Proteostasis in the long-lived *daf-2* mutant of *Caenorhabditis elegans*: the influence of protein turnover and stability

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Summary

Samenvatting

SUMMARY

Ageing is a progressive, deleterious process, involving cellular, structural and functional decline with time, ultimately leading the organism to death. Considering the impact of ageing in the social and economical well being of humans, extensive research has been on the go in this field.

Even though ageing research relies on model organisms from yeast to mice, the application of *Caenorhabditis elegans* as a study organism in this field has proved advantageous. The short life span and short generation time in addition to the small and transparent body and conserved pathways similar to higher eukaryotes are few of the many key aspects of *C. elegans* which makes it a preferred model organism in ageing research. In addition, the availability of several longlived mutants and the completely sequenced genome are a bonus. The most important and well studied of all these mutations, the Insulin/insulin growth factor-1 (IGF-1) *daf-2* receptor gene mutation has shown to extend life span of organisms ranging from worms, fruit-flies to mice. In this present study, we utilize these tools to analyse proteostasis in the long-lived *daf-2* mutant[1].

Proteostasis or protein homeostasis deals with the balance between synthesis, folding, maintenance and degradation of proteins. Ageing at the molecular scale is associated with increased oxidative damage to proteins combined with decreased repair and maintenance in *C. elegans*. Protein damage has been shown to manifest itself as protein aggregation, accumulation and dysfunction which can lead to cellular death. But in the long-lived *daf-2* Insulin/IGF-1 mutants, these alterations seem to be slowed down considerably. Studies have revealed that the *daf-2* mutant is less prone to protein aggregation. Several theories propose increased autophagy in these long-lived mutants which reduces protein damage accumulation by rapid removal and recycling for resynthesis. However as it is already known that reduced protein synthesis induces longevity in many organisms these ideas seems to be in conflict. Considering the fact that protein turnover is a highly energy demanding process, the idea of induced protein turnover seems less likely to be involved in longevity. Hence in an attempt to get some clarity on this issue, we studied the role of protein turnover and autophagy in *daf-2*.

[1] Throughout this thesis, we used a *glp-4*, germlineless mutant background for all strains, but for clarity, we will refer to *daf-2* only in this summary instead of *glp-4;daf-2*.

Protein turnover is the integration of protein degradation and protein synthesis rate. One way to degrade proteins is by autophagy, a catabolic cellular process, conserved in eukaryotes, which involves degradation and recycling of damaged and unnecessary cellular proteins and organelles. Macroautophagy combined with ubiquitin-mediated autophagy is responsible for a large part of the 'household' or bulk protein degradation. On the other hand, autophagy as such is also involved in stress responses, including starvation, cellular immunity, development and cellular signal regulation. The process of autophagy needs strict regulation as hyperactivation or complete inhibition of autophagy are both fatal. Autophagy has been deemed as a process essential for longevity in several organisms including *C. elegans*. In this context we checked the protein turnover rates in the long-lived *daf-2* mutant. We observed that the protein turnover rate was lower in the long-lived mutants compared to the control. These results lead us to investigate the requirement and role of different lysosomal components in the long-lived *daf-2* mutants.

Lysosomal function depends on several hydrolytic enzymes for degradation, membrane proton pumps for lumenal acidification, amino acid permeases for release of digestion products, and biogenic and functional factors such as *glo* and *lmp* genes. We identified a strong reduction in both cathepsin L protease activity and acid phosphatase activity in the long-lived *daf-2* mutant. This data supported our observation of reduced protein turnover in the *daf-2* mutants. By aid of a proteomics experiment, we identified several lysosomal components to be down regulated at the protein level. We further found no effect on longevity in control strains or *daf-2* mutants when specific lysosomal components were knocked down. This was interpreted as a possible redundancy among the different genes involved in similar functions or a possible lack of importance for lysosomes for longevity. Additionally we found that the lysosomes were either less acidic or reduced in quantity in the long lived *daf-2* mutant adding support to the reduced activity of lysosomes. On the other hand we identified that DAF-16, the transcription factor that provides longevity in the *daf-2* mutant, negatively regulates cathepsin L expression. Studies in mammalian cell lines have indicated a role for cathepsin L in insulin receptor regulation. Whether the observed difference in cathepsin L activity has a link to *DAF-2* regulation in *C. elegans* needs to be further investigated.

Although the observed reduced protein turnover suggests efficient energy utilization in long-lived *daf-2* mutants, it does raise questions on the fate of damaged proteins and proteins prone to damage in these mutants. Reduced protein degradation has been shown to induce protein damage accumulation and protein aggregation, leading to cellular dysfunction. On our

quest for the answer we identified chaperons as possible candidates to reduce protein damage in the *daf-2* mutants. Whereas molecular chaperons are involved in protein folding, chemical chaperons are involved in protein stabilization. Several studies have revealed the importance of trehalose, glycerol and amino acids in this phenomenon. Adding to this, studies in *C. elegans* have revealed the necessity of trehalose synthesis for longevity. Hence we chose to investigate the role of trehalose in protein stabilization in the *daf-2* mutants. We identified that protein stability in the long-lived mutant was higher compared to the controls. With knock-down and rescue experiments we were able to identify that this induced protein stability in the long-lived *daf-2* mutant was partially provided by trehalose. On a general note, **we propose that increased protein stability conferred by trehalose in the long-lived *daf-2* mutant might render protein turnover and lysosomal digestion unnecessary for longevity**.

In conclusion, *daf-2* long-lived mutants are characterized by reduced protein turnover and reduced lysosomal components. The fact that *daf-2* worms did not show any life span effect on knockdown of the individual components, whether it is a case of redundancy or a lack of importance for lysosomal activity needs more clarity. The negative effect of reduced protein degradation in the *daf-2* mutant is compensated by induced protein stability rendered by trehalose. Even though overall proteostasis in the long-lived *daf-2* mutants is supposedly governed by protein stability rather than protein turnover, whether this is a common longevity phenomenon needs to be determined.

SAMENVATTING

Veroudering is een progressief, schadelijk proces, waarbij cellulaire, structurele en functionele achteruitgang met de leeftijd uiteindelijk leidt tot de dood van het organisme. Gezien de belangrijke gevolgen van de vergrijzing voor het sociaal en economisch welzijn van de mens, is er reeds heel wat onderzoek uitgevoerd in dit gebied.

Hoewel verouderingsonderzoek op verschillende modelorganismen gebeurt, van gist tot muizen, heeft de nematode *Caenorhabditis elegans* als modelorganisme op dit gebied verschillende voordelen. De korte levensduur en generatietijd, de kleine afmetingen, transparantie en geconserveerde signaalpaden die vergelijkbaar zijn met die van hogere eukaryoten, zijn enkele van de vele voordelen van *C. elegans* die het een uitstekend modelorganisme maakt voor verouderingsonderzoek. Daarbovenop zijn er verscheidene langlevende mutanten beschikbaar en tevens is de volledige genoomsequentie van het model gekend.

De belangrijkste en meest bestudeerde van alle levensduurverlengende mutaties is er een in het insulin/IGF-1 signaaltransductiepad: *daf-2*. Mutatie in dit signaalpad verlengt de levensduur van allerhande organismen, gaande van wormen, fruitvliegen tot muizen. In deze studie maken we gebruik van bovenstaande tools om de proteostase te analyseren in de langlevende *daf-2* mutant[2].

Proteostase of eiwithomeostase is het evenwicht tussen synthese, het opvouwen, onderhoud en afbraak van eiwitten. Op moleculair niveau is veroudering in *C. elegans* geassocieerd met verhoogde oxidatieve schade aan eiwitten, gecombineerd met verminderd herstel en onderhoud. Eiwitschade komt veelal voor onder de vorm van eiwitaggregatie, accumulatie en dysfunctie hetgeen kan leiden tot celdood. In langlevende *daf-2* insulin/IGF-1 mutanten echter, leken deze veranderingen aanzienlijk te worden vertraagd. Studies hebben onder andere aangetoond dat de *daf-2*-mutant minder gevoelig is voor eiwitaggregatie. Verschillende theorieën stellen in deze langlevende mutanten de accumulatie van eiwitschade verminderen door versnelde verwijdering en recyclage van eiwitten via verhoogde autofagie. Nochtans is het gekend dat net een verminderde eiwitsynthese de levensduur kan verlengen in verschillende organismen, hetgeen strijdig lijkt met voorgenoemde theorieën. Gezien

[2] In dit proefschrift hebben we gebruik gemaakt van een *glp-4* kiemcellijnloze achtergrondmutatie voor alle wormstammen, maar voor de duidelijkheid zullen we in deze samenvatting enkel verwijzen naar *daf-2* in plaats van naar *glp-4;daf-2*.

autofagie en eiwitsynthese zeer energieverslindende processen zijn, lijkt het idee van verhoogde eiwit turnover bij langlevende organismen minder waarschijnlijk. In een poging om meer duidelijkheid te krijgen over deze kwestie, hebben we de rol van eiwit turnover en autofagie in de *daf-2* mutant in detail bestudeerd.

Eiwit turover kan worden beschouwd als de integratie van eiwitafbraak en eiwitsynthese. Een manier om eiwitten af te breken is door autofagie, een katabool cellulair proces, geconserveerd in eukaryoten, dat afbraak en recyclage van beschadigde en onnodige eiwitten en organellen regelt. Macroautofagie, samen met ubiquitine-gemedieerde autofagie, is verantwoordelijk voor een groot deel van de 'huishoudelijke' afbraak van eiwitten. Anderzijds is autofagie eveneens betrokken bij stressresponsen, zoals honger en cellulaire immuniteit, maar ook bij ontwikkeling en cellulaire signaaltransductie. Het proces van autofagie moet strikt worden geregeld gezien hyperactivatie of volledige inhibitie van autofagie beide fataal zijn. Autofagie werd beschouwd als een proces dat essentieel is voor een lange levensduur in verschillende organismen, waaronder *C. elegans*. In dit kader hebben we de eiwitturnoversnelheid in de langlevende *daf-2* mutant grondig bestudeerd. We vonden dat deze sterk verlaagd is in de langlevende mutanten vergeleken met de controle. Deze resultaten leidden ons naar verder onderzoek betreffende de verschillende lysosomale componenten in de langlevende *daf-2* mutant.

Lysosomale functie hangt af van verschillende hydrolytische enzymen voor de afbraak, membraan-gebonden protonpompen voor lumenale verzuring, aminozuurpermeasen voor het vrijgeven van de verteringsproducten, en biogene en functionale factoren zoals *glo* en *lmp* genen. In onze experimenten stelden we een sterke vermindering van cathepsine L protease-activiteit en zure fosfataseactiviteit vast in de langlevende *daf-2*-mutant. Deze data ondersteunen onze eerdere waarnemingen omtrent de verminderde eiwit turnover in de *daf-2*-mutanten. Met behulp van een kwantitatief proteomics experiment toonden we aan dat verschillende lysosomale eiwitten neergereguleerd zijn in *daf-2*. Verder vonden we geen effect op levensduur in controle stammen of *daf-2* mutanten wanneer specifieke lysosomale componenten werden uitgeschakeld via RNAi. Dit werd geïnterpreteerd als een mogelijke redundantie binnen de lysosomale genfamilies of het feit dat de lysosomale functie niet bijdraagt aan levensduurverlenging. Daarnaast vonden we dat de *daf-2* lysosomen minder zuur waren of op zijn minst verminderd in aantal hetgeen de eerdere observatie van verminderde lysosomale activiteit ondersteunt. We vonden eveneens dat DAF-16, de transcriptiefactor die de levensduur verlengt in de *daf-2*-mutant, cathepsine L expressie actief

onderdrukt. Studies in zoogdiercellijnen wijzen een rol toe aan cathepsine L in de DAF-2 receptor regulatie. Of de waargenomen daling in cathepsine L-activiteit kan gelinkt worden aan DAF-2 regulatie in *C. elegans* moet verder worden onderzocht.

Hoewel de verlaagde eiwitturnover in langlevende *daf-2* mutanten een goodkopere energiehuishouding suggereert, blijft de vraag wat er gebeurt met de beschadigde en ongewenste eiwitten. Het is aangetoond dat verlaagde eiwitafbraak een accumulatie van beschadigde eiwitten veroorzaakt, hetgeen tot cellulaire disfunctie leidt. Op onze zoektocht naar het antwoord identificeerden we chemische chaperonnes als mogelijke kandidaten ter preventie van eiwitschade in *daf-2* mutanten. Daar waar moleculaire chaperonnes betrokken zijn bij het vouwen van eiwitten, zijn chemische chaperonnes betrokken bij eiwitstabilisatie. Verschillende studies hebben het belang van trehalose, glycerol en aminozuren aangetoond in dit proces. Eerder hadden *C. elegans* studies de noodzaak van trehalosesynthese ter ondersteuning van een lange levensduur reeds aangetoond. Vandaar dat we ervoor gekozen hebben om de rol van trehalose in eiwitstabilisatie in de *daf-2* mutanten dieper te onderzoeken. Vooreerst vonden we dat eiwitstabiliteit in de langlevende mutant effectief hoger was in vergelijking met de controles. Met knock-down- en rescue-experimenten konden we vaststellen dat dit fenomeen inderdaad deels te wijten is aan verhoogde trehalose-aanwezigheid in *daf-2*. Samengevat stellen we dat de **verhoogde trehalose-gemedieerde eiwitstabiliteit in de langlevende *daf-2* mutant een verhoogde eiwit turnover en lysosomale activiteit overbodig maakt**.

We concluderen dat *daf-2* mutanten gekenmerkt worden door verminderde eiwitturnover en verminderde lysosomale activiteit. Het feit dat het uitschakelen van afzonderlijke componenten van het lysosoom geen effect heeft op de levensduur van *daf-2* wormen is mogelijk te wijten aan redundantie van deze componenten of het gebrek aan belang van lysosomale activiteit voor de levensduurverlenging. Het mogelijke negatieve effect van een verlaagde eiwitafbraak in *daf-2*-mutanten wordt gecompenseerd door verhoogde eiwitstabiliteit via de inductie van trehalose. Hoewel proteostase in de langlevende *daf-2* mutanten wellicht wordt gedragen door eiwitstabiliteit eerder dan door eiwitturnover, is nog niet zeker of dit een algemeen geldend fenomeen is bij levensduurverlenging.

PART I

INTRODUCTION

CHAPTER 1

General Introduction, aims and outline of the thesis

1.1 Biology of ageing

1.1.1 What is ageing?

Ageing is an inevitable, progressive deterioration of physiological function, characterized by decreased fecundity and increased mortality with increased age (Bronikowksi and Flatt 2010; (Rose, 1991). In other words, ageing shall be defined as a series of cumulative, progressive, intrinsic and deleterious functional and structural changes that usually begin to manifest themselves at reproductive maturity and eventually culminate in death (Arking, 1998). It has been hypothesised that, as an organism ages, escalated molecular infidelity surpasses the turnover and repair potential and exposes the organism to age associated disorders and pathology (Hayflick 1995; 2000). Ageing has also been associated with the loss of equilibrium between different physiological systems and their ability to respond to changes in the environment, also known as homeostasis. The malfunction of organs is what results in increased disease susceptibility and an exponential increase in mortality. In a nut shell ageing is an irreversible, inevitable and intrinsic age-associated process with increase in vulnerability and loss of viability (Comfort, 1964) and a collection of changes that render an organism progressively more likely to die (Medawar, 1952).

1.1.2 The process of ageing in diverse animals

Even though ageing seems to be a common phenomenon, different organisms age at variable rates. Corals and sponges are reported to survive over 200 and 1000 years respectively. Organisms within the same phylum have shown difference in ageing. For example the nematode *Caenorhabditis elegans* has a normal life span of days to weeks compared to parasitic nematodes like *Ancylostoma duodenale* and *Necator americanus* which can survive up to 15 years (Finch, 1990). Interestingly, the polyphenic nematode *Strongyloides ratti* can survive about one year in a host as a parasitic form compared to the free living form which survives only 5 days (Gardner *et al.*, 2006). In addition nutritive conditions change ageing rates in the same organism like the long-lived invertebrate tubeworm *Lamellibrachia* which shows a shorter life span living in nutrient rich environments whereas the ones living in hydrocarbon seeps show a longevity of 170 to 250 years (Bergquist *et al.*, 2000). This indicates strong genetic control over life span, in addition to environmental modifications and stochastic factors (Finch and Kirkwood 2000). Ants and honeybees display polyphenic ageing phenotypes: the queen has the ability to live 15 years and is fed 10 times more often compared to workers, whereas the worker lives only 1.8 months. Even within the worker population a difference in life span occurs depending on the season, hormonal levels, and the

amount of motor activity. Organisms that lack senescence including *Cyanea capillata* and *Hydra* has been reported (Martinez 1998; Brock and Strehler 1963) but others refuted these claims (Bell 1988).

The remarkable difference in the pace of ageing between similar species suggests different processes that synchronize and accelerate the age-related diseases and weaknesses (Miller 1999; de Magalhaes 2003). Alternatively, there could be a single unified process driving ageing or unique degenerative processes for individual organs each contributing with varying degree to the pathological progression. The identification of the mechanism(s) causing the ageing process still constitutes the main quest in biogerontology. Throughout history, around 300 different hypotheses of ageing, which can be grouped into common themes, have been proposed (Medvedev 1990).

1.1.3 Theories of ageing

1.1.3.1 Evolutionary senescence theories of ageing

The earliest theories of ageing proposed that ageing is naturally selected, to prevent overcrowding and to ensure sufficient resources for the proceeding generation. This ensures resource expenditure on the young population, rather than to the worn-out and less reproductive older population. But this phenomenon of ageing, being genetically programmed, is unlikely as the idea is circular as it fails to explain why and how aged populations are worn out. The answer to the question how ageing could evolve lies on two key aspects of ageing: firstly organisms in the wild are faced with predation and other factors which kill them before they grow old (Moorad and Promislow 2010), and secondly, that the strength of natural selection declines with age (Medawar, 1952).

Group selection, Weismann (1891)

The theory of group selection proposes that ageing evolved on the basis that it benefits the group or species, even though it is detrimental to the specific individual (Weismann *et al.*, 1891). This is not accepted because nature selects more preferably the propagation of generation rather than its termination i.e. selection acts on the individual level rather than on a group.

Mutational accumulation theory, Medwar (1952)

Genes which are beneficial in the early stages of life are favoured over genes that are beneficial in later parts of life. For example, mutations that decrease reproduction and increase mortality in the early part of life (affecting majority of the population) will be more strongly selected against compared to mutations showing the same effect in the later part

(affecting only minority of the population). Hence the deleterious mutations, which act late in life, have the possibility to escape natural selection and accumulate in the offspring.

Antagonistic pleiotropy theory Williams (1957)

In the wild, the probability of an organism surviving to its old age is low due to higher chances of death due to predation and other external causes. Hence, natural selection weakens with age. Being strong in the early part of life, natural selection favours the antagonistically pleiotropic genes which are beneficial during early stages but harmful in older organisms. This explains why genes which benefit development and reproduction which usually occur in early part of life are favoured, even if they have deleterious effects late in life. This is in accordance with other theories which suggest that organisms with elevated risk of death due to external factors, reproduce and age faster (Blanco and Sherman, 2005).

Disposable soma theory, Kirkwood (1977)

The theory by Williams (1957) indicated that ageing occurs only in organisms with a separation of soma and germ line. In most organisms the soma is disposable, after the duty of reproduction is fulfilled by germline. In other words, the soma is supported only till reproduction is achieved. Initially it was considered that bacteria, in addition to some prokaryotes, algae, and protozoans, do not have a distinct germline and soma as they do not possess distinctly delineated age classes (Rose, 1991; Partridge and Barton, 1993). However apart from asymmetrically dividing bacteria (Ackermann *et al.*, 2003), symmetrically dividing *Escherichia coli* was shown to age as it shows a mother-offspring asymmetry (Stewart *et al.*, 2005). The disposable soma theory is an extension of the antagonistic pleiotropy theory and acts as a bridge to the mechanistic theories of ageing.

1.1.3.2 Mechanistic theories of ageing

Mechanistic theories of ageing consider ageing as a result of damage and dysfunction of cellular components rather than focusing on the evolutionary or genetically programmed perspective. These theories are referred to as damage-based theories of ageing.

Rate of living theory, Raymond (1928)

The rate of living theory associates ageing with the pace at which 'life is lived'. The theory hypothesised that, the faster the metabolic or biochemical activity of an organism is, the faster it ages. Accordingly, it was considered that life span of an organism depends on 1. the rate at which energy is utilised (metabolic rate) and 2. a genetically determined quantity of energy utilised during adult life span (metabolic potential). Primary experimental evidence supported this theory including life-span extension by dietary restriction in mice and

prevention of flying activity in housefly (Yan and Sohal, 2000). But the theory failed to answer observations such as the unaltered metabolic rates in flies grown under reduced physiological activity (Miquel *et al.*, 1976; Sohal, 1982) and the unaltered life span of rats kept at cooler temperatures making them consume more food (Holloszy and Smith, 1986). The theory further lost its ground with other observations. Firstly, the hypothesis by Rubner that bigger animals live longer came under attack as it is well evident that bigger animals are less prone to predation rather than ageing slowly (Stearns, 1992; de Magalhaes *et al.*, 2007). Secondly, possessing high metabolic rate, birds and bats are small animals like mice, but live much longer (Austad, 1997). Thirdly, there is no correlation between metabolic rate and longevity in mammals (de Magalhaes *et al.,* 2007). Even though the concept of longevity through energy metabolism involving insulin-like growth factor (IGF) persists (Kenyon 2010), the concept of rate-of-living is now refuted.

Free radical theory, Harman (1956)

The free radical theory relates ageing to accumulation of molecular damage imposed by reactive oxygen species (ROS), the by-products of oxidative metabolism (Harman, 1956). The free radical theory explained the rate of living theory as organisms with increased metabolic rates consume high amounts of oxygen thereby producing and accumulating more ROS, which in turn will cause more molecular damage leading to ageing. Arguments against this theory exist as the damage produced by ROS not only depends on its production but also on its removal and molecular repair. Further studies indicated that oxygen consumption is not proportional to ROS production (Brand, 2000) and the discovery of superoxide dismutase (SOD) (McCord and Fridovich, 1969; Fridovich, 1995) initially seemed to reduce the reliability of this theory.

The free radical theory was further refined to 1. highlighting the role of mitochondria, the key source of ROS as well as superoxide, (mitochondria contribute around 90% of total ROS production (Balaban *et al.*, 2005).2. emphasising the fact that oxidative damage is imposed by ROS which are not necessarily free radicals (peroxides) (Sohal and Weindruch, 1996) and 3. taking into account the presence of ROS removal or counteracting systems. Harman (1972) factored these and proposed the oxidative stress theory of ageing which proposes ageing as an effect of imbalance between ROS production and removal/repair systems (Harman, 1972). Hence, according to this theory, age-associated loss of function is due to the progressive and irreversible accrual of molecular oxidative damage (Sohal and Weindruch 1996).

It should be noted that, even though RS (reactive species) are kept under control, they are not completely eliminated from the system (Halliwell and Gutteridge, 2007). There exists a balance between the production and removal of these RS as they play an important role in redox signalling, regulation and signal transduction (Lander, 1997; Rhee, 1999). Oxidative stress is induced when this delicate balance is interrupted leading to oxidative damage (Sies 1991).

Several modes of defence exist to prevent ROS build-up 1. superoxide dismutase and catalase which can remove superoxide and hydrogen peroxide respectively 2. glutathione and glutathione peroxidase which can reduce H_2O_2 to H_2O. 3. vitamin C and E which can terminate lipid chain reactions employing peroxyl radicals. and 4. peroxiredoxins which are thioredoxin peroxidase enzymes and are shown to render protection against hydrogen peroxide in *C. elegans* (Olahova *et al.*, 2008).

Oxidative stress occurs when the cellular capacity to remove ROS and repair cellular damage is surpassed by concentration of ROS or its production, which results in oxidation of bio-molecules. The key targets of oxidative damage are proteins (Jung *et al.*, 2007). Oxidatively modified proteins increase with age and posses decreased function and altered stability (Gafni, 1997). The thiol group present in the cysteine residues renders them a susceptible target which can be either reversibly (sulfenic acid, disulfide bond formation) or irreversibly (sulfinic acid and sulfonic acids) oxidized (Thomas and Mallis, 2001; Eaton, 2006). Any damage to these cysteine residues may play a physiologically significant role as these residues form the catalytic or regulatory domains in most proteins and enzymes (Thomas and Mallis 2001). Out of all the types of oxidative damage, protein oxidation is the one which can cause a major imbalance in cellular homeostasis. The reason behind this is that these proteins are directly responsible for diverse enzymatic processes and structural support essential for cellular homeostasis (Sohal, 2002). This leads to functional defects, formation of deleterious fragmentations and formation of aggregates when proteins form promiscuous interactions (Sohal 2002). Around half of the intercellular proteins are oxidised (in a non-selective way) in ageing animals (Gafni, 1997). Oxidations involving one or two amino acids usually have a minimal effect on protein function (Levine *et al.*, 1996), but certain proteins are oxidised selectively by ROS at specific sites regulating their function reversibly like calmodulins, calcium pumps, and calcium channels (Eu *et al.*, 2000; Squier and Bigelow, 2000). Functional inactivation of proteins can also occur by oxidation of specific amino acids including Cys, Met, Trp, Tyr, Pro, Lys and His (Berlett and Stadtman, 1997). Carbonyl

addition (carbonylation) is the most common post modification caused by oxidative stress (Dukan *et al.*, 2000). The key proteins and enzymes affected by ROS are the proteins in close proximity to the ROS source. These are usually the mitochondrial proteins like aconitase, an enzyme involved in the citric acid cycle (Yan *et al.*, 1997), whereas in *C. elegans* vitellogenin is a common target (Goto *et al.*, 1999). In addition, ageing induces several other protein modifications including alpha amidation diamide, glutamate oxidation and proline oxidation(Stadman 1995; Rothstein 1975, 1979).

By maintaining the internal cellular environment in a reduced state, thiol groups in cysteine residues are protected from oxidation (Mannervik *et al.*, 1983; Arner and Holmgren, 2000) but problems arise when internal conditions change while the organism starts ageing. Studies in humans have reported the age-induced decrease in plasma redox ratio (Ramirez *et al.*, 2007; Kemp *et al.*, 2008). This can expose the thiol groups for oxidative damage which can then lead to misfolding of proteins. To be able to function normally, a protein has to maintain or regain its tertiary folded structure which is achieved by the help of molecular chaperones and heat shock proteins (Saibil, 2008). Any failure in attaining the folded structure can lead to pathologies as seen in neurodegenerative diseases like Parkinson's, Huntington and Alzheimer's disease.

The loss in physiological stress endurance and cellular function through aggregation of proteins has been attributed to oxidation and nitration of proteins. Once when protein aggregation starts, these sites can act as nucleation sites for further unrelated proteins to aggregate. These aggregations can lead to ordered filamentous structures called amyloid fibrils which are resistant to proteases and still can act as nucleation sites. Essential chaperones and proteases could be removed from service by these nucleation sites. Amyloid toxicity is closely linked to calcium homeostasis and oxidative stress has been shown to modulate calcium homeostasis in ageing animals and in disease conditions (Smith *et al.*, 1997). Proteasomal and lysosomal proteases could be inhibited by these oxidized or cross-linked proteins (Bahr and Bendiske, 2002; Grune *et al.*, 2003). In Alzheimer's disease, protein synthesis rates were found to be greatly reduced (Langstrom *et al.*, 1989; Ding *et al.*, 2005) leading to a great amount of damage to ribosomal complexes (Langstrom *et al.*, 1989; Ding *et al.*, 2005; Honda *et al.*, 2005), which in turn lead to more protein damage and misfolds.

Even though being the most extensively studied theory of ageing, the oxidative stress theory remains unconclusive as it lacks solid experimental evidences to say that oxidative damage is

the reason for ageing (Muller *et al*., 2007). The theory proposes an up regulated antioxidant system and reduced oxidative stress whereas in the longest living rodent, the naked mole rat antioxidative capacity was reduced in addition to increased oxidative damage (Andziak and Buffenstein 2006; Andziak *et al*., 2006). In *C. elegans* experimental inactivation of antioxidant system in both the IIS (mutantion in Insulin/IGF-1 pathway) and DR (dietary restriction/reduced food uptake) conditions failed to shorten lifespan (Yang *et al*., 2007; Doonan *et al*., 2008; Honda *et al*., 2008; Van Raamsdonk and Hekimi 2009; Yen *et al*., 2009). On the other hand intervention studies with administration of pro-oxidants or antioxidants had its drawback. Many ROS species including H_2O_2 and 0_2 functions in molecular signalling (Finkel and Holbrook 2000) and any interference with this essential function would lead us to interpret a change occurring in redox signalling as ROS per se effect on lifespan (Lapointe and Hekimi 2010; Back *et al*., 2012; Gems and Doonan 2009). Moreover in *C. elegans* overexpression of enzymes which render elevated oxidative stress resistance generally failed to extend lifespan (Valentini *et al*., 2012), and in some cases even shortened it (Doonan *et al*., 2008).

Although the relationship between ROS induced damage and ageing is undeniable, the amount of controversies and conflict with the basic predictions of oxidative stress theory led many to conclude the theory unacceptable (Lapointe and Hekimi 2010; Gems and Doonan 2009).

Protein error theory of ageing, Orgel (1963)

The less famous error catastrophe theory, which is filled with controversies, associates senescence to minute alterations in proteins involved in protein synthesis (Orgel, 1963). The central idea of this theory is that translational fidelity decreases with age. Even though alteration in the amino acid sequence of enzymes involved in metabolic pathways may occur, these proteins would be diluted out in the course of protein turnover. However, any alterations in the transcription/translation machinery would spread more errors. Although induction of protein errors was shown to accelerate ageing in bacteria and human cells (Rattan 2003;Holliday, 1996; Rattan 1996; Nystrom, 2002), other evidence suggests that altered proteins are broken-down more rapidly than their normal counterparts (Goldschmidt, 1970). Several groups brought to light that the fidelity of protein synthesis is negligibly affecting ageing (Parker *et al*., 1981; Vanfleteren and De Vreese, 1994).

Hyperfunction theory of ageing, Blagosklonny (2008)

With all the damage-based theories pointing to accumulation of molecular damage as a cause of ageing, the hyperfunction theory proposed by Blagosklonny represents the lack in termination of developmental programme as a cause of ageing. It was also argued that molecular damage does accumulate but contributes less to mortality. It is because hyperfunction induces death far ahead of damage accumulation reaching a suffiecient level to cause life-threatening pathology (Blagosklonny 2008). Experimental evidences supporting this theory include the extension of lifespan when growth promoting insulin/IGF-1 pathway and other protein synthesis pathways are blocked (Kenyon 2010). Additionally the hyperfunction theory is supported by the antagonistic pleiotrophic theory which indicates a lifespan extension when essential developmental genes are inhibited during adulthood. This was evident in *C. elegans* as knock down of several developmentaly essential genes during adulthood induced longevity (Chen et al., 2007). Hyperfunction/ hypertrophy in relation to ageing have been evaluated in *C. elegans*(Gems and la Guardia 2012).Even though several hypertrophy activated malignencies occur in ageing *C. elegans* only overproduction of yolk has a prominant effect on lifespan. Even after cessation of oogenesis yolk accumulates in high quantities in the body cavity (Herndon *et al.*, 2002). Knock-down of yolk synthesis genes which are essential during development and reproduction have shown to extend lifespan (Murphy et al., 2003) indicating that yolk accumulation can lead to mortality. This is interesting as yolk synthesis is shown to be attenuated in *daf-2* mutants (DePina et al., 2011) which are long lived. On the other hand two hallmark charecteristics of hypertrophy induced malignencies, germline atrophy and tumour like mass formation, in *C. elegans* uterus appear not to contribute to ageing process (Gems and de la Guardia 2012) Although hyperfunction seems to be influencing ageing, in *C. elegans* reduced protein synthesis is not sufficient to extend lifespan and requires expression of stress-responsive genes (Wang et al., 2010). Hence the reliability of this theory is still under consideration.

1.2 The model organism *Caenorhabditis elegans*

Caenorhabditis elegans is a free living, non-parasitic nematode which colonizes nutrient and microorganism rich habitats including soil and decomposing vegetation all around the world. It is a eukaryote, transparent, saprophytic nematode that feeds on bacteria and fungi and its habitat varies from soil to degrading leaves and foliage. For laboratory use *C. elegans* is often grown on a lawn of *E. coli* bacteria. It is a pseudocoelomate lacking a distinct respiratory and

circulatory system. After the introduction of this species as potential model for Dev Biol and neurobiology by Sydney Brenner in 1963, its application in various fields has been explored. Thanks to its transparent nature and small size, the developmental pattern of all the 959 somatic cells has been tracked by John Sulston in 1980 (Sulston and White, 1980; Sulston *et al.*, 1983). This served as a tool for studying the genetics of cell fate. In 1998, *C. elegans* was the first multi-cellular organism to have its genome completely sequenced and the resulting genome database is currently freely available online. A important method of gene silencing through RNAi was first identified and applied in *C. elegans* and inferred that around 10% of the total genes are essential (Kamath *et al.*, 2003). The ease of maintaining *C. elegans* cultures in the lab, the fast and convenient 12-hour embryogenesis and 60-hour development to adulthood render them the easiest multicellular model organism. *C. elegans* is used as an important model system in cell biology, ageing, neuroscience, genomics and other biological research fields.

