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The effects of supplemented metabolites on lifespan and stress response pathways in Caenorhabditis elegans

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The Effects of Supplemented Metabolites on Lifespan and Stress Response Pathways in *Caenorhabditis elegans*

by

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DEDICATION

I dedicate this research to Grace Raburn, Carl Darren Blackburn, and Bubba the Bubble Gum Boy. Without each, it would have taken me much longer to realize my goals. Moreover, I dedicate this work to anyone who chooses to battle aging, thus foregoing a peaceful life in return for a rewarding existence.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

Aß: Amyloid beta
AD: Alzheimer's disease
AMPK: AMP-activated protein kinase
ALS: Amyotrophic lateral sclerosis
ATP: Adenosine triphosphate
BDH2: βHB dehydrogenase 2
βHB: beta-hydroxybutyrate
CBP-1: CREB binding protein-1
CCCP: Carbonyl cyanide m-chlorophenyl hydrazone
CL: Cardiolipin
COX-2: Cyclooxygenase-2
CR: Calorie restriction
DNMT: DNA methyltransferase
DR: Dietary restriction
EGCG: (-)-epigallocatechin-3-gallate
eIF-2α: Eukaryotic translation initiation factor-2 alpha
ER: Endoplasmic reticulum
ETC: Electron transport chain
FUdR: 5-fluoro-2′-deoxyuridine
GABA: Gamma-aminobutyric acid
GCN-2: General control nonderepressible-2
GFP: Green fluorescent protein
GPX-1: Glutathione peroxidase 1
GSTK1: Glutathione S-transferase kappa 1
GSTT1: Glutathione S-transferase theta 1
HAT: Histone acetyltransferases
HDAC: Histone deacetylases
HIF-1: Hypoxia-inducible factor 1
HSF: Heat shock factor
HSP: Heat shock protein
Htt: Huntington gene
IDH2: Isocitrate dehydrogenase 2
MMP: Mitochondrial membrane potential
MPP(+):1-methyl-4-phenylpyridinium
MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NAC: N-acetylcysteine
NAD: Nicotinamide adenine dinucleotide
NGM: Nematode growth medium
NRF2: Nuclear factor erythroid 2–related factor 2

OXPHOS: Oxidative phosphorylation
PD: Parkinson's disease
PGC-1α: Peroxisome proliferator-activated receptor gamma coactivator 1α
ROS: Reactive oxygen species
SCFA: Short-chain fatty acid
Sir2: Sirtuin 2
SOD2: Superoxide dismutase 2
STACs: Sirtuin activatin compounds
TCA: Tricarboxylic acid cycle
TDP-43: Tar DNA-binding protein-43
TDO-2: Tryptophan 2,3-dioxygenase
TMRM: Tetramethylrhodamine, methyl ester
TOR: Target of rapamycin
UCP: Uncoupling protein
UPR: Unfolded protein response
YFP: Yellow fluorescent protein

ABSTRACT

Understanding how metabolites contribute to anaplerosis, antioxidant effects, and hormetic pathways during aging is fundamental to creating supplements and dietary habits that may decrease age-associated disease and decline, thus improving the quality of life in old age. In order to uncover metabolic pathways that delay aging, the effects of large sets of metabolites associated with mitochondrial function on lifespan were investigated.

Malate, the tricarboxylic acid (TCA) cycle metabolite, increased lifespan and thermotolerance in *C. elegans*. Addition of fumarate and succinate also extended lifespan and all three metabolites activated nuclear translocation of the cytoprotective DAF-16/FOXO transcription factor and protected from paraquat-induced oxidative stress. The increased longevity provided by malate addition did not occur in fumarase (fum-1), glyoxylate shunt (gei-7), succinate dehydrogenase flavoprotein (sdha-2), or soluble fumarate reductaseF48E8.3 RNAi knockdown worms. Therefore, to increase lifespan, malate must be first converted to fumarate, then fumarate must be reduced to succinate by soluble fumarate reductase and the mitochondrial electron transport chain complex II. Lifespan extension induced by malate depended upon the longevity regulators DAF-16 and SIR-2.1. Malate supplementation did not extend the lifespan of long-lived eat-2 mutant worms, a model of dietary restriction. Malate and fumarate addition increased oxygen consumption, but decreased ATP levels and mitochondrial membrane potential suggesting a mild uncoupling of oxidative phosphorylation.

Each of the twenty amino acids was individually supplemented to *C. elegans* and the effects on lifespan were determined. All amino acids except phenylalanine were found to extend lifespan at least to a small extent at one or more of the 3 concentrations tested with serine, histidine, and proline showing the largest effects. In most cases, amino acid supplementation did not extend lifespan *in eat-2* worms, a model of dietary restriction or in *daf-16*, *sir-2.1*, *rsks-1* (S6 kinase), or *aak-2* (AMPK) longevity pathway mutants or in worms fed RNAi to *skn-1*, the *C. elegans* Nrf2 homolog. Serine and tryptophan addition further protected worms from Alzheimer's amyloid-beta toxicity. Tryptophan and its catabolites nicotinic acid, picolinic acid, and NAD further induced a broad heat shock response. These results indicate that dietary amino acid imbalance and amino acid catabolism affect organismal longevity.

The ketone body beta-hydroxybutyrate (βHB) is a histone deacetylase (HDAC) inhibitor and has been shown to be protective in many disease models, but its effects on aging are not well studied. Therefore we determined the effect of βHB supplementation on the lifespan of *C. elegans*. βHB supplementation extended mean lifespan by approximately 20%. RNAi knockdown of HDACs hda-2 or hda-3 also increased lifespan and further prevented βHB-mediated lifespan extension. βHB-mediated lifespan extension required the DAF-16/FOXO and SKN-1/Nrf longevity pathways, the sirtuin SIR-2.1, and the AMP kinase subunit AAK-2. βHB did not extend lifespan in a genetic model of dietary restriction indicating that βHB is likely functioning through a similar mechanism. βHB addition also upregulated BHB dehydrogenase activity and increased oxygen consumption in the worms. RNAi knockdown of F55E10.6, a short chain dehydrogenase and SKN-1 target gene, prevented the increased lifespan and βHB dehydrogenase activity induced by βHB addition, suggesting that F55E10.6 functions as an inducible βHB dehydrogenase. Furthermore, βHB supplementation delayed Alzheimer's

amyloid-beta toxicity and decreased Parkinson's alpha-synuclein aggregation. The results indicate that D-βHB extends lifespan through inhibiting HDACs and through the activation of conserved stress response pathways.

Aging is a progressive disease caused by the time dependent decline of an organism and is the primary risk factor for many human ailments, including heart disease, cancer, and Alzheimer’s disease. Uncovering metabolic pathways and metabolites that delay the onset of age-related decline was the primary drive of this investigation.

CHAPTER 1

INTRODUCTION AND BACKGROUND

Age-related decline affects nearly every biological system in virtually all species, including humans. Although many theories overlap in the ever evolving biology of aging field, it is apparent that mitochondria are central to life and death. Typically as we age, the function of mitochondria declines in a tissue specific manner and results in a loss of energy production. Understanding how metabolites taken in and broken down in our diet contribute to anaplerosis (the re-filling of metabolites in metabolic pathways), antioxidant effects, and hormetic (beneficial at one dose, but detrimental at a higher dose) pathways during aging is fundamental to creating supplements and dietary habits that may decrease age-associated disease and decline, thus improving the quality of life in old age. In order to discover metabolic pathways that delay aging, the effects of large sets of metabolites on lifespan were investigated.

1.1 Mitochondrial dysfunction

Mitochondria are responsible for the production of ATP through the electron transport chain (ETC) via oxidative phosphorylation (OXPHOS) located within the inner mitochondrial membrane. Because of their importance in cellular physiology, defects in mitochondria are linked to many human diseases and play a central role in aging. Particularly, mitochondrial dysfunction contributes to age-related decline and is itself a consequence of aging. Mitochondria are the major source and the main target of reactive oxygen species and regulate stress response, calcium homeostasis, and apoptosis [1].

Since mitochondria are crucial for biological processes, mitochondrial dysfunction has been postulated to lead to premature aging [2]. Conversely, slight inhibition of mitochondrial respiration extends the lifespan of yeast, *C. elegans*, *Drosophila*, and mice [3-6]. Electrons passing through the mitochondrial ETC leak out and combine with molecular oxygen to form reactive oxygen species (ROS) [7]. Continuous damage to cellular macromolecules via ROS results in the decline of mitochondrial function, and coincides with some age-related disorders [8]. The antioxidant enzymes superoxide dismutase, catalase, and glutathione peroxidase quench oxidants, and are considered oxidant scavengers [9].Glutathione peroxidases rely on the reduced form of glutathione, a 3-amino acid peptide, to convert peroxides to water, while simultaneously oxidizing glutathione. However, levels of small molecule antioxidants and oxidant scavenger enzymes gradually decrease as organisms age, resulting in increased levels of oxidation, accumulation of damaged proteins, DNA, and lipids [10], and decreased mitochondrial function [11, 12]. This decrease in antioxidants as a consequence of aging is of great concern and a current target for anti-aging therapies.

1.2 Mitochondrial disease

ROS are responsible for damaging proteins, DNA, and lipids such as cardiolipin, which is localized in the mitochondrial inner membrane and makes up about 20% of the membrane's mass. Cardiolipin (CL) is considered the glue by which the respiratory complexes are held together and is necessary for preserving the mitochondrial membrane potential [13]. Absence of CL leads to defects in protein import, translocation of cholesterol from outer to the inner membrane [14], and deficiency in major mitochondrial processes [15].

One important process that CL regulates is the release of cytochrome c from mitochondria, which triggers apoptosis [16]. Recent evidence establishes a relationship between

the oxidation and depletion of CL to a decrease of cytochrome c oxidase activity and mitochondrial dysfunction in brain aging, which was alleviated by treatment with the natural antioxidant hormone melatonin. A rise in mitochondrial DNA oxidative damage was also observed in brains derived from Alzheimer's disease patients, likely contributing to neurodegenerative decline [17, 18]. Although much research links mitochondrial dysfunction and Alzheimer's disease (AD), the mechanism is not entirely clear. There is substantial evidence demonstrating progressive accumulation of mitochondrial amyloid-beta, a 40-42 amino acid peptide that forms extracellular plaques in Alzheimer's brains [19], and these levels correspond with the degree of mitochondrial dysfunction [20]. Evidence also suggests that enhanced mitochondrial γ-secretase complex disrupts communication between the mitochondria and the endoplasmic reticulum in models of AD [21]. Huntington's disease is characterized by a mutation in the Huntington gene (Htt), leading to protein aggregation, a reduction in mitochondrial membrane potential, and neurotoxicity resulting in striatal atrophy [22]. Mutated Htt weakens the mitochondrial outer membrane, increasing the sensitivity of the mitochondrial permeability transition pore to apoptotic stimuli such as Ca^{2+} and ROS, resulting in cytochrome c release and caspase activation [23]. Mitochondrial fission is enhanced in Huntington's disease leading to fragmented mitochondria and the degree of fragmentation parallels disease severity [24]. Dopamine treatment increases the vulnerability of striatal neurons to mutant Htt by down-regulating mitochondrial complex II activity [25]. Parkinson's disease is characterized by intracellular α-synuclein-containing Lewy body formation [26], α-synuclein binds to the mitochondrial membrane causing a decrease in complex I activity paralleling an increase in ROS production [27]. Amyotrophic lateral sclerosis (ALS), another neurodegenerative disease commonly referred to as Lou Gehrig's disease, can be caused by mutations in cytoplasic

superoxide dismutase 1, which translocates to mitochondria causing mitochondrial dysfunction [28]. Recent studies using ALS disease models suggest that disruption of the axonal transport of mitochondria along microtubules compromises axonal function [29]. Equally significant to note is that mitochondrial dysfunction is also associated with type 2 diabetes [30], cancer [31, 32], systemic lupus [33], epilepsy, autism, and developmental delay [34]. Interestingly, recent studies using young and aged rats subjected to heterochronic parabiosis (connection of their circulatory systems) indicate an increase in neurogenesis in the aged rats, and that the decline of cognitive abilities in the aged subjects can be reversed by factors in the plasma from the young individual [35, 36]. In a similar study, the protein GDF11 was identified as a blood factor in mice that can induce vascular restoration and increase neurogenesis suggesting a restored capacity of autophagy/mitophagy and mitochondrial biogenesis in aged rodents [37].

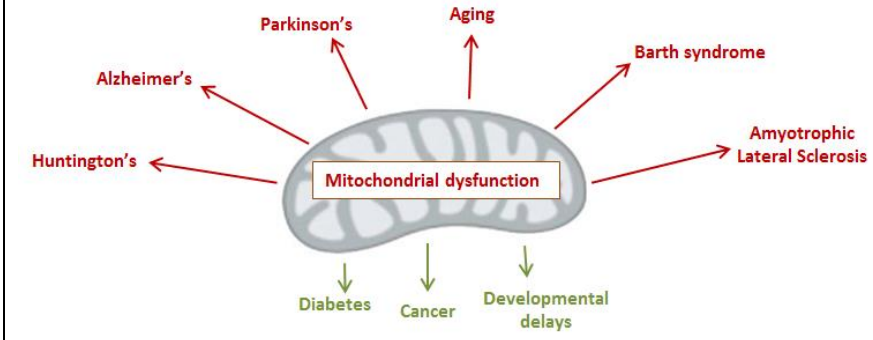

Figure 1.1: Mitochondrial dysfunction underlies multiple age-induced diseases. Diseases in red indicate strong experimental evidence to support malfunctioning mitochondria in disease pathology. Diseases in green exhibit mitochondrial dysfunction as well.

1.3 Calorie restriction

Calorie restriction, a 20-40% lowering of caloric intake, is the most effective method known to extend lifespan in almost every species in which it has been tested, including mammals [38]. Numerous studies recognize calorie restriction (CR) as a successful method to alleviate

age-related decline, combat disease, and increase lifespan in model organisms [39]. CR-mediated lifespan extension suggests a possible conservation of longevity-regulating pathways among species [40]. Researchers have yet to completely unravel how CR extends lifespan, but research spanning the past few decades illustrates a hopeful glimpse at the power of dietary alterations. Some researchers initially hypothesized that CR would decrease overall metabolism. Aging and longevity have been shown to positively correlate with energy metabolism in some mitochondrial electron transport chain mutants. For example, a reduction in mitochondrial energy metabolism and respiration has been shown to increase lifespan in *clk-1* and *isp-1* mutants in *C. elegans* [41]. However, more recent evidence has suggested that such a simple correlation does not always hold as calorie restriction has been shown to not alter or even increase mitochondrial electron transport chain function. Increased resistance to heat and oxidative stress are strong indicators of health and longevity and, as expected, a decline in heat tolerance and an increase in oxyradical damage are both markers of aging [42, 43]. Calorie restriction has been shown to be protective against oxidative and heat stress[43] and the greatest increases in *C. elegans* lifespan have been produced by culturing the worms in a defined axenic media as a type of dietary restriction, which lacks the normal bacterial food source [44].

Currently, there is a considerable amount of interest in finding pharmacological mimetics that act on the same pathways as CR, but compounds acting identical to CR will likely be impossible to identify due to the multiple distinct and overlapping pathways affected by a low calorie regimen. CR mediates an increase in antioxidant response pathways, genes responsible for stress resistance and cellular repair, while also down-regulating inflammatory pathways [45]. Primary fibroblasts cultured with human CR sera induced upregulation of Nrf2, GSTK1, GSTT1, IDH2, GPX1 (glutathione peroxidase 1), and SOD2 (superoxide dismutase 2) [46], emphasizing

the importance of circulating factors in promoting health and longevity. Nrf2 is a transcription factor that induces gene expression protecting from oxidants and xenobiotics. GSTK1 and GSTT1 are glutathione-S-transferases which attach reduced glutathione to xenobiotic substrates for detoxification. IDH2 is a subunit of a citric acid cycle isocitrate dehydrogenase complex that reduces NADP to NADPH, an essential cofactor for the reduction of oxidized glutathione by a glutathione reductase. A wide range of evidence supports the benefits of CR, but for the most part the mechanisms which mediate these benefits have remained elusive.

The hypothesis that CR reduces damage mediated by ROS is supported by research demonstrating an increase in oxidized lipids, proteins, and DNA in the nuclear and mitochondrial genome with age that is decreased by the CR diet. CR has also been shown to delay the decline in superoxide dismutase (SOD) activity and other cellular antioxidant defenses with age [47, 48]. In support of this hypothesis, age-related decline in antioxidant defenses is linked to increased rates of stress-induced apoptosis, which is markedly reduced by CR [49]. CR animals exhibit lower levels of oxidatively damaged DNA and reduced lipid peroxidation [50, 51]. Interestingly, data suggests that CR is responsible for both a decrease in ROS production and also an increase ROS defenses and repair [52]. However an increase in SOD and cellular ROS defenses may not play as large of a role in CR increased longevity as originally theorized, because mice lacking superoxide dismutase show increased oxidative damage, but they do not display signs of premature aging [53]. Pinning down the exact mechanisms through which CR increases longevity has been quite challenging, but there are a few pathways that have been identified that are well established as conserved mediators of longevity.

1.4 Calorie restriction and sirtuins

Studies indicate that CR enhances the intracellular NAD^+/NADH ratio of yeast by up-regulating respiration resulting in decreased NADH levels [54]. Cellular increases in metabolic redox reactions impacting NAD^+/NADH ratios are important to numerous biological processes including apoptosis[55], differentiation [56], and metabolism of fats and carbohydrates [57].

In mammals, disruption of mitochondrial metabolism and NADH shuttles have been associated with age-related diseases such as diabetes [58]. Glucose metabolism controls the secretion of insulin from mouse pancreatic β-cells and reduction of NAD^+ to NADH is responsible for glucose signaling in β-cells [59]. NAD^+ is also an essential pyridine nucleotide that functions in the vital processes of DNA repair and transcription [60]. Further studies in yeast indicate that deleting NADH shuttle components abolishes CR-mediated lifespan extension whereas over-expressing NADH shuttle components extends lifespan [61]. Further studies in yeast showed that decreased NADH levels are associated with CR-induced activation of sirtuins, an event necessary for lifespan extension.

Sir2, the sirtuin or silent information regulator in *Saccharomyces cerevisiae*, is highly conserved from prokaryotes to humans [62], and catalyzes the formation of silent heterochromatin. Sirtuins are NAD^+-dependent protein deacetylases that regulate lifespan in lower organisms and also play a role in mammalian cellular fate [63], and overexpression of sirtuins has been shown to extend the lifespan of some model organisms [64, 65]. By coupling NAD^+ cleavage to modifications of target proteins, sirtuins operate as a possible molecular link between the cellular energy state and the mechanism of lifespan regulation.

The *Caenorhabditis elegans* Sir2 homolog (SIR-2.1) is required for stress resistance, genomic stability, and lifespan extension [66] and increased longevity is seen when *sir-2.1*

expression is increased [67]. Sir2 overexpression in *Drosophila* extends lifespan, however when combined with CR, over-expression did not produce an additive affect [68]. The human homolog of Sir2p, SIRT1, mediates stress resistance, apoptosis, and inflammatory responses by deacetylating transcription factors such as FOXOs [55, 69], nuclear factor Kappa β [70], and p53 [71, 72], giving cells time to repair endogenous damage. Additionally, SIRT1 knock-out mice fed a CR diet did not exhibit the typical lengthened lifespan [73]. SIRT1 overexpression not only extends lifespan and improves healthy aging, but it also reduces tumor formation [74] and regulates insulin secretion [75]. Similarly, resveratrol activates SIRT1 and also improves the health in mice fed a high calorie diet [76].

There are three mitochondrial sirtuins specific to mammals: SIRT3, SIRT4, and SIRT5. SIRT3, a mitochondrial specific sirtuin, is critical for maintaining mitochondrial homeostasis and cellular ROS levels. SIRT3 is responsible for activating isocitrate dehydrogenase by deacetylation during CR resulting in a decrease in oxidative stress and a protection from age-related hearing loss in mice [77]. CR increases activity of SIRT3 and SIRT5 aiding in the metabolic shift from glycolysis to fatty acid oxidation [78, 79] and acetate metabolism [80]. SIRT4 deacetylates glutamate dehydrogenase. SIRT4 expression decreases during CR, removing the break from glutamate dehydrogenase, which allows for the catabolism of glutamine and glutamate into the TCA cycle, providing energy during CR [81]. Recently SIRT1 has emerged as an important regulator of hypothalamus function, which regulates global metabolism and aging in mammals [82]. Histone deacetylases regulate pro- and anti-inflammatory gene expression[83] and pro-inflammatory cyclooxygenase-2 (COX-2) gene expression is suppressed by SIRT1 [84]. Increased SIRT1 activity during CR also reduces inflammation by suppressing the expression of pro-inflammatory genes such as NF-κβ, AP1, and iNOS [85]. SIRT1 is

responsible for deacetylating glycolytic enzymes resulting in the reduction of glycolytic metabolism [86] and repression of HIF-1α [87]. Piceatannol, a structural homolog of resveratrol [88], asserts a stronger effect than resveratrol [89] and acts as an anti-inflammatory compound that decreases the intracellular accumulation of ROS in PC12 cells engineered to produce high levels of Aβ, while preventing Aβ-induced apoptosis [89]. Identifying CR mimetics are highly sought after, but sirtuin activating compounds (STACs) are not the only high rollers in drug discovery.

1.5 Altering epigenetics to increase longevity

There are several compounds that have been shown to extend lifespan through epigenetic modifications DNA methylation status regulates gene expression, maintenance of DNA, and aging [90]. DNA methyltransferases (DNMT) play a crucial role in controlling age-related diseases and DNMT inhibitors have been used in the treatment of cancer [91]. The green tea polyphenol (-)-epigallocatechin-3-gallate (EGCG) reduces DNA hypermethylation induced by cancer-causing agents [92] and several studies reveal that CR decreases DNA methylation and increases genomic stability during aging [93]. Another epigenetic modification of interest is histone modification. Histone modifications include acetylation, methylation, ubiquitination, and ADP ribosylation. Of these, histone acetylation status is the most common modification associated with gene activation.

Histone acetyltransferases (HATs) and histone deacetylases (HDACs) are enzymes that alter histone acetylation. There are four classes of HDACs: class I, II, III (which includes all seven mammalian sirtuins), and IV. HAT modifications depend on nuclear acetyl-CoA concentrations, whereas class III HDAC activity is dependent upon NAD^+ concentrations. In contrast, class I, II, and IV HDACs are zinc-dependent enzymes.

Calorie restriction increases activity of SIRT1, while SIRT1 inactivation abrogates lifespan extension by CR [94]. An increase in HDAC activity during CR suggests the possibility that HDAC inhibitors may be beneficial in the treatment of aging and age-related diseases. HDAC inhibitors such as valproic acid and phenylbutyrate exhibit strong antitumor activity, yet have fairly low toxicity [95]. Curcumin effects histone acetylation status by inhibiting p300/CBP HAT [96] and has also been shown to have anti-cancer properties [97]. As previously referenced, the red grape polyphenol resveratrol is a potent SIRT1 activator and increases the mean lifespan of mice [76]. Butyrate is a short-chain fatty acid (SCFA) that is the end result of bacterial acetyl-CoA fermentation. Butyrate inhibits class I and class II HDACs and increases both *C. elegans* [98] and *Drosophila* [99] lifespans. The ketone D-beta-hydroxybutyrate is structurally very similar to butyrate and is synthesized in the liver from fatty acid oxidation of acetyl-CoA under fasting or ketogenic diet conditions. Beta-hydroxybutyrate has also been shown to be an endogenous HDAC I inhibitor [100] and so therefore may have many uses in the treatment of age-related diseases (Section 1.9). Much more work is needed to fully understand and utilize the benefits of drug induced epigenetic modifications. One exciting possibility is that these modifications are part of a larger phenomenon called hormesis and that epigenetic modifications may be involved with hormetic responses [101].

1.6 Hormesis

Recent studies challenge the theory that mitochondrial reactive oxygen species (ROS) have only a deleterious outcome on cell function and organismal aging. It is becoming clearer that mitochondrial ROS perform vital roles in activating cell survival and disease resistance signaling pathways. ROS may actually act as double-edged swords. This concept is supported by hormesis, a proposed process by which mild stress activates biological stress responses, thereby

improving the health and increasing the lifespan of an organism [102]. Activation of hormetic pathways overcome immediate stresses and may actually repair prior damage in organisms [103] by inducing the expression of genes that encode antioxidant enzymes and cytoprotective proteins (Figure 2) [104]. Proposed examples include exercise, which temporarily produces substantially higher than normal levels of damaging ROS [105], and calorie restriction, which represent a metabolic stress for organisms [106].Other hormetic response activators include low levels of ionizing radiation, which reduces the incidence of cancer [107], consumption of plant phytochemicals, which protect the plant from herbivores and pests, while producing a beneficial health response in humans [108], and exposure to mild heat, responsible for induction of gene expression leading to transcription and translation of heat shock proteins [109, 110]. Ischemic preconditioning also induces the expression of genes encoding protective proteins including heat-shock proteins (HSP) [111]. HSPs are key defense responses that enable proper refolding of damaged protein structure induced by heat, alcohol, heavy metals, age, disease, or oxidative stress, avoiding inactivation of proteins and irregular protein aggregates [112]. The *C. elegans* heat-shock factor (HSF-1) is a transcription factor that regulates response to heat and oxidative stress and serves as a molecular chaperone to slow the rate of aging [113]. More examples of hormetic response pathways include the sirtuin/FOXO pathway [114], the NF-κB pathway [115], the cAMP-response-element-binding protein (CREB) pathway, the AMP kinase pathway [116], the Nrf2–ARE pathway [108], and the DAF-16/Ins/IGF-1 signaling pathway [117]. SKN-1/Nrf is involved with multiple roles in development and cellular homeostasis. The DAF-16/FOXO family of Forkhead transcription factors act as sensors of the insulin signaling pathway and activate protective genes that increase stress resistance and longevity [118-120].

Many flavonoids, such as EGCG from green tea, and other plant-derived polyphenols may promote healthy aging through hormesis. EGCG has been shown to upregulate hemeoxygenase-1 (HO-1) expression in endothelial cells by activation of the Nrf2/ARE pathway, triggering resistance against H_2O_2 induced apoptosis [121]. Likewise, FOXO transcription factors are activated by the polyphenol resveratrol, up-regulating genes involved in energy metabolism and antioxidant pathways [122]. The *C. elegans* transcription factor SKN-1, an ortholog of Nrf2, was shown to mediate the protective responses to oxidative stress and increased lifespan induced by low doses of the plant-derived toxin, plumbagin [123]. As more evidence is published, the concept of hormesis is gaining acceptance as a major regulator of lifespan.

1.7 Hormesis hypothesis of CR

The hormesis hypothesis of CR [124] proposes that food restriction introduces mild stress to an organism and initiates upregulation of cellular defenses, while also altering metabolism, resulting in increased longevity. The hormesis hypothesis of CR was recently altered to include xenohormesis, which refers to the idea that organisms recognize and react to chemical signals produced by other organisms undergoing stress, such as CR.These chemicals can be ingested through food, as in the case of polyphenols [125], and can lead to the activation of internal defense mechanisms [126].The hormesis hypothesis of CR unites much of the unrelated observations originating from different species and laboratories. CR prompts an upregulation of longevity genes that protect an organism and delays reproduction, which are keys to surviving through a shortage of food or specific nutrients.

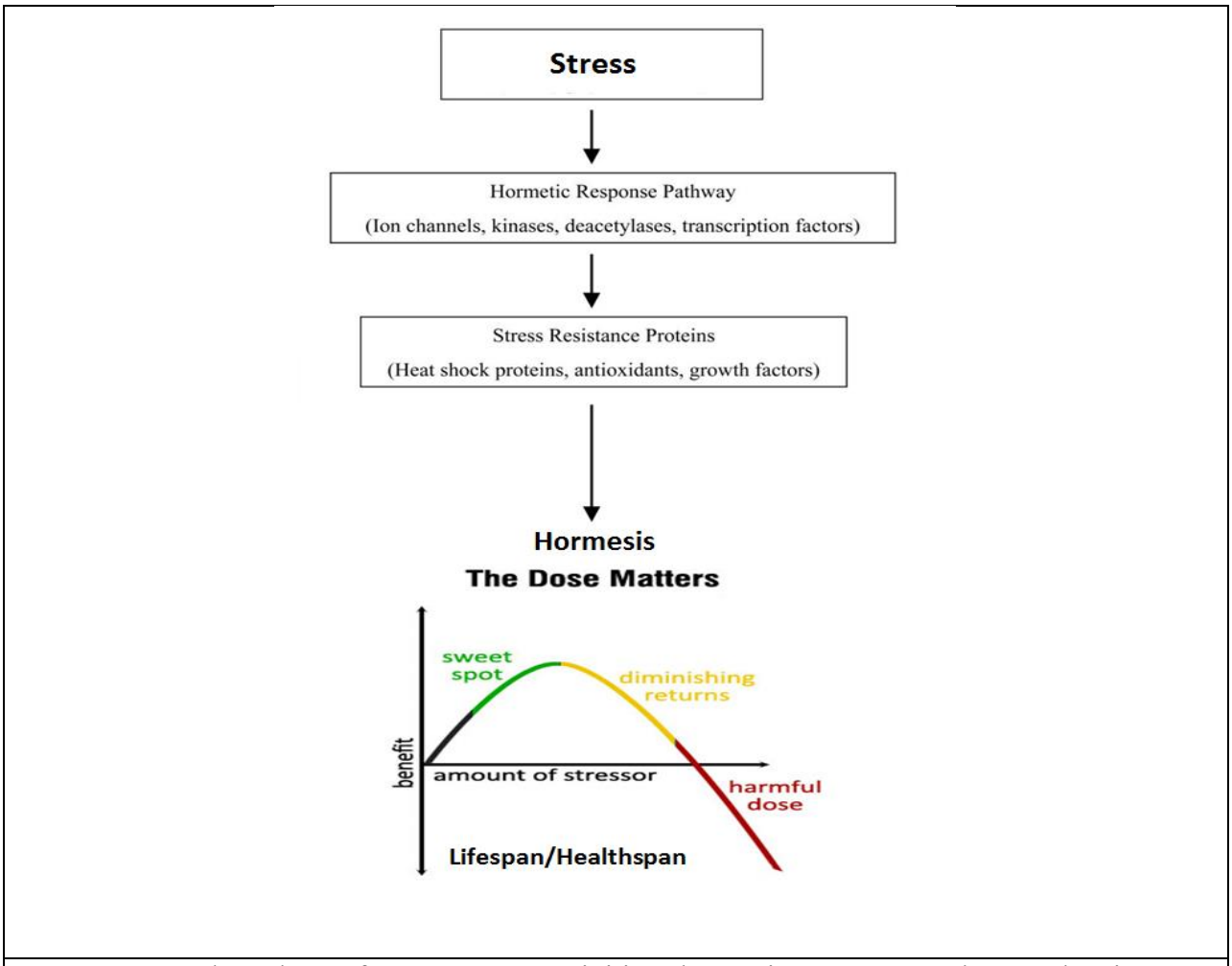

Figure 1.2: A low dose of a stressor may initiate hormetic response pathways that increase lifespan and healthspan of an organism. Too much of the stressor overrides this stress response and is deleterious to the health of the organism.

1.8 Mitohormesis

Mitohormesis is a term used to describe reactive oxygen species (ROS)-induced stress responses within mitochondria [127]. Research strongly suggests that transient endogenous free radical production, caused by glucose restriction, extends the lifespan of *C. elegans* in an *aak-2* (AMPK homolog)-dependent manner. Interestingly, this increase in lifespan is abrogated by antioxidants, suggesting that toxic radicals may mitohormetically extend lifespan and promote overall health [128].This phenomenon has also been observed in insulin-secreting cells following

exposure to sub-lethal levels of H_2O_2 [129]. The mitohormetic protective response is also activated by statins, which generate ROS and lead to an increase in peroxisome proliferator-activated receptor gamma coactivator 1α (PGC-1α) and other mitochondrial-associated genes [130, 131]. Low-dose arsenite has been shown to stimulate resistance against thermal and chemical stressors, while extending the lifespan of *C. elegans* [132] and recent evidence indicates that lipoxidative stress induced by EPA/DHA treatment may contribute to the therapeutic effects observed [133].

Mutations in mitochondrial ETC genes, encoding proteins such as ISP-1 (of complex III) and NUO-6 (of complex I) result in lifespan increases in *C. elegans*. These mutations lead to an increase in superoxide production, although a rise in overall ROS was not detected [134]. Longevity was abolished upon use of antioxidants, again supporting the idea that gradual increases in ROS and minor mitochondrial damage activate mitohormesis [134]. Additionally, oxidative stress-induced hormesis by a transient hypoxia treatment is associated with a reduction of mitochondrial superoxide production through activation of the Ins/IGF-1 signaling pathway [135]. Interestingly, lifespan extension by TCA metabolites may be due to activation of hormetic stress response pathways in conjunction with anaplerosis. The addition of malate to *C. elegans* media leads to lifespan extension and a dramatic increase in NAD+ [136], which may lead to SIRT1-mediated deacetylation and activation of PGC-1α in mammals [137]; PGC-1α upregulates antioxidant and mitochondrial metabolic protective genes in response to CR and exercise [137].

1.9 TCA cycle metabolites

The TCA cycle is a central pathway for the metabolism of sugars, lipids, and amino acids. Although many factors contribute to aging, dysregulation in metabolism has been indicated as a

contributing factor of sarcopenia, the loss of muscle mass, in the elderly [138]. The aging process in *C. elegans* is associated with a disruption in metabolism that could partially be corrected by metabolite supplementation. For instance, the long-lived dauer life cycle stage is accompanied by a metabolic shift toward anaplerosis of the TCA cycle [139]. Metabolomics data from our lab identifies a change in certain *C. elegans* metabolite concentrations with age, including the TCA cycle metabolites citrate, which was upregulated in aged worms, and fumarate and malate, which were downregulated in older worms. Consistent with these findings, we also established that supplementation of fumarate and malate to *C. elegans* diet extends lifespan through the DAF-16 and SIR-2.1 pathways [136]. Fumarate reduction, glyoxylate shunt activity, and mild mitochondrial uncoupling may also contribute to the lifespan extension induced by malate and fumarate by increasing the amount of oxidized NAD and FAD cofactors [136]. Addition of the TCA cycle intermediate oxaloacetate has been shown to extend lifespan in *C. elegans* through an *aak-2*/AMP kinase and *daf-16*/FOXO-dependent mechanism [140] and protect against seizures induced by kainic acid in rodents [141].

The TCA cycle intermediate alpha-ketoglutarate is the entry point in the citric acid cycle for glutamate and glutamine. Alpha-ketoglutarate can cross the blood-brain barrier and then be converted to glutamate, a precursor of the antioxidant glutathione. By inhibiting ATP synthase and target of rapamycin (TOR) kinase, alpha-ketoglutarate dramatically extended the lifespan of *C. elegans*. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a neurotoxin that causes a mitochondrial complex 1 deficit similar to that observed in Parkinson's disease. Alpha-ketoglutarate was found to protect against cell death induced by MPTP [142] and seizure induction by kainic acid [141]. A high dose of arginine alpha-ketoglutarate is found in the Deanna Protocol, which combines a ketogenic diet with natural supplements, and has been

shown to postpone disease progression in an Amyotrophic Lateral Sclerosis (ALS) mouse model [143].

Remarkably, mice overexpressing wild-type frataxin showed an increase in mitochondrial metabolism prompting mitohormesis. This increase in mitochondrial energy metabolism improved survival and protection from doxorubicin-induced cardiac myopathy [144]. Reducing the metabolic requirements of the mitochondria through decreased substrate metabolism is thought to lead to a decrease in cellular damage and ROS production, and CR, which prevents aging-related mitochondrial dysfunction, also increases lifespan. The prevention of aging-related mitochondrial energy deficits or redox imbalance are possible mechanisms of TCA cycle metabolite-induced lifespan extension. Mitochondrial electron transport chain function declines by over 2-fold during aging with a 5-fold decline in cellular ATP levels [145]. Given the role of specific metabolites as activators of hormesis, TCA cycle metabolites may also act in a similar manner.

1.10 Amino Acids, disease, and aging

Of all of the classes of metabolites in the cell, current knowledge suggests that cellular levels of amino acids may affect longevity the most, at least in short-lived model systems. Amino acids are degraded into mitochondrial TCA cycle intermediates [146], many of which have been shown to extend lifespan in *C. elegans* including oxaloacetate [147], malate, and fumarate [136], as well as related metabolites pyruvate [148] and acetate [149], which can be directly converted into the TCA cycle metabolite acetyl-CoA. Amino acids are likely signaling through distinct pathways to affect lifespan. Therefore consuming an optimized ratio of amino acids in the diet is hypothesized to be one possible way for increasing lifespan. Metabolomics analysis of worms has found that many amino acids, including glutamine, valine, and isoleucine

were increased in long-lived *daf-2* insulin receptor mutant worms, while glutamate levels declined [150]. Valine and isoleucine levels returned to wild-type levels in *daf-2*; *daf-16* double mutants as did lifespan. These results suggest that increased valine and isoleucine levels may mediate lifespan extension in *daf-2* mutants in a *daf-16* dependent manner. Amino acids were once commonly thought of as only nutrients and not signaling molecules, but this idea is not only incorrect, it undermines how functionally diverse these compounds really are.

1.10.1 Alanine

Alanine is a non-essential amino acid and also one of 13 exclusively glucogenic amino acids, meaning it is readily converted to glucose within the liver [151]. Alanine is crucial for regulating nitrogen and glucose levels in the body. Following entry into the liver alanine is converted to pyruvate, which participates in gluconeogenesis to supply energy to the TCA cycle. In a fasted state skeletal muscles release profuse amounts of alanine and glutamine into the circulating blood [152]. Additionally, alanine was shown to strengthen the immune system and increase aerobic and anaerobic endurance in long distance runners [153]. More recent studies indicate that alanine assists in the secretion of glucose [154] and supplementation may enhance protection from cardiovascular diseases [155].

1.10.2 Arginine

Arginine is a semi-essential amino acid that plays important roles in nitric acid synthesis, [156] growth hormone secretion [157], and in the removal of excess nitrogenous waste through the urea cycle [158]. Arginine is found in large concentrations in the skin and muscles and is responsible for stimulating the immune system to aid in rapid wound healing [159]. Arginine was also shown to stimulate repair in aged coronary endothelial tissue in humans [160] and also improved depressed macrophage function following trauma-induced hemorrhage in rats [161].

1.10.3 Asparagine

Asparagine is a non-essential amino acid that is important for regulating metabolism and maintaining central nervous system equilibrium. Interestingly, depletion of asparagine by asparaginase resulted in increased survival among lymphoblastic leukemia patients [162], which may be in part due to cell cycle inhibition created by asparagine deprivation [163]. There remains a large gap of knowledge on the effects of asparagine when taken as a supplement.

1.10.4 Aspartic Acid

The non-essential amino acid, aspartic acid, participates in a range of important cellular functions, such as lowering blood ammonia by way of the ornithine cycle and it is often prescribed for fatigue [164]. Aspartic acid is important for generating cellular energy through the TCA cycle by transporting NADH equivalents into the mitochondria, and within the brain aspartic acid acts as a neurotransmitter [165] to regulate synaptic plasticity [166]. Nevertheless increased amounts of aspartic acid may contribute to cognitive impairment by increasing damage to hippocampal neurons [167].

1.10.5 Cysteine

Cysteine is a non-essential amino acid that promotes the production of T4 lymphocytes, which are necessary for immune system activation [168]. An increase in oxidative stress associated with aging results from the decline of antioxidants, such as reduced glutathione [169], which is synthesized from cysteine, glycine, and glutamate. Although these three amino acids parallel the decline of glutathione with age, supplementation with cysteine and glycine resulted in an increase in plasma glutathione levels matching that of young controls. This elevation of amino acids corresponded with a marked decrease in oxidative damage to tissues [170], emphasizing the importance of amino acid supplementation in alleviating age-related decline.

1.10.6 Glutamine

Glutamine is the most abundant essential amino acid in the body and exists as a prominent source of cellular energy as well as an important anaplerotic donator of carbon to the TCA cycle [171]. Glutamine inhibits fatty acid oxidation, reduces blood plasma glucose, and attenuates weight gain in mice prone to obesity [172]. Following prolonged exercise, an increased T-helper cell/T-suppressor cell ratio was seen corresponding to a decrease in infections in athletes supplementing with glutamine [173] and long-term supplementation may be beneficial for maintaining intestinal immunity in the elderly [174]. Glutamine regulates genes involved with cellular proliferation and survival, as well as genes involved with inflammation, such as $NF_K\beta$ [175]. Additionally, glutamine is synthesized from glutamate through glutamine synthetase, which is found in much higher concentrations in the brain [176] and is crucial for both brain development and protection [177]. Glutamine synthetase is increased in AD patients [178] and supplementation with glutamine was shown to be neuroprotective in the disease [179].

1.10.7 Glutamate

Glutamate is a non-essential amino acid often used to enhance the flavor of foods. This amino acid acts as the main player in learning, cognition, and memory and is the most excitatory transmitter in the brain. Glutamate is required for synaptic transmission, but at high levels glutamate induces neuronal excitotoxicity, contributing to neuronal injury in neurodegenerative disorders and diseases including AD [180] ALS [181], mental illness, and brain damage[182], as well as causing myelin damage [183].The crucial balance between low extracellular concentrations and high intracellular concentrations of glutamate are kept in check by glutamate transporters. Much research focuses on finding compounds that can reduce glutamate induced excitotoxicity. Oxaloacetate injection was shown to reduce glutamate levels in rats [184] and in a

separate study it protected the brain following ischemic stroke through the stimulation of oxaloacetate transaminase, responsible for clearing glutamate from the blood [185]. Dopamine, through regulation of calcium hemostasis, was shown to inhibit glutamate excitotoxicity and prevent neuronal death [186]. Although dietary monosodium glutamate (MSG) has gained a bad reputation, it still is not known if the small amount used in food is actually harmful. If so, it is possible that the sodium may be more harmful than the glutamate.

1.10.8 Glycine

Glycine is the smallest of the amino acids and is considered conditionally essential, meaning that there are situations when synthesis of it is limited in the body. One group found that dietary glycine supplementation mimicked lifespan extension by dietary methionine restriction in rats through the clearance of hepatic methionine [187]. Glycine and cysteine supplementation increases glutathione synthesis resulting in a decrease in oxidative stress [188], an increase in insulin sensitivity [189], and may protect tissues from the side effects of radiotherapy [190]. A recent study showed that mitohormesis promoted skeletal muscle cell survival by triggering the serine/1-carbon/glycine pathway to increase protein turnover and amino acid metabolism [191].

1.10.9 Histidine

Histidine is an essential amino acid that acts as a potent anti-glycating agent, free radical scavenger [192], and a powerful metal chelator in plants [193] and mammals [194]. Histidine supplementation is associated with increased insulin secretion and glycemic control, increased glutathione peroxidase activity in diabetic mice, and decreased pro-inflammatory cytokine levels [195]. Histidine has also shown efficacy in treating chronic alcoholic liver injury by increasing glutathione levels and by enhancing catalase activity [196]. In mammals cysteine, histidine, and

glycine addition inhibited NF-κB activation and the expression of E-selectin, which is expressed only on endothelial cells activated by cytokines, suggesting that these amino acids may demonstrate anti-inflammatory effects during endothelial inflammation [197]. Histidine also acts as an antioxidant and scavenger of singlet oxygen and hydroxyl radicals [198] and supplementation was beneficial in treating chronic kidney failure in elderly patients [199]. Although histidine administration shows promise in many areas of health, its supplementation to the diet showed no effect on the mean lifespan of *Drosophila* [200], nor have studies, except my recent study in *C. elegans*, focused on its ability to affect lifespan in other model organisms.

1.10.10 Isoleucine

Isoleucine, another essential amino acid, is part of the branched-chain amino acid (BCAA) family and is best known for its ability to repair injured muscle tissue and stimulate muscle synthesis [201]. Isoleucine stabilizes energy levels by stimulating glucose uptake into skeletal muscle cells, a process mediated by phosphatidylinositol 3-kinase [202]. High blood levels of isoleucine have been associated with hypoglycemia in rats [203], but little research involving life span or health span exists.

1.10.11 Leucine

Like isoleucine, leucine is an essential branch-chained amino acid that works with isoleucine and valine to repair muscle tissue. A diet high in the BCAAs leucine, isoleucine, and valine is associated with longevity and protection from disease [150]. Leucine, isoleucine, valine, and threonine have all been shown to extend chronological longevity in yeast [204]. Leucine is an exclusively ketogenic amino acid, so it is broken down into acetyl-CoA and potentially ketone bodies if glucose levels are low. It is the major amino acid regulating the rate of protein synthesis by the ribosome. The enhancement of mitochondrial biogenesis, cell growth, and differentiation

driven by leucine occurs through the protein kinase mTOR (mammalian target of rapamycin) [205], which stimulates cell growth and division. mTOR also increases PGC-1α levels in some tissues to stimulate mitochondrial function to match the energy supply with the demand [206]. Administration of leucine prevents muscle degeneration *in vitro* [207] and may increase overall muscle anabolism in humans [208, 209]. Leucine promotes fatty acid oxidation, stimulates mitochondrial biogenesis, increases NAD+ levels, upregulates SIRT1 activity, and promotes AMPK signaling in skeletal myotube cells [210]. Although leucine is commonly used by body builders for growth, there is little evidence to support the long-term use of leucine supplementation for the amelioration of sarcopenia in the elderly [211].

1.10.12 Lysine

Lysine, the other fully ketogenic amino acid, is an essential amino acid with the capacity to interrupt replicating viruses and is often used for the treatment of Herpes simplex virus infections [212]. Lysine inhibits Herpes viral growth by blocking and preventing arginine absorption necessary for replication [213]. Another vital job of lysine is the regulation of neural plasticity in the prefrontal cortex and the release of noradrenaline from the hypothalamus [214]. Supplementation with lysine not only improves symptoms of schizophrenia, but increases problem solving speed and capacity in patients [215].

1.10.13 Methionine

Methionine is an essential amino acid. Reducing its dietary levels in rodents has been shown to partially mimic CR and induce lifespan extension. Methionine restriction can also occur when people adhere to a strict ketogenic diet due to the low protein intake prescribed to this diet, and the addition of methionine to the diet of ketogenic fed mice resulted in reversal of weight loss [216]. A diet deficient in methionine is associated with many positive effects such as

enhanced fatty acid oxidation, increased energy, decreased ROS production, reduced oxidative damage [217]. Most notably, methionine restriction extended lifespan in mice by nearly 7% [218], rats by 44% [219], and drosophila by 36% [220]. Similar to CR, methionine restriction produced a lifelong reduction in fatty body mass [221]. Unexpectedly, glycine supplementation mimics the effects of methionine restriction though the clearance of hepatic methionine [222]. Remarkably, when fruit flies were placed on a protein-deficient diet that extended lifespan, the addition of methionine could restore fecundity without decreasing lifespan, suggesting that it may be possible to design a modified amino acid diet that offers the positive effects of dietary restriction without the negative effects [223].

1.10.14 Phenylalanine

Phenylalanine is an essential amino acid that is converted to tyrosine by phenylalanine hydroxylase. Phenylalanine is necessary for proper central nervous system and memory function and was protective against acetylcholine reduction generated by hydroxide radicals [224], which may be beneficial for treating AD related acetylcholine deficiency. Many foods such as meat and most cheese products contain normal and safe amounts of phenylalanine. However, increased phenylalanine has not been associated with benefits; on the contrary, elevated serum phenylalanine has been linked to inflammatory disease [225] and was recently shown to be raised in AD [226].

1.10.15 Proline

Proline is a non-essential amino acid that is broken down into glutamate and, along with glycine, is one of the main components of mammalian collagen. Dietary proline supplementation stimulates the immune system [227]. The topical application of proline to cutaneous wounds led to a rapid acceleration of healing in rats [228]. Further, proline was shown to extend the lifespan

of *C. elegans* by transiently increasing ROS levels, which then stimulated mitohormetic pathways to upregulate superoxide dismutase and catalase [229].

1.10.16 Serine

Serine is a non-essential amino acid that is required for sphingolipid synthesis as well as neuronal survival [230].L-serine is synthesized from 3-phosphoglycerate dehydrogenase, whereas non-proteogenic D-serine is derived from L-serine through serine racemase [231]. Unlike L-serine, the D isoform inhibits sphingolipid synthesis [232].Deficient 3-phosphoglycerate dehydrogenase leads to a decline in L-serine levels in plasma and cerebrospinal fluids resulting in serine deficiency syndrome, which is characterized by seizures, congenital microcephaly, and retardation [233].

1.10.17 Threonine

Threonine is an essential amino acid necessary for glycine and serine production, regulation of the G_1/S phase transition, and proliferation of embryonic cells [234, 235]. Threonine supplementation in laying chickens led to an increase in egg production and improved immune response [236], whereas threonine deficiency was associated with depression and neurological dysfunction in kittens [237]. To date, few if any, studies focus on the efficacy of threonine for the alleviation of age-related decline.

1.10.18 Tryptophan

Tryptophan is an essential amino acid that was long touted as the reason for After Turkey Fatigue Syndrome, the exhaustion that many people feel after eating a large Thanksgiving dinner. Scientists realize that this fatigue syndrome is due to gluttony and not so much the trivial amounts of tryptophan in turkey, which coincidentally is no more than the amounts found in most meats. In any case, tryptophan is a vital precursor of many metabolites, including melatonin

and serotonin, which regulate immunity, inflammation, and circadian cycles. Increased tryptophan boosts neurotransmission and improves memory tests in aged rats [238], while tryptophan enriched cereals improved the sleep/wake cycle in elderly humans [239]. Serotonin production is dependent upon tryptophan levels [240] and tryptophan depletion results in increased aggression in conjunction with a decrease in pain tolerance in humans [241]. As expected, increased tryptophan reduced aggression in pigs [242], alleviated depression and aggression in people [243, 244], acted as a powerful sleep aid [245], and blocking tryptophan catabolism decreased aging and aging-related proteotoxicity in *C. elegans* [246].

1.10.19 Tyrosine

Tyrosine, a non-essential amino acid, is produced from phenylalanine. Tyrosine supplementation may ameliorate cognitive decline induced by stress and fatigue through the sparing of norepinephrine [247]. Supplementation with tyrosine in both hypertensive rats and humans led to a decrease in blood pressure [248], alleviated symptoms of nemaline myopathy [249], and enhanced dopaminergic neurotransmission in Parkinson's disease patients [250].

1.10.20 Valine

Valine is an essential BCAA that together with leucine and isoleucine are broken down to provide the body with energy. BCAA supplementation improves the quality of life and prognosis in patients suffering from hepatitis [251] and may contribute to lower obesity rates in middle-aged adults [252]. BCAA supplementation promotes survival and mitochondrial biogenesis in mice [253], whereas valine supplementation alone was protective against paraquat-induced toxicity in rats [254]. Like isoleucine, little research has been dedicated to this amino acid outside of overall BCAA activity. Elucidating metabolic byproducts and signaling pathways

through which amino acids extend healthy lifespan is not only important for delaying aging itself, but for improving the quality of life for aged individuals.

1.11 Beta-hydroxybutyrate

CR in animals is defined as a daily reduction of food intake. Intermittent fasting (IF) is associated with many of the same benefits as CR, yet IF leads to a greater increase in blood levels of the ketone body β-hydroxybutyrate (□HB) [255], which is produced by the liver when the body is in ketosis. Ketone bodies work to provide energy to the body, most importantly, the nervous system, during ketosis. Through a complex II-dependent mechanism leading to improved mitochondrial respiration and ATP production, mice treated with β-hydroxybutyrate showed partial protection from neurodegeneration and motor deficiency induced by MPTP [256]. Parkinson's disease patients treated with a ketogenic diet for one month improved their Unified Parkinson's Disease Rating Scale scores by a mean of 43% [257]. β-hydroxybutyrate inhibited the growth of malignant cancer [258] and evidence suggests that a low carbohydrate, high ketone diet can halt the growth of cancer completely [259]. Oral ingestion of medium chain triglycerides MCTs increased □HB levels and improved cognitive function in patients with AD [260]. □HB and acetoacetate have been reported to inhibit mitochondrial production of ROS in mouse neocortical neurons following glutamate excitotoxicity, by increasing the NAD+/NADH ratio and improving mitochondrial respiration [261]. Butyrate is a short-chain fatty acid that that inhibits class I and class II HDACs and increases both *C. elegans* [98] and *Drosophila* [99] lifespans. □HB is structurally similar to butyrate and has been shown to be an endogenous class I HDAC inhibitor [100]. In addition, people on a ketogenic diet show increased rates of oxidative metabolism and antioxidant defense due to a rise in fatty acid beta-oxidation [262]. A ketogenic diet, which stimulates the downstream targets of the PGC-1α pathway, protected mice from

neurodegeneration by increasing mitochondrial uncoupling protein levels [263]. The ketogenic diet also delayed progression of motor decline and neuron loss in a murine model of ALS [264]. Although, much more work is needed in the study of the protective effects of ketones and a low carbohydrate diet, there is some evidence that a high fat, medium to low protein, and very low carbohydrate diet is one possible way to delay aging-related disorders.

