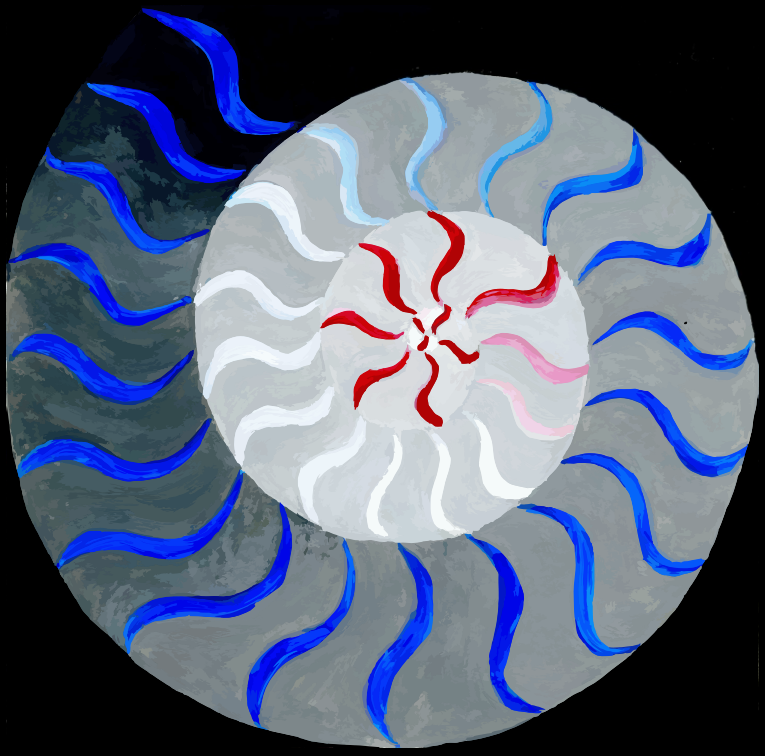
Patterns of protein turnover in aging *Caenorhabditis elegans*

Ineke Dhondt



Promotor: Prof. Dr. Bart Braeckman
Co-promotor: Dr. Geert Depuydt

Academic year 2015-2016

UNIVERSITEIT GENT
fwo
Braeckman Lab

Thesis submitted in fulfillment of the requirements for the degree of Doctor (PhD) in Sciences: Biology

Cover design: Liesbeth Vandendriessche.

Patterns of protein turnover in aging *Caenorhabditis elegans*

Ineke Dhondt

Promotor:

Prof. Dr. Braeckman Bart

Co-promotor:

Dr. Geert Depuydt

Members of the examination committee:

Prof. Dr. Ann Huysseune (Chairman, Ghent University, Belgium)

Prof. Dr. Bart Braeckman (Ghent University, Belgium)

Dr. Geert Depuydt (KU Leuven, Belgium)

Dr. Vladislav Petyuk (Pacific Northwest National Laboratory, USA)

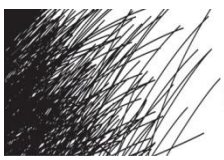
Dr. Tobias Dansen (Utrecht University, Netherlands)

Prof. Dr. Rudi Beyaert (Ghent University, Belgium)

Prof. Dr. Tom Moens (Ghent University, Belgium)

Dr. Filip Matthijssens (Ghent University, Belgium)

Thesis submitted in fulfillment of the requirements for the degree of Doctor (PhD) in Sciences: Biology



UNIVERSITEIT GENT

Research Foundation Flanders
Opening new horizons

Braeckman Lab

DANKWOORD

Ja! Het is gelukt! Vijf jaar geleden belandde ik in dit grandioos avontuur, maar het zou nooit gelukt zijn zonder de steun en hulp van een aantal bijzondere mensen. Daarom wens ik nog enkele personen te bedanken die rechtstreeks of onrechtstreeks hebben bijgedragen tot het tot stand komen van dit doctoraat.

Eerst en vooral wil ik mijn promotor, **Bart**, bedanken om mij de kans te geven dit onderzoek aan te vangen. Gedurende mijn opleiding in de Biologie was je al mijn favoriete lesgever door je aanstekelijk enthousiasme over wetenschap. Ik vond het dan ook een hele eer dat ik in jouw onderzoeksgroep mocht starten. Het record 'op jouw deur kloppen' staat zeker op mijn naam. Dank je wel voor de oneindige feedback, de super fast - binnen de 24 uur - editing van mijn teksten, en het vertrouwen dat je in me stelt om nog verder onderzoek te mogen voeren onder jouw begeleiding.

Mijn co-promotor **Geert**, wil ik bedanken voor alle hulp en raad. Je opende de weg naar de wereld van de proteomics en was een voorbeeld voor mijn eigen onderzoek. Bedankt voor de brainstormmomenten, je kritische vragen en wetenschappelijk inzicht die hebben bijgedragen tot deze thesis.

I wish to thank the examination committee for their critical reading and comments of this PhD thesis. In particular, I would like to thank **Vlad** for the opportunity he gave me to work at PNNL. During my stay in Richland and our ongoing collaboration, you learned me that:

(1) Technical variation is the enemy
(2) Google knows everything
(3) No matter what, you can program it in R
(4) E-mails should be concise and structured, preferably with enumerations.

Vlad, thank you for your infinite patience, your fantastic N14N15 algorithm and clear answers to all my questions. I am very grateful that I had the chance to collaborate with you, this thesis would not exist without your help.

In een labo sta je gelukkig niet alleen. Daarom richt ik graag een woordje van dank aan al mijn vorige en huidige collega's. **Filip, Patricia, Geert, Nilesh, Sasha** en **Arne,** dank je wel voor de voor de toffe momenten en leuke werksfeer.

Natascha, mijn maatje, wat een plezier hebben wij beleefd! Bedankt voor je vriendschap, maar ook voor de vele tips, protocols en feedback. Je bent een voorbeeld voor mij en ik ben ontzettend dankbaar dat ik jouw heb leren kennen. Samen 'ontkoppelen' en Andy's Angels voor altijd!

Andy, ik kan onmogelijk onder woorden brengen wat jij voor mij hebt betekend gedurende mijn doctoraat. De ontspannende koffiepauzes, je luisterend oor, je paraatheid voor elk probleem, … Dank je wel voor de chocomousse, de chocolade, de supergrote eclairs en de bemoedigende post-itjes wanneer ik het nodig had. Je ben een vriend voor het leven en ik kijk al uit naar onze volgende fantastische 'Monkey Splash'.

Renata, **Caroline, Myriam** en **Ives**, dank je wel voor de technische ondersteuning en de hulp bij het uitvoeren van experimenten. **Marjolein**, dank je wel voor de vele micro-injecties. Het maken van transgene wormen op de tonen van Joe FM waren keer op keer een feestje.

Huaihan, thank you very much for your help with the Dendra2 experiments. I have great respect for your enthusiasm and devotion for scientific work. **Madina**, thank you for the great time, all the laugh and fun we had in Madison and Chicago. I wish you both the very best in completing your PhD.

Matthew, mijn pendelgenoot, dank je wel voor de boeiende weetjes van de dag en de toffe babbels tussendoor. Door jou is mijn fietscondities er enorm op vooruit gegaan en is een spurtje om een trein halen al lang geen probleem meer. Ik wens je nog veel succes in je zoektocht naar een fenotype voor je globine mutant!

Lieselot, soms vraag ik mij af of ik mijzelf per ongeluk heb gekloneerd. Jouw bruisende energie, doorzettingsvermogen en motivatie vormen de ideale cocktail voor een vlotte start van je wetenschappelijke carrière. Ik kijk erg uit naar onze verdere samenwerking!

Ook wil ik mijn vrienden, **Margo, Karel, Jeroen, Carolien, Debora, Willem** en **Eske**, bedanken voor de toffe studenttijd in Gent. Het alfabet bracht ons samen en ik hoop dat we nog vele ontspannende reunietjes mogen beleven.

Mijn sportmaatjes, **Esther**, **Saskia** en **Louise**, dank je wel voor de vele ontspannende uurtjes in de fitness en de spinning.

Lies, onze uitjes, de gezellige babbels en je bemoedigende schouderklopjes waren de perfecte ontspanning waar ik steeds weer op kon terugvallen. Dank je wel voor je prachtig ontwerp voor de cover van deze thesis.

Een speciaal woordje van dank gaat uit naar mijn schoonouders, **Els** en **Yves**. Het schrijven van mijn thesis is mede dankzij jullie tot een goed einde gekomen. Dank je wel voor de warme gastvrijheid, de overheerlijke verwenkoffietjes en het verdragen van de vele afleveringen van "Thuis" gedurende mijn meest stressvolle weken.

Woorden schieten me tekort om mijn fantastische ouders te bedanken. Liefste **mama** en **papa**, ik sta nu hier door niemand minder dan jullie. Bedankt voor de immense steun, de oprechte interesse en de eindeloze hulp. Mama, de laatste tijd nam je praktisch mijn hele huishouden over. Dank je wel voor de vele mandjes strijk en de verrukkelijke potjes eten. Papa, dank je wel voor je nieuwsgierigheid en interesse. Ik ben er zeker van dat ik, naast de identieke tenen, mijn 'wetenschappersgen' aan jouw te danken heb. Mama en papa, dank je wel voor de warme thuis, voor de gezellige aperitiefjes en overheerlijke etentjes.

Omaatje, mijn kotmadam, dank je wel voor de vele speculaas en chocolade.

Mijn zus, **Sofie**, mijn soulmate en beste vriendin. Dank je wel dat jij er steeds bent wanneer ik je nodig heb. Tot midden in de nacht was je mijn grootste steun. Jouw humor, speelsheid en relativeringsvermogen hebben mij vaak een hart onder de riem gestoken.

Last but not least, **Thomas**, liefje, wat zou ik doen zonder jou? Ik kan onvoorwaardelijk op je rekenen, ook tijdens mijn doctoraatsonderzoek. Bij de nachtelijke experimenten bleef je graag bij me in het labo, ook al was het slaapbed voor jou veel te klein. Talloze keren ben je mij komen halen in Gent. Op de parking van het INW vroeg je me ten huwelijk, en ik ben ongelofelijk blij dat ik getrouwd ben met het beste ventje ter wereld. De laatste maanden waren voor ons beiden heel druk en we hebben elkaar vaak moeten missen. Ik kijk er enorm naar uit om vanaf nu weer volop met jouw te kunnen leven en te werken aan ons huisje, onze dromen. Hou van je.

