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EFFECTS OF PHARMACEUTICAL MODULATION OF PROTEIN AGGREGATION ON LIFESPAN IN *CAENORHABDITIS ELEGANS*

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Effects of Pharmaceutical Modulation of Protein Aggregation on Lifespan in *Caenorhabditis elegans*

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In Memory of Malin Elisabeth Tolf (1982-2010)

ABSTRACT

Aging is accompanied by a disruption in various homeostatic networks including those devoted to maintaining protein conformation and integrity. Collectively this maintenance is referred to protein homeostasis or proteostasis. Growing evidence suggests that promoting proteostasis may not only be beneficial for protein aggregation-related disorders but may also influence normal aging. We have investigated various pharmacological means of promoting the proteostatic network and the effects on healthspan and longevity in the nematode *Caenorhabditis elegans.*

Here we show that known amyloid binding compounds can prevent protein aggregation and extend lifespan in *C. elegans*. We report that the widely used amyloid-binding compound, Thioflavin-T not only adheres to Amyloid-β (Aβ) but also inhibits its misfolding or aggregation, thereby delaying the accompanying disease state in a *C. elegans* model of Alzheimer's Disease (AD). Our data suggests that Thioflavin-T can prolong the vitality and lifespan of wild type (WT) *C. elegans* by stabilizing the proteostatic machinery. Additionally, we identified three other compounds related to Thioflavin-T that also extend lifespan. Interestingly, these other compounds are known to chelate copper and iron, two metals implicated in several aggregation-related disorders (Paper I).

Many age-related protein aggregation neurodegenerative disorders (NDs) are accompanied by alterations in levels of various metals such as iron and copper. Whether these metals are a cause or consequence of ND is not well understood. In a characterization of metal composition (the metallome) with age we show that normal aging of *C. elegans* is accompanied by accumulation of calcium, iron, copper and manganese. Additionally, we found that increased dietary iron enhances toxicity in both Aβ and PolyQ-associated models of protein aggregation. We were able to reduce endogenous iron levels in *C. elegans* and found that this ameliorated various models of proteotoxic disease. Moreover, we show that CaEDTA exposure increases healthspan and lifespan in WT animals. Taken together, our findings suggest that metal accumulation is an inherent part of the aging process and likely destabilizes the proteostasis of the organism (Paper II).

By using a *C. elegans* model for proteotoxicity we were able to screen a large natural library for additional drugs that could potentially enhance the healthspan and lifespan of *C. elegans*. Subsequently, we investigated the effects of 5-fluoro-2-deoxyuridine (FUdR) in models of protein aggregation. We have found that inhibiting reproduction with FUdR at standard laboratory concentrations improves protein homeostasis, stress resistance and healthspan of *C. elegans* (paper III).

LIST OF SCIENTIFIC PAPERS

I. Alavez S, Vantipalli MC, Zucker DJ, **Klang IM**, Lithgow GJ. (2011). Amyloid-binding compounds maintain protein homeostasis during ageing and extend lifespan. *Nature* **472**, 226-230.

II. **Klang IM**, Schilling B, Sorensen DJ, Sahu AK, Kapahi P, Andersen J, Swoboda P, Killilea D, Gibson BW, Lithgow GJ. (2014). Iron promotes protein insolubility and aging in *C. elegans*. *Aging* **6**, 975-991

III. Angeli S, **Klang IM**, Sivapatham R, Mark K, Zucker D, Bhaumik D, Lithgow GJ, Andersen JK. (2013). A DNA synthesis inhibitor is protective against proteotoxic stressors via modulation of fertility pathways in *Caenorhabditis elegans*. *Aging* **5**, 759-769.

ADDITIONAL PUBLICATION (NOT INCLUDED IN THESIS)

Lucanic M, Held JM, Vantipalli MC, **Klang IM**, Graham JB, Gibson BW, Lithgow GJ, Gill MS. (2011). N-acylethanolamine signaling mediates the effect of diet on lifespan in *Caenorhabditis elegans*. *Nature* **473**, 226-229.

CONTENTS

LIST OF ABBREVIATIONS

Aβ	Amyloid beta
APP	Amyloid precursor protein
AD	Alzheimer's disease
BP	2,2'-Bipyridine
CaEDTA	Calcium disodium ethylenediaminetetraacetic acid
CSF	Cerebrospinal fluid
DR	Dietary restriction
FUdR	5-fluoro-2-deoxyuridine
GSC	Germline stem cell
HSF-1	Heat shock factor 1
HSP	Heat shock protein
HD	Huntington's disease
HIF	Hypoxia-inducible factor
ICP-aes	Inductively coupled plasma atomic emission spectroscopy
IIS	Insulin-like signaling
ND	Neurodegenerative disease
PD	Parkinson's disease
ROS	Reactive oxygen species
ThT	Thioflavin T
UPP	Ubiquitin-proteasome pathway
UPR	Unfolded protein response
YFP	Yellow fluorescent protein

1 INTRODUCTION

1.1 *C. elegans*

Caenorhabditis elegans is a facultative self-fertilizing nematode hermaphrodite with a mean lifespan of ~18 days at 20°C. One self-fertilizing animal produces over 300 progeny over the course of 4-5 days and completes a full lifecycle in as little as 3 days. The adult animal consists of only 959 somatic cells composing many structures and organs including a completely mapped nervous system of 302 neurons (Riddle et al., 1997; White et al., 1986). The transparency of the animal is particularly useful, as one can use fluorescent reporters to visualize many biological processes *in vivo*. The N2 laboratory strain of *C. elegans* has long been defined as the wild-type reference and it can easily be cultured on agar plates with a bacterial lawn of *E. coli* as a food source. Moreover, freezing provides a convenient method of storing stocks of worms . Stocks have remained viable for decades in liquid nitrogen or at -80°C which minimizes the problem of laboratory deviation of WT by spontaneous mutations including transposition events.

