

Growth Signaling in Aging and Disease

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Abstract

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Nutrient sensing and growth signaling pathways are key regulators of healthspan and longevity. Caloric restriction, the most widely studied and well documented intervention in aging, functions largely through effects on nutrient sensing and growth signaling pathways. Genetic approaches to studying aging have further emphasized the central role that growth signaling plays in regulating longevity by identifying key components of growth signaling pathways as critical modifiers of the aging process. Among these genetic factors are the insulin/insulin-like growth factor signaling, AMP kinase, and the mechanistic target of rapamycin (mTOR) complexes. mTOR forms two distinct complexes, mTORC1 and mTORC2, which together act as central coordinators of a variety of intra- and extra-cellular nutrient and growth signals. mTOR integrates extracellular signaling through insulin, IGF, and Akt, intracellular energy status sensing by AMP kinase, intracellular amino acid levels through sensing at the lysosome, and cellular translational capacity through association with the ribosome. As a central regulator of a variety of critical energy sensing pathways mTOR has been demonstrated to significantly contribute to aging and disease. Pathways downstream of mTOR include translation, autophagy, metabolism, and inflammation. The studies presented here examine the relationship between genotype and response to caloric restriction, examining the therapeutic role of mTOR inhibition in a mouse model of a human mitochondrial disease, and investigating the role of a major molecular process regulated by mTOR, autophagy, in the development of cardiac hypertrophy. These studies are diverse but linked by a central focus on the role of growth signaling pathways in aging and human disease and the work presented here provides important and novel insights into disease and aging.

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## CHAPTER 1

# Growth Signaling in Aging and Disease: A Focus on mTOR

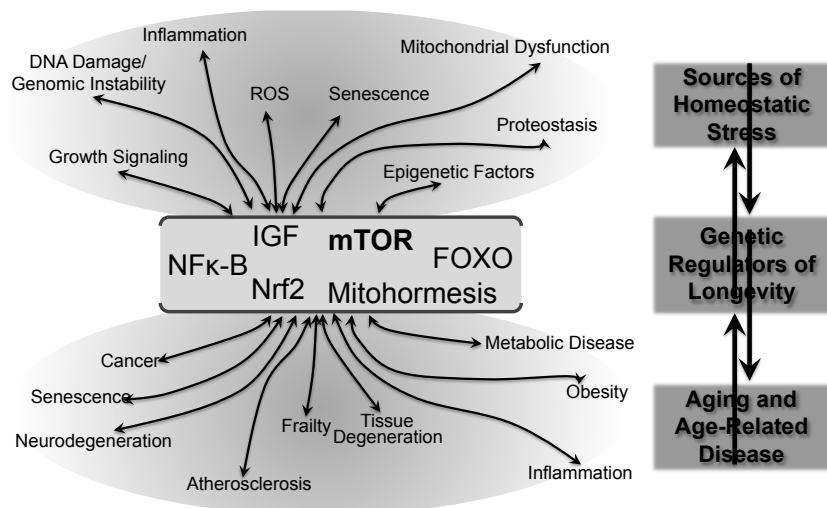
## I. GROWTH SIGNALING AND ENERGY SENSING IN AGING

### Overview

Aging is a highly complex biological process, the mechanistic underpinnings of which are still largely unknown. A number of models have arisen over the years to describe or explain the aging process. The theories that have attempted to simply contribute to the overall picture of the aging process, rather than completely explain it, have generally stood up better over time. Theories that attempted to comprehensively explain aging include the rate of living theory of aging, the reactive oxygen species (ROS) damage theory of aging, and the mitochondrial ROS theory of aging, and the DNA damage accumulation theory of aging [1-6]. While still relevant in specific contexts, all of these models have seen significant challenges over time and likely suffered greatest from their attempt at simplicity. Theories aimed at characterizing specific phenomena of aging, such as the mitochondrial-lysosomal axis theory of aging, models of aging driven by inflammation, and stress response models of aging, such as models of proteostasis in aging, have generally performed more favorably, though suffer from an inability to explain aging as a whole [7-9].

Aging research experienced what could be considered a major breakthrough two

decades ago when researchers first identified genetic factors



**Figure 1 - Genetic Factors Modulate the Aging Process.** While the underlying causes and phenotypic consequences of aging are complex it is clear that genetic factors modulate aging.

involved in regulating longevity in model organisms [10-13]. These studies demonstrated that, whatever the underlying causes, the aging process is modulated by genetic factors and the process of aging can be studied using genetic approaches (**Figure 1**). Furthermore, as genes identified to modulate aging were characterized it became clear that many of the factors involved in the genetic regulation of longevity are involved in nutrient sensing and growth signaling including insulin/insulin-like growth factor (IGF) signaling and the mechanistic target of rapamycin (mTOR) pathway [14-21]. These studies clearly identified nutrient response as a critical component of lifespan regulation, a highly appealing finding given that the single well-documented lifespan extending intervention at the time was caloric restriction (CR), an intervention clearly linked to nutrient availability and nutrient response. Subsequent efforts aimed at characterizing the genetic and mechanistic relationships between CR and nutrient response pathways have solidified the relationship between growth signaling and lifespan. While numerous factors regulate longevity in response to nutrients mTOR has been increasingly recognized to play a central role in the integration of growth signaling and in longevity and health and thus the work presented here is presented from an mTOR-centered perspective. A comprehensive model of the process of aging is undoubtedly still years away, but the work detailed here provides new insights into how genetics, caloric intake, and environmental factors interact to affect lifespan.

### **mTOR in Aging**

A link between mTOR signaling and aging was first established in yeast when studies in *Saccharomyces cerevisiae* demonstrated that deletion of Sch9, the yeast homolog of the mTORC1 substrate S6K results in a significant increase in chronological lifespan [22]. Studies in the nematode *Caenorhabditis elegans* subsequently revealed that mTOR negatively regulates longevity in multicellular organisms; knockdown of *daf-15* (the nematode homolog of Raptor, a component of mTORC1) or *let-363* (the nematode homolog of mTOR) by RNAi can extend lifespan in this model [23, 24]. Reports from *Drosophila melanogaster* and yeast replicative aging studies further supported the role of mTOR in regulating longevity in lower eukaryotes.

Direct evidence of a role for mTOR in mammalian lifespan has been provided by multiple genetic studies in mice showing lifespan extension in mice resulting from deletion of S6K, by double heterozygosity for

mTOR and *mlst8* (a component of mTORC1), as well as murine studies using rapamycin as a pharmaceutical intervention [25-27]. Interestingly, the extent of lifespan extension was sex-specific, with males receiving no longevity benefit from S6K deletion or double heterozygosity of mTORC1 components and female mice also experienced a more robust response to treatment with rapamycin, an 18% increase in median lifespan compared to a 10% increase in male animals [28]. Notably, intervention with rapamycin resulted in an increase in lifespan even when rapamycin treatment began late in life, suggesting that mTOR inhibition may prove an attractive target for intervening in human aging.

## II. mTOR AS A CENTRAL COORDINATOR OF GROWTH SIGNALING

### mTOR in Nutrient and Growth Factor Sensing

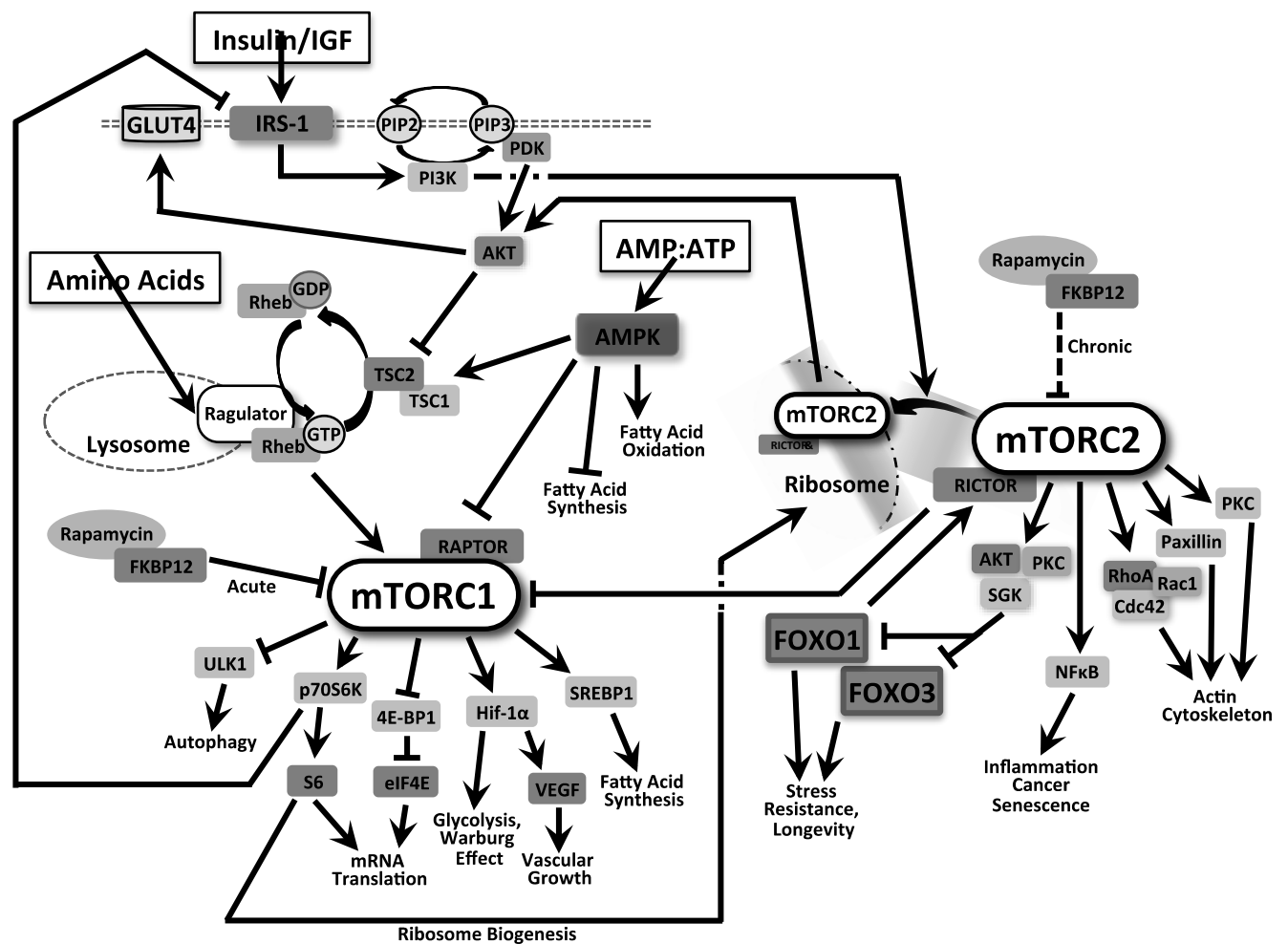
The mechanistic target of rapamycin, mTOR, is a highly conserved serine/threonine kinase that plays a central role in sensing and responding to nutrient availability and growth signaling in eukaryotes (**Figure 2**). mTOR, encoded in mammals by *MTOR*, is an essential component of two distinct multi-protein complexes, mTORC1 and mTORC2. These signaling complexes regulate a variety of basic cellular activities including growth rate, cell size, and metabolism, and act as critical signaling hubs at the interface between nutrient or hormonal cues and cell growth and maintenance.

mTOR was first identified in yeast in studies of the immune-suppressive compound rapamycin.

Rapamycin had previously been shown to act by forming a gain-of-function complex with Fpr1p, with mutations in *FPR1* conferring recessive resistance to rapamycin, and expression of the human homolog FKBP12 (FK-506 binding protein 12) restoring drug sensitivity [29]. Mutations in *TOR1* and *TOR2*, originally designated *DDR1* and *DDR2* (dominant rapamycin resistance), were found to confer resistance to the anti-proliferative effects of rapamycin in yeast [30]. Wild-type Tor1 and Tor2 are bound and inhibited by the Fpr1/rapamycin complex [31], a mechanism that is conserved in mammals with the rapamycin-FKBP12 complex binding to and inhibiting mTOR [32].

mTORC1 and mTORC2 both play essential roles in eukaryotic biology, as complete loss of either Raptor, an mTORC1 specific component, or Rictor, an mTORC2 component, results in embryonic lethality. While

both are essential for development, the two mTOR complexes differ in their components, relative regulatory roles, and upstream regulation of their activity. The upstream regulators and downstream effectors of mTORC1 are generally better characterized than those related to mTORC2. mTOR signaling is affected by numerous intra- and extracellular growth cues and conditions, and it affects numerous downstream pathways and processes. The best characterized of these pathways, in terms of aging and disease, are described below.



**Figure 2 - mTOR Plays a Central Role in Coordinating Growth Signaling and Response to Nutrients.** mTOR, as a critical component of mTORC1 and mTORC2, is central in integrating cellular energy sensing and propagating growth signaling. Critical inputs include insulin/IGF signaling, intracellular AMP:ATP level sensing through AMPK, sensing of intracellular amino acid levels at the lysosome, and coordination with cellular transcriptional capacity at the ribosome. Outputs are diverse and include regulation of mRNA translation, autophagy, metabolism, stress resistance, and inflammation.

**Insulin/IGF/PI3K/AKT.** mTOR is activated by a variety of growth factors and mitogens (**Figure 2**). Among the canonical regulators of mTORC1 signaling in mammals are insulin and the insulin-like growth factors (IGF's). Insulin and IGF's are recognized at the cell surface by tyrosine kinase receptors and provide the primary extracellular regulation of mTOR. Signaling through insulin/IGF is partially through phosphoinositide (PI3) mediated activation PI3-dependant kinase (PDK) and subsequent activating phosphorylation of AKT on T308 [25]. IGF-1 signaling represents a longevity regulating pathway in its own right, acting through both mTOR and through FoxO. IGF receptor loss has been shown to increase lifespan in mice and worms (*daf-12* is the IGF homolog in *C. elegans*), serum IGF levels have been shown to correlate with lifespan among mouse strains, and FOXO gene variants are strongly associated with extreme longevity in humans [33]. IGF-1 is the canonical and best characterized IGF activator of mTOR. AKT stimulates mTORC1 through phosphorylation of the mTORC1 inhibitor tuberous sclerosis complex protein 2 (TSC2). In its active form the TSC1/2 complex is a GTPase activating factor (GAF) for the small guanine nucleotide binding protein Rheb. Stimulation of Rheb by active TSC1/2 results in a conversion of loaded GTP to GDP, inactivating the protein. Active GTP bound Rheb is a necessary component of mTORC1; thus, inhibition of TSC1/2 results in downstream activation of mTORC1 through an increase in active Rheb [34].

**Ribosome Capacity.** While mTORC2 is not activated by insulin and growth factors through canonical signaling events, an intriguing mechanism has been described which couples regulation of mTORC2 activity by extracellular signals to intracellular ribosomal capacity [35]. In this pathway mTORC2 is activated by PI3K through increased physical association with ribosomes. Activated mTORC2 at the ribosome phosphorylates S473 on a hydrophobic motif of Akt, priming it for activation by PDK1. Ribosome associated active mTORC2 also phosphorylates a hydrophobic motif of SGK and PKC. The relationship between mTORC2 and ribosome activity links cellular translation capacity to growth signaling. Simultaneously, ribosomal biosynthesis is also regulated by TORC1 activation of p70S6K and its target, the ribosomal S6 subunit. This crosstalk between mTORC2 and mTORC1 provides another link between intracellular growth capacity sensing and the regulation of growth by mTOR.

mTORC2 thus lies both upstream and downstream of AKT, a relationship that may explain a variety of otherwise inexplicable observations related to mTOR biology. One such case is the uncoupling of mTORC1 and AKT signaling by Foxo induction. Foxo has been found to upregulate levels of Rictor, the mTORC2 specific binding partner of mTOR, and activate AKT while simultaneously causing a decrease in assembled mTORC1. This decrease has been shown to result from sequestration of mTOR to mTORC2, while increased activity of mTORC2 directly activates AKT. The downstream effects of mTORC2 specific signaling are not well characterized in the context of aging but this complex relationship with mTORC1 and AKT demonstrates the importance of mTORC2 in the outcome of mTOR signaling perturbations.

**AMPK.** mTOR activity is modulated not only through extracellular signaling, but also by multiple intracellular energy and nutrient sensing pathways. One well-characterized regulator of mTOR activity is the highly conserved AMP activated kinase, AMPK. AMPK is an ancient sensor of energy status that acts as an intracellular upstream regulator of mTOR. AMPK is sensitive to the AMP:ATP status in the cell and is activated as this ratio increases. Upon activation, AMPK drives catabolic processes, such as fatty acid metabolism, and inhibits anabolic reactions, such as lipid synthesis, for the purpose of balancing intracellular energy status. AMPK acts on mTOR through multiple interactions. TSC2 is activated by AMPK through phosphorylation at T-1227 and S-1345. AMPK also appears to directly inhibit mTORC1 through phosphorylation of Raptor at serine 722 and serine 792 [36].

Multiple pharmaceutical agents that target AMPK signaling are available for research use and AMPK activators have been used clinically in the setting of type II diabetes. Phenformin, a potent and direct AMPK activator, was used for the treatment of type II diabetics for decades before being removed from clinical use. This drug had strong beneficial effects for patients but was found to cause life-threatening, sometimes lethal, lactic acidosis following exercise [37]. The less potent and less characterized drug metformin replaced phenformin in the clinic. Metformin appears to have many of the same biological effects as phenformin (primarily inhibition of glycolysis and regulation of blood glucose) with a much lower frequency of the life-threatening lactic acidosis. The exact mechanism of function for metformin are not well understood, with some recent studies suggesting that the compound functions through inhibition of complex I of the electron transport chain and others demonstrating an effect from the drug even in AMPK

deficient animals, together suggesting that metformin functions in large part through AMPK independent pathways.

**Amino Acid Sensing at the Lysosome.** mTORC1 is also directly activated by amino acids through an interaction at the surface of the lysosome, allowing for intracellular amino acid abundance to regulate growth and metabolism directly through mTOR [38]. This sensing is facilitated by the recently discovered Ragulator complex, a vacuolar ATPase binding complex that contains a guanine nucleotide exchange factor (GEF) for Rheb and is regulated at the lysosomal surface by amino acids [39, 40]. Under conditions of high intracellular amino acids, mTORC1 and Rheb are recruited to the Ragulator complex at the surface of the lysosome where Rheb activates mTORC1 as described above. Together, the regulatory interactions described above define mTORC1 as a central sensor of growth conditions and energy cues.

**Caloric Restriction.** Caloric restriction (CR) is a well-documented lifespan-extending intervention that has been found to be effective in divergent species including yeast, flies, rodents, and non-human primates [41]. Defined as a reduction in nutrient intake in the absence of malnutrition, CR (also referred to as dietary restriction or DR) is a theory but a complicated intervention in practice has not been rigorously standardized. CR strategies differ greatly between organisms, lifespan assay type, laboratory, and field of study [42]. Even within the same organism CR can be implemented in a variety of fashions. In nematodes CR can be accomplished by titrating down the bacterial food available on solid plates or in media or by removing bacteria altogether (referred to as bacterial deprivation or BD) [43]. A variety of CR treatments exist in yeast including variable degrees of glucose deprivation or substitution of glucose (the standard carbon source for yeast on plates) with non-fermentable carbon sources [44, 45]. This is further complicated by the existence of two distinct lifespan models in yeast: the replicative lifespan (RLS) model, where lifespan is represented by replicative fecundity, and chronological lifespan, where lifespan is determined by the duration yeast remain viable when kept in stationary phase [46, 47]. Murine studies of CR vary greatly in terms of food composition, percent CR (mass of food provided compared to *ad libitum* intake), specific nutrient deprivation (such as methionine restriction), and housing conditions, such as implementing CR on singly vs. multiply housed mice [48-50].

Given that CR varies widely in implementation practices it is almost surprising that CR generally works, suggesting that the downstream factors regulating this intervention are robust, well conserved, and may hold real promise in human aging and disease. There has been a significant effort to identify downstream effectors of CR, and mTOR has been often identified as a pathway likely to regulate a portion of the CR response. Given the role of mTOR in nutrient signaling and response to growth cues, it makes sense that mTOR would be involved in the CR response. mTORC1 activity has been shown to be reduced by CR in invertebrate and mammalian studies. Additional evidence implicating mTORC1 in CR comes from reports in which CR fails to additively extend yeast replicative lifespan (RLS) in mutants for mTOR or S6K, as well as from studies in *C. elegans* showing a similar lack of additive extension between a genetic model for CR (the eat-2 mutant) and RNAi against mTOR [51]. S6K and translation initiation factors were, however, found to be additive with CR in this model, suggesting that reducing translation through these mechanisms is either more robust or works to extend lifespan through at least partially distinct mechanisms.

It has become clear over the past decade of aging research in invertebrates, and more recently in murine studies, that classical genetic complementation experiments are often difficult to interpret and are prone to over-interpretation when longevity is the phenotypic readout. Given that longevity is an extraordinarily complex trait and that the regulators of maximum longevity are still poorly understood it has proven difficult to demonstrate true complementarity in lifespan studies. There always exists a possibility that some unrelated factor or process is limiting additive lifespan extension in experiments that appear to demonstrate complementation. Even considering this caveat, there is a large body of literature arising from multiple model organisms that link mTORC1 regulated processes, including autophagy and mRNA translation, to the beneficial effects of CR; together these strongly suggest a role for mTOR in the CR response. Given the clear links of CR to processes downstream of mTOR, the observed decrease in mTORC1 signaling on CR, and the role of mTOR in nutrient signaling, there is a general consensus that altered mTOR signaling plays a significant role in CR, though additional pathways undoubtedly contribute to the overall effects of the intervention.

### **III. mTOR DOWNSTREAM SIGNALING**

As a major energy and growth signaling sensor, mTORC1 acts as a central coordinator of proliferative and maintenance programs. mTORC1 activity drives growth through activation of mRNA translation, regulation of metabolic pathways including glycolysis and fatty acid metabolism, and repression of cellular catabolic pathways, primarily the autophagy/lysosomal degradation pathway. Inhibition of mTORC1 results in reduced mRNA translation, increased catabolic processes, and a shift in metabolic substrate preference. Many of the effects of mTORC1 activity or inhibition are mediated by activation or suppression of downstream transcriptional regulators and the complex crosstalk between these factors. This is particularly apparent in the effects of mTORC1 modulation on metabolism, with the outcomes being highly context dependent on organism, tissue type, duration of intervention, type and severity of intervention, and complex interactions with extracellular or extra-organismal environment. Thus, dissecting out the pathways and targets of key importance in aging and disease is a significant challenge. This is a context in which future work using systems biology approaches may play an especially important role.

While mTOR signaling is complex, some highly conserved and well-defined pathways have been identified downstream of mTORC1 and mTORC2. mTORC1 has been studied more extensively and mTORC1 regulated processes are generally better described. The best-described mTOR driven processes are briefly addressed below.

***mRNA Translation.*** Hormonal signaling and abundant nutrient availability promote mTORC1 activation which up-regulate a variety of cellular processes necessary for growth. One critical process driven by active mTORC1 signaling is mRNA translation, required for protein synthesis and cell growth. mTORC1 kinase activity is known to promote translation through at least two distinct substrates [52]. mTORC1 phosphorylates p70S6K, the 70 kilodalton ribosomal S6 kinase, which is an activator of ribosome biogenesis. The interaction of this level of regulation of translation with the control of ribosomal biogenesis by TORC2 has been previously mentioned. Eukaryotic translation initiation factor 4E-binding protein 1 (eIF4E-BP1 or 4E-BP1) is also directly phosphorylated by mTORC1. Phosphorylation of eIF4E-BP1 results in its release from the eukaryotic translation initiation factor 4E (eIF4E), allowing eIF4E to

associate with mRNA cap binding proteins and form the cap-dependent translation initiation complex. The formation of this cap-binding complex is a key translation initiation event in eukaryotes.

Activation of mRNA translation is a critical function of mTORC1 and likely accounts for many of the phenotypes associated with mTOR driven disease, while decreased translation likely mediates many of the positive effects of mTOR inhibition. The anti-proliferative effects of mTOR inhibitors in cancer and immune diseases may largely be attributed to decreased rates of protein synthesis due to reduced translation. Decreased mRNA translation has been identified as a major pro-longevity intervention in multiple organisms and CR is thought to largely act through decreased translation. Deletion or knockdown of ribosomal components or translation initiation factors has been clearly demonstrated to increase lifespan in yeast, flies and nematodes. Furthermore, deletion of S6K extends lifespan and decreases body size in mice [33], though rates of translation have not yet been directly examined in these animals.

While mRNA translation is globally decreased in the setting of CR, and in at least some reports of mTOR inhibition, multiple studies in model systems suggest that the beneficial effects of reduced translation may result from differential translation of a subset of mRNA's rather than simply being a consequence of reduced global translation. This model has been best established in budding yeast, where lifespan extension resulting from ribosomal protein subunit deletion has been linked to an increase in translation of the transcription factor Gcn4 [53]. Gcn4 regulates a variety of genes including genes coding for proteins necessary for response to low nutrient conditions and genes encoding proteins involved in stress response. In yeast, Gcn4 has been found to be necessary for lifespan extension by ribosomal mutants, and appears necessary for full lifespan extension by mTOR or S6K deletion. Similar observations have been described in nematodes and flies, but this model has not yet found support in mammalian systems. A recent report using the mTORC1 specific catalytic inhibitor Torin 1 in p53  $-/-$  mouse embryonic fibroblasts suggested that differential effects on translation resulting from mTOR inhibition in mammalian cell culture could be largely explained by the presence of a 5' terminal oligopyrimidine (TOP) motif, though the study could not rule out the existence of less abundant differentially regulated 5' or 3' elements [54]. The interpretation of this study is complicated by the authors' claim that all of the effects of mTOR inhibition were mediated through the 4E-BP's. Deletion of individual 4E-BP's has not been

reported to alter translation and has no obvious effect on body size in mice, although it does appear to alter adipose tissue mass [55], while deletion of S6K results in a marked reduction in body mass, demonstrating that S6K regulates growth [26]. These apparent discrepancies may be a result of the cell culture system or mode of mTOR inhibition – it may be that catalytic inhibition of mTORC1 by Torin 1 does not accurately model mTOR inhibition by rapamycins or through genetic modulation, an idea supported by the drive to generate and characterize mTOR kinase domain inhibitors [56].

