The Role of Endoplasmic Reticulum Protein Quality Control in Longevity

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Preface

The work presented in this dissertation was performed in collaboration with Dr. Martin Denzel under the supervision of Professor Dr. Adam Antebi at Max Planck Institute for Biology of Ageing (MPI-Age), in Cologne. Portions of this work were also accomplished in collaboration with Aljona Gutschmidt under supervision of Professor Dr. Torsten Hoppe at the Institute of Genetics, University of Cologne.

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Nadia Jin Storm

Losers quit when they lose – winners keep losing!

Abstract

Aging has become a major challenge for Western countries, as it is estimated that two-thirds of all deaths are related to age-associated diseases. This includes cancer, diabetes, neurodegenerative disorders, cardiovascular diseases, etc. Biological aging is defined as the progressive loss of function over time, and even though the last decades of research have shed light upon many cellular processes involved in aging, many mechanisms remain to be understood.

Cellular quality control mechanisms have been implicated in aging, as proteins involved in these are altered during biological aging. Thus, cellular processes become dysfunctional and normal growth is impaired. The different compartments of cells each have distinct quality control mechanisms to ensure a rapid turnover of proteins, cellular structures and lipids. This includes the mitochondrial unfolded protein response, the endoplasmic reticulum unfolded protein response, the cytosolic heat shock response and autophagy. Protein quality control is an essential mechanism to maintain protein homeostasis. Additionally, it has been found to be associated with aging in a wide range of organisms from yeast to mammals.

The endoplasmic reticulum is the major site for lipid biosynthesis, protein maturation and membrane synthesis. Because of its essential role in protein maturation, the endoplasmic reticulum has a vastly developed protein quality control system, which ensures re-folding or degradation of mis- or unfolded proteins. Some of these pathways, such as the endoplasmic reticulum associated degradation pathways and the endoplasmic reticulum unfolded protein response pathways have been linked to aging. Additionally, autophagy has been suggested to play a role in compartment-specific protein homeostasis. Recent studies have further indicated that these pathways are essential for normal lifespan, and more importantly, that long-lived organisms have elevated activity of one or more of these pathways. This led us to ask whether selecting for mutants with improved protein quality control mechanisms would confer longevity.

Indeed, we found that to be true. By performing a forward genetic screen, selecting for *C. elegans* mutants with increased ability to cope with tunicamycin-induced endoplasmic reticulum stress, we identified four independent gain-of-function

mutations in *glutamine-fructose-6-phosphate amino-transferase 1* (*gfat-1*) that led to lifespan extension. GFAT-1 regulates the rate-limiting step of the hexosamine pathway, which uses fructose-6-phosphate from glycolysis to synthesize UDP-N-Acetylglucosamine (UDP-GlcNAc). UDP-GlcNAc is a precursor for N-glycans. In addition, *gfat-1* gain-of-function mutants had enhanced activity of different cellular degradation pathways, which led to alleviated proteotoxicity of several *C. elegans* models of neurodegenerative disorders, and by supplementing wild type animals with GlcNAc (a derivate of UDP-GlcNAc) we were able to mimic the longevity and amelioration of toxic proteins observed in *gfat-1* gain-of-function mutants. These findings suggest novel therapeutic approaches to promote health and extend lifespan through endogenous molecule modulation of protein quality control, and hence, might be important for future studies of neurodegenerative disorders.

From the tunicamycin resistance screen, we also identified several long-lived mutants with defects in the chemosensory system. Such deficiency has previously been associated with longevity. These mutations also have improved protein quality control mechanisms, as they had decreased levels of aggregated proteins in the endoplasmic reticulum upon introduction of an unstable endoplasmic reticulum resident protein. In addition to the screen, we also found a handful of wild *C. elegans* isolates with improved ability to cope with tunicamycin-induced endoplasmic reticulum stress. Together, these studies may shed light upon novel mechanisms to modulate endoplasmic reticulum quality control mechanisms and induce longevity.

Zusammenfassung

In westlichen Ländern ist das Altern eine große Herausforderung, da bis zu zwei Drittel aller Todesfälle auf altersassoziierte Erkrankungen zurückzuführen sind. Altersasasoziierte Erkrankungen beinhalten Krebs, Diabetes, neurodegenerative Erkrankungen, Herz-Kreislauf-Erkrankungen, usw. Biologisches Altern wird als progressiver Funktionsverlust über die Zeit definiert. Obwohl in den vergangenen Jahren viele dem Altern zugrunde liegende zelluläre Mechanismen erkannt wurden, bleiben noch viele Alterungsprozesse unverstanden.

Die zellulären Mechanismen, die mit dem Alterungsprozess in Verbindung gebracht werden beinhalten die Qualitätskontrolle von Proteinen. Die dabei beteiligten Proteine verändern sich mit dem Altern. Die verschiedenen Zellkompartimente besitzen unterschiedliche Mechanismen zur Qualitätskontrolle. Gemeinsam gewährleisten sie die Erhaltung von Proteinen und Lipiden. Dies beinhaltet die mitochondriale „unfolded Protein Response“ (UPR), die UPR des endoplasmatischen Retikulums, und die cytosolische Hitzeschockantwort, sowie Autophagie. Proteinqualitätskontrolle ist ein wichtiger Mechanismus, um Proteinhomöostase zu erhalten, und sie spielt im Alterungsprozess von Hefe bis hin zu Säugetieren eine wichtige Rolle.

Das endoplasmatische Retikulum spielt in Lipid-Biosynthese, Proteinreifung und Membran-Synthese eine zentrale Rolle. Aufgrund der wesentlichen Rolle bei der Proteinreifung hat das endoplasmatische Retikulum eine stark ausgeprägte Proteinqualitätskontrolle, die eine Neufaltung oder einen Abbau von falsch gefalteten oder ungefalteten Proteinen sicherstellt. Außerdem spielt Autophagie eine wichtige Rolle in der kompartimentspezifischen Proteinhomöostase. Neue Studien haben ferner gezeigt, dass diese Prozesse für die normale Lebensdauer wichtig sind, und besonders in langlebigen Organismen eine essentielle Rolle spielen. Zudem wurde festgestellt, dass die Aktivität von einem oder mehreren dieser Prozesse erhöht ist. Deswegen stellten wir die Frage, ob gezielte Selektion von Mutanten mit verbesserter Protein-Qualitätskontrolle Langlebigkeit vermitteln würde.

Tatsächlich fanden wir heraus, dass dies zutrifft. In einem genetischen Screen, der *C. elegans* Mutanten mit erhöhter Tunicamycinresistenz isolierte, haben wir

gehäuft langlebige Mutanten gefunden. Wir identifizierten vier unabhängige „Gain-of-function“ Mutationen im *glutamin fructose-6-phosphat aminotransferase 1* (*gfat-1*) Gen. GFAT-1 ist für den geschwindigkeitsbestimmenden Schritt des Hexosamin Biosynthesewegs verantwortlich, der Fructose-6-phosphat aus der Glykolyse verwendet, um UDP-N-Acetylglucosamin (UDP-GlcNAc) zu synthetisieren. UDP-GlcNAc ist ein Vorläufer für die N-Glycane. Zudem hatten GFAT-1 „gain-of-function“ Mutanten erhöhte Aktivität von verschiedenen zellulären Abbauwege, und die Mutation verringerte Proteintoxizität in mehreren *C. elegans* Modellen von neurodegenerativen Erkrankungen. Durch Nahrungesergänzung mit GlcNAc (ein Vorläufer von UDP-GlcNAc) konnten in Wildtyp-Tieren ähnliche Verbesserungen festgestellt werden. Diese Ergebnisse deuten auf neue therapeutische Ansätze hin, die die Gesundheit verbessern, und das Leben verlängern könnten.

Aus dem Tunicamycinresistenz-Screen isolierten wir weitere langlebige Mutanten. Diese zeichnen sich durch Defekte im olfaktorischen System aus. Solche Mutationen sind schon vorher mit Langlebigkeit in Verbindung gebracht worden. Diese Mutationen verbesserten ebenfalls die Proteinqualität.

Neben dem Screen identifizierten wir auch einige *C. elegans*-Isolate aus der freien Natur, die sich durch Tunicamyzinresistenz auszeichnen. Gemeinsam zeigen diese Studien, dass die Qualitätskontrolle des endoplasmatischen Retikulums zur Modulierung des Alterunsprozesses beiträgt und Langlebigkeit induzieren kann.

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Abbreviations

AAD	Aging Associated Disease
ABU	Activated in Blocked UPR
ATF	Activating Transcription Factor
ATG	Autophagy (yeast Atg homolog)
BiP	Immunoglobulin-Heavy-Chain-Binding Protein
C. elegans	*Caenorhabditis elegans*
CFP	Cyan Fluorescent Protein
CGC	*C. elegans* Genetics Center
CHIP	C terminus of Hsc Interacting Protein
CHOP	C/EBP homologous protein
CLK	Clock Biological Timing Abnormalities
CMA	Chaperone Mediated Autophagy
CNX	Calnexin
CRT	Calreticulin
D-Arg	D-Arginine
DR	Dietary Restriction
DAF	Abnormal Dauer Formation
Dyf	Dye Filling Phenotype
EAT	Eating: Abnormal Pharyngeal Pumping
eIF	Eukaryotic Initiation Factor
EMS	Ethyl Methanesulfonate
ER	Endoplasmic Reticulum
ERAD	Endoplasmic Reticulum Associated Degradation
FOXO	Forkhead Box Protein
GFAT	Glucosamine 6 Phosphate Amino Transferase
GlcNAc	N-Acetylglucosamin
GFP	Green Fluorescent Protein
GLP	Abnormal Germ Line Proliferation
GNA	Glucosamine Phosphate N-Acetyl Transferase
gof	Gain-of-Function

HDAC	Histone Deacetylase
HIF	hypoxia inducible factor
HRD	HMG-CoA reductase degradation protein
HSF	Heat Shock Factor
HSP	Heat Shock Protein
IFE	Initiation Factor 4E (eIF4E) Family
IGF	Insulin Growth Factor
IRE-1	Inositol-Requiring Enzyme
ISP	Iron-Sulfur Protein
KRI	Krev Interaction Trapped
LC	Liquid chromatography
LGG	LC3, GABARAP and GATE-16 Family
lof	Loss-of-Function
MS	Mass Spectrometry
NF	Nuclear Factor
NHR	Nuclear Hormone Receptor
OGA	O-GlcNAcase
OGT	O-GlcNAc Transferase
OST	Oligosaccharyltransferase
PCR	Protein Chain Reaction
PERK	Pancreatic Endoplasmic Reticulum Kinase
PHA	Defective Pharynx Development
PolyQ	Poly Glutamine
RNase	Endoribonuclease
ROS	Reactive Oxygen Species
S1P/S2P	Site-1 Protease/Site-2 Protease
SEL	Suppressor/Enhancer of LIN-12
SIR	Sirtuin
SRE	Sterol Regulatory Element
SREBP	Sterol Regulatory Element Binding Protein
SRP	Serpin
TCA	Tricarboxylic Acid Cycle
TM	Tunicamycin

TOR	Target of Rapamycin
UDP	Uridindiphosphat
UNC	Uncoordinated
UPR	Unfolded Protein Response
UPS	Ubiquitin-Proteasome System
WB	Western Blotting
WT	Wild Type
XBP	X-box binding protein
YFP	Yellow Fluorescent Protein

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CHAPTER 1. Introduction and Background

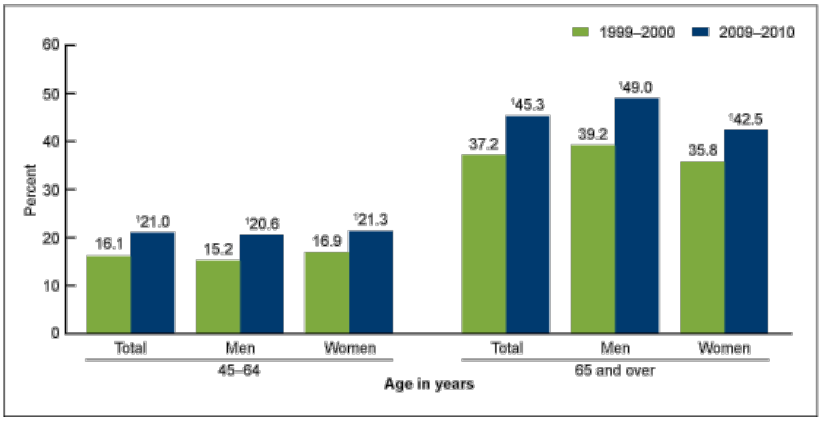
1.A. Aging and society

Aging is defined as the progressive loss of biological function over time, including a decline in cellular homeostasis, making aging a major risk factor for multiple diseases including cancer, cardiovascular disease and neurodegenerative disorders. Many researchers are searching for "the key to youth", however, the underlying proximal mechanisms of aging and the relative contribution of various defense mechanisms essential to longevity remain elusive.

In Western countries, the demography phenomenon is characterized by a decreased fertility, a deceased mortality, and an increased life expectancy (Costello, 2007) (Steyn, 2006). This has led to a major challenge for governments, societies and healthcare systems because of the increased incidence of diseases with age.

Figure 1.1. The total number of both men and women over 65 is increasing.

The percentage of all (total), the number of men and of women between 45 and 64 (left bars) and over 65 (right bars) in 1999-2000 (green) and in 2009-2010 (blue) (Virginia M. Freid, 2012).



A massive increase in the number of humans over 65 years old has occurred over the last 10 years, and this tendency continues (Virginia M. Freid, 2012). In Figure 1.1 the number of humans between 45 and 64 (left), and over 65 (right) are shown. It is striking to notice that the total number of people (men and women together) over 65 has increased by almost 10% in only 10 years, from 2000 to 2010. This puts an enormous pressure on society since many diseases can be categorized as aging-associated diseases (AADs). Recent numbers have suggested that approximately two-thirds of all deaths are related to AADs (Lopez AD, 2006) employing aging as the major risk factor for fatalities in general. AADs are diseases whose frequency increases with biological aging or cellular senescence, which is the state where cells cease to dvivide (Campisi J, 2007). Over the last years, it has been suggested that cellular senescence links multiple pathologies of aging, both degenerative and hyperplastic. Cells undergoeing senescence is unlikely to explain all aging phenotypes, but the role of senescence in AADs should not be ignored. Examples of AADs include cancer, cardiovascular diseases, diabetes, arthritis, and neurodegenerative disorders, which constitute the major reasons for death in the Western world (Lopez AD, 2006). Together, as the human population is getting older and AADs accounts for the major deaths, modern society faces aging as a huge and expensive challenge. Research targeting cellular senescence and biology of aging is therefore essential to shed light on involved mechanisms, and hold the potential to decelerate causes of AADs.

1.B. Cellular aging and longevity

In addition to environmental influences, several genetic components and cellular pathways have been shown to modulate the aging process, many of which are conserved from simple organisms to mammals. These include dietary restriction (DR), reduced insulin/IGF (insulin growth factor) signaling, reduced mitochondrial respiration, and signals from the reproductive system (Kenyon C. J., 2010). The last decades of research have revealed that these pathways modulate diverse downstream processes that decelerate the naturally occurring decline in function and homeostasis leading to cellular aging and senescence. Thus, stress resistance, metabolism, quality control mechanisms and immunity are involved in life- and healthspan.

Genetic mutations known to promote long life across a variety of species also affect stress response or nutrient sensation (Walford RL, 1987). One example of this

is DR, which has been shown to decelerate the aging process in different organisms from yeast to higher primates (Colman, 2009). Recent research has aimed to understand the exact mechanisms of DR-induced longevity. Earlier it was believed that it was caused by a chronic state in which animals experienced life-long hunger. However, Mair et al. were able to show that in the fruit fly *Drosophila,* the lifespan extension came from an acute response to DR (Mair, 2003).

Many other conditions modulate lifespan and life expectancy across various species, and in most, if not all cases, aging modulation is not a stochastic process, but rather is under tight genetic control (Campisi, 2013). For example, single genetic variations in the yeast SIR2 histone deacetylase have been shown to modulate the lifespan of budding yeast. Similarly, genetic modifications in higher organisms have also been found to impact the aging process; knockdown of the *Drosophila* gene *methuselah* led to increased lifespan and enhanced stress resistance in flies (Lin Y. J., 1998), and mutations in the mammalian gene encoding the protein p66shc extend the lifespan of mice about 30% (Migliaccio E., 1999), together supporting the evidence that single genetic mutations can modulate organismal aging.

1.C. *C. elegans* longevity

The roundworm *Caenorhabditis elegans* (*C. elegans*) is a well-studied organism in different aspects of cellular biology and aging. Due to its fast development, high fertilization rate and short lifespan (15-20 days post reproduction), it is widely used in aging research. Hermaphrodites constitute the majority of the population in a reproductively growing laboratory stock, and each worm produces 200-300 eggs during the first five days of adulthood. After hatching, larvae go through 4 larval stages, L1-L4 (L. Byerly, 1976), before reaching adulthood in 72 hours. *C. elegans* development and lifecycle is responsive to environmental factors including temperature, food availability, and population density. If conditions are not optimal, animals may enter one of several alternative stages of arrested development, which are specialized for survival under harsh conditions. These include L1 arrest, occurring if larvae experience unfavorable conditions upon hatching. Best studied is the long-lived dauer diapause, occurring during the third larval stage. These alternative states are not mutually exclusive and in theory, a worm can undergo both stages sequentially (www.wormbook.org).

1.C.1 C. elegans *dauer*

The *C. elegans* dauer stage has been found to display extreme resistance to stress and exceptional longevity (Richard L Russell, 1975) (L. Byerly, 1976). After the discovery of the dauer larva stage, many genes coupled to dauer entry were also found to influence adult behavior and lifespan (Patrice S. Albert, 1988). In 1993 Kenyon and colleagues reported that single genetic mutations in the dauer-constitutive gene *daf-2* (*abnormal dauer formation*), the *C. elegans* homolog to the mammalian insulin receptor, extended the average *C. elegans* hermaphrodite lifespan more than 100%. The lifespan extension was dependent on the activity of the fork-head transcription factor DAF-16 (Kenyon C, 1993), which is also involved in the formation of dauer larvae. Environmental and dietary responses are as well coupled to conserved molecular and cellular pathways, which play important roles in dauer entry and lifespan. These include the neurosensory system, the TGF-β pathway, the insulin/IGF pathway, serotonergic pathways, and the steroid hormone signal transduction pathways (Nicole Fielenbach, 2008). As these pathways are associated with metabolism, growth and survival, regulation of the dauer diapause has been coupled to studies involving cancer, cellular growth and aging. When *C. elegans* larvae enter the dauer diapause, their energy metabolism shifts towards a standby and energy-saving phase. Specifically, cells are downregulating metabolic processes. For example, the activity of the tricarboxylic acid cycle (TCA) that metabolizes fatty acids to provide ATP has been found remarkably decreased (Wadsworth W G, 1989). Since the discovery of single genetic mutations influencing the dauer decision and lifespan extension, many studies have been made to identify other mutations that modulate *C. elegans* lifespan.

1.C.2 Mutations leading to longevity

Some of the most studied *C. elegans* mutant animals with extended lifespans include the germline-deficient animals *glp-1* (*abnormal germline proliferation*), the DR mimick *eat-2 (*eating: abnormal pharyngeal pumping), the insulin/IGF signaling depleted *daf-2*, and mutations in the mitochondrial coenzyme Q homolog, *clk-1* (clock biological timing abnormalities) (Kenyon C. J., 2010).

Decreased energy metabolism is a common phenotype among several long-lived *C. elegans* mutants, such as that observed in the mitochondrial mutant *clk-1* (Wong A, 1995). *daf-2* mutant animals have also shown decreased mitochondrial consumption (own observation). Loss-of-function (lof) mutations in *eat-2* are also associated with decreased oxygen consumption and lowered metabolic rates (Bernard Lakowski, 1998). Conversely, overexpression of CLK-1 activity in WT worms increases mitochondrial activity, accelerates behavioral rates during aging and shortens lifespan (S. Felkai, 1999). Together, the reduced respiration found in different long-lived mutants suggests that longevity is promoted by decreased mitochondrial function, which is conserved among other species (S. Felkai, 1999).
Another mechanism by which lifespan extension may be achieved in *C. elegans* is by depletion of the germ cells either genetically (i.e., by temperature sensitive mutations in the *glp-1* gene) or physically using laser ablation (Nuno Arantes-Oliveira, 2002). Germline longevity is dependent on the nuclear hormone receptor DAF-12/FXR (Nuno Arantes-Oliveira, 2002) and its bile acid-like ligands, the fork-head box A (FOXA) transcription factor PHA-4 and DAF-16 (Honor Hsin, 1999), the latter modulated by the *kri-1* (human KRIT1 (Krev interaction trapped) homolog) (Jennifer R. Berman, 2006). The exact mechanisms remain unknown, however, recent studies have suggested a correlation between germline longevity and miRNAs (Yidong Shen, 2012), lipid metabolism and autophagy (Lapierre LR, 2011) and increased proteasomal activity (Vilchez D, 2012). Furthermore, TOR (target of rapamycin), which in other organisms has been found to modulate the aging process (DE Harrison, 2009) (Matt Kaeberlein, 2011), was found reduced in *glp-1* mutant animals. Downregulation of TOR was associated with an increased level of PHA-4, required for the enhanced autophagy levels seen in these animals. These results were suggested to couple the TOR/PHA-4 pathway to enhanced autophagy in *glp-1* mutant animals, whereas changes in lipid metabolism were suggested to be under control of DAF-16 (Lapierre LR, 2011). Downregulation of TOR and subsequent enhanced transcription of PHA-4 is also seen during DR in *C. elegans*, (Panowski SH, 2007), suggesting that this is an overlapping mechanism between several long-lived *C. elegans* mutants.

1.C.3 C. elegans *vs. higher organisms*

Many of the genes and mechanisms modulating *C. elegans* lifespan have also

been found to play a role in the aging process of higher organisms. This includes modulations of nutrient-signaling pathways, such as the insulin/IGF and the TOR pathways, suggesting that they may induce a physiological state similar to that resulting from periods of food absence (Harrison DE, 2009). Indeed, DR extends lifespan of diverse organisms, including yeast, flies, worms, fish, rodents, and rhesus monkeys (Walford RL, 1987) (Colman, 2009). In addition, upon TOR-mediated and DR-induced longevity, autophagy was found enhanced, and importantly, required to sustain the extended lifespan (Jing Cui, 2013) (Malene Hansen, 2008). A knockdown of components of the autophagic pathway in the remarkably long-lived *C. elegans* strain *daf-2* abrogates its long lifespan (Hars ES, 2007), suggesting that autophagy plays an essential role in longevity, at least in *C. elegans* (Alicia Meléndez, 2003). This was again supported by similar observations in *glp-1* and *eat-2* mutant animals, where inhibition of autophagy decreased longevity (Malene Hansen, 2008) (Lapierre L.R., 2011). In *Drosophila*, inhibition of mTOR signaling extends lifespan, as it does in yeast and *C. elegans* (Kapahi P, 2004), but there is no evidence in flies for a role of autophagy as aging-modulator. In mice, pharmacological approaches using resveratrol, spermidine and rapamycin, an mTOR inhibitor, provide an indication that activation of autophagy, as well as other processes, may be associated with longevity in higher organisms (Eisenberg T, 2009) (Lagouge M, 2006) (Harrison DE, 2009). And recently, research by Pyo and colleagues shows that overexpression of Atg5 in mice activated autophagy and extended their lifespan (Jong-Ok Pyo, 2013), again indicating that autophagy plays an important role in longevity.

1.D. Cellular quality control mechanisms

Resistance to exogenous stress has repeatedly been hypothesized to be involved in longevity of mammalian cells (Mullaart E., 1990) (Finch, 1990), since exposure to environmental stress causes numerous alterations in cellular and extracellular components, resulting in deleterious physiological changes that affect longevity (Kirkwood, 1977) (Mullaart E., 1990). Increased resistance to stress, either by increased prevention of the initial damage or by improved repair of injurious events, leads to reduced damage and increased longevity.

Many mutations associated with *C. elegans* longevity have also been found to play a role in cellular homeostasis and quality control, leading to resistance to exogenous

stress. *daf-2* mutants, for example, are resistant to heat and oxidative stress (Dillin, 2008), *glp-1* mutants are resistant to bortezomib (proteasome inhibiting drug) (Vilchez D, 2012) and *clk-1* mutants show higher resistance to mitochondrial interruptions (Johnson, 1996). Enhanced ability to handle various stress factors arises from increased activity of quality control mechanisms, amplified transcription of genes involved in stress resistance and improved DNA repair mechanisms, which are important cellular processes implicated in aging and senescence (H. Koga, 2011) (Hsu, 2003) (Takashi Tatsuta, 2008).

1.D.1 Protein quality control

Quality control is an evolved ability for the cells to remain "healthy" and to ensure a clean environment. Cells possess specialized pathways to protect against DNA damage, and to turn over and degrade redundant and dysfunctional organelles and proteins. The latter include the ubiquitin-proteasome system (UPS), the mitochondrial unfolded protein response (UPR), the endoplasmic reticulum (ER) UPR and autophagy (L D Osellame, 2013). Lately, autophagy, which by degrading proteins and damaged organelles is involved in cellular quality control, has been suggested to be organelle specific, such as pexophagy, reticulophagy, ribophagy and mitophagy (Sebastián Bernales, 2006) (Dunn WA, 2005) (Kraft C, 2008) (Kim I, 2007). Furthermore, chaperone mediated autophagy (CMA), which turns over proteins containing a specific motif, has become better understood, and is implicated in cellular homeostasis (Cuervo AM, 2004). What these quality control pathways all have in common is that they are all implicated in various diseases, including AADs; dysfunction of one or more of these mechanisms induces or increases the incidence of disease, particularly neurodegenerative disorders and lysosomal dysfunctions (L D Osellame, 2013). Additionally, senescent cells experience a decrease in the activity of these processes, also increasing the risk of cellular dysfunctions due to altered quality control (Hsu, 2003).

A major cellular quality control mechanism involved in biological aging is protein quality control; maintaining protein homeostasis is essential for optimal protein performance and to sustain cellular functions (H. Koga, 2011). Protein quality control mechanisms constitute a major regulatory output of longevity pathways, and many AADs are associated with a decline in protein homeostasis (Jason E. Gestwicki,

2012). This is particularly associated with neurodegenerative conditions, which stem largely from a failure to properly handle protein misfolding, aggregation and toxicity (Kimura, 2007). The protein homeostasis network is required under normal conditions to support chaperone-assisted protein folding and suppression of aggregate formation (W. E. Balch, 2008), however, their importance is more clearly recognized in stress conditions, during biological aging, and conformational diseases (Barral, 2004). In terms of biological aging, work in *C. elegans* and *Drosophila* has suggested that molecular chaperones directly control longevity. Knockdown of the major heat shock factor, *hsf-1,* in *C. elegans* reduces longevity (James F. Morley, 2004). Additionally, disruption of the mitochondrial UPR by inhibition of *hsp-70* (heat shock protein 70) promotes a progeria-like phenotype (Hsu, 2003) (Kimura, 2007) suggesting dependence for protein quality control in lifespan of *C. elegans*. On the other hand, overexpression of HSF-1 promotes longevity in both *C. elegans* (Hsu, 2003) and mouse models, which have also been linked to alleviated proteotoxicity (Mitsuaki Fujimoto, 2005). Additionally, numerous heat shock proteins, such as HSP-16, HSP-70, HSP-27 and mitochondrial HSP-70 have been shown to be directly related to both *C. elegans* and *Drosophila* lifespan (Tatar, 1997) (Yokoyama, 2002). In mammals, molecular chaperones also seem to be required for protein homeostasis. Studies involving *Hsf1* knock-out mice suggest that *Hsf1* is required to protect against demyelination and gliosis that can occur during WT aging (Homma, 2007). Additionally, mutations in molecular chaperone genes, such as *Hsf4*, *sHsp*, and *α-B-crystallin*, have been associated with AADs, such as cataract formation, desmin-related myopathy, and distal motor neuropathy (Bu, 2002) (Vicart, 1998). Lastly, Min et al. showed that inhibition of the ubiquitin ligase/cochaperone, CHIP, whose normal function in mice is to target proteins for degradation via the UPS, led to accelerated aging due to altered protein quality control (Jin-Na Min, 2008).

1.D.2 Mitochondria protein quality control

Reactive oxygen species (ROS) are generated as a result of oxygen consumption by the electron transport chain in the mitochondria. ROS is detrimental, and causes cellular damage. For this reason, the mitochondria contain specific protein quality control systems (Murphy, 2009). Mitochondrial protein quality control mechanisms keep mitochondrial proteins functional until the damage is not

influencing the proteins and components of the system. When cells undergo stress, induced by misfolded proteins, the mitochondrial UPR is turned on. Chaperones (HSP-6 and HSP-60) localized in the mitochondrial matrix are required for protein import, and also facilitate protein folding. On the other hand, proteases that are localized in the inner membrane will degrade proteins that fail to fold or assemble correctly (Tatsuta T. and Langer, 2008). Tom70 is a receptor essential to the import of un- or misfolded proteins, and Hsp90 and Hsp70, were found essential for targeting proteins and translocating them to mitochondria. (Jason C. Young, 2003). During aging and in neurodegenerative diseases components of mitochondrial protein quality control mechanisms, such as the chaperones Hsp90 and Hsp70 as well as the Lon protease, become functionally impaired (Van Dyck L, 1994). The Lon protease family is a family of proteases located in the mitochondrial matrix which are required for mitochondrial function and are suggested to play a role in mitochondrial heat shock response since their activity, regardless of their constitutive activation, is enhanced after thermal stress. It has also been shown that mitochondria play an important role in aging and AADs (Tatsuta T. and Langer, 2008), however, the focus of this project has been on ER protein quality control mechanisms.

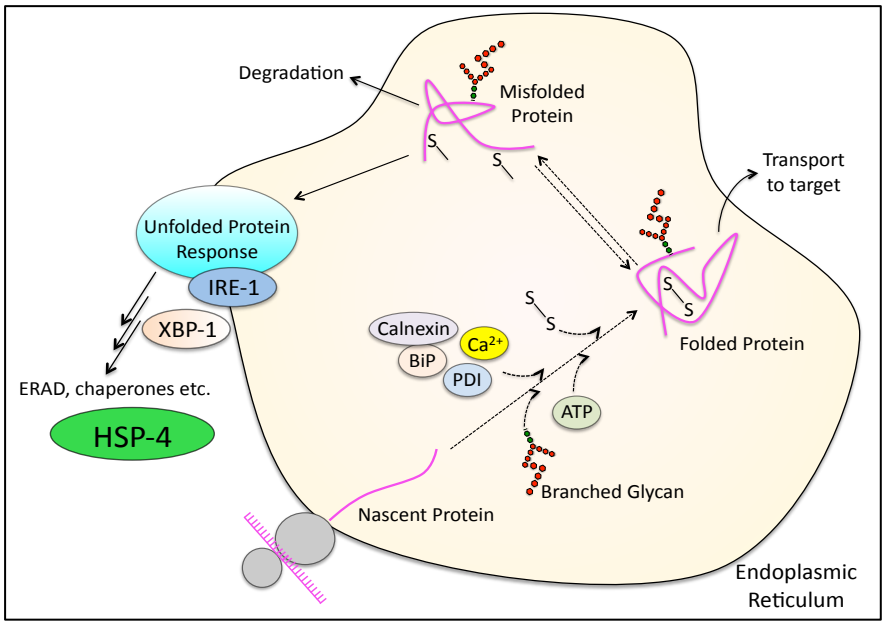
1.E. ER Protein quality control

The ER is the major site of protein synthesis, lipid biosynthesis, membrane biogenesis and various other essential processes. Specifically, the ER is responsible for the structural folding and maturation of proteins of approximately a quarter of the proteins that cross the secretory pathway (Bernd Bukau, 2006), and therefore contains specific protein quality control mechanisms. Protein folding is dependent on a multitude of post-translational modifications that occur in the ER. This includes N-linked glycosylation, disulfide bond formation, lipidation, hydroxylation and oligomerization. N-linked glycosylation is an important, ER- and Golgi-specific event, which is initiated by the transfer of a core oligosaccharide from a dolichol phosphate. The sugar is then transferred from the membrane-bound dolichol phosphate to a consensus Asn-X-Ser/Thr residue in the polypeptide of newly synthesized proteins (S.C. Hubbard, 1981). The attachment of an N-glycan serves several purposes in protein folding. Glycans are hydrophilic molecules and therefore increases the solubility of glycoproteins, and defines the surface for potential protein

interactions. Additionally, due to their large hydrated volume, they guard the attachment area from surrounding proteins, and enable oligosaccharides interactions with the peptide backbone and stabilize its conformation. Lastly, it serves as a lectin machinery to report on the folding status of the protein (M.R. Wormald, 1999) (L. Ellgaard A. H., 2003) (L. Ellgaard M. M., 1999) (Figure 1.2). Together, these mechanisms ensure accurate folding and proper maturation of newly synthesized proteins.

Figure 1.2 Protein maturation in the endoplasmic reticulum (ER).

Proteins structurally mature in the ER, which involves various processes such as formation of disulfide bonds and attachment of complex sugars called N-glycans. If WT or mutated proteins initially fold incorrectly, or if the folding process is subsequently interrupted, the ER posseses various response mechanisms to maintain proper protein homeostasis. This includes induction of ER unfolded protein response and ER associated degradation (ERAD).

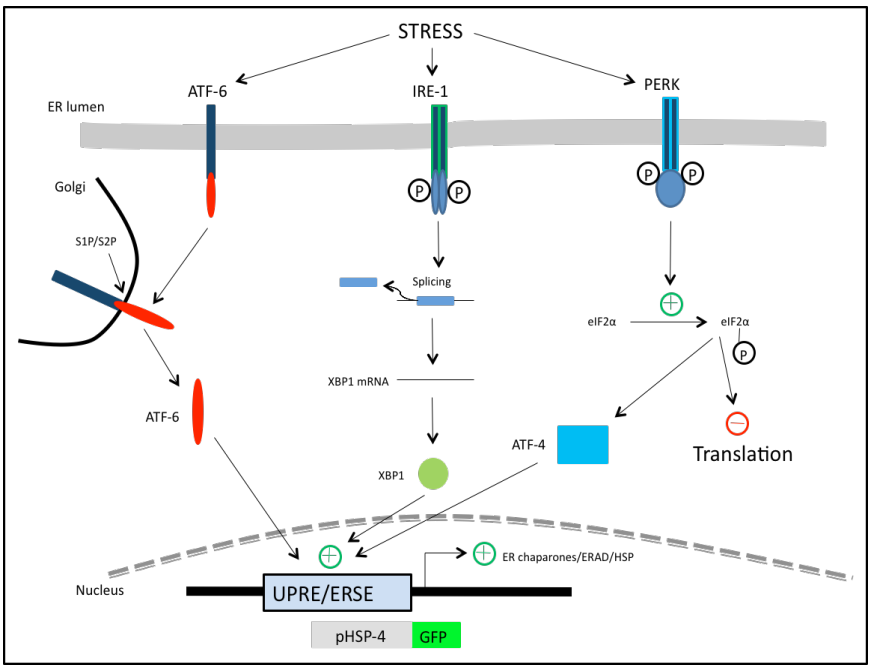


Correspondingly, the ER provides an environment optimized to face challenges occurring from mis- or unfolded proteins. This includes a high concentration of ER resident chaperones that ensures proper folding as well as additional protein quality control mechanisms which coordinate the degradation and refolding of mis- and unfolded proteins (E. Anken, 2005) (L. Ellgaard A. H., 2003) (Roth, 2010). Various mutant polypeptides or WT misfolded proteins associated with human aging and disease are targeted for degradation by an ER protein quality control system, which also requires the cytosolic proteasome for degradation (Jeffrey L. Brodsky, 1999). Together, the ER protein quality control mechanisms are responsible for the removal of mis- and unfolded proteins to ensure protein homeostasis in the ER.

