Overexpression of Sirt1 caused early heart failure with a decrease in oxidative respiration and an increase in degenerated mitochondria (Kawashima et al. 2011). Some have suggested that this interaction might be signaled through ALD2, a mitochondrial encoded gene whose overexpression accentuates myocardial remodeling and contractile dysfunction in aging (Zhang et al. 2014b). Sirt7 deficient mice have shortened lifespans demonstrating cardiac hypertrophy and inflammatory cardiomyopathy (Pillai et al. 2010). Sirt7 deacylelates a protein involved in mitochondrial homeostasis (Vakhrusheva et al. 2008a). When Sirt7 is lacking, apoptosis was shown to increase in primary cultured cardiomyocytes (Vakhrusheva et al. 2008b).

4.8 PROTEOSTASIS AND CARDIAC AGING

Protein homeostasis (proteostasis) is the equilibrium between protein synthesis, maintenance, and degradation. Maintaining the proteome is integral to maintaining cellular functions and organismal health, and many studies have demonstrated that inability to remove unwanted proteins and/or replace them with functional proteins can be detrimental (Koga et al. 2011). Age-related conditions are generally accompanied by a decline in protein quality control mechanisms, thereby causing changes in the global proteome. A few well studied examples include cardiac dysfunction (Hedhli et al. 2005; Christians & Benjamin 2012), neurodegenerative disease (Douglas & Dillin 2010), cataracts (Surguchev & Surguchov 2010), and sarcopenia (de Magalhães 2004; Vinciguerra et al. 2010; Marzetti et al. 2012). While dysfunction of protein quality control mechanisms is a hallmark of aging, interventions that improve protein quality can enhance organismal health and longevity (Morimoto & Cuervo 2009; Douglas & Dillin 2010; Madeo et al. 2010; Koga et al. 2011). For example, the characteristic accumulation of damaged proteins and declines in mitochondrial respiratory capacity with age have been alleviated in models with over-expression of mitochondrial-targeted catalase (Schriner et al. 2005), CR (Kapahi et al. 2010) (Dai et al. 2014a), reduced IGF-1 signaling (Abbas et al. 2008; Puglielli 2008), and rapamycin treatment (Johnson et al. 2013; Dai et al. 2014a). That this mechanism has been implicated in interventions that inhibit mTOR may not be surprising, given its known
effects on protein translation and degradation (see above). Collectively, these studies suggest that dysfunctional proteostasis has a causative role in aging and that restoration of protein homeostasis machinery is protective against aging and age-related disease. However, many mechanistic question of how these processes extend lifespan and healthspan remain unanswered. Fortunately, these processes are receiving increased attention as their roles are becoming more recognized (Madeo et al. 2010; Koga et al. 2011).

The aging cardiac proteome recapitulates most hallmarks of the aged cellular proteome including the appearance of protein aggregates and lipofuscin, increased protein oxidation and damage, increased ubiquitination, and declines in autophagy and the ubiquitin proteasome system (Ravikumar et al. 2002; Wong & Cuervo 2010; Hsieh et al. 2012; Dai et al. 2014a). All of these changes are consistent with altered proteostasis during cardiac aging. Consistent with this, we have observed increased protein ubiquitination and carbonylation in old hearts (Dai et al. 2014a). However, this is not accompanied by increased protein turnover in old hearts; in fact, slower turnover is observed in aging rodents (Niedermüller 1986; Dai et al. 2014a). Together, observation of increased protein ubiquitination and carbonylation, but decreased protein turnover is suggestive of a defect in cardiac proteostasis. These changes may owe to an underlying decline in major protein quality control systems with age, which in turn leads to low quality and damaged proteins which become increasingly unable to perform their roles efficiently. Given the importance of mitochondrial energetics in the heart, mechanisms of mitochondrial quality control are particularly relevant to cardiac aging. The next sections focus on mitochondrial fission, fusion, unfolded protein response and autophagy as critical components of protein quality control.