Since its introduction by Sydney Brenner in 1973 (Brenner, 1973), *C. elegans* has emerged as a powerful model organism with many attributes applicable to genetic and biomedical research. From the study of the genetics of behavior to gene regulation, cell signaling and neuronal development it has also become a crucial avenue for studying the genetics and biology of aging (Hekimi et al., 2001; Kenyon, 2010; Lithgow et al., 1994). The entire genome of *C. elegans* has been sequenced and in addition to the many mutant strains available, RNA interference (RNAi) provides a fast and simple means of reducing the expression of specific genes. In short, RNAi is an endogenous post transcriptional gene silencing mechanism triggered by the presence of double-

stranded RNA (dsRNA). In *C. elegans* dsRNA is generally introduced into the animal through feeding.. Once inside the animal, the enzyme Dicer cleaves dsRNA into shorter dsRNA fragments. After the passenger strand is degraded, the guide strand is incorporated into the RNA-induced silencing complex (RISC). RISC aids in the pairing of the guide strand with a complementary mRNA which is then also cleaved by RISC. Because this process is catalytic and contains associated machinery for propagation and extracellular transport in *C. elegans* one only needs to introduce small amounts of dsRNA for efficient knockdown of a targeted gene. Andrew Fire and Craig C. Mello were awarded the Nobel Prize in Physiology or Medicine for characterizing RNA interference (RNAi) in *C. elegans*. In addition to genetic tractability, many different manipulations make *C. elegans* particularly suitable for aging research. For an example, the nematodes environment can easily be manipulated with the addition of chemical compounds or with various food concentrations. Furthermore, culturing of *C. elegans* is relatively inexpensive in contrast with notoriously expensive mice aging research.

1.2 AGING

In humans, aging is the leading risk factor for many debilitating disorders such as cardiovascular disease, cancer and dementia. These disorders are not only socially deleterious, but also extremely costly. The total estimated worldwide cost of dementia alone is $604 billion in 2010 (Reisberg et al., 1997). The global population is aging at a rapid pace, with an estimated 13 countries being designated as “super-aged” in 2020 with more than 20% of the population over 65. This number is expected to rise to 34 countries by 2030 according to Moody’s Investor Service. This unprecedented pace of global aging will undoubtedly have a significant effect on the economic output over the years. The

current challenge is to prevent the chronologically aging population from becoming a global economic crisis. It is crucial that aging research keep advancing so that the dependency ratio of the human population is kept as low as possible.

Aging is characterized as an entropic breakdown of biological function on most, if not all levels. The biology of aging was long thought to be a random, passive event caused by stochastic deterioration and consequently difficult to control. However, discoveries predominantly made in *C. elegans* have revealed aging as a highly plastic process and several signaling pathways and transcription factors determine longevity (Johnson, 2006; Johnson et al., 1984; Kenyon et al., 1993). Interestingly, many of these pathways and their effect on lifespan have been shown to be evolutionary conserved (Kapahi et al., 2010; Kenyon, 2001). *C. elegans* display a number of age-related changes reminiscent of higher organisms, including humans. For example, worms become less motile with age, have reduced feeding as measured by pharyngeal pumping (Huang et al., 2004) and accumulate insoluble protein (David et al., 2010; Herndon et al., 2002; Reis-Rodrigues et al., 2012). Thus, discoveries of the aging process in *C. elegans* can serve as a platform for research in interventions on human aging.

1.2.2 Manipulation of lifespan in *C. elegans*

C. elegans lifespan can be manipulated via several known genetic pathways (Murakami et al., 2000). The first lifespan modulator gene identified in *C. elegans*, *age-1*, belongs to the *insulin-signaling like pathway* which is a nutritional response program. The *age-1(hx546)* mutant has a life expectancy 65% longer than WT. *Age-1* mutations have little effect on fertility, length of reproduction and rate of development. The mutants are also resistant to various stressors such as excess heat and paraquat (frequently used as an oxidative

stressor in *C. elegans*). Another famous gerontology gene, *daf-2* was identified to have a role in aging in 1993. The DAF-2 receptor lies upstream of *age-1* and bears structural homology with the human insulin/ IGF type receptors. Thus, a *daf-2* mutation also results in a robust lifespan extension, which was found to be dependent on transcription factor DAF-16 (Kenyon et al., 1993). Under favorable conditions DAF-16 is ultimately inhibited by AGE-1 and prevented from nuclear localization. During absence of nutrients or unfavorable conditions, AGE-1 does not signal to phosphorylate DAF-16, which subsequently enters the nucleus to activate many longevity responses. As expected, mutations in *daf-16* lead to a shortening of lifespan.

Many other genetic programs influence survival in *C.* elegans. The disposable soma theory proposes that the energy expenditure for reproduction is costly for the organism in that it diverts resources from somatic cells and thus accelerates aging (Kirkwood and Holliday, 1979). In line with this hypothesis, the loss of germline precursor cells in *C. elegans* results in animals lacking a gonad and interestingly, an extension of lifespan. This lifespan extension is thought to result from a reallocation of resources to somatic cells and it requires DAF-16-mediated regulation of fat metabolism (McCormick et al., 2012; O'Rourke et al., 2009). The entire reproductive system of adult *C. elegans* is produced by the progeny of four precursor cells present in the first larval stage, Z1-Z4 (Kimble and White, 1981). The outermost cells; Z1 and Z4 give rise to the somatic gonad (uterus, spermatheca and other somatic tissues) while Z2 and Z3 are germ-cell precursors. The hermaphrodite germline includes mature sperm and ova with differentiating gametes and totipotent germline stem cells (GSCs). Two cells of the somatic gonad also give molecular signals that promote GSC proliferation (Schedl, 1997). The removal of the germ-cell precursors Z2-Z3 results in a large increase in lifespan of worms (Hsin and Kenyon, 1999).

Interestingly, animals eliminated of Z1-Z4 entirely lack a gonad but are not long-lived. Thus, the somatic gonad also signals to influence longevity. This *germline signaling* has been shown to modulate lifespan in worms, flies and possibly mice (Flatt et al., 2008; Hsin and Kenyon, 1999). In addition to removal of specific germline-precursor cells, there are mutations resulting in germline elimination. The germline is removed in *glp-1* mutants (Austin and Kimble, 1987) and lifespan is extended because of removal of totipotent GSCs that normally produce cues that inhibit longevity (Arantes-Oliveira et al., 2002).

A reduction in nutrients can also extend the lifespan of *C. elegans.* Depending on the type of *dietary restriction* (DR), various "longevity pathways" are involved. A reduced calorie intake, complete absences of food or alternative day feeding all seem to act by various pathways to extend lifespan (Greer and Brunet, 2009). In addition to food manipulation, there are genetic means to induce DR. Mutations which culminate in reduced food intake are called *eat* mutations. Many *eat* mutations cause reduced pharyngeal pumping and thus lead to dietary restriction and increase in lifespan. One can also mimic dietary restriction with molecular compounds. For an example, we previously described a conserved dietary-longevity signaling pathway that can be modulated pharmaceutically (Lucanic et al., 2011).