The presence of luminal unfolded proteins triggers the ER UPR, a signaling cascade that, among others, results in attenuated translation, enhanced ER associated degradation (ERAD), and induced expression of ER-resident proteins such as heat shock protein 4 (HSP-4) (P. Walter, 2011).

Figure 1.3 The ER Unfolded protein response pathways.

Un- and misfolded proteins promote a signaling cascade called the unfolded protein response (UPR) in the ER. ER UPR consists of three "arms" that are all anchored in the ER membrane. Downstream events ensure the transcription of chaperones, ER associated degradation components and attenuated translation. The three pathways are initially regulated by activating transcription factor 6 (ATF-6), inositol requiring enzyme 1 (IRE-1) and protein kinase R-like ER kinase (PERK).



The canonical ER UPR pathway consists of three major arms, which all have sensors anchored in the ER membrane (Figure 1.3): Activating transcription factor 6 (ATF-6), Inositol requiring enzyme 1 (IRE-1) and Protein kinase R-like ER kinase (PERK). In resting cells, the three ER stress sensors are associated with the abundant ER chaperone BiP (immunoglobulin-heavy-chain-binding protein) on their luminal domains. BiP is a member of the heat shock protein 70-chaperone family, and it plays a critical role in ER protein folding and quality control. During ER stress, BiP is dissociated from the receptors, and prefers binding to un- or misfolded proteins initiating one of the three main signaling branches of the UPR (Jingshi Shen, 2005) (David Ron, 2007). BiP has been found to play an important role in natural aging, as well as in lifespan extension. The expression of HSP-4, the *C. elegans* homolog to the mammalian BiP, is altered in aged animals (Rebecca C. Taylor, 2013), and it was even shown that BiP/HSP-4 plays a role in dietary restriction-induced longevity in both worms and flies (Viswanathan M, 2005) (Hyunsun Jo, 2009). In addition, Chen et al. showed that hypoxia inducible factor 1 (HIF-1) is involved in DR mediated longevity, and that both *hif-1* mutants and DR animals fail to extend lifespan when components of the ER UPR are depleted (Di Chen, 2009). Moreover, the ABU (activated in blocked UPR) proteins, which are involved in the non-canonical ER stress response pathways, were suggested as important components of the *sir-2.1* and resveratrol-induced lifespan extension pathway (Ya-Lin Liu, 2009). In fact, the ER stress response pathways have all been implicated in aging and senescence of distinct organisms and cells.

1.E.1 Inositol-Requiring Enzyme 1

An essential mediator of the ER UPR is IRE-1. Induction of the IRE-1-branch of the ER UPR upregulates the expression of genes whose products promote protein folding and degradation of misfolded proteins in the ER (Jonathan H. Lin, 2007). The role of IRE-1 was first observed in *Saccharomyces cerevisiae,* where it was found that knockdown of IRE-1 almost negated the UPR pathway. In addition, this was associated with an attenuation of the expression of a large number of genes including ER chaperones, enzymes involved in maintaining the proper oxidizing environment in the ER and components of the ERAD pathway. This indicated that IRE-1 is an

essential component of the canonical ER UPR pathway (Cox J. S., 1993) (Mori K., 1993) (Fumihiko Urano, 2000). IRE-1 is a bifunctional enzyme possessing both a kinase activity and an endoribonuclease (RNase) activity. Dissociation from BiP triggers IRE-1 oligomerization and activation of its RNase activity by trans-autophosphorylation. Activated IRE-1 induces splicing of mRNA that encodes a transcription factor named X-box binding protein 1 (XBP1 in metazones; in yeast it is named Hac1). The splicing occurs by cutting XBP1 mRNA twice, cutting out an intron. The two remaining mRNA fragments are then ligated, generating a spliced mRNA ready for translation and a subsequent boost of XBP1 protein synthesis (David Ron, 2007) (Jingshi Shen, 2005) (Jonathan H. Lin, 2007). XBP1 is a transcription factor that when active, induces the transcription of ER UPR target genes such as ER chaparones and ERAD proteins (Wu J., 2006).

In organismal and mammalian biology, IRE-1 and the downstream activation of XBP-1 play several essential roles. For example, the pathway is fundamental for bone-morphogenic protein-2 induced osteoblast differentiation (Takahide Tohmonda, 2011), proteasomal and lysosomal degradation (Wei-Chieh Chiang, 2012), cell fate determination (Brandizzi, 2013), and has been implicated in longevity (Sivan Henis-Korenblit, 2010), suggesting a broad and general role in cellular biology for the IRE-1-branch of the ER UPR. Additionally, this indicates that ER protein quality control and protein homeostasis play essential roles in cellular health, and recent research has linked the IRE-1 pathway to multiple diseases including metabolic diseases, neurodegenerative diseases, inflammatory diseases, and cancer (Wang S, 2012).

1.E.2 Activating Transcription Factor 6

Another key regulator of the ER stress-induced transcriptional pathway is ATF-6. ATF-6 is a transcription factor synthesized as an ER membrane precursor, anchored in the membrane of ER. The protein's C-terminal is located in the ER lumen, wheras the N-terminal faces the cytosol – the N-terminal contains the DNA binding domain (Min Hong, 2004). Upon sensation of stress and misfolded proteins, ATF-6 relocates to the Golgi apparatus membrane, a requirement for its activation. Here it is cleaved by the S1P/S2P (site-1 protease/site-2 protease) protease system to generate activated ATF-6 (Jingshi Shen, 2005).

The translocation from ER to the Golgi apparatus and the subsequent cleavage by S1P/S2P is the central step in the activation of ATF-6. The ER chaperone BiP binds to the luminal domain when the cells are unstressed – this will block the intrinsic Golgi apparatus localization signals. When misfolded proteins accumulate in the ER, ATF-6 releases from the BiP association allowing its transport to the Golgi apparatus (Kyosuke Haze, 1999).

When ATF-6 arrives in the nucleus, it functions as a transcription factor and binds to sterol regulatory elements (SRE) and ER stress elements (ERSE) (Min Hong, 2004). Binding to SRE (SRE will be bound to a sterol regulatory element binding protein – SREBP) will recruit histone deacetylase 1 (HDAC1) causing an inhibition of SRE-mediated transcriptional activation. Activatated ATF-6, together with the heteromeric nuclear factor Y (NF-γ), also binds to ERSE and activates the transcription of genes involved in ER UPR such as BiP and grp78 (glucose regulated proteins 78). Grp78 is an ER chaperone protein that protects cells against stress (Jingshi Shen, 2005) (Bertolotti, 2002).

The ER stress-induced ATF-6 pathway is implicated in deceleration of biological aging and cellular senescence since its activation leads to increased expression of chaperones involved in protein quality control and induced transcription of the transcription factor CHOP (C/EBP homologous protein), which is involved in ER stress mediated cell survival and apoptosis (Can E. Senkal, 2009) (Oyadomari S, 2004).

1.E.3 Pancreatic Endoplasmic Reticulum Kinase

The third mechanism of the three ER UPR branches that plays a role in ER stress responses involves phosphorylation of a subunit of the eukaryotic initiation factor 2α (eIF2α), which suppresses translation and thus reduces the folding load in the ER. A family of protein kinases, PERK, phosphorylates eIF2α in response to ER stress conditions. The release of BiP, in response to mis- or unfolded proteins, allows oligomerization and autophosphorylation of PERK. PERK then phosphorylates eIF2α at serine 51 (Ruchira SOOD, 2000). eIF-2 undergoes a guanine exchange reaction which is carried out by eIF-2B. The phosphorylation eIF-2 leads to inhibition of eIF-2B activity, reducing the guanine nucleotide exchange and thus the rate of translation (Yuguang Shi, 1998) (Aparna C. Ranganathan, 2008). This reduces the overall

frequency of messenger RNA translation initiation. However, some mRNAs are preferably translated in the presence of phosporylated eIF2α – such as ATF-4 mRNA. ATF-4 is a transcription factor involved in activating the transcription of ER UPR target genes, encoding factors used in amino-acid biosynthesis, the oxidative stress response and apoptosis (David Ron, 2007).

1.E.4 ER associated degradation

Evidently, cells have to cope with protein damage in order to survive. For this they have evolved a dual response: damaged proteins are either repaired or eliminated by protein quality control mechanisms consisting of large networks of chaperones and proteolytic systems (Alexander Buchberger, 2010). Preferably, cells repair un- or misfolded proteins using ER-resident chaperones, however, irreparable proteins are identified and targeted for ERAD, or degraded in lysosomes by autophagic processes. When proteins in the ER are targeted for degradation, they are transported to ER membrane-bound complexes. Here they are unfolded and translocated out to the cytosol, where they now function as substrates of the UPS. Substrates of the UPS are tagged with ubiquitin in an ATP-consuming process. This requires a catalytic cascade of E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme) and E3 (ubiquitin protein ligase) activities (Hochstrasser, 1996). The ER membrane-bound protein complex that is responsible for translocation is a part of the ERAD pathways, and consists, among others, of two proteins called SEL-1 and SEL-11 in *C. elegans*. These were first recognized in yeast (called Hrd3p and Hrd1, respectively) (Masayuki Kaneko, 2002) (Min Sung Choi, 2010) (Randolph Y. Hampton, 1996).

Polypeptides that do not meet the protein quality control standards are recognized and retained within the ER, and are later delivered to ERAD ligases. This interaction limits immature proteins to the ER and supports their maturation. In mammalian cells, calnexin/calreticulin (CNX/CRT) is an ER specific lectin-type chaperone, which assists re-folding and folding of immature glycoproteins (Helenius, 2004). To prevent blockage of the ER folding machinery, polypeptides that cannot be refolded must be removed from the CNX/CRT protein cycle. This is regulated by EDEM and ER mannosidase I (Jyoti D. Malhotraa, 2007). Proteins fixed in the CNX/CRT protein cycle are eventually trimmed by ER mannosidase I, which leads to their recognition by EDEM. EDEM and CNX interact with each other via their

transmembrane domains, and concurrent binding of CNX and EDEM to a misfolded protein may provide the signal that triggers its degradation (Kazue Kanehara, 2007). Additionally, it was found that overexpression of EDEM enhanced degradation by ERAD due to induced release of proteins from CNX (Oda Y, 2003). This suggests that ERAD is dependent on various ER specific events including ER resident chaperones and EDEM.

ERAD dysfunction has previously been associated with several diseases, and plays an important role in protein quality control. For example, a polymorphism in SEL-1, the ERAD membrane bound protein, has been associated with Alzheimer's disease (Giuliana Saltini, 2006). Other work has pointed out a relationship between p97, a human AAA ATPase, and overload of aggregated proteins in the ER, as a consequence of inhibited ERAD (Kobayashi T, 2002). Excessive amounts of aggregated ER proteins inhibit the ATPase activity of p97, leading to abnormal ER growth and cell death. This is similar to deficits in the neurodegenerative disorder, Machado-Joseph disease (Kobayashi T, 2002).

1.E. 5 Autophagy

Autophagy is an evolutionarily conserved process ensuring cellular homeostasis through degradation of long-lived proteins and functionally unnecessary or damaged intracellular organelles in lysosomes (Cuervo, 2008). Through degradation, autophagy provides energy by turnover of intracellular proteins and organelles to ensure rejuvenation. Autophagy also provides energy necessary for adaptation to various conditions, such as when nutrition is limited or when cells are experiencing stress (i.e., exposure to heat or drugs including tunicamycin, bortezomib, H_2O_2 and others) (Susann Patschan, 2008) (Maiko Ogata, 2006). In addition, autophagy functions as mechanism for removal of unwanted cellular structures by degradation of excess or injured organelles, including damaged mitochondria and ER (Bergamini E., 2007).

At least three types of autophagy are known – CMA, macroautophagy and microautophagy. The difference between these systems is the way that they deliver target structures to the lysosomes. CMA is a mechanism that allows a direct lysosomal import of proteins that contain a particular pentapeptide motif (Majeski, 2004). In contrast, microautophagy and macroautophagy involve membrane

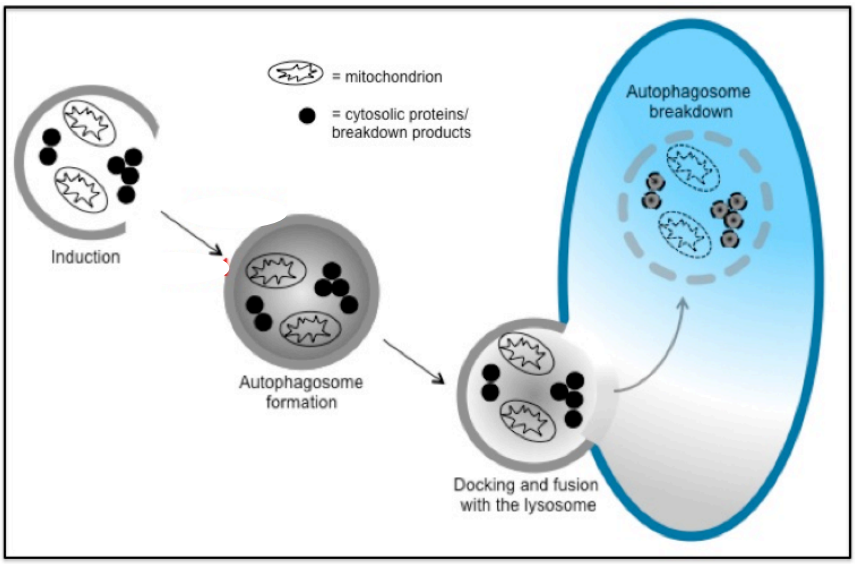
rearrangements that finish at the lysosome and encapsulate proteins.

Macroautophagy (hereafter referred to as autophagy) is the most studied autophagy pathway described in the literature, which involves the sequestration of cytoplasm into a double-membrane cytosolic vesicle referred to as an autophagosomes (Figure 1.4). The autophagosome subsequently fuses with a late endosome or lysosome to form an autophagolysosome. Inside the autophagolysosome, lysosomal hydrolases degrade the sequestered material, which then becomes available to the cell for recycling (www.wormbook.org).

Numerous studies have shown that autophagy plays an important role in diverse physiological and pathological processes, including cell growth, cell differentiation and development, immune responses, cancer, neurodegeneration, infection and metabolic syndromes (Rabinowitz J. D. & White, 2010) (Vellai, 2009). The molecular mechanism of autophagy involves several conserved genes (*atg*), which have multiple functions in various physiological contexts. In particular, Atg5 is important for mammalian cell development, differentiation and maintenance of homeostasis (Kuma A, 2004). *Atg5* is induced during ER stress, and *Atg5*-knockout mice die early. Tissue-specific *Atg5*-deficiencies, especially in neuronal cells, develop progressive insufficiencies in motor function from neurodegeneration. In addition, Atg5 has been implicated in human aging, as the transcriptional level of *Atg5* is altered during natural aging (Rouschop KM, 2010). Accordingly, recent studies in mice have shown that overexpression of *Atg5* is sufficient to extend lifespan, presumably due to alleviated protein aggregation (Jong-Ok Pyo, 2013).

Figure 1.4 Induction of autophagy.

Autophagy is a cellular self-degrading pathway that provides organisms with energy under fasting and unfavorable conditions. By activation of ATG proteins, autophagosomes will be formed. Autophagosomes then dock and fuse with lysosomes. Here, autophagosomes are broken down. The cellular compartments (e.g. mitochondria, ER, proteins and fat) can be recycled, yielding energy for cellular survival.

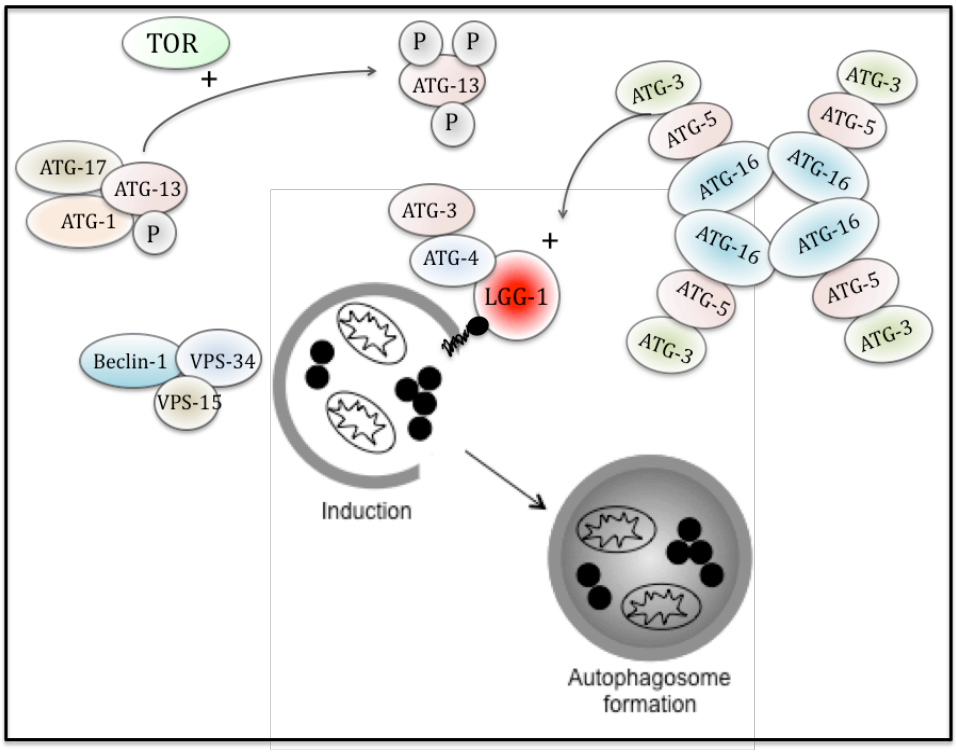


Autophagy is the major regulated cellular pathway for degrading long-lived proteins and the only known pathway for degrading cytoplasmic organelles. As autophagy improves the condition of cells by "cleaning", it is potentially a powerful anti-aging mechanism. Autophagy has been found to be a major provider of fatty acids and other metabolites (Rajat Singh, 2009). Thus, a reduction of autophagy leads to an accumulation of lipids, proteins and non-processed macromolecules, as well as reduced turnover of cellular organelles.

When autophagy is induced, autophagosomes are generated under control of the *atg* genes. These genes were first identified in yeast, but many of them have a *C. elegans* homolog. Figure 1.5 shows an overview of the protein-interactions occurring upon initiation of autophagy; a huge network of proteins is related to the induction and formation of autophagosomes.

Figure 1.5 The autophagy genes.

Induction of autophagy and autophagosome formation is dependent on various proteins that are encoded by the *atg* genes. These create a complex network of proteins that assist in salvaging proteins and components for induction, followed by docking and fusion to lysosomes, and lastly the degradation of cellular waste components.



Autophagosomes that contain cellular building blocks such as lipids, cytosolic proteins, and organelles such as mitochondria and ER, are enclosed by membranes constituting lysosomes. The internal environment in the lysosomes, together with degradative enzymes, degrades the autophagosomal membranes and the contents are broken down and recycled. This will provide cells with energy-yielding molecules such as fatty acids, amino acids, etc. (Rajawat Y.S., 2009) (Kaarniranta, 2009). Autophagy is recognized as an important biological process that functions to promote health and longevity (Czaja et al, 2010) (Cuervo et al, 2008) (Alicia Meléndez, 2003). This is underlined by observations during the aging process where the efficiency of autophagic degradation declines and intracellular waste products accumulate (Kaarniranta, 2009). Additionally, recent *C. elegans* work from Lapierre et al. suggests that both the insulin receptor mutant, *daf-2,* and the germline-less mutant *glp-1* are dependent on autophagy to maintain their long life (Lapierre LR, 2011). Together, autophagy plays an essential role in protein quality control and cellular homeostasis, and, at least in lower organisms, it has been coupled to aging. The mechanism by which autophagy is regulated has been extensively examined in yeast, and due to high conservation of the involved proteins, the mechanism is predicted to be similar (Ohsumi, 2001).

1.F. Protein quality control and aging

During aging, there is a shift in the balance between the protective responses of the ER UPR and the apoptotic pathways, which are pathways induced when cellular damage becomes irreversible (Marishka K. Brown, 2012). Key ER-resident chaperones and enzymes within the ER such as BiP, PDI and calnexin, which are all required for proper folding, are altered during the aging process. Chaperones are progressively oxidized with age, which may contribute to their functional decline (Jonathan E. Nuss, 2008). BiP expression levels, for example, are significantly reduced in several species with age. In the cerebral cortex of aged (22-24 months old) mice compared with that on young (3 months old) mice, BiP proteins levels are decreased 30% (Naidoo N., 2008). BiP mRNA and protein expression levels are also decreased in the hippocampus of aged versus young rats (Paz Gavilan M., 2006). And in *C. elegans*, HSP-4 and especially its ability to respond to ER stress is significantly altered in old worms (Rebecca C. Taylor, 2013). This suggests a broad conservation

of age-associated protein alterations occurring in stress-induced pathways.

Age appears to modify other components of the ER protein quality control mechanisms, in addition to chaperones. PERK mRNA is significantly reduced in the hippocampus of aged rats (Paz Gavilan M., 2006), and another study reported that the kinase activity of eIF2 is downregulated when isolated from aged rat brain tissue. In addition, in both yeast and mammalian neuron-like cells, overexpression of ERAD components ameliorates polyQ toxicity (Martin L. Duennwald, 2008). Due to these findings, it has been suggested that impaired ER protein homeostasis is a broad and highly conserved contributor to polyQ toxicity in yeast, mammalian cells, and human cells expressing full-length polyQ-expanded huntingtin protein, the causative agent of the neurodegenerative disorder Huntington's disease (Martin L. Duennwald, 2008). Additionally, new research has stated that long-term alcohol consumption and aging may promote liver tumor genesis in females through interfering with DNA methylation and expression of genes involved in the ERAD pathways (Hui Han, 2013).

In fact, many human diseases result from altered protein-protein interactions, often occuring due to improper protein folding (Carrell R. W., 1997). Defective protein folding can lead to unstructured accumulation of proteins, which become toxic to the cells. Misfolding leading to toxic aggregation-prone proteins can either occur constitutively, as a result of coding sequence mutations, or be induced by exogenous stress, such as heat shock or hypoxia. Another important group of diseases resulting in formation of toxic protein accumulations are the serpinopathies. These result from mutations in members of the serine protease inhibitor (serpin) family of proteins, which lead to the formation of toxic aggregation-prone proteins.

Conceivably, improved protein quality control mechanisms could provide substantial health benefits by reducing aggregation of toxic protein species. Indeed, recent results show that overexpression of certain genes in these pathtways are able to extend lifespan (Rebecca C. Taylor, 2013) (Hsu, 2003). This includes the ER UPR (Sivan Henis-Korenblit, 2010), components of the proteasomal degradation process (Vilchez D, 2012) and overexpression of the heat shock factor, HSF-1 (Hsu, 2003) (J. F. Morley, 2004).

In addition to ER protein quality control mechanisms, autophagy, the cellular self-degradation process, is essential for many lifespan-extending pathways and was

recently suggested as a target for treatment of various diseases, including neurodegenerative diseases (Frank Madeo, 2010) (Beth Levine, 2008).

1.G. What can we learn from natural variations?

When Sydney Brenner in 1974 described the genetics of *C. elegans*, he revealed novel genetic variations performed using EMS (ethyl methanesulfonate) in a uniform genetic background that changed the animals' behavior and morphology (Brenner S. , 1974). Now, 40 years later, many hundreds of genes have been identified; genes that regulate the roundworms' lifecycle, movement, cellular abilities and much more. However, we might be able to learn from natural genetic variations that have occurred in wild strains of *C. elegans*. The behavior of different wild strains is distinct, due to natural variations in the animals' genome (J Hodgkin, 1997), thus, there is a possibility that behavior, and responses to different types of stress, vary among strains according to their origin. Some laboratories have compared patterns of genetic diversity between mutation accumulation lines to wild isolates. The mutation accumulation lines represent the original mutational process, whereas various differences observed among wild isolates is the result of mutations as well as population structure and phenotypic selection. Denver and colleagues compared mutation patterns in mutation accumulation lines against the wild isolates, and found an excess of transitions over transversions in wild isolates (Denver D.R., 2004). This was due to a mutational bias. In contrast, they found that indels (insertion-deletions) and replacement mutations in coding regions were less frequent among wild isolates than in mutation accumulation lines, suggesting that they are counter selected (Denver D.R, 2000). The genetic diversity in *C. elegans* is generally low compared to other organisms, such as the fruit fly, which is also frequently used for laboratory experiments. This is partly due to *C. elegans'* self-mating abilities (Hartl D.L, 1997).

Phenotypic evolution is the product of mutational and environmental changes, and these evolutionary pressures occur more frequently among *C. elegans* wild isolates compared to inbred laboratory strains (Denver D.R., 2004). The effect of random mutation on the rate and pattern of phenotypic variation can then be compared with the actual natural diversity (Davies E.K, 1999). For example, wild isolates have been found to display differences in developmental programs (Eisenmann D.M., 1998), plugging (some wild isolate males leave a plug after

mating) (Hodgkin J., 1997) and broodsize (Hodgkin J, 1991). Very importantly, some wild isolates of *C. elegans* have enhanced ability to resist toxic environments, such as very high temperatures (J Hodgkin, 1997).

Another significant difference between the primary laboratory strain N2 (Bristol) and many wild isolates is a lack of the *npr* (neuropeptide receptor 1) mutation, which is found in the WT laboratory *C. elegans* strain (Mario De Bono, 1998). NPR-1 is a predicted G-protein-coupled receptor similar to the neuropeptide Y receptors, and is expressed predominantly in the AQR, PQR and URX neurons. Several lof mutations in the *npr-1* gene cause the animals to diffuse across a bacterial lawn. This behavior stems from the animals' ability to sense O_2 and CO_2, which is controlled by the URX neurons (Hallem E.A., 2008). In fact, it was recently shown that ablation of URX neurons in the *npr-1* mutants restores CO_2 avoidance, suggesting that NPR-1 enables CO_2 avoidance by inhibiting the URX neurons (Carrillo MA, 2013). Other wild strains, as for example the Hawaiian WT strain (CB4856), the German strain RC301 and the English strain CB4932, lack this lof mutation, which makes them aggregate on food sources to form clumps.

In nature, conditions are often harsh, and temperature changes, absence of food and population density vary among different origins. Natural variability among different strains dependent on their origin may therefore shed light over novel genes involved in stress response pathways and behavior. The *C. elegans* Genetics Center (CGC) maintains a worm repository (http://www.cgc.cbs.umn.edu/) containing many *C. elegans* wild isolates, collected from many different countries, environments and climates. We decided to order some of these strains, to investigate natural variations that lead to resistance to TM, and to analyze their lifespan. Since they have random natural variations, we might be able to elucidate genetic mutations leading to enhanced stress resistance and longevity from the wild *C. elegans* isolates.

1.H. Summary and hypothesis

In conclusion, aging leads to a decline in function across various cells in different organisms. Defects in protein quality control mechanisms have been implicated in aging, which lead to unbalanced protein homeostasis when interrupted. Genetic studies initiated using yeast have revealed proteins that might play a role in protein homeostasis, aggregation and quality control, and continued research in higher multicellular organisms and in mammalian cellular systems have suggested that there is an increased incidence of AADs when protein quality control mechanisms are impaired. Additionally, we know from *C. elegans* research that long-lived mutants depend on protein quality control mechanisms such as ER UPR and autophagy. Lastly, recent evidence suggests that enhanced autophagy is sufficient to alleviate proteotoxicity and extend lifespan in mice, and that over expression of *hsf-1* or improved degradation by the proteasome alone can extend the lifespan of *C. elegans*.

From the high degree of conservation many of these protein quality control mechanisms have among a wide range of organisms, we aim to utilize the power of genetics in *C. elegans* to better understand lifespan regulation and protein quality control mechanisms. This should provide a particularly excellent tool to further elucidate novel roles of protein quality control in the regulation of proteotoxicity and longevity. We therefore hypothesized that improved ER protein quality control mechanisms in *C. elegans* would confer longevity.

C. elegans natural isolates are typically found in the soil, where they live under very harsh circumstances. Since wild *C. elegans* animals have undergone natural variations that in some cases have led to increased resistance to stress and higher ability to enter the dauer larvae stage, we hypothesized that genetic variations in these animals would reveal new targets and genetic mutations important for quality control mechanisms and longevity.

CHAPTER 2. Experimental Procedures

2.A. Alignment of sequence data

Sequence data was obtained from MPIPZ (Max-Planck-Institute Pflanzenzüchtung) and Cologne Genomic Center, and processed using MAQGene (Henry Bigelow, 2009). The MAQGene output was imported as files readable in Excel spreadsheets, and we piled up the sequences and determined the specific mutations in each mutant. The data indicated at which chromosome the mutation was found, the specific base position mutated, reference and sample base, the depth, what kind of mutation, the class, the amino acid change and in which parent feature the mutation had occurred.

2.B. Autophagy Quantification

Autophagosomes were scored as reported previously (Lapierre L.R., 2011). 15 - 25 transgenic *lgg-1::gfp* animals per condition where whole-mounted and foci were counted using 100x magnification on a Zeiss Axio Imager.Z1 microscope in the seam cells of L3 larvae. For quantification of p62::GFP puncta, 10-20 early L4 larvae were whole-mounted and GFP foci in the posterior pharyngeal bulb were quantified. For quantification we used 100x magnification and additionally 2x zoom on a Leica TCS SP5-X confocal microscope (see section 2.G. Confocal Microscopy).

2.C. *C. elegans* microinjection

DNA plasmids were injected into the distal arm of the gonad in very young adult hermaphrodites, *myo-2::mCherry* was used as co-injection marker. 3-6 animals were placed on 2% agarose pads on glass slides, and covered with injection oil. DNAs were injected at 500 kPa pressure. Worms are recovered using M9 buffer and subsequently placed on NGM (nematode growth media) plates seeded with OP50. Animals successfully receiving the injected DNAs are picked after at least 3 generations.

2.D. *C. elegans* strains and culture

Nematodes were cultured at 20°C on NGM agar plates seeded with the *E. coli* strain OP50, unless indicated otherwise (Brenner S. , 1974). For the genetic dominance test, *gfat-1* mutant animals were crossed with N2 males and TM resistance was scored in the F2 offspring of F1 heterozygous animals.

2.E. *C. elegans* wild isolate collection and isolation

C. elegans wild isolates (Dragon #1, Dragon #9 and Dragon #10) were found in the soil in a vineyard in Drachenfeldt, near Bonn. They were collected in eppendorf tubes. Later they were bleached, isolated on plates and kept under normal laboratory conditions. Wild isolates that were resistant to TM were backcrossed to N2 on TM.

2.F. Compound feeding and developmental TM resistance assay

For developmental TM resistance assays with GlcNAc and UDP-GlcNAc (Sigma) UV-radiation-killed bacteria were transferred to plates containing 10 μg/mL TM. Compounds were then added to the plate surface resulting in a 10 mM compound concentration in the plates. 50 – 80 synchronized WT (N2) eggs per condition were added to the plates and development to L4 or adult stage was scored after 4 and 5 days. For adult exposure experiments for lifespan analyses with D-Arg or GlcNAc, compounds were mixed with the NGM agar to a final concentration of 10 mM unless otherwise noted. UV-killed OP50 bacteria were added on the plates. For motility assays, adult exposure were carried out by maintaining animals on normal NGM agar plates seeded with OP50. The animals were then daily exposed to GlcNAc or D-Arg for 6 hours in S-basal media supplemented with UV killed OP50 bacteria.

2.G. Confocal Microscopy

20-30 animals were collected on 5% agarose pads on glass slides, and anesthetized using 10μL 2mM levamisole. Immediately after mounting, the animals were visualized at a SP5X confocal microscope. In case of quantifying p62 foci, pictures were taken at 100x plus 2.0 times zoom. Number of foci was quantified from the pictures.