4.9 THE ROLE OF FUSION/FISSION DYSREGULATION IN AGE-RELATED CARDIAC BIOENERGETICS DEFICIENCIES

As noted above, mitochondrial dysfunction, and in particular, bioenergetic deficiencies are an important hallmark of cardiac aging. Age-related decline in mitochondrial activity and impaired mitochondrial dynamics offer a potent explanation for deteriorating cardiac performance with age. Dysregulation of mitochondria quality control processes are widely reported in aging and although few studies have focused on the role of dysfunctional mitochondrial dynamics in cardiac senescence, there is extensive evidence to indicate that healthy cardiac performance is highly reliant on precise balance of mitochondrial fission and fusion.

Mitochondria are highly motile organelles that constantly change morphology, fuse, divide, and move depending on energy demands and integrity of individual mitochondria. Following fission, segments of mitochondrial that are dysfunctional, sensed as reduced membrane potential, are targeted for mitophagy (Mouli et al. 2009). This homeostatic process helps to ensure optimal mitochondrial quality and supply of ATP to meet energy demand (Scheibye-Knudsen et al. 2014). The key regulators of mitochondrial dynamics, Mfn1, Mfn2, Opa1, hFis1, Drp1, Mff, MiD49, and MiD51 show high expression in normal cardiac tissue, consistent with their pivotal role in mitochondrial dynamics and bioenergetics (Ong et al. 2013; Palmer et al. 2013) and the high mitochondrial content in this tissue. Genetic defects of proteins regulating fusion/fission are correlated with severe alterations in mitochondria morphology, decreased mtDNA integrity, increased oxidative stress, susceptibility to apoptosis, and metabolic dysregulation (Liesa et al.
Mitofusion 1 and mitofusion 2 null mice are embryonic lethal, while knock-downs have fragmented mitochondria, characteristic of declining mitochondrial fusion; mfn1 deficiency is observed in giant cells similar to those present in age-related cardiac hypertrophy (Chen et al. 2003). Genetic aberrations of mfn1 and 2 are consistent with increased respiratory dysfunction and higher frequencies of mtDNA mutations (Chen et al. 2010). Dysregulation of fission may cause permeabilization of the mitochondrial membrane and release of caspase-3, a key modulator of myopathic apoptosis observed in senescent heart (Beltrami et al. 1994; Narula et al. 1999; Phaneuf & Leeuwenburgh 2002) that can trigger several other cytosolic death pathways (Youle & Karbowski 2005; Suen et al. 2008) likely similar to those observed in heart failure (Narula et al. 1999) and possibly other cardiac pathologies.

Mitochondrial fusion is regulated by Mfn1, Mfn2, and Opa1. Opa1 mice missing one allele, develop cardiomyopathy late in life, and the acetylation of Opa1 has been linked to the development of heart disease when mice are pharmacologically, dietary, or surgically stressed (Samant et al. 2014). The general loss of Opa1 in MEFs has been shown to give fragmented mitochondrial populations. Recently, in a fly model, suppression of Opa1 led to worsened contractility and increased dilation. These challenges were traced back to increased ROS production, and could be reversed by increasing ROS scavenging proteins (Bhandari et al. 2015). Cardiac specific Mfn1/Mfn2 KOs have been shown to develop early onset heart disease (Ikeda et al. 2015a). Mfn1/2 are found on the outer mitochondrial membrane, where they can make hetero- or homo-dimeric interactions with neighboring mitochondria. Mfn1/2, unlike Opa1, are increased in some forms of heart failure (Knowlton et al. 2014). Mfn1/Mfn2 plays a large role in autophagy that is often difficult to separate from their roles in fusion, which is an area of intense research. Mfn2 plays a key role in mitochondria–sarcoplasmic reticulum tethering for calcium signaling. In fact, loss of outer membrane mitofusins (MARF) led to fragmented mitochondria with higher ROS, which was repaired by increasing XBP1 expression, a protein involved in ER stress (Bhandari et al. 2015).