1.12 *C. elegans* as a model to study nutritional effects on lifespan

C. elegans nematode worms are a well-studied model organism often used in biomedical research as a model for human aging and disease and share almost all central metabolic pathways with humans. After reproduction, the worm gradually ages and dies in a similar pattern as humans [265]. Mutations in many mitochondrial-localized proteins in *C.* elegans have been shown to alter lifespan, either increasing or decreasing the rate of aging. These mutations directly impact overall energy metabolism by affecting the ETC complexes. The metabolic pathways studied in these chapters are also present in mammals, so the information gained using *C. elegans* may be applicable to human health and disease. In order to study the effect of metabolites on mitochondrial energy metabolism and the reliance on common longevity pathways, we selected the well-characterized mutant strains of *C. elegans*, *daf-16(mgDf50)*, *daf-2(e1370)*, *age-1(hx546)*, *aak-2(ok524)*, *sir-2.1(ok434)*, *eat-2(ad1116)*, gcn-2, and rsks-1 mutants, and *skn-1*/Nrf and CREB binding protein-1 (*cbp-1*) RNAi worms.

Through the use of six different stress response reporter strains of worms, potential stress responses to dietary supplementation with metabolites were monitored. These reporter strains use GFP driven by stress response promoters. We monitored expression of hsp-70::GFP (HSF-1 target), hsp-16.2::GFP (HSF-1 target), hsp-6::GFP (mitochondrial unfolded protein response), hsp-4::GFP (ER unfolded protein response), sod-3::GFP (DAF-16/FOXO reporter), and gst-

4::GFP (SKN-1/Nrf reporter). All *C. elegans* strains were acquired from The University of Minnesota's *Caenorhabditis Genetics Center.*

Amyloid-β (Aβ) toxicity is responsible for neuronal degeneration seen in AD. The transgenic CL4176 (smg-1(cc546^{ts}) I; dvIs27 [myo-3/ Aβ minigene + rol-6(su1006) marker gene] is a commonly used *C. elegans* model that employs a temperature-sensitive mutation in the mRNA system to initiate muscle expression of the $A\beta_{1-42}$ transgene, resulting in paralysis of the body wall muscle upon temperature upshift from 16 to 25 °C. In conjunction with the CL4176 strain, the CL2006 strain constitutively produces $A\beta_{3-42}$ in the body-wall muscles, resulting in a decreased lifespan and paralysis. The CL2006, CL4176, and CL802 (control) strains all contain the dominant mutant roller phenotype [rol-6 (su1066)] as morphological marker. Parkinson's disease is a neurodegenerative disease associated with the aggregation of alpha-synuclein and death of dopaminergic neurons leading to cognitive and motor decline in patients. The use of transgenic models of *C. elegans* expressing human alpha-synuclein is rapid and economical for exploring the anti-Parkinson's disease effects of compounds. The NL5901 (Punc-54::alpha synuclein::YFP+unc-119), strain is commonly used for drug screens to visualize alpha-synuclein aggregation. Identifying compounds that suppress Aβ-induced paralysis, polyglutamine paralysis, and alpha-synuclein aggregation could lead to the identification of compounds for the prevention and treatment of AD, Parkinson's disease, and ALS.

1.13 Hypothesis and objectives

C. elegans share almost all metabolic pathways with humans. Therefore, we hypothesize that *C. elegans*, both wild-type models and models displaying age-related decline related to humans, are promising candidates for screening dietary metabolites. A wide range of metabolites have been found to have altered concentrations in older *C. elegans,* which may be indicative of

an age-related change in metabolic homeostasis. We therefore proposed the following hypotheses: First, we hypothesized that alterations in metabolite concentrations may represent homeostatic dysregulation. In accordance with this hypothesis, we expected that systematically supplementing the diet of *C. elegans* with metabolites shown to have lower concentrations in older age would compensate for this dysregulation to increase lifespan. Second, we postulated that small changes in many dietary metabolites would result in minor dietary stress, resulting in a hormetic effect. As such, we hypothesized that supplementing the diet of *C. elegans* with metabolites would result in an increase in lifespan and stress resistance, with an accompanying increase in stress signaling and resistance to disease. We have determined that the TCA cycle metabolites fumarate, malate, and oxaloacetate extended lifespan in *C. elegans,* while all of the amino acids except phenylalanine and aspartate extended lifespan at least to a small extent at one or more of the 3 concentrations tested with serine and proline showing the largest effects. Although much is known about the effects of βHB on neurodegenerative and other aging-associated diseases, not much is known about its effects on aging. Moreover, the mechanisms through which βHB are protective are not entirely clear. We hypothesized that βHB would increase the lifespan of *C. elegans* and we determined the cytoprotective signaling pathways required for this effect.

The major aim of this study was to understand the mechanisms by which natural metabolites influence the lifespan of *C. elegans*.

Specific Aim1 (Chapter 3): Determine the signaling pathways through which TCA cycle metabolites increase lifespan and stress tolerance in *C. elegans.*

Aim 1.1: Determine the metabolic pathways through which addition of TCA cycle metabolites to the culture medium extend lifespan.

Aim 1.2: Determine the enzymes and signaling pathways through which TCA cycle metabolites extend lifespan through RNAi knockdown and mutant strains.

Aim 1.3: Determine the effects of TCA cycle metabolites on *C. elegans* models of neurodegenerative disease.

Specific Aim2 (Chapter 4): Determine the metabolic pathways through which the twenty amino acids increase lifespan and stress tolerance in *C. elegans.*

Aim 2.1: Outline the signaling pathways through which individual addition of each of the twenty amino acids to the culture medium at varying concentrations extend lifespan.

Aim 2.2: Determine the enzymes and signaling pathways through which amino acids extend lifespan through RNAi knockdown and mutant strains.

Aim 2.3: Determine the effects of the top performing amino acids on *C. elegans* models of neurodegenerative and diabetic disease.

Specific Aim3 (Chapter 5): Determine the signaling pathways through which βHB increases lifespan and stress tolerance in *C. elegans.*

Aim 3.1: Determine the metabolic pathways through which addition of βHB to the culture medium extend lifespan.

Aim 3.2: Determine the enzymes and signaling pathways through which βHB extends lifespan through RNAi knockdown, enzyme activity assays, and mutant strains.

Aim 3.3: Determine the effects of βHB on *C. elegans* models of diabetic and neurodegenerative disease.

1.14 Impact and significance

The overall goal of this study was to obtain a better understanding of the role of metabolites in the control of *C. elegans* aging. Metabolite supplementation may prove to be a

valuable inducer of hormesis and partially compensate for age-related metabolic dysfunction. The metabolic pathways studied in this proposal are also present in mammals, so the information gained using *C. elegans* is likely applicable to human health and disease.

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CHAPTER 2

MATERIALS AND METHODS

2.1 *C. elegans* strains

All strains used in this study were purchased from the University of Minnesota Caenorhabditis Elegans Center unless otherwise noted in Table 1. Strains were cultured at 20°C in either liquid S media or NGM agar media [1] as indicated.

2.2 Chemicals

L-malic acid was purchased from Chem-Impex International. Succinic acid and fumaric acid were obtained from Fisher Scientific. L-Amino acids were purchased from Acros Organics and Research Products International Corp. Glycine was obtained from Fischer Chemical Company. D-amino acids were purchased from P212121, LLC. When no D or L letter is present before the name of the amino acid (except for glycine), the L isomer was used. Sodium DL-3-hydroxybutyric acid (βHB), sodium butyrate, valproic (2-propylpentanoic) acid, ethidium bromide, and potassium cyanide were purchased from Acros Organics. Sodium D-3-hydroxybutyric acid and L-3-hydroxybutyric acid were obtained from Sigma. 5-fluoro-2′-deoxyuridine (FUdR) was purchased from Research Products International Corp. and Biotang, Inc. Sodium hydroxide (Fischer Scientific) was added to metabolite stock solutions to obtain a pH of 7.0.

Table 2.1 *Caenorhabditis elegans* models. *C. elegans* strains acquired and used for dissertation.

Strain	Genotype	Purchased/Gifted from
N2 Bristol	Wild isolate	University of Minnesota CGC
TJ356	daf-16::GFP zIs356 IV	University of Minnesota CGC
GR1307	daf-16(mgDf50)	University of Minnesota CGC
TG38	aak-2(gt33)	University of Minnesota CGC
DA1116	eat-2(ad1116)	University of Minnesota CGC
TK22	mev-1(kn1)	University of Minnesota CGC
CW152	gas-1(fc21)	University of Minnesota CGC
VC199	sir-2.1(ok434)	University of Minnesota CGC
RB1206	rsks-1(ok1255)	University of Minnesota CGC
RB967	gcn-2(ok871)	University of Minnesota CGC
VC983	hda-2(ok1479)	University of Minnesota CGC
RB1618	hda-3(ok1991)	University of Minnesota CGC
RB758	hda-4(ok518)	University of Minnesota CGC
RB2416	hda-10(ok3311)	University of Minnesota CGC
CL2166	pAF15(gst-4p::GFP::NLS)]	University of Minnesota CGC
SJ4100	hsp-6::gfp(zcIs13)	University of Minnesota CGC
SJ4058	hsp-60::gfp(zcIs9)	University of Minnesota CGC
SJ4005	hsp-4::gfp(zcIs4)	University of Minnesota CGC
ZG449	nhr-57p::GFP + unc-119(+)	University of Minnesota CGC
CL2070	hsp-16-2::gfp(dvIs70)	University of Minnesota CGC
LD1008	skn-1(operon)::GFP + pRF4(rol-6(su1006)	University of Minnesota CGC
PS3551	hsf-1(sy441)	University of Minnesota CGC
SM481	pha-4::GFP::CAAX + (pRF4) rol-6(su1006)	University of Minnesota CGC
CF1874	muIs84 [(pAD76) sod-3p::GFP + rol-6]	University of Minnesota CGC
ZG31	hif-1(ia4)	University of Minnesota CGC
AM140	unc-54p::Q35::YFP	Sandra Westerheide
NL5901	pkIs2386 (unc-54p::alphasynuclein::YFP (+))	University of Minnesota CGC
CL4176	smg-1ts [myo-3::Aβ1–42 long 3′-UTR]	Christopher Link
CL6049	dvls62 (snb-1::hTDP-43 + pCL26 (mtl-2::GFP)	Christopher Link

2.3 Lifespan measurements

All lifespans were cultured at 20°C unless otherwise specified.

2.3.1 Lifespan measurements in 250 mL liquid culture

The worms were bleach-synchronized as follows: 2 mL of 6% NaOCl were mixed with 1 mL of 5 M NaOH per 7.5 mL of concentrated worm suspension, and shaken for 4–7 minutes until the carcasses dissolved as monitored by direct observation. The remaining eggs were then washed 3 times by pelleting at ~1150 *g* for 2 minutes at room temperature, followed by aspiration of the supernatant and resuspension in 50 mL of 0.1 M NaCl. A final pellet of eggs was obtained by centrifugation at ~1150 *g* for 2 minutes at room temperature, followed by aspiration of the supernatant. Eggs were then added to a 250 mL liquid culture, as described above. For experiments without RNAi treatment, bacteria were heat killed at 80°C for 60 minutes. The worms were cultured at 20°C and monitored until they reached adulthood (~72 h), at which time FUdR was added to a final concentration of 400 uM. Worm viability was scored every two days by taking ten 10 µL drops (initially ~20 worms per drop) of the culture and counting the living worms under a microscope. The average number of living worms was then calculated. S medium or deionized water containing 10 mM malate, succinate, or fumarate was added back every three days to compensate for metabolism of the compounds and evaporation, and S medium containing FUdR and bacteria was replaced every 6 days. At least two replicates of each experiment were performed.

When the worm strains were sickly, limiting body movement, and survival in liquid medium, lifespans were performed using standard NGM agar media as described in [2] with 400 uM FUdR under standard conditions [3].

2.3.2 Lifespan measurements in cell culture inserts

Gravid *C. elegans* adults were bleached synchronized as previously described in 2.3.1 to yield eggs in S-medium. Most lifespan experiments were performed in liquid media using 0.4 µM or 3 µM transparent cell culture inserts (BD Falcon #353180 and #353181) in 12-well and 24-well microplates as first described in [4] on an orbital shaker at 135 rotations/min at 20°C. 12-well microplates were found preferable due to the easier visualization of the worms through the suspension of bacteria after swirling the microplate. 3 µM inserts were found preferable due to the increased *E. coli* permeability. Initially 1.25 mL of S-medium containing 9×10^9 HT115 (DE3) *E. coli* per mL was placed in each well of a 12-well microplate. Then bleach synchronized worm eggs were suspended at a concentration of 100–200 eggs/mL in S-medium containing 9×10^9 HT115(DE3) *E. coli* per mL. Lastly, a cell culture insert was placed in each well followed by 0.25 mL of the egg/bacterial suspension (25–50 eggs) into each insert (n = 3–6 wells per condition).

E. coli were grown for 20 hours in 2 L flasks with LB media. The *E. coli* were then spun down, the supernatant poured off, and the pellet frozen until use. The *E. coli* were de-thawed and heat-killed at 80°C for 1 hour with vibration using a Kendal model HB-S-23DHT ultrasonic cleaner. The *E. coli* pellets were then resuspended in an equal volume of S-medium. A 1.3 mL suspension of S-medium containing 9×10^9 HT115 (DE3) *E. coli* per mL was placed in each well of a 12-well or 24-well microplate. Bleach synchronized worm eggs were suspended at a concentration of 100-200 eggs/mL in the suspension of *E. coli* in S-medium. Lastly, a cell culture insert was placed in each well followed by 0.25 mL of the egg/bacterial suspension (25-50 eggs) into each insert (n = 3 wells per condition). Synchronized cultures of worms were placed on an orbital shaker at 135 rotations per minute at 20°C and monitored until they reached adulthood (~72 h), at which time FUdR was added to a final concentration of 400 uM. Worm

viability was scored every two days. Worms that did not respond to repeated stimulus were scored as dead and those that contained internally hatched larvae were excluded. The culture media containing *E. coli* in the 12-well plates below each culture insert (>80% of total culture media volume) was changed every 3 days.

2.3.3 High glucose lifespan measurements

C. elegans lifespan assays as described above with the addition of 40 mM glucose to the culture medium. Worms were scored for viability every day. The media containing bacteria in the wells into which the culture inserts were submerged was changed every two days.

2.4 RNAi feeding experiments

The *E. coli skn-, cbp-1, hda-1, hda-2, hda-3,* F55E10.6, *dhs-2,dhs-16, dhs-20, fum-1, gei-*7, men-*1, sdha-2,* F48E8.3, *qns-1,* and W06B3.1RNAi clones from the Ahringer *C. elegans* RNAi library (Source BioScience LifeSciences) was grown 16 hours and then 1mM IPTG was administered to the *E. coli* for the last 4 hours of growth to induce expression of the double strand RNA. Lifespan experiments were performed using live instead of heat-killed bacteria. The culture media containing bacteria in the 12-well plates into which the culture inserts were submerged was changed daily instead of every 3 days to decrease the chance of *E. coli* metabolism depleting the culture level of the supplemented amino acid.

2.5 GFP reporter strains

The GFP fluorescence of *C. elegans* populations was assayed using a Biotek Synergy 2 microplate reader. Strains were age synchronized and cultured in 12-well microplates as described above. At the L3 stage of larval development animals were treated with βHB or other compounds. Following 24 hours of treatment, worms were washed 3 times in S-medium and approximately 400 worms per 200 μL were added to a clear 96-well microplate and GFP

fluorescence was measured using 485/20 nm excitation and 528/20 nm emission filters (*n*=10 per treatment group).

2.6 Microscopy and quantification

Worms used for microscopy were anesthetized using 1 mM levamisole and transferred to agar pads with glass coverslips and analyzed using an EVOS fluorescence microscope. Comparable results were established in the absence of levamisole (data not shown). Approximately 20 worms per condition were used and all experiments were repeated at least three times. ImageJ™ software was used to quantify pixel intensities.

2.7 Thermotolerance assays

Thermoterance assays were performed on both liquid and solid media as described below.

2.7.1 Thermotolerance assays performed in liquid media

A synchronized population of N2 *C. elegans* eggs was obtained as described in 2.3.1 for the lifespan measurements. Eggs were placed in an aerated longneck glass bottle filled to 250 mL with liquid S medium and 4 g of HT115(DE3) *E. coli* with and without treatment. On day 5 of the lifespan, the worms were removed and diluted to approximately 10 worms per well in a 96 well microplate. The 96 well microplate containing treated and untreated worms was placed in an incubator at 38°C. Worms were scored for movement as a marker of survival every 20–30 minutes for 430 minutes.

2.7.2 Thermotolerance assays performed on NGM agar

A synchronized population of N2 *C. elegans* eggs were placed on treated or non-treated NGM plates and allowed to hatch at 20°C. At the L4 stage of development animals were

transferred to a 35°C incubator. Survival was scored as the number of worms responsive to gentle prodding with a worm pick.

2.8 Alpha-synuclein aggregation assays

Eggs were collected from the NL5901 strain of *C. elegans* following treatment with alkaline bleach and placed in 12 well cell culture inserts as described above, with or without amino acid treatment. Following 2 days of treatment, 400 µM FUdR was added to the inserts to prevent progeny. On day 8 worms were washed 3 times with M9 media and either placed on 1% agarose pads to slow movement or immobilized with 10 mM levamisole. Quantification of the number of inclusions expressing alpha-synuclein-YFP was measured using an EVOS fluorescence microscope. Foci larger than 2 μm^2 were counted for each group (n=30). Image analysis was performed using ImageJ^{TM} software and the assay was completed at least 3 times [5]. Statistical analysis was completed using GraphPad software and calculation of statistical significance between groups was carried out using Student's t-test.

2.9 Aβ-mediated paralysis assays

Paralysis assays were carried out as outlined in [6].Briefly, second generation synchronized gravid *C. elegans* strain CL4176 were placed on treated or untreated 6 cm NGM plates and allowed to lay eggs for 2 hours. After two hours, adults were removed and plates were placed in a 16°C incubator for 48 hours. Following 48 hours, plates were upshifted to a 25°C incubator. Scoring for paralyzed worms began 20 hours after upshift. Animals were scored for movement every two hours. Worms were considered paralyzed if they could not complete a full body movement after stimulation with a worm pick.

2.10 Polyglutamine aggregation assays

AM140 [*unc-54p::Q35::YFP*] worms were synchronized and placed onto NGM plates supplemented with either 1 mM tryptophan, 10 mM serine, or 5 mM proline. Plates were seeded with 90% heat-killed OP50 and 10% live OP50. Images were taken at Day 3 of adulthood. Progeny were avoided by picking daily after day 1. Aggregates were scored for 50 worms per condition in independent biological duplicates.

2.11 Lifespan assays using transgenic TDP-43 nematodes

CL6049 [*snb-1*::hTDP-43 + *mtl-2*::GFP] second generation synchronized gravid animals were placed on treated or untreated 6 cm NGM plates and allowed to lay eggs for 2 hours. After two hours, adults were removed and plates were placed in a 16°C incubator. At the L4 larval stage worms were transferred onto treated or untreated NGM plates with added 5-fluoro-2′-deoxyuridine (FUdR, 0.05 mg/mL) to inhibit growth of progeny. Worms were scored everyday by gentle touching with a platinum wire. Failure to respond to touch or move forward or backwards was scored as dead.

2.12 Protein assay

Protein was assayed essentially as in [7]. Briefly, one mL of worms suspended in S medium or M9 medium was snap frozen in liquid nitrogen and stored at −10°C until analysis. For analysis 500 µL of a worm suspension was sonicated on ice, using a W-380 sonicator (Heat Systems-Ultrasonics, Inc.) (5-second pulses, 50% duty cycle, max power, 12 pulses). 1.5 mL of 1:1 ethanol/acetone was added and the suspension was vortexed, and incubated for 30 minutes at 4°C. The tube was then centrifuged at 15,000×*g* for 10 minutes at room temperature. The supernatant was decanted, and the tube was inverted on a paper towel while the pellet dried. The pellet was then resuspended in 180 µL of 1 N NaOH, and incubated at 70°C for 25 minutes to

degrade lipids that could have interfered with analysis. The NaOH was then diluted with 1.26 mL of deionized water and 360 µL of 10% SDS. The sample was then mixed by inversion and centrifuged at 1,500×*g* for 2 minutes at room temperature. The protein content of the supernatant was then analyzed by the BCA assay (Pierce) according to the manufacturer's protocol.

2.13 NAD, NADH, NAD, and NADPH measurements

The *C.elegans* MH1317strain having genotype kuIs29[unc-119(+) egl-13::GFP(pWH17)] V was used. Worms were synchronized and cultured with heat-killed OP50 *E. coli* as food in the presence of no treatment, 10 mM malate or 10 mM succinate. On day 4 of the lifespan a 2 mL aliquot of each culture was snap frozen in liquid nitrogen. The samples were thawed and 50 µL was added in duplicate to the wells of a 96-well plate. NAD, NADH, NAD, and NADPH measurements were performed using the Elite Fluorimetric NAD/NADH Ratio Assay and Elite NADP/NADPH Ratio Assay Kits (eENZYME, LLC), according to the manufacturer's instructions. Fluorescence was normalized by the GFP expression of each sample.

2.14 GST-4::GFP fluorescence analysis

C. elegans of strain CL2166 having genotype dvIs19[pAF15(gst-4::GFP::NLS)] as described in [8] were used. Approximately 300 age-synchronized worms were grown in cell culture inserts in liquid culture medium containing HT115(DE3) *E. coli* as food as described above. Cultures were supplemented with 10 mM succinate, 10 mM malate, or 10 mM fumarate on day 1 of the lifespan. 10 mM paraquat was added on day 4. 24 hours later on day 5 approximately 20 adult worms from each treatment group were removed and assayed by fluorescence microscopy. Worms in the images were analyzed for fluorescence intensity following background subtraction using NIH ImageJ software version 1.44p.

2.15 DAF-16::GFP and SKN-1::GFP nuclear translocation assays

C. elegans strains N2, TJ356 (DAF-16::GFP), and LD1008 (SKN-1::GFP) were bleach synchronized and eggs were placed in 3 µM cell culture inserts with heat-killed HT115(DE3) *E. coli* in shaken 12-well plates untreated or treated (3 wells per treatment). On day 4 of the lifespan, worms were chilled on ice to slow movement and 40–50 worms per treatment group were photographed and analyzed for nuclear translocation.

2.16 Thrashing measurements

For the thrashing assays N2 worms were grown on NGM agar with live HT115(DE3) control *E. coli* or HT115(DE3) *E. coli* expressing RNAi to malic enzyme (*men-1*) essentially as in [9]. Some worms were grown in the presence of heat-killed HT115(DE3) *E. coli* with 10 mM malate, 10 mM fumarate, or 10 mM succinate, or no addition. Worms were transferred to 50 µL of S medium. After a one minute recovery period thrashes, defined as changes in the direction of bending at the mid body, were counted for 30 seconds (n = 8 for the control N2 worms and the N2 worms feeding on malic enzyme (*men-1*) RNAi-expressing bacteria and n = 16 for malate, fumarate, succinate, and control treated N2 worms).

2.17 Pharyngeal pumping measurements

Pharyngeal pumping assays were performed essentially as in [10]. Briefly, age-synchronized eggs from N2 worms were placed on 6 cm agar plates seeded with OP50 *E. coli* suspended in S medium with or without treatment. Video of 3 day old worms (n = 16 for each group) was recorded with a Scopetek 3.2 megapixel microscope eyepiece camera at a resolution of 1028×764 pixels and quality setting of 50 out of 100 in black and white. Full pumps were manually counted for 20 seconds during reduced speed video playback using the VLC media player.

2.18 Oxygen consumption measurements

Oxygen measurements were performed with live worms using two different methods.

2.18.1 Oxygen measurements using the Clark Oxygen Electrode

N2 worms were grown in 12-well cell culture plates and fed HT115 (DE3) control or F55E10.6 RNAi knockdown-expressing *E. coli* as food for 4 days in the absence or presence of 20 mM βHB. Worms were washed 4 times using M9 buffer to free them of the bacteria and then resuspended in the culture media in which they were grown except without the bacteria. The average concentration of worms was obtained by taking ten 10 μL drops and counting the number of living worms in each drop. The volume of the culture was then adjusted to obtain a final concentration of 2 worms per μL. 300 μL of the worm suspension was then added to the chamber of a Clark oxygen electrode (MT200A chamber, Strathkelvin Instruments) and the respiration was monitored for 5 minutes. The respiratory rate was normalized to protein content by performing a protein assay on the worm suspension.

2.18.2 Oxygen consumption using PreSens OxoPlates

All measurements were acquired using the PreSens OxoPlate protocol supplied with the OxoPlates. Worms were synchronized with and the eggs were placed in 12-well cell culture plates in 1mL of S-medium or appropriate amino acid with live HT115 (DE3) bacteria. The pH and volume of the media were monitored daily. At the L4 stage, worms were washed 3 times with S-medium or appropriate amino acid. Following rinsing, the worms were concentrated to approximately 10 worms per microliter and 200uL of each treatment group was placed in the OxoPlate in replicates of 3-4. Oxygen consumption was measured using 540/650 and 540/590 filters on a Biotek Synergy 2 microplate reader.

2.19 ATP assays

One mL samples of the N2 *C. elegans* cultures grown with either heat-killed *E. coli* or live RNAi-expressing *E. coli* were snap frozen on day 4 of the lifespan in liquid nitrogen. The samples were thawed and then 50 µL was added to a well of a 96-well microplate in a 1:1 ratio with 50 µL of CellTiter Glo solution (Promega, Madison, WI). The plate was shaken for 2 minutes and then incubated at room temperature for 10 minutes. Luminescence of the samples was measured in a Biotek Synergy 2 microplate reader. ATP levels were obtained through the use of a standard curve.

2.20 Mitochondrial membrane potential determination

N2 *C. elegans* were bleach synchronized and 500 eggs were placed in each well of a 12-well shaken microplate along with heat-killed HT115(DE3) *E. coli*. 24 hours later each well was treated with 100 nM tetramethylrhodamine ethylester (TMRE). In addition specific wells were treated with 10 mM malate, 10 mM fumarate, 10 mM succinate, or 10 µM FCCP (trifluorocarbonylcyanide phenylhydrazone) (3 wells per treatment). 24 hours following treatment the worms for each treatment group were washed with 10 mL of S-medium and resuspended in 5 mL of S-medium. 100 µL of worms were added to each well of a 96-well microplate (n = 6) and fluorescence was measured using a 540/30 nm excitation filter and a 590/35 nm emission filter.

2.21 BHB dehydrogenase assays

D-βHB or L-βHB dehydrogenase activity was assayed as in [11], slightly modified from the original method published in [12]. The reaction mix contained 100 mM Tris-HCl pH 8.0, 10 mM $MgSO_4$, 5 mm K^+ EDTA, 400 mM hydrazine hydrate, 1 µM rotenone, 10 mM NAD, 20 mm D-βHB or L-βHB, and 10 µL of Halt protease inhibitor cocktail (Thermo Scientific).Worms were grown with or without D-βHB or L-βHB as indicated. On day 4 of development, worms

were washed 3 times with M9 buffer to free them of bacteria and condensed to 50 worms per microliter. 1 mL of concentrated worms was subjected to 3 freeze-thaws cycles in liquid nitrogen. 50 µL of each sample (~ 2,500 worms) was added to a clear bottomed 96-well microplate, followed by addition of 100 µL of the reaction mix above. NADH fluorescence was measured kinetically for 20 minutes using 360/40 nm excitation and 460/40 nm emission filters (n=5) on a Biotek Synergy 2 microplate reader.

2.22 Statistical analysis

Kaplan-Meier survival analysis and log-rank tests were performed using Sigmaplot version 11.0. Student's t-tests and ANOVA were used in other analyses.

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CHAPTER 3

MALATE AND FUMARATE EXTEND LIFESPAN IN *CAENORHABDITIS ELEGANS*[1]

3.1 Abstract

Malate, the tricarboxylic acid (TCA) cycle metabolite, increased lifespan and thermotolerance in the nematode *C. elegans.* Malate can be synthesized from fumarate by the enzyme fumarase and further oxidized to oxaloacetate by malate dehydrogenase with the accompanying reduction of NAD. Addition of fumarate also extended lifespan, but succinate addition did not, although all three intermediates activated nuclear translocation of the cytoprotective DAF-16/FOXO transcription factor and protected from paraquat-induced oxidative stress. The glyoxylate shunt, an anabolic pathway linked to lifespan extension in *C. elegans*, reversibly converts isocitrate and acetyl-CoA to succinate, malate, and CoA. The increased longevity provided by malate addition did not occur in fumarase (*fum-1*), glyoxylate shunt (*gei-7*), succinate dehydrogenase flavoprotein (*sdha-2*), or soluble fumarate reductase F48E8.3 RNAi knockdown worms. Therefore, to increase lifespan, malate must be first converted to fumarate, and then fumarate must be reduced to succinate by soluble fumarate reductase and the mitochondrial electron transport chain complex II. Reduction of fumarate to succinate is coupled with the oxidation of $FADH_2$ to FAD. Lifespan extension induced by malate depended upon the longevity regulators DAF-16 and SIR-2.1. Malate supplementation did not extend the lifespan of long-lived *eat-2* mutant worms, a model of dietary restriction. Malate and

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fumarate addition increased oxygen consumption, but decreased ATP levels and mitochondrial membrane potential suggesting a mild uncoupling of oxidative phosphorylation. Malate also increased NADPH, NAD, and the NAD/NADH ratio. Fumarate reduction, glyoxylate shunt activity, and mild mitochondrial uncoupling likely contribute to the lifespan extension induced by malate and fumarate by increasing the amount of oxidized NAD and FAD cofactors.

3.2 Introduction

Metabolic control of the aging process is widely accepted, yet little progress has been made in this field due to the complexity of organismal metabolism. Studies of lifespan in model organisms have yielded important roles for organelles [1, 2], especially mitochondria, in regulating the aging process. The mitochondrial electron transport chain (ETC) is the main producer of damaging reactive oxygen species in the cell and therefore has the potential to regulate lifespan as postulated by the free radical theory of aging [3]. However, recently data has accumulated that questions the theory that free radicals are the main regulators of lifespan [4, 5]. Although mitochondrial-derived oxygen radicals have been questioned as the main driving force for the aging process, changes in mitochondrial metabolism almost certainly play a role. Dietary restriction (DR), which extends lifespan [6], also delays the aging-induced ETC dysfunction in rodents [7]. DR increases the NAD/NADH ratio in many tissues [8], which stimulates mitochondrial tricarboxylic acid (TCA) cycle dehydrogenases that utilize NAD as a cofactor. The increased TCA cycle function likely necessitates increased anaplerosis, important for longevity [9].

Alteration of mitochondrial TCA cycle (Fig. 1.1) function influences lifespan in *C. elegans*. For example, RNAi knockdown of aconitase or two of the subunits of mitochondrial NAD^+-dependent isocitrate dehydrogenase have been shown to increase lifespan [10, 11].

Mutations in the thiamine pyrophosphokinase gene, *tpk-1*, which converts thiamine to the essential co-factor thiamine pyrophosphate, essential for pyruvate and α-ketoglutarate dehydrogenases as well as several other enzymes, also extended lifespan [12]. Furthermore, addition of the TCA cycle intermediate oxaloacetate has been shown to extend lifespan in *C. elegans* through an *aak-2*/AMP kinase and *daf-16*/FOXO-dependent mechanism [13]. Supplementation with other metabolites that increase flux through the TCA cycle has also shown beneficial effects on lifespan. Addition of acetate [14] or pyruvate [15] or activation of pyruvate dehydrogenase with dichloroacetate [16] increased lifespan, while the addition of metabolites that feed more upstream into glycolysis, such as glucose or glycerol, decreased lifespan [17], perhaps due to increased methylglyoxal formation [18].

As a soil-dwelling nematode, *C. elegans* has evolved to be more metabolically flexible than many other multicellular organisms. *C. elegans* can survive anaerobically for short periods of time by utilizing a metabolic process known as malate dismutation (Fig. 1.1) or the phosphoenolpyruvate carboxykinase (PEPCK)-succinate pathway [19, 20]. Here, a portion of the intracellular malate is converted to fumarate and then to succinate, which can be excreted from the cell. This process leads to the oxidation of reducing equivalents providing NAD and FAD essential for cellular metabolism. *C. elegans* also has a glyoxylate shunt, not present in mammals, that converts isocitrate and acetyl-CoA to succinate, malate, and CoA using glyoxylate as an intermediate (Fig. 1) [21]. This shunt bypasses two NADH and CO_2 generating steps in the TCA cycle, conserving NAD levels and preventing carbon loss, which is advantageous for biosynthetic reactions in the cell. The glyoxylate shunt is upregulated in many long-lived *C. elegans* mutants [22].

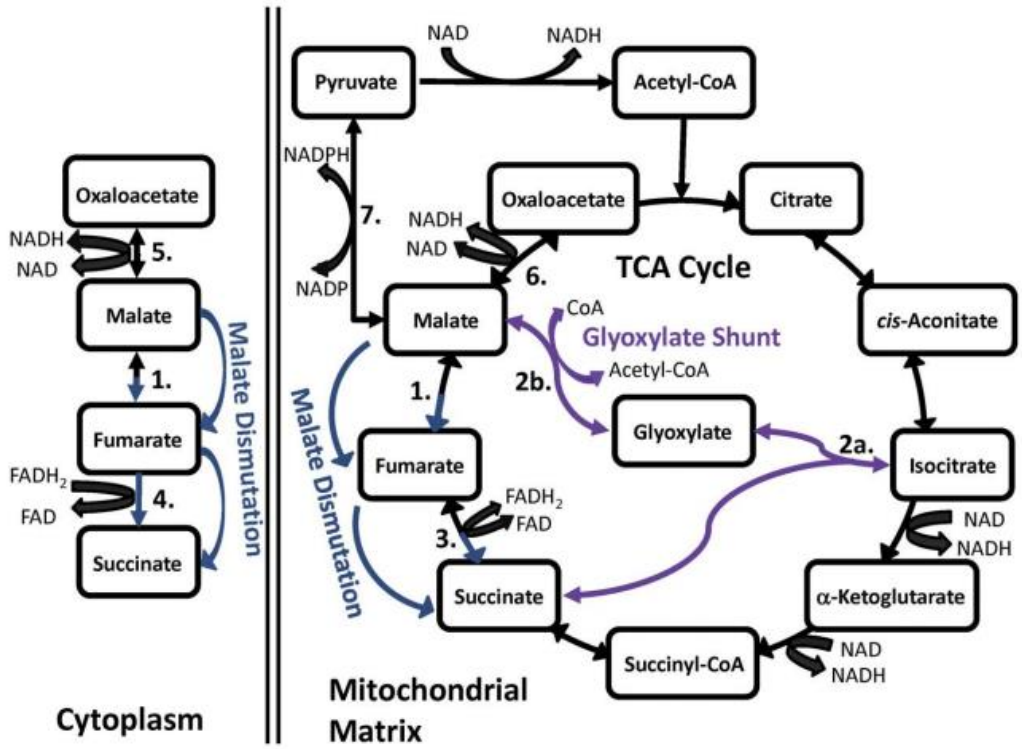

Figure 3.1. The mitochondrial TCA cycle with the glyoxylate shunt and malate dismutation. Numbered reactions are catalyzed by 1.fumarase, 2a. isocitrate lyase, 2b. malate synthase, 3. mitochondrial succinate dehydrogenase/fumarate reductase (complex II), 4. soluble fumarate reductase, 5. cytoplasmic malate dehydrogenase, 6. mitochondrial malate dehydrogenase, and 7. malic enzyme. It is unknown if the glyoxylate shuntis present in mitochondria, peroxisomes, or glyoxysomes in *C. elegans.*

In this report, we tested the effect of added succinate, fumarate, and malate on *C. elegans* lifespan, and determined the effects on mitochondrial function, redox status, and determined which metabolic enzymes and longevity pathways were necessary for lifespan extension.

3.3 Results

3.3.1 Malate extends lifespan in WT but not *eat-2, daf-16, sir-2.1, or hsf-1* mutant worms

In Fig. 3.2A we show that the addition of 10 mM L-malate and 10 mM fumarate, but not 10 mM succinate, to the growth medium of *C. elegans* increased lifespan. A summary of all lifespan experiments is shown in Table 3.1.

Table 3.1. Summary of lifespan experiments

Strain			Treatment	% of untreated mean lifespan	% of N2 mean lifespan	Worms counted[a]	Log-rank p-value
N2			malate	114	114	1,425	<0.001
N2			fumarate	116	116	469	<0.001
N2			succinate	96	96	163	0.72
N2[c]			α-ketoglu[b]	97	97	65	0.21
N2[c]			aspartate	102	102	188	0.57
N2[c]			glyoxylate	96	96	45	0.35
N2 (agar)			malate	110		51	0.04
sir-2.1(ok434)					65	45	<0.001
sir-2.1(ok434)			malate	102		36	0.97
sir-2.1(ok434)			succinate	102		25	0.88
eat-2(ad1116)					120	212	<0.001
eat-2(ad1116)			malate	110		348	0.22
eat-2(ad1116)			succinate	82		193	<0.001
daf-16(mgDf50)					59	54	<0.001
daf-16(mgDf50)			malate	97		56	0.83
daf-16(mgDf50)			succinate	103		43	0.22
aak-2(ok524) (agar)					74	20	<0.001
aak-2(ok524) (agar)			malate	108		17	0.18
aak-2(ok524) (agar)			succinate	111		19	0.10
hsf-1(sy441)[c]					91	181	0.46
hsf-1(sy441)[c]			malate	121		191	0.08
hif-1(ia4)[c]					72	242	<0.001
hif-1(ia4)[c]			malate	125		299	<0.001
hif-1(ia4)[c]			fumarate	110		313	0.02
N2	*skn-1*	RNAi			70	126	<0.001
N2	*skn-1*	RNAi	malate	109		95	0.03
N2	*men-1*	RNAi			53	224	<0.001
N2	*men-1*	RNAi	malate	130		346	<0.001
N2	*mdh-1*	RNAi			66	642	<0.001
N2	*mdh-1*	RNAI	malate	126		800	<0.001
N2	F46E10.10	RNAi			85	230	<0.001
N2	F46E10.10	RNAi	malate	108		180	0.006
N2	*fum-1*	RNAi			66	120	<0.001
N2	*fum-1*	RNAi	malate	101		125	0.49
N2	*fum-1*[c]	RNAi	fumarate	118		545	<0.001
N2	*gei-7*	RNAi			62	183	0.02
N2	*gei-7*	RNAi	malate	112		188	0.24
N2	*gei-7*[c]	RNAi	fumarate	91		115	0.02
N2	*flad-1*	RNAi			71	280	<0.001
N2	*flad-1*	RNAi	malate	160		280	<0.001
N2	*sdha-1*	RNAi			78	466	<0.001
N2	*sdha-1*	RNAi	malate	120		267	<0.001
N2	*sdha-2*[c]	RNAi			92	393	0.12
N2	*sdha-2*[c]	RNAi	malate	95		290	0.95
N2	F48E8.3[c]	RNAi			79	300	<0.001
N2	F48E8.3[c]	RNAi	malate	90		270	0.002
N2	*qns-1*[c]	RNAi			76	177	<0.001
N2	*qns-1*[c]	RNAi	malate	113		185	0.07
N2	W06B3.1[c]	RNAi			94	188	0.46
N2	W06B3.1[c]	RNAi	malate	91		163	0.07

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Malate increased mean lifespan by 14% and the increase was consistently observed ($p<0.001$) in nine total replicates using either live *E. coli* ($n=6$) or heat killed *E. coli* ($n=3$) as the food source. The increased lifespan was not due to reduced food intake from diminished pharyngeal pumping as malate treated (164 pumps per minute ± 4 SEM) showed similar rates as control (168 ± 3 SEM) worms ($p=0.37$). Fumarate increased mean lifespan by 16% ($p<0.001$ $n=4$). Interestingly, a mass spectrometry-based metabolomics analysis identified 188 total worm metabolites and indicated that there was a 2-fold reduction in fumarate levels with aging in *C. elegans* (data not shown). Therefore, added fumarate or malate may compensate for altered TCA cycle function in aged worms. Like succinate, the TCA cycle intermediate α-ketoglutarate failed to extend lifespan. Aspartate, a metabolite of the mitochondrial malate-aspartate NADH shuttle also failed to induce lifespan extension.

Malate addition was unable to extend the lifespan of *sir-2.1(ok434)* mutant worms (Fig. 3.2B). SIR-2.1 is a sirtuin family member and is the closest worm homolog of the mammalian SirT1 NAD-dependent protein deacetylase [23]. In Fig. 3.2C, it is shown that malate did not extend the lifespan of long-lived *eat-2(ad1116)* worms that have reduced pharyngeal pumping rates and are a model of dietary restriction. Treatment with succinate blocked the lifespan extending effects of dietary restriction (DR) in *eat-2* worms, as the maximal lifespan was similar as the N2 control strain and much shorter than untreated or malate treated *eat-2* worms. Fig.3.2D shows that malate did not extend the lifespan of *daf-16(mgDf50)* worms. Therefore, DAF-16 is required for malate-induced lifespan extension. DAF-16 is the worm homolog of mammalian FOXO transcription factors and is required for lifespan extension in several mutant strains, most notably in reduced insulin receptor signaling *daf-2* mutant worms. Malate also failed to significantly extend the lifespan of heat shock factor-1 mutant, *hsf-1(sy441)* worms (log-rank

p=0.08) (Figure 2E), although a protective effect occurred early in life. HSF-1 is required for lifespan extension that occurs in *daf-2* mutants [24] and in some dietary restriction regiments [25, 26].

3.3.2 Malate increased lifespan robustly in *hif-1* mutant worms and slightly in *aak-2* and *skn-1* RNAi worms

Malate and fumarate treatments resulted in increases in the lifespan of hypoxia inducible factor-1 mutant, *hif-1(ia4)* worms. HIF-1 functions downstream of Tor kinase and is necessary for the lifespan extension that occurs in mitochondrial mutants [27, 28]. Malate and succinate treatments did not significantly increase the mean lifespan of *aak-2(ok524)* AMP kinase (AMPK) mutant worms (Fig. 3.2F). But caution should be used when making conclusions from this data, due to the low number of worms used in these experiments. AMPK has been shown to be necessary for lifespan extension in worms following oxaloacetate treatment [13], resveratrol treatment, and under certain DR conditions [25]. SKN-1 is the *C. elegans* homolog of mammalian Nrf transcription factors involved in cellular detoxification, stress defense, and longevity. Malate extended the lifespan of *skn-1* RNAi nematodes by 9% (Fig. 3.3A). Consistent with this, malate, fumarate, and succinate all failed to induce nuclear localization of SKN-1::GFP (data not shown).

3.3.3 Malic enzyme or malate dehydrogenase knockdown did not block malate-induced lifespan extension

Malic enzyme catalyzes the conversion of malate to pyruvate and carbon dioxide with the concurrent reduction of NADP to NADPH.To determine if this reaction is essential for the lifespan extension elicited by malate, we knocked down the sole malic enzyme gene in *C. elegans, men-1* by RNAi and determined the effects of malate addition on lifespan.

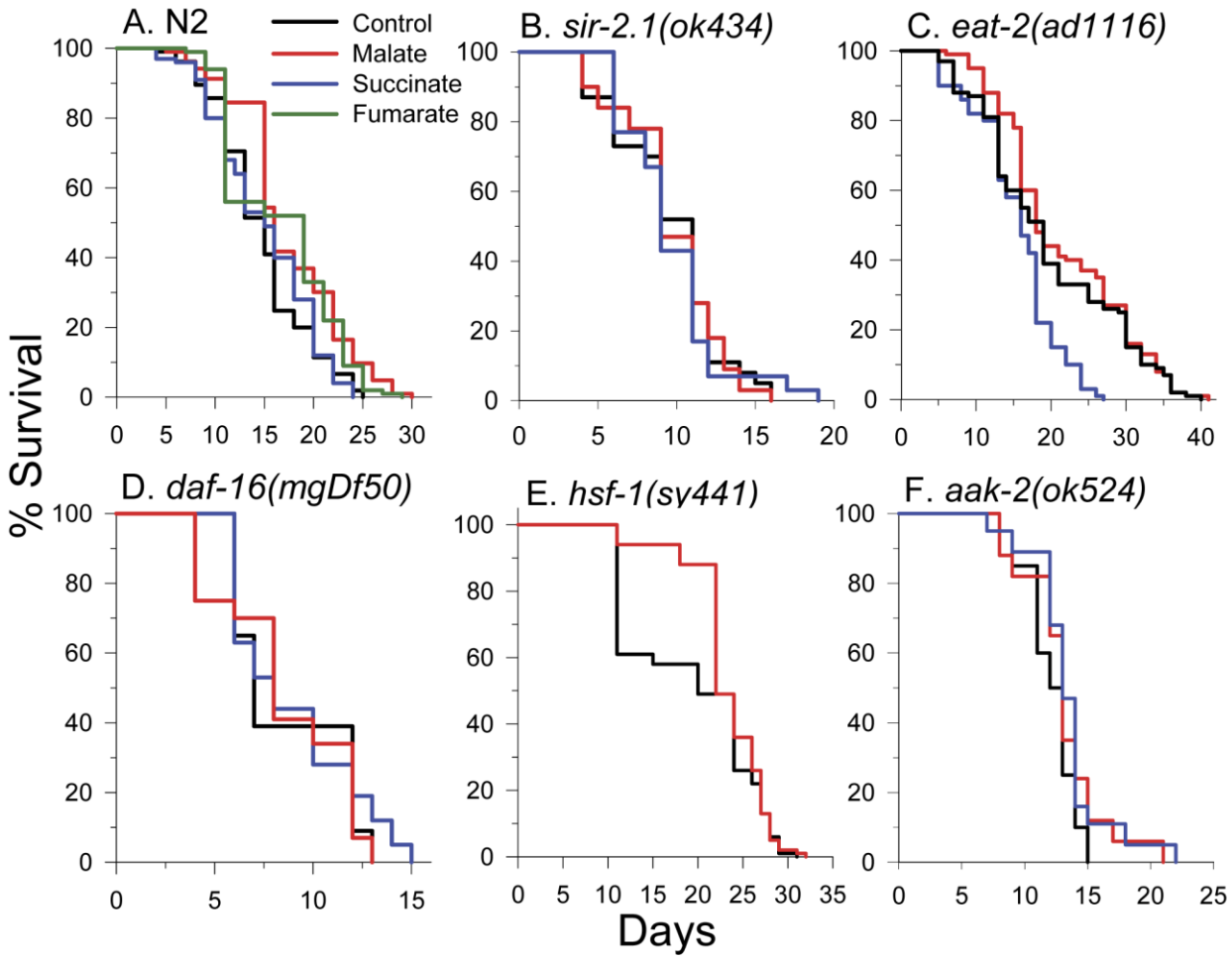


Figure 3.2. Lifespan analysis of *C. elegans* strains in the presence of TCA cycle intermediates**.** 10 mM concentrations of malate, succinate, or fumarate were present where indicated. (**A**) Malate (log-rank $p < 0.001$) or fumarate (log-rank $p < 0.001$), but not succinate (log-rank $p = 0.72$) extended lifespan of N2 worms. (**B**) Neither malate (log-rank $p = 0.97$) nor succinate (log-rank $p= 0.88$) extended lifespan of *sir-2.1(ok434)* worms. (**C**) No effect of malate treatment (log-rank $p= 0.22$) on lifespan and decreased lifespan with succinate treatment (log-rank $p < 0.001$) in *eat-2(ad1116)* worms. (**D**) Neither malate (log-rank $p = 0.83$) nor succinate (log-rank $p = 0.22$) extended lifespan of *daf-16(mgDf50)* worms. (**E**) 10 mM malate (log-rank $p = 0.08$) did not extend the lifespan of HSF-1 mutant *hsf-1(sy441)* worms. (**F**) Neither 10 mM malate (log-rank $p = 0.18$) nor 10 mM succinate (log-rank $p = 0.10$) extended lifespan of AMPK mutant *aak-2(ok524)* worms.

As shown in Fig. 3.3B, the mean lifespan of *men-1* RNAi knockdown worms was only 53% of controls, but malate addition still extended mean lifespan by 30%, suggesting that high malic enzyme activity is not important for malate-induced lifespan extension. Interestingly, malic enzyme RNAi knockdown caused an increased rate of body wall muscle contractility (210 body

bends per minute ± 12 SEM) when compared to control (147 body bends per minute ± 7 SEM) as measured by a thrashing assay ($p < 0.001$).

Malate dehydrogenase catalyzes the reversible conversion of malate to oxaloacetate with the concurrent reduction of NAD to NADH. There are two confirmed malate dehydrogenase genes in *C. elegans*. The *mdh-1* gene codes for a mitochondrial isoform, while the other gene, F46E10.10, encodes a cytoplasmic isoform. F46E10.10 is upregulated in long-lived dauer and *daf-2* worms [19] as well as long-lived mitochondrial mutants [29]. Oxaloacetate, the product of the malate dehydrogenase reaction has been shown to extend lifespan in *C. elegans*. We first knocked down the mitochondrial malate dehydrogenase, *mdh-1,* and determined the effects on lifespan in the absence and presence of malate (Fig. 3.3C).The mean lifespan of *mdh-1* knockdown worms was 66% of controls, but malate addition extended mean lifespan of the RNAi treatment by 26%, nearly back to that observed in the control worms. We then knocked down F46E10.10 and performed lifespan analysis with and without malate (Fig.3.3D). Knockdown worms showed a mean lifespan 85% of controls and malate addition extended mean lifespan of the F46E10.10 knockdown worms by 8%.

3.3.4 The glyoxylate shunt is required for lifespan extension induced by malate or fumarate

The glyoxylate shunt is composed of two enzymes, isocitrate lyase and malate synthase. In *C. elegans* these enzymes are fused into one bifunctional protein named GEI-7 or ICL-1. Isocitrate lyase reversibly converts isocitrate into succinate and glyoxylate. Malate synthase catalyzes the reversible synthesis of malate and CoA from glyoxylate and acetyl-CoA (Fig. 3.1). Since malate can be metabolized by the glyoxylate shunt we determined the lifespan of *gei-7* RNAi worms in the absence and presence of malate. As shown in Fig. 3.4A, the mean lifespan of *gei-7* RNAi knockdown worms was 62% of controls and the lifespan was not statistically different in the presence of malate (log-rank p=0.24).