2.H. Gene alleles and strains

N2 (Bristol)
DR1572: *daf-2(e1368)III*
GR1307: *daf-16(mgDf50)I*
RB1169: *oga-1(ok1207)X*
RB653: *ogt-1(ok430)III*
AA2503: *gfat-1(dh468)II*

AA2560: *gfat-1(dh784)II*

AA2561: *gfat-1(dh785)II*

AA1409: *eat-2 (ad465)II*

MQ887: *isp-1(qm150)IV*

KX15: *ife-2(ok306)X*

CB4037: *glp-1(e2141ts)III*

AA2962: *gfat-1(dh468)II; daf-2(e1368)III*

AA3214: *gfat-1(dh784)II; daf-2(e1368)III*

AA2883: *gfat-1(dh785)II; daf-2(e1368)III*

AA2959: *gfat-1(dh468)II; daf-16(mgDf50)I*

AA2884: *gfat-1(dh784)II; daf-16(mgDf50)I*

AA2885: *gfat-1(dh785)II; daf-16(mgDf50)I*

AA2961: *gfat-1(dh468)II; ogt-1(ok430) III*

AA2886: *gfat-1(dh784)II; ogt-1(ok430) III*

AA3032: *gfat-1(dh785)II; ogt-1(ok430) III*

AA2960: *gfat-1(dh468)II; oga-1(ok1207)X*

AA2911: *gfat-1(dh784)II; oga-1(ok1207)X*

AA2912: *gfat-1(dh785)II; oga-1(ok1207)X*

SJ4005: N2; *zcIs4[Phsp-4::GFP]V*

AA2773: *gfat-1(dh468)II; zcIs4[Phsp-4::GFP]V*

AA3215: *gfat-1(dh784)II; zcIs4[Phsp-4::GFP]V*

AA3216: *gfat-1(dh785)II; zcIs4[Phsp-4::GFP]V*

AM141: N2; *rmIs133[P(unc-54) Q40::YFP]*

AA2988: *gfat-1(dh468)II; rmIs133[P(unc-54) Q40::YFP]*

AA2945: *gfat-1(dh784)II; rmIs133[P(unc-54) Q40::YFP]*

AA2953: *gfat-1(dh785)II; rmIs133[P(unc-54) Q40::YFP]*

AM322: N2; *rmEx135[F25B3.3p::Q86::YFP]*

AA2992: *gfat-1(dh468)II; rmEx135[F25B3.3p::Q86::YFP]*

AA2949: *gfat-1(dh784)II; rmEx135[F25B3.3p::Q86::YFP]*

AA2957: *gfat-1(dh785)II; rmEx135[F25B3.3p::Q86::YFP]*

NL5901: N2; *pkIs2386[Punc-54::alphasynuclein::YFP + unc-119(+)]*

AA2993: *gfat-1(dh468)II; pkIs2386[Punc-54::alphasynuclein::YFP + unc-119(+)]*

AA2950: *gfat-1(dh784)II; pkIs2386[Punc-54::alphasynuclein::YFP + unc-119(+)]*

AA2958: *gfat-1(dh785)II; pkIs2386[Punc-54::alphasynuclein::YFP + unc-119(+)]*

UA44: *baInl1[Pdat-1::α-syn high, Pdat-1::gfp]*

AA3023: *gfat-1(dh468)II; baInl1[Pdat-1::α-syn high, Pdat-1::gfp]*

AA3024: *gfat-1(dh784)II; baInl1[Pdat-1::α-syn high, Pdat-1::gfp]*

AA3025: *gfat-1(dh785)II; baInl1[Pdat-1::α-syn high, Pdat-1::gfp]*

CL2006: N2; *dvIs2[pCL12(Punc-54::human Abeta peptide 1-42 minigene; pRF4(rol-6(su1006)))]*

AA2963: *gfat-1(dh784)II; dvIs2[pCL12(Punc-54::human Abeta peptide 1-42 minigene; pRF4(rol-6(su1006)))]*

AA2963: *gfat-1(dh785)II; dvIs2[pCL12(Punc-54::human Abeta peptide 1-42 minigene; pRF4(rol-6(su1006)))]*

PP657: *unc-119(ed4)III; hhIs76[unc-119(+); unc-54::srp-2(H302R)::yfp]*

AA3116: *gfat-1(dh468)II; unc-119(ed4)III; hhIs76[unc-119(+); unc-54::srp-2(H302R)::yfp]*

AA3118: *gfat-1(dh784)II; unc-119(ed4)III; hhIs76[unc-119(+); unc-54::srp-2(H302R)::yfp]*

AA3119: *gfat-1(dh785)II; unc-119(ed4)III; hhIs76[unc-119(+); unc-54::srp-2(H302R)::yfp]*

DA2123: *adIs2122(lgg-1::GFP; rol-6(su1006))*

AA3129: *gfat-1(dh468)II; adIs2122(lgg-1::GFP; rol-6(su1006))*

AA3128: *gfat-1(dh784)II; adIs2122(lgg-1::GFP; rol-6(su1006))*

AA3131: *gfat-1(dh785)II; adIs2122(lgg-1::GFP; rol-6(su1006))*

AA3173: *N2; dhEx940[gfat-1P::FLAG-HA::cfp::gfat-1 3'UTR; myo-2::mCherry]*
AA3174: *N2; dhEx941[gfat-1P::FLAG-HA::cfp::gfat-1::gfat-1 3'UTR; myo-2::mCherry]*

AA3349: *dhEx940[gfat-1P::FLAG-HA::cfp::gfat-1 3'UTR; myo-2::mCherry]; unc-119(ed4)III; hhIs76[unc-119(+); unc-54::srp-2(H302R)::yfp]*
AA3350: *dhEx941[gfat-1P::FLAG-HA::cfp::gfat-1::gfat-1 3'UTR; myo-2::mCherry]; unc-119(ed4)III; hhIs76[unc-119(+); unc-54::srp-2(H302R)::yfp]*

HZ589: *bpIs151(p62::gfp; unc-76)IV; him-5(e1490)V*
AA3354: *gfat-1(dh468)II; bpIs151(p62::gfp; unc-76)IV; him-5(e1490)V*

KJ5944: *N2; jhEx61 [pcnx1::cnx-1::GFP+pRF4]*
AA3351: *gfat-1(dh468)II; jhEx61[pcnx1::cnx-1::GFP+pRF4]*
AA3352: *gfat-1(dh784)II; jhEx61[pcnx1::cnx-1::GFP+pRF4]*
AA3353: *gfat-1(dh785)II; jhEx61[pcnx1::cnx-1::GFP+pRF4]*

AA3329: *dhEx978[(pRedFlp-Hgr)(sel-1[28094]::S0001_pR6K_Amp_2xTY1ce_-EGFP_FRT_rpsl-_neo_FRT_3xFlag)-dFRT::unc-119-Nat; myo-2::mCherry]*

2.I. Isolation of detergent insoluble protein fraction

Animals were lyzed in RIPA buffer (50 mM Tris pH7.5, 150 mM NaCl, 5 mM EDTA, 0.5% SDS, 0.5% sodium deoxycholate, 1% NP40, 1 mM PMSF, Roche Complete Inhibitors 1x), insoluble material was pelleted by centrifugation at 16,000 x g. Pellet was washed once in RIPA buffer and then solubilized in 8M urea buffer (50 mM Tris pH7.5, 8 M urea, 2% SDS, 50 mM DTT). Protein solutions were then mixed with 2x Laemmli buffer and separated by SDS PAGE.

2.J. LC/MS/MS analysis

Synchronized worm populations were used for analysis. Extraction was done with Methanol/Chloroform. For absolute quantification of UDP-HexNAc an Acquitiy UPLC (Waters) was connected to a Xevo™ TQ (Waters). An Acquity UPLC BEH

Amide 1.7 μm, 2.1 x 30 mm column was used at 15°C. Solvent A was 0.22 μm MilliQ-Water + 0.05% Ammonium Hydroxid (Sigma-Aldrich), 19% filtered through 0.22 μm, and B 95% acetonitrile/5% 0.22 μm MilliQ-Water. A linear gradient from 98% B to 0 % in 2 min at a flow rate of 0.45 ml/min was used. 2 μL of standard and sample were injected. The sample manager was set to 6°C. The TQ was operated in negative ESI MRM (multi reaction monitoring) mode. The source temperature was set to 150°C, desolvation temperature was 650°C and desolvation gas was set to 800 L/h, cone gas to 50 L/h. The following MRM transitions were used for UDP-GlcNAc m/z 302.48 $(M\text{-}H^+)^-$ to 110.95 (quantifier) collision energy 10V, m/z 302.48 to 78.84 (qualifier) collision 30V, m/z 302.48 to 281.89 (qualifier) collision 18V, cone was in all cases 15V. As data management software MassLynx (Waters) and for data evaluation and absolute quantification TargetLynx (Waters) were used. All standards were freshly prepared after two months and dissolved in 50% MeOH and stored at -20°C. With UDP-HexNAc eluting at 0.83 min a standard calibration curve was calculated using following concentrations: 15, 25, 50, 100, 250, 350, 500, 750 ng/mL (daily fresh diluted with 50% MeOH individually from stock solutions 100 μg/ml). Correlation coefficient: $r < 0.990$; response type: internal standard, area; curve type linear; weighting 1/x. The peak integrations were corrected manually, if necessary. A new calibration curve as quality control standards were used during sample analysis and showed between 0.5% and 40% deviation respectively. Blanks after the standards, quality control and sample batch proved to be sufficient. No carry over was detected.

For absolute quantification of Pyruvate and Fructose-6-phosphate with internal standard Uridine-5,6-d2 (Sigma) a Acquitiy UPLC (Waters) was connected to a Xevo™ TQ (Waters). A zic-Hilic column was used at 20°C. Solvent A was 100mM Ammonium Acetate (Sigma-Aldrich) B Acetonitrile. A linear gradient from 60% B to 30 % in 1 min at a flow rate of 0.7 ml/min was used. 2 μL of standard and sample were injected. The sample manager was set to 6°C. The TQ was operated in negative ESI MRM (multi reaction monitoring) mode. The source temperature was set to 150°C, desolvation temperature was 650°C and desolvation gas was set to 800 L/h, cone gas to 50 L/h. The following MRM transitions were used for Pyruvate m/z 86.86 $(M\text{-}H^+)^-$ to 86.86 (quantifier) collision energy 4V, cone was 20V, for Fructose-6-phosphate m/z 258.84 to 96.96 (quantifier) collision energy 12V, m/z 258.84 to 78.89

(qualifier) collision energy 22V, m/z 258.84 to 168.95 (qualifier) collision energy 14V, cone was in all cases 16V, for Uridine-3,5-d2 m/z 244.90 to 111.24 (quantifier) collision 16V, m/z 244.90 to 152.52 (qualifier) collision 14V, cone was 24V. As data management software MassLynx (Waters) and for data evaluation and absolute quantification TargetLynx (Waters) were used. All standards were freshly prepared after two months and dissolved in 40% 100mM Ammonium Acetate/60% Acetonitrile and stored at -20°C. With Pyruvate eluting at 0.49 min using following concentrations: 10, 50, 100, 250, 500, 1000, 2000, 3000 ng/mL and with Fructose-6-phosphate eluting at 0.65 min using following concentrations 200, 400, 800, 1200, 2000, 2500, 3000, 4000 ng/ml a standard calibration curve was calculated with the internal standard Uridine-5,6-d2 eluting at 0.47 min concentration 100 ng/ml for the internal standard (daily fresh diluted with 40% 100mM Ammonium Acetate/60% Acetonitrile individually from stock solutions 100 μg/ml). Correlation coefficient: $r < 0.990$; response type: internal standard, area; curve type linear; weighting 1/x. The peak integrations were corrected manually, if necessary. A new calibration curve as quality control standards were used during sample analysis and showed between 0.5% and 40% deviation respectively. Blanks after the standards, quality control and sample batch proved to be sufficient. No carry over was detected.

2.K. Lifespan assays

Adult lifespan analyses were performed at 20°C on *E. coli* OP50 with young adults as t = 0. 100-150 animals, distributed on 7-10 plates, were used per condition and transferred to fresh plates/scored every day or every second day. The secondary longevity screen after TM selection was done on plates containing 50 μM FUDR. Animals in all RNAi lifespan assays were grown on NGM plates containing OP50 during development, and transferred to RNAi plates as young adults (L4+1 day, t=0). Animals were kept on RNAi bacteria containing plates throughout the experiment. Worms that had undergone internal hatching, vulval bursting, or crawling off the plates were censored. Throughout the experiment strain and/or treatment was unknown to researcher. In some experiments all plates were numbered and randomly mixed. In both cases data were assembled upon completion of the experiment. Statistical analyses were performed with the Mantel-Cox Log Rank method in Excel (Microsoft).

2.L. Mutant mapping and sequence analysis

Rapid SNP mapping was done as previously described (Hammarlund M., 2005). Genomic DNA was prepared using the QIAGEN Gentra Puregene Kit. Whole Genome Sequencing was done on the Illumina HiSeq2000 platform. Sequencing outputs were analysed using the MAQGene pipeline (Bigelow et al., 2009). Output from the MAQGene pipeline was analysed in Excel. 100bp paired-end reads were used with the average coverage larger than 20-fold.

2.M. Paralysis and motility assays

Paralysis of *dvIs2[pCL12(Punc-54::human Abeta peptide 1-42 minigene; pRF4(rol-6(su1006)))]* animals was scored daily by prodding animals at head and tail with a wire on NGM plates. Alive animals that were unable to roll were scored as paralyzed. Motility assays using *rmIs133[P(unc-54) Q40::YFP], baInl1[Pdat-1::α-syn high, Pdat-1::gfp]* and *pkIs2386[Punc-54::alphasynuclein::YFP + unc-119(+)]* were scored at the 7th day of adulthood (L4+8 days). When genetic modifications using RNAi were used, animals were transferred to RNAi plates as young adults (L4+1 day). At L4+8 days, animals were transferred to M9, and motility was determined by counting bodybends over a 30 second interval.

2.N. Plasmid construction and transgenes

gfat-1P::FLAG-HA::cfp::gfat-1-3'UTR

The *gfat-1* promoter of 3.8kb was amplified from genomic DNA by PCR and inserted in front of the *cfp* sequence in the pDC6 vector (kind gift from Dr. Magner, Antebi lab). The *gfat-1* 3'UTR of 517bp was amplified from genomic DNA by PCR and inserted downstream of *cfp* to yield a transcriptional reporter construct.

gfat-1P::FLAG-HA::cfp::gfat-1::gfat-1-3'UTR

The *gfat-1* promoter of 3.8kb was amplified from genomic DNA by PCR and inserted in front of the *cfp* sequence in the pDC6 vector. The *gfat-1* open reading frame and 3'UTR of 3354bp was amplified from genomic DNA by PCR and inserted downstream of *cfp* to yield the translational fusion construct.

gfat-1P::FLAG-HA::cfp::gfat-1::gfat-1-3'UTR and *gfat-1P::FLAG-HA::cfp::gfat-1-3'UTR* were injected into N2 to generate *dhEx941* and *dhEx940*, respectively.

The *sel-1P::gfp* fosmid (Transgenome Project) were injected into N2 to generate *dhEx978*. *myo-2::mCherry* was used as co-injection marker.

2.O. Proteasome Activity Assay

Proteasome chymotryptic and tryptic activity was assayed according to standard procedures (Fredriksson Å., 2012) as the rate of hydrolysis of the fluorogenic peptide suc-LLVY-AMC (Sigma) or ac-RLR-AMC (Enzo), respectively. Extracts were prepared in 25 mM Tris HCl pH7.5 using a dounce homogenizer. 20 μg protein was incubated with 12.5 μM suc-LLVY-AMC in a total volume of 200 μL. AMC fluorescence was measured using 355 nm excitation and 460 nm emission filters with free AMC (Sigma) as standard every 2 minutes for one hour at 25°C.

2.P. qRT-PCR

Synchronized worms at indicated stages were collected in TRIzol (Invitrogen) and frozen in liquid nitrogen. Total RNA was prepared by RNeasy Mini kit (QIAGEN) and cDNA was subsequently generated by iScript cDNA Synthesis Kit (BioRad). qRT-PCR was performed with Power SYBR Green master mix (Applied Biosystems) on a ViiA 7 Real-Time PCR System (Applied Biosystems). *ama-1* was used as internal control. Primer sequences are as listed in Extended Experimental Procedures.

2.Q. qRT-PCR primers

Gene	Primer	Sequence (5'-3')
gfat-1	gfat-1-f	GTCCAACCACGCTACTCATTTGC
	gfat-1-r	TGACAGGTCCAGAATGTTGTTTGG
gna-2	gna-2-f	ACATTCACGAAGCGGGAACACG
	gna-2-r	GTGACAACTTGTAGACGCCAATCG
daf-16	daf-16-f	ATCAGACATCGTTTCCTTCGG
	daf-16-r	TTAACCGTTTCTCTGGACTAGC
sod-3	sod-3-f	CACGAGGCTGTTTCGAAAGG
	sod-3-r	GAATTTCAGCGCTGGTTGGA
dod-3	*dod-3-f*	CGTATATGGACCCAGCTAATG
	dod-3-r	ATGAACACCGGCTCATTC
dod-8	*dod-8-f*	ACAGGATGTCTTCAAAAGGAATATGG

	dod-8-r	TTGCTGGGGTGATAGCTTGG
ama-1	ama-1-f	GGATGGAATGTGGGTTGAGA
	ama-1-r	CGGATTCTTGAATTTCGCGC
hsp-4	hsp-4-f	GTGGCAAACGCGTACTGTGATGA
	hsp-4-r	CGCAACGTATGATGGAGTGATTCT
xbp-1	xbp-1-f	CCGATCCACCTCCATCAAC
	xbp-1-r	ACCGTCTGCTCCTTCCTCAATG
xbp-1	xbp-1-f sp	TGCCTTTGAATCAGCAGTGG
spliced	xbp-1-r	ACCGTCTGCTCCTTCCTCAATG
hsp-3	hsp-3-f	TTTCTCTACCGCCGCTGACAAC
	hsp-3-r	CGTTCTCCTTCGAAGACCTGGATG
R02D3.8	R02D3.8-f	GCTTCTGCGGAGCACACAAATC
	R02D3.8-r	TGCAGCTTGTAGATGGCCAGAAG
B0280.1b	B0280.1b-f	GGAGGATTAAATGGTCGGCCTGAG
	B0280.1b-r	AATGGCGAGAGAAGCGAGAACC
T26F2.2	T26F2.2-f	GGACGAGCCGCTTGATTTAATGC
	T26F2.2-r	AGAAGTCTGCTCCGATCACCTTTG
pdi-2	pdi-2-f	ATGGACTCAACCCTCAACGAGGTC
	pdi-2-r	TCCAGCTGGGAAGAACTTGATGG
pdi-3	pdi-3-f	GATGCCACCGCCAATGATGTTC
	pdi-3-r	GCCAGAAGAGAGTTGGGAATCCTC
cnx-1	cnx-1-f	TACAATGGAGAGTGGAGCATCGG
	cnx-1-r	ATTGCATGATGGCGTGCCTTTG
crt-1	crt-1-f	TGTGGCAGGTCAAGTCAGGAAC
	crt-1-r	TTTCTTCGTCGGCCTTCTCCTTC
pha-4	pha-4-f	GCCAATTTCATGCAAGGAGG
	pha-4-r	GCCAGTGGTAAAACCAAGAGGT
bec-1	bec-1-f	TGCAGTTGCTCGAGTTTTTG
	bec-1-r	TGACACCATTGTCAACCAGTG
lgg-1	lgg-1-f	ACCCAGACCGTATTCCAGTG
	lgg-1-r	ACGAAGTTGGATGCGTTTTC
atg-1	atg-1-f	ACAAATCCCTGTCGTTCCAG

	atg-1-r	AATGAGCCGTTGGATAATGC
lipl-4	lipl-4-f	ATGGCCGAGAAGTTCCTACATCGT
	lipl-4-r	GGTGAATTGGCGACCCAATCGAAA
sel-1	sel-1-f	AGTCAGCGACTCTGGTATCTGC
	sel-1-r	TGCAGTGAGTAACGAGCCTGTG
sel-11	sel-11-f	AGGCTGAGCATCTGTCAGAACG
	sel-11-r	CACGGAATACAGTGAATGCGAGAC
rpn-6	rpn-6-f	ACACTCAGGCTCTTCCACTTGC
	rpn-6-r	TCCTCAAGTTCGACTTCCACGAG
rpn-8	rpn-8-f	TGTACGATCGGTGGTCGCTTTG
	rpn-8-r	TCAGCCTTCTGGAGAGAGATCTTG
hsp-16.2	hsp-16.2f	ACTTTACCACTATTTCCGTCCAGC
	hsp-16.2-r	CCTTGAACCGCTTCTTTCTTT
hsp-70	hsp-70-f	AATGAACCAACTGCTGCTGCTCTT
	hsp-70-r	TGTCCTTTCCGGTCTTCCTTTTG
hsp-6	hsp-6-f	GAACCGGAAAGGAACAACAGATCG
	hsp-6-r	TTTGGTCCTTGGAAAGTCCTCCAG
hsp-60	hsp-60-f	AAATGCCGGTCTTGAGCCATCG
	hsp-60-r	AGAGCTGTGCGAACCACCTTAG
gfp	gfp-f	TTTCACTGGAGTTGTCCCAATTC
	gfp-r	CTTCACCCTCTCCACTGACAGAA

2.R. Quantification of SRP-2^{H302R}::YFP aggregation

SRP-2^{H302R}::YFP puncta were scored in transgenic animals in WT, *gfat-1* gof or Dyf mutant backgrounds. At least 20 animals were whole-mounted per experiment and GFP puncta were counted using a Zeiss Axio Imager.Z1. For clarity of imaging, only the aggregates in the animals' head regions were scored.

2.S. RNAi clones

Gene	Sequence Name	Library
ire-1	C41C4.4	Ahringer

xbp-1	R74.3	Ahringer
pek-1	F46C3.1	Ahringer
atf-6	F45E6.2	Ahringer
hsp-4	F43E2.8	Ahringer
cnx-1	ZK632.6	Ahringer
crt-1	Y38A10A.5	Ahringer
pdi-1	C14B1.1	Ahringer
pdi-2	C07A12.4	Ahringer
sel-1	F45D3.5	Vidal
sel-11	F55A11.3	Vidal
rpn-6	F57B9.10	Ahringer
rpn-8	R12E2.3	Ahringer
stt-3	T12A2.2	Ahringer
ostd-1	M01A10.3	Ahringer
ostb-1	T09A5.11	Ahringer
dad-1	F57B10.10	Ahringer
ribo-1	T22D1.4	Ahringer
ZK686.3	ZK686.3	Ahringer
atg-18	F41E6.13	Vidal
unc-51/atg-1	Y60A3A.1	Vidal
gfat-1	F07A11.2	Ahringer
gna-2	T23G11.2	Ahringer
hsf-1	Y53C10A.12	Ahringer

2.T. RNA interference

RNAi experiments were performed as described earlier (Kamath R.S., 2003) (Rual, 2004). For whole life or developmental knockdown, synchronized eggs were put on corresponding RNAi plates containing IPTG and ampicillin. For adult only knockdown, L4 larvae were picked to NGM plates containing OP50, and 24 hours later transferred to the RNAi plates, L4+1 day. *gfp* (GFP::L4440) RNAi or empty vector (L4440) were used as a non-targeting control. RNAi clones used are listed in 2.S.

2.U. Statistical analysis

Results are presented as mean ± s.e.m. unless noted otherwise. For each experiment, at least 3 biological replicates were carried out and genotypes and/or treatment were blinded during analysis. Statistical tests were performed with One-way ANOVA with Bonferroni post-test (ANOVA) with GraphPad Prism (GraphPad software) unless otherwise noted. Chi-square two-tailed *P*-values were calculated with GraphPad QuickCalcs. Significance levels are *P<0.05, **P<0.01, ***P<0.001 vs. WT control unless otherwise noted.

2.V. Thermo tolerance Test

Thermo tolerance assays were conducted at the indicated temperature and alive worms were scored every or every other hour. Plates were initiated with 20-25 adult animals each, and the plates were mixed in the incubator every time to avoid differences in heat exposure at different locations within the incubator. All plates were numbered randomly.

2.X. Tunicamycin resistance screen

L4 larvae were exposed to 0.3% or 0.1% ethyl methane sulfonate (EMS, Sigma) in M9 buffer for 4 hours. After recovery over night, young adult animals were transferred to new plates to perform an egg-lay (F1). F1 progeny were developed on NGM plates seeded with OP50 and then allowed to lay eggs (F2) on NGM agar plates containing 10 μg/mL tunicamycin (TM, Calbiochem). TM was dissolved in DMSO and nematode development on vehicle control plates did not differ from standard NGM plates. F2 development was scored after 4 and 5 days and resistant mutants, defined as fully developed and fertile animals, were transferred to standard plates and outcrossed 4 times with the parental N2 strain using tunicamycin selection.

2.Y. Western blotting

25 L4 animals were suspended in Laemmli lysis buffer under reducing conditions. Proteins were separated by reducing SDS-PAGE and transferred to PVDF membranes. Membranes were then incubated with specific antibodies to GFP (Clontech), α-tubulin (Sigma), and SEL-1 (produced in the Sommer laboratory).

CHAPTER 3. ER Stress Resistance Screen reveals Longevity Genes

Part of this work has been published in Denzel, Storm et al. (2014). Hexosamine pathway metabolites enhance protein quality control and promote life. *Cell, 13 March issue, 2014*

3.A. Introduction

Several protein quality control mechanisms have been found implicated in aging and AADs. Especially interesting, many long-lived organisms have enhanced ability to cope with various stresses due to improved protein quality control mechanisms (L D Osellame, 2013). This includes mutants of *C. elegans*. For example, the DR mimicked mutant *eat-2*, the insulin signaling depleted mutant *daf-2* and the germline-less mutant *glp-1* do all show enhanced levels of autophagy (Malene Hansen, 2008) (Lapierre LR, 2011). Thus, they depend on autophagy to sustain their extended lifespan. Furthermore, it has been found that the long life of both *glp-1* and *daf-2* mutant animals is dependent on the canonical ER UPR pathways. From various stress resistance-assays, other research groups found that these mutants are more resistant to stress persuaded by heat and various drugs inducing oxidative, mitochondrial and ER stress (Vilchez D, 2012) (Sivan Henis-Korenblit, 2010) (Taylor RC, 2013). This suggests a link between enhanced stress resistance and long life.

We designed a forward genetic screen aiming at selecting mutants with increased resistance to ER stress. ER stress can be induced by pharmacological suppression of N-glycosylation, which can be achieved with the drug tunicamycin (TM). TM blocks the enzymatic assembly of N-glycans and is a well-known inducer of ER stress. TM interrupts the ER functions by inhibiting the addition of complex sugars to the growing sugar-chain on newly synthesized proteins (S-C Kuo, 1976), and in worms, it has been shown that exposure to TM induces ER stress and furthermore triggers the ER stress response pathways through the IRE-1/XBP-1 signaling pathway (Marcella Calfon, 2001) (Xiaohua Shen, 2001). The Kenyon lab showed that this particular branch of the ER UPR pathway is important for the remarkable longevity of the *daf-2* mutants (Sivan Henis-Korenblit, 2010). Interestingly, they also found that *daf-2* mutant animals have a higher threshold for induction of ER UPR upon treatment with lower doses of TM.

Together, previous studies have shown a correlation between enhanced stress resistance and longevity, and we hypothesized that improved ER quality control mechanisms would promote long life.

3.B. Results

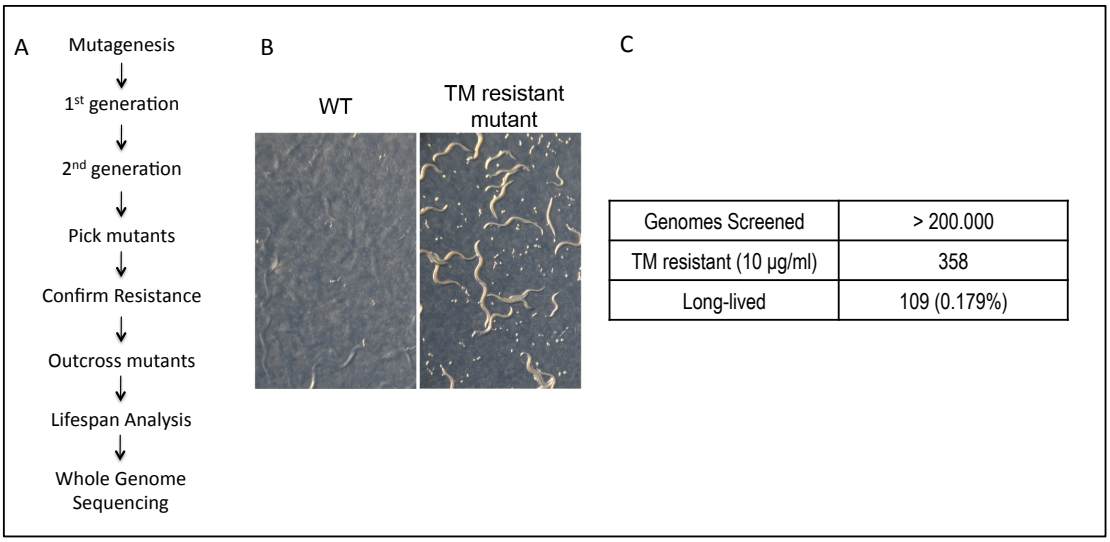
3.B.1 The screen

To identify novel genes involved in ER stress resistance and protein quality control, we designed a forward genetic screen, using developmental abilities on the ER-interrupting drug, TM, as first selection criteria.

Performing a TM killing concentration experiment (data not shown), we decided to use the concentration 10 μg/mL in the screen. Furthermore, we decided to select TM resistant mutants after development, since it made the method easier and thereby allowed us to screen through higher numbers of mutated genomes. The chosen concentration abolished all kinds of WT development, which again eased the factual screen, and gave rise to screen through large numbers of genomes.

Figure 3.1 A developmental TM resistance screen

(A). Screening flow (B). Representative images of WT controls and tunicamycin (TM) resistant *C. elegans* mutants after developmental screen for resistance to 10 µg/mL TM (C). Schematic overview of the screening results



To introduce random genetic mutations in the *C. elegans* genome, we did chemical mutagenesis on young adult hermaphrodites (F_0) using EMS. The young F_0 animals' progeny (F_1) were isolated 4-5 days after the parental egg-lay. Figure 3.1A shows the structure of the screen (see Experimental Procedure for more details about the screen), and in Figure 3.1B (right picture) an example of a TM resistant mutant is shown. From the more than 200.000 mutated genomes, we were able to isolate 358 mutants that completed development on plates containing TM (Figure 3.1). We performed a preliminary survival analysis on these, and found that 109 of tend to live longer (Figure 3.1C), indicating that the screen is enriching for long-lived animals. Of the 109 long-lived mutants, 66 were Dyf (dye filling phenotype) (see Dyf section, chapter 6).

We performed whole genome sequencing on 38 of the long-lived mutants. Most of the candidate hits revealed from sequencing were singletons, however, in a couple of cases we identified independent alleles of the same gene, increasing the likelihood that that specific mutation is the actual mutation causing TM resistance and extended lifespan. Additionally, phenotype specificity has been used to make qualified assumptions on for example the identity of some of the Dyf mutants.

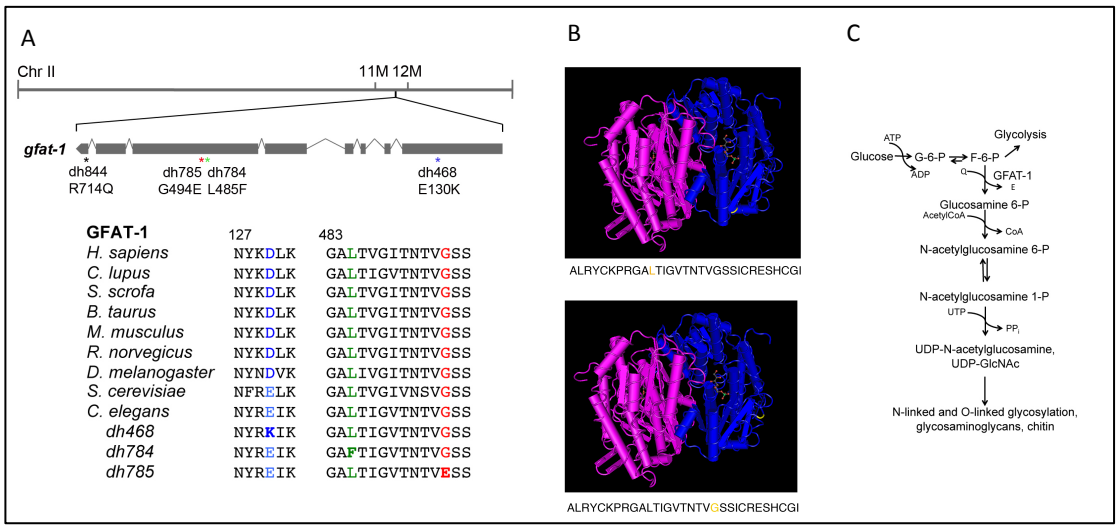
3.B.2 Glutamine-Fructose-6-phosphate Amino-transferase 1

In one case, we had four independent hits in the same gene (F07A11.2), Glutamine-Fructose-6-phosphate Amino-transferase 1 (*gfat-1*). We had four independent point mutations leading to a single amino acid change in each mutant. In Figure 3.2A (top panel) a part of the third *C. elegans* chromosome is shown, specifying the position of *gfat-1*. It is additionally displaying the exact location of the mutations, and indicating the changed amino acid. We excluded the *gfat-1(dh844)* from the following work (for more information, see chapter 10).

GFAT-1 is highly conserved from *C. elegans* to humans, and even the mutated amino acids in the *gfat-1* mutants are conserved (Figure 3.2A, bottom). In Figure 3.2B the mammalian crystal structure of GFAT-1 is shown, and two of the changed amino acids are shown in yellow (top; *dh784* and bottom; *dh785*). In both cases, the amino acid is located on the surface of the protein, suggesting that the mutations are likely to cause a structural change of the protein. However, this is still unknown.

Figure 3.2 Mutations in *gfat-1* result in resistance to TM

(A). Multiple sequence alignment of *gfat-1* protein sequences surrounding the amino acid substitutions of alleles *dh468*, *dh784*, and *dh785* (B). Illustrative structure of GFAT-1, mutated amino acids are indicated in yellow (C). The hexosamine pathway



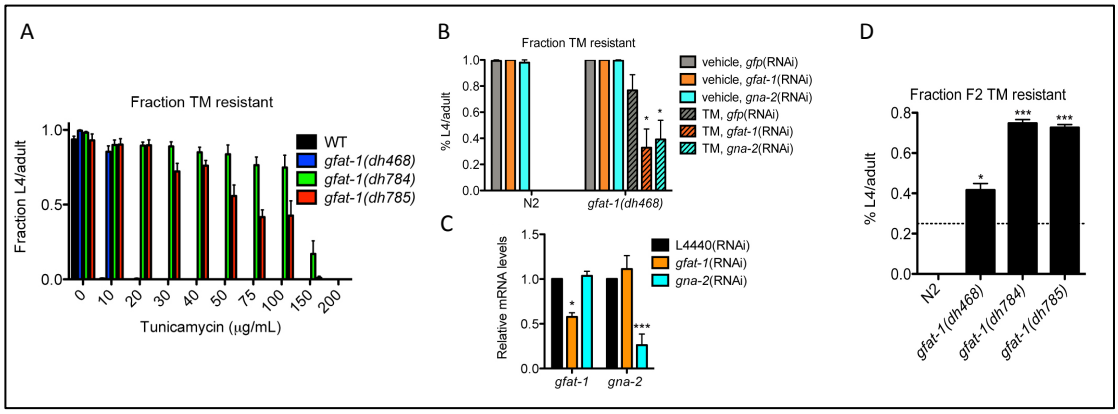
In mammals, GFAT-1 regulates the rate-limiting step of the hexosamine pathway (Figure 3.2C), which in multiple steps converts fructose 6-phosphate into UDP-N-Acetylglucosamine (UDP-GlcNAc). Due to the high similarity between mammalian GFAT-1 and *C. elegans* GFAT-1 we assumed that the functions were equally similar. UDP-GlcNAc is a precursor of N- and O-linked glycosylation of proteins, and therefore serves as a posttranslational modifier of proteins. This makes the hexosamine pathway a key regulator of several pathways, as N- and O-linked glycosylation are essential for many proteins.