Mitochondrial fission is managed by Drp1, Fis1, and Mff. Drp1 is localized to the cytosol until it is attracted to the mitochondrial surface for a fission event (Zepeda et al. 2014). Drp1 has recently been suggested to help protect cardiac cells from IR injury by allowing them to be less reliant on oxidative phosphorylation and delaying or suppressing apoptosis (Givvimani et al. 2014; Zepeda et al. 2014). The depletion of Drp1 in cardiomyocytes or in mouse hearts leads to mitochondrial dysfunction and heart disease, respectively (Ikeda et al. 2015b). A study by Ikeda et al. demonstrated that unchecked mitochondrial fusion, by Drp1 knock out was just as detrimental as is unchecked fission (Ikeda et al. 2015b). MiD49/MiD51 has been shown to recruit Drp1 to the mitochondrial surface (Palmer et al. 2013; Richter et al. 2014; Losón et al. 2015). Fis1 and Mff perform this role, but MiD49/MiD51 only recruit to the mitochondria while Fis1 and Mff are suggested to recruit to the peroxisome as well (Palmer et al. 2013). MiD49/MiD51 overexpression can make up for Drp1 recruitment in Fis1−/−/Mff−/− cells to a normal phenotype (Palmer et al. 2013).

Despite the recent illumination of the roles and mechanisms of fission and fusion, challenges remain in studying these processes in aging cardiac tissue. Much of what is known about mitochondria dynamics and its relationship to energetic deficiencies in the aging heart comes from studies of cultured cardiomyocytes and surgically stressed hearts, not from aging hearts.
Autophagy and Mitophagy

Autophagy is a major quality control pathway essential for the removal of unwanted proteins, macromolecules and organelles to maintain mitochondrial function. Cellular degradation involving lysosomes, a single membrane vesicle containing enzymes for the digestion of macromolecules, is generally categorized under the umbrella term “autophagy” (Madeo et al. 2010). There are three major ways by which proteins can be delivered to a lysosome for degradation, defining the primary categories of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy. Many details of these processes are outside the scope of this review, and readers are referred to detailed reviews on each topic (Madeo et al. 2010; Wong & Cuervo 2010; Koga et al. 2011). This section will focus on macroautophagy and a mitochondrial-specific form of macroautophagy termed “mitophagy”, as these are well characterized processes which may be important in both mitochondrial function and aging. Knocking down components of macroautophagy strongly diminishes mitochondrial function (Ravikumar et al. 2002; Wong & Cuervo 2010; North & Sinclair 2012; Dai et al. 2014a), demonstrating that it plays a key role in mitochondrial maintenance and homeostasis.

Two well characterized regulators of mitophagy are PINK1 and Parkin (Dias et al. 2013; Ashrafi et al. 2014). PINK1, aka phosphatase and tensin (PTEN) homologue-induced kinase 1, is a mitochondria-targeted serine/threonine kinase which serves to protect the cell from mitochondrial dysfunction and apoptosis (Matsuda et al. 2013). Mutations in this protein are the most common cause of recessive familial Parkinsonism in humans (Rochet et al. 2012). In addition, PINK1 KO mice have severe deficiencies in mitochondrial homeostasis accompanied by morphological changes in the mitochondrial network, increased ROS, and susceptibility to heat shock (Matsuda et al. 2013). Together this evidence suggests PINK1 has an important role in Parkinson's disease as well as mitochondrial quality in normal cells, and possibly plays an important role in aging.

Under healthy conditions, PINK1 is imported into mitochondria via the TOM complex and is actively degraded by mitochondrial processing peptidase (MPP) and presenilin-associated rhomboid-like protease (PARL) (Greene et al. 2012; Matsuda et al. 2013; Wohlgemuth et al. 2014a). Upon loss of mitochondrial membrane potential, PINK1 accumulates on the mitochondrial outer membrane and recruits Parkin, an E3 ubiquitin ligase, leading to the poly-ubiquitination of many mitochondrial outer membrane proteins such as Hexokinase I, VCAC1, MFN1/2, and Miro (Hammerling & Gustafsson 2014). These ubiquitinated proteins are recognized by autophagy proteins P62, LC3 II, and BNIP3 to promote fusion with the lysosome and clearance of the dysfunctional organelle via mitophagy (Thomas & Gustafsson 2013; Hammerling & Gustafsson 2014). Many of the details surrounding this pathway and the interactions starting at PINK1 and leading up to mitophagy have been studied in detail and reviewed elsewhere (Dias et al. 2013; Matsuda et al. 2013; Thomas & Gustafsson 2013; Hammerling & Gustafsson 2014).