Global reductions in mRNA translation may directly contribute to the beneficial effects of mTOR inhibition on age-related diseases involving proteotoxic stress. Reduction in rates of translation appear to directly enhance the fidelity of protein synthesis [57, 58] and it is widely accepted that decreases in protein synthesis rates may allow for improved cellular proteostasis, while a loss of proteostasis is a hallmark component of aging and a number of age-related diseases [59-61]. A decreased steady state requirement for protein repair and degradation may result in an increased capacity for cells to respond to transient stresses such as oxidative damage, protein aggregation, and heat or cold shock. It seems that decreased mRNA translation likely promotes longevity at least partially through improved proteostasis and increased protein degradation, though it is difficult to dissect this phenotype away from elevations in autophagy (see below), antioxidant defense, or other biological effects of mTOR inhibition or CR.

**Autophagy.** As a major intracellular recycling pathway in eukaryotes, the autophagy-lysosomal pathway plays an essential role in degrading damaged organelles and macromolecules as well as in regulating intracellular levels of free amino acids through protein catabolism. In addition to promoting protein synthesis and cell growth, active mTOR directly inhibits the intracellular catabolic process of autophagy [62]. Active mTORC1 phosphorylates ULK1 (the mammalian homolog of yeast Atg1), preventing activation autophagosome formation by the ULK1 complex [63, 64]. Active growth signaling through mTOR and through mTOR independent mechanisms also down-regulates the expression of autophagy proteins, including LC3 [65]. Nutrient deprivation decreases mTOR activity through increased AMPK activity, decreased amino acid sensing at the lysosome, and decreased insulin/IGF signaling, relieving the inhibition of autophagy by active mTOR and resulting in an increase in the catabolism of proteins and

organelles. This increased catabolic activity provides free amino acids and allows for cell survival when nutrients are limiting.

Damaged and aggregated proteins, oxidized lipids, and damaged organelles accumulate with age [66, 67], suggesting that basal levels of autophagy decline or are insufficient to adequately prevent this accumulation as time progresses. Lipofuscin, the complex granular pigment that accumulates in aged tissue, is a highly conserved phenotype of cellular aging that has been observed in many multicellular eukaryotes [68]. While the exact composition and functional consequences of lipofuscin remain to be determined, it has become clear that longevity-promoting interventions also slow the rate of lipofuscin accumulation [69]. Thus, lipofuscin is often used as a biomarker of relative age. Given the close correlation between longevity and damaged macromolecule accumulation, the obvious question is if accumulated damaged macromolecules are a driving factor in aging and modulation of this accumulation could attenuate aging. While this hypothesis has proven difficult to test directly given the challenges in selectively inducing the autophagy-lysosomal system, a large body of evidence from yeast and *C. elegans* supports the model that induction of autophagy is a necessary downstream effector of mTORC1 inhibition in mediating lifespan extension [70]. Induction of autophagy has also been shown to be necessary for CR mediated longevity promotion, potentially through mTOR [71]. While the necessity of autophagy for the success of these interventions is broadly accepted, it is not clear whether induction of autophagy alone is sufficient to increase lifespan.

In addition to the role of autophagy in promoting longevity, dysfunction of this pathway has been implicated in a variety of pathologies and activation of autophagy has been demonstrated to attenuate a variety of age-related diseases. The induction of autophagy has been directly implicated as a potential clinical target for treatment of cardiovascular disease, age-related macular degeneration, diabetes, and a variety of neurodegenerative disorders including Parkinson's disease (PD) and Alzheimer's disease (AD) [72-74]. The nervous system appears particularly sensitive to the accumulation of damaged macromolecules and protein aggregates, and increasing autophagic degradation has been shown to prevent neurodegeneration in models of AD and PD as well as in models of Huntington's disease (HD) [59, 75, 76], all progressive neurodegenerative diseases directly associated with proteotoxic insult. An

important caveat to these studies is that, as with the role of autophagy in longevity, it has been difficult to demonstrate that induction of autophagy is sufficient to rescue disease and decreased growth signaling, through mTOR inhibition or CR, are beneficial to a number of neurological disease models through autophagy independent mechanisms (see Chapter 3 for further discussion) [77-79].

**Sirtuins.** The sirtuin family of protein deacetylases and ADP-ribosyltransferases have become among the most intensely studied longevity factors based on their diverse effects on longevity and healthspan in invertebrates and mammals [80]. The best-characterized sirtuins are the SirT1 orthologs (Sir2 in yeast, SIR-2.1 in nematodes, dSir2 in flies, and SirT1 in mammals). While overexpression of Sir2, SIR-2.1 or dSir2 have each been reported to extend lifespan, many recent studies have contradicted these results [81]. Genetic studies in both yeast and *C. elegans* have generally indicated that mTORC1 and SirT1 homologs modulate aging by different mechanisms; CR or mTORC1 inhibition extends lifespan independently of sirtuins in both organisms [51, 82]. One study in yeast reported that inhibition of TORC1 can activate Sir2 under some conditions [83], creating a potential link between these two longevity pathways. In addition, both mTOR and sirtuins are known to interact with common factors, including AMPK, PGC-1 $\alpha$  and FOXO-family proteins, suggesting extensive cross-talk between these longevity pathways within an overall longevity network. Thus, while an abundance of recent evidence has indicated that modulating sirtuins activity alone is insufficient to positively impact lifespan it does appear that sirtuins may play some downstream role in mediating the beneficial effects of CR, AMPK activation, and mTOR inhibition. Recent data also suggests that mTORC2 may be a de-acetylation target of sirtuin, where acetylated mTORC2 activates Akt and sirtuin activation inhibits this feedback[84]. This would provide a novel link between intracellular NADH/NAD<sup>+</sup> status and growth signaling through mTOR.

**Mitochondrial Function and Metabolism: HIF1, NRF2, and Mitophagy.** Mitochondria are key organelles in metabolism, disease, and aging. These organelles are the major producers of energy for most cell types, a primary site of metabolic reactions, a major source of toxic products (both reactive oxygen species and toxic intermediates of metabolism) and provide key cellular signaling regulators. Given the multifaceted role that mitochondria play in eukaryotic biology it is unsurprising that mitochondria have been linked to a variety of pathological states, diseases, and aging [85]. Growth signaling through

mTOR appears to influence mitochondrial function through multiple mechanisms and downstream regulatory factors.

Hypoxia inducible factor 1, Hif-1, is a transcription factor that promotes glycolytic processes and can be activated through mTOR signaling in mammals, best described in the setting of cancer [86]. Activation of this factor is linked to longevity in model organisms through somewhat unclear mechanisms [87]. In mammals, activation of Hif-1 leads to vascular tumor growth [88, 89], “wet” macular degeneration [90], and rheumatoid arthritis [91], all through positive effects on the angiogenic factor VEGF. At the intracellular level, Hif-1 promotes glycolysis, downregulates mitochondrial oxygen consumption, modulates intracellular pH, and at least partially mediates the Warburg effect in mammalian neoplasia [92]. Decreased mTOR signaling, achieved by AMPK activation, attenuates tumor vascularization, tumor metabolism, and the Warburg effect partially through decreased activation of Hif-1 [93]. This pathway likely contributes to the general anti-proliferative effects of mTOR inhibition in preventing mammalian neoplasia.

Consistent with these effects, mTOR inhibition has been associated with increased mitochondrial respiration in yeast and worms and CR has been associated with increased mitochondrial content and respiration in a variety of organisms. This effect has been directly associated with longevity in yeast [94], with adaptive signaling resulting from increased mitochondrial superoxide production being implicated in this response in nematodes, yeast, and mammals [95]. Mice lacking mTORC1 components in white adipose tissue also show an increase in mitochondrial content and respiration, suggesting that this may be conserved in mammals [96]. Mitochondrial metabolism and cellular mitochondrial mass have also been reported to be increased by mTORC1 inhibition, at least in certain conditions, through activation of PGC-1 $\alpha$  and the transcription factor Ying-Yang 1 (YY1) [97]. The exact role of mitochondrial metabolism as a mediator of mTOR driven processes is far from clear, but available data suggests that this is a critical component of aging and disease and thus warrants further attention.

mTOR inhibition has also recently been shown to facilitate activation of retrograde signaling through facilitating nuclear localization and activation of NRF2 increasing levels of NRF1 [98]. NRF2 is an important factor in longevity, playing a key role in the induction of antioxidant enzymes and metabolic

shifts that result from CR[99, 100]. While the link between NRF2 and mTOR is not yet well characterized the relationship between these key players in lifespan regulation warrants further study.

In addition to the above, mTOR affects mitochondrial function through autophagic degradation of mitochondria, a process termed mitophagy. The mitochondrial-lysosomal axis theory of aging suggests that proper maintenance of a functional pool of mitochondria depends on continuous successful removal of damaged and dysfunctional mitochondrial components through fission and mitophagy of fission products [9]. This theory predicts that reducing the rate of turnover of mitochondria, as may occur in aging, would result in an accumulation of dysfunctional mitochondria. This accumulation could lead to an increase in basal ROS production, damage accumulation, loss of tissue homeostasis, and potentially cellular senescence or death. mTOR inhibition increases basal autophagy, as described above, and would thus be predicted to preserve or restore mitochondrial function with age or in disease. While this model remains to be fully assessed it provides an attractive link between mitochondrial function and mTOR signaling in aging and disease and is supported by evidence presented here (see Chapter 5).

***Stem Cell Maintenance.*** Stem cell loss and dysfunction are likely a significant factor in mammalian aging and age-related diseases. This is particularly likely in proliferative tissues such as dermis, the immune system, and the gastrointestinal system, as well as in wound repair or response to ischemic injury, settings where proliferative capacity is required. While the exact role for stem cells in aging is currently unknown, evidence suggests that mTORC1 is central in the maintenance of stem cells with age. mTOR inhibition has been shown to protect immune function with age in murine models of infection (discussed below) and this has been attributed to enhanced hematopoietic stem cell capacity. mTOR inhibition with rapamycin has also been recently reported to improve intestinal stem cell function, although in this case the improvement was linked to alterations in mTOR signaling in the adjacent Paneth cells, which are responsible for maintaining the stem cell niche, rather than a direct effect on intestinal stem cells [101]. CR has been shown to enhance the function of skeletal muscle stem cells, presumably related, at least in part, to the concomitant decrease in mTOR activity [102]. The role of stem cells in aging and of mTOR in regulating their function remains an exciting and largely uncharted avenue of research.

## IV. MTOR AND DISEASE

### Longevity and Healthspan

Extension of healthspan, defined as the duration of life for which an organism is free from major age-related disease or loss of function, is considered by many to be the critical goal of aging research. Longevity studies in model organisms intrinsically include healthspan components. *C. elegans* viability determination in standard plate or liquid based lifespan studies is based on the ability of the animals to respond to mechanical stimulus [43]. Yeast studies, both replicative and chronological, depend on the cell capacity to successfully produce progeny. In both cases the individual organism may remain viable beyond the point that they are considered deceased by the assay standards. Thus, the nematode and yeast models are tied to neurological and muscular health and reproductive capacity, respectively. In the murine model animal welfare regulations prevent expiration of mice by natural causes (even natural age-related deaths), requiring euthanasia if animals decline past a set cutoff in body mass, appear immobile, hunched, or in pain, or if they show signs of painful and untreatable diseases such as ulcers or cancers. These restrictions may complicate accurate determination of lifespan in a longevity study but they also compel longevity promoting interventions to be those that also protect healthspan.

Considering these restrictions it is perhaps unsurprising that current data suggests longevity-enhancing interventions extend healthspan and decrease or delay the incidence of age-related disease rather than increasing longevity of sick individuals [103]. Conversely, relatively few examples exist in the literature where healthspan is benefited in the absence of longevity benefits, perhaps a result of using lifespan as a primary endpoint. A noted exception is the recent (2013) NIA study of CR in rhesus monkeys, which observed a significant decrease in the appearance of age-related diseases without a change in survival[104]. While this, and studies like it, show that it may be possible to uncouple the healthspan and longevity effects of CR, they are generally limited by a lack of positive controls and or a clear definition of what constitutes baseline healthspan. The rhesus study, for example, stands in contrast to a prior study that reported an increase in both lifespan and healthspan [105]. Differences in diet, housing conditions,

and severity of the CR protocol may be factors distinguishing the two studies and the lack of a positive control limits the conclusions that can be drawn from this work. It is also possible that longevity promoting interventions may more sensitively and broadly affect healthspan than lifespan so that they improve health at a lower effective dose than that needed to improve longevity. In any case, healthspan benefits, even in the absence of effects on lifespan, are of significant clinical worth. While the ultimate goal of aging research is to understand the mechanisms driving the aging process and develop interventions targeting this decline the human health and socioeconomic impact of age-related diseases would benefit greatly from any intervention that could effectively improve healthspan.

There has been a recent emphasis on defining and characterizing the effects of longevity promoting interventions on age-related health parameters. Each model used for aging studies has a set of health parameters that have been used to explore the relationship between lifespan, healthspan, and aging interventions. In yeast the primary healthspan parameters are replicative capacity (in this case the actual readout for lifespan), mitochondrial function, and cell morphology. Nematodes have typically been used to study proteostasis, the clearance of damaged macromolecules during the aging process and in neurological decline, diseases of proteotoxicity, and age-related changes in muscle function. *Drosophila melanogaster* has been useful in studying neuronal, muscular, sensory, stem cell, and cardiac function with age. Mammalian systems have been used to examine a variety of age-related physiological and health parameters relevant to the biology of human aging.

### **mTOR and Disease**

Concurrent with demonstrations of a role for mTOR in regulating longevity it became increasingly apparent that mTOR signaling plays a central role in regulating healthspan and a variety of age-related and non age-related pathologies. mTOR inhibition attenuates many age-related changes in lower organisms, as described above. In addition, mTOR inhibition slows or delays many age-related and age-associated changes that are broadly conserved from lower eukaryotes to mammals including lipofuscin accumulation, age-related mitochondrial dysfunction [106, 107], and loss of proteostasis. Age related cardiac, neural/cognitive, and stem cell functions are improved in murine models through inhibition of mTOR [101, 108-111]. While the efficacy of mTOR inhibitors in attenuating age-related pathologies in

humans is yet to be determined there is an abundance of literature suggesting that mTOR is a clear potential target for treating diseases of aging.

**Heart disease.** There is evidence that mTOR inhibition may be generally protective against cardiomyopathies, including age-related cardiomyopathy [112]. Zebrafish heterozygous for mTOR are protected against two models of cardiomyopathy [113]. Administration of rapamycin markedly suppresses cardiac hypertrophy in the trans-aortic constriction model of pressure-overload induced heart failure [114] and rapamycin treatment has been shown to result in regression of established pressure overload induced cardiac hypertrophy, fibrosis, and dysfunction. Perhaps the greatest impact of mTOR-targeted pharmacotherapy in cardiac disease has emerged through the widespread use of stents that elute rapamycin or rapamycin derivatives (e.g. everolimus, temsirolimus, ridaforolimus, umirolimus, zotarolimus, collectively referred to as “rapamycins”) to inhibit cell proliferation and restenosis following angioplasty, with significant decreases in major adverse cardiovascular events during the first few years after implantation [115].

While decreased mTOR signaling has been clearly demonstrated to attenuate a variety of cardiac myopathy and failure models, the precise mechanisms of importance are less clear. Decreased mRNA translation, inflammation, and hypertrophic growth signaling, increased autophagy, improved mitochondrial function or content, and altered metabolic preference have all been independently linked to improved outcome resulting from mTOR inhibition in cardiac models making it difficult to parse out the key functions downstream of mTOR crucial to the benefits observed [112].

**Neurodegenerative disease.** As noted above, it has been suggested that mTORC1 inhibition mediated enhancement of autophagy may lead to improved degradation of aberrant or misfolded proteins and reduced proteotoxic stress in neurodegenerative diseases such as PD, AD, and HD. Thus, inhibition of mTOR could prove to be a successful therapeutic strategy in neurodegenerative disease. Evidence of such effects has been seen in fly and murine models of PD as well as in fly, murine, and cell culture models of HD. Positive effects of rapamycin on disease progression have been reported in two different mouse models of AD (see above).

The evidence of a benefit of rapamycin in neurodegenerative diseases raises the question of whether mTORC1 inhibition might also attenuate age-related declines in cognitive function in the absence of a more severe neurological disorder. Recent studies assessing the effects of chronic mTOR inhibition on cognitive function during aging in mice have reported that old animals treated with rapamycin performed substantially better on tasks measuring spatial learning and memory than did untreated, age-matched animals [116]. Intriguingly, there were indications that rapamycin also enhanced cognitive function in young mice and had anti-anxiety and anti-depressive effects at all ages. mTOR has also been identified as a potential target for treatment of seizures and epilepsy of diverse etiologies [117-120], suggesting that growth signaling inhibition may have broad neurological benefits. The combined complexity of the central nervous system and processes downstream of mTOR makes it a challenge to isolate the mechanisms underlying the beneficial effects of decreased mTOR signaling but the clear therapeutic potential makes it a very attractive target for testing mTOR inhibitors in a clinical setting.

**Cancer.** A majority of tumors show evidence for activation or upregulation of mTOR signaling, and mTOR inhibition has been studied extensively as a potential therapy for a wide variety of cancers [121].

Rapamycins potently inhibit growth of solid tumor cell lines but have shown disappointing efficacy in several clinical trials, though certain rare cancers, including renal cell carcinomas and glioblastomas, may respond to mTOR inhibition. Mutations in tuberous sclerosis complex proteins (TSC1 and TSC2), which are upstream inhibitors of mTOR, cause a variety of hyperplastic diseases including tuberous sclerosis, directly linking hyperplasia to mTOR over-activity. Dysplasias driven by TSC1 or TSC2 loss are currently being evaluated as clinical targets for mTOR inhibitors. Clinical data for trials involving tuberous sclerosis patients who were not candidates for surgery are striking – 35% of patients treated with everolimus responded to treatment compared to 0% of controls [122]. Rapamycin has been shown delay or reduce deaths due to age-related and age-associated cancers in several murine studies, with the effects on age-related cancers likely a major contributing factor in observed lifespan extending effects in mice [123].

**Diabetes and obesity.** mTOR signaling has been implicated in the development of age-associated metabolic disorders such as obesity and type II diabetes. Inhibition of mTORC1 inhibits, while mTORC1 activation stimulates, adipogenesis in mice [124, 125]. Obesity results in chronic activation of mTOR in

adipose tissue, a state that has been linked to obesity-associated cancers, inflammation, beta cell adaptation preceding type II diabetes, non-alcoholic fatty liver disease, and many other complications [124]. Multiple downstream effectors of mTOR signaling, such as S6K-1, 4EBP1, and SREBP, act as mediators between nutrient signaling and the development of obesity and type II diabetes. SREBP, a transcription factor that induces the expression of lipogenic genes, is activated by mTORC1 but not mTORC2. S6K-1 null mice display reduced body fat mass and resistance to diet induced obesity, while mice lacking 4EBP1 show increased sensitivity to diet induced obesity and adipogenesis, possibly through hyper-activation of S6K-1. Mice lacking Raptor in adipose tissue are lean with fewer and smaller adipocytes, have increased insulin sensitivity, and show resistance to diet induced obesity [96]. Conversely, mice with adipose specific knock out of Rictor have normal body fat mass and glucose tolerance but are hyperinsulinemic and have enlarged organs [126]. Mice lacking S6K1 in all tissues or lacking Raptor specifically in adipose tissue are resistant to diet-induced obesity [96].

The relationship between mTOR signaling and age-associated metabolic disorders such as type II diabetes and obesity is complicated. Rapamycin can protect mice from diet-induced obesity through the inhibition of adipocyte differentiation [127]. However, mice and rats chronically treated with rapamycin demonstrate altered insulin sensitivity and glucose tolerance, effects of chronic mTOR inhibition that have been attributed to effects on mTORC2 [27]. Blood hyperlipidemia is also observed with chronic rapamycin treatment in mouse and human studies [125]. The S6K1 knockout mice are also hypoinsulinemic and glucose intolerant, apparently due to a decrease in beta cell size and function [26]. Despite these symptoms S6K1 knockout mice and rapamycin treated mice are long-lived and have improved healthspans, suggesting that these effects are not detrimental to health or survival. It has also been pointed out that the “starvation induced diabetes” associated with mTORC1 inhibition differs substantially from type II diabetes, which is caused by insulin resistance resulting from over-nutrition in association with mTOR activation. Thus, additional study is needed to determine whether targeted inhibition of mTORC1 and the observed changes in lipid profile, insulin sensitivity, and glucose homeostasis represent a health risk or whether they represent an altered metabolic state consistent with the promotion of longevity and health.

**Immune Function.** Rapamycins are used clinically as immunosuppressive or immunomodulatory drugs. There are, however, also reports that rapamycins can enhance immune system efficacy in certain settings, including tuberculosis, anti-tumor vaccine responses in mice, and vaccinia vaccination in non-human primates [128]. In the context of age-related immune function, rapamycin restored self-renewal capacity of hematopoietic stem cells (HSC) and allowed for an effective response to influenza vaccination in old mice, which was protective against subsequent infection, while deletion of Tsc1 in young mice led to an old-mouse HSC phenotype [129]. The apparent contradiction between these observations and the use of rapamycins as immunosuppressive drugs may be explained by fact that mTOR can exert divergent immunoregulatory functions during immune cell activation and differentiation, depending on the cell subset type. Furthermore, while rapamycins may limit immune activation and proliferation in the setting of an immunogenic insult they appear to have a robust effect in preventing age-related declines in immune function, thus preserving immune function later in life. Thus, rapamycins' functions in immune biology are more complex than previously recognized with outcomes depending on dose, duration of treatment, immune cell type, and specific immune challenge. The long-term effects of rapamycins on age-related declines in immune function stand in stark contrast to those of short-term responses.

**Inflammation.** Inflammation is strongly associated with aging and drives a multitude of age-associated disorders. Cardiovascular disease, obesity and metabolic disorders, cancer, and neurodegenerative diseases all include inflammatory components and attenuation of inflammation has been implicated as a clinical target in each of these disease settings[130]. Hyperactive mTOR has been linked to inflammation and inhibition of mTOR by rapamycins has been demonstrated to be anti-inflammatory in renal disease, lung infection, and in vascular inflammation in atherosclerosis and following angioplasty, and inflammation is a major component of "overnutrition-induced" diseases [131]. CR strongly attenuates age-related inflammatory signaling, an effect at least partly mediated through mTOR [132]. mTOR appears to be a good candidate for treating or preventing age-related inflammatory processes while reduced inflammation seems to play a mechanistic role in the pro-longevity effects of mTOR inhibitors.

**Renal Disease.** Rapamycins are used clinically to reduce nephrotoxicity in chemotherapy, prevent allograft rejection, and as a treatment for renal cell carcinoma. Activation of mTOR signaling has been

associated with several common forms of kidney disease, suggesting that inhibition of mTOR might have broad therapeutic benefits for renal health. Consistent with this, rapamycins have been shown to reduce kidney fibrosis, attenuate diabetic nephropathy, and improve outcome in animal models of polycystic kidney disease[133].

***Age-related Macular Degeneration.*** Age-related macular degeneration is the leading cause of blindness in Western countries. Capillary overgrowth in the choroid layer of the eye, which is a contributing factor, has been attributed to excessive production of VEGF. Rapamycin has been shown to reduce VEGF expression in retinal pigment epithelium and inhibit angiogenesis in vitro [134]. In a rat model of age-related macular degeneration, rapamycin decreased the incidence and severity of retinopathy [135] and in human patients rapamycin appeared to decrease the need for anti-VEGF intravitreal injections by approximately half [136]. Thus, age-related macular degeneration appears to be a promising clinical target for mTOR inhibiting interventions.

***Hutchinson-Gilford Progeria and Laminopathies.*** Hutchinson-Gilford progeria syndrome (HGPS) is typically caused by a *de novo* mutation in the lamin A/C gene (LMNA) that activates a cryptic splice site, producing an abnormal lamin A protein termed progerin. Accumulation of progerin leads to aberrant nuclear morphology in vitro and is believed to be the causal factor in the pathogenesis of disease. The precise mechanism linking progerin accumulation to the phenotypes associated with this disease is unclear but it is generally thought to involve disruption of nuclear DNA binding proteins, including transcription factors and DNA repair components, as a result of aberrant nuclear scaffold structure. It has been reported that treatment of cells from HGPS patients with rapamycin corrects the nuclear morphology defect, delays the onset of cellular senescence, and enhances the clearance of progerin through autophagic degradation [137]. No effective treatment currently exists for HGPS and these data suggest that rapamycins might slow disease progression in HGPS patients. Any success in HGPS would strongly suggest that rapamycins might show efficacy in patients diagnosed with atypical Werners' Syndrome, often caused by non-HGPS mutations in LMNA, as well as in patients with muscular dystrophies resulting from lamin mutations or other laminopathies.

## V. SUMMARY

Nutrient sensing and growth signaling are fundamental biological processes that regulate normal biological function but also drive a variety of diseases and aging. mTOR is a central regulator of nutrient sensing and signaling, integrating intracellular amino acid levels, ribosome capacity, intracellular energy status downstream of AMPK, and a variety of extracellular signals. As a central component of growth and metabolism mTOR is a key regulator of aging and disease. Genetic and pharmaceutical intervention data have firmly established a role for mTOR in aging and disease and first generation mTOR inhibitors have already begun providing new and potent therapies in a wide variety of disease settings.