Veel liefs,
Ineke

TABLE OF CONTENTS

SUMMARY

The aging process is a recognizable phenomenon as we all experience its inescapable impact. Over the last centuries, mankind has tried to understand the decay of our body, to delay the onset of age-related pathologies and ultimately, to extend its lifespan. Aging starts at the end of the reproductive age and encompasses the progressive decline in cellular maintenance, repair and defense processes. This results in the gradual failure of homeostasis and functionality of cells, tissues and organs over time, and finally leads to inevitable death. Worldwide, the number of elderly people is escalating rapidly, which has a severe social and economical impact on the society. Knowledge on the biological basis of aging advances at an accelerating pace, however, the true nature of the genetic, biochemical and physiological mechanisms at the heart of the aging process remains one of the greatest unsolved mysteries in science.

The free-living nematode *Caenorhabditis elegans* is a popular model organism in many research disciplines (more than 1000 laboratories around the world make use of this model), including aging research. This transparent worm has a short lifespan and generation time, which permits fast screening for lifespan-affecting mutations and conditions. In addition, its 100 million base pair genome is fully sequenced and well-annotated and numerous mutant strains are available, several of which are long-lived. Specific genome editing, through the targeted insertion or deletion of DNA in a worm's genome, allows the generation of transgenic worms to monitor or manipulate biological processes *in vivo*.

In this thesis, we have used *C. elegans* to study proteome dynamics during aging in wildtype worms and in the long-lived *daf-2* insulin/IGF-1 signaling (IIS) mutant. The proteome is the entire set of proteins expressed by an organism at a certain time. It represents the true functionality of a system. Hence, its analysis is crucial to understand biological processes, including aging. Proteins in the cell are not static, but are continually degraded and replaced by new copies, a process called protein turnover. Protein synthesis and degradation slow down with age in many species. This observation led to the formulation of the protein turnover hypothesis, stating that the increase of protein dwell-time with age results in the accumulation of damaged and misfolded proteins, likely driving the aging process. In this vein, increasing protein turnover seems a plausible strategy to slow down aging and extend

Summary

lifespan. However, conflicting studies report an overall down-regulation of protein synthesis in long-lived species and a recent study using classical ^{35}S-labeling showed reduced bulk turnover in the long-lived *daf-2* IIS mutant.

Previous methods often neglect the heterogeneous nature of protein turnover among functionally different and spatially separated proteins. To uncover changing patterns in protein turnover at the level of individual proteins, we used a combination of Stable Isotope Labeling by Nitrogen in *Caenorhabditis elegans* (SILeNCe) and accurate mass spectrometry technology.

Firstly, we explored protein turnover patterns in aging worms on a proteome wide scale. We observed a gradual slowdown of protein turnover for a considerable part of the aging proteome, whereas only a minor fraction showed increased turnover with age. Disparate protein turnover changes within the majority of functionally and spatially related protein groups indicate the stochastic impact of aging on protein turnover. This mirrors the randomness observed in the pathobiology of aging worms. In particular, the slowdown of protein turnover of a seemingly random set of proteins involved in the translation machinery indicates de-regulation of anabolic processes with age. Additionally, we found slower protein turnover rates for constituents of the folding machinery, which is tightly associated with the translation apparatus and safeguard the proteome upon misfolding and accumulation of protein aggregates. Taken together, the widely ranging dwell-times of the translation and folding machineries likely impose a proteome imbalance and ultimately its collapse with age.

Intriguingly, aging worms seem to maintain their (protein) degradation mechanisms. Proteins associated with the ubiquitin/proteasomal system and antioxidant machinery preserve high turnover rates, possibly to manage the proteotoxic escalation and oxidative stress with age. However, this maintenance fights a losing battle, eventually resulting in the ultimate collapse of the proteome.

Secondly, we analyzed shifts in individual protein turnover rates upon reduced IIS. Our data confirmed previous work, showing an overall slowdown of protein turnover in long-lived *daf-2* worms. Subsets of proteins with specifically enhanced protein turnover could not be detected, which further questions the reliability of the protein turnover hypothesis of aging.

The most prominent observation was the drastic and systematic slowdown of the turnover of the translation machinery. Reducing ribosome biogenesis likely saves much cellular energy, which can be invested in lifespan promoting stress-response systems. Furthermore, IIS-driven anabolic processes result in hypertrophy with age. Hence, inhibition of biosynthesis, due to mutation in *daf-2*, seems to prevent the accumulation of irrelevant macromolecules, subsequently retarding the aging process.

In conclusion, de-regulation of anabolic processes during aging likely drives the aging process. The strictly controlled downturn of protein turnover in long-lived *daf-2*, especially for proteins involved in the translation machinery, is a key factor to allocate cellular energy towards stress-resistance pathways and to avoid the accumulation of age-promoting hypertrophy-related pathologies.

SAMENVATTING

Het verouderingsproces is een herkenbaar fenomeen aangezien iedereen de onvermijdelijke impact ervan ervaart. Over de eeuwen heen hebben mensen getracht om de aftakeling van ons lichaam te begrijpen, de aanvang van verouderingsziekten uit te stellen, en uiteindelijk zelfs levensduur te verlengen. Veroudering start op de reproductieve leeftijd en omvat de progressieve aftakeling van cellulaire processen die verantwoordelijk zijn voor onderhoud, herstel en verdediging. Dit resulteert in het gradueel falen van homeostase en functionaliteit van de cellen, weefsels en organen met de tijd, hetgeen onvermijdelijk leidt tot de dood. Wereldwijd groeit het aantal ouderen exponentieel en dit heeft een sterke sociale en economische impact op de samenleving. De kennis over de biologische basis van veroudering neemt snel toe, echter de ware aard van de genetische, biochemische en fysiologische mechanismen die de kern van het verouderingsproces vormen, blijft één van de grootste onopgeloste mysteries binnen de wetenschap.

De nematode *Caenorhabditis elegans* is een populair modelorganisme in veel onderzoeksdisciplines (meer dan 1000 laboratoria wereldwijd werken met dit model), alsook in verouderingsonderzoek. De korte levensduur en generatietijd van deze transparante worm laten toe om snel en herhaaldelijk te screenen naar mutaties en condities die een effect hebben op de levensduur. Verder is het genoom, dat bestaat uit 100 miljoen base paren, volledig gesequeneerd en grotendeels geannoteerd en zijn er vele mutanten beschikbaar, waaronder ook langlevende. De specifieke aanpassing van het genoom, door doelgerichte insertie of deletie van DNA, laat toe om transgene wormen te maken waardoor biologische processen *in vivo* opgevolgd en gemanipuleerd kunnen worden.

In deze thesis hebben we *C. elegans* gebruikt om de dynamiek van het proteoom te bestuderen gedurende veroudering en in een langlevende *daf-2* insulin/IGF-1 signalisatie (IIS) mutant. Het proteoom omvat de volledige set aan eiwitten die tot expressie komt in een organisme op een gegeven moment. Het representeert de ware functionaliteit van het systeem, waardoor proteoomanalyse cruciaal is om biologische processen te begrijpen, zoals ook veroudering. Eiwitten in de cel zijn niet statisch, maar worden voortdurend afgebroken en vervangen door nieuwe kopieën in een proces dat eiwitturnover heet. Eiwitsynthese en degradatie vertragen met de leeftijd in vele organismen. Deze observatie leidde tot de

formulering van de eiwitturnoverhypothese, die zegt dat de langere aanwezigheid van eiwitten in de cel resulteert in de accumulatie van eiwitschade en verkeerd gevouwen eiwitten, een proces dat mogelijks veroudering in de hand werkt. In dit opzicht kan men veronderstellen dat een toename in eiwitturnover een aannemelijke strategie is om veroudering te vertragen en levensduur te verlengen.

Echter, experimentele studies rapporteren een algemene neerregulatie van eiwitsynthese in langlevende organismen en een recente studie, die gebruik maakte van een klassiek ^{35}S-labeling methode, werd de verminderde eiwitturnover aangetoond in de langlevende *daf-2* IIS mutant.

De voorgaande analyses negeerden vaak het heterogene karakter van eiwitturnover tussen functioneel verschillende en ruimtelijk gescheiden eiwitten. Om patronen in eiwitturnover op het niveau van individuele eiwitten te analyseren, maakten we in deze thesis gebruik van Stable Isotope Labeling by Nitrogen in *Caenorhabditis elegans* (SILeNCe) in combinatie met accurate massa spectrometrie technologie.

Vooreerst gingen we patronen na in veranderende eiwitturnover in verouderende wormen. We observeerden de graduele vertraging in eiwitturnover voor een aanzienlijk deel van het verouderende proteoom, waarbij enkel een minderheid aan gedetecteerde eiwitten een toename in eiwitturnover vertoonde met de leeftijd. De meerderheid van functioneel en ruimtelijk gerelateerde eiwitten vertoonden uiteenlopende patronen van veranderende eiwitturnover, welke de stochastisch impact van veroudering aantoont op dit proces. Dit wordt mede gereflecteerd in de willekeurigheid waarmee verouderingsverschijnselen optreden binnen verouderende wormen. De vertraging van eiwitturnover van een random set van eiwitten die verbonden zijn aan de translatiemachinerie duidt op een deregulatie van anabole processen met de leeftijd. Een gelijkaardige vertraging werd geobserveerd voor componenten van de opvouwmachinerie, die nauw verbonden is aan de translatiemachinerie en instaat voor de algemene handhaving van het proteoom inzake foutieve eiwitconformaties en aggregaatvorming. Veroudering resulteert in uiteenlopende halfwaardetijden van eiwitten betrokken in de translatie- en opvouwmachinerie en brengen het proteoom wellicht uit evenwicht en veroorzaakt uiteindelijk zijn ondergang.