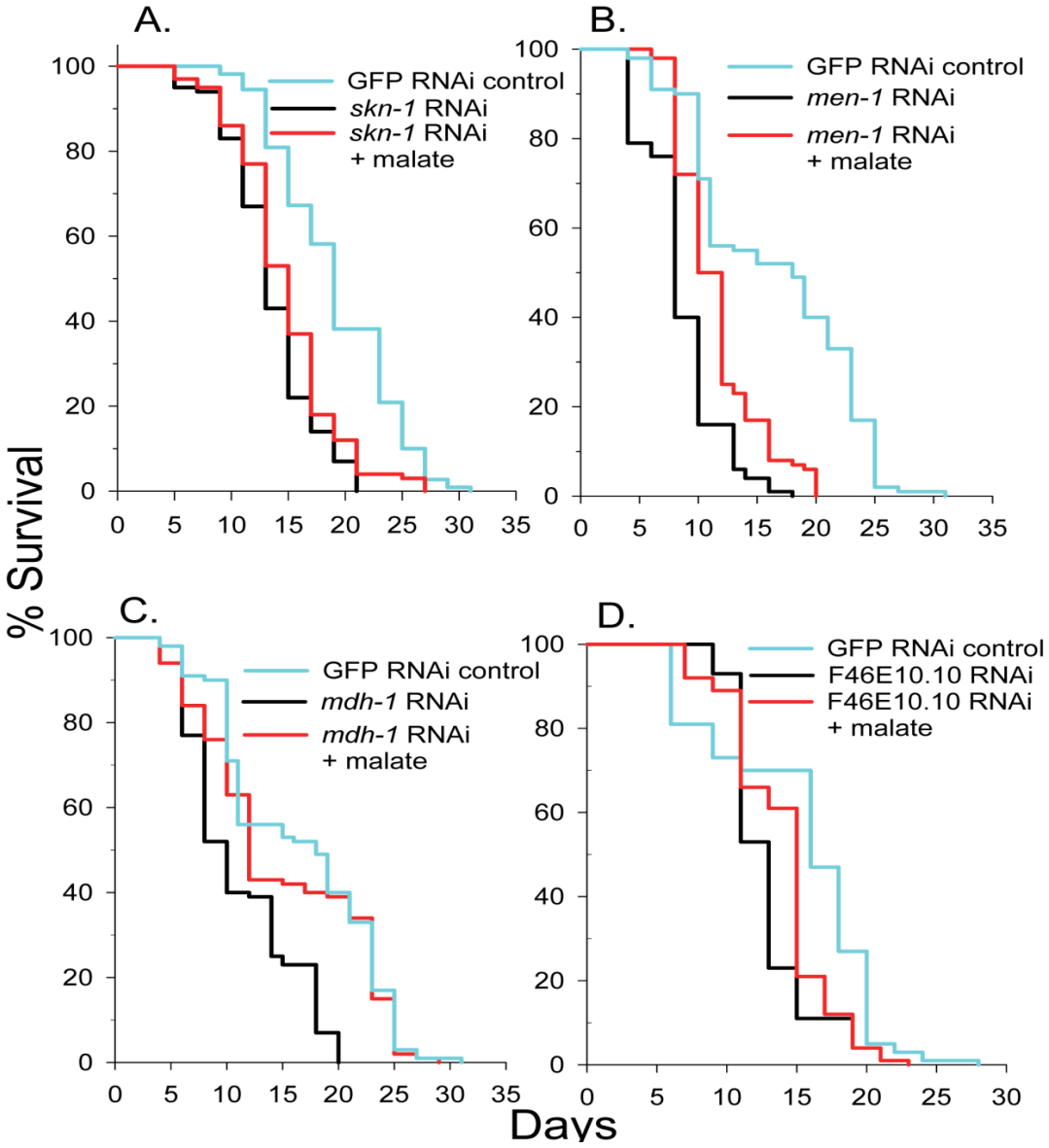


Figure 3.3. Adding malate to the media increased lifespan of C. elegans RNAi knockdown strains. (A) 10 mM malate increased lifespan of skn-1 RNAi knockdown N2 worms (log-rank p = 0.03). (B) 10 mM malate increased the lifespan of malic enzyme (men-1) RNAi knockdown N2 worms (log-rank $p < 0.001$). (C) 10 mM malate increased the lifespan of mitochondrial malate dehydrogenase (mdh-1) RNAi knockdown N2 worms (log-rank $p < 0.001$). (D) 10 mM malate increased the lifespan of cytoplasmic malate dehydrogenase F46E10.10 RNAi knockdown N2 worms (log-rank p = 0.006).

Fumarate addition also did not increase lifespan and even decreased the lifespan of the *gei-7* RNAi knockdown worms by 9% (Fig. 3.4B). Therefore, the glyoxylate shunt appears to be essential for the lifespan extension mediated by malate or fumarate treatment.

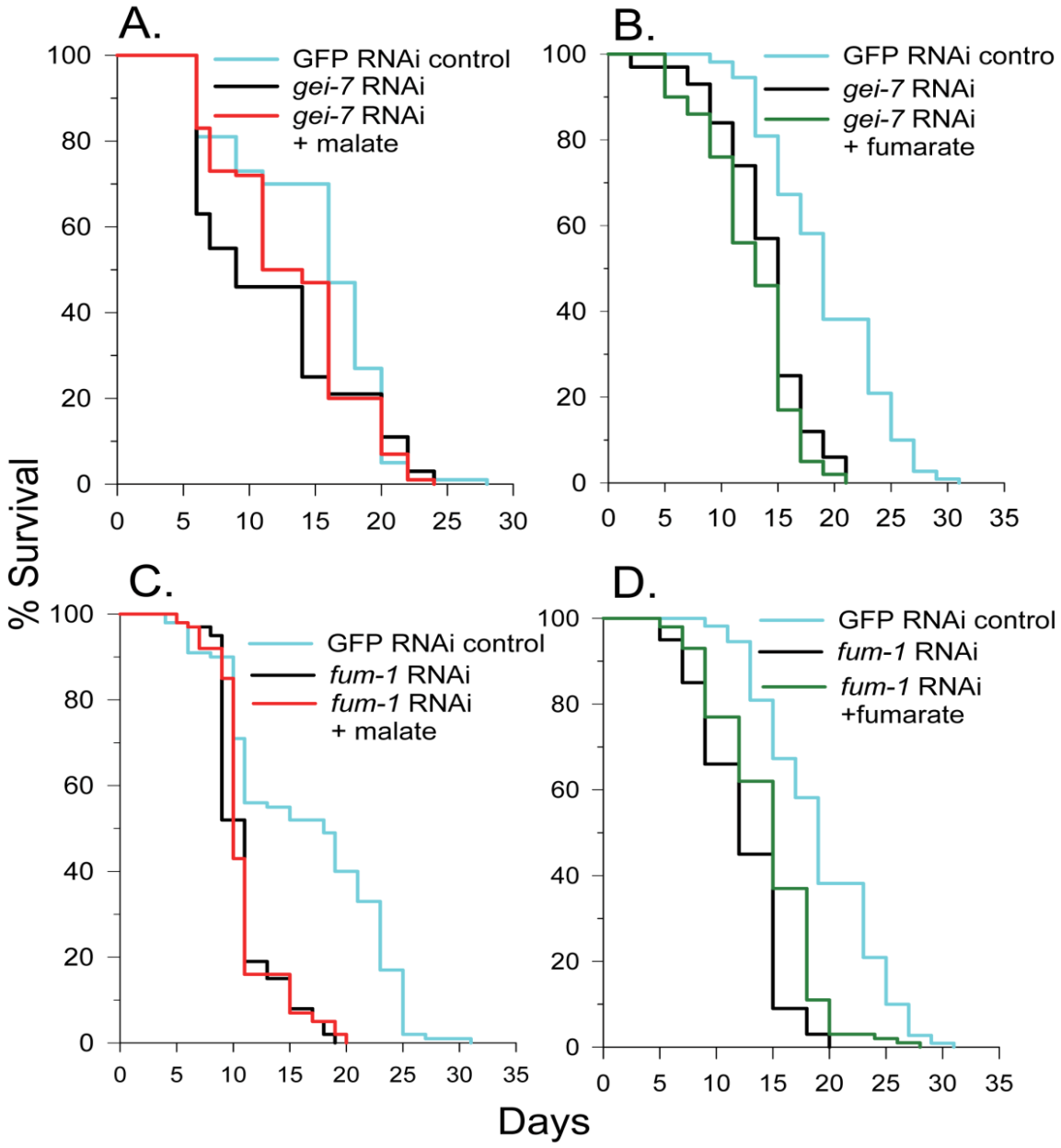


Figure 3.4. Malate-induced lifespan extension requires fumarase and the glyoxylate cycle. (**A**) 10 mM malate treatment did not alter the lifespan of glyoxylate cycle *gei-7* RNAi knockdown N2 worms (log-rank $p = 0.24$). (**B**) 10 mM fumarate treatment decreased the lifespan of glyoxylate cycle *gei-7* RNAi knockdown N2 worms (log-rank $p = 0.02$). (**C**) 10 mM malate treatment did not alter the lifespan of fumarase (*fum-1*) RNAi knockdown N2 worms (log-rank $p = 0.49$). (**D**) 10 mM fumarate treatment increased the lifespan of fumarase (*fum-1*) RNAi knockdown N2 worms (log-rank $p < 0.001$).

Interestingly, 10 mM glyoxylate failed to extend lifespan, suggesting a limited conversion of glyoxylate to malate in the worms under these culture conditions or alternatively, that the acetyl-CoA consumed during conversion of glyoxylate to malate may prevent lifespan extension.

3.3.5 Fumarase is required for lifespan extension induced by malate but not by fumarate

Fumarase catalyzes the reversible conversion of fumarate to malate and is dually targeted to both the cytoplasm and mitochondria [30]. We performed a lifespan assay using fumarase (*fum-1*) RNAi knockdown worms in the presence and absence of malate (Fig. 3.4C). The mean lifespan of *fum-1* knockdown worms was 66% of controls. Strikingly, *fum-1* knockdown prevented malate from increasing lifespan (log-rank, p=0.49). But in contrast to malate addition, fumarate addition, which is still able to be converted to succinate, did extend lifespan by 18% in *fum-1* RNAi knockdown worms (Fig. 3.4D). Therefore, added malate must be metabolized by fumarase to form fumarate, running a portion of the TCA cycle backwards, to extend lifespan.

3.3.6 Malate increased lifespan in *sdha-1* RNAi knockdown worms, but not in *sdha-2* and F48E8.3 RNAi knockdown worms

There are three separate fumarate reductase isoforms in *C. elegans*. Two of them, *sdha-1* and *sdha-2*, are exchangeable subunits of the mitochondrial fumarate reductase/succinate dehydrogenase complex II of the respiratory chain and the other is a soluble cytoplasmic fumarate reductase F48E8.3. There has been a report of a large decrease in *sdha-2* expression in long-lived dauer larvae [20], but another group found no change [19]. SDHA-2 protein levels were downregulated in long-lived *eat-2* worms [31]. *Sdha-1* expression levels were unchanged [20] or slightly decreased [19] in dauers, while F48E8.3 was strongly upregulated [19, 20]. F48E8.3 protein levels were not changed in *eat-2* worms [31]. Proteomics analysis showed an

increase in *sdha-1* levels with aging (data not shown). It was hypothesized that the *sdha-1*/*sdha-2* ratio may influence lifespan by regulating the relative fumarate reductase to succinate dehydrogenase activity of complex II [19]. However, others hypothesized that complex II flavoprotein phosphorylation may play a role in controlling the relative fumarate reductase to succinate dehydrogenase activities [32].

To determine if *sdha-1* plays a role in lifespan extension mediated by malate, we knocked it down by RNAi treatment and determined the lifespan in the absence and presence of malate. As shown in Fig. 5A, *sdha-1* knockdown worms had a mean lifespan 78% of controls and malate treatment increased mean lifespan by 20%. The data indicate that *sdha-1* does not likely play a role in lifespan extension by malate. We next knocked down *sdha-2* by RNAi and determined the effect of malate treatment. Malate addition did not significantly alter the lifespan (log-rank p=0.95) (Fig.3. 5B). We also knocked down the soluble fumarate reductase F48E8.3 by RNAi. These worms only lived 79% as long as controls. Once again, malate addition did not extend lifespan and even caused a 10% decrease in lifespan (Fig. 3.5C). These experiments further confirm the role of fumarate reduction and the malate dismutation pathway in the lifespan extending effects of malate.

3.3.7 The effect of malate on lifespan in flavin adenine dinucleotide (FAD) synthase RNAi knockdown worms

One possible difference between succinate and malate metabolism and their different effects on lifespan is that following malate conversion to fumarate, fumarate is metabolized to succinate by fumarate reductase to increase the FAD/$FADH_2$ ratio in the cell, while succinate conversion to fumarate has the opposite effect on the ratio. To gain insight into a possible role for FAD levels on lifespan extension by malate, we determined if malate could extend lifespan

when the *flad-1* gene encoding FAD synthase, the terminal step in FAD synthesis, was knocked down by RNAi. As shown in Fig. 5D, *flad-1* RNAi knockdown had a mean lifespan of 71% of the control, and malate treatment completely restored the lifespan back to that of the control. Therefore, FAD levels do not appear to be the limiting factor for malate-mediated lifespan extension. It is possible; however, that a low FAD/$FADH_2$ ratio limits normal *C. elegans* lifespan under these growth conditions and malate treatment increases this ratio to increase lifespan.

3.3.8 Malate, fumarate, and succinate treatment increased stress resistance

Since many treatments and mutations that extend lifespan also increase stress resistance, we determined if malate, fumarate, or succinate could also increase the thermotolerance of the worms or decrease oxidative stress. As shown in Fig. 3.6A, malate increased the thermotolerance, the survival time of the worms at 38°C, by 32%, while succinate (log-rank p=0.03) and fumarate (log-rank p=0.12) were less protective, only increasing thermotolerance by 13% and 10% respectively. The transcription factor SKN-1/Nrf is upregulated in *C. elegans* in response to oxidative stress and activates transcription of antioxidant genes such as glutathione-S-transferase-4 (*gst-4*). By monitoring the fluorescence of a *gst-4::gfp* oxidative stress reporter worm strain [33], we found that malate, fumarate, and succinate all decreased endogenous oxidative stress and the oxidative stress following treatment with paraquat, a stimulator of mitochondrial reactive oxygen species production (Fig. 6B). Consistent with this protection, malate, fumarate, and succinate treatment all induced the nuclear translocation of DAF-16::GFP (Fig. 3.6C).

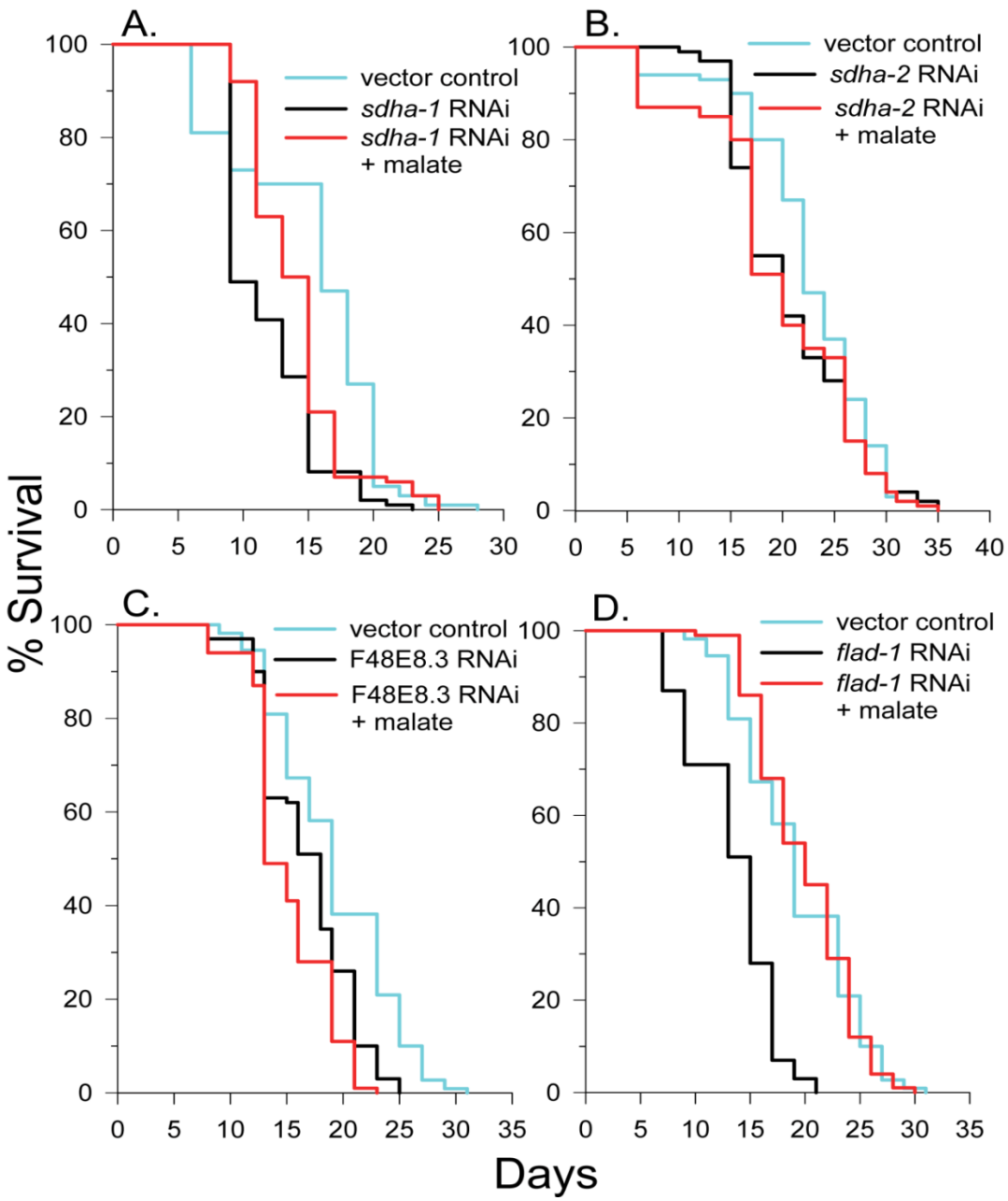
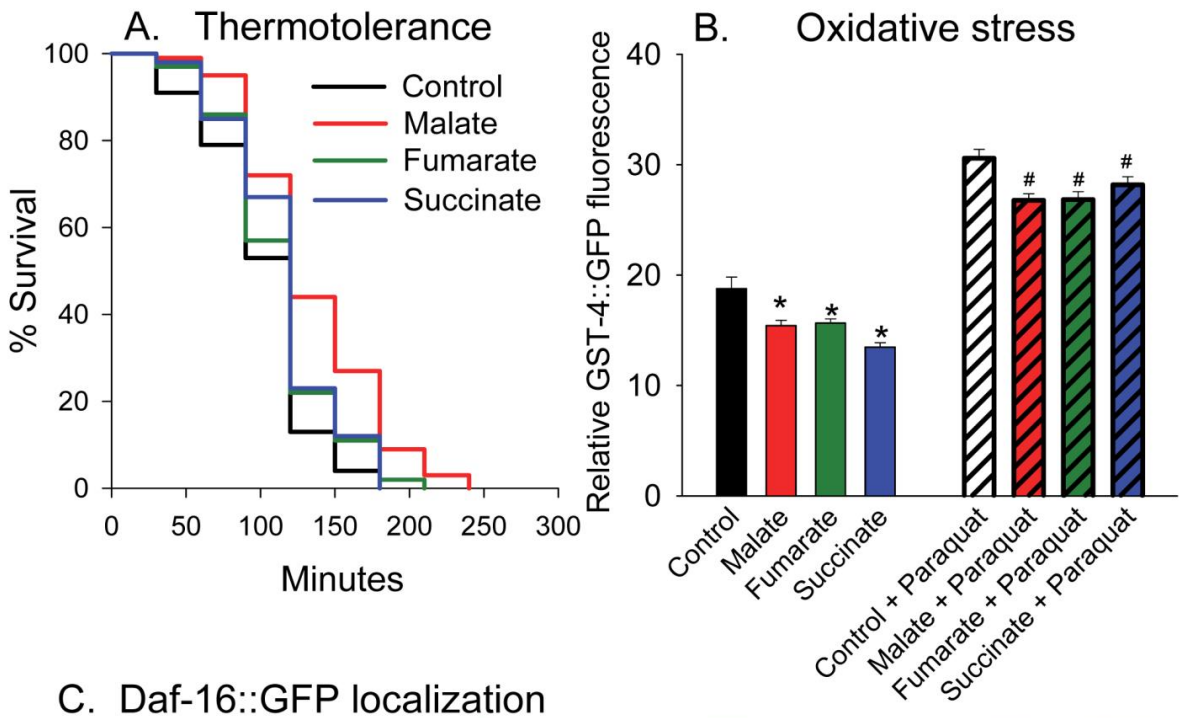
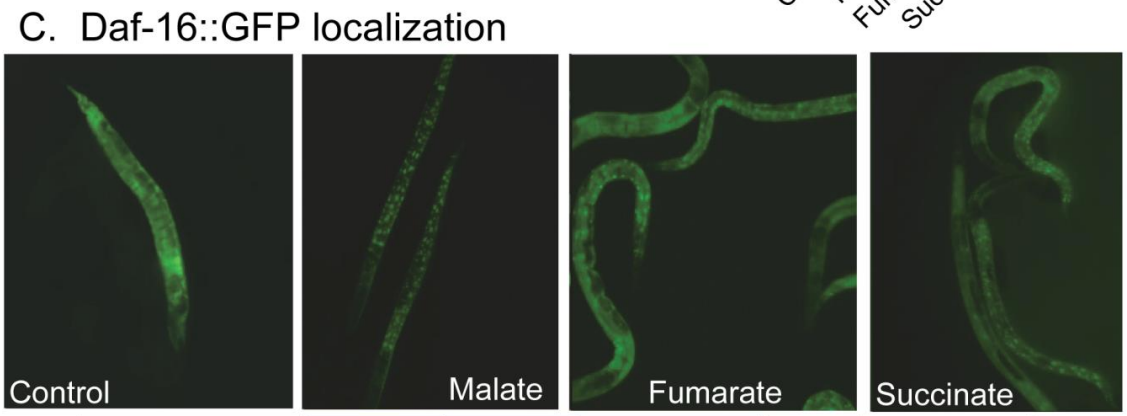


Figure 3.5. Malate treatment did not increase the lifespan of *sdha-2* and F48E8.3 RNAi knockdown worms. (**A**) 10 mM malate increased the lifespan of complex II flavoprotein (*sdha-1*) RNAi knockdown N2 worms (log-rank $p < 0.001$). (**B**) 10 mM malate did not increase the lifespan of complex II flavoprotein (*sdha-2*) RNAi knockdown N2 worms (log-rank $p = 0.95$). (**C**) 10 mM malate decreased the lifespan of soluble fumarate reductase F48E8.3 RNAi knockdown N2 worms (log-rank $p = 0.002$). (**D**) 10 mM malate increased the lifespan of FAD synthase (*flad-1*) RNAi knockdown N2 worms (log-rank $p < 0.001$).





	Cytosol	Nuclear	Intermediate		Total assayed
Control	84.2%	0.0%	15.8%		57
10 mM malate	27.7%	33.8%	38.5%		65
10 mM fumarate	28.1%	28.1%	43.8%		64
10 mM succinate	33.3%	35.2%	31.5%		54

Figure 3.6. Effects of malate, fumarate, and succinate on thermotolerance, oxidative stress, and DAF-16::GFP nuclear translocation. (**A**) 10 mM malate increased thermotolerance (log-rank $p < 0.001$), while 10 mM succinate (log-rank $p = 0.03$) and 10 mM fumarate (log-rank $p = 0.12$) had smaller protective effects. *C. elegans* were grown at 20°C and then upshifted to 38°C. (**B**) 10 mM malate, fumarate, or succinate treatment decreased GST-4::GFP fluorescence in the absence (* $p < 0.05$ compared to untreated N2) and in the presence of 10 mM paraquat (# $p < 0.05$ compared to paraquat treated N2). (**C**) 10 mM malate, fumarate, or succinate treatment increased the nuclear translocation of DAF-16::GFP.

3.3.9 Malate treatment increased the NAD/NADH ratio and decreased the NADP/NADPH ratio

An enhanced NAD/NADH ratio occurs in certain tissues during DR in rodents [8], and this ratio may be important for lifespan extension by activating sirtuins [34]. Therefore we cultured the worms for 4 days with malate or succinate and then measured NAD and NADH levels. As shown in Fig. 3.7A, malate addition greatly increased the NAD/NADH ratio.

This result was surprising given that malate metabolism through the enzyme malate dehydrogenase converts NAD to NADH, which would yield opposite results. Malate addition also strongly increased total NAD+NADH levels, which also occurs in certain tissues during DR in mice [8]. Succinate, which did not extend lifespan, showed a smaller increase in NAD levels.

To determine if normal NAD(H) levels are required for malate-induced lifespan extension we individually knocked down two enzymes in the NAD synthesis pathway by RNAi and monitored lifespan (Table 3.1). We knocked down the NAD synthetase gene *qns-1* and the *nmnat-2* gene (W06B3.1) by RNAi. Knocking down W06B3.1 decreased lifespan by 6% and fully prevented lifespan extension by malate addition, suggesting normal NAD(H) levels may be necessary for malate-mediated lifespan extension. However, *qns-1* knockdown decreased lifespan by 24%, but malate addition increased lifespan by 13% (p=0.07). This somewhat inconclusive data indicates further research is necessary to determine the exact role that NAD(H) levels play in malate-mediated lifespan extension.

We also measured NADP and NADPH levels following growth of *C. elegans* for 4 days with malate or succinate (Fig 3.7B). Malate treatment greatly increased NADPH levels to decrease the NADP/NADPH ratio, while succinate treatment also increased NADPH levels, but to a lesser extent than malate. Both treatments also increased total NADP+NADPH levels.

3.3.10 The effects of malate, fumarate, and succinate on oxygen consumption, ATP levels, and mitochondrial membrane potential

To determine if malate, fumarate, or succinate treatment had an effect on mitochondrial function, we measured worm oxygen consumption (Fig. 3.8A), ATP levels (Fig.3.8B), and mitochondrial membrane potential (Fig 3.8C). Growth in the presence of malate for 4 days led to an increase in the rate of oxygen consumption. Growth in the presence of fumarate also increased respiration, while growth in the presence of succinate decreased respiration. Culture of the worms with malate or succinate greatly decreased ATP levels, while the decrease of ATP following culture with fumarate was small. To determine if TCA cycle metabolite-treated worms increased muscle contraction to burn more ATP, we conducted thrashing experiments. Malate treated worms showed a slightly decreased rate of thrashing (84.8 ± 2.2 SEM body bends

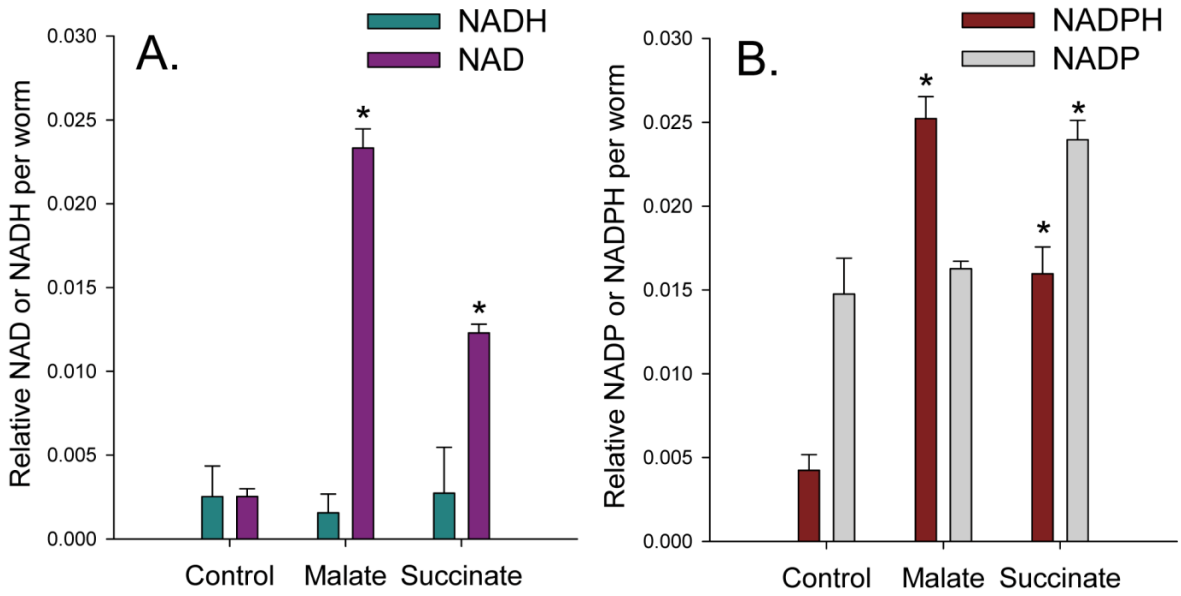


Figure 3.7. Malate treatment increased *C. elegans* NAD and NADPH levels more than succinate. **(A)** Relative NAD and NADH levels in day 4 worms cultured with 10 mM malate, 10 mM succinate, or no addition (* $p < 0.05$ compared to control). **(B)** Relative NADP and NADPH levels in day 4 worms cultured with 10 mM malate, 10 mM succinate, or no addition (* $p < 0.05$ compared to control).

per minute) compared to controls (94.4 ± 4.0 SEM body bends per minute) (p=0.04) while fumarate and succinate treatment resulted in no significant difference in the rate of thrashing (87.6 ± 3.3 and 92.8 ± 3.4 SEM body bends per minute, respectively) compared to controls. Therefore, decreased ATP levels are not a result of increased thrashing.

One possible interpretation of the respiratory and ATP results is that malate and fumarate are inducing a mild uncoupling of oxidative phosphorylation. Therefore, we monitored the mitochondrial membrane potential ($\Delta\Psi$) with the cationic fluorescent dye TMRE (Fig. 3.8C). Worms grown in the presence of malate, fumarate, or the uncoupler FCCP showed decreased $\Delta\Psi$ compared to control, with malate showing a robust decline, almost to the extent of FCCP. A non-toxic 10 μM concentration of uncoupler, a concentration that was previously shown to extend lifespan [35] was chosen. Succinate treatment showed a non-significant decrease in $\Delta\Psi$. Therefore, mitochondrial uncoupling is likely the cause for the decreased $\Delta\Psi$ and ATP levels occurring following treatment with malate and fumarate, while decreased respiratory activity is likely responsible for the decreased ATP levels in succinate treated worms.

3.3.11 Prevention of the malate-induced drop in ATP levels in *aak-2, sir-2.1,* and *hif-1* mutants and in *gei-7* RNAi knockdown worms

To discern more about the malate-induced drop in ATP levels and to gain insight into possible important upstream players of malate-induced signaling pathways, we measured ATP levels of different mutant and RNAi knockdown worms grown in the presence or absence of malate (Table 3.2). We discovered that malate addition caused a large increase in the ATP levels in *aak-2(ok524)* and *sir-2.1(ok434)* mutants, while a small increase in ATP levels was observed in the *hif-1(ia4)* mutant and *gei-7* RNAi knockdown worms. Malate treatment decreased ATP to varying extents in the *daf-16(mgDf50)* and *hsf-1(sy441)* mutant strains and the *skn-1*, *sdha-2*, and

fum-1 RNAi knockdown worms. This data suggests that SIR-2.1 and AAK-2 may be important upstream transducers of malate signaling.

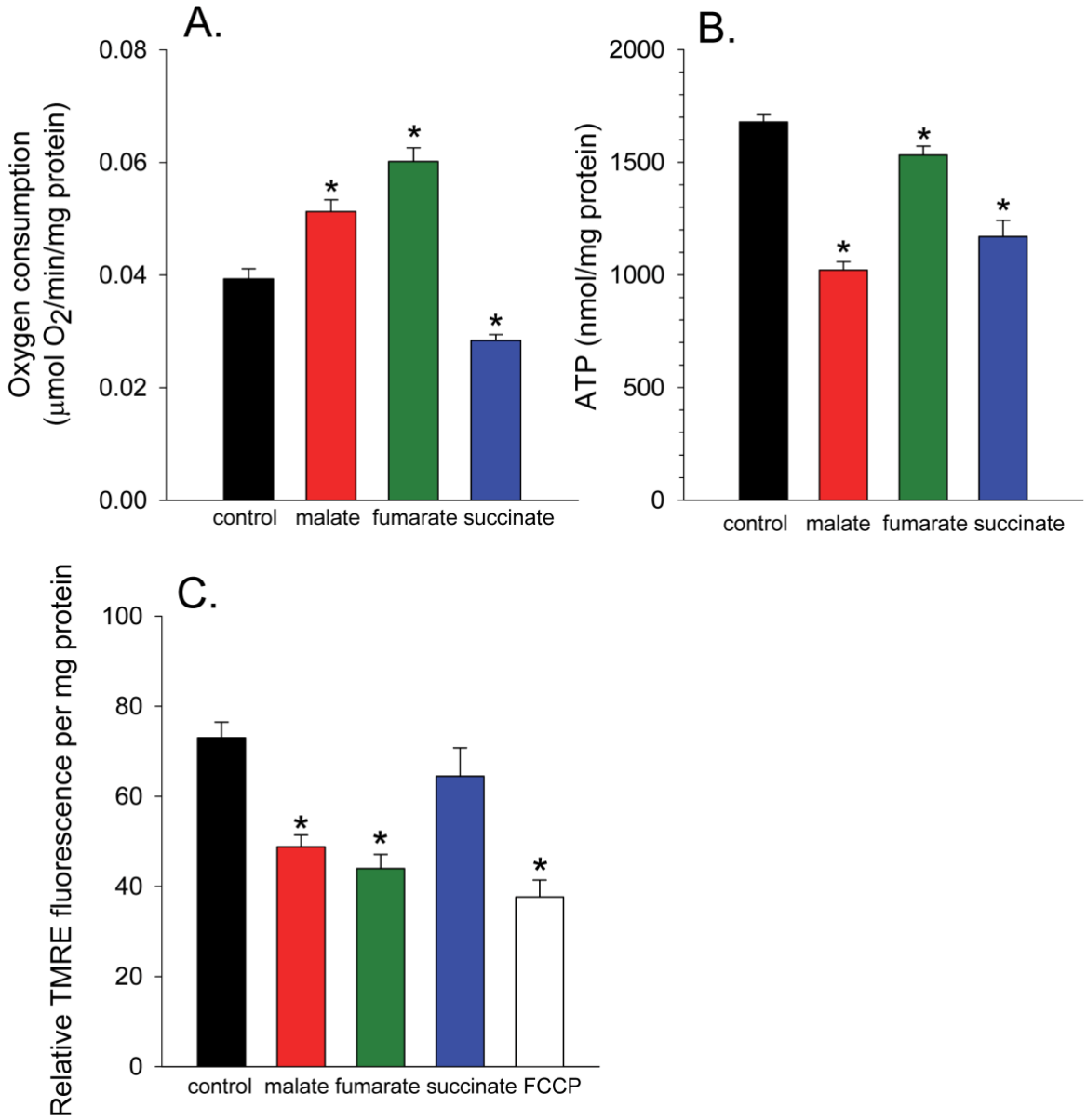


Figure 3.8. The effect of malate, fumarate, and succinate on respiration, ATP, and ΔΨ in *C. elegans*. (**A**) The effect of 10 mM malate, fumarate, or succinate treatment on oxygen consumption in day 4 N2 worms ($p < 0.001$). (**B**) The effect of 10 mM malate, fumarate, or succinate treatment on ATP levels in day 4 N2 worms ($p < 0.001$). (**C**) The effect of 10 mM malate, fumarate, or succinate or 10 µM FCCP treatment on ΔΨ in day 2 N2 worms.

Table 3.2 Effect of 10 mM malate on *C. elegans* ATP levels

Strain	RNAi knockdown	ATP following 10 mM malate (% of same strain untreated)	Standard error
N2 control		60.4%	3.3%
daf-16(mgDf50)		38.7%	1.8%
N2	*skn-1*	62.4%	1.9%
N2	*sdha-2*	64.4%	4.1%
hsf-1(sy441)		84.5%	3.0%
N2	*fum-1*	88.4%	2.7%
N2	*gei-7*	114.6%	2.4%
hif-1(ia4)		118.2%	2.5%
sir-2.1(ok434)		149.9%	2.8%
aak-2(ok524)		166.0%	5.1%

[a]Worms counted refers to the sum of the numbers counted on the first count day.
[b]α-ketoglu = α-ketoglutarate. [c]Performed in cell culture inserts.
ATP levels were measured as indicated in the Methods.
doi:10.1371/journal.pone.0058345.t002

3.4 Discussion

Mitochondrial electron transport chain function, which oxidizes NADH and $FADH_2$, decreases with age across species [36]. This leads to a decreased cellular NAD/NADH ratio in specific tissues in aged organisms. Anti-aging therapies such as DR increase the NAD/NADH ratio in many tissues as a possible mechanism to delay aging. We show for the first time that malate and fumarate addition extend lifespan in *C. elegans*, while succinate addition did not. Addition of the TCA cycle intermediates increased the NAD/NADH ratio, which may be important for the mechanism of lifespan extension. Malate and fumarate treatment also increased oxygen consumption and decreased $\Delta\Psi$, suggesting a mild mitochondrial uncoupling, while succinate treatment did not. The glyoxylate shunt and malate dismutation/fumarate reduction metabolic pathways were also necessary for lifespan extension. Activation of these pathways together with induction of mitochondrial uncoupling likely result in increased cellular NAD levels. Increased NAD levels have been described to activate the histone deacetylase SIR-2.1 [23] and AMP kinase [37] to increase lifespan.

3.4.1 Flavin and pyridine nucleotide levels in aging and lifespan extension

Since fumarate conversion to succinate by fumarate reductase also oxidizes $FADH_2$ to FAD, an increased $FAD/FADH_2$ ratio may play a role in lifespan extension. Malate and fumarate likely induce large increases in the $FAD/FADH_2$ and NAD/NADH ratios to extend lifespan, while succinate has a smaller effect on the NAD/NADH ratio and likely an opposite effect on the $FAD/FADH_2$ levels. In this regard, administration of 5 μM FAD to a short-lived *C. elegans* frataxin RNAi knockdown strain extended lifespan to an extent that surpassed the untreated control worms [38]. We also have obtained data that FAD addition to the medium extends lifespan (manuscript in preparation). FAD levels have been shown to decrease in many different tissues with age in rats [39], and levels were restored by exercise [40], which extends mean lifespan [41, 42].

Fumarate reductase has been shown to be essential for the growth of *Sacchromyces cerevisiae* under anaerobic conditions for the re-oxidation of $FADH_2$ [43]. During the dauer state and other conditions that extend lifespan, *C. elegans* transitions to a metabolic state very similar to the one it enters during anaerobic conditions [44]. In dauer larvae, fumarate reductase activity and the glyoxylate cycle protein GEI-7 are upregulated [20], which decreases the amount of NAD reduced to NADH in the TCA cycle. However, oxygen is present under these conditions and electron transport chain complex I function continues to oxidize NADH. This metabolic transition increases the NAD/NADH ratio and may result in lifespan extension.

3.4.2 Malate and fumarate may increase lifespan through increasing the NAD/NADH ratio

Malate likely increases NADPH levels through the action of malic enzyme, converting malate to pyruvate with reduction of NADP to NADPH. Malate, as a TCA cycle intermediate, increases TCA cycle flux and electron transport chain activity to increase oxygen consumption.

However, the results that malate increased the NAD/NADH ratio and decreased ATP levels were quite unexpected and may be key to the mechanism of lifespan extension induced by malate. Since oxygen consumption was increased and $\Delta\Psi$ was decreased by malate and fumarate, they likely induce mitochondrial uncoupling. Uncoupling decreases $\Delta\Psi$, which often leads to reduced reactive oxygen species production. Mitochondrial uncouplers have been shown to extend lifespan in *C. elegans* [35, 45], consistent with the "uncoupling to survive" hypothesis of longevity [46].

Malate and fumarate may also increase lifespan by increasing mitochondrial respiration. Increased electron transport chain function relative to TCA cycle function will increase the NAD/NADH ratio, which may extend lifespan. In this regard, one research group has found a positive correlation between *C. elegans* oxygen consumption and lifespan. By examining lifespan following RNAi knockdown of the frataxin gene, the authors proposed that 73% of the lifespan decline following frataxin knockdown was due to decreases in the oxygen consumption rate [47]. This research group suggests that high rates of respiration are necessary to produce the normal reactive oxygen species-mediated cell signaling required for a normal lifespan. They further went on to show that glucose restriction increases lifespan by stimulating mitochondrial respiration [48] and that *daf-2* mutants show reduced glucose uptake, which stimulates mitochondrial oxidation of L-proline to increase oxygen consumption and increase lifespan [49]. Malate and fumarate could also extend lifespan by decreasing the rate of decline of oxygen consumption over the lifespan. In this regard, a research group using eight different long and short-lived mutant strains, found a strong correlation between the inverse of the rate of decline of oxygen consumption with age and the lifespan [50]. For example, long-lived *daf-2* worms showed a very slow rate of loss of oxygen consumption over their lifespan.

Another mechanism through which malate may increase the NAD/NADH ratio is through increasing the activity of the ETC, so more NADH is oxidized by complex I. This may be possible by activating the NADH-fumarate reductase (malate dismutation) system. Using this system, following oxidation of NADH by complex I, electrons can be passed to rhodoquinone instead of ubiquinone. Rhodoquinone passes electrons to membrane bound fumarate reductase (complex II), which terminally reduces fumarate to succinate. In order for this activity to lead to oxidation of NADH at a faster rate, complex I activity must be limited by the amount of oxidized coenzyme Q (ubiquinone). If this is true, increasing the amount of oxidized rhodoquinone by increasing fumarate levels could increase complex I activity to increase the NAD/NADH ratio. Using fumarate as a terminal electron acceptor would also result in decreased ATP levels as only one proton is pumped per NADH oxidized instead of 3 protons being pumped when oxygen is used as the terminal electron acceptor. Decreased electron flow through complex III of the ETC could decrease ROS production and be a mechanism of lifespan extension, as complex III is an important generator of ROS [51]. However, since malate addition increased oxygen consumption in the worms, fumarate reduction likely only plays a minor role in total ETC function under these conditions.

Glyoxylate shunt activity also increases the NAD/NADH ratio as the shunt bypasses two of the three NADH generating reactions of the TCA cycle. We have also shown that glyoxylate shunt activity is required for the malate or fumarate-mediated increase in lifespan. The glyoxylate shunt gene *gei-7* has been shown to be required for lifespan extension mediated by *daf-16* in *daf-2* insulin receptor mutants [52]. So it is not surprising that the glyoxylate shunt is also required for the lifespan extension mediated by TCA cycle metabolites, which is also *daf-16* dependent.

3.4.3 Does malate increase acetyl-CoA levels to increase lifespan?

The glyoxylate shunt conversion of malate and CoA to glyoxylate and acetyl-CoA may be important for malate-mediated lifespan extension. Other metabolites that potentially increase acetyl-CoA levels, such as pyruvate [15] and acetate [14], have also been shown to increase lifespan. Further support of an important role of increased acetyl-CoA levels in lifespan extension is that glyoxylate addition did not extend lifespan. Glyoxylate can be converted to malate, but at the expense of decreasing acetyl-CoA levels. DR induces a metabolic shift from glucose oxidation to fatty acid oxidation that would also increase acetyl-CoA levels. Histone acetyltransferases (HATs) utilize acetyl-CoA as a cofactor for acetylation of histone tails. In this regard the HAT *cpb-1*/p300 is induced in *daf-2* worms and by DR and is essential for full lifespan extension by these interventions in *C. elegans* [53]. The histone deacetylase inhibitors sodium butyrate and trichostatin A also increased lifespan in *C. elegans*. In yeast it has been demonstrated that acetyl-CoA levels regulate protein acetylation [54] and that prevention of the acetylation of the gluconeogenic enzyme PEPCK blocks chronological lifespan extension induced by water starvation [55].

3.4.4 A proposed mechanism of how malate metabolism results in increased lifespan

We hypothesize that addition of malate or fumarate to *C. elegans* somehow leads to activation of the glyoxylate shunt. Regulation of the glyoxylate shunt has not been well studied in eukaryotes. In Gram-negative bacteria, a dual function kinase/phosphatase AceK responds to changes in carbon source to control phosphorylation-induced inactivation of isocitrate dehydrogenase, which induces flux into the glyoxylate shunt [56]. Lysine acetylation of isocitrate lyase and AceK also regulate shunt activity [57]. Upregulation of shunt activity would increase NAD levels, which are known to activate AMP kinase [37] and sirtuins [58]. AMP

kinase activation can further increase NAD levels and sirtuin activity [59]. However, metabolism under these conditions likely becomes limited by FAD levels, so malate dismutation is activated to oxidize $FADH_2$ to FAD. SIR-2.1 is known to activate DAF-16 activity [60], which can lead to lifespan extension [61] and further upregulation of *gei-7* expression [52] to amplify the lifespan extending signaling pathway.

3.4.5 Does mitochondrial uncoupling play a role in malate-mediated lifespan extension?

Malate-mediated mitochondrial uncoupling may be essential for lifespan extension. But 3 experimental results are inconsistent with this suggestion. First, malate addition resulted in lifespan extension in *hif-1* mutant worms, where ATP levels remain high, suggesting uncoupling is not occurring to a great extent in this strain, yet lifespan was still extended. Second, malate addition to *daf-16* mutants resulted in a large decrease in ATP levels, which may indicate mitochondrial uncoupling was occurring, when no lifespan extension was induced. However, one must be careful in ascribing decreases in ATP levels to decreases in oxidative phosphorylation. Changes in glycolysis and buffering ATP into phosphocreatine can also cause relatively quick changes in ATP levels without altering oxidative phosphorylation. And third, lifespan extension mediated by the uncoupler CCCP was described to be *daf-16* independent [35], whereas the lifespan extension mediated by malate requires *daf-16*. Further research needs to be performed to determine if activation of mitochondrial uncoupling, or, minimally, a decreased $\Delta\Psi$, is a common pathway of lifespan extension for compounds that extend *C. elegans* lifespan. In this regard we have found that a blueberry/green tea extract mixture that extended *C. elegans* lifespan also increased oxygen consumption and decreased ATP levels (data not shown).

3.4.6 Malate-induced lifespan extension compared to oxaloacetate-induced lifespan extension

Unsurprisingly, the lifespan extension observed following malate addition was similar to that observed with oxaloacetate treatment [13]. For example, both required *daf-16*. However, there were slight differences. Oxaloacetate was reported to extend median lifespan by 25%, while we report malate only increased mean lifespan by 14%. Under our liquid culture conditions we found that 10 mM oxaloacetate extended mean lifespan by 49% (data not shown). This larger effect than malate or fumarate may be due to a higher NAD/NADH ratio in oxaloacetate fed worms. Also, we found that malate-induced lifespan extension was completely dependent upon the presence of *sir-2.1,* while oxaloacetate-induced lifespan was still increased in the absence of *sir-2.1* [13]. This may be due to different growth conditions, either in liquid or on agar medium.

The worms in most of our experiments were cultured in liquid S medium, which differs slightly in nutrient composition from nematode growth media (NGM) commonly used for culturing worms on agar. The liquid S medium contains 10 mM citrate (a TCA cycle metabolite), in addition to phosphate as a buffer, while the NGM agar lacks citrate, but contains peptone powder (2.5 g/l) absent in S medium. The added citrate may not be required for malate-mediated lifespan extension as we found that malate extended the lifespan of worms grown on NGM agar plates by 10% (see Table 1), but this should be further verified due to the small number of worms used in the experiment. Also, it has been reported that adding citrate to the culture medium did not extend lifespan [14]. The worms grown in liquid medium are not dietarily restricted as *eat-2* worms showed a robust increase in lifespan in liquid medium, as they do on agar plates.

3.4.7 TCA Cycle function is a key determinant of longevity

Much data implicate TCA cycle function in the control of longevity. Many TCA cycle genes are upregulated in long-lived Ames dwarf and Little mice [62]. Brown Norway rats, a long-lived strain, do not shown declines in brain TCA cycle function with age in contrast to short-lived strains [63]. In yeast, glucose limitation increases chronological lifespan and upregulates TCA cycle gene expression [64]. Yeast mitochondrial ETC gene knockouts do not show extended chronological lifespan under DR conditions, but most TCA cycle gene knockouts showed even greater extension of lifespan than the wild-type yeast undergoing DR [65]. Yeast mutants with increased lifespan had increased levels of TCA cycle metabolites [66]. A downregulation of TCA cycle and ETC gene expression occurs in long-lived *C. elegans* dauer larvae [19] while long-lived *daf-2* insulin receptor mutants show either unchanged [19] or decreased [67] TCA cycle gene expression with either unchanged [19] or increased [67] ETC gene expression. Mutations in the *Drosophila* Indy gene [68], a sodium coupled TCA cycle dicarboxylate and tricarboxylate carrier in the plasma membrane extend lifespan. Similar results were found when two of the three *C. elegans* homologs of Indy were knocked down [69, 70]. But others failed to replicate these findings [71]. Knockdown of the mouse homolog of Indy resulted in DR-like phenotypes as well [72]. As a whole, there appears to be little consistency in these observations in different experimental models. However, proper coordination between ETC function and TCA function is likely necessary to maintain a normal to slightly high NAD/NADH ratio conducive to long life. High TCA cycle function with low ETC function is not favorable for extended lifespan because this would drive down mitochondrial and cellular NAD/NADH slowing important NAD-driven reactions likely necessary for extended lifespan. As another example, dietary restriction in mammals likely decreases flux through the TCA cycle while ETC

function is maintained throughout lifespan, resulting in an increased NAD/NADH ratio in several important tissues and lifespan extension.

3.4.8 Succinate as a blocker of DR-induced longevity

Since succinate, but not malate addition blocked lifespan extension in *eat-2* worms, it is possible that reduction of fumarate to succinate or maintaining a high $FAD/FADH_2$ ratio is essential for DR-induced longevity in *C. elegans*. Increased succinate levels likely decrease fumarate reductase activity through product inhibition. However, since long-lived *eat-2* worms were shown to have a 21-fold increase in the rate of [2-^{14}C] acetate oxidation as measured by $^{14}CO_2$ release [73], perhaps *eat-2* worms increase both TCA cycle activity (at least the CO_2 generating portion of the cycle) and fumarate reduction to extend lifespan. Interestingly, proteomic experiments revealed that the glyoxylate cycle protein GEI-7 was down-regulated slightly in *eat-2* worms [73], while GEI-7 is upregulated in long-lived dauer and *daf-2* worms [19]. Therefore distinct metabolic programs may be activated to extend lifespan under these different conditions.

3.4.9 Malate treatment has beneficial effects in mammals

Although we showed an important role for the glyoxylate shunt and malate dismutation, metabolic pathways absent in mammals, in malate-mediated lifespan extension in *C. elegans*, malate treatment has been shown to be very beneficial in mammals as well. Malate is found at high concentrations in unripened fruit, most notably in apples, and may contribute to some of the beneficial effects when these fruit are consumed. In addition, livers excised from aged rats that had been administered malate for 30 days displayed increased activities of complexes I, III, and IV of the electron transport chain (ETC) [74]. Malate also improved antioxidant function, leading to increased superoxide dismutase, glutathione peroxidase, reduced glutathione, and

decreased lipid peroxidation [75]. Malate supplementation also increased the activity of malate-aspartate shuttle components [76]. However, malate had no effect on the decreased mitochondrial membrane potential measured in aged rat liver [74]. Therefore, many of the protective effects of malate treatment seem to be conserved from nematodes to mammals.

3.4.10 Conclusion

Malate and fumarate increased the lifespan of *C. elegans*, while succinate did not. The glyoxylate shunt and malate dismutation/fumarate reduction pathways and SIR-2.1 were required for malate-mediated lifespan extension. DAF-16 translocation to the nucleus and transcription of DAF-16 target genes also plays an essential role in malate-mediated lifespan extension. However, since succinate addition can also induce DAF-16 nuclear translocation without lifespan extension, other factors are also involved. In this regard, further research should aim to elucidate the mechanisms through which addition of malate and fumarate to the culture medium lead to an uncoupling of mitochondrial oxidative phosphorylation and also determine if the $FAD/FADH_2$ ratio plays a role in lifespan determination. Since fumarate, malate, and oxaloacetate extend lifespan in *C. elegans* through a mechanism similar to dietary restriction, an anaplerotic cocktail of these compounds may be useful for the treatment of human aging-associated disorders.

3.5 Supporting information

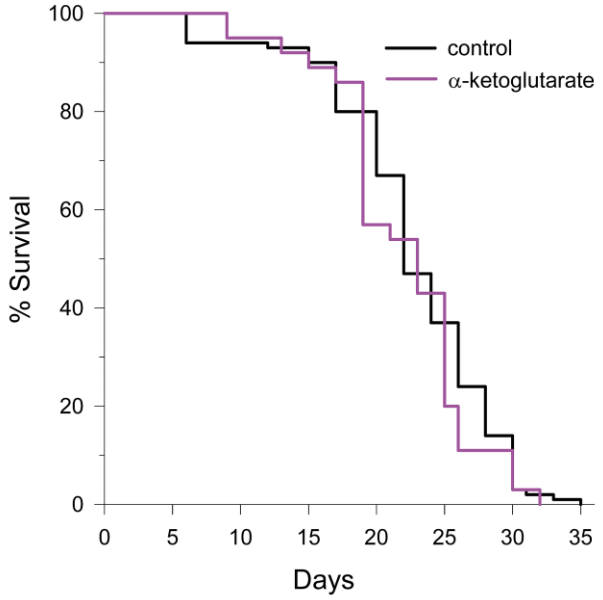


Figure 3.9. α-ketoglutarate addition did not alter the lifespan of *C. elegans*. *C. elegans* N2 worms were grown in cell culture inserts in 12-well microplates fed heat-killed *E. coli* with media change every 3 days in the absence or presence of 10 mM α-ketoglutarate (log-rank p =0.21 vs. untreated control).

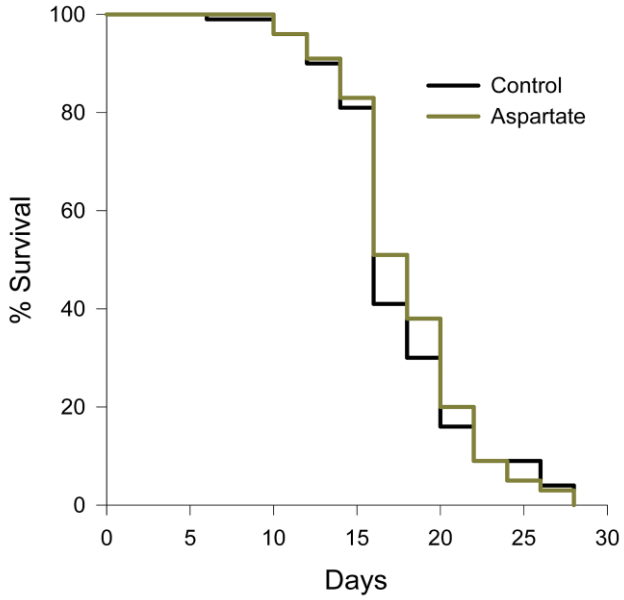


Figure 3.10. Aspartate addition did not alter the lifespan of *C. elegans*. *C. elegans* N2 worms were grown in cell culture inserts in 12-well microplates fed heat-killed *E. coli* with media change every 3 days in the absence or presence of 10 mM aspartate (log-rank p = 0.57 vs. untreated control).