3.B.3 gfat-1 gain-of-function mutations protect against TM

The three different *gfat-1* mutants showed distinguishable resistance to TM (Figure 3.3A). The *gfat-1(dh468)* showed lowest resistance, and as the concentration of TM was increased to 20 μg/mL, all development was abolished. On the other hand, *gfat-1(dh784)* and *gfat-1(dh785)* had developing larvae even at 150 and 100 μg/mL respectively, indicating that specific alleles cause concentration-specific resistance to TM. To confirm the *gfat-1* mutants' resistance to TM, we knocked down *gfat-1* or *gna-2* (*glucosamine 6-phosphate N-acetyltransferase 2*), which regulates the conversion of glucosamine 6-phophate to N-Acetylglucosamine 6-phosphate (the following step of the hexosamine pathway), and scored the animals' resistance to TM. Surprisingly, the N2 WT (wild type) animals were not resistant to TM when *gfat-1* or *gna-2* were knocked down by RNAi, and along the same line, *gfat-1(dh468)* mutant became sensitive to TM when treated with RNAi against *gfat-1* or *gna-2* (Figure 3.3B). We measured the mRNA level of *gfat-1* and *gna-2* after RNAi treatment, and did however find that mRNA levels were reduced upon the RNAi treatment (Figure 3.3 C).

Figure 3.3 Knockdown of *gfat-1* causes TM sensitivity in *gfat-1* mutants

(A). Quantification of developmental TM resistance assay of alleles *dh468*, *dh784*, and *dh785* using different concentrations, 0 μg/mL TM to 200 μg/mL TM (B). Developmental TM resistance assay was performed on 10 μg/mL TM-supplemented RNAi plates. Eggs were transferred to *gfat-1* or *gna-2* RNAi plates so TM exposure and RNAi mediated knockdown occurred simultaneously (n=3) (C). Quantitative real-time PCR measurements of *gfat-1* and *gna-2* mRNA levels after developmental RNAi treatment of either gene in WT. Analysis was done in day 1 adults (n=3) (D). Developmental TM resistance assay with the offspring of heterozygous animals reveals that alleles *dh468*, *dh784*, and *dh785* were statistically significant different from a distribution expected for a recessive allele (data are means±s.e.m., n=18, *P<0.05, ***P<0.001 by chi-squared test)

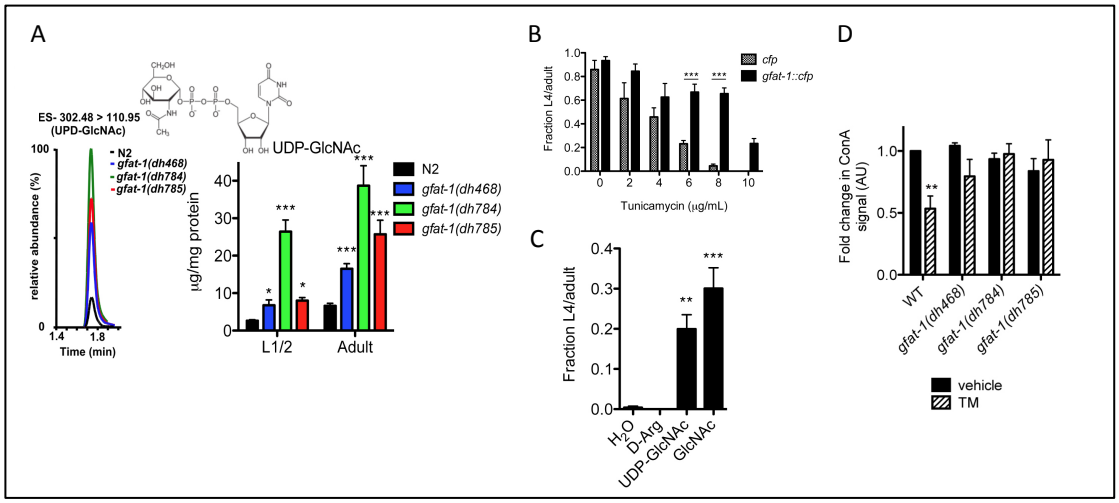


Earlier it has been found that knockdown of *gfat-1* (F07A11.2) by RNAi in worms causes embryonic lethality and developmental arrest (www.wormbase.org), however, we did not observe any changes in developmental timing, pumping rate or brood size (Supplemental Figure 1A, B, C) in the *gfat-1* mutants. That led us to speculate whether the mutations we had detected were gain-of-function (gof) mutations instead of lof mutations. Additionally, we observed that the *gfat-1* mutations were dominant, leading to at least partial resistant in heterozygous animals (Figure 3.3D). gof mutations are usually dominant, again indicating that the *gfat-1* mutations were gof mutations. Together, these results suggested that GFAT-1 lof causes TM sensitivity, and we therefor hypothesized that the *gfat-1* mutations in the mutants from the screen were gof mutations.

Conceivably, resistance to TM in the *gfat-1* mutants stems from gof mutations, and we would therefore expect these mutants to have an increased flux through the hexosamine pathway, resulting in a higher level of the end product, UDP-GlcNAc. To test this, we measured the levels of UDP-GlcNAc by Liquid-Chromatography/Mass-Spectrometry (LC/MS) and found that the three independent *gfat-1* mutants all had significantly increased levels of UDP-GlcNAc in both early larvae stages (L1/L2) and in young adults (Figure 3.4A). We additionally created a transgenic worm strain, overexpressing *gfat-1* under its own promoter and found that these animals were resistant to TM (Figure 3.4B). Furthermore we were able to feed UDP-GlcNAc and GlcNAc (a UDP-GlcNAc precursor) to WT animals, and rescue their TM sensitivity (Figure 3.4C).

Figure 3.4 *gfat-1* gof mutations lead to resistance to TM.

(A). Representative LC/MS scans of adult *C. elegans* extracts (left) and quantitative LC/MS analysis of pooled L1/L2 larvae and adult animals for UDP-HexNAc levels in *dh468*, *dh784*, and *dh785* alleles of *gfat-1* (n=8) (right) (B). Developmental TM resistance assay using transgenic animals expressing *cfp* or *gfat-1::cfp* fusion under *gfat-1* regulatory elements (n=3) (C). Quantification of developmental TM resistance assay in adult animals after supplementation of indicated compounds on TM plates (n=6 **P<0.01, ***P<0.001 vs. D-Arg) (D). Quantification of western blot from control and 10 µg/mL TM treated WT and *gfat-1* gof mutant animals stained with HRP-coupled ConcavalinA. TM treatment was initiated at L4 and done for 18 hours (n=4)



Elevated levels of UDP-GlcNAc are likely to be the reason for the resistance to TM observed in the *gfat-1* mutants. UDP-GlcNAc serves as a precursor for N-glycans, and we speculated whether the resistance to TM toxicity observed when UDP-GlcNAc is elevated was due to the animals' ability to maintain proper levels of N-glycans. To test this we used Concavalin A staining, and measured bound proteins by western blot (WB). Concavalin A is a lectin, and binds all N-glycosylated proteins. Interestingly, we found that the level of N-glycosylated proteins was decreased in WT animals when they were exposed to TM, which suggests that TM toxicity in WT animals stems from inhibited N-glycosylation of proteins. Conversely, no effect was observed in the *gfat-1* mutants, suggesting that they are able to maintain a proper level of N-glycosylation during TM treatment (Figure 3.4D). All together these results suggest that *gfat-1* mutant animals from the TM resistance screen have gof mutations in *gfat-1*. GFAT-1 gof presumably leads to an increased flux through the hexosamine pathway, which results in resistance to TM.

3.C. Discussion

We have used a forward genetic screening method to select mutants with enhanced abilities to cope with TM-induced ER stress. From the screen we have identified four independent mutations in *gfat-1*, which encodes GFAT-1 that catalyzes the rate-limiting step of the hexosamine pathway. We identified all the mutations as gof mutations, and detected an increased flux through the hexosamine pathway resulting in higher levels of the pathway's end product, UDP-GlcNAc. In addition, WT animals became resistant to TM when we overexpressed *gfat-1* under its own promoter. Consistently, WT animals were able to develop in the presence of TM when we supplemented them with UDP-GlcNAc or GlcNAc. Together, these results suggest that GFAT-1 gof or supplementation with hexosamine pathway metabolites enhance the ability to cope with TM-induced ER stress.

We hypothesize that the TM resistance stems from mechanisms downstream of GFAT-1, however, we can not exclude that *gfat-1* gof or overexpression of *gfat-1* downregulate the glycolysis pathway. Previously it has been suggested that caloric restriction enhance the resistance to various stresses (Byung P. Yu, 2006) and Schulz et al. showed in addition that glucose restriction extended the lifespan of WT animals, whereas increased levels of glucose shortened the lifespan (Schulz TJ, 2007). We did, however, not detect any changes in pyruvate levels in the *gfat-1* gof mutants, suggesting that glucose metabolism is unchanged, but we cannot completely exclude it.

CHAPTER 4. Hexosamine Pathway Metabolites enhance Protein Quality Control and Prolong Life

Part of this work has been published in Denzel, Storm et al. (2014). Hexosamine pathway metabolites enhance protein quality control and promote life. *Cell, 13 March issue, 2014*

4.A. Introduction

In the previous chapter, I have presented our results suggesting that GFAT-1 gof leads to increased levels of UDP-GlcNAc and that this results in resistance to TM-induced ER stress. ER stress resistance and ER protein quality control mechanisms have previously been found to play a role in *C. elegans* longevity (Rebecca C. Taylor, 2013) (Sivan Henis-Korenblit, 2010). Additionally, previous studies have indicated that these mechanisms decline with age in *C. elegans* (Rebecca C. Taylor, 2013), again suggesting them to be involved in aging.

Improved protein homeostasis can result from enhanced folding and re-folding or from increased turnover of proteins, as a consequence of amplified activity of the degradation pathways. The ER has a specific degradation mechanism termed ERAD. ERAD recognizes un- or misfolded proteins in the ER lumen, and transfers them to protein complexes located in the ER membrane. Here, the proteins will be unfolded and translocated out to the cytoplasm, where they after ubiquitination are substrates for the proteasome. Inhibition of ERAD is associated with a higher incidence of AADs such as Alzheimer's disease, and point at an important link between altered protein homeostasis and aging (Kobayashi T, 2002) (Giuliana Saltini, 2006). Furthermore, autophagy, which also plays a role in protein homeostasis (Cuervo, 2008), has been related to aging. Together, this suggests that improved protein quality control mechanisms play a role in aging.

4.B. Results

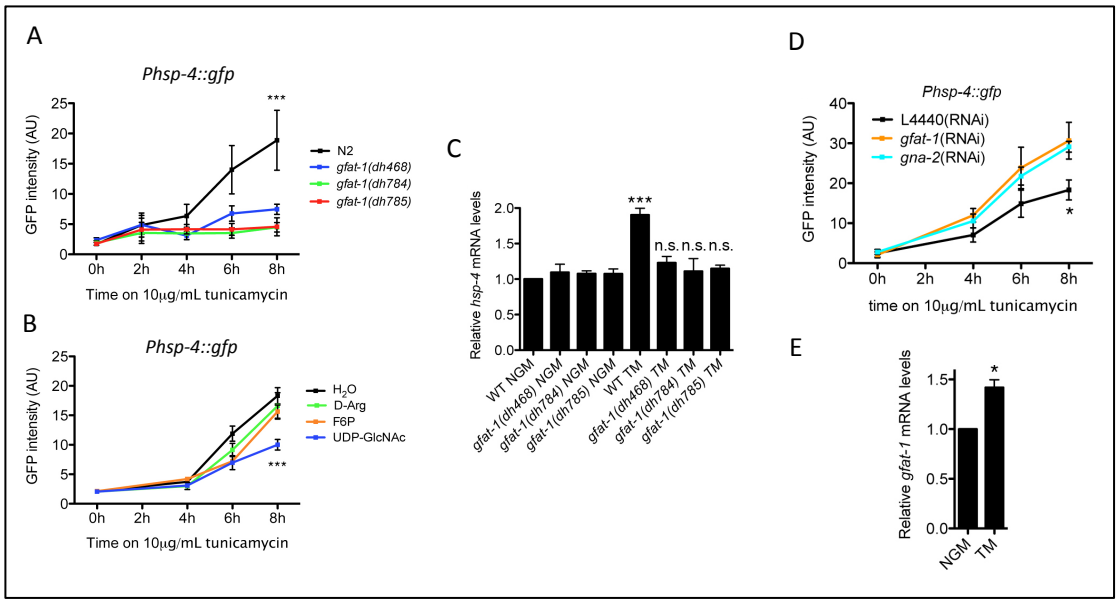
4.B.1 GFAT-1 gof protects against ER stress and extends lifespan

gfat-1 gof mutations in *C. elegans* led to resistance to a WT lethal concentration of TM, suggesting that these mutants have improved protection against TM-induced ER stress. We therefore looked into the possible connection between GFAT-1 gof and ER stress response pathways. A known target of the ER UPR is the ER chaperone HSP-4 (heat-shock protein 4), which expression is highly increased upon ER stress. We used a *C. elegans* transgenic strain that has *gfp* expressed under control of the *hsp-4* promoter, giving us the opportunity to determine the expression levels of *hsp-4* by measuring GFP intensity (Figure 4.1). Interestingly, we found that all *gfat-1* gof mutant animals showed lower expression of *hsp-4* after TM-treatment for 4, 6 and 8

hours compared to WT controls (Figure 4.1A), suggesting that they are protected against TM in a way that they never register the ER stress. Alternatively, they are dealing with the TM toxicity upstream of the ER UPR. Consistently, we observed similar effects when treating WT animals with UDP-GlcNAc upon TM exposure (Figure 4.1B), again suggesting that increased levels of hexosamines are protecting the cells against TM-induced ER stress. To confirm the results we measured the *hsp-4* mRNA expression level by qPCR and found that during normal conditions, the WT and the *gfat-1* gof mutants' *hsp-4* levels are similar. However, after 8 hours exposure to TM we observed a doubling of *hsp-4* mRNA level in WT animals, whereas no changes were observed in the *gfat-1* gof mutants (Figure 4.1C).

Figure 4.1 *gfat-1* gof mutants show less induction of ER stress responses

(A). WT or *gfat-1* gof mutants carrying the *Phsp-4::gfp* reporter were exposed to 10 μg/mL TM on day 1 of adulthood. Quantitative analysis show that the *gfat-1* alleles *dh468*, *dh784*, and *dh785* significantly suppressed GFP induction after 8 hours (n=3) (B). Transgenic animals carrying the *Phsp-4::gfp* reporter were exposed to 10 μg/mL TM on day 1 of adulthood on plates seeded with UV-killed OP50 bacteria and supplemented with 10 mM of indicated compounds. (n=3, ANOVA ***P<0.001 vs. D-Arg) (C). Quantitative real-time PCR measurements of *hsp-4* mRNA in control and 10 μg/mL TM treated WT and *gfat-1* gof mutant animals. Treatment was done as in (B) (D). *Phsp-4::gfp* transgenic animals were exposed to 10 μg/mL TM on day 1 of adulthood after RNAi mediated knockdown of *gfat-1* or *gna-2* during development. *gfat-1* or *gna-2* knockdown hyper sensitizes WT animals to TM-induced *Phsp-4::gfp* expression (n=3, *P<0.05 vs. *gfat-1* or *gna-2* RNAi treatment) (E). Quantitative real-time PCR measurements of *gfat-1* mRNA in control and 10 μg/mL TM treated animals. Treatment was done as in (B) (t-test)

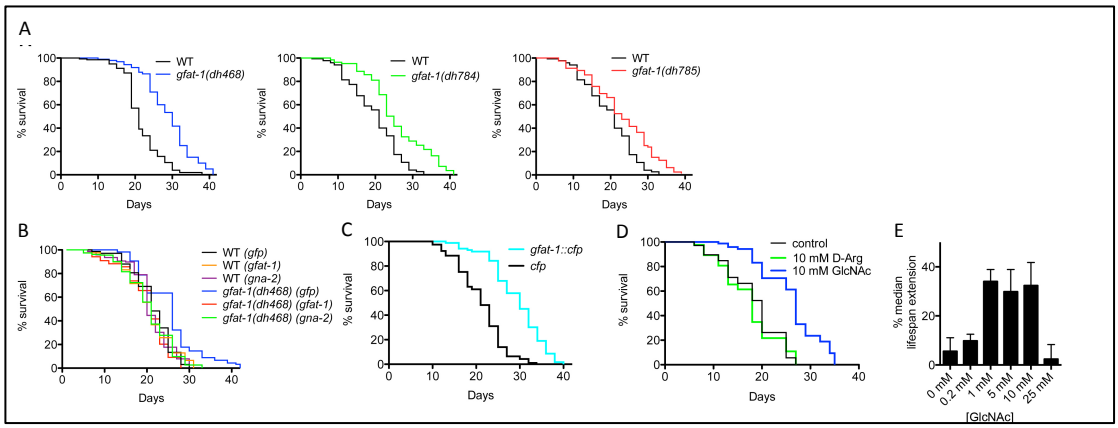


Employing GFAT-1 and the hexosamine pathway upstream of the ER UPR, and assuming that GFAT-1 is also the rate-limiting enzyme in the hexosamine pathway in *C. elegans,* as it is in mammals, we asked whether WT *hsp-4* expression would be affected upon depletion of components of the hexosamine pathway. Interestingly, we found an additive increase in *hsp-4* expression after 8 hours of TM exposure in WT animals, when *gfat-1* or *gna-2* were knocked down by RNAi (Figure 4.1D). Additionally, *gfat-1* mRNA expression levels were increased in WT animals when exposed to TM. Together, these results suggest the hexosamine pathway as a key-regulator of the ER stress response pathways, since blocking the hexosamine pathway enhance the stress response, and activation of the hexosamine pathway decreases the response. ER stress response pathways are also turned on when animals are exposed to heat. We did however not detect any resistance to heat in the *gfat-1* gof mutants (Supplemental figure 4.1D).

Previously, Henis-Kohrenbilt and colleagues showed that long-lived *daf-2* mutant animals are protected against ER stress, and that the ER stress response pathways are essential for their remarkable lifespan extension (Sivan Henis-Korenblit, 2010). We carefully tested the lifespan of the *gfat-1* gof mutants, and interestingly, we found them all to be significantly long-lived (Figure 4.2A). To make sure that the longevity arises directly from mutations in *gfat-1*, we knocked down *gfat-1* or *gna-2* by RNAi in one of the *gfat-1* gof mutants (*dh468*), and observed an attenuation of lifespan extension, without significant changes in WT lifespan (Figure 4.2B). This suggests that the lifespan extension is directly related to enhanced flux through the hexosamine pathway resulting from *gfat-1* gof mutations.

Figure 4.2 Enhanced hexosamine pathway flux extends lifespan

(A). Kaplan-Meier survival curves of *gfat-1* alleles *dh468*, *dh784*, *dh785* show significant lifespan extensions (left panel: WT median lifespan 21 days, *gfat-1(dh468)* median lifespan 30 days, P<0.0001; middle-left panel: WT median lifespan 21 days, *gfat-1(dh784)* median lifespan 25 days, P<0.0001; middle-right panel: WT median lifespan 21 days, *gfat-1(dh785)* median lifespan 23 days, P=0.0004; right panel: WT median lifespan 21 days (B). Kaplan-Meier survival curves of WT and *gfat-1(dh468)* animals using the indicated RNAi treatment (C). Lifespan assay using transgenic animals expressing *cfp* or *gfat-1::cfp* fusion under *gfat-1* regulatory elements (*Pgfat-1::cfp* median lifespan 21 days, *Pgfat-1::gfat-1::cfp* median lifespan 30 days, P<0.0001) (D). Survival curves of WT animals on NGM plates supplemented with the indicated compounds. Animals were maintained on UV-killed OP50 *E. coli*. D-Arg was used as osmolarity control. (10 mM D-Arg median lifespan 18 days, 10 mM GlcNAc median lifespan 27 days, P<0.0001) (E). Average median lifespan extension observed supplementing the indicated GlcNAc concentrations to WT animals, n=3



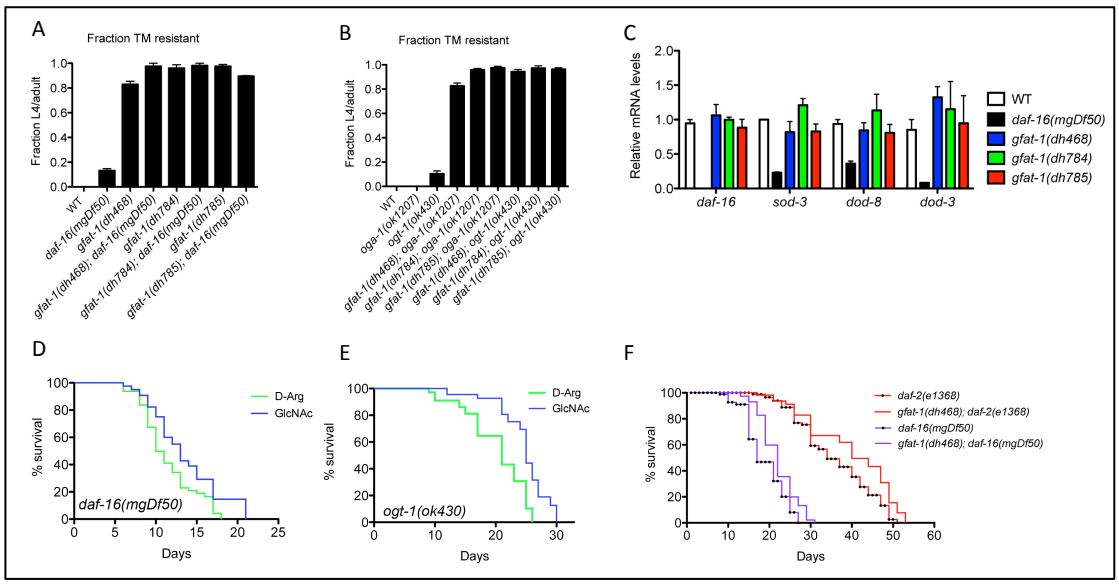
GFAT-1's ameliorating role in aging was additionally supported when we observed that overexpression of *gfat-1* in WT animals mimicked the longevity phenotype found in *gfat-1* gof mutants (Figure 4.2C). Lastly, however very interestingly, we were able to extend WT animals' lifespan by supplementing GlcNAc to the media (Figure 4.2D) even down to 1mM GlcNAc (Figure 4.2E). Hence, 25mM GlcNAc reversed the lifespan extension, but did not cause a decrease in lifespan compared to WT N2 animals. This suggests that the lifespan extension is concentration-dependent.

4.B.2 GFAT-1 gof-induced lifespan extension is independent of already known longevity pathways

Several longevity pathways in *C. elegans*, including reduced insulin/IGF signaling pathway and germline removal, depend on the FOXO transcription factor DAF-16 to maintain their long life (Kenyon C. J., 2010). DAF-16 also mediates resistance to various types of cellular stress, so we tested the role of DAF-16 in the *gfat-1* gof mutants. We found that GFAT-1 gof-induced TM resistance was independent of DAF-16 in all three *gfat-1* gof mutant alleles (data not shown). Additionally, expression of DAF-16 target genes such as *sod-3*, *dod-3* and *dod-8* were unchanged in the gof mutants (Figure 4.3A, C), at least when measured by qPCR. Notably, GlcNAc supplementation and *gfat-1* gof mutation extended the lifespan of *daf-16(mgDf50)* and *daf-2(e1368)* mutants (Figure 4.3D, F), pointing at a longevity mechanism at least partially independent from the DAF-16/FOXO and DAF-2 longevity pathways. No changes in UDP-GlcNAc levels were observed in other tested long-lived animals, including those carrying mutations in genes affecting the insulin signaling pathway (*daf-2*), gonadal outgrowth (*glp-1*) and mitochondrial function (*isp-1*), dietary intake (*eat-2)*, and protein translation (*ife-2)* (data not shown). Collectively, these results indicate that known longevity pathways do not obviously activate the hexosamine pathway, implying that GFAT-1 gof is a novel mechanism to extend lifespan in the roundworm *C. elegans*.

Figure 4.3 *gfat-1* gof mutants are long-lived, independent of the DAF-2/DAF-16 longevity pathway

(A). Developmental TM resistance assay using *gfat-1* gof and *daf-16* single and double mutants (B). Developmental TM resistance assay using *gfat-1* gof, *oga-1* and *ogt-1* single and double mutants (C). Quantitative real-time PCR measurements of DAF-16 target genes in adult animals from indicated genotypes shows no induction in *gfat-1* gof mutants (D), (E). Kaplan-Meier survival curves of *daf-16(mgDf50)* (D) and *ogt-1(ok430)* (E) treated with indicated compounds (F). Kaplan-Meier survival curves of *gfat-1* gof, *daf-2* and *daf-16* single and double mutants (G). Kaplan-Meier survival curves of *gfat-1* gof, *ogt-1* and *oga-1* single and double mutants (H). Kaplan-Meier survival curves of *gfat-1(dh468)* using indicated RNAi bacteria.



In addition to N-glycosylation, which is specifically inhibited by TM, the hexosamine pathway gives rise to precursors used for O-glycosylation. O-glycosylation was previously implicated in *C. elegans* lifespan: O-GlcNAc transferase (*ogt-1*) lof mutants are short-lived while O-GlcNAcase (*oga-1*) mutants are modestly long-lived (Rahman M.M., 2010). This suggests that enhanced O-glycosylation of client proteins is associated with longevity. However, neither *oga-1* nor *ogt-1* affected the *gfat-1* mutants' developmental TM resistance (Figure 4.3B). Moreover, GlcNAc supplementation extended the lifespan of *ogt-1* mutants (Figure 4.3E), and both the *ogt-1* and *oga-1* mutants lived longer when crossed into the *gfat-1(dh468)* mutant background (Figure 4.3F). These results indicate that O-glycosylation does not play an appreciable role in developmental TM resistance or longevity of *gfat-1* gof mutants.

HSF-1 is a key regulator of *C. elegans* stress response pathways, and we therefore looked at *hsf-1* mutants' lifespan when introduced into the *gfat-1* gof mutant background. We did not observe any differences between the lifespan of *gfat-1(dh468)* mutant animals on RNAi against *hsf-1* and *gfp* (data not shown). However, *hsf-1* RNAi made all animals very short-lived, making it hard to detect any specific changes in lifespan.

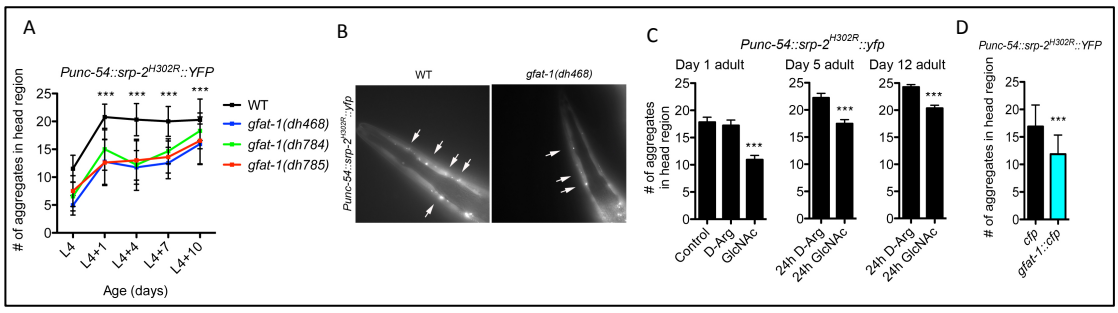
4.B.3 GFAT-1 gof improves ER protein quality control

Given that we observed resistance to TM-induced ER stress, we hypothesized that improved ER protein homeostasis might contribute to the observed lifespan extension. We therefore asked whether elevation of hexosamine pathway metabolites causes changes in the protein-folding milieu within the ER lumen. To this end, we made use of the neuroserpin homolog and folding sensor serpin 2 (SRP-2), which is fused to YFP (SRP-2^{H302R}::YFP). SRP-2 accumulates in the ER lumen due to a point mutation, and the aggregation is modulated by the heat shock response and the UPR (Schipanski A., 2013). *srp-2::yfp* is expressed in the body wall muscles due to co-expression with the *unc-54* promoter. 0 the head region as a read-out for ER degradation and/refolding functions. A time course analysis revealed an increase in SRP-2^{H302R}::YFP puncta from the L4 to adult stage and no further increase after the first day of adulthood in WT animals. At all measured time points, SRP-2^{H302R}::YFP

accumulation was significantly reduced in *gfat-1* gof mutants (Figure 4.4A) and in Figure 4.4B representative photos are shown.

Figure 4.4 Enhanced hexosamine pathway flux alleviates SRP-2^{H302R} aggregation in the ER

(A). Time course of SRP-2^{H302R}::YFP aggregation. Quantification was done in head region of ≥ 20 animals (data are mean ± s.d., representative result from n=3 experiments, ***P<0.001 vs. *gfat-1*) (B). Representative fluorescent images of *Punc-54::srp-2^{H302R}::yfp* transgenic animals crossed to the indicated strains. Head regions are shown and arrows point to SRP-2^{H302R}::YFP aggregates (C). Quantification of SRP-2^{H302R}::YFP aggregates after exposure to the indicated compounds at indicated stages of life. Supplementation with compounds was done for the 24 hours prior to quantification (n=3) (D). Quantification of SRP-2^{H302R}::YFP in *cfp* and *gfat-1::cfp* transgenic animals (representative result from n=3 experiments, t-test)

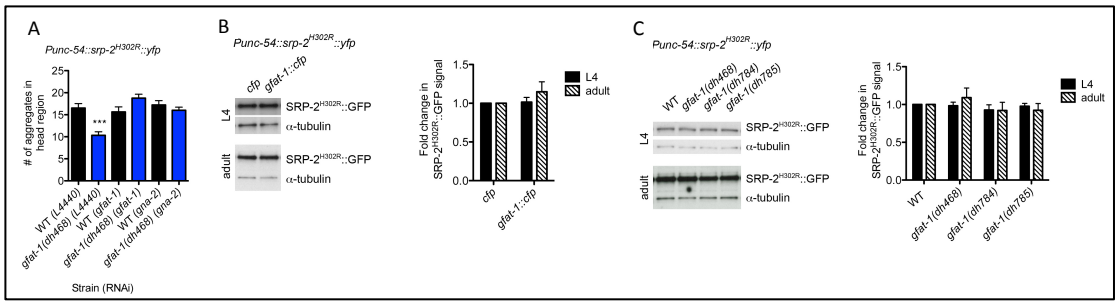


Next, we tested if GlcNAc supplementation might alleviate WT SRP-2 aggregation. Interestingly, GlcNAc treatment ameliorated protein aggregation in young adults (Figure 4.4C, left) when supplemented with GlcNAc from L1. More importantly, we were able to clear already established SRP-2^{H302R}::YFP aggregates when provided at a later time during adulthood. Remarkably, 24h exposures to GlcNAc reduced SRP-2^{H302R}::YFP puncta at various adult time points until day 12 of adulthood (Figure 4.4C). Together these data reveal that increased hexosamine pathway metabolite levels not only improve the ER's capacity to prevent accumulation of aggregated proteins, but also reverse aggregation. Lastly, we also observed reduced aggregation of SRP-2 in *gfat-1::cfp* transgenic animals (Figure 4.4D), again indicating that enhanced hexosamine pathway flux, induced genetically or pharmacologically, improve ER protein homeostasis.

To make sure that the improved prevention and removal of SRP-2 aggregates is directly linked to enhanced hexosamines, we knocked down *gfat-1* and *gna-2* in *gfat-1(dh468)* gof mutants by RNAi and quantified the number of SRP-2 aggregates. We observed a decrease in SRP-2 aggregates in *gfat-1(dh468)* gof mutant animals upon treatment with control RNAi (empty vector) compared to WT animals. Conversely, the decrease was rescued when *gfat-1* or *gna-2* were knocked down (Figure 4.5A), supporting the hypothesis that improved ER protein quality control is linked to the *gfat-1* gof mutation. Additionally, we measured the overall protein levels of SRP-2, and found no changes (Figure 4.5B, C), suggesting that reduced protein aggregation is a result of improved protein homeostasis and not a general downregulation of protein-occurrence.

Figure 4.5 GFAT-1 gof improves SRP-2^{H302R} aggregation without affecting overall SRP expression

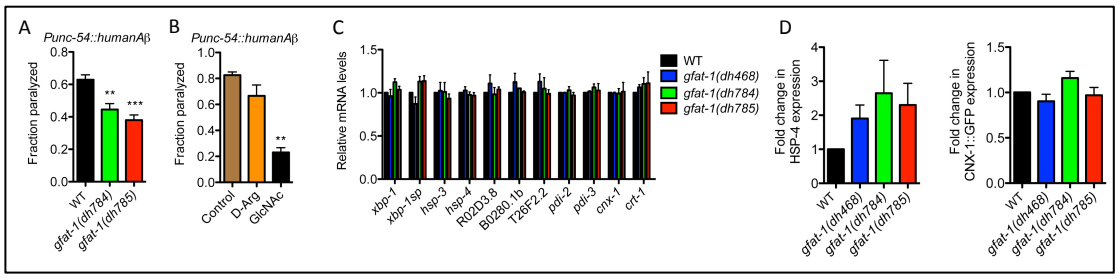
(A). Quantification of SRP-2^{H302R}::GFP aggregates in WT and *gfat-1(dh468)* animals after treatment with indicated RNAi. Knockdown of *gfat-1* or *gna-2* abolishes the suppression of SRP-2^{H302R}::GFP aggregated formation in *gfat-1(dh468)* animals (B). Western blotting of SRP-2^{H302R}::GFP and α-tubulin in *cfp* and *gfat-1::cfp* transgenic animals detected no significant differences in relative SRP-2^{H302R}::GFP expression in the transgenic lines (C). Western blots showing protein expression levels of SRP-2^{H302R}::GFP and α-tubulin in L4 and adult animals. Densitometry and quantification of signals (right) shows no difference in relative SRP-2^{H302R}::GFP levels in WT and *gfat-1* gof mutant animals.



As an additional toxic aggregation prone protein, we turned to animals expressing human ER-targeted Aβ42 peptide in the muscles (Link, 1995) (Link C.D., 2001). These animals undergo progressive muscle-paralysis during adult aging due to Aβ toxicity. When crossed with *gfat-1* gof mutants, transgenic Aβ animals showed significant improvements in motility at day 8 of adulthood (Figure 4.6A). Similarly, WT Aβ animals showed delayed paralysis after GlcNAc treatment compared to control animals that were fed supplemented with D-Arg (Figure 4.6B). These results support the idea that increased hexosamine pathway metabolites, either from GFAT-1 gof or GlcNAc supplementation, alleviate proteotoxicity in the ER.

Figure 4.6 GFAT-1 gof improves ER functions in a post-translational manner

(A). Fraction of paralyzed *Punc-54::humanAβ* transgenic animals in WT of *gfat-1* gof mutant background at day 7 of adulthood (n=4) (B). *Punc-54::humanAβ* transgenic animals were treated with indicated compounds in a bath for 6 hours daily in the presence of UV killed OP50 and otherwise maintained under standard culture conditions; fraction paralyzed worms were quantified at day 7 of adulthood. (n=3 **P<0.01 vs. D-Arg) (C). Relative mRNA levels of indicated UPR genes in WT animals and *gfat-1* mutants (n=3) (D). Quantified protein levels of HSP-4 (left) and CNX-1::GFP (right) determined by Western blotting (n=4 (left) and n=3 (right)).