DR signaling pathways are by no means exclusive to pharmaceutical intervention. Well over 70 compounds have been demonstrated to extend lifespan in *C. elegans* by various mechanisms of action (Lucanic et al., 2013; Ye et al., 2014). Many compounds that extend lifespan in *C. elegans* are FDA approved and are widely used in human pharmacology including aspirin (Wan et al., 2013), vitamin E (Harrington and Harley, 1988) and lithium (McColl et al., 2008). There is a wide class of compounds that promote longevity in *C. elegans*

with mechanisms including stress-resistance, autophagy, DAF-16-dependency, antioxidant properties, hormesis, proteostasis and TOR-dependency. The target of rapamycin (TOR) pathway is a major, highly conserved nutrient-sensing pathway that, when down-regulated lead to lifespan increase in worms, flies and mice (Bjedov et al., 2010; Chen et al., 2013; Selman and Partridge, 2012; Spong and Bartke, 2012; Wilkinson et al., 2012). It is named after the drug rapamycin, which inhibits the central component of the pathway.

Metformin is another drug that has been shown to extend lifespan in *C. elegans*. There are several mechanisms suggested for metformin-mediated lifespan extension in *C. elegans.* Early reports suggested that metformin improves healthspan and lifespan by activating DR and antioxidant defense longevity pathways (Onken and Driscoll, 2010) but it has also been shown to alter folate and methionine metabolism in the bacterial food source, thereby extending lifespan (Cabreiro et al., 2013). Another suggested mechanism of metformin is a hormetic response, in which metformin-induced production of ROS increases lifespan (De Haes et al., 2014). Indeed, mounting evidence suggests that multiple mechanisms of action of pharmaceuticals might be the norm, rather than the exception (Imming et al., 2006) and *C. elegans* provides an excellent platform in which to identify and investigate compounds that can be further explored for beneficial effects on aging in mammals.

Table 1. Example of the diversity of compounds that increase lifespan in *C. elegans*

Compound	Type of Compound	Potential mechanism
Tocotrienol	Natural product	Antioxidant
EUK-8/EUK134c	Synthetic	Stress resistance
Tamarixetin	Natural flavonoid	Stress resistance
Resveratrol	Natural product	Stress resistance, autophagy, TOR
Ethosuxumide	Synthetic, anticonvulsants	Serotonin dependent, DR
Blueberry extract	Natural product	HSR, osmotic pathway
Cocoa	Natural product	Antioxidant, metal chelator
Lipoic Acid	Natural metabolite	Stress resistance
Valproic acid	Synthetic psychoactive	DAF-16 dependent
Acetic acid	Reagent, natural product	DAF-16 dependent
Catechin	Natural flavonoid	DAF-2, NHR-8, MEV-1 dependent
Spermidine	Natural polyamine	Becline-1 dependent
Trehalose	Natural disaccharide	Stress resistance, DAF-16 dependent
Metformin	Biguanide, antidiabetic	DR mimetic, SKN-1 dependent
Thioflavin T	Synthetic, benzothiazole	Protein homeostasis
Curcumin	Natural product	Protein homeostasis
CaEDTA, EDTA	Synthetic	Metal chelator, protein homeostasis
Diallyl trisulfide	Natural product	SKN-1 dependent
Royal jelly	Natural product	DAF-2/DAF-16 dependent
Vitamin E	Natural product	Antioxidant
LiCl	Chemical mood stabilizer	GSK-3 and LSD-1 dependent
Damask Rose extract	Natural product	Antioxidant
Rapamycin	Natural product	TOR
Astragalan	Natural product	DAF-16 dependent
Reserpine	Natural product	Stress resistance
Celecoxib	Synthetic COX2 inhibitor	DAF-16 dependent
Oxaloacetate	Krebs cycle metabolite	DAF-16/AMPK/ dependent

Table adapted from (Lucanic et al., 2013)

1.3 PROTEIN AGGREGATION

Proteins typically fold into specific three-dimensional conformations after synthesis. This native state is imperative for the functionality of the protein and a misfolded protein will be more likely to aggregate, often due to hydrophobic residues being improperly exposed. Therefore, the biological pathways within cells that control the biogenesis, trafficking, folding and degradation of proteins are critical for overall cellular and organismal functionality and survival.

Organisms respond to insults such as heat, oxidative stress and environmental toxins by activating transcriptional and post-transcriptional programs that promote proteostasis. Molecular chaperones are proteins that assist the non-covalent folding or unfolding of macromolecules. In many cases, chaperones prevent newly synthesized proteins from misfolding into non-functional structures. Heat Shock Proteins (HSPs) are a class of chaperones induced upon thermal stress. Many HSPs are activated by various stressors that otherwise cause aggregation (Li and Srivastava, 2004; Wu, 1995). In addition to molecular chaperones, the ubiquitin-proteasome pathway (UPP) plays a major role in maintaining proteostasis (Goodsell, 2003; Hershko and Ciechanover, 1982). The UPP degrades misfolded proteins in two well-characterized steps; tagging of the substrate protein with ubiquitin molecules and degradation of the tagged protein by the proteasome (Ciechanover et al., 1980; Haas et al., 1982; Hershko et al., 1980; Pickart and Eddins, 2004). In this manner the UPP controls the timed destruction of cellular proteins and allows for recycling of oligopeptides. When chaperone activity and the UPP are not sufficient at restoring proteostasis a more global disruption to protein folding ensues. In these scenarios the Unfolded Protein Response (UPR) is activated. The UPR is a

highly conserved pathway that is triggered by an accumulation of misfolded or aggregated proteins in the lumen of the endoplasmic reticulum. The UPR activates three mechanisms; first it halts protein translation as to reduce buildup of aggregates, secondly it works to degrade misfolded proteins and activate chaperones. Lastly, if balance is not restored, the UPR can initiate apoptosis. The mitochondrial unfolded protein response (UPR^{mt}) is a conceptually similar stress-response pathway that activates transcription of mitochocondrial chaperone genes to promote proteostasis within the mitochondrion.

1.3.2 Protein Aggregation in Disease

There are numerous causes for protein aggregation. Several known mutations cause improper aggregation-prone tertiary structures and result in accumulation of aggregates. A failure in the chaperone network or the ubiquitin-proteasome pathway due to aging and/or metabolic and environmental stressors can exacerbate proteostasis-related disease (Forsberg et al., 2010; Jansen et al., 2014; Rodriguez et al., 2014). Thus, many aggregation-related disorders are also aging diseases.