The studies presented here represent work that has contributed to advancing our understanding of growth and nutrient signaling in aging and disease. These studies include work uncovering the molecular mechanisms underlying genotype specific responses to reduced nutrient signaling through caloric restriction (Chapters 2) and applying this knowledge to identify novel treatment strategies for currently untreatable human genetic disorders (Chapter 3). As a result of these studies we have made significant progress in understanding the pharmacodynamics of the commonly used mTOR inhibitor rapamycin and have gained novel insights into the dose-dependency of physiological effects resulting from rapamycin treatment (Chapter 4). Also presented here is work that characterizes the role of a major pathway downstream of mTOR, autophagy, in a model for pressure-induced cardiac disease (Chapter 5). Finally, we have uncovered a novel phenotype involved in cardiac aging that links functionally links mitochondrial dysfunction, ROS, autophagy, and mTOR and provides support for the lysosomal-mitochondrial theory in cardiac aging (Chapter 6). Together this work provides significant new insights into the molecular processes underlying aging, age-related disease, interventions in aging and disease, and growth signaling.

## CHAPTER 2

# Molecular Mechanisms Underlying Genotype Specific Responses to Caloric Restriction

### OVERVIEW

Caloric restriction (CR) increases lifespan and attenuates age-related phenotypes in many organisms; however, the effect of CR on longevity of individuals in genetically heterogeneous populations is not well characterized. Here we describe a large-scale effort to define molecular mechanisms that underlie genotype-specific responses to CR. The effect of CR on lifespan was determined for 166 single-gene deletion strains in *Saccharomyces cerevisiae*. Resulting changes in mean lifespan ranged from a reduction of 79% to an increase of 103%. Vacuolar pH homeostasis, superoxide dismutase activity, and mitochondrial proteostasis were found to be strong determinants of the response to CR. Proteomic analysis of cells deficient in prohibitins revealed induction of a mitochondrial unfolded protein response (mtUPR) which has not previously been described in yeast. Mitochondrial proteotoxic stress in prohibitin mutants was suppressed by CR via reduced cytoplasmic mRNA translation. A similar relationship between prohibitins, the mtUPR, and longevity was also observed in *Caenorhabditis elegans*. These observations define conserved molecular processes that underlie genotype-dependent effects of CR that may be important modulators of CR in higher organisms.

### INTRODUCTION

Caloric restriction (CR, also referred to as dietary restriction or DR) is the most widely studied intervention for slowing aging and improving healthspan in diverse species [138]. Defined as a reduction in nutrient availability without malnutrition, CR can increase lifespan in yeast, nematodes, fruit flies, and other invertebrates. In both mice and rats, CR has been found to increase lifespan and delay the onset of a variety of age-related phenotypes. Positive health effects of CR have also been reported in a primate, the rhesus macaque, where CR reduced age-related mortality and lowered the incidence of age-related

disease [105]. If, and to what degree, CR will slow aging in humans remains to be determined, though studies to suggest that CR provides at least some benefits to human subjects consistent with a delaying of the aging process [139].

Several genetic pathways have been suggested to play a role in mediating the health and longevity effects of DR. These include growth hormone and insulin/IGF-1-like signaling, activity of the NAD<sup>+</sup> dependent deacetylase enzymes sirtuins, and signaling through the mechanistic target of rapamycin (mTOR), which is a component of insulin/IGF signaling but is also responsive to a variety of other intracellular energy signals [25, 33]. mTOR signaling in particular has become an intensely studied longevity pathway (see **Chapter 1**), as inhibition of mTOR by the drug rapamycin is sufficient to increase lifespan in mice, even with treatment begun at 600 days of age [28]. Genetic studies indicate that inhibition of mTOR is likely involved in lifespan extension by CR in yeast, nematodes, and flies, though the relationship between mTOR and CR in mammals is yet to be determined [25].

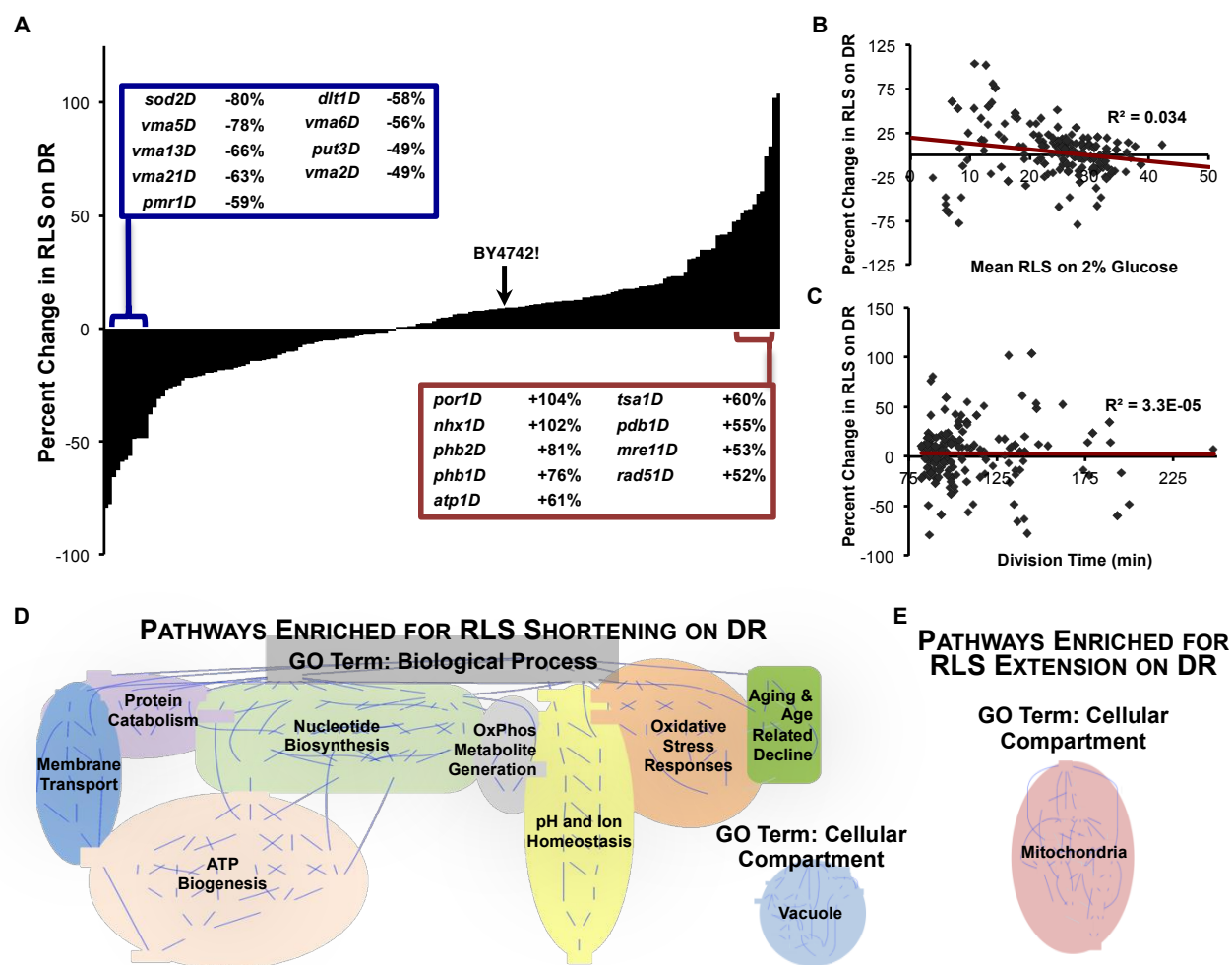
Despite abundant data indicating that CR can slow aging across evolutionarily divergent species there exist reports where DR has had no effect or has caused a reduction in lifespan. For example, one study that examined a mouse strain recently derived from the wild found that mean lifespan of the strain was not extended by CR [140]. A study which characterized the effect of CR on 42 recombinant inbred mouse lines resulted in a distribution of responses ranging from 98% extension to 68% reduction in lifespan [141]. Recently, two high-profile and independent studies of CR in rhesus macaque, each spanning more than three decades, resulted in markedly different outcomes: one reported significant reductions in mortality due to age-related causes as well as a significant reduction in the rate of appearance of age-related disease in the CR group [105] while the other found no change in survival, though CR still improved measures of healthspan [142]. These studies are complicated by multiple factors that likely influenced the outcome, ranging from food composition to housing conditions, but together they support the idea that genotype is a critical factor in determining the effect CR has on longevity. The molecular processes underlying genotype-dependent responses to CR remain largely unexplored.

In this study we used the budding yeast *Saccharomyces cerevisiae* as a model to explore the interaction between genotype and the effect of CR on replicative lifespan. Yeast replicative lifespan (RLS) is defined as the number of daughter cells a mother cell is capable of producing before irreversibly exiting the cell cycle [143]. As budding yeast divide asymmetrically it is possible to measure the RLS of individual cells and report a population based survival curve as would be reported for a multicellular organism survival study. CR in *S. cerevisiae* is achieved by reducing the glucose concentration in the medium from 2% to 0.5% or lower and has been shown to extend RLS in multiple strain backgrounds [144]. Here we examined the effect of CR at 0.05% glucose on the RLS of 166 strains selected from a single gene deletion library, each strain lacking a single non-essential gene. Similarly to the aforementioned inbred mouse study [141] we observed a wide distribution in response to CR from dramatic decreases to substantial increases in lifespan relative to that observed for wild-type cells (**Figure 3A**). Gene ontology (GO) analysis of genes associated with significantly positive or negative changes in RLS by CR revealed multiple conserved molecular processes associated with the CR response (**Figure 3D-E**). Among the strongest responders were subunits of the vacuolar ATPase (v-ATPase), superoxide dismutases, and the mitochondrial prohibitins. Disruption of vacuolar/lysosomal components or superoxide dismutases (*SOD1* and *SOD2*) lead to a dramatic shortening of RLS by CR, while cells lacking mitochondrial prohibitins (*PHB1*, *PHB2*) are short-lived under control conditions and show a large lifespan extension of RLS in response to CR, a relationship that we found to be conserved in the nematode *Caenorhabditis elegans*.

## GENOTYPE SPECIFIC RESPONSES TO CALORIC RESTRICTION

To investigate the interaction between genotype and CR, we analyzed the effect of CR, accomplished by reducing glucose concentration from 2% to 0.05%, on approximately 100 randomly selected single gene deletion strains. Additional strains were added to the screen either randomly or by selecting within previously tested functional categories (e.g. vacuolar ATPases) to validate earlier results. Strains showing growth properties inconsistent with those annotated in the *Saccharomyces* Genome Database ([www.yeastgenome.org](http://www.yeastgenome.org)) were excluded from the screen and not included in subsequent analysis. In total, RLS data for 166 strains are reported here. The resulting distribution showed a median

CR-induced increase in mean RLS of 3.9% with changes ranging from a 79% reduction to a 103% increase (**Figure 3A**). CR resulted in statistically significant changes to RLS (multiple testing corrected  $q < 0.05$ ) for 82 (49%) strains and non-significant effects from CR for the remaining 84 strains. The absolute effect of CR (mean RLS on CR medium minus mean RLS on control medium) shows a similar distribution (**Figure S1A**).

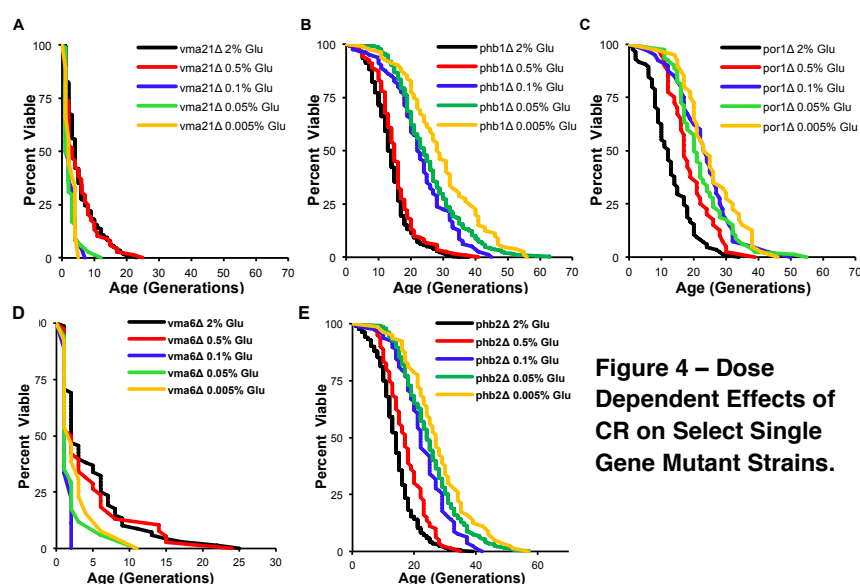


**Figure 3 - Genotype-Dependent variation in Yeast Replicative Lifespan Response to Caloric Restriction.** (A) RLS of 166 single gene deletion strains subjected to CR at 0.05% glucose. RLS varied from 80% reduction to 104% extension. Percent change in RLS on DR did not correlate with general fitness as measured by mean lifespan on 2% glucose (B) or division time (C). Gene ontology analysis of mutants with RLS significantly shortened (D) or extended (E) by CR revealed specific molecular processes involved in determining the direction of the response.

We considered the possibility that the effect of CR could be influenced by the overall fitness of a given strain. To test this we compared the effect of CR on RLS in each strain to two measures of fitness: the RLS of the strain under control conditions and maximal growth rate (an indicator of general fecundity).

In both cases there is no significant correlation between fitness and the effect of CR measured by either percent or absolute change (**Figure 1B-C, Figure S1B-C**).

In contrast to the aforementioned studies in mice and primates, the genetic variation in each yeast strain is known and limited to a single gene, allowing for the study of mechanisms underlying differential responses. Gene ontology (GO) analysis of genes associated with either significant positive or significant negative response to CR revealed an enrichment of a unique set of specific cellular processes for each case. Analysis of the 166 gene set controlled for any enrichment in the set independent of response to CR. Genes involved in pathways regulating cellular and organellar pH, ion homeostasis, and oxidative stress response were significantly enriched in strains whose RLS was reduced by CR (**Figure 1D**), while genes involved in mitochondrial function were highly enriched in strains where RLS was increased by CR (**Figure 1E**). Intriguingly, while multiple molecular genetic pathways show enrichment among negative responders to CR mitochondrial function was the only GO category enriched for positive response to CR in this gene set. While additional pathways are likely to be revealed as coverage of the



**Figure 4 – Dose Dependent Effects of CR on Select Single Gene Mutant Strains.**

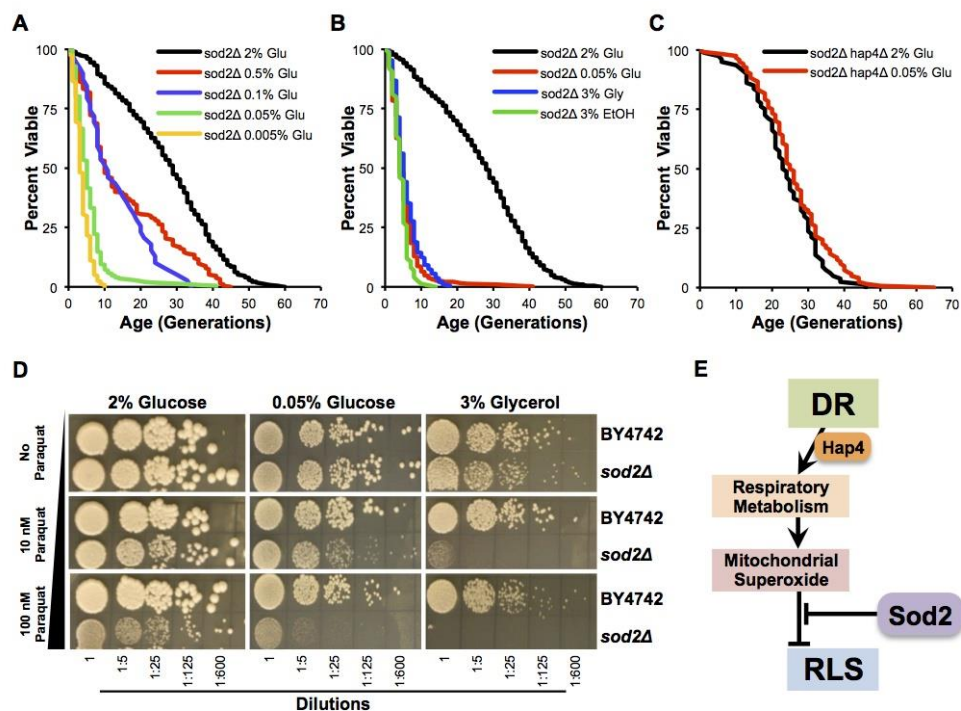
yeast genome is expanded in future studies this observation suggests a robust positive relationship between CR and mitochondrial mutants, an observation we pursued in subsequent work (see **Chapter 3**).

To further characterize the nutrient response profile of the strains strongly affected by CR, we examined RLS for select single gene mutants across a range of glucose concentrations from 2% to 0.005% (**Figure 4**). These include gene deletions corresponding of the mitochondrial porin, *POR1*, the mitochondrial inner membrane (MIM) chaperone complex component, *PHB1*, the vacuolar ATPase

subunits encoded by *VMA21* and *VMA6*, and the mitochondrial superoxide dismutase, *SOD2* (Figure 5A). Each of these strains shows a dose-dependency in their response to DR.

## MECHANISM OF LIFESPAN REDUCTION BY CR IN SUPEROXIDE DISMUTASE MUTANTS

To determine the molecular mechanisms underlying these genotype-specific responses to CR, we first examined *sod2Δ* cells. Sod2 is an antioxidant enzyme that is localized to the mitochondria and neutralizes the toxic superoxide radical by converting it into hydrogen peroxide [145, 146]. Sod2 expression or activation are reportedly increased by CR in murine, invertebrate, and yeast models and is often implicated as contributing to the protective antioxidant properties of CR [147-149]. One effect of CR in yeast is to increase in mitochondrial respiration, a major source of reactive oxygen species, suggesting that Sod expression is increased in order to compensate for increased intracellular oxidative stress.



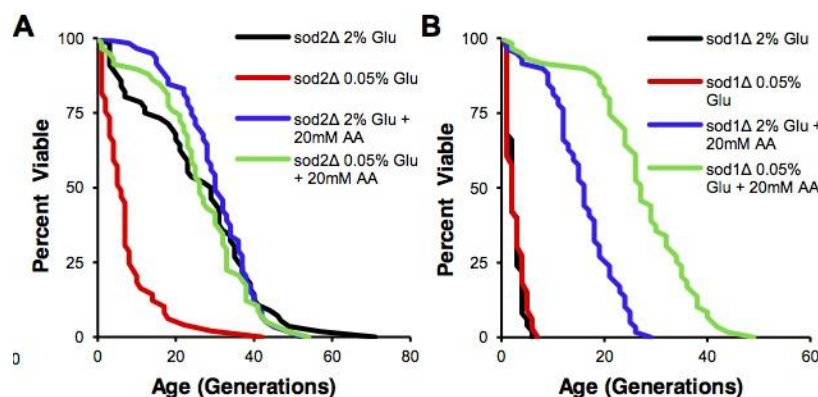
**Figure 5 – Sod2 Mutant RLS is Decreased by CR Because of an Inability to Detoxify Increased ROS Levels.** (A) Dose-response of *sod2Δ* cells to CR. (B) Non-fermentable carbon source media mimics the RLS effects of CR on Sod2 cells. (C) Preventing the induction of respiration through loss of Hap4 rescues Sod2 mutants on CR. (D) Sod2 mutants are sensitive to paraquat and CR increases the relative sensitivity of these cells. (E) Our data supports a model whereby ROS decrease RLS in Sod2 mutant cells on CR.

Considering this relationship we considered the possibility that the effects of CR on *sod2Δ* cells may result from a failure to adequately detoxify superoxide radicals resulting from increased oxidative

metabolism. We observed a similar reduction in the RLS

of *sod2Δ* cells on non-fermentable carbon sources (**Figure 5B**), consistent with this model.

The induction of respiration in yeast is largely mediated by Hap4, a transcription factor which regulates the expression of many nuclear encoded mitochondrial proteins [150]. In otherwise wild-type cells, deletion of *HAP4* has little effect on the response to CR (**Figure S1C**). In contrast, deletion of *HAP4* fully suppresses the lifespan shortening effect of CR in *sod2Δ* cells (**Figure 5C**), strongly suggesting that increased oxidative metabolism is causal in the effect of CR on *sod2Δ* cells. Deletion of *SOD2* also results in sensitivity to the superoxide generating compound paraquat under control conditions and *sod2Δ* cells show a marked increase in sensitivity to paraquat compared to wild-type cells when subjected to CR (**Figure 5D**). Together these data demonstrate that mitochondrial antioxidant capacity is critically important for lifespan extension when respiration as defects in these processes cause CR to become



**Figure 6 – Rescue of Sod Mutants by Antioxidants.** (A) *Sod2* RLS on CR is rescued by addition of ascorbic acid while (B) *Sod1* cell RLS is rescued on CR and dramatically attenuated on 2% glucose media by addition of ascorbic acid

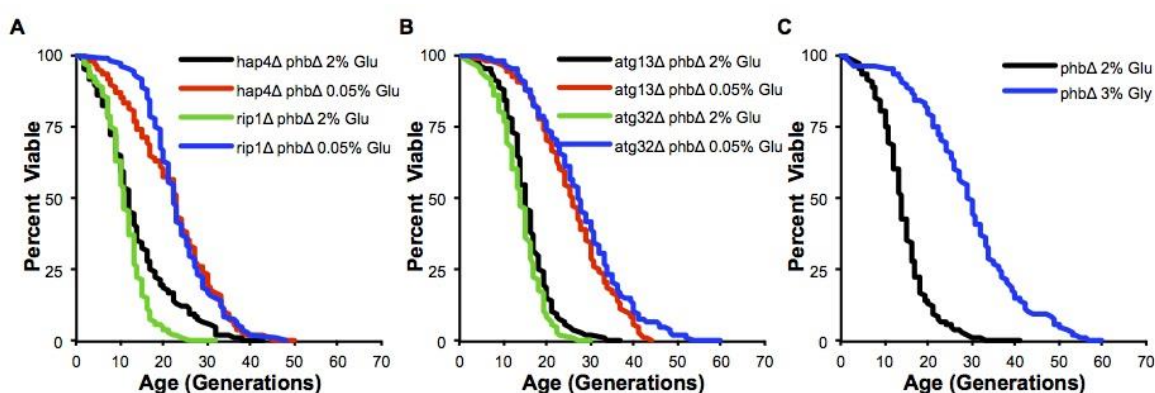
harmful (**Figure 5E**). Finally, we found that addition of the antioxidant ascorbic acid (AA) to the media suppresses the short RLS of both *sod2Δ* and *sod1Δ* cells on 2% glucose and CR media (**Figures 6A-B**), strongly supporting this model.

## MECHANISM OF LIFESPAN EXTENSION BY CR IN PROHIBITIN MUTANTS

We next chose to examine the mechanisms underlying RLS extension by CR in a strain showing a strong positive response. Among the strains showing the most positive response to CR were many genes coding for proteins involved in mitochondrial function. These include the mitochondrial voltage gated ion channel *Por1*, a homolog of mammalian VDAC [151], and both subunits of the mitochondrial prohibitin complex, *Phb1* and *Phb2* (**Figure 3A, inset, Figure 4B,C, and D**), which behaved identically in each assay performed in these studies (referred to as *phbΔ* hereafter). CR thus appears to be particularly

beneficial in the context of at least certain forms of mitochondrial dysfunction. We focused on understanding the mechanistic basis for robust lifespan extension in *phbΔ* cells.

The prohibitin complex is an inner mitochondrial membrane complex highly conserved from yeast to mammals. This complex participates in mitochondrial protein folding, electron transport chain assembly, and regulation of mitochondrial proteases [152]. Yeast lacking prohibitin have been previously reported to have short RLS and reduced mitochondrial membrane potential [153], phenotypes confirmed in this study (**Figures 3A, Figure S1D**). Because the prohibitin complex functions in the mitochondria, and in light of our observations in the *Sod2* deficient cells, we first tested if increased mitochondrial respiration was the cause of the RLS extension from CR in *phbΔ* cells. We generated double mutant cells lacking the prohibitin complex and the transcription factor Hap4, discussed above. Although these cells are deficient for growth on a non-fermentable carbon source, they show a robust lifespan extension from DR at 0.05% glucose and the resultant lifespan is indistinguishable from prohibitin single mutant cells (**Figure 7A**). A similar effect is seen when prohibitin deficiency is combined with deletion of the gene encoding the Rieske iron-sulfur protein, Rip1 (homolog of human UQCRFS1), which is the catalytic subunit of ETC complex III and thus required for mitochondrial electron transport chain function [154] (**Figure 7A**). Together these data demonstrate that the mechanism of RLS extension by CR in the *phbΔ* mutants is distinct from that underlying decreased RLS in *sod2Δ* cells.



**Figure 7 – The increase in *phbΔ* RLS by CR is not Dependent on Respiration or Autophagy.** (A) Blocking the induction of respiration through deletion of Hap4 or Rip1 does not prevent the increase in RLS of prohibitin deficient cells on CR. (2) Deletion of genes involved in autophagy also fails to attenuate the effects of CR in *phbΔ* cells. (C) CR on 3% Glycerol has a similar effect on the RLS of *phbΔ* cells as 0.05% Glucose.

