Oxidative stress in *C. elegans*: Discovery of a mechanistic role for γ-ketoaldehyde lipid peroxidation products in the Free Radical Theory of Aging

By

Thuy Tuong Nguyen

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Approved:

L. Jackson Roberts, II, M.D

Michael Aschner, Ph.D.

John A. Oates, M.D.

Sean S. Davies. Ph.D.

Michael L. Freeman, Ph.D.

To my faithful friend, Levi, for his constant companionship,

And

To my husband, Kristian – without you, none of this would have been possible.

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CHAPTER I

INTRODUCTION TO ISOKETAL-MEDIATED OXIDATIVE DAMAGE AND ITS EFFECTS ON AGING

Introduction

Free radical attack of tissue biomolecules, such as lipids, are major indicators of oxidative stress. (Gardner 1989, Niki 2009). Upon oxidant injury, free radicals readily attack lipid biomolecules which forms of a wide variety of peroxidation products. Accumulation of lipid peroxidation products has been thought to contribute to the pathogenesis in a number of human diseases, including, but not limited to atherosclerosis, cancer, and neurodegenerative diseases (Halliwell and Gutteridge 1990, Gutteridge 1995, Butterfield 1997). The formation of secondary products of lipid peroxidation, such as the reactive aldehydes, play a pivotal role in the propagation of oxidative damage (Poon, Calabrese et al. 2004). Lipid peroxidation byproducts, including malondialdehyde (MDA), a potential crosslinking agent, and the reactive hydroxyalkenals, are known to serve a dual role in propagating and partially mediating the deleterious effects of lipid peroxidation (Comporti 1998, Poon, Calabrese et al. 2004).

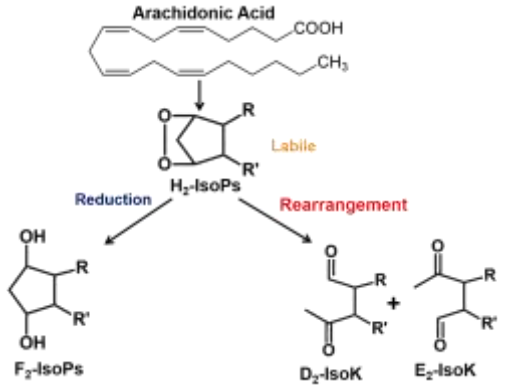

Figure 1.1. Isoketal synthesis from the non-enzymatic oxygenation of arachidonic acid

A family of 64 highly reactive levuglandin-like γ-ketoaldehyde (γ-KA, or isoketals, IsoK) regio- and stereo-isomers are formed as products of the isoprostane pathway via rearrangement of prostaglandin H_2-like endoperoxide intermediates (H_2-isoprostanes) (Morrow, Hill et al. 1990, Brame,

Salomon et al. 1999) (Fig. 1.1). H_2-IsoPs undergo non-enzymatic rearrangement to form F_2-IsoPs, D_2-IsoPs, E_2-IsoPs, and highly reactive IsoKs (Davies, Amarnath et al. 2004).

Although F_2-IsoPs and IsoKs are formed through the same pathway, both species play vastly differing functions in the body. According to the Biomarkers of Oxidative Stress Study (BOSS), quantification of F_2-IsoPs is one of the most reliable approaches to assess oxidative stress *in vivo*, which has allowed for establishment of the important role of oxidant injury in numerous human diseases (Morrow, Hill et al. 1990, Kadiiska, Basu et al. 2013). In contrast, IsoKs irreversibly and rapidly react with the ε-amino lysyl residues of proteins, forming stable adducts and intramolecular cross-links. Therefore, IsoK generation and subsequent adduction can produce deleterious effects, consequently resulting in negative attenuation of biological function via protein modification. As stated previously, F_2-IsoPs and IsoKs are generated in the same pathway, formed in tissues from arachidonic acid esterified to phospholipids, upon an acute oxidative injury (Morrow, Harris et al. 1990, Brame, Boutaud et al. 2004). Both F_2-IsoP and IsoK levels rise swiftly, in a matter of hours after oxidative injury, yet clearance occurs on different orders of magnitude for the two species. F_2-IsoPs have a half-life of less than 20 minutes, and it is due to this rapid clearance that F_2-IsoPs can readily be measured in plasma or urine (Moore 2004). However, in direct contrast to the rapid clearance of F_2-IsoPs, IsoKs are not immediately cleared from the body due to its proclivity to forming protein adducts.

There is considerable interest in the biological relevance of IsoKs, partially because of their potential in modifying proteins to cause cellular dysfunction, along with initial findings suggesting a likelihood of IsoK involvement in the mechanisms of disease. This interest is in part due to basic features of their biochemistry: IsoKs rapidly adduct to solvent-accessible lysine residues of proteins (Brame, Salomon et al. 1999), have a propensity to cross-link proteins (Iyer, Ghosh et al.

1989, Boutaud, Li et al. 2001, Boutaud, Ou et al. 2002, Davies, Amarnath et al. 2002), and lastly, known to adduct to aminophospholipids (Sullivan, Matafonova et al. 2010).

Discovery of γ-Ketoaldehydes

In 1990, Morrow, Roberts, and colleagues reported the formation of prostaglandin (PG)-like compounds *in vivo*, termed isoprostanes, by free radical-induced peroxidation of biologically relevant poly unsaturated fatty acids (PUFAs) such as arachidonic acid (AA, C20:4, ω-6) and eicosapentaenoic acid (EPA, C20:5, n-3) (Morrow, Harris et al. 1990). Highly labile, PGH_2-like intermediates in the IsoP pathway are unstable and undergo non-enzymatic rearrangement *in vivo* to form E_2-IsoPs, D_2-IsoPs, and isothromboxanes along with F_2-IsoPs (Morrow, Minton et al. 1994, Morrow, Awad et al. 1996).

Studies indicate IsoKs could be the most prevalent H_2-IsoP product rearrangement product *in vivo*, which was discovered through research conducted to assess the percent yield of levulinaldehyde product formation upon lipid peroxidation. If IsoKs were the major agents of oxidant-mediated cellular dysfunction, it would be reasonable to predict a high abundance of γ-ketoaldehydes formation in the event of arachidonic acid peroxidation. To investigate the yield of IsoK formation from the rearrangement of H_2-IsoPs, the precursor PGH_2 was oxidized in a neutral phosphate buffer solution. PGH_2 was used instead of arachidonic acid because of the relative convenience in obtaining PGH_2 enzymatically rather than the labor-intensive efforts in isolating the H_2-IsoPs from the mixture formed during oxidation of arachidonic acid. Thus, incubation of PGH_2 in phosphate buffered solution results in 22% yield of γ-ketoaldehyde (Salomon, Miller et al. 1984). Although this unexpected low yield indicate little probability of IsoK-mediated oxidant damage, Salomon and colleagues postulated this result was due to utilizing a solvent dissimilar to its endogenous environment. The rationale was due to the fact H_2-IsoPs form in a lipophilic

environment, considering the arachidonic acid (or other PUFA moiety) is still esterified to the phospholipids (Morrow, Awad et al. 1992). Therefore, repeating the experiment in a more hydrophobic buffer would result in a different percent yield of IsoK which more closely mimics the endogenous environment during its rearrangement. When the reaction was repeated in the more hydrophobic organic solvent, dimethyl sulfoxide (DMSO), 70% of yield was γ-ketoaldehyde (Salomon, Miller et al. 1984, Salomon 1985), which is remarkable because this suggests that IsoKs could be the major product of H_2-IsoP rearrangement *in vivo*.

The question of whether γ-ketoaldehydes are biologically relevant lipid peroxidation products still remains. Considering that the IsoP endoperoxides undergo rearrangement to form various eicosanoids, Brame and colleagues sought to evaluate the relevance of γ-ketoaldehydes by first quantifying the formation of these products upon oxidant insult. Although their biochemical reactivity to proteins suggest a compelling contribution to disease development, Brame et al. probed whether level of IsoK generation after acute oxidative injury was sufficient in mediating deleterious effects from protein adduction. In order to determine the relative abundance of IsoK formation relative to other H_2-IsoP rearrangement products, arachidonic acid was oxidized using iron/ascorbate/ADP in a neutral phosphate buffer. The percent composition of the H_2-IsoP rearrangement products was then analyzed by gas chromatography-mass spectrometry (GC/MS) after derivatization (Fig. 1.2). For every 1 mg of arachidonic acid oxidized, 1.8 μg of E_2/D_2-IsoP is formed, along with 1.2 μg IsoK and 0.8 μg F_2-IsoPs (Brame, Salomon et al. 1999). From this, Brame and colleagues established that IsoK formation occurs at comparable rates to F_2-IsoP formation. Considering the relative amount of IsoK and F_2-IsoP formation, one can predict conditions that increase F_2-IsoP levels will likely raise IsoK levels in tandem.

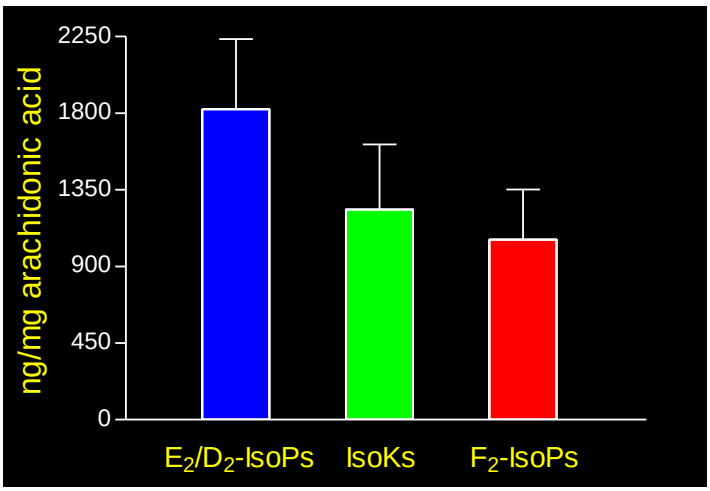

Figure 1.2. IsoK formed in tandem with other H_2-IsoP rearrangement products upon oxidation of arachidonic acid

γ-Ketoaldehydes Cross-link Proteins

IsoKs react covalently to lysyl residues of proteins to form a stable adduct and intramolecular cross-links (Iyer, Ghosh et al. 1989, Boutaud, Brame et al. 1999, Brame, Salomon et al. 1999). These molecules react at a rate that far exceeds other well studied products of lipid peroxidation, including 4-hydroxynonenal (4-HNE) (Brame, Salomon et al. 1999). One of the earliest conundrums facing investigators was the lack of detectable IsoK in tissues such as plasma and urine, despite the wealth of F_2-IsoPs as determined by GC/MS. In addition, although large quantities of F_2-IsoPs were found in iron/ADP/ascorbate treated liver microsomes, investigators failed to detect IsoKs in the samples. Keeping in mind the relative ratio of IsoKs and F_2-IsoPs formed upon arachidonic acid peroxidation, detection of F_2-IsoPs in biological samples should in theory also indicate the occurrence of IsoK formation. In order to understand this phenomenon, Brame and colleagues carried out studies to determine if rate of reactivity of IsoKs with proteins could potentially explain the lack of IsoK detection in biological samples.

Brame and colleagues incubated synthetic IsoK with a protein source, bovine serum albumin, and observed a swift depletion in free IsoK, where within 20 s less than 50% of the free

compound remained (Brame, Salomon et al. 1999). When another fast reacting lipid alkenal, 4-hydroxynonenal (4-HNE), was incubated with albumin, the investigators observed a period of 80 min for the level of free 4-HNE to reduce to 50%, which corroborates with previously published reaction rates. Bearing in mind the fast rate of IsoK depletion, these results suggest that IsoK is largely found as an adduct on any macromolecule containing a solvent-accessible amine group. The reaction of isoketals with lysine forms pyrrole adducts which then rearrange into lactam, hydroxylactam, or cross-linked adducts under oxidizing conditions. In addition, when oxidation of protein pyrrole adducts occur in the proximity of other nucleophiles, such as cysteine, cross-linkage occurs between the amino acid residues. (Amarnath, Valentine et al. 1994).

Seeing that highly reactive γ-ketoaldehydes are known to react with amine groups to form protein crosslinks, Davies and colleagues set out studies assaying the conditions in which intramolecular crosslinkage occurs. Considerate attention was given in observing which conditions gave rise to IsoK-protein crosslinkage, such as IsoK to protein ratio, protein concentration, and the concentration of other nucleophiles in close proximity. When IsoK is added to ovalbumin (OVA) in phosphate buffer in a 1:1 ratio, a slight fraction of monomer is converted to high molecular weight oligomers via intramolecular cross-links (Davies, Amarnath et al. 2002). Isoketals crosslink proteins more potently and rapidly than other known reactive lipid aldehydes (Davies, Amarnath et al. 2002), to where HNE and MDA concentrations required at least 100-fold higher levels to achieve the same extent of crosslinking (Iyer, Ghosh et al. 1989).

Formation of isoketal crosslinks can alter the conformation and function of proteins, therefore the extent crosslinking contributes to isoketal action needs to be further explored. Due to the rapidness of IsoK reactivity towards lysine, free IsoKs are not detectable *in vivo* where protein is present (Iyer, Ghosh et al. 1989, Brame, Salomon et al. 1999). Although free IsoKs cannot be

detected in biological samples, the formation of isoketals *in vivo* can be quantified by measuring highly stable isoketal-lysyl-lactam adducts using mass spectrometry (Davies, Amarnath et al. 2007). Due to their long half-life, isoketal-lysyl-lactam adduct levels in tissues serve as a measure of oxidative stress over time (Poliakov, Brennan et al. 2003).

Biological relevance of γ-Ketoaldehydes

Significantly increased levels of IsoK-lysyl-lactam adducts can be found in several pathological conditions including cardiovascular disease (atherosclerosis, end-stage renal disease, *etc*.), neurodegenerative diseases such as Alzheimer's disease, and is an important factor in the etiology of hypertension (Salomon, Batyreva et al. 2000, Zagol-Ikapitte, Masterson et al. 2005). Elevated levels of isoketal adducted proteins are predicted in a wide variety of conditions associated with oxidative injury and inflammation (Reilly, Delanty et al. 1996, Montine, Markesbery et al. 1998, Dworski, Murray et al. 1999, Laight, Desai et al. 1999, Voutilainen, Morrow et al. 1999, Davies, Amarnath et al. 2002). In fact, experimental models of oxidative injury and inflammation show increased isoketal adduct formation, which can be seen in carbon tetrachloride treated rats (Brame, Boutaud et al. 2004), hyperoxia-treated mice (Davies, Talati et al. 2004), septic mice (Poliakov, Brennan et al. 2003), and *ex vivo* activation of platelets (Boutaud, Li et al. 2003). Despite IsoKs having a demonstrated cytotoxic effect, a causal role in induction of protein aggregation, and a detrimental impact on enzymatic function, (Schmidley, Dadson et al. 1992, Boutaud, Ou et al. 2002, Davies, Amarnath et al. 2002), concerted efforts in discovering and developing pharmacological probes are essential for *in vivo* studies to reveal the contribution of IsoK adduction to proteins in disease. Additionally, such probes could prove therapeutically useful as preventative measures against IsoK-mediated pathology.

Pyridoxamine, the First Small-Molecule Scavenger of Isoketals

A lead compound, pyridoxamine (PM) was first discovered as a carbonyl scavenger in 1996 by Billy G. Hudson and colleagues (Booth, Khalifah et al. 1996). Pyridoxamine is a vitamer in the vitamin B_6 family. Pyridoxamine was subsequently identified as an isoketal scavenger through initial screens looking at primary amines that protected radiolabeled lysine from modification by synthetic isoketal. Subsequently, Amarnath and colleagues measured second-order reaction rates for a series of primary amines relative to *N*-α-acetyl-lysine (Amarnath, Amarnath et al. 2004). Reaction of γ-ketoaldehyde with pyridoxamine was 2,000 times greater than that of *N*-α-acetyl-lysine (Amarnath, Amarnath et al. 2004). Delivery of high concentrations of pyridoxamine is tolerated well *in vivo* (Degenhardt, Alderson et al. 2002), and can neutralize multiple α-ketoaldehydes generated during lipid and sugar metabolism (Onorato, Jenkins et al. 2000, Voziyan, Metz et al. 2002). Despite the plausibility of PM as surrogate probe of isoketal function *in vivo*, lipid peroxidation forms IsoKs esterified in situ to phospholipids (Brame, Boutaud et al. 2004), therefore lipophilic scavengers are expected to be more efficacious than the hydrophilic pyridoxamine. Utilizing structure-activity relationships (SAR), the critical moiety needed to still allow for scavenging of IsoKs was determined to be a phenolic amine composed of the hydroxyl group adjacent to the methyl amine. Consequently, alternative lipophilic phenolic amines such as salicylamine (SA) and pentylpyridoxamine (PPM) show comparable potencies and selectivity as PM for scavenging isoketals (Davies, Brantley et al. 2006).

Selective Scavengers of Isoketals

All three scavengers show selectivity towards γ-ketoaldehydes over other dicarbonyls and do not significantly scavenge α,β-unsaturated carbonyls such as HNE (Amarnath, Amarnath et al. 2004, Davies, Brantley et al. 2006). From these results, Amarnath et al. concluded that PM and

related phenolic amines preferentially scavenge IsoKs compared to other lipid aldehydes. Selectivity of these phenolic amines for IsoKs is thought to be attributed to the ability of the hydroxyl group adjacent to the methylamine to catalyze pyrrole formation (Amarnath, Amarnath et al. 2004). Reduction of IsoK levels by PM, SA, and PPM is due to direct IsoK scavenging, and not through inhibition of lipid peroxidation; *in vitro* studies of arachidonic acid oxidation in the presence of the three scavengers did not show significantly reduced levels of F_2-IsoPs, indicating normal occurrence of lipid peroxidation (Davies, Brantley et al. 2006). All three scavengers significantly decreased IsoK-Lys-lactam adduction in aqueous solutions, however in platelets, PM reduces levels of IsoK protein adducts to a lesser extent than that of SA and PPM (Davies, Brantley et al. 2006). In intact cells challenged with hydrogen peroxide, pre-incubation of PM did not protect cell viability, in contrast, SA and PPM shielded cells against death (Davies, Brantley et al. 2006). Likewise, SA and PPM exhibited protective effects against oxidant induced inhibition of sodium currents, whereas PM lacks protective effects (Nakajima, Davies et al. 2010). SA is orally bioavailable (Zagol-Ikapitte, Matafonova et al. 2010), and delivery of SA in the drinking water of an Alzheimer's mouse model prevented the age-associated decline in working memory (Davies, Bodine et al. 2011). Accordingly, IsoK scavengers could be valuable tools for probing the contribution of IsoKs in the etiology of human diseases. Therefore, the primary goal of this project is to reveal the molecular processes involved in IsoK-mediated oxidative injury, thereby opening new avenues of therapeutic interventions to limit oxidative damage.

Putative Role of IsoKs in Aging

The aging field has actively embraced the role of oxidative stress in the development of aging and age-related disease. Although great advances and breakthroughs have informed the field, the exact molecular events involved in aging are unclear (Voss and Siems 2006). In order to study

the effect of oxidative stress on aging at the cellular level, several biomarkers of oxidative damage have been identified, including but not limited to lipid peroxidation and protein oxidation products, antioxidative acting enzymes, and glutathione among many other substances. Although there is a scarcity of data relating to the bioactivity of IsoK adducts in aging, recent studies suggest a potential role for IsoK adduction in mediating some of the aging effects of lipid peroxidation. For instance, in studies with young and aged rats, quantification of free circulating F_2-IsoPs in aged rats (22 – 24 month) showed a mean of 20.3-fold (range 4.3 – 42.9-fold) increase compared to levels measured in young animals (Roberts and Morrow 2000). The fact that levels of F_2-IsoPs in plasma were increased with age adds legitimacy to the prevailing thought that aged animals are under a remarkable amount of general oxidant stress. This data also suggests a similar increase in IsoK production with age, considering previous studies indicate F_2-IsoP formation occurs in relative amounts to IsoK formation (Figure 1.2).Hence, there is an open question over the potential part that IsoKs may intervene in the aging process and if so, how scavengers of IsoKs may be potentially influence normal aging.

The State of Health and Aging in the World

People worldwide are living longer. For the first time in history, the proportion of people aged over 60 years is expanding faster than any other age group, as result of longer life expectancies and declining fertility rates (Public health report: The Department of Economic and Social Affairs of the United Nations Secretariat; http://www.un.org/en/development/desa/policy/wess/wess_archive/2007wess.pdf). In low- and middle-income countries, this demographic shift is largely due to massive reductions in mortality at younger ages from infectious diseases. In high-income countries, increases in life expectancy are due to positive outcomes of medical progress. Due to these technological advances, the World

Health Organization (WHO) predicts that between 2015 and 2050, the percentage of the world's population over 60 years will nearly double from 12% to 22%, and by 2020, the number of individuals aged 60 years and older will outnumber children younger than 5 years. (World Report on Health and Ageing: World Health Organization; http://apps.who.int/iris/bitstream/10665/186463/1/9789240694811_eng.pdf?ua=1).

The current pace of an aging population is exponentially faster than in the past, and with this all countries face major challenges to ensure their public health, social services, and health care systems are ready to service this growing demographic. Encouragingly, public health initiatives in America are being launched to promote healthy aging, improve quality of life in older adults, and identify pharmacological therapeutics to promote healthy aging (termed health span) with the goal of postponing the onset of geriatric syndromes and chronic health conditions (Centers for Disease Control and Prevention (CDC); https://www.cdc.gov/aging/pdf/state-aging-health-in-america-2013.pdf).

The Concept of Aging

According to a recent review in the previous year by Riera and Dilin, "aging is considered to be the biggest risk factor for developing several of the world's most prevalent chronic diseases" (Riera and Dillin 2015). These aging and age-related diseases include metabolic syndromes, cardiovascular and neurodegenerative diseases, and most prevalent forms of cancer (Kennedy, Berger et al. 2014). Typically, there is a high incidence of chronic diseases and co-morbidities in the elderly. In the United States, more than a quarter of its citizens and two out of every three older individuals have multiple chronic conditions, and disease management for this population accounts for over 60% of the country's health care budget. Generally, aging is viewed as an overall decline

in cell and tissue function, which increases susceptibility to disease, and ultimately leading to tissue failure and death.

Denham Harman characterizes aging as the aggregation of changes to the body over time (Harman 1956). American gerontologist, Bernard Strehler defines aging as universal, progressive, and deleterious (Baker 1965). According to Strehler, aging is considered to be universal, because it is a "phenomenon that occurs in different degrees in all individuals of a species". Aging must be progressive, through which it leads to changes that additively occur throughout the lifespan. Aging is lastly defined to be deleterious, due to the changes associated with aging tend to be harmful to the individual (Rose, Flatt et al. 2012).

Therefore, it is crucial to define the underlying molecular events through which aging occurs, understand how these changes lead to the diseases of old age, and how researchers can utilize this knowledge to devise methods for diagnosis, treatment, and prevention of aging and age-related diseases. As aging occurs for inexplicable reasons and unfolds in complex ways, theory plays a remarkably fundamental role in its research. Considering human aging remains a largely unexplored area of research, strides should be taken to identify small molecules that extend lifespan in tandem with healthy aging (healthspan), as well as understanding the mode of action of these drugs.

General Theories of Aging

Aging is defined by deteriorating changes that progressively diminish function, resulting in a gradual decline in the capacity to respond to environmental challenges and an increasing vulnerability to disease and death. It is also associated with a progressive loss of tissue and function over time (Flatt 2012). Nearly all physiological functions lose efficiency with aging, in which aged individuals lose the capacity to maintain homeostasis when faced by changes in external

environment. However, the exact molecular events occurring in this process and are responsible for aging are yet to be defined. There are many theories of aging; Medvedev counted over 300 theories of aging in 1990 (Medvedev 1990). New classes arise constantly as research in molecular and cellular biology uncover previously unimagined phenomena.

Evolutionary Programmed and Non-Programmed Aging/Longevity

Programmed aging implies that aging follows a biological timetable, in which a set of genes control a signaling cascade to initiate cellular senescence, leading to death (Giaimo and d'Adda di Fagagna 2012). In this theory, the regulation of aging occurs through changes in gene expression to alter molecular pathways involved in cellular maintenance and defense systems. The theory of programmed aging proposes that organismal fitness declines in favor of natural selection for genetic programs with beneficial effects on reproductive health early in life.

There are two main classes of evolutionary programmed aging theories: 1.) Modern programmed theories, state that species have an evolutionary need to live for a particular time, and once the time has passed organisms need to limit their lifespans and therefore developed aging programs as mechanisms to limit organism lifespan in order to obtain a species-specific optimum lifespan (Libertini 1988, Skulachev 1997, Goldsmith 2010), and 2.) Modern non-programmed theories, such as antagonistic pleiotropy theory (Williams 2001) and disposable soma theory (Kirkwood and Holliday 1979), which contend that a species has an evolutionary need to live for a particular minimum lifespan and therefore only evolved the capability for overcoming natural progression toward decline to the extent necessary to achieve said lifespan.

Modern programmed aging theory suggests an innate program in the genome, which is initiated at a given life stage in the organism's life cycle. The activation of this biological timetable leads to a death cascade in various bodily systems (Libertini 2015). Within the programmed theory

of aging lies three major avenues of investigation which encompass the activation of this biological timetable; 1.) Programmed longevity, 2.) Endocrine theory, and 3.) Immunological theory.

Programmed Aging. Programmed longevity posits an evolutionary bias for genes controlling the activation and rate of senescence, which leads to disease states, and eventually death. A central tenant of this theory involves the concept of a programmed gene control of development that exists to control a genetic cascade responsible for senescence. Most hypotheses to explain the occurrence of programmed aging pertain to the notion that aging benefits species by preventing overcrowding and/or facilitating further evolution by turnover of generations. These ideas can be traced back in earlier days, with Weismann (Weismann, Poulton et al. 1891) stating "Worn out individuals are not only valueless to the species, but they are even harmful, for they take place of those which are sound." With the thought of a programmed gene control cascade driving the occurrence of aging, the general consensus is to discover and develop an agent that can interfere with the activity of any gene belonging to the death cascade, particularly via mutation in order to modulate the lifespan of the organism. The goal of such a therapy is to extend longevity with a minimal effect on development and reproduction of the organism.

Endocrine Theory of Aging. The endocrine theory of aging is at the moment, one of the most studied genetic regulatory networks that impacts aging through a biological clock which acts through hormones. The evolutionarily conserved insulin/insulin growth factor 1(IGF-1)-like endocrine system is a major component in the hormonal regulation of aging. Genetic mouse models of decreased insulin/IGF-1 signaling (IIS) extends lifespan and healthspan in mice (Bluher, Kahn et al. 2003, Holzenberger, Dupont et al. 2003, Taguchi, Wartschow et al. 2007, Selman, Lingard et al. 2008, Ortega-Molina, Efeyan et al. 2012, Foukas, Bilanges et al. 2013, Nojima, Yamashita et al. 2013, Xu, Gontier et al. 2014). In addition, inhibition of the IIS in *Caenorhabditis*

elegans results in a constitutive dauer formation during development. Dauer formation in this critical developmental period results in increased lifespan in adult animals (Kenyon, Chang et al. 1993). In addition, IIS mutants show abnormal egg-laying, increased lipid storage, and robustness during cellular stress (Kimura, Tissenbaum et al. 1997, Paradis and Ruvkun 1998, Kenyon 2005). With all the research on the role and mechanisms of insulin/insulin-like growth factor signaling on aging, the prevalent therapeutic strategy is to use natural activators and inhibitors of the insulin/insulin-like growth factor signaling pathway to design drug therapies. Currently, three proteins have demonstrated lifespan extension possibly via IGF-1 (Kurosu, Yamamoto et al. 2005, Zhang, Xie et al. 2012).

Immunological Theory of Aging. The immunological theory of aging, or immunosenescence, advances the idea that there are genetic programs in place to degrade the immune system over time. The gradual decline of the immune system leads to loss of antibody efficacy, and with the body's defense mechanisms against new diseases compromised, the body becomes more susceptible to cellular stress and eventual death (Cornelius 1972). The immunological theory of aging was proposed by leading pathologist, Roy Walford in 1964 (Walford 1964), based on the observation that optimal functioning of the immune system occurs at reproductive maturation and gradually lessens with age. This age-associated immune deficiency is ubiquitous and found in both long- and short-lived species (Ginaldi, Loreto et al. 2001), and found to be under genetic control rather than as a deteriorative occurrence (Franceschi, Valensin et al. 1999). Wolford states there is a growing amount of evidence that many age-related diseases result from malfunctioning immune system in conjunction with heightened inflammation (Caruso, Candore et al. 2005). Although direct relationships depicting causation between dysregulated

immune response and age-related diseases have not been fully elucidated, a role for indirect involvement of the immune system exists.

Non-Programmed Evolutionary Aging. In contrast, modern non-programmed evolutionary aging considers genes have only evolved the capability of overcoming progressive deterioration processes for an organism to live only for a particular minimum lifespan. One central premise to this theory is the existence of genes with dual functions which may be both beneficial and detrimental to the organism depending on the life cycle stage. One example of this evolutionary theory of aging is the disposable soma hypothesis, where one of the basic tenets contends that there are physiological and/or biochemical restrictions that limit and hamper the preservation of an optimal maintenance system at advanced ages. Due to limited resources, the body must choose between reproductive capacity and a more efficacious maintenance/homeostatic system. Thus, this limiting factor makes the maintenance of an optimum efficiency vulnerable at advanced ages.

The antagonistic pleiotropy theory was proposed by Williams in 1957, whereby Williams suggest a specific type of genetic inter-trait linkage, pleiotropy, as an evolutionary explanation for senescence (Williams 2001). Pleiotropy, or inter-trait linkage exists in a way to make it difficult for the evolution process to remove an individually adverse trait, such as aging, without simultaneously removing one or more beneficial traits. To explain further, inter-trait linkage is the phenomenon where one gene produces more than one phenotypic trait in an organism which may produce dichotomizing results. The gene could potentially produce a trait beneficial to the organism's fitness and also result in one or more trait adverse to fitness. In summation, the antagonistic pleiotropy hypothesis proposes aging is caused by genes that are simultaneously advantageous in the young or adult stage and unfavorable in the older ages, and are consequently, only partially countered by natural selection.