A few studies have shown that PINK1/Parkin mediated mitophagy is important for heart function, particularly in the context of adaptation and recovery from stress. Parkin KO rats, in contrast to wild type, lack cardioprotection following ischemic preconditioning (Huang et al. 2011). Parkin deficient mice exhibit impaired recovery of cardiac function after sepsis.
(Piquereau et al. 2013) and have reduced survival and larger infarct size following myocardial infarction (Kubli et al. 2013). All of these studies noted that Parkin deficient animals show disorganized mitochondrial networks, small or fragmented mitochondria, and an increase in cardiomyocyte cell death. The infarct study additionally showed that overexpression of Parkin in isolated cardiomyocytes protects against hypoxia mediated cell death (Kubli et al. 2013). Interestingly, protein ubiquitination and LC3II, markers of mitophagy, were not higher in control mice than in Parkin deficient animals after sepsis or in the remote zone after myocardial infarction. However, there was evidence of compensatory increases in macroautophagy, and possibly an induction of alternative BNIP3-mediated mitophagy, where Parkin-dependent mitophagy is absent. Recent work by the Dorn lab suggests that Parkin is not required for routine maintenance of the mitochondria, but is important in stress-reactive pathways (Song et al. 2015). This comes with the caveat that the work was performed on mice that were between nine and ten months old, well into adulthood, but not necessarily recapitulating aging (Song et al. 2015). A more detailed review details the role of mitophagy, including the less known role of BNIP3, in the heart (Jimenez et al. 2014b).

Even though there were obvious morphological differences in the mitochondria of Parkin deficient animals, one common observation of these studies was that under normal conditions there was no apparent difference in cardiac function compared to wild type mice until advanced age or animals were first subjected to stress. PINK1 KO mice also show increased vulnerability to ischemic injury (Siddall et al. 2013), but unlike Parkin deficiency, loss of PINK1 has been reported to show signs of cardiac dysfunction in mice as young as 2 months (Billia et al. 2011). By six months of age, PINK1 KO and heterozygous mutants show increased heart weight, cardiomyocyte hypertrophy, decreased fractional shortening, and increases in hypertrophic gene expression (Billia et al. 2011). Again, in contrast to Parkin deficient mice under normal conditions, this study also reported reductions in mitochondrial biogenesis and bioenergetics starting at 2 months of age. Collectively, studies in PINK1/Parkin have shown that these mediators are important for cardiac function, particularly in response to stressors, and compensatory increases in other degradation pathways may alleviate the dysfunction resulting in reduced mitophagy. However, considerable uncertainty remains in understanding the relative roles of mitophagy vs. other proteostatic processes in maintaining mitochondrial protein quality control. A key observation is that half-lives of different respiratory chain complexes and even different proteins within a complex are highly variable, including in the heart (Kim et al. 2012; Karunadharma et al. 2015a). This would not be expected on the basis of the common perception of mitophagy was a bulk recycling process. It has been suggested, however, that damaged proteins can be preferentially segregated to the mitochondrial components that are degraded by mitophagy (Abeliovich et al. 2013), but there is also evidence that proteosomal activity correlates with respiratory chain protein half-lives (Dai et al. 2014a; Karunadharma et al. 2015a). Further studies will be needed to more clearly determine the relative roles of mitophagy and other proteostatic mechanisms in mitochondria, including their importance in age-related declines in the heart.