Samenvatting

Een intrigerende waarneming is dat oude wormen de turnover van eiwitten betrokken in eiwitdegradatie waarborgen. Eiwitten geassocieerd met het ubiquitine/proteosoom systeem en de antioxidantmachinerie behouden hun hoge turnover snelheden, waarschijnlijk in een poging om de accumulatie van toxische eiwitten en oxidatieve stress te beheren. Desondanks is deze inspanning onvoldoende om de uiteindelijke neergang van het proteoom te weerstaan.

In een twee luik van deze thesis analyseerden we de individuele eiwitturnover snelheden onder verminderde IIS. Een algemene daling in eiwitturnover kon worden aangetoond in de langlevende *daf-2*, en bevestigt daarbij de data van een voorgaande studie. Tegen onze verwachting, konden we geen specifieke subsets van eiwitten aantreffen met een verhoogde eiwitturnover, waardoor de eiwitturnover hypothese volledig wordt weerlegd. De belangrijkste observatie was de drastische en gecontroleerde vertraging van eiwitten van de translatiemachinerie. Gereduceerde ribosoomsynthese bespaart veel cellulaire energie die kan worden geïnvesteerd in levensduurverlengende stress-reponse systemen. Daarenboven stimuleert IIS anabole processen die resulteren in hypertrofie met toenemende leeftijd. Bijgevolg voorkomt de inhibitie van eiwitsynthese, door mutatie in *daf-2*, de accumulatie van irrelevante macromoleculen, waardoor het verouderingsproces wordt vertraagd.

Tot besluit stellen wij dat een geleidelijke deregulatie van anabole processen met de tijd mede aan de basis ligt van het verouderingsproces. De strikt gecontroleerde vermindering in eiwitturnover, voornamelijk voor eiwitten betrokken bij het translatieproces, is mogelijks een sleutelfactor in de allocatie van energie naar stressresistentiesystemen. Daarenboven worden verouderingsbevorderende hypertrofie-gerelateerde aandoeningen voorkomen onder gereduceerde IIS.

PART I INTRODUCTION

Chapter 1. Introduction and outline of the thesis

1.1 Aging

1.1.1 What is aging?

Aging is a nearly universal and complex biological phenomenon. It is a longstanding enigma that every person can relate to as we all will experience it unavoidably. We must face aging with a rational attitude of being a horrible disease on its own and therefore the main cause of human illness and death (Gems 2015).

Aging is defined as the intrinsic (from within the organism or cell) physiological degeneration, leading to an increase in age-specific mortality rate and a decrease in age-specific reproductive rate (Arking 1998). Intriguingly, there is a profound variation in aging rates among and within species. A general observation is the correlation between body size and lifespan across different species: larger animals tend to live longer compared to smaller animals (Promislow 1993). Several outliers exist with the naked mole-rat (*Heterocephalus glaber*) as best-known example as it lives about ten times longer compared to other rodents with similar body size (Sherman & Jarvis 2002). Other species seem to neglect senescence, such as the bristlecone pine (*Pinus longaeva* – 4862 years), rougheye rockfish (*Sebastes aleutianus* – 205 years), tortoise (*Aldabrachelys gigantea* – 150 years) and quahog clam (*Arctica islandica* – 507 years) (Finch 1998; Butler *et al.* 2013). Aging does not only differ widely between species, it can also vary within a species. Interestingly, an inverse correlation can be found between body size and lifespan comparing individuals within the same species: smaller individuals are likely to live longer. For instance, the small Chihuahua dog lives approximately two times longer as the larger Great Dane. This relationship also exists in humans, although to a smaller extent compared to dogs (Pitt & Kaeberlein 2015). Nevertheless, variation in lifespan within and between species is often independent of body size but rather dependent on ecological factors and mode-of-life traits, which result in a different extrinsic mortality (death caused by external factors such as disease, predation, food shortages or accidents) (Healy *et al.* 2014). The difference in lifespan expectancy of honey bees is one of the most remarkable examples of natural plasticity of aging. Queens of the honey bee (*Apis mellifera*) can live up to 7 years, while lifetimes of summer and winter

honeybee workers are only 60 and 300 days, respectively (Finch 1998). External mortality is highly reduced for queens, since they never leave the safety of the nest center and receive high-quality food. Difference in lifespan expectancy between winter and summer bees can be explained by the distinct transition from in-hive workers to foragers (Rueppell *et al.* 2007). Flight capability, activity period and foraging environment are other factors influencing maximum lifespan between species. Volant, nocturnal (active at night and dusk) and arboreal (foraging in trees) or fossorial (adapted to digging) species tend to live longer, because they can more easily evade predators or possess means to escape predation and unfavorable conditions (Healy *et al.* 2014). The fact that species and individuals age at profoundly diverse rates reveals the enormous complexity of the aging process. Hence, the major focus of gerontologists is to unravel the different factors underlying individual rates of aging.

1.1.2 Why do we age?

The "why" of this enigmatic problem is based on the idea of the often-quoted geneticist and evolutionary biologist Theodosius Dobzhansky, that "nothing in biology makes sense except in the light of evolution" (Dobzhansky 1964). In 1889, Weismann proposed the programmed death theory, stating that aging might be a beneficial programmed process evolved to avoid competition for resources between older organisms and their own offspring. Yet the idea that aging has a purpose is incorrect. For instance, it is unlikely that aging has evolved to eliminate worn out elderly people to benefit the human species, as this should prevent us from curing Alzheimer's disease and cancer (Gems 2015).

A new evolutionary concept, proposed in the mid-20th century, implies that aging is not an adaptation in any sense (Haldane 1941; Medawar 1952; Williams 1957). In general, these new ideas rely on the fact that reproductive success drives evolutionary fitness, and not individual long-term survival. In this vein, Medawar (1952) proposed The Mutation Accumulation Theory, which hypothesizes that natural selection acts early in life, while evolutionary pressure decreases at older age. Hence, mutations with deleterious effects later in life might accumulate over many generations driving the aging process (Medawar 1952). Williams (1957) further extended this concept by invoking the pleiotropic capacity of gene mutations, as proposed in his Antagonistic Pleiotropy theory. Gene mutations can

cause beneficial changes early in life that enhance reproductive success, but in later life have detrimental effects (Williams 1957). Therefore, animals with higher mortality risk caused by extrinsic factors (such as predation, starvation and disease) will reproduce and age faster. This even implies that in the wild, few individuals survive long enough to develop senescent pathologies, which consequently have negligible impact on evolutionary fitness (Williams 1957; Gems 2015).

Another non-programmed aging theory is the Disposable Soma Theory (Kirkwood 1977; Kirkwood & Rose 1991), which focuses on the physiological aspect of aging. This theory suggests the optimized allocation of metabolic resources between reproduction, growth and cellular maintenance processes. Recent findings support the presence of early-late life trade-offs, even in the wild, suggesting that limited resources force individuals to trade somatic maintenance later in life for high allocation to reproduction early in life (Lemaitre *et al.* 2015). Hence, somatic damage will progressively accumulate with age as organisms invest less in maintenance and repair later in life (Kirkwood & Rose 1991).

1.1.3 How does aging occur?

Mechanistic theories, in contrast to the evolutionary theories (1.1.2), focus on the molecular changes and mechanisms underlying the aging process. Over the past centuries, gerontologists have proposed many mechanistic theories, most of which focus on the accumulation of macromolecular damage.

The telomere theory of aging

In the 1930s, McClintock and Muller suggested that chromosome ends are required to ensure chromosome stability and faithful segregation of genetic material upon cell division (Muller 1938; McClintock 1939; McClintock 1941). These functional chromosome ends consist of DNA tandem repeats (TTAGGG) and are termed telomeres from the Greek for “end” (telos) and “part” (meros). Chromosomes in somatic cells appear to lose about 50 to 200 nucleotides from their telomeres as cells divide (Bettelheim *et al.* 2015). Olovnikov linked telomere shortening to the observation that cells lose replicative capacity after approximately 50 cell divisions, commonly termed the Hayflick limit (Hayflick 1965;

Olovnikov 1971; Harley *et al.* 1990; Olovnikov 1996). Laboratory evidence confirmed Olovnikov's telomere hypothesis of aging and led to the idea that telomere length acts as a mitotic clock (Harley *et al.* 1990; Olovnikov 1996). Telomeric DNA can be restored in immortal cells, such as germline cells, stem cells and tumor cells, by the expression of telomerase, an DNA polymerase that does not exist in normal somatic cells (Blackburn *et al.* 1989; Greider & Blackburn 1989; Wright *et al.* 1996). The absence of telomerase in somatic cells is thought to be a potent anticancer protection mechanism for long-lived species such as humans (Shay & Wright 2011). Numerous studies showed that telomere shortening contributes to aging and age-related disorders. The loss of these protective caps can lead to induction of chromosomal instability and to the initiation of tumors (Shay & Wright 2011; Meena *et al.* 2015). Furthermore, telomeric shortening appears to drive the activation of the tumor suppressor p53, causing the ultimate loss of cell's proliferative ability (von Zglinicki 1998). However, the loss of telomeres does not seem to be the key universal reason of aging for several reasons. First, telomere length does not correlate with lifespan (Raices *et al.* 2005). The common laboratory mice (*Mus musculus*) have much longer telomeres compared to humans, yet they live much shorter (Greider 1996). Secondly, the telomeres of cells in post-mitotic tissues of some animals, such as *C. elegans* and the fruit fly *Drosophila melanogaster,* cannot shorten during adulthood, although these animals do age. Thirdly, knock-out mice lacking telomerase show lifespan reduction, but only after three generations (Kipling & Faragher 1999; Rudolph *et al.* 1999). Therefore the relevance of these results to normal aging remains unconvincing. Fourthly, many cells undergo moderate number of cell divisions during the life of an individual. Hence, they will not be affected adversely by telomere shortening (Goyns & Lavery 2000). Furthermore, human fibroblasts taken from elderly people do not reach the limit of their proliferation potential (Schneider & Mitsui 1976; Bierman 1978).