Alzheimer's Disease (AD) is the most common form of dementia and there is no cure to date. Roughly 30 million people suffer from AD in the world and it is predicted to affect 1 in 85 people by 2050 (Brookmeyer et al., 2007). The neuropathological hallmarks of AD are neurofibrillary tangles (NFT) and plaques composed of amyloid peptide (Aβ) processed from the amyloid precursor protein (APP). APP is a neuronal transmembrane protein that is critical to neuron growth and post-injury repair (Kamenetz et al., 2003; Korte et al., 2012; Young-Pearse et al., 2008). In AD, APP is divided into smaller fragments, some of which give rise to Aβ fibrils that form extracellular senile plaques (Tiraboschi et al., 2004). Aβ is neurotoxic at non-physiological concentrations *in*

vitro and $A\beta_{1-42}$ is known to rapidly self-aggregate in solution (Hilbich et al., 1991; Jarrett et al., 1993). The overproduction of Aβ is often regarded as the major contributor to the disease and evidence point toward the soluble, but not the fibrillar forms, as the cause for the pathology (Lue et al., 1999; McLean et al., 1999). In addition to Aβ plaques, AD brains also contain tau aggregates. Tau is a microtubule-associated protein that normally stabilizes the cytoskeleton and fosters the neuronal transport system. Tau is hyperphosphorylated in AD, creating sticky threads that culminate in neurofibrillary tangles and a disruption of microtubular transport (Mendoza et al., 2013). The prognosis for AD patients is poor. The symptoms generally progress from mild short-term memory loss to severe cognitive problems that render the individual dependent on full-time care. In addition to cognitive decline, AD is also accompanied by psychological effects such as depression, irritability and aggression (Burke et al., 1988; Burns et al., 1990; Shuttleworth et al., 1987). Therapies for AD include behavioral interventions but the majority of diagnosed patients are on pharmaceutical medications. Two classes of medications are in use for AD; a range of acetylcholinesterase inhibitors and one NMDA receptor antagonist. The acetylcholinesterase inhibitors lead to an increase in acetylcholine which is decreased in AD brains, and the NMDA receptor antagonist inhibits glutamate overstimulation and resulting excitotoxicity (Lipton, 2006). Both classes of drugs are only moderately effective in advanced stages and neither has proven to delay onset of the disease (Raina et al., 2008).

Huntington's Disease (HD) is a neurodegenerative genetic disorder causing cognitive decline, muscle twitching (chorea) and behavioral symptoms. HD is quite unique among age-related ND in that all cases are caused by mutation in a single gene for which genetic testing is available (Reilmann et al., 2014). HD is caused by mutation of the *HTT* gene, producing excessive CAG

trinucleotide repeats. Thus, HD belongs to the polyglutamine tract (polyQ) disease family. PolyQ is subject to increased aggregation and a progressive degeneration of nerve cells with age is a common pathology of PolyQ diseases. Symptomatic treatment options for HD include dopamine receptor antagonist that reduce dopamine levels but long-term use of these drugs have adverse effects and are not beneficial in later stages of HD.

Parkinson's Disease results in debilitating motor symptoms, which are collectively called Parkinsonism (Pilger et al.). The disease is idiopathic but as with AD, there are controversial reports of risk factors such as exposure to environmental toxins (Tanner et al., 2014). PD is characterized by the accumulation of alpha-synuclein and other proteins into Lewy bodies, proteinaceous aggregates within dopamine cells in the substantia nigra region of the brain. The mechanism behind formation of Lewy bodies is unclear but it seems that impairment of the UPS could be partially responsible (Feany and Bender, 2000; Masliah et al., 2000). Treatments of PD include levodopa and dopamine agonists that are effective at managing the early motor dysfunctions caused by the decline in dopamine. Unfortunately, in later stages of the disease drugs are ineffective and can contribute to dyskinesia, the involuntary twitching often displayed by PD patients.

1.3.3 *C. elegans* Models of Proteotoxic Disease

In addition to serving as a platform for studying the genetics of normal aging, *C. elegans* has become useful for the study of age-related aggregation disorders. The worm genome contains homologs of two-thirds of all human disease genes (Remm and Sonnhammer, 2000; Sonnhammer and Durbin, 1997) and transgenic animals have been created to express human peptides to study the formation of aggregation *in vivo*. Several AD models have been created by

transgenic expression of the human Amyloid β protein in *C. elegans* (Lublin and Link, 2013; McColl et al., 2012; Minniti et al., 2009). For example, CL4176[*smg-1(cc546ts); dvIs27(myo-3::Aβ3–42 let 39UTR(pAF29))*] is a strain expressing the human Aβ peptide in the body wall muscle which form intracellular aggregates (Drake et al., 2003). It is a temperature-sensitive strain in which Aβ is expressed in muscle upon a temperature upshift, leading to eventual paralysis of the animal. The paralysis phenotype provides a straightforward read-out when assessing a drugs effect on Aβ aggregation or toxicity. In addition, the Aβ aggregates can be visualized by immunohistochemistry and quantified by western blot (Minniti et al., 2009).

There are also HD worm models, including strain AM141[*rmIs133(P(unc-54) Q40::YFP)*] in which an extended polyglutamine stretch is expressed in the muscle of the animal (Morley et al., 2002). Expression of a huntingtin fragment containing polyQ expansions leads to protein aggregate formation, easily visualized by the yellow fluorescent protein (YFP) tag. This model can be used to address the effects of compounds on polyglutamine expansions by quantifying the number of aggregates by microscopy or by assessing the functional physiology of the animals by measuring movement. HE250 [*unc-52(e669su250)II*]) is a strain expressing a metastable mutant perlecan protein. Perlecan is a heparin sulfate proteoglycan core protein of the mammalian basement membrane. In HE250, perlecan misfolds, resulting in destabilization of muscle tissue with ensuing paralysis (Mackenzie et al., 1978). In contrast to the more specific AD and HD model strains, HE250 is used as an indicator of the general protein homeostatic network capacity (Gidalevitz et al., 2006).

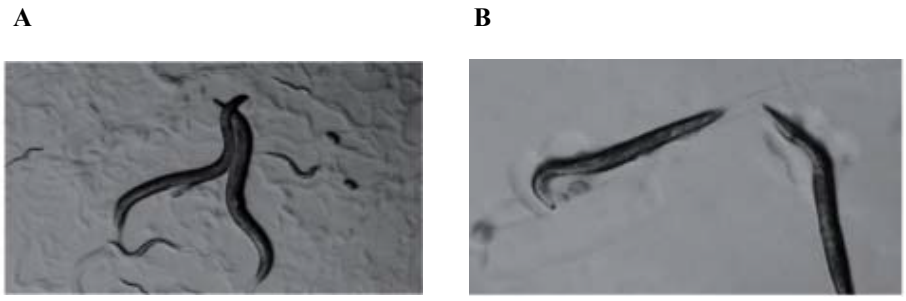

Figure 1. Paralysis phenotype in *C. elegans* model of proteotoxic disease

A) Adult HE250[*unc-52(e669su250)II*] exhibiting normal movement

B) Adult HE250[*unc-52(e669su250)II*] paralysis phenotype after 25°C upshift

1.3.4 Proteomic Studies in *C. elegans*

In addition to models of ND, one can employ a global approach in identifying and quantifying aggregation-prone proteins in *C. elegans*. Proteins such as tau and Aβ have long been separated using strong detergent buffers. The aggregation-prone property of these proteins renders them insoluble in most buffers but subsequent solubilization is possible with strong acids. A similar approach can be used to quantify insoluble proteins in *C. elegans*. One can visually analyze this 'insolublome' on SDS-PAGE gels and further identify and quantify the fraction using mass spectrometry-based proteomic analysis. Once the aggregation-prone proteins are identified utilization of software such as DAVID (The Database for Annotation, Visualization and Integrated Discovery) allows for discovery of common functional or structural features (Huang da et al., 2009)