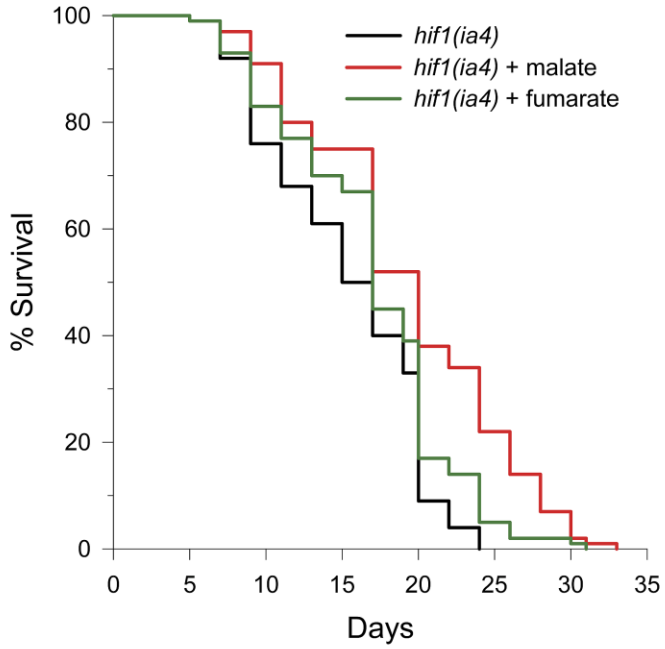


Figure 3.11. Malate and fumarate increased the lifespan of *hif-1* mutant worms. *C. elegans hif-1(ia4)* worms were grown in cell culture inserts in 12-well microplates fed heat-killed *E. coli* with media change every 3 days in the absence or presence of 10 mM malate (log-rank $p < 0.001$ vs. untreated control) or 10 mM fumarate (log-rank $p = 0.02$ vs. untreated control).

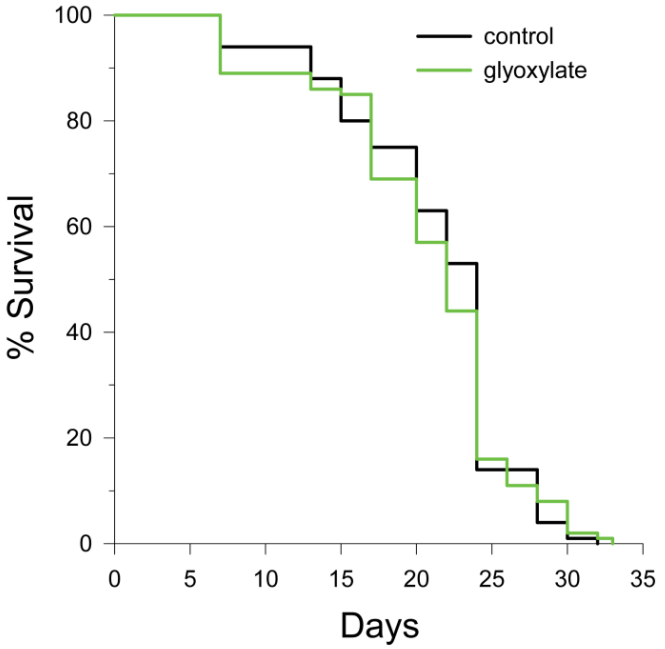


Figure 3.12. Glyoxylate addition did not alter the lifespan of *C. elegans*. *C. elegans* N2 worms were grown in cell culture inserts in 12-well microplates fed heat-killed *E. coli* with media change every 3 days in the absence or presence of 10 mM glyoxylate (log-rank $p = 0.35$ vs. untreated control).

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3.7 Author contributions

Conceived and designed the experiments: PB CE NC. Performed the experiments: CE NC AB JC. Analyzed the data: CE NC. Contributed reagents/materials/analysis tools: CE NC PB. Wrote the paper: PB.

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CHAPTER 4

MECHANISMS OF AMINO ACID-MEDIATED LIFESPAN EXTENSION IN *CAENORHABDITIS ELEGANS*[2]

4.1 Abstract

Little is known about the role of amino acids in cellular signaling pathways, especially as it pertains to pathways that regulate the rate of aging. However, it has been shown that methionine or tryptophan restriction extends lifespan in higher eukaryotes and increased proline or tryptophan levels increase longevity in *C. elegans*. In addition, leucine strongly activates the TOR signaling pathway, which when inhibited increases lifespan.

Therefore each of the 20 proteogenic amino acids was individually supplemented to *C. elegans* and the effects on lifespan were determined. All amino acids except phenylalanine and aspartate extended lifespan at least to a small extent at one or more of the 3 concentrations tested with serine and proline showing the largest effects. 11 of the amino acids were less potent at higher doses, while 5 even decreased lifespan. Serine, proline, or histidine-mediated lifespan extension was greatly inhibited in *eat-2* worms, a model of dietary restriction, *daf-16*/FOXO, *sir-2.1*, *rsks-1* (ribosomal S6 kinase), *gcn-2*, and *aak-2* (AMPK) longevity pathway mutants and in *bec-1* autophagy-defective knockdown worms. 8 of 10 longevity-promoting amino acids tested activated a SKN-1/Nrf2 reporter strain, while serine and histidine were the only amino acids from those to activate a hypoxia-inducible factor (HIF-1) reporter strain. Thermotolerance was increased by proline or tryptophan supplementation, while tryptophan-mediated lifespan

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extension was independent of DAF-16/FOXO and SKN-1/Nrf2 signaling, but tryptophan and several related pyridine-containing compounds induced the mitochondrial unfolded protein response and an ER stress response. High glucose levels or mutations affecting electron transport chain (ETC) function inhibited amino acid-mediated lifespan extension suggesting that metabolism plays an important role. Providing many other cellular metabolites to *C. elegans* also increased longevity suggesting that anaplerosis of tricarboxylic acid (TCA) cycle substrates likely plays a role in lifespan extension.

Supplementation of *C. elegans* with 18 of the 20 individual amino acids extended lifespan, but lifespan often decreased with increasing concentration suggesting hormesis. Lifespan extension appears to be caused by altered mitochondrial TCA cycle metabolism and respiratory substrate utilization resulting in the activation of the DAF-16/FOXO and SKN-1/Nrf2 stress response pathways.

4.2 Background

In *C. elegans* nematodes free amino acid concentrations change with age [1] and are altered in long-lived worms [2]. In humans, altered plasma amino acid concentrations are biomarkers of several diseases [3] such as type 2 diabetes [4]. Calorie restriction has long been known to delay aging [5] and protein restriction may be responsible for around half of this effect [6]. Methionine [7, 8] or tryptophan [9, 10] restriction partially mimics protein restriction to extend lifespan and delay aging-related disease in rodents. But the role that other amino acids play in longevity and disease has been harder to elucidate. In this regard, experiments with yeast, worms, and fruit flies are increasingly being used to address this issue.

Using the yeast *Saccharomyces cerevisiae*, it was first discovered that supplementation with the branched chain amino acids (leucine, isoleucine, or valine) or threonine extended

chronological lifespan by downregulating the general amino acid control (GAAC) pathway [11]. Others found that glutamate supplementation extended chronological lifespan [12]. Consistent with the ability of glutamate to extend lifespan, deletion of genes involved in converting glutamate to gamma-aminobutyric acid (GABA) increased replicative lifespan [13] and led to increased conversion of glutamate to alpha-ketoglutarate and other TCA cycle intermediates, which may be involved in lifespan extension by maintaining mitochondrial respiratory function. Others using different conditions found that supplementation with serine, threonine, or valine decreased chronological lifespan [14] while limitation of asparagine [15], methionine, aspartate, or glutamate [12] extended lifespan. Further research using yeast deletion strains of differing lifespans found that intracellular levels of many amino acids positively correlated with lifespan [16].

In *Drosophila*, dietary restriction (DR) or protein restriction [17] extends lifespan and supplementing methionine in combination with one or more of the essential amino acids decreased the lifespan back to the fully fed level [18]. Interestingly, adding methionine by itself to DR flies increased protein translation [19] and fecundity [18] without decreasing lifespan, uncoupling these events. Increased levels of amino acids, especially leucine [20, 21], activate the TOR kinase, which leads to an increased rate of translation. Inhibition of the TOR kinase with rapamycin [22] or expressing a dominant negative p70-S6 kinase, a kinase downstream of TOR, extended organismal longevity [23]. Metabolism of sulfur containing amino acids was shown to be essential for DR-mediated longevity in *Drosophila* [19], but supplementation of cysteine or methionine failed to extend lifespan in fully fed *Drosophila* [24, 25]. However, supplementing casein and methionine together led to lifespan extension [24].

In *C. elegans*, proline supplementation extended lifespan that relied upon its catabolism and a transient increase in reactive oxygen species (ROS) production from the mitochondrial electron transport chain [26]. Increased tryptophan levels also increased longevity in *C. elegans* as knockdown of an enzyme that catabolizes tryptophan increased lifespan [27]. Unexpectedly, knockdown of an aromatic amino acid transporter also extended lifespan [28], suggesting that decreased tryptophan or other aromatic amino acid levels may also boost longevity. Others found that decreased tyrosine degradation led to increased longevity, but surprisingly supplementation of tyrosine to the culture medium did not extend lifespan [29]. The majority of amino acid pool sizes are upregulated in long-lived worms [2]. In *daf-2* insulin-receptor deficient worms, for example, the levels of 8 of the 12 measured amino acids were increased, including the 3 branched chain amino acids. The branched chain amino acids are of special interest for longevity research, since their levels decreased to wild-type levels in the normal-lived *daf-2*/*daf-16* double mutants [2].

Feeding mice a diet high in branched chain amino acids led to increased mitochondrial biogenesis in muscle, decreased ROS production, and increased average lifespan of males [30]. However, branched chain amino acid levels declined in long-lived metformin-treated worms [31], and increased plasma levels of branched chain amino acids are correlated with the development of insulin resistance and type 2 diabetes in humans [32]. Furthermore, studies correlating high levels of free amino acids with longevity must be interpreted with caution as a decreased rate of translation is frequently associated with or even required for longevity and the increased amino acid pools may just be a result of that decreased rate of protein synthesis [33, 34].

Due to the incomplete knowledge of the effects of amino acids on longevity as well as the widespread use of amino acid and protein supplementation in the human diet we determined the effects of individual amino acid supplementation on *C. elegans* lifespan. We found that the vast majority of amino acids extended lifespan and further determined many of the signaling pathways required. We then tested the ability of several amino acids or tryptophan catabolites to induce a heat shock response, the ER stress response, or the mitochondrial unfolded protein response, which frequently accompany lifespan extension. The amino acids that extended lifespan to the greatest extent were then tested for effects on stress resistance and proteotoxicity.

4.3 Results

4.3.1 The effects of individual L-amino acids on the lifespan of *C. elegans*

We determined the effects of individually supplementing the 19 L-amino acids or glycine on the lifespan of *C. elegans* at 1 mM (Figure 4.1A), 5 mM (Figure 4.1B), and 10 mM (Figure 4.1 C) concentrations. The percent change of mean lifespan compared to that of untreated controls performed at the same time is also shown as a table. *C. elegans* worms were grown in liquid S medium with heat-killed *E. coli* as food. Heat killing prevented or at least greatly reduced bacterial catabolism of the added amino acid. Unlike nematode growth medium (NGM) which is standardly used, the S medium contains no peptone, so the bacterial food source and the supplemented amino acid are the only sources of dietary amino acids. The worms feeding on heat-killed bacteria had a mean lifespan of 17.2 +/- 0.3 days. At a 1 mM concentration, the amino acids that extended lifespan to the greatest extent (14-17%) were proline, leucine, glutamine, glutamate, and tryptophan. At a 5 mM concentration, the greatest lifespan extension was observed with proline, serine, cysteine, and glutamine (16-19%). At this concentration phenylalanine decreased lifespan (8%). Lastly, at a 10 mM concentration, the greatest increases

in longevity were observed with serine, proline, arginine, and methionine addition (14-22%). Asparagine, aspartate, phenylalanine, glutamine, and glutamate decreased lifespan at this concentration (6-25%). 5 of the amino acids increased lifespan with increasing concentration from 1 to 10 mM (arginine, histidine, methionine, threonine, and serine), while 7 of the amino acids decreased lifespan with increasing concentration in this range (aspartate, glutamate, glycine, phenylalanine, tryptophan, tyrosine, and valine). 3 of the amino acids had the greatest lifespan extension at the 5 mM concentration (asparagine, cysteine, and glutamine), while the 5 mM concentration yielded the least lifespan extension for alanine. Example lifespan curves for serine, proline, histidine, and tryptophan at concentrations that yielded the greatest effects on mean lifespan are shown.

4.3.2 The rate of amino acid uptake may limit the effect on lifespan

To determine if the rate of transport of amino acids into the worms may have limited their effects on lifespan, we administered the cell-permeable histidine analogs N-acetyl-histidine or histidine methylester, which get cleaved by intracellular enzymes to form histidine and monitored lifespan (Table 4.1). These compounds yielded greater lifespan extension than histidine at the 1 mM dose, suggesting that the rate of transport of the amino acids into the worms is likely limiting their effect on lifespan. The highest concentration of histidine methyl ester (10 mM) did not extend lifespan as expected for a hormetic dose response. If the same observation made for histidine holds for other amino acids, then the rate of amino acid absorption by the intestine may be an important factor controlling their ability to extend lifespan. The rate of transport of hydrophilic antioxidant compounds into *C. elegans* has also been shown to limit their effect on lifespan [35].

4.3.3 The effects of D-amino acids on the lifespan of *C. elegans*

To determine if the effects on lifespan were specific for L-amino acids, we also determined if there were effects on longevity when supplementing the 4 D-amino acids found endogenously in *C. elegans* [36], D-alanine, D-serine, D-aspartate, or D-glutamate (Table 4.1) as well as D-proline (Table 4.1), which is found naturally at low concentrations in mammals [37]. D-alanine and D-asparatate showed greater lifespan extension then their corresponding L-isomers. D-glutamate addition yielded effects on lifespan somewhat similar to its corresponding L-isomer. In contrast to the strong pro-longevity effects observed with L-serine, D-serine supplementation did not lead to lifespan extension at any of the concentrations added and even slightly decreased lifespan at the higher concentrations. And lastly, D-proline supplementation did not extend lifespan as well. These lifespan results are consistent with metabolism of the D-amino acids being required for lifespan extension as D-alanine, D-aspartate, and D-glutamate are present in the *E. coli* food source and have been shown to be catabolized by the products of the *C. elegans* genes *daao-1*, *ddo-1*, and *ddo-3*, respectively. D-serine was not found to be present in the *E. coli* diet, but was instead found to be synthesized endogenously by *C. elegans*. However, no *C. elegans* enzyme has yet been found to mediate D-serine [36] or D-proline degradation.

4.3.4 Amino acid-mediated lifespan extension, except for tryptophan, is DAF-16 dependent

To determine if lifespan extension induced by amino acids requires specific longevity pathways, individual amino acids were administered to mutants of known longevity pathways at the concentration that maximally extended lifespan. First, the amino acids alanine, cysteine, glutamine, histidine, lysine, proline, serine, tryptophan, and tyrosine were individually supplemented to short-lived *daf-16(mgDf50)* mutants (Table 4.2). DAF-16/FOXO is a central transcription factor that translocates to the nucleus to activate a stress response program in

insulin-receptor signaling-deficient worms [38]. Tryptophan was the only supplemented amino acid that extended lifespan in the *daf-16* mutant strain indicating tryptophan activates a longevity pathway independent of *daf-16*, while the other 8 amino acids require DAF-16 mediated gene expression for the increased longevity. Cysteine and histidine even decreased lifespan when supplemented to this strain.

To confirm that amino acids activate DAF-16 transcriptional activity, we measured the fluorescence of a *sod-3p::gfp* DAF-16 reporter strain of worms following culture in the presence of individual amino acids (Figure 4.2A). As expected from the lifespan data, serine and proline increased fluorescence of these worms. The presence of tryptophan also increased fluorescence, so tryptophan likely activates both DAF-16-dependent and DAF-16-independent pathways for lifespan extension. There was also a strong trend for leucine to increase fluorescence. Unexpectedly, histidine did not increase expression of *sod-3p::gfp*. The reasons for this finding are unclear as we found that DAF-16 was required for histidine-mediated lifespan extension.

4.3.5 Most amino acids activate SKN-1 transcriptional activity

The SKN-1/Nrf2 signaling pathway increases cellular antioxidant and detoxification gene expression to extend lifespan. We determined the effect of serine, tryptophan, histidine, or proline addition on lifespan of *skn-1* knockdown worms. As shown in Table 4, tryptophan or serine supplementation extended lifespan, but not to the extent as in control worms, while there were only insignificant trends toward increased lifespan following histidine or proline addition.

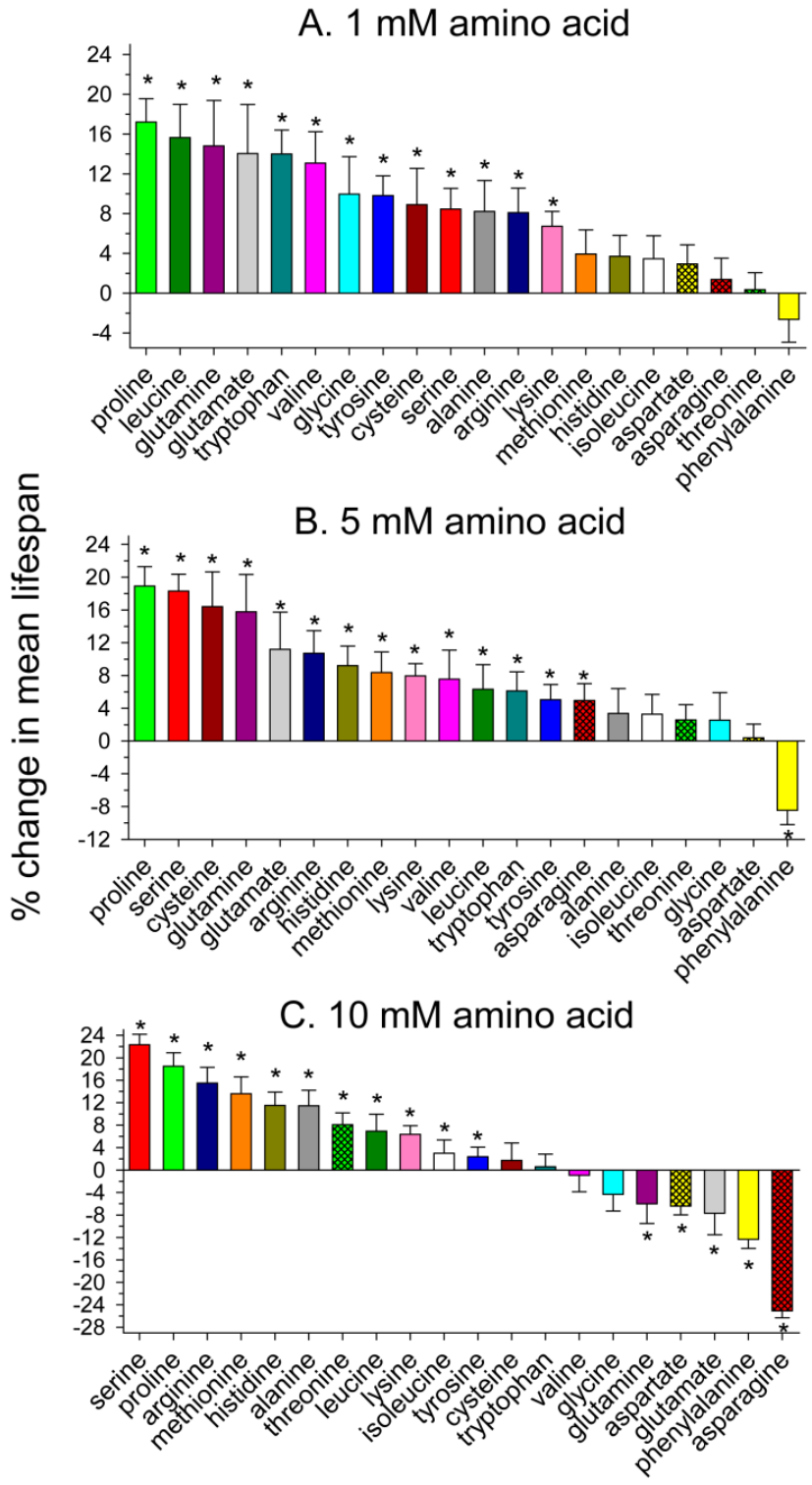

Figure 4.1. Individual supplementation of most amino acids extends mean lifespan in *C. elegans*. Mean lifespan of *C. elegans* supplemented with a **(A)** 1 mM, **(B)** 5 mM, or **(C)** 10 mM concentration of each of the 20 amino acids (* log rank $p < 0.05$ vs. control).

Table 4.1 The effects of D-amino acids and membrane-permeable L-histidine analogs on *C. elegans* N2 lifespan.

Treatment	Concentration	% of untreated mean lifespan	p-value	# of worms	Replicates
D-alanine	1 mM	114	<0.001	219	2
	5 mM	116	<0.001	273	2
	10 mM	116	<0.001	240	2
D-aspartate	1 mM	107	0.024	233	2
	5 mM	118	<0.001	269	2
	10 mM	108	<0.001	228	2
D-glutamate	1 mM	114	<0.001	207	2
	5 mM	118	<0.001	249	2
	10 mM	97	0.165	232	2
D-serine	1 mM	100	0.600	209	2
	5 mM	91	<0.001	198	2
	10 mM	93	<0.001	220	2
D-proline	5 mM	101	0.734	113	2
N-acetyl-L-histidine	0.1 mM	103	0.383	142	2
	1 mM	112	0.002	188	2
	10 mM	110	0.008	196	2
L-histidine methyl ester	0.1 mM	108	<0.001	215	2
	1 mM	109	<0.001	246	2
	10 mM	104	0.11	193	2

Table 4.2. The effects of amino acids on lifespan in *daf-16* mutant and *skn-1* knockdown *C. elegans*

Strain	Treatment	% of N2 mean lifespan	% of untreated mean lifespan	p-value	# of worms	Replicates
daf-16(mgDf50)	control	73		<0.001	608	7
	5 mM histidine		92	<0.001	286	2
	5 mM proline		106	0.0871	133	2
	1 mM alanine		102	0.111	107	2
	1 mM tryptophan		118	0.008	183	2
	10 mM serine		105	0.104	161	2
	10 mM glutamine		95	0.107	125	2
	5 mM cysteine		95	0.107	115	2
	10 mM cysteine		81	<0.001	105	2
	5 mM tyrosine		96	0.578	140	2
	5 mM lysine		99	0.907	130	2
N2 (*skn-1* RNAi) with live bacteria	control	59		<0.001	156	3
	1 mM tryptophan		107	0.042	125	3
	10 mM serine		107	0.01	175	3
	5 mM histidine		103	0.296	139	3
	5 mM proline		105	0.088	111	2
N2 with live bacteria	control	-			298	5
	1 mM tryptophan		109	<0.001	317	5
	10 mM serine		109	<0.001	375	5
	5 mM histidine		110	<0.001	277	5
	5 mM proline		108	<0.001	268	4

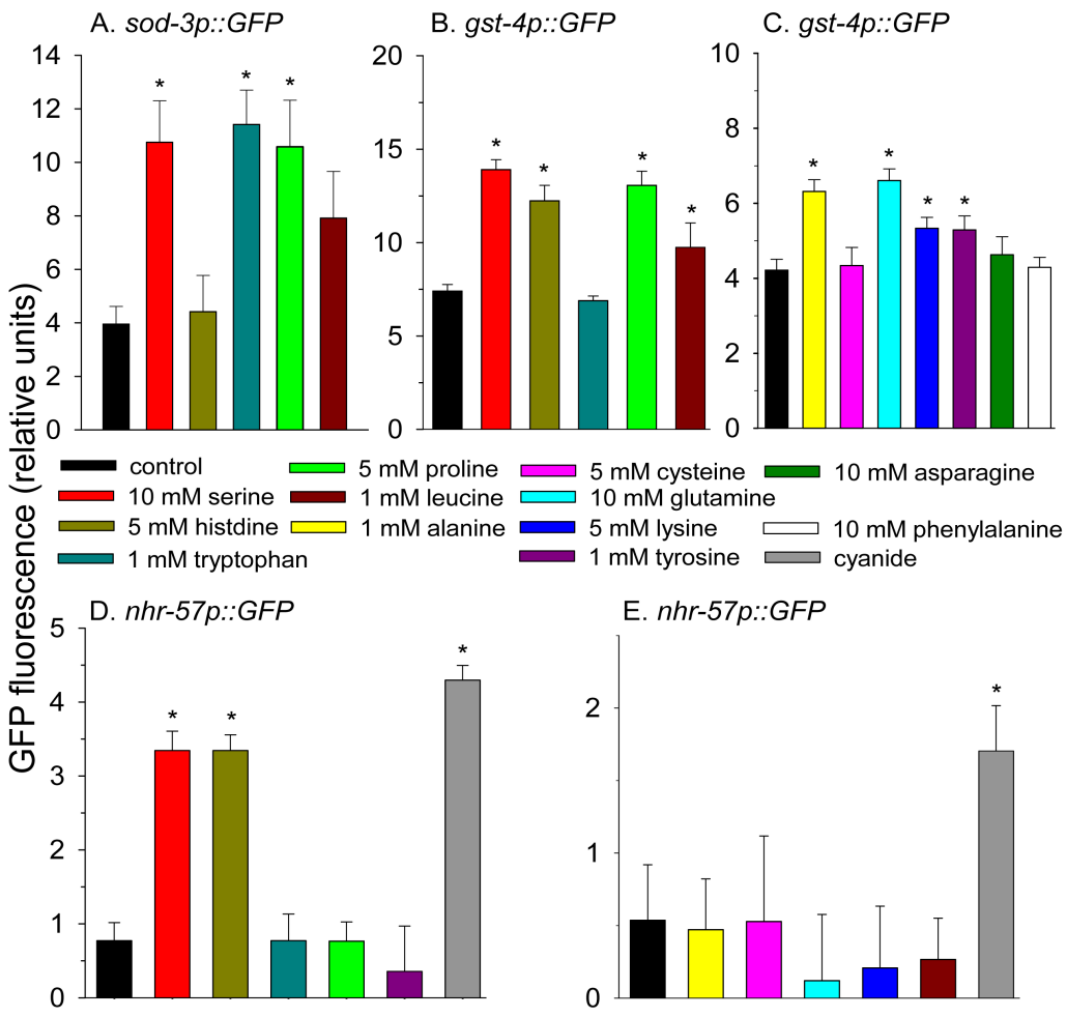

Figure 4.2. The effects of amino acid addition on DAF-16, SKN-1, and HIF-1-mediated gene expression. **(A)** The effects of amino acid addition on *sod-3p::GFP* fluorescence as a measure of DAF-16 transcriptional activity. **(B)** and **(C)** The effects of amino acid addition on *gst-4p::GFP* fluorescence as a measure of SKN-1 transcriptional activity. **(D)** and **(E)** The effects of amino acid addition on *nhr-57p::GFP* fluorescence as a measure of HIF-1 transcriptional activity. 20 µM potassium cyanide was used as a positive control (* $p < 0.05$).

To further check the ability of amino acids to activate SKN-1, we used a *gst-4p::GFP* SKN-1 reporter strain of worms (Figure 4.2B and Figure 4.2C). We found that 8 of the 10 amino acids tested that increased lifespan increased GFP expression of the reporter strain. Amino acids that activated *gst-4p::GFP* expression included serine, proline, glutamine, alanine, leucine, lysine, and tyrosine, while tryptophan and cysteine did not. We also tested the effects of 2 amino acids,

phenylalanine and asparagine, which decreased lifespan on the fluorescence of this reporter strain and observed no induction of expression. Overall, there was a small correlation between the amount of SKN-1 activity and the extent of lifespan extension as serine and proline extended lifespan to the greatest extent and also increased fluorescence of the SKN-1 reporter strain to the greatest extent. It has previously been hypothesized that proline catabolism transiently increases ROS production that leads to SKN-1 activation [26]. Our results are consistent with this hypothesis. Cysteine is a strong antioxidant and likely quenched ROS required for SKN-1 activation likely explaining the lack of activation by this amino acid.

The RNAi feeding experiments require live bacteria, while heat-killed bacteria were used in all other lifespan experiments. It is possible that the live bacteria used in the SKN-1 RNAi lifespan experiments metabolized the added amino acids dampening the degree of lifespan extension. Therefore, we performed control experiments supplementing amino acids to *C. elegans* feeding on live control HT115(DE3) *E. coli*. *C. elegans* fed live control bacteria had a mean lifespan of 16.1 +/- 0.2 days, slightly less than worms fed heat-killed bacteria (mean lifespan of 17.2 +/- 0.3 days). Histidine extended lifespan to a similar extent in the presence of live or heat-killed bacteria as shown (Table 4.2). However, tryptophan-induced lifespan extension was slightly blunted by the use of live bacteria, and serine or proline-induced lifespan extension was blunted by roughly 50% by the use of live bacteria. A faster rate of *E. coli* catabolism of serine and proline than tryptophan and histidine likely explain these observations.

4.3.6 Histidine and serine increase HIF-1 target gene expression

The hypoxia-inducible factor-1 (HIF-1) protein is degraded quickly during standard conditions, but is stabilized during hypoxia or by other specific stresses to increase lifespan in *C. elegans* [39]. Therefore, we tested the HIF-1 reporter strain *nhr-57p::GFP* [39] for amino acid-

induced changes in GFP fluorescence (Figure 4.2D and Figure 4.2E). Cyanide was used as a positive control as it inhibits cytochrome c oxidase, the protein complex which binds molecular oxygen, the terminal electron acceptor in the electron transport chain, to mimic the effects of hypoxia on mitochondria. We found that histidine or serine increased fluorescence, while tryptophan, proline, tyrosine, alanine, cysteine, glutamine, lysine, or leucine did not. These data indicate that stabilization of HIF-1 may be one of the mechanisms through which histidine and serine extend lifespan, although lifespan experiments with HIF-1 mutant worms are needed to confirm this hypothesis.

4.3.7 Amino acid-mediated lifespan extension is AAK-2 (AMPK) dependent

Next we individually administered *C. elegans* our test set of 10 amino acids except leucine to *aak-2(gt33)* worms, which are depleted of one of the two catalytic subunit of AMP-activated protein kinase (AMPK) and performed lifespan analysis (Table 4.3). AMPK signaling inhibits target of rapamycin (TOR) kinase signaling to stimulate autophagy to recycle cellular components. AMPK also stimulates the sirtuin deacetylase SIR-2.1, SKN-1/Nrf2, and DAF-16/FOXO pro-longevity pathways [40]. None of the amino acids extended lifespan in this mutant strain. Tyrosine decreased lifespan while the other 8 amino acids tested had no significant effect. Therefore, AAK-2 is required for the longevity benefits provided by the amino acids.

4.3.8 Many amino acids require SIR-2.1 for lifespan extension

Next the effects of individual supplementation of these same 9 amino acids as well as phenylalanine on lifespan of the *sir-2.1(ok434)* NAD-dependent sirtuin deacetylase mutant were determined (Table 4.3). Small to moderate lifespan increases occurred with cysteine, glutamine, lysine, and low dose phenylalanine supplementation, but there were no significant effects of 6 other amino acids tested on the lifespan of this strain. Therefore SIR-2.1 was required for

lifespan extension mediated by slightly more than half of the amino acids tested. Surprisingly, high dose (10 mM) phenylalanine supplementation did not lead to a decreased lifespan in this strain as it did in the N2 control worms.

4.3.9 Amino acids do not significantly extend lifespan in long-lived DR worms

Restricting a specific amino acid such as methionine from the diet can be utilized to extend lifespan and gain some of the benefits of dietary restriction (DR) [8], but there is not much evidence that specific amino acid supplementation can yield enhanced longevity effects. Therefore, we administered individual amino acids to *eat-2(ad1116)* mutants that are dietarily restricted and long-lived because of reduced pharyngeal pumping. The non-treated control *eat-2* mutants had a mean lifespan 44% longer than N2 controls indicating that our control worms were not dietarily restricted under our growth conditions. None of the 5 amino acids tested yielded statistically significant lifespan extension (Table 4.3). However, 4 of the amino acids yielded strong trends toward lifespan extension (p-values between 0.08 and 0.10). Therefore the individual amino acids are likely utilizing some portion of the DR signaling pathway for lifespan extension.

4.3.10 Autophagy is required for the lifespan extension induced by serine, proline, or histidine supplementation, but not by tryptophan

Since autophagy has been shown to be required for DR-mediated lifespan extension [41], we determined if autophagy was also required for amino acid-mediated longevity. To block autophagy we knocked down *bec-1*, the *C. elegans* Beclin-1 homolog and monitored lifespan following supplementation with serine, proline, histidine, or tryptophan.

Table 4.3. The effects of amino acids on lifespan in aak-2, *sir-2.1* and *eat-2* mutant and *bec-1* knockdown worms

Strain	Treatment	% of N2 control mean lifespan	% of untreated mean lifespan	p-value	# of worms	Replicates
aak-2(gt33)	control	80		<0.001	786	8
	5 mM histidine		100	0.877	189	2
	5 mM proline		100	0.919	187	2
	1 mM alanine		100	0.943	209	2
	1 mM tryptophan		102	0.060	220	2
	10 mM serine		101	0.137	204	2
	5 mM lysine		87	0.373	180	2
	5 mM cysteine		85	0.952	140	2
	10 mM cysteine		90	0.122	145	2
	5 mM tyrosine		80	<0.001	200	2
	5 mM glutamine		94	0.436	180	2
	10 mM glutamine		103	0.126	200	2
sir-2.1(ok434)	control	86		<0.001	299	4
	5 mM histidine		100	<0.830	154	2
	5 mM proline		104	0.211	114	2
	1 mM alanine		98	0.225	165	2
	1 mM tryptophan		102	0.673	121	2
	10 mM serine		101	0.482	136	2
	10 mM glutamine		110	<0.001	180	2
	5 mM cysteine		105	0.244	192	2
	10 mM cysteine		110	<0.001	190	2
	5 mM tyrosine		93	0.231	131	2
	5 mM lysine		107	0.017	146	2
	1 mM phenylalanine		105	0.049	155	2

Table 4.3 (Continued)

Strain	Treatment	% of N2 control mean lifespan	% of untreated mean lifespan	p-value	# of worms	Replicates
	10 mM phenylalanine		101	0.402	176	2
eat-2(ad1116)	control	142		<0.001	135	2
	10 mM serine		108	0.078	161	2
	1 mM tryptophan		105	0.099	138	2
	5 mM glutamine		100	0.841	134	2
	5 mM histidine		106	0.091	153	2
	5 mM proline		107	0.076	176	2
N2 (*bec-1* RNAi) with live bacteria	control	123		<0.001	184	2
	1 mM tryptophan		107	<0.001	162	2
	10 mM serine		101	0.316	152	2
	5 mM histidine		99	0.274	117	2
	5 mM proline		98	0.09	102	2

Knockdown of *bec-1* by RNAi feeding increased lifespan as has previously been shown in [42] and prevented lifespan extension induced by supplementation with serine, proline or histidine, but not by tryptophan (Table 4.3).Therefore, the majority of amino acids, but not tryptophan, require autophagy for lifespan extension, once again suggesting that tryptophan extends lifespan through a mechanism distinct from other amino acids.

The PHA-4/FOXA transcription factor is required for induction of autophagy and lifespan extension in response to DR. In addition, expression of the PHA-4 transcription factor has been shown to be upregulated by roughly 50% by DR [43]. Therefore, we determined if individual amino acid administration could increase PHA-4 protein levels by using a strain of

worms engineered to express PHA-4:GFP:3xFLAG using the endogenous *pha-4* promoter [44]. Surprisingly, we found that serine, histidine, or tryptophan addition did not alter the GFP fluorescence of this strain. However, leucine addition resulted in a strong trend toward increased fluorescence (p=0.08). On the whole, individual amino acid supplementation did not appear to have much of an effect on fluorescence in this strain. However, we cannot yet rule out the possibility that changes in PHA-4 localization or post-translational modification play a role in individual amino acid-induced longevity. It is also possible that the added GFP and FLAG tags affect the stability of the protein. Lifespan studies using *pha-4* RNAi are needed to determine a role, if any, for PHA-4 in amino acid-mediated lifespan extension.

4.3.11 Inhibition of TOR signaling plays a role in amino acid-mediated lifespan extension

Specific amino acids, most notably leucine, but also to a lesser extent arginine and glutamine can be activators of the TOR signaling pathway that limits lifespan [45]. Administering rapamycin, a TOR inhibitor, or feeding TOR RNAi to *C. elegans* induces autophagy and extends lifespan [46, 47]. More recently it was found that alpha-ketoglutarate supplementation can lead to TOR inhibition to extend lifespan [48]. Knockout or knockdown of the ribosomal S6 kinase, which is downstream of TOR kinase in the signaling pathway, also extends lifespan [49]. Part of this effect may rely on a decreased rate of protein translation as inhibitors of protein translation can also extend lifespan [49]. Therefore, we determined the effects of specific amino acids on lifespan in long-lived *rsks-1(ok1255)* ribosomal S6 kinase mutants, where this arm of the TOR signaling pathway is inhibited (Table 4). Unlike the results with N2 control worms, addition of serine or tryptophan did not alter the lifespan of this strain, while proline or histidine addition slightly decreased lifespan, and tryptophan addition decreased lifespan by 20%. Therefore, these amino acids appear to use inhibition of TOR signaling to mediate lifespan extension, as the amino acids did not extend lifespan in long-lived mutant worms where TOR signaling was already disrupted.

4.3.12 A decreased rate of translation is required for the full lifespan extending effects of amino acids

GCN-2 (general control nonderepressible-2) kinase can slow the rate of translation initiation by phosphorylating eukaryotic translation initiation factor-2 alpha (eIF-2α) when tRNAs become uncharged due to low amino acid levels [34] or in times of mitochondrial metabolic stress [33]. We hypothesized that amino-acid supplementation causing amino acid imbalance could result in inefficient tRNA charging or cause metabolic stress signaling through GCN-2 to extend lifespan. Therefore, we determined the lifespan of *gcn-2(ok871)* mutants supplemented with individual amino acids (Table 4.4). We found that histidine or tryptophan supplementation did not lead to increased longevity when using this strain, while only a 4% or 7% lifespan extension occurred when serine or proline, respectively, were supplemented. To further determine a role for decreased translation in amino acid imbalance-mediated longevity, we performed lifespan analysis using the *ife-2(ok306)* strain [50], which is deficient in an isoform of the translation initiation factor eIF4E and shown to be long-lived. Surprisingly, under our liquid culture conditions using heat-killed bacteria as food, the lifespan of this strain was not significantly different than the control. However, supplementation of serine, proline, histidine, or tryptophan to this strain did not lead to extended lifespan, while tryptophan addition even slightly decreased lifespan. Therefore signaling to slow the rate of translation is likely a general mechanism involved in individual amino acid-mediated increased longevity.

4.3.13 The effect of individual amino acids on the lifespan of mitochondrial ETC complex I and II mutants

To test the hypothesis that mitochondrial ETC activity is important for amino-acid induced lifespan extension we supplemented serine, histidine, or proline to either short-lived mitochondrial ETC complex I defective *gas-1(fc21)* mutant worms or to short-lived mitochondrial ETC complex II defective *mev-1(kn1)* mutant worms (Table 4.4). We found that proline supplementation extended the lifespan of *gas-1* mutants, but that serine or histidine were unable to extend lifespan, although there was a strong trend with histidine (p=0.09). When we supplemented each of these 3 amino acids to *mev-1* mutants, we found opposite effects. Proline did not extend lifespan, although a strong trend was observed (p=0.10), while serine and histidine extended lifespan. Therefore normal complex I (NADH dehydrogenase) activity is required for the full serine and histidine-mediated lifespan extension, while normal complex II activity is required for proline-mediated lifespan extension. This data may be explained in that proline dehydrogenase generates $FADH_2$ which feeds electrons into the ETC at complex II and histidine and serine catabolism generates NADH that feeds electrons into the ETC at complex I.

4.3.14 Most supplemented metabolites extended lifespan at an optimal concentration

Since supplementation with an optimal concentration of most amino acids extended the lifespan of the worms, it is possible that their breakdown to TCA cycle intermediates may play a role in lifespan extension. It has previously been shown that supplementation with pyruvate [51], acetate (that can be readily metabolized to the TCA cycle substrate acetyl-CoA) [52], or the TCA cycle intermediates malate, fumarate [53], oxaloacetate [54], and alpha-ketoglutarate [48] extended lifespan in *C. elegans*. We therefore determined the lifespan of the worms individually

supplemented with 1, 5, or 10 mM concentrations of the TCA cycle intermediates citrate, isocitrate, alpha-ketoglutarate, or succinate (Table 4.1).

We previously found that 10 mM succinate did not extend lifespan, but did induce translocation of the pro-longevity factor DAF-16 to the nucleus [53]. But here we find that lowering the concentration of succinate to 5 mM or 1 mM resulted in lifespan extension (Figure 4.3A), consistent with a recent report of a longevity benefit [48]. Citrate is present at 10 mM in all of our experiments as a standard buffer component of the S-medium. We found that removing it did not affect the lifespan (Figure 4.3B). Previous findings also failed to find an extension of lifespan with citrate supplementation [48, 52]. We found that alpha-ketoglutarate at any of the 4 concentrations tested from 0.1 to 10 mM extended lifespan (Figure 3C), as recently reported for an 8 mM dose [48]. Adding DL-isocitrate to the medium led to an increase in lifespan at the 5 mM concentration, but a decrease in lifespan at the 10 mM concentration (Figure 4.3D). It is unknown if the non-naturally occurring L-isomer contributed to this effect, but since we found the non-naturally occurring isomer D-malate to decrease lifespan at all 3 concentrations tested (Table 4.1), it is a strong possibility. Another group observed no effect of 8 mM isocitrate on lifespan [48]. Therefore, of the 7 TCA cycle intermediates that we have tested, 6 were able to extend lifespan at an optimal dose.

We hypothesized that catabolism of the amino acids for anaplerosis or energy production was likely playing a role in the lifespan extension. If this is true supplementing other common cellular metabolites should also extend lifespan. Therefore, we performed lifespan analysis of worms supplemented with sugars or other metabolites lacking nitrogen atoms.

Table 4.4 The effects of amino acids on lifespan of rsks-1, gcn-2, ife-2, gas-1, and mev-1

strain	treatment	% of N2 control mean lifespan	% of untreated mean lifespan	p-value	# of worms	replicates
rsks-1(ok1255)	control	109		<0.001	362	4
	1 mM tryptophan		71	<0.001	130	2
	5 mM histidine		93	0.031	135	2
	5 mM proline		94	0.042	140	2
	10 mM serine		101	0.236	162	2
gcn-2(ok871)	control	90		<0.001	479	4
	1 mM tryptophan		99	0.489	160	2
	5 mM histidine		100	0.335	163	2
	5 mM proline		107	0.001	205	2
	10 mM serine		104	0.006	183	2
Ife-2(ok306)	control	97		0.245	112	2
	1 mM tryptophan		94	0.029	138	2
	5 mM histidine		98	0.597	110	2
	5 mM proline		102	0.352	117	2
	10 mM serine		99	0.791	96	2
gas-1(fc21)	control	69		<0.001	119	2
	5 mM histidine		104	0.092	110	2
	10 mM serine		98	0.327	106	2
	5 mM proline		109	0.003	115	2
mev-1(kn1)	control Table 4.6 (Continued)	69		<0.001	273	2
	5 mM histidine		104	0.021	215	2
	10 mM serine		109	<0.001	258	2
	5 m		102	0.102	256	2

Table 4.5 The effects of TCA cycle intermediates on *C. elegans* lifespan

treatment	concentration	% of untreated mean lifespan	p-value	# of worms	replicates
succinate	1 mM	111	<0.001	590	4
	5 mM	110	<0.001	570	4
	10 mM	104	0.098	607	4
citrate[1]	10 mM	98	0.414	229	2
α-ketoglutarate	0.1 mM	110	<0.001	261	3
	1 mM	115	<0.001	345	3
	5 mM	111	<0.001	337	3
	10 mM	107	0.042	333	3
DL-isocitrate	1 mM	103	0.202	452	4
	5 mM	113	<0.001	523	4
	10 mM	72	<0.001	217	4
D-malate	1 mM	85	<0.001	106	1
	5 mM	76	<0.001	77	1
	10 mM	79	<0.001	142	1

[1]compared to a medium lacking citrate. All other experiments contain 10 mM citrate as part of the standard culture media.

Glucuronolactone, glyceraldehyde, fructose, propionate, or dihydroxyacetone did not extend lifespan and the last 4 of these compounds even decreased lifespan by 13-25% at the 10 mM dose. Glyceraldehyde, fructose, and dihydroxyacetone are readily converted into glycolytic intermediates leading to the formation of toxic methylglyoxyl from glyceraldehyde phosphate or dihydroxyacetone phosphate, which could contribute to their toxicity, while propionic acid is known to be neurotoxic at high levels [28]. Since many amino acids activated SKN-1, while TCA cycle intermediates did not, we hypothesized that nitrogen-containing metabolites might be slightly more potent inducers of lifespan extension. The effects of many nitrogen-containing metabolites on lifespan are shown in 4.13. At an optimal dose carnosine, beta-alanine, betaine, homocysteine, ornithine, agmatine, putrescine, taurine, and theanine extended lifespan.

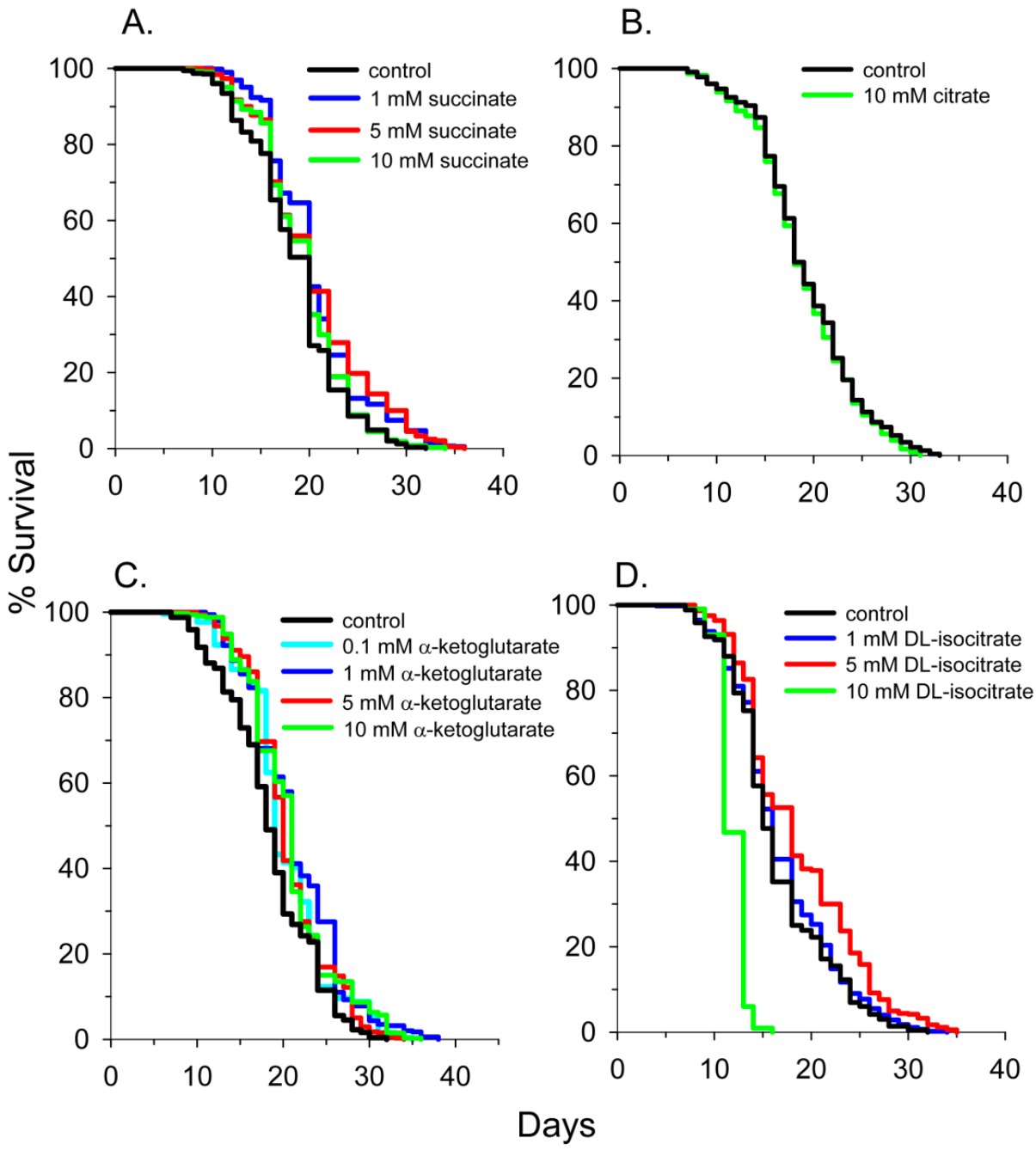

Figure 4.3. Individual supplementation of many TCA cycle metabolites extends mean lifespan in *C. elegans*. **(A)** Succinate extends lifespan, **(B)** citrate does not extend lifespan, **(C)** alpha-ketoglutarate extends lifespan, and **(D)** isocitrate extends lifespan at one or more of the concentrations tested. 10 mM citrate is a standard component of the S-medium. It was removed to determine the effect of citrate on lifespan.

For the majority of these compounds, the lowest concentration, such as 1 mM, yielded greater lifespan extension than the highest 10 mM concentration suggesting a hormetic dose response. Supplementation with creatine, or the histidine catabolites histamine or urocanic acid did not extend lifespan at any of the 3 concentrations tested. Although most of the nitrogen-containing compounds extended lifespan at an optimal dose, the extent of lifespan extension was not noticeably different than when supplementing with compounds lacking nitrogen.

4.3.15 *C. elegans* lifespan was not limited by nitrogen availability

We used heat-killed *E. coli* as a food source to prevent the bacteria from metabolizing the added metabolites, but we have found that heat-killing *E. coli* causes the loss of one or more essential growth-limiting nutrients during heating. So lowering the concentration of heat-killed bacteria in the growth media by just a factor of 2 did not allow completion of larval growth into adulthood, but instead led to dauer formation. The concentration of live bacteria could be reduced by 30-40 fold before dauer formation during larval development. To test if the worms may have been nitrogen limited under our culture conditions, we supplemented the worms with peptone or other nitrogen containing compounds and measured the lifespan. Interestingly, peptone at 1.25 g/L, half the concentration present in nematode growth media (NGM) decreased lifespan by 22% (Additional file 6: Table S3). This concentration contains roughly 10 mM total amino acids. These results support published findings where 5 g/L (2x NGM) and 10 g/L (4x NGM) peptone also decreased *C. elegans* lifespan in liquid S medium [55]. Lowering the peptone concentration to 0.125 g/L (0.1x NGM) yielded a similar lifespan as untreated controls.

We next added ammonium chloride as a nitrogen source. Concentrations of ammonium chloride from 1 to 10 mM did not extend lifespan. So amino acids do not increase lifespan solely by providing nitrogen to the worms.

4.3.16 Phenylalanine and alpha-ketoglutarate activate a HSF-1 reporter strain

Since supplementation of many of the amino acids and other metabolites showed less lifespan extension at higher concentrations, we hypothesized that *C. elegans* mounted a stress response that resulted in lifespan extension at lower amino acid levels, but at higher levels the stress response was overwhelmed leading to decreased lifespan. Therefore, we determined if amino acid supplementation activates a *Phsp-16.2::GFP* heat shock reporter strain of worms. HSP-16.2 is a small cytoplasmic heat shock protein and target of the HSF-1 transcription factor [56]. We first tested the effects of glutamine, histidine, methionine, serine, tryptophan, or tyrosine, amino acids that extended lifespan, or phenylalanine, an amino acid that decreased lifespan on GFP fluorescence in the *Phsp-16.2::GFP* reporter strain using heat shock as a positive control (Figure 4.4 A). Of these amino acids, only phenylalanine activated GFP reporter gene expression.