The molecular damage theories

The Rate-of-Living theory (live fast, die young) of Pearl, popular in the early 20th century, asserts that lifespan is inversely related to metabolic rate as the total amount of metabolic energy is the same for every species (Pearl 1928). Hence, high metabolic rates would be

supposed to accelerate aging. Later, this theory was integrated with the long-standing Free Radical Theory of Aging (FRTA), postulated by Denham Harman in 1956. According to this theory, aging is the result of the accumulation of macromolecular damage, driven by reactive oxygen species (ROS), such as the superoxide ($O_2^{\bullet-}$) and hydroxyl (•OH) radicals. Since endogenously produced ROS are normal by-products of oxidative metabolism, an association between the FRTA and the Rate-of-Living theory was suggested: higher metabolic rate, thus higher oxygen consumption, will result in more ROS-induced oxidative damage, thereby driving the aging process (Harman 1956). However, this concept has been rejected as the metabolic rate of long-lived animals, due to dietary restriction or a mitochondrial mutation, was shown not to be decreased (Braeckman *et al.* 2002a; Houthoofd *et al.* 2002; Van Voorhies *et al.* 2004; Van Raamsdonk *et al.* 2010).

The FRTA received more attention with the discovery of superoxide dismutase (SOD), an enzyme which catalyzes superoxide radicals (McCord & Fridovich 1969). In 1972, a role for mitochondria in the aging process was proposed as these organelles are the major site of ROS production (Harman 1972). Additionally, the detection of other ROS that are not free radicals, for instance hydrogen peroxide (H_2O_2), expanded the FRTA even further (Chance *et al.* 1979) and is now often referred as the oxidative damage/stress theory of aging (Sohal & Weindruch 1996). It postulates that antioxidants are not sufficient to cope with oxidative stress over time, resulting in the progressive accumulation of damaged macromolecules, ultimately leading to aging and death (Fig. 1).

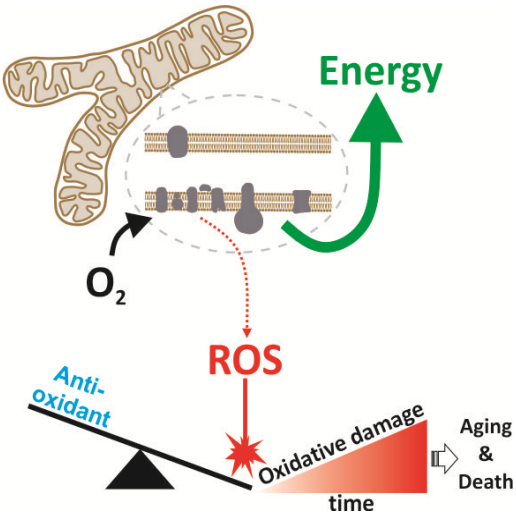


Fig. 1 Schematic representation of the oxidative stress theory of aging. Reactive oxygen species (ROS) are byproducts of normal energy metabolism. The imbalance between ROS production and removal by antioxidant defense systems, leads to the progressive accumulation of oxidative damage over time, which is in turn responsible for the progressive functional decline of various cellular processes and eventually results in aging and death.

Oxidative stress theory in crisis

The oxidative stress theory is probably the most extensively studied theory of aging over the past several decades. However, increasing number of studies challenge the veracity of this theory as conflicting evidence is piling up in recent years querying the causal role of ROS in aging (Van Raamsdonk & Hekimi 2010). For example, impaired mitochondrial Mn superoxide dismutase (SOD) in *C. elegans* results in higher levels of protein oxidation and hypersensitivity to oxidative stress, yet lifespan of these mutants is unchanged or even increased (Yang *et al.* 2007; Doonan *et al.* 2008; Van Raamsdonk & Hekimi 2009). Although oxidative stress plays a role in many age-associated diseases, including Parkinson's disease (Zhang *et al.* 2000), Alzheimer's disease (Qin *et al.* 2006), and nearly all cardiovascular diseases (Dhalla *et al.* 2000), it seems not to be the primary or initial cause of aging.

ROS are best known as the toxic by-products of oxidative metabolism causing damage to macromolecules. However, we need to move away from this conservative image as ROS are involved in many vital physiological processes too (Brieger *et al.* 2012). Evidence reveals a central role of ROS in pathogen killing (Snelgrove *et al.* 2006), regulation of inflammation (Tschopp 2011), hormone production (Erdamar *et al.* 2008), brain and memory functioning (Massaad & Klann 2011), and cell-to-cell communication (Sorce & Krause 2009). Finding new key functions for ROS remains an attractive field of research, whereby novel approaches using *in vivo* ROS biosensors, such as HyPer and Orp-roGFP2, will definitely enhance the understanding of ROS signaling in health, disease and aging (Back *et al.* 2012).

Protein turnover hypothesis

An alternative for the oxidative stress theory was the Error Catastrophy theory, formulated in 1963 by Orgel (Orgel 1963). The basic idea relies on the competence of the protein-synthetic apparatus. It states that an error in a protein molecule, for example responsible for some metabolic function, will have no severe effect since this single error protein can be degraded with no further impact on the soma. However, errors in proteins functional in protein synthesis (ribosomal proteins), will lead to escalating error frequency, resulting in the exponential accumulation of erroneous molecules and ultimately an error catastrophe. However, 2D gel electrophoresis analysis of proteins from young and old animals (mice, rats, *D. melanogaster* and *C. elegans*) revealed no higher frequency of errors in the latter

(Vaughan & Calvin 1977; Wilson *et al.* 1978; Parker *et al.* 1981; Johnson & McCaffrey 1985; Takahashi & Goto 1988; Vanfleteren & De Vreese 1994). Much evidence contradicts the Error Catastrophe Hypothesis, excluding the error feedback in translation as a major factor in the aging process.

Nevertheless, extensive evidence demonstrates the accumulation of aberrant proteins over time, especially due to oxidative damage (Martin *et al.* 1996). Increased levels of damaged, inactive or partially active, post-translationally modified and misfolded proteins in aged organisms are well-reported (Rothstein 1979; Stadtman 1988; Rothstein 1989; Gafni 1990; Rosenberger 1991; Stadtman 1992). Approximately 20-30% of all cellular proteins become carbonylated with age (Starke-Reed & Oliver 1989; Stadtman 1992), while even 40-50% are oxidatively damaged when all other possible forms of protein alterations are considered (Stadtman 1992).

To manage these damaged proteins, cells can degrade and replace them with newly synthesized copies in a process called protein turnover. It were Reiss and Rothstein (1974) who proposed a slow-down in the rate of protein turnover as a major cause of protein alterations (Reiss & Rothstein 1974). The observation of increased dwell-times of proteins in old free-living nematodes confirmed this idea (Prasanna & Lane 1977; Prasanna & Lane 1979; Sharma *et al.* 1979; Rothstein 1982). Hence, age-related decrease in protein turnover, due to declines in the rates of both protein synthesis and protein degradation, is now referred to as the protein turnover hypothesis of aging or protein damage catastrophe (Fig. 2). According to this concept, the progressive accumulation of misfolded, posttranslationally altered and damaged proteins leads to the functional collapse of aged cells and ultimately death (Ryazanov & Nefsky 2002).

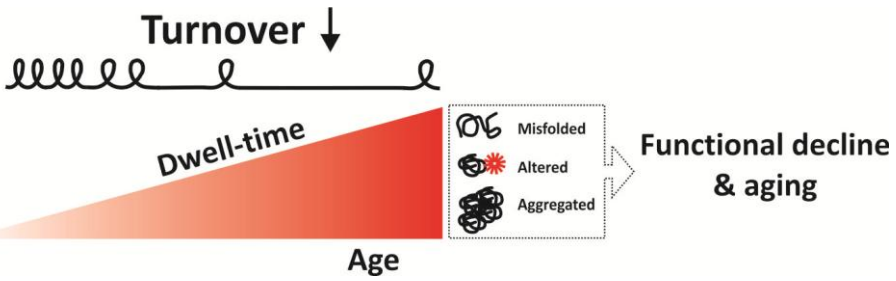


Fig. 2 Schematic representation of the protein turnover hypothesis of aging. Slow-down of protein turnover results in the increased dwell-times of proteins with age. Hence, proteins become increasingly vulnerable for misfolding, posttranslational alterations and aggregation, which lead to functional collapse of aged cells.

Recent work corroborated the decrease in bulk protein turnover in aging *C. elegans* (Depuydt *et al.* submitted), however, turnover of individual proteins with age is still poorly understood **(section 1.4.1)**. A first objective of this thesis is to survey different patterns in individual protein turnover and their changes over time. Results are discussed in **chapter 2**.

Increasing protein turnover seems a plausible strategy to slow down aging and extend lifespan. However, classical pulse-chase ^{35}S-labeling contradicts this hypothesis as long-lived worms show reduced protein turnover (Depuydt *et al.* submitted). Since protein turnover is considered to be one of the major energy-consuming maintenance processes (Russell & Cook 1995), long-lived worms may save energy by lowering protein turnover and invest in other lifespan-extending mechanisms. Although aging and longevity has little effect on evolutionary fitness, these longevity promoting mechanisms could evolve because they have an evolutionary benefit early in life. The insulin/IGF-1 signaling (IIS) pathway regulates the generation of the dauer larvae, a long-lived development variant that arise from second-stage larvae when conditions are harsh (Larsen *et al.* 1995; Vanfleteren & Braeckman 1999). Reduced IIS by mutation in the DAF-2 receptor prolongs lifespan in postreproductive adults by triggering the nuclear location of transcription factor DAF-16 (**Section 1.3.2**). Recent evidence points to the investment in the stabilization of the proteome in *daf-2* mutant worms, involving chemical chaperones such as trehalose (Depuydt *et al.* submitted). A second aim of this thesis was to further elucidate the protein turnover patterns at the level of individual proteins in long-lived *daf-2* animals compared to controls with normal lifespan. Results are discussed in **chapter 3**.