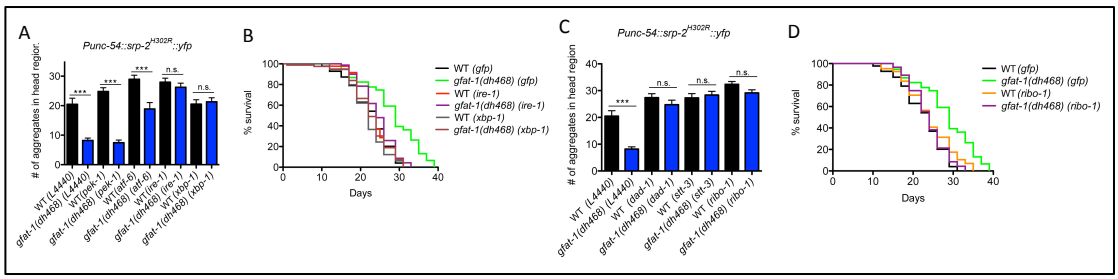


Conceivably, GFAT-1 gof leads to increased aggregation removal, prevention of aggregation and improved motility by upregulating one or several of the ER stress response pathways. Unexpectedly, however, we did not observe any changes in the transcriptional regulation of known UPR target genes by qPCR (Figure 4.6C), including *hsp-4*, spliced *xbp-1*, and target genes of the ATF-6 and PEK-1 UPR branches (Shen, 2005). HSP-4 protein levels were slightly increased without reaching statistical significance, and CNX-1 protein levels were unchanged (Figure 4.6D), suggesting that ER UPR is not upregulated in the *gfat-1* gof mutants.

To further explore ER functions in *gfat-1* gof mutant animals we investigated the effects of inhibiting ER UPR components and proteins imvolved in N-glycosylation in the aggregation dynamics of SRP-2^{H302R}::YFP. Downregulation of the ER UPR pathway using RNAi against *ire-1* and *xbp-1* increased SRP-2^{H302R}::YFP aggregates in *gfat-1* gof mutants (Figure 4.7A). Knockdown of *atf-6* only partly suppressed the beneficial effects from the *gfat-1* gof mutation, and *pek-1* knockdown had no effect. Correlatively, GFAT-1 gof-induced longevity was completely dependent on the *ire-1* and *xbp-1* (Figure 4.7B). Together, these results suggest that specific branches of the ER UPR pathway co-assist the improved protein homeostasis and longevity of the *gfat-1* gof mutants. So even though we did not observe any changes at the transcriptional level, these results suggest that a functional ER UPR is required but not activated in *gfat-1* gof mutant animals.

Figure 4.7 Improved ER functions in *gfat-1* gof mutants are dependent on ER-UPR and N-glycosylation components

(A). SRP-2^{H302R}::YFP aggregate count in WT and *gfat-1(dh468)* mutants after RNAi-mediated knockdown of indicated UPR genes (n=3) (B). Representative survival demography of WT and *gfat-1(dh468)* animals treated with indicated RNAi targeting UPR genes. (WT(*gfp*) median lifespan 24 days, *gfat-1(dh468)*(*gfp*) median lifespan 29 days, P<0.0001; WT(*ire-1*) median lifespan 22 days, *gfat-1(dh468)*(*ire-1*) median lifespan 24 days, P<0.005 vs. *gfat-1(dh468)*(*gfp*); WT(*xbp-1*) median lifespan 22 days, *gfat-1(dh468)*(*xbp-1*) median lifespan 22 days, P<0.001 vs. *gfat-1(dh468)*(*gfp*)) (C). SRP-2^{H302R}::YFP aggregate quantification in WT and *gfat-1(dh468)* animals treated with RNAi targeting indicated oligosaccharyltransferase (OST) complex genes (n=3) (D). Representative lifespan analysis of WT and *gfat-1(dh468)* animals treated with RNAi targeting the OST complex gene *ribo-1* (WT(*gfp*) median lifespan 24 days, *gfat-1(dh468)*(*gfp*) median lifespan 29 days, $p < 0.0001$; WT(*ribo-1*) median lifespan 24 days, *gfat-1(dh468)*(*ribo-1*) median lifespan 24 days, $p < 0.0013$ vs. *gfat-1(dh468)*(*gfp*))

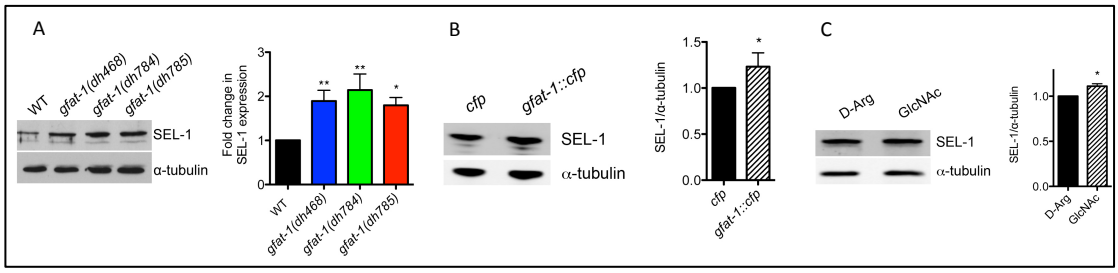


Another pathway required for the *gfat-1* gof mutants, is the N-glycosylation pathway. Preventing N-glycosylation by inhibiting components of the oligosaccharyltransferase (OST) complex suppressed the reduction of SRP-2^{H302R}::YFP puncta in *gfat-1* gof mutants. Additionally, the lifespan extension was dependent on the OST complex (Figure 4.7C, D). These data suggest that N-glycosylation is required for improved protein homeostasis and longevity in *gfat-1* gof mutants.

We were surprised to find no evidence for induction of the ER UPR or the N-glycosylation pathways, and thus, we hypothesized that ERAD activity might be induced. ERAD client proteins are retro-translocated from the ER lumen to the cytosol before proteasomal degradation. The SEL-11 ubiquitin ligase complex controls this translocation and ubiquitin-dependent degradation process (Bordallo et al., 1998) in conjunction with the ER membrane glycoprotein SEL-1 that serves as a cofactor (Hampton et al., 1996). Interestingly, we found a 2-fold increase in SEL-1 protein expression in all three *gfat-1* gof mutants using Western blot analysis of worm lysates (Figure 4.8A). Similarly, we detected increased levels of SEL-1 protein expression in *gfat-1::cfp* transgenic animals and in WT animals supplemented with GlcNAc (Figure 4.8B, C). Together, this points at evidence that enhanced hexosamine pathway metabolites increase the expression of SEL-1 protein levels, and suggests a possible longevity-mechanism.

Figure 4.8 Enhanced flux through the hexosamine pathway increases the expression of SEL-1

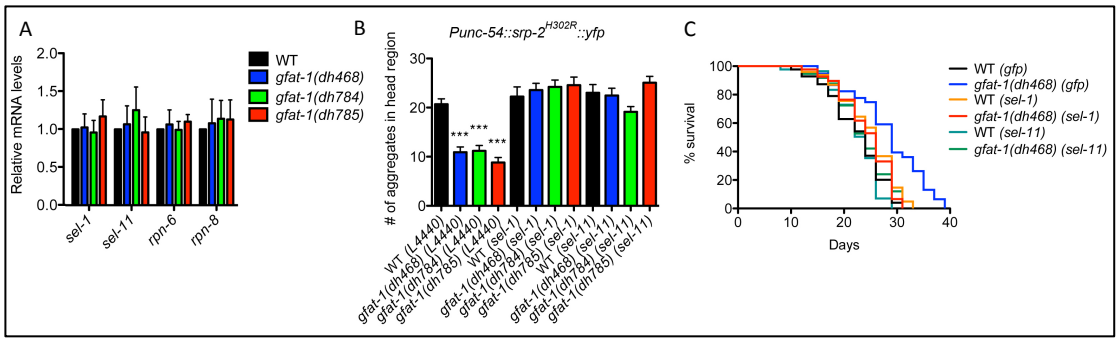
(A). Representative Western blot from L4 WT and *gfat-1* gof mutant animals detecting SEL-1 and α-tubulin (left) and analysis of SEL-1 Western blots from n=3 independent experiments. SEL-1 levels were normalized to α-tubulin (right) (B). Representative Western blot from L4 *cfp* and *gfat-1::cfp* transgenic animals detecting SEL-1 and α-tubulin (left) and analysis of SEL-1 Western blots from n=3 independent experiments. SEL-1 levels were normalized to α-tubulin (n=4, t-test) (right) (C). Representative Western blot from L4 WT animals supplemented with indicated compounds detecting SEL-1 and α-tubulin (left) and analysis of SEL-1 Western blots from n=3 independent experiments. SEL-1 levels were normalized to α-tubulin (n=7, t-test) (right)



We did not detect any transcriptional changes of *sel-1* or *sel-11* (Figure 4.9A), again suggesting that the increased expression level of SEL-1 stems from post-translational modifications. We next tested the role of ERAD in SRP-2^{H302R}::YFP aggregation and lifespan extension of *gfat-1* gof mutants. RNAi experiments identified a strict requirement for *sel-1* and *sel-11*, as knockdown suppressed the *gfat-1* mutants' alleviated ER luminal protein aggregation (Figure 4.9B). Additionally, the longevity of the *gfat-1* gof mutants was completely abolished upon knockdown of *sel-1* or *sel-11*, again suggesting a strict requirement of ERAD (Figure 4.9C).

Figure 4.9 ERAD is required for GFAT-1 gof-induced longevity

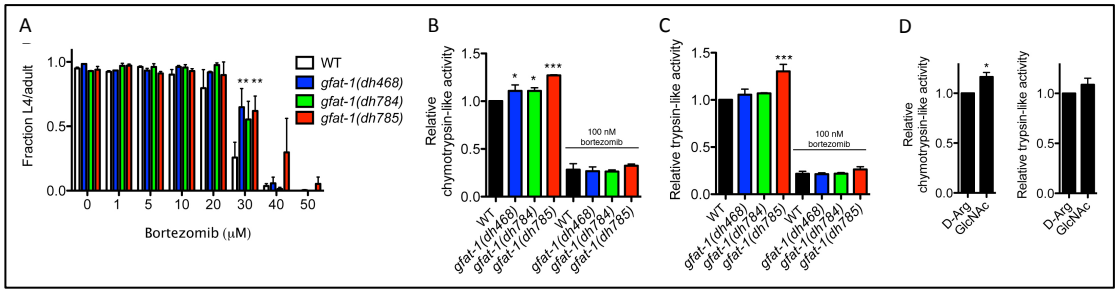
(A). Quantitative real-time PCR measurements of ERAD genes in L4 animals from indicated genotypes (B). SRP-2^{H302R}::YFP aggregation in WT and *gfat-1* gof mutants treated with RNAi targeting ERAD genes *sel-1* and *sel-11* (n=3) (C). Lifespan analysis of WT and *gfat-1(dh468)* animals treated with indicated RNAi targeting ERAD gene expression (WT(*gfp*) median lifespan 24 days, *gfat-1(dh468)*(*gfp*) median lifespan 29 days, P<0.0001; WT(*sel-1*) median lifespan 22 days, *gfat-1(dh468)*(*sel-1*) median lifespan 22 days, P<0.005 vs. *gfat-1(dh468)*(*gfp*); WT(*sel-11*) median lifespan 24 days, *gfat-1(dh468)*(*sel-11*) median lifespan 24 days, P<0.005 vs. *gfat-1(dh468)*(*gfp*))



When luminal aggregated proteins are translocated to the cytoplasm, they will be ubiquitinated and broken down by the proteasome. We were unable to detect any transcriptional changes in *rpn-6*, which is an essential subunit for the activity of the 26S/30S proteasome, and in *rpn-8* that also serves as a proteasome regulatory particle (Figure 4.9A). However, since we had observed an apparent involvement of the ERAD machinery, we speculated whether GFAT-1 gof also led to resistance to bortezomib, which is a proteasome inhibitor. Indeed this was the case; we found that all *gfat-1* gof mutants exhibited increased resistance to 30μM bortezomib (Figure 4.10A).

Figure 4.10 Increased hexosamine pathway metabolites improve protein degradation by the proteasome

(A). Developmental bortezomib resistance assay with indicated genotypes. *gfat-1* gof alleles show significantly increased fraction of developed animals compared to WT at 30 μM bortezomib (n=3) (B). Relative chymotrypsin-like proteasome activity assay measuring LLVY-AMC turnover at 25°C in lysates of *gfat-1* gof mutant L4 larvae. The proteasome inhibitor bortezomib served as a negative control (n=4) (C). Relative trypsin-like proteasome activity assay measuring LLVY-AMC turnover at 25°C in lysates of *gfat-1* gof mutant L4 larvae. The proteasome inhibitor bortezomib served as a negative control (n=3) (D). Relative chymotrypsin-like (left) and trypsin-like (right) proteasome activity assays measuring LLVY-AMC turnover at 25°C in lysates of WT L4 larvae exposed to indicated compound. The proteasome inhibitor bortezomib served as a negative control (n=3)



Bortezomib inhibits the 26S proteasome, suggesting that *gfat-1* gof mutants are able to overcome this inhibition by either directly increasing the activity or indirectly by enhancing degradation of proteins in for example lysosomes. To test this, we measured the activity of the proteasome, and observed a mild, yet significant, increase in chymotrypsin-like proteasome activity, as measured by turnover of an LLVY-tagged fluorescent substrate, in all *gfat-1* gof alleles (Figure 10B). Additionally, *gfat-1(dh785)* showed a significant increase in trypsin-like activity (Figure 10C). Lastly, we supplemented WT animals with GlcNAc and measured their proteasome activities; supplementation stimulated both the chymotrypsin-like and the trypsin-like proteasome activities in WT animals, although the trypsin-like activity did not reach significance (Figure 10D). Together, this suggests that enhanced hexosamine pathway metabolites increase degradation of proteins by the proteasome, and that it is, at least partly, allele-specific.

Knockdown of *rpn-6* and *rpn-8* using RNAi, abrogated the lifespan extension of *gfat-1* gof mutants, however, it also shortened WT lifespan, and it is therefore impossible to say anything about the specificity and importance of *rpn-6* and *rpn-8* in GFAT-1 gof longevity. Altogether, increased expression of SEL-1 and enhanced proteasome activity suggest that improved ER protein homeostasis in *gfat-1* gof mutants is linked to longevity and enhanced proteolytic capacity.

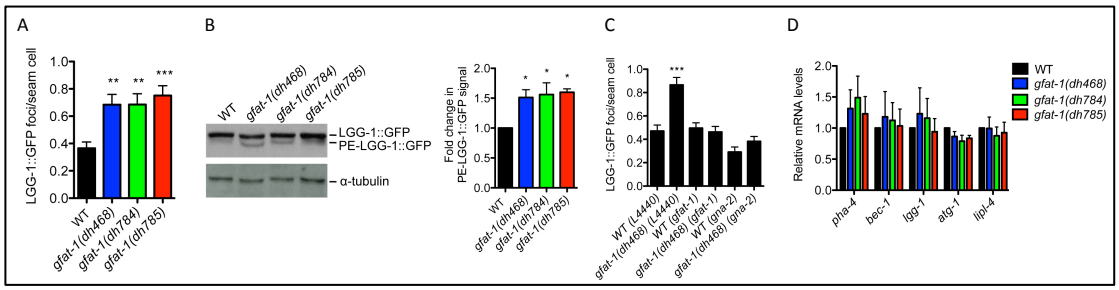
4.B.4 GFAT-1 gof enhances autophagy

Autophagy has been suggested to be involved in protein quality control (Menzies F.M., 2011), and indeed also in longevity. Autophagy is, however, not an ER specific degradation pathway, but we speculated that it might also be affected, since a recent study has indicated that the hexosamine metabolite, glucosamine, induces autophagic events in cell cultures (Shintani T., 2010). When autophagosomes are formed, the nematode Microtubule-associated protein light chain 3 (LC3) homologue LGG-1 condenses into puncta. The number of puncta is reflecting the activity of autophagy, or at least the presence of formed autophagosomes (Melendez, 2003). We used a transgenic *C. elegans* strain that has *lgg-1* fused to *gfp*, and quantified the number of formed puncta (foci) in epidermal seam cells of young

animals. Interestingly, we found a 60-70% increase of LGG-1::GFP foci formation in *gfat-1* gof mutants relative to WT animals (Figure 11A). Consistently, this increase was confirmed by WB where we detected an increase in the lipidated form of LGG-1::GFP in all *gfat-1* gof mutant alleles (Figure 11B). This enhancement was indeed due to increased flux through the hexosamine pathway since a knockdown of *gfat-1* or *gna-2* by RNAi reduced the increased puncta formation (Figure 11C). These results suggest that GFAT-1 gof induces autophagy, and that the increase in autophagic events is due to increased hexosamines. However, no significant changes in mRNA expression of known autophagy genes were observed, indicating that autophagy is induced post-transcriptionally (Figure 11D).

Figure 4.11 GFAT-1 gof enhances the level of autophagy

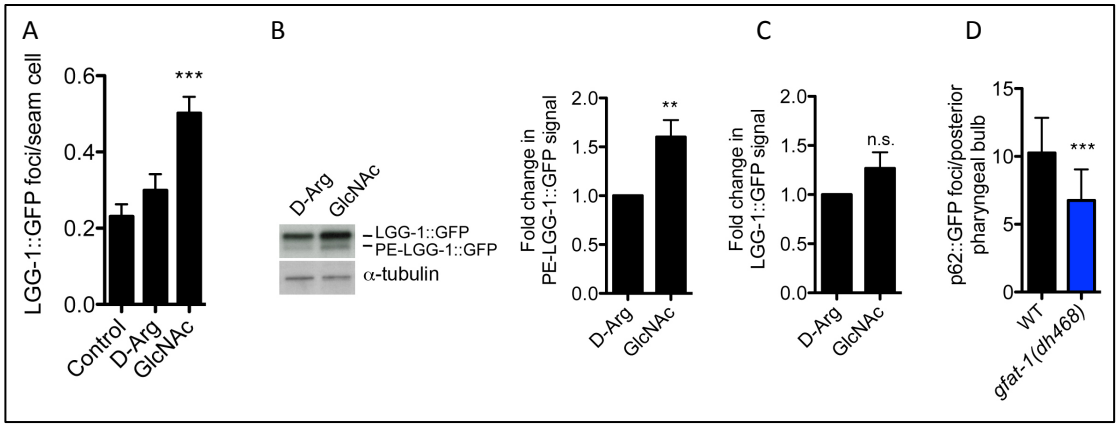
(A). Average number of LGG-1:GFP foci per seam cell of L3 larvae in indicated genotypes shows elevated autophagosome formation in *gfat-1* gof mutants, **P<0.001, ***P<0.001 vs. WT (B). Representative (left) and analysis (right) of western blot using anti-GFP antibodies to detect LGG-1::GFP fusion protein in L4 animals, PE-LGG-1::GFP levels were normalized to α-tubulin (n=6) (C). Quantification of LGG-1::GFP puncta in WT and *gfat-1(dh468)* animals after knockdown of *gfat-1* or *gna-2* (n=3) (D). Quantitative real-time PCR measurements of autophagy genes in L4 animals from indicated genotypes (n=5)



Conceivably, autophagy is enhanced in the *gfat-1* gof mutants due to increased flux through the hexosamine pathway leading to a higher level of hexosamines. To confirm this idea, we supplemented WT animals with GlcNAc and measured the level of autophagy. Consistent with earlier results, we found the number of LGG-1::GFP puncta increased in WT animals supplemented with GlcNAc compared to control animals supplemented with M9 or D-Arg as a control for osmolality (Figure 4.11A). The same was observed when we quantified LGG-1::GFP by WB, without changing the overall GFP expression significantly (Figure 4.11B, C).

Figure 4.12 GlcNAc induces autophagic degradation

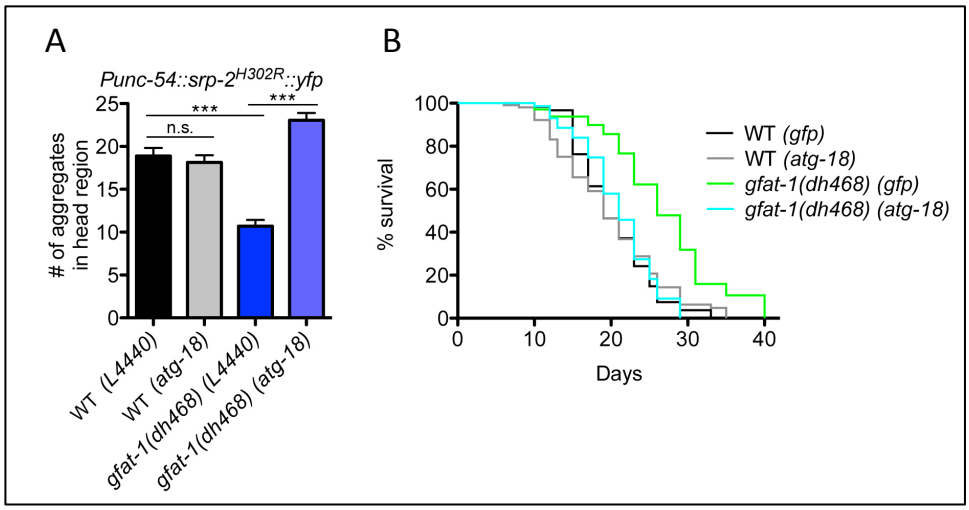
(A). Quantification of LGG-1::GFP puncta in seam cells of L3 WT animals after indicated treatment (n=3) (B). Representative western blot (left) and analysis (right) of western blot using anti-GFP antibodies to detect LGG-1::GFP fusion protein in L4 WT animals after indicated treatment (C). Analysis of LGG-1::GFP Western blots as in (B) from n=4 experiments. LGG-1::GFP levels were normalized to α-tubulin (D). Average number of p62::GFP foci per posterior pharyngeal bulb of L4 larvae in ≥ 15 WT and *gfat-1(dh468)* animals (t-test, n=3)



We interpreted increased levels of LGG-1::GFP foci as enhanced levels of autophagy, but to be sure that increased LGG-1::GFP expression is a measure for enhanced activation and increased degradation, we quantified the turnover of p62. p62 is also called sequestosome 1, and is an ubiquitin-binding scaffold protein and a physiological substrate selectively removed by autophagy (Tian Y., 2010). We observed a reduction in p62::GFP foci in the posterior pharyngeal bulb when the transgene was expressed in the *gfat-1(dh468)* gof mutant background (Figure 4.11D) compared to WT controls. This result supports the previous presented data and all together, the findings suggest that enhanced hexosamines, as a result of genetically or pharmacologically modifications, increase the activity and turnover of proteins by autophagy.

Figure 4.13 *gfat-1* gof mutants are dependent on autophagy to remain protected against SRP-2^{H302R} aggregation and to sustain longevity.

(A). Quantification of SRP-2^{H302R}::YFP aggregates in L4 animals after developmental exposure to *atg-18* RNAi or L4440 empty vector control (n=3) (B) Kaplan-Meier survival curves of N2 and *gfat-1(dh468)* animals exposed to indicated RNAi from young adulthood (WT(*gfp*) median lifespan 19 days, *gfat-1(dh468)*(*gfp*) median lifespan 26 days, P<0.0001; WT(*atg-18*) median lifespan 19 days, *gfat-1(dh468)*(*atg-18*) median lifespan 21 days, P<0.0001 vs. *gfat-1(dh468)*(*gfp*))

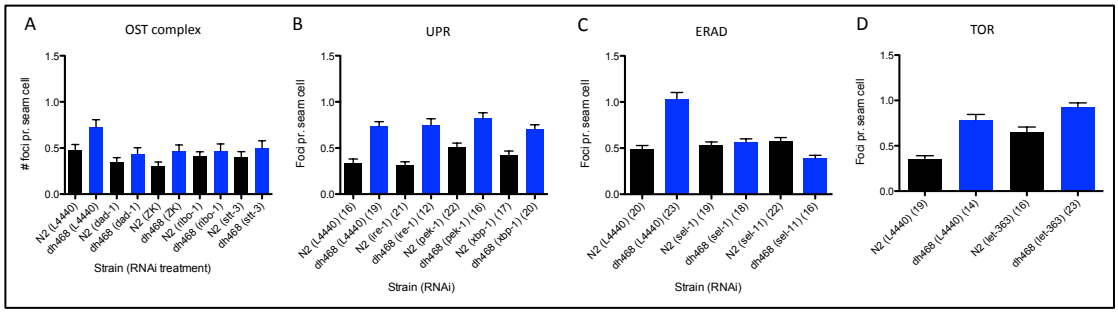


Since autophagy was induced as a result of increased hexosamine pathway flux, we tested if improved ER protein homeostasis and lifespan extension of *gfat-1* gof mutants depend on autophagy. Interestingly, we found that prevented SRP-2 protein aggregation in *gfat-1* gof mutants was abolished by RNAi mediated knockdown of *atg-18*, which is an autophagy-specific and essential gene (Figure 4.12A). WT SRP-2 aggregation levels did not change significantly, suggesting a specific dependence of autophagy in *gfat-1* gof mutants. Furthermore, *atg-18* RNAi shortened the lifespan of *gfat-1* gof mutants without having an effect on adult WT controls (Figure 4.12B). Taken together, *gfat-1* gof mutations induce autophagy, which supports ER protein homeostasis and lifespan extension.

Conceivably, protein degradation by autophagy contributes to a cleaner milieu in the ER lumen, and as shown in Figure 4.13, autophagy is additionally required for GFAT-1 gof longevity. To test different possibilities for the upregulated activity of autophagy, we knocked down components of the N-glycosylation pathway (Figure 4.14A), the ER UPR (Figure 4.14B), ERAD (Figure 4.14C) and in addition, TOR (target of rapamycin) (*let-363*) (Figure 4.14D).

Figure 4.14 GFAT-1 gof-induced autophagy increase is dependent on N-glycosylation components and ERAD

(A, B, C, D). Quantification of LGG-1::GFP puncta in seam cells of L3 WT and *gfat-1* gof mutant animals after inhibition of components of the OST complex (A), the UPR pathway (B), the ERAD pathway (C) and TOR (D)



When we impaired N-glycosylation in *gfat-1* gof mutants by knocking down components of the OST complex, we observed a decrease in LGG-1::GFP puncta, suggesting that the autophagy-induction is dependent on N-glycosylation. This was not a general effect, as WT levels were not significantly changed upon knockdown of OST complex components (Figure 4.14A). In contrast, we did not find any changes in the formation of LGG-1::GFP foci in the *gfat-1* gof mutants when we knocked down components of the ER UPR pathways. This suggests that the upregulation of autophagy is independent on ER UPR (Figure 4.14B). When we, on the other hand, knocked down components of the ERAD pathway, we completely abrogated the increased number of LGG-1::GFP foci, indicating a strict dependence of ERAD (Figure 4.14C). Lastly, we tested the interplay with TOR, which is known to inhibit autophagy; knockdown of *tor* (*let-363*) increases autophagy, which for example is observed in DR animals (Malene Hansen, 2008). When we knocked down *tor* in *gfat-1* gof mutants, we did observe a modest increase in the number of LGG-1::GFP puncta, suggesting that GFAT-1 gof, at least partly, enhances the activity of autophagy in a TOR independent manner (Figure 4.14D). Together, these results indicate that several protein quality control mechanisms are affecting each other. Additionally, GFAT-1 gof-induced autophagy is independent of the ER UPR and of the TOR-mediated upregulation of autophagy.

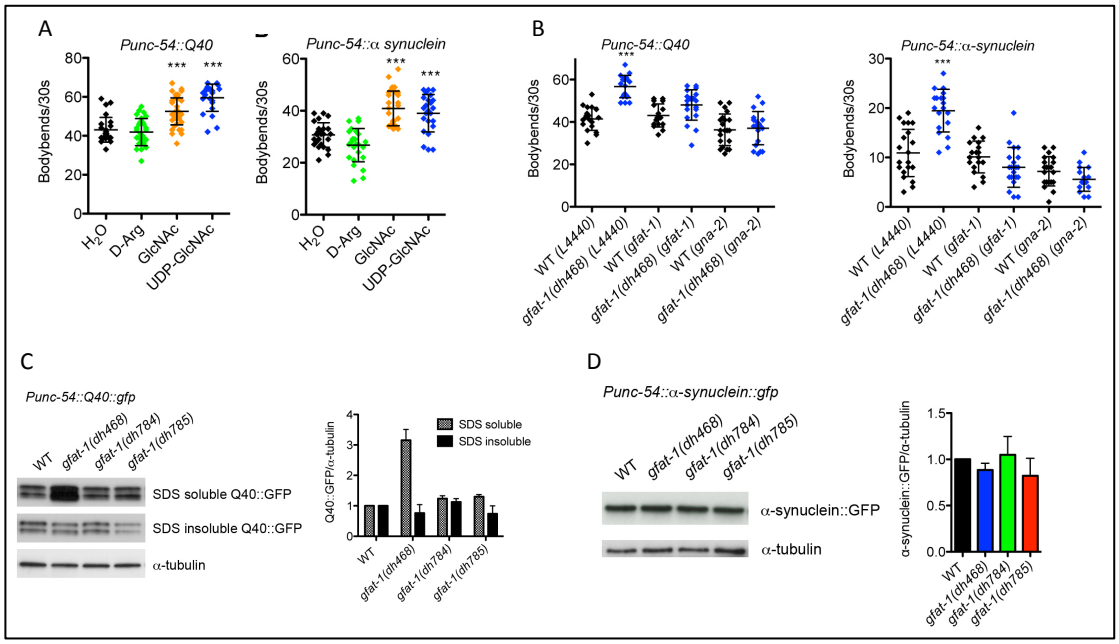
4.B.5 Increased hexosamine pathway metabolites alleviates proteotoxicity

Protein quality control mechanisms are not only important for ER protein homeostasis, but are comprehensive mechanisms essential for cellular maintenance, functions and survival. Since we had observed increased degradation by autophagy and elevated proteasome activity in *gfat-1* gof mutants and in WT animals supplemented with GlcNAc, we wondered whether these changes might go beyond the ER. To test this, we made use of additional protein aggregation models, including polyglutamine (polyQ40) repeats and α-synuclein, which are both cytosolic aggregation prone protein models. PolyQ repeats are found in aggregation-prone proteins such as Huntingtin, implicated in Huntington's disease, whereas α-synuclein is associated with Parkinson's and Alzheimer's disease. Because polyQ40 and α-synuclein transgenic animals express the ectopic toxic protein species in muscle cells

(under control of the *unc-54* promoter), they have enhanced muscle paralysis starting from day 6 of adulthood. This allows us to use motility as readout for toxicity, by counting the number of bodybends in liquid.

Figure 4.15 GFAT-1 gof alleviates proteotoxicity

(A). Motility assays using 7 days old animals with *Punc-54* driven muscle specific expression of (left) polyQ40-YFP fusion protein, and (right) α-synuclein-GFP after treatment with indicated compounds (B). Motility assay using *Punc-54::polyQ40* (left) and *Punc-54::α-synuclein* (right) transgenic animals at day 7 of adulthood after exposure to indicated RNAi starting from day 1 of adulthood (C). Representative (left) and analysis (right) of GFP Western blots of SDS soluble and SDS insoluble material in *Punc-54::Q40::gfp* transgenic animals with the indicated genotypes (D). Representative (left) and analysis (right) of GFP Western blots of SDS soluble and SDS insoluble material in *Punc-54::α-synuclein::gfp* transgenic animals with the indicated genotypes

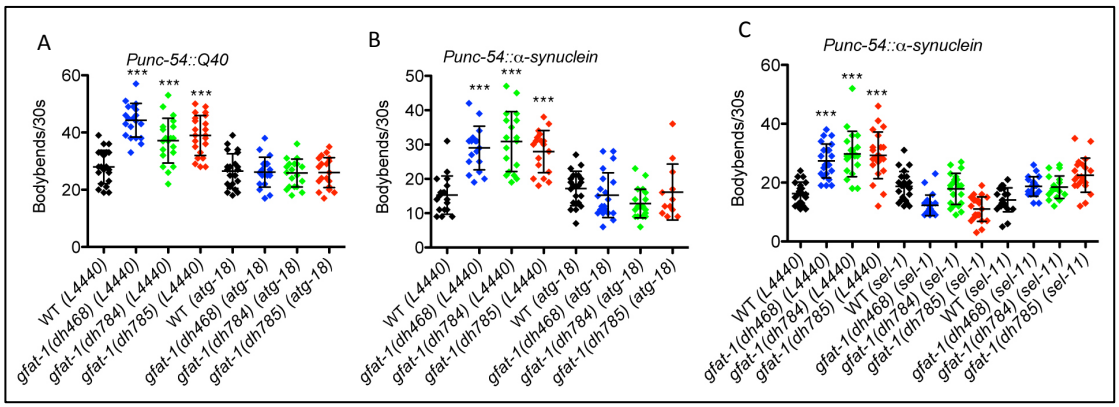


Interestingly, we found significant motor function improvements in both proteotoxic disease models upon a 6-day treatment initiated from young adulthood. We treated the animals for 6 hours per day with 10 mM GlcNAc or 10 mM UDP-GlcNAc supplemented in liquid (Figure 4.15A). Consistently, we observed increased motility in both aggregation prone protein models when they were crossed into the *gfat-1* gof mutant background (Figure 4.15B). The hexosamine pathway specificity was confirmed by RNAi knockdown experiments targeting *gfat-1* or *gna-2*; we rescued the motility improvement in *gfat-1* gof mutants upon inhibition of *gfat-1* or *gna-2* without changing the WT motor function (Figure 4.15B). These results suggest an extensive role of the hexosamine pathway in protection from proteotoxicity. Overall abundance of Q40::GFP and α-synuclein::GFP was not reduced in the *gfat-1* gof mutants (Figure 4.15C, D), suggesting that improved motility is due to amelioration of proteotoxicity and not an unspecific decrease in protein occurrence.

Conceivably, GFAT-1 gof improves motility due to improved protein quality control mechanisms. To test this hypothesis, we knocked down *atg-18*, *sel-1* and *sel-11* in *gfat-1* gof mutants and measured their proteotoxicity, again using motility as readout.

Figure 4.16 GFAT-1 gof alleviates proteotoxicity in manner dependent on autophagy and ERAD

(A, B, C). Motility assays using 7 days old animals with *Punc-54* driven muscle specific expression of (A) polyQ40-YFP fusion protein, and (B, C) α-synuclein-GFP. Genotypes and RNAi treatment are indicated



Surprisingly, however interestingly, we found that both autophagy and ERAD are required for GFAT-1 gof-improved motility. Knockdown of *atg-18, sel-1* or *sel-11* completely abolished the amended bodybends in both polyQ40 and α-synuclein disease models in all three *gfat-1* gof alleles (Figure 4.16A, B, C). Together, these data show that hexosamine pathway metabolites induce a broad-spectrum protection against proteotoxicity in various cellular compartments.