1.3.5 Protein Aggregation: An Aging Hallmark

As many protein aggregation-associated diseases increase in frequency with age, it seems clear that cells generally lose the ability to maintain proteostasis over time. The imperative functions of protein degradation and disposal as well as chaperone efficiency all decline with age (Cuervo and Dice, 2000; Lund et al., 2002; Tonoki et al., 2009). Aging is also accompanied by an increase in oxidative stress, resulting in modifications such as oxidation and nitration, ultimately contributing to an increase in protein aggregation (Poon et al., 2006; Squier, 2001). We and others have shown that aging of *C. elegans* results in an accumulation of insoluble proteins, limiting the lifespan of the organism independent of a particular disease state (David et al., 2010; Reis-Rodrigues et al., 2012). Therefore, multiple lines of evidence suggests that protein aggregation is a key pathological feature of the aging process.

Interestingly, many transcription factors and responses that regulate proteostasis also govern aging in *C. elegans*. For example, overexpression of UPR mediator XBP-1 increases longevity (Taylor and Dillin, 2013) while a reduction of the IRE-1/XBP-1 UPR branch shortens the long lifespan caused by reduced IIS in *C. elegans* (Henis-Korenblit et al., 2010). Furthermore, a reduction in IIS provides protection from toxic protein aggregation (David et al., 2010). This protection is dependent upon both DAF-16 and HSF-1(Hsu et al., 2003; Morley and Morimoto, 2004). HSF-1 is a conserved transcription factor which upregulates the transcription of many chaperone genes in response to heat stress. This Heat Shock Response (HSR) is crucial in maintaining proteostasis during exposure to environmental stressors. Notably, overexpression of *hsf-1* extends lifespan in *C. elegans* (Hsu et al., 2003) as does overexpression of HSF-1 target genes *hsp-16* and *hsp-70* in both *C. elegans* and *D. melangaster.*

(Khazaeli et al., 1997; Walker and Lithgow, 2003; Walker et al., 2001). Much like a reduction in IIS, DR also fosters proteostasis and has been found to promote protection from Aβ aggregation via HSF-1 activity (Steinkraus et al., 2008). There are such numerous examples of lifespan governing mechanisms playing a role in proteostasis and vice versa that it indeed would seem as if proteostasis and longevity, at least in part, depend upon each other.

1.4 METALS

Many metals function as osmolytes, structural fulcrums and signal transduction agents. As such, they are required cofactors for an estimated one quarter to one third of the human proteome and are involved in key biological processes including respiration, gene regulation, DNA replication and neurotransmission (Lippert, 1992; Osterberg, 1974; Williams, 1988). Copper is an essential trace metal with a crucial role in mitochondrial respiration and neurotransmission (Opazo et al., 2014).

In addition, a family of copper-dependent amine oxidases functions in determining the integrity of connective tissues (Turski and Thiele, 2009). Manganese is an essential component of human Mn-superoxide dismutase, pyruvate decarboxylase and glutamine synthase (Christianson, 1997) but is seemingly interchangeable with either magnesium or zinc in most processes (Andreini et al., 2008). Iron is the most active metal used in biological systems and is most famous for being part of heme moieties; the oxygen carriers in proteins such as hemoglobin (Tsiftsoglou et al., 2006). Iron-sulfur clusters are a chemical species utilized in a wide range of processes such as the mitochondrial electron transport chain, the TCA cycle and gene expression.

Conversely to copper, manganese and iron, non-biologically essential metals (e.g. aluminium, cadmium, mercury) often act to disrupt normal metabolism and cause numerous types of toxic effects (Kozlowski et al., 2014).

1.4.2 Iron Homeostasis in *C. elegans*

Iron is essential in primary life functions for all eukaryotes due to its use as an electron donor and acceptor. The most abundant and stable oxidation states of iron are Fe^{2+} and Fe^{3+}, which are readily convertible. The ability of iron to satisfy redox potential reactions is what makes it absolutely essential for life, however, this property is also a detriment. Fe^{2+} reacts with oxygen intermediates such as H_2O_2 to produce highly reactive free radical species. In turn, the reduction of Fe^{3+} regenerates the active form of Fe^{2+} that can re-enter the redox cycling with the continuous deleterious production of free radicals in the *Fenton reaction* chain(Stohs and Bagchi, 1995). Therefore, the tight regulation of iron is imperative for the health of the organism.

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^-$ Fenton Reaction

$Fe^{3+} + O_2^- \rightarrow \ + Fe^{2+} + O_2$

C. elegans are heme auxotrophs and are as such dependent on acquiring heme from the environment (Rao et al., 2005). *C. elegans* iron export is facilitated by ferroportins FPN-1.1, FPN-1.2 and FPN-1.3 which shuttles iron to the interstitial space while iron import is facilitated by a mammalian DMT-1 ortholog; SMF-3. SMF-3 is highly expressed in the intestinal epithelium (Au et al., 2009) and is transcriptionally activated during iron deficiency to restore balance (Romney et al., 2011).

The control of iron availability in the cells is largely dependent on ferritins, ubiquitous proteins with iron storage and detoxification capacity. In

mammals, cytosolic ferritins are composed of two types of subunits, the Heavy and the Light chain, forming a spherical cage. Heavy chain ferritins (H-ferritins) contain a ferroxidase center which has the oxidation capacity to alter Fe^{2+} to Fe^{3+}(Lawson et al., 1989). In mitochondria, ferritin is present in the form of a complex with 24 identical chains (Finazzi and Arosio, 2014). *C. elegans* have two forms of H-ferritin genes, *ftn-1* which is predominantly expressed in the intestine and *ftn-2* which is expressed in most cell types (Kim et al., 2004; Romney et al., 2008). Similarly to mammalian H-ferritin, both FTN-1 and FTN-2 contain ferroxidase active-site residues (Gourley et al., 2003). The organism regulates its iron storage capacity in response to its environment; iron chelation decreases *ftn-1* expression and iron supplementation increases *ftn-1* expression. As expected, *ftn-1* mutants are sensitive to iron exposure (Kim et al., 2004; Valentini et al., 2012).