Hyperfunction hypothesis

Currently, a radical shift in the overall perception of the aging process is occurring. Traditional long-standing theories, involving the accumulation of molecular damage, are overwhelmed by conflicting evidence (Van Raamsdonk & Hekimi 2010). Therefore, new alternatives need to be considered outside the damage-maintenance box to explain the underlying mechanisms of the aging process. Blagosklonny was one of the first gerontologists who proposed a theory independent of molecular damage, thereby opposing the oxidative damage and turnover dogmas (Blagosklonny 2006; Blagosklonny 2007b; Blagosklonny 2008). According to the hyperfunction theory, aging is caused by a

continuation of deregulated developmental processes in late life, resulting in hypertrophy which implies the pathological character of senescence (Blagosklonny 2007b; Gems & de la Guardia 2013; Gems 2015). Indeed, a clear association has been made between several hyperfunctions, including hypertrophy, and diseases such as obesity, cancer, hyperglucemia, hyperlipidemia, hyperinsulinemia, hyperprolactinemia, hypertension and many other "hypers" (Blagosklonny 2012). Whereas development is tightly regulated, aging is the purposeless ongoing of programs that are not switched off upon its completion (Blagosklonny 2013). Thereto, Blagosklonny expressed his idea using a metaphor in which aging is depicted as the shadow (quasi-program) of actual programs (Fig. 3).

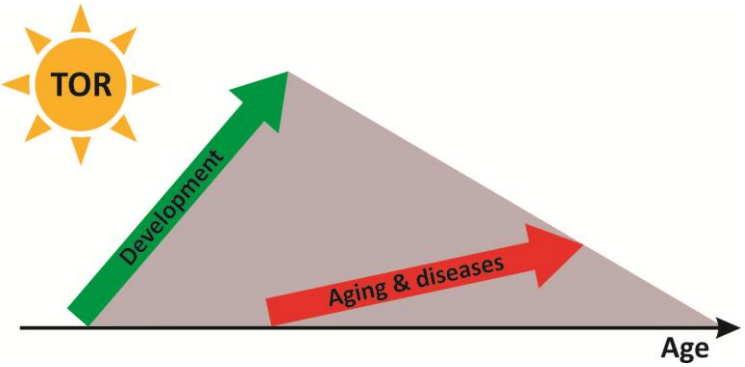


Fig. 3 From development growth (program) to aging (shadow). Adapted from Blagosklonny (2013).

Interestingly, the hyperfunction hypothesis provides the missing mechanic evidence to support the Antagonistic Pleiotropy theory of aging (**Section 1.1.2**). The ultimate examples of this evolutionary theory are the growth-signaling and nutrient-sensing pathways, such as the insulin/IGF-1 and TOR (target of rapamycin) signaling pathways (**Section 1.3.2**). These pathways are essential early in life, while they are well-known to be pro-aging later in life (Demidenko *et al.* 2009; Schug 2010; Leontieva *et al.* 2011). Natural selection favors the beneficial program of these anabolic processes in developing organisms. However, aging and disease are not programmed. Hence, natural selection is incapable of selecting against aging as it is the quasi-program (shadow) of aimless continuations of developmental programs, resulting in hyperfunctions later in life (Blagosklonny 2010b; Blagosklonny 2013).

Many observations and ideas support the hyperfunction theory:

- Attenuation of insulin/IGF-1 signaling pathways or TOR decelerate aging in many species, including yeast, *C. elegans, Drosophila* and mice (Kapahi *et al.* 2010; Kenyon 2010; Dominick *et al.* 2015; Scialo *et al.* 2015)
- Reduction of protein synthesis (drives hypertrophy) or activation of autophagy (opposing hypertrophy) leads to lifespan extension in yeast and *C. elegans* (Kapahi *et al.* ; Kapahi *et al.* 2010; Kaeberlein & Kennedy 2011).
- Hypertrophy underlies many age-related disorders, thereby implying the fundamentally pathological nature of aging (Gems 2015).
- Hypertrophy related diseases, such as cancer and autoimmunity, may be treated with rapamycin (pharmacological inhibitor of TOR), which seems to act anti-proliferative (Blagosklonny 2006; Blagosklonny 2015). It has been shown that rapamycin has anti-tumor effects (inhibits cell proliferation) and posses certain immunostimulatory activities (e.g. elimination of malignant cells) (Blagosklonny 2007a; Bravo-San Pedro & Senovilla 2013).

Changes occurring during *C. elegans* aging reflect several hypertrophic pathologies, such as neurite outgrowths, cuticle thickening, uterine tumors and escalating levels of lipid and yolk inclusions (Gems & de la Guardia 2013). Yet, only the latter seems to contribute to mortality in aging worms. Yolk (vitellogenin), the storage molecule for developing oocytes, is synthesized in the intestine, whereafter it is transferred across the body cavity to the gonads (Kimble & Sharrock 1983). Production of yolk continuously goes on, even in postreproductive worms, resulting in excess of purposeless yolk which accumulates throughout the body cavity (Herndon *et al.* 2002). Long-lived worms, due to mutation in insulin/IGF-1 signaling pathway or dietary restriction, show reduced levels of vitellogenins (DePina *et al.* 2011; Depuydt *et al.* 2013). Additionally, knockdown of vitellogenin genes results in lifespan extension (Murphy *et al.* 2003). These observations further corroborate the link between yolk production as a hyperfunction-driven pathology and mortality.

1.2 *Caenorhabditis elegans* as a model organism

Caenorhabditis elegans (Rhabditida) is a free-living terrestrial nematode introduced by Sydney Brenner as a model organism to study the genetics of development and behavior (Brenner 1974). In the wild, this roundworm survives predominantly on micro-organisms that develop on decomposing plant material.

C. elegans comes in two sexes: self-fertilizing hermaphrodites with two sex chromosomes (XX) and single X male worms (XO) (Fig. 4). Males are rare as they arise infrequently due to a spontaneous error in chromosome disjunction during meiosis in the hermaphrodite germline. However, higher frequency may be obtained through mating (up to 50%). One single hermaphrodite is capable of laying 300 self-fertilized eggs, while this number can exceed 1000 via insemination by a male (Altun & Hall 2009).

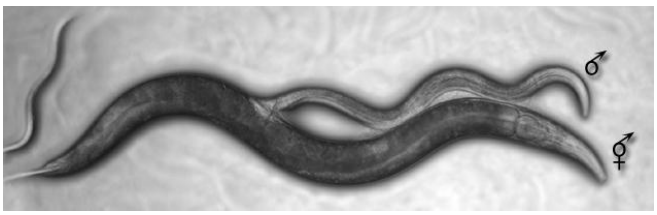


Fig. 4 *C. elegans* arises in two sexes: self-fertilizing hermaphrodites and males (Altun & Hall 2009).

1.2.1 Lifecycle and development

The lifecycle of *C. elegans* starts with an embryonic stage, inside the hermaphrodite uterus, approximately until gastrula stage, which further develops *ex utero*. A nematode worm develops through four larval stages (L1-L4), each separated by a molt. An arrested state, called dauer larva, may be entered at the end of the L2 stage if the environmental conditions are not optimal for further growth (overcrowding, absence of food, high temperature).

In such case pharyngeal pumping is completely suppressed and the worm becomes lethargic although rapid movement is still possible upon mechanical stimulation. The extremely long-lived dauer is a non-aging state and its duration has no impact on post-dauer lifespan. Dauers show increased stress resistance, are hypometabolic, and survive on internally stored carbohydrate and lipid reserves (Lant & Storey 2010).

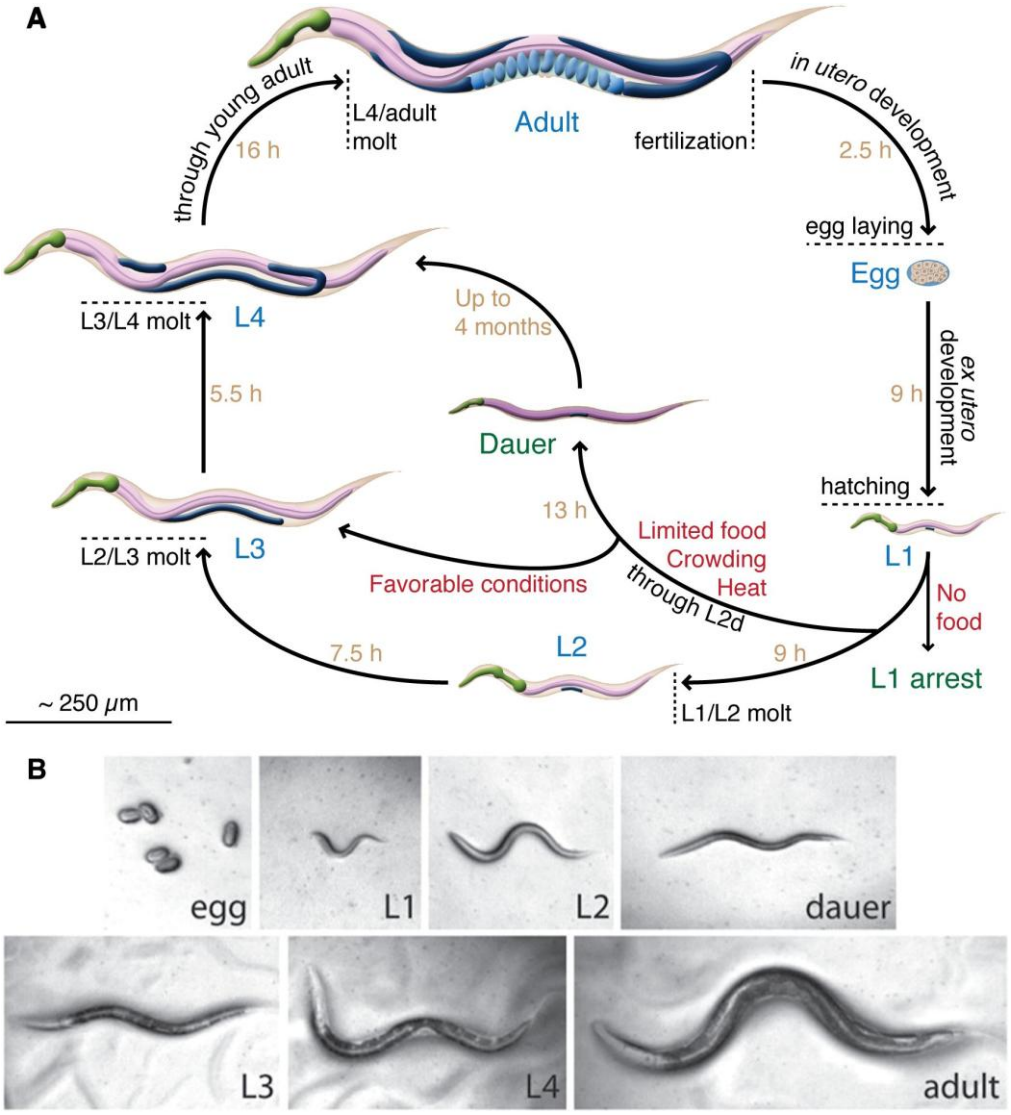


Fig. 5 (A) Lifecycle of *C. elegans* (Modified from Altun and Hall (2012)). (B) Micrographs of laid eggs, larval stages (L1-L2-dauer-L3-L4) and adult stage (Modified from Fielenbach and Antebi (2008)), (Erkut, Cihan (2014).