Modulation of macroautophagy has shown a mix of positive and negative results in various heart disease models; however, numerous lines of evidence have shown that macroautophagy has an important role in organisinal and cardiac aging. A recent report found that genetic over-expression of ATG5, a vital autophagy protein involved in autophagosomal formation, improved
mitochondrial morphology, respiratory rates, and extended lifespan in mice (Pyo et al. 2013). ATG5 has been shown to have a pro-apoptotic function, and this activity in reducing cancer deaths C57BL/6 mice may be a longevity-promoting component. Cardiac-specific knockdown of ATG5 in mice has conversely been shown to accelerate aspects of aging in the heart, suggesting that autophagy plays an important role in maintaining normal heart function and mediating cardiac aging. Like normally aging mice, cardiac specific ATG5 mutants develop left ventricular hypertrophy, but they also develop accelerated heart failure with decreased fractional shortening, abnormal mitochondrial morphology, decreased respiratory capacity, and die prematurely (Taneike et al. 2010; Dutta et al. 2013; Wohlgemuth et al. 2014a). While the mechanism by which autophagy maintains cardiac function is not fully understood, fragmentation of mitochondria and accumulation of ubiquitinated proteins and p62 in mice lacking ATG5 suggests that this is an essential protective mechanism (Jana 2012; Dutta et al. 2013). In agreement with this, a study performed on cardiomyocyte cell lines found that induction of autophagy was protective against oxidative stress-induced protein aggregation, reduced levels of protein ubiquitination, improved mitochondrial function, and reduced cell death (Jana 2012; Wohlgemuth et al. 2014a).

Inhibition of the mTOR pathway (see above) is well known to increase autophagy and extend lifespan. In fact, the mTOR inhibitor rapamycin is one of the few drugs available which can be used to increase autophagy. Longevity studies with rapamycin and other forms of mTOR inhibition have reported increased autophagy in animals across many studies (Puglielli 2008; Morimoto & Cuervo 2009; Madeo et al. 2010; Johnson et al. 2013), and offer further evidence that autophagy may play a central role in aging. Even so, due to the difficulty of specifically over-expressing autophagy components without targeting non-specific processes, direct evidence that activating autophagy can extend lifespan is not yet available.

4.11 MITOCHONDRIAL UNFOLDED PROTEIN RESPONSE

The mitochondrial unfolded protein response (UPRMT) is another aspect of protein quality control implicated in cardiac aging via its effects on mitochondrial function. UPRMT was first proposed in 1996, and described as a stress response involving mitochondrial chaperones and heat shock proteins (Martinus et al. 1996). Various models have been investigated to help reduce the amount of stress that occurs in the mitochondria, helping to decrease the UPRMT (Dai et al. 2012b; Yang et al. 2013). Dietary supplementation with taurine, a key nutrient for cardiac health, was shown to decrease oxidative stress and inhibit mitochondria-dependent cell apoptosis (Yang et al. 2013). Prohibitins (Phb), highly conserved proteins in the mitochondria, have pivotal roles in the UPRMT. Phb make hetero-multimeric ring complexes that help with proper mitochondrial protein folding, ETC. assembly, and the regulation of proteases (Schleit et al. 2013; Richter-Dennerlein et al. 2014). Phb2 helps ensure that OPA1 is functional for mitochondrial fusion. In a complex with DNAJC19, Phb2 is responsible for maintaining cardiolipin and mitochondrial cristae structure for healthy mitochondrial function (Richter-Dennerlein et al. 2014). Other proteins involved in this process are mtShp70, Hsp60, and Hsp10. Mitochondrial Unfolded Response Elements 1 and 2 (MURE1 and MURE2) help upregulate Hsp60 and ClpP during mitochondrial homeostasis (Pulliam et al. 2014).