In evolutionary biology, the fitness trade-offs from antagonistic pleiotropy is one of the reasons to explain why organisms are never able to reach perfection through natural selection. Within this theory, mutational changes to a gene that can result in a beneficial effect (e.g. increased lifespan) would likely also cause adverse changes to other phenotypic traits in a linkage that would prevent the evolution process from accomplishing the beneficial effect (greater longevity) without incurring the adverse penalties. According to Williams' evolutionary antagonistic pleiotropy aging theory, there is an evolutionary need to live and produce for a certain species-specific minimum time and after that age, there is no net evolutionary advantage or disadvantage from reproducing longer. Proponents of this theory believe the inter-trait linkage prevents the discovery of finding an anti-aging agent due to the large number of independent factors that contribute to aging.

Error Theories of Aging

The error theories of aging highlights environmental damage to living organisms over time, which causes an accumulation of errors in cellular structures to reach a catastrophic level that is incompatible with cellular survival. Environmental factors, such as extreme temperatures, malnutrition, oxidations, and hypoxia affects maintenance required for stress response, signal transduction, and metabolism. The damage accrued gradually causes attrition in the efficacy of organismal function, leading to death.

DNA damage theory. This theory posits cellular deterioration and dysregulation occurs from an accumulation of DNA damage associated with age. Healthy individuals are able to respond and repair DNA damage that occurs constantly in living organisms, however some DNA defects accumulate as damage outpaces the rate of repair. Of note, errors in mitochondrial DNA may result in mitochondrial dysfunction. Maintenance of metabolic homeostasis along with appropriate stress responses absolutely requires proper mitochondrial function. Mitochondrial dysfunction is

known to play a critical role in aging and as well as other numerous human diseases, for example cancer and neurodegenerative disorders (Wallace 2005). The causes of the onset and progression of these pathologies are a result of metabolic disruption and oxidative injury.

Metabolic 'Rate of Living' Theory. In the early 1900s, work of Max Rubner suggest a role for oxygen in determining lifespan. Rubner noted that large animals tend to outlive smaller animals, and speculated this phenomena was due to an inverse relationship in metabolic rate to organismal size (Rubner 1908); shorter lifespans were seen in organisms with a greater rate of oxygen basal metabolism (Brys, Vanfleteren et al. 2007). The inverse relationship between basal metabolism and longevity provided the groundwork for Raymond Pearl's rate-of-living theory of aging (Pearl 1928). As originally proposed by Pearl, this theory stated that the lifespan of an organism is reliant on upon the depletion of a fixed amount of a "vital substance," or energy expenditure occurs at a rate proportional to the metabolic rate of the organism. There are two distinct factors thought to govern length of life, according to the rate of living theory of aging: 1.) a genetically determined metabolic potential, and 2.) rate of metabolism (Sohal 1986).

This theory predicts that long-lived species or individuals should have a reduced rate of basal oxygen metabolism as compared to shorter-lived organisms. Or in other words, as summarized in a common popular phrase, the rate of living theory of aging is likened to "live fast and die young" of which is still prevalent in common perception, although this theory cannot explain many exceptions to its rule (e.g. extreme longevity of some eusocial animals such as naked mole rats and ant queens). Furthermore, the rate-of-living theory of aging does not comprehensively explain the maximum lifespan of organisms (Hulbert, Pamplona et al. 2007). Modern studies propose an adapted version of Pearl's rate of living theory underscoring the role of growth (TOR) and stress resistance (FOXO) pathways (Rollo 2010).

Free Radicals Theory of Aging. Based from observations made in the early 1950s, prominent biogerontologist, Denham Harman proposed that the most prevalent molecular explanation for aging – whereby aging is due to a decline in cellular and tissue function – was a result from oxygen radicals produced by respiratory enzymes causing additive damaging effects to macromolecules (Harman 1956). Breaking down this theory in a linear fashion, it proposes that free radicals released during respiration cause damage to macromolecules comprising the cell, which results in an accumulation of damage to ultimately cause the cells, and eventually organs, to cease functional activity. This chain of events leads to the aging phenotype (Sohal and Weindruch 1996, Clancy and Birdsall 2013). Although free radicals are generated constantly throughout the life of the cell, oxidative damage to macromolecules occurs when antioxidant systems are overwhelmed with an influx of free radicals generated at a rate faster than antioxidant systems can detoxify.

In a broad assortment of tissues and animal models of aging (yeast, nematodes, fruit flies, mice, *et cetera*), increases in oxidative damage to macromolecules (nucleic acids, lipids, and proteins) have been reported (Agarwal and Sohal 1994, Bokov, Chaudhuri et al. 2004, Stadtman 2006). Furthermore, in animal models with increased lifespan, either a reduction in oxidative damage and/or protection against oxidant injury was observed; in *Caenorhabditis elegans*, strains containing mutations in the insulin/IGF-1 signaling pathways, such as *age-1, daf-2, and daf-16* mutants, resulted in reduction in oxidant injury and increased resistance to oxidative damage (Lithgow, White et al. 1995, Johnson, Lithgow et al. 1996, Honda and Honda 1999, Ishii, Goto et al. 2002). The inverse is also observed, where increased production of ROS shortens lifespan. Lastly, early studies on caloric restriction showed reductions in oxidative injury to lipids, nucleic acids, and proteins in caloric restricted rodents compared to free-feeding rodents (Sohal and

Forster 2014), along with greater resistance to oxidative stress (Sohal and Weindruch 1996, Yu 1996, Barja 2002). Among the many theories of aging, the free radical theory of aging carries the most extensive body of literature (Blagosklonny 2008, Doonan, McElwee et al. 2008, Mockett, Sohal et al. 2010, Cabreiro, Ackerman et al. 2011)

Mitochondrial Theory of Aging. After years of research investigating the Free Radical Theory of Aging, Harman revised this theory to incorporate the role of mitochondria in aging. This "mitochondrial theory of aging", (Lee and Wei 2012, Bereiter-Hahn 2014) encompasses the effects of cumulative oxidative damage to mitochondria induced by ROS toxicity in the organism's lifetime. Regarding the role of mitochondria in aging, the electron transport chain (ETC) leaks electrons during normal metabolic respiration which then generates ROS. Oxidative damage occurs in times when environmental stressors increase the rate of ROS production to overwhelm antioxidative response elements and/or compromised antioxidative response elements lead to imbalances in ROS equilibrium.

Mitochondrial damage in aged cells is well characterized. Notable damage observed includes a steady drop in respiratory chain capacity, elevated oxidative damage, lower levels of mitochondrial content, and irregular mitochondrial morphology (Kirkwood 2005, Gaziev, Abdullaev et al. 2014, LaRocca, Hearon et al. 2014). Mitochondrial dysfunction can lead to severe damage in an organism. A vast majority of research on why mitochondrial dysfunction gradually develop with time has focused on mtDNA integrity. Mutations and deletions in mtDNA increase with age, and areas of aged tissue that show mitochondrial ETC dysfunction contain abundant mtDNA damage (Xie, Lu et al. 2014). These findings and many earlier studies indicate that continuous accumulation of mtDNA damage may be responsible in aging.

Proteostasis and Aging. A living organism exhibiting protein homeostasis (proteostasis) possesses a proteome in functional balance, where cells, tissues, and organs are properly functioning due to the taut regulation of protein maintenance. Stress-response transcription factors, molecular chaperones, and protein degradation responses constitute the proteostasis network (PN), whose main function is to sense and react to "proteotoxic stress" and occurrences of protein misfolding to ensure cell viability (Balch, Morimoto et al. 2008). Maintenance of proteome balance is valuable for preservation of normal protein quality control and cellular function. It requires a multiplex web of cellular components, primarily mechanisms of the production and maintenance of proteins synthesis (Balch, Morimoto et al. 2008, Hartl, Bracher et al. 2011).The proteostasis network consists of ~1,400 proteins in human cells (Kim, Hipp et al. 2013), which includes molecular chaperones (Brehme, Voisine et al. 2014) in addition to proteins comprising the oxidative stress defense, along with of the proteolytic degradation network. These quality control mechanisms is central to the health of the proteome.

Chronic stressors challenge the proteostasis balance, causing an accumulation of intracellular damage, and allowing for proteotoxicity to develop (Vilchez, Saez et al. 2014). The ability of cells and organs to preserve proteostasis is gradually compromised with age (Labbadia and Morimoto 2015), and the cell's ability to manage misfolded proteins declines (Ben-Zvi, Miller et al. 2009, Douglas and Dillin 2010) with an increase in protein oxidation as defenses against ROS declines (Finkel and Holbrook 2000), mislocalization, and protein aggregation observed in aged organisms (Morimoto 2008, David, Ollikainen et al. 2010, Reis-Rodrigues, Czerwieniec et al. 2012, Rana, Rera et al. 2013). In order to untangle the complex network of responses, careful examination of the evolution in proteome architecture and regulation occurring during aging is needed. In 2015, studies using quantitative mass spectrometric methods, Walther and his cohorts

discovered that aging in *C. elegans*, is accompanied by degrading protein homeostasis, resulting in widespread alterations in the proteome (Walther, Kasturi et al. 2015). In addition, protein degradation pathways have also been shown to cause proteotoxicity and reduction of lifespan in *Drosophila*, *C. elegans*, and mice (Finley 2009, Li, Zhao et al. 2013, Tsakiri, Sykiotis et al. 2013). All things considered, the idea that reduced function in the protein degradation pathway could contribute to proteostasis collapse and aging remains a viable theory.

Risk analysis of Parkinson's, Alzheimer's, and Huntington's and other neurodegenerative diseases find aging as a primary indicator of disease emergence associated with aggregate deposition (Balch, Morimoto et al. 2008, Knowles, Vendruscolo et al. 2014, Labbadia and Morimoto 2015). Increased levels of aberrant proteins place great stress on the apparatuses of proteostasis and as a result may hasten aging (Gidalevitz, Ben-Zvi et al. 2006, Balch, Morimoto et al. 2008, Olzscha, Schermann et al. 2011, Hipp, Park et al. 2014).

Summary

Aging is a complex and multiplex process that has induced countless new and modified theories to explain the aging process. Despite a robust literature on several theories of aging, no consensus on a comprehensive and unifying theory of aging exists; in fact, numerous proposed theories overlap in non-trivial ways. Several of the aforementioned theories have been found to interact and influence each other in a multifactorial manner. A comprehensive approach to aging research is critical to further advances.

Caenorhabditis elegans as a Model of Aging

The nematode *Caenorhabditis elegans* (*C. elegans*) is a genetically tractable and powerful model organism useful for exploring aging research (Herndon, Schmeissner et al. 2002, Golden, Hubbard et al. 2006)`. *C. elegans* has been a model system at the leading edge of aging research

ever since the first long-lived mutant was discovered (Klass 1983, Kenyon, Chang et al. 1993, Lithgow, White et al. 1995). *C. elegans* is a small, free-living, soil-dwelling nematode of about 1 mm in length that feeds primarily on bacteria. This nematode has a number of features that makes it a powerful tool in aging research. First, its small size allows for easy and inexpensive storage and culturing in the laboratory on a diet of *Escherichia coli*. Second, *C. elegans* is hermaphroditic, allowing genetically identical populations to be maintained in a laboratory setting. Third, it reproduces rapidly and prolifically, with a short generation time, and approximately 300 progenies per self-fertilizing hermaphrodite. Most importantly, *C. elegans* have a short life-cycle (~3 weeks), which is significant for whole life-span assays (Johnson and Wood 1982), are sophisticated multicellular animals, with 959 somatic cells that form multiple organ systems including nervous tissue, musculature, and epidermis, and contain 302 neurons, capable of demonstrating complex behaviors. Due to their transparency, the exact anatomical position and fate of all of their somatic cells is known, which allows for the relatively easy identification of cellular abnormalities. The somatic cells of the adult animals are post-mitotic, and thus makes *C. elegans* an ideal animal model for aging research in non-dividing cells. Nematodes have been genetically tractable since completion of sequencing on the entire nematode genome; these worms can be 1.) manipulated by genetic tweaks to provide strains with either loss-of-function mutations or complete gene knockout (Brenner 1974) or 2.) inactivated through RNA interference (RNAi) by feeding bacteria that express double-stranded RNA for the gene of interest to the animals (Fire, Xu et al. 1998). Studies conducted by the *C. elegans* Sequencing Consortium found the *C. elegans* genome contains homologs of approximately two-thirds of all human disease genes (Consortium 1998), and *C. elegans* aging shares many characteristics with human aging. Taken together, all of the features mentioned make the *C. elegans* worm a clearly beneficial animal model in the study of aging.

Early major breakthroughs in aging research were derived from *C. elegans* studies. In 1983, Klass identified mutants that altered lifespan (Klass 1983). All mutants identified by Klass were subsequently found to be in one locus, termed *age-1* (Friedman and Johnson 1988, Johnson 1990, Morris, Tissenbaum et al. 1996). From this finding, aging researchers discovered aging is modulated by genes which can attenuate gene function to either extend or shorten life. A second breakthrough was the identification of another mutant that alters lifespan (Age mutant), *daf-2*, which similarly to *age-1*, lengthened the life of adult worms (Kenyon, Chang et al. 1993). Subsequent molecular cloning and identification of identification of *daf-2* showed it to be an insulin/IGF-1 receptor (Kimura, Tissenbaum et al. 1997).

The genetic tractability of this nematode and its ease in molecular cloning is one of the main rationale for the popularity of *C. elegans*; particularly, the discovery of the mutations in the insulin/IGF-1 signaling pathway propelled the popularity of using worms in aging studies. Other experimental techniques are being applied to *C. elegans* aging research. Taken together, the *C. elegans* system has provided several major advances in the identification of molecular mechanisms of aging.

Significance and Central Premise

Clearly, taken all together from earlier studies, countering IsoK reactivity to proteins has extensive beneficial biological effects. Yet, little is known about and/or which molecular processes that are being altered by the IsoK scavengers. Specifically, the potential role that oxidative injury from IsoKs may play in the aging process, and how IsoK scavengers may attenuate normal aging is an open question. In order to answer this question, we looked to one of the most well characterized pathways involved in the intersection of metabolic control, redox regulation, and aging: the sirtuin pathway. Sirtuins are a highly conserved family of nicotinamide adenine

dinucleotide (NAD^+)-dependent protein deacetylases that was historically discovered through its association with aging in yeast and other lower eukaryotic organisms (Blander and Guarente 2004, Sauve, Wolberger et al. 2006). Sirtuins play a crucial part in countering nutritional and environmental stressors such as fasting, DNA damage, and oxidative stress (Ciccia and Elledge 2010, Majmundar, Wong et al. 2010, Sengupta, Peterson et al. 2010, Wellen and Thompson 2010). Furthermore, it has been shown that sirtuins deacetylate lysine residues (Tao, Coleman et al. 2010), which suggests a molecular target for isoketal-protein adducts. **Specifically, we hypothesize that sirtuin activity may be negatively attenuated by isoketal adduction, whereby administration of selective scavengers of IsoK could prevent inactivation of sirtuins to attenuate the lifespan of an organism.**

A Therapeutic Role for Sirtuins in Diseases of Aging

As stated above, sirtuins are key regulators of organismal longevity. Genes encoding proteins of whose increased activity positively affects longevity are considered to be anti-aging genes. Out of the multitude of genes identified that influence aging in model organisms, genes encoding sirtuins are considered to be one of the only anti-aging genes due to the fact their gene dosage level is correlated with increases in lifespan. Manipulating gene dosage of Sirtuin 1 (SIRT1) orthologs show a retardation of the aging phenotype in *Saccharyomyces cerevisiae*, *Caenorhabditis elegans*, and *Drosophila melanogaster* (Kaeberlein, McVey et al. 1999, Tissenbaum and Guarente 2001, Wood, Rogina et al. 2004). To date, most interventions targeting processes that contribute directly to aging have generally failed (Le Couteur, McLachlan et al. 2012), yet attenuation of SIRT1 orthologs demonstrate a consistent deceleration of aging and lifespan extension across taxa. Sirtuin activity in aging is of particular interest, due to their characteristic of being a druggable class of enzymes. Opening up the possibility of using small

molecules to target sirtuin modulation could positively affect a range of aging and age-related diseases.

Sirtuin Biology: Structure, Function, and Diversity of Sirtuins

Sirtuins are deacetylases or ADP-ribosyltransferases that requires NAD^+ for their enzymatic activity. As a broad overview, sirtuin activation initiates a transcriptional cascade that promotes a more optimal mitochondrial oxidative metabolic function which results in increased resilience to oxidative stress (Haigis and Sinclair 2010). Sirtuins armor against oxidative stress by 1.) increasing antioxidant pathways via deacetylation to boost catalytic activity of superoxide dismutase 2 (SOD2) and isocitrate dehydrogenase 2 (IDH2), both located in mitochondria, and 2.) promoting DNA damage repair through deacetylation or ADP-ribosylation of DNA repair proteins (Tennen and Chua 2011). This conserved family of enzymes contain seven family members found in mammals, SIRT1-7, which are categorized by their highly conserved NAD^+-binding and catalytic domain, termed the sirtuin core domain (Frye 2000).

Crystal structures of sirtuins from a variety of organisms have shown these proteins consist of a catalytic core of approximately 250 amino acids, which is made up of a NAD^+-binding domain and a Zn^{2+}-binding domain containing four highly conserved cysteine residues (Finnin, Donigian et al. 2001, Min, Landry et al. 2001, Avalos, Celic et al. 2002, Chang, Kim et al. 2002, Zhao, Harshaw et al. 2004). The catalytic site sits in a hydrophobic interface of the two domains. The hydrophobic channels places the acetyl-lysine side chain optimal to catalyze the reaction with NAD^+. Although sirtuins are relatively conserved across the seven family members, significant departures can be observed in their N and C termini. With differences in enzymatic activities, unique binding partners and substrates, and distinct subcellular localization and expression patterns, the seven sirtuin family members are likely to impact differing biology.

Mammalian sirtuins are found in numerous compartments located within the cell and various organ systems (Haigis and Sinclair 2010), of which a summary of distributions can be seen in Table 1.1. SIRT1, SIRT6, and SIRT7 are found predominantly in the nucleus (even though SIRT1 is known to have significant cytoplasmic functions as well) (Michan and Sinclair 2007), whereas SIRT3-5 reside in mitochondria, and lastly SIRT2 is primarily cytoplasmic (with some known associations between SIR2 and nuclear proteins). Broadly speaking, sirtuins can be found in several compartments of the cell, with a fluid localization that can be determined by cell type and its physiology.

Table 1.1: Summary of mammalian sirtuins

Sirtuin	Organ/Tissue	Location	Interactions	Biology
SIRT1	L, B, P, H, M, A	Nucleus	FOXO, PGC-1α NF-κB, Ku70, etc.	Metabolism, stress
SIRT2	H, B, SM	Cytosol	Tubulin, H4, FOXO	Cell cycle
SIRT3	K, B, H, L, BA, MM	Mitochondria	AceCS2, GDH complex I	Thermogenesis, ATP production
SIRT4	H, LVSM, SRM, Pβ	Mitochondria	GDH, IDE, ANT	Insulin secretion
SIRT5	B, H, M, L, K	Mitochondria	CPS1	Urea cycle
SIRT6	M, B, L	Nucleus	Histone H3, NF-κB	Base excision repair, metabolism
SIRT7	L, S	Nucleolus	Pol I	rDNA transcription

Abbreviations: L, Liver; B, Brain; P, Pancreas; Pβ, Pancreatic β–cells; H, Heart; M, Muscle; SM, Skeletal Muscle; VSM, Vascular Smooth Muscle; SRM, Vascular Striated Muscle; A, Adipose; BA, Brown Adipose; K, Kidney; MM, Mitochrondrial Matrix; S, Spleen; AceCS2, acetyl-CoA-synthetase 2; ANT, adenine nucleotide translocator, CPS1, carbamoyl phosphate synthetase 1; FOXO, forkhead box, subgroup O; GDH, glutamate dehydrogenase; IDE, insulin degrading enzyme; NF-κB, nuclear factor kappa B; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; Pol 1, DNA polymerase 1, rDNA, recombinant DNA.

Caenorhabditis elegans Sirtuins

In *C. elegans*, there are four *C. elegans* yeast Sir2 and human SIRT paralogs; *sir-2.1*, *sir2-2*, *sir-2.3*, and *sir-2.4*, each encode proteins with regions of homology to the sirtuin core domain (Frye 2000, Greiss and Gartner 2009). Of the four *C. elegans* SIRT homologs, *sir-2.1* is the most

comprehensively studied worm sirtuin homolog, in part due to its close similarity to both yeast Sir2p and mammalian SIRT 1 according to phylogenetic analysis. It is widely expressed in muscle cells, intestine, hypodermis, nerve cells in the head, in the tail, and the ventral nerve cord (Berdichevsky, Viswanathan et al. 2006, Wang and Tissenbaum 2006, Bamps, Wirtz et al. 2009).

As mentioned earlier, *sir-2.2* encodes one of the four *C. elegans* proteins with similarity to yeast Sir2p. It is thought to play a role in genomic stability by protecting the genome against mutations, as RNAi knockdown of SIR-2.2 function produces an elevated level of spontaneous mutagenesis in the animal (Tissenbaum and Guarente 2001). Additionally, *sir-2.3* shares similarities to yeast Sir2p and SIRT1, but little else is known about this particular family member. Lastly, *sir-2.4* is an ortholog of human SIRT6 and is thought to be involved in P granule assembly (perinuclear RNA granules specific to germline) and response to heat. SIR-2.4 is expressed in the head and tail neurons, along with the spermathecal-uterine valve cell. It is also thought to be localized in the P granule, cytoplasmic stress granule, and nuclear pore (Chiang, Tishkoff et al. 2012, Jedrusik-Bode, Studencka et al. 2013). Earlier nematode sirtuin role in aging studies were mostly dedicated to phenotypic analysis of sirtuin gene dosage manipulation. More recently, research focus pivoted to utilizing various interdisciplinary techniques to establish the role of sirtuins in longevity.

Sirtuin Biochemical Activity

Most sirtuin catalyze NAD^+-dependent deacetylation (Imai, Armstrong et al. 2000, Landry, Slama et al. 2000, Tanner, Landry et al. 2000, North, Marshall et al. 2003). More specifically, regarding their biochemical properties (Fig. 1.3)[1], SIRT1, SIRT2, SIRT3, and SIRT6 display

[1] Figure 5, adapted from May, Olivia. "Sirtuins, to your health." *Cayman Chemicals Article Library*. n. d., n. pag. Web. 14 July 2016. Reprinted with permission of Cayman Chemicals

NAD-dependent deacetylase activity, although their catalytic efficiency and substrate specificities vary (Vaziri, Dessain et al. 2001, North, Marshall et al. 2003). SIRT4 and SIRT6 are ADP-ribosyltransferases, with SIRT6 known to perform both auto-ADP-ribosyltransferase and substrate-specific deacetylase activities (Michishita, McCord et al. 2008), whereas SIRT4 possesses NAD^+-dependent mono-ADP-ribosyltransferase activity (Liszt, Ford et al. 2005, Haigis, Mostoslavsky et al. 2006). Fascinatingly, SIRT5 was discovered to have desuccinylase and demalonylase activity that acted as more physiologically relevant than its modest deacetylase activity (Du, Zhou et al. 2011). SIRT7 was described as a tumor suppressor p53 deacetylase (Vakhrusheva, Smolka et al. 2008), but this finding contradicted an earlier study (Michishita, Park et al. 2005) thus, the catalytic activity of SIRT7 remains unknown pending fuller characterization. In summary, sirtuin activity may be determined by the quantity of sirtuin molecules, availability of its NAD^+ co-substrate, and local concentration of its regulatory inhibitor, nicotinamide. Furthermore, sirtuin activity may be influenced by other intracellular proteins (Kim, Kho et al. 2007, Zhao, Kruse et al. 2008).

Regulation of Sirtuin Activity via NAD^+ Salvage Pathway

NAD^+ is a well-studied coenzyme mediating many redox reactions; it plays an important role in the regulation of NAD^+ consuming enzymes, which not only include sirtuins, but poly-ADP-ribose polymerases (PARPs) and CD38/157 ectoenzymes as well. The absolute requirement of the co-substrate NAD^+ in sirtuin reactions places sirtuins at the nexus of cellular energy metabolic regulation and provides a probable linkage between cytosolic energy status and nuclear signaling. While the NAD^+/NADH ratio always favors NAD^+, in conditions with an abundance of energy, intracellular NADH levels will rise and NAD^+ levels will drop concurrently (Nogueiras, Habegger et al. 2012). In low energy/fasting states or during certain cellular stressors, the

NAD^+/NADH ratio increases, therefore decreasing nicotinamide levels, which then activates sirtuins (Lin, Kaeberlein et al. 2002, Anderson, Bitterman et al. 2003). The NAD^+ concentration in cells is maintained in equilibrium between its synthesis and its consumption.

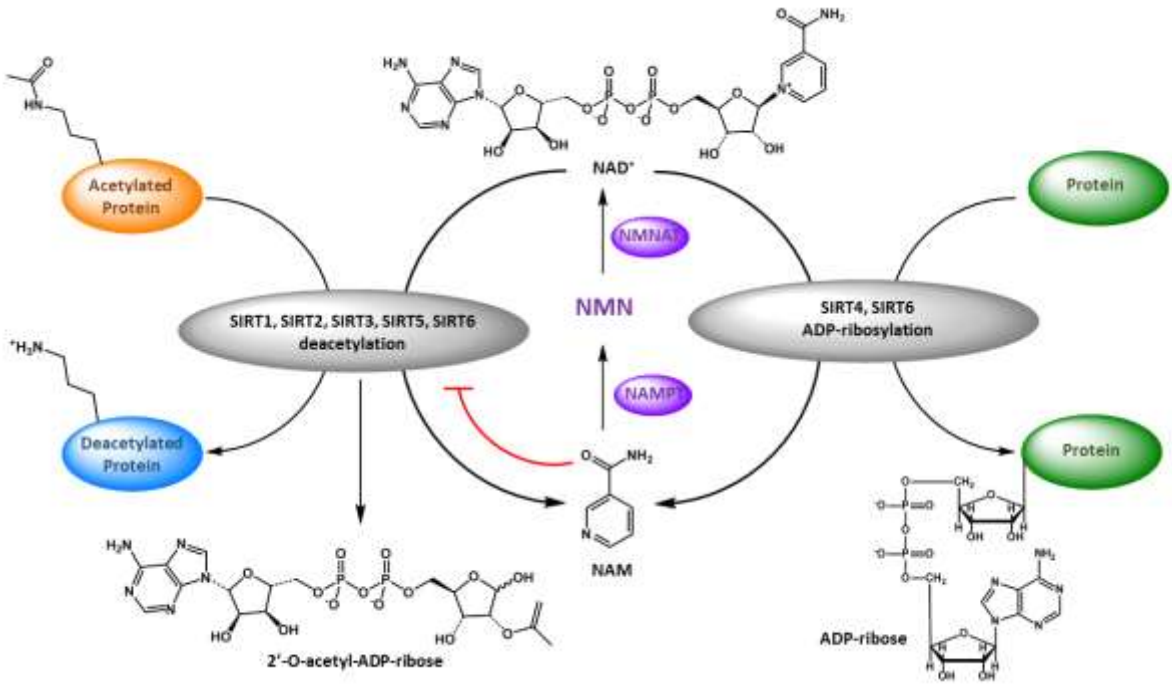

Figure 1.3. Sirtuin biochemistry. Sirtuins catalyze either ADP-dependent deacetylation or ADP-ribosylation reactions. Both mechanisms cleave NAD to release nicotinamide (NAM). Abbreviations: ADP, adenosine diphosphate; NAD^+, nicotinamide adenine dinucleotide; NAM, nicotinamide; NAMPT, nicotinamide phosphoribosyltransferase; NMN, nicotinamide mononucleotide; NMNAT, nicotinamide mononucleotide adenylytransferase; *O*-AADPR, 2'-*O*-acetyl-ADP-ribose. Reprinted with permission from Cayman Chemicals.

In humans, NAMPT (nicotinamide phosphoribosyltransferase) converts nicotinamide to nicotinamide mononucleotide, which is the rate-limiting step of the NAD^+ salvage pathway (Magni, Amici et al. 2004, Revollo, Grimm et al. 2004). NAMPT expression is upregulated during caloric restriction and cellular stress (Magni, Amici et al. 2004, Iqbal and Zaidi 2006, Yang, Lavu et al. 2006), and is especially elevated in ties of prolonged fasting (Yang, Yang et al. 2007). Several lines of evidence show caloric restriction implicated in decreasing intracellular NADH and

nicotinamide concentrations, as well as increasing Sir2 activity and lifespan (Lin, Defossez et al. 2000, Lin, Kaeberlein et al. 2002, Anderson, Bitterman et al. 2003, Lin, Ford et al. 2004, Sauve, Moir et al. 2005). Lastly, biochemical studies confirmed that the NAD+ to nicotinamide ratio is the main regulatory mechanism of sirtuins (Schmidt, Smith et al. 2004).

Sirtuins in Calorie Restriction and Aging

Notwithstanding the multitude of nutrient-sensing pathways implicated in longevity (e.g., insulin signaling (Kenyon 2010), targets-of-Rapamycin (TOR) (Johnson, Rabinovitch et al. 2013), AMP kinase (Kahn, Alquier et al. 2005), and sirtuins (Guarente 2000)) at present, the NAD^+-dependent sirtuin pathway is one of the mechanisms which characterize the effects of caloric restriction (CR) on aging. Eighty years ago, Clive McCay first demonstrated that a decrease in energy intake by 30-40% increased up to 50% longer median and maximum lifespans in white rats (McCay, Crowell et al. 1935). Caloric restriction, i.e. a reduction of calorie consumption to 60-90% of a normal balanced diet without malnutrition, increases longevity in a wide variety of species, from unicellular yeast to multicellular fungus and metazoans including fruit flies, worms, fish, rodents, non-human primates, and presumably humans as well (Roth, Lane et al. 2002, Smith, McClure et al. 2007, Smith, Kaeberlein et al. 2008, Anderson, Shanmuganayagam et al. 2009, Finley and Haigis 2009, Wang, Neretti et al. 2009, van Diepeningen, Maas et al. 2010). However, caloric restriction did not mediate longevity effects in a few species, namely wild mice and houseflies (Cooper, Mockett et al. 2004, Harper, Leathers et al. 2006).