1.2.1 Morphology

C. elegans is a multi-cellular animal and its size ranges between 1 – 1.2 mm as an adult. The body is pseudocoelomate and has two cylinders: the outer one, which includes the muscle cells, hypodermis and cuticle, and an inner cylinder which includes the pharynx, gut, and rectum. The gonads form an additional tubular structure in the pseudocoel. The hermaphrodites are proterandrous in which the two gametogenetic phases follow each other in the same ovotestis (Mounier Brun 1980). The hermaphrodite has two bilaterally symmetrical gonad arms which include the germline (covered by the gonadal sheath), oocytes and a chamber where oocytes are fertilized called spermatheca. A complete anatomical description with electron microscopy has been established on the cell lineage level (Brenner 1973; Lewis and Fleming 1995). Males occur less frequently by spontaneous non-disjunction in the hermaphrodite germ line and constitutes around 0.1% of the wild-type population. In males, a single armed gonad forms the reproductive system and opens to the exterior through the epithelial chamber called the proctodeum ending in a cloaca. Two sclerotic spicules are present in the proctodeum and are used to locate and open the vulva of the hermaphrodite during sperm transfer (Garcia *et al.*, 2001). In the hermaphrodite 302 neurons forming around 8000 synapses construct the nervous system. There exists a so called brain which is a ring of neurons circling the pharynx acting as sensors of the worm. The male has 89 additional neurons including several classes of tail sensilla.

1.2.2 Life cycle and development

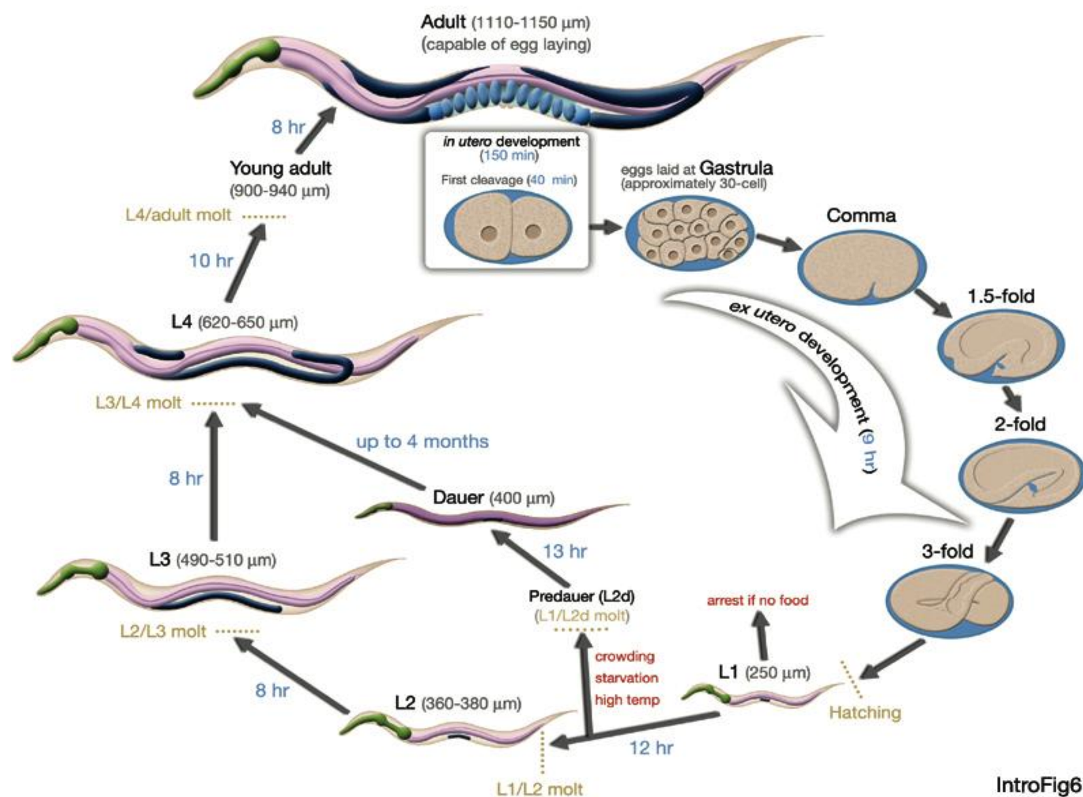

Figure 1: A representation of the lifecycle of *Caenorhabditis elegans* at 22°C (Altun and Hall 2005 http://www.wormatlas.org/ver1/handbook/contents.htm).

Two stages constitute embryogenesis in *C. elegans* namely 1. proliferation and 2. organogenesis/morphogenesis (Sulston *et al.*, 1983). In the proliferation stage a single cell divides to give 558 undifferentiated cells (Zuckerman and Dusenbery, 1980; Wood, 1988). Organogenesis dictates the stop of cell division and initiation of differentiation of cells. This leads to a threefold elongation of the embryo leading to a fully differentiated animal. Feeding post hatching acts as a trigger for the postembryonic development. Three hours after hatching, the postembryonic developmental programme starts and cell division resumes (Ambros, 2000). Absence of food post hatching leads to developmental arrest which can be survived up to 6 to 10 days. On availability of food the hatchlings proceed normal development (Johnson *et al.*, 1984; Slack and Ruvkun, 1997). Larvae pass through 4 stages (L1 to L4) to adulthood (Fig.1). Each stage is clearly demarked by the appearance of a moulting lethargus when the old cuticle is shed and a new stage-specific cuticle synthesised. Three steps govern the cuticle formation. At first, the cuticle is separated from the hypodermis (apolysis), followed by the synthesis of a new cuticle from the hypodermis and

finally the shedding of the old cuticle (ecdysis). At each moult, the protein composition and ultrastructure of the cuticle was found to be different (White, 1988).

Under unfavourable environmental conditions, at the end of L2 stage, the animal can go into an arrested state of growth called the dauer larva (Klass and Hirsh, 1976). Unfavourable conditions vary from lack of food, high temperature or the presence of dauer pheromone indicating overcrowding. The presence or absence of these factors dictates the L2 larvae to moult to an L3 or to get arrested in dauer state (Riddle, 1988). Dauer larvae are marked by a very thin morphology with an altered thick cuticle. A cuticle block seals the buccal and anal cavity. The intestinal and pharyngeal lumens are shrunken showing small and indistinct microvilli in the intestine and the gut cells show a dark appearance. The dauer decision has no effect on the post-dauer life span as the dauer stage is a non-ageing state (Klass and Hirsh, 1976). Dauers show reduced movement and feeding is completely arrested. During the diapauses as these animals stop feeding, they consume their reserves transforming fat into glucose with the help of glyoxylate cycle (Riddle and Albert 1997). In general aerobic respiration (citric acid cycle and mitochondrial respiratory chain) is down regulated and privilege is given to glycolysis and fermentative metabolism (glyconeogenesis, glyoxylate pathway and trehalose biosynthesis) (Holt and Riddle 2003). Dauer larvae exhibit a reduced metabolic rate including oxygen consumption (Houthoofd *et al.*, 2002; Vanfleteren and De Vreese 1996), reduced ATP levels and increased intracellular pH (Wadsworth and Riddle 1988). They show around 11.6 fold reduction in flux of metabolites through the TCA cycle relative to adults (O'Rior-dan and Burnell 1989) in addition to 3.8 fold reduction in oxygen consumtion rate relative to L3 stage (Vanfleteren and De Vreese 1996). Eventhough dauer larvar possess reduced transcriptional activity relative to other larval stages (Snutch and Baillie 1983) they are competent to initiate and elongate transcripts. In this stage *C. elegans* can survive for more than 3 months before reserves are emptied (Klass 1977). On return of favourable conditions and food, the worm exits dauer stage, starts feeding 2-3 hours later and gradually moults to L4 stage around 10 hours later.

1.2.3 Ageing in *C. elegans*

Ageing in *C. elegans* is distinct both in appearance and behaviour. Aged worms move, feed and defecate slowly compared to younger animals (Klass, 1977; Bolanowski *et al.*, 1981; Duhon and Johnson, 1995). Morphologically, aged animals appear distorted, rough and lumpy whereas physiologically they progressively fail to respond to touch, move and pump

food (Huang *et al.*, 2004). Their sensitivity to thermal stress (Lithgow *et al.,* 1995) and oxidative stress increases (Honda *et al.*, 1993; Larsen, 1993; Darr and Fridovich, 1995). The intestine of the worm was thought to slowly accumulate the ageing pigment lipofuscin leading to auto-fluorescence and structurally become flaccid (Garigan *et al.*, 2002; Gerstbrein *et al.*, 2005). However, this was refuted by a recent study showing that the blue fluorescent material in the worm gut is not age pigment and does not accumulate gradually over age in individuals (Coburn *et al.*, 2012). In *C. elegans* in addition to ageing associated degeneration of intestine and muscle cells, morphological changes including blebbing and beading have been reported in the neuronal tissue (Tank *et al*., 2011; Toth *et al*.,2012; Pan *et al*.,2011). Although the molecular mechanisms underlying this morphological changes have not been uncovered, the conserved pathways involved (insulin and MAPK) have been established (Chew *et al*., 2013). The body cavity tends to accumulate yolk protein whereas the muscle sarcomeres are disorganized in aged worms (Herndon *et al.,* 2002).

1.3 Signaling pathways regulating ageing in *C. elegans*

1.3.1 Insulin/IGF-1 pathway

The insulin/insulin-like growth factor 1 (IGF-1) pathway is a life span-regulating, evolutionarily conserved pathway common among many species including *Drosophila melanogaster* (Clancy *et al.*, 2001; Tatar *et al.*, 2001), *C. elegans* and mammals (Benyoucef *et al.*, 2007). Homologous to the mammalian Insulin and IGF-1 pathways, the *C. elegans daf-2* signalling pathway is part of the endocrine system responsible for development, metabolism and longevity (Finch and Ruvkun, 2001). *daf-2* is the receptor that mediates the only insulin-like signalling pathway in *C. elegans* (Fig 2) and the signal is channelled through the phosphatidyl 3-OH kinase AGE-1, and kinases PDK-1, AKT-1 and AKT-2 to DAF-16, an ortholog of human FOXO fork-head transcription factor (Morris *et al.*, 1996; Finch and Ruvkun, 2001; Garofalo, 2002; Nakai *et al.*, 2002; Bluher *et al.*, 2003; Holzenberger *et al.*, 2003).

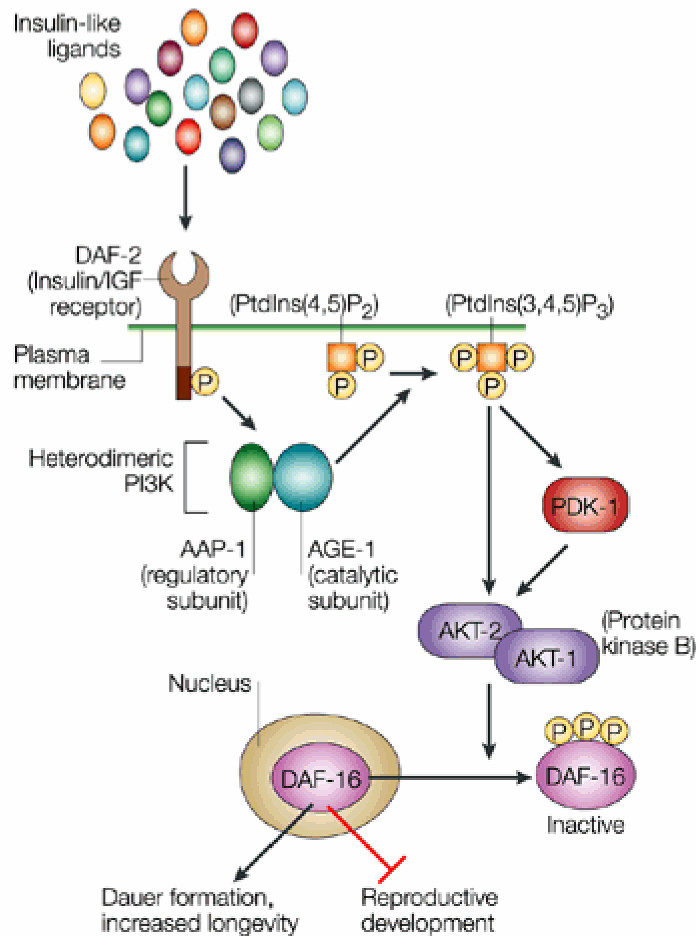

Figure 2: Representation of the *daf-2* IGF-1 pathway in *Caenorhabditis elegans* (Partridge and Gems, 2002)

Reduced *daf-2* signalling has been shown to extend *C. elegans* life span up to twofold (Wadsworth and Riddle, 1989; Kenyon *et al.*, 1993; Kimura *et al.*, 1997) and characterised by biochemical changes like increased fat storage, increased expression of antioxidant enzymes (Larsen, 1993; Vanfleteren and De Vreese, 1994; Honda and Honda, 1999), increased resistance to heat (Lithgow *et al.*, 1995), hypoxia (Scott *et al.*, 2002), bacterial pathogens (Garsin *et al.*, 2003) and heavy metals (Barsyte *et al.*, 2001). DAF-16, the downstream mediator of life span extending genes is negatively regulated by the Ins/IGF-1 pathway by phosphorylation and nuclear exclusion (Riddle *et al.*, 1981; Kenyon *et al.*, 1993; Lin *et al.*, 1997; Ogg *et al.*, 1997). Mutation of specific components of the Ins/IGF-1 pathway leads to DAF-16 nuclear translocation mediating transcription of life span-extending genes (Lin *et al.*, 1997; Ogg *et al.*, 1997; Lee *et al.*, 2001). Although DAF-16 binding sites are identified in many genes, only few are responsive to mutation in *daf-2* and few genes mediate life span

extension under low *daf-2* signalling (Furuyama *et al.*, 2000; Lee *et al.*, 2003). It is interesting that none of the DAF-16 target genes has shown similar life span extension when repressed or expressed all alone. Hence the life span extension observed in the Ins/IGF-1 mutants proves to be a combined expression and repression of several genes involved in different pathways that influence life span extension (Murphy *et al.*, 2003; McElwee *et al.*, 2004). The Ins/IGF-1 pathway is also involved in sensing stress, food availability and crowding during development and reversibly channels development to enter a stress-resistant larval dauer stage. On return of favourable conditions, the worms exit this stage and develop into adults (Cassada and Russell, 1975; Riddle *et al.*, 1981). *daf-2* mutants show an up-regulation of stress responsive genes like *ctl-1* (catalase), *sod-3* (manganese superoxide dismutase), *hsp-16.2* and pathogen resistance genes like cytochrome P450s, genes involved in carbohydrate, lipid and steroid metabolism whereas a down regulation was found for genes involved in apolipoprotein binding, neuronal signalling, DNA replication, RNA binding and protein turnover (Murphy *et al.*, 2003). In addition to these, *daf-2* repression also causes disaggregation and therefore protects the worms from polyQ and Aβ (1-42) toxicity (Morley *et al.*, 2002; Hsu, 2003; Florez-McClure *et al.*, 2007).

Around 40 insulin-like peptides are identified in *C. elegans*, and they were found to be primarily expressed in the neurons and few in the intestine (Pierce *et al.*, 2001; Li *et al.*, 2003; Murphy *et al.*, 2003; Murphy *et al.*, 2007). In response to environmental signals these peptides antagonise (INS-1 and INS-18) or agonise (INS-7 and DAF-28) signalling through the DAF-2 receptor. In response to environmental cues, the sensory neurons express INS, which induce the IGF-1 pathway in peripheral tissues. On the other hand, the intestine, the central nervous system and the gonad express secondary proteins which regulate DAF-16 in the whole worm. The neuronal expression/repression of *daf-2* is a deciding factor for life span extension (Apfeld and Kenyon, 1998; Wolkow *et al.*, 2000) whereas *daf-16* expression in other tissues in addition to neurons plays a key role in life span extension (Libina *et al.*, 2003). In addition to these controls in *C. elegans*, a feedback loop is present by which the expression of *ins-7* (a *daf-2* agonist) in the intestine is repressed by DAF-16 which further propagates DAF-16 activity (Libina *et al.*, 2003; Murphy *et al.*, 2007).

IGF-1 independent pathways converge in DAF-16

Several pathways modulate DAF-16 and its life span-extending effect independently of *daf-2* (Fig. 3). For example, life span extension via mutation in the stress responsive *sir-2.1* gene is DAF-16 dependant (Tissenbaum and Guarente, 2001). This pathway requires two 14-3-3

proteins that were shown to interact with DAF-16 in the cytosol after which this complex interacts with SIR-2.1 in the nucleus, which then leads to expression of the MnSOD *sod-3*, thioredoxins and phase II detoxification genes of the *gst* family (Berdichevsky *et al.*, 2006).

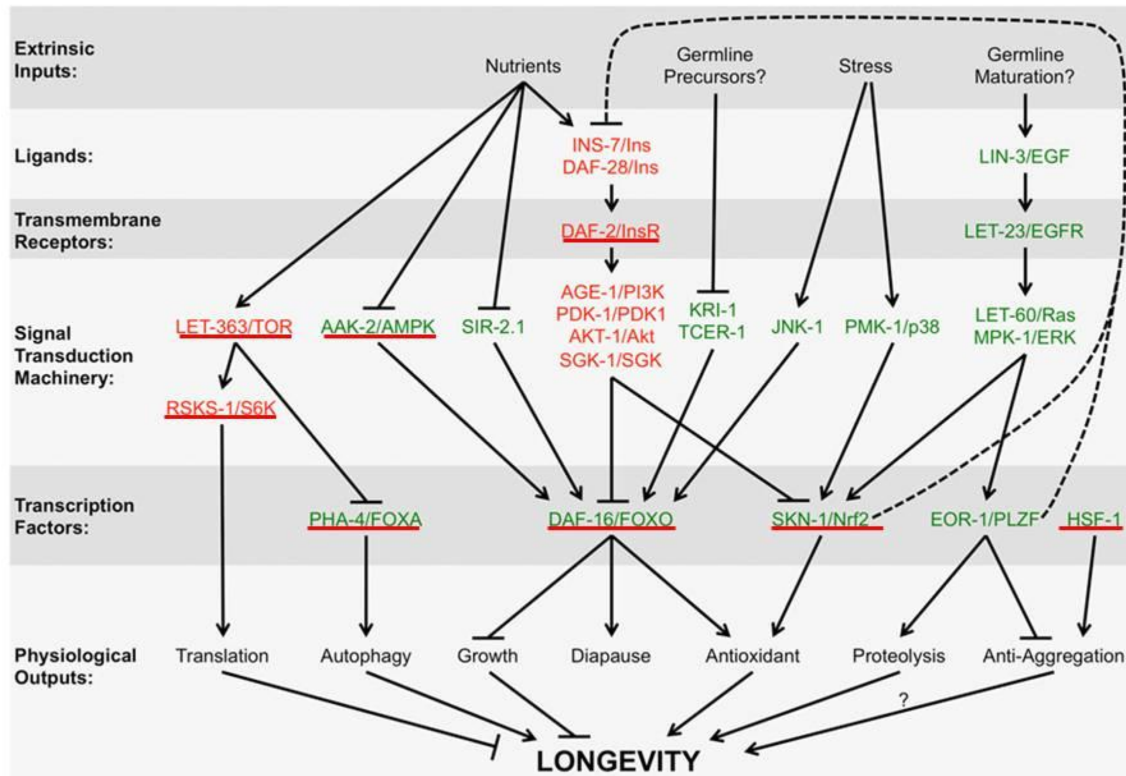

Figure 3: A schematic representation of multiple pathways regulating longevity (Rongo, 2011). Genes whose wild-type function ultimately acts to shorten lifespan are represented in red letters, whereas genes whose wild-type function ultimately acts to enhance lifespan are represented as green letters. Pathways important to this work are highlighted with red line.

In another parallel pathway interactions happens between the 14-3-3 proteins and phosphorylated DAF-16 which is retained in the cytosol (van der Heide *et al.*, 2004; Berdichevsky *et al.*, 2006). When the Ins/IGF-1 signal is repressed, dephosphorylation occurs and the interaction between the 14-3-3 proteins and DAF-16 is countered and DAF-16 starts translocation to the nucleus.

Another pathway indicates specific phosphorylation of DAF-16 by JNK-1 (c-Jun N terminal Kinase) on stress cues, leading to DAF-16 nuclear translocation and binding to SIR-2.1 in a 14-3-3 protein dependent manner, which in turn leads to the expression of DAF-16 downstream genes.

JNK-1 can specifically phosphorylate DAF-16, independently of the IGF signalling pathway, mediating DAF-16 nuclear translocation with subsequent increases in stress resistance and life span. Activation of JNK-1 requires phosphorylation by JKK-1 (Oh *et al.*, 2005).

The MST-1 like protein CST-1 works similar to JNK-1; it can phosphorylate DAF-16, breaking its interaction with 14-3-3 proteins and allowing DAF-16 to translocate to the nucleus. CST-1 has the ability to further extend life span of *daf-2* mutant worms (Lehtinen *et al.*, 2006).

The transcription factor SKN-1 is also involved in stress tolerance by induction of phase II detoxification response. One isoform, localized to ASI neurons, is involved in dietary restriction induced longevity and the other isoform, localized in the intestine, is responsible for stress resistance (An and Blackwell, 2003). GSK-3 and IGF kinases AKT-1, AKT-2 and SGK-1 phosphorylate SKN-1 preventing its nuclear accumulation whereas reduced IGF translocates it to the nucleus (Tullet *et al.*, 2008). The life span extension effect of SKN-1 over-expressing worms is independent of DAF-16 and thus acts in parallel (Tullet *et al.,* 2008).

In addition to these germline signalling pathways converge on DAF-16 regulating longevity. Hormones synthesised by the reproductive system can influence ageing in *C. elegans* (Yamawaki et al., 2010). *C. elegans* lacking germline have been shown to live 60% long compared to fertile worms (Hsin and Kenyon 1999). This lifespan extending effect requires an intact somatic gonad and DAF-16 nuclear localization in the intestinal cells (Hsin and Kenyon 1999; Lin *et al*., 2001). The longevity effect is lost if the entire gonad is removed indicating the channelling of longevity signals through this tissue to the intestine in particular (His and Kenyon 1999). Unlike DAF-16 nuclear localization during low IIS, germline mutants require the nuclear receptor DAF-12 for the translocation (Berman and Kenyon 2006). DAF-16 in the nucleus intereacts with elongation factor and RNA binding protein TCER-1 and PHI-62 respectively to extend lifespan (McCormick et al., 2012; Ghazi et al., 2009).

1.3.2 Dietary Restriction (DR)

Reduced intake of food without malnutrition has been identified to induce life span extension and better health in various species including *Saccharomyces cerevisiae* (Jiang *et al.*, 2000), *C. elegans, Drosophila melanogaster* (Partridge *et al.*, 1987; Chapman and Partridge, 1996), fish, mice (Weindruch *et al.*, 1986) and rhesus monkeys (Lane *et al.*, 2002).

A reduction in fertility was observed in dietary restricted worms (Holehan and Merry, 1986; Chapman and Partridge, 1996) which could be an adaptation to increase fitness by diverting nutrients away from reproduction towards somatic maintenance (Holliday, 1989) or to avoid malnourished offspring. To confer dietary restriction on *C. elegans* three major techniques are used: 1. bacterial dilution, 2. behavioural mutants which are slow feeders, and 3. axenic or semi-defined liquid medium without bacteria. Dietary restriction by an approximate tenfold bacterial dilution induced life span extension (Houthoofd *et al.*, 2003; Bishop and Guarente, 2007). Eat mutants show defective pharyngeal pumping, resulting in reduced feeding, was shown to extended life span as well (Avery, 1993; Lakowski and Hekimi, 1998; Hsu, 2003). Axenic medium is a sterile semi-defined (Houthoofd *et al.*, 2002) or completely defined (Szewczyk *et al.*, 2003) liquid culture medium without *E. coli* and has been shown to increase *C. elegans* life span by 50-100% (Vanfleteren and Braeckman, 1999); Vanfleteren 1980).

1.3.2.1 PHA-4 as a mediator of the dietary restriction effect

The extension in life span due to caloric restriction could be because of two reasons: 1. reduced caloric intake may lead to reduced metabolic rate and a concomitant reduction in ROS production, leading to less damage and increased life span, or 2. the effect of caloric restriction on several signalling pathways. Upon dietary restriction, SMK-1 interacts with a fork head transcription factor PHA-4, an ortholog of human FOXA family of transcription factor to regulate transcriptional response. Loss of PHA-4 abrogates the dietary restriction-induced life span extension and was identified to act independently of other life span extending pathways (Houthoofd *et al.*, 2003; Panowski *et al.*, 2007). Over-expression of PHA-4 increased life span independently of DAF-16 even though both transcription factors compete for overlapping target genes. PHA-4 is involved in the expression of *sod-1, 2, 4* and *5* whereas DAF-16 is involved in expression of *sod-1, 3* and *5*. During embryogenesis and larval stages, PHA-4 is expressed in the pharynx and intestine, whereas in adulthood it is expressed in the intestine and few neurons in the head and tail. During development, adulthood and dietary restriction the expression pattern is unchanged and in all cases PHA-4 localizes to the nucleus. An 80% increase in expression levels of PHA-4 was observed in dietary restricted worms (Panowski *et al.,* 2007).

1.3.2.2 The role of SKN-1 in dietary restriction

The transcription factor SKN-1, which acts in the two sensory ASI neurons located in the head of the worm, is also indispensable for life span extension caused by dietary restriction

(Fig 4). The neurons then signal with a cell non-autonomous endocrine mechanism to the non-neuronal body tissues to increase metabolism (Bishop and Guarente, 2007). Some isoforms of SKN-1 are expressed from downstream operons of *bec-1*, a gene which is involved in autophagy induction during caloric restriction (Yue *et al.*, 2003). Role of SKN-1 in regulation of nutritional stress was evident with studies indicating impairment of response to dietary restriction on mutation of four SKN-1 alleles. In the neurons SKN-1 acts independently of the *daf-2* pathway as mutation in *skn-1* was not able to impair longevity induced by *daf-2* mutation (Bishop and Guarente 2007). Lifespan extension by SKN-1 is independent of DAF-16 (Tullet *et al.,* 2008).

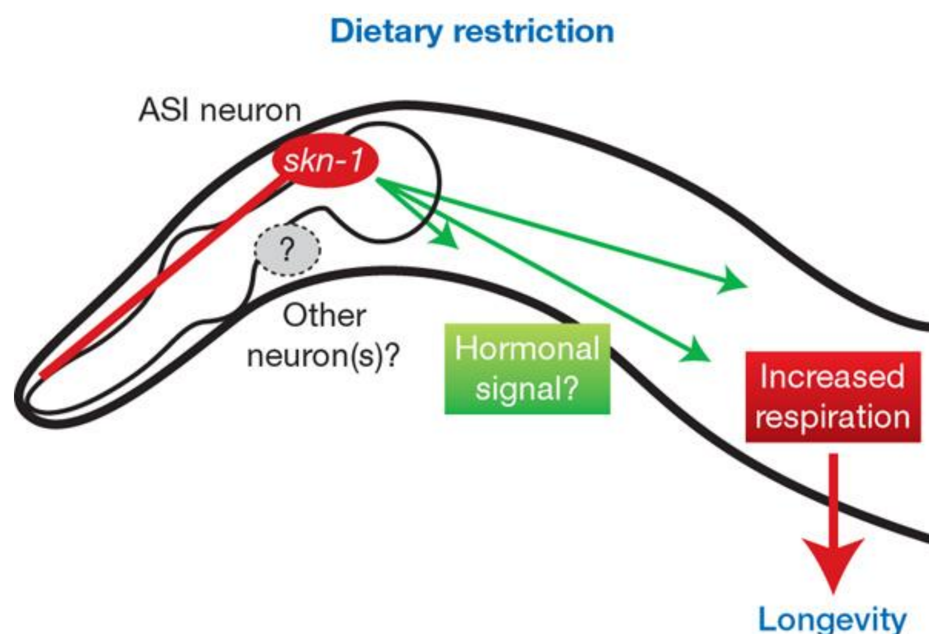

Figure 4: SKN-1 channels signals through the ASI neurons during dieterary restriction (Bishop and Guarente, 2007).

1.3.2.3 SIR-2.1 in dietary restriction

The NAD^+ dependent histone deacetylases named sirtuins and related molecules have been implemented in dietary restriction-induced life span extension (Blander and Guarente, 2004) in yeast (Lin *et al.*, 2000) and fruit fly (Rogina and Helfand, 2004). *sir-2.1*, a *C. elegans* sirtuin homologue, was shown to be essential for caloric restriction induced protection against dopaminergic neurodegeneration (Jadiya *et al.*, 2011). However, this data has been the subject of much debate as studies have raised concerns about the robustness of the effects of sirtuins in *C. elegans* and *Drosophilla* (Burnett *et al.*, 2011; Viswanathan and Guarente, 2011). These studies indicate abrogation of *sir-2.1* over expression induced longevity on

standardisation of background and use of appropriate controls (Burnett *et al.*, 2011) and proposed *sir-2.1* overexpression not sufficient to increase lifespan.

1.3.2.4 TOR signaling

Dietary restriction, heat/oxidative/hypoxic stress and Ins/IGF signalling interact synergistically in extending life span which suggests that these pathways run in parallel, possibly ending up in a common mechanism controlling life span (Houthoofd *et al.*, 2003; Wullschleger *et al.*, 2006). TOR kinase, which plays a role in the amino acid sensing pathway controlling cell growth (Oldham and Hafen, 2003; Hay and Sonenberg, 2004), is a candidate pathway induced by dietary restriction. Translation is induced by TOR through S6 kinase (S6K) and 4E-BP phosphorylation when levels of available amino acids are high. Reduced TOR signalling during low levels of available amino acids induces macroautophagy. S6K is involved in translation elongation and elF4E released by 4E-BP initiates mRNA translation (Wullschleger *et al.*, 2006). Macroautophagy provides the worm with the essential amino acids required to maintain translation of house-keeping genes and plays a key role in protein and organelle turnover (Levine and Klionsky, 2004). Cross talk between Ins/IGF and TOR does exist as inhibition of the *C. elegans* TOR and RAPTOR (regulatory associated protein of TOR) homologues *let-363* and *daf-15* results in a mid-larval dauer like phenotype. If there was no cross talk, Ins/IGF signalling would have prevented dauer larva formation (Long *et al.*, 2002; Jia *et al.*, 2004; Walker *et al.*, 2005). The complex interactions between Ins/IGF signalling and TOR is further exemplified by the fact that DAF-16 negatively regulates DAF-15 (RAPTOR) (Jia *et al.,* 2004) and that TOR knockdown (*let-363* RNAi) does not further extend the long life span of the *daf-2* Ins/IGF mutants. However, both pathways often seem to work independently: life span extension caused by *let-363* RNAi (TOT knockdown) is independent of DAF-16 (Vellai *et al.*, 2003).

As can be expected from its function as an aminoacid sensor controlling cell growth, TOR (*let-363*) mutants have reduced protein synthesis (Vellai *et al.*, 2003; Hansen *et al.*, 2007). In concert with this, TOR mutants also show increased autophagy as seen by up-regulation of *lgg-1*::GFP foci, which is a reporter of autophagic function. A similar induction of autophagy was also seen under dietary restriction (Hansen *et al.*, 2008). Both TOR and PHA-4 are required for autophagy induction under DR conditions and autophagy is required for DR induced life span extension (Panowski *et al.*, 2007). Even though PHA-4 is required for life span extension in TOR/*let-363* or S6K/*rsks-1* mutants, PHA-4 was not required for the life

span extension in eIF4E/*ife-2* mutants (Sheaffer *et al.*, 2008) suggesting that they act on longevity through distinct mechanisms.

Induction of autophagy was observed when FOXO was activated in *C. elegans,* mammals (Mammucari *et al.*, 2007), *Drosophila* (Juhasz *et al.*, 2007) and mouse (Zhao *et al.*, 2007) and conversely, a reduction in starvation-induced autophagy when FOXO was knocked-down (Juhasz *et al.,* 2007). DAF-16-induced autophagy is independent of mTOR (Mammucari *et al.,* 2007) however knockdown of mTORC2/ RICTOR showed FOXO-mediated autophagy induction, suggesting that DAF-16 acts downstream of TOR-mediated autophagy.