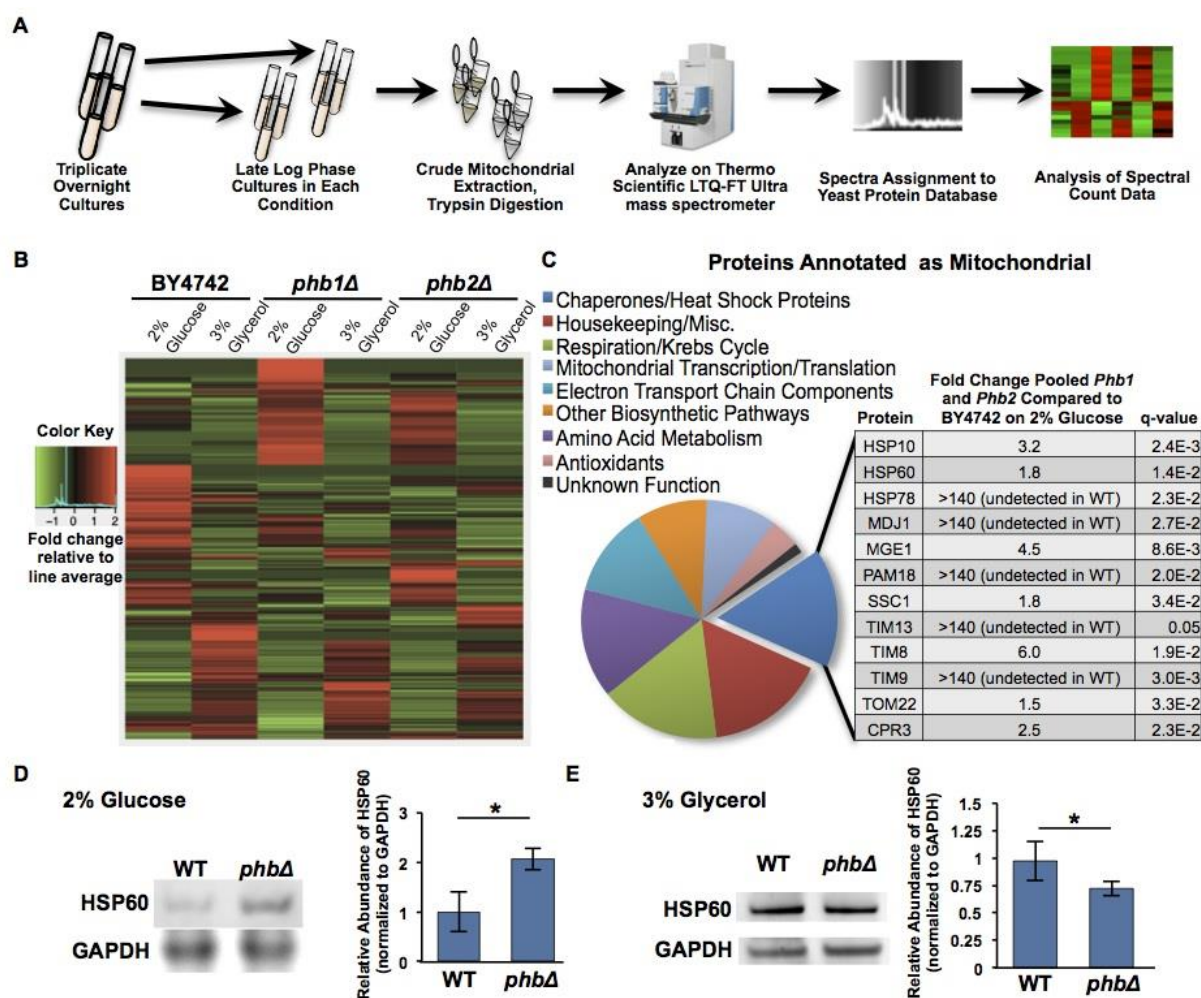
In addition to a shift toward mitochondrial respiration, CR is associated with induction of autophagy in yeast as well as mammals [155] (see also **Chapter 1**). We considered the possibility that induction of autophagy by CR might mediate the observed increase lifespan in prohibitin mutants. We found, however, that deletion of ATG13 or ATG12, genes important and essential for autophagy, respectively [156, 157], had no effect on the increase in RLS of *phbΔ* cells on CR (**Figures 7B**). Thus, we conclude that the robust RLS increase by CR does not result from increased autophagy in *phbΔ* cells.

To further characterize the mechanism of by which CR extends RLS in prohibitin deficient yeast we examined the effect of prohibitin deficiency on the proteome. Here, CR was accomplished by growing cells in 3% glycerol (CR-Gly), a non-fermentable carbon source that induces many of the same physiological effects as 0.05% glucose including robust RLS extension of *phbΔ* cells (**Figure 7C**). Given that the prohibitin is a mitochondrial protein we sought to ensure that we would be able to adequately examine the mitochondrial proteome and thus performed sub-cellular fractionation and used mitochondria enriched fractions for our proteomic analysis (**Figure 8A**). These conditions were necessary to prevent exhaustion of the carbon source during liquid culture and allow for growth of sufficient cell mass to harvest mitochondria for mass spectrometry, a complicating factor that makes liquid culture CR using 0.05% glucose technically unfeasible.

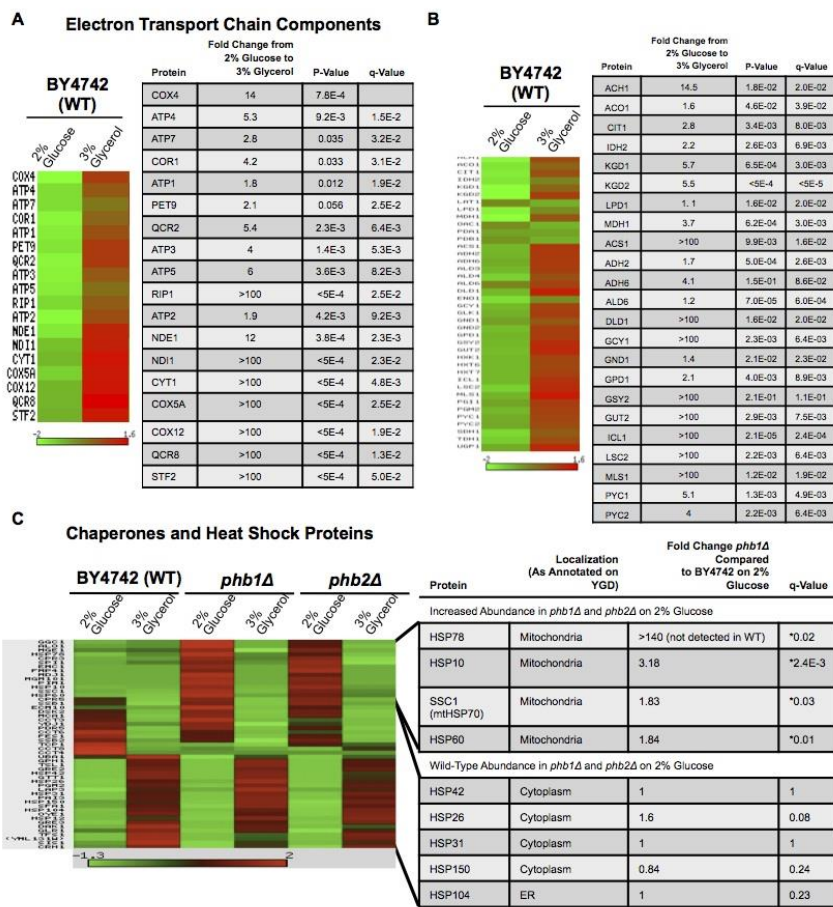
As expected, peptides corresponding to proteins reported to be up-regulated by the metabolic shift to respiration were higher in both wild-type cells and prohibitin mutants in CR-Gly samples (**Figure 8B, Figure 9A-B**). In addition, several peptides were differentially abundant in mitochondria from prohibitin mutants relative to wild-type cells under control conditions but not CR-Gly (**Figure 8B-C, Figure 9C**), demonstrating a rescue of phenotype at the level of the proteome. Prohibitin mutants in control conditions had elevated levels of mitochondrial heat shock proteins and chaperones associated with the mitochondrial unfolded protein response (mtUPR), a response that has been described in nematodes and mammals [158] but, to our knowledge at the time of this writing, has not previously been identified in yeast. The induction of chaperones appeared to be restricted to the mtUPR, as chaperones associated with cytoplasmic or endoplasmic reticulum (ER) proteotoxic stress were not induced (**Figure 9C**). In organisms with a recognized mtUPR the response involves an induction of nuclear-encoded

mitochondrial stress proteins including Hsp60 (Cpn60), Hsp10 (Cpn10), and mtHsp70 (Ssc1 in yeast, Hsp-6 in worms) [158, 159]. Each of these was significantly increased in mitochondrial fractions from *phbΔ* samples grown in control medium but not in cells grown in CR-Gly (Figure 8A, Figure 9C).

Elevation of Hsp60 in prohibitin mutants under control but not CR conditions was verified by western blot using whole-cell lysates (Figure 8D-E). These data demonstrate that a mitochondrial specific response, similar to the mtUPR observed in other species, occurs in prohibitin deficient yeast cells and that DR suppresses this phenotype.



**Figure 8 – Shotgun Proteomic Analysis of Mitochondria Enriched Samples Reveals an Induction of the Mitochondrial Unfolded Protein Response.** (A) Log-phase cultures of biological replicates were grown in 2% glucose or 3% glycerol and a crude mitochondrial extraction was performed to enrich for this organelle. Samples were proteolytically digested and analyzed by protein mass spectrometry. (B) Heat-maps of the proteomic data reveals a marked proteomic difference between BY4742 (WT) and *phbΔ* cells on grown in 2% glucose but no difference between WT and prohibitin mutant cells grown in 3% glycerol. (C) Analysis of the proteomic data revealed an induction of proteins associated with the mitochondrial unfolded protein response (mtUPR). (D) Western blot analysis of Hsp60 verified an induction of this marker of the mtUPR in *phbΔ* cells grown in 2% glucose but no difference between *phbΔ* cells and WT in 3% glycerol.



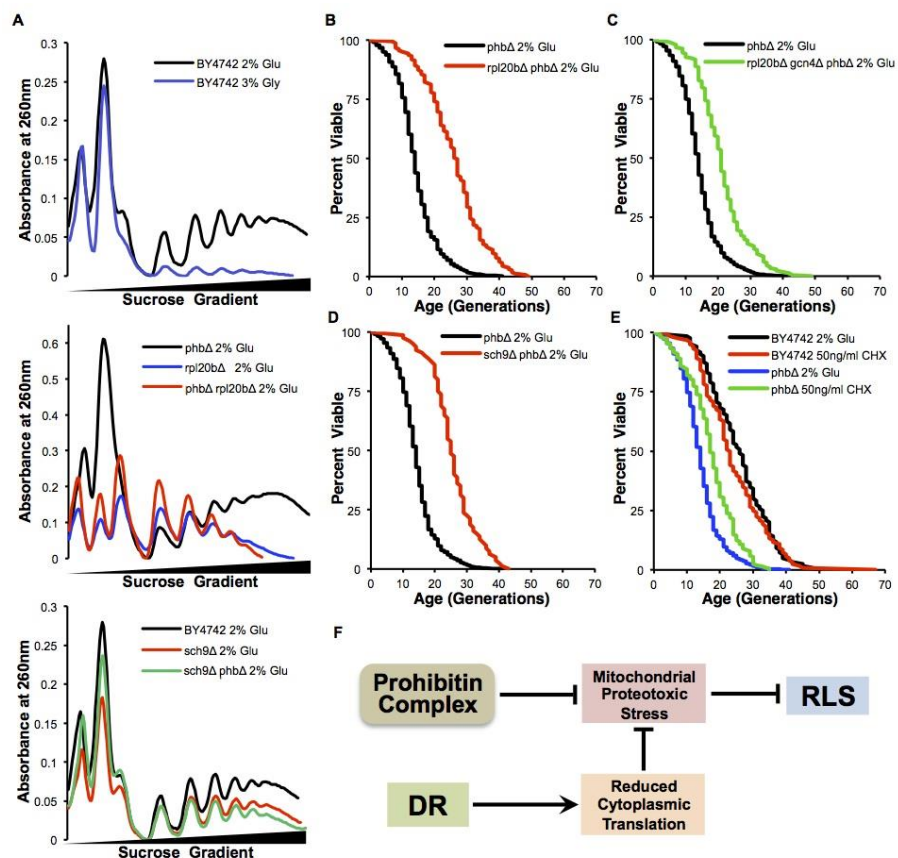
**Figure 9 – Proteomic Data Demonstrated an Induction of Respiratory Proteins in CR-Gly and the mtUPR in Prohibitin Mutants in 2% Glucose.** (A) Electron transport chain and (B) enzymes involved in respiration were induced in CR-Gly. (C) Mitochondrial unfolded protein response chaperones, but not ER or cytoplasmic unfolded protein response components, were induced in prohibitin deficient cells in 2% glucose and rescued to WT levels in CR-Gly.

CR has previously been associated with reduced cytoplasmic translation [160], a response we also observed in wild-type cells grown in glycerol (Figure 10A). We considered the possibility that CR might reduce mitochondrial proteotoxic stress and extend the RLS of prohibitin mutants through reduced translation of cytoplasmic proteins destined for import into the mitochondria. Deletion of the large ribosomal subunit *RPL20B* provides a robust genetic model for reduced cytoplasmic translation (Figure 10A). We found that

deletion of *RPL20B* in *phbΔ* cells increased lifespan to the same degree as CR (Figure 10B).

The RLS of wild-type cells can be extended by deletion of genes encoding components of the large ribosomal subunit but generally not by deletion of small ribosomal subunit genes [53]. This is thought to occur through activation of the transcription factor Gcn4, which appears to be activated by large but not small subunit disruption. RLS extension by deletion of *RPL20B* in otherwise wild-type cells requires Gcn4 [53]. In contrast, loss of *GCN4* did not prevent RLS extension by *RPL20B* deletion in *phbΔ* cells (Figure 10C). Together these results suggest that reduced global mRNA translation associated with *RPL20B* deletion, rather than activation of *GCN4*, is responsible for RLS extension in *phbΔ* cells.

To further test this model we examined the effect of deletion of the gene encoding the ribosomal S6 kinase homolog, Sch9, on RLS in *phbΔ* cells. Sch9 promotes mRNA translation in response to nutrient availability [161]. Deletion of *SCH9* is sufficient to increase RLS and dramatically reduce translation under control conditions (**Figure 10A**), and lifespan extension by DR is partially mediated by reduced Sch9 activity [82]. Similar to CR, deletion of *SCH9* robustly reduced levels of translation and increased RLS in *phbΔ* cells (**Figure 10A, 10D**).



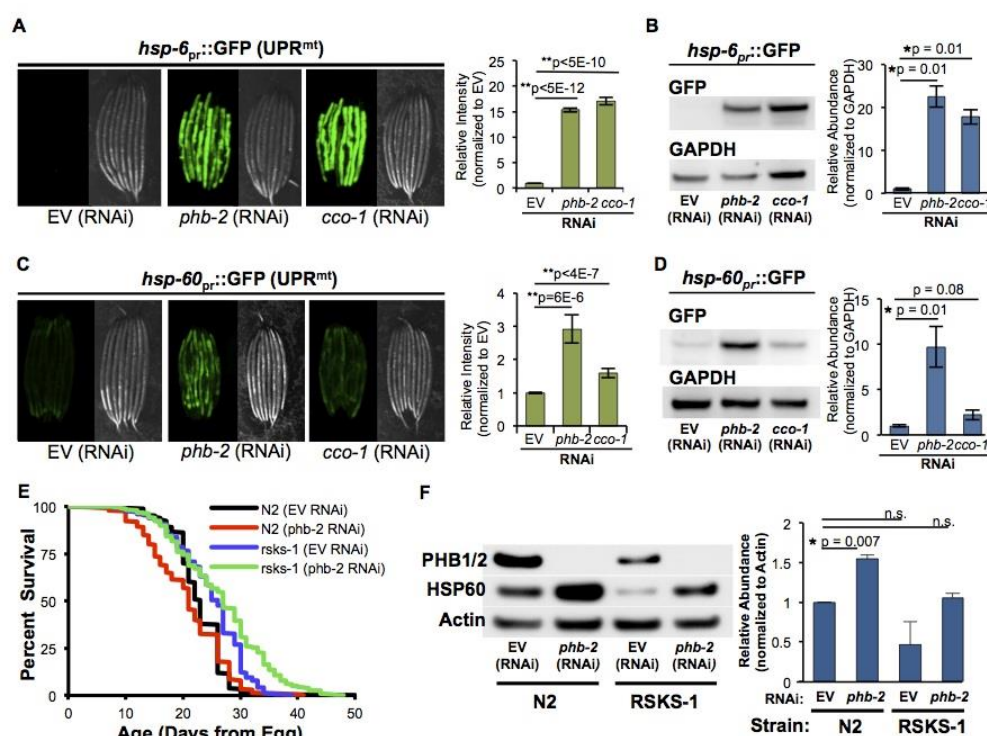
**Figure 10 – Interventions that Rescue RLS in the Prohibitin Mutants is Associated with Decreased Cytoplasmic Translation.** (A) Loading of polysomes is greatly reduced in CR-Gly. Deletion of *RPL20B* or *SCH9* decreases cytoplasmic translation in the prohibitin mutants and (B-D) confers an RLS extension similar to CR. (C) Deletion of *GCN4* does not prevent the RLS extension resulting from deletion of *RPL20B* in the prohibitin mutants. (E) Cycloheximide (CHX), an inhibitor of cytoplasmic translation, also extends lifespan in *phbΔ* cells. (F) This data supports a model where loss of prohibitin results in a mitochondrial proteotoxic stress which is repressed by decreased cytoplasmic translation.

Finally, we examined the effect of the translation inhibitor cycloheximide on RLS. Cycloheximide binds to the

E site of eukaryotic ribosomes, inhibiting mRNA translation through a mechanism distinct from loss of ribosomal subunits and having no effect on mitochondrial translation [162]. 50 ng/mL cycloheximide had little effect on the RLS of wild-type cells, as previously reported [53], but significantly (although to a modest degree compared to genetic manipulation of translation) increased the RLS of prohibitin-deficient cells (**Figure 10E**). Together this data demonstrate that a reduction in cytoplasmic mRNA translation is sufficient to increase the RLS of cells deficient in mitochondrial prohibitins and that, although regulation of

mRNA translation is important for RLS extension in both wild-type and prohibitin deficient cells, the mechanisms are at least partially distinct (**Figure 10F**).

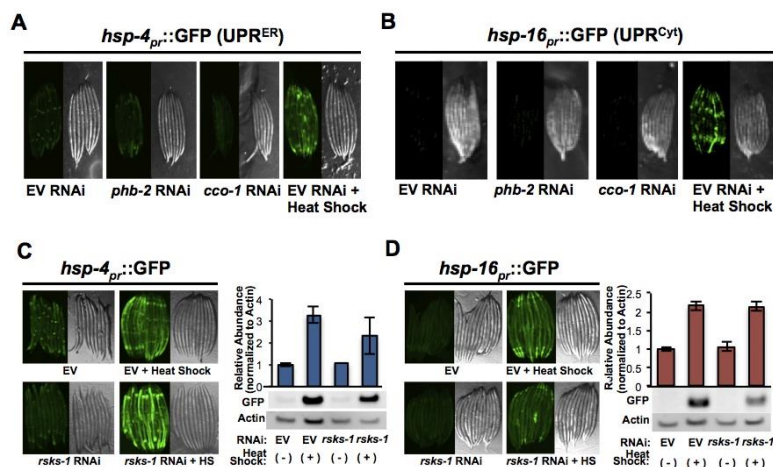
RNAi knockdown of the worm ortholog of PHB2 shows a synergistic lifespan extension when combined with a genetic model of CR in *Caenorhabditis elegans* [163], suggesting that the effect of prohibitin deficiency on the response to CR is conserved. We therefore asked whether the mechanistic basis for this interaction is conserved. We performed knock-down of *phb-2* by RNAi in the nematode *C. elegans* and observed a robust induction of both commonly used mtUPR reporter strains which express GFP under the control of the *hsp-6* and *hsp-60* promoters [164] (**Figure 11 A-D**). No induction was observed using *hsp-4* or *hsp-16* promoter driven GFP, reporters of the cytoplasmic and ER UPR's respectively (**Figure 12 A-B**). The basal level and induction of *Hsp60* was attenuated by deletion of *rsks-1* (**Figure 11F**), which encodes the nematode ortholog of Sch9 and, as in yeast [53], reduces mRNA translation in *C. elegans* [165]. *Rsks-1* RNAi did not attenuate induction of the cytoplasmic or ER UPR's by heat shock, indicating that the effect on *hsp-60* induction was unlikely to be a direct result of the general reduction in translation caused by knockdown of *rsks-1* loss (**Figure 12 C-D**). Finally, prohibitin knockdown shortens the lifespan of wild-type (N2) animals but extends lifespan in animals lacking *rsks-1*,



**Figure 11 – Conservation of the Effects of Prohibitin Deficiency in *C. elegans*.** (A-B) *phb-2* RNAi robustly induces the *hsp-6pr::GFP* reporter in *C. elegans*. (C-D) *phb-2* RNAi also induces the Hsp60 promoter driven GFP reporter, in this case even more robustly than the well-reported *cco-1* RNAi positive control. (E) *Rsks-1* mutants are slightly long-lived and further extended by *phb-2* RNAi, whereas WT animals lifespan is slightly shortened by *phb-2* RNAi. (F) Basal levels and induction of Hsp60 are reduced in the *rsks-1* mutants.

which are already long-lived (**Figure 11E**). Thus the effect of prohibitin deficiency on lifespan and mitochondrial stress, the response to CR, and the underlying mechanisms for that response appear to be shared between yeast and *C. elegans*.

**Figure 12 – Phb-2 RNAi does not Induce the ER or Cytoplasmic UPR Responses and Rsk-1 RNAi Fails to Attenuate Induction of these Responses by Heat Shock.** (A) *phb-2* RNAi does not induce the ER UPR reporter *hsp-4<sub>pr</sub>::GFP* or the cytoplasmic UPR reporter *hsp-16<sub>pr</sub>::GFP* (B). These data were produced in the same experiment as Figure 11. (C-D) *Rsk-1* RNAi does not attenuate induction of the cytoplasmic or ER UPR responses when induced by heat shock and does not significantly affect levels of these reporters in control animals.



## DISCUSSION

CR attenuates aging in a number of organisms but it is clear that the effects of CR are strongly influenced by genetic background. While the influence of genetic background on the response to CR is clear the relationship between genotype and CR response has not yet been directly addressed. Presented here are the effects of a single method of CR on the lifespans of 166 yeast strains, each lacking a single non-essential gene, data that represent the first unbiased probe into the genetic regulation of CR effects. The data presented here are consistent with a study in mice examining effects of 40% DR on survival in 41 recombinant inbred mouse strains [141] and, although that study examined a small number of animals for each genotype (5 per condition), the distribution of effects from CR is strikingly similar to our data from the 166 yeast deletion strains.

While our study examined only single gene deletions, which undoubtedly underrepresents genetic variation in natural populations, this approach has the distinct advantage that the primary genetic factor is known for each strain examined. The data presented clearly indicates that genetic context plays a fundamental role in determining the effect of CR on lifespan. Furthermore, we have begun to elucidate the molecular processes that influence this response. Using GO analysis we have defined specific molecular processes that underlie both positive and negative responses to CR. Each of the molecular

processes identified here is highly conserved from yeast to humans, suggesting that similar mechanisms could underlie genotype-specific responses to CR in higher eukaryotes, even in cases where the individual components of a molecular pathway are poorly conserved. In the case of the prohibitins, their shared effects on mitochondrial protein homeostasis and longevity in *C. elegans* further support this notion, and murine studies of superoxide dismutase KO animals suggest that the CR response observed here is conserved in mammals [166] (see below). Due to the extraordinary amount of work needed to perform a study of this nature this report includes only a limited number of single deletion strains. Further study of additional strains will likely reveal additional cellular processes that modify the response to CR and provide novel insight into the mechanisms underlying this intervention.

#### *Conserved features of the mitochondrial unfolded protein response in yeast*

Evidence for a conserved mtUPR had not previously been extended to yeast [167] despite the high conservation of the canonical mitochondrial stress response proteins Hsp60, Ssc1 (mtHSP70), Hsp10, and Mdj1 (mtDnaJ) [168]. In *C. elegans*, studies of the mtUPR have relied almost exclusively on the *hsp-6<sub>pr</sub>::GFP* and *hsp-60<sub>pr</sub>::GFP* reporter strains used in this study. Induction of these reporters in response to mitochondrial proteotoxic stress requires several factors, including ubiquitin-like protein Ubl-5, the matrix peptide exporter Haf-1, and the Dve-1 transcription factor [158]. Interestingly, we were unable to detect a change in mRNA levels of *HSP60* in prohibitin-deficient cells, suggesting that in yeast the regulation of the mtUPR may involve post-transcriptional regulation of protein levels (data not shown). Although the factors that mediate this response appear to be different in yeast, the degree to which the signaling and regulatory components of the mtUPR are conserved will be important to assess.