Dauer larvae tend to stand on their tails, while waving their heads back and forth, a behavior called nictation. This action permits attachment to passing invertebrates, so they can be dispersed to new locations (Cassada & Russell 1975). Finally, dauer larvae molt to L4 stage upon favorable conditions and, after one final molt, become adult mature hermaphrodites. Under advantageous conditions, the egg-to-egg cycle lasts only three days. After the reproductive period of three-to-four days, worms may live approximately two more weeks (Fig. 5) (Altun & Hall 2009).

1.2.2 General morphology

C. elegans has an unsegmented, cylindrical body shape that is tapered at both ends. Its simple body plan consists of an outer tube and an inner tube separated by the pseudocoelomic fluid, which causes an internal hydrostatic pressure to maintain the body shape. An overview of the anatomy is shown in figure 6.

The body wall comprises the cuticle and underlying hypodermis, an excretory system, neurons and body wall muscles for locomotion. The inner tube (alimentary canal) includes the pharynx (food uptake), intestine (digestion), rectum and anus (defecation).

The worm is protected by a collagenous cuticle which surrounds the outside of the body wall and also lines the pharynx and rectum. The cuticle consists of an surface coat, an epicuticle, cortical zone, medial zone and basal zone. The underlying hypodermis and seam cells secrete the cuticle. Several tissues open via pores to the outside, such as alimentary (mouth and anus), reproductive (vulva), sensory (openings for sensilla) and excretory systems (excretory pore).

The adult *C. elegans* is a eutelic post-mitotic animal with 959 somatic nuclei (male has 1031), including 302 neurons which communicate through approximately 6400 chemical synapses. Neuronal processes run along the ventral or dorsal nerve cord to the nerve ring in the head region (also called the 'brain'). *C. elegans'* nervous system is capable of regulating a wide range of behaviors and responses, despite this low number of neuronal cells. Muscles receive their input via muscle arms that extend to the nerve cords. The striated body wall muscles are organized into strips in four quadrants, two dorsal and two ventral, along the whole length of the worm. Smaller, non-striated muscles are present in the pharynx and around the intestine, rectum and vulva.

The twenty intestinal cells form the inner tube of the worm which runs from head to tail. The lumen of the pharynx, a nearly autonomous organ, is continuous with the lumen of the intestine which carries numerous microvilli. Bacterial food is grinded in the pharynx, digested in the intestine and egested to the outside through the rectum and anus during defecation.

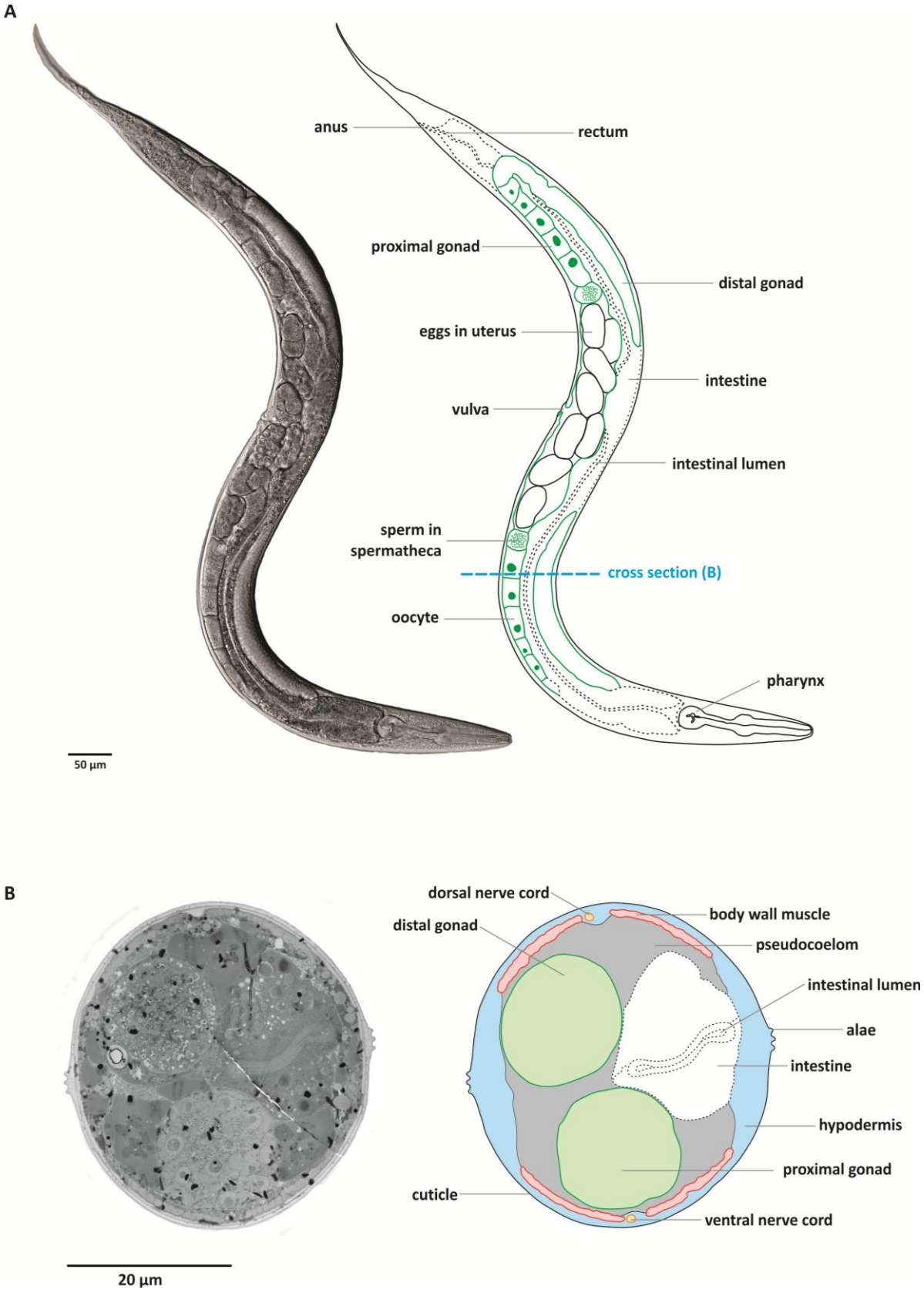


Fig. 6 Anatomy of an adult hermaphrodite. (A) Nomarski (DIC) image of an mature hermaphrodite with schematic representation of the anatomical structures (Via Nikon TiE-C2 40x CFI Plan Apochromat). (B) Cross section through the mid body with schematic labeling of the major tissues. The approximate level is indicated with a blue dashed line in (A). (Via transmission electron microscopy, 1500x).

Introduction

The gonads occupy most of the pseudocoelomic space in mature worms. Two U-shaped bilaterally symmetric arms are connected to a central uterus via spermathecae. The distal part of the gonad (ovary) is a syncytium with germline nuclei in cup-shaped open compartments, organized around a central cytoplasmic core, the rachis. As they pass through the gonad arms and move towards the proximal end, oocytes separate from the syncytium, enlarge and become fertilized by the sperm in the spermatheca. Diploid zygotes are stored in the uterus, surrounded by a vitelline membrane and chitinous egg shel, and deposited through the vulva, which protrudes at the ventral midline (Altun & Hall 2009).

The male reproductive system consists out of the somatic gonad, the germline and the proctodeum (male posterior gut, modified rectum) with associated neurons and muscles. The male gonad forms a single J-shaped arm and produces during the entire life of the animal only male gametes or sperm. The cloaca spicules probe for the vulval opening and insert into the vulva fully. This triggers the ejaculation or sperm release from the gonad (Altun & Hall 2009).

1.2.3 Advantages and disadvantages of the model organism *C. elegans*

Today, *C. elegans* has become a powerful experimental system in many scientific disciplines, such as aging research. The *C. elegans* community counts 1,135 registered laboratories working with this model organism, and this number is continuously increasing (17 new labs joined the community in September 2015; blog.wormbase.org).

Working with *C. elegans* has many advantages. Its small size, short lifespan and high fecundity allow easy and inexpensive maintenance. In the lab, homozygous and genetically identical worms can be maintained via self-fertilization of hermaphrodites, whereas crossing with males facilitates the generation of new mutant combinations.

Large cultures can be grown very rapidly and worms can be frozen in glycerol for long-term storage in liquid nitrogen. This little worm harbors many major tissues and cell types, including muscle cells, neurons, intestinal cells, germ cells and epidermis. In addition, *C. elegans*' invariant cell lineage is completely mapped (Sulston *et al.* 1983), which allows studies at single-cell resolution within a whole organism.

C. elegans was the first multi-cellular eukaryote to have its whole 100 million base pairs genome sequenced (The *C. elegans* Genome Sequencing Consortium 1998). The number of protein-coding genes is predicted to be 20,375 (www.wormbase.org; release WS249) and approximately 44 percent of human genes have a *C. elegans* ortholog (Shaye & Greenwald 2011). Moreover, introduction of human gene orthologs often rescues the functional phenotype in mutant worms (Calahorro & Ruiz-Rubio 2012).

The introduction of the RNA interference (RNAi) technique, together with the whole genome sequence information, provided a real 'Big Bang' for research in this model organism (Ceron & Swoboda 2008). RNAi feeding clones can be easily administered to silence particular genes of interest and are collected in libraries (Ahringer and Vidal libraries) which target roughly 94 percent of the *C. elegans* genome (Kamath & Ahringer 2003; Rual *et al.* 2004; Ahringer 2006). Additionally, a huge collection of mutant strains is available through the Caenorhabditis Genetics Center and a detailed description of these strains is available in the online Wormbase website (Thompson *et al.* 2013).