4.B.6 GFAT-1 gof does not change the transcriptional output of the ER quality control mechanisms

From the presented data, we find a clear enhancement of ER protein quality control mechanisms and autophagy, and our results also suggest that they are required for the *gfat-1* gof mutant's lifespan extension. We were however not able to detect any changes in mRNA levels of these genes, and performed RNA sequencing to reveal possible changes in the transcriptome. Very surprisingly, expression levels in the *gfat-1* gof mutants of genes involved in any of the studied pathways were unchanged compared to WT controls. In general, only a small number of genes showed significant expression changes. Among this group, it was noteworthy that *pmk-1*, *pmk-2*, and *pmk-3*, orthologues of the human p38 mitogen-activated protein kinase (MAPK) showed significant up-regulation. In worms, the MAPK pathway has been found to play a role in stress resistance via the transcription factor SKN-1 (Inoue H, 2005) that plays a role in oxidative stress resistance and xenobiotic detoxification. Additionally, latest research point at the MAPK pathway to play a role in SKN-1 and DAF-2 longevity mediated pathways (Okuyama T, 2010).

4.C. Discussion

In this chapter, I show results indicating that enhanced levels of hexosamine pathway metabolites either by gof mutations in *gfat-1*, by enhancing the copy number of WT *gfat-1* or by supplementing WT animals with GlcNAc extend lifespan and alleviates proteotoxicity of various toxic aggregation prone proteins, which leads to improved motility. Our results also suggest that the protein degradation is enhanced by elevated levels of SEL-1, higher activity of the proteasome and by increased formation of autophagosomes. Lastly, the GFAT-1-induced longevity was dependent on these pathways and our results clearly point towards a global improvement in protein quality control.

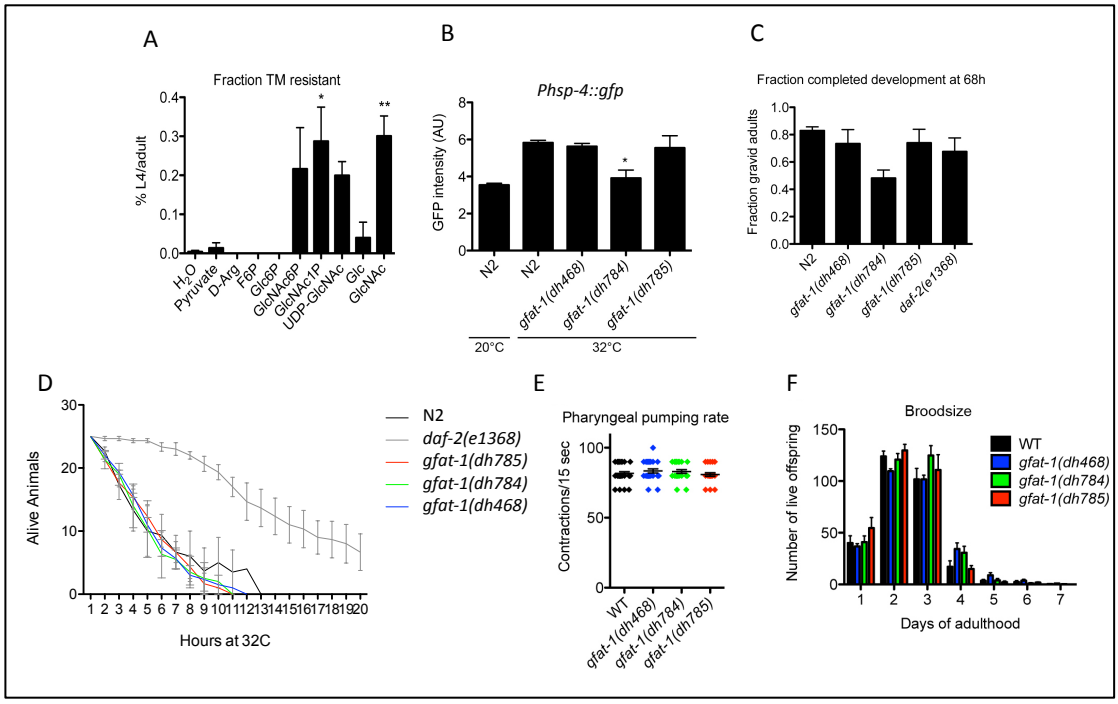
The data indicate that the improved protein homeostasis stems largely from post-transcriptional modifications leading to enhancement of ERAD, proteasome activity as well as autophagy, as we found no transcriptional changes in genes of these pathways. We furthermore failed to observe any transcriptional changes in stress response genes of for example the mitochondrial heat shock response, again suggesting that the observed improvements are not due to transcriptional changes. We did however observe transcriptional changes in genes regulating the MAPK pathway in *C. elegans*, which is involved in stress resistance and longevity modulated by SKN-1 and DAF-16. Lifespan studies using mutants with inhibited DAF-16 did conversely indicate that GFAT-1 gof-mediated longevity is, at least partly, independent of DAF-16.

GFAT-1-induced longevity seems to be a novel mechanism to prolong life, as elevated levels of UDP-GlcNAc were not detected in several other long-lived *C. elegans* mutants, including mutants with reduced insulin signaling, decreased mitochondrial activity, inhibited gonadal signaling or dietary restriction. Earlier studies have suggested that O-glycosylation plays a role in *daf-2* longevity, and that inhibition of *oga-1* (O-GlcNAcase) or *ogt-1* (O-GlcNAc transferase) extend and shortens *C. elegans* lifespan, respectively. We did not observe any changes, different from WTs, in *gfat-1* gof mutants when modulating these genes. Additionally, we were able to extend the lifespan of these mutants by supplementing them with GlcNAc, suggesting that GFAT-1 induced longevity is independent of the O-glycosylation dependent lifespan modulations.

Together, these data suggest a novel mechanism to extend *C. elegans* lifespan that presumably is a result of post-transcriptional modifications of proteins involved in cellular protein quality control mechanisms.

See chapter 9 for a more detailed discussion about GFAT-1.

Supplemental Figure 1. Additional Characterization of the *gfat-1* gof mutants (A). Fraction animals that completed development on TM plates after treatment with indicated compound (B). GFP intensity of *Phsp-4::gfp* transgenic animals after heat exposure. Genotypes are indicated under the bars (C). Developmental timing detected in *gfat-1* gof mutants (D). Heat stress resistance assay using gfat-1 gof mutants, estimating the survival at 32C (E). Pumping rate mesured in young adults in indicated *gfat-1* gof mutants (F). Number of live offspring at different days in indicated *gfat-1* gof mutants



CHAPTER 5. Increased *sel-1* Expression is Sufficient to Extend Life

5.A. Introduction

In the presented data, and in Denzel, Storm et al., we showed that GFAT-1 gof increases the protein expression level of the ERAD component, SEL-1. Additionally, we find that *sel-1* is required for GFAT-1 gof-induced longevity and for ameliorated clearance of aggregation prone proteins. We also show that GFAT-1 gof and supplementation with GlcNAc increase the activity of the proteasome, and from earlier published work, we learn that altered components of the ERAD pathway are involved in neurodegenerative disorders (Giuliana Saltini, 2006), which conceivably stems from a decline in protein homeostasis caused by inhibited degradation of proteins.

Together, these results suggest an important role for ERAD in modulation of longevity.

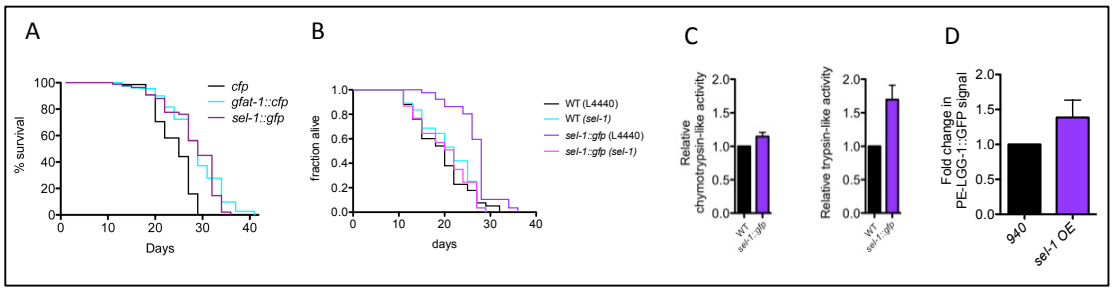
5.B. Results

5.B.1 Enhanced ER protein quality control is sufficient to promote longevity and enhance proteasome activity

To confirm the specificity of the *sel-1* dependency in *gfat-1* gof mutant animals, and to test whether SEL-1 was not only dependent, but also sufficient to extend lifespan, we created a transgenic *C. elegans* strain overexpressing *sel-1* (*sel-1::gfp*). Interestingly, the strain showed a remarkable longevity phenotype, comparable to the longevity phenotype induced by GFAT-1 gof (Figure 5.1A). To ensure that the extended lifespan of *sel-1::gfp* transgenic animals did not arise from a random side effect, we knocked down *sel-1* using RNAi. Inhibition of *sel-1* did not affect WT lifespan significantly during adulthood, but abolished the lifespan extension in *sel-1::gfp* transgenic animals (Figure 5.1B). This suggests that the prolonged life observed as a consequence of GFAT-1 gof might be specific to SEL-1 overexpression, as *sel-1* overexpression was sufficient to extend WT lifespan.

Figure 5.1 *sel-1* over expression is sufficient to extend lifespan and enhance autophagy

(A). Representative Kaplan-Meier survival curves of indicated transgenic animals (B). Representatve Kaplan-Meier survival curves of indicated animals with indicated RNAi treatment (C). Relative chymotrypsin-like (left) and trypsin-like (right) proteasome activity assays measuring LLVY-AMC turnover at 25°C in lysates of WT and *sel-1::gfp* transgenic L4 larvae (n=3) (D). Analysis of western blot using anti-GFP antibodies to detect LGG-1::GFP fusion protein in L4 animals, PE-LGG-1::GFP levels were normalized to α-tubulin



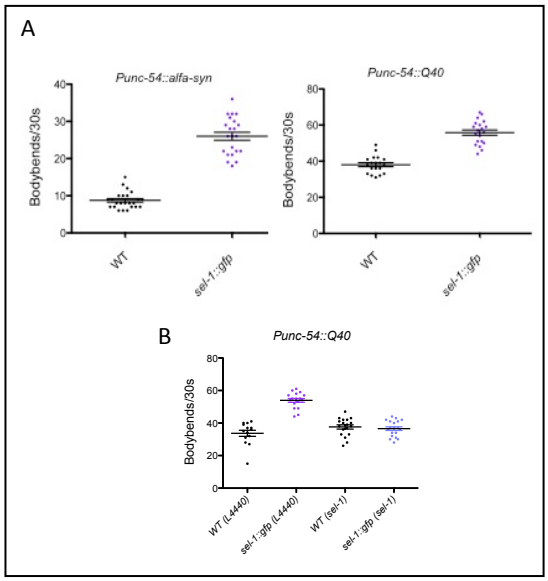
As stated earlier, SEL-1 is a membrane complex involved in retro-translocation of unfolded proteins from the ER lumen to the cytosol, where the proteins after ubiquitination are substrates of the proteasome. We therefore speculated whether proteasome activities would be increased in *sel-1::gfp* transgenic animals. Indeed, we found both the chymotrypsin- and the trypsin-like activities enhanced in *sel-1::gfp* transgenic animals (Figure 5.1C), suggesting that protein degradation, due to enhanced ERAD, is increased. Earlier results (Figure 4.14) indicated that enhanced autophagy in *gfat-1* gof mutants was dependent on ERAD, *sel-1* and *sel-11* specifically. We therefore asked whether *sel-1::gfp* transgenic animals also had elevated levels of autophagy. Surprisingly, we observed increased levels of lipidated LGG-1::GFP in *sel-1::gfp* animals compared to WT control animals and to *dh940*, which carries only the promoter of *gfat-1* fused to *cfp* (Figure 5.1D). We used this strain as a control for transgenic introduction to WT animals. This suggests a direct relationship between ERAD and autophagy, and all together we indicate that ERAD not only is important for the *gfat-1* gof mutants, but also is sufficient to induce autophagy and promote long life.

5.B.2 sel-1 overexpression improves motility of C. elegans protein aggregation models

In Denzel, Storm et al., we have reported that both ERAD and autophagy are essential for GFAT-1 gof-induced motility improvements using the aggregation prone protein disease models, α-synuclein and polyQ40. Conceivably, ERAD and autophagy are collaborating on removing aggregated toxic proteins in these models, and in this way slowing muscle paralysis.

Figure 5.2 *sel-1* over expression is sufficient to alleviate proteotoxicity

(A). Motility assays using 7 days old transgenic *sel-1::gfp* animals with *Punc-54* driven muscle specific expression of (left) α-synuclein-GFP fusion protein, and (right) polyQ40-YFP (B) Same setup as (A) right with inhibition of indicated genes using RNAi



We tested the motility, by counting bodybends in liquid over a 30 seconds interval, in both α-synuclein and polyQ40 transgenic worms when crossed into the *sel-1::gfp* transgenic background. Very interestingly, we found improved motility at day 7 of adulthood in both models (Figure 5.2A), indicating that an increased expression of *sel-1* is sufficient to alleviate proteotoxicity. To confirm that the improved motor function is a direct result of *sel-1* overexpression, we knocked down *sel-1* by RNAi. When *sel-1* is inhibited, the motility improvement is ameliorated, without changing the WT motility significantly (Figure 5.2B), supporting the hypothesis that *sel-1* overexpression is sufficient to alleviate proteotoxicity.

5.C. Discussion

In this chapter, I show results suggesting a direct link between ERAD and longevity, as *sel-1* overexpression is sufficient to extend WT lifespan. Additionally, this chapter shows that *sel-1* overexpression alleviated proteotoxicity, enhanced the activity of the proteasome, and increased the level of autophagy. These results suggest that elevated SEL-1 alone is sufficient to mimic the improved protein quality control mechanisms observed in *gfat-1* gof mutants, which was dependent on *sel-1*. Taken together, the results indicate a direct relationship between ERAD and autophagy, and the increased expression of SEL-1 detected in the *gfat-1* gof mutants might therefore point towards a more direct mechanism for the mutant's prolonged life.

The yeast homologue to SEL-1 in *C. elegans* is called Hrd3p. Strikingly, Hrd3p is a glycosylated protein, and was found required for optimal translocation of unfunctional proteins from the ER to the cytosol (Richard K. Plemper, 1999). This provides a possible link between N-glycosylation and ERAD. Presumably, increased levels of UDP-GlcNAc enhance N-glycosylation of proteins, and this could be the link to increased levels of SEL-1. This hypothesis is additionally supported by the fact that *gfat-1* gof mutants were dependent on functional glycosylation; inhibition of OST complex components shortened the lifespan extension observed in *gfat-1* gof mutant animals, and abolished the improved motility as a consequence of advanced ER protein homeostasis.

Whether the transgenic strain overexpressing *sel-1* is dependent on GFAT-1 and N-glycosylation is not yet known, and we aim to address that in the future. SEL-1 is required for the retro-translocation of proteins from the ER to the cytosol, but how that enhances the activity of the proteasome, we likewise aim to look at. By now we do not have any results indicating that increased delivery of proteins to the cytosol will enhance ubiquitinaion, but we are currently trying to address that.

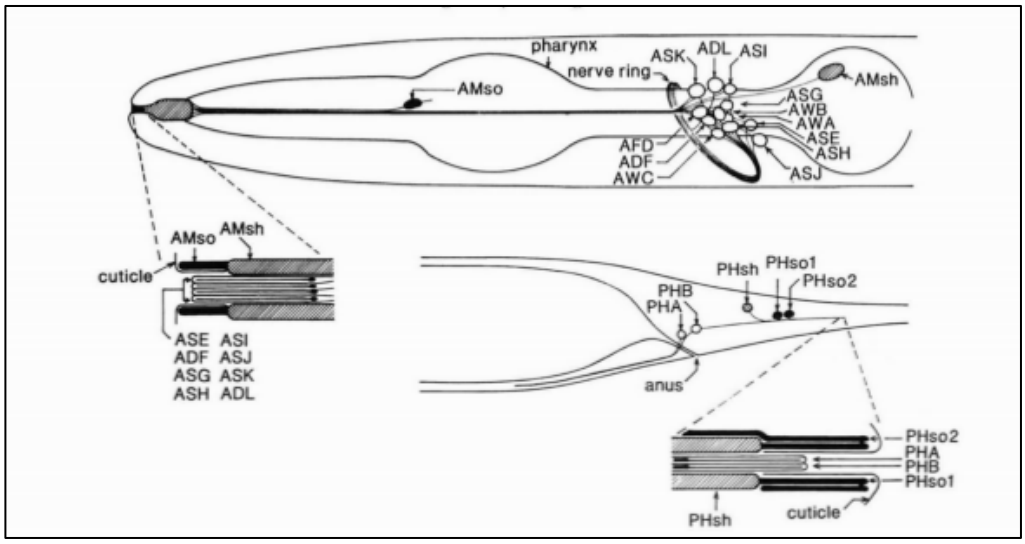
CHAPTER 6. Chemosensory-Deficient Mutants are Resistant to Tunicamycin and Long-Lived

6.A. Introduction

Besides the *gfat-1* gof mutants, we were able to identify a handful of other TM-resistant mutants from the screen. As early selection criteria in the screen, we stained the worms with DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, Molecular Probes), to seperate the mutant animals into two groups. *C. elegans* senses signals from the environment through ciliated sensory neurons located primarily in sensory organs in the head and tail. Cilia function as sensory receptors, and mutants with defective sensory cilia have impaired sensory perception (E L Peckol, 1999). DiI stains six amphid neurons, ASI, ADL, ASK, AWB, ASH and ASJ, and two phasmid neurons, PHA and PHB (Figure 6.1), which is absent when the animals carry a mutation that makes them sensory deficient (J Collet, 1998).

Figure 6.1 *C. elegans* sensory system.

The sensory system in *C. elegans* consists of different neurons, of which amphid neurons are required for their chemosensory-abilities (ASI, ADL, ASK, ASH, AWB and ASJ). Their cell bodies are located near the pharyngeal bulb. (www.wormbook.org)



Mutants with inhibited ability to take up DiI, are referred to as Dyf mutants (Dye filling defective). They are not uninteresting, however, it has previously been shown that mutants with a sensory deficiency are long-lived and more resistant to distinct stress for example heat (Riddle, 2003) (Kenyon J. A., 1999). Additionally, it was shown that Dyf-induced longevity is dependent on DAF-16, suggesting an already known downstream modulation of aging.

daf-2 mutant animals are as well dependent on DAF-16, but are not resistant to TM at the concentration used in the presented screen. Additionally, DAF-16 does not seem to play a role in the improved protein homeostasis observed in the *gfat-1* gof mutants, suggesting that the mechanism leading to TM-induced ER stress resistance is independent of DAF-16. So even though the Dyf mutants are known to be long-lived and stress resistant, there might be unknown mechanisms functioning to ensure Dyf-induced resistance to TM.

6.B. Results

6.B.1 TM resistance screen enrich for Dyf mutants

Of the 109 long-lived mutants from the screen, we found a sensory deficiency in approximately 60% (66 of 109) of the mutants, determined by the animals' ability to take up DiI in the amphid neurons. Apfeld and Kenyon showed that the Dyf mutants are long-lived due to activation of DAF-16 – at least, they found that they are dependent on DAF-16 to sustain their long life, and the authors are additionally suggesting that the lifespan extension found in the cilia deficient mutants is somehow dependent on the DAF-2 signaling pathway. *daf-2* mutant animals are known to be long-lived, and more resistant to stress compared to WT animals (P L Larsen, 1995) (C Kenyon, 1993), however, *daf-2(1368)* is not able to develop on 10 ug/mL TM, which is the concentration we used in our TM resistance screen. This suggests that the Dyf mutants (at least the ones that we had identified in our screen) had an increased ability to resist ER stress independent of the DAF-2/DAF-16 signaling pathway.

Table 6.1 Schematic overview of the sequenced Dyf mutants from the developmental TM resistance screen.

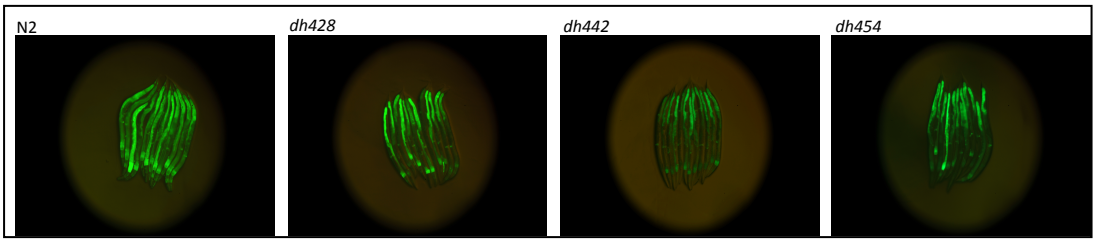
Various mutations leading to a premature stop mutation are listed. Additionally, *likely hit* refers to genetic mutations that are likely to cause the observed phenotypes (lifespan extension, ER stress resistance and/or dye filling defective).

Mutant	DiI result	Premature Stop Mutations	Likely hit	Other hits
dh428	Dyf		*che-3* - missense in pos. 8062111	
dh467	Dyf		*dyf-2* - 5'UTR in pos. 13686249	
dh431	Dyf	*dyf-2* - pos. 13676150		
dh435	Dyf	*tat-6* - pos. 3131801, *crp-1* - pos. 10480295		*srv-6* - pos. 4258064 (also a hit in *dh454*)
dh438	Dyf		*nhr-214* - missense in pos. 12701758	
dh440	Dyf		No mutations that pile with any	
dh441	Dyf	Y76B12C.6 - pos. 1990628, *osm-3* - pos. 3796925	*osm-3* - pos. 3796925	
dh442	Dyf		*che-3* - missense in pos. 8072943	
dh443	Dyf	*glh-2* - pos. 6514897, ZK858.8 - pos. 9125432		*nhr-214* - missense in pos. 12701753
dh447	Dyf	*srz-43* - pos. 15636436, F14D7.6 - pos. 14305952	*rbc-1* (F54E4.1b) - nrRNA in pos. 14617426 (also hit in *dh461*)	
dh448	Dyf		No mutations that pile with any	
dh461	Dyf		*rbc-1* - indel insertion in pos. 14614978 (also a hit in *dh447*)	
dh449	Dyf		*dyf-2* 5'UTR in pos. 13679167	
dh451	Dyf		No mutations that pile with any	
dh454	Dyf			*srv-6* - pos. 4258255 (also a hit in *dh435*)
dh456	Dyf	*dyf-17* - pos. 19129038		*egl-30* (M01D7.7b) 5'UTR in pos. 1837821 (also a hit in *dh443*)
dh457	Dyf		*dyf-2* - missense in pos. 13679167	

Of the 66 Dyf mutants found in our screen, we have sequenced 17 (Table 6.1), and in many of them, we have recognized mutations in genes that previously have been associated with Dyf mutants. These include *dyf-2*, *che-3* and *osm-3*. Conceivably, the Dyf mutants' extended lifespan is dependent on DAF-16, at least partly. Other factors may play a role as well, and we therefore looked at the HSP-4 expression levels. Surprisingly, we did not find increased levels of HSP-4 expression in the Dyf mutants under baseline conditions, suggesting that they do not have higher levels of ER UPR. Exposure to TM induced the expression of HSP in WT animals, but not in the Dyf mutants. Conversely, we found that the expression of HSP-4 is lower in the Dyf mutants when exposed to TM (Figure 6.2), suggesting that they are protected against TM due to decreased sensitivity, and therefore higher threshold.

Figure 6.2 Dyf mutants are protected against TM induced ER stress

HSP-4::GFP expression in WT (N2) and indicated Dyf strains. The worms have been treated with TM for 24 hours starting from late L4.



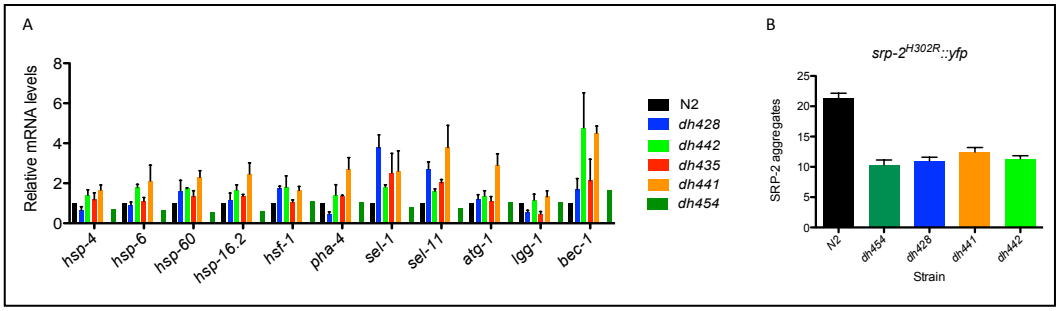
We speculated that a defect in the sensory cilia makes the animals senseless, and in this way makes the threshold for TM-induced ER stress higher. A similar response to TM was observed in the *gfat-1* gof mutants, also suggesting that they never "feel" stressed. However, in the *gfat-1* gof mutants, the TM resistance and the low levels of HSP-4 expression seem to impinge on a direct competition between TM and the N-glycan precursor, UDP-GlcNAc. Thus, by increasing the concentration of UDP-GlcNAc the toxicity of TM would be outcompeted, whereas the mechanism in the Dyf mutants seems to be different.

Very pleliminary results analyzing WB from whole worm lysates (data not shown) again suggested distinct mechanisms leading to TM resistance in the *gfat-1* gof mutants and the Dyf mutants. Exposure to TM does not affect the N-glycan level in *gfat-1* gof mutants, suggesting that the mechanism leading to TM resistance is upstream of N-glycosylation. In the Dyf mutants, however, it seems to be different, as N-glycan levels are decreased upon treatment with TM. This indicates that the resistance to TM is downstream of N-glycosylation. The Dyf-induced protection against TM toxicity does not seem to be dependent on increased ER UPR (Figure 6.2), so instead we measured the relative mRNA levels of other genes known to be activated during stress, including genes involved in ERAD and autophagy (Figure 6.3A).

Interestingly, we did not observe any significant changes in expression of any heat shock proteins (*hsp-4*, *hsp-6*, *hsp-60* or *hsp-16.2*). There was a slight tendency that one of the Dyf mutants, *dh441*, had increased levels, but it was not significant.

Figure 6.3 Dyf mutants have improved protein aggregation conditions

(A). Relative mRNA levels of indicated genes in WT (black bars) and some of the Dyf mutants (colored bars) (B). Quantification of SRP-2 aggregates in young adults in indicated Dyf mutants



On the other hand, we found a strong tendency pointing at an increased expression of genes related to ERAD and autophagy pathways in the Dyf mutants. This suggests that Dyf mutants have improved protein quality control mechanisms. Previously we had found both ERAD and autophagy to be required for alleviated protein aggregation formation in the ER, and asked whether the Dyf mutants also showed improved clearance of SRP-2 aggregation formation. Interestingly, we observed fewer SRP-2 aggregates in the 4 tested Dyf mutants (Figure 6.3B), suggesting that the activity of ERAD is enhanced in these mutants.

Together, these results indicate that the sensory deficient mutants are resistant to TM, long-lived and have improved protein homeostasis. Additionally, the data suggest that Dyf mutants resist TM in a manner different from the *gfat-1* gof mutants.

6.C. Discussion

Lee et al. showed that chemosensory deficiencies are likely to cause resistance to stress, for example heat and oxidative stress. Additionally, these mutants have been found to be long-lived in a DAF-16 dependent manner. Because of the link to the insulin/IGF signaling pathway, it was surprising that they were identified in this screening setup, as *daf-2* mutants are not resistance to TM at the concentration used. It is possible that parts of the phenotypes observed in the Dyf mutants are dependent on DAF-16, and it is also likely that DAF-16 plays a major role in the lifespan extension. But regarding *daf-2* mutant's missing ability to develop on TM, there must be a DAF-2/DAF-16 independent mechanism modulated in the Dyf mutants.

In our screen, we have identified 66 Dyf mutants, which all showed significant lifespan extensions. Additionally, we hypothesize that the TM resistance stems from a mechanism distinct from the mechanism that makes the *gfat-1* gof mutants resistant to TM. The Dyf mutants further showed changes in genes involved in stress response pathways, and lastly, we observed improved prevention of protein aggregation in the ER. Whether it is the same mechanism improving the ER protein quality control mechanisms in the Dyf mutants, as for the *gfat-1* gof mutants, is not clear. However, we do not expect so.

From literature, and from personal communication with Dr. Sueng-Jae Lee, it is believed that the Dyf-induced longevity is completely dependent on DAF-16, however, our results indicate differently, at least partly. A DAF-2/DAF-16 independent mechanism must be modulated additionally, making the Dyf mutants able to develop on TM. This mechanism is however still unknown, and we would have to do further experiments to come to a conclusion. In the presented data we have used the *daf-2* mutant allele *e1368,* however, the missing TM resistance in this *daf-2* mutant can be an allele specific thing. Therefore, it is only possible to indicate that one mutant allele, *daf-2(1368),* fails to develop on TM.

One possibility when suggesting relationships between the chemosensory defects and longevity, besides regulating DAF-16, is that Dyf mutants are slightly dietary restricted or stimulate the release of molecules that signals a DR state. DR worms are known to be long-lived and more resistant oxidative and thermal stress (Koen Houthoofd, 2002), due to enhanced quality control mechanisms, for example autophagy. The major transcription factor required for autophagy and longevity in DR

animals, PHA-4, could therefore be hypothesized to play a major role in the Dyf mutants. Additionally, the transcription factors DAF-16 and PHA-4 have been shown, at least in *C. elegans*, to be simultaneously enhanced in other long-lived mutants, and to be involved in distinct mechanisms. Whether PHA-4 plays a role in the Dyf mutants' improved stress resistance and longevity, is however, not known to this date.

CHAPTER 7. *C. elegans* Wild Isolates Display Improved Stress Resistance

7.A. Introduction

Under laboratory conditions, *C. elegans* WT animals live on average 20 days post reproduction. In addition, they are sensitive to TM. Due to variable conditions in nature, such as overcrowding, temperature changes and limited food availability, we wondered whether natural genomic variations in wild *C. elegans* isolates would reveal new targets and knowledge about protein quality control mechanisms. To test this we asked whether animals found at different origins in the wild would be resistant to TM-induced ER stress and confer longevity.

In most *C. elegans* research laboratories, the N2 (Bristol) strain is used as a WT control. This is also true for our laboratory, and in the following this strain will be referred to as N2.

7.B. Results

7.B.1 The strains

In Bonn (Germany), we collected three (presumably) independent *C. elegans* strains in a vineyard; Dragon #1, Dragon #9 and Dragon #10. Furthermore we received another 29 strains from the *C. elegans* genetics center (CGC) from different origins and climates around the world (see 7.1).

Table 7.1 Wild *C. elegans* isolates

Schematic overview of the 3 *C. elegans* strains collected in Bonn (Dragon #1, Dragon #9 and Dragon #10) and the wild isolates obtained from the *C. elegans* Genetics Center. In the columns are shown (from left) name of the strain, origin, type of environment when known and the F_1 generation's ability to develop in the presence of TM.

	Origin	**Environment**	**F1 development on TM**
Europe			
Dragon #1	Bonn, Germany	Vineyard	No
Dragon #9	Bonn, Germany	Vineyard	Yes
Dragon #10	Bonn, Germany	Vineyard	Yes
JU829	Tübingen, Germany	Compost heap	No
MY6	Münster, Germany	Compost heap	No
CB4932	Taunton, England	Mushroom farm	No
ED3005	Edinburgh, Scotland	Compost bin	Yes
JU262	Le Blanc, Indre, France	Vegetable garden	No
JU393	Hermanville, France	Compost pile	No
JU258	Madeira, Portugal	Fruit and vegetable garden	No
JU775	Lisbon, Portugal	Botanical garden	No
JU1400	Sevilla, Spain	Rotting orange fruits	No
JU1401	Carmona, Spain	Isolated from a snail	No
JU1440	Barcelona, Spain	Rotting palm fruit	No
North America			
CB4507	Palm Canyon, CA		No
CB4555	Pasadena, CA	Flower bed	No
CB4855	Palo Alto, CA	Compost heap	No
CB4856	Hawaii	Pineapple field	No
DH424	El Prieto Canyon, CA		No
PB306	Connecticut Valley, CT		Yes
PS2025	Altadena, CA	Garden	No
PX174	Lincoln City, OR	Forest	No
PX176	Eugene, OR	Grass compost	No
TR388	Madison, WI		No

South America			
JU1171	Concepcion, Chile	Compost sample	No
JU165	Montevideo, Uruguay	Compost	No
Africa			
ED3040	Johannesburg, South Africa	Compost	No
ED3042	Ceres, South Africa	Plant nursery	Yes
ED3072	Limuru, Kenya	Mushroom farm	No
LKC34	Madagascar		Yes
Australia			
JU1615	Melbourne, Australia	Compost heap	No
Asia			
JU1088	Kakegawa, Japan	soil	No

Most of the *C. elegans* wild isolates behaved differently from N2 animals, showing social behavior when cultured under laboratory conditions. This included population aggregation on the plates, which is known to originate from lack of a point mutation in *npr-1* (Mario De Bono, 1998). There were no obvious correlations between the social behavior and the origin of the strains (supplemental table 7.1). DH424, TR388, CB4507 and CB4555 originate from warmer climates (compared to Germany) in North America, and JU1615 from Melbourne, Australia. These strains showed "N2 like" behavior in terms of absent population aggregation on plates, suggesting that *C. elegans* isolates from these warmer climates have a mutation in *npr-1*, comparable with the N2 laboratory strain. However, this was not a general observation in all strains from warm climates. It is therefore not possible to draw any conclusions about the animals' behavior from their origin. The three Dragon strains collected in Bonn also showed aggregation behavior.

7.B.2 Natural variations in wild isolates result enhanced stress resistance

Next, we tested the animals' ability to cope with TM-induced ER stress, and interestingly, we found that the F_1 generation of ED3005, ED3042 PB306 and LKC34 were able to develop normally on plates containing TM. This was also the case for Dragon #9 and Dragon #10. Since N2 worms are completely sensitive to TM resulting in complete inhibition of all F_1 development, these observations suggest that natural variations in some wild *C. elegans* isolates improve the animals' ability to cope with TM-induced ER stress. We did not, however, observe any correlations between the TM resistance and geographic origin.