Intriguingly, known lifespan and healthspan modulators are involved in ferritin regulation in *C. elegans*. The hypoxia-inducible factor (HIF-1) plays a role in oxygen homeostasis, tumor formation, cell survival and the inflammatory response. It is also a known lifespan modulator in *C. elegans* as stabilization of the HIF-1 protein extends longevity (Leiser et al., 2011; Leiser and Kaeberlein, 2010; Mehta et al., 2009; Zhang et al., 2009). *Hif-1* also plays a role in iron homeostasis, as HIF-1 transcriptionally inhibits *ftn-1* and *ftn-2* during iron deficient scenarios. 2'2-Bipyridine (BP) is an iron chelator to which *C. elegans* responds by down regulating ferritin. *Hif-1(ia04)* mutant animals lack the appropriate reduction of *ftn-1* and *ftn-2* mRNA levels with exposure to BP. In addition, constituent expression of HIF-1 results in reduced *ftn-1* and *ftn-2* levels comparable to wild-type animals exposed to BP (Romney et al., 2008). HIF-1 also participates in iron homeostasis by activating *smf-3* transcription during iron deficiency in order to increase iron uptake. In addition to HIF-1, DAF-16 has

been shown to modulate iron levels in *C. elegans* as long-lived *daf-2* mutants show a DAF-16-dependent increase of *ftn-1* expression (Ackerman and Gems, 2012). Increased available iron promotes growth but also increases ROS production. Therefore, it is plausible that the IIS regulation of *ftn-1* modulates a trade-off between growth and stress resistance partly by iron accessibility.

1.4.3 Metal Imbalances and Neurodegenerative Disease

Neurodegenerative disorders (ND) share similar pathological features and are often associated with disruption in analogous metabolic processes such as protein aggregation and oxidative stress, both of which are associated with the involvement of metal ions. In addition, these aggregation-related disorders often have a more clear-cut association with metals as they frequently are accompanied by metal accumulation in the patients' brain. Copper, manganese and iron are all linked to a number of neurological conditions (Danilo Milardi, 2011).

Copper has been linked to ND in various ways. The blood serum and Cerebral Spinal Fluid (CSF) of patients with AD have significantly higher levels of copper compared with age-matched controls (Basun et al., 1991; Squitti et al., 2002). Copper seems to be abnormally redistributed in AD brain regions with an overall reduction in Cu levels and high concentrations in senile plaques (Deibel et al., 1996; Loeffler et al., 1996; Lovell et al., 1998). There is some evidence to suggest that copper in combination with a high fat diet increases the risk for AD (Morris et al., 2006) and small amounts of Cu in drinking water can induce Aβ plaques and learning deficits in an AD rabbit model (Sparks and Schreurs, 2003). Copper is also implicated in PD. Similarly to AD, plasma levels of PD patients contain higher levels of Cu compared with controls (Kumudini et al., 2014) and Cu seems to aggravate the aggregation and toxicity caused by α-synuclein (Wang et al., 2010). Amyotrophic Lateral Sclerosis is a motor neuron disease in

which metals long have been implicated. Copper and other metals have been found to be elevated in tissue, serum and bone from ALS patients (Ihara 2005) as well. In addition to an involvement in ND, copper homeostasis is perturbed with normal aging; plasma copper levels have been reported to increase in blood serum and plasma of humans with age (Iskra et al., 1993; Madaric et al., 1994; McMaster et al., 1992).

Manganese has mainly been linked to PD. To date, four PD genes have been shown to have a connection with Manganese regulation (Chen et al., 2014). Metal related PD hypotheses are also based on a similarity between Mn exposure induced neurodegeneration and PD. Specifically, Manganism is a neurological disorder that is caused by high exposure to Mn resulting in motor dysfunction, cognitive and behavioral problems akin to those of PD. Another class of neurodegenerative diseases that are influenced by manganese are prion misfolding diseases. Prions cause neurodegeneration by aggregating and forming extracellular amyloid plaques in the CNS and manganese levels have been shown to be elevated in patients with prion disease as well (Thackray et al., 2002).

1.4.4 Iron and Neurodegenerative Disease

In addition to extracellular plaques and intra-neuronal tangles, AD is accompanied by an impaired ubiquitin-proteasome system and increased oxidative stress (Mandel et al., 2007). Iron accumulates in specific regions of AD patients' brains, with higher concentrations in the hippocampus and cerebral cortex (Danilo Milardi 2011). The high oxidative buildup in AD neurons could be explained by the iron surplus in these regions as histo-chemical studies have shown that there is a presence of Fe^{2+} deposits and Fe^{3+} in senile plaques and neurofibrillary tangles of AD patients (Smith et al., 1997). Iron overload increases production of both APP and Amyloid β in mice, and chronic iron

treatment causes accelerated cognitive impairment in transgenic mice model of AD (Becerril-Ortega et al., 2014). Furthermore, *in vitro* studies have also shown that iron can induce the formation of neurotoxic fibrils of Aβ (Bush, 2003).

In addition to the pathological involvement of iron in AD, there is a regulatory relationship between iron levels and Aβ as iron has been found to modulate the expression of APP through an iron-responsive element (IRE) in its 5' UTR (Avramovich-Tirosh et al., 2008). This pathway has been investigated as a potential avenue for pharmaceutical treatment of AD patients and several iron chelators have been found to suppress Aβ accumulation by modulating translation of APP (Danilo Milardi, 2011; Venti et al., 2004). However, the mechanism of Aβ-mediated toxicity remains elusive as large amounts of amyloid plaques have been observed with minor neuronal alterations in both mouse AD models and human aging brains suggesting that Aβ accumulation alone is not sufficient to drive AD (Danilo Milardi, 2011). Indeed, in what has been speculated as a compensatory neuroprotective response, Aβ possesses iron-binding sites, which subsequently would lower the generation of ROS. The complicated relationship between iron and Aβ in neurodegeneration is unclear and requires further investigation.

Parkinsonian brains have elevated levels of iron in microglia, astrocytes, oligodendrocytes and dopaminergic neurons (Gaeta and Hider, 2005). The degeneration of the substantia nigra region in PD could be attributed to oxidative stress; there is an abnormal accumulation of reactive iron in this region of PD patients' brains as well (Faucheux et al., 2003; Zecca et al., 2004). In addition to a perturbation of overall iron levels, there is also an abnormal distribution of Fe^{2+} to Fe^{3+} ratio associated with PD. Post mortem parkinsonian brains have higher levels of reactive Fe^{2+} compared with controls (Sofic et al., 1988). Interestingly, a reduction in reactive iron by either genetic or pharmacological means has been

found to result in protection against Parkinson-inducing toxin 1-methyl-4-phenyl-1,2,3,6-tetrapyridine (Kaur et al., 2003) suggesting that iron chelation might be a potential therapy for PD.