1.3.3 Mitochondrial metabolism regulates lifespan

Lifespan increase on mild inhibition of mitochondrial electron transport chain has been observed in several species including yeast (Kirchman *et al*., 1999), *Drosophila* (Copeland *et al*., 2009), *C. elegans* (Lakowski and Hekimi 1996) and mice (Liu *et al*., 2005). Mutations in the electron transport chain genes such as *clk-1*, encoding a mitochondrial hydroxylase involved in ubiquinone synthesis (Lakowski and Hekimi 1996), *gro-1*, encoding tRNA transferase (Lemieux et al., 2001), *isp-1*, encoding Rieske iron sulphur protein (Feng et al., 2001) and *nuo-6*, encoding a subunit of complex I (Yang and Hekimi 2010) extend lifespan. Inhibition of the several other components of the electron transport chain by RNAi extend lifespan independent of DAF-16 and IIS pathway (Dillin *et al*., 2002). Even though reduced ROS levels due to reduced respiration was reasoned as the mechanism for longevity of *isp-1* mutant (Feng et al., 2001) other longlived mit mutants (*nuo-6, clk-1* and *isp-1*) show increased ROS levels (Yang and Hekimi 2010; Lee *et al*., 2010).

1.3.4 Other pathways involved in life span extension in *C. elegans*

Over-expression of the AMP activated protein kinase (AMPK) α subunit *aak-2* extends life span in *C. elegans* (Apfeld *et al.*, 2004). Increase in the AMP:ATP ratio, indicating energy shortage, causes activation of the AAK-2 protein, which can be considered as an energy sensor. The AMP:ATP ratio can be caused by oxidative stress, ischemia, hypoxia, heat shock and normal ageing (Apfeld *et al.*, 2004; Towler and Hardie, 2007). Evidently, DR also activates AAK-2 and the life span extension is DAF-16 independent (Apfeld *et al*., 2004). It was also shown that AAK-2 activity is fully required for the longevity of *daf-2* mutants and *sir-2.1* over-expressors, and partially required for longevity of the mitochondrial mutants *isp-1* and *clk-1*.. In contrast, germline stem cell mutants *glp-1* and food-restricted *eat-2* mutants did not require AAK-2 for their long life (Curtis *et al.*, 2006). DAF-16 up-regulates *aak-2*

transcription (Schuster *et al.*, 2010) and both genes have overlapping effects on longevity (Curtis *et al.,* 2006).

Age dependent regulations of several genes are taken care of by the GATA transcription factors ELT-6, ELT-5 and ELT-3. Knockdown of *elt*-5 and *elt-6* result in an extension in life span. In old worms, *elt-5* and *elt-6* repress *elt-3*, a gene which is responsible for expression of several genes involved in stress resistance. Thus, the gradual repression of *elt-3*, which is considered as an ageing marker, is not attributed to age-related accumulation of damage or stress or inflammation. Knockdown of *elt-3* in long-lived *daf-2* and *eat-2* mutants suppresses the longevity phenotype (Budovskaya *et al.*, 2008). The regulation of this pathway relies on the circuit driven by drift of developmental pathways rather than accumulation of damage (Budovskaya *et al.,* 2008).

1.4 Life span extension – Enhanced protein turnover or protein stability?

1.4.1 Proteostasis

Protein homeostasis or proteostasis is the functional maintenance of the proteins, by regulating their localization, concentration, interactions, conformation in relation to each other. The key factors involved in this maintenance are gene transcription, RNA metabolism and protein synthesis, folding, assembly, trafficking, disassembly and degradation (Balch *et al.*, 2008; Cohen and Dillin, 2008). Enhanced proteostasis is essential for longevity.Wide scale loss of proteostasis is considered to be one of the hallmarks of ageing (Lopez-Otin *et al*., 2013). Further loss of proteostasis can exacerbate misfolding and ageing by imbalance and sequestration of quality control machinery components (Della David *et al*., 2010; Kirstein-Miles *et al*., 2013). It is argued that proteostasis collapse is not a gradual process but a rather sudden event in the early part of life that triggers proteome mismanagement (Labbadia and Morimoto 2014). Proteostasis can be achieved by either increased protein turnover or stability (Neuhofer and Beck, 2005a, b). Both topics will be described in detail below. Investigation of the mode by which proteostasis is achieved in *daf-2* mutants is the core of this study.

1.4.2 Protein turnover

Protein turnover is one of the mechanisms by which protein homeostasis or proteostasis is achieved. In a first line of defense, oxidative damage to proteins is prevented by the

antioxidant system. If oxidative damage to proteins does occur, protein removal and resynthesis are of major importance to remove the damaged proteins. The combination of protein synthesis and protein degradation is called protein turnover which would be studied in this thesis investigating its role in *daf-2* longevity.

The importance of protein synthesis in ageing is evident as reduced translation extends lifespan and enhances stress resistance through TOR inhibition in *C. elegans* and flies (Bjedov and Partridge 2011). Age-associated decline in protein turnover is common in nematodes (Prasanna and Lane, 1979; Sharma *et al.*, 1979) and other organisms, as they age (Makrides, 1983; Van Remmen *et al.,* 1995). This decline in protein turnover has been indicated as a cause for accumulation of damaged proteins accompanied by ageing (Stadtman, 1988; Rattan and Clark, 1996). Aggrevation of this accumulation may occur as lipofuscin, the ageing pigment, which inhibits the proteasome (Sitte *et al.*, 2000). However, later this ageing pigment with similar spectral properties to lipofuscin was found to be Anthranilic acid, a derivative of tryptophan by action of kynurenine pathway (Coburn *et al.*, 2013). Chaperone function also declines with age (Soti and Csermely, 2000), which may be yet another factor that adds to the accumulation of damaged proteins with age.

1.4.2.1 Autophagy- cellular function and molecular mechanism

A major conserved catabolic process which involves degradation of cellular components with the help of the lysosomal system is called macroautophagy or autophagy in general (Levine and Klionsky, 2004). Autophagy is the only mode of degradation of cellular organelles (Dunn, 1994; Klionsky and Emr, 2000; Klionsky, 2007). The global turnover of cellular material within the cell relies on autophagy and the ubiquitin-proteasome system (UPS). A basal level of autophagy is required in most eukaryotes such as yeast (Tsukada and Ohsumi, 1993; Wang and Klionsky, 2003), *C. elegans* (Melendez *et al.*, 2003; Hansen *et al.*, 2008) and mammals (Hara *et al.*, 2006). On the other hand, under conditions of stress (high temperature, hormones, hypoxia, starvation) autophagy is induced by mechanisms which sense extracellular and intracellular factors and transduce appropriate signals to regulate autophagy components (Levine, 2004; Levine and Klionsky, 2004). Mechanistically autophagy induction is the synthesis of autophagosomes. Autophagy is a physiological process involved in adaptive and innate immunity and development (Levine and Kroemer 2008; Levine and Klionsky 2004). Their role in anti-ageing, cell death and tumor suppression has also been established (Mizushima 2005).

In nematodes, autophagy plays a key role in survival during starvation (Kang *et al.*, 2007). The autophagy marker *lgg-1* was found to be activated in starved *C. elegans*. On the contrary, excessive autophagy is lethal. An increased amount of autophagy was observed in the dauers and the presence of autophagy was found to be essential for dauer development (Melendez *et al.*, 2003). Autophagy is essential for reproductive development, as autophagy-impaired *bec-1* mutants of *C. elegans* are sterile (Takacs-Vellai *et al.*, 2005). Interestingly, life span extension in *daf-2* mutants requires autophagy as well, suggesting that this process is of ultimate importance for somatic maintenance. In neurodegenerative diseases like Huntington's disease, Alzheimer's disease and Parkinson's disease autophagy is involved in the degradation of aggregate prone proteins (Morley *et al.*, 2002; Jia *et al.*, 2007).

Autophagy involves a sequence of well-regulated steps initiating with the formation of a double membrane vesicle called autophagosome, and ending up with the fusion with a lysosome forming an autophagolysosome. In the autophagolysosome, degradation of cellular components and macromolecules takes place with the help of the hydrolytic enzymes under acidic conditions and the digested products are channelled out back into the cytoplasm.

The dynamic process of autophagy can be differentiated into several steps: 1. induction 2. cargo selection and packageing 3. nucleation or vesicle formation 4. vesicle expansion and completion 5. retrieval 6. fusion and docking of the completed vesicle with the lysosome/vacuole and 7. breakdown of the intraluminal vesicle and its cargo and recycling of macromolecular constituents (Fig.5). The protein machinery involved in the different processes has been identified in *Pichia pastoris*, *Hansenula polymorpha* and *Saccharomyces cerevisiae*.

1.4.2.2 Autophagy in *C. elegans*

Autophagy is involved in longevity regulation in *C. elegans*. Long lived *daf-2* mutants show increased autophagy indepently of DAF-16 (Hansen *et al.,* 2008). In dietary-restricted worms TOR is downregulated, resulting in the increase of the autophagic marker *lgg-1* (Hansen *et al.,* 2008). The latter study showed that both PHA-4 and TOR are required for the induction of autophagy in DR worms. The process of autophagy was shown to be indispensable for life span extension under different forms of DR (Panowski *et al.,* 2007; Kang *et al.,* 2007;Hansen *et al.,* 2008). Also in case of the long lived Ins/IGF mutant *daf-2*, autophagy is necessary for its long life (Melendez *et al.*, 20003). However, induction of autophagy by itself alone is not sufficient for life span extension as autophagy is high in *daf-2;daf-16* double mutants that

have normal life span. This suggests that DAF-16 activity and autophagy are both necessary for life span extension (Hansen *et al.,* 2008) Even though inactivation of autophagy genes *bec-1, atg-18, lgg-1, atg-7, lgg-3* and *unc-51* also showed to reduce life span in *daf-2* mutants (Hars *et al.*, 2007; Toth *et al.*, 2008) the need for autophagy to support longevity is not well understood. Autophagy in *daf-2* mutants is activated through AMPK and its and downstream targets ULK-1/hATG-1 (Egan *et al.*, 2011).

1.4.2.3 Lysosomes

Lysosomes are essential components in a cell and are required for normal turnover of proteins and organelles. They are cytoplasmic acidic vesicles found in all cells except the mammalian erythrocytes and were discovered by Christian De Duve and his coworkers (de Duve, 2005). Lysosomes are filled with hydrolytic enzymes which are involved in 1. digestion of cellular nutrients 2. protein turnover 3. tissue remodelling 4. lysis of invaders 5. autolysis of dead cells 6. surviving during temporary starvation. The lysosomes are used in the processes of heterophagy (or endocytosis, which includes phagocytosis and pinocytosis) and autophagy (self-eating). In order to fulfill their catabolic function, lysosomes house phosphatases, glycosidases (Bolanowski *et al.*, 1983) and proteinases (Sarkis *et al.*, 1988). This large family of hydrolytic enzymes (about 50) are targeted to primary lysosomes through the trans-golgi network (TGN). These vesicles fuse with autophagosomes and late endosomes (Dunn, 1990) digesting the substrate in them. In this thesis the activity of these organells will be studied in the longlived *daf-2* mutant. Several of the components tested and important for lysosomal function,will be described below.

1.4.2.4 Lysosome-associated proteins

Proteases

Proteases are broadly classified as exopeptidases and endopeptidases (proteinases). Based on the structure of the active site and catalytic mechanism, proteases or proteolytic enzymes are into five groups: aspartyl-, serine-, cysteine-, metallo-, and threonine proteases.

Proteolysis plays an important role in activation/inactivation of enzymes and regulatory proteins and degradation of unnecessary damaged proteins. Both in neurodegeneration and ageing, the regulatory and degrading mechanisms of proteases play a key role. These proteases are a key as the integrity of the lysosomal membrane declines during ageing and causes leakage of hydrolytic enzymes to the cytosol (Chondrogianni *et al.*, 2002). This fact was supported by studies which indicate a high activity of these enzymes in the cytosol

compared to the lysosome during ageing. The lysosomal proteases have cell- and species-specific functions. For example, yolk processing in chicken involves aspartyl protease cathepsin D (Retzek *et al.*, 1992), whereas in *Xenopus* and fish this process is supported by cathepsin D and a cysteine protease cathepsin L (Yoshizaki and Yonezawa, 1998; Carnevali *et al.*, 1999; Kwon *et al.*, 2001), and in insects the presence of cathepsin B and L are essential for yolk processing (Yamamoto and Takahashi, 1993; Liu *et al.*, 1996; Cho *et al.*, 1999). In *C. elegans*, cathepsin D was found to be the major proteolytic enzyme followed by two thiol proteases which are leupeptin-sensitive and posses properties similar to cathepsin L and B of vertebrates and another leupeptin-insensitive thiol independent proteolytic enzyme (Sarkis *et al.*, 1988). These proteases are utilized by parasitic nematode species for penetration of the host: a study by McKerrow *et al.,* (1990) indicated the involvement of metallo-protease in skin penetration by nematode larvae of *Strongyloides stercoralis* (McKerrow *et al.*, 1990).

Cysteine proteases

These enzymes are involved in the processes of protein breakdown in the lysosome, processing of pro-hormones and pro-enzymes, antigen presentation, cell proliferation, differentiation, apoptosis and fertilization (Chapman *et al.*, 1997; Grzelakowska-Sztabert, 1998; Berdowska and Siewinski, 2000) and their functions are tissue-specific. Cysteine proteases include cathepsin B, L, F, C, K, H, S, W and V, some of which are amino or carboxy exopeptidases and others endopeptidases. All cathepsins are active and stable at acidic pH (indicating their intralysosomal function) except for cathepsin S which is stable at pH 7 (Turk *et al.*, 2002). The role of cysteine proteases as intracellular lysosomal enzymes involved in protein turnover has been documented in mammals. These enzymes also posses extracellular activity related to metastasis and tumour invasion (Sloane *et al.*, 1987). In nematodes, cysteine proteases are found to be the major class of proteases secreted by parasitic species and these proteases are involved in the parasite-host interaction (Knox and Kennedy, 1988; McGinty *et al.*, 1993; Richter *et al.*, 2002). The *C. elegans* cysteine protease *ced-3* is expressed abundantly in the embryonic stages and is involved in controlling programmed cell death (Yuan *et al.*, 1993). Cystein proteases cathepsin L, S, B, H ,K and C were also defined as key enzymes in mammalian apoptosis (Guicciardi *et al.*, 2000; Reiners *et al.*, 2002; Cirman *et al.*, 2004), aspartic protease cathepsin D (Kagedal *et al.*, 2001; Bidere *et al.*, 2003) and lactoferrin (Katunuma *et al.*, 2004) which further leads to activation of cytosolic factors Bid/Bak leading to mitochondrial permeabilization (Boya *et al.*, 2003) and ultimately, apoptosis.

Cathepsin B

Cathepsin B has the ability to be both an endopeptidase and a peptidyl-dipeptidase (Aronson and Barrett, 1978). In addition to protein degradation in lysosomes, cathepsin B is also involved in other cellular functions like antigen processing (Berzofsky *et al.,* 1988), fragmentation of MHC class II molecule prior to antigen binding (Xu *et al.*, 1994), and extracellular processes such as bone resorption (Delaisse *et al.*, 1984). Cathepsin B is also of importance in oncology as it is involved in extracellular matrix degradation during tumour cell invasion (Recklies *et al.*, 1982).

Cathepsin B is synthesized as a propeptide which gets activated by cleavage in the lysosome (Menard *et al.*, 1998; Rozman *et al.*, 1999). This process of activation can happen auto-catalytically or by other proteases like cathepsin D and pepsin (Turk *et al.*, 2001). Glycosaminoglycans (Caglic *et al.*, 2007) were shown to induce conformational changes to the cathepsin zymogen, speeding up autocatalytic activation.

Four *C. elegans* cathepsin B genes *cpr-3, cpr-4, cpr-5* and *cpr-6* were shown to be very divergent from vertebrate counterparts but show glycosylation sites that are typical for proteins that are targeted to the lysosomes (Larminie and Johnstone, 1996). Each of these *cpr* genes shows different temporal expression patterns during development. Post hatch, all four genes were up-regulated, with *cpr-4* and *cpr-5* having an elevated transcript level in all larval stages and a decline during adulthood. By contrast, *cpr-6* is up-regulated during the L4 and adult stages (Laminie and Johnstone 1996).

It has been suggested that, as animals age, the cytosolic pH drops and this acidification can result in lysosomal membrane permeability leading to release of cathepsin B and L into the cytosol inducing degradation (Cuervo and Dice 1998). Cathepsin B activity was shown to increase with age in rat liver and human serum (Keppler *et al.*, 2000; Wyczalkowska-Tomasik and Paczek, 2012). However, reports on age-related changes in cathepsin B are discordant; in aged rat nerve cells a low cathepsin B and L activity was measured while cathepsin D was increased (Amano *et al.*, 1995). Yet another study reports increased levels of cathepsin B, E and D in aged rat brain tissues together with a decrease in cathepsin L activity.

Cathepsin B-like activity was observed in parasitic species like *Schistosoma mansoni* (McKerrow and Doenhoff, 1988) and *Plasmodium falciparum* (Rosenthal *et al.,* 1988) which use hemoglobinase. In *Haemonchus contortus* the major intestinal transcript was found to be the cathepsin B-like cysteine protease *cbl* (Jasmer *et al.*, 2001) probably functioning as a

digestive enzyme (Rhoads and Fetterer, 1995). Cathepsin B activity was shown to be increased in the neostriatum in aged rats (Nakanishi *et al.*, 1994).

Cathepsin L

Cathepsin Ls are well-regulated lysosome-specific enzymes that are extremely unstable at neutral or alkaline pH (Turk *et al.*, 1999). Cathepsin L is known for its elastic fibre degenerative function similar to cathepsin S and K which are associated with pathologies of the cardiovascular system and are inhibited by cystatins (Novinec *et al.*, 2007). Cathepsin L is also involved in the regulation of insulin signalling by selective breakdown of intracellular insulin-like growth factor binding proteins (IGFBPs) in lysosomal and endosomal vacuoles (Busby *et al.*, 2000; Zwad *et al.*, 2002). Ageing rats have shown a decrease in cathepsin L activity in all the brain regions compared to young counterparts which possess higher activity (Nakanishi *et al.*, 1994; Amano *et al.*, 1995). Several groups of cathepsins seem to compensate for proteolytic function in the absence of others. Selective inhibition of cathepsin L and B activity has been shown to increase levels of cathepsin D and lysosome-related dense bodies which did not emit auto-fluorescence (Bednarski *et al.*, 1997).

CPL-1 is a well studied cathepsin L gene expressed in *C. elegans* and in animal and plant parasitic nematodes. Cathepsin L, a key player in moulting, is found to be more efficient in collagen solubilisation compared to its counterparts cathepsin B and S (Maciewicz *et al.*, 1987). In *C. elegans*, *cpl-1* is required during embryogenesis, larval development and moulting (Britton and Murray, 2002; Hashmi *et al.*, 2002) and is provided maternally to the offspring. Although in *cpl-1* mutants, endocytosis and platelet formation in yolk proceeds normally, abnormal aggregation and accumulation of yolk platelets in the cytoplasm occur during the 8-12 cell stage of embryogenesis (Britton and Murray, 2004). This platelet fusion is brought about by conformational changes and aberrant processing of the yolk proteins in the mutants. In addition, cathepsin L mutation in mice resulted in abnormal aggregation and fusion of lysosomes leading to lysosomal disorders (Stypmann *et al.*, 2002).

Cathepsin Z

Proteases are important in worm moulting as the inner part of the old cuticle should be degraded before ecdysis (Samuels and Paterson, 1995; Yochem *et al.*, 1999)(Lee 2002). In *C. elegans,* cathepsin Z is one of the several enzymes involved in moulting. Two cathepsin Z enzymes have been identified in *C. elegans*; CPZ-1 and CPZ-2 (Hashmi *et al.*, 2002). The activity of cathepsin Z is partially required during embryogenesis as cathepsin Z mutation

leads to 10-20% embryonic lethality (Hashmi *et al.*, 2004). As expected, knockdown of *cpz-1* also interferes with moulting and leads to morphological defects in the heads and tails of larvae and defective gonadal development in adults. Signifying its importance in moulting, high *cpz-1* expression was reported in the new and old cuticle during moulting (Lustigman *et al.,* 1996). It is also hypothesised that *cpz-1* is involved in extracellular matrix and cuticular assembly processes and indirect processing or activation of other proteins and pro-enzymes required for moulting and cuticular assembly (Hashmi *et al.*, 2004). *cpz-1* is expressed in all the developmental stages in the hypodermal cells, cuticle, gonads and pharynx (Hashmi *et al.,* 2004).

Aspartyl proteases

The aspartyl proteases include pepsins, chymosins, cathepsin E, D and renins. The pathology of neurodegenerative diseases like Alzheimer's, Parkinson's and Huntington's and the normal process of ageing depend on the functionality of this proteolytic system. Hence, the activation of lysosomal aspartyl proteases and calcium dependent calpain proteases have been implicated as key determinants of cellular destruction during ageing and neurodegeneration (Samara and Tavernarakis, 2003).

Studies indicate a high proteolytic activity of *C. elegans* extracts at acidic pH and total inhibition of this activity by the specific aspartyl protease inhibitor pepstatin (Sarkis *et al.*, 1988). Even though cDNA analysis identified at least 12 putative aspartyl proteases in *C. elegans* that were homologuous with those of mammalian parasites (Tcherepanova *et al.*, 2000), only 5 (ASP-1 to ASP-6) of the aspartyl proteases were isolated using pepstatin affinity chromatography (Geier *et al.*, 1999; Tcherepanova *et al.*, 2000). This group includes both lysosomal and secreted proteases. The lysosomal targeting factors (N-glycosylation and four conserved cysteine residues) were found in ASP-1, ASP-2, ASP-5 and ASP-6 whereas a lack of N-glycosylation suggests a different localization and function for ASP-3 and ASP-4. In addition to their role in neurodegeneration, ASP-3 and ASP-4 mediate necrosis (Syntichaki *et al.*, 2002). Both are predominantly localized to the intestine and to lower extent in the hypodermis, muscle cells and neurons. ASP-6 is highly expressed in the intestine, muscle cells, pharynx hypodermal cells and epithelial cells and are secreted in the entire body cavity. Excretion of this enzyme in a phosphorylcholine-substituted form has been observed in *C. elegans* (Lochnit *et al.,* 2006). In late embryonic and larval stages, ASP-1 is reported to be expressed in the lysosomes of the intestinal cells and it renders partial protection against cell death (Syntichaki *et al.,* 2002). Interestingly, knockdown of *asp-18* in long lived *daf-2*

mutants reduced the life span by around 35% compared to wild type strain. As *asp-18* up-regulation is DAF-16-dependent and this is a non-proteasomal protease, this suggests a function of proteolytic activity for life span extension in *daf-2* mutants (McElwee *et al.,* 2003). Reduction of overall aspartic protease activity by chemical inhibitors, mutation or starvation, reduces necrosis thereby protecting proteins against neurodegeneration in *C. elegans* motor neurons (Samara and Tavernarakis, 2003).

Cathepsin D

The carboxyl protease cathepsin D is sensitive to pepstatin and has an optimal pH of 3. These enzymes are converted from the single to double chain in the lysosome by cysteine protease cleavage or by autocatalytic activity of cathepsin D (Bi *et al.*, 2000). Cathepsin D's are the major lysosomal aspartate proteases and are involved in endopeptidase activity degrading long lived proteins (Dean, 1977; Jones *et al.*, 1982) and α-synuclein (Hossain *et al.*, 2001).

Typical to aspartyl proteases, cathepsin D's role in ageing and neurodegeneration is significant. In aged mice and guinea pigs, cathepsin D was found to be activated in specific brain regions which are susceptible to ageing related neurodegenerative diseases (Lee *et al.*, 2000; Jiang *et al.*, 2001a; Vohra *et al.*, 2002). Cathepsin D was found to be up-regulated in the brain even in the early stages of AD (Cataldo *et al.*, 1997; Adamec *et al.*, 2000). Cathepsin D activity plays a key role in clearance of AD β amyloid proteins (Hamazaki, 1996; McDermott and Gibson, 1996). In Cathepsin D deficient mice, CNS neurons were shown to accumulate ceroid lipofuscin in lysosomal structures and exhibit autofluorescence (Koike *et al.*, 2000). In ovine, cathepsin D mutation leads to profound neurodegeneration caused by congenital lysosomal storage disease (Tyynela *et al.*, 2000). In a *C. elegans* model expressing α-synuclein in neurons, overexpression of cathepsin D (and not B and L) reduced aggregate formation (Qiao *et al.*, 2008).

Cathepsin D is also involved in controlling multiple proteolytic pathways as reduced cathepsin D levels reduce macroautophagy and proteasomal activity. A study by Adamec *et al.* (2000) showed that inducing necrosis or apoptosis, increased expression of cathepsin D and activated the lysosomal endosomal system, which was similar to observations during ageing and Alzheimer's disease in neurons of humans and rats (Crawford *et al.*, 2000). Cathepsin D, on contrast, can also induce protein aggregation as it possesses the ability to be active in the neutral pH of the cytoplasm causing cytoskeletal damage and generating phosphorylated *tau* from which neurofibrillary tangles form (Bi *et al.*, 2000). This phenomenon strongly depends on lysosomal enzyme leakage which occurs during ageing.

The *C. elegans* cathepsin D mutant *cad-1* shows low cathepsin D activity and displays altered morphology such as clear or bubbled intestinal cells with high vacuolation (Jacobson *et al.*, 1988). Ageing in *C. elegans* caused a 10-fold decline in cathepsin D levels but also significant activity decreases of thiol protease cathepsins Ce1 and Ce2 (Sarkis *et al.*, 1988). Ageing leads to oxidation of proteins and aggregation, but with a reduced proteolytic activity in older worms, the aggregation and ageing process could be magnified. However, in *C. elegans*, 4 to 8 hours of starvation reduces cathepsin D by 65% as well as thiol cathepsins Ce1 and Ce2 (Hawdon *et al.*, 1989). A decrease in cathepsin D levels (to 15%) was also observed in mutants with compromised food uptake. This decrease was the result of an auto-digestion effect due to lack of protein substrate in lysosomes (Hawdon *et al.,* 1989).

Protease inhibitors

Protease inhibition is a process required for regulation of enzyme activity. Imbalances between the protease and their inhibitors have been shown to result in uncontrolled proteolysis leading to inflammatory diseases and tumour growth (Henskens *et al.*, 1996; Berdowska and Siewinski, 2000), osteoporosis, diabetes, renal failure, multiple sclerosis, rheumatoid arthritis, and neurodegenerative diseases. A precise regulation of these proteases is essential for appropriate functioning of cells and organisms. This regulation is achieved at various levels, from expression levels, secretion and further maturation, and by enzyme inhibitors.

Cystatins – The endogenous cysteine protease inhibitors, cystatins, are potentially important for their regulatory functions and are the largest described group of natural cysteine protease inhibitors by diversity (Bode *et al.*, 1990). Cystatins are found in cytosol but most abundantly extracellularly as secreted proteins (Abrahamson *et al.*, 1986; Kopitar-Jerala, 2006). They are involved in inhibiting cathepsin L, V and legumain. Cystatins act by binding close to the catalytic site hindering substrate binding (Bode and Huber, 2000). Even though the inhibitor binds the enzyme similarly to a substrate, it points away from the active centre at position P1 avoiding cleavage, hence remaining intact and binding the enzyme non-productively (Stubbs *et al.*, 1990).

The expression of two cystatins CPI-1 (K08B4.6) and CPI-2a (R01B10.1) has been described in *C. elegans* and both possess a considerable amount of sequence homology with *Brugia malayi, Acanthocheilonema viteae, Onchocerca volvulus, Litomosoides sigmodontis* cysteine inhibitors (Lustigman *et al.*, 2002). Cathepsin B, L and S have been identified to be inhibited by these 2 inhibitors with K_i ranging from 0.01 to 33.88 (Schierack *et al.*, 2003). Studies

indicate the differential expression of both inhibitors along *C. elegans* development. CPI-2a was shown to be essential during development and reproduction and they interact with cathepsin enzymes CPL-1 and CPZ-1 (Hashmi *et al.*, 2006). At the transcript levels, *cpi-2a* was found to be expressed throughout development whereas at the onset of each moult a peak in expression was reported. It is indeed noteworthy that an increase in transcript levels of *cpl-1/cpz-1* was reported before each moult. This indicates a systematic regulation of activity of these enzymes at various stages and tissues. In addition to developmental stages, the transcript levels of *cpi-2*a stayed moderately elevated during adulthood corresponding to elevated levels of *cpl-1* and *cpz-1* (Hashmi *et al.,* 2006). *cpi-2a::lacZ* assays had shown a high expression in the intestinal and hypodermal cells, pharyngeal cells including the pharyngeal gland cells in the L1-L4 stage whereas in adults the expression was confined to uterine embryos, pharyngeal muscles, pharyngeal gland cells and few hypodermal cells in the tail region (Hashmi *et al.,* 2006). Anti-CPI-2a immunoelectron microscopic assays has shown the localization of CPI-2a to the cuticle of all stages. During embryogenesis and oogenesis, localization was pertained to the gonadal sheath, oocytes and embryo, yolk granules, yolk platelets, egg shells, and sperm.

In addition to cystatins, other protease inhibitors are characterized in non-*C. elegans* species. These inhibitors include propeptides, serpins, *P35*, thyropins and chagasins, and inhibitors of the apoptosis protein family (IAP's). It is very likely that the *C. elegans* genome encodes homologues for at least a few of these inhibitors.

Acid phosphatase

Acid phosphatases are high molecular weight enzymes involved in dephosphorylation of phosphomonoester substrates (Van Etten, 1982; Vincent *et al.*, 1992; Bull *et al.*, 2002). In many species, acid phosphatases are represented as large gene families that are partially redundant such as mammalian lysosomal acid phosphatase functional redundancy in bone resorption (Suter *et al.*, 2001). *C. elegans* also has a large family of acid phosphatases. Here, the intestinal acid phosphatase *pho-1* does not show redundancy as mutants exhibit extreme embryonic lethality and this phosphatase acts maternally. As knock-out of *pho-1* induced embryonic arrest and because this lethality is maternal it was proposed that the presence of *pho-1* might be required by the maternal intestine to process specific nutrients or cleavage products that are to be transferred to the embryos (Fukushige *et al.*, 2005). PHO-1, a histidine acid phosphatase (Vincent *et al.,* 1992; Van Etten 1982; Bull *et al.,* 2002) similar to human prostatic acid phosphatase, localizes to the lumenal surface of the *C. elegans* intestine in the

vicinity of the brush border in all but the anterior 6 cells (Beh *et al.*, 1991). Acid phosphatase activity was observed to be reduced in the early stages (occytes and embryos) of development but a gradual increase in activity from the embryonic stages was reported (Beh *et al.,* 1991). *pho-2* and *pho-3* were detected with isoelectric focusing and shown to be expressed in oocytes, early embryos and L4, while absent during the L1 to L3 stages. This L4 up-regulation was suspected to be for a later maternal transfer of *pho-2* and *pho-3* to the embryos (Beh *et al.,* 1991). *pho-11* possesses a DAF-16 binding site (DBE) and is differentially expressed in the *daf-2* and *daf-16;daf-2* mutatnts (McElwee *et al.*, 2003).

Amino acid transporters

Under normal physiological conditions, hydrolysed products in the lysosomes are channelled to the cytosol through specific transporters. Any defect in the lysosomal efflux could lead to lysosomal storage diseases such as sialic acid storage disorder and nephropathic cystinosis (Mancini *et al.*, 2000) which are caused by mutations in the transport protein sialin and cystinosin respectively (Gahl *et al.*, 1982; Renlund *et al.*, 1986; Tietze *et al.*, 1989; Havelaar *et al.*, 1998; Town *et al.*, 1998; Mancini *et al.*, 2000). Amino acids not only serve as organic nitrogen source for eukaryotic cells, they can also act as neurotransmitters and hormones involved in communication between cells and tissues and some are involved in stress adaptation (Clark and Amara, 1993; Fischer *et al.*, 1995; Bennett *et al.*, 1996; Rentsch *et al.*, 1996). Even though several systems of amino acid transport have been described in vacuoles (Ohsumi and Anraku, 1981; Sato *et al.*, 1984b, a) and lysosomes (Pisoni and Thoene, 1991) the specific proteins responsible have not yet been studied in detail.

Each group of amino acid transporters are substrate specific. This is important as vacuoles occupy 25% of the cell volume in *S. cerevisiae* and function as storage compartments for specific basic amino acids (Wiemken and Nurse, 1973; Wiemken and Durr, 1974; Matile, 1978; Huberwalchli and Wiemken, 1979; Kitamoto *et al.*, 1988).