We next tested the effects of TCA cycle intermediate or pyruvate supplementation on the *Phsp-16.2::GFP* reporter strain, as amino acids are broken down into TCA cycle intermediates when they are present in excess. When administered to the *Phsp-16.2::GFP* reporter strain alpha-ketoglutarate, but none of the other TCA cycle intermediates supplemented increased GFP fluorescence (Figure 4.5A). Many of the amino acids showing the largest stimulatory effects on lifespan (proline, arginine, histidine, glutamine, and glutamate) are catabolized through glutamate into alpha-ketoglutarate in the TCA cycle.

4.3.17 Histidine, tryptophan, and citrate induce an ER stress response

We next determined if amino acids activated the endoplasmic reticulum (ER) stress response by using a reporter strain of worms engineered to contain a heat shock protein-4 (*hsp-4*) promoter driving expression of green fluorescent protein (GFP) [57]. We tested the effect of

glutamine, histidine, methionine, serine, tryptophan, or tyrosine supplementation on expression of GFP in the *Phsp-4::GFP* reporter strain of worms using heat shock as a positive control (Figure 4.4B). Histidine and tryptophan induced GFP expression, methionine and glutamine reduced GFP expression, while the other amino acids had no effect.

Subsequently we determined the effects of supplemented TCA cycle intermediates and pyruvate on the *Phsp-4::GFP* ER stress response reporter strains of worms (Figure 4.5B). Citrate activated *Phsp-4::GFP* reporter gene expression, and there was also a strong trend ($p=0.08$) for increased expression with alpha-ketoglutarate, while the other TCA cycle intermediates had no effect. Chelation of calcium or other metal ions is a possible mechanism as to how some of these compounds or their metabolites activate ER stress.

4.3.18 Tryptophan induces the mitochondrial unfolded protein response

We further tested for activation of the mitochondrial unfolded protein response using *Phsp-6* and *Phsp-60* reporter strains and ethidium bromide treatment as the positive control. HSP-6 is the worm homolog of mammalian mitochondrial hsp-70, while HSP-60 is also localized to the mitochondrion. Both mitochondrial heat shock proteins play a role in the mitochondrial unfolded protein response, but this response is not always associated with longevity [58]. For the *Phsp-6::GFP* reporter strain we tested glutamine, histidine, serine, phenylalanine, and tryptophan (Figure 4.4C). We found phenylalanine and tryptophan to robustly increase expression, while the other amino acids did not increase expression. We further tested the effects of glutamine, histidine, phenylalanine, proline, serine, tryptophan, and tyrosine on GFP expression in the *Phsp-60::GFP* reporter strain (Figure 4.4D). We found only tryptophan to increase expression. There was also a strong trend for proline to increase expression ($p=0.06$),

while serine slightly decreased expression. Overall, most amino acids do not rely upon the mitochondrial unfolded protein response pathway for lifespan extension.

Next, we determined the effects of the TCA cycle intermediates and pyruvate on the mitochondrial unfolded protein response reporter strains. None of the metabolites affected expression of the *Phsp-6::GFP* reporter (Figure 4.5C), while pyruvate, succinate, and malate slightly decreased expression of the *Phsp-60::GFP* reporter strain (Figure 4.5D). Overall, the data suggest that TCA cycle intermediate supplementation does not require the mitochondrial unfolded protein response pathway for lifespan extension.

4.3.19 Tryptophan metabolites nicotinic acid, nicotinamide, and picolinic acid induce both ER stress and mitochondrial unfolded protein responses

Since tryptophan activated expression of the ER stress response and 2 mitochondrial unfolded protein response reporter strains, we determined if one or more of its breakdown products or structurally related metabolites could also induce these responses or activate expression in the HSF-1 reporter strain. Therefore we added many of the tryptophan degradation products or tryptophan-related cellular metabolites including serotonin, anthranilic acid, nicotinic acid, nicotinamide, NAD, glutaric acid, kynurenic acid, quinolinic acid, and picolinic acid to the HSF-1 reporter strain, the ER stress reporter strain, and the 2 mitochondrial unfolded protein response reporter strains (Figure 6A-D). Strikingly, nicotinic acid activated expression of the same ER stress response and mitochondrial unfolded protein response reporters as tryptophan, while picolinic acid and nicotinamide activated expression of all 4 GFP reporter strains, although activation of *hsp-60::GFP* by nicotinamide was low ($p=0.07$). Picolinic acid is an isomer of nicotinic acid and an important endogenous metal chelator [59]. Quinolinic acid induced expression of the 2 non-mitochondrial heat shock protein reporters. Therefore, we performed

lifespan experiments adding 1 mM picolinic acid or 1 mM quinolinic acid to the culture medium (Additional file 6: Table S3). Picolinic acid addition showed a trend (p=0.13) toward increased lifespan. In contrast, quinolinic acid addition decreased worm lifespan by 26%, as might be expected from its known neurotoxicity [60]. NAD precursors have previously been shown to induce the mitochondrial unfolded protein response [61]. Here, we added a 0.1 mM dose of NAD and found it to only activate expression of *hsp-4,* the marker of ER stress. Although tryptophan metabolic byproducts could contribute to the protective effects of supplemental tryptophan, others have shown data suggesting that tryptophan itself may be the protective metabolite in a *C. elegans* model of alpha-synuclein toxicity [27].

4.3.20 Proline and tryptophan increase *C. elegans* thermotolerance

Amino acids could have induced expression of other heat shock or stress-inducible genes to extend lifespan not assayed in our reporter experiments described above. Therefore, we determined the effect of individual amino acid supplementation on the thermotolerance of *C. elegans*. We supplemented the growth medium with glutamine, histidine, proline, serine, or tryptophan and monitored the viability of the worms following transfer from 20°C to 35°C. Proline provided a 30% increase in thermotolerance and tryptophan provided a 10% increase in thermotolerance, while there was no significant effect of the other amino acids. The effect of proline is not too surprising given that proline stabilizes proteins and membranes and is overproduced to protect plants and some microorganisms from osmotic, salinity, and temperature stresses [62].

We next monitored the resistance to oxidative stress by monitoring the viability of worms following administration of paraquat. Paraquat is an inducer of superoxide production through redox cycling. None of the tested amino acids including proline, serine, histidine, tryptophan, or leucine significantly protected the worm viability against this stress, although there was a strong trend for protection with histidine (p=0.08).

4.3.21 Little effect of amino acid supplementation on Alzheimer's amyloid-beta toxicity

Since many of the amino acids extended lifespan when they were supplemented to the culture medium, we also determined if individual amino acid supplementation could delay toxicity in *C. elegans* models of human neurodegenerative disorders. We first determined the effects of serine, histidine, proline, tryptophan, or methionine supplementation on the rate of paralysis development when the amyloid-beta peptide, which builds up in Alzheimer's disease brain, is expressed in worm body wall muscle from a temperature-inducible promoter [63]. Overall, amino acid supplementation only had a minimal effect on the rate of paralysis. We found that serine (p=0.07) (Figure 4.7A) or histidine (p=0.05) (Figure 4.7B) supplementation gave strong trends to delay muscle paralysis, while no significant effects were found with proline, methionine, or tryptophan (Additional file 4.8).

4.3.22 Tryptophan protects from alpha-synuclein and polyglutamine toxicity

We next determined the effects of supplementation with histidine, proline, serine, or tryptophan on alpha-synuclein aggregation in worms expressing an alpha-synuclein-green fluorescent protein fusion (Figure 4.7C). Tryptophan was highly protective giving a 17% reduction in aggregate fluorescence, while the other amino acids were without effect. We also tested the effects of tryptophan, serine, and proline on aggregates formed from the expression of a polyglutamine (Q35)–GFP fusion protein [64] (Additional file 4.9).

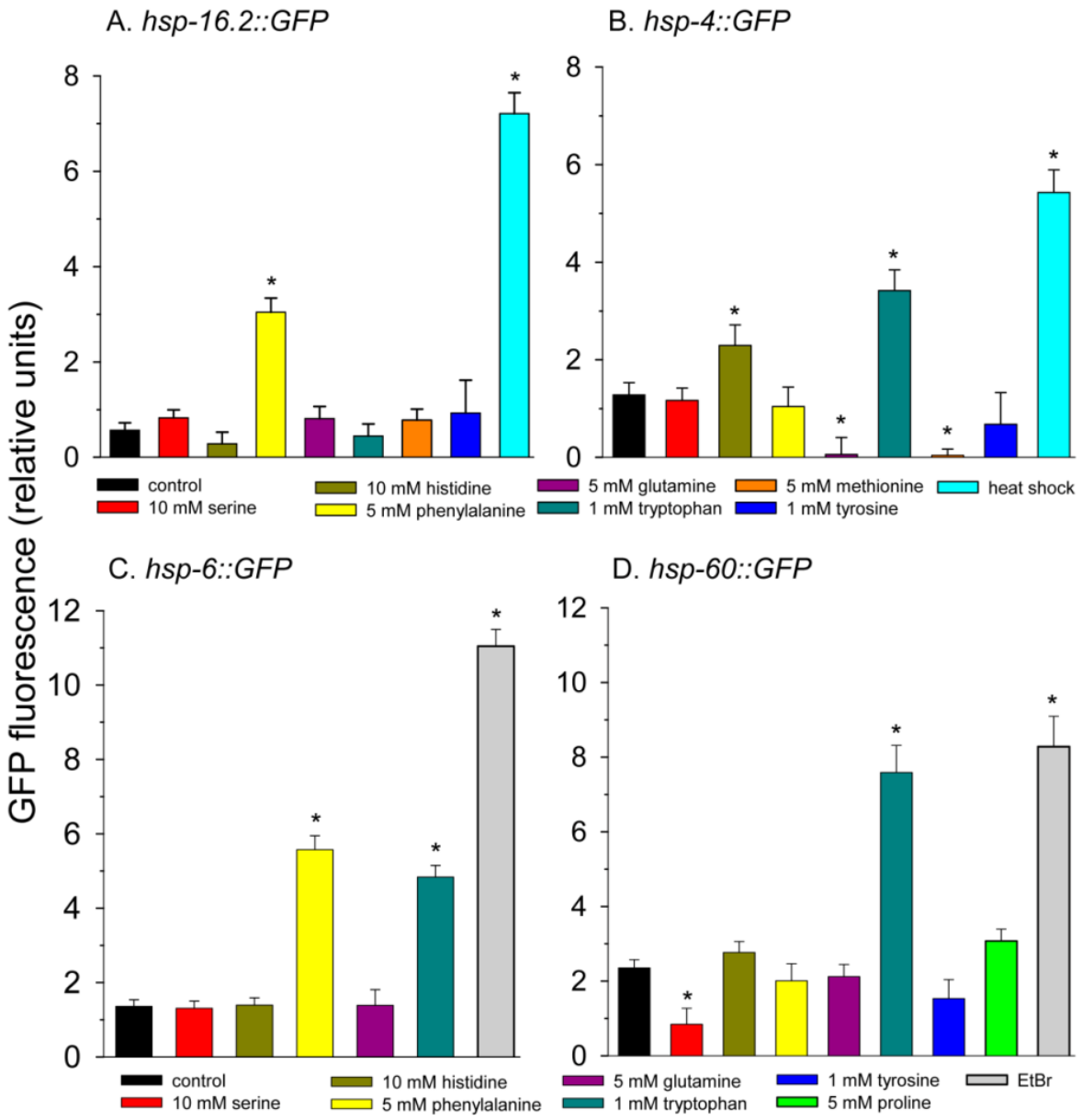

Figure 4.4. The effects of amino acids on heat shock, mitochondrial unfolded protein response, and ER stress response. Amino acids were added at the concentration tested that yielded maximal lifespan extension except phenylalanine, which did not extend lifespan. **(A)** *Phsp-16.2::GFP* **(B)** *Phsp-4::GFP* **(C)** *Phsp-6::GFP* **(D)** *Phsp-60::GFP*. For panels A and B heat shock at 35°C for 2 hours was used as a positive control. For panels C and D 50 µg/ml ethidium bromide treatment for 2 days was used as a positive control (* $p < 0.05$ vs. control).

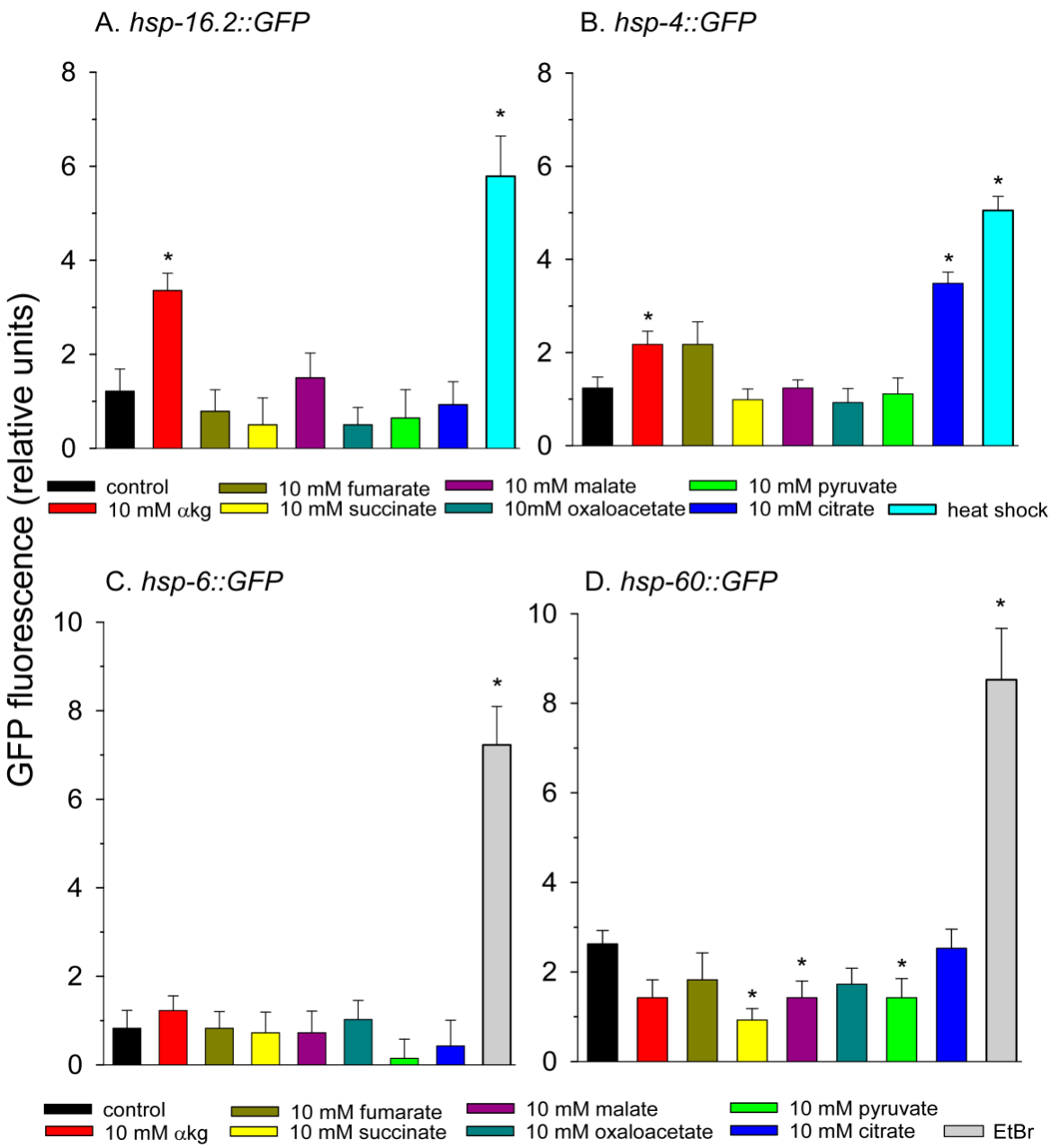

Figure 4.5. The effects of TCA cycle metabolites and pyruvate on heat shock, mitochondrial unfolded protein response, and ER stress response. **(A)** *Phsp-16.2::GFP* **(B)** *Phsp-4::GFP* **(C)** *Phsp-6::GFP* **(D)** *Phsp-60::GFP* (* $p < 0.05$ vs. control). For panels A and B heat shock at 35°C for 2 hours was used as a positive control. For panels C and D 50 µg/ml ethidium bromide treatment for 2 days was used as a positive control (* $p < 0.05$ vs. control).

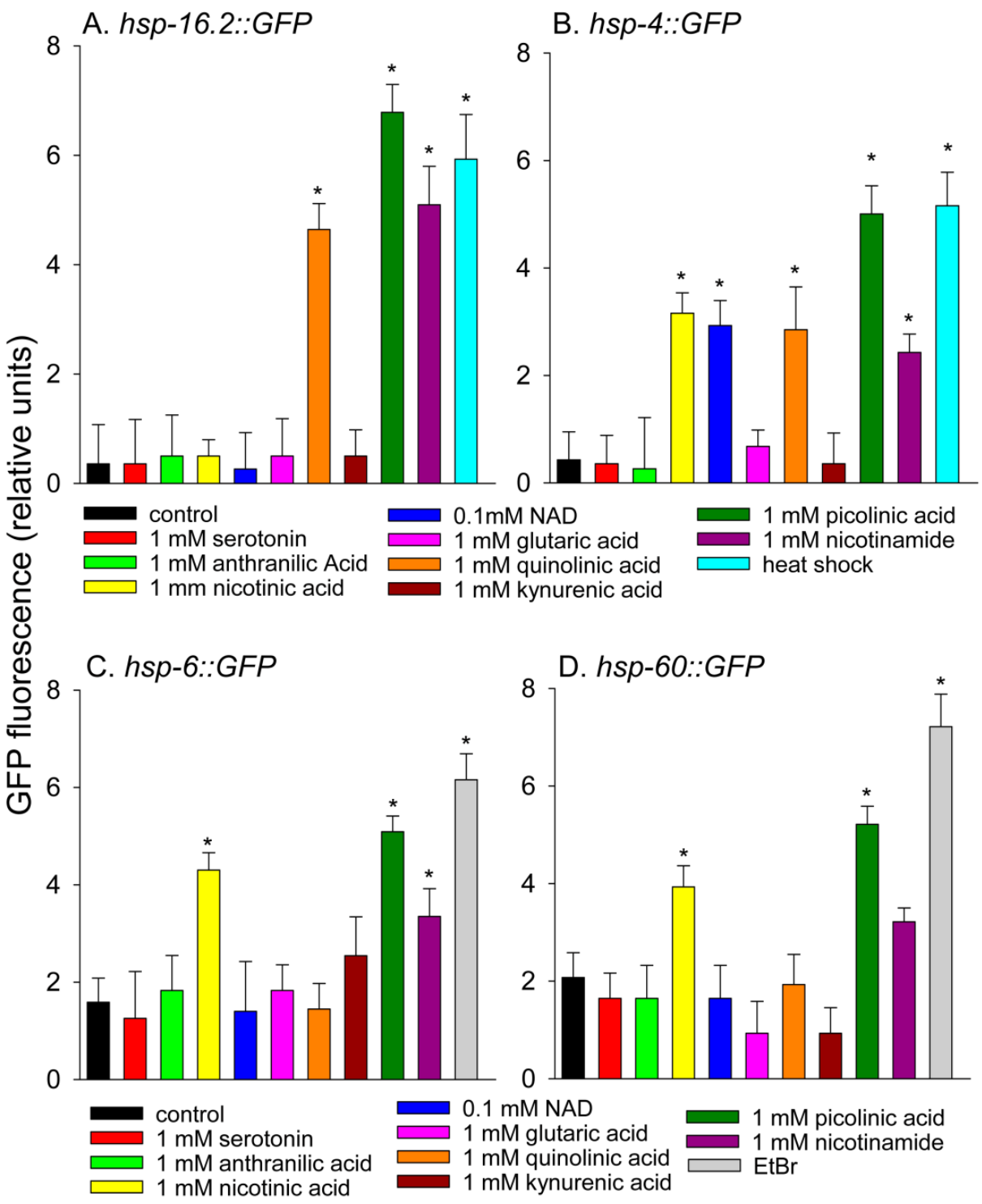

Figure 4.6. The effects of tryptophan metabolites on heat shock, mitochondrial unfolded protein response, and ER stress response. **(A)** *Phsp-16.2::GFP* **(B)** *Phsp-4::GFP* **(C)** *Phsp-6::GFP* **(D)** *Phsp-60::GFP* (* $p < 0.05$ vs. control). For panels A and B heat shock at 35°C for 2 hours was used as a positive control. For panels C and D 50 µg/ml ethidium bromide treatment for 2 days was used as a positive control (* $p < 0.05$ vs. control).

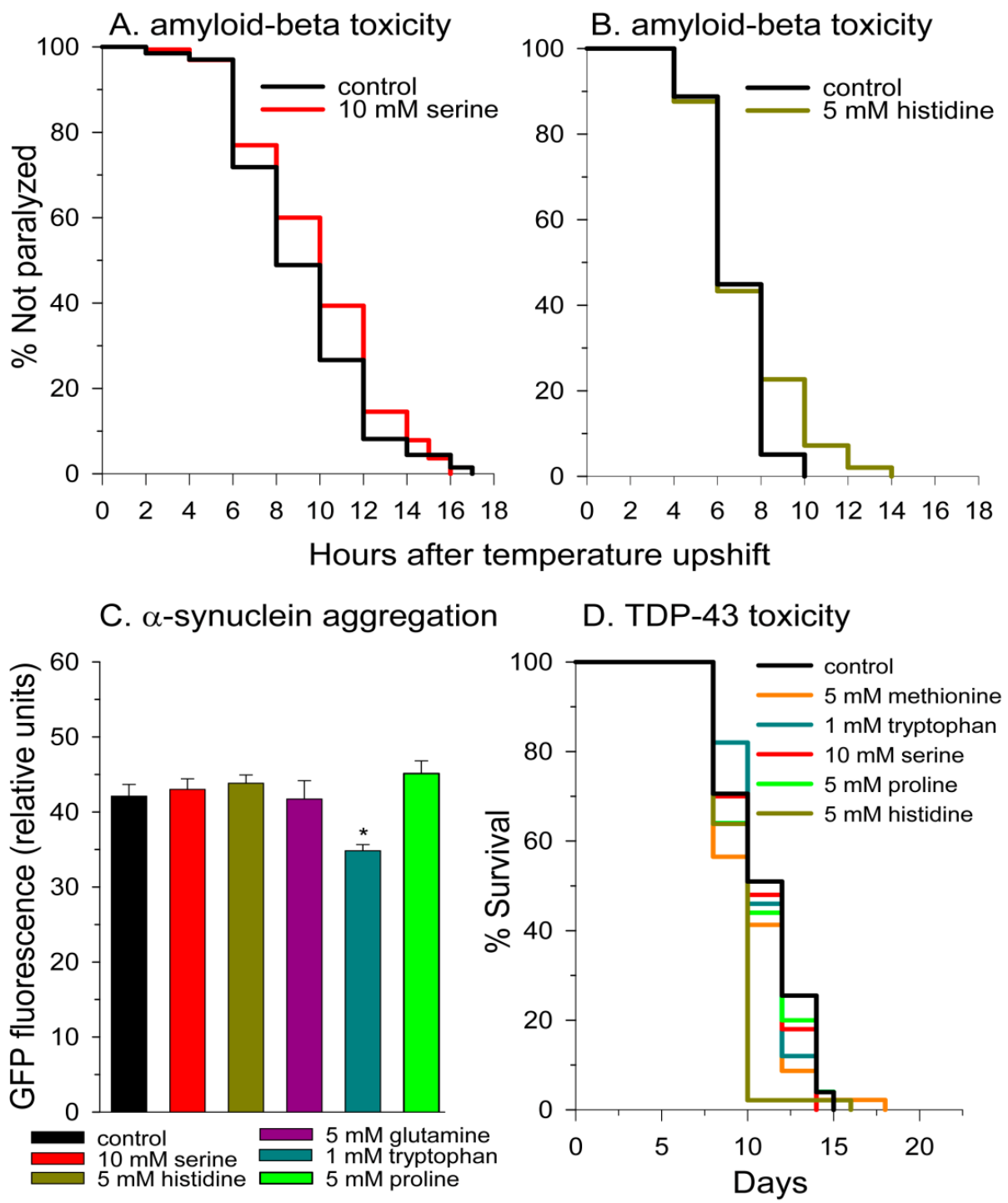

Figure 4.7. Amino acids do not provide much protection in models of proteotoxicity. **(A)** Serine and **(B)** histidine supplementation yield slight protection against muscle amyloid-beta toxicity. **(C)** Tryptophan supplementation decreases alpha-synuclein aggregate fluorescence. **(D)** Amino acid supplementation does not protect against TDP-43 toxicity.

We found a strong trend toward a decreased number of aggregates with the addition of tryptophan ($p = 0.07$), but no significant effect of the other two amino acids tested. It is not surprising that tryptophan supplementation was protective in these *C. elegans* proteotoxicity models as depletion of the TDO-2 enzyme that degrades tryptophan has been shown to increase worm tryptophan levels and be protective in several worm models of proteotoxicity [27]. It was also shown that tryptophan supplementation was able to increase the motility of Q40 polyglutamine expressing worms.

4.3.23 Alpha-ketoglutarate, but not amino acids, protects *C. elegans* expressing TDP-43

Tar DNA-binding protein-43 (TDP-43) is mostly a nuclear protein under normal conditions, but cytoplasmic accumulation is associated with ALS and other neurodegenerative diseases. Overexpression of TDP-43 leads to toxicity in *C. elegans* and is used as an ALS model. We therefore tested if several amino acids including histidine, methionine, proline, serine, and tryptophan could alter the reduced lifespan of TDP-43 overexpressing worms (Figure 4.7D and Additional file 4.10). However, none of these amino acids protected against the reduced lifespan, while histidine addition even further reduced the lifespan. But, we did find a protective effect of alpha-ketoglutarate supplementation in this model, supporting data from a study in which alpha-ketoglutarate in combination with other metabolites was protective in the SOD1-G93A mouse model of ALS [65].

4.3.24 Serine and tryptophan partially block lifespan reduction in high glucose culture media

High glucose in the bloodstream is a marker of insulin resistance and diabetes and high levels of glucose in *C. elegans* culture medium is known to reduce lifespan [66, 67]. Therefore high glucose supplementation has been used as a model of diabetes in *C. elegans*. Since

alterations of amino acid levels also accompany diabetes, we determined if select amino acids could alter the decreased lifespan resulting from the addition of 50 mM glucose to the culture medium. We found glucose addition decreased lifespan by 30 percent and that serine (Figure 4.8A) or tryptophan (Figure 4.8B) supplementation partially blocked lifespan reduction. Histidine, proline, or tyrosine addition had no significant effect, while glutamine addition decreased lifespan to a greater extent than glucose by itself.

4.3.25 Amino acid supplementation does not significantly alter *C. elegans* oxygen consumption and ATP levels

Since amino acid supplementation may transiently increase ROS production for the activation of SKN-1, we hypothesized that the added amino acids may be catabolized to increase mitochondrial ETC function. Therefore we measured worm oxygen consumption and ATP levels.However, we did not find significantly altered oxygen consumption or ATP levels following addition of the individual amino acids serine, proline, histidine, tryptophan, leucine, asparagine, or phenylalanine to the worms. Therefore, the supplemented amino acids are either not being metabolized at a substantial rate or are being metabolized in replacement of respiratory substrates present in the *E. coli* food source preventing an overall increase in metabolic rate.

4.4 Discussion

Individual supplementation with 18 of the 20 proteogenic amino acids at an optimal concentration increased the lifespan of *C. elegans*. This metabolite-induced lifespan extension was not specific for only amino acids as supplementation with TCA cycle intermediates and many different classes of cellular metabolites also led to significant lifespan extension, suggesting altered cellular metabolism or TCA cycle anaplerosis plays a prominent role.

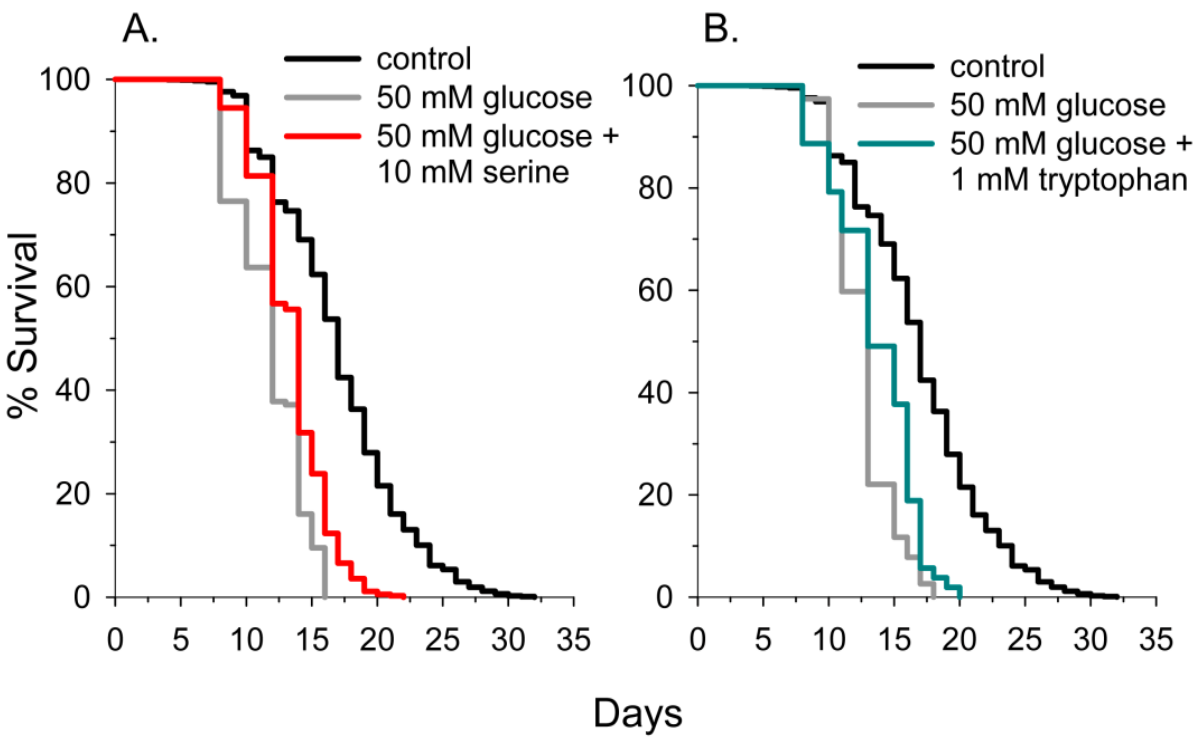

Figure4. 8. Serine or tryptophan partially prevent high glucose from reducing *C. elegans* lifespan. **(A)** Serine extends *C. elegans* lifespan in the presence of high glucose ($p < 0.001$). **(B)** Tryptophan extends *C. elegans* lifespan in the presence of high glucose ($p < 0.001$).

Of the 20 amino acids, serine and proline extended lifespan to the largest extent. Strikingly, these and other top amino acids did not significantly extend the lifespan of long-lived dietary restricted *eat-2* mutants. If similar effects occurred in humans, imbalanced amino acid diets could be developed as a dietary restriction mimetic to delay aging and aging-related disease. In this regard a methionine restricted diet has been used clinically to treat metabolic syndrome [68]. However, whether or not amino acid supplementation strategies will apply to higher eukaryotes remains to be determined as individual supplementation of methionine failed to extend the lifespan of fruit flies [24], while supplementation of methionine decreased the lifespan of mice [69].

4.4.1 Individual amino acid supplementation may decrease the rate of translation to extend lifespan

Most of the well-studied longevity pathways in *C. elegans* such as DAF-16, SKN-1, AAK-2, SIR-2.1, and HIF-1 appear to be involved in the lifespan extension mediated by histidine, serine, and proline, with the exception of the HIF-1 pathway for proline. However, tryptophan extends lifespan in a SKN-1 and DAF-16 independent manner, although tryptophan did increase fluorescence of a DAF-16 reporter strain of worms. These different longevity pathways may converge to decrease the rate of translation by the ribosome for lifespan extension. A small to moderate amount of amino acid imbalance could hinder correct aminoacyl-tRNA synthetase charging of tRNAs slowing the rate of translation in a GCN-2 dependent manner to increase lifespan, while a higher amount of amino acid imbalance may lead to a toxic reduction in translation rates decreasing lifespan. Previously, a decreased rate of translation has been shown to be essential for lifespan extension in mitochondrial mutants [33], during TOR inhibition, or during dietary restriction [34]. Furthermore, long-lived *daf-2* mutants were also shown to have a reduced rate of translation [70]. Reduced mitochondrial translation causing mitonuclear protein imbalance has also been shown to lead to increased lifespan [71]. High rates of translation during aging may overwhelm protein chaperone systems resulting in proteotoxicity. Slowing translation or increasing heat shock protein activity may delay this proteotoxicity to increase longevity.

The branched chain amino acid leucine most strongly activates TOR kinase, which can increase the rate of translation and lead to decreased lifespan. Therefore, we were surprised to find that supplementation with 1 mM leucine greatly increased lifespan. In fact, leucine was the second most potent amino acid at this concentration for promoting longevity. Under our standard

growth conditions TOR signaling may already be highly activated by amino acids present in the bacterial food source and so may not be able to be further activated. Instead, we hypothesize that leucine metabolism or the amino acid imbalance caused by excess leucine may have activated other signaling pathways leading to TOR inhibition. In this regard, the lack of lifespan extension by amino acids in *rsks-1* mutants suggests that individual amino acid supplementation inhibits TOR signaling to extend lifespan. Of the other two branched chain amino acids, valine supplementation showed similar lifespan trends as leucine, even though it is not a potent inducer of TOR activity, and isoleucine supplementation showed little effect on lifespan. At higher concentrations, branched chain amino acids did not affect lifespan as much as at the lower concentrations. Therefore, high levels of branched chain amino acids do not always directly correlate with lifespan extension. Consistent with this hypothesis, decreased levels of branched chain amino acids were found in long-lived Ames dwarf mice [72]. However, amino acid imbalance cannot explain the increased longevity conferred upon supplementation with many other diverse cellular metabolites. Therefore, TCA cycle anaplerosis leading to a reprogramming of mitochondrial metabolism may be the molecular mechanism responsible for lifespan extension.

4.4.2 NAD precursors and tryptophan induce the ER stress response and the mitochondrial unfolded protein response

Another significant finding in this report is that supplementation with tryptophan, nicotinamide, nicotinic acid, or its isomer picolinic acid resulted in the activation of both the mitochondrial unfolded protein and the ER stress response pathways, while NAD addition induced only the ER stress response. In mouse muscle and heart, decreased nuclear NAD levels with aging cause a hypoxia inducible factor-1α (HIF-1α)-induced decrease in mitochondrial

transcription leading to mitochondrial dysfunction that was reversed by supplementation with an NAD precursor [73]. In addition to reversing this aging-induced mitochondrial dysfunction, supplementing NAD precursors (or tryptophan) can further protect cells by inducing an ER stress response and a mitochondrial unfolded protein response, although it has already been shown that NAD precursors induce a mitochondrial unfolded protein response [61]. Picolinic acid is a strong metal chelator that is frequently taken with chromium as a possible treatment for metabolic syndrome, although the efficacy of this treatment has been questioned [74]. But picolinic acid itself has been shown to be neuroprotective [59]. It will be important to determine if the protective effects of picolinic acid are due to metal chelation and if picolinic acid, nicotinic acid, and nicotinamide also induce the ER stress response or mitochondrial unfolded protein response in mammalian cells.

4.4.3 Is catabolism of amino acids important for their longevity effects?

The catabolism of supplemented amino acids likely mediates their effects on longevity, but there are likely exceptions to this rule. We were surprised to find no increase in oxygen consumption or ATP levels as markers of increased metabolism when amino acids were added to the culture medium. It was previously shown that proline catabolism and the resulting transient increase in ROS production by the electron transport chain were essential for its pro-longevity effects [26]. Most other amino acids may also be metabolized to emit a transient ROS signal leading to SKN-1 dependent lifespan extension. Possible evidence for this includes that enzymes for proline, tryptophan, phenylalanine, glutamine, and D-alanine degradation are upregulated in long-lived *daf-2* insulin receptor mutant worms [26], where SKN-1 is also activated [75]. However, alpha-ketoglutarate-mediated lifespan extension was shown to be independent of ROS production [48]. Therefore more downstream TCA cycle catabolites such as alpha-ketoglutarate,

succinate, malate, and fumarate extend lifespan through SKN-1independent, but DAF-16 dependent mechanisms [53]. In addition, cysteine and tryptophan-induced lifespan extension were independent of SKN-1, so supplementation with these amino acids did not likely alter mitochondrial metabolism to increase ROS production. Consistent with this, knockdown of the tryptophan 2,3-dioxygenase (TDO-2) enzyme that degrades tryptophan led to increased lifespan and increased tryptophan levels, but no changes in the levels of many of the downstream metabolites of tryptophan degradation suggesting that increased tryptophan levels and not altered levels of the catabolic byproducts were responsible for the increased lifespan and protection from proteotoxicity [27].

Future studies will aim to determine the metabolic mechanisms through which amino acids activate SKN-1 activity and through which serine and histidine activate HIF-1. For example, enzyme knockdown studies using RNAi may be used to determine whether serine catabolism is required for serine-mediated lifespan extension, as serine can be directly deaminated to pyruvate, which extends lifespan [51]. However, serine can also be used as a one-carbon donor for methylation events such as histone methylation, which can alter gene expression patterns leading to increased longevity as well [76].

4.4.4 TCA cycle metabolism and longevity

Different amino acids are catabolized and enter the TCA cycle through different intermediates of the cycle. We did not find that the amino acids yielding the greatest effects on lifespan were degraded through one common catabolic pathway. Additional file 13: Figure S7, Additional file 14 Figure S8, and Additional file 15: Figure S9 show the specific TCA cycle intermediate into which each of the amino acids is catabolized and how lifespan was affected by 1 mM, 5 mM, and 10 mM amino acid concentrations, respectively. As mentioned previously, the

only trend in the data set is that amino acids broken down into alpha-ketoglutarate yielded larger than average lifespan extensions. However, this cannot explain the large lifespan extension induced by serine addition, which is catabolized to pyruvate. Surprisingly, we previously discovered that malate or fumarate supplementation increased total pyridine (NAD + NADH) nucleotide levels and also induced mild mitochondrial uncoupling increasing the NAD/NADH ratio, both of which may have been involved in the lifespan extension induced by these TCA cycle intermediates [53]. Catabolism of other metabolites may result in similar effects.

Knockdown of mitochondrial aconitase or a subunit of mitochondrial NAD-dependent isocitrate dehydrogenase increased lifespan in *C. elegans* [77]. The increased longevity of these mutants is likely due in part to decreased flux through this portion of the TCA cycle leading to increased NAD levels, which has been shown to extend lifespan [78]. The isocitrate dehydrogenase and alpha-ketoglutarate dehydrogenase enzymes reduce mitochondrial NAD to NADH. Citrate supplementation may not increase lifespan due to the increased flux through this portion of the TCA cycle leading to a decrease in NAD levels, although in the cytoplasm citrate is metabolized to acetyl-CoA that has been shown to inhibit autophagy, which can also prevent lifespan extension [79]. However, isocitrate supplementation would also not be expected to increase longevity as its normal metabolism is predicted to lower NAD levels. However, there is also an NADP-dependent isocitrate dehydrogenase isoform present that may help prevent declines in NAD levels and allow for isocitrate-mediated lifespan extension at least in a narrow range of concentrations.

It is possible that supplementation with amino acids broken down into alpha-ketoglutarate may extend lifespan by running isocitrate dehydrogenase in the opposite direction of its normal mode to oxidize NADH to NAD while alpha-ketoglutarate and carbon dioxide are

metabolized into isocitrate. Some cancer cells have been shown to use this metabolism when oxidizing glutamine as a primary energy substrate [80]. The citrate produced from isocitrate is then exported to the cytoplasm where acetyl-CoA and oxaloacetate are formed by ATP citrate lyase. This metabolic flexibility in TCA cycle metabolism may be required for specific amino acid and TCA cycle metabolite-mediated longevity and is a hypothesis for future testing, as running the TCA cycle in the reverse of its normal direction was shown to be needed for malate and fumarate-mediated lifespan extension [53].

4.4.5 Glycolysis as a lifespan shortening metabolic pathway in *C. elegans*

Since supplementation with glucose and other glycolytic precursors decrease lifespan in *C. elegans* as shown here and in [66, 67], there are likely specific metabolic pathways such as glycolysis that decrease lifespan. We propose that many of the supplemented metabolites that increased lifespan are catabolized by mitochondria to increase TCA cycle metabolite levels. The DAF-16/FOXO longevity pathway has been shown to be activated by increased TCA cycle metabolite levels [53]. So the TCA cycle appears to be lifespan-extending metabolic pathway in *C. elegans*. In addition increased TCA cycle flux could also transiently increase mitochondrial ETC ROS production to activate the SKN-1 longevity pathway as previously suggested [26]. This metabolite oxidation for energy production would also decrease reliance on lifespan-shortening glycolysis as a source of mitochondrial respiratory substrates.

4.4.6 Refinements in *C. elegans* culture media for lifespan experiments by limiting peptone levels and using heat-killed bacteria during adulthood

When performing lifespan experiments, sources of stress should be removed from the environment, so control animal lifespan is not limited. Unfortunately when working with *C. elegans*, this has proven difficult due to the slight toxicity of their live *E. coli* food source. We

and others [55] have also found that the peptone present in nematode growth media (NGM) also induces a type of stress that decreases lifespan. It is unclear which component of peptone decreases lifespan as it contains not only proteolyzed proteins (amino acids and oligopeptides), but also fats, metals, salts, vitamins, and other compounds. Because of the slight toxicity associated with the use of peptone and live *E. coli*, we chose to perform *C. elegans* lifespan experiments using heat-killed *E. coli* in liquid S-medium, which lacks peptone. Due to partial degradation of one or more nutrients in the *E. coli* food source needed for larval development during the heat-killing treatment, it may be advantageous, especially in certain mutant backgrounds, to treat the worms with live *E. coli* during larval development to ensure adequate nutrition, and then switch them to heat-killed *E. coli* during adulthood to prevent the bacteria from metabolizing added nutrients. Further refinements in experimental methods will allow *C. elegans* to become an even more valuable model for investigating the effects of altered metabolism on lifespan.

4.5 Conclusions

Individual amino acid supplementation increased the lifespan of *C. elegans* and increased stress resistance with serine, proline, and tryptophan showing the greatest effects. Many longevity pathways including DAF-16, SKN-1, AAK-2, SIR-2.1, GCN-2, heat shock, autophagy, DR, and inhibition of TOR signaling are involved in these protective effects. Anaplerosis and altered mitochondrial metabolism transiently increasing ROS production to activate SKN-1 appear to be involved in the longevity signaling. The exact pathways involved vary slightly from one amino acid to the next. For example, serine and histidine stimulated transcriptional activity of HIF-1, while 6 other amino acids did not. Likewise 8 lifespan-extending amino acids increased the transcriptional activity of SKN-1, but not tryptophan, cysteine, or the lifespan-

decreasing amino acids phenylalanine and asparagine. Uniquely, tryptophan activated the ER stress response and mitochondrial unfolded protein response pathways and lifespan extension was independent of SKN-1 and DAF-16. Future experiments will aim to develop an improved axenic medium that can be used to determine the effects of amino acid restriction on the lifespan of *C. elegans*. Through the use of both amino acid supplementation and restriction, a diet may one day be developed that can substantially increase stress resistance and slow aging and the onset of aging-associated disorders.

4.6 Supporting information

Table 4.6 The effect of amino acid supplementation on *C. elegans* lifespan

Strain	Concentration	Amino acid	% of control mean lifespan	*n*	# of worms	*p*-value
N2	1 mM	alanine	108	3	264	0.015
N2	5 mM		104	3	277	0.305
N2	10 mM		111	3	274	0.003
N2	1 mM	arginine	108	4	428	0.007
N2	5 mM		111	4	412	<0.001
N2	10 mM		115	4	438	<0.001
N2	1 mM	asparagine	101	3	350	0.331
N2	5 mM		105	3	319	0.032
N2	10 mM		75	3	297	<0.001
N2	1 mM	aspartate	103	3	477	0.15
N2	5 mM		100	3	495	0.052
N2	10 mM		94	3	544	0.001
N2	1 mM	cysteine	109	2	200	0.029
N2	5 mM		116	2	190	<0.001
N2	10 mM		102	2	195	0.513
N2	1 mM	glutamate	114	2	156	0.005
N2	5 mM		111	2	191	0.034
N2	10 mM		92	2	172	0.005
N2	1 mM	glutamine	115	2	184	0.002

Table 4.6 (Continued)

Strain	Concentration	Amino acid	% of control mean lifespan	*n*	# of worms	*p*-value
N2	5 mM		116	2	196	0.002
N2	10 mM		94	2	214	0.001
N2	1 mM	glycine	110	3	321	0.008
N2	5 mM		103	3	298	0.538
N2	10 mM		96	3	327	0.09
N2	1 mM	histidine	104	3	338	0.155
N2	5 mM		109	3	269	<0.001
N2	10 mM		112	3	282	<0.001
N2	1 mM	isoleucine	103	3	344	0.079
N2	5 mM		103	3	290	0.066
N2	10 mM		103	3	360	0.033
N2	1 mM	leucine	116	2	265	<0.001
N2	5 mM		106	2	238	0.042
N2	10 mM		107	2	240	0.032
N2	1 mM	lysine	107	4	562	<0.001
N2	5 mM		108	4	583	<0.001
N2	10 mM		106	4	547	<0.001
N2	1 mM	methionine	104	2	160	0.094
N2	5 mM		108	2	162	0.002
N2	10 mM		114	2	148	<0.001
N2	1 mM	phenylalanine	97	2	167	0.491
N2	5 mM		92	2	165	<0.001
N2	10 mM		88	2	164	<0.001
N2	1 mM	proline	117	3	369	<0.001
N2	5 mM		119	3	322	<0.001
N2	10 mM		118	3	329	<0.001
N2	1 mM	serine	108	5	495	<0.001
N2	5 mM		118	5	501	<0.001
N2	10 mM		122	5	607	<0.001
N2	1 mM	threonine	100	3	317	0.901
N2	5 mM		103	3	326	0.179
N2	10 mM		108	3	279	<0.001
N2	1 mM	tryptophan	114	3	289	<0.001
N2	5 mM		106	3	234	0.003

Table 4.6 (Continued)

Strain	Concentration	Amino acid	% of control mean lifespan	*n*	# of worms	*p*-value
N2	10 mM		101	3	203	0.643
N2	1 mM	tyrosine	110	2	198	<0.001
N2	5 mM		105	2	186	0.001
N2	10 mM		102	2	168	0.014
N2	1 mM	valine	113	3	338	0.155
N2	5 mM		108	3	269	<0.001
N2	10 mM		99	3	282	<0.001

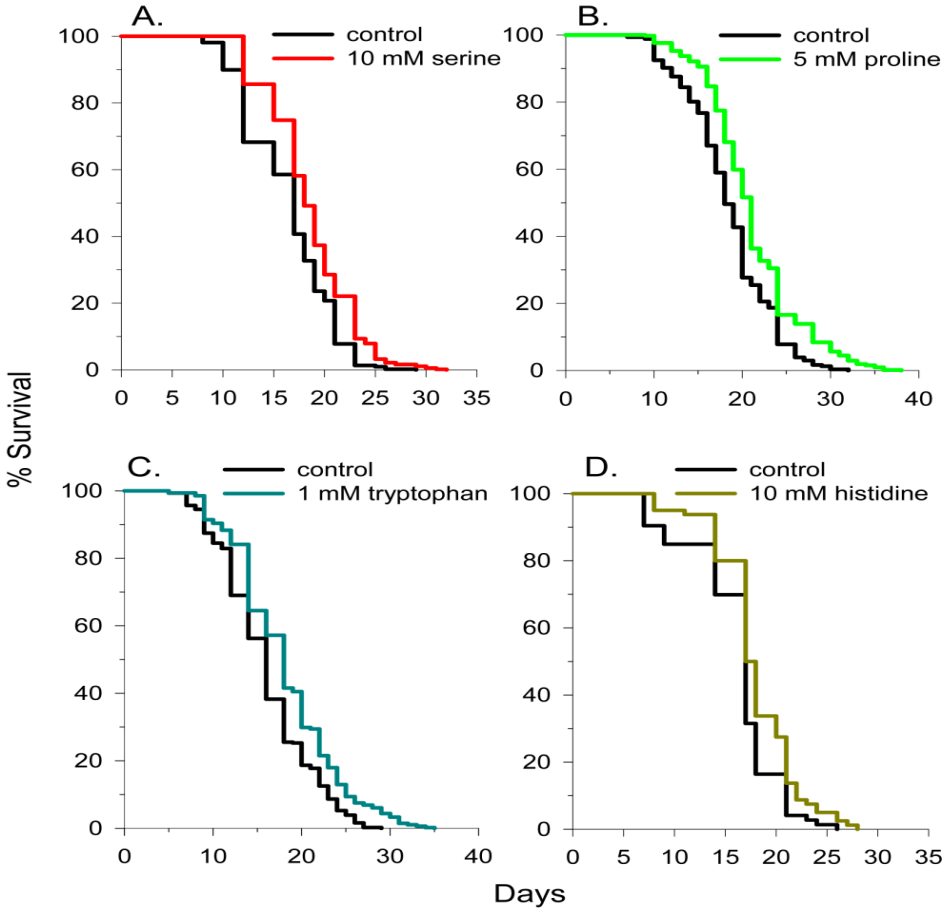

Figure 4.9. Example lifespan curves for several amino acids that strongly extended lifespan in *C. elegans*. (A) serine, (B) proline, (C) tryptophan, and (D) histidine. Concentrations chosen for display were those that stimulated lifespan extension to the greatest extent (log rank $p < 0.001$).

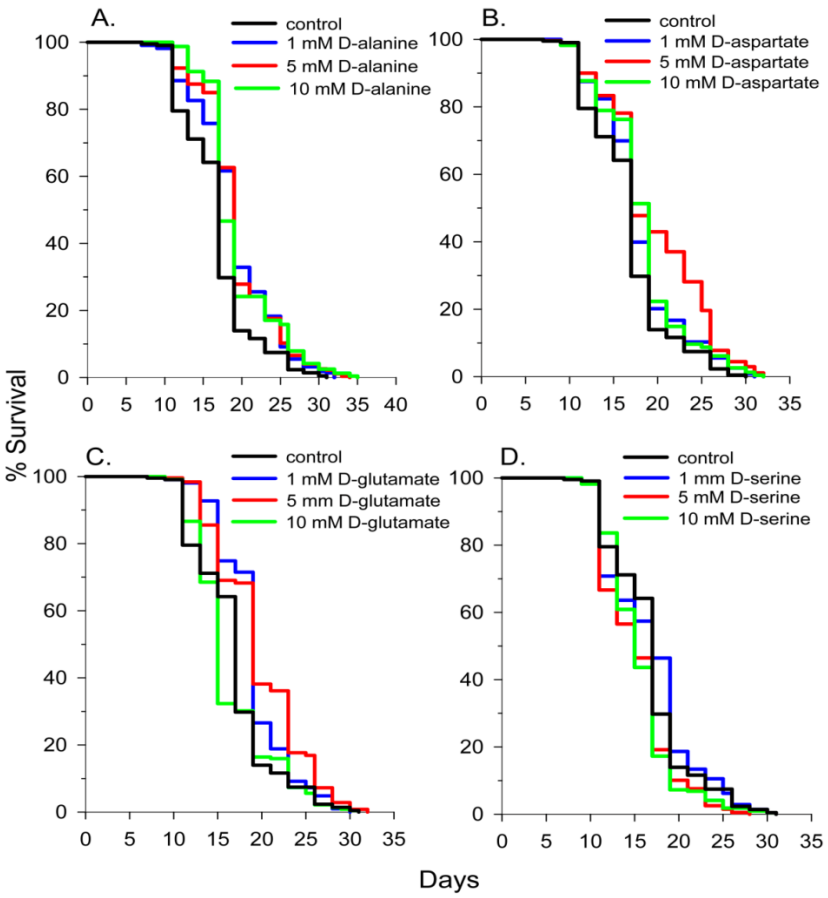

Figure 4.10. Supplementation of several D-amino acids found endogenously in *C. elegans* extends lifespan. (A) D-alanine, B) D-aspartate, or C) D-glutamate extended lifespan at one or more of the concentrations tested (log rank $p < 0.05$), while (D) D-serine supplementation did not extend lifespan at any of the concentrations tested.