The observation that prohibitin deficiency induces a mitochondrial proteotoxic stress response and shortens lifespan in both yeast and *C. elegans* is of particular interest. The importance of nuclear-mitochondrial signaling in aging was first demonstrated by the retrograde response in yeast [169]. More recently, the mtUPR has been shown to be required for lifespan extension following electron transport chain (ETC) RNAi in *C. elegans* [170]. This raises the question of why induction of the mtUPR is

associated with enhanced longevity in some cases, such as knockdown of cytochrome c oxidase 1 (*cco-1*) by RNAi [170], and reduced longevity in other cases, such knockdown of *phb-2* ([163] and **Figure 11E**). One possibility is that the nature of the mitochondrial proteotoxic stress may differ between prohibitin-deficient cells and cells deficient in components of the ETC. An alternative explanation is that situations where induction of the mtUPR is associated with extended lifespan are examples of hormesis. In these scenarios, induction of the mtUPR in the absence of a lifespan-shortening defect may result in an increase in chaperones that promote or facilitate longevity. Prohibitin loss may represent a case where the window of hormesis has been exceeded and the underlying defect reduces longevity. This explanation is consistent with the fact that prohibitin deletion is developmentally lethal in *C. elegans* and mice [171] and that conditional post-developmental brain-specific knockout of prohibitin in mice results in neurodegeneration [172]. This type of dosage-dependent hormetic relationship has been described for the ER-UPR and heat shock, where mild stress is capable of extending lifespan in a setting where more intense stress is lifespan limiting.

Our data suggest that induction of the mtUPR in yeast and nematodes deficient for prohibitins indicates a failure to maintain protein homeostasis in the MIM coupled with a robust induction of the mtUPR. Prohibitins are localized to the MIM where they have been implicated in a variety of processes. Notably, prohibitins negatively regulate the mitochondrial AAA (m-AAA) protease comprised of Afg3 and Yta12 in the MIM [173]. The m-AAA proteases are producers of mitochondrial peptides in yeast and worms that act to mediate mitochondria to nucleus signaling. The peptides produced by these proteases are exported from the mitochondria by Mdl1 and Mdl2 in yeast [174], while peptides produced in mitochondria in worms are exported by Haf-1, an exporter reported to be necessary for induction of the mtUPR [175]. Intriguingly, it has recently been demonstrated that induction of the mtUPR in *C. elegans* can be accompanied by a reduction in cytoplasmic translation through a parallel pathway involving GCN-2 [176]. This implies that reduced cytoplasmic translation is a homeostatic process that evolved to promote proteostasis in the face of a mitochondrial proteotoxic stress. Interestingly, deletion of *AFG3* extends RLS and decreases cytoplasmic mRNA translation in wild-type yeast [177], while prohibitin deletion does not reduce translation (this study) and is synthetically lethal with both *AFG3* and *YTA12* ([178] and in our study, data not shown). Together, this suggests that a functional complex of the m-AAA

proteases may be necessary for induction of the mtUPR in yeast, that disruption of this complex prevents the response to mitochondrial proteotoxic stress and leads to inviability in *phbΔ* cells, and that the translation reduction mediated by loss of *AFG3* may be mechanistically related to the reduced translation resulting from mitochondrial proteotoxic stress in worms. The availability of a yeast model in which to study the mtUPR should facilitate addressing these important questions.

#### *Genotype determines the mechanisms by which DR modulates longevity*

Another important observation from this study is that, in addition to the magnitude and direction of effect, the mechanisms by which CR influences longevity is critically depending on genetic context. In *sod2Δ* cells, for example, induction of mitochondrial respiration dramatically shortens lifespan while in wild-type [179] or prohibitin mutant cells (this study) induction of respiration has little or no effect on RLS extension by CR. The effect of respiratory deficiency on RLS under both control and CR conditions varies among different laboratory yeast strains [150, 169]. It seems likely that natural diversity in antioxidant systems may contribute these varied responses.

Our observations in *sod2Δ* cells are similar to previous reports examining the effects of CR in a transgenic mouse model of amyotrophic lateral sclerosis (ALS). G93A animals express a mutant form of human *SOD1*, have increased free radical levels, and display progressive limb weakness and paralysis due to loss of spinal motor neurons similar to human ALS patients. When subjected to a 40% reduction in caloric intake, G93A animals experienced increased oxidative stress and accelerated disease onset and progression [166, 180]. In our screen, *sod1Δ* cells subjected to CR also had a reduced RLS (**Figure 6B**), although the extremely short RLS of these cells on both normal and CR media prohibited epistasis experiments. We did, however, observe a robust increase in the RLS of both *sod1Δ* and *sod2Δ* cells when ascorbic acid or NAC were added to either standard or CR media (**Figure 6A-B**). Together, the findings in yeast and mice support a model where, under conditions of reduced antioxidant capacity, CR is detrimental to both life span and health span by increasing oxidative damage. Of note, mice lacking *SOD1* completely are also short lived but die from neoplasia, do not suffer from neurodegenerative

symptoms, and CR has been reported to rescue the lifespan of these animals [181]. The differences between these models, and their response to CR, are striking and warrant further attention.

Recently, Hughes *et al* [182] reported a reduction in RLS in vacuolar protein deficient (*vmaΔ*) cells subjected to CR. This report provides an independent confirmation of the data reported herein for some strains in our screen. In contrast to our findings in *sod2Δ* cells, the CR-mediated decrease in RLS of *vmaΔ* cells was reported to result from altered amino acid storage within the vacuole rather than increased respiration. Therefore, although both *sod2Δ* and *vmaΔ* cells both experience reductions in RLS in response to CR, it appears that two distinct mechanisms are responsible.

Another example of mechanistic differences in the longevity effect from CR is evident from the fact that a general decrease in mRNA translation is sufficient to extend the RLS of prohibitin mutants but not wild-type cells. Gcn4 is required for RLS extension from mutants with reduced mRNA translation in wild-type yeast [53], but not in prohibitin mutants. We propose that mitochondrial proteotoxic stress limits the RLS of prohibitin mutants and that a general inhibition of cytoplasmic mRNA translation alleviates this defect. This model is consistent with a prior study reporting that inhibition of cytoplasmic mRNA translation can suppress the short RLS and mitochondrial degeneration of cells expressing a dominant-negative allele of the adenine nucleotide translocase Aac2 [183]. Thus, although CR extends lifespan in both WT and prohibitin mutants, the relative mechanistic underpinnings for lifespan extension are unique.

In addition to providing insight into the role of genetic variation in the response to CR, the data and approach presented here may also suggest novel therapeutic strategies for a subset of genetic diseases. For example, the robust positive effect of CR in several strains lacking a nuclear encoded mitochondrial protein (15 of the top 20 responders) may indicate that CR, or CR mimetics such as the mTOR inhibitor rapamycin, may be beneficial in mitochondrial disorders. Such a scenario could potentially result in a paradigm shift in the way mitochondrial disease is viewed and treated as no efficacious interventions are currently available. To address this possibility we pursued this idea in a murine model of a human mitochondrial disease, studies which are detailed in **Chapter 3**. In the case of the prohibitin mutants in yeast and worms, the mechanistic basis for the positive effects of CR appear to involve attenuation of a proteotoxic stress in the mitochondria mediated by a global reduction in mRNA

translation. It will be important to determine whether a similar mechanism is at play in the other mitochondrial mutants showing a robust response to CR, as well as whether the mtUPR is induced in diseases of mitochondrial dysfunction, or whether additional mechanisms are at work.

In addition to providing the first unbiased and large-scale examination of the molecular mechanisms underlying genotype specific responses to CR the work presented here has significant implications into how CR, or CR mimetics, should be viewed from a clinical and intervention standpoint. Given the strong genotype dependency on the response to CR it is likely that human genetic variance will play a major role in the efficacy and safety of CR on an individual-to-individual basis. While the bulk of the human population would likely be free of significant genetic modifiers of CR there is evidence here that certain genetic deficiencies may lead to dramatically negative outcomes when combined with caloric restriction. A number of disease-causing genetic variants of Sod2 have been described in humans, for example, with clinical manifestations including deafness, coronary artery disease, adrenoleukodystrophy, and cancer [166, 184-186]. While the complete deletion of Sod2 studied here is undoubtedly quite different from the described gene variants in humans (both in terms of the genetic manipulation and the unique phenotypic outcomes in humans and yeast) it would nevertheless be prudent to show caution in suggesting CR or CR mimetics to individuals with genetic abnormalities. With that consideration, this data (and that shown in **Chapter 3**) also strongly suggests that certain human diseases may greatly benefit from CR or CR mimetics. Perhaps the take-home message from this study is that CR is a powerful intervention with numerous genetic modifiers and while it may be greatly beneficial when employed in the proper setting, which includes WT yeast and is very likely to include the bulk of the general human population, it should be treated as any intervention and all potential contraindications, both known and unknown, should be considered.

## **METHODS**

### **Strains and Media**

Yeast strains were derived from the BY4742 (*MAT $\alpha$*  *MET15*, *his3D1 $\Delta$* , *leu2D $\Delta$* , *lys2D $\Delta$* , *ura3D $\Delta$* ) and BY4741 (*MATa* *LYS2*, *his3D1 $\Delta$* , *leu2D $\Delta$* , *lys2D $\Delta$* , *ura3D $\Delta$* ) backgrounds. The *MATa* and *MAT $\alpha$*  ORF

deletion collections and parental strains were obtained from Open Biosystems. Additional strains were generated by PCR-based gene disruption as previously described [53].

Cells were grown on standard YEP agar containing 1% yeast extract and 2% peptone with 2% glucose (YPD) unless otherwise noted. Unless stated otherwise, DR was accomplished by reducing the glucose content of the YEP medium to 0.05%, as described [187]. Alternative forms of DR used in this study included 0.5% glucose, 0.1% glucose, 0.005% glucose, and 3% glycerol in YEP. Cycloheximide (Sigma-Aldrich, St. Louis MO) was dissolved in 100% ethanol at 100mg/ml and cycloheximide, ascorbate, and NAC were added to YEP-agar as previously described [53].

All *C. elegans* strains used in this study were obtained from the Caenorhabditis Genetic Center (CGC). Strains used were SJ4100 (*zcls13[hsp-6pr::GFP]*), SJ4058 (*zcls9[hsp-60pr::GFP]*), CL2070 (*dvls[hsp-16.2pr::GFP]*), RB1206 (*rsk-1(ok1255)* III) and N2 wild-type.

### **Replicative Lifespan (RLS) Assay**

RLS experiments were performed as described [188]. Frozen stocks were streaked onto YPD plates two days prior to the start of the experiments. All strains were grown on experimental media prior to start of RLS experiments. Virgin daughter cells obtained from an overnight patch. Cells were incubated at 30°C and examined for the formation of new daughter cells every 2-4 hours. Statistical significance for individual experiments was determined using a Wilcoxon Rank-Sum test and defined as  $p < 0.05$ . q-value multiple testing correction was utilized to determine significance across the 166 single gene mutant set using the open source software R (R Development Core Team, 2008) with the plug-in QVALUE [189]. All experiments were blinded so that individuals performing microdissection had no knowledge of the genotype or media composition.

### **Polysome Profiles**

Polysome profiles were performed as previously described [53]. Briefly, log phase cultures were arrested by quick chilling and addition of 100µg/ml cycloheximide and pelleted. Pellets were washed once in 10ml lysis buffer (25mM Tris-HCl pH 7.5, 40mM KCl, 7.5mM MgCl<sub>2</sub>, 1mM DTT, 0.5mg/ml heparin, 100µg/ml cycloheximide), resuspended in 1ml lysis buffer, and lysed by shearing using glass beads.

Triton X-100 and sodium deoxycholate were each added to a final concentration of 1%. Samples were incubated 5 min on ice and centrifuged to separate cell debris. Protein content was determined by absorbance at 260nm on a Nanodrop spectrometer (Thermo Scientific). 20 OD units were resuspended in 1ml Lysis Buffer containing 1% Triton X-100 and 1% sodium deoxycholate. Samples were loaded onto 11ml linear 7%-47% sucrose gradients (50mM Tris-HCl pH 7.5, 0.8M KCl, 15mM MgCl<sub>2</sub>, 0.5mg/ml heparin, and 100µg/ml cycloheximide) and centrifuged 2 hours at 39,000rpm at 4°C. Gradients were collected from the top and analyzed at 254nm.

### **Isolation of Mitochondrial Proteins and Mass Spectrometry**

Mitochondrial proteins were isolated from log phase cultures as described previously [190]. Briefly, cell pellets were washed once in DI water and resuspended in DTT buffer (100mM Tris-H<sub>2</sub>SO<sub>4</sub> pH9.4, 10mM dithiothreitol). Samples were incubated 15 minutes at 30°C, resuspended in Lyticase Buffer (1.2M sorbitol, 20mM potassium phosphate pH 7.4, 1U/ml lyticase), and incubated for 10 minutes at 30°C. Following incubation, samples were resuspended in homogenization buffer (0.6M sorbitol, Tris-HCl pH 7.4, 1mM EDTA), homogenized using a dounce homogenizer, and centrifuged 10 min at 600g. The supernatant was removed and spun for 10min at 6500g.

Samples were normalized to 20µg/100µL and mixed at a 1:1 with 0.1% RapiGest/50mM Ammonium Bicarbonate pH 7.8/5mM DTT and incubated at 50°C for 30 min. IAA was added to 15mM and samples were incubated, covered from light, at RT for 30 min. Samples were digested by adding trypsin to a final concentration of 1:50 enzyme:protein and incubated for one hour at 37°C with shaking. HCl was added to a final concentration of 200mM, samples were incubated at 37°C for 45 min with shaking, then spun at 14,000rpm for 10min. The supernatant containing peptides was moved to a new tube and stored at -20°C until analysis. LC-MS method and proteomic data analysis was performed as described in Supplemental Methods.

### **C. elegans UPR Reporter Induction**

Synchronized eggs were obtained by treating adult animals with hypochlorite. Eggs were grown on EV, *phb-2*, or *cco-1* RNAi at 20° C on Nematode Growth Media (NGM) containing AMP and IPTG [43]. At day

3, animals were assayed for GFP expression by fluorescent microscopy using a Zeiss SteREO Lumar.V12 (Thornwood, NY, USA) microscope. Image analysis was performed using ImageJ.

### **Western Blotting**

Yeast protein was extracted by freeze-fracture and homogenization in RIPA buffer (50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% deoxycholate, 0.1% SDS, and 1X protease inhibitor cocktail (Cat. #05892791001; Roche)). Yeast Hsp60 was detected by immunoblot using anti-Hsp60 (SPA-807, Stressgen) and GAPDH using anti-GAPDH (ab9485) both at a 1:1,000 dilution in 1% milk TBST. GFP reporter worms were homogenized in RIPA buffer using dounce homogenizers. Proteins were detected by immunoblot using anti-GFP (ab69312, Abcam, or sc-9996, Santa Cruz Biotechnology) at a 1:2,500 dilution in 1% milk TBST and anti-GAPDH antibodies (ab9485; Abcam).

### ***C. elegans* Lifespan Experiments**

*C. elegans* lifespans were performed as previously described [43]. Nematodes were maintained at 20°C on NGM. Synchronized eggs obtained by treating adult animals with hypochlorite were grown on NGM plates containing 1 mM IPTG, 25 µg/ml carbenicillin, seeded with HT115(DE3) bacteria transformed with either the pL4440 vector or the *phb-2* RNAi construct. At larval stage 4 worms were transferred to plates with 50 µM FUDR to prevent hatching of progeny. Lifespans were performed at 20°C. Cohorts were examined every 1-3 days using tactile stimulation to verify viability of animals. Ruptured animals were not censored from experiments. Animals lost due to foraging were not included in the analysis.

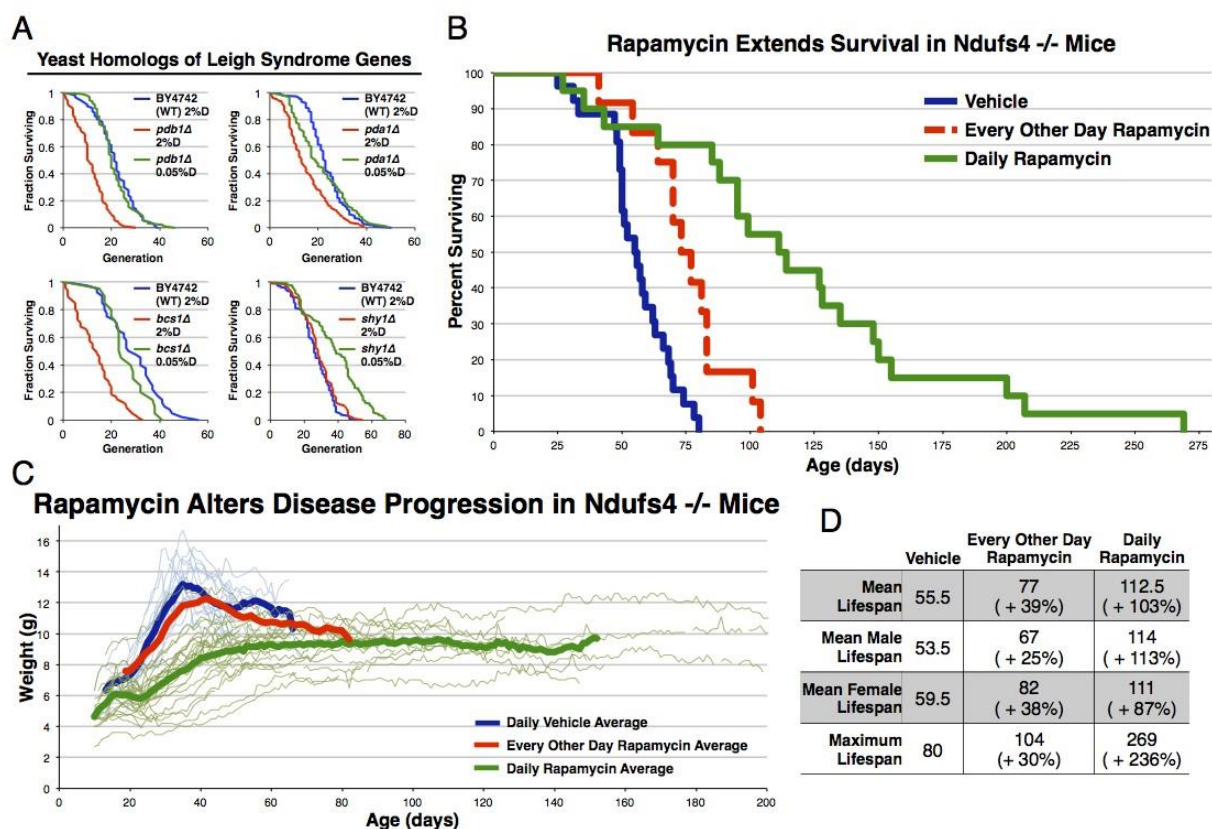
## CHAPTER 3

# MTOR INHIBITION ATTENUATES MITOCHONDRIAL DISEASE THROUGH METABOLIC ADAPTATION

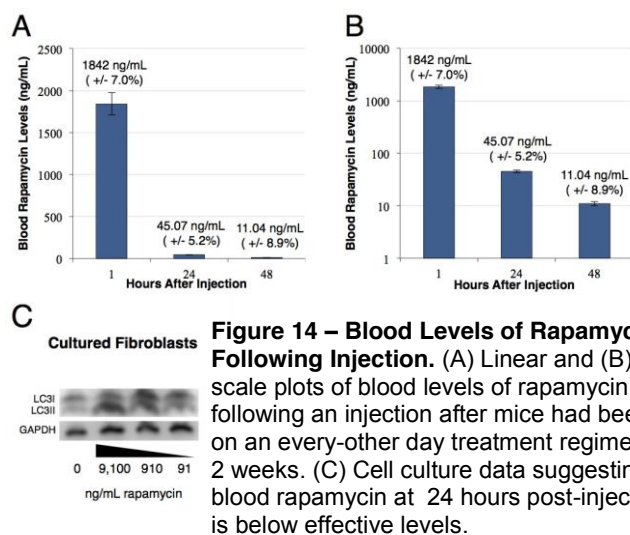
### INTRODUCTION

Leigh Syndrome is a clinically defined disease that results from several distinct genetic defects that disrupt mitochondrial function. It is the most common mitochondrial disorder in children, appearing in 1:40,000 newborns in the US[191]. The disease is characterized by retarded growth, low body weight, lactic acidosis, myopathy, dyspnea, and progressive encephalopathy localized primarily to the vestibular nuclei, cerebellum, and olfactory bulb[192, 193]. Affected patients typically succumb to respiratory failure resulting from the neuropathy, with average age of death at 6-7 years[191]. Thiamine and high-fat diet are also commonly prescribed, though no convincing clinical evidence exists to suggest that these interventions influence disease progression or severity. As no effective treatment exists, intervention is limited to palliative care and bicarbonate infusions for management of lactic acidosis[194].

In a recently published study, we reported that reduced nutrient signaling is sufficient to suppress short replicative lifespans (RLS) in several budding yeast mutants defective for mitochondrial function[45]. Reduced nutrient sensing was accomplished through mutations in the mechanistic target of rapamycin (mTOR) pathway or by glucose restriction, which inhibits mTOR in yeast[195]. This led us to ask whether reductions in nutrient sensing through mTOR inhibition might alleviate mitochondrial disease. To test this, we first examined four yeast mutants corresponding to human mitochondrial disease genes: PDHA (*pda1Δ*), PDHB (*pdb1Δ*), BCSL1 (*bcs1Δ*), and SURF1 (*shy1Δ*). These genes encode the E1 alpha and beta subunits of pyruvate dehydrogenase, a mitochondrial chaperone involved in assembly of electron transport chain (ETC) complex III, and a complex IV assembly factor, respectively. Mutations in these genes are associated with several human mitochondrial disorders including PDH deficiency, lactic acidemia, Bjornstad Syndrome, GRACILE Syndrome, and Leigh Syndrome[196-198]. In every case glucose restriction robustly increased survival of the mitochondrial mutant yeast cells, suppressing the RLS defect in the three short-lived mutants and further extending the fourth (**Figure 13A**).

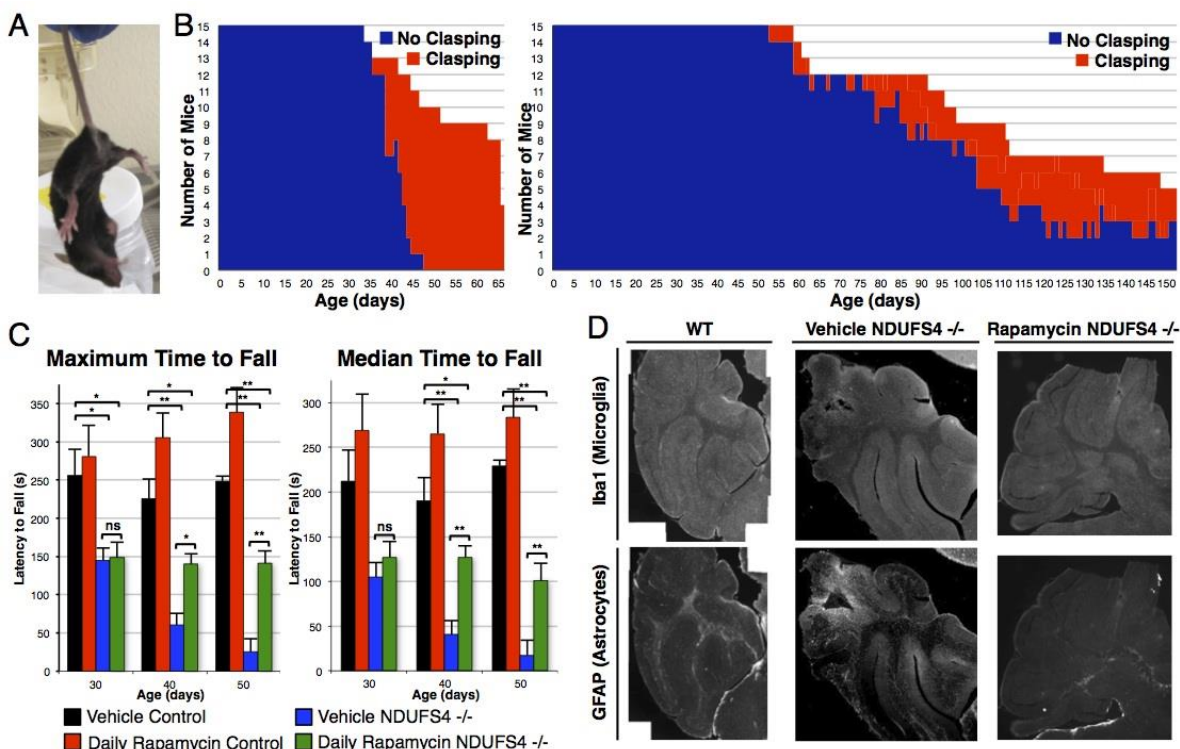


**Figure 13 – Effects of CR on Leigh Syndrome Homolog Mutant Yeast and Rapamycin on the *Ndufs4* <sup>-/-</sup> Murine Model of Leigh Syndrome.** (A) RLS of single-gene mutants of Leigh Syndrome homologs in yeast were significantly extended (log-rank  $p < 0.005$  in each case) by CR. (B) Survival of *Ndufs4* <sup>-/-</sup> mice is extended by every-other-day rapamycin injection and dramatically extended by daily treatment with rapamycin (log-rank  $p = 0.0002$  and  $p < 0.0001$ , respectively). (C) Weight plots of *Ndufs4* <sup>-/-</sup> mice treated given daily vehicle, every-other-day rapamycin, or daily rapamycin injections. (D) Mean and maximum lifespan data for *Ndufs4* <sup>-/-</sup> mice, separated by gender.