Around 20 amino acid transporter families have been identified (Saier *et al.*, 1999) based on the amino acids they transport. Initially, the eukaryotic amino acid/auxin permease AAAP family (Young *et al.*, 1999) was identified as a family of H^+/amino acid symporters in plant cell plasma membranes (Frommer *et al.*, 1993; Ortiz-Lopez *et al.*, 2000). Later studies showed that this family possesses transporters for loading inhibitory amino acids (gama-amino butyric acid and glycine) into animal nerve cell synaptic vesicles (McIntire *et al.*, 1997; Sagne *et al.*, 1997) through an H^+ antiport mechanism. This is similar to the vesicular GABA transporter (VGAT) transporters, identified in the mammalian system, which mediate

transport of gamma amino butyric acid (GABA) into vesicles and also relies on H^+ (McIntire *et al.,* 1997). The transport systems A and N (SN1) are associated with VGAT and are important for nitrogen metabolism and the glutamine-glutamate cycle; both systems require Na^+ (Chaudhry *et al.*, 1999; Albers *et al.*, 2001; Chaudhry *et al.*, 2002). Also included in this family is LYAAT-1, a H^+ coupled plasma membrane and synaptic vesicle amino acid transporter which is expressed at highest levels in the brain neurons in the rat and is mostly active in acidic pH. Additionally, over-expression of these transporters results in the neuronal accumulation of GABA, L-proline and L-alanine via H^+/amino acid symport. In all the regions of rat brain, LYAAT-1 colocalises with the lysosmal marker cathepsin D, signifying its lysosomal function (Agulhon *et al.*, 2003). Being an proton symporter, LYAAT-1 activity is dependent on proton pumps elsewhere in the membrane. It is also note worthy that LYAAT does not show sequence homology with sialin or cystinosin (lysosomal sialic acid and cysteine transporters respectively) nor does it correspond to the two transport systems for L-proline transport, the f and p systems, found in fibroblasts as these systems are pH independent (Sagne *et al.*, 2001).

In *C. elegans* there are several close homologues of catalytic heteromeric amino acid transporter (HAT) proteins. Basically, the amino acid transporter structure is distinguished as a transmembrane subunit and a type II glycoprotein subunit (Verrey *et al.*, 1999; Chillaron *et al.*, 2001; Wagner *et al.*, 2001). When expressed in *Xenopus laevis,* LAT-1, the first characterised HAT, proved to be a large neutral amino acid exchanger (Kanai *et al.*, 1998; Mastroberardino *et al.*, 1998). Each HAT is selective for a specific amino acid (Verrey, 2003). In *C. elegans*, nine HAT subunit homologs have been identified and they are named *aat-1* to *aat-9*. Two HAT glycoprotein homologues have also been identified in *C. elegans* and were suggested to be named *atg-1* and *2* by Veljkovic *et al.*, 2004 (Veljkovic *et al.*, 2004b). Close to the transmembrane region a cys residue is present which, in mammals, forms a disulfide linkage to the catalytic subunit (Pfeiffer *et al.*, 1998). AAT-1 or 3 when co-expressed with ATG-2 functions in Na^+-independent amino acid transport of small neutral amino acids in *Xenopus* oocytes (Veljkovic *et al.*, 2004b). AAT-1/ATG-2 and AAT-3/ATG-2 showed similar transport characteristics as the mammalian L and *asc-1* transport systems and were reported to be shifted more towards small neutral L-amino acids like Ala, Ser, Val, Thr, His, Gly, compared to KAT2-4F2hc (Pineda *et al.*, 1999; Rossier *et al.*, 1999; Segawa *et al.*, 1999; Meier *et al.*, 2002).

The *C. elegans* HATs are not only involved in import of amino acids but also export and are obligatory amino acid exchangers similar to the vertebrate L type LAT1 LAT2 and *asc-1* transporters (Fukasawa *et al.*, 2000; Meier *et al.*, 2002) indicating a well preserved function of amino acid transporters in metazoans. The expression levels of these amino acid transporters did not show any difference during or post development, is expressed over the entire life span of *C. elegans* (Hill *et al.*, 2000; Jiang *et al.*, 2001b; Veljkovic *et al.*, 2004a). RNAi silencing of ATG-1,2 and AAT-1,3 did not show any phenotypical change (Kamath *et al.*, 2003). In contrast to AAT-1 and 3, AAT-9 which does not have a conserved cysteine residue, was shown to reach the cell surface of *Xenopus* oocytes without the heavy chain subunit while still functioning as an aromatic amino acid exchanger (Veljkovic *et al.*, 2004a). However, L-Phe uptake efficiency was increased about 85% when the heavy chain ATG-1 or ATG-2 was co-expressed in *C. elegans* with AAT-9. L-Phe was observed in studies as a preferred amino acid for AAT-9 and the uptake is Na^+ dependent. AAT-9 is localized to neurons close to the pharyngeal bulbs and the wall muscles near the mouth (Veljkovic *et al.*, 2004a).

H+V-ATPase

Similar to synaptic vesicles, lysosomes and vacuoles can generate a proton electrochemical gradient and maintain an acidic internal lumen with the help of vacuolar H^+VATPase (Kakinuma *et al.*, 1981). Vacuolar membrane bound H^+V-ATPase has been identified in several acidic compartments in yeast and plant vacuoles, in coated vesicles, chromaffin granules, golgi apparatus, and proton transporting microsomal vesicles of eukaryotic cells (Forgac *et al.*, 1983; Glickman *et al.*, 1983; Yamashiro *et al.*, 1983; Gluck and Caldwell, 1987; Hirata *et al.*, 1990; Puopolo and Forgac, 1990). Maximal H^+V-ATPase activity was observed at pH6.8 (Harikumar and Reeves, 1983; Wang and Gluck, 1990) and these proton pumps can be specifically inhibited by N-ethylmaleimide and nitrate (Ohkuma *et al.*, 1982; Harikumar and Reeves, 1983).

Similar to the ATP synthases of mitochondria and chloroplasts (Cross, 2000; Fillingame *et al.*, 2000; Weber and Senior, 2000), H^+V-ATPase complexes are composed of two domains: the peripheral V_1 domain, housing three ATP binding sites and made of 8 subunits responsible for ATP hydrolysis, and the integral V_0 domain made of 5 subunits responsible for proton translocation (Stevens and Forgac, 1997; Forgac, 1999; Bowman and Bowman, 2000).

The acidification of vesicles through H^+V-ATPase is important for several cellular functions including 1. endosomal acidification which is required for release of receptors for recycling

from the internalized receptor-ligand (Tycko and Maxfield, 1982) and/or activation of lysosomal enzymes (Ghosh *et al.*, 2003; Maxfield and McGraw, 2004) 2. vesicle budding providing an acid lumen for endocytosis (Hurtado-Lorenzo *et al.*, 2006) 3. V_0 domain is involved in membrane fusion via SNARE complex formation (Peters *et al.*, 2001; Hiesinger *et al.*, 2005; Liegeois *et al.*, 2006).

Regulation of H^+V-ATPase occurs by reversible dissociation of the V_0 domain from the V_1 domain which disrupts the ATP hydrolysis function (Kane, 2006). A decrease in glucose level triggers this phenomenon in a call to conserve energy in yeast (Kane 2006) and insect cells (Beyenbach and Wieczorek, 2006). The reassembly process requires the RAVE complex (regulator of the ATPase of vacuolar and endosomal membranes) which belongs to the SCF ubiquitin ligase family and involves binding with subunit C (Seol *et al.*, 2001; Smardon and Kane, 2007). Several subunits of the pump are involved in glucose sensing. A glycolytic enzyme aldolase acts as a glucose sensor by inducing H+V-ATPase assembly in presence of glucose (Lu *et al.,* 2004) and inducing a disassembly when it loses interaction with subunit B (Lu *et al.*, 2007). In subunit A also specific regions are involved in glucose sensing and disassembly as mutations in this region reduces glucose sensing (Shao *et al.*, 2003) and reduced glucose levels reduce the assembly of this subunit with the V_0 subunit (Shao and Forgac, 2004). Knock-out of H^+V-ATPase subunits in yeast have shown to accelerate ageing by increase pH of vacuoles (Hughes and Gottschling, 2012) and a resultant decrease of aminoacid import (Molin and Demir) 2014).

In *C. elegans*, the 19 different genes encoding the subunits of H^+V-ATPase have been identified and named as; *vha-1* to *4* which constitute the V_0 domain (Oka *et al.*, 1997, 1998), four isoforms (*vha-5* to *7* and *unc-32*) which constitute the A subunit and are expressed cell specifically (*vha-6* in the intestine, *vha-7* in the hypodermis, *vha-5* in excretory cells) (Oka *et al.*, 2001), *vha-11* encoding the C subunit (Oka and Futai, 2000) and *vha-12* which encodes the B subunit (Syntichaki *et al.*, 2005). The *vha-8* subunit is identified as the E subunit of the V_1 domain (Choi *et al.*, 2003) and is essential for intestinal cell acidification, larval development and survival (Ji *et al.*, 2006). A failure in yolk uptake was reported on knockdown of *vha-8,* signifying its role in receptor mediated endocytosis (Ji *et al.,* 2006). Other phenotypes were necrosis and swollen vacuoles in the hypodermis of the head. The subunit encoded by *vha-6* is found to be essential for larval survival and is expressed in the apical membrane of intestinal epithelial cells (Oka *et al.*, 2001). The requirement of *vha-6* for

acidification of the intestinal lumen and for cellular pH recovery post defecation in the intestine has been reported by Allman *et al.,* 2009 (Allman *et al.*, 2009).

Lysosome Associated Membrane Protein (LAMP)

LAMPs (Lysosome-associated membrane proteins) are a group of integral membrane proteins which are highly glycosylated, and predominantly present in endosomes and lysosomes (Chen *et al.*, 1985; Lippincott-Schwartz and Fambrough, 1986) but they can also be found on cell surfaces (Hughes and August, 1982) as characterized in avian and mammalian cells. In vertebrates, only two genes LAMP-1 and LAMP-2 encode LAMP family (Fukuda *et al.*, 1988)(Fukuda *et al.,* 1988). A relative of the LAMP family, CD68/macrosialin possesses a high degree of structural similarity to LAMPs and is also localized to the endosome/lysosome (Holness *et al.*, 1993; Holness and Simmons, 1993) but differs from LAMPs by its presence which is restricted to the immune system (da Silva *et al.*, 1996; Van Velzen *et al.*, 1997). The function of LAMPs is not yet well established. LAMP-1 was proposed to function in phagosome maturation (Garin *et al.*, 2001) in the late stages of phagolysosome formation in mammals (Vieira *et al.*, 2002).

The *C. elegans* LMP-1 shows sequence similarity to LAMP/CD68s. LMP-1 is the only *C. elegans* membrane protein which possesses a vertebrate lysosome targeting GYXXπ sequence where π is a large hydrophobic residue. In *C. elegans*, expression of LMP-1 was found in intestinal cells of later embryonic stages (Kostich *et al.*, 2000). Knockdown of *lmp-1* in *C. elegans* displays no developmental defects or aberrant developmental rates but adult worms reveal less dense gut granules in the intestine (Kostich *et al.,* 2000). Further, electron microscopy of the *lmp-1* mutants revealed the absence of a subset of granules which are large, round and smooth. Instead, irregular shaped granules, involved in engulfment of other granules, were found. LMP-2 is an ortholog of mammalian LAMP-2a and is involved in chaperone-mediated autophagy (Cuervo, 2004; Zhang and Cuervo, 2008) which is responsible for around 30% of the cytosolic protein degradation (Arias and Cuervo, 2011). Over-expression of LMP-2 in *C. elegans* increases life span by around 34% and it was hypothesised that this could be due to increased protein degradation and hence reduced protein damage (Sagi and Kim, 2012).

Gut granules

Derived from the endosomal system, lysosome related organelles (LRO) differ from lysosomes in morphology, composition and function. LROs are compartments with

specialized cellular function like signalling, storage and secretion (e.g. blood clotting signals by α-granules, acrosomes in sperm cells, Weibel-Palade bodies, and pigment production and storage by melanosomes (Raposo and Marks, 2007).

In *C. elegans*, LROs are found in the intestine and referred to as gut granules (Hermann *et al.*, 2005). These gut granules are involved in accumulation of auto-fluorescent pigment (Klass, 1977; Gerstbrein *et al.*, 2005) which is a death marker (Coburn *et al.*, 2013). Gut granules in *C. elegans* were initially identified to be involved in fat storage (Ashrafi *et al.*, 2003; Schroeder *et al.*, 2007). Later studies identified LROs as not the major fat storage organelles, putting forward oil red O as the better staining method (O'Rourke *et al.*, 2009). Gut granules also function in zinc storage (Roh *et al.*, 2012) and are mobilized through the membrane by CDF and ZIP proteins (Cragg *et al.*, 2005; Feeney *et al.*, 2005).

Staining analysis and electron microscopy revealed the presence of three types of gut granules: one which displays autofluorescence, is acidic, birefringent and stains with acridine orange, Nile red but not with oil red O. This first type expresses the markers GLO-3, PGP-2, FUS-1(Treusch *et al.*, 2004; Hermann *et al.*, 2005; Nunes *et al.*, 2005; Schroeder *et al.*, 2007). A second type displays no autofluorescence, is pH neutral (Nunes *et al.,* 2005) and does not stain with acridine orange and oil red O but can be stained with Nile Red. This type of granules expresses the late endosomal membrane markers LMP-1, RAB-7 and the early markers RME-1 and RAB-5 (Treusch *et al.*, 2004; Nunes *et al.*, 2005; Rabbitts *et al.*, 2008) and UNC-108 (Lu *et al.*, 2008). The last type of gut granule is only present in *C. elegans* and is electron dense, does not stain with Nile Red, and is not autofluorescent.

In *C. elegans*, *glo-1,* a member of the Rab family of small GTPases, is involved in gut granule/LRO biogenesis (Hermann *et al.*, 2005) similar to Rab38/GLO-1 orthologs in mammals (Loftus *et al.*, 2002) and *Drosophila* (Ma *et al.*, 2004). *glo-1* is mainly expressed in the gut and neurons in *C. elegans* embryonic and adult stages (Grill *et al.*, 2007). *glo-1* mutants lack gut granules as well as gut autofluorescence that is generated by a reduced anthranilic acid glucosyl ester, a product of the kynurenine pathway (Coburn *et al.*, 2013). A significant number of connections between life span, lysosomes and gut granules exist. The release of high levels of excitatory neurotransmitters by kynurenines (Saito *et al.*, 1993) can trigger calcium influx which can further lead to necrotic cell death by cathepsins (Rothman and Olney, 1995). In *Drosophila*, life span is extended by inhibition of the kynurenine pathway enzyme tryptophan 2,3 dioxygenase (TDO) (Oxenkrug *et al.*, 2011) and in *C. elegans*, knockdown of the *tdo-2* homologue as well as the lysosomal cathepsin protease

reduces granule fluorescence (Coburn *et al.,* 2013). In vertebrate cells, many proteins involved in lysosomal synthesis like AP-1, AP-3 and HOPS complex are also required for LRO synthesis (Raposo *et al.*, 2007). Interestingly, in *C. elegans* the Glo phenotype (lack of autofluorescence) is observed in mutants lacking the Rab38 homologue GLO-1, ABC transporter PGP-2, putative guanine nucleotide exchange factor GLO-4 and subunits of HOPS and AP-3 adaptor complex (Hermann *et al.*, 2005; Schroeder *et al.*, 2007). The induction of gut granule formation in *C. elegans* is controlled by expression of GATA transcription factors END-1/END-3 which are promoted by wnt signalling (Maduro, 2006).

During gastrulation, *glo-3* is expressed in the intestine precursor cells and later in life in the intestine, (Rabbitts *et al.*, 2008). *glo-3* was found to function in parallel or downstream of *pgp-2* and the AP-3 complex, similar to *glo-1*. GLO-3 is responsible for regulating the formation, maturation and stability of gut granules and selection of cargo trafficking. Even though GLO-3 does not have any sequence homology with LIMP-2, a lysosomal associated protein involved in lysosomal cargo sorting in mammals (Reczek *et al.*, 2007), it posseses a similar domain structure. Interestingly, *glo-3* mutants were able to develop gut granules during adulthood indicating an alternative pathway supporting this formation (Rabbitts *et al.,* 2008). A sequence similarity search of *glo-3* revealed no similarity to proteins outside the nematode phylum, which might imply a novel adaptation as in nematodes as for the assembly of LRO.

1.4.2.5 Lysosomes and ageing

Ageing reduces lysosomal activity and causes leakage of the lysosomal membrane. Also lysosomal morphology tends to change with age: under normal conditions, lysosomes in *C. elegans* neurons are scattered in the cytoplasm whereas under conditions of neurodegeneration, lysosomes are found to be enlarged and arranged close to the nucleus progressively fusing and forming a huge vacuole (Hall *et al.*, 1997; Artal-Sanz *et al.*, 2006).

Ageing induces damage to the membrane of these lysosomes in vivo causes leakage of the hydrolytic enzymes into the cytoplasm which could lead to damage or digestion of the major cellular components. In primates the ‘calpain cathepsin hypothesis’ indicates the role of μ-calpain in neuronal ageing-associated lysosomal disruption. Ischemic insult activates, calpain translocation to lysosomal membrane leading to lysosomal disruption and spillage of cathepsins into cytoplasm dismantling the whole neuron (Yamashima *et al.*, 2003). In addition, down regulation of vesicular trafficking can increase rate of ageing. In fact vesicular

trafficking genes are essential for *sod-3* expression in *daf-2* mutants in addition to their requirement for life span extension (Samuelson *et al.*, 2007).

Lysosomes are involved in the ageing process by 1. alterations in the membrane permeability of the lysosomes which cause leakage of hydrolytic enzymes or elevated autophagy. 2. accumulation of damaged useless proteins and disruptive dead weight (lipofuscin pigment) due to inadequate digestion or lysis. This may interfere in normal metabolic and lytic processes. 3. extrusion of digestive enzymes due to membrane damage or cell death resulting in digestion of extracellular structures leading to connective tissue injuries, vascular changes, collagen formation, autoantibody production and other degradative alterations.

Many macromolecules contain iron which when degraded in the lysosome creates a iron-rich environment. These iron-rich lysosomes are sensitive to oxidative stress whereas less active lysosomes are not (Kurz *et al.*, 2008). This is because of the Fenton-type reaction between hydrogen peroxide and iron inside the lysosome which leads to accumulating lipofuscin. On oxidative stress this process is rapid and releases pigment called ceroid. This accumulation renders lysosomes unfunctional. The degree of lysosomal damage, which shall lead to growth arrest, autophagy, apoptosis or necrosis, is governed by the amount of oxidative damage on it. Mutations in the lysosomal enzymes could result in accumulation of substrates of those specific enzymes, which leads to lysosomal storage diseases. Lipofuscin is produced due to low level diffusion of hydrogen peroxide into iron rich lysosomes. As ageing occurs lipofuscin starts to occupy a substantial part of the lysosome leading to diversion of the produced enzymes away from the autophagosomes. This can cause more damage to the mitochondria and other proteins leading to cell death. In earlier studies these lipofuscin filled lysosomes were considered inactive bodies while later studies proved that these lysosomes receive new hydrolytic enzymes by fusing with other lysosomes (Brunk and Ericsson, 1972; Luzio *et al.*, 2007).

1.4.2.6 Proteasome

In addition to macroautophagy, the ubiquitin proteasomal system is involved in protein degradation. In this system, specific proteins are selected and marked for degradation with ubiquitin molecules, which are then degraded by the proteasome. The proteasome, representing 1% of the total cellular protein, is responsible for about 30% of the cellular protein degradation and is localized in the nucleus and cytosol. It occurs in two major forms: the 20S and 26S proteasome and the proportion is modulated by intracellular calcium levels (Voges *et al.*, 1999). The 26S proteasome is involved in degradation of the majority of

proteins by employing an ATP dependent ubiquitin-mediated pathway (Voges *et al.,* 1999); in contrast, the 20S proteasome is involved in ATP independent selective degradation of partially unfolded and oxidised proteins (Grune *et al.*, 1996; Davies, 2001; Ferrington *et al.*, 2005). In the 26S ubiquitin mediated pathway, ubiquitin ligase is involved in attaching ubiquitin molecules to specific proteins, tagging them for clearance thereby regulating diverse molecular pathways (Sarikas *et al.*, 2008).

In yeast, proteasome activation leads to life span extension (Kruegel *et al.*, 2011). A strong correlation between life span and proteasomal activity exists. Knocking out proteasomal subunits decreases *C. elegans* life span (Yun *et al.*, 2008). Several genes and factors influence the ubiquitin/proteasome system (UPS) activity. In *C. elegans*, UPS activity in the somatic cells of germline-ablated worms is high and considered to be the cause of life span extension in these worms. Enhancing *rpn-6* expression, a proteasome subunit stabilizing the interaction between the 20S core and 19S cap, was sufficient to elevate the UPS activity and hence the life span extension (Vilchez *et al.*, 2012). On the other hand contradicting views also exist. In *daf-2* mutants, the increased DAF-16 activity lowers proteasomal activity (Dillin *et al.*, 2002; Stout *et al.*, 2013). Supporting this theory, *bec-1*, which has been shown to be essential for longevity in *daf-2* mutants, has been shown to negatively regulate proteasomal activity in *C. elegans* (Fitzenberger *et al.*, 2013).

The functional capability of the 26S subunit of the proteasome deteriorates as age progresses in whole organisms, senescent primary cultures, and human tissues (Friguet *et al.*, 2000; Sitte *et al.*, 2000; Friguet, 2002; Shringarpure and Davies, 2002; Morimoto and Cuervo, 2009; Baraibar and Friguet, 2012). Several neurodegenerative diseases have been identified in rodents and humans due to mutations in the UPS system (Mizuno *et al.*, 2001; Zhai *et al.*, 2003), which is logical as this system acts against formation of protein aggregates.

1.4.2.7 Endoplasmic reticulum associated degradation (ERAD)

Misfolded or damaged proteins in the endoplasmic reticulum, both soluble and oligomeric, are degraded by the ER associated degradation (ERAD) (Vembar and Brodsky, 2008). Different sensors detect the location (lumen or membrane) of the misfolded protein in the ER (Vembar and Brodsky, 2008; Hoseki *et al.*, 2010) and function in retro-translocation of these proteins to the cytoplasm where they are marked for proteolysis by ubiquitination and degraded by the proteasomal system (Meusser *et al.*, 2005; Nakatsukasa *et al.*, 2008).

1.4.3 Protein stability

Besides protein turnover, increased protein stability is a second factor which could be involved in maintaining proteostasis.

Chaperones interact with around 15 to 30% of freshly synthesised proteins and refold them to a native structure and the proteasome acts on around 20% of the newly synthesised proteins degrading them instantly (Wickner *et al.*, 1999; Turner and Varshavsky, 2000). Even though the degradative path can wastefully degrade these misfolded proteins, minimising their appearance and preventing immune response and protein aggregation (Wickner *et al.*, 1999), age-associated increase in oxidised and misfolded proteins or decreased function of chaperones or proteases can lead to accumulation of these erroneous proteins. This age associated decrease in protein degradation causes protein aggregation and interferes in cellular function acting as nucleation sites for other unrelated proteins to aggregate. Ageing leads to increased protein damage in worms, fungi, bats, flies, rodents, birds and humans (Stadtman *et al.*, 1992; Barja, 1998; Levine *et al.*, 2002; Nystrom, 2005; Koga *et al.*, 2011; Baraibar and Friguet, 2012). Chemical stabilizers like trehalose and glycerol are efficient in preventing this age associated decline in protein stability. In this study protein stability will be assessed in *daf-2* mutants for their influence on proteostasis.

1.4.3.1 Protein stabilizers

Oxidation of proteins causes carbonylation which leads to inappropriate protein cross links including misfoldings which in turn may lead to protein aggregation (Grune *et al.*, 1997; Mirzaei and Regnier, 2008). Misfolded proteins are stabilized to prevent further damage and loss of function. Protein stability is brought about by molecular chaperones (heat shock factors) and chemical chaperones (osmolytes).

Molecular chaperones

Molecular chaperons and heat shock factors play an important role in maintaining proteostasis especially in response to stress (Wu 1995; Nollen and Morimoto 2002; Morimoto 2002). Molecular chaperones are involved in de novo protein folding, maintenance of native form of pre-existing proteins (Hartl 1996; Bukau and Horwich 1998; Frydman 2001) and degradation. Other functions include protein quality control, including protein disaggregation and unfolding and targeting misfolded proteins for proteolysis (Kim *et al.*, 2013).

The requirement of these chaperones for longevity has been studied in *C. elegans*. In *C. elegans* and *Drosophila* overexpression of molecular chaperons which includes HSP-70 and

other small heat shock protein families have shown to induce longevity (Yokoyama *et al*., 2002; Walker and Lithgow 2003). In addition HSF-1 knockdown in *C. elegans* have shown to decrease longevity and accelerate ageing phenotype (Garigan *et al*., 2002) whereas overexpression of HSF-1 have shown to extend lifespan (Hsu *et al*., 2003; Morley and Morimoto 2004). Though the role of molecular chaperones had been well studied in *C. elegans* the role of chemical chaperones are less understood.

Chemical chaperones

Chemical chaperones fall into three major categories: 1. polyols (glycerol, sucrose, trehalose, other sugars) 2. amino acids (proline and glycine), and 3. particular methylamines (sarcosine, trimethylamine N-oxide (TMAO) and betaine).

Different classes of osmolytes can be selected by an organism for the protection against different stresses. Polyols protect against desiccation, dehydration and temperature stress, amino acids are used against ionic stress, whereas methylamine osmolytes operate against urea accumulation (Yancey *et al.*, 1982). It should be noted that not all organic osmolytes are involved in protection during stress (e.g. urea). The presence of methylamine osmolytes protects cells which are enriched with urea (Forster and Goldstein, 1976; Yancey and Somero, 1979). High amounts of urea have been shown to accumulate in intracellular fluids of cartilaginous fishes (sharks, rays), coelacanth and freshwater fish exposed to water stress (Janssens and Cohen, 1968). These high urea concentrations interfere with protein–ligand interactions. These organisms protect their proteins by accumulating the nitrogenous osmolyte, methylamine. Other osmolytes like TMAO and betaine were also shown to accumulate ranging from one-half to one-third of the urea concentrations (Yancey *et al.*, 1982). The other osmolytes will be discussed in detail below.

Glycerol

In response to ionic stress, glycerol levels increase to 10-20 fold in *C. elegans*, and this small metabolite possibly functions in protein stabilization. Supporting studies have shown an increase in glycerol-3-phosphate dehydrogenase expression upon hyper-tonicity (Lamitina *et al.*, 2004). In addition to its rapid accumulation during stress, high amounts of glycerol are excreted ranging on recovery from hypertonic conditions (Lamitina *et al.,* 2004). This signifies its precise regulation. In *C. elegans*, any disruption or damage to the cuticle is monitored by genes like *osm-7, osm-11* and many *dpy* genes and induce glycerol biosynthesis on osmotic stress (Wheeler and Thomas, 2006). *osr-1* mutants posses 30-40 fold higher

glycerol compared to wild type *C. elegans* and this property protects worms from shrinkage on acute exposure to NaCl (Solomon *et al.*, 2004). However, inhibition of glycerol synthesis genes nor constitutive elevation of glycerol levels did not show to alter protein aggregation (Burkewitz *et al.*, 2012) signifying that it is a less potent protein stabilizer.

Proline and glycine

Many studies found accumulation of amino acids such as proline and glycine in freeze-tolerant animals (Storey and Storey, 1996; Neufeld and Leader, 1998). The exact physiological action of these amino acid in the protective process is a question as their levels are very low compared to the proportion of proteins they should stabilize. In addition to protein stabilization, proline (and trehalose) were shown to replace water molecules by binding to head groups of membrane phospholipids and hence rendering membrane stability when the cell shrinks osmotically (Rudolph and Crowe, 1985; Storey and Storey, 1996). Unlike other osmolytes involved in stabilizing proteins, proline was shown to be involved in maintaining redox state in water-stressed plants (Cushman, 2001). Even with their stabilizing capability, the level of stability these minute quantities of amino acids could render on a larger volume of damaged proteins is questionable in an energy-conserved environment.

Trehalose

The presence of trehalose has been reported in a variety of organisms like yeast, fungi, bacteria, insects, invertebrates and plants (Elbein *et al.*, 2003). Presence of trehalose in the animal kingdom is widespread in invertebrates and it even occurs in some vertebrates (Arguelles, 2000). In most higher vertebrates, trehalose is absent, but trehalase - an enzyme that digests trehalose - is present (Ishihara *et al.*, 1997).

Trehalose is a bis-acetal non reducing homodisaccharide in which the two glucose molecules are linked together in a α-1, 1-glycosidic linkage. Several specific chemical and physical properties of trehalose render them suitable for stabilizing proteins. These properties include: low chemical reactivity, high glass transition temperature (above 100ºC), non-reducing nature, existence of a number of polymers, high affinity to water molecules, high melting temperature (above 200ºC), high stability in extreme pHs etc. Trehalose does not have the ability to traverse through the plasma membrane although it can be taken up by cells by fluid-phase endocytosis and pinocytosis (Ma *et al.*, 2005).

Glycogen is the most proximal glucose source for trehalose synthesis. Two glucose molecules, UDP-glucose synthesized from glucose-1-phosphate and glucose-6-phosphate,

undergo a condensation reaction to form trehalose (Fig-7). The enzyme trehalose-phosphate synthase catalyses this reaction. In *C. elegans* there are two putative trehalose-6-phosphate synthase genes (*tps-1* and *tps-2*). The glucose regulatory mechanism is similar in both vertebrates and invertebrates and involves the insulin signalling and glucagon/ hypertrehalosemic (HTH) (an adipokinetic neuropeptide) signal systems.. Trehalose is broken-down to glucose by the enzyme trehalase which is regulated at the transcriptional level depending of the availability of food, at post-transcription level by phosphorylation and also regulated post-translationally by trehalase inhibitors (Thompson, 2003). In *C. elegans* there are five trehalase (*tre*) genes known. *C. elegans* trehalase was reported to be an integral membrane protein, similar to mammals (Ruf *et al.*, 1990), which is attached to membranes by a glucosylphosphatidyl-inositol (GPI) anchor (Behm, 1997). In addition to TRE-2 and TRE-5 possesing the DGPI site for GPI anchorage, TRE-2 and TRE-4 possess transmembrane regions whereas TRE-1, TRE-3 and TRE-5 do not have any transmembrane regions. The presence of N terminal signal peptides shows that TRE-4 and TRE-5 are secreted (Pellerone *et al.*, 2003). The presence of soluble and membrane bound enzymes allows breakdown of intracellular and extracellular trehalose.

The possible function trehalose may provide in eukaryotes are: 1. protein stabilization during osmotic, thermal, or oxidative stress; 2. cryopreservation, keeping the hemolymph in liquid state, even at freezing temperatures; 3. as an energy source; 4. for anoxia tolerance, and 5. as a structural component in bacteria and plant cell wall polysaccharide and chitin synthesis.

The role of trehalose in oxidative, hypoxic, thermal, and desiccation stresses has been studied in detail (De Virgilio *et al.*, 1994; Watanabe *et al.*, 2002; Elbein *et al.*, 2003; Jagdale *et al.*, 2005; Sakurai *et al.*, 2008). High osmolarity induces trehalose synthesis in *Escherichia coli* which is necessary for its survival (Kempf and Bremer, 1998). Trehalose renders protection against oxidative damage on proteins. In wild type *Candida albicans* exposure to H_2O_2 induces trehalose production and improves life span. This effect was suggested to be caused by prevention of aggregate formation; mutants lacking trehalose are sensitive to H_2O_2 exposure (Gonzalez-Parraga *et al.*, 2003). Polyglutamine (PolyQ) and polyalanine (PolyA) mediated protein aggregation is prevented by trehalose (Tanaka *et al.*, 2004; Davies *et al.*, 2006). Aggregation of denatured proteins after heat shock was also shown to be prevented by trehalose (Hottiger *et al.*, 1994).