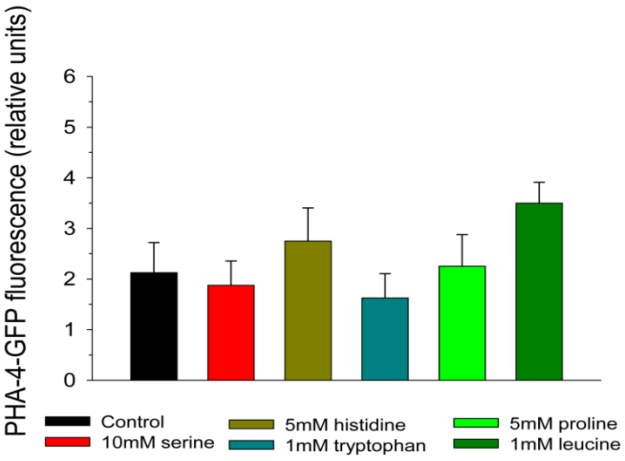

Figure 4.11. The effect of amino acids on the fluorescence of a *pha-4p::gfp:pha-4* reporter strain of *C. elegans*. (* $p < 0.05$)

Table 4.7 The effect of sugars and other metabolites lacking nitrogen on *C. elegans* lifespan

Treatment	% of N2 control mean lifespan	p-value	# of worms	Replicates
1 mM ribose	109	<0.001	333	3
5 mM ribose	109	<0.001	345	3
10 mM ribose	101	0.726	377	3
10 mM glycerol	121	<0.001	134	1
1 mM dihydroxyacetone	77	<0.001	275	2
10 mM dihydroxyacetone	75	<0.001	269	2
1 mM glyceraldehyde	74	<0.001	113	1
10 mM glyceraldehyde	87	<0.001	186	1
1 mM inositol	103	0.0397	220	1
10 mM inositol	117	<0.001	235	1
10 mM xylose	106	0.038	133	1
1 mM galactose	101	0.887	296	2
10 mM galactose	106	0.0349	250	2
1 mM gluconate	108	0.0263	127	1
5 mM gluconate	117	<0.001	124	1
10 mM gluconate	116	<0.001	120	1
1 mM glucuronolactone	102	0.717	290	1
5 mM glucuronolactone	103	0.829	300	1
10 mM glucuronolactone	103	0.831	310	1
1 mM propionate	85	<0.001	104	1
5 mM propionate	87	<0.001	106	1
10 mM propionate	82	<0.001	110	1
1 mM fructose	96	0.010	251	2
10 mM fructose	86	<0.001	233	2
1 mM phosphoenolpyruvate	112	<0.001	180	1
5 mM phosphoenolpyruvate	112	<0.001	170	1
10 mM phosphoenolpyruvate	108	0.002	149	1
0.01 mM caprylate	101	0.356	192	2
0.1 mM caprylate	107	0.019	165	2
1 mM caprylate	95	0.001	186	2
5 mM caprylate	82	0.001	165	2
1 mM DL-lactate	103	0.35	213	1
5 mM DL-lactate	106	0.03	220	1
10 mM DL-lactate	102	0.62	189	1

Table 4.8 The effects of nitrogen containing metabolites on *C. elegans* lifespan

treatment	% of control mean lifespan	p-value	# of worms	replicates
0.125 g/L peptone	101	0.176	231	2
0.625 g/L peptone	90	<0.001	232	2
1.25 g/L peptone	77	<0.001	226	2
1 mM ammonium chloride	99	0.78	185	2
5 mM ammonium chloride	98	0.727	205	2
10 mM ammonium chloride	104	0.0789	238	2
1 mM creatine	96	0.001	267	2
5 mM creatine	80	<0.001	170	2
10 mm creatine	82	<0.001	118	2
0.01 mM carnosine	101	0.833	149	2
0.1 mM carnosine	114	<0.001	187	2
1 mM carnosine	111	0.0163	186	2
1 mM beta-alanine	113	<0.001	130	1
5 mM beta-alanine	105	0.871	228	1
0.1 mM ornithine	103	0.261	226	2
1 mM ornithine	108	<0.001	218	2
10 mM ornithine	90	<0.001	206	2
0.1 mM agmatine	101	0.627	223	2
1 mM agmatine	116	<0.001	212	2
10 mM agmatine	80	<0.001	220	2
0.1 mM putrescine	101	0.754	205	2
1 mm putrescine	110	<0.001	250	2
10 mM putrescine	87	<0.001	239	2
1 mM taurine	111	<0.001	261	2
5 mM taurine	106	0.0113	273	2
10 mM taurine	96	0.0237	231	2
0.1 mM theanine	114	0.007	130	1
1mM theanine	108	<0.001	225	1
5mM theanine	110	<0.001	172	1
10mM theanine	85	<0.001	183	1

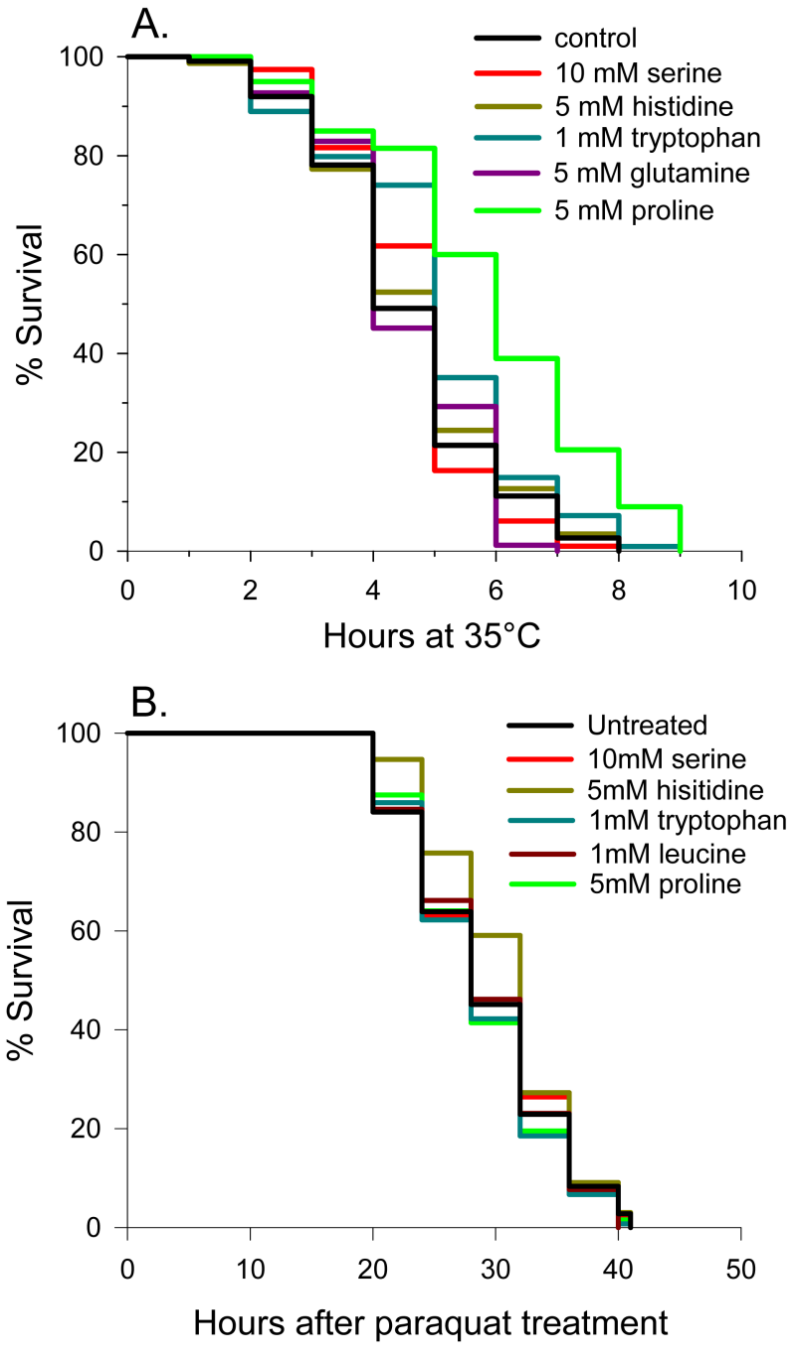

Figure 4.12. Thermotolerance and paraquat-induced toxicity. (A) Proline or tryptophan supplementation increases thermotolerance in *C. elegans*. (log rank $p < 0.001$) Serine, histidine, or glutamine supplementation did not significantly affect thermotolerance. (B) Amino acid supplementation did not significantly delay paraquat-induced toxicity. However, there was a strong trend toward protection with histidine ($p=0.08$), but no effect with serine, proline, tryptophan, or leucine.

Table 4.9 The effects of amino acids on amyloid-beta-induced muscle paralysis

treatment	% of mean control time to paralysis	p-value	# of worms	replicates
10 mM serine	108.05	0.073	172	3
1 mM tryptophan	104.24	0.207	185	3
5 mM histidine	106.20	0.05	97	2
5 mM proline	99.14	0.896	85	1
1 mM methionine	98.99	0.739	163	3
30 mM methionine	81.02	0.001	88	1

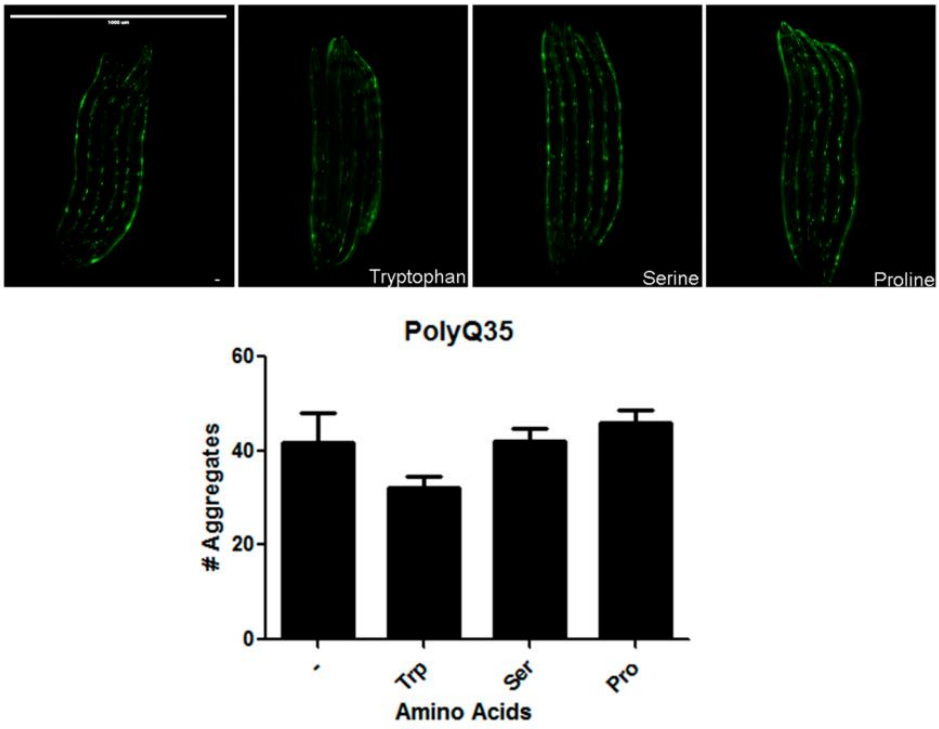

Figure 4.13. Tryptophan slightly decreased polyglutamine aggregates in *C. elegans*. The GFP fluorescence of five worms placed side by side are shown in each photo. There were on average 7 less aggregates in tryptophan treated worms than in controls ($p = 0.07$).

Table 4.10 The effects of amino acids on lifespan in human TDP-43 transgenic *C. elegans*

treatment	% of mean control lifespan	p-value	# of worms	replicates
10 mM serine	98	0.373	50	2
1 mM tryptophan	98	0.287	50	2
5 mM histidine	86	<0.001	47	2
5 mM proline	97	0.484	50	2
5 mM methionine	93	0.113	46	2
25 mM alpha-ketoglutarate	110	0.008	100	2

Table 4.11 The effects of amino acids on *C. elegans* lifespan in the presence of 50 mM glucose.

treatment	% of mean lifespan in the presence of 50 mM glucose	p-value	# of worms	replicates
10 mM serine	114	<0.001	365	2
5 mM histidine	97	0.2910	334	2
5 mM glutamine	92	<0.001	120	2
5 mM proline	101	0.546	205	2
1 mM tyrosine	96	0.0870	172	2
1 mM tryptophan	110	<0.001	143	2
10 mM tryptophan	102	0.7340	112	2

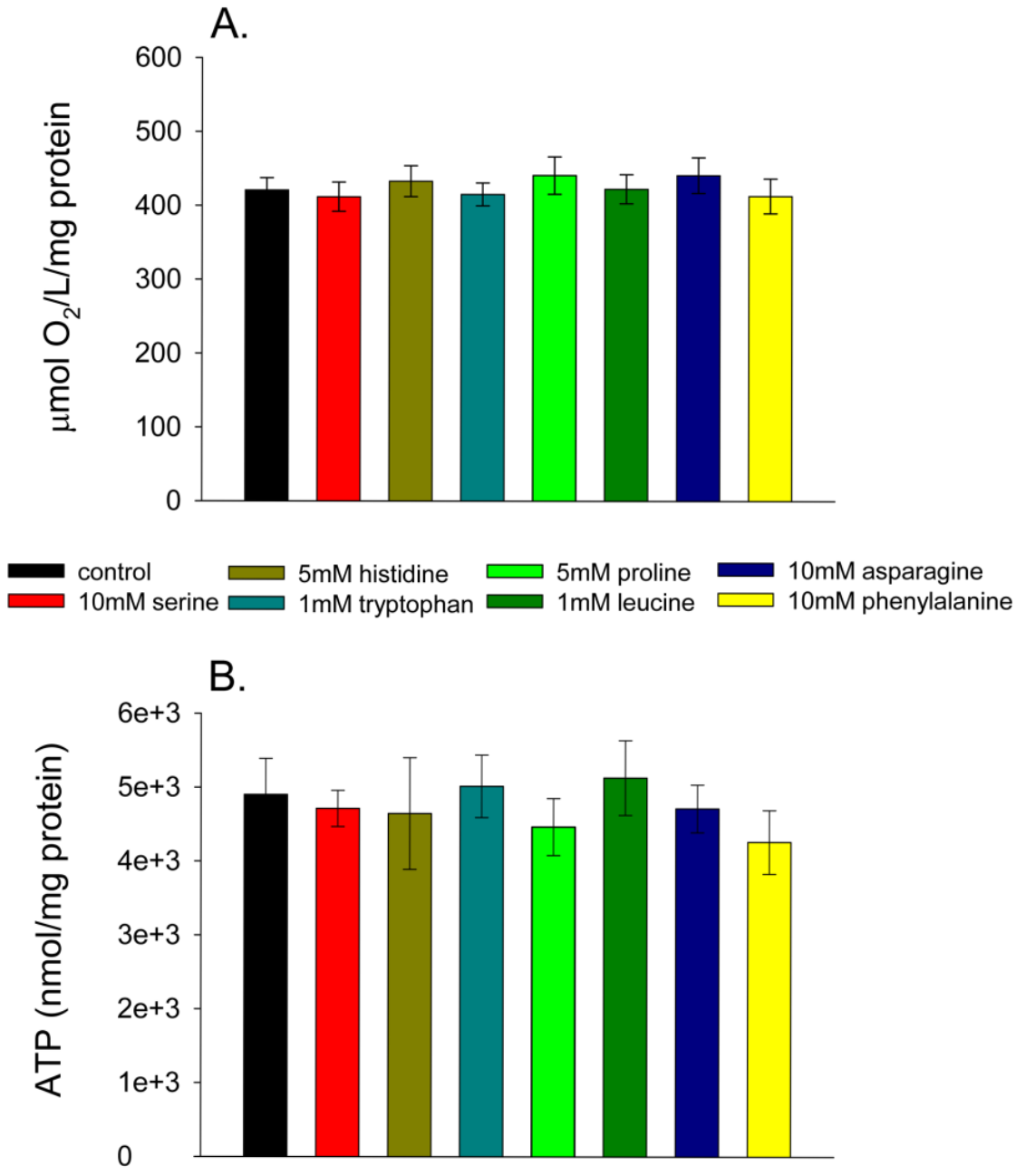

Figure 4.14. Amino acid supplementation did not significantly alter *C. elegans* oxygen consumption or ATP levels. A) The amount of oxygen in the medium following a 30 minute incubation in the well of an Oxoplate. B) ATP levels.

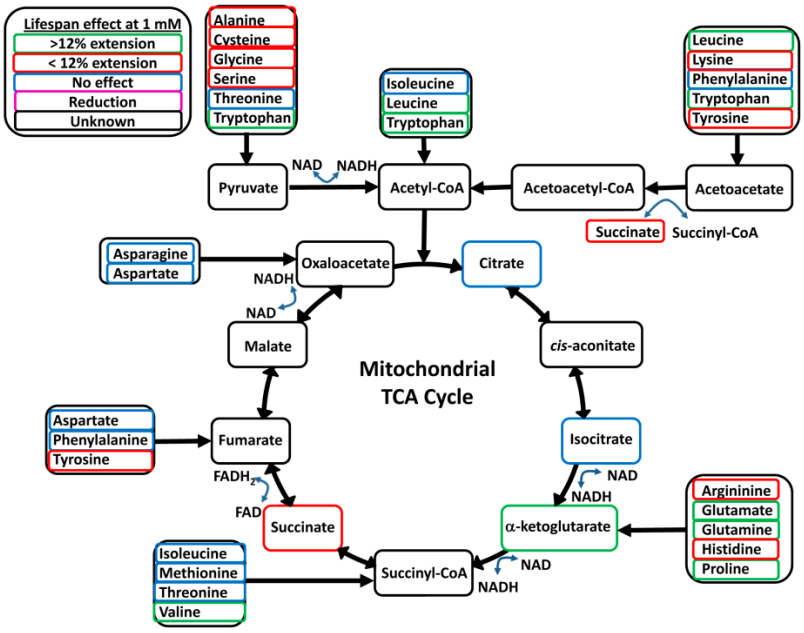

Figure 4.15. Metabolism and effects on lifespan of a 1 mM dose of amino acids. A diagram of the TCA cycle metabolites to which the 20 amino acids are catabolized is shown. It is also shown how supplementation of a 1 mM concentration of the amino acids or some of the TCA cycle metabolites affected *C. elegans* lifespan.

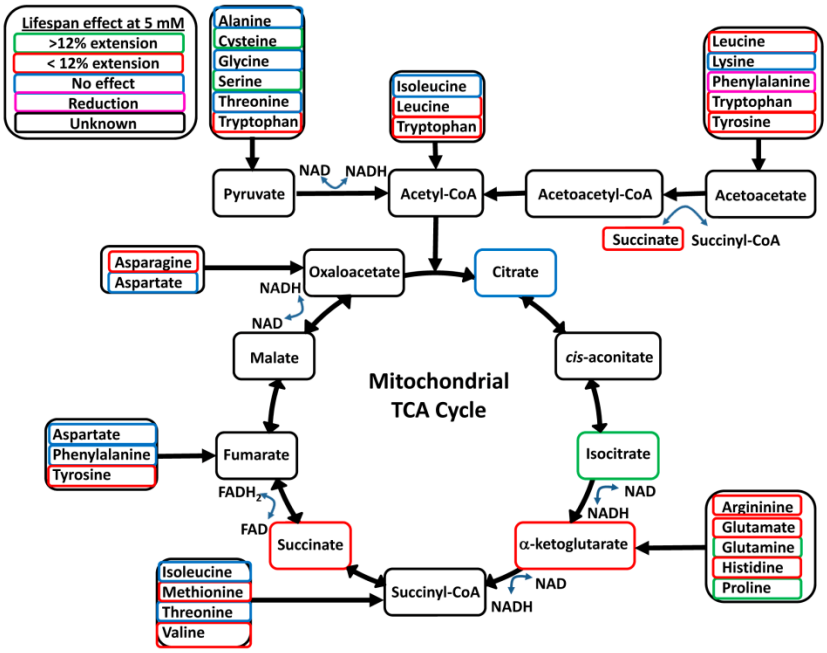

Figure 4.16. Metabolism and effects on lifespan of a 5 mM dose of amino acids. A diagram of the TCA cycle metabolites to which the 20 amino acids are catabolized is shown. It is also shown how supplementation of a 5 mM concentration of the amino acids or some of the TCA cycle metabolites affected *C. elegans* lifespan.

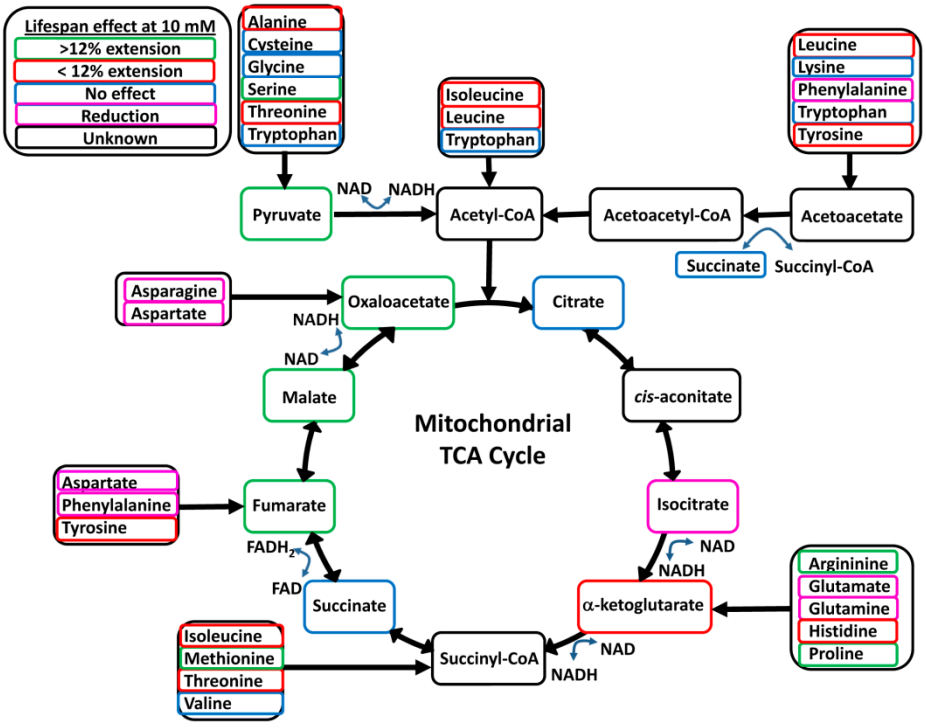

Figure 4.17. Metabolism and effects on lifespan of a 10 mM dose of amino acids. A diagram of the TCA cycle metabolites to which the 20 amino acids are catabolized is shown. It is also shown how supplementation of a 10 mM concentration of the amino acids or some of the TCA cycle metabolites affected *C. elegans* lifespan.

4.7 Competing interests

The authors declare that they have no competing interests.

4.8 Acknowledgements

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4.9 Authors' contributions

CE performed most of the lifespan, GFP reporter strain, proteotoxicity experiments, and data analysis. JC performed data analysis. NC performed GFP reporter strain experiments and data analysis. AB, MR and DL performed a few lifespan and GFP reporter strain experiments. JB and SW planned the experiments with the polyglutamine-expressing worms, while JB performed these experiments. CE and PB conceived the studies and drafted the manuscript. All authors read and approved the final manuscript.

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CHAPTER 5

D-BETA-HYDROXYBUTYRATE EXTENDS LIFESPAN IN *C. ELEGANS*[3]

5.1 Abstract

The ketone body beta-hydroxybutyrate (βHB) is a histone deacetylase (HDAC) inhibitor and has been shown to be protective in many disease models, but its effects on aging are not well studied. Therefore we determined the effect of βHB supplementation on the lifespan of *C. elegans* nematodes. βHB supplementation extended mean lifespan by approximately 20%. RNAi knockdown of HDACs *hda-2* or *hda-3* also increased lifespan and further prevented βHB-mediated lifespan extension. βHB-mediated lifespan extension required the DAF-16/FOXO and SKN-1/Nrf longevity pathways, the sirtuin SIR-2.1, and the AMP kinase subunit AAK-2. βHB did not extend lifespan in a genetic model of dietary restriction indicating that βHB is likely functioning through a similar mechanism. βHB addition also upregulated BHB dehydrogenase activity and increased oxygen consumption in the worms. RNAi knockdown of F55E10.6, a short chain dehydrogenase and SKN-1 target gene, prevented the increased lifespan and βHB dehydrogenase activity induced by βHB addition, suggesting that F55E10.6 functions as an inducible βHB dehydrogenase. Furthermore, βHB supplementation increased worm thermotolerance and partially prevented glucose toxicity. It also delayed Alzheimer's amyloid-beta toxicity and decreased Parkinson's alpha-synuclein aggregation. The results indicate that D-βHB extends lifespan through inhibiting HDACs and through the activation of conserved stress response pathways.

[3] This chapter have been previously published in *Aging,* 2014, 6(8): 621–644 and have been reproduced with permission from *Aging*

5.2 Introduction

Aging leads to a progressive decline of cell and tissue function and is the primary risk factor for many ailments, including the prevalent neurodegenerative disorders Alzheimer's disease (AD) and Parkinson's disease (PD). Mitochondria are the central hub of cellular metabolism and mitochondrial dysfunction, especially in stem cells [1], has been shown to cause the development of premature aging phenotypes in mice [2]. Paradoxically, slight inhibition of mitochondrial respiration can also lead to small increases in reactive oxygen species (ROS) production and extend the lifespan of yeast, *C. elegans*, *Drosophila*, and mice [3-6]. Even in young animals, roughly 0.15% of electrons passing through the mitochondrial electron transport chain (ETC) combine with molecular oxygen to form superoxide [7, 8]. Mitochondrial ROS production increases with age and leads to progressive damage of cellular macromolecules as outlined in the mitochondrial free radical theory of aging [9].

Dietary restriction (DR) increases the lifespan of many organisms including *C. elegans* [10]. As interest in the molecular mechanisms responsible for the effect of DR on lifespan have expanded, so has the discovery of the pathways involved and the search for DR mimetic compounds that promote survival and stress resistance [11, 12]. The ketone body beta-hydroxybutyrate (βHB) has been described as a DR mimetic compound [13], in part because it increases in the plasma during DR and when administered exogenously leads to decreased levels of oxidative stress [14]. In mammals, βHB is produced in the liver, primarily from the catabolic breakdown of fatty acids, and is used as an alternative energy source when blood glucose is low. This is especially important in the brain where only a very limited amount of fatty acid beta-oxidation takes place [15]. In mitochondria βHB is catabolized to acetoacetate by βHB dehydrogenase 1 (BDH1). The reaction is linked to the reduction of NAD to NADH, which fuels

mitochondrial ETC complex I. The resulting acetoacetate is catabolized to acetoacetyl-CoA and then to acetyl-CoA, which is metabolized as part of the TCA cycle. The βHB dehydrogenase 2 (BDH2) enzyme localizes to the cytoplasm, but no changes in ketone body metabolism were found in BDH2 knockout mice suggesting BDH2 plays a limited role, if any, in ketone body metabolism [16].

Researchers are investigating DR mimetics not only for their possible lifespan extending capabilities, but also for their potential ability to delay the onset and progression of age-associated diseases such as AD. [17, 18]. Phenotypes of AD brain include extracellular senile plaques containing Aß peptide as well as intracellular neurofibrillary tangles consisting of hyperphosphorylated tau protein [19]. Intracellular Aß can inhibit mitochondrial ETC complex IV and increase ROS production [20]. βHB has shown some efficacy in the protection against AD-mediated neurodegeneration in animal models and human trials. βHB protected cultured hippocampal neurons from Aß $_{1–42}$ toxicity [21]. βHB or a ketogenic diet has shown mixed effects on disease phenotypes in mouse models of AD. For example in one study, a ketogenic diet lowered Aß levels, but did not affect cognitive impairment [22]. In another study a ketogenic diet improved motor function, but did not affect cognition or tau or Aß pathology [23]. However, a further study found that supplementation of βHB methyl ester to AD mice was able to restore cognitive function and decrease Aß levels, likely due to the fact that the methyl ester is transported through the blood-brain barrier more efficiently than the free acid [24]. There is also evidence for the clinical use of ketone bodies to treat neurodegenerative disorders as oral ingestion of medium chain triglycerides, which are catabolized in part to ketone bodies, increased plasma levels of βHB and led to improved cognitive function in human patients with AD [25].

PD, another aging-associated disorder, is characterized by an accumulation of Lewy bodies in the substantia nigra region of the brain. The alpha-synuclein protein is a major component of Lewy bodies and can also localize to mitochondrial membranes [26] causing decreased ETC complex I activity with an accompanying increase in ROS production [27]. This may be partly responsible for the increased mitochondrial oxidative damage that has been observed in brains from autopsied PD patients [28, 29]. βHB has also been shown to be efficacious in several animal models of PD. Mice treated with βHB showed partial protection against neurodegeneration and motor deficiency induced by the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which induces PD-like symptoms [30]. Additionally, βHB protected cultured neurons from toxicity induced by the structurally related ETC complex I inhibitor 1-methyl-4-phenylpyridinium (MPP(+)) [21]. In human clinical trials, PD patients treated with a ketogenic diet for one month improved their Unified Parkinson's Disease Rating Scale scores by a mean of 43% [31].

Although much is known about the effects of βHB on neurodegenerative and other aging-associated diseases, not much is known about its effects on aging. Moreover, the mechanisms through which βHB are protective are not entirely clear. However, recent evidence suggests that βHB protects against oxidative stress through its action as a class I and class IIa histone deacetylase inhibitor to increase expression of stress response genes such as FoxO3A and MT2 [14]. In this report we determined the effect of βHB on lifespan in *C. elegans* and determined the cytoprotective signaling pathways required for this effect. We then determined the effects of βHB on proteotoxicity in nematode models of AD, PD, and amyotrophic lateral sclerosis (ALS).

5.3 Results

5.3.1 D-beta-hydroxybutyrate extends the lifespan of *C. elegans*

Addition of 2, 10, or 20 mM DL-beta-hydroxybutyrate (βHB) to the culture medium of *C. elegans* feeding on heat-killed E. coli increased lifespan with 20 mM having the greatest effect, increasing mean lifespan by 26%, from 17.2 to 21.7 days (Figure 5.1A). 50 mM and 100 mM concentrations decreased lifespan. Therefore a 20 mM concentration was used in further experiments. When *C. elegans* were fed live *E. coli*, 20 mM βHB only extended mean lifespan by 14%, from 16.0 to 18.3 days. This is likely due to catabolism of a portion of the βHB by the bacteria. To determine if the lifespan extension was due to D-βHB or L-βHB, we performed lifespan experiments with each isomer separately and found that only D-βHB addition resulted in lifespan extension (Figure 5.1B).

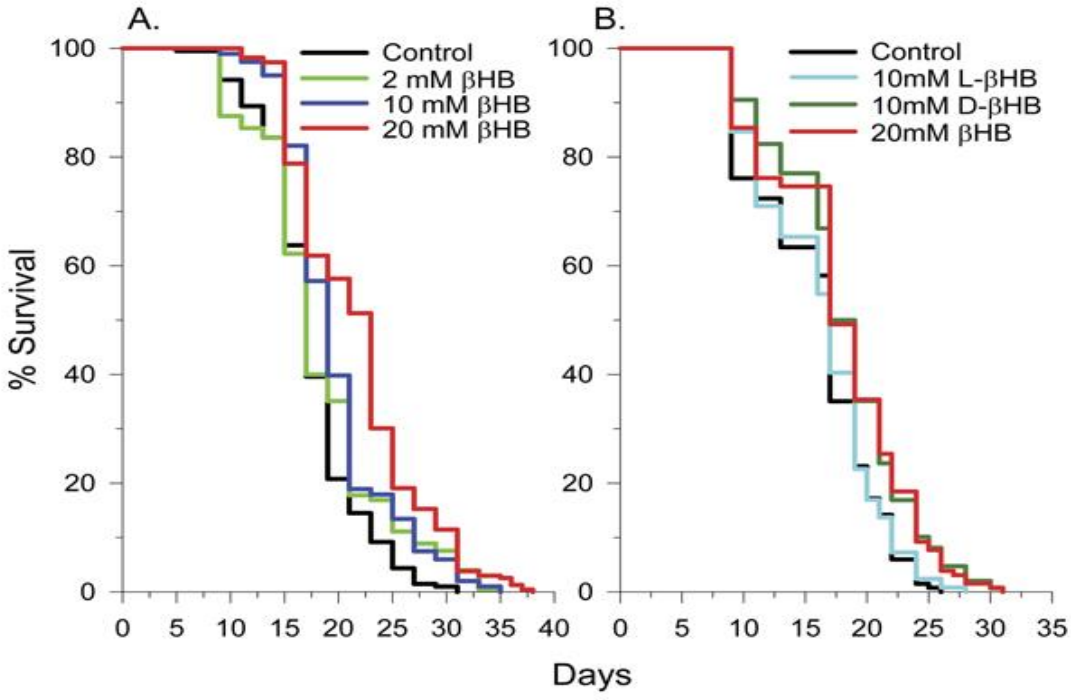

Figure 5.1. D-βHB extends the lifespan of N2 *C. elegans* worms. **(A)** Concentration dependency of βHB-mediated extension of lifespan. **(B)** D-βHB, but not L-βHB addition led to lifespan extension. When no D or L prefix is present, βHB refers to DL-βHB.

5.3.2 βHB or butyrate individually, but not when combined, extend the lifespan of *C. elegans*

The histone deacetylase (HDAC) inhibitors sodium butyrate and valproic acid have been shown to extend lifespan in *C. elegans* [32, 33]. Since βHB has a similar chemical structure as butyrate and since βHB has been shown to inhibit class I and IIa histone deacetylases (HDACs 1, 3, and 4) in mammals with a K_i of 2-5 mM [13], we determined if βHB could further extend the lifespan of sodium butyrate treated worms. As shown in Figure 2A, and as previously found by others [33], sodium butyrate extended lifespan, but strikingly the combination of sodium butyrate and βHB led to a slightly decreased lifespan. This data is consistent with the possibility that βHB is functioning as an HDAC inhibitor as HDAC inhibitors such as valproic acid are known to cause decreased lifespan at higher concentrations in *C. elegans* ([32]. The combination of sodium butyrate and βHB likely has an additive inhibitory effect on HDAC activity, thereby decreasing lifespan. βHB addition also decreased the lifespan of worms treated with valproic acid (Supplementary Table 5.1), likely through a similar mechanism.

5.3.3 Inhibition of HDA-2 and HDA-3 play a role in βHB-mediated lifespan extension

Many general HDAC inhibitors inhibit both class I and class II HDAC enzymes [13, 34]. The *C. elegans* genome has 3 class I HDACs, *hda-1*, *hda-2*, and *hda-3*. In addition there are 5 class II HDACs, *hda-4*, *hda-5*, *hda-6*, *hda-10*, and *hda-11*, with *hda-4* being the only member of class IIa [35]. To determine if HDAC inhibition plays a role in βHB-mediated lifespan extension, we knocked down the 3 class I HDACs in *C. elegans* by RNAi in the presence or absence of βHB and determined the effect on lifespan. RNAi knockdown of *hda-1* had no effect on lifespan and βHB addition extended lifespan, but to a lesser extent than in the absence of knockdown (Fig. 5.2B). However, RNAi knockdown of either *hda-2* (Fig. 5.2C) or *hda-3* (Fig. 5.2D)

extended *C. elegans* lifespan, by 13% and 16% respectively, and prevented βHB from further extending lifespan. Therefore βHB likely extends lifespan in part through inhibition of *hda-2* and *hda-3*. We also performed lifespan analysis using *hda-2(ok1479)*, *hda-3(ok1991)*, *hda-4(ok518)*, and *hda-10(ok3311)* mutant worms (Supplementary Figure 5.9 A-D). All of the HDAC mutant strains had roughly 30% decreased mean lifespans indicating that a partial knockdown, but not full knockout of HDA-2 or HDA-3 activity promotes lifespan extension. Consistent with the RNAi knockdown results, βHB addition did not extend the lifespan of the *hda-2* or *hda-3* mutant animals. However, βHB addition did lead to lifespan extension in the *hda-4* and *hda-10* mutants, suggesting βHB primarily extends lifespan through inhibiting the class I HDACs in *C. elegans*.

5.3.4 F55E10.6 is likely a D-βHB inducible βHB dehydrogenase gene

There is no strong homolog of human mitochondrial BDH1 in *C. elegans*. However, a BLASTP search identified 4 candidate genes with 38-41% protein sequence identity with BDH1 (*dhs-2*, *dhs-20*, *dhs-16* (a 3-hydroxysteroid dehydrogenase [36]), and F55E10.6 (similar to human microsomal retinol dehydrogenase [37] and hydroxysteroid dehydrogenases [38]). DHS-2, DHS-16, and DHS-20 have been predicted to have a mitochondrial localization [39] like BDH1, however DHS-20 has also been predicted to have an ER localization [40] as has F55E10.6 [41]. Therefore, we knocked down each of the 4 candidates individually in the worms grown in the absence or presence of D-βHB and measured D-βHB dehydrogenase activity in the worm extracts. None of the knockdowns showed decreased basal D-βHB dehydrogenase activity. However, we found that adding D-βHB to the growth medium resulted in a 2-fold increase in D-βHB dehydrogenase activity and knockdown of F55E10.6 largely prevented this increased D-βHB dehydrogenase activity (Fig. 5.3A), suggesting that F55E10.6 likely encodes the D-βHB

inducible D-βHB dehydrogenase activity. Knockdown of either *dhs-2* or *dhs-16* increased the ability of D-βHB to upregulate D-βHB dehydrogenase activity.

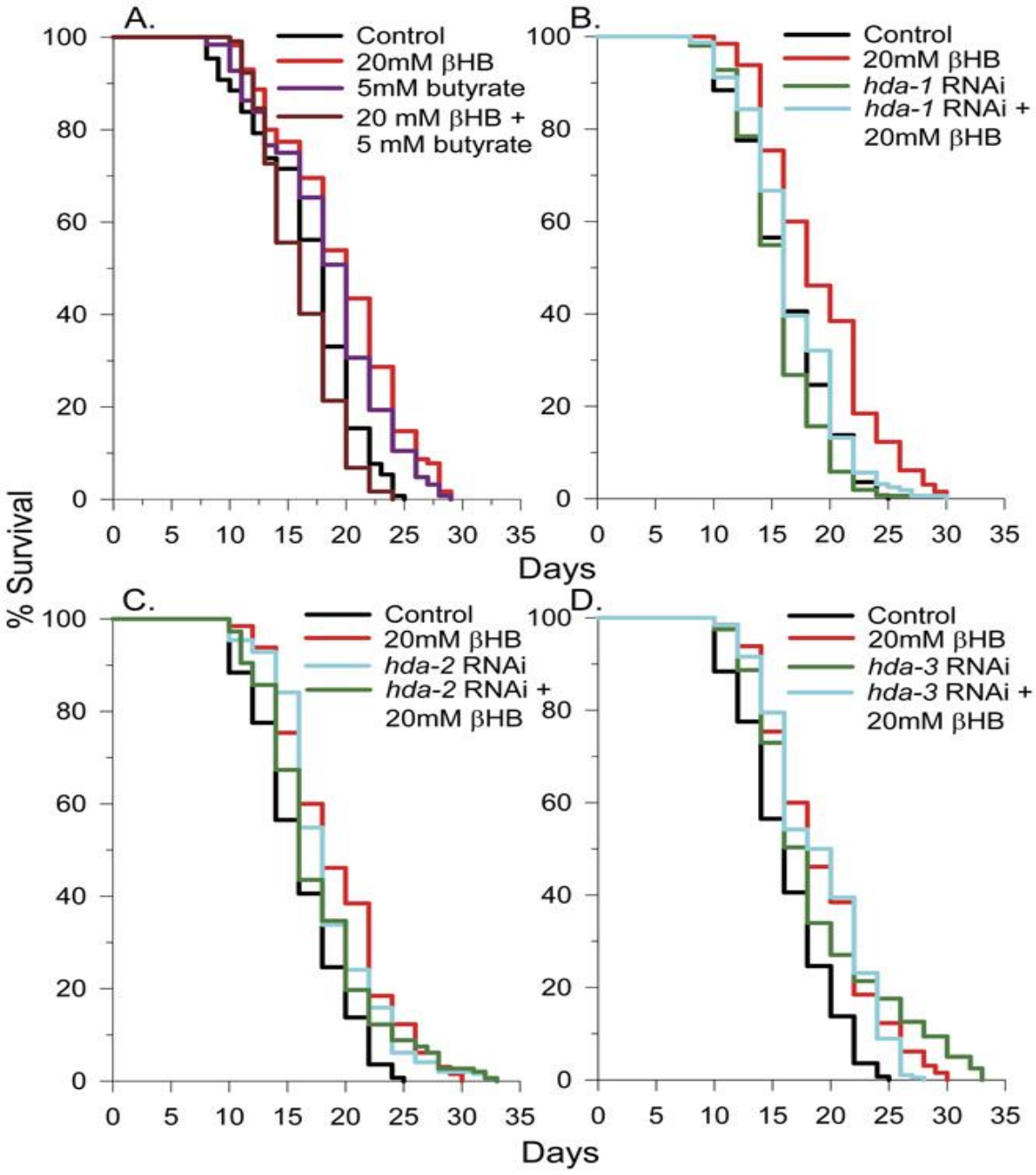

Figure 5.2. βHB-mediated HDAC inhibition plays a role in lifespan extension. **(A)** Survival of N2 worms in the presence of βHB, butyrate, or both compounds together. **(B)** Effects of *hda-1*, **(C)** *hda-2*, or **(D)** *hda-3* RNAi knockdown on *C. elegans* lifespan in the presence or absence of 20 mM βHB.

We also found that adding L-βHB to the culture medium resulted in a roughly 3-fold increase in D-βHB dehydrogenase activity in the worm extracts that was independent of the expression of F55E10.6, *dhs-2*, *dhs-16*, or *dhs-20* (Figure 5.3B), suggesting L-βHB induces a separate D-βHB dehydrogenase enzyme. Consistent with this data, when worms were cultured with a racemic mixture of 20 mM βHB (DL-βHB) we found an almost additive 4.5-fold increase in D-βHB dehydrogenase activity (data not shown).

We also measured L-βHB dehydrogenase activity in the worm extracts (Supplementary Figure 2). There was roughly 5-fold lower basal L-βHB dehydrogenase activity than D-βHB dehydrogenase activity in the worm extracts (data not shown). Adding D-βHB to the culture medium yielded a roughly 50% increase in L-βHB dehydrogenase activity in control worm extracts that was almost completely blocked by RNAi knockdown of F55E10.6. Therefore, the F55E10.6 βHB dehydrogenase activity can likely utilize either D-βHB or L-βHB as substrates, but the activity with D-βHB appears roughly 10-fold higher than with L-βHB. Interestingly, knockdown of *dhs-2*, *dhs-16*, or *dhs-20* decreased basal L-βHB dehydrogenase activity in the extracts (Supplementary Figure 5.10). Determining whether any of these genes play a direct role in L-βHB metabolism, or if a gene encoding an enzyme with L-βHB dehydrogenase activity is down-regulated by knockdown of these genes awaits further investigation.

5.3.5 F55E10.6 is required for βHB-mediated longevity

To determine if βHB-mediated upregulation of F55E10.6 was essential for the effect of βHB on longevity, lifespan was monitored in worms in which expression of F55E10.6 was knocked down by RNAi feeding. RNAi knockdown of F55E10.6 increased lifespan by 7% and unexpectedly completely prevented lifespan extension induced by βHB supplementation (Figure 5.4A). F55E10.6 is a SKN-1 transcriptional target [42].

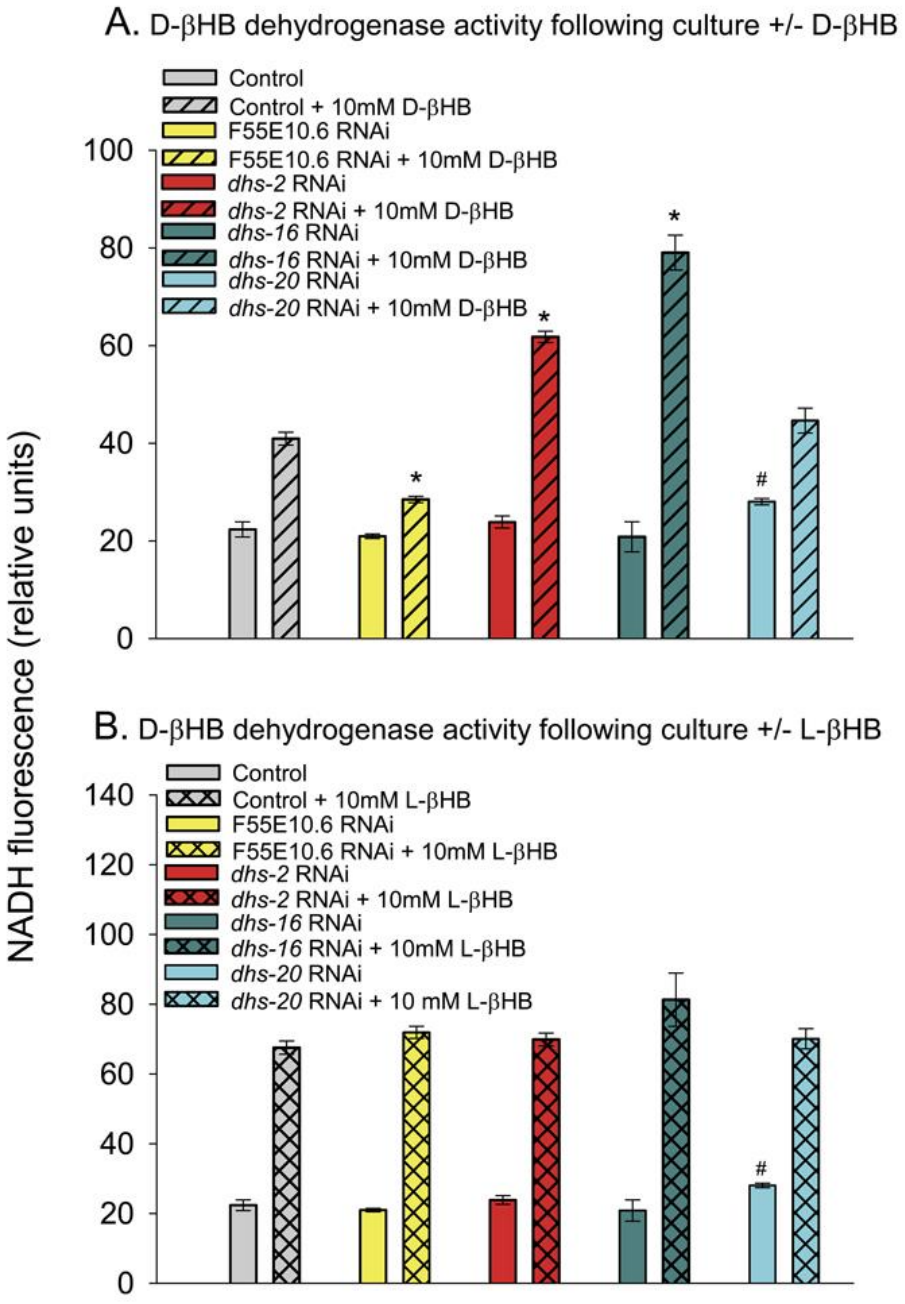

Figure 5.3. D-βHB dehydrogenase activity in worm extracts following RNAi-mediated gene knockdown. **(A)** D-βHB dehydrogenase activity following worm culture in the absence or presence of 10 mM D-βHB. Conditions in the legend refer to the culture conditions. The genes F55E10.6, *dhs-2*, *dhs-16*, or *dhs-20* were knocked down by RNAi feeding (* $p < 0.05$ compared to Control + 10 mM D-βHB; # $p < 0.05$ compared to Control). **(B)** D-βHB dehydrogenase activity following worm culture in the absence or presence of 10 mM L-βHB. The assay conditions were the same as panel A (# $p < 0.05$ compared to Control).

SKN-1 is a homolog of mammalian Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and a transcriptional regulator that induces the expression of genes involved in antioxidant defense and xenobiotic metabolism to promote longevity. Therefore, addition of βHB to the culture media activates SKN-1, which induces expression of F55E10.6. F55E10.6 could either metabolize βHB or metabolize another endogenous substrate leading to lifespan extension.

It is possible that metabolism of βHB, either dependent or independent of F55E10.6, is required for lifespan extension. This increased βHB metabolism may increase TCA cycle and electron transport chain (ETC) activity increasing ROS production, which has been shown to lead to lifespan extension in *C. elegans* [43]. Therefore we determined if administration of the antioxidant and glutathione precursor N-acetylcysteine (NAC) prevented the lifespan extension induced by βHB. As shown in Figure 5.4B, NAC by itself moderately increased lifespan, but NAC supplementation did not prevent lifespan extension mediated by βHB. Therefore, βHB is likely extending lifespan through a mechanism that does not require increased ROS production.

5.3.6 Knockdown of F55E10.6 does not prevent the βHB-mediated increase in oxygen consumption

Since F55E10.6 expression was essential for βHB-mediated lifespan extension, we wished to determine if supplemented βHB was being utilized as a respiratory substrate by the worms and whether knocking down F55E10.6 would decrease βHB-induced respiratory metabolism. Therefore, we determined the effect of βHB supplementation on worm oxygen consumption (Figure 5.4C). βHB supplementation increased oxygen consumption by 2.3 fold indicating that βHB is being metabolized by the worms. Unexpectedly, we found that RNAi knockdown of F55E10.6 in the absence of βHB also increased oxygen consumption by around 2.3 fold, suggesting that F55E10.6 represses mitochondrial biogenesis or respiratory function.

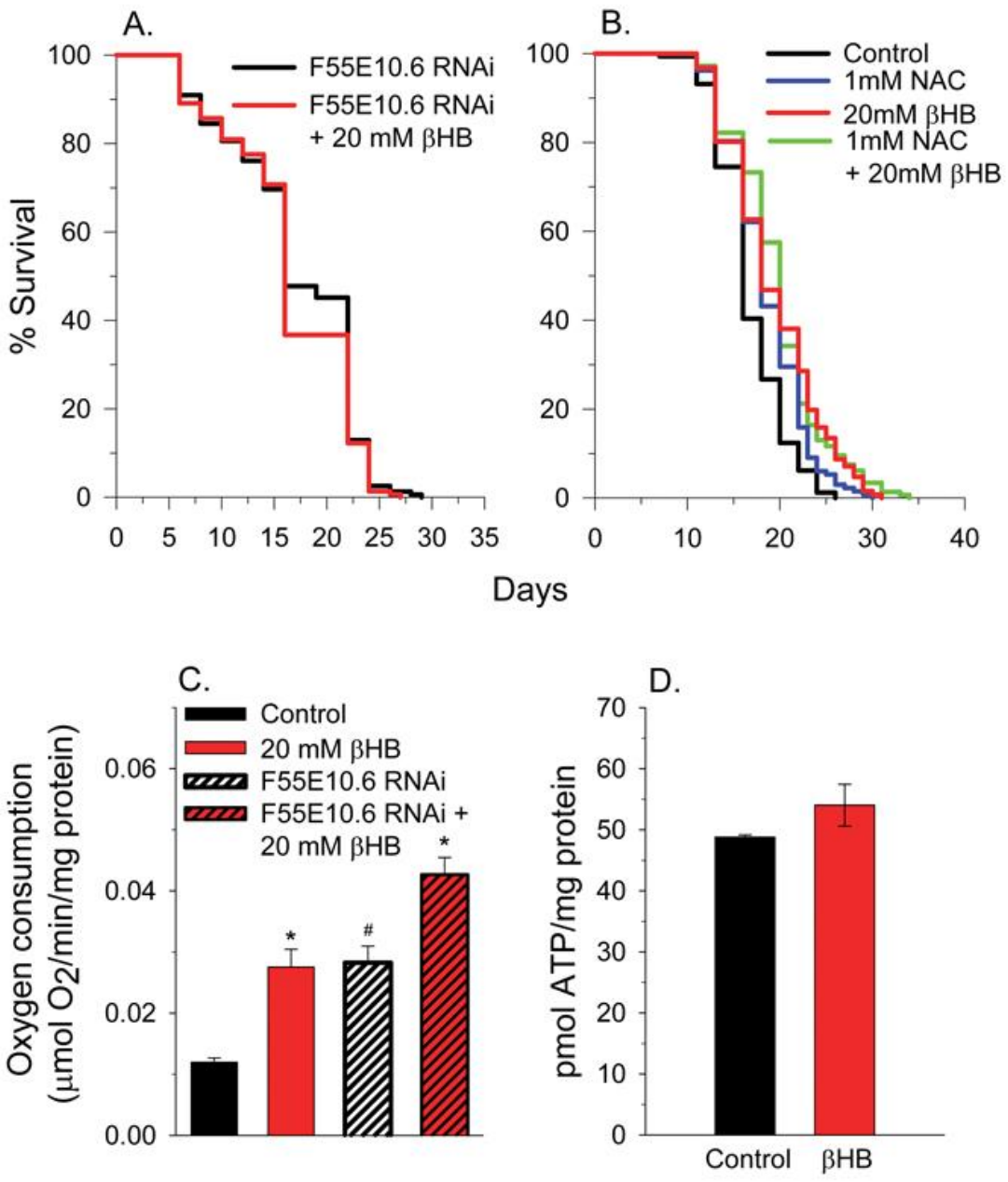

Figure 5.4. F55E10.6 is required for βHB-mediated lifespan extension, but not for βHB-induced oxygen consumption. (**A**) Treatment with βHB did not increase the lifespan of N2 worms fed RNAi to F55E10.6. **(B)** The addition of N-acetyl-L-cysteine (NAC) did not decrease the lifespan of βHB treated worms. **(C)** The effect of 20 mM βHB and RNAi knockdown of F55E10.6 on oxygen consumption (* $p < 0.05$ vs. untreated; # $p < 0.05$ vs. Control). **(D)** The effect of 20 mM βHB treatment on ATP levels in day 4 N2 worms ($p = 0.202$). Data are represented as mean +/- SEM.