Based on these results, we decided to investigate the effects of rapamycin, a specific inhibitor of mTOR, in a mammalian model of Leigh Syndrome, the *Ndufs4* knockout mouse[199]. *Ndufs4*, the murine homolog of human *NDUFS4*, encodes a subunit of complex I of the electron transport chain (ETC) and is involved in assembly and stability of the complex[200]. *Ndufs4* <sup>-/-</sup> mice show a profound

neurodegenerative phenotype along with retarded growth, lethargy, ataxia, weight loss, and ultimately death at a median age of 50 days[201]. Neuronal deterioration and gliosis is most pronounced in the

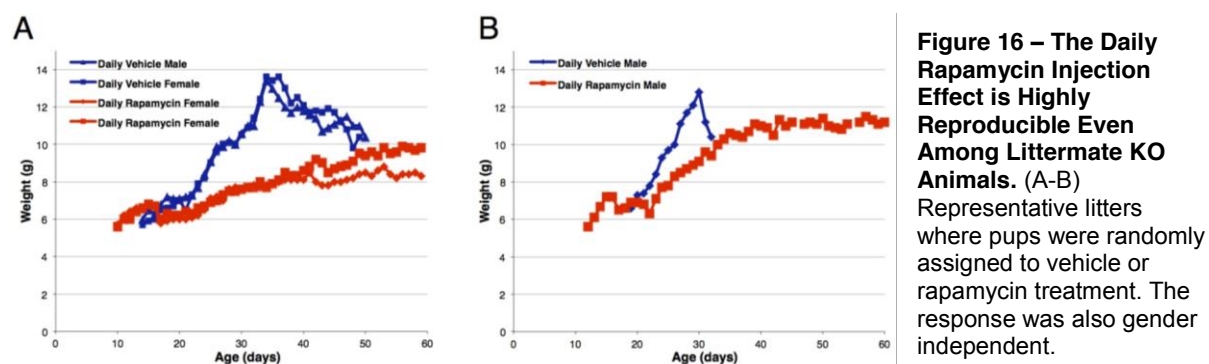


**Figure 15 – Rapamycin Attenuates Disease in *Ndufs4*<sup>-/-</sup> mice.** (A) Claspings behavior, a widely used sign of neurological degeneration. (B) Claspings is apparent in *Ndufs4*<sup>-/-</sup> mice as disease progresses and is dramatically attenuated by daily rapamycin treatment. A total of 15 mice were observed for claspings daily in each treatment group. Animals are not included in the total following death. These plots are significantly different ( $p < 0.001$  by log-rank test). (C) Rotarod performance steadily declines as disease progresses in the *Ndufs4*<sup>-/-</sup> mice, while daily rapamycin prevents this decline. (D) Staining for lesions in 55- to 60- day old animals. All vehicle treated KO animals show lesions at this age while we were unable to detect lesions in daily rapamycin treated age-matched mice.

olfactory bulb, cerebellum, and vestibular nuclei. We first examined the effects of delivering 8 mg/kg of rapamycin every other day by intraperitoneal (IP) injection beginning at weaning (approximately postnatal day 20, P20). This treatment regimen is sufficient to reduce mTOR signaling in several tissues in wild-type (WT) animals[202] and provided significant increases in median survival of both male (25%) and female (38%) *Ndufs4*<sup>-/-</sup> mice (**Figure 13B, D**). A slight reduction in growth and maximum body size and a delay in age of disease onset were also observed (**Figure 13 C**). While these results demonstrated that mTOR inhibition benefits *Ndufs4*<sup>-/-</sup> animals we noted that levels of rapamycin in the blood potentially fell below physiologically relevant levels by about 24 hours after injection (**Figure 14**). We therefore performed a follow-up study delivering 8 mg/kg rapamycin by daily IP injection starting at P10. In this cohort we observed a striking extension of median and maximum lifespan, with the longest-lived mouse surviving to 269 days, a greater than three-fold increase in survival. Median survival of male and female mice was 114 and 111 days, respectively (**Figure 13D**).

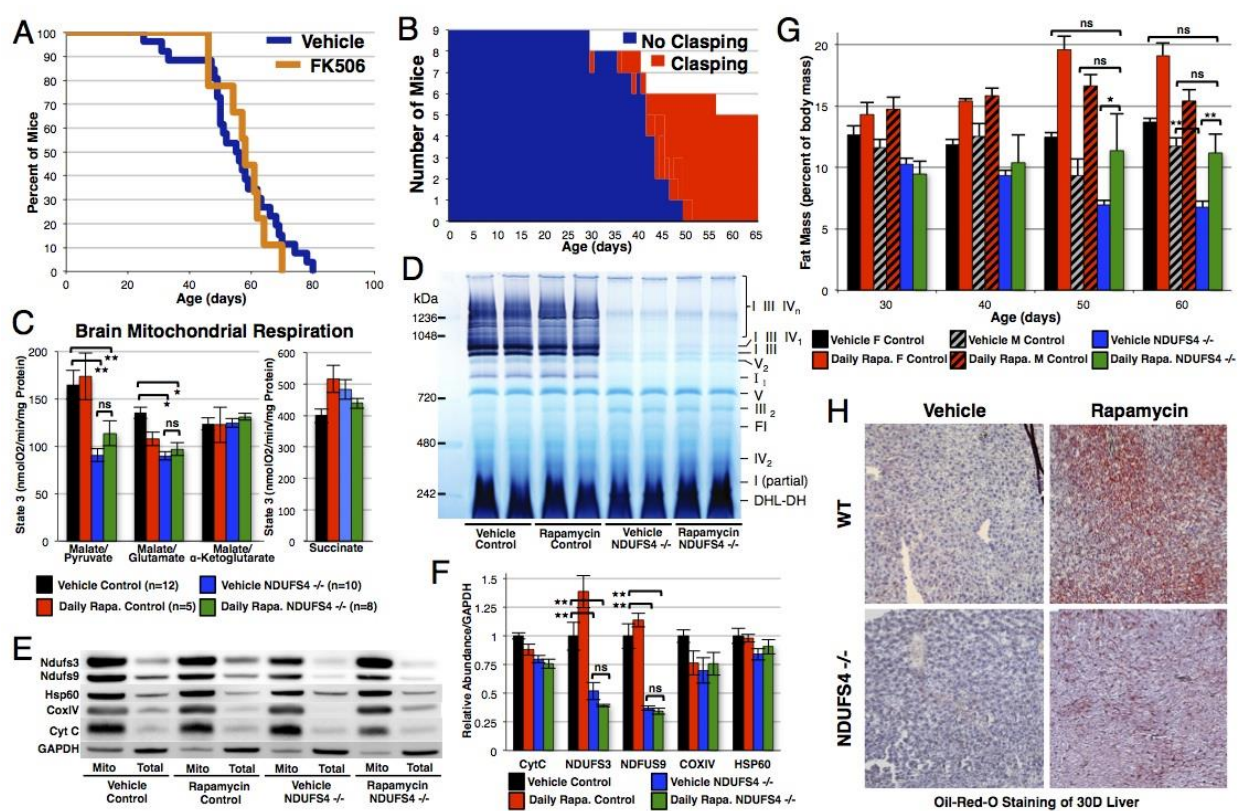
Vehicle injected KO mice begin to display neurological symptoms between P35 and P40,

coinciding with a peak in body weight (**Figure 13B-C, Figure 15B**). Following this peak in weight, disease symptoms progressively worsen and weight declines, generally culminating in death within 1-2 weeks (end-of-life criteria described in detail in methods). Daily rapamycin injection dampens developmental weight gain in both KO and control mice and prevents the progressive weight loss phenotype in the KO mice (**Figure 13C, Figure 15B**). Assignment of littermate KO mice to different treatments demonstrated that this effect is robust and highly reproducible even in mice from the same litter (**Figure 16**). Appearance and severity of clasping, a commonly reported and easily scored phenotype that generally progresses in severity coordinately with weight loss and neurological decline, was also greatly attenuated in the rapamycin treated mice (**Figure 15A-B**). Many treated mice survived to advanced age without showing signs of neurological disease. Performance on a rotarod assay, which measures balance, coordination, and endurance, was also assessed for a separate cohort of animals. Both vehicle and rapamycin treated KO animals showed decreased performance compared to control mice; however, while the performance of vehicle treated KOs progressively worsened during disease progression, rapamycin treated KOs maintained their performance with age (**Figure 15C**). KO mice treated with rapamycin were also spared from the characteristic neurological lesions (**Figure 15D**). We have been unable to conclusively determine the new cause of death but suspect iatrogenic infection caused by daily IP injections to be a major limiting factor on survival rapamycin treated KOs rather than neurological decline. Together these data indicate that daily high-dose rapamycin treatment fundamentally alters disease onset and progression in this model.



Given the pleiotropic effects of mTOR inhibition (reviewed in [25, 125, 203]) we sought to determine the downstream mechanisms associated with attenuation of disease in rapamycin treated

*Ndufs4*<sup>-/-</sup> mice. Rapamycin has well-documented immune-modulatory effects, so we first considered the possibility that inhibition of neuroinflammation was mediating the observed rescue. To test this model, we treated mice with the calcineurin inhibitor FK-506 (tacrolimus). FK-506 is a clinically approved immunosuppressive drug that binds the same physical target as rapamycin, FKBP12, but disrupts calcineurin signaling rather than mTOR[204]. This drug has been reported to attenuate neurodegeneration in stroke, Huntington's disease, and prion disease models through immunomodulation and effects on calcineurin signaling[204, 205]. We found, however, that FK-506 had no detectable effect on disease onset or progression in the *Ndufs4*<sup>-/-</sup> mice (Figure 17A,B), indicating that neither immunosuppression nor off-target disruption of calcineurin signaling through binding of FKBP12 are likely to account for the effects of rapamycin.



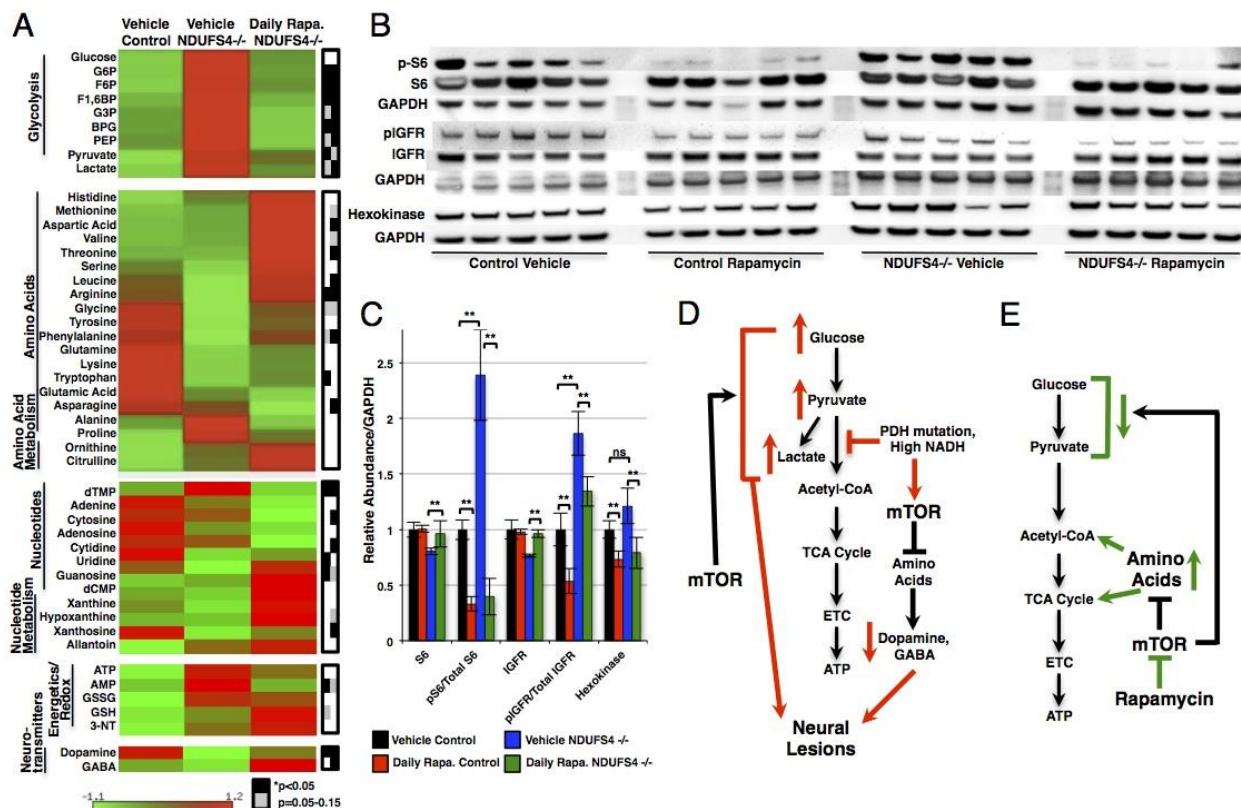
**Figure 17 – Rescue of the *Ndufs4*<sup>-/-</sup> Mice is Associated with an Attenuation of Liver Fat Storage but not Changes in Mitochondrial Function or Immune-Modulation.** (A-B) FK-506, delivered at the highest-tolerated dose, failed to extend lifespan (A) or attenuate disease (B) in *NDUFS4*<sup>-/-</sup> mice. (C) Rapamycin has no observed affect on respiratory activity or complex I deficiency of mitochondria isolated from 60 day old *NDUFS4*<sup>-/-</sup> mice (see also Figure S7). (D) In-gel activity of ETC complex I. Mitochondrial complex I assembly and stability was unchanged by rapamycin (see also Figure S8). (E-F) Complex I subunits are significantly reduced in *NDUFS4*<sup>-/-</sup> mice and rapamycin has no effect on total levels (F) or localization (E) of mitochondrial proteins (see also Figure S9). (G) Total body fat progressively decreases in *NDUFS4*<sup>-/-</sup> but is maintained in rapamycin treated animals. Fat mass differs by gender in control but not KO mice. Total fat mass is also increased in control animals. (H) Liver fat droplets are deficient in vehicle treated *NDUFS4*<sup>-/-</sup> animals and partially rescued by rapamycin. Rapamycin increases hepatic lipid droplet levels. (\* $p < 0.05$ , \*\* $p < 0.005$ )

We next considered that rapamycin could be improving mitochondrial function through increased macroautophagy or mitophagy, the degradation of mitochondria through the autophagy-lysosome pathway. Such a mechanism would be predicted to enhance mitochondrial function and improve the overall quality of mitochondria by removal of the least-functional components of the mitochondrial network. Although we were able to detect evidence for induction of autophagy in liver and brain homogenates of KOs treated with rapamycin (data not shown), there was no corresponding rescue of complex I deficiency or of mitochondrial functional capacity (**Figure 17C-F**). Complex I assembly and stability were also found to be unaltered by rapamycin treatment (**Figure 17D**), as were the levels and localization of mitochondrial ETC proteins (**Figure 17E-F**). HSP60, a component of the mitochondrial unfolded protein response (mtUPR) was not induced in brain by either *Ndufs4* loss or by rapamycin (**Figure 17F**). Together, these data suggest that the attenuation of Leigh Syndrome progression in *Ndufs4* *-/-* mice by rapamycin does not act primarily through increased autophagy, complex I assembly or stability, or improvements in bulk mitochondrial function.

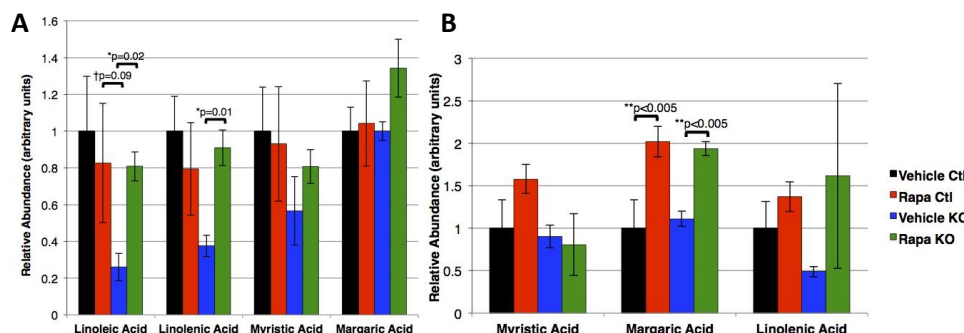
As a central coordinator of nutrient sensing and growth status, mTOR plays a key role in regulating metabolism by integrating intracellular sensing of amino acids at the lysosome, energetic status downstream of AMPK and in response to changes in redox status, and extracellular signaling through insulin/IGF signaling[25, 195]. We reasoned that loss of NDUFS4 might perturb metabolic signaling such that mTOR is aberrantly activated, which could be alleviated by rapamycin. Consistent with this idea, Western blotting of whole brain lysates revealed that phosphorylation of ribosomal protein S6, a downstream target of mTORC1 activity, was significantly increased in the KO mice versus controls (**Figure 18B**). Rapamycin dramatically reduced phosphorylation of S6 in both control and KO animals. IGFR phosphorylation was also increased in *Ndufs4* *-/-* animals, with rapamycin attenuating this effect. Total S6 and total IGFR were both slightly but significantly decreased in vehicle treated *Ndufs4* *-/-* animals, perhaps through feedback inhibition resulting from chronic activation of mTOR, as has been previously been reported for IGFR[206]. Using whole-body quantitative magnetic resonance we observed a significant reduction and progressive loss in body fat in the *Ndufs4* *-/-* mice, consistent with metabolic dysfunction playing a role in disease progression. Daily rapamycin treatment prevented fat loss (**Figure 17G**). Furthermore, Oil-Red-O staining and metabolomic analysis of liver demonstrated that *Ndufs4* *-/-*

have a marked deficiency in liver fat droplets and free fatty acids that is partially rescued by rapamycin

(Figure 17H).



**Figure 18 – *Ndufs4*<sup>-/-</sup> mice have a distinct metabolic signature that is partially rescued by rapamycin.** (A) Metabolites measured from whole-brain isolates of 30 day old animals. Values are row-normalized with variance adjusted to 1. Raw data provided in Tables S1-2. p-values of individual metabolites are indicated on the right of the heat map. (B-C) mTOR activity, as indicated by phosphorylation of rpS6, is increased in *NDUFS4*<sup>-/-</sup> mouse brain and liver (Figure S10). Decreased levels of total IGFR and S6 in *NDUFS4*<sup>-/-</sup> animals suggest feedback inhibition from chronic mTOR activation. Rapamycin potently inhibits phosphorylation of rpS6 and rescues levels of IGFR and S6. (D) Metabolic model of disease in *NDUFS4*<sup>-/-</sup> mice. (E) Data presented here supports a model by which increased levels of intracellular amino acids, as well as glycolysis, prevent neurological decline in the *NDUFS4*<sup>-/-</sup> mice.



**Figure 19 – Detected Free Fatty Acids are Rescued by Rapamycin in Brain and Liver.** (A) Liver and (B) brain free fatty acids detected by metabolomics assay.

Mitochondrial encephalopathies are associated with metabolic alterations including defective ATP production, increased oxidative damage, decreased antioxidant capacity, and in some cases, the accumulation of metabolites such as NADH, pyruvate, and lactic acid[207]. Whole-brain metabolomics

performed on 30 day old animals revealed a clear metabolic phenotype in the *Ndufs4* *-/-* mice that includes an accumulation of all detected glycolytic intermediates, as well as pyruvate and lactate, findings consistent with clinical reports of Leigh Syndrome [192, 196] (**Figure 18A**). The metabolomic signature of the *Ndufs4* *-/-* mice was also characterized by a decrease in free amino acids, nucleotides, and products of nucleotide catabolism, increases in oxidative stress markers, and significantly reduced levels of GABA and dopamine (**Figure 18A**). Levels of free fatty acids in the brain of KO mice were also reduced, consistent with the findings in liver (**Figure 19**). Rapamycin treatment rescued many of these defects associated with NDUFS4 deficiency including levels of GABA, dopamine, and free fatty acids. Rapamycin treatment resulted in a decrease of glycolytic intermediates to levels similar to those in control animals, although pyruvate and lactate remained slightly elevated. Hexokinase, the first enzyme in glucose metabolism through glycolysis, was also increased in *Ndufs4* *-/-* animals and decreased by rapamycin in both KO and control animals, consistent with the decrease in glycolytic intermediates observed by metabolomics. An increase in free amino acids, metabolic markers of amino acid catabolism, markers of nucleotide catabolism, and fatty acids accompanied the decrease in glycolytic intermediates. Dopamine and GABA were also rescued to control levels, while markers of oxidative stress were unchanged.

## DISCUSSION

Taken together, the data presented here demonstrate that the inhibition of mTOR dramatically increases survival and healthspan in the NDUFS4 *-/-* model of Leigh Syndrome and suggest that these effects are mediated largely through a shift in metabolic state (**Figure 18A, D-E**). Our data suggest that it is the accumulation of toxic metabolites, such as glycolytic intermediates and lactate, and perhaps decreased abundance of free amino acids that are critical rapamycin-responsive aspects of the disease, while rapamycin had no direct effect in ETC function or Complex I deficiency. Furthermore, the finding that baseline energy sensing through mTOR is aberrant in the *Ndufs4* *-/-* mice suggests that modulating energy sensing, rather than caloric intake, may be key to treating mitochondrial disease. It appears that interventions designed around increasing calories, such as high-fat diets, may fail because an accumulation of toxic products, rather than an energy deficiency, underlies the pathogenesis of Leigh Syndrome mutations and perhaps other mitochondrial disorders. It may be that targeting the derailment of

intracellular signaling is necessary to accomplish shifts in substrate preference. This model also provides an explanation for the shared clinical presentation of Leigh Syndrome genes such as PDHB and NDUFS4 and the striking similarities in metabolic phenotypes and clinical presentation between Leigh Syndrome and Wernicke's encephalopathy, a nutritional encephalopathy resulting from thiamine deficiency[208]. The intracellular sensing mechanism responsible for increased mTOR activity in the NDUFS4 *-/-* animals remains to be determined, though increased NADH levels would appear to be a likely candidate. It will also be important to determine whether dietary alterations are effective if performed in a context of reduced mTOR activity. Perhaps interventions that have thus far proven ineffective will have synergistic effects when combined with interventions targeting nutrient sensing. Collectively, our data identifies nutrient sensing in general, and the mTOR pathway in particular, as novel and potent therapeutic targets for treating mitochondrial disease.

## **METHODS**

### *Mouse Care*

KO mice were always housed with littermate control animals. All animals in this study were provided food on the floor of the cage and gel in order to eliminate ability to access food or water as a confounding factor. Euthanasia criteria were as follows: loss of 20 percent of maximum body weight, immobility, or general moribund appearance. Nearly every mouse in the vehicle and every-other-day rapamycin groups reached the 20% body weight loss cutoff, while daily rapamycin injected mice all died spontaneously and without warning, appearing healthy and active until the day they expired. All mice were euthanized by cervical dislocation and tissues were rapidly collected and processed as needed.

### *Western Blotting*

Whole-organs were rinsed of blood and flash-frozen in liquid nitrogen. Tissues were cryohomogenized on dry ice and the homogenized frozen powder was split for protein extraction. Protein was extracted by sonicating powdered tissue in RIPA buffer (10mM Tris-HCl pH 8.0, 1mM EDTA, 1%

Triton X-100, 0.1% Sodium deoxycholate, 0.1% SDS, 140mM NaCl) with Roche cComplete Ultra protease inhibitor and PhosSTOP phosphatase inhibitor tablets added prior to use.

### *Statistical Analysis*

All data were presented as means +/- SEM. Comparisons between 2 groups were performed using student t tests. All tests are two-tailed except Figure S1 and S3, which are one-tailed. P<0.05 was considered significant. Regression statistics in excel and graphpad were used to calculate statistics for the correlation plots.

### *Mitochondrial Isolation*

Mitochondria were isolated using the crude-prep method described in *Wang., et al., 2011. Isolation of brain mitochondria from neonatal mice. Journal of Neurochemistry.*

### *Respiration Assays*

Respiration of freshly isolated mitochondria was measured with a Seahorse XF24 flux analyzer (Seahorse BioScience, Billerica, MA) following the manufacturers guidelines (<http://www.seahorsebio.com/resources/tech-writing/iso-mito-xf24.pdf>). Briefly (□): 5ug of mitochondria were adsorbed to the bottom of each sample well by spinning 50ul of mitochondrial suspension at 2000g for 20min at 4C. The medium was MAS (70mM sucrose, 220mM mannitol, 10mM KH<sub>2</sub>PO<sub>4</sub>, 5mM MgCl<sub>2</sub>, 2mM HEPES, 1mM EGTA, 0.02% fatty acid-free BSA, pHed to 7.2 with KOH at 37C) supplemented with electron donor substrate. Electron donor combinations for complex I dependent respiration were 5mM malate plus either 10mM pyruvate, 10mM glutamate or 10mM  $\alpha$ -ketoglutarate. For measuring complex I-independent respiration the complex II substrate succinate (13mM) in combination with the complex I inhibitor rotenone (2 $\mu$ M) was utilized. Before loading the plate into the Seahorse the assay volume all wells, sample and blanks was adjusted to 500ul by adding more of the same medium (MAS plus electron donors). The instrument was programmed to execute the following protocol at 37C:

Wait 10min, Mix 1min, Wait 3min, Mix 1min, Wait 3min, Mix 1min, Measure 3min, Mix 1min, Measure 3min state2 respiration, Mix 1min, Inject ATPsynthetase substrate ADP (4mM, pHed to 7.2), Mix 1min,

Measure 3min state3 respiration, Mix 1min, Inject ATPsynthetase inhibitor oligomycin (2.5ug/ml), Mix 1min, Measure 3min state4 respiration, Mix 1min, Inject uncoupler carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (= FCCP) (5uM), Mix 1min, Measure 3min uncoupled respiration, Mix 1min, Inject complex III inhibitor antimycin A (4uM), Mix 1min, Measure 3min respiratory chain-independent respiration. Concentrations given signify concentrations after injection in assay. Respiration rates for each well were determined using the Akos algorithm for OCR (oxygen consumption rate) (2009 Gerencser et al. AnalChem81 6868 - Seahorse - Quantitative microplate-based respirometry with correction for oxygen diffusion), background corrected and normalized to ug of loaded protein. State3 and uncoupled respiration rates declined during the 3min measurement intervall and were therefore determined as the initial (maximum) respiration rate measured. State2 and state4 rates were stable and therefore reported as the average over the respective measurement interval. For each mitochondrial preparation these parameters were reported as the mean of 5 technical replicates.