AD and PD are not the only neurodegenerative disorders in which perturbed iron homeostasis is involved. There are more direct examples of iron accumulation as a causative factor in ND. Ferritin mutations can cause human disorders by perturbing iron homeostasis. Neuroferritinopathy is such a disease and is mainly characterized by chorea, tremor and ataxia but cognitive decline has also been reported (McNeill and Chinnery, 2012). The disorder is thought to be caused by cerebral iron accumulation particularly in the basal ganglia (Crompton et al., 2002). Another example is the autosomal dominant disease, aceruloplasminemia. It is caused by a mutation in the *Cp* gene which results in the complete absence of Cp ferroxidase activity. The inability to oxidize Fe and remove its excess results in peroxidation and ROS formation within the cell, ultimately causing severe CNS degeneration.

1.4.5 Elemental Analysis in *C. elegans*

Inductively coupled plasma-atomic emission spectroscopy (ICP-AES) is a well-established method of measuring the levels of elements in a sample by exploiting the fact that thermally ionized atoms emit characteristic photons at given wavelengths when the electrons within the atom transition from excited to ground states. The instrument measures the intensity of photon yield at specific calibrated wavelengths for each element of interest and then quantifies the elemental composition within the unknown sample using reference standards. A stable plasma of ~7000 °K is generated using neutral argon which is ionized by an intense electromagnetic field within a high-intensity radio frequency coil. The rate of release of new electrons in collision is balanced by the rate of

recombination of electrons with argon ions to generate the stable plasma. The sample is then introduced into the plasma through a nebulizer and carried by an argon carrier gas flow. Photons from the ionized sample are collected at either axial or radial angles, directed through the instrument via prisms and mirrors, and rastered onto a charge-coupled device (CCD) with solid-state sensors for wavelengths from infrared to ultraviolet electromagnetic energy. Other elements for weak electronegative ionization suppression (cesium) and internal standard measurement (yttrium) are run in-line with unknown samples. A robotic autosampler assists in the delivery and wash-out between each sample (Boumans, 1987).

ICP-AES analysis requires a sample to be in solution. For our purposes, we prepared *C. elegans* material by washing off large cultures of synchronous populations and determining the dried weight of samples before acid digestion and subsequent dilution. Once the concentrations were analyzed, we normalized the values for dry weight and could thus determine elemental composition of young versus old animals, as well as animals subjected to treatment with the metal chelator, EDTA.

2 AIMS

The overall objective of this thesis has been to elucidate the relationship between protein aggregation and aging by utilizing compounds with varied mechanisms of action in stabilizing proteostasis in *C. elegans*.

Specifically

Paper I: First, investigate whether known aggregation-binding molecules can slow aging in *C. elegans.* Secondly, investigate if the compounds inhibit protein aggregation *in vivo* through examination of the chemical's effects on *C. elegans* models of proteotoxic disease and ultimately investigate if known longevity modulator genes are required for the compounds' efficacy.

Paper II: Examine the metallome with age to elucidate any endogenous changes in metal levels. Subsequently, investigate whether iron, which was found to accumulate with age, can drive protein aggregation and aging phenotypes. Thirdly, investigate if pharmacological reduction of the iron load slows protein aggregation, and ultimately aging.

Paper III: Screen a 640-compound natural-product library for additional small molecules that could slow protein aggregation. One of the strongest hit in our paradigm was analogous to a drug frequently used in *C. elegans* research; FUdR. We therefore sought to investigate if FUdR protects the proteostatic network and thereby influence healthspan and lifespan.

3 RESULTS AND DISCUSSION

3.1 PAPER I: Amyloid-binding compounds maintain protein homeostasis during ageing and extend lifespan.

Protein homeostasis has been shown to be a critical component of longevity through extensive genetic studies. For an example, proteostatic stabilization by overexpression of chaperones or transcription factors can extend lifespan in *C. elegans*. Pharmaceutical means of promoting proteostasis could therefore provide a useful tool in treating age-related diseases. We reasoned that compounds with known protein-fibril and protein-aggregate-binding properties could affect age-related changes to protein homeostasis and possibly slow aging. In order to test this hypothesis, we investigated compounds traditionally used in histopathology to stain amyloid tissues and bind protein fibrils *in vitro* and in cell culture. In this paper, we were indeed able to show that exposure of adult *C. elegans* to compounds that promote proteostasis can prolong health- and lifespan.

First, we investigated the effects of exposing adult *C. elegans* to the fibril-binding dye flavonoid ThT (4-(3,6-dimethyl-1,3-benzothiazol-3-ium-2-yl)-N,N-dimethylaniline chloride). We found that exposure to 50 μM or 100 μM ThT throughout adult lifespan increases median lifespan by 60% and maximal lifespan up to 78%. Although lifespan is a common measurement in aging research one could argue that healthspan might be of greater importance. *C. elegans* exhibits a decline in movement with age and the vigor of the animal can be measured in the number of body bends within a given timeframe. We investigated the effects of the compounds on healthspan by measuring

spontaneous movement throughout adulthood. Exposure to ThT at concentrations that extend lifespan slowed age-related decline in movement, suggesting that ThT also extends healthspan of *C. elegans*.

In order to investigate the commonality of aggregation-binding molecules we asked whether other compounds with similar properties, including curcumin and rifampicin also could slow aging and found this to be the case, albeit at slightly lower extents compared with ThT. We then asked whether ThT and Curcumin act via different mechanisms by combining the treatments and found no additive effect of the compounds, suggesting that they act in the same pathway/manner to extend lifespan. In order to further confirm that ThT binds to aggregation-prone proteins, we performed immunohistochemical assays on worms exposed to ThT. Both immunohistochemical and phenotypical data showed that ThT significantly suppressed Aβ aggregation, polyQ aggregation and WT age-related aggregation of oligomeric protein. In order to improve our understanding of the mechanism by which ThT extends lifespan we investigated the involvement of genetic pathways by either exposing mutant worm to ThT or by utilizing RNAi. A loss of effect in a mutant background suggests that this gene is required for the drugs response. HSF-1 and SKN-1 are both transcription factors in stress response pathways, involved in stabilization of misfolded proteins and lifespan regulation and our findings suggest that ThT depend on HSF-1 and SKN-1 for its effect on lifespan.