In primates, there are conflicting results in the two large-scale primate studies reported thus far; CR did not increase the average lifespan of the rhesus monkey cohort at the NIH, while a cohort of similar monkeys housed in Wisconsin were reported to live longer on a CR diet (Colman, Anderson et al. 2009, Mattison, Roth et al. 2012). It is possible differences in food composition,

genetic background, and experimental design may account for these discrepancies. Even so, CR is the only non-genetic strategy known to extend not only mean and maximum lifespan but also healthspan by delaying the onset of age-related disorders such as cardiovascular disease, inflammation, arthritis, cancer, diabetes, diverticulosis, neurodegeneration, and cognitive and motor impairment (Weindruch, Walford et al. 1986, Nikolich-Zugich and Messaoudi 2005, Colman, Anderson et al. 2009, Mattison, Roth et al. 2012).

Amassed data points to the role of sirtuins in modulating longevity, chiefly in CR-mediated longevity across various organisms. In 2000, Leonard Guarente proposed the following hypothesis in a review in *Genes & Development;* sirtuins were activated upon calorie restriction to slow the rate of aging and decline in health in organisms (Guarente 2000). This theory was put forth in part because of the following key early evidence; the discovery that sirtuins require NAD^+ for its biochemical activity (Imai, Armstrong et al. 2000) in conjunction with the revelation that extra gene copies of sirtuins results in increased longevity in yeast, nematodes, and flies (Kaeberlein, McVey et al. 1999, Tissenbaum and Guarente 2001, Wood, Rogina et al. 2004). In addition, another crucial evidence for a sirtuin role in CR-mediated lifespan extension rose from the correlation of replicative aging (the number of times a mother cell divides) in yeast to longevity, whereby lifespan extension by CR requires *SIR2* (Kaeberlein, McVey et al. 1999). Studies of asymmetric division in yeast mutants lacking *sir2* have shown fewer than normal division times (20-30 times), which resulted in a shortened lifespan. Mutant lacking *sir2* also displayed premature aging, which can be evidenced by an increase in extrachromosomal rDNA. This was thought to be a sign of rDNA recombination dysregulation in the *sir2* mutants (Gottlieb and Esposito 1989, Sinclair and Guarente 1997, Smith and Boeke 1997).

Other evidence was brought forth, connecting sirtuins to CR and aging. In these studies, investigators found that a simple glucose starvation regimen (0.5% glucose) in yeast resulted in effects similarly seen in models with increased sirtuin activity. Glucose starvation promoted transcriptional changes in metabolism away from normal fermentation towards respiration, which resulted in Sir-2 dependent shift in mother cell longevity (Lin, Kaeberlein et al. 2002). This suggests that sirtuins were activated by NAD^+/NADH ratios increasing. An alternative explanation for the observed shift hypothesizes that CR-induced stress concurrently activates both yeast pyrazinamidase/nicotinamidase 1 (PCN1), which is one of the early initiators in the NAD^+ salvage pathway (catalyzes deamidation of nicotinamide), in conjunction with increased Sir2 activation in order to promote longevity. Despite the plausibility of either or both mechanisms in obliquely explaining sirtuin's nutrient sensing regulation of CR, a direct molecular mechanism is still needed.

However, there is some debate about the exact role of sirtuins in CR-mediated lifespan extension. Kaeberlein and colleagues demonstrated a more austere CR regimen (0.05% glucose) also prolongs yeast replicative lifespan, but surprisingly does not require either Sir2p activity or mitochondrial respiration (Kaeberlein, Kirkland et al. 2004, Kaeberlein, Hu et al. 2005). *SIR2* was also found to have no effect on yeast survival and chronological lifespan under starvation conditions, along with appearing to reduce survival of certain exceptionally long-lived mutant strains (*sch9*) (Fabrizio, Gattazzo et al. 2005). Other challenges to the robustness of the sirtuin control on CR-induced lifespan extension have arisen in other model organisms; perhaps, the greatest contest to the sirtuin/CR-mediated longevity hypothesis was Burnett and colleagues' study which illustrated the lack of lifespan extension in both worms and flies transgenic for the respective *SIR2* orthologs following adjustment of genetic bias (Burnett, Valentini et al. 2011). While

Burnett's main thesis hinged on the thought that controlling for genetic background explains for the differences in sirtuin mediated effects in CR and aging, a few of the earlier studies did due diligence in controlling for genetic background (e.g. Bauer, Morris et al. 2009). Taken all together, this set of conflicting results points to a critical re-assessment of the staying power of the sirtuin/CR-mediated longevity hypothesis.

Burnett's paper in 2011 challenged the dogma of the field and provoked a reexamination of earlier published findings. Subsequently, Stumpferl and other groups replicated the original findings after additional in-depth considerations in a myriad of studies performed in yeast and nematodes. Stumpferl and colleagues set out to integrate the paradoxical reports of sirtuin role in CR and lifespan in yeast, along with reaffirming the pivotal role of sirtuins in longevity (Stumpferl, Brand et al. 2012). Within this study, genome-wide quantitative trait locus (QTL) analysis was performed on contradictory yeast strains which originated from different sources (i.e. a laboratory strain and a clinical isolate). Crossing these divergent strains for QTL analysis pinpointed five codon differences in the *SIR2* alleles in the different strains. The five codon differences were attributed to more than half of the reported inconsistences between the strains and sirtuin role in aging. In addition, studies from the Guarente group, Rizki et al., Ludewig et al., Mouchiroud et al., and Schmeisser et al. rebutted Burnett's claim (Rizki, Iwata et al. 2011, Viswanathan and Guarente 2011, Ludewig, Izrayelit et al. 2013, Mouchiroud, Houtkooper et al. 2013, Schmeisser, Mansfeld et al. 2013). Under a number of conditions manipulation of the SIR-2.1 showed increase in lifespan while controlling for genetic background, granted, not to the similar level as previously published (Tissenbaum and Guarente 2001). In sum, the collected work presented above robustly reaffirms the paradigm that sirtuins are conserved mediators of longevity.

Small Molecule Modulators of Sirtuins

Shortly following the fundamental studies establishing the sirtuin/CR-mediated longevity field, (Kaeberlein, McVey et al. 1999, Imai, Armstrong et al. 2000), medicinal chemistry efforts opened up the field of CR mimetic drugs by developing small molecule activators of mammalian SIRT1 (Howitz, Bitterman et al. 2003). During the past decade, over 3,500 sirtuin activating compounds (STACs), like the first widely studied small polyphenol resveratrol, have been synthesized and analyzed (Lavu, Boss et al. 2008). The discovery and development of STACs arose from the finding that sirtuin regulation occurs on both transcriptional and posttranslational level. All of this evidence assembled into a cogent theory formed a rational drug design paradigm where researchers aim to discover small molecules and protein interactors that can mimic the benefits of CR without undergoing a caloric restriction regimen.

The detection of sirtuin activating compounds is an exciting frontier in drug discovery. Through the use of medicinal chemistry, STACs with improved specificity for sirtuin activation along with enhanced bioavailability have been developed. Considering SIRT1 and its orthologs are the most widely studied sirtuin family member, it is not surprising the number of small-molecule modulators of SIRT1 currently being characterized. A few of these sirtuin modulating compounds targeting SIRT1 include the following inhibitors: splitomycin, sirtinol (Solomon, Pasupuleti et al. 2006), and EX-527 (Solomon, Pasupuleti et al. 2006), as well as sirtinol analogs which target yeast SIR2, SIRT1, and SIRT2 (Mai, Massa et al. 2005). Resveratrol, SRT1720, SRT2183, and analogs of nicotinamide (NAM) can activate SIRT1 activity (Howitz, Bitterman et al. 2003, Milne, Lambert et al. 2007, Smith, Kenney et al. 2009). NAM analogs interfere with NAM inhibition of sirtuins (Sauve, Moir et al. 2005), whereas SIRT1 activators achieve efficacy through two modes. First, via lowering the binding affinity for the substrate and for NAD^+, and

second, in a more limited capacity through an increase in enzyme velocity (Howitz, Bitterman et al. 2003, Milne, Lambert et al. 2007).

A high-throughput screen using a fluorophore-linked peptide reporter identified resveratrol and other polyphenol small molecules as sirtuin activators. This particular assay used low concentrations of the fluorophore-linked substrate, NAD^+, and SirT1 enzyme in order to maximize signal of compounds which activate SirT1. A set of well-known polyphenol compounds with high sirtuin activation was identified from this screen. These particular polyphenol compounds were identified in part due to their known biological and antioxidant activities. Furthermore, these compounds showed activation levels two- to eight-fold higher than the best activation level observed for resveratrol. From the group of activators identified from the assay, quercetin and other catechins were of particular interest due to their similarity to the most widely known sirtuin activator, resveratrol. Resveratrol was found to produce many of the same beneficial outcomes consistent with CR-mediated SIRT1 activation– for example, it has been reported in suppressing tumor growth and inflammation, which in turn promotes cardiovascular health and protect against neurodegenerative diseases (Jang, Cai et al. 1997, Baur, Pearson et al. 2006, Baur and Sinclair 2006, Richard, Pawlus et al. 2011). Most of all, resveratrol administration produces transcriptional profiles similar to those obtained with caloric restriction (Barger, Kayo et al. 2008, Pearson, Baur et al. 2008) and prevents early mortality in obese mice (Baur, Pearson et al. 2006).

Nonetheless, the proposed mechanism of sirtuin activating compounds was challenged due to several studies indicating *in vitro* substrate-specific effects on SIRT1 activity (Kaeberlein, McDonagh et al. 2005, Dai, Kustigian et al. 2010, Huber, McBurney et al. 2010, Pacholec, Bleasdale et al. 2010). These studies dispute the claim that resveratrol and other STACs are direct modulators of sirtuin activity due to loss in reporter activity in the *in vitro* assay upon removal of

the fluorescent tag on the substrate peptide. (Kaeberlein, McDonagh et al. 2005). Likewise, the finding of Park et al. pointed to an indirect activation via cAMP signaling cascades (Park, Ahmad et al. 2012). In the wake of these challenges, Dai et al., Price et al., and Hubbard et al. each supplied clarification of resveratrol and other STACs functioning directly on SIRT1. Three following studies provide strong evidence that resveratrol and other STACs work by directly activating SIRT1. First, Dai et al. demonstrated activation can occur with peptides lacking moieties (Dai, Kustigian et al. 2010), and activation transpires with aromatic amino acids at residues proximal to the deacetylated lysine. This necessary configuration was conceivably overlooked in earlier studies focusing more attention to the apparent requirement for fluorescent tags. Second, a study conducted by Price and colleagues established a link between SIRT1 and resveratrol along with other STACs. This study outlined the requisite for SIRT1 in imparting the beneficial physiological effects of resveratrol and other STACs administration; researchers found that deletion of SIRT1 in adult mice abrogated drug effects (Price, Gomes et al. 2012). Third, site-directed mutagenesis pinpointed a single mutation in SIRT1 that abrogated resveratrol and SIRT1 interaction (Hubbard, Gomes et al. 2013). More specifically, this mutation described in the study lies nearby to the outer portions of the catalytic domain and delineates the allosteric site in the protein for activation by small molecules. En masse, all of these findings in a wide variety of models highly suggest resveratrol and other STACs directly activate sirtuins to achieve favorable lifespan and healthspan outcomes.

Conclusions

Currently, there are no known pharmaceutical interventions that definitively retard aging in humans. Effort continues to provide further insight into the multi-faceted process of normal and diseased aging, which with every new discovery allows for different avenues of pharmacotherapies

to retard the development of aging. Hypothetically, isoketal scavengers could pose as a potential modulator of sirtuin activity – a proposed mechanism of action might entail activation of sirtuin activity via prevention of oxidative damage-mediated IsoK adduction to sirtuins. Working from this supposition, normal sirtuin function could be inhibited by IsoK adduction upon an outside stressor such as oxidative injury. By preventing IsoKs from adducting to the protein, sirtuins will be able to signal and regulate supposed pathways involved in the aging process. Therefore, IsoK scavengers could be a small molecule modulator of sirtuins, and potentially be a pharmacotherapy for averting age-related diseases, extending life, and improving the quality of life in individuals of advanced years.

Overview of Specific Aims[2]

Highly reactive acyclic γ-ketoaldehydes termed isoketals (IsoKs), are formed via the isoprostane pathway of lipid peroxidation. IsoKs covalently adduct ε-amino groups in lysyl residues of proteins, forming stable adducts and intramolecular cross-links. IsoK-lysyl-lactam adducts are increased in atherosclerosis, end-stage renal disease, and Alzheimer's disease. A selective IsoK scavenger, salicylamine (SA), was developed and tested in *Caenorhabditis elegans* to elucidate the molecular processes in isoketal-mediated oxidative injury, and in doing so to provide the basis for the development of novel rational pharmacological interventions to limit oxidative damage. The overarching hypothesis of this thesis is that the biochemical reactivity of the lipid peroxidation products of polyunsaturated fatty acids (PUFAs) (such as arachidonic acid

[2] Portions of this section have been published in a research article in *Aging* written by Nguyen, T. T., S. W. Caito, W. E. Zackert, J. D. West, S. Zhu, M. Aschner, J. P. Fessel and L. J. Roberts, 2nd (2016). "Scavengers of reactive gamma-ketoaldehydes extend caenorhabditis elegans lifespan and healthspan through protein-level interactions with sir-2.1 and ets-7." Aging (Albany NY) **8**(8): 1759-1780.

or the more abundant PUFA in *C. elegans*, eicosapentaenoic acid), the reactive IsoKs, adversely affect the function of key lifespan regulating proteins, ultimately resulting in normal and diseased aging. Administration of the selective IsoK scavenger, SA, is hypothesized to delay and reverse the signs of aging, in addition to illuminating the pathophysiological process of aging.

Chapter II. The optimization and characterization of the chromosomally integrated, dual fluorescence-based reporter strain, *Pgst-4::GFP* (VP596 dvls19[pAF15(Pgst-4::GFP::NLS)];vsIs33[Pdop-3::RFP]) will be discussed. This chapter establishes a use for the *Pgst-4::GFP* transgenic reporter strain in qualitative observations of *in vivo* oxidative stress in *C. elegans*, whereas measurements of F_3-isoprostanes is more conducive to quantitative measures of oxidative stress.

Chapter III. The development of a quantitative gas chromatography-negative ion chemical ionization-mass spectrometry (GC-NICI-MS) approach to measure *in vivo* oxidative stress in C. elegans will be discussed. The methods described in this chapter have been optimized and validated to provide the most sensitive and selective assay for quantification of lipid peroxidation from *C. elegans* lysates.

Chapter IV. The role of IsoKs, SIR-2.1, and ETS-7 in salicylamine-mediated lifespan extension will be discussed. Notably, beneficial SA longevity effects appear to operate primarily at the protein level, placing SA broadly in the role of a proteostasis mediator. SA administration preserves SIR-2.1 and ETS-7 function to enhance normal antioxidant defenses, while effects on mitochondrial function and gene expression were found to be small to nonexistent. Markedly, salicylamine was shown to be a potent anti-aging intervention, with a high translational potential for affecting mammalian models of disease.

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CHAPTER II

DEVELOPMENT AND CHARACTERIZATION OF a FLUORESCENT-BASED ASSAY FOR *in vivo* MEASUREMENT OF OXIDATIVE STRESS

Introduction

The free radical theory of aging as proposed by Denham Harman theorizes that reactive oxygen species (ROS) accumulates over time, causing damage which leads to aging. Harman expanded the theory to propose that cells and tissues decline in activity due to the cumulative effects of macromolecular damage caused by byproducts of the normal oxidative metabolism, the oxygen radicals produced by respiration (Harman 1956). Oxidative stress was generally thought to occur due to an imbalance between ROS production and elimination. Later, Harman adapted his theory to incorporate the role of mitochondria [and specifically the electron transport chain (ETC) activity] in aging since ROS was considered to be largely produced by mitochondria (Harman 1972).

This model for aging is attractive, considering the mechanism of ROS production is universal among animals; but most of all, it is a mechanism that is universal throughout all organisms, seeing as this damage is a by-product of ordinary living (Stadtman and Levine 2000). There is abundant research that establish that ROS and oxidative damage increase with age (Stadtman 2006), increased production of ROS shortens lifespan (Kirkwood and Kowald 2012), and consequently, reducing oxidative damage extends the lifespan of various model organisms (yeast, nematodes, fruit flies, mice, *etc.*). The relationship of energy metabolism and ROS production can be observed at the mitochondrial level, in which accumulation of ROS is generated as an aftermath of normal and diseased ETC activity. Not only is the link between ROS and energy

metabolism seen at the mitochondrial level, studies have shown a connection at the nuclear level by means of the nuclear factor E2-related factor 2 (Nrf2)/antioxidant response element (ARE) pathway.

The Nrf/CNC (Cap'n'collar) family of transcription regulators are commonly known to initiate and mediate cellular defense against environmental stressors resulting in increased oxidative damage. The Nrf/CNC transcription factor is located in the cytoplasm until disturbances that increase oxidative stress cause this transcription factor to translocate to the nucleus. Nrf/CNC binds to the enhancer ARE sequence, which is present in genes that encode for proteins involved in antioxidative defenses such as glutathione synthesis (Kobayashi and Yamamoto 2006, Sykiotis and Bohmann 2010). Nrf/CNC nuclear translocation results in the expression of a host of phase II detoxification and antioxidants, including thioredoxins and glutathione-synthesizing enzymes to maintain the redox balance, enzymes to detoxify xenobiotics such as glutathione *S*-transferases, proteasomal subunits, and various other cytoprotective proteins (Lee, Calkins et al. 2003, Lee, Shih et al. 2003, Shih, Johnson et al. 2003, Oliveira, Porter Abate et al. 2009). Collectively, these enzymes work to mitigate the harmful effects of ROS.

The inducible CNC transcription factors are conserved in worms, insects, fish, birds, and mammals, but absent in plants and fungi. In the nematode *C. elegans*, the Nrf/CNC functional ortholog is termed SKN-1 (Walker, See et al. 2000, An and Blackwell 2003). Similar to Nrf/CNC function in mammals, nematode SKN-1 plays a highly regulatory role in pathways which promote longevity. A condensed lifespan can be observed in loss-of-function *skn-1* mutants (An and Blackwell 2003), and although overexpression of SKN-1 results in adverse effects in nematodes, modest SKN-1 overexpression has been shown to extend lifespan significantly (Tullet, Hertweck et al. 2008).

In light of the significant role free radical damage in aging, measures to assess *in vivo* oxidative stress may shed insight into disease etiology. There are several approaches to assess the role of oxidative damage in *C. elegans* – one could measure levels of ROS in either whole worm (living or lysates) or specifically in mitochondria, or quantify signs of oxidative damage (e.g. measuring levels of lipid and DNA oxidation products). Here, we describe usage of a GFP (green fluorescent protein) transgenic reporter strain, VP596, to relay activity of the nematode transcription factor SKN-1. The response to oxidative stress is regulated by the SKN-1 transcription factor, which regulates cytoprotective genes that encode enzymes which initiate phase II detoxification and antioxidants (Lee and Johnson 2004, Mathers, Fraser et al. 2004). This assay utilizes a chromosomally integrated, dual fluorescence-based reporter for a core SKN-1 activated gene, *gst-4*, which encodes for glutathione-S transferase (Choe, Przybysz et al. 2009, Leung, Deonarine et al. 2011). In the present study, we tested the fitness of the transcriptional reporter of *gst-4* (*Pgst-4::GFP*) in monitoring *in vivo* oxidative damage in *C. elegans* by way of indirect SKN-1 signaling, through challenge with known pro-oxidant compounds (e.g. juglone, paraquat, *etc*.). We compared the results to other methods of oxidative damage measurement, such as isoprostane collection, and highlighted the strengths and weaknesses between these two approaches.

Materials & Methods

C. elegans Strains and Maintenance

C. elegans strains were cultured at 20°C on standard nematode growth media (NGM) agar plates seeded with *Escherichia coli* strain NA22. The following strains were used in this work: wild-type *C. elegans* Bristol strain N2, and VP596. N2 strains were provided by the *Caenorhabditis* Genetics

Center (University of Minnesota, St. Paul, MN) and VP596 strain was provided by Dr. Keith P. Choe (University of Florida). Synchronous L1 populations were obtained by isolating embryos from gravid worms by alkaline hypochlorite (0.5N NaOH, 1% hypochlorite; 8 min at 23°C), and segregating eggs from worm and bacterial debris by floatation on a sucrose gradient. The recovered eggs were rinsed in M9 buffer and placed on fresh agar plates seeded with *E. coli* strain OP50 and maintained at 20°C until late-L4/young adult stage.

Reagents

Unless otherwise stated all reagents were obtained from Sigma-Aldrich (St. Louis, MO).

Pro-oxidant Exposure

Synchronized (Lewis and Fleming 1995) late stage L4 larvae were exposed to varying concentrations of either 5-hydroxy-p-naphthoquinone (juglone), hydrogen peroxide (H_2O_2), tert-Butyl-hydroperoxide (t-BOOH), or paraquat diluted in 85 mM NaCl supplemented with 0.1% Tween-20 medium (addition of 0.1% Tween-20 to liquid medium discourages mechanical shearing stress of animals) for 1 h, rotating at 23°C. After 1 h treatment, animals were removed from the liquid, transferred to agar plates spread with *E. coli* bacterial strain OP50, and allowed to recover for 4 h before fluorescence was measured.

Lethality

Synchronized late-L4/young adult stage worms were acutely treated with juglone (0-200 µM) in glass tubes for 1 hr at 23°C. Worms were then pelleted by centrifugation at 1200 rpm for 1 minute and washed twice with 85 mM NaCl + 0.1% Tween-20 buffer. 30-50 worms were counted and transferred to NGM agar plates seeded with E. coli bacterial strain OP50 in triplicate. Survival of

worms was assessed by touch-provoked movement using a platinum wire (Gill, Olsen et al. 2003) 48 h post-treatment.

Oxidative Stress Reporter Assay and Microscopy

Pgst-4::GFP fluorescence was quantified using a microplates reader (FLUOstar Optima microplate reader, BMG Labtechnologies). Synchronized (Lewis and Fleming 1995) late stage L4 worms were exposed to pro-oxidants (juglone, t-BOOH, H_2O_2, and paraquat) for 1 h, removed from liquid, transferred to OP50-seeded bacterial plates, and allowed to recover for 4 h before fluorescence was measured. A sub-set of worms (~10-15 worms per condition) were mounted onto 4% agar pads (in M9 medium) and anesthetized with 0.2% tricaine/0.02% tetramisole in M9 buffer. Representative images of *Pgst-4::GFP* fluorescent intensity at varying concentrations of juglone was imaged using an epifluorescence microscope (Nikon Eclipse 80i) equipped with a Lambda LS Xenon lamp (Sutter Instrument Company) and Nikon Plan Fluor 20x dry and Nikon Plan Apo 60x 1.3 oil objectives.

F_3-Isoprostane Collection

F_3-Isoprostane levels were quantified from pro-oxidant treated worms using a gas-chromatography-negative ion chemical ionization-mass spectrometry (GC-NICI-MS) approach (Milne, Sanchez et al. 2007), as previous described (Nguyen and Aschner 2014) in whole worm extracts from synchronized late L4 stage worms treated with various concentrations of either juglone, t-BOOH, H_2O_2, or paraquat. Protein concentration of nematode homogenates were determined using the bicinchoninic acid (BCA) protein assay as described by the manufacturer (Pierce Protein Biology, Waltham, MA).

Statistics

All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, Inc.). Dose-response lethality curves were generated using a sigmoidal dose-response model with a top constraint at 100% and determination of LD_{50} values (concentration at which drug induces 50% reduction in survival).

Results & Discussion

Establishing a SKN-1 response with xenobiotic juglone

As oxidative stress and its resulting damage is involved in aging and age-related diseases, we examined whether the transgenic fluorescent reporter strain, VP596 would be a suitable measure of *in vivo* oxidative stress in *C. elegans* via indirect measurement of SKN-1 signaling. As described in depth in Leung et al. (2011), the transgenic strain VP596 (dvls19[pAF15(*gst-4*::*GFP::NLS*)];vsls33[*dop-3::RFP*]) contains two fluorescent constructs to report SKN-1 activity [*Pgst-4::GFP* (Link and Johnson 2002)] and normalize worm number across all assay wells [*Pdop-3::RFP* (Chase, Pepper et al. 2004)], which is then expressed as a ratio of GFP/RFP (Leung, Deonarine et al. 2011). To activate the SKN-1 response, a reactive oxygen-generating napthoquinone, juglone, was used. Juglone has previously been utilized in oxidative stress studies in *C. elegans* (Strayer, Wu et al. 2003, de Castro, Hegi de Castro et al. 2004, Przybysz, Choe et al. 2009).

To assess the effect of juglone toxicity on nematode survival, juglone lethality curves were established for Bristol N2 WT and VP596 strains (Fig. 2.1A and 2.1B). Toxicity was assessed in order to conduct following fluorescent studies in a sub-lethal dose range. Dose-response survival curves following acute juglone exposure revealed N2 WT and VP596 had similar sensitivities to

juglone-induced lethality (LD_{50} = 48.6 µM and LD_{50} = 45.0 µM, respectively). All following experiments were conducted in the sub-lethal dose range of juglone (~30 – 50 µM).

In order to establish the juglone concentration response curve, late-stage larval stage 4 worms were exposed to juglone in liquid (85 mM NaCl + 0.01% Tween-20) for 1 h and then transferred to agar plates for recovery. GFP fluorescence intensity was then measured 4 h after plating worms at a concentration of 200 worms/well and 5 replicates per juglone concentration, using a FLUOStar Optima (BMG LabTech, Ortenberg, Germany). Maximal efficacy of a ~4.5-fold increase in *Pgst-4::GFP* fluorescence intensity was found to be ~35 µM juglone, and half-maximal efficacy was 16.6 µM (Fig. 2.1C). VP596 worms express GST-4::GFP in the intestinal lining of the worm, which can be seen visualized in worms treated with 0, 10, 30, and 50 µM juglone. Representative epifluorescence microscopic images depicting intestinal expression of GST-4::GFP (Fig. 2.1D) shows dose-dependent increase in fluorescent intensity, up to 30 µM juglone. Concentrations higher than 30 µM show a slight decrease in fluorescent intensity, possibly due to lethality issues at this particular dose, considering LD_{50} was found to be in the same concentration range.

Methods to quantify oxidative stress in *C. elegans*

Juglone (5-hydroxy-1,4-naphthoquinone) is a potent redox active compound with the potential to generate superoxide anion radicals (Bolton, Trush et al. 2000). This naphthoquinone and has been used previously in *C. elegans* to isolate genetic programs to defend against deleterious effects of oxidative damage (Strayer, Wu et al. 2003), identify genes that promote resistance to oxidative stress (de Castro, Hegi de Castro et al. 2004), and to establish a hormetic response (Przybysz, Choe et al. 2009). In addition, juglone is also an electrophilic xenobiotic that can react with either superoxide ($O_2^{-\bullet}$), hydroxyl radical ($^{\bullet}OH$), and hydrogen peroxide (H_2O_2), of

which is a concern because hydroxyl radicals are powerful oxidizing agents that could potentially cause damage to essential macromolecules. For example, oxidation of solvent-accessible cysteine residues in proteins create disulfide bonds which can modify protein structure and impede function (Bolton, Trush et al. 2000).

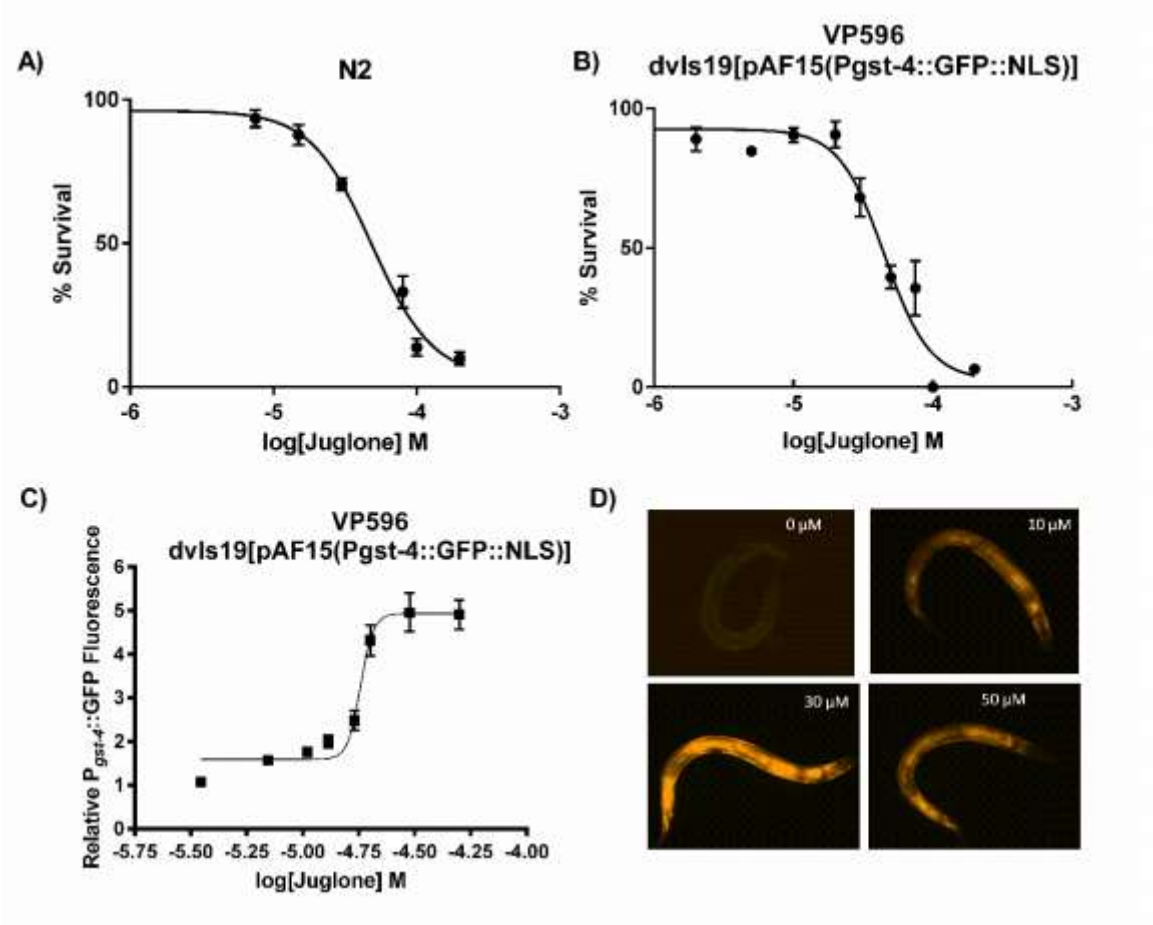

Figure 2.1. Establishing a juglone dosing regimen with the *Pgst-4::GFP* reporter strain. (A) Dose response lethality curves following acute juglone exposure. Late-L4 stage Bristol N2 WT worms were treated for 1 hr with increasing concentrations of 5-hydroxy-1,4-naphthoquinone (juglone). LD_{50} was found to be 48.6 µM (B) Lethality curve for *Pgst-4::GFP* (VP596) reporter strain. LD_{50} = 45.0 µM. (C) Concentration response curve for *Pgst-4::GFP* reporter strain treated with increasing concentrations of juglone for 1 hr. Fluorescent intensity of *gst-4* expression was measured from VP596 after a 4 hr recovery time. EC_{50} = 16.6 µM and EC_{MAX} ~35 µM. (D) Representative epifluorescence images from juglone-treated *Pgst-4::GFP* animals. Data are expressed as means ± SEM from three independent experiments.