#### *Blue Native Gels (BNG)*

BNG-PAGE was performed as described by Wittig et al. (2006 Nature Protocols 1(1) 418) with the modification that precast minigels (Novex, NativePAGE 3-12% Bis-Tris, BN2011BX10, 1mm thick) were used in an XCell SureLock Mini-Cell (Invitrogen). Key features of the sample treatment: 150ug of previously frozen mitochondria samples were extracted with either digitonin (6:1 detergent:protein w:w) or Triton X-100 (5:1 detergent:protein w:w) and protein complexes in the 15,000g supernatant were negatively charged with Coomassie Blue G-250 (1:8 detergent:dye w:w). After 1h electrophoresis at 100V blue cathode buffer was replaced by cathode buffer without Coomassie blue and 300V were applied for an additional 1.5h. At the end of the run the overall protein distribution in the gel could be imaged without any additional Coomassie staining.

Complex I in-gel activity (IGA) staining: Gels were incubated at room temperature with 2.5mM NADH and 0.5mM nitroblue tetrazolium (mod. of Sabar et al. PlantJ44 893). The purpelish hue of the formazan reaction product identifies bands with NADH dehydrogenase activity were. (A prominent cross reactivity at about 200 kD is probably caused by dehydrolipoamide dehydrogenase (2009 EH Meyer et al. PlantPhysiol151 603)).

## CHAPTER 4

### **Evidence that Defective Autophagy Precedes Hypertrophy and is Associated with Cellular Dysfunction in Angiotensin II Induced Cardiac Injury**

#### **INTRODUCTION**

Autophagy is a fundamental cellular process in eukaryotes that mediates degradation and recycling of cellular organelles and macromolecules. While the necessity of autophagy for normal cellular function and survival is well established the precise role autophagy plays in disease settings is often ambiguous [209-216]. In addition to the canonical role autophagy plays in promoting homeostasis through catabolism of cellular components the autophagic process has also been proposed to regulate or mediate cell death in certain settings [217]. Although the existence of a programmed autophagic cell death pathway is controversial, it is clear that activation of autophagic pathways is often associated with cell death through both apoptosis and necrosis [218, 219]. This apparent context-dependent function of autophagy in promoting cell death versus promoting homeostasis has complicated studies of the role of autophagy in the pathogenesis of human disease and continues to contribute to conflicting reports [212].

Interventions that promote autophagy such as caloric restriction, exercise, and pharmaceutical intervention with the mechanistic target of rapamycin (mTOR) inhibitor rapamycin have been shown to prevent or attenuate cardiac injury in a variety of disease models [25]. Conversely, genetic disruption of autophagy induces cardiac dysfunction in murine and fly models[112], as well as in human genetic disorders associated with disrupted autophagic or lysosomal function, including Danon Syndrome (LAMP2 $\alpha$  cardiomyopathy) [220, 221], Fabry Disease [222], and Pompe Disease [223]. While these studies clearly demonstrate the importance of functional cellular degradation machinery for normal cardiac function the roles of autophagy in age, diet, and hypertension induced cardiomyopathies have not been clearly defined [224]. Here we have examined the role of autophagy in chronic Angiotensin II (AngII) induced cardiac hypertrophy, a model for hypertensive cardiomyopathy[112]. Since increased levels of AngII have been observed with age in heart [112] this model may also be of relevance to cardiac aging.

Using an early, pre-hypertrophic timepoint after AngII administration, we have identified disrupted autophagy as a characteristic feature of AngII treated mouse hearts. Our data suggests that the accumulation of dysfunctional autophagosomes may play a role in the development of AngII induced cardiac injury by directly disrupting cardiomyocyte structure, thus mediating some of the effects of AngII in inducing cardiac hypertrophy. This work assigns a disease-promoting role to the accumulation of autophagy markers associated with cardiac hypertrophy that have been previously reported in this model [225] and may explain some of the cardioprotective effects of AngII blocking therapies which extend beyond simple anti-pressor effects [226-228].

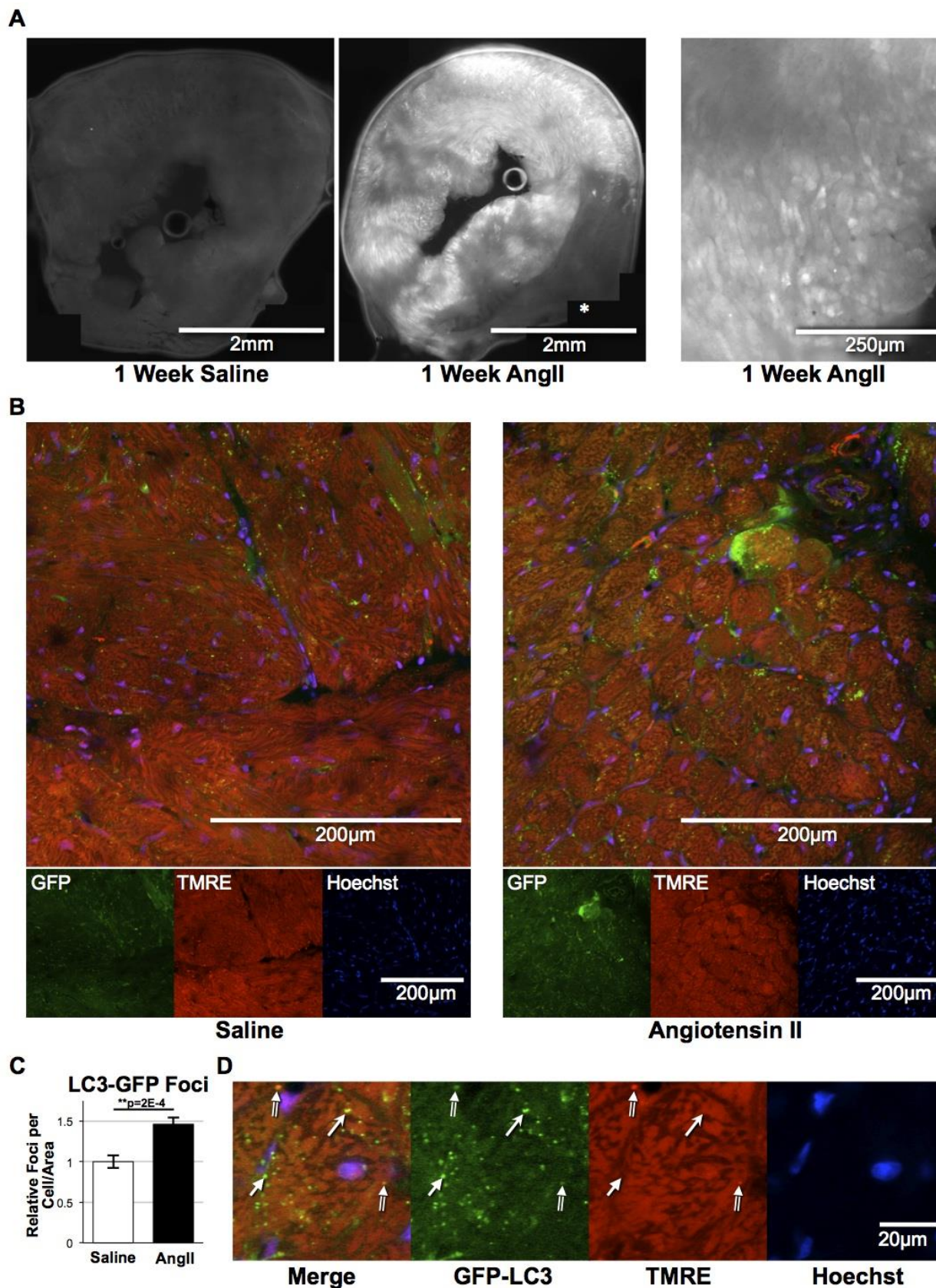
## RESULTS

### *Angiotensin II induces a dramatic increase GFP-LC3 and GFP-LC3 foci in murine cardiac tissue*

To investigate the role of autophagy in the development of AngII induced cardiac hypertrophy we examined tissue from mice expressing the autophagy marker microtubule-associated protein light-chain 3 (LC3), the mammalian homolog of yeast Atg8, tagged with green fluorescent protein (GFP) [229]. Ex-vivo imaging of live tissue slices revealed an unexpected and dramatic accumulation of GFP-LC3 following 1 week of AngII infusion, a pre-hypertrophic time-point (**Figure 20A-C**). Interestingly, the increased GFP signal in AngII treated hearts largely spared the right ventricle, which does not become hypertrophic with AngII treatment [230-232]. In addition to the regional difference there was heterogeneity among cells within a given region (**Figure 20A-C**). Imaging of GFP-LC3 expressing tissue by ex-vivo confocal microscopy, as previously described [233], revealed that GFP-LC3 accumulates in AngII treated hearts in both autophagic foci and as diffuse GFP-LC3 (**Figure 20B-C**). Thus the increased fluorescence by low power microscopy is a result of both autophagosome associated and free GFP-LC3.

### *Microscopic analysis reveals highly dysmorphic cells characterized by extreme accumulation of GFP-LC3*

A small subset (less than 2%) of cardiomyocytes in AngII treated hearts were found to substantially accumulate GFP-LC3 and appeared dysmorphic (**Figure 20B, 21A-B**). These cells are characterized by disruption of mitochondria and contractile units with GFP-LC3 positive foci and high-GFP regions appearing to physically disrupt the normal subcellular organization of the cardiomyocytes. Subsarcolemmal

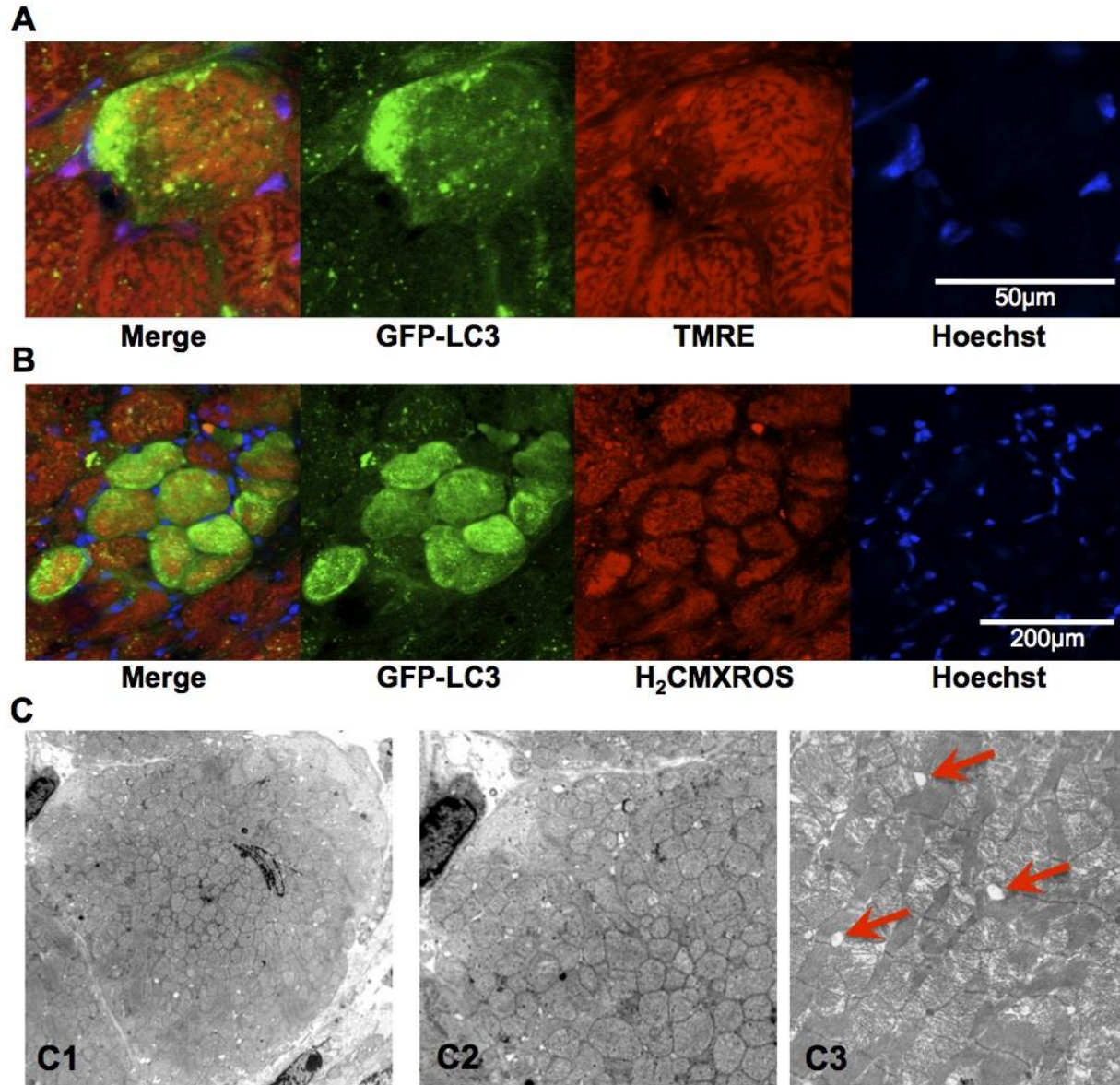


**Figure 20 - GFP-LC3 Accumulates in AngII Induced Cardiac Hypertrophy.** (A) Imaging of GFP-LC3 in fresh heart slices ex-vivo shows an increase in GFP following 1 week AngII. Images shown are representative of slices from homozygous transgenic mice. This increase in GFP is also apparent in heterozygous transgenic mice (Figure S2). (B-C) Confocal imaging of shows increased diffuse and focal GFP in AngII treated mouse hearts. Open arrows indicate engulfed mitochondria with maintained membrane potential, closed arrows indicate engulfed mitochondria with reduced membrane potential (determined by staining with TMRE).

mitochondria appeared to be dissociated from the cell periphery with contractile units pushed out of center of the cells (**Figure 21A-B**). These high-GFP staining cells did not appear in the saline treated animals.

Staining with TMRE, a mitochondrial membrane potential sensitive dye, revealed that some of the GFP-LC3 foci are associated with mitochondria that have diminished membrane potential (**Figure 20C, Figure 21A**). In addition, staining with the reactive oxygen species (ROS) sensitive mitochondria localized dye H<sub>2</sub>CMXRos revealed that the mitochondria in these disrupted cells produced higher levels of ROS than their normal neighboring cells (**Figure 21B**). Together these observations suggest that the presence of accumulated autophagosomes not only physically disrupts cardiomyocyte structure but also alter mitochondrial function by increasing ROS production and decreasing membrane potential, both consistent with changes that have been reported to occur in cardiomyopathy [234].

Using electron microscopy (EM) we examined tissue from non-transgenic animals treated with AngII for one week in order to eliminate the possibility that the observed disruption of cellular structure could be a result of the presence of the exogenous protein in the transgenic mice. We observed highly dysmorphic cells filled with autophagic material as seen by live tissue imaging (**Figure 21C**). The accumulated autophagic/lysosomal organelles were observed to physically disrupt cardiomyocyte structure and organization by EM, as was suggested by confocal microscopy in GFP-LC3 expressing mouse tissue. Together this data demonstrates that autophagic/lysosomal vesicles accumulate and physically disrupt cardiomyocyte structure following one week of AngII infusion.



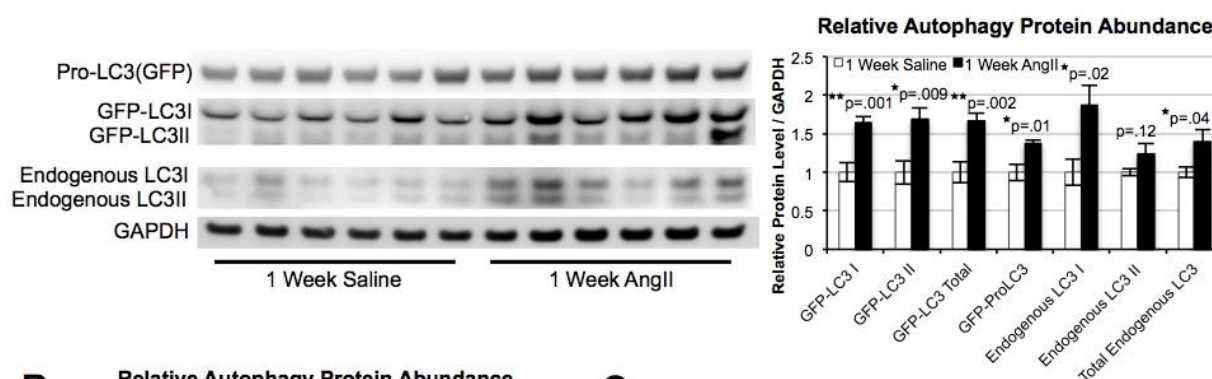
**Figure 21 – Accumulated Autophagosomes Physically Disrupt Cellular Structure.** In some cells in AngII treated hearts mitochondria (stained red) and contractile units (absence of staining) are displaced. Low membrane potential mitochondria (A) and high-ROS producing mitochondria (B) are present in these cells. (C) EM imaging verifies this subcellular effect of 1 week AngII treatment in WT animals including cells with mislocalized contractile units and mitochondria and large vacuolated spaces (C1-2) and autophagosomes disrupting contractile unit structure (C3).

*Autophagic markers accumulation occurs without an increase in mRNA levels of autophagy proteins in AngII treated cardiac tissue*

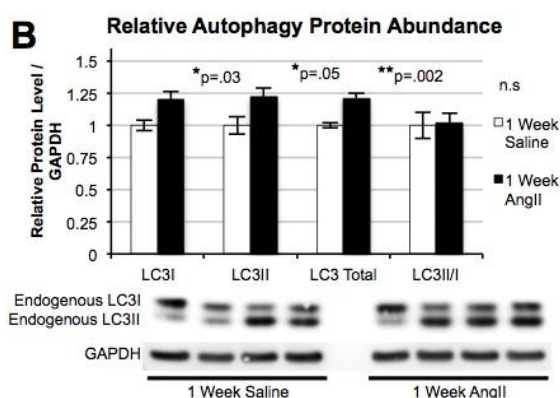
The accumulation of GFP-LC3 was verified by western blotting of whole tissue lysates, which also revealed an accumulation of endogenous LC3 and demonstrated that both the inactive and conjugated

forms of the protein (LC3 I and II, respectively) accumulate (**Figure 22A**). We considered the possibility that the accumulation was an artifact of the presence of the GFP-LC3 fusion protein but found that LC3I and II were increased in abundance in non-transgenic mice treated with 1 week of AngII (**Figure 22B**), consistent with prior reports of AngII induced cardiac hypertrophy and eliminating this possibility [225].

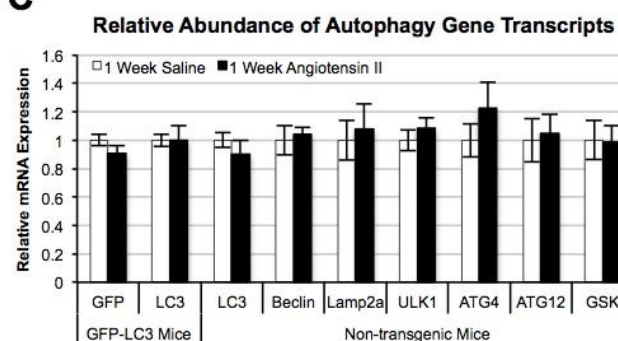
**A**



**B**



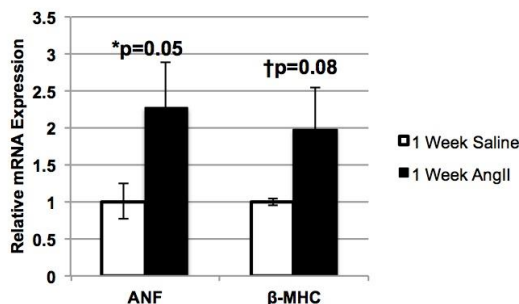
**C**



**Figure 22 - Western blotting verifies the accumulation of LC3 observed by microscopy.** (A) Endogenous and GFP-tagged LC3 accumulate in 1 week AngII treated mouse hearts. (B) LC3 also accumulates in WT animal tissue. (C) The increased protein levels are not the result of increased gene expression.

The GFP-LC3 transgenic mice express the modified protein under the CAG promoter and the transgene cassette has been mapped to the distal region of chromosome 2, 106 basepairs upstream of a pseudogene [235]. Thus, the expression of this transgene is unlikely to be upregulated by AngII treatment, suggesting that the exogenous protein is accumulating rather than being produced at higher levels. qPCR analysis of transcript levels verified that transcriptional activation of the transgene was not induced by AngII treatment (**Figure 22C**). Furthermore, endogenous LC3 transcript levels were also unaltered by one week of AngII. The observed accumulation of LC3 thus appeared to result from a disruption in the normal turnover of the protein rather than an increase in production. We extended our

analysis of transcript levels to additional endogenous autophagic proteins and found that none were increased by one week of AngII. mRNA levels of the hypertrophic markers ANF and  $\beta$ -MHC were slightly increased by one week of AngII (**Figure 23**). Together these data suggested that a decrease in the elimination of LC3, rather than an upregulation of autophagy by increased translation, is the driving factor behind the observed accumulation of this marker.



**Figure 23 – Control for qPCR Analysis.** Transcript levels for hypertrophic markers ANF and  $\beta$ -MHC were increased and trending up at 1 week of AngII treatment.

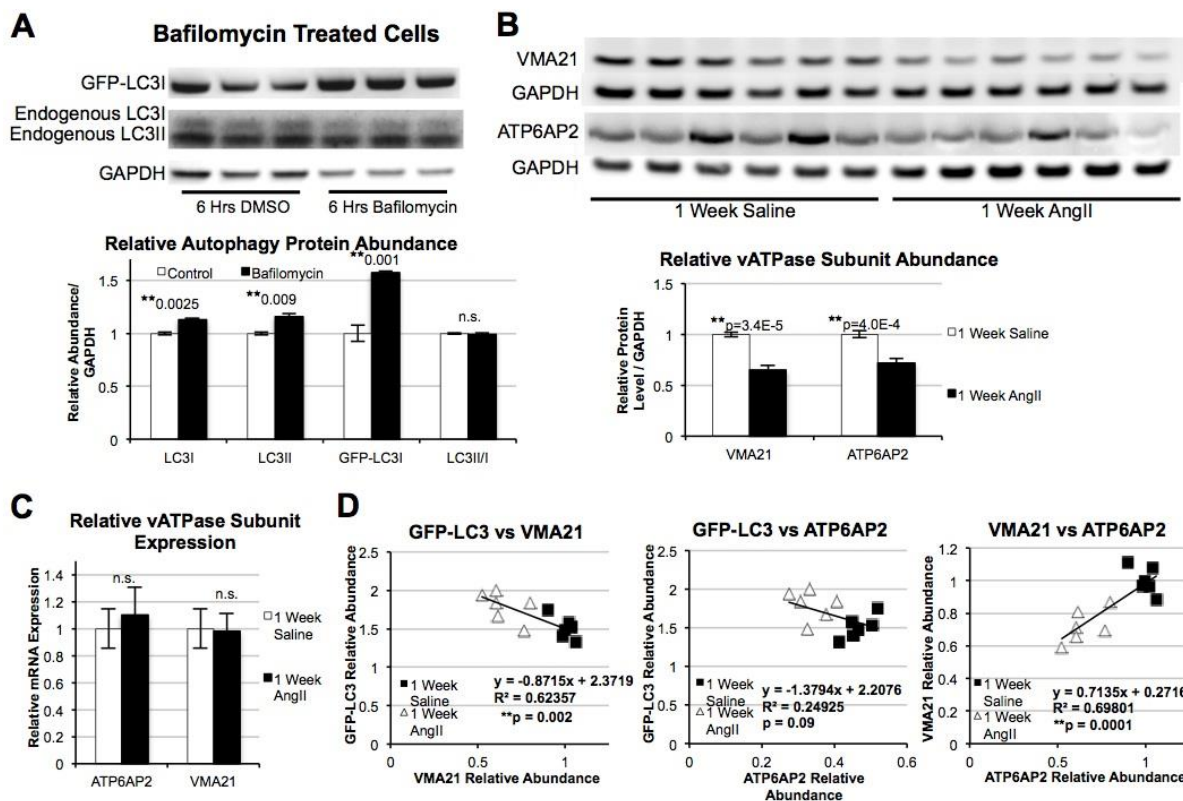
*Inhibition of lysosomal acidification by bafilomycin mimics the GFP-LC3 and LC3I and II accumulation seen in Angiotensin treated cardiac tissue*

In order to establish whether the accumulated autophagic proteins observed in AngII treated mouse tissue was consistent with a decrease in completion of successful autophagy we derived primary fibroblast cell lines from GFP-LC3 expressing mice and treated them with the autophagy inhibitor bafilomycin. Bafilomycin inhibits lysosomal ATPases (vacuolar ATPases, v-ATPases), preventing the acidification of mature autophagosomes and autolysosomes. Failure to acidify autophagosomes results in an accumulation of autophagic vesicles that cannot be properly cleared [236]. Using bafilomycin in primary cultures we found that pharmaceutical inhibition of autophagic clearance results in an accumulation of markers of autophagy similar to that seen in AngII treated hearts (**Figure 24A**).