This study further solidifies protein aggregation as a detrimental feature of the aging process and validates the theory that proteostatic protection extends the vitality and life of *C. elegans* and it illustrates the multifaceted utility of *C. elegans* in basic aging research. Moreover, several life-extending compounds are identified which could be further tested in higher organisms such as mice.

3.2 PAPER II: Iron promotes protein insolubility and aging in *C. elegans*

When we were investigating the effects of compounds with molecular similarity to ThT (paper I) we came across several compounds that also extended lifespan but at much lower concentrations; 2-(2-hydroxyphenyl)-benzoxazole (HBX) and 2-(2-hydroxyphenyl) benzothiazole (HBT). Interestingly, these compounds were previously shown to bind divalent metals in addition to Aβ. Because we were aware of the involvement of metals in aggregation-related disorders, we wanted to investigate the role of metals in proteostasis with age. There are several studies that have focused on single metals in the context of aging or disease but few that have approached this relationship in an integrative way.

We first aimed to establish a novel picture of aging by studying the elemental composition of *C. elegans* during aging, which would facilitate the identification of new targets for age-related therapies. We investigated whether the metallome is altered with age in *C. elegans* by measuring several metals in synchronous populations of aged animals with ICP. After determining that iron accumulates as a function of organismal aging we asked whether iron supplementation could accelerate aging-phenotypes. We discovered that addition of iron to the food source leads to acceleration of age-related aggregation phenotypes in *C. elegans* models of AD and HD. After extracting the SDS-insoluble proteins a mass spectrometry approach allowed us to identify 681 proteins for control worms and 1068 proteins for iron-treated worms suggesting that iron potentiates the tendency of numerous proteins to aggregate. Interestingly, we observed a strong similarity in the functional classes of aggregation-prone proteins in aged and iron-treated animals, suggesting that iron supplementation and aging have very similar effects on protein insolubility.

To find out if we could decelerate aging with a reduction of metal load, we tried several chelators. In order to screen for bioavailability we measured the growth rate and fertility of animals exposed to various chelators. Knowing that metals are essential for growth and reproduction we reasoned that we would impair these processes if the compounds were bioavailable to the animals. After establishing that CaEDTA significantly slowed growth and delayed reproduction we went on to investigate if metal content of the animals were altered utilizing ICP. Our data suggests that CaEDTA exposure significantly reduces the iron load, and to a lesser extent, zinc, in *C. elegans*. We set out to investigate if a reduction in the *in vivo* iron load would promote proteostasis by utilizing the ND *C. elegans* models that were exacerbated in response to iron. We were able to show that exposure to 2.5 mM CaEDTA reduced protein aggregation phenotypes in all paradigms tested. Importantly, CaEDTA exposure from day one of adulthood not only extended lifespan of WT *C. elegans* but also improved healthspan in a dose-dependent manner.

Based on these observations we conclude that metal homeostasis is perturbed in aging of *C. elegans* and maintenance of metallostasis could provide a useful avenue in maintaining proteostasis with age in other organisms. We also deduce that elevated iron levels can lead to an acceleration of several aging phenotypes, such as the accumulation of insoluble protein.

3.3 PAPER III: A DNA Synthesis Inhibitor is Protective Against Proteotoxic Stressors via Modulation of Fertility Pathways in *Caenorhabditis elegans.*

In paper I we investigated the effects of known amyloid-binding molecules and found they had advantageous effects on lifespan in *C. elegans*. In order to identify additional compounds with similar effects we screened a library

of small molecules in a general model of protein misfolding (HE 250). The natural product library that was screened contains 640 small molecules that are mostly derived from plant, bacteria, fungi and animal sources. A compelling case for natural products is that their activity has been selected for through evolution with a reduced rate of cytotoxicity. Based on previous knowledge and findings (paper I and II) we reasoned that compounds that slow protein aggregation are also likely to extend healthspan and lifespan.

We identified 5-fluorouridine (FU) as a strong hit and recognized its similarity with 5-fluoro 2-deoxyuridine (FUdR). FUdR is widely used in *C. elegans* research and thus we set out to investigate if FUdR also ameliorates protein misfolding. We examined the effects of FUdR in the *unc-52* mutant model HE250 and found that similarly to FU, FUdR significantly inhibited proteotoxicity as measured by paralysis in this strain. To determine if FUdR confers protection in age-dependent models of aggregation, similarly to compounds identified in paper I and II, we again examined the temperature-sensitive Aβ and PolyQ models, CL4176 [*smg-1 (cc546ts); dvIs27(myo-3::Aβ3–42 let 39UTR(pAF29))*] and AM141[*rmIs133(P(unc-54) Q40::YFP)*], respectively. We exposed these animals to FUdR at the L4 stage, during spermatogenesis. Together, our data suggests that FUdR confers protection in models of protein aggregation by inhibiting fertilization.

Next, we tested whether FUdR treatment leads to stress resistance. We examined the survival of animals exposed to FUdR at higher temperatures and also utilized a transcriptional reporter strain, *$_p$hsp-16.2::GFP*, in which GFP is under the transcriptional control of the *hsp-16.2* promoter (Link et al., 1999). Together our data suggests that FUdR may confer thermotolerance in part by upregulating the heat-stress response. Loss of germline precursor cells in *C.*

elegans result in sterile animals with improved protein homeostasis and extended lifespan. We attempted to validate whether FUdR relies on the germline to confer protection by utilizing *glp-1(e2141)* mutant animals that lack a germline. Because previous reports have shown that DAF-16 and DAF-12 (a steroid hormone receptor) are required for lifespan extension in animals lacking a germline we sought to find out whether FUdR relies on these pathways to confer thermotolerance. Our findings suggest that FUdR does not act by inhibiting these germline signals, but is acting in an unknown, germline dependent manner. In order to determine where in the reproductive pathway FUdR is acting to confer stress resistance we examined several mutants deficient in sperm production, meiotic maturation of oocytes and ovulation. FEM-3 mutant animals are phenotypically female, laying unfertilized oocytes (Doniach and Hodgkin, 1984; Hodgkin, 1986). This mutant was found resistant to FUdR treatment, but restoration of ovulation (by introduction to WT males) in this strain was sufficient to reinstate the effects of the drug. Thus, our data suggest that FUdR confers stress resistance by inhibiting oocytic maturation. The observation was further validated by studies in which FEM-3 knockdown via RNAi resulted in upregulation of the heat shock response as well as protection in the polyQ model AM141.

In conclusion, we show that the widely used DNA synthesis inhibitor, FUdR is implicated in the heat stress response via a sex-determining pathway. Our data suggests a model in which FUdR acts via a germline signal to confer protection against proteotoxicity. Furthermore, our findings signify the importance of caution while utilizing FUdR in *C. elegans* research.

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