From these earlier studies, juglone has been found to form harmful adducts to alter proteins (O'Brien 1991, Bolton, Trush et al. 2000), lipid peroxidation (Fong, McCay et al. 1973), and DNA damage (Bjelland and Seeberg 2003). Furthermore, quinones such as juglone, also act as Michael acceptors; mechanistically, damage to macromolecules could occur through covalent binding of quinones with cellular nucleophiles. An example of this occurrence can be seen through quinone reaction with glutathione (GSH) or solvent-accessible cysteine residues of proteins; these reactions lead to a reduction of GSH levels (Bolton, Trush et al. 2000). The possibility of juglone reacting with cysteine residues is troubling, bearing in mind the *Pgst-4::GFP* relies on fluorescent intensity reporting the activity of glutathione-s transferase activity.

With these considerations in mind, concerns about off-target effects of juglone in the *Pgst-4::GFP* reporter assay were raised, due to the potential for juglone interference in SKN-1 regulation. To address these concerns, the transgenic fluorescent strain (VP596, dvls19[pAF15(*gst-4*::*GFP::NLS*)];vsls33[*dop-3::RFP*]) was exposed to various pro-oxidants typically used in *C. elegans* oxidative stress studies (juglone, H_2O_2, paraquat, and t-BOOH). A concentration response curve (CRC) was observed for juglone, with maximal efficacy over a 4.5-fold increase concentrations higher than 30 µM, tert-Butyl hydroperoxide, with a maximal efficacy ~2.5-fold increase at 1 mM t-BOOH, and paraquat, with a maximal efficacy ~1.5-fold increase at 85 mM paraquat – but a CRC could not be observed for hydrogen peroxide (Fig. 2.2A). Of the pro-oxidants tested, juglone induced the strongest fold-change in fluorescent intensity.

F_3-isoprostanes (F_3-IsoPs) were collected from pro-oxidant exposed transgenic GST-4::GFP nematodes as a comparative method to test whether the *Pgst-4::GFP* assay is an adequate measure of oxidative damage. F_3-IsoPs are the non-enzymatically rearranged lipid peroxidation products of eicosapentaenoic acid (EPA), and are known to be a highly sensitive and accurate

marker of oxidative damage in *C. elegans* (Gao, Yin et al. 2006, Labuschagne, Stigter et al. 2013, Nguyen and Aschner 2014). Late-stage L4 worms were treated in the same manner as described previous (pro-oxidants: 0 and 30 M juglone; 0 and 1 mM t-BOOH; 0 and 1 mM H_2O_2; 0 and 85 mM paraquat). Samples were homogenized using the BioSpec Mini-Beadbeater-16 with zirconium oxide beads (1 mm) at 4°C. Homogenates were prepared for GC/MS using protocols established previously in the lab (Nguyen and Aschner 2014). F_3-IsoP sample collection from VP596 worms exposed to the various pro-oxidants all show a significant increase in F_3-IsoP levels when compared to vehicle control (Fig. 2.2B), with juglone showing a ~1.8-fold increase in oxidant injury, an observed ~11-fold increase in oxidant injury upon administration of t-BOOH, a ~1.5-fold increase in oxidative damage with paraquat, and an expected increase in oxidant injury with H_2O_2 dosing (~2.1-fold increase). Therefore, this suggests that F_3-IsoP collection is a more appropriate measure of oxidative damage than the transgenic *Pgst-4::GFP* reporter strain, VP596.

Conclusion

The *C. elegans* GFP reporter strain, VP596 dvls19[pAF15(Pgst-4::GFP::NLS)];vsIs33[Pdop-3::RFP], was utilized for the exploration of the role oxidative stress plays in aging. For the first time, the current study provides evidence that while the *Pgst-4::GFP* reporter strain may be used for qualitative evaluation of *in vivo* oxidative stress in *C. elegans*, quantification of F_3-Isoprostanes (F_3-IsoPs) should be preferred in quantitative studies. From the data provided in this study, a discrepancy in sensitivity to measuring oxidative damage can be seen

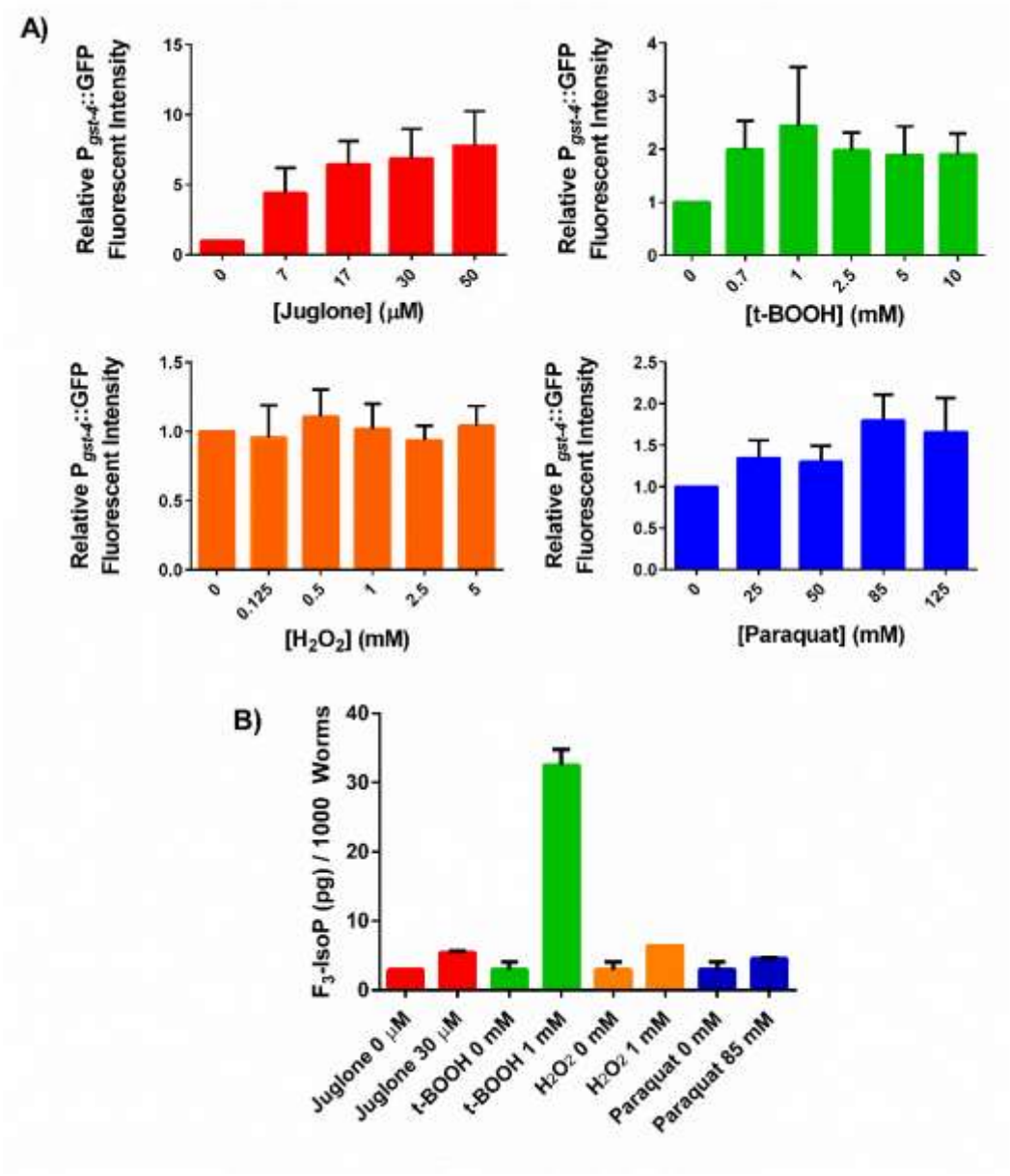

Figure 2.2. *Pgst-4::GFP* reporter strain is a qualitative measure of oxidative stress. (A) Relative fluorescent intensity of *Pgst-4::GFP* reporter treated with juglone, t-BOOH, H_2O_2, and paraquat for 1 hr. Maximum fluorescent intensity was observed: juglone, ~4.5-fold increase at 30 M; t-BOOH, ~2.5-fold increase at 1 mM; paraquat, ~1.5-fold increase at 85 mM; no change in fluorescent intensity observed with H_2O_2. (B) F_3-IsoP measurement of *Pgst-4::GFP* reporter strains treated with juglone, t-BOOH, H2O2, and paraquat. An increased fold-change in F_3-IsoP levels were detected for all pro-oxidants: juglone, ~1.8-fold increase at 30 μM, t-BOOH, ~11-fold increase at 1 mM; H_2O_2, ~2.1-fold increase at 1 mM; paraquat, ~1.5-fold increase at 85 mM. Data are expressed as means ± SEM from two independent experiments.

between the two approaches used: 1.) *Pgst-4::GFP* reporter strain, and 2.) F_3-IsoP measurements. Higher responses to pro-oxidant treatment can be seen in quantification of endogenous F_3-IsoP levels when compared to the responses of the *Pgst-4::GFP* assay when treated with pro-oxidants. In addition, juglone is an arylating agent that reacts with thiols on cysteine residues of protein to form covalent bonds resulting in quinone-thiol Michael adducts (O'Brien 1991, Bolton, Trush et al. 2000), which may potentially interfere with *Pgst-4::GFP* fluorescent intensity signals. In conclusion, we recommend use of the *Pgst-4::GFP* reporter strain in qualitative studies of *in vivo* oxidative stress in *C. elegans*, and to discourage the usage of juglone as a pro-oxidant in conjunction with this reporter strain.

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CHAPTER III

F_3-ISOPROSTANES AS a MEASURE OF *in vivo* OXIDATIVE DAMAGE IN *Caenorhabditis elegans*[3]

Introduction

Numerous environmental toxins cause oxidative damage to lipids, proteins, and DNA. Lipid peroxidation is a hallmark of oxidative stress, in part due to its susceptibility to free radical attack (Gardner 1989, Niki 2009). Damage to tissue biomolecules, including lipids, proteins and DNA by reactive oxygen species (ROS) is thought to be a major contributor to pathophysiology of oxidative stress (Halliwell and Gutteridge 1990, Gutteridge 1995, Butterfield 1997). ROS are chemically reactive, oxygen-containing molecules typically generated during aerobic metabolism, with the mitochondria being a major source of endogenous ROS (Chen, Vazquez et al. 2003, Muller, Liu et al. 2004). Some of the most common ROS produced in living systems are superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals (·OH). Reactive nitrogen species (RNS) are related reactive species formed in cells, which include peroxynitrite ($ONOO^-$), nitric oxide (·NO) and other carbon-centered radicals. Excess ROS accumulation can cause injury to surrounding biomolecules; therefore the cell has developed a series of antioxidant defense mechanisms to mitigate the effects of free radical-induced oxidative damage and maintain redox homeostasis. The primary antioxidant defenses include antioxidant enzymes to degrade ROS, such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase, along with low

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molecular weight antioxidant molecules to serve as free radical scavengers and/or reducing agents. Oxidative stress occurs when there is an imbalance between production of free radicals and antioxidant defenses to favor an environment where excess ROS can cause oxidant damage to biomolecules.

Measuring oxidative stress requires accurate quantification of either free radical production or damaged biomolecules, such as lipid peroxidation products. Several analytical approaches [electron paramagnetic resonance (EPR), chemiluminescence, fluorescence] have been used to detect levels of intraworm ROS and their oxidation products; however many of these techniques suffer from lack of sensitivity and specificity, as well as being methodologically difficult to execute. In a recent multi-investigator study sponsored by the National Institutes of Health (NIH), the Biomarkers of Oxidative Stress Study (BOSS) found that the most accurate method to assess oxidant stress *in vivo* is through quantification of plasma or urinary F_2-Isoprostanes (F_2-IsoPs) (Kadiiska, Basu et al. 2013). The predominant mammalian polyunsaturated fatty acid, arachidonic acid (AA), can give rise to non-enzymatic formation of F_2-IsoPs through free radical-induced lipid peroxidation (Morrow, Hill et al. 1990). Because of their chemical stability and prevalence in all human tissues and biological fluids, IsoPs are regarded as the gold standard for quantification of oxidative damage in mammals. In *C. elegans* the major fatty acid eicosapantaenoic acid (EPA) forms F_3-IsoPs, which were also recently identified as markers for oxidative damage (Gao, Yin et al. 2006, Chang, Patel et al. 2008, Song, Paschos et al. 2009).

This unit will describe methods to measure F_3-IsoPs in *C. elegans* using a gas chromatography-negative ion chemical ionization-mass spectrometry (GC-NICI-MS) approach employing a stable isotope dilution (see Fig. 3.1). This includes solid-phase extraction (SPE), thin-layer chromatography (TLC) purification, chemical derivatization, and MS detection using

negative ion chemical ionization (NICI) coupled with GC. The advantages of GC/MS include the high resolution of GC separation on fused silica capillary columns and the specificity and sensitivity of MS, which allows the lower limit of detection of the F_3-IsoPs to be in the low pictogram range. This assay is a robust and sensitive method that enables quantification of endogenous levels of oxidative damage in *C. elegans*. A goal of this unit is to introduce a sensitive and accurate measure of endogenous oxidative damage in *C. elegans* for oxidant stress studies. Several considerations of this protocol and alternative approaches are discussed in the Commentary section below. Methods are described for collection of whole worm lysates, lipid extraction for measurement of total F_3-IsoPs, and sample preparation with chemical derivatization and extraction for GC-MS analysis.

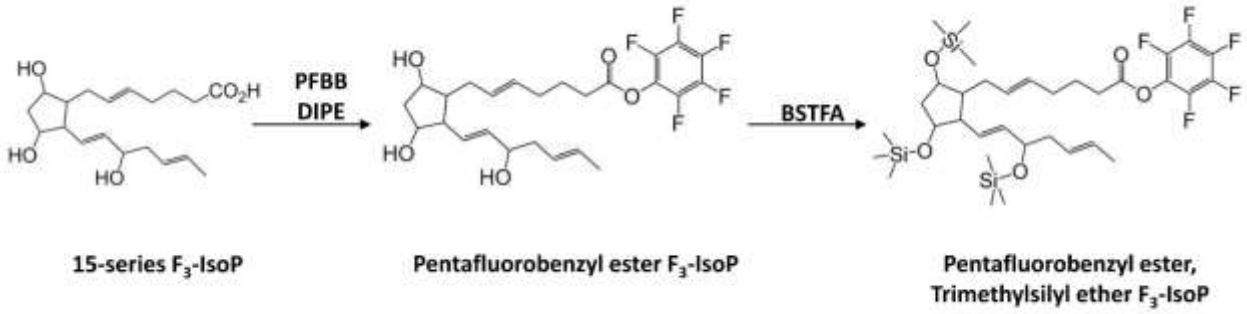

Figure 3.1. Derivatization of 15-series F_3-Isoprostanes for GC/MS analysis.
F_3-IsoPs must undergo chemical transformation to the pentafluorobenzyl ester, trimethylsilyl ester derivatives prior to GC/MS analysis. After separation phase extraction/purification, F_3-IsoPs are first derivatized by PFBB, which attacks the carboxyl group of the F_3-IsoP. The pentafluorobenzyl ester moiety of the resulting product is an ideal leaving group for formation of the carboxylate anion detected by the mass spectrometer. Trimethylsilyl groups are added to the hydroxyl groups through derivatization by BSTFA, which functions to lower polarity of the molecule and significantly lowers the boiling point of the resultant derivatives, for ease of vaporization when injected into the gas chromatograph. During ionization process, the pentafluorobenzyl ester is cleaved and the carboxylate anion is the resulting species detected by the mass spectrometer.

Sample Collection and Preparation

This protocol describes the method for nematode collection and homogenization for quantification of F_3-IsoPs as a measure of lipid peroxidation. A critical consideration in the accurate measurement of oxidant damage is decreasing the *ex vivo* oxidation of eicosopentaenoic acid, which can generate F_3-IsoPs outside of the desired experiment. This may be addressed by flash-freezing washed samples in liquid nitrogen directly following collection if not used for sample preparation and analysis immediately.

Table 3.1: Materials for sample collection and preparation

NaCl
1X Tris-buffered saline, pH 7.4 (Fisher, acidify to pH 6.8 with HCl)
1x Protease Inhibitor Cocktail (Sigma, cat. no. P8465) 1% Triton X-100 (Sigma, cat. no. T8787)
2-ml sterile polypropylene screw-cap tubes
1.0 mm zirconia beads (BioSpec Products, cat. no. 11079110zx)
High-energy cell disruptor, (BioSpec Products, cat. no. 607)

Worm collection

Harvest healthy synchronized nematodes by washing plates with room temperature 85 mM NaCl, using 10-12 mL per plate. Transfer resuspended nematodes to 15 mL conical tubes using sterile pipettes and centrifuge to pellet worms for 1 minute at 400 x *g*, 23°C. Repeat washes at least 3 times or until aqueous phase is clear. Centrifuge worms at 1600 x *g*, 23°C, for 5 minutes. Aspirate the supernatant down to 100 μL and transfer worm solution into 2-mL polypropylene microvials.

Preparation of homogenization solutions

Prepare lysis buffer: (0.0497 g NaCl, 100 μL of 1% Triton X-100, 100 μl of 1M Tris buffered saline, pH 6.8, 100 μl of 1X protease inhibitor cocktail, and 9.7 mL of sterile water).

Aliquot 2 mL into 15 mL conical tubes and store at -20 °C. Keep aliquot of freshly prepared lysis buffer in an ice bucket for immediate use. To prepare samples for homogenization, add 400 μL cold lysis buffer to 2-mL polypropylene microvial containing nematodes. Eicosapentaenoic acid in tissue samples can oxidize ex vivo to generate F_3-IsoPs. If worm samples are not to be used immediately after collection, flash freeze the sample in liquid nitrogen and store at -80 °C. To add zirconia beads to sample vials, cut the tip off the plastic twist-top for easier pouring. Add 300 μL 1.0 mm zirconia beads into samples for homogenization. Bring total lysate volume of each sample to ~700 μL by adding 200 μL cold 85 mM NaCl. Prepare stirring ice water on stir plate to cool samples in between homogenization.

Homogenization of nematodes

To homogenize worms, all following steps should be performed in a 4 °C cold room. Homogenize worms with a beadbeater at full speed for 20 s. Remove samples from beadbeater and place in stirring ice water for 1 min. Repeat homogenization and cooling steps for a total of 7 cycles. Centrifuge for 30 min at 3500 x *g* in a tabletop centrifuge at 4 °C to separate aqueous and organic layers. After centrifugation, carefully pipette off aqueous layer and transfer to a new 2-mL polypropylene microvial. Lysates can be stored at -80 °C until ready for sample purification.

Cleaning of zirconium beads

Transfer used zirconium beads from sample tubes into a clean, open stainless steel or glass tray. Soak beads in diluted bleach mixture (1:10 bleach:H_2O) for 5 minutes. Rinse beads in distilled water until aqueous phase is clear. Dry beads in glass tray at 40 – 70C oven. Beads can be autoclaved for 30 minutes and stored in dispensing container before use. Zirconium beads can be reused for approximately 10 uses before beads wear down in size.

Lipid Extraction and Hydrolysis of F_3-IsoP-containing Phospholipids in C. elegans Lysates

F_3-IsoPs are formed *in situ* esterified to phospholipids and are subsequently released by phospholipases. In tissue samples and worm lysates, this phenomenon creates two forms of F_3-IsoPs; one form that remains esterified in the membrane, and a second that is hydrolyzed and released in free form. Both free and esterified F_3-IsoPs are quantified for total F_2-IsoP formation in the worm lysates. In order to quantify total F_3-IsoPs, it is necessary to extract the phospholipids from the tissue and release esterified F_3-IsoPs from the phospholipids via base hydrolysis. A free radical scavenging agent, BHT, is added to the organic solvent during extraction of phospholipids in order to decrease the amount of *ex vivo* oxidation of EPA.

Table 3.2: Materials for lipid extraction and hydrolysis of *C. elegans* lysates

15% KOH, wt/v (see recipe)
Methanol containing BHT (0.005% v/v) (see recipe)
pH 3 water (see recipe)
1 N HCl
1.7-ml microcentrifuge tube
15-mL plastic tube (Denville, cat. no. T8173)

Lipid extraction and base hydrolysis of *C. elegans* lysates

Let samples thaw on ice. Once thawed, aliquot 30 μL of lysate into a new 1.7-mL microcentrifuge tube for protein content normalization. To each sample add 500 μL of KOH (15%, wt/v). Mix gently by inversion, making sure to not vortex samples and incubate at 37 °C for 30 min. To quantify total F_3-IsoPs formation, both free and esterified F_3-IsoPs are analyzed by releasing F_3-IsoPs from the phospholipids via base hydrolysis. Add 500 μL of methanol containing butylated hydroxytoluene (BHT) to each sample. Adding a free radical scavenging agent like butylated hydroxytoluene (BHT) to the lipid extracts is important to inhibit ex vivo formation of F_2-IsoPs. Total lipids are dissolved in 500 μL methanol containing BHT (0.005% v/v) to saponify

lipid extracts and release esterified isoprostanes. Mix gently by inversion and centrifuge for 2 min at maximum speed in a tabletop centrifuge at room temperature. Following centrifugation, a semisolid protein layer should have formed between the upper (aqueous) and the lower (organic) layers. Carefully pipette off semisolid protein layer and discard. Transfer aqueous layer to a 15-mL plastic tube by pipet and place on ice. Acidify the mixture to pH 3 with 1 ml HCl (1N) and bring total volume to 10 mL with pH 3 water in preparation for purification of F_2-IsoPs with solid-phase extraction (SPE). It is important that samples are corrected to pH 3 or slightly lower (down to pH 2.5) in order for the carboxylate moiety to be protonated in order to chromatograph correctly on the Sep-Pak cartridges. Note, it is important to continue immediately with the purification steps below. The sample should not be stored in this form because of the potential for oxidation.

Sample Purification for Mass Spectrometric Analysis

This GC/MS method is based on those described by Morrow, Roberts, and colleagues (Morrow, Zackert et al. 1999, Gao, Yin et al. 2006, Milne, Sanchez et al. 2007) and involves derivatization of carboxyl moieties on the F_3-IsoPs to the pentafluorobenzyl ester, trimethylsilyl ether derivatives. Separation of compounds is achieved by solid-phase extraction using both C18 and silica Sep-Pak to remove the contaminants. Pentafluorobenzyl (PFB) moiety is then reacted with the molecule to enhance detection using electron capture chemical ionization techniques[15]. Lastly, trimethylsilyl (TMS) is introduced to add to cap the hydroxyl groups to lower its boiling point for ease of vaporization in the gas chromatograph. Analysis of the derivatives of 15-F_{3t}-IsoPs and the internal standard is carried out using the selective-ion monitoring (SIM) techniques, where the ions monitored are *m/z* 567 for F_3-IsoPs and *m/z* 571 for the internal standard, respectively.

Table 3.3: Materials for sample purification for GC/MS analysis

[$^2H_4$]-15-F_{2t}-IsoP (8-iso-$PGF2_\alpha$) (Cayman Chemical, cat. no. 316351)
Methanol (Fisher, cat. no. A4544)
pH 3 Water (see recipe)
1 N HCl
Heptane (Fisher, cat. no. H3504)
Ethyl acetate (Fisher, cat. no. E1964)
Acetonitrile (Fisher, cat. no. A9984)
10% Pentafluorobenzyl bromide (PFBB) (Sigma, cat. no. 10105-2, see recipe)
10% *N,N'*-Diisopropylethylamine (DIPE) (Sigma, cat. no. D3887, see recipe)
Calcium hydride (Sigma, cat. no. 208027)
Chloroform (VWR, EM-CX1059-1)
Ethanol, absolute, 200 proof (Fisher, AC61509040)
10-ml Disposable plastic syringe (Laboratory Supply, cat. no. SMJ5512878)
15-ml plastic tube (Denville, cat. no. T8173)
5-ml plastic vial with cap (Denville, cat. no. T8200)
C_{18} Plus solid-phase extraction (Sep-Pak) cartridge (Waters Associates, cat. no. WAT036575)
Silica Plus solid-phase extraction (Sep-Pak) cartridge (Waters Associates, cat. no. WAT036580)
Supelco Visiprep SPE vacuum manifold (Sigma, cat. no. 57265)
Analytical evaporation unit with water bath at 37°C (Organomation, cat. no. 11634-P)
TLC filter paper (VWR, cat. no. 28298-020)
TLC silica plates (Analtech, cat. no. 43931-2)
Glass TLC tank (VWR, cat. no. 21432-761)

Sample purification using Separation-Phase Extraction (SPE)

To sample accurately add 248 pg of the internal standard ([$^2H_4$]-15-F_{2t}-IsoP) and vortex. For reverse phase, connect one C-18 Sep-Pak cartridge to a 10-ml disposable syringe per sample and place on vacuum manifold. Precondition each cartridge with 5 ml methanol and 5 ml pH 3 water. Use a pressure < 5 mm Hg for column conditioning. Dispense acidified sample to the preconditioned Sep-Pak cartridge. During sample loading, the vacuum manifold should be set at < 5 mm Hg for a flow rate through the column at 1-2 ml per min, such that individual drops emerge from the Sep-Pak. This is to avoid loss of IsoPs when applying sample to the cartridge. Wash cartridge first with 5 mL pH 3 water followed by 5 mL heptane. Heptane removes nonpolar contaminates including un-oxidized EPA. Elute F_3-IsoPs with 10 mL of ethyl acetate/heptane

(50:50 v/v) into a 15-mL plastic tube. Dry ethyl acetate/heptane eluate with anhydrous sodium sulfate. Add 1-5 g Na_2SO_4 to vial and swirl gently for 5 s. This step removes residual water from the eluent. Remove used C-18 Sep-Pak cartridges from syringes and discard. Connect one silica Sep-Pak cartridge to each syringe per sample and place on vacuum manifold. Precondition each silica cartridge with 5 mL ethyl acetate/heptane (50:50 v/v). Apply eluent from C-18 Sep-Pak cartridge to silica Sep-Pak cartridge. Care should be taken to decant eluent and not to transfer any sodium sulfate to the extraction cartridge. Wash cartridge with 5 mL ethyl acetate, then elute F_3-IsoPs from the silica Sep-Pak cartridge with 5 mL ethyl acetate/methanol (45:55 v/v) into a 5-mL collection vial. Evaporate eluent under nitrogen in the analytical evaporation unit in a fume hood.

Conversion of F_3-IsoPs to corresponding pentafluorobenzyl esters

Add 40 μL of 10% (v/v) PFBB in acetonitrile and 20 μL of 10% (v/v) DIPE in acetonitrile to the sample vial, vortex briefly, and incubate for 20 min at 37 °C. Convert F_3-IsoPs to the corresponding pentafluorobenzyl esters. This derivatization facilitates compound analysis by GC/MS. Do not work outside of a well-ventilated fume hood due to PFBB being a potent lachrymator. Dry sample thoroughly under nitrogen in an analytical evaporation unit and resuspend material in 50 μL methanol/chloroform (3:2, v/v). Vortex briefly.

Thin-Layer Chromatography (TLC)

Prepare TLC tank by adding 92 mL chloroform, 8 mL ethanol, 1 mL acetic acid, and TLC filter paper to saturate the tank. Allow tank to equilibrate for 30 min. Mark solvent front line and label sample lanes (see Fig. 3.2). Silica plates contain a pre-adsorbent zone towards the bottom of the plate and four pre-scored lanes. Measure 13 cm from top of the pre-adsorbent zone (origin line) and mark. Lightly score solvent front across plate. Above marked solvent front, label individual samples per each four lane. Apply mixture from each sample to a silica TLC plate. Dry samples

for 5-10 s with a hair dryer. Samples are applied to the upper half of pre-adsorbent zone in four pre-scored lanes, taking care to apply only one sample per lane. Avoid adding sample to the first 1 cm of the plate. Using a separate TLC plate, apply approximately 2-5 µg of the methyl ester of prostaglandin F2α (PGF2α) to one lane for use as a standard. After ensuring that the application solvent has dried, place TLC plates into the TLC tank. Solvent is allowed to migrate 13 cm, and plates are removed from tank for solvent to evaporate. Not all TLC plates may reach 13 cm at the same time. Visualize the TLC standard by spraying the standard plate with the phosphomolybdic acid solution and then heating on a hot plate. Do not spray sample plates. Scrape silica from the sample TLC plates in the region of the TLC standard (Rf should be approximately 0.18), scraping from 0.5 cm above the middle of the visualized standard to 1 cm below the standard. Keep sample plates until GC/MS analysis is complete. Place scraped silica from each sample into separate 1.7-mL microcentrifuge tubes and add 1 mL ethanol in ethyl acetate (15%, v/v). Vortex vigorously for

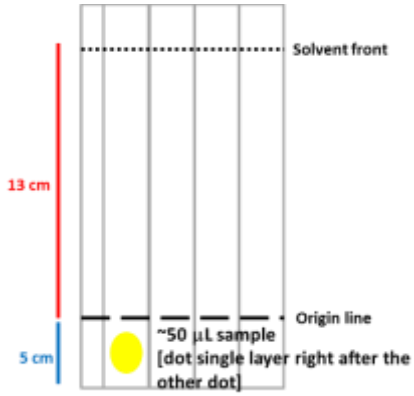

Figure 3.2. TLC plate set-up. Solvent front should be marked lightly in pencil 13 cm from origin line (top of the pre-adsorbent zone) on a silica TLC plate. Sample is applied in separate lanes in the pre-adsorbent zone.