Recently, the UPRMT was investigated in an in vivo model of electron transport deficiency mice. Surf1−/− and litter mate control hearts were investigated for their induction of UPRMT proteins.
under the constant stress of COX assembly deficiency. The authors found significant increases in Lon and Trx2, with a trend of increased CHOP, all implicated in the UPR\textsuperscript{MT}, demonstrating the role that the mitochondrial UPR\textsuperscript{MT} can play to help relieve mitochondrial dysfunction in hearts in a stressed environment (Pulliam \textit{et al.} 2014). This study was performed in young mice, leaving the exact role of the UPR\textsuperscript{MT} in aging still open; however, this remains an area of active investigation (Haynes & Ron 2010; Bennett & Kaeberlein 2014). CHOP, Lon, and Trx2 are key proteins in the stress response pathways. CHOP is activated by the marking of unfolded proteins by BiP/GRP78, helping to prevent aggregation of the misfolded proteins (Collins \textit{et al.} 2014). Lon and Trx2 have recently been shown to play a key role in decreasing ROS in the mitochondria and preventing apoptosis within the cardiomyocytes. Recent work suggests that Lon helps prevent ROS induced apoptosis in a hypoxia model, and one can hypothesize that Lon would perform this role in any stressed environmental situation, not only in hypoxia (Kuo \textit{et al.} 2015). Trx2 has been known to be important in preventing apoptosis, since Trx2 knockout mice are embryonically lethal. Work from the Min lab shows that there is a decrease in Trx2 expression in human dilated cardiomyopathy patients, and that mice with Trx2 deleted from their heart also develop this disease (Huang \textit{et al.} 2015). The loss of Trx2 increased oxidative stress, apoptosis, fibrosis, and contractile dysfunction, due to Trx2 not being around for decreasing ROS production or binding ASK1 to prevent apoptosis (Huang \textit{et al.} 2015). This encourages the notion that an increased expression of Trx2 helps maintain a stable and healthy environment in stressed hearts by decreasing ROS production and blocking mass apoptotic cell death (Huang \textit{et al.} 2015). Another study using BXM mice demonstrated that the transcriptional regulation and protein regulation of the same protein can vary in the UPR\textsuperscript{MT} in opposite directions (Wu \textit{et al.} 2014a). Thus, while there is considerable interest in UPR\textsuperscript{MT} as a new and potentially underappreciated mechanism of proteostasis, it is too early to know its significance in normative cardiac aging.

4.12 MITOCHONDRIAL TARGETED THERAPIES

Due to the critical importance of mitochondria in insuring adequate cellular energetics and function, there has been great interest in discovering mitochondrially targeted therapies for various diseases and conditions, including cardiac dysfunction and aging. Some of these therapies attempt to decrease the oxidative stress in the mitochondrial environment (Gómez \textit{et al.} 2014), while others focus on structural components of mitochondria (Kloner \textit{et al.} 2012; Birk \textit{et al.} 2014; Jiang \textit{et al.} 2014; McLachlan \textit{et al.} 2014; Szeto 2014). It can be argued that CR and inhibition of mTOR signaling can do both, and thus, these two interventions, described above, and may also be considered to be mitochondrial therapies.

4.12.1 Mitochondrial antioxidants

The triphenylalkyl phosphonium ion (TPP +) has been conjugated to coenzyme Q (MitoQ) and plastoquinone (SkQ1) to deliver these redox-active compounds into the mitochondrial matrix, utilizing the negative potential gradient across the inner mitochondrial membrane. MitoQ has been shown to help maintain eNOS availability and reduce hypertension. MitoQ has been given together with losartan, an angiotensin receptor blocker, that did not decrease ROS production in the mitochondria, but the combined therapy did lead to an improvement in cardiovascular function (McLachlan \textit{et al.} 2014). SkQ1 is another mitochondrial targeted antioxidant that has been reported to extend lifespan in male BALB/c mice and dwarf hamsters (Anisimov \textit{et al.} 2014).
In the BALB/c mice there was also a reduction in age-related cardiac hypertrophy (Anisimov et al. 2011; Manskikh et al. 2014). Pretreatment with MitoQ and SkQ1 have both been shown to have beneficial effects in animal models of ischemia-induced cardiac dysfunction (Adlam et al. 2005; Antonenko et al. 2008). The role that mitochondrial-targeted antioxidants might be able to play in protecting or repairing cardiac mitochondrial dysfunction in aging is thus a promising area of study. CoQ10, a mimic of a naturally occurring antioxidant of the electron transport chain has also showed promise improving mitochondrial function in the heart. Mouse studies involving ApoA1−/− mice demonstrate that addition of CoQ10 improves infarct size to that of a wild-type mouse (Dadabayev et al. 2014). Current human studies using CoQ10 in dietary supplements in adults have hint at improved health with an optimal diet, and clinical study in children with primary mitochondrial diseases is underway (Stacpoole et al. 2012; González-Guardia et al. 2015).