As *C. elegans* is a transparent worm, every cell can be studied in live animals using Nomarski imaging. Furthermore, *in vivo* GFP labeling methods allow spatial and temporal analysis of gene expression using fluorescence microscopy (Chalfie *et al.* 1994). Transgenic worms can be easily generated via microinjection of DNA or biolistic bombardment (Hochbaum *et al.* 2010; Katz 2015). Recently, innovative genome engineering techniques have been developed, such as MosSCI and CRISPR/Cas (Frokjaer-Jensen *et al.* 2014; Waaijers & Boxem 2014). These excellent experimental methods allow genome editing by the targeted insertion or deletion of DNA into defined locations of the worm's genome.

The enormous amount of data (especially the -omics data, **Section 1.4**) generated in thousands of *C. elegans* studies, is collected in well-organized online databases, further promoting the use of this model organism in the study of complex molecular biology (Table 1).

Table 1 Overview of online *C. elegans* resources.

Name database	URL	Information
WormBase	http://www.wormbase.org/	Central database of *C. elegans* biology
WormBook	http://www.wormbook.org/	Open collection of peer-reviewed chapters on *C. elegans* biology
WormAtlas	http://www.wormatlas.org/	Database of behavioral and structural anatomy of *C. elegans*
WormImage	http://www.wormimage.org/	Database on anatomy (tissues), collecting electron microscopy images of *C. elegans*
WORFDB	http://worfdb.dfci.harvard.edu/	ORFeome clones and associated data
Hope Laboratory Expression Pattern Database	http://worfdb.dfci.harvard.edu/promoteromedb/	Promoter::GFP fusion expression patterns
***C. elegans* Gene Knockout Consortium**	http://celeganskoconsortium.omrf.org/	Gene knockout strains
Caenorhabditis Genetics Center	http://cbs.umn.edu/cgc/	Collection of mutant strains
RNAiDB	http://www.rnai.org/	RNAi phenotypes
PhenoBank	http://www.worm.mpi-cbg.de/phenobank/cgi-bin/MenuPage.py	RNAi phenotypes and associated data (Sonnichsen *et al.* 2005)
Tissue-specific expression prediction for *C. elegans*	http://worm-tissue.princeton.edu	Prediction tool for tissue-specific expression of genes in *C. elegans* (Chikina *et al.* 2009)
OpenWorm Browser	http://browser.openworm.org/	Virtual anatomical model of *C. elegans*

Despite all these advantages, *C. elegans* has some limitations. First of all, its small size hampers the analysis of separate tissues. In addition, often large populations need to be cultured to perform molecular and biochemical experiments, in particular if subcellular organelles are studied. This worm lacks some defined organ types and their corresponding age-related diseases present in higher organisms, such as brain (Alzheimer's disease), heart (cardiovascular disease), lungs (lung disease/cancer), kidneys (declining renal function) and liver (fatty liver disease). In this vein, it is not clear to what extent conclusions can be drawn from a simple invertebrate model organism that diverged from other animals between 600 and 1300 million years ago (Gruber *et al.* 2015). Yet, the existence of universally conserved pathways challenge this drawback (Partridge & Gems 2002), however, it is important to clearly distinguish between conserved (public) and private aspects of aging (Partridge & Gems 2002).

In conclusion, despite a few drawbacks, *C. elegans* has a very large potential to push the frontiers of knowledge much further, making it a top model organism.

1.2.4 Aging phenotype in *C. elegans*

The age-related changes of *C. elegans* are well described, including the alteration in behavior, reproduction, morphology and macromolecules (Fig. 7) (Herndon *et al.* 2002; Collins *et al.* 2008; Gems & de la Guardia 2013).

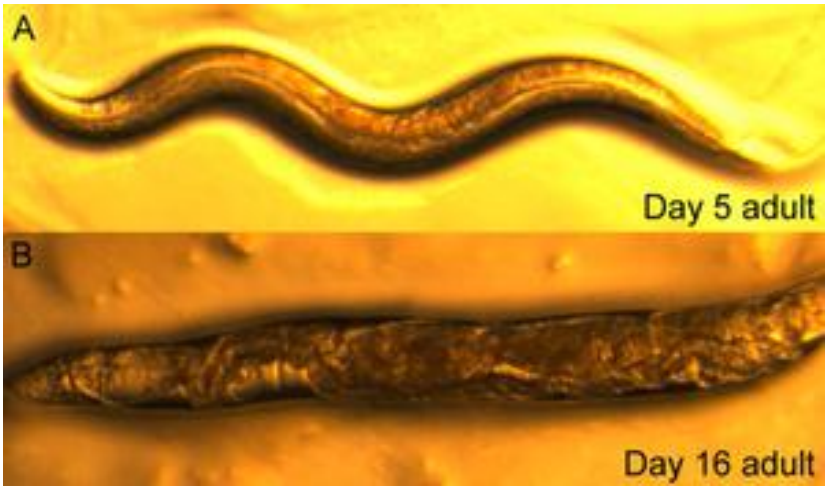


Fig. 7 The overall morphology of youthful day 5 *C. elegans* worms (A) and old day 16 worms (B). Aging in senescing worms is characterized by declines in motility and the ability to sense and respond to environmental stimuli (Kaletsky & Murphy 2010).

Aging *C. elegans* progressively lose muscle mass and function over time, mirroring human sarcopenia. Sarcomeres become disorganized and show less myosin thick filaments in old muscles. In addition, muscle cells tend to shrink with age due to progressive cytoplasmic loss. Muscle deterioration is also observed in the pharynx, which appearance becomes more irregular in old worms. Overall, loss of muscle function is reflected in a decreased locomotion and pharyngeal pumping with age. Remarkably, senescing worms show an intact nervous system at the cellular level. Although changes in biophysical properties (nerve conduction, synaptic transmission) cannot be excluded, no age-related neurodegeneration could be observed in old nematodes. Other tissues, such as hypodermis and intestine, can show age-related weakening of plasma membranes. Hence, these tissues can suffer from random disruptions that contribute to the death of an animal (Herndon *et al.* 2002).

Introduction

A number of pathologies associated with hyperfunction, particularly hypertrophy, are observed in aging worms. Age-associated atrophy is observed in the germline, while the uterus shows clear hypertrophy due to the development of uterine masses (tumors) (Gems & de la Guardia 2013). Additionally, old worms show accumulation of purposeless yolk throughout the body cavity, an aging phenotype that may well contribute to mortality (Herndon *et al.* 2002; Gems & de la Guardia 2013). Another example of hypertrophy is the striking thickening of the cuticle with age. The cells of the underlying hypodermis continuously synthesize cuticle collagens, which results in a cuticle up to 10 times thicker in old worms compared to youthful animals (Herndon *et al.* 2002; Gems & de la Guardia 2013).

Most apparent changes at the biochemical level are the increase in single-strand DNA breaks and carbonylated proteins (Klass *et al.* 1983; Adachi *et al.* 1998; Yasuda *et al.* 1999). The decline in oxygen consumption, heat production and ATP levels indicate an exponential decrease of metabolic activity in aging worms (Braeckman *et al.* 2000; Braeckman *et al.* 2002a).

1.3 Protein homeostasis and aging in *C. elegans*

The proteome is the entire pool of proteins expressed by an organism at a given time. The cellular proteome is highly dynamic and maintained by quality control pathways to fine-tune its overall integrity and balance, a process called protein homeostasis or proteostasis. Protein turnover is a major mechanism to (i) allow rapid adaptation to changing extracellular conditions, (ii) to renew proteins in order to preserve their functionality and to cope with intracellular damage (Morimoto & Cuervo 2009). The main components of the protein quality control (PQC) pathways are the chaperones and proteolytic systems, which are responsible for proteome surveillance (identify altered proteins), protein repair (if damage is reversible) and protein degradation (if repair is not possible) **(Section 1.3.1)** (Koga *et al.* 2011).

Many studies demonstrate the loss of proteostasis with age (Morimoto & Cuervo 2009; Koga *et al.* 2011; Taylor & Dillin 2011; Lopez-Otin *et al.* 2013). Two main factors may contribute to the age-related proteome changes: increased intracellular proteotoxicity due to altered metabolism in aging organisms and the progressive decline of PQC systems (Koga *et al.* 2011). Additionally, it has been shown that proteostasis mechanisms play an important role in long-lived mutants, as their proper function is required for the increase of lifespan (Hsu *et al.* 2003; Morley & Morimoto 2004; Wadhwa *et al.* 2005) **(Section 1.3.3)**. Failures of these PQC systems are often associated with severe human diseases, known as protein conformational disorders or aggregopathies (Morimoto 2008).

1.3.1 Lifecycle of a protein

The coordinated action of three cellular machineries - translation, folding or assembly and clearance - is responsible for proteome maintenance and renewal under normal conditions. The interacting activities of the proteostasis regulators face the acute and chronic challenges to manipulate the concentration, conformation, binding interactions (quaternary structure) and allocation of proteins (Balch *et al.* 2008). The life-cycle of a protein starts with the copying of a specific section of DNA (encoding a gene) into messenger RNA (mRNA) by RNA polymerase enzymes, a process called transcription (Fig. 8) (Brown & Brown 2003).