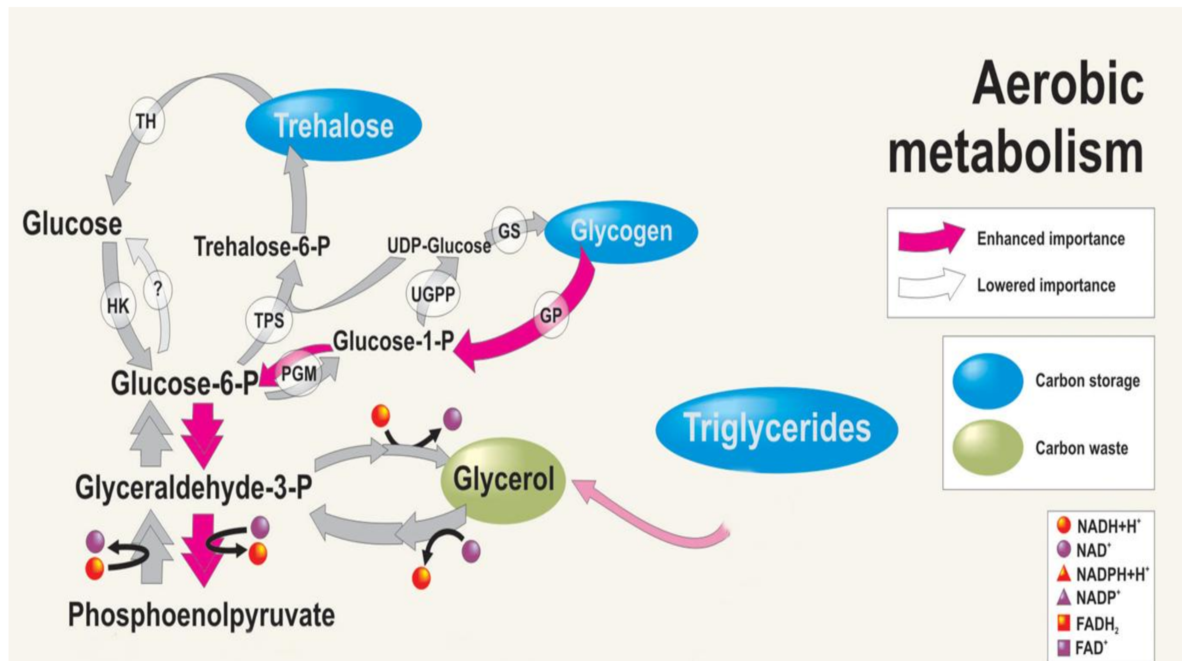

Figure 7: Trehalose biochemistry in *C. elegans*. This diagram is primarily based on the work of (Holt and Riddle, 2003; Burnell *et al*., 2005; McElwee *et al*., 2006). Adapted from Braeckman *et al.,* (2009) wormbook (Braeckman *et al*., 2009).

In addition to stabilizing proteins, trehalose also assists molecular chaperones during their folding process (Elliott *et al*., 1996; Singer and Lindquist, 1998; Viner and Clegg, 2001). Trehalose induces expression of the heat shock factor HSF-1 as well as other molecular chaperones (Bulman and Nelson, 2005; Conlin and Nelson, 2007). Partially folded proteins in yeast are stabilized by trehalose during heat shock and when these proteins are reactivated by chaperones, trehalose levels rapidly decrease by specific hydrolysis (Singer and Lindquist, 1998). Evidence correlating the amount of trehalose and the levels of thermo-tolerance and life span exist in yeast and *C. elegans* respectively (Hottiger *et al.*, 1987; Hottiger *et al.*, 1994; Honda *et al.*, 2010).

Autophagy may protect cells from accumulation of damaged proteins and aggregates by rapid proteolysis of unwanted proteins. Trehalose is shown to induce mTOR-independent autophagy, thereby enhancing clearance of proteins associated with human late-onset Huntington disease (HD) and Parkinson disease (Sarkar *et al.*, 2007).

Trehalose seems to be a preferred biological osmolyte. Compared to other sugars (fructose, sucrose and glucose) trehalose has a higher protective effect on yeast pyrophosphatase exposed at 50ºC (Sola-Penna and Meyer-Fernandes, 1998). The reason for this property of trehalose could be attributed to the lower amount of water in trehalose solutions compared to the same concentration of other sugar solutions. In *Drosophila*, unlike glucose, trehalose

concentrations were found to vary largely (Becker *et al.*, 1996). Unlike monosaccharides such as glucose, which engage in a “browning” (Maillard) reaction, trehalose does not undergo browning which can be damageing to the protein when dried (Tunnacliffe and Lapinski, 2003).

Trehalose increased resistance to several other conditions which induce protein damage and accumulation. *Drosophila* overexpressing trehalose synthase (*tps-1*) resists hypoxia for up to 4 hours without any tissue damage. Under normal conditions these animals suffer irreversible tissue damage on exposure to hypoxia for 5 minutes (Haddad and Ma, 2001; Chen and Haddad, 2004). Similarly, increased survival rate after hypoxic stress was observed in HEK-293 cells transfected with the *Drosophila tps-1* gene. This was an effect of the high expression of trehalose which resulted in lower amounts of aggregation and ubiquitination. *In vitro* protein aggregation caused by anoxia is also prevented by trehalose (Chenn and Haddad 2004). A reduced level of intra-nuclear protein aggregation in several organs was also reported in a Huntington’s disease mouse model orally administered with trehalose (Tanaka *et al.*, 2004). Trehalose has also been shown to be a promising cure for Alzheimer’s disease where it acts by preventing β-amyloid aggregate formation which normally leads to amyloid plaques (Liu *et al.*, 2005).

1.4.3.2 Protein stability, life span extension and ageing

Trehalose provides protein stability by reducing oxidative protein damage and aggregation and is therefore a potential candidate for life span extension in *C. elegans*. Indeed, trehalose treatment extends *C. elegans* life span upto 30%. Examination of these worms revealed that this life span extension coalesces with a reduced age-independent vulnerability, increased reproductive span and pharyngeal-pumping rate, a reduced the amount of autofluorescence accumulation, reduced polyglutamine aggregation, and enhanced thermo-tolerance. Interestingly, trehalose addition in long lived *daf-2* mutants did not extend life any further (Honda *et al.*, 2010). Another long lived IIS mutant strain, *age-1*, was found to posses higher levels of trehalose (Lamitina and Strange, 2005). The relationship between increased thermo-tolerance and life span extension has been studied in detail (Lithgow *et al.*, 1994; Gems *et al.*, 1998; Arantes-Oliveira *et al.*, 2003; Munoz and Riddle, 2003) which also suggests the involvement of trehalose.

In line with this, the trehalose-related genes *tps-1*, *tps-2* (trehalose 6 phosphate synthases) and *tre-4* (trehalase) are upregulated in *daf-2* mutants (Lamitina and Strange, 2005). DAF-16, which is reported to bind to several stress responsive genes, binds the DBE domain of *tps-1,*

gpd-2 (glyceraldehydes-3-phosphate dehydrogenase), *hsp-12.6* (small heat shock protein) leading to their up-regulation (Schuster *et al.,* 2010). This binding phenomenon was confirmed by Zhang *et al.,* 2013 with chromatin immune-precipitation experiments (ChIP) (Zhang *et al.*, 2013). Most importantly, the life span-extending effect of *daf-2* was partially lost when the trehalose 6 phosphate synthase-1 (*tps-1*) and *tps-2* genes, involved in trehalose biosynthesis, were knocked down (Honda *et al.,* 2010).

Hanover *et al.,* 2005 showed that O-GlcNAc transferase (OGT), when mutated in *C. elegans*, increased trehalose and glycogen levels 3-fold compared to wild type. This increase was complimented with a 3-fold decrease in triglycerides. O-GlcNAc transferase is involved in attaching O-GlcNAc (O-linked-N-acetylglucosamine) to serine and threonine residues of intracellular proteins. They catalyse posttranslational modification of RNA polymerase II and transcriptional factors (Chen *et al*., 2013). It is interesting that OGT mutants show a decrease in life span in *C. elegans* adults, suggesting that high trehalose levels per se are not sufficient to extend life span. However, it is possible that OGT-1 is responsible for vital functions in *C. elegans* and strongly reduces life span irrespective of the concomitant trehalose induction.

Finally, studies have reported an elevation in trehalose levels in the dauer stage. During this stage, the worms stop feeding so it is essential that they metabolize stored macronutrients, chiefly lipids but also glycogen. It is essential that energy is transported from these lipid and glycogen containing cells (intestine and epidermis) to other energy demanding tissues like muscles and neurons (Ogg *et al.*, 1997). Trehalose is thught to be used for this transport rather than glucose as *C. elegans* lacks the functional glucose-6-phosphatase (McElwee *et al.*, 2004).

1.5 Aims and outline of the thesis

It is generally accepted that proteostasis or protein homeostasis is essential for longevity in several organisms. In *Caenorhabditis elegans*, the insulin IGF-1 signalling mutants have been shown to have extended life span and increased proteostasis has been hypothesised to be the underlying reason. Our main goal was to further explore proteostasis in *daf-2* mutants and its role in longevity. Cellular proteostasis may result either from elevated protein turnover or from higher protein stability; the mechanism underlying *daf-2* proteostasis is not known. We hypothesise that, in the *daf-2* mutant, which has a restructured energy metabolism, the proteome is stabilized rather than turned over at high rate, the latter being energetically very expensive. We approached this goal step by step as follows:

Objective 1: In **chapter 3**, we test whether protein synthesis and degradation rates in the IIS mutant are increased to maintain proteostasis. We are interested in knowing this because previous studies have proposed increased protein turnover rates in *daf-2* mutants based on the genetic necessity of autphagy genes for life span extension in *daf-2*. Although reasonable, we consider this unlikely as protein turnover is an expensive process. We will use the radiolabel ^{35}S pulse chase methodology to investigate the protein turnover rates.

Objective 2: An alternative approach to study the importance of bulk autophagy in life span extension of *daf-2* mutants is to investigate their lysosomal activity. We will focus on this organelle in **chapter 2** of the thesis. We will generate transcriptional (qPCR), translational (iTRAQ proteomics), as well as activity (enzyme assays) data from lysosomal components and verify whether their expression and activity are up- or downregulated in the *daf-2* longevity mutant. Next, we will also knockdown key lysosomal components by RNAi and study its effect on life span of the *daf-2* and control strains.

Objective 3: We want to use an easy, straightforward technique to check *daf-2* proteome stability. We will apply TCA-mediated acid precipitation, in which a large part of the proteome is removed by precipitation. Well-stabilized proteins, however, resist precipitation

and can be quantified. In **chapter 3**, we will test whether this precipitation-resistant fraction is larger in *daf-2* mutants.

Objective 4: We will also focus on candidate factors enhancing *daf-2* proteome stability. Previous studies found high trehalose levels in *daf-2* mutants and up-regulated expression of trehalose synthase genes. Therefore, trehalose will be the candidate compound of choice and we will investigate its role in *daf-2* proteome stability. The results of this study will also be integrated in **chapter 3**.

Finally, in the **discussion section**, we will integrate all our new findings and propose a new molecular model that underlies the longevity of the *daf-2* insulin/IGF receptor mutant.

PART II

RESULTS

CHAPTER 2

Lysosomal activity is down-regulated in the long lived *C. elegans daf-2* mutant

CONTRIBUTION

Nematode cultures and sampling

Cathepsin L and acid phosphatases enzyme assays

RNAi culturing and experimentation

Acridine orange staining and confocal microscopy

Writing the manuscript

To be submitted as

Lysosomal activity is down-regulated in the long lived *C. elegans daf-2* mutant

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2.1 Abstract

In *daf*-2, a long lived *C. elegans* insulin/IGF receptor mutant, life span extension is said to be supported by autophagy, a major conserved bulk degradative pathway. This leads to the assumption that protein turnover in this mutant may be upregulated which may prevent the accumulation of damaged proteins and a deceleration of the ageing process. Recent studies, however, pointed out that protein turnover is strongly decreased in *daf-2*, contradicting earlier assumptions. In this study we focus on the functional relevance of the lysosome, a major organelle involved in autophagy, in *daf-2* longevity. Previous investigations with iTRAQ protein profiling and qPCR mRNA level profiling in our lab showed a global downregulation of the lysosomal components in long lived *glp-4;daf-2* mutants. As a follow-up my analysis showed lysosomal cathepsin L and acid phosphatase enzyme activities were downregulated as well. This global silencing of lysosomal activity raises questions about their requirement for longevity. In this study, knock-down of several lysosomal components did not influence *C. elegans* life span. Taking into account the general lysosomal downregulation, we propose that *C. elegans daf-2* longevity does not rely on gross lysosomal function that supports high protein turnover. Nevertheless, the process of autophagy may be essential at low levels in specific cells to support *daf-2* longevity.

2.2 Introduction

Ageing is commonly considered as a result of gradual accumulation of cellular damage, caused by malfunctions of biological processes involved in turnover, repair and maintenance (Sohal *et al.*, 1994). Autophagy is a key housekeeping process, conserved in eukaryotes, that helps to maintain cellular homeostasis by efficient degradation of damaged or unwanted proteins and organelles. Therefore, it is not surprising that autophagy has been put forward as a central player in life span extension of *Caenorhabditis elegans* (Tóth *et al.,* 2008; Hansen *et al.,* 2008). One of the most pronounced and best studied mechanisms of life span extension in *C. elegans* is mutation in the insulin/IGF signalling receptor *daf-2*, and its longevity is dependent on autophagic activity (Melendez *et al.,* 2002; Hansen *et al.,* 2008). However, the role of the lysosome, the major organelle involved in macroautophagy. has not been studied in detail in this remarkable mutant.

Lysosomes occur as single membrane organelles and possess over 50 different acid hydrolases involved in degradation of biological polymers including nucleic acids, proteins, lipids and carbohydrates. Even though lysosomal enzymes have been studied in great detail, most nematode studies are limited to gastrointestinal parasitic nematodes which require proteases for host penetration (Robinson *et al.*, 2009) or in *C. elegans* embryogenesis and moulting (Hashmi *et al.*, 2002). Studies on their role in longevity are very limited.

Considering that 1. autophagy clears the cell from damaged and unwanted proteins, 2. damaged molecules tend to accumulate during ageing and 3. autophagy is said to be required to support the long life span of the *C. elegans* Ins/IGF mutant *daf-2*, it is tempting to directly correlate these phenomena and hypothesize that *daf-2* mutants are long lived due to increased bulk autophagic activity that clears the damage in the worm and rejuvenates the proteome. Therefore, we expect a gross increase in lysosomal activity in *daf-2* mutants.

In this study we focused on the differential regulation of a variety of lysosomal proteins in the *daf-2* mutant lysosome. Counter to our expectations, we found a total absence acid proteolytic activity in *daf-2* worms with a concomitant activation of a protease inhibiting system, general downregulation of most protease genes, a decrease in acid phosphatase activity and lowered lysosomal acidification. These data suggest that overall lysosome activity is very low in *daf-2*. Life span analysis indicates that lysosomal function and even lysosomal formation are not required for *daf-2* longevity. This casts serious doubt on the assumption that *daf-2* longevity is supported by high rates of damage clearing via autophagy.

2.3 Results

In a recent study, we have found that protein synthesis and degradation is slowed down in *daf-2* mutants (Depuydt et al, 2013; own unpublished data). These results contradict the protein turnover hypothesis (Gafni, 1990; Ryazanov and Nefsky, 2002; Brunk and Terman, 2002; Tavernarakis and Driscoll, 2002) but are in line with the findings that genetic inhibition of protein synthesis machinery extends life span in *C. elegans* (Pan *et al.,* 2007; Synthichaki *et al.,* 2007; Hansen *et al.,* 2007). Like our previous studies, we here use the worm strains that allow us to focus specifically on the FOXO transcription factor DAF-16, which is required and sufficient for the longevity of the *daf-2* mutant, in an exclusively somatic context. Therefore, experiments were performed in a germline-deficient *glp-4* background. The reference strain, with normal life span was *glp-4 daf-16;daf-2* and the long lived strain tested was *glp-4;daf-2*.

Cathepsin L is deactivated by a cysteine protease inhibitor in *glp-4;daf-2*

In order to break down proteins, lysosomes are equipped with a large family of proteases, including aspartic, cysteine and serine protease subfamilies (Brix, 2005). We tested the activity of the ubiquitous lysosomal cysteine protease cathepsin L in homogenates of young adult worms (second day of adulthood) as a proxy of lysosomal protease activity. Surprisingly, we found that cathepsin L activity is virtually absent in the long lived *glp-4;daf-2* mutants while in the *glp-4 daf-16;daf-2* reference strain, we found increased cathepsin L activity over the *glp-4* background control (**Fig 1A**). These data suggest that DAF-16 is a strong repressor of cathepsin L activity. A similar pattern was observed in strains lacking the *glp-4* background mutation, although the effect was less outspoken, which suggests that some interaction with the *glp-4* mutation may exist (**Fig 1B**). The cathespin L activity of *glp-4 daf-16;daf-2* mutants rapidly decreases over age and falls to background levels around day 10 of adulthood. In the long lived *glp-4;daf-2* mutant, no significant cathepsin activity was measured over the entire adult life span (**Fig 1C**). We showed that the low cathepsin L activity in *glp-4;daf-2* mutants was not due to a shift in pH optimum in the mutant compared to the reference strain (**Fig 1D**), suggesting that the enzyme is actively downregulated.

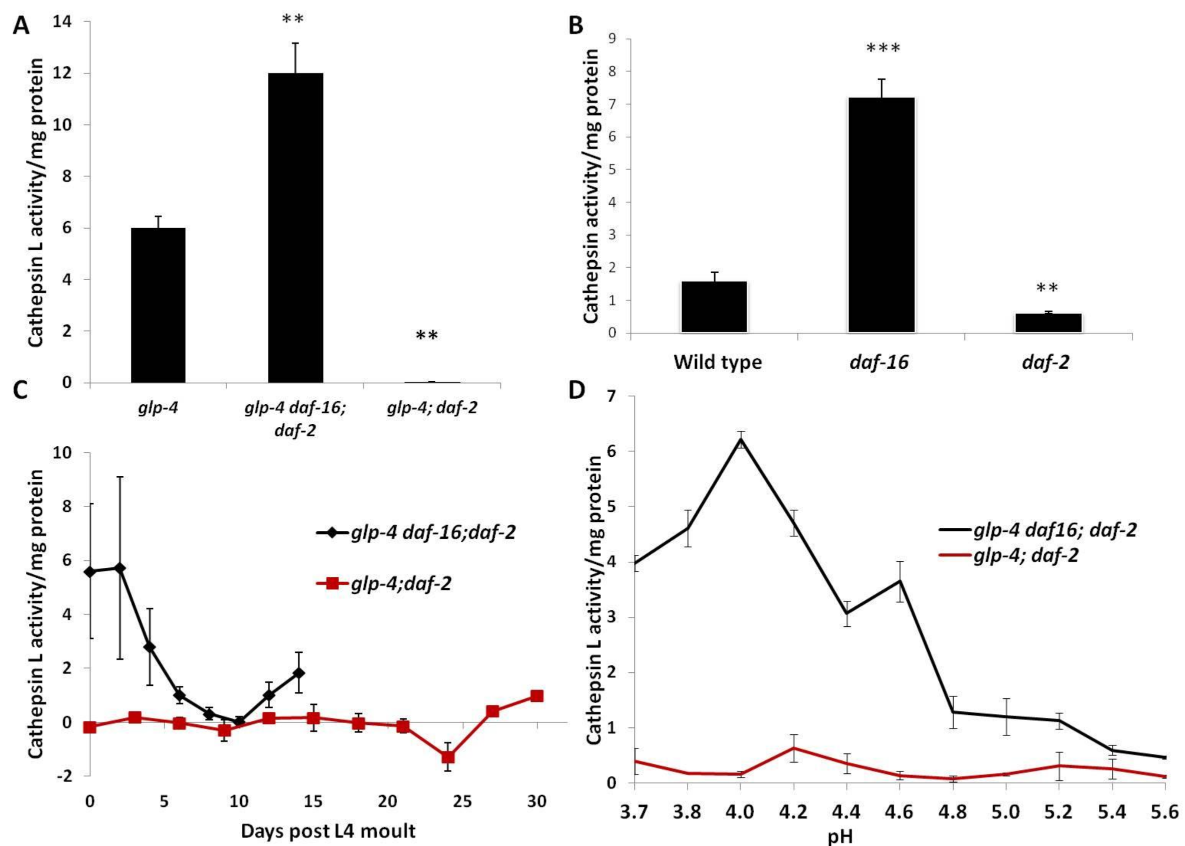

Figure 1: Cathepsin L enzyme expression and activity in *glp-4;daf-2* and the correlation between increasing age and reduced cathepsin L activity. A. Cathepsin L activity at young adult stage in control *glp-4*, *glp-4 daf-16;daf-2* and the longlived *glp-4;daf-2* mutants (P-Value *<0.05, **<0.01, ***<0.001). **B**. Cathepsin L activity in the non *glp-4* background mutants. **C**. Age associated cathepsin L activity in the long lived *glp-4;daf-2* and control strains**. D.** Influence of pH on cathepsin L enzymatic activity**.** P-Value *<0.05, **<0.01, ***<0.001).

Next, we asked which members of the *C. elegans* cathepsin family contributed to the activity that was obtained in the enzymatic assays described above. RNAi knockdown of these members in the *glp-4 daf-16;daf-2* reference strain showed that *cpl-1*(RNAi) caused a significant 2.5 fold decrease in activity compared to the L4440 empty vector control (**Fig 2A**). All other cathepsin knockdowns did not clearly contribute to the total cathespin L activity signal. This relative contribution pattern was largely reflected in the abundance of cathepsins in *glp-4;daf-2* mutants as determined by an iTRAQ proteomics approach (**Fig 2B**). Thus, in *glp-4;daf-2*, the most active cathepsins are downregulated at the protein level. Only ASP-1 shows a deviation from this pattern: this protein is clearly downregulated in *glp-4;daf-2* mutants while it does not contribute strongly to the overall cathepsin activity in control worms.

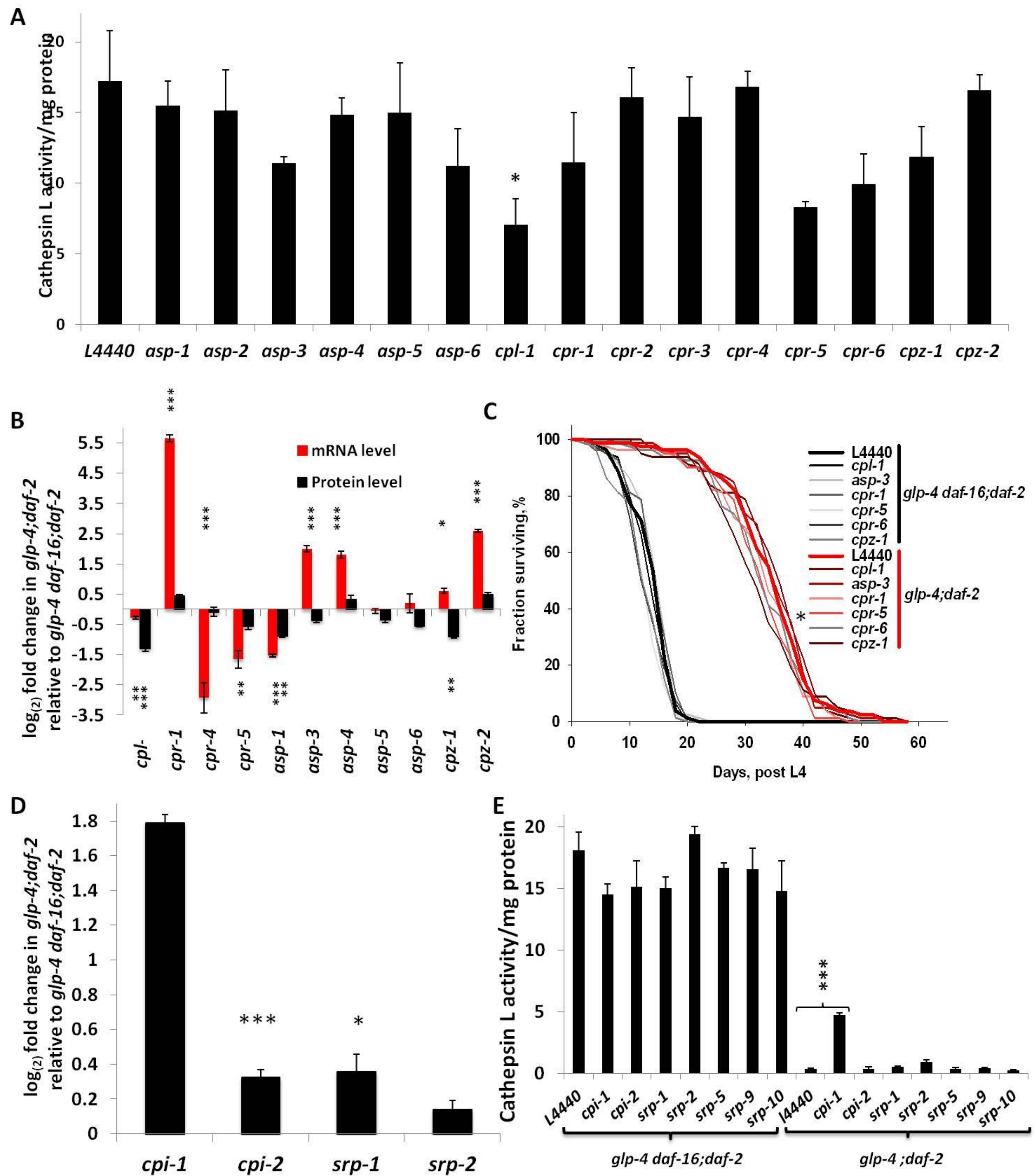

Figure 2: Cathepsin L activity on RNAi knock-down of specific cathepsin genes and its effect on life span. **A**. Cathepsin L activity after RNAi knock-down of specific cathepsin genes in the *glp-4 daf-16;daf-2* reference strain. **B**. mRNA data and iTRAQ data on relative protein level of different cathepsins in *glp-4 daf-16;daf-2* and *glp-4;daf-2* mutants. **C**. Life span analysis of *glp-4 daf-16;daf-2* and *glp-4;daf-2* mutants combined with RNAi knockdown of specific cathepsin enzymes. **D.** iTRAQ quantification of protease inhibitor relative levels in *glp-4 daf-16;daf-2* and *glp-4;daf-2* mutants. **E**. Partial rescue of cathepsin L activity in long lived *glp-4;daf-2* mutants on *cpi-1* RNAi. No significant change in cathepsin L levels in *glp-4 daf-16;daf-2* strain on serpine and cystatin RNAi. (P-Value *<0.05, **<0.01, ***<0.001)

Strikingly, Q-PCR data show no straight correlation with the quantitative proteomics data: some cathepsin proteins are downregulated in *glp-4;daf-2* while their transcripts are more abundant or remain nearly unaltered (*asp-3, asp-6, cpl-1, cpz-1*). In other cases, cathepsin protein and mRNA levels match (*asp-1, asp-4, cpz-2*). Yet, some genes show strong regulation at the transcriptional level, while no change occurs as the protein level (*cpr-1* and *cpr-4*). These patterns indicate that cathepsin expression is under clear transcriptional and (post)translational control.

Is the strong downregulation of cathepsin, as seen in *glp-4;daf-2* mutants, required and sufficient for life span extension? We tested this by knocking down all cathepsins that contribute to general cathepsin activity in the *glp-4 daf-16;daf-2* reference strain. No life span extension was observed under these conditions, suggesting that reduction of cathepsin activity is not required to support Ins/IGF-induced longevity or that lysosomal cathepsins are redundant in *C. elegans*. As expected, further knockdown of cathepsins in the long lived *glp-4;daf-2* mutant did not alter life span either (**Fig 2C**).

The striking absence of cathepsin L activity in *glp-4;daf-2* mutants and the (post)translational control of cathepsin activity lead to the analysis of protease inhibitors. Our iTRAQ proteomics screen revealed a strong upregulation of the cystein protease inhibitor CPI-1 and a weaker upregulation of CPI-2 (**Fig 2D**). Also weak upregulation of the serine protease inhibitors SRP-1 and SRP-2 was detected (**Fig 2D**). RNAi knockdown of these protease inhibitors shows that *cpi-1*(RNAi), but not RNAi of *cpi-2* or *srp* genes, lead to significant derepression of cathepsin L activity in *glp-4;daf-2* mutants (**Fig 2E**). These data suggest that, in *glp-4;daf-2* mutants, DAF-16 actively suppresses cathepsin L activity by upregulation of the protease inhibitor *cpi-1*.

Acid phosphatase activity is repressed in long lived *glp-4;daf-2* mutants

Next, we wondered whether the strong protease phenotype of *glp-4;daf-2* mutants extends to other lysosomal hydrolases as well. Acid phosphatase activity was also significantly reduced in *glp-4;daf-2* compared the the reference strain, although not to background levels, as was the case for cathepsin L activity (**Fig 3A**). Counter to the pattern found for cathepsin L activity, acid phosphatase activity does not decrease with age in the *glp-4 daf-16;daf-2* reference strain, but gradually decreases with time in the long lived *glp-4;daf-2* strain (**Fig 3B**).

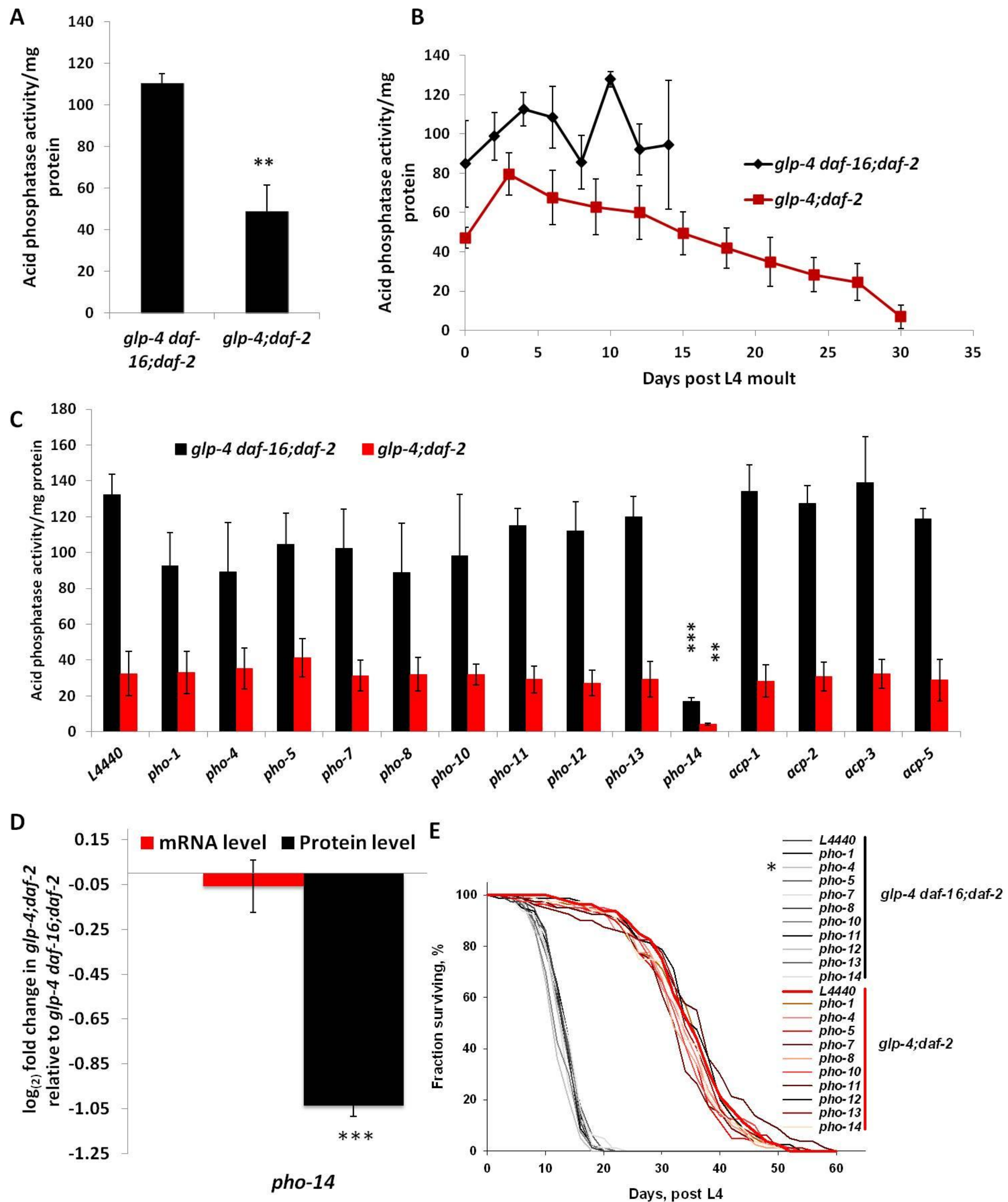

Figure 3: Acid phosphatase activity and expression in *glp-4;daf-2* and effects on life span. A. Acid phophatase activity in young adult population of the longlived *glp-4;daf-2* and normal lived *glp-4 daf-16;daf-2* mutants. **B**. Acid phosphatases along the age of *glp-4;daf-2* and *glp-4 daf-16;daf-2* mutants. **C**. Acid phosphatase activity in the two mutants on RNAi knock-down of specific acid phosphatase genes. **D**. Relative transcript and protein abundance of *pho-14* in the longlived versus control mutants. **E**. Life span analysis of *glp-4 daf-16;daf-2* and *glp-4;daf-2* mutants with specific acid phosphatases knocked-down by RNAi. (P-Value *<0.05, **<0.01, ***<0.001)

RNAi inhibition of individual phosphatases shows that PHO-14 is responsible for approximately 80% of the total acid phosphatase enzyme activity in both *glp-4;daf-2* and *glp-*

4;daf-16;daf-2 (**Fig 3C**). The low acid phosphatase activity in *glp-4;daf-2*, is at least partially dependent on lowered PHO-14 levels, as detected in our iTRAQ proteomics dataset (**Fig 3D**). RNAi knockdown of other phosphatases did not significantly alter overall acid phosphatase activity although slight decreases that did not reach the 0.05 significance level were found for *pho-1, pho-4, pho-5, pho-7, pho-8* and *pho-10* (**Fig 3C**).