10-30 s to extract analytes from the silica, then centrifuge in a table-top centrifuge at maximum speed (126,600 x g) for 2 minutes at room temperature. Carefully transfer ethanol in ethyl acetate and place in a new 1.7-mL microcentrifuge tube. Silica may affect instrument sensitivity; therefore

take care not to disrupt the silica pellet in the bottom of the tube. Dry under nitrogen in the analytical evaporation unit in a fume hood.Formation of trimethyl silyl ether derivatives

Once dried, dissolve samples in 20 μL bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 6 μL dry dimethylformamide (DMF). This reaction converts analytes to the trimethylsilyl ether derivatives. Vortex well and incubate sample at 37 °C for 5 min. Dry reagents under nitrogen in the analytical evaporation unit.

Quantification of F_3-IsoPs by GC-MS

Resuspend sample in 20 μL dry undecane and vortex briefly. Transfer sample to an autosampler vial for GC/MS analysis. GC-NICI-MS approach employing stable isotope dilution will be utilized for F_3-IsoP quantification. The major ions generated in the NICI mass spectra of the pentafluorobenzyl ester, tristrimethylsilyl ether derivatives of F_3-IsoP are *m/z* 567 and corresponding ion for the $[^2H_4]$15-F_{2t}-IsoP internal standard, *m/z* 573. To quantify, the height of the peak containing the derivatized F_3-IsoP is compared to the height of the deuterated internal standard peak. Levels of F_3-IsoPs in nematodes are reported in nanograms per gram of total protein content.

Commentary

Background information

Given the amount of evidence implicating oxidative stress in the etiology of cancer, neurodegenerative disease, and aging, studying the role of oxidative damage will broaden knowledge of disease. In order to collect a comprehensive examination of the role of oxidative stress in disease, methods to directly quantify the amount of oxidative damage and ROS generation are in demand.

Fluorescent or chemiluminescent dyes, such as dichlorodihydrofluorescein (DCFH-DA), dihydroethidium, and dihydrorhodamine are used frequently for measuring H_2O_2, $O_2^{\bullet-}$ and $ONOO^-$ either in whole worm or isolated mitochondria (Vanfleteren 1993, Schulz, Zarse et al. 2007, Dingley, Polyak et al. 2010). The most prevalently used fluorescent probe for detecting intracellular H_2O_2 and oxidative stress is DCFH-DA. DCFH-DA is cell-permeable and is hydrolyzed intracellularly to the DCFH carboxylate anion, which is retained in the cell. Two-electron oxidation of DCFH forms the fluorescent product, dichlorofluorescein (DCF), which can be monitored by several fluorescence-based techniques, such as fluorescent microplate readers, confocal microscopy, and flow cytometry. Despite the user-friendly appeal of DCFH-DA dye, there are several limitations and artifacts associated with the DCF assay due to the complex intracellular redox chemistry of DCFH (Hempel, Buettner et al. 1999, Burkitt and Wardman 2001, Bonini, Rota et al. 2006). A serious caveat to consider is the indirect nature of DCF fluorescence; DCF is not formed through the direct reaction between DCFH and H_2O_2. Therefore, DCF fluorescence is not a direct measure of H_2O_2 levels in the worm. In addition, several oxidizing species will oxidize DCFH to DCF, making the dye nonspecific for a particular form of ROS. And lastly, the intermediate radical, $DCF^{\bullet-}$, rapidly reacts with O_2 to form $O_2^{\bullet-}$. The dismutation of $O_2^{\bullet-}$ yields additional H_2O_2 (Folkes, Patel et al. 2009), which can establish a redox-cycling mechanism leading to artificial amplification of fluorescence signal intensity. Therefore, it is important to recognize the limitation of the dyes and avoid erroneous interpretations; DCFH-DA cannot reliably quantify intracellular H_2O_2 and ROS as a measure of oxidative stress.

As previously discussed, real-time quantification of ROS in living organisms is limited with redox-sensitive probes due to the non-specific and disruptive nature of the fluorescent dyes. To overcome the limitation of the dyes, recent advances in constructing genetically encoded redox-

sensitive sensors allows for new avenues for investigating redox signaling (Back, De Vos et al. 2012, Knoefler, Thamsen et al. 2012). Use of redox sensors via either a hydrogen peroxide sensor protein coupled with a glutathione redox potential sensor (HyPer and Grx1-roGFP2, respectively) (Back, De Vos et al. 2012) or quantitative redox proteomics (OxICAT) (Knoefler, Thamsen et al. 2012) can overcome limitations of the fluorescent dye to provide quantification of ROS in an intact organism. HyPer and Grx1-roGFP2 are ratiometric biosensors that can be used to determine the oxidized-to-reduced ratios of H_2O_2 and GSSG/2GSH. OxICAT is a redox proteomic technique, which monitors the oxidation status of various redox-sensitive protein thiols with a thiol-reactive affinity tag (ICAT). The use of genetically encoded redox-sensitive sensors and redox proteomics provide efficient methods to study ROS generation.

The discovery of isoprostanes as products of non-enzymatic lipid peroxidation has been a major breakthrough in the field of free radical chemistry. The quantification of F_2-IsoPs and related analogues has extended our understanding the role of free radicals in the physiological processes and has established the occurrence of oxidative stress in a wide variety of disease states. As stated previously, lipids react readily with free radicals, resulting in production of stable peroxidation products. F_2-IsoPs and related analogues form *in situ* in the phospholipid bilayer, which then gets released in free form upon hydrolysis by phospholipases, presumably by the action of phospholipase A_2 (Morrow, Awad et al. 1992) and in part, by platelet-activation factor (PAF) acetylhydrolysases I and II (Stafforini, Sheller et al. 2006). F_2-IsoPs are stable, robust molecules detectable tissues and biological fluids, including plasma, urine, cerebrospinal fluid, and bile (Morrow, Zackert et al. 1999). Studies have shown that quantification of isoprostanes in either plasma urine is representative of their endogenous production, therefore measuring lipid peroxidation via IsoP quantification is an accurate index of *in vivo* oxidative stress (Kadiiska, Basu

et al. 2013). As EPA is the most abundant PUFA in *C. elegans*, quantifying the resultant free-radical catalyzed oxidation product of EPA, the F_3-IsoPs, is a highly sensitive and accurate representation of endogenous oxidant damage in the worm.

Numerous approaches have been developed to quantify IsoPs either through use of mass spectrometry (Morrow and Roberts 1999, Song, Paschos et al. 2009, Labuschagne, Stigter et al. 2013) or immunological approaches (Sodergren, Vessby et al. 2000). The methodology described herein uses a gas chromatography – negative ion chemical ionization – mass spectrometry (GC-NICI-MS) approach, where F_3-IsoPs are quantified through measurement of the 15-F_{3t}-IsoP. The advantages of this technique over other methods include its sensitivity, specificity, and reproducibility, with quantitative yields in the low picogram range. One drawback to this particular method is the labor-intensive nature and requirement for considerable equipment expenditures. Several alternative mass spectrometric assays have been developed to quantify F_3-IsoPs; Labuschagne and colleagues developed a liquid chromatography – mass spectrometry assay to measure F_3-IsoPs in *C. elegans* (Labuschagne, Stigter et al. 2013*)*. An advantage to the LC-MS method is that the sample preparation for LC-MS analysis does not require derivatization of the molecule, and therefore is simpler than sample preparation for GC-MS. One drawback to the LC-MS assay is the higher limits of detection in contrast to the more highly sensitive nature of GC-MS. Other methods to quantify IsoPs involve immunological approaches, employing antibodies against 15-F_{2t}-IsoP, with at least three immunoassay kits available commercially (Sodergren, Vessby et al. 2000). The disadvantage of this assay is that precision and accuracy are limited. The sensitivity and/or specificity of these kits might vary substantially between manufacturers. In addition, currently there is a lack of commercially available immunoassay kits for detecting F_3-IsoPs.

In conclusion, we present a robust GC/MS approach for accurate, sensitive and selective quantification of endogenous oxidative damage in *C. elegans*. The equipment used for purification, derivatization and GC/MS analysis is readily available in most pharmacology laboratories. Robust quantification of F_3-IsoPs can be detected for a wide-range of pathologies affected by ROS, therefore our method should be applicable to studying oxidative damage in the development of pathologies and aging.

Critical parameters

As F_3-IsoPs can be readily generated *ex vivo* in biological materials containing polyunsaturated fatty acids, it is imperative to take care when the samples are obtained and during processing to prevent *ex vivo* oxidation, which can generate IsoPs. For best results, samples should be flash frozen in liquid nitrogen immediately upon collection and not thawed until sample preparation and analysis. Samples should be stored at -70°C until analysis.

Troubleshooting

For troubleshooting, if the peak signal is extremely low or if no peaks are detected by the mass spectrometer, the sample should be removed from the autosampler vial, dried thoroughly under nitrogen, and steps for "formation of trimethylsilyl ether derivatives" should be repeated. If peaks are still not detected, another likely point of error would be problems with the thin-layer chromatography. Scrape all silica from the bottom half of sample lane, place in a microcentrifuge tube, and extract with ethyl acetate as described earlier. Continue by finishing the assay as described above. If no result is obtained, it will be necessary to repeat the entire analysis with a new sample. If the internal standard is detected with a strong signal at *m/z* 573 but there are very low or nonexistent peaks at *m/z* 567, then the level of F_3-IsoPs is below the limit of detection. The limit of detection for this assay is 5 pg.

Anticipated results

A representative chromatogram obtained from the analysis of F_3-IsoPs in *C. elegans* is shown in Fig. 3.3. The peaks in upper panel of Fig. 3.3 represent endogenous F_3-IsoPs in worm lysates. The

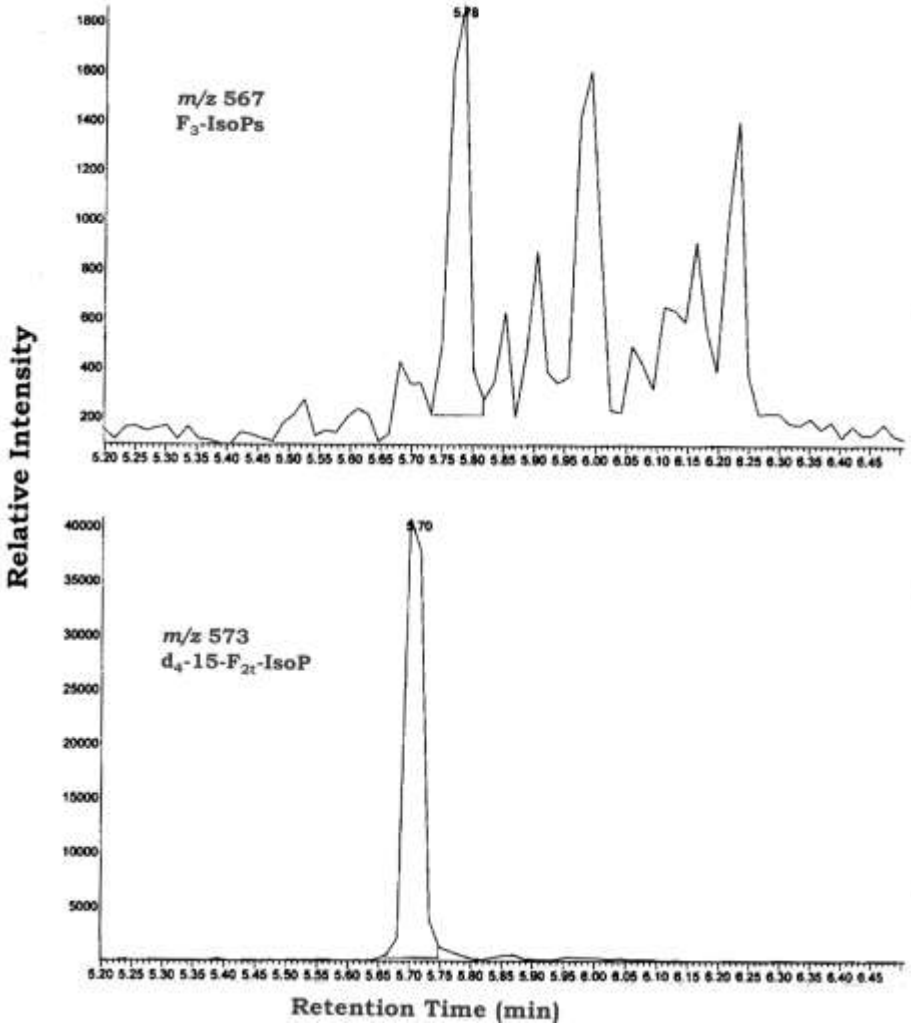

Figure 3.3. Representative chromatogram showing the elution of endogenous F3-IsoPs in *C. elegans* lysates. A representative selective ion monitoring chromatogram obtained from the analysis of F3-IsoPs in *C. elegans* lysates is shown in the top panel. The chromatogram peak in the lower *m/z* 573 ion current chromatogram represents the internal standard d_4-15-F_{2t}-IsoP. The series of peaks in the upper *m/z* 567 ion current chromatogram represent putative F_3-IsoPs eluting over approximately a 1-min interval. These compounds possess a molecular mass predicted for F_3-IsoPs and elute at the predicted retention time. F_3-IsoPs are predicted to elute at later retention times than the deuterated 15-F_{2t}-IsoP internal standard due to F_3-IsoPs containing one more double bond than F_2-IsoPs. The concentration of F_3-IsoPs is calculated using the ratio of the intensity of this peak to that of the internal standards. Levels of F_3-IsoPs are reported in picograms per milligram of protein content.

single peak in the lower *m/z* 573 chromatogram represents the [$^2H_4$]-15-F_{2t}-IsoP internal standard. For quantification purposes, the height of the peak containing the derivatized F_3-IsoP (*m/z* 567) is compared to the height of the deuterated internal standard peak (*m/z* 573). Levels of F_3-IsoPs in whole worm lysates are reported in picograms per milligram of total worm protein.

Time considerations

In general, 12-15 samples can be assayed in approximately 10 hrs by an experienced investigator. Homogenization, lipid extraction, and hydrolysis of this number of worm samples requires ~3 hrs; Sep-Pak purification takes ~2 hrs; drying, derivatization, and TLC purification requires ~3 hrs; and extraction, drying, and silyation requires ~2 hrs. Although this method is more time intensive when compared to other assays of oxidative stress, quantifying F_3-IsoPs through GC/MS analysis results in an accurate, sensitive, and linear assessment of endogenous oxidative damage.

Reagents and Solutions

85 mM NaCl
10 g NaCl
2 liter H_2O
Autoclave 45 min
Store up to 1 year at 4°C

pH 3 Water
500 ml H_2O
500 µL 1 N HCl
Store up to 1 year at 23°C

15% wt/v KOH
15 g KOH
100 ml H_2O
Store up to 1 year at 23°C

10% v/v PFBB
1 ml PFBB
9 ml Dry acetonitrile
Store over calcium hydride to keep solution free of water.

Methanol containing BHT (0.005% v/v)
5 mg BHT
100 ml methanol
Store up to 1 year at 23°C in an amber-colored bottle

10% v/v DIPE
1 ml DIPE
9 ml Dry acetonitrile
Store over calcium hydride to keep solution free of water.

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CHAPTER IV

SCAVENGERS OF REACTIVE γ-KETOALDEHYDES EXTEND *Caenorhabditis elegans* LIFESPAN AND HEALTHSPAN THROUGH PROTEIN-LEVEL INTERACTIONS WITH SIR-2.1 AND ETS-7[4]

Introduction

Peroxidation of polyunsaturated fatty acids is a hallmark of oxidative stress, in part due to their susceptibility to free radical attack (Gardner 1989, Niki 2009). Accumulation of lipid peroxidation products has been implicated in the pathogenesis of a number of human diseases, such as atherosclerosis, cancer, and neurodegenerative diseases (Halliwell and Gutteridge 1990, Gutteridge 1995, Butterfield 1997). This phenomenon plays a critical role in the propagation of oxidative damage and in cell death cascades, in part through the formation of reactive aldehydes (Poon, Calabrese et al. 2004). These secondary products of lipid peroxidation, which include malondialdehyde (MDA) and the reactive hydroxyl-alkenals, are known to contribute to and partially mediate the effects of lipid peroxidation (Comporti 1998, Poon, Calabrese et al. 2004).

Recent work at Vanderbilt has identified highly reactive levuglandin-like γ-ketoaldehydes (γ-KA, or isoketals, IsoK) comprised of 64 regio- and stereo-isomers. Isoketals are formed as products of the isoprostane pathway via rearrangement of prostaglandin H_2-like endoperoxide intermediates (H_2-isoprostanes) (Morrow, Hill et al. 1990, Brame, Salomon et al. 1999). IsoKs covalently adduct ε-amino groups in lysyl residues of proteins to form stable adducts (structurally

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characterized as lactam rings) and intramolecular cross-links (Iyer, Ghosh et al. 1989, Boutaud, Brame et al. 1999, Brame, Salomon et al. 1999). IsoK-lysyl-lactam adducts have been shown to be significantly increased in atherosclerosis, end-stage renal disease, Alzheimer's disease, and as a significant contributing cause of hypertension (Salomon, Batyreva et al. 2000, Zagol-Ikapitte, Masterson et al. 2005, Kirabo, Fontana et al. 2014). While the potent cytotoxicity of IsoKs and their ability to induce protein aggregation and to disrupt enzymatic function indicate a strong pathologic potential (Murthi, Salomon et al. 1990, Schmidley, Dadson et al. 1992, Boutaud, Ou et al. 2002, Davies, Amarnath et al. 2002), meaningful investigation into the extent to which formation of IsoK adducts on proteins contributes to disease requires methods to selectively reduce the formation of IsoK adducts *in vivo*.

To better define the biological role of isoketals in oxidative injury and potentially prevent their detrimental effects, studies were performed to identify selective scavengers of IsoKs. A lead compound, pyridoxamine (PM) was identified through initial screens (Amarnath, Amarnath et al. 2004). Structure-activity relationship studies identified the critical moiety to be a phenolic amine with the hydroxyl group adjacent to the methyl amine. Therefore, other phenolic amines such as salicylamine (SA) are similarly potent and as selective as PM for scavenging isoketals, but are more lipophilic. SA protects cellular viability in intact cells exposed to hydrogen peroxide, with SA pre-treatment leading to 5% occurrence in cell death, compared to 95% cell death in vehicle control treated cells, which suggests that IsoKs are major effectors of oxidative mediated death (Davies, Brantley et al. 2006). SA is orally bioavailable (Zagol-Ikapitte, Matafonova et al. 2010), and administering SA in mice prevents the age-related loss of working memory and the development of angiotensin II-induce hypertension (Davies, Bodine et al. 2011, Kirabo, Fontana et al. 2014). Although preventing IsoKs from adducting to proteins has broad and remarkable

beneficial biological effects, the precise molecular processes that are being altered by the IsoK scavengers are not clearly defined. More precisely, the potential role that IsoKs may play in the aging process, and how IsoK scavengers may be able to influence normal aging, is an open question currently.

Aging is characterized by progressively diminishing function at the molecular, cellular, tissue, and whole organism levels (Kirkwood 2005, Jin 2010, Rose, Flatt et al. 2012, Wilkinson, Taylor et al. 2012). The overall results are a gradual decline in the capacity to respond to environmental challenges and an increasing vulnerability to disease and death (Haigis and Yankner 2010, Flatt 2012, Murshid, Eguchi et al. 2013). The mechanisms contributing to the multi-level, multisystem changes that we recognize as aging are a matter of fairly intense debate. Broadly, mechanisms underlying aging can be divided into theories of programmed aging (Lockshin and Beaulaton 1974, Forciea 1989) and theories of cumulative damage (Gladyshev 2014) and failed homeostasis (Comfort 1968), though it must be recognized that the two may be related (Hartl 2016). At least for the cumulative damage/failed homeostasis hypotheses (e.g., "rate of living"/metabolic theory (Lints 1989, Brys, Vanfleteren et al. 2007), free radical theory (Harman 1956), failed proteostasis (Labbadia and Morimoto 2015), cumulative DNA damage (Maynard, Fang et al. 2015), etc.), pathways controlling molecular metabolism and redox homeostasis repeatedly emerge as being central to the aging process (Hartl 2016). One of the best studied pathways lying at the intersection of metabolic control, redox regulation, and aging is the sirtuin pathway.

Sirtuins are a highly conserved family of proteins that play major roles in adapting physiology to dietary extremes, as well as being implicated in countering aging and diseases associated with aging (Lin, Defossez et al. 2000, Donmez and Guarente 2010, Bell and Guarente

2011). Sirtuins are nicotinamide adenine dinucleotide (NAD^+)-dependent protein deacetylases and/or ADP-ribosyltransferases. Due to the requirement of NAD for biochemical activity, sirtuins sense and respond to the metabolic status of the cell. Indeed, this is thought to be a key mechanism by which caloric restriction extends natural lifespan – namely, by increasing NAD^+ availability, which increases sirtuin activity. Numerous studies in model organisms, including yeast, worms, and flies, suggest that manipulation of sirtuin Sir2 (silent information regulator 2) and its homologs can extend lifespan (Kaeberlein, McVey et al. 1999, Guarente 2000, Lin, Defossez et al. 2000, Tissenbaum and Guarente 2001, Rogina and Helfand 2004). Over-expression of the closest homolog to yeast Sir2 in *C. elegans*, *sir-2.1*, leads to extension of lifespan, and deletion or knockdown of the gene shortens lifespan (Tissenbaum and Guarente 2001, Lee, Wilson et al. 2006, Wang and Tissenbaum 2006, Hashimoto, Ookuma et al. 2009). Although it is a family of seven mammalian sirtuins (SIRT1-7) that play various roles in the regulation of stress resistance, metabolism, and cell survival, their roles in the regulation of mammalian lifespan are still unresolved. Despite the uncertainty, many studies suggest that sirtuins are a linchpin, regulating multiple homeostasis and stress response pathways – antioxidant defenses, energy metabolism, mitochondrial genomic maintenance, and others – that contribute to aging and age-related diseases.

In the present study, we tested the hypothesis that age-related oxidant injury and accumulation of IsoKs leads to adduction and inactivation of key proteins that regulate lifespan and healthspan. Specifically, we hypothesized that SIR-2.1 and downstream targets are inactivated by IsoK adduction, and that treatment with a scavenger of IsoKs would preserve protein function, extend natural lifespan and diminish degenerative changes in physical health. We found that treating wild-type Bristol N2 with SA, a potent scavenger of IsoKs, significantly extended lifespan and healthspan. We showed that SA's effects on longevity are dependent upon SIR-2.1, as

knocking out its protein biochemical function abolished drug effect. In subsequent experiments, we have defined a SIR-2.1/*ets-7* axis that is preserved by SA to regulate lifespan and healthspan along with classical markers of aging and oxidant injury, largely through maintaining proteostasis.

Materials & Methods

C. elegans Strains and Maintenance

C. elegans strains were cultured at 20°C on standard nematode growth media (NGM) agar plates seeded with *Escherichia coli* strain NA22. The following strains were used in this work: wild-type *C. elegans* Bristol strain (N2), *sir-2.1(ok434) IV, F19F10.5(ok888) V,* and *daf-16(mu86).* Strains were obtained from the *Caenorhabditis* Genetics Center (University of Minnesota, St. Paul, MN). For generating cultures of 15-day-old (Day 15) adult worms, synchronized late-stage L4s/early young adult worms (Lewis and Fleming 1995) were transferred to peptone enriched 15 cm plates containing UV-irradiated OP50 *E. coli* and 0.12 mM 5-fluoro-2'-deoxyuridine (FUDR) to inhibit progeny production(Mitchell, Stiles et al. 1979) with or without drug until harvest.

Salicylamine Exposure

Nematodes grown on NGM-agar plates containing 0.5% peptone, were harvested, and eggs were isolated by alkaline hypochlorite with 0.5 N NaOH, 1% hypochlorite; 8 min at 23°C. The recovered eggs were rinsed in M9 buffer and placed on fresh agar plates seeded with *E. coli* strain OP50 and maintained at 20°C until late-L4/young adult stage. After the late L4/young adult molt, worms were transferred to peptone enriched 15 cm plates containing 0.12 mM FUDR, OP50 *E. coli*, and varying concentrations of SA. Salicylamine drug plates were made fresh before transfer by spreading SA on top of the agar and plates were allowed to dry. *E. coli* strain OP50 was exposed to UV radiation for 30 minutes to kill the bacteria before seeding onto the SA-FUDR NGM agar

plates. Worms were exposed to SA throughout its life until harvest by transferring worms to fresh SA-FUDR-OP50 NGM plates every other day.

Longevity Assays

Survival cultures were grown on 60-mm agar plates; after the late-stage L4/young adult molt, approximately 100 adults were transferred onto SA-OP50-seeded NGM plates. Salicylamine drug plates were made fresh before transfer by spreading SA on top of the agar. Plates were allowed to dry before seeding with UV-irradiated OP50 bacteria. Worms were maintained at 20°C and live worms were counted during transfer to freshly made SA-OP50-NGM agar plates every 2-3 days. Survival was scored as movement upon slight touch with the platinum wire. Worms were maintained until death.

Autofluorescence Measurement

Synchronized late L4/early young adult worms were plated on FUDR containing SA-OP50-seeded NGM plates and worms were maintained at 20°C. Every fifth day, 10-15 worms were mounted onto 2% agar pads and anesthetized with 3 mM levamisole in DMSO. Images were taken at 250-ms exposure under a DAPI filter using an epifluorescence microscope (Nikon Eclipse 80i) equipped with a Lambda LS Xenon lamp (Sutter Instrument Company) and Nikon Plan Fluor 20x dry and Nikon Plan Apo 60x 1.3 oil objectives. The fluorescence was calculated using ImageJ software(Gavet and Pines 2010).

Pharyngeal Pumping

C. elegans pharyngeal pumping rate assays were performed on 60-mm agar plates with bacterial lawns at room temperature. Every fifth day, worms were transferred to fresh bacteria-seeded NGM plates, and incubated at 25°C for 10 min in order to equilibrate feeding rates before measurement.

After 10 min incubation, worms were observed under the Zeiss TLB 3.1 microscope with focus on the pharynx. The number of contractions in the terminal bulb of the pharynx was counted for 20 s and then plotted.

Oxygen Consumption Analysis

Oxygen consumption rate for whole *C. elegans* was measured using a Seahorse Bioscience XF^{e}96 Analyzer. Worms were harvested from Day 0, 2, and 15 colonies maintained on FUDR containing SA-OP50-seeded NGM plates by washing in M9 medium, followed by floatation on an ice-cold 60% w/v sucrose gradient to segregate clean bacteria-free adult worms from bacterial debris. Worms were seeded at 1,000 worms/well in M9. After 20 min equilibration, a 2-min measurement was performed to obtain basal OCR for all experimental conditions and strains.

Genome Copy Number Analysis

Relative mitochondrial and nuclear copy number were measured by quantitative, real-time PCR (Bratic, Hench et al. 2009). Primers for NADH dehydrogenase unit 1 (*nd1*) and a 164bp region of the *cox-4* gene were used in determination of mtDNA copy number. The *nd1* forward primer 5' – AGCGTCATTTATTGGGAAGAAGAC – 3' and reverse primer 5' AAGCTTGTGCTAATCCCATAAATGT – 3'. *Cox-4* forward primer 5' – GCC GAC TGG AAG AAC TTG TC – 3' and reverse primer 5' – GCGGAGATCACCTTCCAGTA – 3'. Real-time PCR conditions were 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, and 60 s at 63°C. Amplified products were detected with SYBR Green (iQ™ SYBR® Green Supermix, Bio-Rad) and fluorescent signal intensities were determined by CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad) by software CFX Manager™ (version 3.1). Crude worm lysate was harvested from Day 0, 2, and 15 stage nematodes grown on FUDR containing SA-OP50-seeded

NGM plates and used as template DNA for real-time PCR based determination of mtDNA and nucDNA copy numbers.

NAD^+-dependent Deacetylation in Bioluminescence Assay

Relative activity of the NAD+-dependent histone deacetylase (HDAC) class III enzymes (sirtuins) was measured using the SIRT-Glo™ Assay and Screening System (Promega Corporation, Madison, WI) according to the manufacturer's instructions with minor modifications. This assay uses an acetylated, luminogenic peptide substrate that can be deacetylated by SIRT activities. Deacetylation of the peptide substrate is measured using a coupled enzymatic system with a protease in the reagent provided and then cleaves the luminogenic peptide to liberate aminoluciferin. Free aminoluciferin can be quantified using the Ultra-Glo™ firefly luciferase reaction to produce a stable, persistent emission of light. Purified recombinant human SIRT1 (R&D Systems, Biotechne) activity was assayed in HEPES-buffered saline (10 mM HEPES, 150 nM NaCl, 2 mM $MgCl_2$) in the presence and absence of 15-E_2-IsoK. 15-E_2-IsoK was synthesized by the method of Armanath et al (Amarnath, Amarnath et al. 2005). Luminescence was detected by a microplate reader (FLUOstar Optima microplate reader, BMG Labtechnologies).

Sample preparation and detection of endogenous F_3-IsoPs by GC/MS

F_3-Isoprostanes were quantified from SA-treated worms using a gas-chromatography-negative ion chemical ionization-mass spectrometry (GC-NICI-MS) approach (Milne, Sanchez et al. 2007). Worms maintained on FUDR containing SA-OP50-seeded NGM plates were harvested at Day 15 by washing in M9 medium, followed by floatation on an ice-cold 60% w/v sucrose gradient to segregate clean bacteria-free adult worms from bacterial debris. Clean worms were transferred to Eppendorf tubes and homogenized using the Mini-Beadbeater-24® (BioSpec, Bartlesville, OK) with zirconium oxide beads (1.0 mm), at 4°C. Homogenates were then hydrolyzed by 15% w/v

KOH, containing 57 µM BHT (5% w/v BHT:MeOH) for 30 min at 37°C. Next, samples were centrifuged at max speed to pellet worm debris and supernatant was transferred to a 16-mL polypropylene tubes (Denville Scientific, Inc., Holliston, MA).