*Vacuolar ATPase subunits are significantly decreased by one week of Angiotensin treatment*

Given the effect of bafilomycin on autophagic markers in cultured cells we considered the possibility that altered expression of v-ATPase subunits might contribute to the accumulation of undigested autophagosomes in angiotensin treated mouse hearts. Consistent with this hypothesis we found protein levels of Vma21, a highly conserved chaperone required for proper assembly of the v-

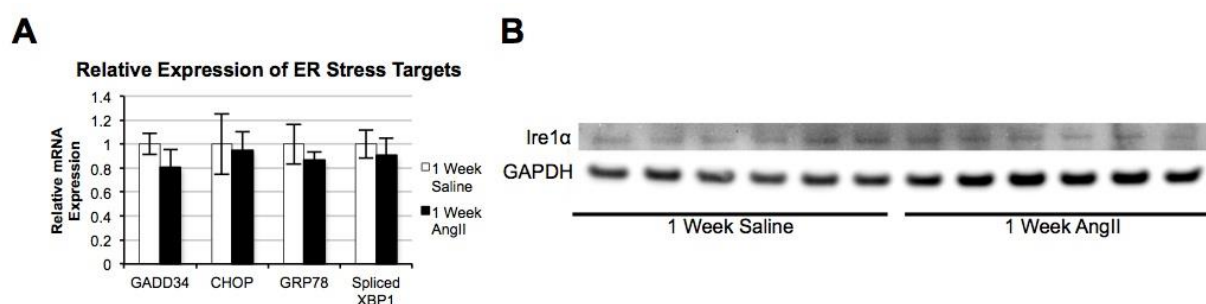
ATPase complex, were significantly decreased (**Figure 24B**). Mutations in Vma21 can result in x-linked myopathy with excessive autophagy (XMEA), a disease where skeletal muscle cells are disrupted by accumulated LC3 and Lamp2a positive autophagic/lysosomal material in a fashion similar to that observed in the 1 week AngII treated hearts[237]. While mutations in Vma21 that cause XMEA do not appear to affect cardiac function the similarities of cellular phenotype are striking and suggest that decreases in Vma21 may play a causal role in the accumulation of LC3 observed in our model.



**Figure 24 – Vacuolar inhibition and decreased levels of v-ATPase subunits correspond to accumulated LC3.** (A) Inhibition of v-ATPase function by bafilomycin mimics the observed LC3 phenotype in cultured cells. (B) Vma21 and Atp6ap2 are both significantly decreased in 1 week AngII treated mouse hearts. (C) The decrease in Vma21 and Atp6ap2 is not the result of decreased expression. (D) Vma21 and Atp6ap2 levels negatively correlate with LC3 levels and positively correlate with each other.

Inclusion body myositis (IBM) is another genetic disorder characterized by the accumulation of undegraded vacuoles within skeletal muscle cells which results in a disruption of intracellular structure, cell death, and muscle wasting. The genetic factors underlying IBM are unknown, but decreased lysosomal acidification is a generally accepted mechanistic factor [238]. One proposed mechanism underlying the pathogenesis of IBM is an ER stress mediated decrease in the expression of Vma21 [239].

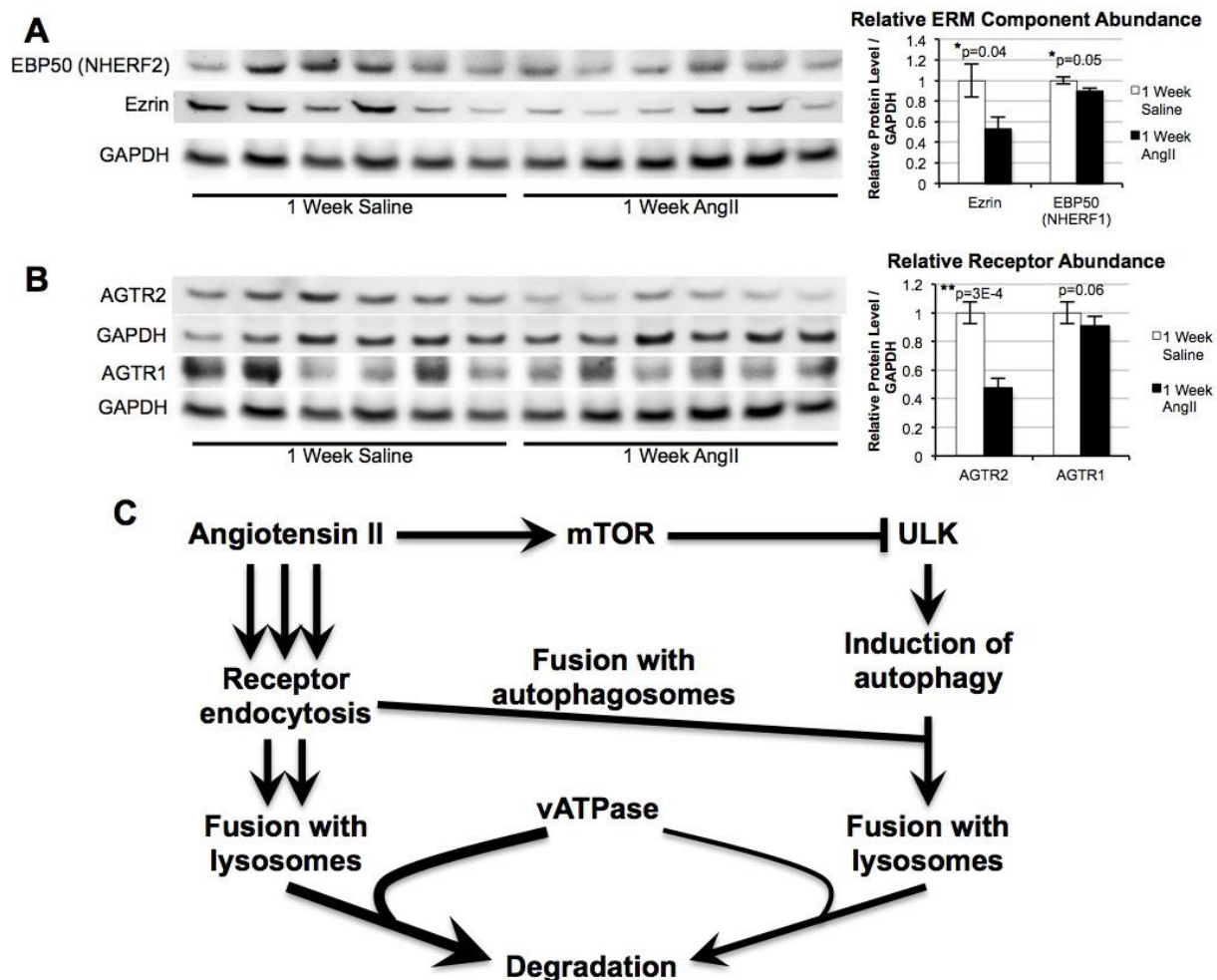
Based on the striking cellular and molecular similarities between the observed blockage of autophagy in our model and the reported phenotype of IBM we considered the possibility that 1 week AngII induces ER stress and down-regulates the vacuolar protein Vma21. We found, however, no evidence for induction of the ER stress markers Ire1 $\alpha$ , Gadd34, Chop, Grp78, or spliced Xbp-1 mRNA (**Figure 25**). Furthermore, transcript levels of Vma21 were unchanged by 1 week AngII,



**Figure 25 – Markers of ER Stress are not Induced by 1 Week AngII Treatment.** (A) Transcript levels of GADD34, CHOP, GRP78, and XBP1 were unchanged as were protein levels of Ire1 $\alpha$  (B), all widely used markers of ER stress.

We next considered that the (pro)renin receptor Atp6ap2, which functions as a renin receptor, a component of the vacuolar ATPase, and a regulator of endosomal trafficking, might be mediating of the observed phenotype. This dual-function protein has been shown to be an essential component of the v-ATPase in murine cardiomyocytes and loss of Atp6ap2 results in a lethal cardiomyopathy with subcellular accumulation of undegraded autophagic vesicles [240]. Given the potential for a direct mechanism linking humoral signaling to lysosomal acidification we examined the expression and abundance of the Atp6ap2. At one week of treatment protein levels of Atp6ap2 were significantly decreased in AngII treated mice compared to saline treated controls while mRNA levels appeared unchanged (**Figure 24C**), corresponding to the changes observed with Vma21. Interestingly, protein levels of Vma21 and Atp6ap2 both show strong negative correlations ( $p=0.002$  and  $p=0.09$ , respectively) with total levels of LC3 among all mice, regardless of treatment (**Figure 24D**), suggestive of a mechanistic link between decreased levels of v-ATPase components and increased LC3.

*Western blot and analyses suggest endolysosomal pathway activation is responsible for the decreased abundance of lysosomal ATPase subunits and accumulated autophagosome markers*



**Figure 26 – Increased endocytic degradation of AngII responsive receptors may explain the observed phenotype.** (A) EBP50 and Ezrin, two components of endocytic vesicle trafficking, are decreased by 1 week AngII. (B) AGTR2 and AGTR1 are both decreased by 1 week AngII. (C) A model consistent with the observations described here. Increased receptor uptake and degradation may deplete available v-ATPase units and result in an accumulation of undegraded autophagic vesicles, LC3I, and LC3II. mTOR activation by AngII inhibits the induction of autophagy and may contribute to the increase in free LC3 and LC3I.

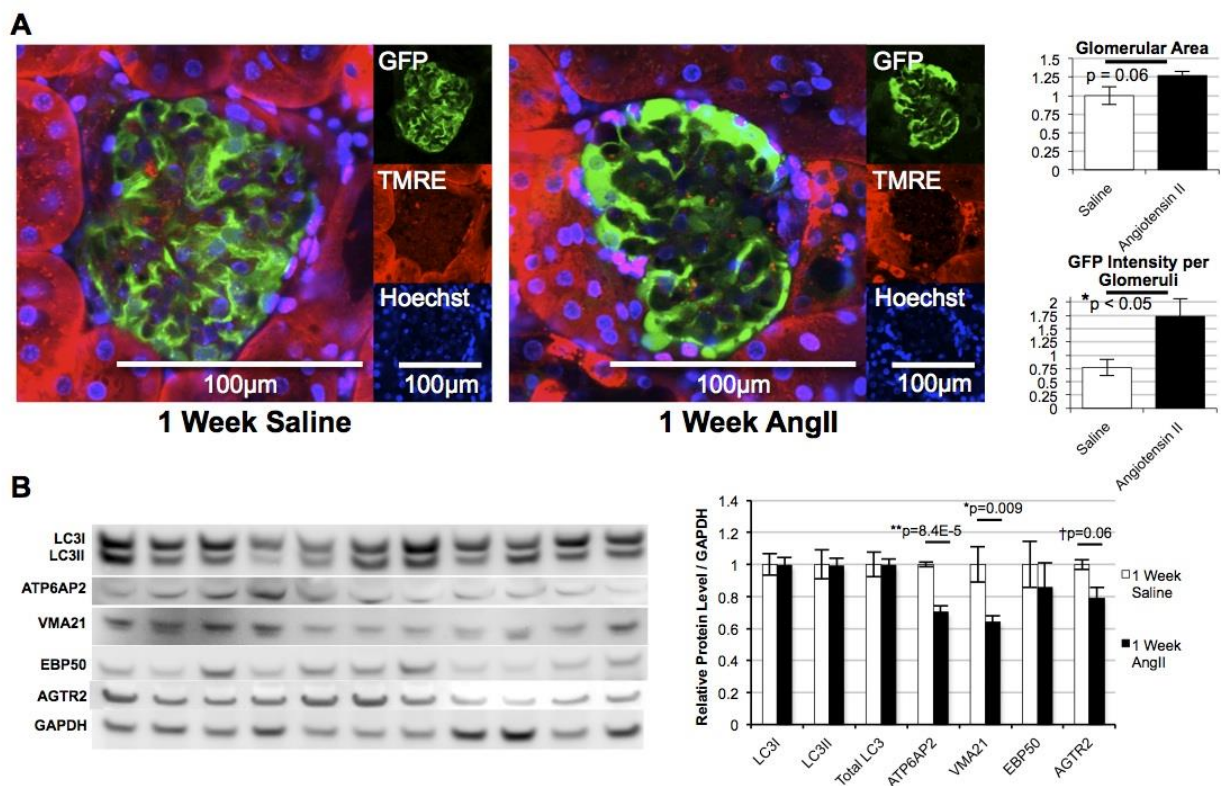
Given the apparent discordance in autophagic (LC3 I and II) and lysosomal (Lamp2a) proteins versus v-ATPase components we considered that a pathway leading to increased v-ATPase turnover might account for these observations. Receptor endocytosis induced by AngII treatment has been well documented and has been reported to play important regulatory roles in signal transduction, feedback, and sensitization [241, 242]. Endocytic vesicles formed by receptor endocytosis can meet a variety of fates, one of which is degradation through fusion with an early endosome, maturation into multivesicular body (MVB), fusion with an autophagosome to form an amphisome, and finally amphisome-lysosome fusion and lysosomal degradation. Importantly, late endosomes can also directly fuse with lysosomes through the endolysosomal pathway, a process that bypasses the late amphisome, and thus autophagic

vesicles [243]. Thus, we reasoned that chronic stimulation by AngII may result in receptor endocytosis and lysosomal degradation of MVB's. In this scenario endosomes undergoing lysosomal degradation may deplete v-ATPase components while decreasing the availability of lysosomes for fusion with autophagosomes. The consequence would be an accumulation of autophagosomes and autophagolysosomes in parallel with the observed decrease in v-ATPases. The observed increase in

Angiotensin receptor uptake and recycling during treatment with AngII is well documented [242, 244]. To assess endocytic pathway activation in our AngII treated mice we examined levels of NHERF-1 (EBP-50) and Ezrin, adaptor and scaffold proteins that are part of the Ezrin/Radixin/Meosin (ERM) complex and involved in the endocytic recycling of G-protein coupled receptors [245, 246]. We found that both of these targets were significantly decreased in AngII treated mouse hearts (**Figure 26A**). Furthermore, western blotting for the AngII receptors AGTR2 and AGTR1 revealed a significant decrease in the abundance of AGTR2 and a strong trend towards a decrease in AGTR1 (**Figure 26B**). As previously stated, Atp6ap2 (the (pro)renin receptor and vATPase component) is also AngII responsive and was decreased by AngII treatment. Together this data supports a model by which increased receptor degradation through the endocytic pathway depletes the available pool of vATPase subunits and leads to an accumulation of un-degraded autophagosomes (**Figure 26C**).

#### *Renal response to AngII similar to that observed in heart tissue*

Based on the observations in heart we predicted that we should see similar changes in autophagy, vacuolar ATPase subunits, and AGTR2 in kidney, a tissue that is also sensitive to AngII induced injury [247, 248]. Live tissue imaging revealed that GFP-LC3 does in fact accumulate in the glomeruli of AngII treated animals (**Figure 27A**), though protein levels of LC3I and II were unchanged by whole tissue western blotting (**Figure 27B**). Interestingly, the vATPase subunits ATP6AP2 and VMA21 were significantly decreased with AngII treatment and EBP50 and AGTR2 trended down, consistent with the effects seen in heart. Together this data suggests that while similar mechanisms are at play in kidney, the overall effects of AngII treatment on autophagic marker accumulation are cell and tissue specific. Within the kidney the glomeruli appear to be the most sensitive to AngII induced changes to autophagy.



**Figure 27 – Effects of 1 week AngII on kidney autophagy.** (A) 1 week AngII results in autophagic dysfunction in kidney glomeruli and (B) decreased levels of v-ATPase subunits, AGTR2, and EBP50, as was observed in heart.

## DISCUSSION

The accumulation of autophagosomes observed after 1 week of AngII appears to directly affect cellular structure and function through disruption of mitochondrial localization and may play a causal role in the cardiac oxidative stress, hypertrophic, and cell death associated with AngII treatment. In this work we have demonstrated that the increased autophagy markers seen in AngII induced cardiac hypertrophy results from an accumulation of autophagic proteins rather than increased production. Our data also suggests that in this setting constitutive receptor endocytosis may drive cellular dysfunction through a depletion of lysosomal vATPase units and concomitant increase in un-degraded autophagic material.

Inducing autophagy is a well-established and potent therapeutic intervention in a variety of models of cardiomyopathy [25, 112]. Rapamycin, a pharmaceutical inhibitor of mTOR that induces autophagy, has shown efficacy in pressure-overload and obesity induced cardiac hypertrophy [249, 250]. Conversely, mTOR overactivation appears to be a driver of hypertrophy in the hearts of obese animals through decreased autophagic flux [251]. Preconditioning is a cardioprotective intervention that is

autophagy dependent [252]. Similarly, the efficacy of caloric restriction (CR), which has been shown to ameliorate AngII induced cardiac hypertrophy, is dependent on induction of autophagy [253]. Together this data implicates autophagy as a process critical to the development or prevention of cardiac hypertrophy from diverse causes.

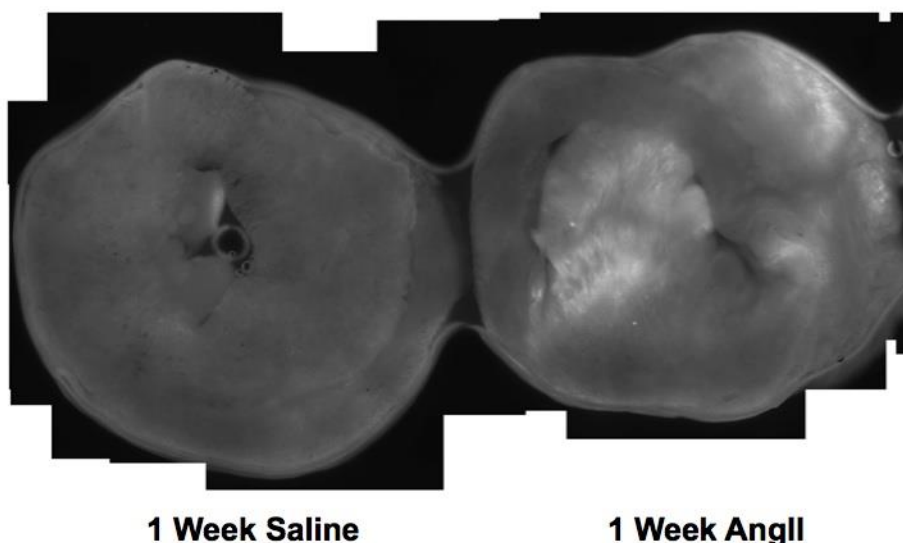
Angiotensin converting enzyme (ACE) inhibitors or ATR antagonists are known to have beneficial cardiac effects in human patients that are at least partially independent of their anti-pressor activity [226, 254]. The data presented here suggests that these agents may act to prevent disease at least partially through a reduction in receptor endocytosis, an effect that would be secondary to ACE inhibition and has been documented with ATR antagonists [255]. While this idea remains to be directly tested the potential implications are would be broadly important in human disease. An intriguing recent development supporting this model is provided by prospective studies showing that treatment with ATR antagonists provides a dramatic protection against Alzheimer's disease (AD) symptoms and pathology [256, 257]. While many of the effects on cognitive function may be ascribed to normalization of blood pressure it is surprising that ATR antagonist use was associated with a significant increase in large vessel hemorrhage or infarct incidence while also associated with a significant decrease in amyloid plaque neurofibrillary tangle abundance in both control and AD patients. Amyloid metabolism appears to be directly altered by ATR antagonists, conceivably through alterations in proteostasis driven by changes in autophagy. It is possible that receptor endocytosis may be an important clinical target beyond the model demonstrated here.

## **MATERIALS and METHODS**

### *Animal Care*

All experiments were performed on young (4-6 months old) male C57Bl/6CR mice. AngII was administered via Alzet subdermal osmotic minipumps that were inserted subdermally. Control animals underwent identical treatment and received pumps containing saline only. AngII was purchased from MP Biosciences. All experiments using GFP-LC3 mice were performed using mice with a single copy (heterozygous) of the transgene except the whole-heart slices in **Figure 20A** which were obtained using

mice homozygous for the transgene. Heart slices from heterozygous mice show the same phenotype (**Figure 28**) but we chose to include the homozygous slices in **Figure 20A** as the phenotype is more readily visible in the higher-GFP expression animals.



**Figure 28 – GFP-LC3 Levels in Heterozygous Mice Treated for 1 Week.** Mice heterozygous for the GFP-LC3 transgene show the same phenotype as homozygous mice (Figure 20A) but with a lower intensity of fluorescence as is expected.

#### *Live Tissue Imaging*

Live tissue imaging was performed as previously described [233]. Briefly, mice were euthanized by cervical dislocation and tissues were rapidly moved into low glucose DMEM (Invitrogen) on ice and sliced into 2mm thick slices using a heart-cutting block (Zivic instruments). When stained, slices were placed in staining buffer (DMEM containing dyes) and incubated, protected from light, on ice for 30 min followed by a 10 min destain in dye free DMEM. Low power images were collected using a Nikon Eclipse E600 microscope and consist of multiple microscope fields stitched using Adobe Photoshop 11. Confocal images were collected on a Zeiss LSM510 microscope using chambered coverslides. Dye concentrations, filter settings, and image collection were performed as described [233].

#### *Western Blotting and Quantitative Polymerase Chain Reaction*

Whole-organs were rinsed of blood and flash-frozen in liquid nitrogen. Tissues were cryohomogenized on dry ice and the homogenized frozen powder was split for protein and RNA extractions. Protein was extracted by sonicating powdered tissue in RIPA buffer (10mM Tris-HCl pH 8.0, 1mM EDTA, 1% Triton X-100, 0.1% Sodium deoxycholate, 0.1% SDS, 140mM NaCl) with Roche cOmplete Ultra protease inhibitor and PhosSTOP phosphatase inhibitor tablets added prior to use. RNA was extracted using the Trizol method. Each sample was run in triplicate by and median values used for analysis. qPCR data was collected using a 4 point relative dilution standard curve for each transcript and for GAPDH.

#### *Electron Microscopy*

Electron microscopy was performed on a JEOL JEM 1200EXII transmission electron microscope.

#### *Statistical Analysis*

All data were presented as means +/- SEM. Comparisons between 2 groups were performed using student t tests. All tests are two-tailed except Figure S1 and S3, which are one-tailed.  $P < 0.05$  was considered significant. Regression statistics in excel and graphpad were used to calculate statistics for the correlation plots.

## CHAPTER 5

### Conclusions and Future Work

In the studies described here we have made significant contributions to the understanding of the genetics underlying differential response to caloric restriction, identified inhibition of growth signaling through mTOR as a novel therapeutic strategy for a devastating and untreatable human childhood disorder, and described the role of dysfunctional autophagy in promoting cardiac dysfunction and hypertrophy in the angiotensin II induced model of hypertrophy, as well as providing evidence in support of a possible mechanism underlying the observations made.

Significant work remains to be done in order to provide a comprehensive view of the mechanisms underlying genotype specific responses to caloric restriction and our work presented here represents only the first step toward that goal. Outlying the interactions between genotype and nutrient availability is a complex but critically important task. In addition to the importance of understanding the genotype-CR interaction from the perspective of aging research these studies have direct implications for human health and disease. In the discussion of Chapter 2 we highlight work done in a mouse model of ALS that suggests the genotype-CR interaction at Sod1 is conserved between yeast and mammals. This example illustrates the direct impact that this work, and subsequent studies, may have on human health. If this relationship is conserved to humans it could prove highly beneficial for disease onset or progress of ALS for patients to avoid situations of nutrient deprivation such as fasting or dieting. This is just one example where the yeast genetic data may provide insight into human health.

The work outlined in Chapter 2 is a direct test of the hypothesis that insights gained in the yeast screen are valuable to our understanding of mammalian biology. Here, using a mouse model that very accurately models a devastating childhood genetic disease, we have demonstrated that knowledge gained from examining the CR screen was sufficient to predict and provide a novel therapeutic strategy for treating this disease. In addition to the novelty of the approach of inhibiting growth signaling to treat a mitochondrial disorder this study has uncovered the first effective pharmacological intervention strategy in a mammalian model of this disease, and thus represents major progress in treating this and, if the

relationship holds true for other genetic deficiencies, possibly other mitochondrial disorders. A great deal of additional work will be required to determine how broad these observations are but the potential clinical value is clear. Furthermore, our efforts to not only determine if mTOR inhibition would benefit the Leigh syndrome mouse model but to uncover the mechanistic underpinnings of the response have led to a model whereby shifts in metabolic state mediate the beneficial effects. This has provided novel insights into the pathogenesis of the disease and also provides a clear mechanistic target that is more specific than broad mTOR inhibition. Targeting metabolic state more directly may provide additional therapeutic strategies, allowing for combined therapies and novel agents which can be designed to limit off-target effects.

In Chapter 2 we also provide evidence that dosage methods of treatment with rapamycin are key to the outcome. While this seems an obvious observation there has been no consensus or rigorous study into the dose-dependency of rapamycin responses, with many unique treatment methods currently utilized. The fact that certain treatment methods are likely extremely suboptimal, and that these suboptimal methods are flooding the literature with accounts of effects or absent effects which may not fairly represent true mTOR inhibition, suggests that determining the dose-dependency of mTOR inhibition through rapamycin, and other mTOR inhibitors, is an extremely important task. This work is currently being undertaken by our group as a follow-up to the study presented here.

Finally, we examined the role of autophagy in a commonly used model system for inducing left ventricular cardiac hypertrophy. Autophagy is a key process downstream of mTOR (see Chapter 1) that has been suggested to be crucial for the beneficial effects of mTOR inhibition and nutrient inhibition (through caloric restriction) more broadly. While often implicated in cardiac dysfunction the exact role of autophagy in AngII induced cardiac hypertrophy has been elusive. Using a novel live ex-vivo imaging technique we developed we have been able to assign a pathogenic role to dysfunctional autophagy in this setting. Furthermore, we have uncovered a possible mechanistic cause for dysfunctional autophagy in AngII treated hearts which both explains the beneficial effects of rapamycin and caloric restriction in this model and has broad implications into the potential mechanisms whereby ACE inhibitors and AGTR antagonists provide benefits outside of their pressor-effects. Specifically, our data may explain why these

agents have been found, in large prospective studies, to attenuate the risk of Alzheimer's disease. While largely speculative at this point the model we have proposed warrants further investigation and may prove to have significant impact on human health outside of cardiovascular biology.

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