But RNAi knockdown of F55E10.6 did not decrease the βHB-mediated increase in oxygen consumption. The results suggest that the metabolism of βHB by F55E10.6 does not play a significant role in the use of βHB as an energy substrate for respiration, and so other mechanisms likely explain the requirement of F55E10.6 for lifespan extension.

We also determined the effect of βHB addition on worm ATP levels (Figure 5.4D). ATP levels were not significantly altered by βHB addition, although oxygen consumption rates increased suggesting that βHB either stimulates energy utilization pathways or decreases the coupling efficiency of oxidative phosphorylation. In this regard, we have previously shown that growth of *C. elegans* in the presence of the TCA cycle metabolites malate or fumarate resulted in a partial uncoupling of mitochondria [44].

5.3.7 SKN-1 and DAF-16 are required for βHB-mediated longevity

To determine other molecular pathways through which βHB functions to extend lifespan, we performed lifespan experiments using worms deficient in common longevity pathways. In *C. elegans* the SKN-1 transcriptional activator is normally sequestered in the cytoplasm by WDR-23 and the DDB1/CUL4 ubiquitin ligase complex until the presence of specific xenobiotics or reactive oxygen species leads to a disruption of the interaction. This allows nuclear translocation of SKN-1 leading to the activation of a phase II detoxification transcriptional response and lifespan extension [45, 46]. We next determined if βHB extended the lifespan of worms in which SKN-1 levels were knocked down by RNAi feeding. Consistent with a role for SKN-1 and SKN-1 transcriptional targets such as F55E10.6 in βHB-mediated longevity, βHB did not extend the lifespan in these SKN-1 RNAi worms (Figure 5.5A). Additionally βHB was able to increase GFP fluorescence in the *gst-4*::*gfp* SKN-1 reporter strain of worms (Figure 5.5B) supporting this assertion. βHB was also able to induce expression of this reporter strain following knockdown of

the F55E10.6 gene suggesting that F55E10.6 functions downstream of SKN-1 in the longevity pathway, as is expected for a SKN-1 transcriptional target.

Disruption of the DAF-2 insulin receptor signaling pathway is known to extend lifespan through activation of the DAF-16 transcriptional activator. DAF-16 is homologous to mammalian FOXO genes. βHB supplementation to worms homozygous for the *daf-16*(*mgDf50*) null allele did not lead to lifespan extension (Figure 5.5C). Furthermore, βHB supplementation slightly increased fluorescence of the *sod-3::gfp* DAF-16 reporter strain of worms (Figure 5.5D), supporting the ability of βHB treatment to activate DAF-16 activity for lifespan extension. Butyrate treatment also led to a similar small increase in GFP fluorescence of the *sod-3::gfp* worms suggesting a similar mechanism through which βHB and butyrate extend lifespan.

Another transcriptional regulator linked to longevity is hypoxia inducible factor-1 (HIF-1) [47]. We hypothesized that βHB catabolism would increase the concentration of TCA cycle metabolites that inhibit the alpha-ketoglutarate-dependent degradation of HIF-1 by the proteasome [48, 49] initiated by the EGL-9 prolyl hydroxylase [50]. However, we found that βHB supplementation did not increase fluorescence in the *nhr-57::gfp* reporter strain [47] for HIF-1 transcriptional activity (Supplementary Figure 3A). In addition supplementation with 10 mM pyruvate, or the TCA cycle metabolites citrate, succinate, fumarate, malate, or oxaloacetate also failed to induce GFP expression (Supplementary Figure 5.12B) suggesting that *C. elegans* HIF-1 may be regulated slightly differently than mammalian HIF-1.

5.3.8 βHB increases thermotolerance

Since lifespan extension, and DAF-16 and SKN-1 activation in particular, has been linked to stress resistance, we determined the effect of βHB supplementation on thermotolerance in *C. elegans*.

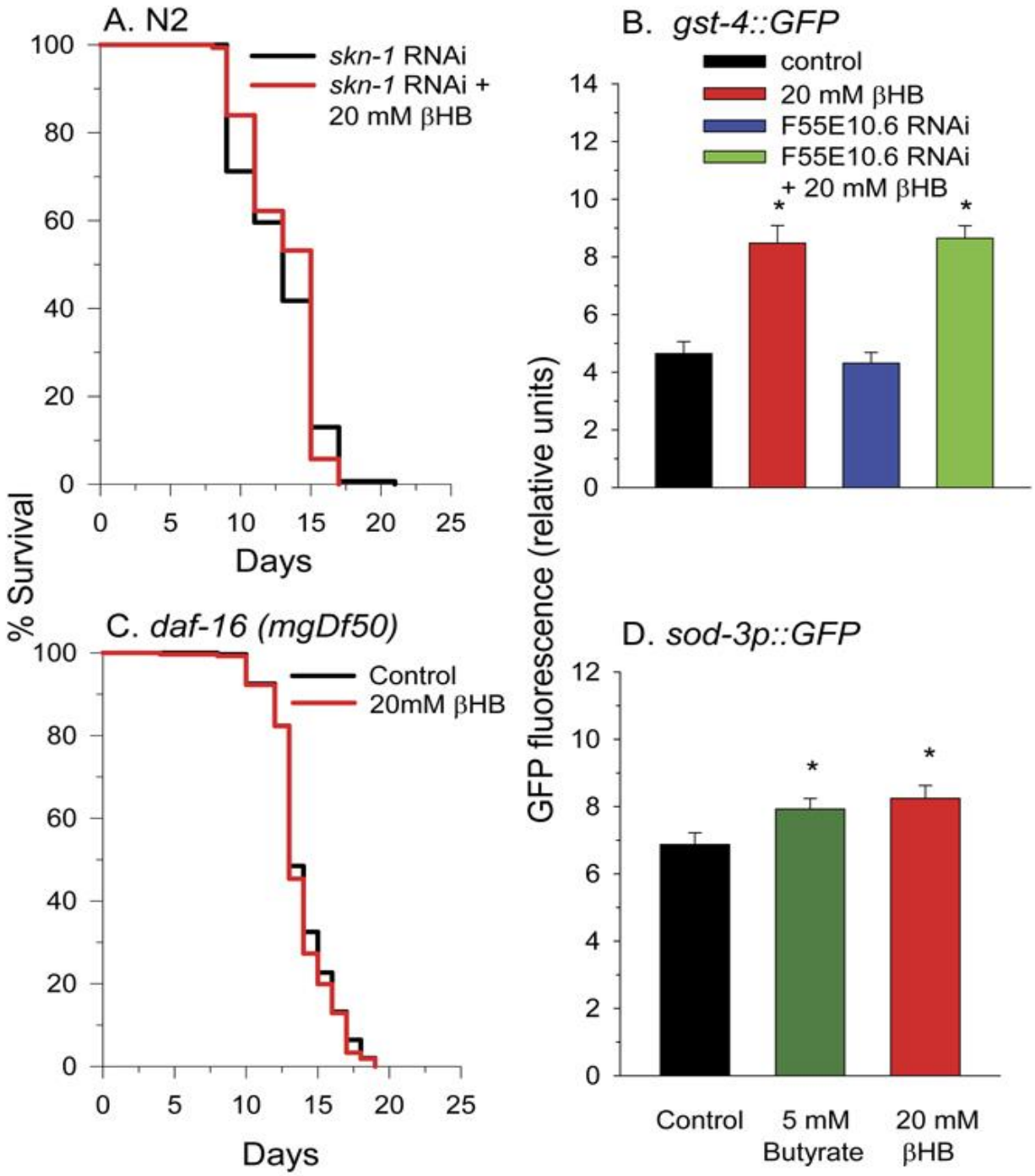

Figure 5.5. SKN-1 and DAF-16 are required for βHB-mediated lifespan extension. (**A**) βHB addition did not increase the lifespan of N2 worms fed RNAi to knockdown expression of *skn-1*. **(B)** βHB addition increased fluorescence of the *gst-4::gfp* SKN-1 reporter strain. Data are represented as mean +/- SEM (* $p < 0.05$). **(C)** βHB addition did not increase lifespan in *daf-16(mgDf50)* mutant worms. **(D)** βHB or butyrate increased fluorescence when administered to the *sod-3::gfp* DAF-16 reporter strain. Data are represented as mean +/- SEM (* $p < 0.05$).

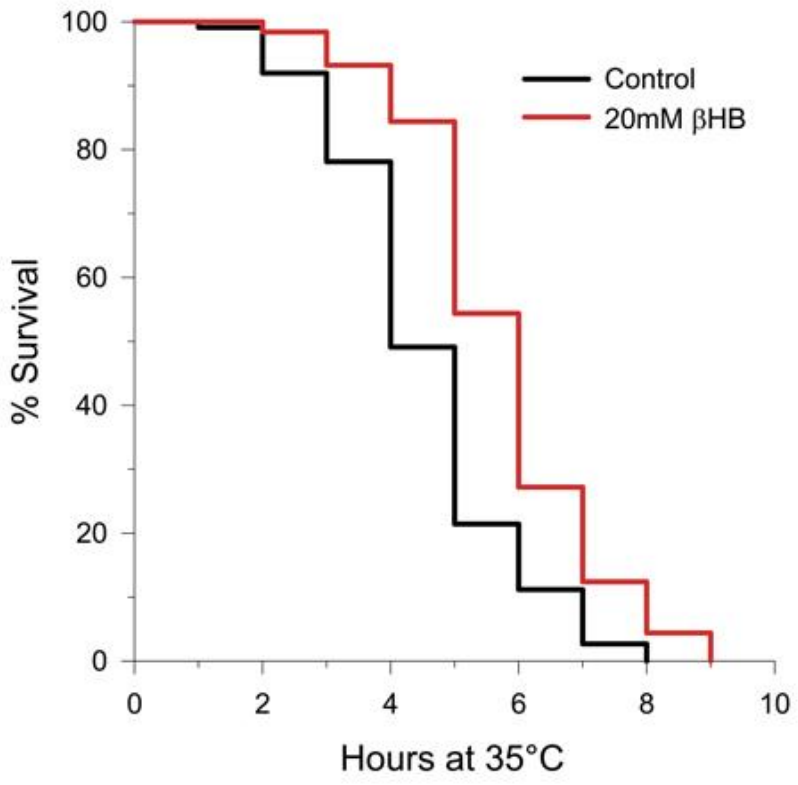

Figure 6. Treatment with βHB increases thermotolerance (log-rank $p < 0.001$) in N2 worms when upshifted from 20°C to 35°C. βHB mean survival time = 5.7 hours. Control mean survival time = 4.5 hours.

As shown in Figure 5.6, βHB administration extended the mean survival time of the worms after they were shifted to an elevated temperature by 22%. Due to the increased thermotolerance we hypothesized that heat shock proteins were induced by βHB supplementation. Therefore we monitored GFP fluorescence in 4 heat shock reporter strains of worms following βHB treatment.

We used the strains *hsp-6::gfp* and *hsp-60::gfp* to monitor the mitochondrial unfolded protein response [51], *hsp-4::gfp* to monitor ER stress, and *hsp-16.2::gfp* to monitor heat shock factor-1 (HSF-1)-mediated gene expression [52]. βHB supplementation did not induce expression of any of these four reporter strains. Therefore βHB supplementation does not induce a broad heat shock response, even though thermotolerance was increased.

5.3.9 Decreased protein synthesis rates likely contribute to βHB-mediated lifespan extension

Recently it has been recognized that several of the common longevity pathways converge to decrease the rate of translation initiation to extend lifespan [53, 54]. This can occur through several mechanisms including preventing phosphorylation of ribosomal protein S6 by S6 kinases of the TOR signaling pathway, blocking phosphorylation of eukaryotic initiation factor 4E (eIF4E) binding proteins (4E-BPs) by the TOR kinase, or by activation of general control nonderepressible 2 (GCN2) kinase. GCN2 activation can occur in the presence of uncharged tRNAs due to amino acid restriction [55] or during times when mitochondria produce high levels of reactive oxygen species [56]. *C. elegans* appears to lack close functional homologs of mammalian 4E-BPs [57] (although one distant homolog has been reported [58]), so they likely control the rate of translation initiation mainly through the p70 S6 kinase homolog RSKS-1 and the worm GCN2 homolog GCN-2. Therefore, we obtained the mutant strains *rsks-1(ok1255)* and *gcn-2(ok871)* and performed lifespan analysis in the absence or presence of βHB. The untreated lifespan of the *rsks-1* mutant was greater than that of the N2 control (Fig. 5.7A) as expected, while the untreated lifespan of the *gcn-2* mutant was less than the N2 control (Fig. 5.7B). With either strain, we found that βHB-mediated lifespan extension was greatly blunted compared to the effect on the N2 control strain. There was a 5% mean lifespan extension in the βHB-treated *rsks-1* mutant and a 8% mean lifespan extension in the βHB-treated *gcn-2* mutant compared to the 26% lifespan extension in the βHB-treated N2 control. Therefore, the ability to decrease translation rates through both the TOR/RSKS-1 and GCN-2 pathways likely allows for full βHB-mediated lifespan extension in the wild-type N2 animals.

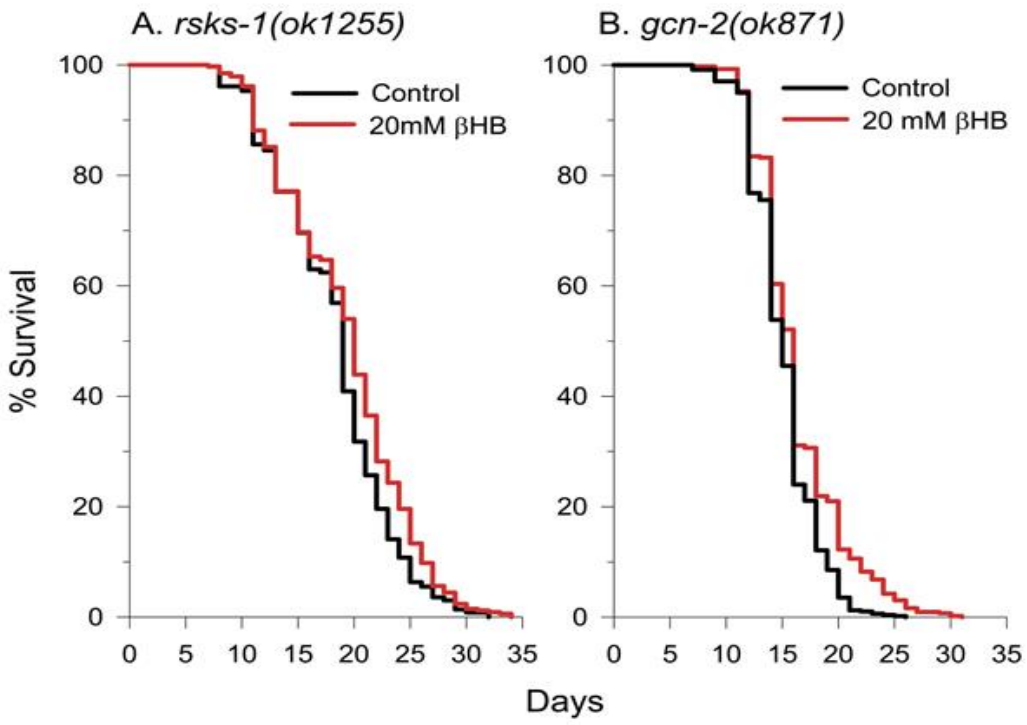

Figure 5.7. A decreased rate of protein synthesis contributes to βHB-mediated longevity. (**A**) βHB-mediated lifespan extension was blunted in *rsks-1(ok1255)* mutant worms. **(B)** βHB-mediated lifespan extension was also blunted in *gcn-2(ok871)* mutant worms.

5.3.10 ETC Complex I function is needed for full βHB-mediated lifespan extension

Following mitochondrial βHB dehydrogenase function, acetoacetate is converted to acetoacetyl-CoA with the concurrent conversion of succinyl-CoA to succinate as a byproduct of the succinyl-CoA: 3-ketoacid CoA transferase reaction. Because of this succinate production, it has been suggested that βHB protected a PD cell model by increasing mitochondrial ETC complex II (succinate dehydrogenase) activity, bypassing the ETC complex I deficits present in the disease [30]. To determine if normal mitochondrial ETC complex I or complex II activity is required for βHB-mediated lifespan extension, we determined the effect of βHB supplementation on the lifespan of short-lived complex I-defective *gas-1(fc-21)* mutants [59] (Figure 5.8A) and short-lived complex II defective *mev-1(kn1)* mutants [60] (Figure 5.8B). βHB extended the

lifespan of the *gas-1* mutant by 11%, but not to the full 26% extent observed in wild-type worms. Therefore, normal ETC complex I function is necessary for the full effect of βHB on longevity. βHB supplementation fully extended the lifespan of *mev-1* mutants indicating that βHB does not require normal ETC complex II function to extend lifespan.

5.3.11 βHB-mediated longevity requires AAK-2, SIR-2.1, CBP-1, and may occur in a similar manner as in DR

To identify if other important longevity regulators are required for βHB-mediated longevity, βHB was supplemented to AMP kinase (AMPK) *aak-2(TG38)* mutant worms (Figure 5.9A) and *sir-2.1(ok434)* NAD-dependent protein deacetylase mutant worms (Figure 5.9B) and lifespan was monitored. βHB addition did not extend the lifespan of either strain suggesting that both proteins play a role in βHB-mediated longevity. Since ketone body levels rise during caloric restriction (CR) in mammals and increased βHB levels may be responsible for some portion of the increased stress and disease resistance conferred by CR, we determined the effect of βHB supplementation on lifespan in the nematode *eat-2(ad1116)* model of dietary restriction (DR) in which pharyngeal pumping is slowed (Figure 5.9C). We found that treatment with βHB had no significant effect on the longevity of *eat-2* worms suggesting that βHB extended lifespan using some of the same downstream effectors activated in DR. The CREB binding protein-1 (CBP-1) transcriptional co-activator and protein acetyltransferase has been shown to be essential for DR-mediated longevity in *C. elegans* [33]. Therefore we determined the effect of βHB on lifespan in worms where *cbp-1* expression was knocked down by RNAi (Fig. 5.9D). Consistent with βHB extending lifespan in a manner similar to DR, knocking down *cbp-1* prevented lifespan extension induced by βHB treatment.

Many of the same compounds that extend lifespan in *C. elegans* in a CBP-1 dependent manner also protect against glucose toxicity [61]. It's been shown that *C. elegans* shows a reduced lifespan when grown in a high glucose containing media [62-64]. When we grew worms in 50 mM glucose, lifespan was decreased by roughly 30% (Figure 5.10A). βHB supplementation to the glucose-containing media partially restored the lifespan, resulting in a lifespan reduction of only 21% compared to the non-glucose treated controls.

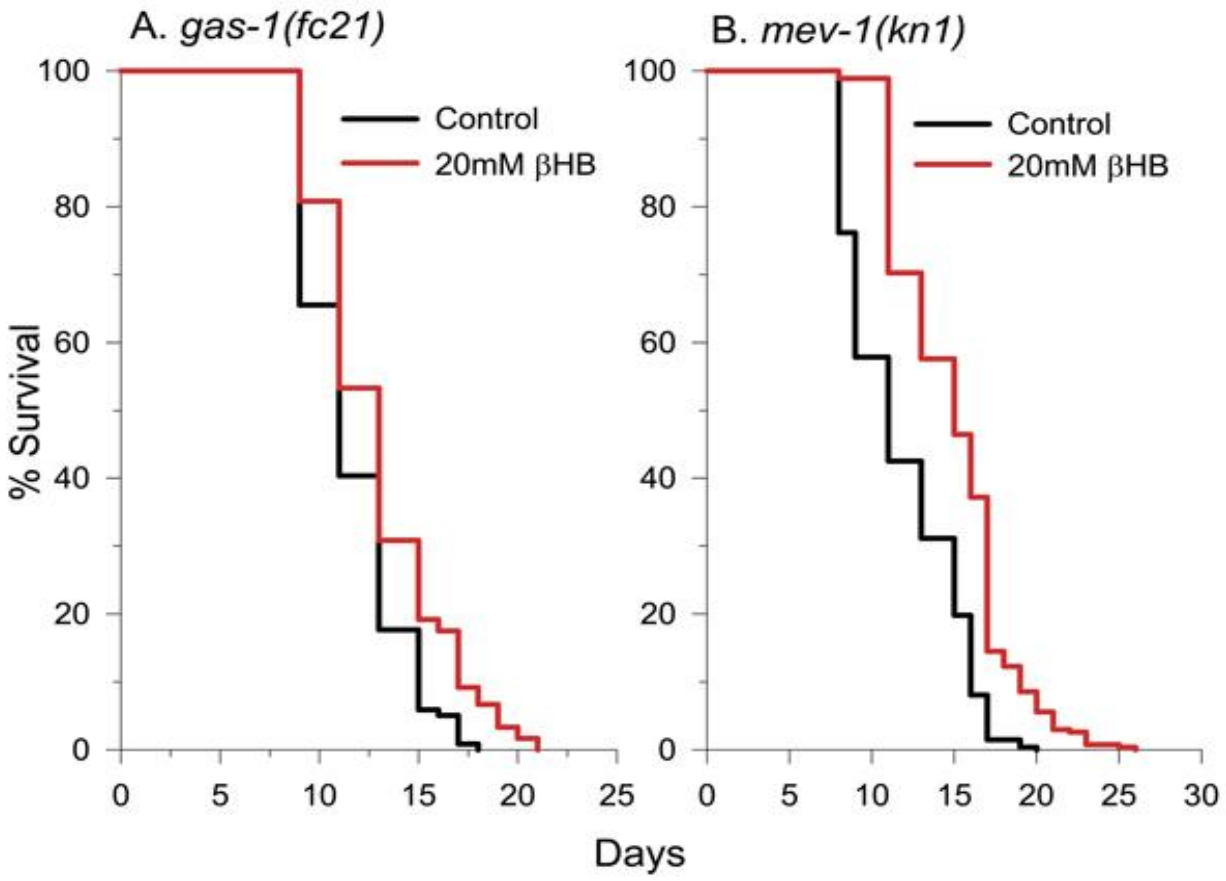

Figure 5.8. βHB extends the lifespan of short-lived mitochondrial ETC complex I and complex II mutants. **(A)** *gas-1(fc21)* survival in the absence and presence of βHB. **(B)** *mev-1(kn1)* survival in the absence and presence of βHB.

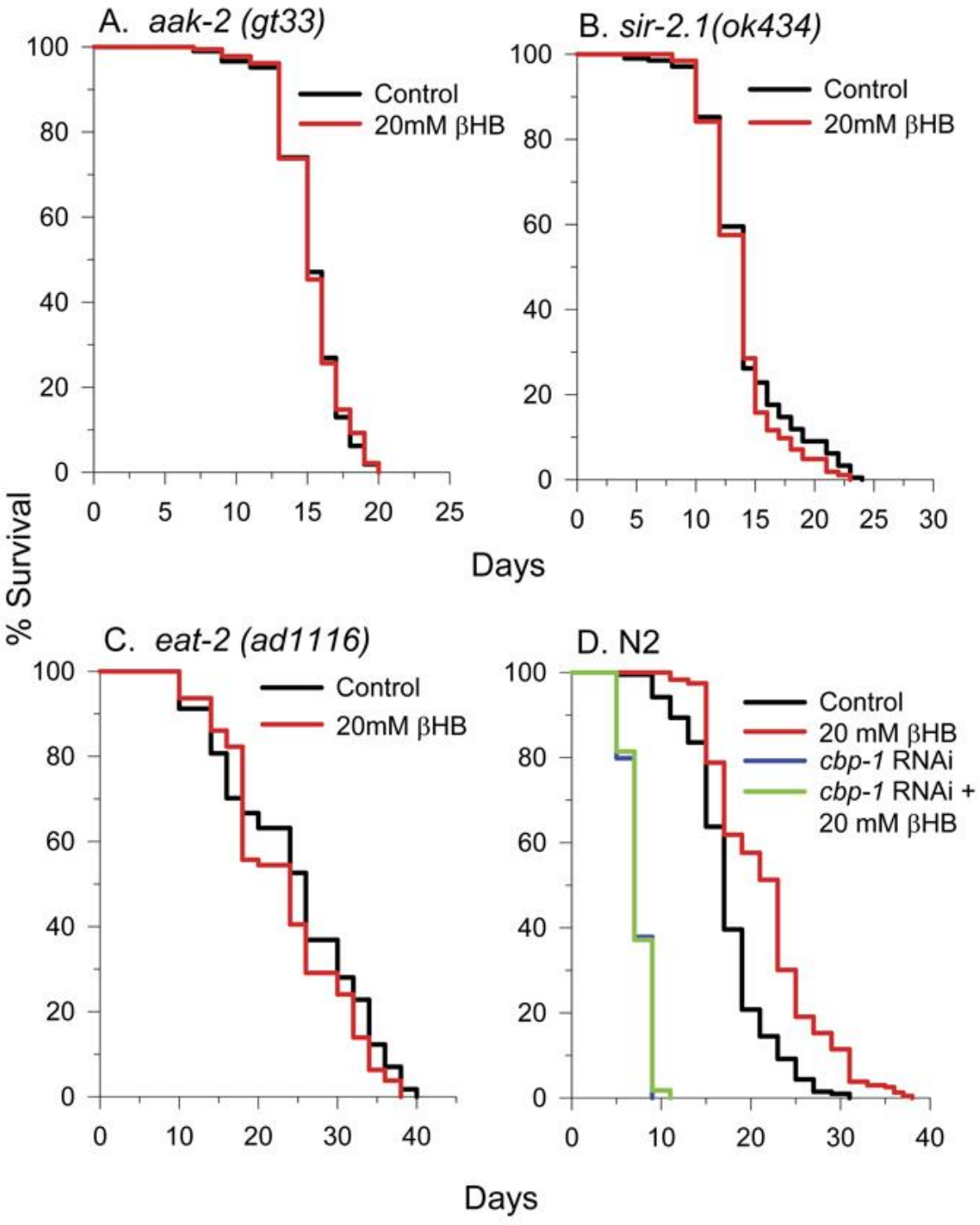

Figure 5.9. βHB extends lifespan in a similar manner as DR and requires AAK-2, SIR-2.1, and CBP-1. (**A**) βHB does not extend lifespan of AMPK mutant *aak-2(TG38)* worms, (**B**) *sir-2.1(ok434)* worms, or (**C**) *eat-2(ad1116)* worms. (**D**) Additionally, βHB does not extend the lifespan *cbp-1* RNAi knockdown N2 worms.

5.2.12 βHB delays Aß-induced paralysis and decreases alpha-synuclein aggregation

We next performed experiments using a strain of worms engineered to express human AD-associated Aß peptide within body wall muscle upon temperature upshift from 16° to 25°C, which leads to paralysis of all worms by 32 hours after upshift [65]. Figure 10B shows the paralysis over time in these worms in the presence and absence of βHB treatment. βHB increased the mean paralysis time following Aß induction by 15%, from approximately 26 to 30 hours. Since βHB supplementation was beneficial in this model of proteotoxicity, we next determined the effects of βHB administration on a PD-model worm strain expressing human α-synuclein fused to yellow fluorescent protein (YFP) in the body wall muscle [66]. Alpha-synuclein protein is prone to aggregation and is the major protein constituent of Lewy bodies in PD brain [67]. YFP aggregation and fluorescence was decreased by 35% in worms treated with βHB for 8 days, indicating a protective decrease in the levels of alpha-synuclein aggregates (Figure 5.10C).

A ketogenic diet has been shown to delay loss of motor performance and loss of spinal cord motor neurons in the SOD1-G93A mouse model of amyotrophic lateral sclerosis (ALS) [68]. So lastly, we performed experiments using worms overexpressing human TDP-43 [69], which forms insoluble aggregates in the nervous system of patients with ALS and other neurodegenerative disorders [70] and when expressed in the nervous system of worms [69]. TDP-43 expression caused a greatly reduced lifespan in *C. elegans* both when grown at 20°C and when grown at 16°C (Supplementary Table 1). 20 mM βHB supplementation was unable to prevent the reduction in lifespan (Figure 10D). Concentrations of βHB from 2 mM to 200 mM were also tested. Only 30 mM βHB was found to be effective at delaying toxicity and the increase in longevity at this concentration was only 5%.

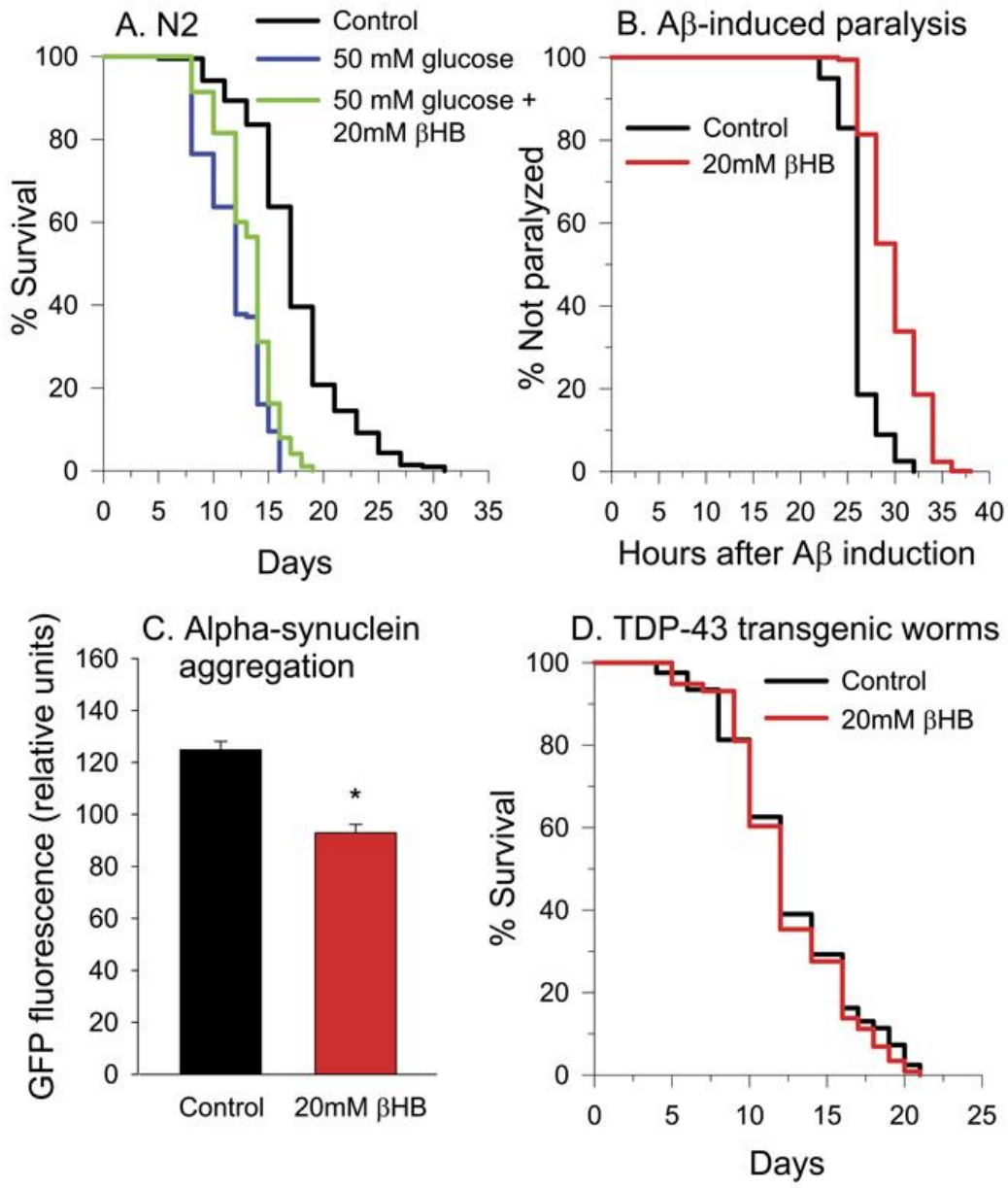

Figure 5.10. βHB protects against glucose toxicity and proteotoxicity. **(A)** Treatment with βHB partially protects against 50 mM glucose-induced reduction of lifespan in N2 worms. **(B)** Survival of the CL4176 strain of *C. elegans* expressing Aβ in muscle following temperature upshift. Treatment with βHB increases the time to paralysis (log-rank $p < 0.001$). βHB-treated mean lifespan = 29 hours, untreated control mean lifespan = 26 hours. The curves are generated from the results of six assays ($n > 500$ for both groups). **(C)** Treatment with βHB decreases α-synuclein-GFP aggregation in the NL5901 strain GFP fluorescence readings were taken on day 8 of worm lifespan. Data are represented as mean +/- SEM. (log-rank $p < 0.001$) **(D)** 20 mM βHB did not protect against the shortened lifespan induced by human TDP-43 overexpression when the worms were grown at 16°C.

5.4 Discussion

Administering βHB to *C. elegans* extended lifespan and delayed proteotoxicity and glucose toxicity. βHB extended *C. elegans* lifespan in a SIR-2.1 and AMPK-dependent manner that also required the stress-responsive transcription factors DAF-16 and SKN-1. Since βHB did not extend lifespan in *eat-2* pharyngeal pumping mutants, βHB likely acts as a dietary restriction mimetic, as previously hypothesized for its effects in mammals [71]. Even though protective effects of D-βHB on rodent disease models are known, this is the first report to identify D-βHB as a positive modulator of organismal longevity in wild-type animals. We also identified many of the signaling pathways and genes required for this effect. A key finding is that D-βHB-mediated lifespan extension requires SKN-1 and its transcriptional target F55E10.6, a short-chain dehydrogenase/reductase with βHB dehydrogenase activity, although inhibition of HDACS HDA-2 and HDA-3 are also required for the increased longevity.

5.4.1 The role of F55E10.6 in βHB-mediated lifespan extension

The βHB dehydrogenase enzyme assay data suggest that F55E10.6 is a D-βHB-inducible βHB-dehydrogenase enzyme. However, D-βHB may not be the preferred physiological substrate for the enzyme or the substrate required for lifespan extension. Since knockdown of F55E10.6 did not affect the increased oxygen consumption following βHB supplementation, the enzyme does not likely possess a mitochondrial localization producing NADH for ETC complex I function. In this regard in addition to sharing homology with mitochondrial BDH1, F55E10.6 shares homology with microsomal retinol dehydrogenases [37] and microsomal hydroxysteroid dehydrogenases [38]. Due to these homologies and the role that SKN-1 plays in controlling the ER stress response [41], F55E10.6 has been putatively assigned an ER localization [41],

although no signal peptide was found [40]. Key to its localization, F55E10.6 is predicted to have a transmembrane domain [40].

It is of interest that knockdown of F55E10.6, a SKN-1 target gene increased *C. elegans* oxygen consumption, suggesting that SKN-1 signaling may decrease mitochondrial biogenesis or function. Expression of F55E10.6 has been shown to decline with aging [72], likely due to the aging-related decline in activity of SKN-1 [42]. The mammalian SKN-1 homolog Nrf2 is also known to play a role in mitochondrial biogenesis. When overexpressed Nrf2 was shown to be a negative modulator of mitochondrial mass and membrane potential in a high throughput screen using C2C12 myoblast cells [73]. However, when upregulated under physiological conditions Nrf2 was found to be a positive regulator of mitochondrial biogenesis by inducing nuclear respiratory factor-1 (NRF-1) and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1□) expression in heart [74], liver [75], and lung [76].

5.4.2 Full βHB-mediated lifespan extension requires mitochondrial ETC complex I function

βHB-induced lifespan extension was partially blocked in ETC complex I mutant worms and was unaffected in mitochondrial ETC complex II mutant worms. This likely suggests that the lifespan extension is driven partly by βHB metabolism-independent effects and partly by metabolism-dependent effects. Normal complex I activity may be needed to maintain a high NAD/NADH ratio beneficial for maximal lifespan extension [77]. In this regard, it has been shown that rotenone can induce a roughly 10-fold reduction in *C. elegans* respiration, but only a 2-fold reduction occurred in the presence of 10 mM βHB [78]. Therefore, βHB either stabilized complex I function in the presence of rotenone or stimulated complex II-dependent respiration to bypass this block of complex I function. In mammals βHB has been shown to stabilize and increase the efficiency of ETC complex I [68, 79]. The increased rate of NADH oxidation in the

presence of βHB led to decreased ROS levels in mouse neocortical neurons following glutamate excitotoxicity [79]. βHB may also enhance complex I activity in worms, but it may not be able to fully do so in the *gas-1* mutants preventing full lifespan extension.

5.4.3 Proposed mechanism for βHB-mediated lifespan extension

We propose 2 possible mechanisms for lifespan extension mediated by βHB supplementation. In the first mechanism (see Supplementary Figure 5), we propose that βHB directly inhibits HDACs to increase histone acetylation [14] causing gene expression changes leading to S□□□□ activation, independent of βHB catabolism. The metabolism-independent activation of SKN-1 is consistent with our previous data showing that stimulation of metabolism by supplementation of several TCA cycle metabolites did not activate SKN-1 transcriptional activity [44]. Next, SKN-1 activity induces expression of F55E10.6 [42, 45], required for proper execution of the SKN-1 longevity program. SKN-1 activation has been shown to repress expression of the insulin-like peptides DAF-28 and INS-39, decreasing DAF-2 insulin receptor signaling to activate DAF-16 [80]. βHB catabolism also likely increases the level of specific TCA cycle intermediates, which may contribute to the DAF-16-mediated lifespan extension. We and others have shown that supplementation of the TCA cycle metabolites fumarate, malate, and oxaloacetate activated nuclear translocation of DAF-16 to extend lifespan in an AMPK and SIR-2.1-dependent manner [44, 81]. Others have also found that the TCA cycle metabolite alpha-ketoglutarate extends lifespan through a TOR kinase-dependent mechanism [82]. Although this model is consistent with our data, is also possible that βHB-mediated HDAC inhibition causes a direct transcriptional upregulation of DAF-16, as βHB-mediated HDAC inhibition directly upregulates expression of the DAF-16 homolog FOXO3A in mammals [14].

In addition, βHB metabolism may increase acetyl-CoA levels that serve as a substrate for histone acetyltransferases to increase histone acetylation [83], which could strengthen the effects of HDAC inhibition to extend lifespan. However, increased cytoplasmic acetyl-CoA levels have also been shown to inhibit autophagy [84], which could potentially dampen lifespan extension. However, the acetyl group from mitochondrial acetyl-CoA can be transferred to carnitine to form acetylcarnitine and exported from mitochondria to the nucleus, where acetyl-CoA is reformed and used for nuclear histone acetylation [85]. This mechanism may allow for increased histone acetylation without decreased rates of autophagy.

The second proposed model of how βHB may extend lifespan is through inhibition of the insulin signaling pathway. In mammals, it has been shown, contrary to expectations, that βHB administration or a ketogenic diet blocks the insulin signaling pathway in muscle leading to insulin resistance [86]. This adaptation likely evolved to allow the brain preferential use of the bloodstream glucose during starvation. However, a ketogenic diet has also been shown to be effective at lowering blood glucose in patients with type II diabetes due to the decreased carbohydrate intake [87]. In mouse studies, βHB administration yielded a 50% reduced phosphorylation and activity of Akt/protein kinase B downstream of the insulin receptor decreasing insulin signaling [86]. The mechanism for this βHB-mediated inhibition of Akt and the insulin signaling pathway was not fully elucidated, but it relied upon administration of D-βHB and not L-βHB, suggesting that mitochondrial metabolism of D-βHB may be involved. In *C. elegans,* inhibition or decreased expression of Akt or other proteins of the insulin signaling pathway have been shown to activate both DAF-16 and SKN-1 leading to lifespan extension [88], thereby providing a potential mechanism for the effect of βHB on longevity.

5.4.4 βHB does not induce a broad heat shock response, but still increases thermotolerance

Although βHB did not induce expression of four specific heat shock proteins monitored (Supplementary Fig. 3), it did activate the DAF-16 and SKN-1 signaling pathways, which are both likely responsible for the increased thermotolerance observed following βHB treatment. Previous research has shown that RNAi knockdown of either *skn-1* or *daf-16* decreased thermotolerance [89]. DAF-16 is known to induce expression of several heat shock proteins including *hsp-12.6*, *sip-1*, and *hsp-16.1*, which may play a role in the increased thermotolerance. The factors that SKN-1 induce to confer thermotolerance are less clear, although SKN-1 function has been implicated in the induction of *hsp-4* expression and activation of the ER stress response [41]. However, we did not find βHB to induce *hsp-4::GFP* expression, but a positive trend was observed ($p = 0.18$).

5.4.5 Neuroprotective effects of ketone bodies

In an AD cell model, βHB has been shown to protect hippocampal neurons from Aß toxicity [90]. The protection may have occurred through decreasing ROS levels as decreased ROS production is known to lower expression levels of beta-secretase (BACE1), a protease that contributes to toxic Aß generation [91]. This mechanism may be responsible for the ketogenic diet-induced reduction of Aß levels in a mouse model of AD [22]. The brain's ability to utilize glucose decreases in AD. To prevent deficits in brain ATP levels, βHB has been used as an alternative metabolic energy source for patients with AD [25]. Increased inflammation accompanies brain aging and may contribute to the development of AD. Increased levels of ketone bodies have been shown to reduce inflammation [71, 92] and this may result from increased mitochondrial efficiency and decreased ROS production [93].

PD is associated with aggregation of alpha-synuclein and death of dopaminergic neurons leading to motor decline. Mice treated with βHB showed partial protection against neurodegeneration and motor deficiency induced by MPTP [30]. Surprisingly, this was not due to increased NADH generation fueling complex I, but was described to be due to an increased supply of succinate, a substrate for ETC complex II [94]. As mentioned previously, the stimulation of complex II activity by βHB metabolism depends on the increased succinate produced as a byproduct of the mitochondrial succinyl-CoA: 3-ketoacid CoA transferase reaction. However, since βHB fully extended lifespan in complex II-defective *mev-1* mutants, it is unlikely that this mechanism plays a substantial role in βHB-mediated lifespan extension. The mechanism of βHB-mediated protection in PD models may be similar to the mechanism by which βHB supplementation increases lifespan in the complex I-defective *gas-1* worms.

5.4.6 Study limitations and future directions

Although we were able to dissect many of the pathways through which βHB extends lifespan in *C. elegans*, many questions remain. For example, is the reason that D-βHB but not L-βHB extended lifespan due to the increased ability of D- βHB to be metabolized or due to the higher efficiency of D-βHB as an HDAC inhibitor? Is HDAC inhibition required for βHB-mediated upregulation of SKN-1 or DAF-16 activity? In addition to extending lifespan as shown here, RNAi knockdown of the class I HDAC *hda-3* was shown to protect against polyglutamine-mediated toxicity in a *C. elegans* Huntington's disease, while knockdown of most other HDACs increased toxicity [95]. Is HDAC inhibition or SKN-1 activity required for DAF-16 activation by βHB? Also is βHB catabolism required for βHB-mediated SKN-1 or DAF-16 activation? Furthermore, is the transcription factor PHA-4/FoxA, which is required for DR-mediated longevity [96], also required for βHB-mediated longevity? In this regard mammalian Foxa2 is

known to induce expression of BDH1 [97]. Do βHB levels increase in DR worms and if so does this play a role in DR-mediated longevity? Lastly, are the same signaling pathways required for longevity also required for βHB-mediated protection in the *C. elegans* models of Aß and alpha-synuclein toxicity? Future experiments will provide answers to these questions and elucidate the molecular mechanisms responsible for the protective effects of βHB. This knowledge will allow for a broader use of βHB as a therapy for aging-related disorders.

5.5 Conclusions

βHB treatment extended lifespan and protected against metabolic, proteotoxic and thermal stress in *C. elegans*. βHB-mediated lifespan extension occurred through induction of the DAF-16 and SKN-1 signaling pathways and was dependent upon βHB-mediated inhibition of HDACs HDA-2 and HDA-3. Our data support the hypothesis that βHB is a DR mimetic and that βHB treatment will likely be useful in the treatment of many human aging-associated disorders.

5.6 Supporting information

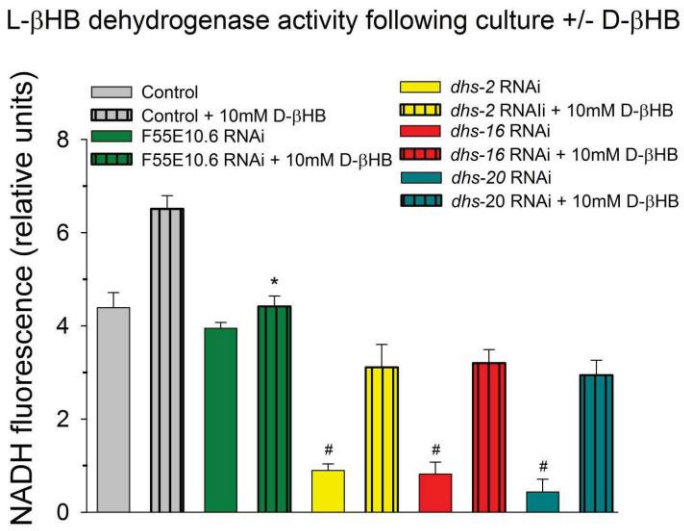

Figure 5.11. L-βHB dehydrogenase activity in worm extracts grown in the absence or presence of 10 mM D-βHB. Knockdown of F55E10.6 almost completely prevented the increased L-βHB dehydrogenase activity induced by culturing with D-βHB (* $p < 0.05$ vs. (Control + 10mM D-βHB)). Knockdown of *dhs-2*, *dhs-16*, or *dhs-20* decreased endogenous L-βHB dehydrogenase activity in the extracts (# $p < 0.05$ vs. Control).

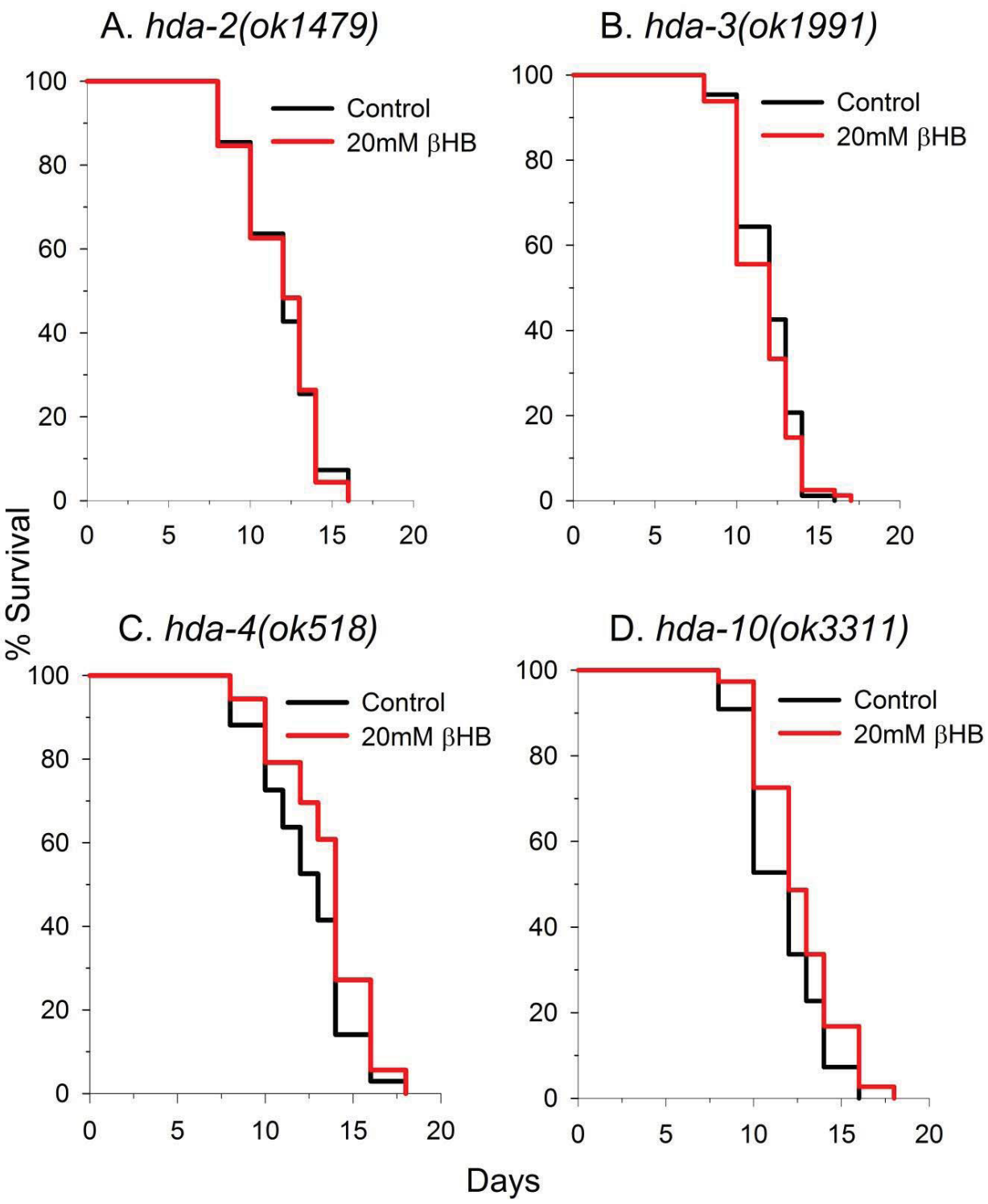

Figure 5.12. The effect of βHB on lifespan in histone deacetylase mutants. βHB addition did not extend the lifespan of **(A)** *hda-2(ok1479)* mutant worms or **(B)** *hda-3(ok1991)* mutant worms. βHB addition extended the lifespan of **(C)** *hda-4(ok518)* mutant worms (log-rank p =0.001) and **(D)** *hda-10(ok3311)* mutant worms (log-rank p =0.002).

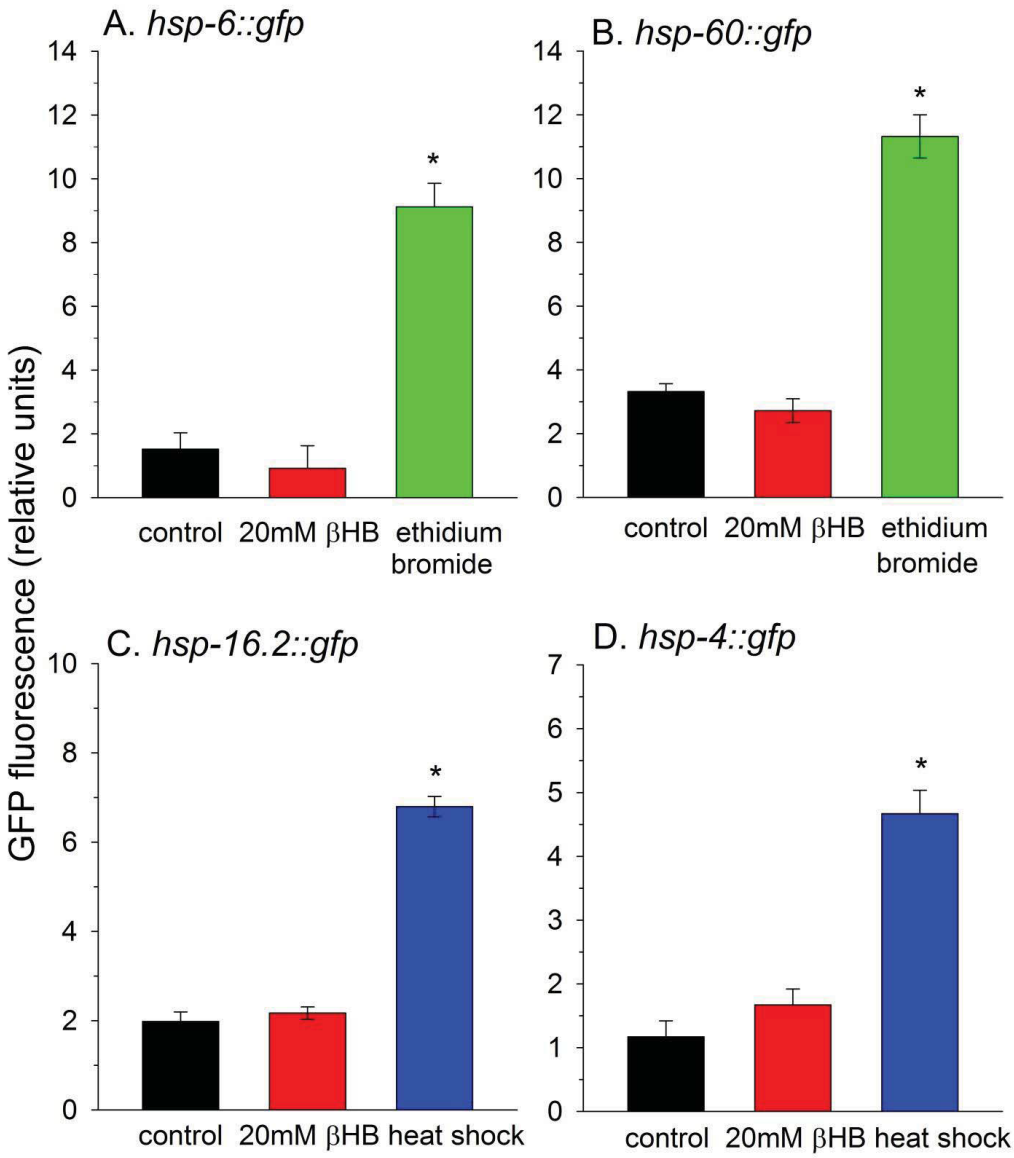

Figure 5.13. βHB addition does not induce expression of several heat shock proteins. The effect of βHB addition on the fluorescence of **(A)** *hsp-6::gfp*, **(B)** *hsp-60::gfp*, **(C)** *hsp-16.2p::GFP*, or **(D)***hsp-4::GFP* worms. For (A) and (B) 50 _g/ml ethidium bromide treatment for 2 days was used as a positive control. For (C) and (D) heat shock at 35°C for 2 hours was used as a positive control. (* $p <$ 0.05).