4.12.2 Cardiolipin-targeted therapies

Two CL targeted drugs have been studied, TPP-n-ISA and SS-31. TPP-n-ISA, studied primarily in brain injury and radiation, helped maintain CL in a structural arrangement that makes peroxidation more difficult (Jiang et al. 2014). The Szeto–Schiller (SS) compounds are tetrapeptides that preferentially concentrate in the mitochondrial inner membrane independent of the mitochondrial potential gradient. SS-31 (or as an acetate salt MTP-131, aka Bendavia™), the best studied of these, has been shown to reduce ROS levels and prevent ischemia–reperfusion injury in a variety of infarct models (Szeto & Schiller 2011; Kloner et al. 2012). In our laboratory, we have found SS-31 to be protective of angiotensin II induced cardiac hypertrophy, as well as G alpha q-induced cardiac failure (Dai et al. 2011a). The protective effect of SS31 in the TAC model of heart failure was as great as that of mCAT, and conferred an even more complete protection of failure-related proteomic alterations than did mCAT (Dai et al. 2013). It has recently been shown that SS-31 targets CL, altering the CL/cytochrome c interaction to optimize electron transfer, inhibit ROS generation and cytochrome c peroxidase activity. In a number of disease models SS-31 appears to help maintain mitochondrial cristae density, presumably by preserving the tetralinoleoyl isoform of CL which is vital to maintaining cristae curvature (Birk et al. 2014; Szeto 2014; Szeto & Birk 2014). By stabilizing the CL-cytochrome c interaction, SS-31 may also prevent the pro-apoptotic activity of cytochrome c, although this has not been proven. In its clinical formulation, Bendavia, SS-31 is currently in multiple phase II studies, including a study to examine its impact to improve outcome in patients with acute myocardial infarction (Chakrabarti et al. 2013), as well as for treatment of patients with mitochondrial myopathy in primary mitochondrial disease, including Barth syndrome (NCT02367014).

The application of mitochondrially targeted therapies appears to be an exciting area of growth, as additional druggable targets to protect or improve the function of this important organelle are discovered.

4.12.3 Signaling pathway therapies

As previously mentioned above, the regulation of NAD is key to allowing the sirtuins pathways to maintain their function, which helps with healthspan and lifespan. With this idea, more NAD+/NADH therapies are being created. In a study of gas-1 mutant worms, nicotinic acid (NA)
and resveratrol both improved survival of the worms (McCormack et al. 2015). NA helped decrease the amount of ROS in the worms system and it was suggested that the NA increased NAD$^+$ pools which can help maintain Sirt3 acetylation patterns. Nicotinamide riboside can also increase the NAD$^+$ pool without activating Sirt3, but providing comparable benefits (Felici et al. 2015). PARP-1 inhibitors, Phe and PJ34, demonstrated improved mitochondrial content and membrane potential in Complex I mutant human fibroblasts (Felici et al. 2015). Both inhibitors increase the transcription of mitochondrially encoded respiratory complexes, relaying a better survival of the cells.

4.13 CONCLUSIONS

Mitochondrial dysfunction is a hallmark of cardiac aging, with a multitude of interactions that are involved in health and disease. Because of its central role in cellular energetics, mitochondria play important roles in multiple signaling pathways. While we have described some of these functions in mTOR, IGF-1, and sirtuin signaling, the complexities of these interactions leave many questions for future investigation. A better understanding of the underlying biology will help to elucidate the multiple roles that mitochondrial dysfunction may play in cardiac aging and disease. Greater mechanistic insights will also allow development of novel mitochondrial targeted therapies to attenuate or reverse mitochondrial dysfunction and cardiac aging.


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