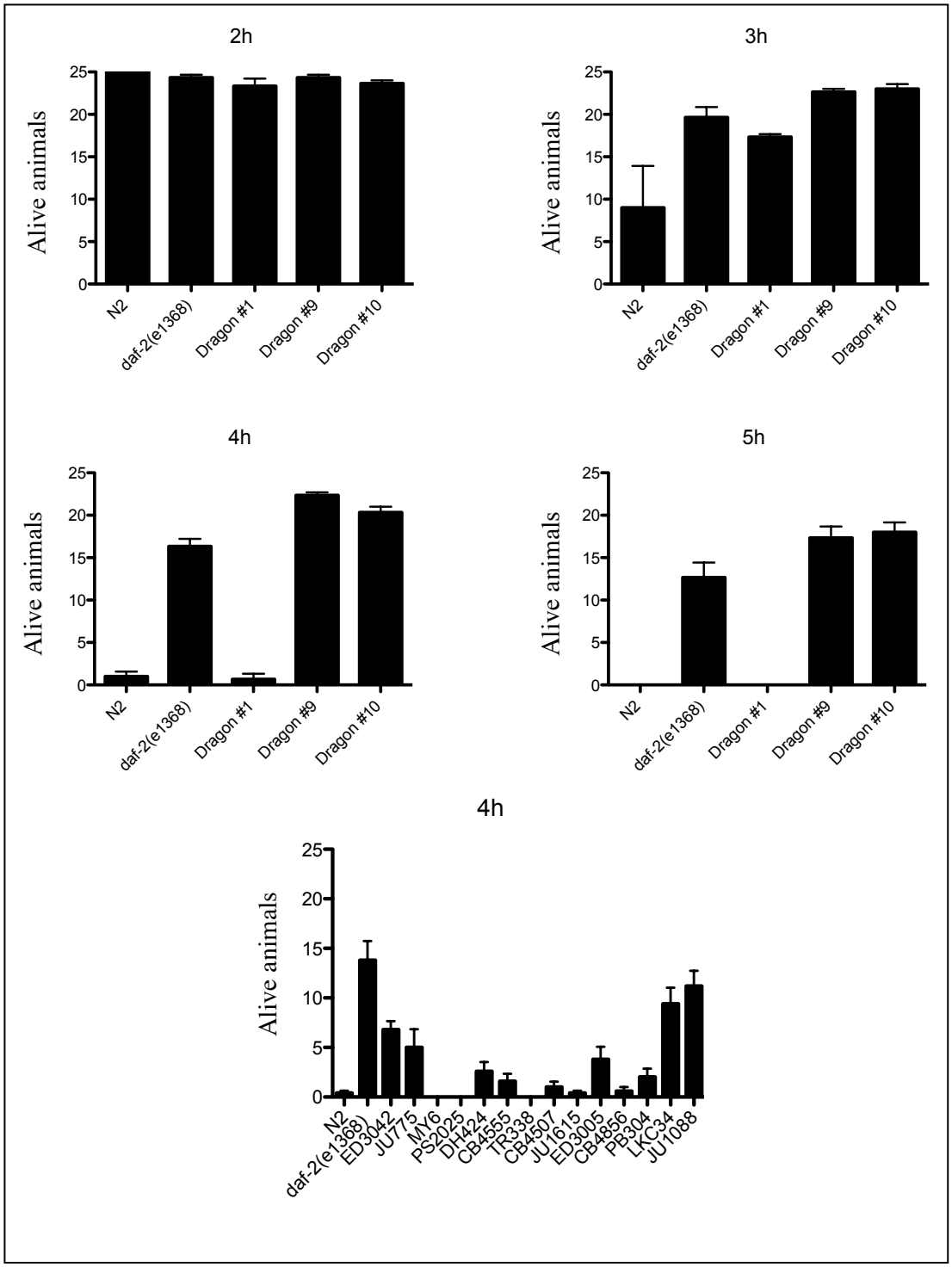
Conceivably, the TM-resistant Dragon and CGC wild *C. elegans* isolates may have enhanced protection of the ER arising from natural genetic variations. To further test this, we conducted thermotolerance tests byincubating the animals at 37°C for 5 hours. We scored the number of live worms every hour starting after two hours. After 4 hours, almost all N2 control animals were dead (Figure 7.1, 4h), whereas more than 60% of the *daf-2(e1368)* mutant animals were alive, which was expected since these mutants are known to be more resistant to heat. Interestingly, Dragon #9 and Dragon #10 showed strong resistance to heat as well, and after 4 hours around 80% and 75% were still alive, respectively (Figure 7.1). The same tendency was observed after 5

hours of heat incubation, suggesting that Dragon #9 and Dragon #10 not only are protected against TM-induced ER stress, but also against heat-induced ER stress.

Figure 7.1 Natural variations in *C. elegans* animals found in the nature result in ER stress resistance.

Heat stress resistance test performed at 37°C. The animals were scored after 2, 3, 4 and 5 hours, and indicated with the bar graphs are the number live worms at the various time points in the indicated strains. The bottom figure shows some of the CGC strains that were tested only at the 4-hour time point.

All experiments have been repeated at least twice with similar results, representative bar graphs shown.


2h
Alive animals
25
20
15
10
5
0
N2
daf-2(e1368)
Dragon #1
Dragon #9
Dragon #10
3h
Alive animals
25
20
15
10
5
0
N2
daf-2(e1368)
Dragon #1
Dragon #9
Dragon #10
4h
Alive animals
25
20
15
10
5
0
N2
daf-2(e1368)
Dragon #1
Dragon #9
Dragon #10
5h
Alive animals
25
20
15
10
5
0
N2
daf-2(e1368)
Dragon #1
Dragon #9
Dragon #10
4h
Alive animals
25
20
15
10
5
0
N2
daf-2(e1368)
ED3042
JU775
MY6
PS2025
DH424
CB4555
TR338
CB4507
JU1615
ED3005
CB4856
PB304
LKC34
JU1088

Furthermore, we also tested the heat resistance of some of the wild strains obtained from the CGC. Again, we observed a slight correlation between the TM resistant strains and heat resistance. The wild *C. elegans* isolates that showed improved resistance to TM (ED2005, ED3042, PB306, LKC34) also showed improved resistance to heat (Figure 7.1, bottom). Additional strains tested show enhanced resistance to heat compared to N2 controls without being resistant to TM, however, suggesting that the modulations causing TM-induced and heat-induced ER stress are different. This was also supported from our observations using *gfat-1* gof mutants; they show advanced resistance to TM without being resistant to heat.

7.C. Discussion

From the wild strains isolated in Bonn (Dragon strains) and from the strains we received from CGC, we were able to confirm the hypothesis that natural variations indeed occur, and that it leads to phenotypic changes.

Dragon #9 and Dragon #10 were both collected in a vineyard in Bonn, and they showed changed behavior when cultured under laboratory conditions, compared to N2 animals. Additionally, they showed enhanced resistance to TM and heat, altogether suggesting that natural variations in their genomes have occurred. The lifespan of these strains were, however, not significantly increased (data not shown). If anything, it seemed that Dragon #9 and Dragon #10 were short-lived compared to N2.

In the *C. elegans* wild isolates obtained from CGC we also detected a handful of strains with increased resistance to TM or to heat. But we were not able to draw any connections between their phenotypic characteristics and their origin. Likewise, we did not observe any relationships between original location and social behavior possibly arising from a genetic mutation in the *npr-1* gene.

What kind of genetic variations that have occured in these strains is still unknown, but it would be very interesting to sequence their genomes in the attempt to identify the mutations leading to increased ability to cope with TM and heat.

Our results support previous published work indicating that natural phenotypic variations are detectable in wild isolates, but whether the stress resistance seen is due to the same mutation in all strains, is unknown. I would, however, find it highly unlikely that all strains, independent of origin, climate and environment, have undergone the same natural variations, although we detected similar phenotypes in some of the wild isolates. Whether the increased resistance to TM- and heat-induced ER stress occurs from changes in the hexosamine pathway is also unknown. However, due to relatively small similarities between the wild isolates and the *gfat-1* gof mutants (besides the resistance to TM), the chances are not any more likely than other random mutations leading to stress resistance.

Supplemental Table 7.1 Behavior remarks of the wild C. elegans isolates

This figure gives a schematically overview over the social behavior of the different C. elegans isolates. In the left column the name of the strain is indicated and in the right is any remarks about behavior and place of egg-lay indicated

Strain	Remark
Europe	
JU829	Lay eggs mainly in edge of bacteria
MY6	Accumulations of L1/2/3 worms, nearly no eggs in the middle of the plate
CB4932	Accumulation of grown up worms on crowded plates also without starvation, on empty plates worms and eggs mainly in edge of bacteria
ED3005	Worms and eggs mainly in edge of bacteria
JU262	Accumulations of worms of all ages in edge of bacteria together with eggs
JU393	Accumulations of L1/2/3 worms and eggs only in edge of bacteria
JU258	Eggs mainly in edge of bacteria
JU775	L1/2 mainly in accumulations in the edge of the bacteria, older worms all over the plate
JU1400	All eggs mainly in the edge of the bacteria
JU1401	L1/2 mainly in accumulations in the edge of the bacteria together with eggs, L3/4/adult worms all over the plate
JU1440	Accumulations of L1/2/3, nearly no eggs in the middle of the plate
North America	
CB4507	N2 like
CB4555	N2 like
CB4855	Accumulations of small worms
CB4856	Eggs only in the edge of the bacteria
DH424	N2 like
PB306	Eggs mainly in edge of the bacteria
PS2025	Accumulations of worms of different ages, adult single worms also mainly in edge together with eggs
PX174	Eggs mainly in the edge of the bacteria
PX176	Eggs mainly in the edge of the bacteria
TR388	N2 like
South America	
JU1171	Worms and eggs mainly in the edge of the bacteria
JU1652	Eggs only in the edge of the bacteria
Africa	
ED3040	Accumulations of L1/2/3 worms and eggs mainly in the edge of the bacteria
ED3042	Accumulations of adult worms without starvation and worms and also eggs mainly in the edge of the bacteria

ED3072	Eggs mainly in the edge of the bacteria
LKC34	Eggs mainly in the edge of the bacteria
Australia	
JU1615	N2 like
Asia	
JU1088	Accumulations of L1/2 worms, adult worms all over the plate

CHAPTER 8. NHR-62-Induced Autophagy is Required for the Dietary Restriction-Induced Longevity

Part of this work has been published in Heestand et al. (2013).
Dietary Restriction Induced Longevity Is Mediated by Nuclear Receptor NHR-62 in *Caenorhabditis elegans*. *Plos Genetics*

8.A. Introduction

Lifespan extension is a well-known consequence of DR (Colman, 2009), either by long-term restriction or as an acute response to reduced nutrients (Mair, 2003). The recent decades of research in the pathways modulating DR-induced lifespan extension have revealed many genes involved in these processes. These include down-regulation of TOR (Kapahi P, 2004) (Eisenberg T, 2009), increased transcription of *pha-4*/*Foxa* (Panowski SH, 2007) and enhanced degradation by autophagy (Malene Hansen, 2008). TOR, PHA-4/Foxa and genes required for autophagy are additionally believed to be involved in fat metabolism, development and response to hormonal signaling (Rajat Singh S. K., 2009) (Jing Cui, 2013).

Nuclear hormone receptors (NHR) are transcription factors, and have been found to be involved in the same cellular mechanisms (Wollam J, 2011) (Magner DB, 2008). Bree Heestand hypothesized that there was a common regulatory mechanism, and initiated a project where he searched for regulators of the DR-induced longevity pathways among the 284 NHRs in *C. elegans*. In this work the *eat-2* mutant was used. *eat-2* mutant animals have altered pharyngeal pumping leading to downstream processes mimicking DR (Avery, 1993).

In this work, Heestand et al. found that especially NHR-62 was required for *eat-2* longevity, without changing the WT lifespan when knocked down (Bree N. Heestand, 2013). Additionally, they found that NHR-62 was essential for the DR-induced changes in fat metabolism, especially the changes in fatty acid composition during DR. Lipases are involved in the release of fatty acids from triglycerides, and have in addition previously been found to play an important role in longevity (Lapierre LR, 2011). Heestand et al. therefore screened through the 34 predicted lipases, and found that knockdown of C40H1.8 abolished the *eat-2* longevity, at least partly. More interestingly he found an NHR-62-dependent increase in mRNA levels of C40H1.8 and *lgg-1*, suggesting that autophagy might also be upregulated in a NHR-62 dependent manner.

8.B. Results

8.B.1 NHR-62 regulates C40H1.8 and autophagy

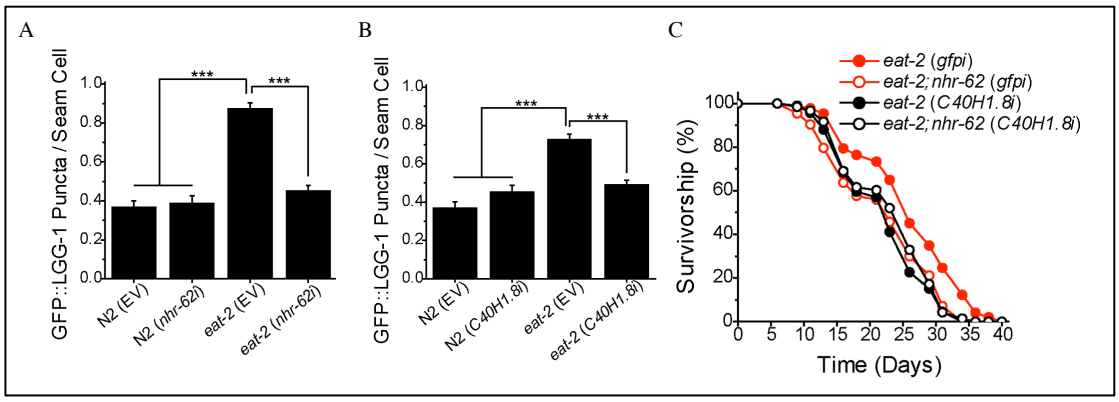
To measure autophagy we made use of a transgenic *C. elegans* strain expressing *lgg-1* fused to *gfp*. LGG-1::GFP is being used to estimate the level of autophagy by quantifying the number of LGG-1::GFP forming puncta in seam cells of young larvae.

Knockdown of *nhr-62* using RNAi did not change the WT level of LGG-1::GFP puncta, however, when knocked down in *eat-2* mutant animals, the enhanced level of autophagy detected, was completely abolished (Figure 8.1A). This suggests that the DR-induced autophagy is dependent on NHR-62.

Expression of C40H1.8 was also found increased in *eat-2* mutant animals. Lapierre et al. previously reported that *glp-1* mutant animals are dependent on the lipase *lipl-4* to maintain their long life and increased autophagy levels (Lapierre LR, 2011). We therefore asked whether the *eat-2*-induced autophagy increase was dependent on C40H1.8. Interestingly, we found that a knockdown of C40H1.8 shortened the lifespan of *eat-2* mutant animals (Figure 8.1B), suggesting that DR-induced longevity is dependent on functional lipolysis.

Figure 8.1 NHR-62-induced C40H1.8 expression is required for autophagy under DR

(A, B). LGG-1::GFP quantification of indicated strains using indicated RNAi bacteria, n>3, ***P<0.001, EV, empty vector (L4440) (C). Lifespan analysis of indicated strains using indicated RNAi bacteria. Median lifespan; *eat-2(gfpi)*: 27, *eat-2;nhr-62(gfpi)*: 22, *eat-2*(C40H1.8): 22, *eat-2;nhr-62*(C40H1.8): 23. *eat-2;nhr-62(C40H1.8)* vs. *eat-2;nhr-62*(*gfpi*). (log-rank test p = 0.4798)



Next, we asked whether C40H1.8 also plays a role in *eat-2* lifespan extension. Indeed it does; knockdown of C40H1.8 using RNAi completely reversed the *eat-2* lifespan back to WT levels (Figure 8.1C). More interestingly, we did not observe any further decrease in the lifespan of the double mutant *eat-2;nhr-62* (Figure 8.1C), suggesting that the lipase C40H1.8 could be a target of NHR-62, which is modulated by DR.

Together, these results suggest that DR-induced longevity is under control of at least one of the NHRs. Additionally, our results indicate that an NHR-62 dependent induction of autophagy in the long-lived mutant *eat-2*. Whether this induction of autophagy is dependent for the *eat-2* longevity is not completely clear, but since autophagy previously has been found to be required, and since NHR-62 and C40H1.8 are dependent to maintain the extended life, we would assume that the NHR-62-induced autophagy enhancement is also essential for the *eat-2* longevity.

CHAPTER 9. Discussion and Future Perspectives

9.A. Discussion

Here, I have reported our findings on how we, by performing a forward genetic screen, revealed novel correlations between protein quality control mechanisms and longevity. I show how increased levels of hexosamines, either gained by genetic modifications of *gfat-1* or pharmacologically by supplementing GlcNAc to the medium, result in resistance to TM-induced ER stress, extend *C. elegans* lifespan and improve protein quality control mechanisms. Additionally, I show data indicating that the stress resistance and longevity of *gfat-1* gof mutants is dependent upon a functional ERAD pathway, ER UPR and autophagy.

Additional mutants identified in the screen show increased stress resistance due to loss of chemosensory functions, which also leads to extended lifespan and improved ER quality control. However, this seems to be independent of the hexosamine pathway, as these mutants show distinct expression patterns of stress response genes, and also show at least partial dependence upon the insulin/IGF-signaling pathway to maintain their extended lifespan (Kenyon J. A., 1999). Furthermore, we isolated some still unidentified long-lived mutant animals from the TM screen, which may be independent of the hexosamine pathway, as they do not contain mutations in any of the genes known to be involved (See section 10.C).

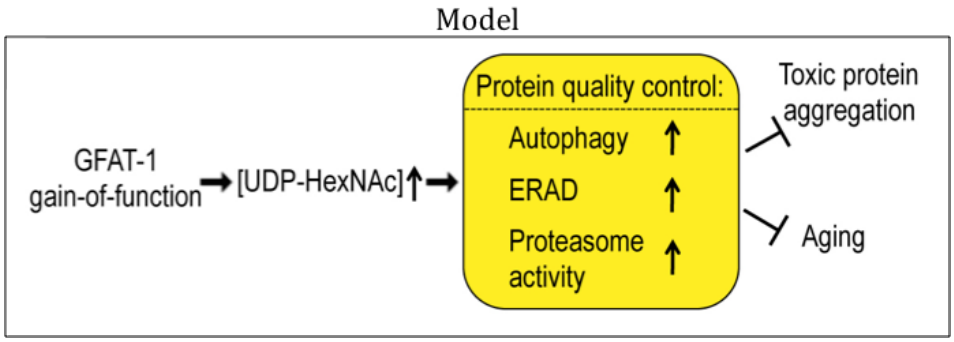
In data unrelated to the TM screen, I show that natural variations in the *C. elegans* genome lead to TM resistance, and that there might be a broader protection against ER stress in the wild, as some of the *C. elegans* wild isolates also show resistance to heat.

All together, the results suggest a novel association between protein quality control and longevity, and open up new possibilities to combat proteotoxic diseases. Additionally, the presented data point at new targets for understanding the relationship between improved stress resistance and longevity.

9.A.1 GFAT-1 gof

Using *gfat-1* gof mutants and activation of the hexosamine pathway, we demonstrate that elevated levels of the endogenous metabolite UDP-GlcNAc extend WT lifespan and ameliorate various proteinopathies. Lifespan extension induced by

GlcNAc or *gfat-1* gof required multiple aspects of ER function, including N-glycosylation, ERAD, and ER UPR signaling. Importantly, GlcNAc supplementation or GFAT-1 gof alleviated, and in some cases reversed, proteotoxicity in models of neurodegenerative diseases in distinct tissues and cellular compartments. This suggests a broad-spectrum improvement in protein quality control. Moreover, elevated levels of UDP-GlcNAc are not seen in several other major models of longevity such as reduced insulin signaling, mitochondrial function, gonadal longevity or dietary restriction, suggesting that GFAT-1 gof invokes a novel mechanism promoting long life.



ER function, particularly related to its stress response pathways, have previously been linked to health and lifespan (Brown and Naidoo, 2012; Parodi, 2000). Many age-related diseases and conditions, including diabetes, chronic inflammation, heart disease and neurodegenerative diseases, have also been associated with ER stress responses (Cao and Kaufman, 2013; Ozcan and Tabas, 2012; Walter and Ron, 2011). In *C. elegans*, the ER UPR has recently been found to be essential for the longevity of *daf-2* mutants. At the same time, Taylor and Dillin reported that constitutive activation of the ER UPR component XBP-1 was sufficient to extend the lifespan of *C. elegans*. Together with these results, our data support the hypothesis that improved protein quality control promotes longevity. Although *gfat-1* gof mutants showed requirement for ER UPR basal levels to sustain their extended lifespan and ameliorated proteotoxicity, and also displayed increased levels of SEL-1 protein expression, enhanced autophagy degradation, and higher proteasomal activity, we surprisingly did not detect any changes in transcriptional induction of the ER UPR, as measured by *xbp-1* splicing and mRNA levels of downstream target genes such as *hsp-4*. Instead, GlcNAc or GFAT-1 gof appear to coordinate post-

transcriptional stimulation of protein quality control mechanisms, ERAD and autophagy. We also did not detect any transcriptional changes in mitochondrial UPR genes (*hsp-6, hsp-60*) or cytosolic heat shock response genes (*hsp-16.2, hsp-70*) when measured by qPCR. Furthermore, transcriptome analysis of *gfat-1* gof mutants did not reveal any changes in mRNA levels of other known stress response genes. All together, these results support a post-transcriptional and proximal mechanism for longevity that works at the level of metabolites and their interaction with client proteins.

9.A.2 Links between metabolites, ER, and autophagy

How are hexosamine metabolites influencing ERAD, proteasome activity and autophagy? One possibility is that the phenotypes result from broad changes in the secretory apparatus, presumably by modulation of N-glycosylation. However, we did not observe any global changes in steady state protein glycosylation as measured by N-glycan labeling with ConcavalinA. Another possibility is that specific secretory proteins may be affected. Indeed, the observation that increased UDP-GlcNAc results in increased expression of SEL-1, involved in ERAD, may provide an important hint. Conceivably, elevated UDP-GlcNAc stimulates the covalent N-glycosylation of SEL-1 and other ERAD components, thereby affecting their activity and/or stability. Consistent with this idea, increased transcription of *sel-1* is sufficient to extend lifespan. In addition, we detected a dependence of *gfat-1*-induced aggregate clearance and longevity on components of the N-glycosylation machinery. It is noteworthy that Hrd3p, the yeast homologue of SEL-1, is itself a glycoprotein (Saito et al., 1999), which functions in a complex with the Hrd1p/SEL-11 ubiquitin ligase, and has been shown to autoregulate its own activity (Plemper et al., 1999). Presumably, elevated SEL-1 enhances ubiquitination, ERAD, and proteasome activity to improve protein degradation. Future experiments should clarify the possible mechanisms at work.

Are ERAD and autophagy linked? In certain pathological serpinopathy models, ERAD has been shown to be stimulated concomitantly with autophagy, resembling our observations with GFAT-1 gof (Kroeger et al., 2009). We, however, failed to uncover evidence for endogenous protein misfolding stress in our mutants; animals had normal developmental timing and brood sizes, and demonstrated better

handling of misfolded proteins. Another view is that organelle homeostasis within the ER may require intimate coordination of these processes. Autophagosomes form at ER-mitochondria contact sites and thus ER membranes might be an origin for autophagic vesicle formation (Hamasaki et al., 2013). Such sites may be used to initiate normal macroautophagy. Alternatively, they may facilitate so-called ERAD tuning, in which ERAD components are selectively removed from the ER to diminish the degradation response. During ERAD tuning, the unlipidated form of LC3 physically associates with SEL-1 to mediate this process (Bernasconi et al., 2012). In *gfat-1* gof mutants, however, we observed an increase in the lipidated form of LC3, consistent with canonical autophagy.

Several other small molecule metabolites have been shown to stimulate autophagy including spermidine, trehalose, and others (Eisenberg et al., 2009) (Honda et al., 2010). It is unclear if they work in concert with GlcNAc, or influence ERAD function. Conceivably, these metabolites work in a unified or parallel pathway. It is striking that a single endogenous metabolite can stimulate both ER quality control and autophagy, suggesting a potential role as a signaling molecule. GlcNAc finds wide acceptance as a food supplement thought to improve osteoarthritis, albeit little is known about the mechanistic underpinnings and a metaanalysis did not reveal benefits. Our data strongly suggest that beyond these effects, it might improve protein homeostasis, promote health and increase lifespan. This study therefore provides promising potential to identify new strategies to ameliorate diseases caused by interruptions in protein homeostasis.

9.A.3 Dyf mutants and improved protein quality control

Lee et al. showed that chemosensory deficiencies are likely to cause resistance to stress, such as heat and oxidative stress. Additionally, these mutants have been found to be long-lived in a DAF-16 dependent manner. Because of the link to the insulin/IGF signaling pathway, it was surprising that they were identified in this screen, as *daf-2* mutants are not resistant to TM at the concentration used. It is possible the phenotypes observed in the Dyf mutants are only partially dependent on DAF-16, and it is also likely that DAF-16 plays a major role in the lifespan extension. Regardless, the inability of *daf-2* mutants to develop on TM suggests that there must be a DAF-2/DAF-16 independent mechanism modulated in the Dyf mutants.

In the screen, we identified 66 Dyf mutants, all of which showed significant lifespan extensions. The Dyf mutants also showed transcriptional changes in genes involved in stress response pathways, and we observed ameliorated protein aggregation in the ER. Whether the same mechanisms are involved for improving ER protein quality control in the Dyf mutants, as for the *gfat-1* gof mutants, is not clear. However, we do not expect so.

From the literature, and from personal communication with Dr. Sueng-Jae Lee, it is believed that the Dyf-induced longevity is completely dependent upon DAF-16, however, our results indicate only partial dependence. A DAF-2/DAF-16 independent mechanism must also be modulated, making the Dyf mutants able to develop on TM. This mechanism remains to be discovered, however, and further experiments are required to confirm this. In the presented data we used the *daf-2* mutant allele *e1368,* and the absence of TM-resistance in *daf-2* mutant animals may be allele-specific. We can therefore only indicate that *daf-2(1368)* mutant animals not are resistant to TM.

Besides modulating DAF-16, it is possible that the chemosensory defects stimulate the release of molecules that signal a DR state. DR worms are known to be long-lived, and are also characterized by resistance to oxidative and thermal stress (Koen Houthoofd, 2002). The major transcription factor required for autophagy and longevity in DR animals, PHA-4, might therefore play a major role in protein quality control mechanisms in the Dyf mutants. Additionally, the transcription factors DAF-16 and PHA-4 have previously been shown, at least in *C. elegans*, to be simultaneously enhanced in other long-lived mutants, although they are involved in distinct mechanisms. Whether PHA-4 plays a role in the Dyf mutants' improved stress-resistance and longevity, however, is not known.

9.A.4 What can we learn from nature?

From the wild strains that we isolated in Bonn (the Dragon strains) and from the strains we received from CGC, we were able to confirm the hypothesis that natural variations in stress resistance do indeed occur.

Dragon #9 and Dragon #10 were both collected in a vineyard in Bonn, and displayed behavioral differences when cultured under laboratory conditions, compared to N2 animals. Additionally, they showed enhanced resistance to TM and heat, suggesting

that natural variations in their genomes have occurred. The lifespan of these strains were, however, not significantly different compared to N2 (data not shown). It even seemed that Dragon #9 and Dragon #10 were short-lived, if changed at all. The unchanged (or decreased) lifespan may also be due to the wild isolates' nature; they are not adjusted to laboratory conditions.

In the *C. elegans* wild isolates obtained from CGC, we also detected a handful of strains with increased resistance to TM or heat. We were, however, not able to draw any connections between their phenotypic characteristics and their origin. Relative to the surrounding substrates of the wild isolate's origin (i.e. moisture, fruit, grass), we considered the possibility that some wild isolates probably live mostly as dauers during long periods of famine with only sporadic episodes of feasting. We did, however, fail in observing any correlation between environmental origin and phenotypes. Likewise, we did not observe any relationships between original geographic location and social behavior possibly arising from genetic mutations in the *npr-1* gene.

What kind of genetic variations that have occured in these strains is still unknown, but it would be very interesting to sequence their genomes in the attempt to identify the mutations leading to increased ability to cope with TM- and heat-induced stress.

Our results support previously published work indicating that natural phenotypic variations are detectable in wild isolates, however, the finding that some of these wild isolates are resistant to TM is novel. This open up new possibilities that natural variations in the genome can reveal important information about stress resistance. Whether increased stress resistance is due to the same mutation in all strains, is unknown. It would be highly unlikely that they all strains, independent of origin, climate and environment, have undergone the same natural variations in their genomes, although we detected similar phenotypes in some of the wild isolates. Further experiments will be made to test the animals' lifespan. In addition, we will investigate the mechanism(s) leading to resistance to TM in the wild isolates. We do not expect the wild isolates to be resistant to TM due to mechanisms involving the hexosamine pathway, but we can not exclude it completely.

9.B. Future Perspectives

All together, this data reveals a novel modulator of *C. elegans* lifespan through control of ER stress response pathways, although further investigation is necessary to clarify the exact mechanisms involved. It will be important to understand how changes in endogenous metabolite levels can bring about global improvements in protein homeostasis to influence lifespan. To this end, the three major future perspectives will be to perform in-depth molecular characterization of GFAT-1 and its regulation, to describe how N-glycosylation affects stress response pathways, and finally to address the role of the hexosamine pathway in human cells. Additionally, further investigation of the Dyf mutants to reveal their dependence on other longevity pathways such as the DR-induced longevity pathway will raise the opportunity to learn more about the interplay between chemosensation and the ER longevity pathway.

9.B.1 GFAT-1 structural changes

GFAT-1 is regulated by product inhibition and phosphorylation, and it would therefore be of great importance to study the specific point mutations identified in the *gfat-1* gof mutants. At least two of the *gfat-1* gof mutations (*dh784* and *dh785*) lead to changes in amino acids that are located on the surface of the GFAT-1 protein. Investigating the exact changes of GFAT-1 could reveal information about whether the mutations might cause structural changes that lead to constitutive activation due to inability of UDP-GlcNAc to bind or structural changes that lead to inhibited phosphorylation. The significance of this also stems from the importance of identifying other regulators and components of the ER longevity pathway.

9.B.2 GFAT-1 repressor screen

GFAT-1 gof enhances the hexosamine pathway to promote longevity and alleviate proteotoxicity, but to shed light on other genes involved in the ER longevity pathway, it would be interesting to perform a GFAT-1 repressor screen. By randomly mutating *srp-2::gfp* transgenic animals crossed into the *gfat-1* gof mutant background, we would select for mutants with reduced ability to prevent SRP-2 aggregation. In this way, we might be able to identity both up- and downstream regulators of the ER

longevity pathway, which would be of great importance in understanding howenhanced flux through the hexosamine pathway leads to alleviated protein aggregation. Along the same line, a GFAT-1-enhancement-screen could be interesting: screening different groups of small molecules and measuring the expression and activity of GFAT-1. This could include groups of sugars, sterols and fatty acids, and would be of great importance as it has the potential to identify small molecule metabolites that modulate ER stress and lifespan.

9.B.3 GFAT-1 longevity pathway

Looking downstream of the hexosamine pathway, a lot of questions remain to be answered. In particular, how elevated UDP-GlcNAc levels have the ability to improve protein quality control pathways, and how these pathways are all regulated and inter-regulated, will be an important focus of future studies. For example, is N-glycosylation of specific proteins modulated in the *gfat-1* gof mutants, and is this essential for the lifespan extension? Additionally, there is a chance that UDP-GlcNAc is affecting other metabolic pathways – in this case, which? And lastly, is there cross-talk between ERAD and autophagy? In the current study, we have primarily focused on the degradation pathways, assuming that the GFAT-1 gof-improved protein homeostasis is due to increased degradation of mis- and unfolded proteins, preventing aggregation and toxicity. There is, however, also the possibility that mis- and unfolded proteins are re-folded. We did not observe any expression changes in the folding machinery including CNX and HSP-4, but investigating the protein folding pathways might be important to understand the exact mechanisms promoting healthy life in *gfat-1* gof mutants.

9.B.4 SEL-1 overexpression

Expression of SEL-1 was increased in gfat-1 gof mutants, and further investigation showed that *sel-1* over expression was sufficient to extend lifespan and ameliorate protein homeostasis. Conceivably, *sel-1* overexpression leads to increased degradation of proteins in the ER, but we also detected enhanced autophagy turnover upon *sel-1* overexpression. Using confocal and electron microscopy, it would be interesting to look at co-localization of ER resident proteins and proteins involved in

autophagy. This would shed light upon the correlation between these pathways, and give us the opportunity to investigate the possible reticulophagy, which is ER-specific autophagy degradation.

9.B.5 Neuronal signalling and protein quality control

The work in the Dyf mutants opens new doors to understand how neuronal signalling affects distinct cellular tissues. Firstly, it would be interesting to investigate the role of the transcription factors, DAF-16 and PHA-4 in the downstream signaling events. If DAF-16 is the main regulator of the stress-reponse outputs in the Dyf mutants, we would expect similar transcriptional changes in *daf-2* mutant animals, as observed in the Dyf mutants. We will therefore perform a comprehensive qPCR analysis of genes involved in ER stress response pathways in *daf-2* mutant animals and compare it to the expression patterns obtained from the Dyf mutants. Additional links are however still missing, and some of them could presumably be revealed by answering the following questions: Are DAF-16 and PHA-4 required for the resistance to TM and the alleviated aggregation of SRP-2? Is TOR modulated in the Dyf mutants? Is DR sufficient to further extend the Dyf-induced longevity?

9.B.6 The Role of ER Stress Pathways in Mammalian Health and Longevity

GFAT-1 is highly conserved across various organisms. In fact, at the level of amino acid sequence, human GFAT-1 is 85% similar to the worm GFAT-1, and the function is presumably the same. It would therefore be interesting to further investigate the role of GFAT-1 gof in mammalian and even in human cells, to test whether the observed phenotypes found in *C. elegans* are conserved as well. Very preliminary data show that polyglutamine repeats are degraded in the presence of GlcNAc in cultured medium. However, more details about the role of hexosamines in modulating stress resistance pathways in mammalian cells remain to be determined.

Glucosamine and GlcNAc have already reached wide acceptance as food supplements sold in various drug stores around the world. In the USA glucosamine is promoted almost as a panacea, to alleviate inflammation, chronic intestinal diseases, cancer, diabetes, lung diseases and even as a skin lightening agent. Whether GlcNAc or glucosamine can be considered as a multifunctional drug is not known. We do,

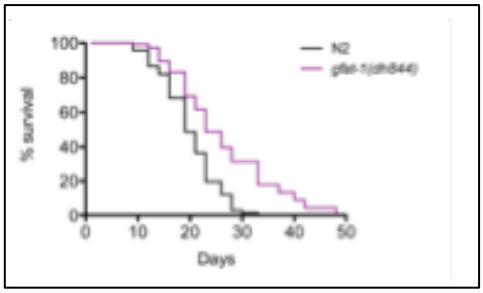
however, provide evidence for its role in protein homeostasis and lifespan extension. I present data that support the hypothesis that GlcNAc treatment is sufficient to ameliorate proteotoxic diseases by clearing or preventing protein aggregation formation in the roundworm *C. elegans*. Furthermore, our results suggest that several protein quality control mechanisms are enhanced upon treatment with GlcNAc, presumably playing a role in GlcNAc-induced lifespan extension.