These small differences from the empty vector control were absent in the *glp-4;daf-2* background, suggesting that these *pho* genes may be downregulated in this mutant, adding to its low acid phosphatase activity phenotype. At the transcriptional level, we found a general upregulation of most *acp* genes in *glp-4;daf-2*, while most *pho* mRNA levels were not altered drastically, except for the clear downregulation of *pho-1* and *pho-13* (**Fig 4**).

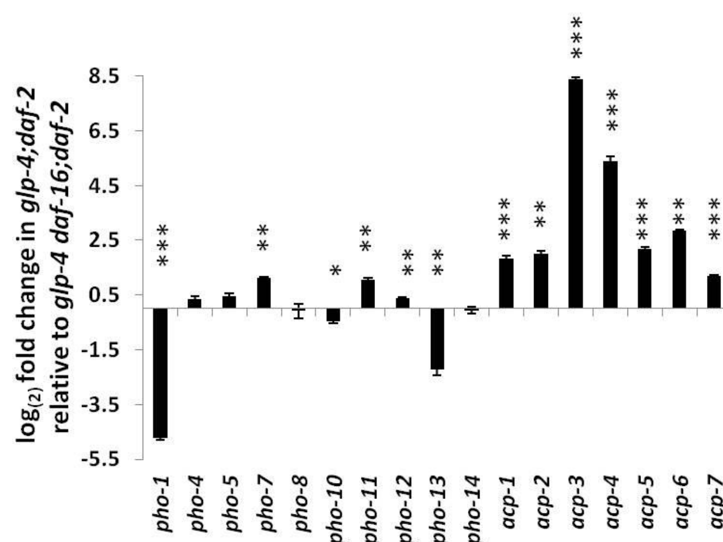

Figure 4: Individual acid phosphatases mRNA levels. Relative mRNA levels of different acid phosphatases in *glp-4;daf-2* and *glp-4 daf-16;daf-2* mutants.

Unfortunately, these transcriptional patterns cannot be compared to protein levels as only two acid phosphatases were picked up in the iTRAQ experiment (2-fold decrease of PHO-14 and 2-fold increase of ACP-6). RNAi knockdown of *pho* genes does not alter life span in both *glp-4;daf-2* and *glp-4 daf-16;daf-2* (**Fig 3E**). Similar to the cathepsin effect on life span, acid phophatase knockdown is also not sufficient to extend life span or *C. elegans* phosphatases are (partially) redundant.

glp-4;daf-2 mutants lack efficient lysosomal acidification machinery

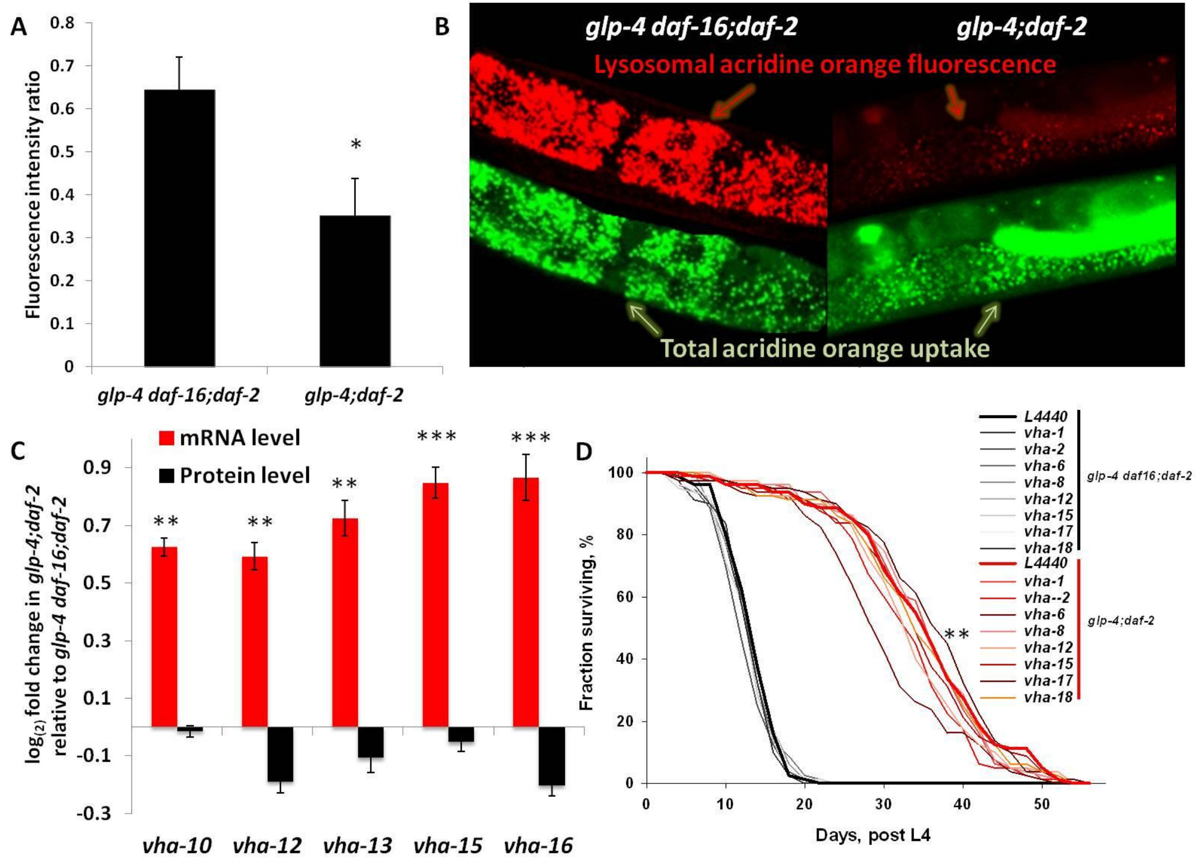

Figure 5: Reduced acridine orange staining and down-regulation of H⁺VATPase in the long lived *glp-4;daf-2* mutants. A. Acridine orange fluorescence intensity ratio measurement in long lived and control worms. **B**. Confocal microscopy images of acridine orange stained *glp-4 daf-16;daf-2* and *glp-4;daf-2* worms. **C**. iTRAQ and qPCR data on the relative protein and mRNA expression levels of H^+VATPase subunits in *glp-4;daf-2* worms relative to *glp-4 daf-16;daf-2* worms **D**. Life span changes on knock-down of specific genes representing individual H^+VATPase subunits.(P-Value *<0.05, **<0.01, ***<0.001).

Lysosomal hydrolases operate exclusively in a narrow acidic pH range, which was confirmed for *C. elegans* cathepsin L in this study (**Fig 1D**). We asked whether, besides general downregulation of lysosomal enzymes, lysosomal acidification was also inhibited in long lived *glp-4;daf-2* worms. In a first approach, we used the metachromatic lysosomotropic weak base acridine orange as a lysosomal marker. Acridine orange shows red fluorescence when trapped in the lysosomes (highly protonated oligomeric form) and green fluorescence in regions which are neutral or slightly alkaline conditions (deprotonated monomeric form). As the *daf-2(e1370)* allele causes an Eat phenotype, with reduced pharyngeal pumping, we used the green fluorescence as a measure of acridine orange uptake and normalised the red lysosomal signal to this green signal. In the *glp-4;daf-2* mutant, the red-to-green fluorescence

ratio was significantly decreased compared to the reference strain (**Fig 5A, B**). This decrease may signify a lower degree of lysosomal acidification. On the contrary, these subunits were significantly upregulated at the transcript level (**Fig 5C**). Post transcriptional regulation might be a reason for the reduced protein levels observed (**Fig 5C**). Knock-down of VHA proton pump subunits is not sufficient to extend life span of the *glp-4 daf-16;daf-2* reference strain, nor does it have a significant effect on *glp-4;daf-2* longevity, except for *vha-6(RNAi)*, which slightly reduces life span of *glp-4;daf-2* (**Fig 5D**). *vha-6* is found to be essential for larval survival and is expressed in the apical membrane of intestinal epithelial cells (oka et al., 2001).

Amino acid permeases and other lysosomal compounds

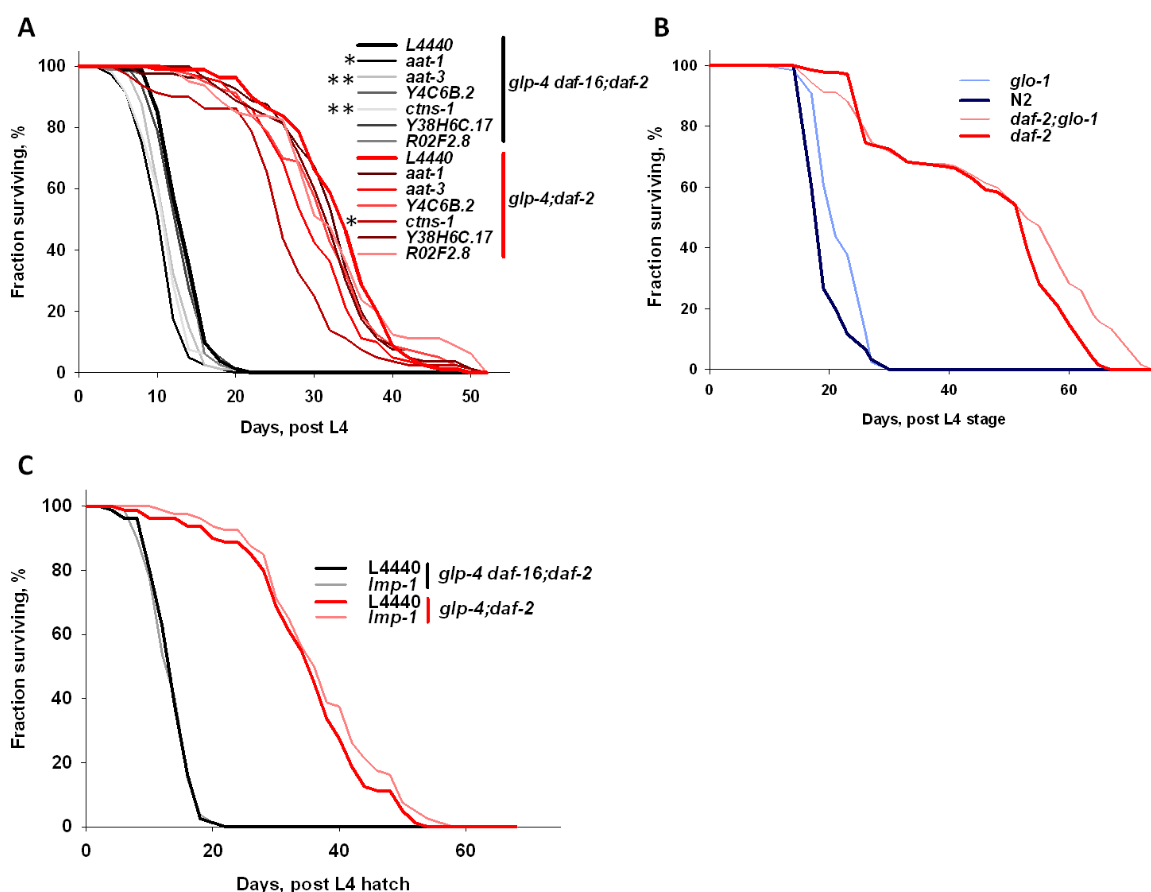

Figure 6: Life span change upon RNAi knockdown of different lysosomal components. A. Life span of *glp-4 daf-16;daf-2* and *glp-4;daf-2* treated with RNAi against amino acid permease genes. **B.** Effect of *glo-1* mutation on wild type and *daf-2 C. elegans* life span **C.** Life span changes upon *Imp-1(RNAi)* in *glp-4 daf-16;daf-2* and *glp-4;daf-2*. (P-Value *<0.05, **<0.01, ***<0.001).

As the lysosomal amino acid permeases are involved in transport of amino acids in and out of the lysosomes, their influence on life span extension was also assessed. Knock-down of the amino acid permeases *aat-1, aat-3* and *ctns-1* shortened life span significantly in *glp-4 daf-*

16;daf-2 control worms (**Fig 6A**), whereas only *ctns-1* remained that phenotype in *glp-4; daf-2*.

The influence of *glo-1*, an essential component for gut granule biogenesis, on life span of worms was assessed. Acridine orange staining is strongly reduced in these mutants (Hermann et al., 2005). Mutation in *glo-1* did not result in any significant change in life span in wild type and *daf-2* (**Fig 6B**). Knock-down of another lysosomal component involved in phagosome maturation, *lmp-1*, showed a reduction in acridine orange staining (personal observation) but no significant reduction in life span of both mutants (**Fig 6C**).

2.4 Discussion

Ageing is commonly regarded as the result of accumulation of some sort of damage. The most prominent mechanistic theory of ageing is the oxidative damage theory, in which it is assumed that reactive oxygen species (ROS) cause damage to proteins, lipids and nucleic acids, resulting in physiological decline and, ultimately, death of the organism. Several processes are predicted by this theory to ameliorate age-related functional decline: prevention of ROS formation, ROS scavenging by antioxidants, damage repair or breakdown and resynthesis (turnover) of damaged molecules. Indeed, it has been suggested that high protein turnover could support life span extension (Gafni, 1990; Ryazanov and Nefsky, 2002; Tavernarakis and Driscoll, 2002) and the finding that autophagy is required for the life span extension in several *C. elegans* mutants, including the Ins/IGF mutant *daf-2* (Melendez *et al.,* 2002; Tóth *et al.,* 2008; Hansen *et al.,* 2008) seemed to convincingly support this notion. On the other hand, inhibition of protein synthesis in *C. elegans* extends life span (Hansen *et al.,* 2007;Pan *et al.,* 2007; Synthichaki *et al.,* 2007), which seems to contradict the protein turnover hypothesis. More recently, we and others found that, in *daf-2* mutants, protein synthesis and breakdown, as well as expression of ribosomal subunits is strongly decreased (Depuydt *et al.,* 2013; Stout *et al.,* 2013; unpublished observations). This argues against increased protein turnover and purging of oxidative damage in long lived *daf-2* mutants.

In this study, we set out to focus on an essential organelle in the autophagic process: the lysosome. We expect that, if bulk protein turnover is enhanced in *daf-2* mutants by autophagy, lysosomal presence and activity should be strongly enhanced in these mutants. We used the germlineless *glp-4;daf-2* longlived mutant in this study and compared it with the normal-lived *glp-4 daf-16;daf-2* reference strain. This allowed us to focus on the somatic function of DAF-16 in extending *C. elegans* life span. Expression and functional activity of a

series of lysosomal key enzymes and proteins were studied, including proteases, acid phosphatases, the proton pump, and amino acid permeases,

We identified cathepsin L, a key lysosomal proteolytic enzyme, to be down-regulated in expression and activity in the long lived *glp-4;daf-2* mutant compared to its *glp-4 daf-16;daf-2* and *glp-4* counterparts. This is in line with our previous findings that total protein degradation rate, determined with ^{35}S pulse-chase labelling, was lowered in *glp-4;daf-2* compared to the control (Depuydt *et al.,* 2013). Comparison of cathepsin L activity in the three strains showed that DAF-16 is a negative regulator of cathepsin L activity.

Several studies in nematodes have linked ageing to decreased protein turnover (Prasanna and Lane, 1979; Sharma *et al.*, 1979) and cathepsin D activity (Sarkis *et al.*, 1988). In line with these studies we found an age-dependent decrease in cathepsin L activity in the reference strain. Supportive to our results, ageing in rats has been shown to decrease cathepsin L activity in all brain regions (Nakanishi *et al.*, 1994; Amano *et al.*, 1995). *cpl-1* and *cpz-1* which were identified in our study as important contributors of cathepsin L activity in the reference strain, are reported to be involved in embryonic and postembryonic development and disruption of these genes induced sterility in adult nematodes (Hashmi *et al.*, 2002; Britton and Murray, 2004; Hashmi *et al.*, 2004; Hashmi *et al.*, 2006). Knockdown of *cpr-1*, another contributor of cathepsin activity, was shown to induce dauer formation (Jensen *et al.*, 2010), a phenotype which is also linked to Ins/IGF signalling. ASP-4, one of the few upregulated cathepsins in *glp-4;daf-2*, is involved in necrotic cell death and neuro-degeneration (Syntichaki *et al.*, 2002), although no significant cathepsin L activity is detected in this strain. Although our proteomics dataset indicated weak to strong down-regulation of most cathepsin , a complete lack of cathepsin L activity in the long lived mutant was very surprising. Apparently, cathepsin activity was silenced post-translationally. Indeed, we found increased expression of cystatins and serpins *cpi-1, srp-1, srp-2* in *glp-4;daf-2* mutants along with partial cathepsin L activity rescue upon *cpi-1* knock-down. Even though CPI-2a was shown to be essential during development and reproduction and to be interacting with CPL-1 and CPZ-1 (Hashmi *et al.*, 2006) *cpi-2* did not seem to be involved in the bulk cathepsin inhibition in the long lived mutants, nor were *srp* genes.

Acid phosphatases, involved in recycling of inorganic phosphates, play a key role in the regulation of several metabolic reactions (Osagie 1992). Similar to what we observed with cathepsin L activity; acid phosphatase enzyme activity was reduced in the longlived mutants compared to the control hinting at a general down-regulation of lysosomal enzymes in *glp-4;daf-2* mutants. Even though studies have pointed out *pho-1* as the major and most

important lysosomal acid phosphatase (Fukushige *et al.*, 2005), we observed *pho-14* to be responsible for the majority of acid phosphatase activity in both mutants.

Contrary to earlier studies that reported a 2.5-fold increase of acid phosphatase activity over age in wild type (Bolanowski *et al.,* 1983), we observed nu such increase, nor a decrease in our *glp-4 daf-16;daf-2* reference strain. In the long lived *glp-4;daf-2*, this activity gradually decreases over time. Although *pho-11* possesses a DAF-16 binding element (DBE) and its transcripts are up-regulated in *daf-2* mutants compared to *daf-16*; *daf-2* controls (McElwee *et al.,* 2003), we only found a weak two-fold upregulation of this transcript in our qPCR analysis of *glp-4;daf-2*. More generally, we found a stronger upregulation of the family of *acp* transcripts (not resulting in higher overall acid phosphatase acivity) in *glp-4;daf-2*, while *pho* transcripts did not change drastically, except for *pho-1* and *pho-13*, which were strongly downregulated in *glp-4;daf-2*.

As lysosomal enzyme function is clearly affected in *glp-4;daf-2* we decided to analyze lysosomal abundance and acidification in this longevity mutant with the lysosomotropic agent acridine orange. We observed lower red fluorescence intensity, indicating a decrease of the lysosomal system. Key components of the lysosomal proton pumps (H^+V-ATPase subunits) showed reduced abundancy according to our proteomics experiment, a regulation that was not reflected at the transcriptional level. Lysosomal acidification is also regulated by reversible dissociation of the V_0 from the V_1 domain of the VHA proton pump, resulting in a disruption of the ATPase activity of the complex. This dissociation is brought about by reduced glucose in yeast (Kane, 2006) and insect cells (Beyenbach and Wieczorek, 2006) and is an energy-preserving strategy. This may happen in the long lived *glp-4;daf-2* worms as well, although we have no direct experimental evidence that supports this hypothesis.

Overall, we found a general decrease of lysosomal components and activity in the long lived *glp-4;daf-2* mutant. This observation makes it very unlikely that life span in this Ins/IGF mutant is extended because of massively increased autophagy and cellular renewal. On the other hand, is lysosomal downregulation sufficient to obtain longevity? An extensive set of life span experiments showed that knocking down cathepsins, acid phosphatases and proton pump subunits does not extend life span of the *glp-4 daf-16;daf-2* reference strain, nor does it change life span of the long lived *glp-4;daf-2*. Thus, lysosomal activity is irrelevant to *C. elegans* adult life span or the lysosomal components act redundantly. In order to overcome the latter problem, we tested the life span of worms in which biogenesis of lysosomal-related organelles was halted. These worms showed unaltered life span, even in the presence of a

daf-2 mutation. These data strongly suggest that downregulation of, and even absence of lysosomes does not influence life span. Nevertheless, the clear inhibition of lysosomal function in the long lived *glp-4;daf-2* may fit in a general decrease of energy expenditure, allowing the worms to survive longer with their internal energy reserves (high glycogen and fat content – Depuydt *et al.,* 2014), just like dauers do. In recent studies (Honda and Honda, 2010; own unpublished observations) it was found that synthesis of the chemical chaperone trehalose is required for the life span extension seen in *daf-2* mutants. Thus, it seems that protein stabilization rather than enhancement of turnover is key to *daf-2* longevity. Finally, the process of autophagy may be also crucial to *daf-2* longevity, yet not as a tool for large-scale protein turnover and clearance of damage.

2.5 Materials and Methods

The strains used in this study are: Bristol N2 (wild type strain), *glp-4(bn2*ts*)I, glp-4(bn2*ts*)I daf-16(mgDf50)I;daf-2(e1370*ts*)III, glp-4(bn2*ts*)I;daf-2(e1370*ts*)III, daf-16(mgDf50)I, daf-2(e1370*ts*)III*, *daf-2(e1370*ts*)III;glo-1(zu391)*X which were obtained from David Gems and the *Caenorhabditis* Genetics Centre. All strains were maintained on nutrient agar (NA) plates plated with a lawn of freshly prepared *E.coli* K12 bacterial cells and cultured at 17ºC.

Culturing and sampling for enzyme assays

Worms were synchronized by isolating eggs from gravid worms by using an alkaline hydrochlorite bleach (Wood 1988) and letting them hatch overnight in S-buffer (0.1M NaCl, 0.05M Potassium phosphate, pH 6) at 17ºC. The hatched L1s were seeded onto NA plates with an *E. coli* K12 bacterial lawn. The worms were grown at 16ºC for three days till they reached L3 stage, and then transferred to 24ºC for the rest of the experiment. When worms reached the young adult stage, they were sampled immediately.

For the longitudinal enzyme assays, worm samples were collected every other day beginning from the young adult stage for *glp-4 daf-16;daf-2* mutants and once every three days for *glp-4;daf-2* mutants.

For measuring enzyme activity post RNAi, L4 worms were transferred from NA plates to NGM plates with a lawn of the specific bacterial RNAi clone after a fivefold wash with S-buffer. RNAi clones were obtained from Ahringer library (Source Bioscience Lifesciences, Nottingham, UK). Worms were cultured for 2 days on the RNAi plates before sampling.

All sampling was done by rinsing off plates and washing worms five times with S-buffer in rapid succession. The worm samples were homogenized in 50mM Na/K phosphate buffer pH 7.8 with a Precellys24 homogenizer (Bertin Technologies, Aix-en-Provence, France) for 1 minute at 5000 cycles. The homogenate was centrifuged at 14000 rpm for 10 minutes to remove debris and the supernatant used for enzyme analysis.

Cathepsin L enzyme assay

Cathepsin L activity was measured using the Magic Red™ Cathepsin L detection kit (AbD Serotec, Oxford, UK). The substrate of this kit, MR-$(FR)_2$, contains two copies of phenylalanine-arginine (FR) coupled to cresyl violet, a photostable red fluorophore. The enzymatic breakdown of the two dipeptide cathepsin targeting sequence allows cresyl violet to become fluorescent upon excitation. The method was optimised to be used in 96-well microtiter plates. Each well consists of 190µl of cathepsin reaction buffer (400mM sodium acetate, 4mM EDTA and 8mM DTT at pH 5.4), 10µl sample homogenate and 8µl of 26x Magic Red cathepsin L reagent stock. A positive control of human liver cathepsin L (Sigma-Aldrich®) was used and a mixture of sample homogenate and buffer was used as a negative control. The emission kinetics was measured immediately after filling the plate for up to 20 minutes in a SpectraMax Gemini microplate reader (Molecular Devices, CA, USA) used at 592-nm excitation / 628-nm emission wavelengths. Enzyme activity normalized to protein content, measured using a standard bicinchoninic acid (BCA) assay (Pierce Biotechnology, Rockford, IL).

Acid phosphatase enzyme assay

Acid phosphatase activity was measured using 4-methylumbelliferyl phosphate (4-MUP) as substrate. The assay was performed in a 96-well microtiter plate and each well contained 90 µl reaction mixture of 4-MUP (97.5µM final concentration in 50mM citrate buffer, pH 4.6) and 10 µl sample homogenate. Emission was measured for 20 minutes at 450 nm while excited at 360 nm using the SpecraMax Gemini microtiter plate reader.

Life span analysis

Nutrient growth medium (NGM) plates were prepared with a central lawn of the specific RNAi bacteria. Synchronised young adult worms were brought on the plates and scored for survival every other day till the all worms were dead. Live worms were transferred to fresh

RNAi plates once every four days till motility and feeding was reduced. For survival analysis the online tool OASIS was used (Yang *et al.* 2011).

Acridine orange staining

Worms were incubated for one hour on an *E. coli* K12 lawn containing 0.5 mg/ml acridine orange dissolved in S-buffer. Post staining, worms were recovered on a lawn without acridine orange to remove stained bacteria associated with the gut and body wall. Worms were washed five times with S-buffer before fixed on slides with glycerol. Imageing was done with a Nikon TiE-C2 confocal microscope. Image intensity of ROIs was analyzed with ImageJ. Lysosomal acridine orange uptake (Exc 488nm / Em 515nm) was normalized to total acridine orange uptake (Exc 543nm / Em 570 nm) and is represented as fluorescence intensity ratio.

qRT-PCR and proteomics

For mRNA quantification, quantitative real-time PCR was performed as described in Depuydt *et al.* (2013). Protein abundances were measured in an iTRAQ (isobaric tag for relative and absolute quantification) approach. Samples for iTRAQ were prepared as described in Depuydt *et al.* (2013) and iTRAQ mass spectromtry analysis was performed as described in Petyuk *et al*. (2007) and Yi *et al*. (2014).

Data analysis

Data was assessed for normal distribution using Shapiro and homogeneity of varience was tested using Levene tests. Exceptionally in two situations data lacked normal distribution but a log transformation restored normal distribution. Significance was assessed by using ANOVA and Tukey's honest significance post-hoc test. For lifespan analysis Kaplan-Meier estimates was used as described in Yang *et al*., (2011).

CHAPTER 3

Increased protein stability and decreased protein turnover in the *C. elegans* Ins/IGF-1 *daf-2* mutant

CONTRIBUTION

Nematode cultures and sampling

Trehalose assays and stability assay

RNAi experimentation

Writing the manuscript

Will be submitted as

Increased protein stability and decreased protein turnover in the *C. elegans* Ins/IGF-1 *daf-2* mutant

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3.1 Abstract

In *Caenorhabditis elegans* cellular proteostasis is essential for longevity. As it was shown that autophagy is essential to the life span extension of the insulin/IGF mutant *daf-2*, it is expected that it achieves this phenotype by increasing turnover. However, this strategy can have its toll on total energy reserves of these animals. By using classical ^{35}S pulse-chase labelling, we observed that protein synthesis and degradation rates are decreased in the *glp-4;daf-2* longlived mutant. Although reduction of protein turnover may be energetically favourable, it may lead to accumulation and aggregation of damaged proteins. As this has been shown not to be the case in *daf-2*, this strain applies another strategy to maintain proteostasis. We observed higher stability of the *daf-2* proteome upon acid precipation. We identified that the chemical chaperone trehalose is responsible for this increased resistance to acid precipitation. Thus, *daf-2* mutants save energy by decreasing protein turnover rates and stabilize their proteome by trehalose.

3.2 Introduction

Progressively declining rates of both protein synthesis and degradation with age have been observed in many species from yeast to mammals, including nematodes (Van Remmen *et al.*, 1995; Rattan, 1996). These declining protein turnover rates are accompanied by increased accumulation and aggregation of biochemically altered and misfolded proteins (Hipkiss, 2006), which are in turn linked to the development of age-related pathologies and suggested to contribute to the ageing process (Lindner and Demarez, 2009). The autophagic-lysosomal and ubiquitin-proteasome system (UPS) are primarily responsible for the proteolytic clearance of aberrant proteins and their age-associated impairment is thought to be the main cause of the progressive loss of proteostasis (Carrard *et al.*, 2002; Martinez-Vicente *et al.*, 2005; Terman *et al.*, 2007). Interventions that promote longevity such as dietary restriction and reduced insulin/IGF-1 signaling (IIS) are therefore thought to stimulate proteolytic turnover of proteins (and whole organelles), thereby delaying the accumulation of cellular damage and slowing-down ageing (Bergamini *et al.*, 2003; Terman *et al.*, 2010). Consistent with this notion, dietary restriction was found to stimulate macroautophagy (Cavallini *et al.*, 2001; Donati *et al.*, 2001) and proteasome function (Hayashi and Goto, 1998; Radak *et al.*, 2002), and to increase turnover of proteins in ageing rats (Goto *et al.*, 2001) and liver mitochondria in mice (Miwa *et al.*, 2008).

In *C. elegans,* dietary restriction or reduced IIS is said to increased activation of macroautophagy as observed by a GFP-tagged reporter protein that localizes to the autophagosomal membrane upon autophagic induction (Melendez *et al.*, 2003; Hansen *et al.*, 2008). Although autophagic activation is required for increased longevity in these animals, whether bulk protein degradation is also increased under these conditions has not been tested to date. On the other hand, inhibition of overall protein synthesis rates is now well-established to increase longevity from yeast to mice, but is difficult to reconcile with the turnover paradigm (Kaeberlein and Kennedy, 2011). Moreover we have recently shown that protein synthesis rates are strongly decreased in the IIS receptor defective *daf-2* mutant (Depuydt *et al.*,2013).

Using a classical pulse-chase approach, we have attempted to assess the effect of ageing on overall protein synthesis and bulk degradation on normal-lived compared to long lived insulin/IGF-1-like signaling (IIS) mutants of *C. elegans*. As expected, we found a strong age-dependent decline in protein turnover rates in normal-lived *C. elegans.* Counter to the

turnover paradigm, long lived IIS mutants display very low protein synthesis and degradation levels throughout life. Instead we found that their proteomes are much more resilient to trichloroacetic acid-induced protein precipitation, suggesting that increased proteostasis in these animals does not depend on protein turnover. We show that, in the IIS mutant, this increased resilience of the proteome is mediated to large extent by increased levels of trehalose, requiring the trehalose 6-phosphate synthase genes *tps-1* and *tps-2*.

Our work thus implies that enhanced proteostasis in the long lived ISS mutant is obtained by stabilizing the proteome with protectants such as trehalose, rather than by enhancing protein turnover rates to minimize damage accumulation.

3.3 Results

Reduced insulin/IGF-1 signaling reduces protein synthesis.

Increased protein turnover is thought to be beneficial for the animal because this process removes and replaces damaged protein molecules, thereby delaying the progressive accumulation of protein damage - a hallmark of ageing (Ryazanov and Nefsky, 2002). When worms are fed bacteria that were grown in the presence of [^{35}S]sulfate, newly formed nematode proteins are labeled with ^{35}S. This allows determining the rate of protein synthesis- or degradation by measuring respectively the increase or decrease (after pulse-labeling) of radioactivity in trichloroacetic acid (TCA)-precipitated proteins as a function of time. We applied this method in a normal-lived reference strain *glp-4 daf-16;daf-2* and the long lived *glp-4;daf-2* strain. As the DAF-16 protein is necessary and sufficient to cause the life span-extending effect of *daf-2* mutants, the *daf-2;daf-16* double mutation was used in the reference strain.Comparision of protein synthesis/degradation rates in these strains would provide us the DAF-16 effect over these processes. The *glp-4* mutant background, causing sterility, was used in both strains to avoid purging of radioactive signal by egg laying. It should be noted that the *glp-4* mutants show a *daf-16* dependent lifespan extension which we compromised for avoiding the more misleading radioactive purging. We found that the overall rate of protein synthesis declines rapidly with age in the normal-lived reference strain (slope = -13.5 ± 2.5; $P < 0.001$; linear mixed model (LMM)) (**Fig 1A**). **Fig 1 B**, is a magnified representation of one data point, showing the six hour protein synthesis rate. Surprisingly, the rate of protein synthesis in young adult (adult day 2) IIS mutants is approximately five times lower compared to the young adult control population ($P < 0.0001$, LMM), and this low level of ^{35}S incorporation is maintained during the first quarter of their life span (slope = -0.5 ±

1.35; P = 0.72; LMM). *daf-2* mutants that do not carry the *glp-4* background mutation and mutants carrying the less pleiotropic *daf-2(m577)* allele were also confirmed to show reduced protein synthesis levels (**Fig 1C**).