Samples were spiked with 248 pg of deuterated internal standards, [$^2H_4$]-15-F_{2t}-IsoP, quantified and calibrated by the method of Milne et al. (Milne, Sanchez et al. 2007) and acidified to pH < 3 with HCl in preparation for further Separation Phase Extraction (SPE). C_{18} Sep-Pak cartridges (Waters, Milford, MA) were preconditioned with 5 mL of MeOH, followed by 5 mL of pH 3 water and subjected to vacuum to obtain a flow rate of 1 mL/min. Samples were applied to the cartridges and allowed to flow through completely before adding equal volume of pH 3 water and heptane to wash columns before eluting with ethyl acetate: heptane (1:1 v:v). Anhydrous sodium sulfate was then added to each sample to absorb excess water from samples and then applied to silica Sep-Pak cartridges (Waters, Milford, MA) preconditioned with ethyl acetate. Samples were transferred to the silica Sep-Pak columns and allowed to pass through before washing with ethyl acetate, and eluted with ethyl acetate: MeOH (45:55 v:v).

Eluates were dried under nitrogen and F_3-IsoPs and resuspended in MeOH for separation by Thin Layer Chromatography (TLC). The free acid TLC standard, 8-iso-Prostaglandin $F_{2\alpha}$ methyl ester (8-iso-$PGF_{2\alpha}$, Cayman Chemicals, Ann Arbor, MI) and samples were spotted on pre-washed silica TLC plates, placed in a TLC tank containing chloroform: MeOH: Acetic acid (84.5:14.5:1 v:v:v), and allowed to run until reaching solvent front. The free acid TLC standard was visualized by spraying standard plate with phosphomolybdic acid solution, and samples were scraped from the TLC plate in the region of the TLC standard (R_f ~ 0.35). Samples were extracted from silica by resuspension in ethyl acetate: EtOH (1:1 v:v) and dried under nitrogen. All steps from this point followed the F_3-IsoP measurement protocol as described by the method of Nguyen

et al. (Nguyen and Aschner 2014). Deuterated F_2-IsoP standard was measured at *m/z* 573. F_3-IsoP was measured at *m/z* 567. Endogenous F_3-IsoP levels were quantified by comparing the height of the peak containing the derivatized F_3-IsoP to the height of the deuterated internal standard peak.

Protein concentration of nematode homogenates were determined using the bicinchoninic acid (BCA) protein assay as described by the manufacturer (Pierce Protein Biology, Waltham, MA).

Quantification of isoketal protein adducts using LC/MS

Worms grown on FUDR containing SA-OP50-seeded NGM plates were collected at Day 15 adult stage by washing in M9 medium, followed by an ice-cold 60% w/v sucrose gradient to segregate clean bacteria-free adult worms from bacterial debris. Clean worms were transferred to Eppendorf tubes and flash-frozen in liquid nitrogen and thawed at 37°C 3x. Samples were homogenized using a handheld homogenizer (Polytron PT 1200E, KINEMATICA AG), in buffer containing antioxidants (100 µM indomethacin, 220 µM butylated hydroxytoluene, and 5 mM triphenylphosphine) and 100 µM pyridoxamine dihydrochloride to prevent artifactural generation of IsoK protein adducts during sample processing. Levels of IsoK-lysyl-lactam adduct was measured as previously described (Davies, Amarnath et al. 2007).

In brief, IsoK protein adducts are measured after enzymatic proteolysis and separation as IsoK-lysyl-lactam adducts by liquid chromatography tandem mass spectrometry (LC/MS/MS) using a heavy isotope labeled internal standard for quantification. Samples were treated with 15% KOH to hydrolyze esterified isoketals and then subjected to complete proteolytic digestion using pronase protease (*Streptomyces griseus*, Calbiochem, San Diego, CA) and aminopeptidase M (Calbiochem, San Diego, CA), consecutively, to release the IsoK-lysyl-lactam adduct. After digestion, 500 pg of a ($^{13}C_6$)-IsoK-lysyl-lactam internal standard was added to each sample,

followed by partial purification of lysyl adducts by solid-phase extraction (SPE) and further purification by preparative HPLC (2690 Alliance HPLC system, Waters, Milford, MA). Isok-lysyl-lactam adducts were then quantified by selective reaction monitoring LC electrospray tandem mass spectrometry for transition from *m/z* 477 → 84 and *m/z* 487 → 90 for internal standard (ThermoFinnigan Surveyer MS pump coupled to TSQ quantum triple-quadrupole mass spectrometer, Thermo Fischer Scientific, Waltham, MA).

Protein concentration of nematode homogenates were determined using the Thermo Scientific Pierce BCA Protein Assay as described by the manufacturer (Pierce Protein Biology, Waltham, MA).

Western Blot

Day 15 adult worms grown on FUDR containing SA-OP50-seeded NGM plates were harvested in M9 medium, followed by floatation on an ice-cold 60% w/v sucrose gradient to segregate clean bacteria-free adult worms from bacterial debris. Clean worms were transferred to Eppendorf tubes containing radioimmunoprecipitation assay (RIPA) buffer with protease inhibitor, trichostatin A, nicotinamide, and phosphatase inhibitors and flash-frozen in liquid nitrogen and thawed at 37°C 3x. Twenty to thirty µg of protein were loaded onto a 10% SDS-PAGE acrylamide gel. Proteins were electroblotted onto nitrocellulose membranes, blocked with 0.1% Tween PBS with 5% nonfat milk and 0.05% sodium azide, and western blots were performed with the primary antibodies anti-MnSOD (ab13533, AbCam, Cambridge, MA), anti-acetyl-lysine 122 MnSOD (a generous gift of D.R. Gius, Northwestern University at Chicago, IL, USA; Epitomics, Inc, Burlingame, CA), anti-acetyl-lysine 68 MnSOD (a generous gift of D.R. Gius, Northwestern University at Chicago, IL, USA;Epitomics, Inc, Burlingame, CA), and anti-β-actin (A5316, Sigma, St. Louis, MO). Proteins were visualized by species-appropriate secondary antibodies labeled with horseradish peroxidase

(Santa Cruz Biotechnology, Dallas, TX) and chemiluminescent substrate (Amersham ECL Prime Western Blotting Detection Reagent, GE Healthcare, Pittsburgh, PA). Densitometry was obtained with ImageJ.

Microarray Analyses

Total RNA was isolated via the Trizol method. Worms maintained on FUDR containing SA-OP50-seeded NGM plates were harvested at Day 15 by washing in M9 medium, followed by floatation on an ice-cold 60% w/v sucrose gradient to segregate clean bacteria-free adult worms from bacterial debris. Clean worms were transferred to Eppendorf tubes containing Trizol (Life Technologies) and then snap-frozen in liquid nitrogen and thawed at 37°C 3x. Chloroform was added to each sample, followed by precipitation using isopropanol and washing with 75% ethanol. The supernatant was then transferred to an RNeasy MinElute (Qiagen Inc., Valencia, CA) spin column and all steps from this point followed the RNA purification protocols described in the manufacturer's instructions.

This mixture was then vortexed and transferred to a Shredder Column (Qiagen Inc., Valencia, CA) and centrifuged. Eluate from the Shredder Column was transferred to a Preclear Column contained in the Versagene Kit and all steps following protocols described in the kit manual. After isolation, total RNA was reverse transcribed to double-stranded cDNA, amplified, labeled, and fragmented using the NuGEN Ovulation Biotin Kit (San Carlos, CA). Fragmentation was confirmed using an Agilent Bioanazlyer 2100 (Santa Clara, CA) and fragmented, labeled product was hybridized to an Affymetrix *C. elegans* Gene 1.0 ST GeneChip (Santa Clara, CA) according to the manufacturer's protocols.

Microarray data analysis was performed on arrays normalized by Robust Multi-chip Analysis (RMA). The quality controls on samples and on probe sets were performed stepwise to

detect the outlying samples and poor probe sets. The Principal Components Analysis (PCA) score plot and hybridization controls plot were applied for sample detection, with at least one sample with log2(expression)>7. Filtering for high-quality data resulted in 109 genes with at least 25% change in expression, which were defined as salicylamine responsive genes. Independent validation of microarray results was performed by examining changes in mRNA expression using RT-PCR methods as described below.

TaqMan Gene Expression Assay

Total RNA was isolated via the Trizol method, as described previously. Following isolation, 2 µg total RNA was used for cDNA synthesis using the High Capacity cDNA Reverse Transcription Kit (Life Technologies), per manufacturer's instructions. Quantitative real time PCR (Bio-Rad) was conducted using TaqMan Gene Expression Assay Probes (Life Technologies) for each gene. Amplified products was normalized to housekeeping gene, *ama-1* (RNA polymerase II) after determining fold difference using the comparative $2^{-\Delta\Delta Ct}$ method (Latif, Bauer-Sardina et al. 2001). The following probes were used: *ama-1* (Assay ID: Ce2462269_m1), *ets-7* (Ce02477624_g1), *F13D12.6* (Ce02439540_m1), *siah-1* (Assay ID: Ce02462269_m1), and *sma-4* (Assay ID: Ce202447346_g1).

Statistics

All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, Inc.). Concentration response curves were generated using a sigmoidal dose-response model with a top constraint at 100%. Statistical significance of the lifespan experiments were assessed using Mantel-Cox log-rank test, a nonparametric measure that assesses differences in entire survival curves. Comparisons between two groups were performed using a two-tailed Student's *t*-test assuming equal variances. Multiple group comparisons at different time points was done using

two-way ANOVA with repeated measures, followed by Bonferroni's multiple comparison post-hoc tests. Values of $P < 0.05$ were considered statistically significant.

Results

Salicylamine increases lifespan and healthspan of wild-type adult *C. elegans*

The oxidative stress theory of aging postulates that ROS formed by normal metabolic processes play a role in the aging process (Harman 1956). The imbalance between pro-oxidants and antioxidants leads to an accumulation of oxidative damage in a variety of macromolecules with age, resulting in a progressive loss in functional cellular processes. Given our observation that the reactive γ-ketoaldehydes termed IsoKs play a critical role in oxidative injury by adducting to and inactivating multiple proteins, we postulated that SA administration would extend natural lifespan by scavenging IsoKs and preventing age-related inactivation of key protein targets. Starting at Day 1 of adulthood, N2 WT *C. elegans* were continuously exposed to increasing concentrations of SA until natural death (Fig. 4.1A). SA produced a significant dose-dependent increase in median lifespan (Fig. 4.1B), with 50 µM increasing median lifespan by 18% from 16 days to 19 days, 100 µM increasing lifespan by 32% from 16 days to 21 days ($p < 0.05$), and 500 µM increasing median lifespan by 56% from 16 days to 25 days ($p < 0.01$). These data show a significant lifespan extension effect of SA in adult WT *C. elegans*.

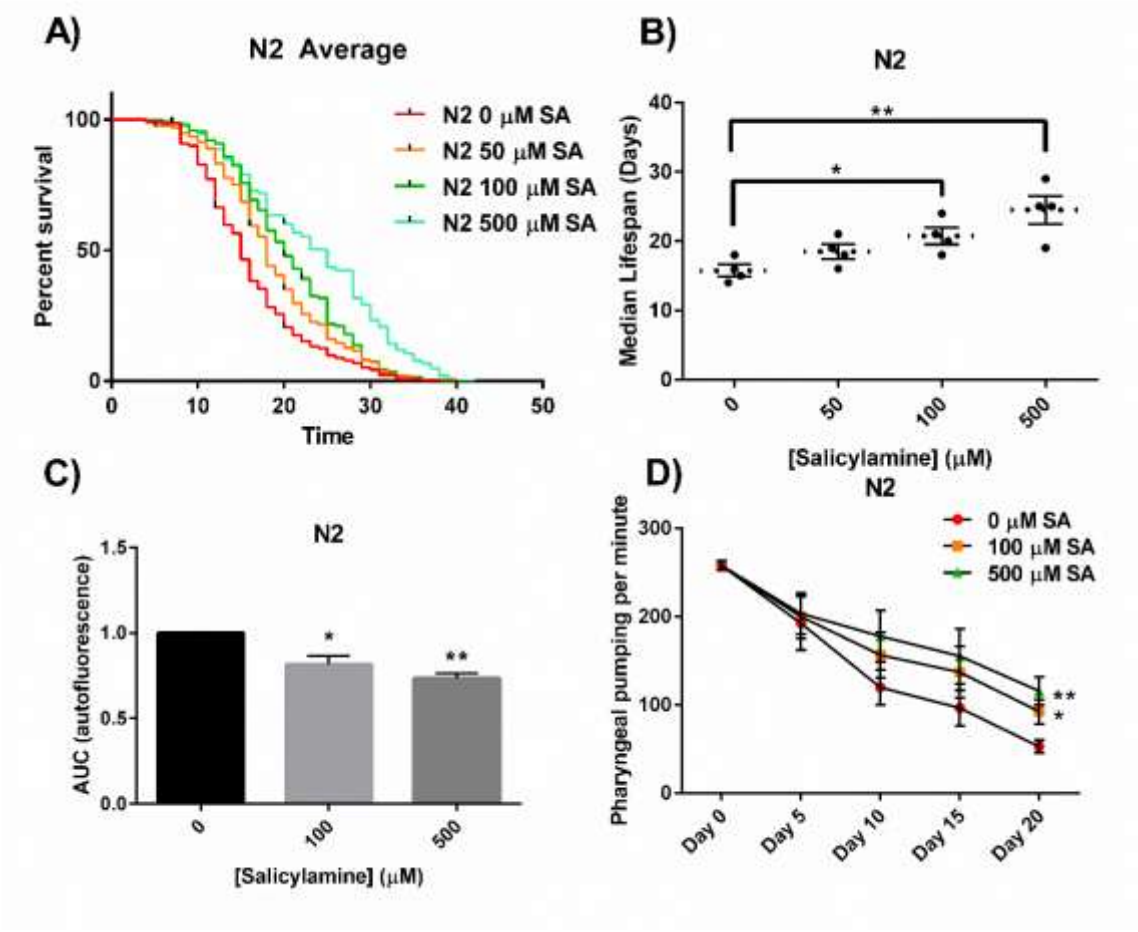

Figure 4.1. SA extends the lifespan of N2 *C. elegans* worms. (A) Kaplan-Meier survival curves for concentration dependency of SA-mediated N2 lifespan extension. Upon day 1 of adulthood, SA was administered every 2 days and survival was assessed every other day until all the worms died. (B) Summary of SA treated N2 median lifespans. SA administration shows a dose-dependent increase in median lifespan. Data are expressed as means ± SEM from four independent experiments. *P < 0.05 as compared with vehicle control, **P < 0.01 as compared to vehicle control. (C) Effects of SA-mediated decreases in lipofuscin autofluorescence accumulation with age. SA response profiles were generated from integrating the area-under-the-curve (AUC) of fluorescent intensity as a function of time. Compared with N2 vehicle control, treatment with SA shows a significant reduction in autofluorescence. Data are expressed as means ± SEM from five independent experiments. *P < 0.01 as compared with vehicle control, **P < 0.005 as compared to vehicle control. (D) Changes in pharyngeal pumping rate of aging worms. Pumping rate declines with age, however SA administration retards decline in pumping rate. Data are expressed as means ± SEM from five independent experiments. *P < 0.05 as compared with vehicle control, **P < 0.01 as compared to vehicle control.

We next sought to demonstrate that SA administration would not only prolong natural lifespan in adult worms, but that the longer-lived worms would also exhibit prolonged healthspan – i.e., that they would be phenotypically more youthful (Bansal, Zhu et al. 2015). To quantify this, we chose both a biochemical endpoint (lipofuscin autofluorescence) and a behavioral measure (pharyngeal pumping) that change predictably with aging and are associated with healthspan. The accumulation in the nematode intestinal epithelium of autofluorescent lipofuscin granules, a heterogeneous mixture of oxidized and crosslinked lipids and proteins and advanced glycation end products, is a known conserved phenomenon observed to increase with age (Clokey and Jacobson 1986, Yin 1996). Visualization of lipofuscin granules is often used as an age-related assessment of healthspan. We quantified lipofuscin autofluorescence over time (every 5 days) in N2 adult nematodes (10-20 per colony) in the presence of increasing doses of SA (representative confocal images depicting nematode autofluorescence over age shown in Supplemental Figure 1). SA response profiles were generated from integrating the area-under-the-curve (AUC) of fluorescent intensity as a function of time (Fig. 4.1C). Treatment with either 100 µM or 500 µM SA showed a significant reduction in age-associated lipofuscin accumulation compared with vehicle control in WT animals ($p < 0.01$). For a behavioral/functional measurement of health, we quantified pharyngeal pumping rates. Worms ingest bacteria using the pharynx, requiring constant pumping by the pharyngeal bulb (Wolkow 2006). The rate of pharyngeal pumping declines reliably with age and has been attributed to multiple age-related processes (Herndon, Schmeissner et al. 2002, Huang, Xiong et al. 2004). We quantified pharyngeal pumping rates in WT N2 worms established on OP50-seeded NGM agar plates with increasing concentrations of SA. The frequency of pharyngeal pumping was measured and recorded every fifth day as the animals aged. Overall, WT N2 worms showed dose-dependent protection against age-associated decline in pharyngeal

pumping rate (Fig. 1D) ($p < 0.05$). Taken together, these data demonstrate that SA not only dose-dependently prolongs lifespan but also healthspan when administered to adult N2 *C. elegans*.

SA administration deceases formation of IsoK-lysyl-lactam protein adducts

The mechanism by which IsoKs inactivate protein targets involves covalent adduction of the ε-amine group on the side chains of lysine residues within target proteins (Fig. 4.2A). The initial product of this covalent adduction is a lactam ring structure composed of the lysyl side chain and the isoketal. SA's amine group is much more reactive toward IsoKs and preserves protein function by more rapidly forming an adduct with the IsoK, preventing the adduction of lysyl side chains. If this mechanism is operative *in vivo* when *C. elegans* are treated with SA, a dose-dependent reduction in IsoK-lysyl-lactam adducts should be observed. To test this hypothesis directly, WT N2 worms were treated with vehicle or increasing concentrations of SA until day 15, then collected for IsoK-lysyl-lactam adduct quantification by liquid chromatography tandem mass spectrometry (LC/MS/MS) using a heavy isotope-labeled internal standard for quantification (Davies, Amarnath et al. 2007). SA treatment resulted in a significant, dose-dependent reduction in IsoK-lysyl-lactam adduct levels compared with vehicle control in WT animals ($p < 0.01$) (Fig. 4.2B).

SIR-2.1 is a critical protein whose function is preserved by salicylamine

Though SA treatment should prevent IsoK adduct formation on many different protein targets, and many of these proteins could have some impact on lifespan and healthspan, we hypothesized that there would be specific proteins of particular importance in mediating SA's beneficial effects. Specifically, we hypothesized that SIR-2.1 would be one of these "high value targets" for several reasons. Increased activity of SIR-2.1 and its orthologs have been significantly associated with increased lifespan in multiple studies. Sirtuins are lysine deacetylases that are normally found in membrane-bound and membrane-rich subcellular compartments (e.g., mitochondria), so they are

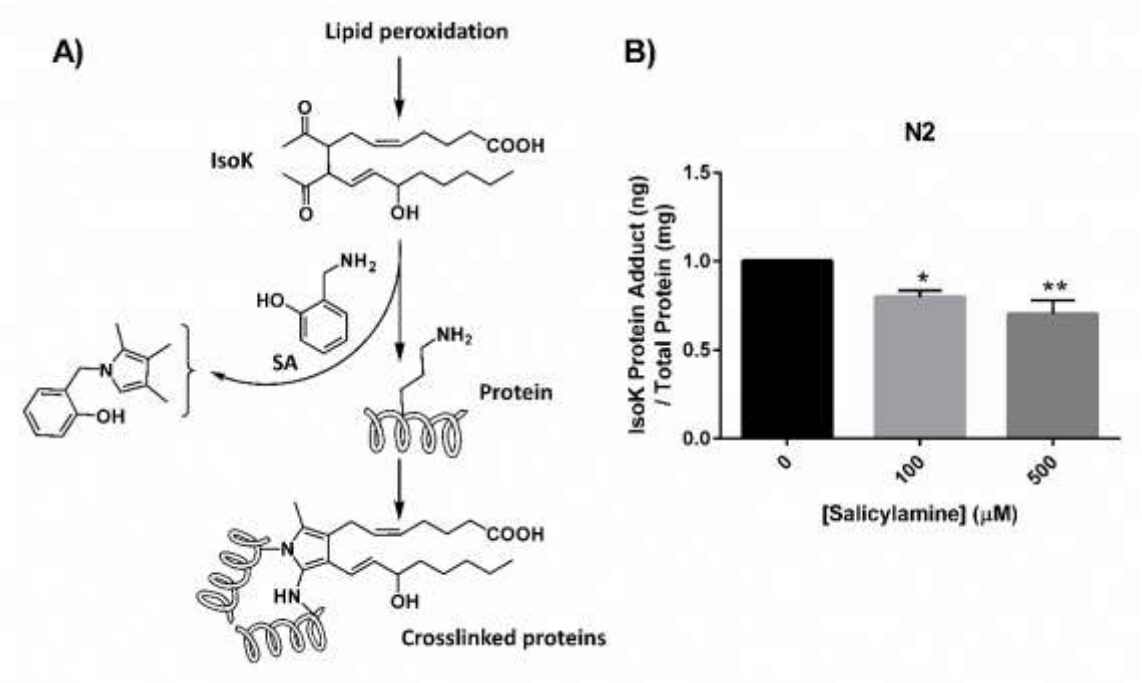

Figure 4.2. SA administration decreases formation of IsoK-lysyl-lactam protein adducts. (A) Schematic illustrating lipid peroxidation and formation of IsoKs. Isoks react with ε-amino in lysyl residues of proteins to form stable lactam adducts. Addition of the IsoK scavenger, SA, prevents IsoK adduction. (B) IsoK-lysyl-lactam adduct quantification by LC/MS/MS. IsoK-lysyl-lactam adducts were decreased with SA treatment. Data are expressed as means ± SEM from four independent experiments. *P < 0.01 as compared with vehicle control, **P < 0.005 as compared to vehicle control.

in very close proximity to the membrane lipids that give rise to IsoKs and to the lysyl residues that are their targets. Finally, some sirtuin isoforms have been shown to have greatest deacylase activity toward longer acyl chain lysyl moieties, raising the possibility of an enhanced likelihood of SIR-2.1 being present in close proximity to IsoKs and protein lysyl side chains (Zhu, Zhou et al. 2012, Choudhary, Weinert et al. 2014). First, we wanted to directly test the hypothesis that isoketals are chemically capable of inactivating sirtuin proteins, as this would lend plausibility to a direct interaction in vivo and would support the hypothesis that sufficient oxidative stress, rather than activating sirtuins (Bokov, Chaudhuri et al. 2004, Caito, Rajendrasozhan et al. 2010, Someya, Yu et al. 2010, Tao, Coleman et al. 2010, Bell and Guarente 2011), will actually lead to inhibition.

Recombinant human SIRT1 was incubated with increasing concentrations of synthetically pure IsoK, and enzymatic deacetylase activity was assessed using the luminescence based Sirt-Glo assay (Promega). Purified isoketals dose-dependently inhibited recombinant human sirtuin 1 (Fig. 4.3A), with an IC_{50} of 97.8 μM.

We next tested the hypothesis that, *in vivo*, SIR-2.1 is a critical protein that is functionally preserved from time-dependent oxidative inactivation when *C. elegans* are treated with increasing doses of SA. We treated a nematode strain carrying a non-functional SIR-2.1 mutation, VC199 [*sir-2.1*(ok434)], with the same concentrations of SA as were used in lifespan experiments with WT nematodes. Starting at day 1 of adulthood, SIR-2.1 mutants were grown on SA-coated OP50-seeded NGM plates. SIR-2.1 mutant worms were transferred to freshly made SA-OP50-NGM agar plates every 2 days and survival was assessed using a platinum wire until all worms died; survival was scored as movement upon slight touch with the platinum wire (Fig. 4.3B). When SA was administered to the VC199 strain, the effect of extending median lifespan that had been observed in the N2 strain was entirely abolished (Fig.3C; VC199 median lifespan: 16 days, 0 μM SA; 15 days, 50 μM SA; 15 days, 100 μM SA; 15 days, 500 μM SA; $p = 0.70$). This suggests that SA-mediated lifespan extension acts in part through preservation of sirtuin activity. In addition, we observed SA-mediated lifespan extension operates outside canonical *C. elegans* longevity pathways, such as the insulin/IGF-1-like signaling pathway (Supplemental Figure 2).

We next wanted to test the hypothesis that SIR-2.1 is required for the healthspan extending effects of SA in addition to the longevity effects. As with the N2 strain, we treated VC199 nematodes with increasing concentrations of SA and assessed the age-dependent accumulation of autofluorescent lipofuscin granules and decrease in pharyngeal pumping rate. In worms lacking SIR-2.1, none of the doses of SA used were able to decrease the accumulation of lipofuscin (Fig.

3D) ($p = 0.50$) or to preserve pharyngeal pumping rates (Fig. 4.3E) ($p = 0.50$) at day 15. Taken together, these data strongly suggest that SIR-2.1 is a critical target for SA's effects on healthspan as well as lifespan in adult *C. elegans*.

SIR-2.1 preservation enhances resistance to oxidant stress but does not affect mitochondrial function

Sirtuins represent a regulatory hub for a variety of cellular processes that lie at the heart of molecular aging, including energy metabolism, mitochondrial structural and genomic integrity maintenance, and redox balance. Indeed, one of the major stimuli for activation of sirtuins is oxidative stress. Multiple sirtuin isoforms in multiple different species have been shown to play an important role in cellular defenses against oxidant injury (Caito, Rajendrasozhan et al. 2010, Someya, Yu et al. 2010, Bell and Guarente 2011). The mitochondrial antioxidant manganese superoxide dismutase (MnSOD) has been identified as one of the major protein targets that is deacetylated by sirtuin isoforms, with deacetylation enhancing the enzymatic activity of MnSOD (Tao, Coleman et al. 2010). If SA indeed preserves SIR-2.1 enzymatic function in vivo, we hypothesized that SA treatment would dose-dependently decrease biomarkers of oxidant injury in a SIR-2.1-dependent manner.

To quantify oxidant injury, we measured F_3-isoprostanes (F3-IsoPs). F3-IsoPs are the products of free radical-mediated peroxidation of eicosapentaenoic acid (EPA), and are known to be a highly sensitive and accurate marker of oxidative damage in *Caenorhabditis elegans* (Gao, Yin et al. 2006, Labuschagne, Stigter et al. 2013, Nguyen and Aschner 2014). F_3-IsoPs were collected from WT N2 and from VC199 (SIR-2.1 deficient strain) nematodes grown on OP50-NGM agar plates containing increasing concentrations of SA from Day 1 of adulthood until collection at Day 15. Quantification of F_3-IsoPs from WT N2 worms (Fig. 4.4A) exhibited a dose-

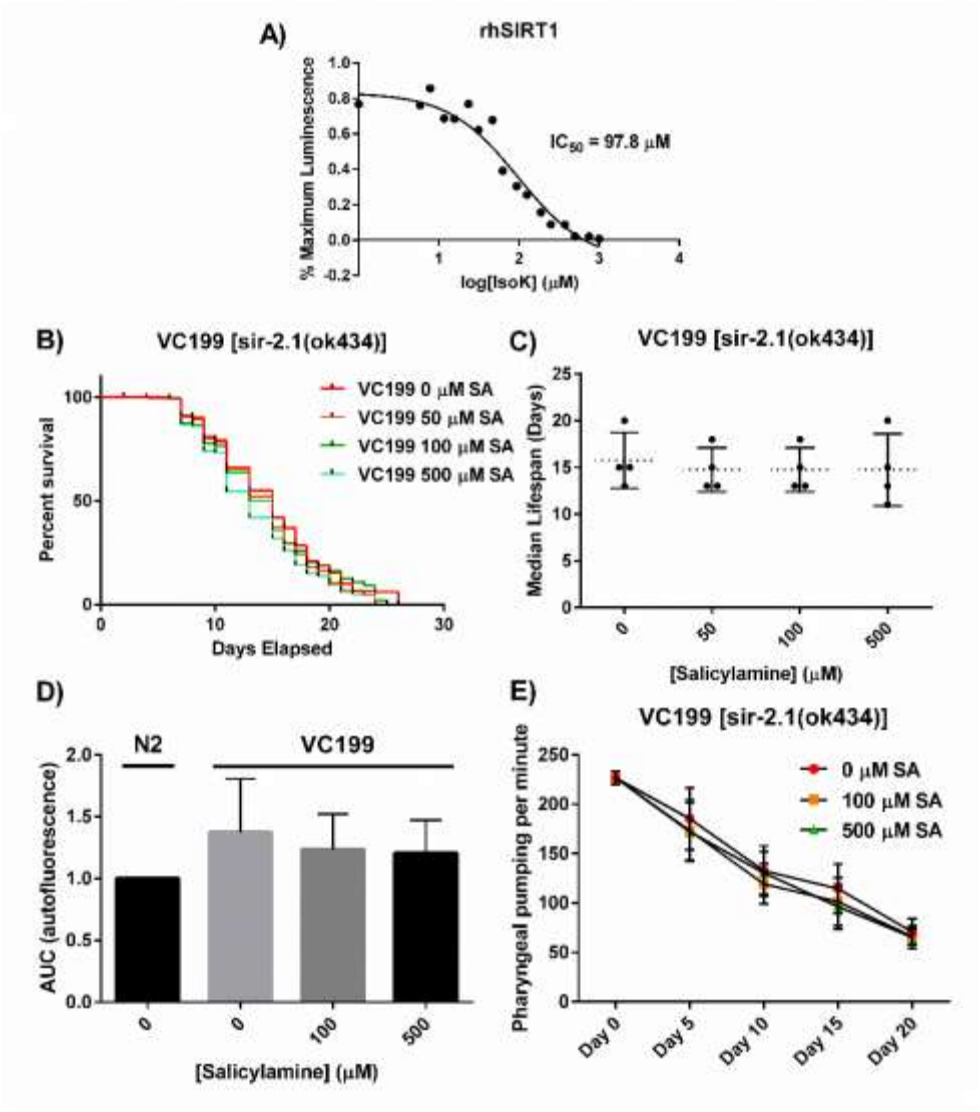

Figure 4.3. SIR-2.1 is required for SA-mediated lifespan extension. (A) Synthetically purified IsoKs decrease biochemical activity of rhSIRT1. Recombinant human SIRT1 was incubated with increasing concentrations of IsoK and enzymatic activity was assessed using a luminescence based assay. Concentration-response curves were generated and IC50 values were calculated from three independent experiments. (B) Kaplan-Meier survival curves depicting effects of SA administration on lifespan of non-functional SIR-2.1 mutant. (C) Summary of SA-treated SIR-2.1 mutant median lifespan. SA administration does not affect median lifespan of SIR-2.1 mutants. Data are expressed as means ± SEM from four independent experiments. P = 0.70. (D) Changes in lipofuscin autofluorescence accumulation with age. Compared to vehicle control in WT animals, SA response profiles indicate neither dose of SA were able to decrease the accumulation of lipofuscin. Data are expressed as means ± SEM from four independent experiments. P = 0.5. (E) Changes in pharyngeal pumping rate in SA-treated SIR-2.1 mutants. Administration of SA failed to preserve pharyngeal pumping

dependent decrease in F_3-IsoP levels, with 100 μM SA decreasing F_3-IsoP production by 29% ($p < 0.01$) and 500 μM displaying a 44% decrease in F_3-IsoP levels ($p < 0.005$). In sharp contrast, SIR-2.1 deficient nematodes showed a slightly higher baseline level of F3-IsoPs that did not significantly decrease at any dose of SA tested (Fig. 4.4B), confirming that SA itself does not act as a direct antioxidant and supporting the hypothesis that SIR-2.1 enzymatic activity is preserved by SA treatment with the predicted positive effect on cellular defense against oxidant injury. To further characterize SA's ability to preserve SIR-2.1 function, we assessed acetylation of MnSOD at Lys 122. At the highest dose of SA (500 μM) on day 15 of adult life, WT N2 nematodes show a trend toward lower acetyl-Lys 122 in MnSOD compared to the VC199 strain treated with the same SA dose (Fig. 4.4C and 4.4D), supporting at least a modest positive effect of SA treatment on MnSOD acetylation state and function, mediated by SIR-2.1.