We aim to translate these findings into mammalian cellular systems, and in the future, propose a clinical trial investigating the therapeautic usage of GlcNAc in human patients suffering from neurodegenerative disorders like Alzheimer's disease. If successful, such translational studies would highlight the ability of basic genetic investigation in *C. elegans* to inform mammalian biology and positively impact human health and disease.

CHAPTER 10. Addenda

10.A. Another *gfat-1* gof mutant

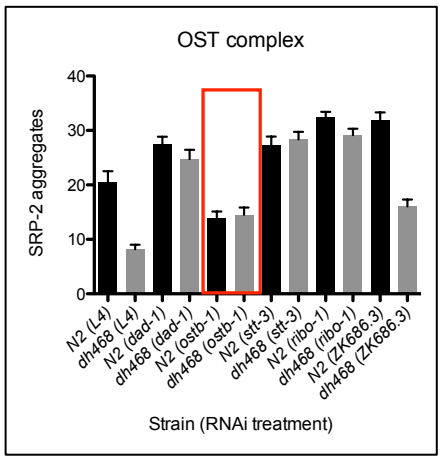
In the previous presented material, and in Denzel, Storm et al., we show that GFAT-1 gof extends lifespan and alleviates proteotoxicity of various models of neurodegenerative disorders. We demonstrate 3 independent alleles of *gfat-1*, all resulting in gof of the gene, and all extending lifespan. We did however identify an additional allele of *gfat-1* (*gfat-1(dh844)*). The lifespan of *gfat-1(dh844)* was also remarkable extended (*gfat-1(dh844)* median lifespan 26 days, P<0.0001).



10.B. Additional results

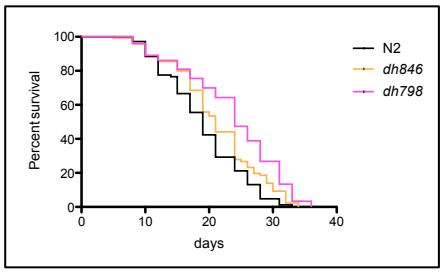
From our results, we hypothesize that GFAT-1 gof extends lifespan through elevated levels of UDP-GlcNAc that are used as precursors for N-glycans. This would suggest a mechanism where N-glycosylation is increased, and in this way either improve the folding or re-folding of proteins in the ER. To test this, we knocked down components of the OST (oligosaccharyltransferase) complex, which carries out the N-glycosylation of proteins. When we knock down components of this complex by RNAi in adulthood, the long-lived *gfat-1* gof mutants lost their lifespan extension. Furthermore, the lowered levels of SRP-2 aggregation (presumably as a consequence of enhanced ERAD function) were rescued when components of the OST complex were knocked down (Figure 4.3).

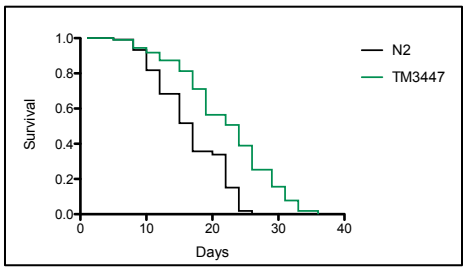
Interestingly, we observed that RNAi against *ostb-1* did not change the level of SRP-2 aggregates. We repeated the experiment, and did again not see any changes. This can of course be due to a defective RNAi bacteria clone, and we will test if this is so. However, it could also open an interesting part and possible noven function of the *C. elegans* OST complex.



10.C. A hexosamine pathway independent increase of UDP-GlcNAc?

An additional interesting mutant from our screen is *dh798*. *dh798* has several mutations in the genome, and they seem to be concentrated on chromosome III and X (see table). The *dh798* mutant animals were long-lived (see lifespan curve, pink) and non-Dyf.





In addition, they are not obviously associated with the hexosamine pathway (genetic mutations are shown in the two tables). Among the mutations identified in *dh798,* we found a genetic mutation in C56G2.1b, which encodes a protein called AKAP-1 (A-kinase anchor protein). A mutation in this gene was additionally detected in *dh846* mutant animals, increasing the likelihood that this mutation causes the observed phenotypes. *dh846* is also long-lived (see lifespan curve, yellow). We ordered a mutant that has a lof mutation in C56G2.1b and analyzed the lifespan. Interestingly, this mutant was long-lived (right lifespan curve, green), suggesting that C56G2.1b plays a role in longevity.

Chromosome	Location	Reference Base	Sample Base	Variant Type	Classes	Description
III	785763	G	A	point	three_prime_UTR	none
III	4640495	A	G	point	missense	AAT->AGT[Asn->Ser]
IV	5187902	T	A	point	missense	TCT->ACT[Ser->Thr]
III	5765139	X	X	indel	nongenic,nongenic	-2688 upstream,626 into
III	5969453	X	X	indel	nongenic,nongenic	-1798 downstream,-1977 downstream
X	6283374	G	C	point	missense	CCG->GCG[Pro->Ala]
III	6362931	T	C	point	five_prime_UTR	none

X	9463969	T	G	point	missense	GAT->GAG[Asp->Glu]
IV	10112497	X	X	indel	SNP	none
X	16461402	T	G	point	ncRNA	none
X	17441958	X	X	indel	nongenic	-10349 upstream

This table (upper) shows the genetic mutations in *dh798* compared to WT animals. The lower table shows the parental features, and contains a description of what the mutated gene is encoding, when available.

Parent Features	**Possible protein**
{F42G9.1a}	Protein Phosphatase, Mg2+/Mn2+ dependent
{F10F2.5}	C-type LECtin
{W03F8.3}	T23B5.4 Isoform 1 of Probable peptide chain release factor C12orf65, mitochondrial
{B0244.11},{B0244.2}	phogrin
{F25B5.1a},{F25B5.1b}	DAF-16/FOXO Controlled, germline Tumor affecting
{T07H6.4}	lev-9 encodes a novel extracellular protein; lev-9 was identified in screens for mutants that are resistant to the anthelmintic levamisole; lev-9 mutations result in weak levamisole resistance with respect to locomotion, but strong resistance with respect to egg laying.
{C56G2.1b}	AKAP-1
{ZK899.6}	UN
{uCE4-1092}	
{F38E9.t2}	UN
{C36E6.2}	Isoform 1 of Uncharacterized protein C6orf106 (phosphoserine domain)

We do, however, not have further evidence that these mutants are associated, and we additionally, do not have further evidence that the mutations in C56G2.1b make the animals resistant to TM and make them long-lived.

We are currently analyzing the sequences of these mutants again, to look for other mutations, as we observed an increased level of UDP-GlcNAc in the *dh798* mutant animals. From our first analysis, these mutants have no hits in the hexosamine pathway, so we were surprised to detect an enhanced level of UDP-GlcNAc. The mechanisms and further correlations are however still unknown.

10.D. Lifespan table

This table contains all lifespan experiments conducted with relation to the paper "Hexosamine pathway metabolites enhance protein quality control and prolong life".

Strain	Treat-ment (RNAi)	Median LS	Difference from cntl	Animals	Max LS	Difference from cntl	P value	Reference cntl
N2 (WT)		21		104/150	32			
gfat-1(dh784)		24	14.29%	108/150	38	18.75%	<0.0001	vs. N2
gfat-1(dh785)		24	14.29%	100/150	40	25.00%	<0.0001	vs. N2
N2		21		88/150	31			
gfat-1(dh784)		25	19.05%	63/150	41	32.26%	<0.0001	vs. N2
gfat-1(dh785)		23	9.52%	88/150	39	25.81%	0.0004	vs. N2
N2		19		79/150	30			
daf-2(e1368)		30	57.89%	102/150	53	76.67%	<0.0001	vs. N2
gfat-1(dh468)		24	26.32%	94/150	38	26.67%	<0.0001	vs. N2
gfat-1(dh784)		24	26.32%	88/150	42	40.00%	<0.0001	vs. N2
gfat-1(dh785)		21	10.53%	85/150	40	33.33%	0.005	vs. N2
N2		20		82/150	30			
daf-2(e1368)		32	60.00%	79/150	52	73.33%	<0.0001	vs. N2
gfat-1(dh468)		24	20.00%	104/150	34	13.33%	0.00016	vs. N2
gfat-1(dh784)		25	25.00%	110/150	36	20.00%	<0.0001	vs. N2
gfat-1(dh785)		23	15.00%	89/150	34	13.33%	0.0017	vs. N2
N2		20		103/150	26			
gfat-1(dh468)		22	10.00%	98/150	34	30.77%	0.0042	vs. N2
gfat-1(dh784)		24	20.00%	88/150	36	38.46%	0.0003	vs. N2
gfat-1(dh785)		24	20.00%	101/150	30	15.38%	0.0051	vs. N2
N2		19		104/150	31			
ogt-1		17	-10.53%	67/150	33	6.45%	ns	vs. N2
gfat-1(dh468)		23	21.05%	85/150	39	25.81%	0.0005	vs. N2
daf-2(e1368)		29	52.63%	88/150	51	64.52%	<0.0001	vs. N2
N2		21		90/150	38			
gfat-1(dh468)		30	42.86%	49/150	41	7.89%	<0.0001	vs. N2
daf-2(e1368)		37	76.19%	55/150	61	60.53%	<0.0001	vs. N2
N2		20		95/150	32			
gfat-1(dh468)		28	40.00%	74/150	46	43.75%	<0.0001	vs. N2
N2		21		103/150	29			
gfat-1(dh468)		27	28.57%	87/150	37	27.59%	<0.0001	vs. N2
daf-2(e1368)		34	61.90%	64/150	51	75.86%	<0.0001	vs. N2
daf-16(mgDf50)		17	-19.05%	91/150	27	-6.90%	<0.0001	vs. N2
ogt-1(ok430)		23	9.52%	72/150	27	-6.90%	ns	

dh468;daf-2		40	90.48%	63/150	53	82.76%	0.00042	vs. *daf-2(e1368)*
dh468;daf-16		22	4.76%	67/150	31	6.90%	<0.0001	vs. *daf-16(mgDf50)*
dh468;ogt-1		25	19.05%	81/150	39	34.48%	0.0056	vs. *ogt-1*
N2		19		98/150	33			
gfat-1(dh468)		28	47.37%	91/151	42	27.27%	<0.0001	vs. N2
N2		20		37/150	33			
gfat-1(dh468)		27	35.00%	30/150	39	18.18%	0.0001	vs. N2
daf-2(e1368)		31	55.00%	54/150	46	39.39%	P<0.0001	vs. N2
daf-16(mgDf50)		15	-25.00%	63/150	22	-33.33%	P<0.0001	vs. N2
ogt-1(ok430)		20	0.00%	66/150	29	-12.12%	0.2827	vs. N2
oga-1(ok1207)		23	13.04%	71/150	31	-6.06	P<0.05	vs. N2
dh468;daf-2		35	75.00%	55/150	46	39.39%	0.0042	vs. *daf-2(e1368)*
dh468;daf-16		20	0.00%	72/150	29	-12.12%	<0.0001	vs. *daf-16(mgDf50)*
dh468;ogt-1		22	10.00%	78/150	31	-6.06%	0.0074	vs. *ogt-1(ok430)*
dh468;oga-1		23	0.00%	83/150	31	0.00%	ns	vs. *oga-1(ok1207)*
N2		21		110/122	32			
daf-2(e1368)		32	52.38%	107/120	46	43.75%	<0.0001	vs. N2
gfat-1(dh784)		23	9.52%	113/121	29	-9.38%	<0.0001	vs. N2
gfat-1(dh785)		23	9.52%	113/108	32	0.00%	0.0709	vs. N2
N2		21		96/152	33			
gfat-1(dh468)		23	9.52%	94/143	33	0.00%	0.4014	vs. N2
daf-2(e1368)		31	47.62%	84/141	49	48.48%	<0.0001	vs. N2
N2 live OP50		21		84/152	31			
N2 10 mM D-Arg		24		79/144	32			
N2 0 mM GlcNAc		24	0.00%	69/150	33	3.13%	0.5264	vs. N2 D-Arg 10 mM
N2 0.2 mM GlcNAc		27	12.50%	91/150	32	0.00%	0.0243	vs. N2 D-Arg 10 mM
N2 1 mM GlcNAc		31	29.17%	85/151	39	21.88%	<0.0001	vs. N2 D-Arg 10 mM
N2 5 mM GlcNAc		29	20.83%	112/150	32	0.00%	0.0007	vs. N2 D-Arg 10 mM
N2 10 mM GlcNAc		31	29.17%	89/150	38	18.75%	<0.0001	vs. N2 D-Arg 10 mM
N2 25 mM GlcNAc		26	8.33%	78/148	32	0.00%	0.0411	vs. N2 D-Arg 10 mM
N2 live OP50		20		91/168	30			
N2 10 mM D-Arg		28		16/106	33			
N2 0.2 mM GlcNAc		30	7.14%	22/101	37	12.12%	0.1995	vs. N2 D-Arg 10 mM
N2 1 mM GlcNAc		30	7.14%	20/139	35	6.06%	0.0237	vs. N2 D-Arg 10 mM
N2 5 mM GlcNAc		29	3.57%	29/126	45	36.36%	0.0252	vs. N2 D-Arg 10 mM
N2 10 mM GlcNAc		33	17.86%	27/143	40	21.21%	0.0002	vs. N2 D-Arg 10 mM
N2 25 mM GlcNAc		27	-3.57%	23/124	44	33.33%	0.4141	vs. N2 D-Arg 10 mM
N2 live OP50		18		59/150	27			
N2 10 mM D-Arg		18		52/150	27			
N2 0 mM GlcNAc		20	11.11%	64/150	27	0.00%	0.5243	vs. N2 D-Arg 10 mM
N2 1 mM GlcNAc		25	38.89%	30/150	32	18.52%	0.0000	vs. N2 D-Arg 10 mM
N2 5 mM GlcNAc		25	38.89%	34/150	36	33.33%	0.0000	vs. N2 D-Arg 10 mM
N2 10 mM GlcNAc		27	50.00%	30/150	36	33.33%	0.0000	vs. N2 D-Arg 10 mM

Pgfat-1::cfp		26		92/138	37			
Pgfat-1::gfat-1::cfp		28	7.69%	65/122	39	5.41%	0.0008	vs. Pgfat::CFP
Pgfat-1::cfp		21		97/119	34			
Pgfat-1::gfat-1::cfp		30	42.86%	71/94	40	17.65%	<0.0001	vs. Pgfat::CFP
Pgfat-1::cfp		23		63/72	32			
Pgfat-1::gfat-1::cfp		27	17.39%	53/75	36	12.50%	<0.0001	vs. Pgfat::CFP
N2	L4440	17		107/150	32			
N2	*atg-1*	19	11.76%	117/150	31	-3.13%	0.7721	vs. N2
N2	*atg-18*	19	11.76%	49/90	31	-3.13%	0.7496	vs. N2
gfat-1(dh468)	L4440	26	52.94%	67/180	38	18.75%	<0.0001	vs. N2
gfat-1(dh468)	*atg-1*	26	0.00%	97/150	34	-10.53%	0.0986	vs. *gfat-1(dh468)*
gfat-1(dh468)	*atg-18*	22	-15.38%	103/150	34	-10.53%	<0.0001	vs. *gfat-1(dh468)*
N2	L4440	20		75/86	33			
N2	*sel-1*	23	15.00%	68/77	31	-6.06%	0.9525	vs. N2 (L4440)
N2	*sel-11*	23	15.00%	46/54	29	-6.45%	0.9656	vs. N2 (L4440)
N2	*rpn-6*	16	-20.00%	82/86	23	-20.69%	<0.0001	vs. N2 (L4440)
N2	*rpn-8*	17	-15.00%	69/83	33	43.48%	0.0006	vs. N2 (L4440)
N2	*pek-1*	23	15.00%	56/85	33	0.00%	0.1328	vs. N2 (L4440)
N2	*atf-6*	20	0.00%	58/70	31	-6.06%	0.6697	vs. N2 (L4440)
N2	*ire-1*	23	15.00%	63/78	31	0.00%	0.2499	vs. N2 (L4440)
N2	*xbp-1*	20	0.00%	68/77	31	0.00%	0.9325	vs. N2 (L4440)
gfat-1(dh468)	L4440	25	25.00%	52/87	37	19.35%	<0.0001	vs. N2 (L4440)
gfat-1(dh468)	*sel-1*	23	-8.00%	58/70	33	-10.81%	<0.0001	vs. *dh468* (L4440)
gfat-1(dh468)	*sel-11*	23	-8.00%	47/76	31	-16.22%	0.0011	vs. *dh468* (L4440)
gfat-1(dh468)	*rpn-6*	16	-36.00%	80/80	23	-37.84%	<0.0001	vs. *dh468* (L4440)
gfat-1(dh468)	*rpn-8*	17	-32.00%	77/95	35	-5.41%	0.0415	vs. *dh468* (L4440)
gfat-1(dh468)	*pek-1*	25	0.00%	59/70	37	0.00%	0.2592	vs. *dh468* (L4440)
gfat-1(dh468)	*atf-6*	25	0.00%	58/72	39	5.41%	0.7259	vs. *dh468* (L4440)
gfat-1(dh468)	*ire-1*	20	-20.00%	48/59	31	-16.22%	0.003	vs. *dh468* (L4440)
gfat-1(dh468)	*xbp-1*	20	-20.00%	56/80	31	-16.22%	0.001	vs. *dh468* (L4440)
N2	*gfp*	24		43/87	31			
N2	*sel-1*	22	-8.33%	32/66	29	-6.45%	0.9325	vs. N2 (*gfp*)
N2	*sel-11*	24	0.00%	29/87	29	-12.12%	0.8705	vs. N2 (*gfp*)
N2	*dad-1*	24	0.00%	26/76	33	0.00%	0.0559	vs. N2 (*gfp*)
N2	*ribo-1*	24	0.00%	37/87	35	6.06%	0.1522	vs. N2 (*gfp*)
N2	ZK686.3	26	8.33%	37/107	31	-6.06%	0.0088	vs. N2 (*gfp*)
N2	*ostb-1*	24	0.00%	32/86	33	0.00%	0.2433	vs. N2 (*gfp*)
N2	*ire-1*	22	-8.33%	30/92	31	-6.06%	0.3529	vs. N2 (*gfp*)
N2	*xbp-1*	22	-8.33%	31/79	35	6.06%	0.6335	vs. N2 (*gfp*)
gfat-1(dh468)	*gfp*	29	20.83%	33/89	39	25.81%	<0.0001	vs. N2 (*gfp*)
gfat-1(dh468)	*sel-1*	22	-24.14%	36/85	29	-25.64%	0.0007	vs. *dh468 (gfp)*
gfat-1(dh468)	*sel-11*	24	-17.24%	29/81	31	-20.51%	0.0019	vs. *dh468 (gfp)*
gfat-1(dh468)	*dad-1*	26	-10.34%	30/74	35	-10.26%	0.0156	vs. *dh468 (gfp)*
gfat-1(dh468)	*ribo-1*	24	-17.24%	29/68	33	-15.38%	0.0013	vs. *dh468 (gfp)*
gfat-1(dh468)	ZK686.3	26	-10.34%	36/92	35	-10.26%	0.0279	vs. *dh468 (gfp)*
gfat-1(dh468)	*ostb-1*	24	-17.24%	19/58	29	-25.64%	0.002	vs. *dh468 (gfp)*

gfat-1(dh468)	*ire-1*	24	-17.24%	33/85	33	-15.38%	0.0048	vs. *dh468 (gfp)*
gfat-1(dh468)	*xbp-1*	22	-24.14%	33/81	31	-20.51%	0.0001	vs. *dh468 (gfp)*

10.E. Selected sequencing files

Sequencing file showing mutant variations for the *dh468* mutants presented in the previous (*gfat-1* mutation is highlighted with **bolded** font).

Chromosome	Location	Reference Base	Sample Base	Variant Type	Classes	Description	
II	3902182	A	G	point	missense	AGT->GGT[Ser->Gly]	{F53C3.4}
II	4181214	C	T	point	missense	CTT->TTT[Leu->Phe]	{R03H10.1}
II	4870234	C	T	point	missense	GAG->AAG[Glu->Lys]	{F11G11.11}
II	6559526	A	T	point	three_prime_UTR	none	{F18C5.10.1}
II	9260428	C	T	point	missense	GGA->GAA[Gly->Glu]	{C01G6.1a.1,C01G6.1a.2,C01G6.1b.1,C01G6.1b.2}
II	10714155	C	T	point	missense	GAA->AAA[Glu->Lys]	{C09G5.2}
II	**11594909**	**C**	**T**	**point**	**missense**	**GAA->AAA[Glu->Lys]**	**{F07A11.2a,F07A11.2b.1,F07A11.2b.2,F07A11.2b.3}**
II	14286245	C	T	point	missense	GCT->ACT[Ala->Thr]	{Y54G11A.12}
II	14474753	C	T	point	ncRNA	none	{C13B4.t1}
III	5467696	T	C	point	three_prime_UTR	none	{F48E8.2}
X	391641	G	A	point	missense	GCC->GTC[Ala->Val]	{R04A9.7}
X	5289823	G	A	point	three_prime_UTR	none	{F32A6.4a}
X	5897509	G	A	point	missense	GAT->AAT[Asp->Asn]	{F49E10.1}
X	6598276	G	A	point	missense	CCG->TCG[Pro->Ser]	{T28B4.4.1,T28B4.4.2}
X	7113751	G	A	point	missense	TGC->TAC[Cys->Tyr]	{C36B7.1}
X	8895158	G	A	point	missense	GCT->ACT[Ala->Thr]	{C13E3.1}
X	13083431	G	A	point	missense	GGA->AGA[Gly->Arg]	{R03G8.5}
X	16855333	G	A	point	missense	CAC->TAC[His->Tyr]	{T20F7.5}
X	16878162	G	A	point	missense, three_prime_UTR	TCT->TTT[Ser->Phe],none	{T20F7.7},{T20F7.1}

10.F. Pile-up table

Table showing possible hits and selected mutations in other TM resistant mutants from the screen.

Mutant	Institution	DiI	Premature Stop Mutations	Other hits	Comments
dh428	CCG	Dyf		che-3 - missense in pos. 8062111	
dh467	CCG	Dyf		dyf-2 - 5'UTR in pos. 13686249	
dh431	CCG	Dyf	dyf-2 - pos. 13676150		
dh435	CCG	Dyf	tat-6 - pos. 3131801, crp-1 - pos. 10480295	srv-6 - pos. 4258064 (also a hit in dh454)	crp-1 (RB855) was ordered at CGC, and was tunicamycin resistant first time, and sensitive the second.
dh468	CCG	Filling		GFAT (F07A11.2) - confirmed by LC/MS	
dh438	CCG	Dyf		nhr-214 - missense in pos. 12701758	nhr-214 (VC1356) was ordered at CGC. But was NOT resistant to TM
dh440	CCG	Dyf		No mutations that pile with any	
dh441	CCG	Dyf	Y76B12C.6 - pos. 1990628, osm-3 - pos. 3796925	osm-3 - pos. 3796925	
dh442	CCG	Dyf		che-3 - missense in pos. 8072943	
dh443	CCG	Dyf	glh-2 - pos. 6514897, ZK858.8 - pos. 9125432	C13E3.1 - missense in pos. 16333 (also hit in 468), nhr-214 - missense in pos. 12701753	glh-2 (KB2) will be ordered
dh447	CCG	Dyf	srz-43 - pos. 15636436, F14D7.6 - pos. 14305952	rbc-1 (F54E4.1b) - nrRNA in pos. 14617426 (also hit in dh461)	
dh448	CCG	Dyf		No mutations that pile with any	
dh461	CCG	Dyf		rbc-1 - indel insertion in pos. 14614978 (also a hit in dh447)	
dh449	CCG	Dyf		dyf-2 5'UTR in pos. 13679167	
dh451	CCG	Dyf		No mutations that pile with any	
dh454	CCG	Dyf		srv-6 - pos. 4258255 (also a hit in dh435)	
dh456	CCG	Dyf	dyf-17 - pos. 19129038	egl-30 (M01D7.7b) 5'UTR in pos. 1837821 (also a hit in dh443)	
dh457	CCG	Dyf		dyf-2 - missense in pos. 13679167	
dh784	CCG	Filling		GFAT (F07A11.2) - confirmed by LC/MS	
dh785	CCG	Filling		GFAT (F07A11.2) - confirmed by LC/MS	
dh786	CCG	Filling	vit-2 - pos. 5104020, K01B6.3 - pos. 9303527	vit-2 - pos. 5104020	Strain is requested from Carmen
dh799	CCG	Filling			
dh798	CCG	Filling		Has a hit in C56G2.1 (6362931) - also a hit in dh846 (6363870)	
dh844	MPIZ	Filling	exp-1 - pos. 6157373, T15D6.10 - pos. 12395126, cho-1 - pos. 13213269	GFAT (F07A11.2) - pos. 11592503	exp-1 (3 strains available) and cho-1 strains are ordered
dh846	MPIZ	Filling		Has a hit in C56G2.1 (6363870) - also a hit in dh798 (6362931)	
dh848	MPIZ	Filling		Y69A2AR.14 - pos. 2555780 (also a hit in dh856)	
dh856	MPIZ	Filling		Y69A2AR.14 - pos. 2555862 (also a hit in dh848)	
dh861	MPIZ	Filling	che-3 - pos. 8072875 - FILLS (05.05.12)	Has a couple of interesting hits; elo-6, cdh-12, sri-40 and a couple of unc's	elo-6 and cdh-12 strains are ordered

dh852	MPIZ	Filling			
dh820	MPIZ	Filling			
dh824	MPIZ	Filling			
dh825	MPIZ	Filling		D1046.5 - pos. 8957612, TPRA-1 (human TRansmembrane Protein, Adipocyte associated 1) ortholog	
dh783	MPIZ	Filling			
dh802	MPIZ	Filling		C23F12.2 - completely unknown; nothing available or known	It encodes a putative diacylglycerol kinase
dh867				Has a hit in F08B1.1a, which was also a hit in 301A (dh844)	F08B1.1a is vhp-1, which is a MAP kinase phosphatase that is known to be resistant to pathogens when knocked down
dh868	MPIZ	Filling		Has 2 independent hits in C56G2.5	
dh860	MPIZ	Filling		Has a hit in K04B12.1 that was also a hit in 327G	the gene is called plx-2 and is one of two C. elegans plexins, conserved transmembrane proteins that function as semaphorin receptors

10.G. Hexosamine Pathway Metabolites enhance Protein Quality Control and Prolong Life

Cell

Hexosamine Pathway Metabolites Enhance Protein Quality Control and Prolong Life

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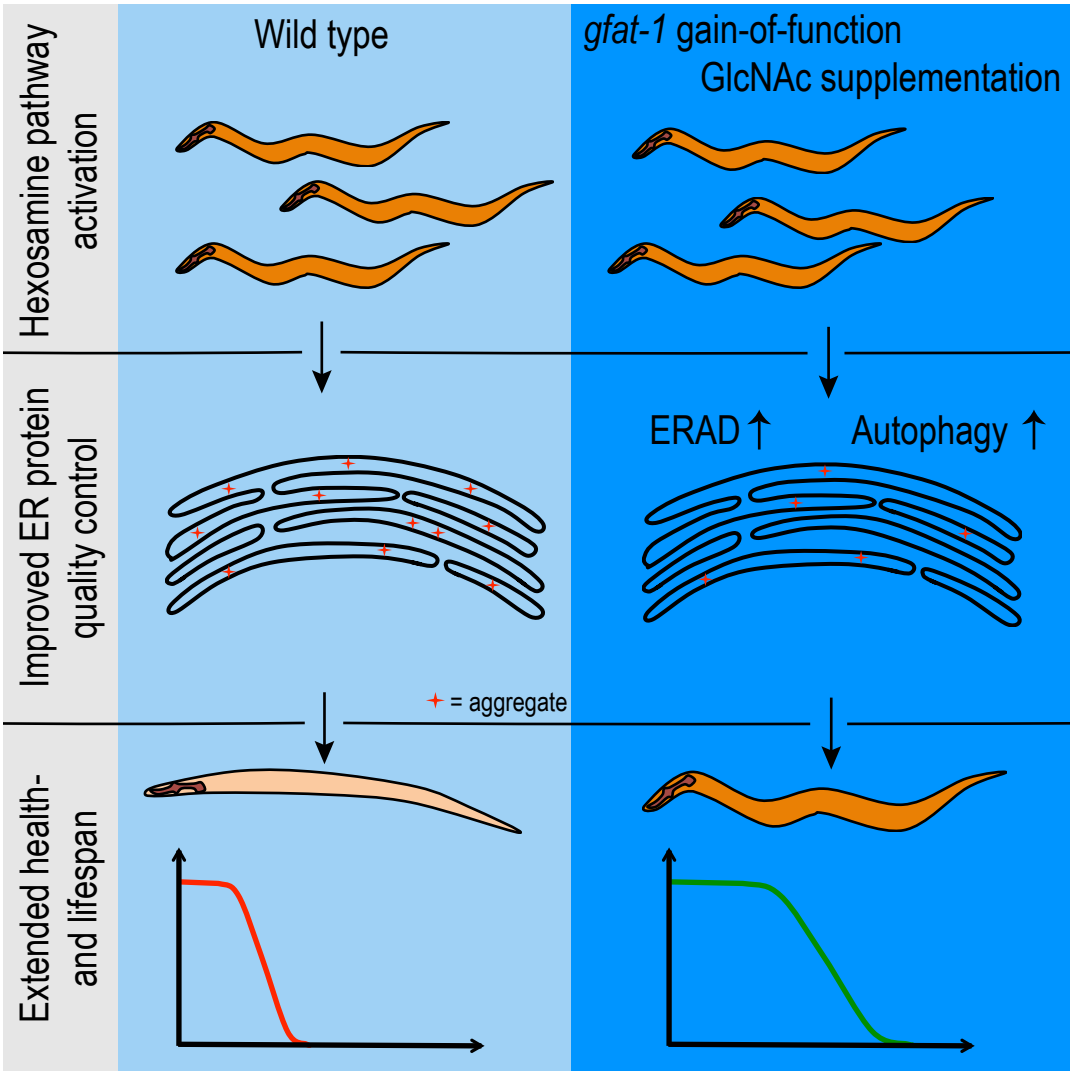
Graphical abstract



10.H. Dietary Restriction Induced Longevity Is Mediated by Nuclear Receptor NHR-62 in Caenorhabditis elegans

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Dietary Restriction Induced Longevity Is Mediated by Nuclear Receptor NHR-62 in *Caenorhabditis elegans*

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Abstract

Dietary restriction (DR) extends lifespan in a wide variety of species, yet the underlying mechanisms are not well understood. Here we show that the *Caenorhabditis elegans* HNF4α-related nuclear hormone receptor NHR-62 is required for metabolic and physiologic responses associated with DR-induced longevity. *nhr-62* mediates the longevity of *eat-2* mutants, a genetic mimetic of dietary restriction, and blunts the longevity response of DR induced by bacterial food dilution at low nutrient levels. Metabolic changes associated with DR, including decreased Oil Red O staining, decreased triglyceride levels, and increased autophagy are partly reversed by mutation of *nhr-62*. Additionally, the DR fatty acid profile is altered in *nhr-62* mutants. Expression profiles reveal that several hundred genes induced by DR depend on the activity of NHR-62, including a putative lipase required for the DR response. This study provides critical evidence of nuclear hormone receptor regulation of the DR longevity response, suggesting hormonal and metabolic control of life span.

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10.I. Dissertation work contributions

Figure 1.1 The total number of both men and women over 65 is increasing.
Figure 1.2 The endoplasmic reticulum (ER). NS
Figure 1.3 The ER Unfolded protein response pathways. NS
Figure 1.4 Induction of autophagy. NS
Figure 1.5 The autophagy genes. NS
Figure 3.1 A developmental TM resistance screen. A. NS B. NS + MD C. NS + MD
Figure 3.2 Mutations in gfat-1 result in resistance to TM. A. NS + MD B. MD C. NS + MD
Figure 3.3 Knockdown of gfat-1 causes TM sensitivity in *gfat-1* mutants. MD
Figure 3.4 *gfat-1* gof mutations lead to resistance to TM. MD
Figure 4.1 *gfat-1* gof mutants show less induction of ER stress responses. MD
Figure 4.2 Enhanced hexosamine pathway flux extends lifespan. NS
Figure 4.3 gfat-1 gof mutants are long-lived, independent of the DAF-2/DAF-16 longevity pathway. A, B, C. MD D, E, F. NS
Figure 4.4 Enhanced hexosamine pathway flux alleviates SRP-2^{H302R} aggregation in the ER. NS
Figure 4.5 GFAT-1 gof improves SRP-2^{H302R} aggregation without affecting overall SRP expression. A. NS B, C. MD
Figure 4.6 GFAT-1 gof improves ER functions in a post-translational manner. A, B. NS + MD C, D. MD
Figure 4.7 Improved ER functions in *gfat-1* gof mutants are dependent on ER-UPR and N-glycosylation components. NS
Figure 4.8 Enhanced flux through the hexosamine pathway increases the expression of SEL-1. AG
Figure 4.9 ERAD is required for GFAT-1 gof-induced longevity. NS
Figure 4.10 Increased the hexosamine pathway metabolites improve protein degradation by the proteasome. A. NS + MD B, C, D. MD
Figure 4.11 GFAT-1 gof enhances the level of autophagy. A. NS B. MD C. NS
Figure 4.12 GlcNAc induces autophagic degradation. A. NS B. MD C, D. NS
Figure 4.13 *gfat-1* gof mutants are dependent on autophagy to remain protected against SRP-2^{H302R} aggregation and to sustain longevity. NS
Figure 4.14 GFAT-1 gof-induced autophagy increase is dependent on Nglycosylation components and ERAD. NS
Figure 4.15 GFAT-1 gof alleviates proteotoxicity. A, B. NS C, D. MD
Figure 4.16 GFAT-1 gof alleviates proteotoxicity in manner dependent on autophagy and ERAD. NS
Supplemental Figure 4.1 Additional Characterization of the *gfat-1* gof mutants. A. MD B, C, D, E, F. NS
Figure 5.1 sel-1 over expression is sufficient to extend lifespan and enhance Autophagy. A, B. NS C, D. MD
Figure 5.2 *sel-1* over expression is sufficient to alleviate proteotoxicity. NS

Figure 6.1 C. elegans sensory system. NS
Figure 6.2 Dyf mutants are protected against TM induced ER stress. NS
Figure 6.3 Dyf mutants have improved protein aggregation conditions. NS
Figure 7.1 Natural variations in *C. elegans* animals found in the nature result in ER stress resistance. NS
Figure 8.1 NHR-62-induced C40H1.8 expression is required for autophagy under DR. NS

NS, Nadia Storm
MD, Martin Denzel
AG, Aljona Gutschmidt

LC/MS experiments were performed in collaboration with Yvonne Hinze, qPCR experiments were performed by Ruth Baddi, survival assays were confirmed by Ruth Baddi, figure 6.1, 6.2 and 6.3 were assisted by Rebecca Osterauer.

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