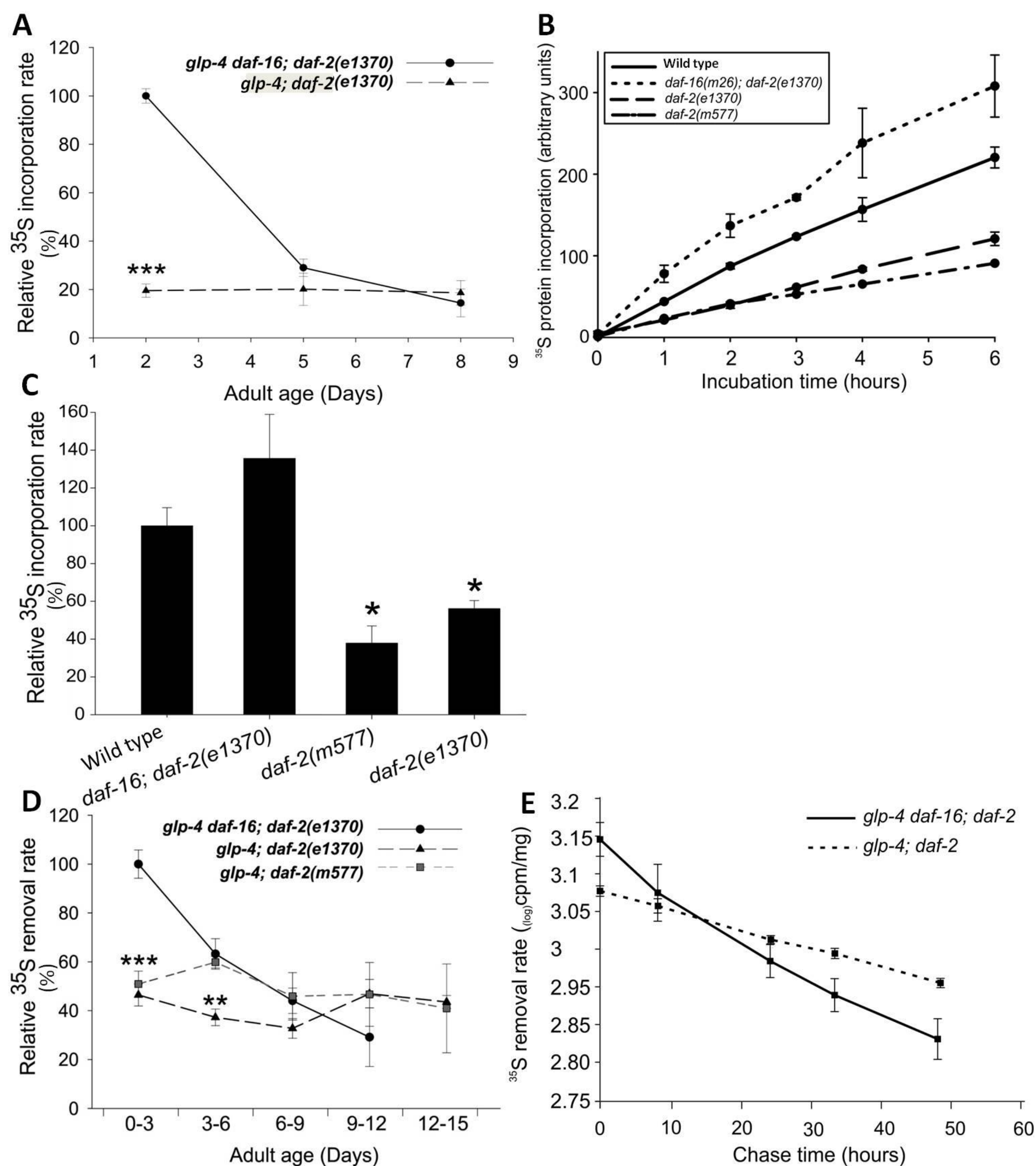

Figure 1: Protein synthesis and degradation is decreased in IIS mutants. A. Relative rate of ^{35}S incorporation into newly formed proteins with age by feeding ^{35}S-labeled bacteria to controls (*glp-4 daf-16; daf-2(e1370)*) and the insulin/IGF-1 receptor mutant *glp-4; daf-2(e1370)*. ('***' = P < 0.0005, LMM). **B.** A representation of one data point, showing the six hour protein synthesis rate. **C.**Protein synthesis levels measured at the second day of adulthood in *C. elegans* without the *glp-4(bn2*ts*)* mutant background allele. ('*' = P < 0.05). **D.** Relative rate of ^{35}S removal from TCA precipitated proteins in function of age. ('*' = P< 0.05, '**' = P< 0.005, '***' = P< 0.0005, LMM). Averages ± SEM are from four biological replicates. **E**. A representation of one data point showing the 48 hour protein degradation rate.

daf-2 **mutants show reduced protein degradation.**

We optimized the ^{35}S radioactive pulse-chase protocol for measuring protein degradation rates in *C. elegans*. In this protocol, adult worms are pulse-labeled by overnight feeding ^{35}S radioactive K12 bacteria. During the chase period, worms are kept in fresh culture medium supplemented with non-radioactive K12 and 5 samples were taken over a 48 hrs chase period. The rate at which ^{35}S label is lost from the TCA-precipitated (pTCA) protein is a measure for the respective protein degradation rate. Surprisingly**,** we found that both young *daf-2(e1370)* and *daf-2(m577)* mutants retain ^{35}S much longer in the pTCA protein fraction compared to controls ($P < 0.0001$; LMM, **Fig 1D**). **Fig 1 E,** is a magnified representation of one data point showing the 48 hour protein degradation rate. The low level of protein degradation remains fairly constant during the first quarter of *daf-2*'s life span (*daf-2(e1370)* slope = -0.97 ± 2.74; $P = 0.72$; *daf-2(m577)* slope = -1.1 ± 0.86; $P = 0.16$; LMM). Due to reduced lifespan of *daf-16 ;daf-2* mutant, our measurement in this strain was restricted to day 12.

The *daf-2* proteome is more resistant to TCA-mediated precipitation.

When the ^{35}S activity in the TCA soluble (sTCA) fraction was measured we found that the normalized ^{35}S activity in this compartment was much higher in the long lived *daf-2(e1370)* mutant compared to the reference strain, irrespective of age (**Fig 2A**). To eliminate the influence of the allele we tested both a less pleiotrpic class I *daf-2(m577)* and a more pleiotropic classII *daf-2(e1370)* allele. The class I allele posses constitutive dauer formation, increased longevity of adult, increased intrinsic thermotolerance and display low levels of L1 arrest. The more pleiotropic class II allele in addition to possessing the class I defects show reduced mortality, high levels of L1 and embryonic arrest, abnormal adult body and gonad morphology, reduced brood size and progeny production late in life. In addition classII mutants are not suppressed by *daf-12* (Gems *et al*., 1998). A similar trend was also observed for *daf-2(m577)* mutant allele, albeit with only borderline statistical significance. Measurement in the *daf-2 (m577)* and *daf-16; daf-2* strains were terminated at day 11 due to lack of samples. Intrigued by this finding we decided to further investigate the nature of this ^{35}S increase in the non-precipitable fraction. Preliminary investigation using the bincinchonic acid (BCA) protein quantitation assay clearly indicated that the increase in sTCA ^{35}S activity in *daf-2* nematodes was proteinaceous in nature (**Fig 2B**).

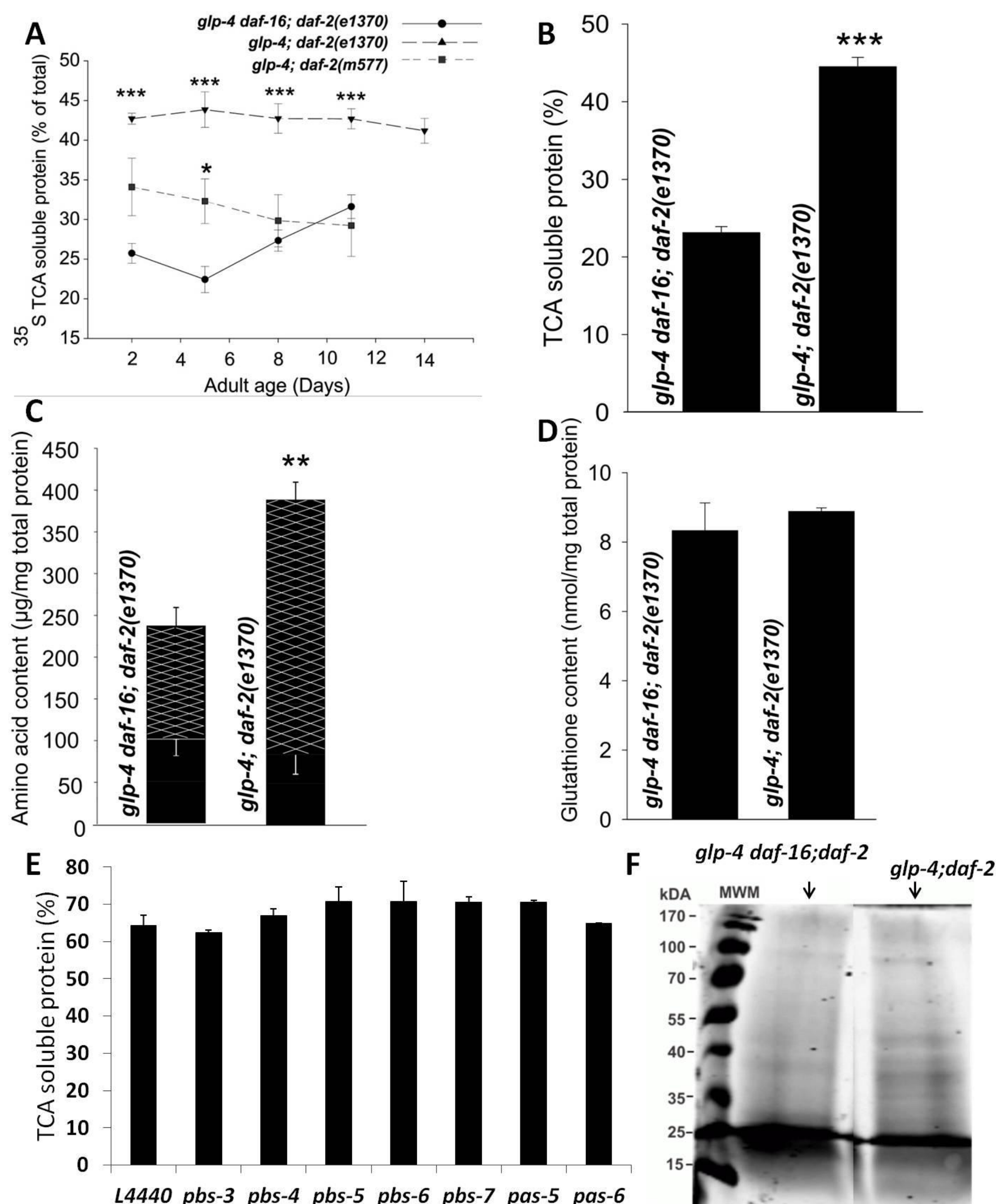

Figure 2: Increased protein solubilization in the *glp-4;daf-2* mutant. A. Percentage of total ^{35}S-activity remaining soluble in the presence of TCA (10% f.c.) measured after an eight hours chase period as a function of adult age. Mean values ± s.e.m. of three to six biological replicates are shown ('*'= P <0.05 and '***' = *P* < 0.0005). **B.** Percentage of total proteinaceous material remaining soluble in the presence of TCA (10% f.c.) as determined by bicinchoninic acid assay (BCA). Mean values ± s.e.m. of three biological replicates. **C.** Total amino acid content determined by HPLC analysis of free- (solid stacked bars) and bound- (hatched stacked bars) amino acids in the sTCA fraction. Bound amino acids originate from complete acid hydrolysis of peptides and proteins that remained soluble in the sTCA fraction. Full bar height is indicative for total proteinaceous content (amino acids, peptides and proteins) in the sTCA fraction. Mean values ± s.e.m. of three biological replicates are shown. '**' = *P* < 0.005. **D.** Glutathione levels in *glp-4 daf-16;daf-2* and *glp-4;daf-2* mutants. **E.** Effect of knock-down of proteasomal subunits on protein precipitation in *glp-4;daf-2* mutants. **F.** SDS-PAGE gel separation of the TCA soluble fraction of control *glp-4 daf-16;daf-2* and *glp-4;daf-2* mutants (lanes with additional treatments not relevant to this study were left out).

The thiol-containing tripeptide glutathione (GSH) is maintained at higher levels in middle-aged and old *daf-2* mutants compared to wild-type (Brys *et al.*, 2007). Therefore, we asked whether the high ^{35}S levels in the sTCA of *daf-2* worms could be due to elevated GSH levels. We did not find elevated glutathione levels in young *daf-2* worms, confirming earlier findings by Brys et al. (**Fig 2D**).

Next, we reasoned that intracellular recycling of amino acids (*e.g.* by autophagy) may be up-regulated in *daf-2* mutants, resulting in high protein turnover rates that may not be readily detected by classical pulse-chase experiments. We therefore wondered if the rise in sTCA proteinaceous content was the result of increased standing levels of free amino acids in *daf-2* worms. To test this hypothesis we determined amino acid content in the sTCA fraction by HPLC separation of o-phtaldialdehyde derivatized amino acids followed by fluorometric detection. However, we found no significant overall change in the standing levels of free amino acids between the control and long lived worms (solid bars in **Fig 2C**). Adding support knock-down of the several proteasomal subunits did not change the sTCA fraction in the *daf-2* mutants (**Fig 2E**). Hydrogen chloride acid hydrolization was used to break down peptide bonds and release the amino acid constituents of proteins and peptides present in the sTCA fraction. The amount of bound amino acids (*i.e.* amino acids released from peptides or proteins in the sample) was significantly elevated in *daf-2* mutants (hatched bars in **Fig 2C**). In addition we checked the TCA soluble fraction for presence of proteins on SDS-PAGE gels. We clearly observed proteins in the TCA soluble fraction, suggesting that TCA is not able to precipitate all proteins in a biological homogenate (**Fig 2F**). To eliminate the influence of the method of precipitation used, other precipitation methods including ammonium acetate and polyethylene glycol (PEG) were tested on *glp-4;daf-2* and *glp-4 daf-16;daf-2* homogenates. We observed no influence of the method on the precipitation resistance of *glp-4;daf-2* (**Fig 3 A,B**). We therefore conclude that the observed increase in sTCA ^{35}S activity in the *daf-2(e1370)* mutant is the result from peptides and proteins that resist precipitation in the presence of TCA.

Increased *daf-2* TCA resistance is mediated by *tps-1* and *tps-2*

Insulin-like signaling mutants are characterized by elevated levels of the glucose dissacharide trehalose that result from the increased expression of the trehalose-6-phosphate synthase genes *tps-1* and *tps-2* (McElwee *et al.*, 2003; Murphy *et al.*, 2003; Lamitina and Strange, 2005; Fuchs *et al.*, 2010; Honda *et al.*, 2010; Depuydt *et al.,* 2014). Besides its role in

carbohydrate storage and transport, trehalose also acts as a cytoprotectant against cold, heat, dehydration, hypoxic and oxidative insult in invertebrates, most likely by stabilizing the proteome and lipid membranes (Hottiger *et al.*, 1994; Jain and Roy, 2009; Erkut *et al.*, 2011). High trehalose levels contribute to *daf-2* longevity as RNAi knock-down of *tps-1* and *tps-2* shortens *daf-2* life span significantly (Honda *et al.*, 2010).

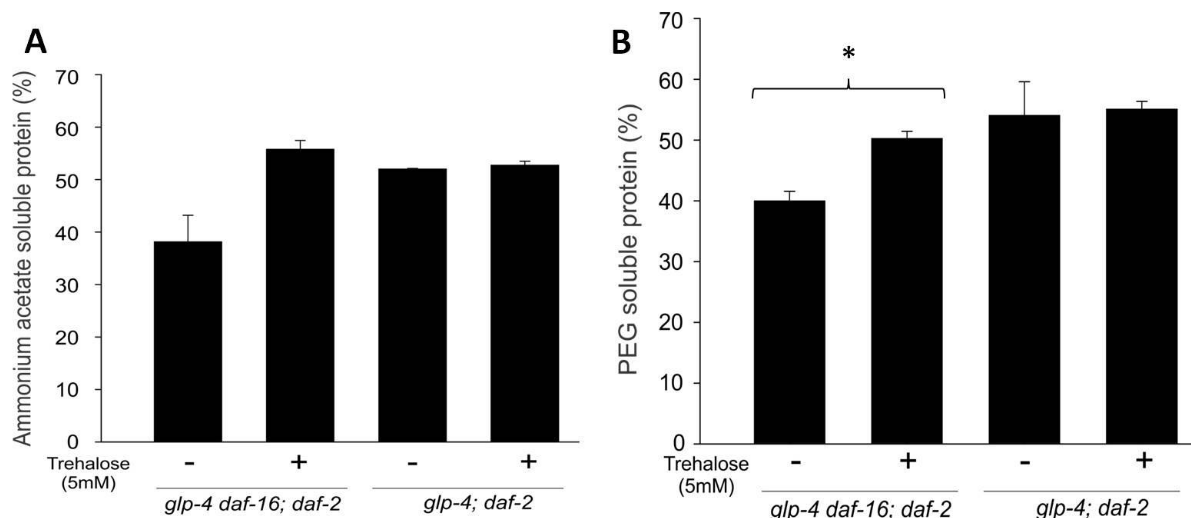

Figure 3*: glp-4;daf-2* proteome resistance to different precipitation methods. A. Precipitation of *glp-4;daf-2* and *glp-4 daf-16;daf-2* proteome by ammonium acetate mediated precipitation. **B.** Precipitation of *glp-4;daf-2* and *glp-4 daf-16;daf-2* proteome by polyethylene glycol (PEG) mediated precipitation. ('*' = $P < 0.05$).

To test whether trehalose can protect proteins from TCA-mediated precipitation, different concentrations of trehalose were added to worm homogenates. Addition of 5mM trehalose results in a significant increase in protein solubility in the presence of 10% TCA, but higher trehalose concentrations had no additional effect on solubility (**Fig 4A**)., It is known that DMSO can extend *C. elegans* life span independently of IIS signaling, and ameliorates paralysis induced by amyloid-β aggregation (Pellerone *et al.*, 2003). The latter suggests DMSO could improve protein homeostasis. Indeed, adding DMSO to our worm homogenates increased protein solubility significantly in the presence of 10% TCA (**Fig 4A**). Similar result were obtained by adding glycerol (**Figure 4A**), a common co-solvent for the storage of proteins, inhibiting their aggregation (Vagenende *et al.*, 2009), and produced in *C. elegans* upon osmotic stress (Lamitina *et al.*, 2004; Burkewitz *et al.*, 2012). To test whether the high resistance to TCA-mediated protein precipitation in the *glp-4;daf-2* mutant is dependent on trehalose, we measured sTCA protein content upon treating *daf-2* with *tps-1* or *tps-2* RNAi. Both *tps-1* and *tps-2* RNAi resulted in a strong decrease in worm trehalose levels, mirrored by decreased protein solubility in the presence of 10% TCA (**Figure 4B**). It is noteworthy

that *tps-1* RNAi of *glp-4;daf-2* worms leads to comparable levels in both trehalose and protein solubility as in the *glp-4 daf-16; daf-2* reference strain fed RNAi bacteria expressing an empty vector. This indicates that protein solubility of *daf-2* worms in the presence of TCA is mainly determined by trehalose levels.

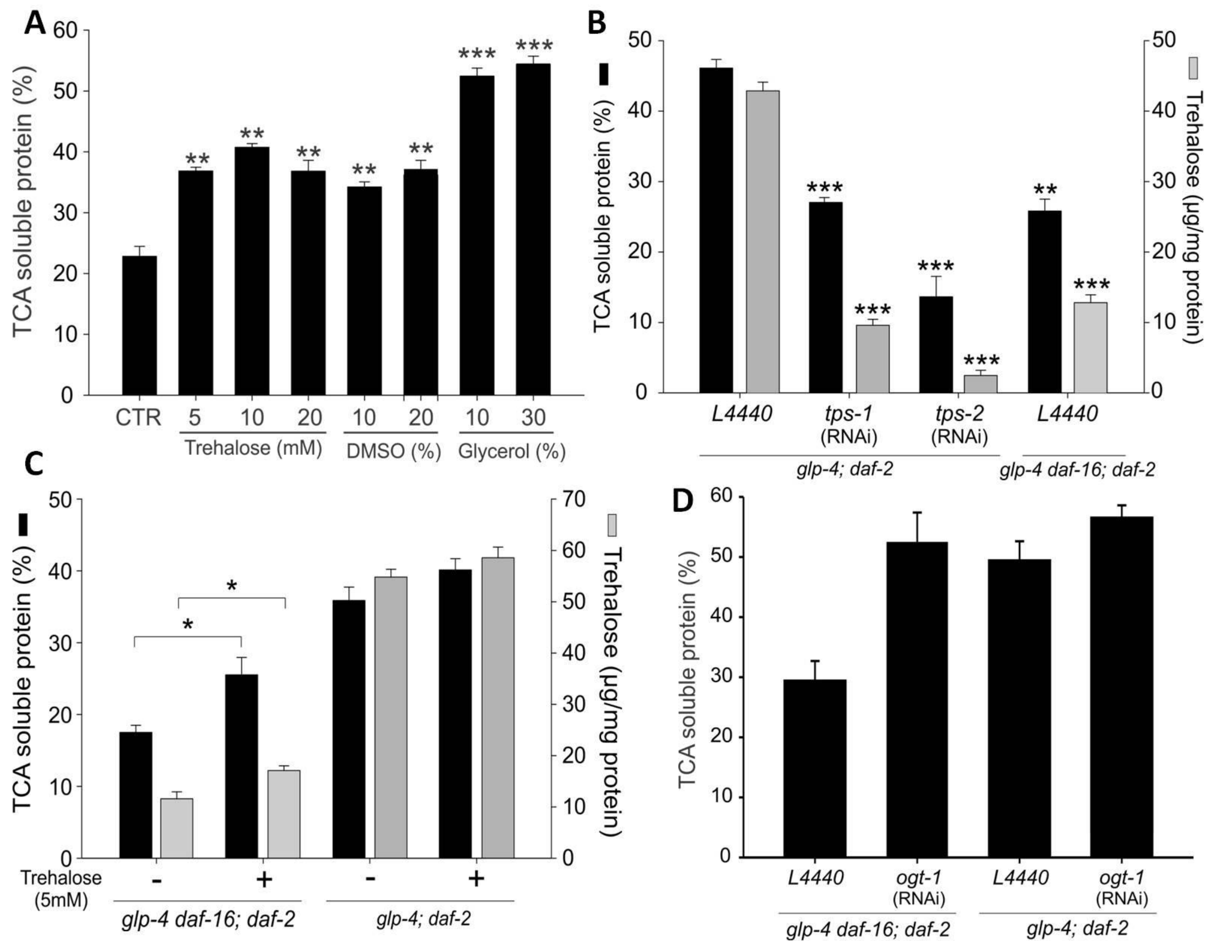

Figure 4 Trehalose elevates protein resistance against TCA precipitation (A) The effect of trehalose, DMSO and glycerol on protein solubility in the presence of 10% TCA. **(B)** Protein solubility and trehalose content of worms (*glp-4 daf-2* and *glp-4 daf-16; daf-2*) either fed RNAi bacteria expressing *tps-1*, *tps-2* or empty vector (L4440). **(C)** The effect of adding 5mM trehalose to the culture medium on both protein solubility and worm trehalose concentration. (D) Effect of RNAi on O-GlcNAc transferase *ogt-1* on protein stability. ('*' = $P < 0.05$, '**' = $P < 0.005$, '***' = $P < 0.0005$)

Adding trehalose to the nutrient agar culture medium (5mM f.c.) also results in an increased solubility of *glp-4 daf-16; daf-2* worm proteins after homogenization and exposure to 10% TCA as a result of a small but significant increase in intra-animal trehalose levels (**Fig 4C**). In *glp-4;daf-2* mutant nematodes no additional effect in protein solubility is seen upon adding trehalose to the culture medium, since intra-animal trehalose levels remained unchanged under these conditions (**Fig 4C**). To find out whether high trehalose levels are sufficient to

render precipitation resistance to the proteome, we knocked down *ogt-1*, which has been shown to increase trehalose levels in *C. elegans*. We observed an increase in protein stability when *ogt-1* was knocked-down in *glp-4 daf-16;daf-2*. Interestingly, we observed no further increase in protein stability in the *glp-4;daf-2* mutants compared to the control L4440 fed population (**Fig 4D**).

3.4 Discussion

Protein turnover decreases over age

Classical pulse-chase labeling showed that protein synthesis as well as degradation rates drastically decrease over age in the *C. elegans* reference strain used in this study. Age-related decreases in protein synthesis rates have been known for long and were described for the vinegar eel *Turbatrix aceti* (Prasanna and Lane, 1979) as well as many other species (ref Brown-Borg *et al.*, 1996). The reason for this decline is still unclear but a decrease in translation efficiency or ribosome abundance may be involved (Guarente, 1997; Payao *et al.*, 1998; Southworth *et al.*, 2009). We observed that the decrease in protein synthesis was paralleled by a similar decrease in protein degradation. This may point to age-related deterioration of the lysosomal and/or proteasomal system. Indeed, three lysosomal protease activities decline 2.5 to 10-fold with age in *C. elegans* (Sarkis *et al.*, 1988). Also impairment of the ubiquitin proteasome system (UPS) was reported in the dorsorectal neurons of aged *C. elegans* worms although ageing did not affect the UPS in the body wall muscle cells (Hamer *et al.*, 2010). It is often assumed that the age-related reduction in protein turnover is causal to increased protein damage and aggregation that is observed in old individuals (Van Remmen *et al.*, 1995; Rattan, 1996; Shringarpure and Davies, 2002).

***daf-2* mutants show very low protein turnover irrespective of age**

If protein damage and aggregation, both hallmarks of ageing, can be cleared by increased protein turnover, it is expected that long lived *daf-2* mutants display increased turnover rates. However, our results do not support this prediction. On the contrary, *daf-2* mutants showed severely reduced protein synthesis and degradation rates. This downregulation was observed in class I allele *m577* as well as in the more pleiotropic class II allele *e1370*. Our pulse-chase experiments confirm earlier findings of proteomic studies describing the global down-regulation of protein synthesis machinery including ribosomal subunits, tRNA synthetases, s-adenosyl methionine synthase-I (SAMS-1), Vigilin and RACK-1 in IIS mutants (Depuydt *et al.*, 2013, Stout *et al.,* 2013). Conversely, when protein synthesis is downregulated by genetic intervention, life span of *C. elegans* is increased (Hansen *et al.*, 2007; Synthichaki *et al.*,

2007; Pan *et al.*, 2007; Wang *et al.*, 2010; Tohyama *et al.*, 2008). Thus, life span extension in *daf-2* mutants is not caused by overall increased protein turnover.

***daf-2* proteins are more resistant to TCA precipitation**

The *daf-2* mutants showed a higher retention of ^{35}S radiolabel in the TCA soluble protein fraction (sTCA) compared to the control. Interestingly correlation between increased lifespan and increased protein solubility was observed between the class I and class II mutants. The more pleiotropic classII *daf-2 (e1370)* mutant was reported to have a mean lifespan of 31.4±2.3 days compared to 20.8±0.4 days for the less pleiotropic classI *daf-2 (m577)* mutant (Vartiainen *et al*., 2006).The observed high ^{35}S radiolabel signal in *daf-2* mutants may represent increased levels of free amino acids and may point at efficient internal recycling of proteins in *daf-2* mutants. Label reutilization is a blind spot in radioisotope pulse chase studies and there are no straightforward solutions to this artifact (Miwa *et al*., 2010). Furthermore, the possibility of high internal recycling is supported by the observation that autophagic activity in *C. elegans daf-2* mutants is a prerequisite for their longevity (Melendez *et al*., 2003; Hansen *et al*., 2008). Also, increased proteasomal activity may underlie an elevated free aminoacid pool in *daf-2* animals (Matilainen *et al*., 2013). However, we found that RNAi inhibition of proteasomal subunits did not lower the elevated S^{35} sTCA levels in the *daf-2* mutants (supplemental 3). Moreover, we showed that the overall free aminoacid pool, as measured by HPLC analysis of the sTCA fraction, was not increased in *daf-2* mutants, indicating that the high ^{35}S signal must originate from other molecular sources. Indeed, the levels of aminoacids bound in peptides and proteins were significantly increased in the *daf-2* sTCA fraction. This increase was not due to elevated GSH levels as shown earlier (Brys *et al.,* 2010) and confirmed in this study. Additionally gel electrophoresis of sTCA fraction of *daf-2* mutant indicated the presence of large proteins. The resistance of the *daf-2* proteome to precipitation with TCA, and also other precipitants, suggests increased protein stability in *daf-2* worms. Hence, protein stability rather than protein turnover may be key to *daf-2* longevity.

This result is in line with the finding that the *daf-2* proteome is less prone to aggregation (David *et al*., 2010). Protein stabilization or protection is governed by chemical or molecular chaperones (e.g. trehalose, glycerol, sucrose, proline, heat shock proteins). Because it was found earlier that trehalose (Fuchs *et al*., 2010; Lamitina and Strange, 2005) and trehalose synthase (Depuydt *et al*., 2014) levels are increased in IIS mutants and that their life span extension is partially dependent on the trehalose synthase genes *tps-1* and *tps-2* (Honda *et al*., 2010), we extended our functional analysis to this protective disaccharide. Moreover, a

fruitfly study (Belgacem and Martin, 2005) suggests that the link between defective IIS signaling and trehalosemia seemed to be evolutionary conserved, at least in ecdysozoa. Also, reduced protein degradation may induce trehalose, as was seen in *Saccharomyces cerevisiae* treated with proteasome blockers (Lee and Goldberg, 1998).

Trehalose stabilizes the worm proteome

It was shown earlier that addition of trehalose extends life span of wild-type but not *daf-2* mutant worms and that life span extension in *daf-2* worms is partially dependent on trehalose synthesis (Honda *et al.,* 2010). We found that trehalose, added directly to worm homogenates, can act as a stabilizing agent for the *in vitro* worm proteome, thus making it more resistant to TCA precipitation. Similar effects were obtained with other stabilizers such as DMSO and glycerol. Control worms, cultured on trehalose-enriched medium, showed significant uptake of this disaccharide and a concurrent increase in sTCA protein content. Additional trehalose uptake and proteome stabilization was not observed in *daf-2* mutants, suggesting that these animals reach maximal proteome stability via endogenous trehalose synthesis. This stability can be decreased by knocking down the trehalose synthase genes *tps-1* and *tps-2*, confirming that the stabilization is trehalose-specific.

All previous results seem to point at the importance of trehalose to proteome stability and life span extension in *C. elegans*, but is increased proteome stability by trehalose sufficient to extend life span? We show that knock-down of O-GlcNAc transferase *ogt-1* does provide increased protein solubility in TCA while others have shown that *ogt-1* mutant contains increased levels of trehalose (Hanover et al, 2005) but it is not long lived (Rahman *et al.,* 2010). This seems to indicate that resistance of the proteome to TCA is not a prerequisite to longevity. However, it cannot be ruled out that this mutant is shortlived due to pleiotropic effects caused by *ogt-1* knockout. It would be interesting to check the influence of the increased trehalose and proteome stability levels on lifespan under the less severe *ogt-1* RNAi knock-down conditions rather in a knock-out. In addition, does the increased protein stability in this mutant reduce turnover? Answer to which would through light on the relationship between these two proteostasis processes in *C. elegans*.

3.5 Materials and Methods

C. elegans **strains and culturing:**

The following strains were used*: glp-4(bn2*ts*)I; daf-2(e1370)III*, *glp-4(bn2*ts*)I; daf-2(m577)III and glp-4(bn2*ts*)I daf-16(mgDf50)I; daf-2(e1370*ts*)III*. The temperature-sensitive *glp-4(bn2)* background mutation disrupts normal post-embryonic proliferation of the

germline resulting in sterile adults without eggs (Beanan and Strome, 1992). This prevents the loss of ^{35}S-labeled protein due to egg-laying. Synchronized cohorts of worms were grown as described previously (Braeckman *et al.*, 2002). Worms were grown on *E. coli* K12-seeded nutrient agar plates until third larval stage (L3) at 16°C and then shifted to 24°C for the remainder of the experiment. At fourth larval stage, worms were transferred into Fernbach flasks containing 250 ml S-buffer at densities not exceeding 1500 worms/ml and shaken at 120 rounds per minute. Frozen *E. coli* K12 cells were added at approx. 3×10^9 cells mL^{-1} ($OD_{550} = 1.8$). *E. coli* was added twice daily to the culture medium to maintain desired OD_{550} levels.