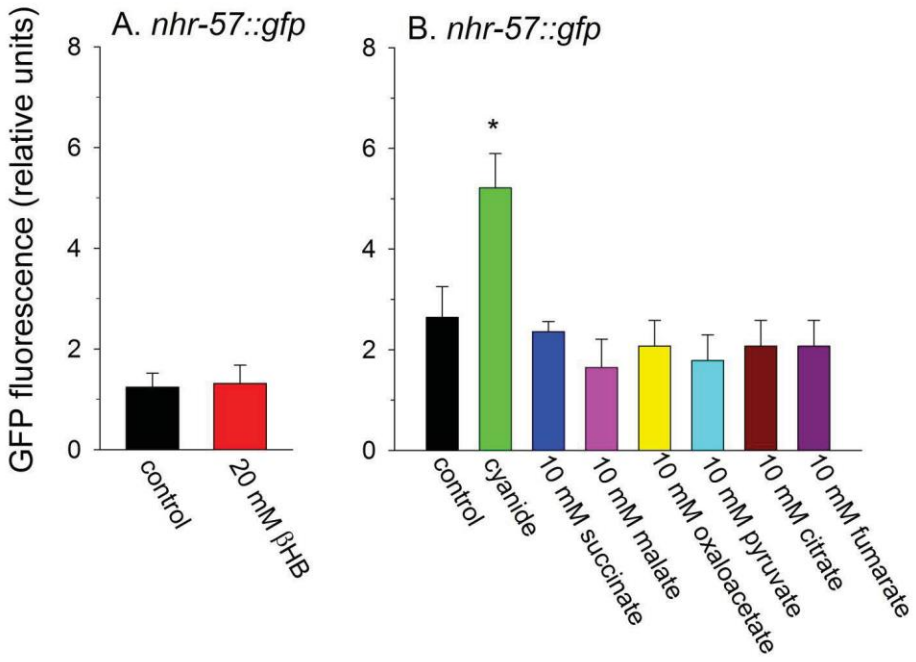

Figure 5.14. βHB, pyruvate, or TCA cycle metabolites do not activate HIF-1 transcriptional activity. The effect of **(A)** βHB, **(B)** pyruvate, or TCA cycle metabolites on GFP fluorescence in *nhr-57::gfp* worms. 20 _M potassium cyanide was used as a positive control. (* $p < 0.05$).

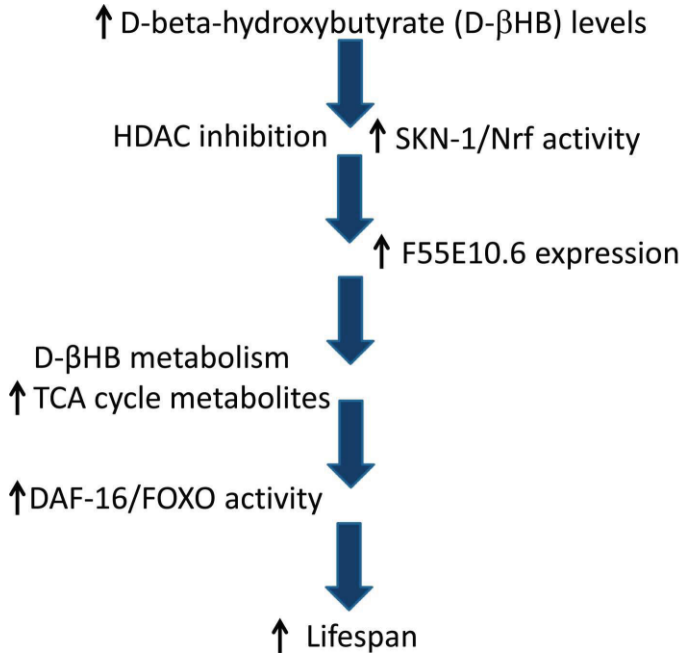

Figure 5.15. One possible mechanism through which βHB may extend lifespan in *C. elegans*.

Table 5.1 Lifespan data

Strain	RNAi	Treatment	% of untreated mean lifespan	% of N2 mean lifespan	*n*	# of worms	*p*-value
N2		2 mM βHB	105	105	2	224	0.024
N2		10 mM βHB	113	113	2	201	<0.001
N2		20 mM βHB	126	126	6	586	<0.001
N2		50 mM βHB	81	81	1	100	<0.001
N2		100 mM βHB	69	69	1	100	<0.001
N2		6 mM valproic acid	93	93	2	118	<0.001
N2		6 mM valproic acid + 20 mM βHB	84	84	2	126	<0.001
N2		5 mM butyrate	110	110	2	124	<0.001
N2		5 mM butyrate + 20 mM βHB	95	95	2	117	0.023
N2		1 mM NAC + 20 mM βHB	117	117	2	146	<0.001
N2		1 mM NAC	109	109	2	132	0.001
N2		50 mM glucose	70	70	4	336	<0.001
N2		50 mM glucose + 20 mM βHB	79	79	4	362	<0.001[#]
N2	F55E10.6 RNAi			107	3	375	0.008
N2	F55E10.6 RNAi	20 mM βHB	100		3	403	0.527
N2	*skn-1* RNAi			76	2	145	<0.001
N2	*skn-1* RNAi	20 mM βHB	103		2	156	0.557
N2	*cbp-1* RNAi			44	2	118	<0.001
N2	*cbp-1* RNAi	20 mM βHB	101		2	112	0.803
N2	*hda-1* RNAi			99	2	153	0.425
N2	*hda-1* RNAi	20 mM βHB	108		2	159	0.002
N2	*hda-2* RNAi			112	2	396	<0.001
N2	*hda-2* RNAi	20 mM βHB	96		2	362	0.018
N2	*hda-3* RNAi			111	2	316	<0.001
N2	*hda-3* RNAi	20 mM βHB	102		2	380	0.648
hda-2(ok1479)				69	1	110	<0.001
hda-2(ok1479)		20 mM βHB	100		1	91	0.98
hda-3(ok1991)				69	1	87	<0.001
hda-3(ok1991)		20 mM βHB	97		1	81	0.29
hda-4(ok518)				72	1	135	<0.001
hda-4(ok518)		20 mM βHB	108		1	125	0.001
hda-10(ok3311)				67	1	110	<0.001
hda-10(ok3311)		20 mM βHB	109		1	113	0.002
daf-16(mgDf50)				83	2	295	<0.001
daf-16(mgDf50)		20 mM βHB	99		2	270	0.361
aak-2(gt33)				88	2	208	<0.001
aak-2(gt33)		20 mM βHB	100		2	184	0.793
sir-2.1(ok434)				84	2	210	<0.001
sir-2.1(ok434)		20 mM βHB	98		2	265	0.113
eat-2(ad1116)				146	2	114	<0.001
eat-2(ad1116)		20 mM βHB	95		2	157	0.114

Table 5.1 (Continued)

gas-1(fc21)				69	2	119	<0.001
gas-1(fc21)		20 mM βHB	111		2	120	<0.001
mev-1(kn1)				70	2	273	<0.001
mev-1(kn1)		20 mM βHB	127		2	269	<0.001
rsks-1(ok1255)				106	3	362	<0.001
rsks-1(ok1255)		20 mM βHB	105		3	337	0.002
gcn-2(ok871)				88	3	479	<0.001
gcn-2(ok871)		20 mM βHB	108		3	428	<0.001
CL6049 *(16°C) snb-1::TDP-43*				57	1	123	<0.001
CL6049 *(16°C) snb-1::TDP-43*		20 mM βHB	99		1	114	0.413
CL6049 *(16°C) snb-1::TDP-43*		30 mm βHB	105		1	117	0.006
CL6049 *snb-1::TDP-43*				61	1	90	<0.001
CL6049 *snb-1::TDP-43*		50 mM βHB	101		1	93	0.951
CL6049 *snb-1::TDP-43*		100 mM βHB	103		1	53	0.791

[a]Compared to 50 mM glucose treated worms or untreated worms.

5.7 Competing interests

No conflict of interest could be disclosed for any author.

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5.9 Authors' contributions

CE performed most of the lifespan, GFP reporter strain, proteotoxicity experiments, and data analysis. JC performed data analysis. NC and CE performed enzyme assays, oxygen

experiments, and ATP assays. MR and DL performed a few lifespan experiments and data analysis. CE and PB conceived the studies and drafted the manuscript. All authors read and approved the final manuscript.

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CHAPTER 6

SIGNIFICANCE OF FINDING COMPOUNDS THAT INFLUENCE AGING

6.1 Summary

Our studies focus on discovering supplemented metabolites that attenuate aging and age-related decline and elucidating the genetic pathways involved. In Chapter 3 we show that the TCA cycle metabolites malate and fumarate increased the lifespan of *C. elegans* via the non-mammalian glyoxylate shunt and malate dismutation/fumarate reduction pathways. Further, we identified that the SIR-2.1 and DAF-16 proteins were necessary for malate-mediated lifespan extension. The next step was to examine (in Chapter 4) the effects on the rate of aging of amino acids that catabolize into TCA metabolites. We show that individual amino acid supplementation increased the lifespan of *C. elegans* and the pathways involved vary from one amino acid to the next. Various longevity pathways including DAF-16, SKN-1, AAK-2, SIR-2.1, GCN-2, heat shock, autophagy, CR, and inhibition of TOR signaling were involved in protective effects. Finally, we looked at alternative mitochondrial energy sources and found that βHB treatment extended lifespan and protected against metabolic, proteotoxic, and thermal stress in *C. elegans*. βHB-mediated lifespan extension occurred through induction of the DAF-16 and SKN-1 signaling pathways and relied upon βHB-mediated inhibition of HDACs. Our data support the hypothesis that βHB is a CR mimetic and that treatment with βHB will likely be beneficial in the management of many human aging-related disorders.

Although TCA cycle metabolites, most amino acids, and βHB are produced naturally within cells, this does not denote that the metabolites, when administered through feeding, will act in the same manner. One can assume that CR induced ketosis, when compared to βHB supplementation, activates alternate signaling pathways and ketones are catabolized for energy in

various tissues to different extents. The same rationale applies to TCA cycle metabolites and amino acids.

6.2 TCA Cycle metabolites and aging

As described in Chapter 3, changes in energy metabolism influence epigenetic mechanisms and can alter the aging process. The TCA cycle is a central pathway for metabolism and much data implicate its function in the control of longevity [1]. Proper coordination between the electron transport chain and the TCA cycle is necessary to maintain a normal NAD^+/NADH ratio beneficial to long life. The function of the mitochondrial electron transport chain, which oxidizes NADH and $FADH_2$, decreases with age across species [2], which leads to a reduced cellular NAD^+/NADH ratio during aging. Anti-aging therapies such as CR increase the NAD^+/NADH ratio in many tissues as a possible mechanism to delay aging.

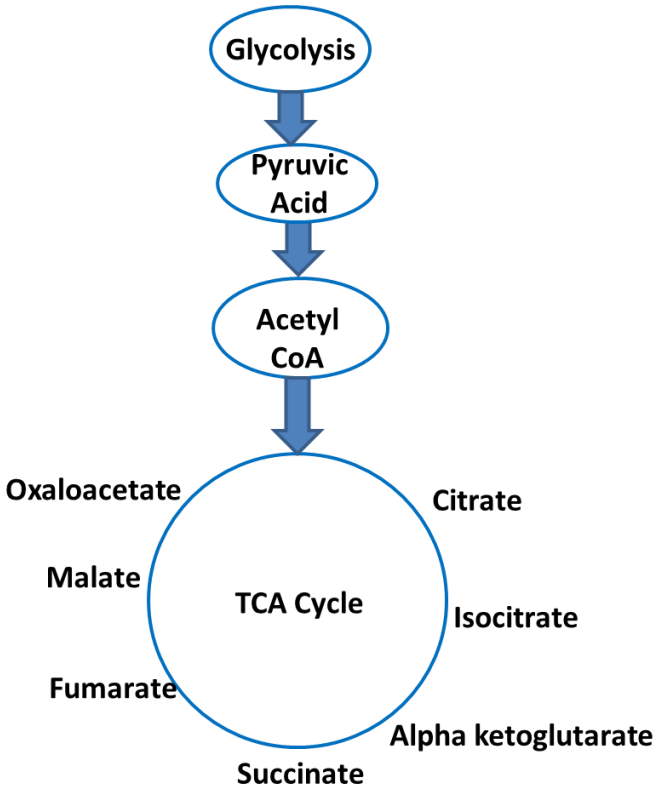

Figure 6.1. The TCA Cycle

In an effort to understand if TCA cycle intermediates could rescue mitochondrial dysfunction during the aging process in *C. elegans*, we performed lifespan assays supplementing TCA cycle metabolites into the worm's diet. Yeast mutants with increased lifespan have been shown to have increased levels of TCA cycle metabolites [3]. In Chapter 3, we showed that malate and fumarate addition extend lifespan in *C. elegans* and the addition of TCA cycle intermediates increased the NAD^+/NADH ratio, likely contributing to lifespan extension. Increased NAD^+ levels activate the histone deacetylase SIR-2.1 [4] and AMP kinase [5], both known to interact with DAF-16 to increase lifespan. Through knockdown models we showed that malate-mediated lifespan extension is dependent upon SIR-2.1 and AMP kinase. Malate supplementation resulted in DAF-16 translocation to the nucleus and transcription of DAF-16 target genes, which likewise plays an essential role in lifespan extension. In Chapter 4 we show that a lower concentration of succinate (when compared to malate) results in lifespan extension, which supported data from Chapter 3 indicating that succinate addition induces DAF-16 nuclear translocation.

We also hypothesized that addition of malate or fumarate to *C. elegans* leads to activation of the glyoxylate shunt. Glyoxylate shunt activity increases the NAD^+/NADH ratio as the shunt bypasses two of the three NADH generating reactions of the TCA cycle resulting in a conservation of NAD^+. Isocitrate is reversibly converted into succinate and glyoxylate by the isocitrate lyase activity. The glyoxylate shunt malate synthase enzymatic activity catalyzes the reversible conversion of malate and CoA to glyoxylate and acetyl-CoA that may also be important for malate-mediated lifespan extension. Upregulation of shunt activity would increase NAD^+ levels, though metabolism under these conditions likely becomes limited by FAD levels,

so malate dismutation is activated to oxidize $FADH_2$ to FAD. Further research should target the role of the FAD/$FADH_2$ ratio in lifespan determination. We did find that addition of FAD to worm media increased lifespan (data not shown).

6.3 NAD^+ metabolism and aging

Mammals convert tryptophan to NAD^+ through quinolinic acid in the de novo NAD^+ synthesis pathway [6]. When looking at the C. elegans genome, the first gene in the pathway for quinolinic acid conversion to NAD^+ appears to be absent. Therefore it was first assumed that worms must utilize the NAD^+ salvage pathway rather than direct de novo synthesis. The first enzyme in the NAD^+ salvage pathway is nicotinamidase that catalyzes the conversion of nicotinamide to nicotinic acid. There are three nicotinamidases found in *C. elegans*, two PNC-1 isoforms and PNC-2. Male worms with mutations in PNC-1 are infertile while hermaphrodites with the same mutation display muscle impairment [7]. Knockdown of the NAD^+ salvage pathway in worms decreases lifespan, but since it is not lethal there may be additional genes that play a role in quinolinic acid conversion to NAD^+. So therefore, there is likely a mechanism present in worms to convert tryptophan to NAD^+ (see Fig. 2), which I hypothesize to play a role in tryptophan-mediated longevity. During CR in yeast, PNC-1 activity and expression is upregulated to convert the Sir2 inhibitor NAM to nicotinic acid, resulting in increased Sir2 activity [8]. Given that malate supplementation increased lifespan through SIR-2.1, it is plausible that PNC-1 is upregulated through the addition of TCA cycle metabolites (Fig 2). This is supported by our data that malate supplementation greatly increased NAD(H) levels in *C. elegans* (see Chapter 3).Therefore further investigation of TCA cycle metabolite supplementation in conjunction with RNAi knockdown of PNC-1 would further elucidate the mechanism by which malate, fumarate, oxaloacetate, and alpha-ketoglutarate extend lifespan.

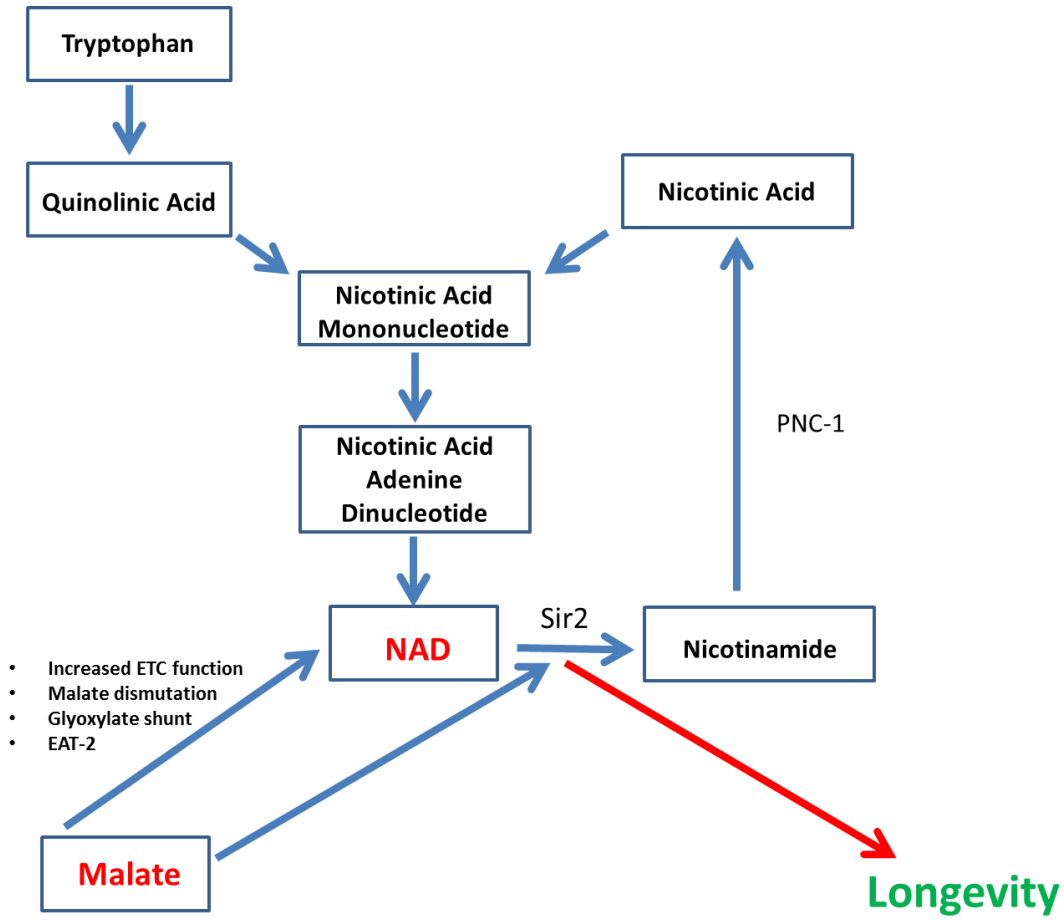

Figure 6.2. The *C. elegans* NAD salvage pathway. Malate may be increasing NAD through the indirect upregulation of pnc-1.

Although increased NAD^+ levels are important for lifespan extension, the assays used for NAD^+ quantification in *C. elegans* are difficult. Moreover, when assays optimized for mammalian cell culture or tissues are changed and utilized with *C. elegans*, the possibility of errors in the results are increased. The main reason for this is that *C. elegans* possess a hard cuticle that may be incompletely disrupted when performing assays to release small molecule metabolites such as NAD^+. Therefore, the results may be difficult to replicate. Being that *C. elegans* have become a popular model for aging, the need for nematode specific protocols is

necessary for accurate and reproducible results. A recent study provided evidence that altered NAD^+ homeostasis and mitochondrial dysfunction accompany age-related decline using a novel, non-invasive, resonance-based, *in vivo* NAD^+ assay to monitor NAD^+ concentrations in the human brain [9]. This non-invasive method could be adapted for use on *C. elegans* to follow NAD^+ homeostasis throughout their lifespan. Further, this method could more accurately verify if addition of metabolites, such as malate, are increasing NAD^+ levels in long-lived worms.

6.4 Mitochondrial membrane potential and aging

The pumping of protons by the electron transport chain together with phosphate-proton symporters in the inner mitochondrial membrane creates a mitochondrial membrane potential (MMP) across the inner membrane. Decreased MMP has been shown to increase lifespan in *C. elegans* [10] and yeast [11]. This is not completely intuitive as the magnitude of the MMP is directly proportional to the amount of ATP that can be made by ATP synthase. However, a high MMP also increases ROS production and this ROS production, not ATP levels, may limit lifespan. Many long-lived mutants and RNAi knockdown of genes that are associated with lifespan extension exhibit a lower MMP when compared to wild-type nematodes. Diminished MMP of DAF-2 mutants is reliant on DAF-16, suggesting that DAF-16 is essential for lifespan regulation and mitochondrial energy homeostasis. Additionally, studies show that protection by mitochondrial uncoupling against neuronal excitotoxicity is dependent on the increased phosphorylation state of AMPK [10]. We showed in Chapter 3 that *C. elegans* longevity by malate supplementation is dependent upon AMPK and DAF-16 activation. Malate also increased oxygen consumption, decreased ATP, and was associated with a decline in MMP. Therefore, malate may act as a mild mitochondrial uncoupler, leading to the activation of DAF-16 by AMPK stimulating increased longevity. Although the use of TMRE and TMRM are common

assays used to quantify MMP in *C. elegans*, results are challenging to reproduce since conditions of animal culture vary considerably in the literature. One way to follow up and verify the effects of metabolites on MMP is to use multiple types of assays that are developed specifically for worms, such as the recently developed assay that incorporates the fluorescent cationic, lipophilic carbocyanine dye, DiSC3(3) [12]. DiSC3(3) is a membrane potential sensitive dye used for examining *in vivo* changes in MMP and exhibits fluorescence fluctuations upon membrane hyperpolarization (when the matrix space of the mitochondrion becomes more negative). Multiple techniques and *C. elegans* specific assays will improve reproducibility of the results.

6.5 Mitohormesis and alternate mechanisms of metabolite-mediated longevity

Small deviations in cellular metabolism such as through metabolite supplementation can activate multiple stress response pathways and upregulate genes associated with longevity such as HSF-1, SKN-1, and heat shock proteins. In Chapters 4 we investigate the role of amino acids in lifespan extension and identify that many amino acids are acting as hormetics and a few as mitohormetics, such as tryptophan and proline. To answer some questions left from Chapter 1, we also found that alpha-ketoglutarate supplementation upregulates HSF-1 and the ER stress response in GFP reporter strains of worms. Most of the amino acids that extended lifespan the greatest: proline, arginine, histidine, glutamine, and glutamate, are catabolized through glutamate into alpha-ketoglutarate in the TCA cycle. It was recently shown that alpha-ketoglutarate greatly extends lifespan of *C. elegans* by inhibiting ATP synthase and TOR [13]. Citrate also activates an ER stress reporter, however, no lifespan extension was seen when citrate was added to worm medium. GFP reporter strains can be dim and heat shock proteins are only one part of the hormesis story. Understanding the effect of these metabolites on the ROS defense enzymes (SOD, catalase) is necessary and can be achieved by western blot analysis or enzymatic assays

specific for catalase and SOD. Monitoring ROS levels in TCA cycle treated young and old worms would allow for a better understanding of how the mitochondria is affected and if homeostasis is being influenced.

Although we showed an important role for the glyoxylate shunt and malate dismutation in malate-mediated lifespan extension in *C. elegans*, malate treatment has been shown to be beneficial in mammals as well. Therefore, many of the protective effects of malate treatment seem to be conserved from nematodes to mammals. Since fumarate, malate, succinate, alpha-ketoglutarate, isocitrate, and oxaloacetate extend lifespan in *C. elegans* through a mechanism similar to calorie restriction, an anaplerotic cocktail of these compounds may be useful for the treatment of human aging-associated disorders.

6.6 Amino acids function through diverse mechanisms to extend lifespan

Amino acids can be deaminated into keto acids and then directly or indirectly fed into the TCA cycle. In *C. elegans*, the mitohormetic effect of proline supplementation extended lifespan [14] and knockdown of the enzyme that catabolizes tryptophan also led to lifespan extension [15]. Feeding mice a diet high in branched chain amino acids led to increased mitochondrial biogenesis in muscle, decreased ROS production, and increased average lifespan of males [16]. Due to the incomplete knowledge of the effects of amino acids on longevity as well as the widespread use of amino acid and protein supplementation in the human diet we determined the effects of individual amino acid supplementation on *C. elegans* lifespan. In Chapter 4, we establish that individual amino acid supplementation increased the lifespan of *C. elegans* and increased stress resistance with serine, proline, and tryptophan showing the greatest effects. Perhaps these amino acids are transported into the gut at a faster rate and therefore act as quick anaplerotics for the TCA cycle intermediates. Because so little is known about many of the

amino acids, especially serine, future work should aim at investigating how these metabolites contribute to longevity in more complex models.

Of the 20 amino acids, serine and proline extended lifespan to the largest extent. Proline was recently found to be mitohormetic in *C. elegans* and our data confirms this finding. In Chapter 4, we show that the well-studied longevity pathways including DAF-16, SKN-1, AAK-2, SIR-2.1, GCN-2, heat shock, autophagy, calorie restriction, and inhibition of TOR signaling are involved in these protective effects. These different longevity pathways may converge to decrease the rate of translation by the ribosome for lifespan extension. In addition, cysteine and tryptophan-induced lifespan extension were independent of SKN-1, so supplementation with these amino acids did not likely alter mitochondrial metabolism to increase ROS production.

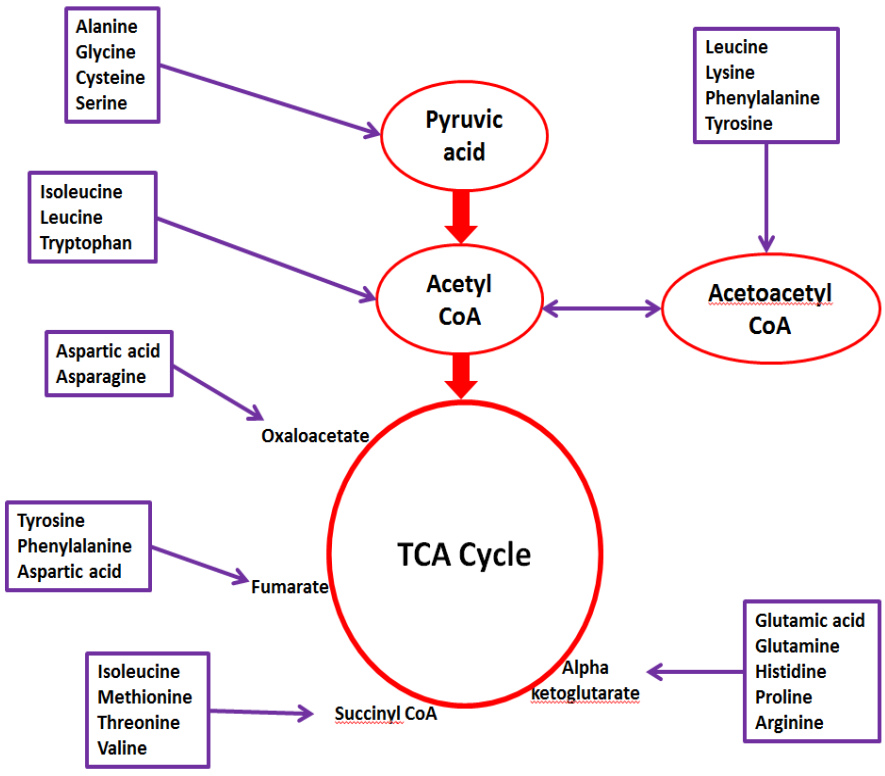

Figure 6.3. Entry of amino acids into the TCA cycle.

As discussed in Chapter 4, the pathways involved with lifespan extension vary from one amino acid to the next, but the DAF-16 and AMP kinase longevity pathways appeared to be activated by supplementation most, if not all, lifespan extending amino acids. Examples of other longevity pathways activated include, serine and histidine stimulating transcriptional activity of HIF-1, while other amino acids did not. Eight lifespan-extending amino acids increased the transcriptional activity of SKN-1, but not tryptophan, cysteine, or the lifespan-decreasing amino acids phenylalanine and asparagine. Proline has been previously shown to extend the lifespan of worms through mitohormesis and it showed the greatest lifespan extension of all the amino acids. Another mechanism of lifespan extension observed with proline supplementation may be FOXO activation of glutamine synthetase, which inhibits TOR signaling by blocking its lysosomal translocation and stimulates autophagy to recycle cellular components [17]. Inhibition of TOR activity through upregulation of glutamine synthetase expression may also explain how alpha-ketoglutarate, glutamine, arginine, and histidine influence lifespan. Proline can be catabolized to glutamate through proline dehydrogenase and 1-pyrroline-s-carboxylate dehydrogenase. Prior studies show that an increase in proline dehydrogenase increases mitochondrial ROS production, which activates stress signaling genes to extend lifespan. Perhaps, the impressive lifespan extending ability of proline is due to a combination of mitohormesis and inhibition of TOR signaling. Interesting, plants exposed to various stressful conditions accumulate abundant proline reserves. Proline acts as a metal chelator and overproduction of proline supports osmotic balance and prevents oxidative damage in plant cells [18]. Similarly, this same trend of proline accumulation is seen in *E. coli* when undergoing hypertonic stress [19]. Therefore, additional proline may protect *C. elegans* from protein misfolding during normal aging and during times of stress. Future research should aim to

identify a role for proline in protecting nematodes from osmotic imbalance. Additionally, proline precursors have been shown to sustain collagen synthesis in mammals [20]. Proline is hydroxylated into collagen, which is necessary for the development and maintenance of the exoskeleton (cuticle) in *C. elegans* [21] and collagen remodeling is required for longevity in *C. elegans* [22]. Therefore, proline may not only be protecting through mitohormesis, chelation, and osmotic regulation, but it may also contribute to collagen sparing and synthesis in aged worms, reducing the rate of cuticle breakdown.

Of the top lifespan extending amino acids, perhaps the least is known about how serine levels affect signaling pathways in mammals. In Chapter 4 we found serine among the top amino acids to extend lifespan and the lifespan extension required HIF-1, DAF-16, SKN-1, and inhibition of TOR signaling, which, like proline, may converge to decrease the rate of translation by the ribosome for lifespan extension. In human endothelial cells serine acts as an antioxidant and cytoprotectant through upregulation of Nrf2 and heme oxygenase-1 [23]. Serine is a precursor for tryptophan, glycine, cysteine, and pyruvate, which all demonstrate lifespan extending capabilities in *C. elegans*. In tumors, HIF-1 activation increases glycolysis, including an increase in pyruvate production [24]. Perhaps serine addition acts on HIF-1 to increase pyruvate levels resulting in TCA cycle anaplerosis in aging worms. Serine is also an essential building block of phospholipids, such as phosphatidylserine, and therefore necessary for cellular membrane composition. The phospholipid cardiolipin accounts for 20% of mitochondrial phospholipids and deterioration of cardiolipin is associated with age-related disease and decline. Serine supplementation may increase phosphatidylserine synthesis and participate in cardiolipin sparing. Quantifying phosphatidylserine and cardiolipin in serine treated worms and isolated mitochondria would elucidate any role for serine supplementation in phospholipid metabolism.

Interestingly, in human endothelial cells, serine acts as an antioxidant and cytoprotectant by increasing expression of Nrf2, its transcriptional target HO-1, and NO. Being that both histidine and serine were tested at 10 mM concentrations (while others were tested at 1 or 5 mM concentrations), homeostatic dysregulation could also explain why these two metabolites, but not the others**,** induced HIF-1 activity. Careful consideration should be taken when using GFP reporter gene fusion animals. Multiple assays should be utilized to verify findings to make certain that results are reproducible under all growth conditions. Amino acid supplementation in liquid culture is susceptible to bacterial and fungal contamination, which is only sometimes readily detectable. Therefore, any results should be explored with uncertainty and verified by multiple techniques and labs.

As mentioned in Chapter 1, histidine is a zinc and nickel chelator that protects against metal toxicity in *C. elegans* [25] and human astrocytes [26]. Cobalt-induced HIF-1 expression is inhibited by histidine in cultured mammalian cells [27], yet HIF-1 activation has been shown to be regulated by the conversion of histidine to histamine through histidine decarboxylase expression under hypoxic conditions [28]. Future research should identify if histamine, histidine analogs, or histidine breakdown products, such as urocanic acid, increase HIF-1 expression. Unlike histidine, N-acetyl-histidine and histidine methylester are cell permeable and both were slightly more effective than histidine at increasing lifespan when supplemented at a low concentration. These results suggest that the rate of amino acid absorption by the intestine may be a limiting factor in lifespan extension.

Another significant finding from my research is that supplementation with tryptophan, nicotinamide, quinolinic acid, nicotinic acid, or its isomer picolinic acid resulted in the activation of both the mitochondrial unfolded protein and the ER stress response pathways, while NAD^+

addition induced only the ER stress response. Although there is no obvious homolog for the first gene in the pathway for quinolinic acid conversion to NAD^+ in *C. elegans*, knockdown of the NAD^+ salvage pathway is not lethal to worms indicating that other genes may be involved in tryptophan catabolism. As mentioned above, *in vivo* quantification of NAD^+ in tryptophan treated worms could directly answer if tryptophan alone or its breakdown products are responsible for increasing NAD^+ levels. NAD^+ precursors and tryptophan can also protect cells by inducing an ER stress response and a mitochondrial unfolded protein response. Picolinic acid is a strong metal chelator and it will be important to determine if the protective effects of picolinic acid are due to metal chelation or increased NAD^+ levels.

Different amino acids are catabolized and enter the TCA cycle through separate intermediates. We did not find that the amino acids yielding the greatest effects on lifespan were degraded through one common catabolic pathway. We suggest that through anaplerosis, supplemented metabolites that increased lifespan may be catabolized by mitochondria to increase TCA cycle metabolite levels. The DAF-16/FOXO longevity pathway has been shown to be activated by increased TCA cycle metabolite levels [29]. As shown in Chapter 3, the TCA cycle appears to be a lifespan-extending metabolic pathway in *C. elegans*.

Future experiments will aim to develop an axenic medium that can be used to determine the effects of amino acid restriction on the lifespan of *C. elegans*. Currently used axenic medium appears to induce dietary restriction yielding long-lived worms. Future studies will also aim to determine the metabolic mechanisms through which amino acids activate SKN-1 activity and through which serine and histidine activate HIF-1. It should also be identified whether serine catabolism is required for serine-mediated lifespan extension, as serine can be converted to pyruvate, which extends lifespan. Since lifespan extension by serine is so robust, further research

is warranted to identify the exact mechanistic details. Research should continue to focus on the individual signaling pathways by which amino acids affect health and lifespan. In our studies we focused on 1, 5, and 10 mM concentrations, but further research should examine concentrations below and above these parameters to identify a proper dose for amino acids that had little to no effect on lifespan. It is hoped that an optimal dose will be identified for each amino acid. Through the use of both amino acid supplementation and restriction, a diet may one day be developed that can significantly increase stress resistance and slow aging and the onset of aging-associated disorders.

6.7 Beta-hydroxybutyrate as an alternative energy source

Under standard dietary conditions, the human body uses glucose to fuel glycolysis, which is used as energy or stored as glycogen when present in excess. Glucose oxidation is used together with fatty acid and amino acid oxidation for energy production. During times of starvation or restricted carbohydrate consumption when glycogen stores are reduced, triglycerides in adipose tissue are broken down into fatty acids and transported to peripheral tissues to be broken down by mitochondrial beta-oxidation into acetyl-CoA for energy production through the TCA cycle. In the liver**,** fatty acids experience a different fate. There acetyl-CoA is converted into the ketone bodies acetoacetate and βHB**,** which are exported from the liver to other tissues as an alternative energy source. This is especially important for neurons, which are unable to catabolize fatty acids at an appreciable rate. The ketogenic diet is a high fat, low protein and low carbohydrate diet that mimics calorie restriction to provide the body with energy when glucose is not freely available. Many of the benefits produced by CR, especially at the mitochondrial level, can be attributed to the ketones generated by a low calorie state [29]. Like CR, βHB has shown some effectiveness in the protection against Alzheimer's and

Parkinson's disease-mediated neurodegeneration in animal models and human trials, but until now little was known about its effects on lifespan. Because βHB can feed into the TCA cycle, in Chapter 5 we determined if it can also extend lifespan in *C. elegans*.

We were the first to identify D-βHB as a positive modulator of organismal longevity. We showed that administering βHB to *C. elegans* extended lifespan and delayed proteotoxicity and glucose toxicity in a SIR-2.1 and AMPK-dependent manner that also required the stress-responsive transcription factors DAF-16 and SKN-1. The mammalian SKN-1 homolog Nrf2 has been shown to play a limited role in mitochondrial biogenesis in some tissues. We also found that βHB did not extend lifespan in *eat-2* pharyngeal pumping mutants and therefore acts as a dietary restriction mimetic. An important finding in Chapter 5 is that D-βHB-mediated lifespan extension required SKN-1 and its transcriptional target F55E10.6, a short-chain dehydrogenase/reductase with βHB dehydrogenase activity, although inhibition of HDACS HDA-2 and HDA-3 were also required for the increased longevity. βHB-induced lifespan extension was partially blocked in ETC complex I mutant worms and was unaffected in mitochondrial ETC complex II mutant worms. This suggests that the lifespan extension is driven partly by mitochondrial metabolism-dependent effects, specifically though NAD(H) metabolism.

Multiple physiological conditions are associated with a reduction in the cellular $NADH/NAD^+$ ratio. As discussed in Chapter 1, CR decreases the $NADH/NAD^+$ in yeast, which activates Sir2 (NADH is a competitive inhibitor of Sir2), resulting in an increase in lifespan [30]. Another situation where a decline in NADH is seen is during the use of metformin, a drug prescribed for the treatment of Type II diabetes, which accompanies increased SIRT1 activity [31]. Activation of SIRT1 by metformin was associated with lowered blood glucose and recovered insulin sensitivity.

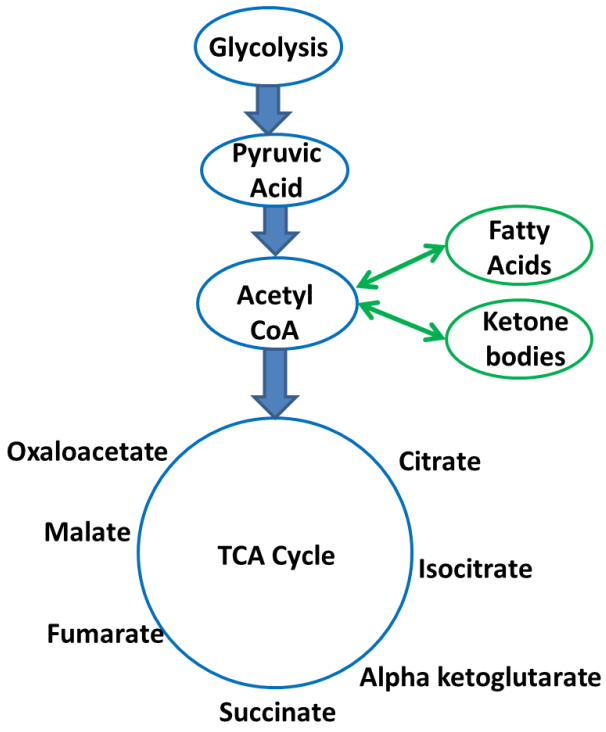

Figure 6.4. Ketone bodies entry in to the TCA cycle.

Like CR, the ketogenic diet reduces the NADH/NAD^+ ratio by boosting mitochondrial respiration through increased NADH oxidation [32]. So similar are the affects seen during CR and the administration of βHB (or the ketogenic diet) that some researchers speculate that CR may be beneficial at least in part through increasing ketone levels, which act as HDAC inhibitors and increase expression of stress response genes. Not only do both CR and the ketogenic diet extend lifespan, reduce the NADH/NAD^+ ratio, and activate SIRT1 [33], they are associated with a reduction in ROS production [29]. Both CR and the ketogenic induce mitochondrial biogenesis, which partially compensates for the decline in ATP observed during aging and dysfunction [34]. Prior to the irreversible conversion of acetoacetate to acetone, acetoacetate and βHB exist in a reversible equilibrium. So, in theory, administration of acetoacetate to *C. elegans*

would increase that NAD^+/NADH ratio and extend lifespan. Unfortunately, acetoacetate is quickly decarboxylated and is very unstable, making its use as a supplement difficult.

In Chapter 5 we propose two overlapping pathways for lifespan extension mediated by βHB supplementation as shown in Figure 4. In the first, βHB directly inhibits HDACs to increase histone acetylation leading to DAF-16/FOXO transcription and activation. The second proposed protective pathway involves mitochondrial metabolism of βHB, leading to increased citric acid cycle metabolites and electron transport chain (ETC) activity, increased reactive oxygen species production, and activation of the SKN-1 antioxidant response pathway.

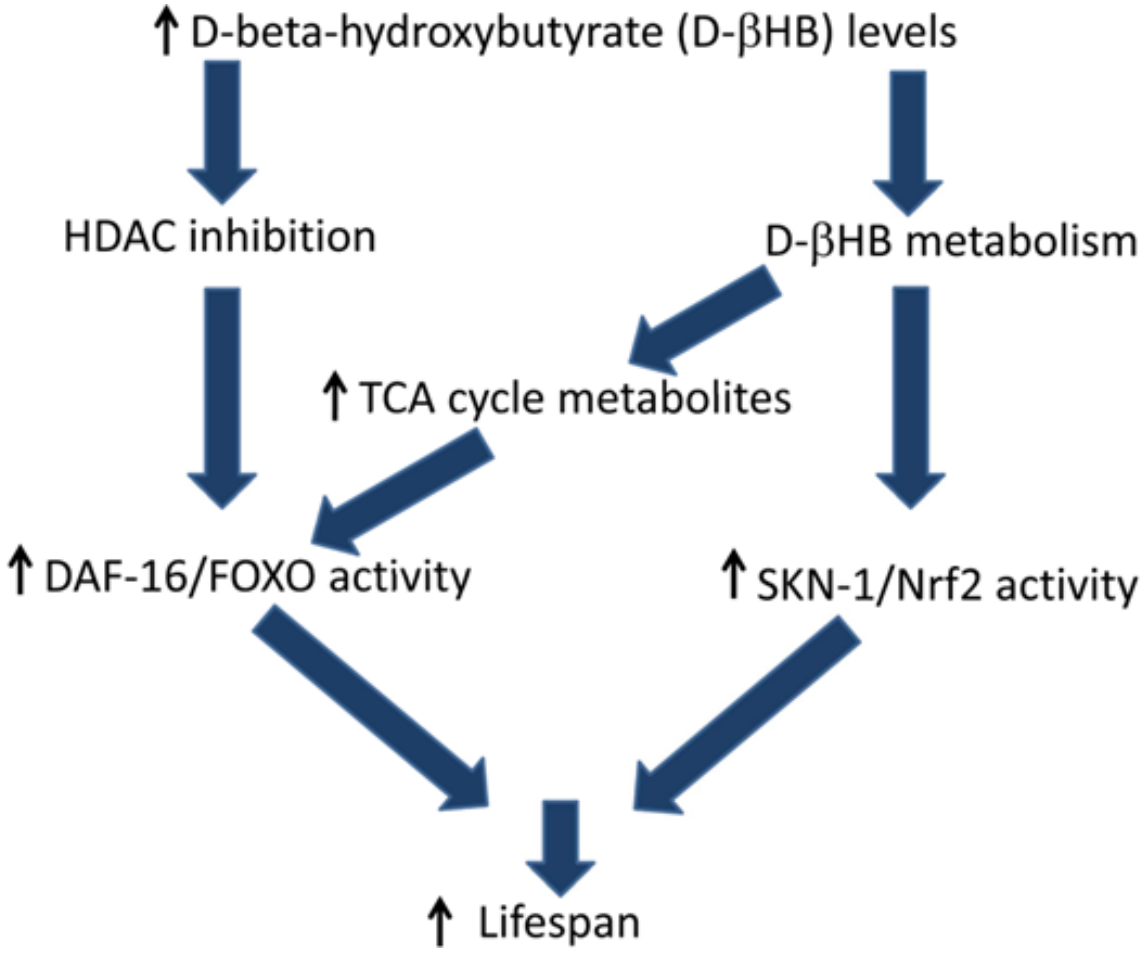

Figure 6.5. Proposed mechanisms of D- βHB mediated lifespan extension in *C. elegans.*

In Chapter 5 we also showed that βHB increased thermotolerance and delayed Aβ-induced paralysis and decreased alpha-synuclein aggregation in *C. elegans* models of human disease. Although we show compelling evidence for the use of D-βHB as a prophylactic for disease in nematode models, questions remain. For example, are the same signaling pathways required for longevity also required for βHB-mediated protection in the *C. elegans* models of amyloid beta and alpha-synuclein toxicity? Future experiments will elucidate the molecular mechanisms responsible for the protective effects of βHB and this knowledge will allow for a broader use of D-βHB as a therapy for aging-related disorders.

6.8 Metabolites that delay aging

In order to discover metabolic pathways that delay aging, the effects of large sets of metabolites on lifespan were investigated. *C. elegans* share almost all metabolic pathways with humans. We hypothesized that changes in mitochondrial metabolite levels could result in an increased lifespan and protection from proteotoxic stress. We have determined that the TCA cycle metabolites fumarate, malate, succinate, alpha-ketoglutarate, isocitrate, and oxaloacetate extended lifespan in *C. elegans*, while all of the amino acids except phenylalanine and aspartate extended lifespan at least to a small extent at one or more of the 3 concentrations tested with serine and proline showing the largest effects. We also found that the ketone body, βHB, extends lifespan in *C. elegans* and we determined the cytoprotective signaling pathways required for this effect. Figure 5 highlights the many pathways associated with longevity.

The major aim of this study was to understand the mechanisms by which natural metabolites influence the lifespan of *C. elegans*. From our studies we have identified many metabolites that can increase lifespan and prevent age-related decline.

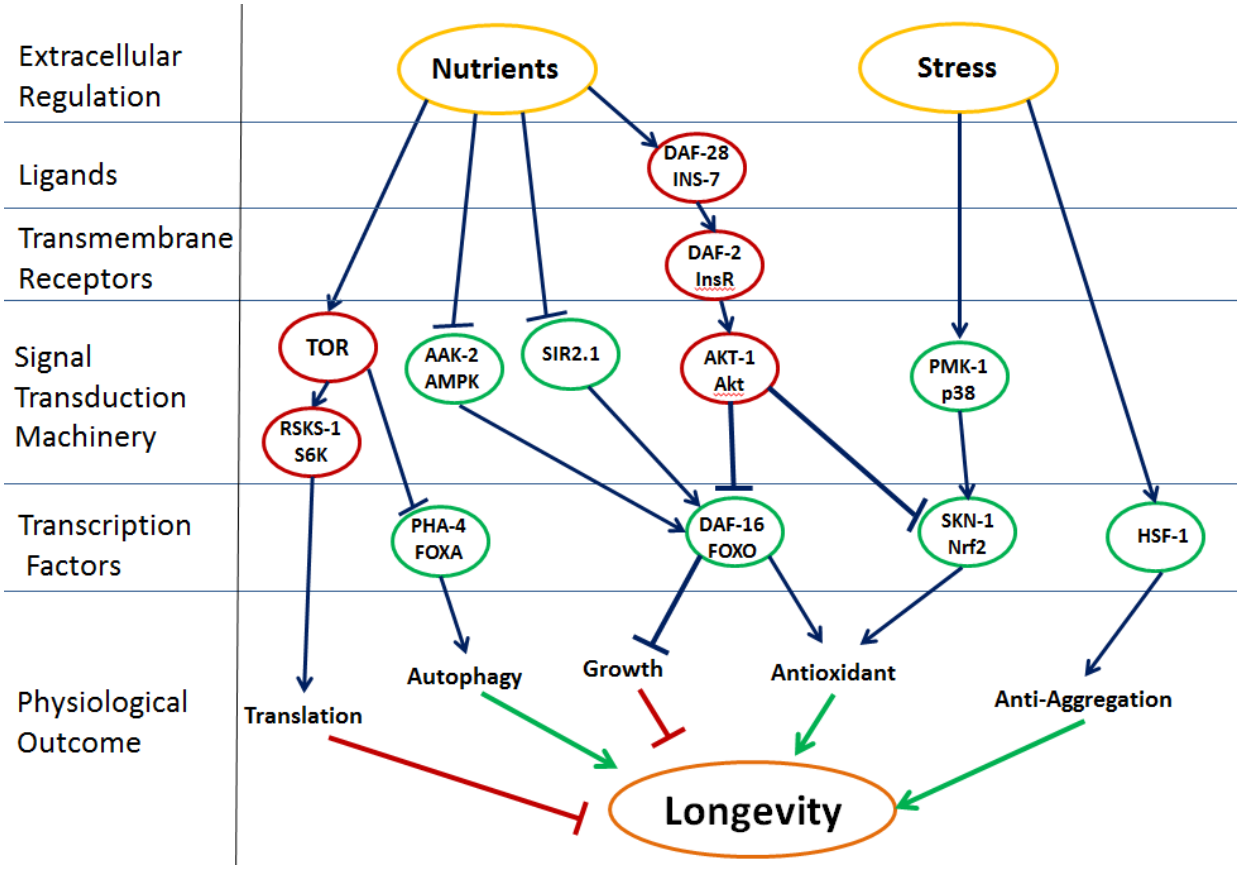

Figure 6.6. The availability of nutrients and external stress roles in longevity.

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CHAPTER 7

APPENDICES

7.1 Aging journal

Dear Editors,

I am writing my dissertation to fulfill my requirements for a PhD. I would like to include the article published on 8/7/2014. Vol 6, No 8, pp 621-644.
I am the first author of the article titled "D-beta-hydroxybutyrate extends lifespan in C. elegans."
Many thanks for your time and help.

Best regards,
Clare Edwards

Olga Krasnova Jan 31 (4 days ago) to me

Dear Clare,There are no objections for your request to include the AGING article in your PhD dissertation.

Best regards,
Olga Krasnova
Production editor

7.2 PLOS ONE

Dear Editors,

I am writing my dissertation to fulfill my requirements for a PhD. I would like to include the article published in the March 2013 edition of PLOS ONE. DOI: 10.1371/journal.pone.0058345.
I am the first author of the article titled "Malate and Fumarate Extend Lifespan in Caenorhabditis elegans.

Many thanks for your time and help.

Best regards,
Clare Edwards
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7.4 IUCAC and IRB Approval

The invertebrate nematode, *C. elegans*, was used in this dissertation. Invertebrate animal models and are not protected by the Animal Welfare Act (AWA). Therefore, IUCAC and IRB approval are unnecessary.