Mitochondria lie at the center of aging biology, playing crucial roles in energy production, carbon substrate metabolism, apoptosis regulation, and redox balance and signaling (Finkel and Holbrook 2000, Wallace 2005, Ryan and Hoogenraad 2007). Since sirtuins play a major role in regulating mitochondrial function, we next wanted to investigate whether SA was exerting any of its effects via protection of mitochondrial processes. To investigate SA's effects on mitochondrial respiration, we administered SA to WT N2 (Fig. 4.5A) and non-functional SIR-2.1 mutant worms (Fig. 5B) and measured oxygen consumption rate (OCR) in whole worms over several days. OCR decreased with age in both N2 and VC199 strains, and SA showed no effect on mitochondrial OCR in either strain ($p > 0.05$). We also examined the effect of SA treatment on mitochondrial DNA (mtDNA) integrity. Using a quantitative polymerase chain reaction (qPCR) assay to measure mtDNA content relative to nuclear DNA, WT N2 (Fig. 4.5C) and VC199 SIR-2.1 mutants (Fig. 4.5D) showed no significant difference in mtDNA copy number with age or with SA treatment.

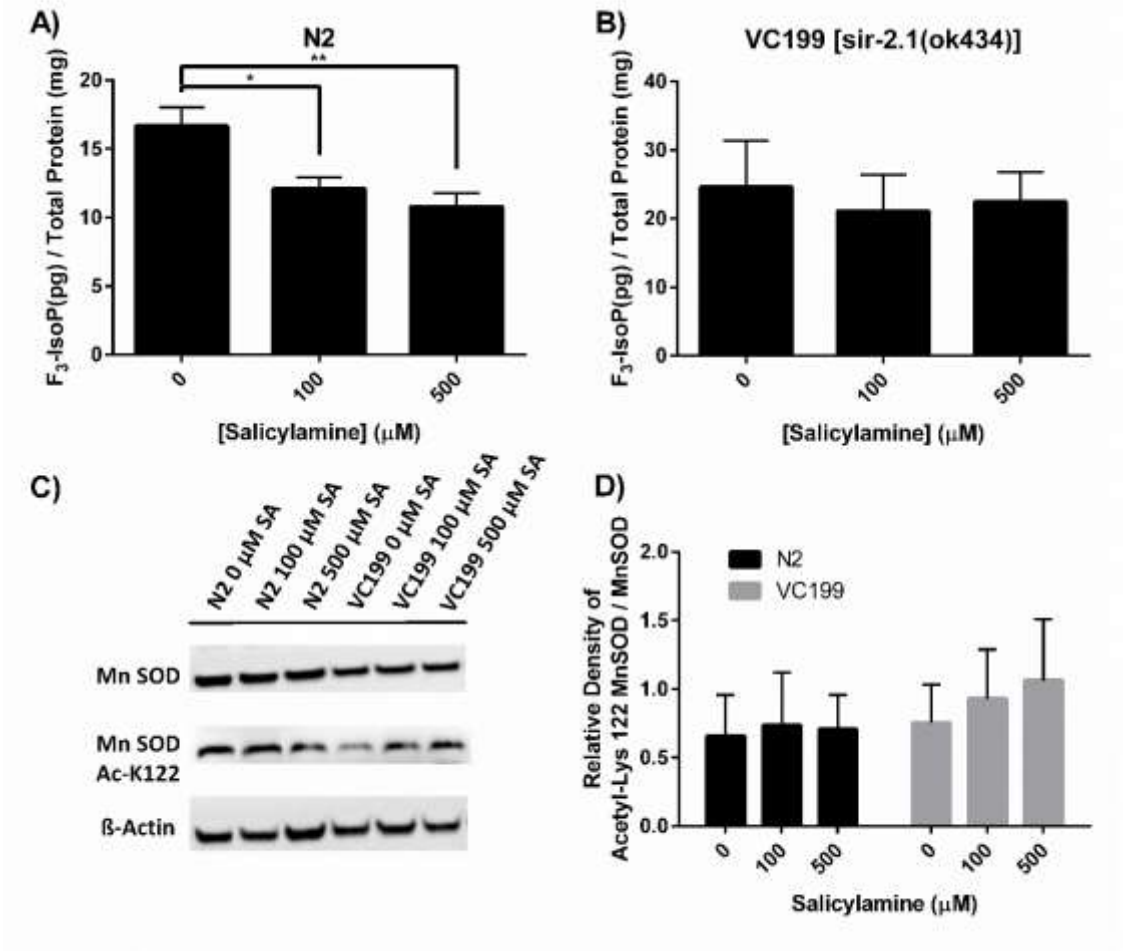

Figure 4.4. SA treatment dose-dependently decreases biomarkers of oxidant injury in a SIR-2.1-dependent manner. (A, B) Quantification of oxidant damage via F_3-IsoP measurement. N2 WT and SIR-2.1 mutant animals were given SA from day 1 of adulthood until collection. Lysates were collected at day 15 of adulthood and F_3-IsoPs were measured by GC/MS. Data are expressed as means ± SEM from four independent experiments. * $P < 0.01$ as compared with vehicle control, **$P < 0.005$ as compared to vehicle control. (C) Levels of acetyl-Lys 122 MnSOD was measured from N2 WT and SIR-2.1 mutant protein extracts and analyzed by Western blot. (D) Quantification of acetyl-Lys 122 MnSOD. Treatment with SA in WT N2 nematodes show a trend toward lower acetyl-Lys 122 MnSOD compared to SIR-2.1 mutant animals. Data are expressed as means ± SEM from four independent experiments. $P > 0.05$.

Taken together, these data suggest that the SIR-2.1 dependent effects of SA treatment are not mediated through significant changes in mitochondrial function.

Gene expression analysis reveals *ets-7* as an important effector of salicylamine

Programmed aging is one of the major theories of aging, wherein this theory implies there is a built-in program in the genome that activates senescence, which leads to death (Gershon and Gershon 2000). In addition to the functions discussed above, sirtuins can have powerful regulatory effects on gene expression programs by virtue of their proposed histone deacetylase activity. With supporting evidence that SA's effects are at least in part SIR-2.1 mediated, we sought to better define the role of changes in global gene expression following SA treatment in WT N2 worms. We hypothesized that there would be fairly broad changes in gene expression that would converge on one or more specific pathways. To assess whether SA alters an aging gene transcriptional program, we carried out microarray analysis on adult WT N2 worms exposed to SA (0, 100, 500 μM SA) for 15 days. Gene expression arrays showed that, surprisingly, SA treatment exerted a relatively minor effect on gene expression, with the major variable impacting on gene expression being aging itself. From the microarray, we identified 26 genes upregulated by both 100 and 500 μM SA (Group I), 38 genes more strongly downregulated by 500 μM SA than 100 μM SA (Group II), 15 genes with variable downregulation (Group III), and 30 genes downregulated by both doses of SA (Group IV) (Fig. 4.6A). Of the 8,902 probe sets with unigene identifiers, only 109 probe sets showed at least a 25% change in expression with SA administration at Day 15. Thus, the major effect of SA treatment appears to be at the post-translational level, as would be predicted by SA's proposed mechanism of action.

Nonetheless, there were some significantly changed genes downstream from SA treatment. First, to validate the microarray results, we performed real-time RT-PCR on four genes, *siah-1,*

sma-4, *F13D12.6*, and *ets-7* (Fig. 6B). The genes *siah-1* and *sma-4* showed downregulation by SA in Day 15 WT N2 worms, and *F13D12.6* and *ets-7* showed upregulation by SA. Messenger RNA levels for *siah-1* and *sma-4* were decreased by nearly 25%, and *F13D12.6* and *ets-7* message levels were increased by nearly 25% by SA administration ($p < 0.05$). We did not observe SA effects on *sir-2.1* mRNA levels from the gene expression arrays. Real-time RT-PCR confirmed administration of SA did not appreciably increase or decrease *sir-2.1* transcript levels in aged

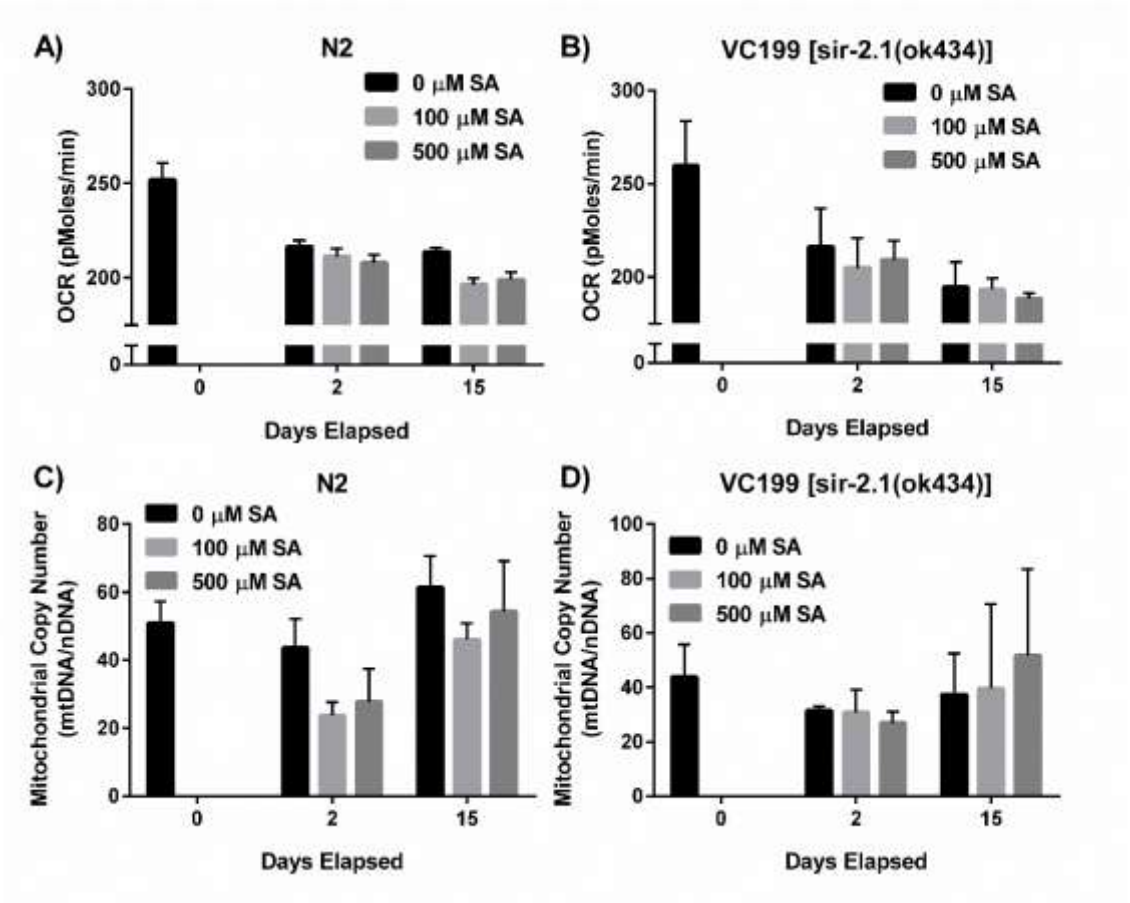

Figure 4.5. SIR-2.1 preservation does not affect mitochondrial function. (A, B) SA administration does not alter oxygen consumption rate (OCR). OCR of N2 WT and SIR-2.1 mutation in the presence and absence of SA was measured over time via XF Seahorse Biosciences Analyzer™. Data are expressed as means ± SEM from four independent experiments. P = 0.1 and P = 0.3, respectively. (C, D) SA treatment does not alter mtDNA integrity. Analysis of mtDNA content collected over time from lysates of SA-treated N2 WT and SIR-2.1 mutant animals. Data are expressed as means ± SEM from four independent experiments. P = 0.1 and P = 0.6, respectively.

animals (Supplemental Figure 3), supporting the hypothesis that SA-mediated lifespan extension is acting primarily via preservation of protein biochemistry.

Pathway Analysis using Gene Ontology (GO) with WEB-based Gene SeT AnaLysis Toolkit (WEBGESTALT < http://bioinfo.vanderbilt.edu/webgestalt/>) highlighted the metabolic process, lipid metabolic process, proteolysis pathways among many others as being altered favorably by the administration of SA (Supplemental Figure 4). From the array data confirmed by RT-PCR, we identified the ETS class transcription factor ETS-7 as a protein of interest. ETS factors are known to be involved in regulating lipid metabolism and regulate lifespan in both *Drosophila melanogaster and C. elegans* (Thyagarajan, Blaszczak et al. 2010, Alic, Giannakou et al. 2014).

To investigate the role of ETS-7 in regulating lifespan downstream from salicylamine, SA was administered to the *ets-7* gene knock-out strain, RB981 [F19F10.5(ok888) V]). Similar to the SIR-2.1 deficient VC199 strain, loss of *ets-7* showed no SA-mediated effect on lifespan extension (RB981 median lifespan: 16 days, 0 μM SA; 15 days, 500 μM SA, $p > 0.05$) (Fig. 4.6C and 4.6D). We originally hypothesized that upregulation of *ets-7* depends on SIR-2.1, which could result in enhanced longevity. In order to test this, we carried out real-time RT-PCR quantification of *ets-7* in non-functional SIR-2.1 mutant nematodes treated with increasing doses of SA (Fig. 4.6E). Messenger RNA levels for *ets-7* were increased by 32% in Day 15 WT N2 worms by 500 μM SA administration ($p < 0.05$), and similarly, a dose-dependent increase in *ets-7* transcriptional level can be observed in Day 15 non-functional SIR-2.1 mutants, VC199, with the highest dose of SA showing a significant 442% increase in mRNA expression ($p < 0.01$). Taken together, these findings suggest that *ets-7* upregulation in non-functional SIR-2.1 mutant may be an attempt at compensation for loss of SIR-2.1, which is further enhanced by SA-administration, but is

ultimately insufficient in extending lifespan in the absence of SIR-2.1. Our data suggest that *ets-7* is necessary for SA-dependent increase in lifespan, but is insufficient without the presence of functional SIR-2.1.

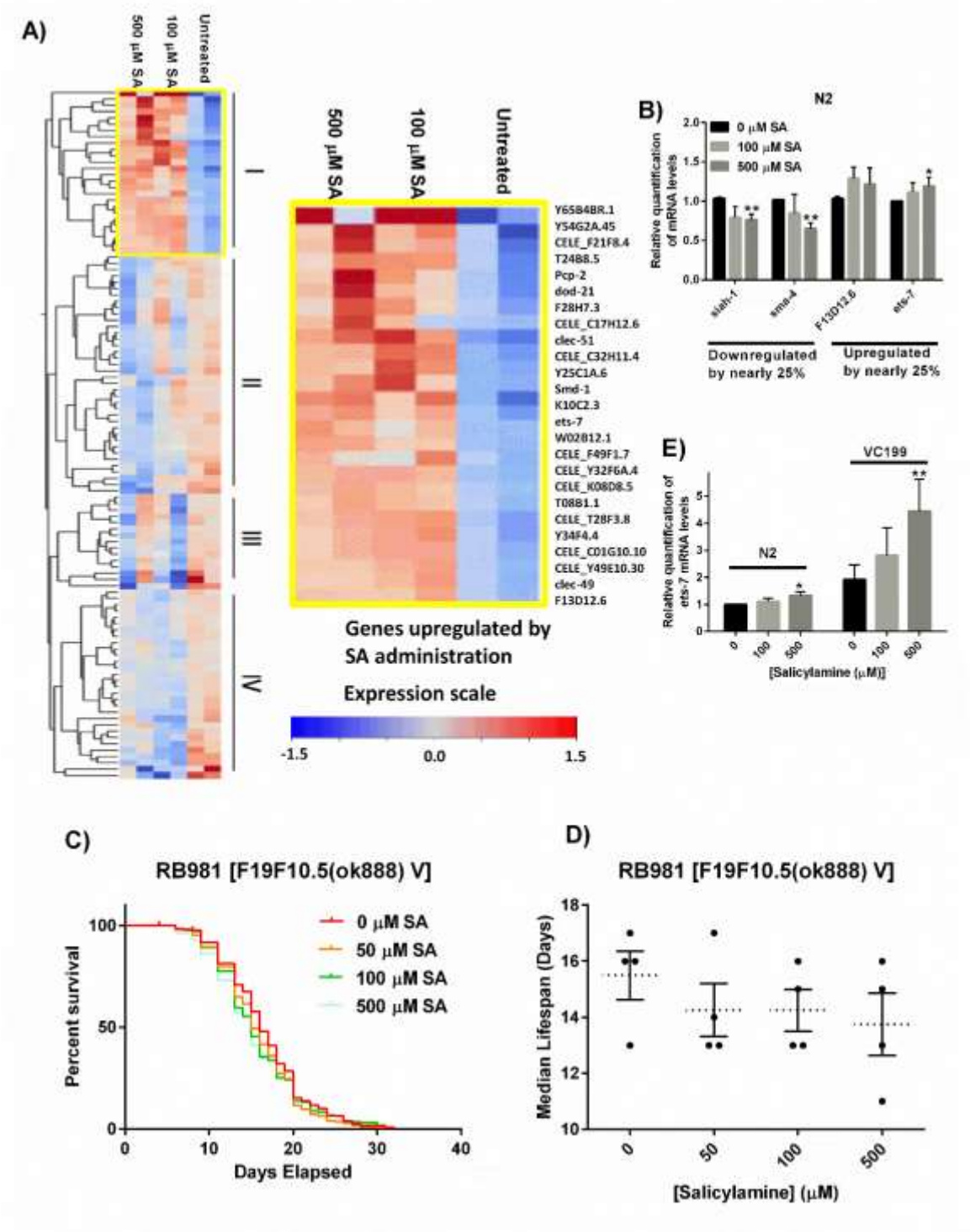

Figure 4.6. Gene expression analysis reveals *ets-7* as an important effector of SA. (A) Heat map of genes differentially regulated by treatment in 15 day CE. 109 probe sets had at least a 25% change in expression concordant in both samples. These include 26 genes upregulated by both doses of SA (group I), 38 genes more strongly downregulated by 500 μM SA than 100 μM SA (Group II), 15 genes with variable downregulation (Group III), and 30 genes downregulated regardless of dose of SA (Group IV). (B) Real-time RT-PCR validation of microarray results on selected genes. The genes, *siah-1* and *sma-4* showed downregulation by SA in day 15 WT N2 worms, and *F13D12.6* and *ets-7* showed upregulation by SA. Data are expressed as means ± SEM from five independent experiments. *P < 0.05 as compared to vehicle control, **P < 0.01 as compared to vehicle control. (C) Kaplan-Meier survival curves for concentration dependency of SA-mediated *ets-7* knock-out mutant lifespan extension. (D) Summary of SA-treated *ets-7* knock-out mutant median lifespan. SA administration does not affect median lifespan of *ets-7* knock-out mutants. Data are expressed as means ± SEM from five independent experiments. P = 0.40. (E) Real-time RT-PCR quantification of *ets-7* in non-functional SIR-2.1 mutant nematodes treated with increasing doses of SA. Transcriptional levels for ets-7 were increased by 25% in day 15 N2 WT worms by SA administration, and a dose-dependent increase in *ets-7* mRNA levels can be observed in day 15 SIR-2.1 mutants. Data are expressed as means ± SEM from four independent experiments. *P < 0.05 as compared to vehicle control, and **P < 0.01 as compared to vehicle control.

Discussion

In the present study, we have shown that treatment with salicylamine, a scavenger of some of the most damaging products of oxidant injury – namely, γ-ketoaldehydes generally, and isoketals specifically – can prolong natural lifespan and healthspan in *C. elegans*, and that these effects are dependent upon SIR-2.1 and ETS-7. We have shown that the effects of SA operate primarily at the protein level, in keeping with what is known about the biochemical mechanism of action and placing SA broadly in the role of a proteostasis mediator. We further show that, although SIR-2.1 and ETS-7 are important targets preserved by SA treatment, the major biochemical effect of preserving the activities of these two targets is to enhance normal antioxidant defenses, with effects on mitochondrial function, mtDNA integrity, and gene expression being small to nonexistent.

Several aspects of this study are worth particular attention. First, SA treatment was begun and exhibited its effects in adult worms. This is distinct from some other longevity-extending interventions which rely on genetic manipulation or application of a particular treatment or stressor during a critical developmental period (Chin, Fu et al. 2014, Edwards, Canfield et al. 2015, McCormack, Polyak et al. 2015, Rauthan, Ranji et al. 2015). Such interventions are certainly informative but are likely to be of limited direct translation potential. Second, though SA exhibited predictable effects on oxidative stress by way of SIR-2.1 mediated activation of antioxidant enzymes (e.g., MnSOD), the lack of effect on gene expression and on mitochondrial function was striking and informative. This suggests that SA is not working through maintenance or modification of large gene expression programs, nor probably through maintenance of overall nuclear and/or mitochondrial genomic integrity, nor through large effects on mitochondrial function. The lack of any apparent effect on mitochondrial oxygen consumption is perhaps a bit surprising, given the central role of SIR-2.1 in mediating the effects of SA treatment. However, as previously mentioned, SIR-2.1 is known to exert a wide range of pleiotropic effects beyond metabolic regulation (Tissenbaum and Guarente 2001, Wang and Tissenbaum 2006, Guo and Garcia 2014, Pant, Saikia et al. 2014, Furuhashi, Matsunaga et al. 2016). Our data could be consistent with preservation of any number of these other functions ascribed to SIR-2.1, though in these investigations, the major effector mechanism identified downstream from SIR-2.1 was antioxidant defense. Finally, SA treatment does not appear to induce any of the stress/hormesis loops (Calabrese, Dhawan et al. 2015, Edwards, Canfield et al. 2015, Monaghan and Haussmann 2015, Hartl 2016) that have been shown to impact upon longevity when activated or inhibited.

The identification of ETS-7 by gene expression array and the subsequent finding that ETS-7 is necessary but not sufficient for SA to exert its effects deserves specific mention. We focused

on ETS-7 particularly because there is precedent in the literature for ETS family transcription factors regulating longevity through effects on lipid metabolism (Thyagarajan, Blaszczak et al. 2010, Alic, Giannakou et al. 2014, Sun, Sun et al. 2015). Given that other lipid metabolic genes were shown to be significantly regulated by SA treatment (Supplemental Tables 1 and 2), we reasoned that ETS-7 may be an important regulatory gene, with the other lipid metabolism genes identified being downstream from ETS-7. Future investigations will focus on the specific gene targets regulated by ETS-7. The finding that ETS-7 is required for SA's effects, but DAF-16 is not (Supplemental Figure 2), also suggests that there are indeed specific protein targets modulated by SA. Furthermore, the observed interactions between SA, SIR-2.1, and ETS-7 strongly suggest that extension of natural lifespan by SA treatment occurs through preservation of the biochemical activities of multiple regulatory proteins, with SIR-2.1 serving as a primary node in the signaling pathway and ETS-7 playing a secondary role. We have certainly not exhaustively investigated all of the possibilities, with other targets (e.g., PCH-2 (Qian, Xu et al. 2015)) existing in the published literature that may be of particular interest. Further delineation of all of the important signaling nodes impacted by SA treatment and elucidation of the signaling hierarchy will be major areas of focus for future investigations.

Our study does have some important limitations. Our data were generated exclusively in *C. elegans*. While a powerful model system for aging research, and though SA treatment has been shown to have substantial beneficial effects in a variety of mammalian models of disease, the findings in the present study will need to be verified in more complex model organisms. A precise dose-effect relationship with SA treatment cannot be accurately determined from the studies we report here. The dose-dependent reduction in IsoK-lactam adducts quantified by mass spectrometry (Figure 4.2B) confirms that we are in the pharmacologic range, but the precise

location on the dose-response curve is not clear. As mentioned above, we also know that there are other protein targets that are likely being preserved with SA treatment, but a full characterization of the proteome-level effects of SA is beyond the scope of the present study.

The translation potential for SA as a clinically useful anti-aging therapy is fairly high. Salicylamine is orally bioavailable in mammals (Zagol-Ikapitte, Matafonova et al. 2010). Long-term administration (approximately one year) in mice via drinking water has shown no evidence of intolerance and no evidence of excess adverse events (Davies, Bodine et al. 2011). This is particularly important when considering an anti-aging intervention, as we would anticipate that SA would need to be administered on an ongoing basis over a long period of time given its mechanism of action. No excess tumor formation was observed in mice with long-term SA treatment (McMaster, Kirabo et al. 2015, Egnatchik 2016, Wu, Saleh et al. 2016), an important negative given recent reports regarding negative effects of nonspecific antioxidant therapies with regard to tumor metastasis (Prasad, Gupta et al. 2016, Wang, Liu et al. 2016). Finally, the translation of SA into human studies should be able to proceed fairly rapidly, as it is a naturally occurring small molecule found in buckwheat seeds and is currently awaiting Generally Recognized as Safe (GRAS) designation for use as a natural supplement.

Supplemental Information

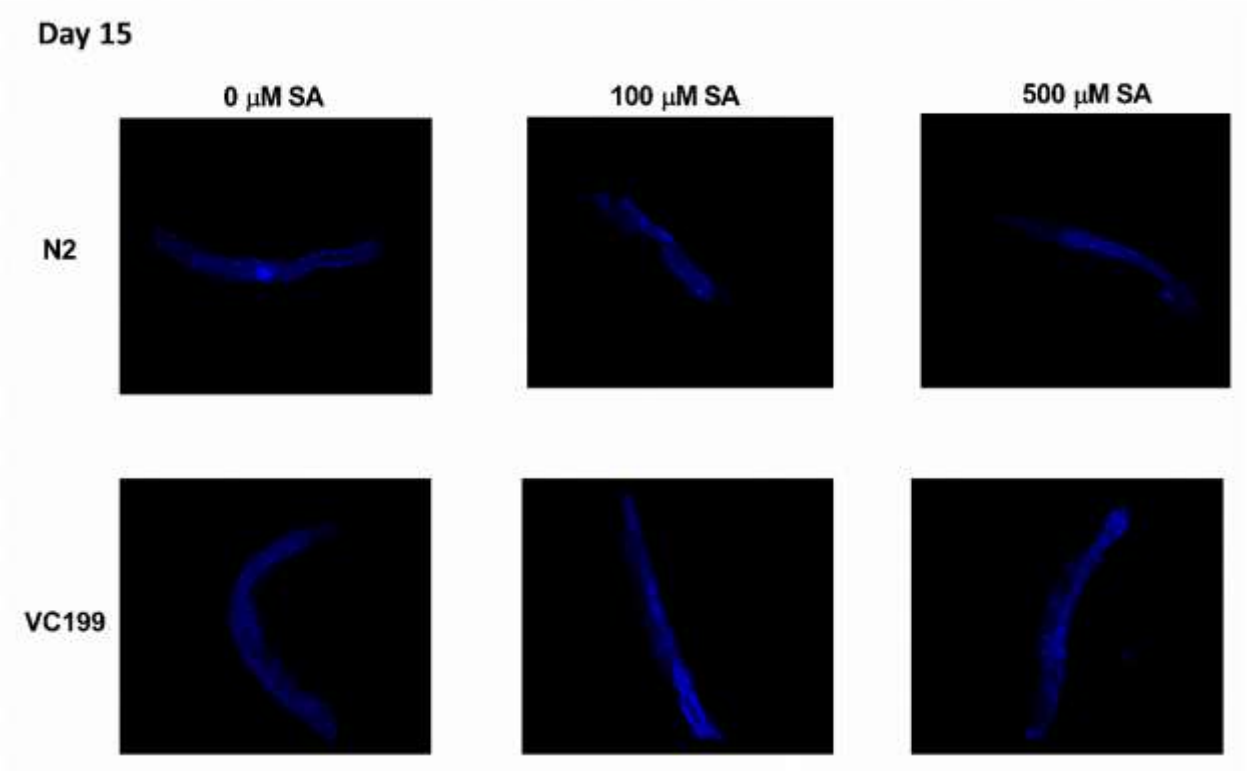

Figure S1. Change in lipofuscin autofluorescence with age. (A) Representative confocal images are shown from four experiments. Synchronized late L4/early young adult worms were plated on FUDR containing SA-OP50-seeded NGM plates and worms were maintained at 20°C. Every fifth day, 10-15 worms were mounted onto 2% agar pads and anesthetized with 3 mM levamisole in DMSO. Representative confocal images of each treatment condition were captured through Plan-Aprochromat 20x objective on an LSM510 confocal microscope (Carl Zeiss MicroImaging, Inc) scanning every 200 nm for XZ sections. Images were processed with the Zeiss LSM Image Browser. Figure S1 relates to manuscript figure 1C and 3D.