^{35}S protein assays:

^{35}S-labeled bacteria were obtained by growing *E. coli* K12 overnight at 37°C in low-sulfate medium (44 mM Na_2HPO_4, 22 mM KH_2PO_4, 85 mM NaCl, 20 mM NH_4Cl, 1.25 mg/L Thiamine, 0.1% (w/v) glucose, 2 mM $MgCl_2$) (Lewis and Fleming, 1995) supplemented with LB-medium (1% f.c.) and 5µCi/mL [^{35}S]Sulfate (PerlinElmer, Waltman, MA). These quantities were carefully chosen as they optimize the balance between bacterial growth and efficient label incorporation. Bacterial concentrations were determined by measuring optical density at 550nm. To label worms for protein synthesis, ^{35}S-bacteria (for concentrations used see main text) were fed to worms (adult day 2) cultured in 10mL S-buffer in tissue culture flasks (approx. 1000 worms/mL). The rate of protein synthesis was calculated as the upward slope of ^{35}S protein incorporation from six samples taken over a time-period of 6 hours. For measuring protein degradation, worms (adult day 1) were first pulse-labeled by feeding ^{35}S bacteria overnight. ^{35}S-bacteria were diluted (1:1) with non-radioactive. Worms were then cleansed from radioactive bacteria (c.f. sampling procedure below) and kept in liquid culture containing non-radioactive K12 (*i.e.* chase period). The protein degradation rate was calculated as the downward slope of log-transformed protein radioactivity from five samples taken over a 48-hour chase period. To prevent reincorporation of excreted ^{35}S, the chase medium was refreshed twice daily. During the sampling process, worms were washed five times over a period of 15 minutes in S-buffer supplemented with non-radioactive *E. coli* K12 to purge the intestine from undigested ^{35}S-labeled bacteria. Negative controls were produced by incubating worms in ^{35}S bacteria for < 1 min. To isolate proteins, worms were first boiled for 15 minutes in 50% Tris-SDS buffer (25 mM Tris, 250 mM NaCl, 5% SDS pH 7.4) and debris was pelleted by centrifugation for 5 minutes at 20,000 rcf. To precipitate proteins in the supernatant, trichloroacetic acid (TCA, final concentration 9.3%) was added to the

supernatant and allowed to incubate at room temperature for 1 hour. Precipitated proteins were centrifuged at 20,000 rcf for 5 minutes and washed once with 1 mL of 10% TCA. The protein pellet (TCA insoluble fraction) was dissolved in 150 µL 350 mM NaOH for at least 1 hour at room temperature. 100µl was added to 5 mL Ultima Gold LSC-cocktail (PerkinElmer, Waltman, MA) for liquid scintillation counting in a Tri-Carb 2800TR Liquid Scintillation Counter (PerlinElmer, Waltman, MA). Counts per minute (cpm) were normalized to total protein concentration as determined by BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). Normalizing to protein content has its own draw backs especially when *daf-2* strain, which posses reduced protein content, is compared with strains having higher protein content. Since every norlization method has its own drawbacks (Braeckman et al., 2002) we employed this normalization method, widely accepted, keeping in mind the possibilities of being mislead. Additional normalization methods could empower the fairness of the data.

Determination of free and bound amino acid content:

Determination of amino acid concentrations by HPLC was performed as described before (Kerkaert *et al.*, 2011). Free amino acids were extracted by treating worm homogenates with 15% trichloroacetic acid and taking the supernatant. Bound amino acids were released by acid hydrolysis in 12 M HCl containing 0.1% phenol and 0.1% Na_2SO_3 for 24h at 105°C followed by neutralization of the mixture. All amino acids were derivatized with o-phtaldialdehyde (OPA) in the injector of the Agilent 1100 system HPLC (Agilent Technologies, Switzerland) and quantified by fluorometry (340ex./450em. nm). Free and bound amino acid content was normalized to the total amount of protein (TCA soluble and insoluble fraction) in each sample determined by BCA Protein Assay Kit (Thermo Scientific, Rockford, IL).

Glutathione and trehalose levels:

Worms were grown till their young adult stage (day 2) on agar plates. Samples taken on this stage were frozen immediately. The thawed and homogenized samples were used for trehalose measurement. Trehalose was measured using trehalose assay kit (Megazyme, Wicklow, Ireland) on microplates (Erkut *et al.*, 2011). Glutathione was measured using the glutathione assay kit (Oxford Biomedical Research) on microplates (Anderson 1996).

Regression analysis:

Regression analysis was performed to assess whether rates of protein degradation and synthesis change with age using the mixed linear regression model (LMM) PROC MIXED in SAS 9.2 (SAS Institute Inc. 2002-2003, Cary, NC, USA). Considering that we have to compare the slopes of protein synthesis and degradation in ageing strains of *C. elegans* the linear mixed model was used.

Trehalose and protein stability data was assessed for normal distribution using Shapiro and homogeneity of varience was checked with Levene tests. Significance was assessed by using ANOVA and Tukey's honest significance post-hoc test.

PART III

DISCUSSION

CHAPTER 4

GENERAL DISCUSSION

General discussion

In general, a major mechanism that determines ageing is loss of protein homeostasis or proteostasis (Taylor and Dillin, 2011; Lepez-Otin *et al.*, 2013). The state of proteostasis depends on regulated gene transcription, RNA metabolism and protein synthesis, folding, assembly, trafficking, disassembly and degradation (Balch *et al.*, 2008; Cohen and Dillin, 2008) of specific proteins in relation to the others. In any organism proteostasis is achieved by either 1. an efficient protein turnover – eliminating damaged proteins and organelles, synthesising new ones, or by 2. Stabilizing the proteome – rendering the need of wasteful degradation and energy expensive synthesis unnecessary (Neuhofer and Beck, 2005). In this study we analysed the mechanism by which proteostasis is achieved in the long lived *daf-2* mutants.

4.1 Reduced protein turnover in the long lived worm

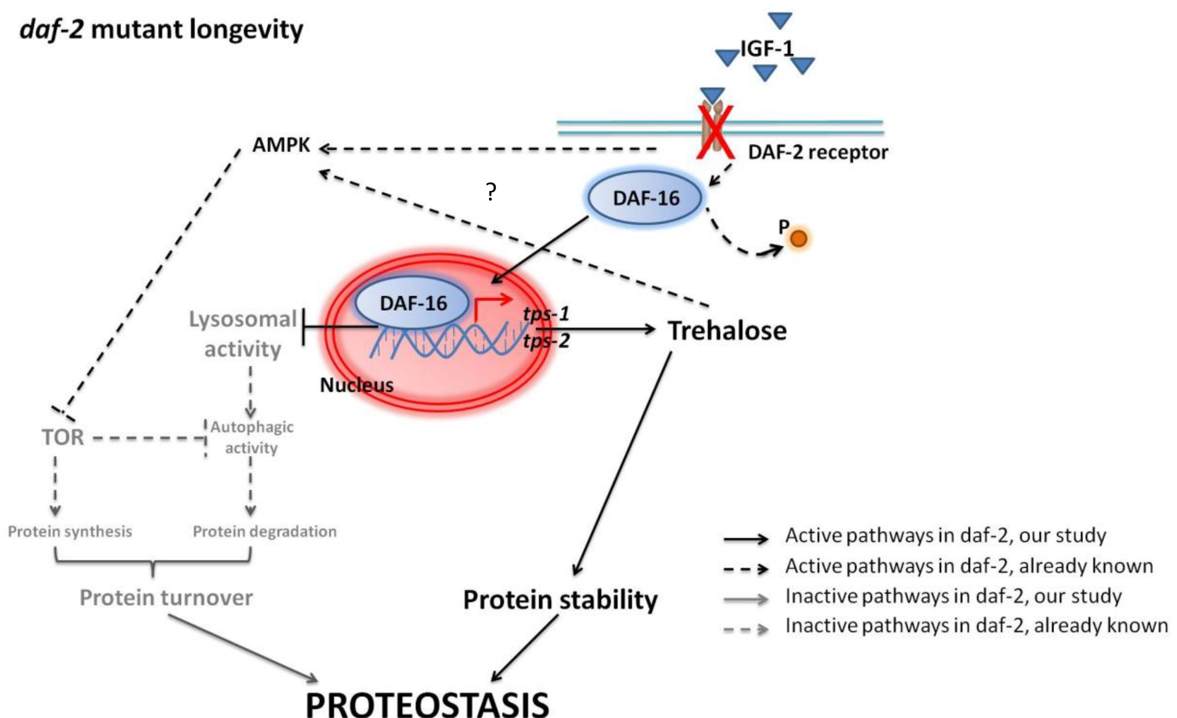

Figure 1. An overview of proteostasis rendered by reduced turnover and increased stability in the longlived *daf-2* mutant.

The rapid synthesis of new proteins in combination with elevated degradation of the damaged proteins could increase proteostasis. On the other hand, protein synthesis as such is an energy expensive process and consumes a large amount of ATP, for example in amphibians this process is estimated to consume around 10% of the total energy (Fuery *et al.*, 1998). Such an

energetic investment is not evident in *daf-2(e1370)*, as it shows an Eat phenotype. Moreover, studies involving knock-down of several components required for protein synthesis showed to induce longevity in *C. elegans* (Hansen *et al.*, 2007; Pan *et al.*, 2007; Syntichaki *et al.*, 2007; Wang *et al.*, 2010) rather than the vice-versa. With reduced protein synthesis rate, one could expect an increased protein degradation rate because, in IIS mutants, reduced TOR results in reduced protein synthesis and increased autophagy (Hansen *et al.*, 2007; Hansen *et al.*, 2008). Both protein synthesis and degradation are tightly linked and TOR acts as link between them. In *daf-2* mutants silencing of TOR kinase signaling can occur by 1. DAF-16 dependent suppression of *daf-15*/Raptor (Jia et al., 2004) or 2. DAF-16 independent activation of AMPK and its downstream target ULK-1/hATG-1 (Hansen *et al.*, 2008; Egan *et al.*, 2011). On the other hand, protein synthesis extends life span by inducing SKN-1 dependent oxidative stress resistance which in turn up-regulates TORC1 inhibiting autophagy (Hansen *et al.*, 2008; Yu *et al.*, 2010; Robida-Stubbs *et al.*, 2012). Adding to the confusion, IIS mutants have been shown to have high ubiquitin-proteasome system (UPS) activity (Oliveira *et al.*, 2009; Li *et al.*, 2011; Matilainen *et al.*, 2013). To get a clear picture we checked the protein turnover rate in the long lived *daf-2* mutant.

It is a general perspective that protein degradation can enhance proteostasis by rapid removal of damaged proteins. However, in case of reduced protein synthesis in the *daf-2* mutants (Stout *et al.*, 2013), high rates of protein degradation would be energy inefficient. Interestingly our ^{35}S pulse chase study showed a reduced protein degradation rate in the *daf-2* mutants. Hence, the overall protein turnover, which is the combination of both protein synthesis and protein degradation rates, was found to be lower in the long lived *daf-2* mutants indicative of an increased dwell time of all the protein in the organism.

Contrasting with our observation, studies in *C. elegans* have indicated that autophagy is essential for longevity in the *daf-2* mutant (Melendez *et al.*, 2003). To further investigate this, we measured the activity of the major lysosomal cysteine protease cathepsin L. Adding support to our hypothesis, we observed reduced cathepsin L protein abundancy and activity in the long lived *daf-2* mutants. Another interesting finding was that in *daf-2*, cathepsin L activity was partially inhibited by cystatin (cystein protease inhibitor) and to a lesser degree by serpines (serine protease inhibitors). This strongly correlates with our observation of reduced protein turnover in the *daf-2* mutants. Taking into account the huge population of lysosomal proteases, we focused our study on cathepsin L considering the fact that it is one of the major and well studied lysosomal protease. On the contrary there exists extensive

variation in activity among the various groups of lysosomal enzymes during ageing. This is well evident in rat nerve cells (Amano *et al.*, 1995) whereas in *C. elegans* it is not well studied. Further expression and activity studies of other proteases in *C. elegans daf-2* and aged animals might provide more insights into proteostasis. Cathepsin D a major lysosomal aspartate protease involved in endopeptidase activity degrading long lived proteins (Dean, 1977; Jones *et al.*, 1982) and α-synuclein (Hossain *et al.*, 2001) is an interesting candidate.

4.2 Reduced protein turnover is an ageing hallmark

It is known that protein carbonylation increases with age (Starke-Reed and Oliver, 1989; Stadtman *et al.*, 1992). The increased protein damage along with the decreased removal leads to further protein damage and dysfunction. The two major hallmarks of ageing are, reduced protein synthesis rate (Makrides, 1983; Johnson and Mccaffrey, 1985; Rattan, 1996) and reduced lysosomal and proteasomal activities (Grune, 2000; Keller *et al.*, 2000). We identified a similar age associated decrease in protein synthesis and degradation/protein turnover rate in the control worms. This change was negligible in the long lived mutant which has already a reduced turnover rate. Similar results were obtained with cathepsin L activity which displays reduced activity in aged worms. Supporting our data, other studies have shown the decrease in cathepsin L activity in the brain tissues of aged rats (Nakanishi *et al.*, 1994). It is interesting that a similar reduced protein turnover phenotype manifests in both the ageing population and the long lived population in *C. elegans*. Even though the decrease in protein turnover in aged animals shall be linked to damage of any rate-limiting component of protein synthesis like the elongation factor, the decline in *daf-2* is proposed to be due to active regulation by cellular signalling.

4.3 The sleeping lysosomes of the *daf-2* mutant

The major protein degrading systems in *C. elegans* are the proteasomal system and the autophagic system. The proteasomal system was found to be unchanged on *daf-2* mutation in *C. elegans* (Vilchez *et al.*, 2012), whereas studies have indicated increased autophagy induction in *daf-2* mutants (Melendez *et al.*, 2003; Hansen *et al.*, 2008). This makes autophagy a more interesting and preferred degradative pathway in our study. With the ^{35}S studies and the cathepsin L expression and activity indicating down-regulated protein turnover in the long lived *daf-2* mutants, we further questioned the need for autophagy in longevity as studies indicate autophagy genes *bec-1, lgg-1, atg-18, atg-7, vps-34* and *unc-52* to be required for life span extension in *daf-2* mutants (Melendez *et al.*, 2003; Hansen *et al.*, 2008; Hars *et al.*, 2007; Toth *et al.*, 2008). Here the mere localization of *lgg-1* to

autophagosomal membrane was considered as an increase in autophagic activity. Reduction in lysosomal trafficking can lead to autophagosomal accumulation (Klionsky 2012) which shall be misinterpreted as reduced autophagy. On the other hand autophagy induction might be essential in the IIS mutant for lipid and organelle turnover rather than protein turnover. These arguments are supported by a study, which propose that autophagy is not sufficient for life span extension as long lived *daf-2* mutants requires both autophagy and DAF-16 for life span extension (Hansen *et al.*, 2008). As the lysosome is the major organelle involved in autophagy, we further studied the role of its components in longevity.

Consistent with our previous finding, the activity of another major class of lysosomal enzymes, acid phosphatases, involved in protein dephosphorylation (Bull *et al.,* 2002), was also found to be reduced in the long lived mutants. In addition, we identified *pho-14* as the major acid phosphatase in *C. elegans*, whereas earlier studies suggested *pho-1* to be the major acid phosphatase (Fukushige *et al.,* 2005). We identified that the knock-down of several lysosomal components did not change life span of the long lived *daf-2* mutants. This hints at a strong redundancy among the lysosomal genes or a possible lack of necessity of these genes in *daf-2* life span extension.

In addition to the lack of any effect on life span when lysosomal components were knocked-down in *daf-2* mutants, these components were generally down-regulated at the protein level in *daf-2* mutants. Our studies with the lysosomal stain acridine orange also indicated a less acidic or less pronounced lysosomal machinery in the *daf-2* mutant. The reduced acidification of the lysosomes in the *daf-2* mutants compliment with the reduced cathepsin L activity which needs acidic lumen for activity. Signifying this we also identified that the optimal pH for these cathepsin L in *C. elegans* to be pH4 and this optimum was not different in the *daf-2* mutant. The observed reduction in acidification offers more support to the needlessness of lysosome in longevity rather than to the enzymatic redundancy which still needs investigation.

On the other hand is the general decrease in lysosomal activity cause of the reduced protein turnover in the *daf-2* mutants. Generally lysosomes account for about 50-70% of cellular protein turnover (Pollard *et al.*, 2007). Eventhough the observed down regulation of the lysosomal activity could in part contribute to the overall reduction in protein turnover, in this study, only, one of the several lysosomal proteases, cathepsin L, was analysed. Additionally a study on proteasome activity in *daf-2* mutant has shown no significant deviation from control

population (Vilchez *et al.*, 2012). Whereas another study have revealed an increase in proteasome activity (Oliveira *et al.*, 2009; Li *et al.*, 2011; Matilainen *et al.*, 2013). Further analysis of the several lysosomal enzymes and the proteasome system is required for a strong conclusion.

4.4 Cathepsin L might regulate Insulin/IGF signalling

One interesting observation was that, in the long lived *daf-2* mutants, cathepsin L activity was reduced to almost undetectable levels. In addition to the primary regulation by down regulation of protein expression we observed a DAF-16 dependent up-regulation of cathepsin inhibitors which is an efficient and stringent regulation. This was considered as a possible mode of insulin/IGF-1 signalling regulation as cathepsin L is known for insulin receptor/ligand degradation and recycling. Studies on mice have indicated that cathepsin L is involved in degradation of the insulin receptor IR and insulin-like growth factor-1 receptor IGF-1R, leading to increased glucose uptake in the preadipocytes (Yang *et al.*, 2007) while other studies indicate its role in glucose tolerance (Huang *et al.*, 2003). Additionally studies on isolated rat adipocytes have shown internalization of insulin receptors, post binding insulin, for digestion (Marshall *et al.*, 1981). Observing our data, we propose a similar negative feedback mechanism in *C. elegans*. In *daf-2* mutants the endocytosed DAF-2 receptors might remain undigested due to their reduced lysosomal activity. In general the cell trys to regain normal physiological state i.e. reduced DAF-16 nuclear localization, in this case. In an attempt to achieve this, the cell might increase its DAF-2 receptor synthesis. Whereas, in this case with a mutant receptor, it would not be possible to further signals to DAF-16. On the other hand *daf-16;daf-2* mutants have higher lysosomal activity and might efficiently degrade DAF-2 receptors. In addition to this digestion, the cells in an attempt to regain normal physiological state might attempt to reduce the synthesis of DAF-2 receptors. This would be done in an attempt to increase DAF-16 nuclear localization, which in this case (*daf-16* mutant) would not be possible (**Fig 2**) leaving DAF-16 cytosolicaly localized. With basal level of evidences, this hypothesis needs further in depth investigation.

4.5 Is reduced protein turnover not healthy?

The most widely accepted ageing theory, the oxidative stress theory, relates ageing to to accumulation of oxidative (protein) damage (Stadtman and Berlett, 1998). A common mode of increasing longevity is by stimulation of protein renewal which was found in longevity phenotypes including DR (Van Remmen *et al.*, 1995; Stadtman 1992). Many longevity

inducing pathways work by either, up-regulating protein synthesis and down-regulating degradation, or vice versa (Vellai and Takacs-Vellai, 2010).

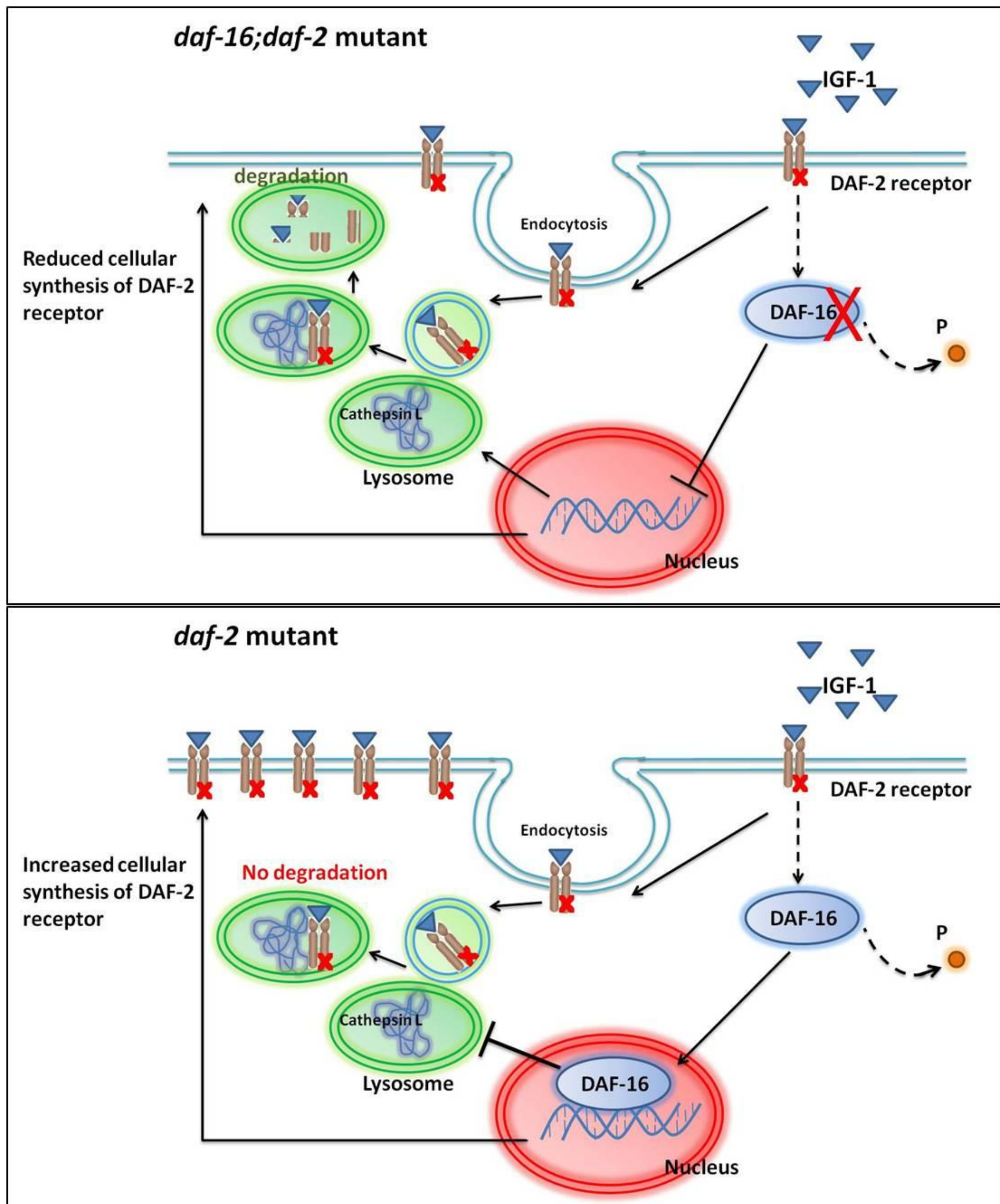

Figure 2. Cathepsin L regulation of Insulin/IGF signaling in the *daf-16;daf-2* and *daf-2* mutants.

A common problem with decreased protein turnover is damaged protein accumulation and aggregation. These aggregations lead to development of neurodegenerative disorders similar to pathological conditions of Parkinson's disease and Alzheimer's disease (Alves-Rodrigues

et al., 1998; Andersen *et al.*, 2000). The other problem of reduced protein synthesis is increased protein half-life which was studied in nematode *Turbatrix aceti*, (Prasanna and Lane 1979). In general, damaged protein accumulation increases the rate of ageing (Stadtman and Berlett 1998). Even though several other longevity interventions like DR have shown to reduce protein damage by reducing the protein dwell time, it does not seem to be the path of preference in the *daf-2* worms as we observed reduced turnover rates and reduced lysosomal activity. With an age-associated increase in oxidative damage (Dubey *et al.*, 1996; Forster *et al.*, 2000) this reduced protein turnover in *daf-2* would possibly be lethal. On the other hand, studies have indicated that the *daf-2* proteome is less susceptible to these kinds of protein aggregations (David *et al.*, 2010). This led us to explore *daf-2* proteome stability.

4.6 Trehalose stabilizes *daf-2* proteome

Organisms utilize molecular and chemical chaperones which are involved in protein folding and stabilization, respectively.

Even though several chemical chaperones were described and are expressed during stress in *C. elegans*, trehalose a bis-acetal, non-reducing disaccharide was shown to reduce polyglutamine (PolyQ) and polyalanine (PolyA) mediated aggregation in the mouse model (Davies *et al.*, 2006; Tanaka *et al.*, 2004). It is also expressed during stress in *C. elegans*, which makes it an interesting candidate. Another candidate, glycerol, which was also shown to be induced during stress in *C. elegans* was not a good candidate as studies reveal inhibition of glycerol synthesis not sufficient to increase protein damage in *C. elegans* (Burkewitz *et al.*, 2012). Trehalose was also found to be synthesised in higher levels (Lamitina and Strange, 2005; Fuchs *et al.*, 2010) and trehalose synthase genes *tps-1* and *tps-2* are upregulated in the *daf-2* mutant (Depuydt *et al.*, 2014). In another study, these genes were identified to be essential for longevity in the *daf-2* mutants (Honda *et al.*, 2010). In our study, we observed that the proteome of *daf-2* mutants is more stable and resistant against acid precipitation. We also showed that this stability was caused by trehalose as addition of trehalose and knock-down of trehalose synthase genes rendered increase and decrease in stability respectively. One could question why trehalose might be the preferred osmolyte by *daf-2*. Its antioxidant properties (Herdeiro *et al.*, 2006) along with its role in stress resistance to desiccation, hypoxia, oxidation, thermal stress (De Virgilio *et al.*, 1994; Watanabe *et al.*, 2002; Elbein *et al.*, 2003) and its resistance to acidic environment (Reyes-Dela Torre *et al.,* 2012) possibly make it an ideal stabilizer. Additionally, trehalose can induce autophagy by an mTOR-independent pathway by involving the AMP-activated protein kinase (AMPK) and TORC1

inhibition, as was shown in human disease models (Sarkar *et al*., 2007). Even though this can benefit removal of damaged proteins, in *C. elegans daf-2* mutants, the autophagy-inducing property of trehalose might be weak or inactive due to the low lysosomal activity. The fact that proteasome inhibition induces trehalose synthesis in *Saccharomyces cervisiae* (Lee and Goldberg, 1998), confirms the link between decreased protein degradation and induction of chemical chaperones.

It is speculated from our study that the increased levels of trehalose in the *daf-2* mutant renders its proteome stabilized from any damage. In this shielded state, damage attained should be very low and the damaged proteins shall be further prevented from aggregation by trehalose. In addition to this, cathepsin L activity and lysosomal activity were regulated through expression of cathepsin inhibitors and reduced acidification respectively, both of which can be expressionally modulated at any point. The ubiquitin proteasome system is also shown to be up-regulated in IIS mutants (Oliveira *et al*., 2009; Matilainen *et al*., 2013). These degradative pathways might also be involved in the selective degradation of damaged and non-functional proteins during ageing. Though these are highly speculative, only further experimental interventions shall bring in clarity.

Interestingly we observed a saturation point for protein stability in *C. elegans* and the proteome of long lived *daf-2* mutants might reach this point. We observed that any addition of trehalose to *daf-2* did not increase stability above the 40-60% limit under the experimental conditions. Adding support to this, the trehalose overexpressing mutant *ogt-1* also failed to stabilize the proteome beyond this limit. This was similar to what was observed in earlier studies that showed no further life span extension on addition of trehalose in the *daf-2* mutants whereas other mutants did attain longevity (Honda *et al*., 2010). This suggests that the proteome of *C. elegans* has a saturation point for its stability and this point, together with a possible maximum life span extension is reached in IIS mutants. Preliminary SDS-PAGE studies indicate that heavier proteins are less stabilized; further analysis to identify the specific proteins stabilized might provide us more insights into the longevity proteome.

A similar increase in trehalose levels and stability was reported and observed in long lived *age-1* IIS mutants (Lamitina and Strange, 2005). Further investigation is needed to establish whether trehalose and stability up-regulation is a common longevity phenomenon or a specific IIS signalling pathway-mediated longevity phenomenon.

It is interesting that several longevity traits observed in *daf-2* mutant is complementary to the dauer phenotype (Kenyon *et al.*, 1993). Both the systems have shown to divert energy consumption away from anabolic processes and towards detoxification and cellular maintenance (Vanfleteren and De Vreese 1995; Houthoofd *et al.*, 2002; McElwee *et al.*, 2004; Halaschek-Wiener *et al.*, 2005). Major complementary components, identified by transcriptomics and proteomics, include expression of alpha-crystallin family of small heat shock proteins, anti-ROS defense systems, enhanced cell maintanence and reduced protein turnover (Burnell *et al.*, 2005; Jones *et al.*, 2009). In addition studies have revealed an almost identical proteome between L3 and dauer with only 8 differentially expressed proteins (Madi *et al.*, 2008). Similar to *daf-2*, autophagy genes are essential for dauer development in addition to *daf-2* lifespan extension (Melendez *et al.*, 2003). Trehalose was also observed to be upregulated several folds in dauers (Erkut *et al.*, 2011). With such similarities between the two phenotypes, is trehalose also involved in stabilizing dauer proteome?

With protein turnover down-regulated and stability increased by chemical chaperones, we wondered whether molecular chaperones could be involved in this alliance. The heat shock factor 1 (HSF-1) which is active in *daf-2* mutants, activates transcription of molecular chaperones in *C. elegans* (Barna *et al.*, 2012). Chaperones are heat shock proteins involved in protein refolding and prevent protein denaturation and aggregation (Hartl and Hayer-Hartl, 2002) and are well-studied in *C. elegans* Alzheimer's model too (Cohen *et al.*, 2006). On the other hand studies indicate that HSF-1 and other molecular chaperones are induced by trehalose in yeast in a dose dependent way (Conlin and Nelson 2007; Bulman and Nelson 2005). Future investigation is required to identify whether trehalose compensates for molecular chaperone-induced stability.

4.7 Conclusion

Though it is generally accepted that longevity is achieved by reducing age-associated protein damage and restoring protein function, the mode of achieving this seems to vary among long lived animals. Whereas longevity induced by DR benefits from a reduced ageing-associated decline in protein turnover (Lewis *et al.*, 1985; Miwa *et al.*, 2008), we propose longevity in *daf-2* is supported by downregulation of protein turnover and increased protein stability. Although we accept that autophagy induction is essential for turnover of house-keeping proteins in *daf-2* mutant, we propose that it is not sufficient for longevity as supported by study of Hansen *et al.*, 2008. We clearly observed that protein turnover and lysosomal activity

is down-regulated in this mutant. However, alhough these proteostatic functions are down-regulated, protein stability is increased by trehalose synthesis.

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Curriculum vitae

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Publications

Depuydt G, Xie F, Petyuk VA, **Shanmugam N**, Smolders A, Dhondt I, Brewer HM, Camp DG, Smith RD and Braeckman B (2013) Reduced insulin/IGF-1 signaling and dietary restriction inhibit translation but preserve muscle mass in *Caenorhabditis elegans., MCP* 3624-3639

Meelkop E, Temmerman L, Janssen T, Suetens N, Beets I, Rompay LV, **Shanmugam N**, Husson SJ, Schoofs L (2012) PDF receptor signaling in *Caenorhabditis elegans* modulates locomotion and egg-laying., *Mol Cell Endocrinol* 361(1-2):232-40

Shanmugam N, Depuydt G, Braeckman B (2014) Increased protein stability and decreased protein turnover in the *Caenorhabditis elegans* Ins/IGF-1 *daf-2* mutant (Will be submitted)

Shanmugam N, Depuydt G, Rasulova M, Petyuk VA, Braeckman B (2014) Lysosomal activity is down-regulated in the long lived *Caenorhabditis elegans daf-2* mutant (Will be submitted)

Abstracts

Depuydt G, Xie F, Petyuk V, Brewer H, Smolders A, Dhondt I, **Shanmugam N**, Camp DG, Smith R, Braeckman B, “LC-MS Proteomics Analysis Reveals Both Common and Unique Changes in the Proteome of Insulin/IGF-1 Receptor Mutant and Dietary Restricted *C. elegans*”, Ageing, metabolism, stress, pathogenesis and small RNAs in *C. elegans*, Madison, Wisconsin, USA, 2012 July 13

Shanmugam N, Depuydt G, Smolders A, Braeckman B, “Proteostasis in *C. elegans*: protein turnover or protein stability?” EMBO Workshop on: The Regulation of Ageing and Proteostasis, Maále Hachamisha, Israel, 2014 September 7.

Teaching related activities

Master thesis supervision

2010-2012 Madina F Rusulova, The ageing lysosome of *Caenorhabditis elegans*.

2012-2013 Elien Van Wallendael, Lysosomale enzymactiviteit en eiwitstabiliteit bij de langlevende *Caenorhabditis elegans glp-4; daf-2* mutant.

Bachelor thesis supervision

2012-2013 Ruud Merkx Vergelijkende, Studie van Lysosomale Enzymen in *glp-4;daf-2, glp-4 daf-16; daf-2, glo*-Mutanten en Wild type van *Caenorhabditis elegans*.

Practical courses

Nematodes as model organisms (Master of Nelatology), (2010-2013) Prof. Dr. Bart P Braeckman.

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