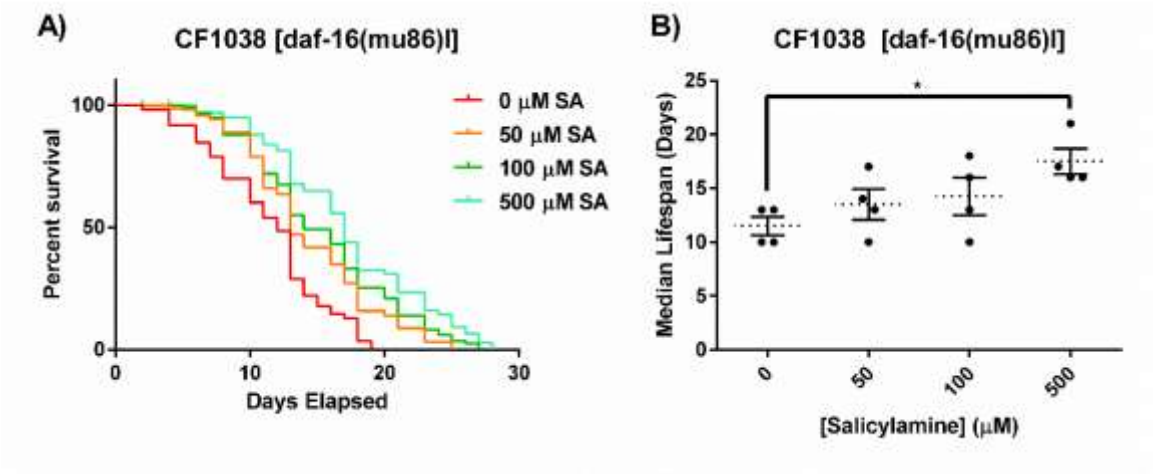

Figure S2. SA extends the lifespan of *daf-16* gene knockout mutant strain. (A) Kaplan-Meier survival curves depicting effects of SA administration on *daf-16* gene knockout mutant strain. Starting at day 1 of adulthood, animals were transferred to OP50-seeded NGM-SA plates every 2 days. Survival was assessed every 2 days until all the worms died. (B) Summary of SA treated *daf-16* knockout mutant median lifespans. SA increased maximum and median lifespan in *daf-16* knockout worms. Data are expressed as means ± SEM from four independent experiments. *P < 0.01 as compared to vehicle control. Figure S2 relates to manuscript figure 3B.

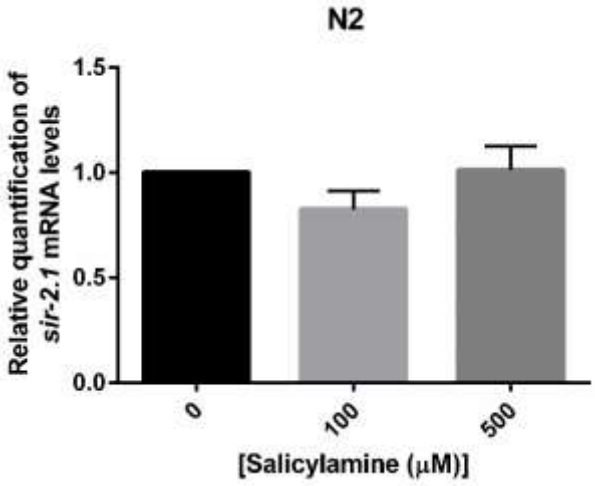

Figure S3. SA does not attenuate *sir-2.1* mRNA levels. Real-time RT-PCR quantification of *sir-2.1* in wild-type N2 nematodes treated with increasing doses of SA. Data are expressed as means ± SEM from five independent experiments. P = 0.08 and P = 0.2, respectively.

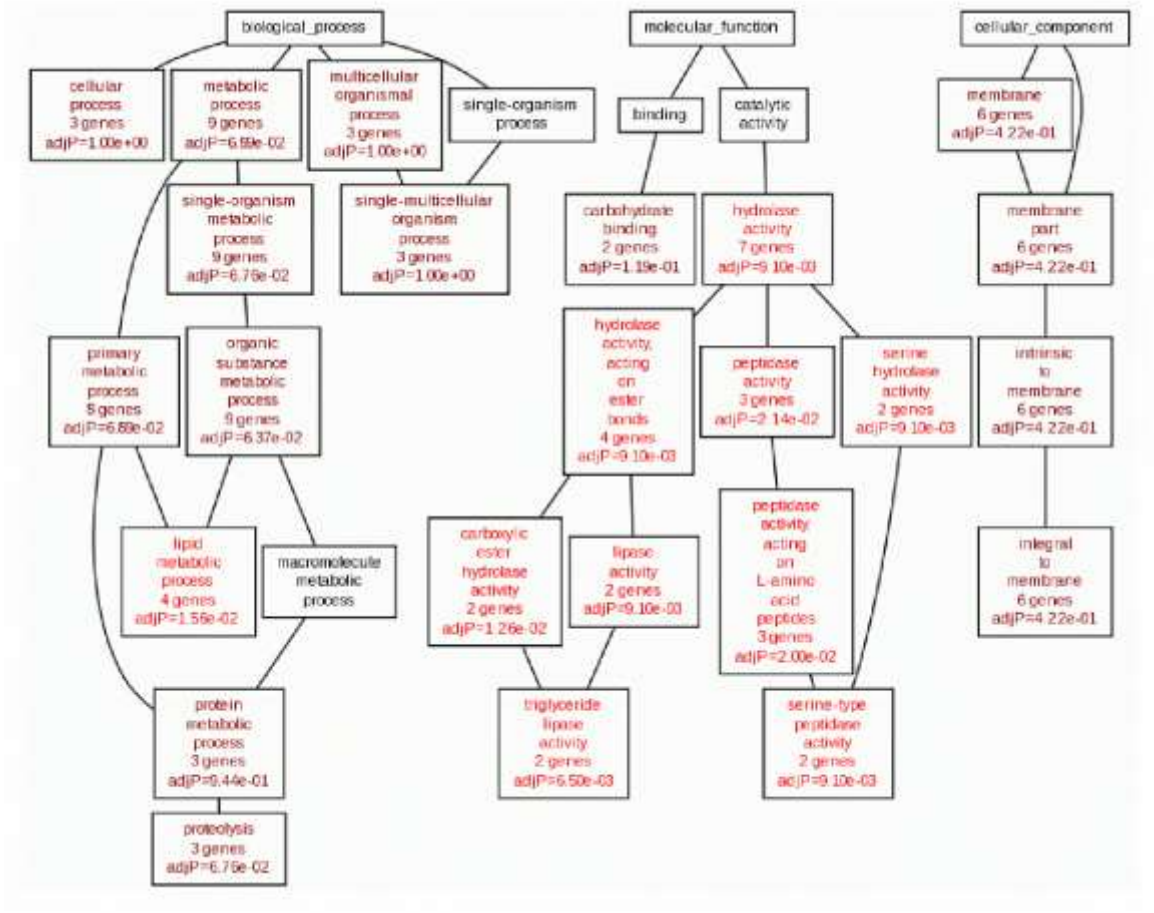

Figure S4. Gene Ontology enrichment via WEBGESTALT. Pathway analysis of SA-mediated genomic changes in day 15 N2 WT worms. To further explore the genomic effects of SA administration on N2 WT worms, Gene Ontology (GO) enrichment was performed using WebGestalt, an approach which incorporates information from different public resources and provides graphical depiction of large gene sets from functional genomic, proteomic, and large-scale genetic studies. Biological relationships among Directed acyclic graphs (DAG) were generated using GOView, a web-based application to allow users to visualize and compare multiple provided GO term lists to identify common and specific biological themes. (A) DAG of Group I genes upregulated by SA administration. Chart highlights the metabolic process, lipid metabolic process, and proteolysis pathways among many others as being altered favorably by SA administration.

Supplemental Table 1: Lipid metabolism genes identified by Gene Ontology/WebGestalt analysis. List of lipid metabolism genes identified by Gene Ontology/WebGestalt analysis that are significantly upregulated by salicylamine treatment. This is the subset of genes most likely to represent downstream targets of *ets-7*.

Supplemental Table 1: Lipid Metabolism Genes Identified by GO/WebGestalt

Lipid Metabolic Process			**ID: GO: 0006629**
Gene Symbol	**Gene Name**	**EntrezGene**	**Ensembl**
Y65B4BR.1	Protein Y65B4BR.1	190488	CELE_Y65B4BR.1
W02B12.1	Protein W02B12.1	174746	CELE_ W02B12.1
F28H7.3	Protein F28H7.3	179490	CELE_ F28H7.3
Y54G2A.45	Protein Y54G2A.45	3896802	CELE_ Y54G2A.45

Supplemental Table 2: Metabolic process genes identified by Gene Ontology/WebGestalt analysis. The larger list of genes exhibiting significant changes with salicylamine treatment, and reorganized as representing metabolic processes more broadly by GO/WebGestalt. Notably, this list includes all of the genes in Supplemental T1 and captures *ets-7* itself.

Supplemental Table 2: Metabolic Process Genes Identified by GO/WebGestalt

Metabolic Process			**ID: GO: 0008152**
Gene Symbol	**Gene Name**	**EntrezGene**	**Ensembl**
Y65B4BR.1	Protein Y65B4BR.1	190488	CELE_Y65B4BR.1
pcp-2	Protein PCP-2	177741	CELE_F23B2.12
W02B12.1	Protein W02B12.1	174746	CELE_ W02B12.1
ets-7	Protein ETS-7	184687	CELE_F19F10.5
Y54G2A.45	Protein Y54G2A.45	3896802	CELE_ Y54G2A.45
smd-1	Protein SMD-1	173269	CELE_F47G4.7
F13D12.6	Protein F13D12.6	174802	CELE_F13D12.6
F28H7.3	Protein F28H7.3	179490	CELE_ F28H7.3
K10C2.3	Protein K10C2.3	180917	CELE_K10C2.3

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CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

Introduction

The focus of this dissertation project was to elucidate the molecular processes involved in reactive γ-ketoaldehyde/isoketal-mediated oxidative injury, and in doing so to provide the basis for the development of new rational therapeutic interventions to limit oxidative damage in aging. This is a reasonable avenue to pursue in view of the multitude of studies suggesting that reactive oxygen species (ROS) play a role in normal aging by causing random deleterious oxidative damage to tissue biomolecules (e.g. lipids, proteins, and DNA) (Halliwell and Gutteridge 1990, Gutteridge 1995, Butterfield 1997, Muller, Lustgarten et al. 2007). Previous studies have shown that lipid peroxidation leads to the formation of reactive aldehydes, amongst which the γ-ketoaldehydes formed via the isoprostane pathway of lipid peroxidation are the most reactive and injurious. Reactive γ-ketoaldehydes adduct to proteins, and increased IsoK-protein adducts have been reported in a number of disease conditions, including atherosclerosis, myocardial infarction, end-stage renal disease, hypoxia, among other disease conditions (Brame, Boutaud et al. 2004, Fukuda, Davies et al. 2005, Davies, Brantley et al. 2006, Guo, Chen et al. 2011). The development and characterization of selective scavengers of IsoKs have proven to be a remarkable protective intervention against oxidative damage in a variety of animal models of oxidative stress (Davies, Brantley et al. 2006, Davies, Bodine et al. 2011).

Throughout the work presented in this dissertation, we have dedicated efforts in investigating the role of IsoK-mediated oxidant injury and its effects on aging in the model

organism, *Caenorhabditis elegans*. We first set out to establish an *in vivo* measure of oxidative damage using the chromosomally integrated, dual fluorescence-based reporter strain, *Pgst-4::GFP* (VP596 dvls19[pAF15(Pgst-4::GFP::NLS)];vsIs33[Pdop-3::RFP]). After results indicated *Pgst-4::GFP* reporter strain was best used for qualitative measurements, we refocused efforts into optimizing a quantitative measure of oxidative damage in *C. elegans*. During the course of this thesis work, a highly sensitive and specific gas chromatography-mass spectrometry (GC/MS) technique was developed to measure endogenous lipid peroxidation in *C. elegans* via measurement of the lipid peroxidation product F_3-isoprostanes (F_3-IsoPs) by GC/MS approaches; specifically in the context of this thesis work, for the purpose in investigating the role of oxidative stress in aging. The core of this dissertation revolved around the discovery of two major nodes through which IsoK-mediated oxidative damage contributes to the aging process. We discovered that SIR-2.1 and ETS-7 play important roles in the progressive molecular development of aging, and that selective scavengers of IsoKs (i.e. salicylamine, SA) preserves the biochemical activity of these proteins to enhance normal antioxidant defenses against aging. Taken together, this body of work supports the potential for IsoK scavengers, such as SA, as a clinically useful anti-aging therapeutic intervention

Summary of Findings

Development of a fluorescent-based assay for *in vivo* measurement of oxidative stress

The development of tools for sensitive, specific, and accurate measures of oxidative damage is an important area of current research. In Chapter II, we reported the adaptation of the *Pgst-4::GFP* transgenic reporter strain for studying the role of oxidative stress in aging. Initial efforts in establishing an oxidative stress paradigm utilized the potent redox-generating agent, 5-hydroxy-1,4-naphthoquinone (juglone) to generate a stress environment to activate antioxidant

response elements with the hopes in capturing a protective effect against oxidative stress when pre-treated with the selective IsoK scavenger, salicylamine. In fact, after establishing a juglone dosing paradigm and a concentration range within maximum efficacy (EC_{max}), preliminary studies showed the effect of salicylamine pre-treatment on attenuating the SKN-1 response in *C. elegans* (Fig. 5.1).

Although this data seemed promising, the oxidative stress model used in the *Pgst-4::GFP* reporter strain was troubling, as juglone is an arylating agent known to react readily with sulfur nucleophiles. Concern was raised for the veracity of this reporter strain in indirectly measuring

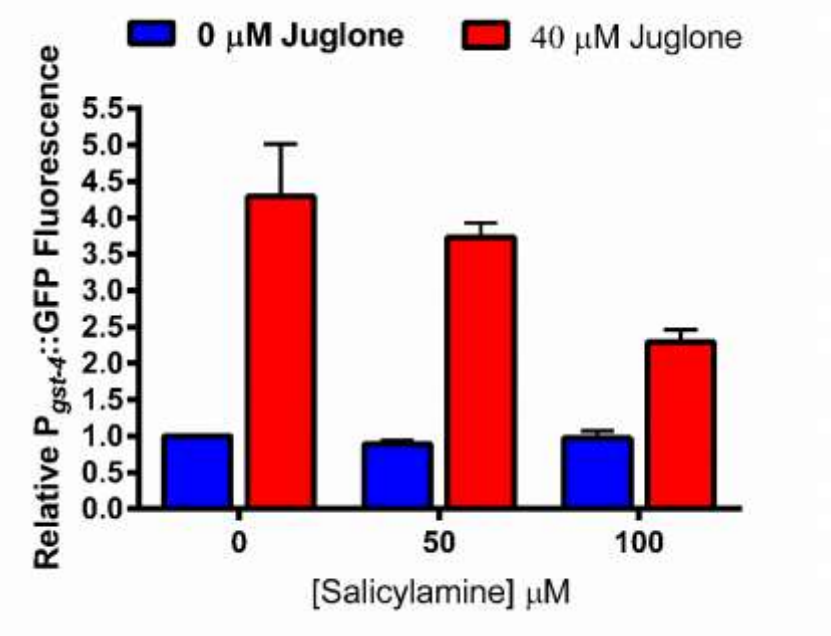

Figure 5.1. SA protects against juglone-mediated oxidative stress. Salicylamine (0, 50, 100 μM) was plated onto nematode growth media (NGM) agar plates and allowed to dry before plating with UV-killed *E. coli* OP50. Worms were grown on foil-covered SA-NGM-OP50-seeded agar plates at 20C for 2 days in the dark, as salicylamine is light-sensitive (Zagol-Ikapitte, Matafonova et al. 2010). A maximal dose (EC_{max} = 40 μM) of juglone and vehicle control was administered for 1 hr in liquid and then transferred to OP50-seeded NGM agar plates for 4 hr recovery. Juglone-exposed worms were transferred to 96-well plates at a concentration of 200 worms/well with 5 replicate wells per treatment condition, and GFP/RFP fluorescence intensity was measured with a FLUOStar Optima (BMG LabTech, Ortenberg, Germany). Data are expressed as means ± SEM from 5 technical replicate wells per treatment condition from one independent experiment.

SKN-1 activation, due to the potential for juglone reaction with cysteine residues on the proteins that regulate SKN-1 activation, which may produce a false positive fluorescent signal. Bearing these concerns in mind, other pro-oxidants typically used in *C. elegans* were tested on the transgenic fluorescent reporter strain in order to establish a juglone-free oxidative stress paradigm. Comparison of fluorescent intensity results to GC/MS analysis of F_3-IsoPs collected from the *Pgst-4::GFP* animals treated with various pro-oxidants (juglone, hydrogen peroxide, tert-Butyl-hydroperoxide, and paraquat) lead to the discovery *Pgst-4::GFP* reporter strain was a less sensitive measure of oxidative stress. In the *Pgst-4::GFP* reporter strain, the pro-oxidant juglone produced the greatest effects in fluorescent intensity; possibly due to its ability to create additive fluorescence when reacting with regulators of SKN-1. In the GC/MS approach, F_3-IsoP collection resulted in the predicted increased oxidative damage responses in the presence of all pro-oxidants tested. Not surprisingly, tert-Butyl hydroperoxide (t-BOOH) generated the greatest response in increasing lipid peroxidation products. This is possibly due to the fact t-BOOH is a more hydrophobic agent and could more readily undergo lipid peroxidation in the hydrophobic environment in which F_3-Isoprostanes are generated within.

In sum, *Pgst-4::GFP* is best used in qualitative studies of in vivo oxidative stress, and the use of juglone as a pro-oxidant in conjunction with this reporter strain is highly discouraged. Studies needing quantitative measures of oxidative damage are best serviced by measurement of F_3-Isoprostanes in the animals.

F_3-Isoprostanes as a measure of *in vivo* oxidative damage in *C. elegans*[5]

Oxidative stress has been associated with development of a wide variety of disease processes, including cardiovascular disease and neurodegenerative diseases, as well as progressive and normal aging processes. Isoprostanes (IsoPs) are prostaglandin-like compounds that are generated *in vivo* from lipid peroxidation of arachidonic acid (AA, C20:4, ω-6) and other polyunsaturated fatty acids (PUFA). Since the discovery of IsoPs by Morrow and Roberts in 1990, quantification of IsoPs has been shown to be excellent biomarkers of *in vivo* oxidative damage. Eicosapentaenoic acid (EPA, C20:5, ω-3) is the most abundant PUFA in *C. elegans* and gives rise to F_3-IsoPs upon nonenzymatic free radical-catalyzed lipid peroxidation. In Chapter III, the protocol presented is the current methodology that our laboratory uses to quantify F_3-IsoPs in *C. elegans* using gas chromatography/mass spectrometry (GC/MS). The methods described herein has been optimized and validated to provide the best sensitivity and selectivity for quantification of F_3-IsoPs from *C. elegans* lysates.

One major addendum to the assay that was not originally included in the time of its printing is the inclusion of an additional thin-layer chromatography (TLC) separation modification to strengthen signal strength of the individual samples collected. This particular modification was historically used as an additional extraction step to purify urine or plasma samples from human patients that received the anesthetic, Propofol. Although an additional separation step decreases the overall yield of the sample, this extraction services as a method of tweaking the signal-to-noise ratio. Utilizing this second TLC separation step has led to sharper *m/z* 567 peaks in the GC/MS

[5] Excepts taken from Thuy Nguyen and Michael Aschner "F_3-Isoprostanes as a measure of *in vivo* oxidative damage in *Caenorhabditis elegans" Current Protocols in Toxicology* 62:11 **(2014)** : 11.17.1-11.17.13. © 2014 John Wiley & Sons, Inc. Reprinted with permission of John Wiley & Sons, Inc.

chromatogram with an increased signal-to-noise ratio. Considering the loss in yield of sample due to the presence of a second TLC extraction/separation step, it is recommended to having a starting material of 2-3 mg total protein concentration (~30,000 – 40,000 worms) per sample condition. In summary, it is highly recommended to utilize this modification when measuring F_3-isoprostanes in *C. elegans* (Appendix A).

Scavengers of γ-KAs extend *C. elegans* lifespan through interactions with SIR-2.1 & ETS-7[6]

As stated earlier, IsoKs are highly reactive γ-ketoaldehyde products of lipid peroxidation that covalently adduct lysine side chains in proteins, impairing their function. In Chapter IV, we tested the hypothesis that IsoKs contribute to molecular aging through adduction and inactivation of specific protein targets, and that this process can be abolished using the selective IsoK scavenger, SA. Administration of SA extends adult nematode longevity by nearly 56% and prevents multiple deleterious age-related biochemical and functional changes. Testing of a variety of molecular targets for SA's action revealed the sirtuin SIR-2.1 as the leading candidate. When SA was administered to a SIR-2.1 knockout strain, the effects on lifespan and healthspan extension were abolished. Further investigations into the mechanisms of SIR-2.1-dependent SA-mediated lifespan extension indicated these beneficial effects were not mediated by large changes in gene expression programs or by significant changes in mitochondrial function. However, expression array analysis did show SA-dependent regulation of the transcription factor *ets-7* and associated genes. In *ets-7* knockout worms, SA's longevity effects were abolished, similar to *sir-2.1* knockouts. However, SA dose-dependently increases *ets-7* mRNA levels in non-functional SIR-

[6] Excerpts taken from Thuy Nguyen, Samuel Caito, William Zackert, James West, Shijun Zhu, Michael Aschner, Joshua Fessel, and L. Jackson Roberts, II "Scavengers of reactive γ-ketoaldehydes extend *Caenorhabditis elegans* lifespan and healthspan through protein-level interactions with SIR-2.1 and ETS-7" *Aging* **(2016)** *In press*.

2.1 mutant, suggesting that both are necessary for SA's complete lifespan and healthspan extension.

In this study, we identified the ETS transcription factor, ETS-7, as a longevity determinant in *C. elegans*. ETS factors are members of one of the largest families of evolutionarily conserved transcriptional regulators, which play fundamentally important roles in differentiation, development, transformation, and cellular proliferation (Findlay, LaRue et al. 2013). The oncogenic *v-ets* was the first member of its family to be studied; discovered in 1983, this family member was found to be a part of the transforming fusion protein (p135, gag-myb-ets) expressed by the avian retrovirus E26 (Leprince, Gegonne et al. 1983, Klempnauer and Bishop 1984). This particular family member, the *v-ets* oncogene, is known to cause mixed erythroid and myeloid leukemias (Blair and Athanasiou 2000). It is from this founding member that the name *ets* is derived from; ETS stands for the E26 transforming sequence or E-twenty-six specific sequence.

All ETS family members are characterized by a conserved sequence that encodes the DNA-binding (ETS) domain. The ETS family of transcription factors is one of the largest families, consisting of 28 ETS genes in humans, 27 in mice, 11 in sea urchin, 10 in *Caenorhabditis elegans*, and 9 in *Drosophila* [see reviews, (Seth and Watson 2005, Gutierrez-Hartmann, Duval et al. 2007, Hollenhorst, McIntosh et al. 2011)]. The ETS family of transcription factors was of particular interest in this work due to a number of recently published manuscripts implicating ETS family members in regulation of longevity through effects on lipid metabolism (Thyagarajan, Blaszczak et al. 2010, Alic, Giannakou et al. 2014, Sun, Sun et al. 2015).

In 2014, Thyagarajan and colleagues identified ETS-4 as a longevity determinant in *C. elegans* through gene knock-out studies, wherein worms that lack ETS-4 lived significantly longer; this group demonstrated that *ets-4* is required in the post-developmental stage to regulate adult

lifespan (Thyagarajan, Blaszczak et al. 2010). Using bioinformatics, this group identified seventy ETS-4-regulated genes that were enriched for known longevity effectors in the function of lipid transport and metabolism, as well as immunity. The *C. elegans ets-4* is the ortholog of the vertebrate SPEDEF/SAM pointed domain containing ETS transcription factor. Studies in human cell lines have implicated SPDEF function in tumorigenesis, where SPEDEF was found to have alterations in mRNA levels in breast and prostate tumors (Feldman, Sementchenko et al. 2003, Gu, Zerbini et al. 2007), as well as alterations in cell migration and invasion pathways of tumor cell lines (Oettgen, Finger et al. 2000, Chen, Nandi et al. 2002, Gunawardane, Sgroi et al. 2005, Gu, Zerbini et al. 2007, Turner, Moussa et al. 2007). Thus, the findings in this study provided a link between ETS factor, physiological aging and tumor susceptibility.

In *Drosophila*, two studies from the Partridge group found a role for the ETS transcriptional repressor, *Anterior open (Aop)* in extending adult lifespan (Alic, Giannakou et al. 2014, Slack, Alic et al. 2015). *Aop* is the functional ortholog of human *Etv6* gene, and in *Drosophila*, it is found in the adult gut and is regulated by the dFOXO, along with being known to counteract the activity of the ETS activator, *Pointed (Pnt)*. In 2014, Alic, Partridge, and colleagues showed that dFOXO activates *Aop* in adult fly gut to antagonize the ETS activator, *Pnt*, by AOP directly displacing PNT from a dFOXO gene promoter/enhancer bound region to extend lifespan. In addition, using Gene Ontology (GO) analysis, this group found that the most represented GO category was "lipid particle" which encapsulates both lipid droplets and sites of cellular lipid storage. From this, the group proved through genetic, biochemical, and functional studies that PNT/AOP and dFOXO together regulate lipid metabolism. Taken together, this indicates that *Aop/Pnt* coupling have metabolic and longevity functions in the fly. Most importantly, in 2015, the same group identified a potent small-molecular inhibitor of the Ras-Erk-ETS signaling as an anti-aging intervention,

which solidifies the "druggability" of pathways involving ETS signaling as a pro-longevity mechanism.

In summary, we found that SIR-2.1 and ETS-7 are major nodes that play important roles in selective scavengers of IsoK-mediated lifespan extension in *Caenorhabditis elegans*. Most importantly, this work indicates the translation potential for the selective scavenger of isoketals, salicylamine, is a clinically useful anti-aging therapy. The translation of SA into studies of human longevity can proceed relatively fast, as it is a naturally occurring compound found in buckwheat seeds and is currently awaiting approval for use as a natural supplement by Generally Regarded as Safe (GRAS).

Future Directions

While the data presented in this dissertation clearly suggests a role for lipid peroxidation-mediated oxidative stress in aging, and consequently salicylamine-mediated protective effects against aging via an undefined pathway involving SIR-2.1 and ETS-7 in *Caenorhabditis elegans*, the exact mechanism which SA administration affects longevity regulation remains to be determined. Considering the mechanism of action for SA scavenging of IsoKs, we are well aware that SA functions to protect against deleterious effects of isoketal adduction on the protein level, therefore it is within reason to assume there are other protein targets likely to be preserved with SA treatment which may also function in the regulation of longevity. A full characterization of the proteome-level effects of SA was beyond the scope of this present dissertation, but a next logical step in order to flesh out the findings from this dissertation.

The dosing regimen from the study presented is also of slight concern. The precise drug exposure response with SA administration cannot be obtained from the studies reported in this dissertation. Animals were exposed continually with SA throughout its lifetime, but biochemical

measures were taken at a snapshot of its life time. To develop a more accurate SA exposure response profile in future directions, we would recommend measuring drug exposure over time for every experimental approach, similar to the drug exposure over time profiles generated in the progressive autofluorescent lipofuscin experiments (Fig. 4.1C and 4.3D). By measuring drug exposure over time and generating area-under-the-curve (AUC) analysis we were able to develop a more comprehensive profile for drug administration for that particular assay. Developing drug exposure profiles with AUC analysis for our biochemical measures (such as F_3-IsoP collection, western blot analysis, *etc*.) would have given us more powerful tools to analyze the pharmacologic range of SA in *C. elegans* and determine a dose-effect relationship with SA administration.

Lastly, all studies conducted in this dissertation was generated exclusively in *C. elegans*. While the genetic tractability and short lifespans makes *C. elegans* a powerful model for aging research, the anti-aging effects of SA administration will need to be verified in a more complex model organism. Considering the beneficial effects of SA treatment in a variety of mammalian models of disease (reviewed more in-depth in Chapter I), the translation into other mammalian models of aging is feasible and the next logical progression of this research.

Conclusions

The overall goal of this thesis research was to examine the molecular and biophysical processes of IsoK-mediated oxidative damage that contribute to aging, and to consequently provide the basis for the development of new rational therapeutic interventions, such as salicylamine, to limit oxidative damage. Through these studies, we have developed an analytical approach to assess endogenous levels of oxidative stress in *C. elegans*, and identified a major pro-longevity pathway in which pharmacological manipulation could extend organismal lifespan.

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APPENDIX A

F_2-Isoprostane Assay – Additional Steps Required for Samples from Patients Receiving Propofol Anesthetic

Introduction

The following scheme is to be performed after samples are extracted using the silica Sep-Pak cartridge, and dried under nitrogen at 37°C. After TLC chromatography is performed, proceed with the existing protocol by reacting samples with PFBB. This modification is in courtesy of William E. Zackert from the Clinical Pharmacology Department in Vanderbilt University Medical Center.

Thin-Layer Chromatography Protocol

Dry samples eluted from silica Sep-Pak in normal assay, under N_2 at 37°C. Re-suspend in 50ul methanol. Spot on a pre-washed silica TLC plate and place in tank containing 100mls of solvent consisting of:

Supplementary Table 3: TLC chromatography tank composition

Chloroform	85 %
Methanol	15 %
Acetic Acid	1 %
Water	0.8 %

Spot a standard plate with 5ug PGF_{2a}, free acid TLC standard, place plates in tank and run to 13cm above the origin. Spray standard plate with phosphomolybdic acid and place on hot plate until standard band appears. Scrape 2cm of silica off each sample plate from 0.5cm above the **top** of the F_{2a} band, to 1.5cm below the **top** of the band. Extract the silica with 1ml, 1:1 ethyl acetate: ethanol. Centrifuge, transfer supernatant to another microcentrifuge tube and dry under N_2. Perform PFBB and 2nd TLC (93:7) steps as normal.