Introduction

Translation of mRNA, either in the cytosol or endoplasmic reticulum (ER), supplies cells with newly synthesized proteins. Most cytosolic proteins fold spontaneously after translation, however, proper folding is often achieved by the interaction with chaperones which provide a favorable folding environment. In the ER, proteins (mostly destined for secretion) reach their folding state upon interaction with ER specific chaperones. Additionally, partial or complete unfolding/refolding is often necessary to translocate proteins across organelle membranes (Koga *et al.* 2011). Chaperones also act as a surveillance mechanism to safeguard proteome integrity, preventing the accumulation of faulty proteins caused by molecular damage, mostly oxidative, and undesired posttranslational modifications (Hipp *et al.* 2014). Irreversibly damaged proteins are targeted for degradation via intracellular proteolytic systems, including the proteasomal and autophagosomal degradation pathways. However, if repair is possible, chaperones assist misfolded proteins to reacquire a stable conformational state. Folding and unfolding events cause the exposure of hydrophobic areas of proteins normally hidden from other cellular components. Conformational changes would have no negative effects in absence of other protein interactions. Yet, the high protein density in the cytosol and lumen of organelles likely cause accidental contact with these proaggregation regions, which is mostly prevented by chaperones (True 2006). Nevertheless, if chaperones do not intervene, protein aggregates (due to self-association and interaction with nearby proteins) will be formed (Balch *et al.* 2008). Protein aggregates often act as cellular sinks for proteins, as well as for molecular chaperones as they bind these protein aggregates in an attempt to prevent further growth (Ravikumar *et al.* 2002). Degradation through the autophagic system can occur according to the subcellular location of these aggregates. For example, protein aggregates in the nucleus usually persist whereas cytosolic aggregates are amenable to degradation (Tanaka *et al.* 2004). In mitochondria and ER, organelle-specific responses arise upon protein unfolding in their lumen (unfolded protein response or UPR), thereby increasing the level of chaperones to facilitate refolding (Koga *et al.* 2011). The balance within the proteostasis network is crucial for proper cellular functioning and provides a reason why any intervention that causes an imbalance often has a major impact on cellular homeostasis (Morimoto & Cuervo 2009).

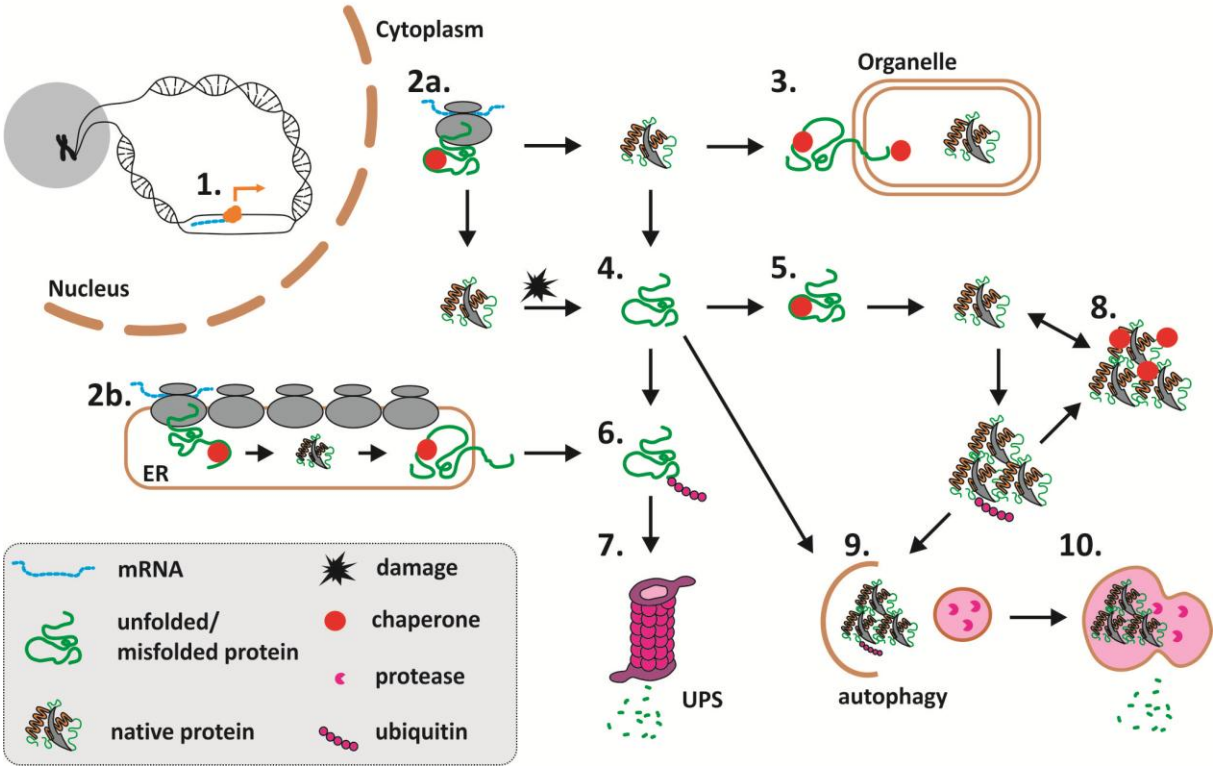


Fig. 8 Life-cycle of proteins. After transcription of DNA to mRNA (1), translation can occur either in the cytosol (2a) or endoplasmic reticulum (ER) (2b) where chaperones may assist in protein folding. Intervention of chaperones is necessary to allow intracellular protein trafficking and passage across organelle membranes (3). Chaperones can assist in the remodeling of misfolded proteins (5), however, irreversible damaged proteins will be destined for degradation by the ubiquitin-proteasome system (UPS) (6,7) or autophagy (9). Protein alterations can lead to the formation of proteotoxic aggregates (8), which are bound by chaperones in an attempt to prevent further growth. This intervention may lead to disaggregation, however, these aggregates are likely sequestered into autophagosomes (9) and degraded in autolysosomes (10).

1.3.2 Mechanisms of lifespan extension in *C. elegans*

Lifespan extending mechanisms have been associated with stress and nutrient sensing pathways. When conditions are favorable, anabolic processes are promoted for growth and reproduction. However, stressful conditions and periods of food scarcity triggers a physiological shift towards cell maintenance and protection. The insulin/IGF-1 (IIS) signaling pathway and some other important longevity-promoting pathways involved in stress and nutrient sensing in *C. elegans* will be introduced.

The insulin/IGF-1 signaling pathway

The insulin/IGF-1 signaling (IIS) cascade was the first pathway shown to influence aging in *C. elegans* (Kenyon 2010). Reduced activity of IIS extends lifespan in many organisms, both invertebrate and vertebrate species (Bartke 2008; Kenyon 2010) and correlates with increased lifespan in humans (Suh *et al.* 2008; Flachsbart *et al.* 2009), implying the conserved effects of this pathway on aging (Kenyon 2010). In *C. elegans*, it was found that lifespan was doubled upon mutation in the *daf-2*, ortholog of the mammalian tyrosine kinase insulin/IGF-1 receptor (InR) (Kenyon *et al.* 1993). Mutation in *age-1*, its downstream phosphatidiylinositol 3-kinase (PI3K), extends lifespan with 60% (Friedman & Johnson 1988). All mutations in the IIS cascade rely on a functional Forkhead (FOXO) transcription factor DAF-16 to induce longevity (Kenyon *et al.* 1993; Larsen *et al.* 1995; Murakami & Johnson 1996; Lin *et al.* 1997; Ogg *et al.* 1997), which strongly demonstrates the genetic propensity of senescence. This idea launched the extensive unraveling of the aging process at the genetic level in many model organisms, including *C. elegans*, resulting in the characterization of most of the IIS components (Fig. 9) (Kenyon 2010).

C. elegans expresses approximately 40 insulin-like peptides, at least two of which act as agonists (DAF-28 and INS-7) and at least one operates as an antagonist (INS-1) of DAF-2 (Malone *et al.* 1996; Pierce *et al.* 2001; Li *et al.* 2003). Upon ligand binding, the DAF-2 receptor can initiate a phosphorylation cascade, thereby preventing the nuclear localization of DAF-16 (Fig. 9a).

Reduced IIS, for example due to mutation in *daf-2*, facilitates the accumulation of DAF-16 in the nucleus, which regulates downstream genes that extend lifespan and increase stress resistance (Kenyon *et al.* 1993). Once in the nucleus, multiple co-regulators converge on DAF-16 to modulate its activity in long-lived insulin/IGF-1 pathway mutants (Fig. 9b) (Lapierre & Hansen 2012).

The subcellular localization of DAF-16 co-regulator HSF-1 (heat-shock factor-1) is negatively regulated by the formation of a DDL-1 containing HSF-1 inhibitory complex (DHIC), consisting of HSF-1, HSB-1, DDL-1 and DDL-2. IIS signaling promotes the repression by this complex, whereas reduced IIS activity disrupts DHIC formation and enhances HSF-1 activity for stress resistance, including the up-regulation of heat-shock proteins involved in proteostasis (Hsu

et al. 2003; Li *et al.* 2008; Chiang *et al.* 2012). The transcription factor Skinhead SKN-1 also interplays with DAF-16 to regulate oxidative stress and expression of detoxification genes under reduced IIS, although it extends lifespan independently of DAF-16 (Tullet *et al.* 2008). The subcellular localization of SKN-1 is regulated by phosphorylation via the evolutionary conserved p38 mitogen-activated protein kinase (MAPK) cascade (Inoue *et al.* 2005). DAF-16 nuclear localization can also be regulated by an intestinal ankyrin-repeat protein KRI-1, orthologous to the human disease gene KRIT1/CCM1. This protein plays an instructive role in promoting longevity by reflecting the status of the germline (Berman & Kenyon 2006). Another longevity regulator is HCF-1 (host cell factor 1), which shows a ubiquitous nuclear localization and physically associates with nuclear DAF-16. This negative regulator limits DAF-16 to access specific target genes, whereas inactivation of HCF-1 causes DAF-16-dependent longevity (Li *et al.* 2008). The genetic interaction of DAF-16 with SMK-1 (suppressor of MEK) leads to regulation of genes specific for the protection against oxidative stress, UV stress and microbial infections (Wolff *et al.* 2006).

IIS pathway mutants accumulate DAF-16 in the nuclei of many cell types to modulate a wide variety of metabolic changes and stress responses, promoting longevity (Lee *et al.* 2003; McElwee *et al.* 2003; Murphy *et al.* 2003). The IIS pathway regulates reproduction, lipid metabolism, dauer formation and aging independently of each other (Kenyon *et al.* 1993; Larsen *et al.* 1995; Kimura *et al.* 1997; Riddle & Albert 1997; Gems *et al.* 1998; Dillin *et al.* 2002), and these processes may require tight regulation between different tissues. Indeed, it was shown that DAF-2 acts cell non-autonomously and coordinates expression of downstream longevity genes in different tissues via cross-communication and feed-back regulation, thereby specifying the rate of aging of the animal as a whole (Apfeld & Kenyon 1998; Wolkow *et al.* 2000; Libina *et al.* 2003).

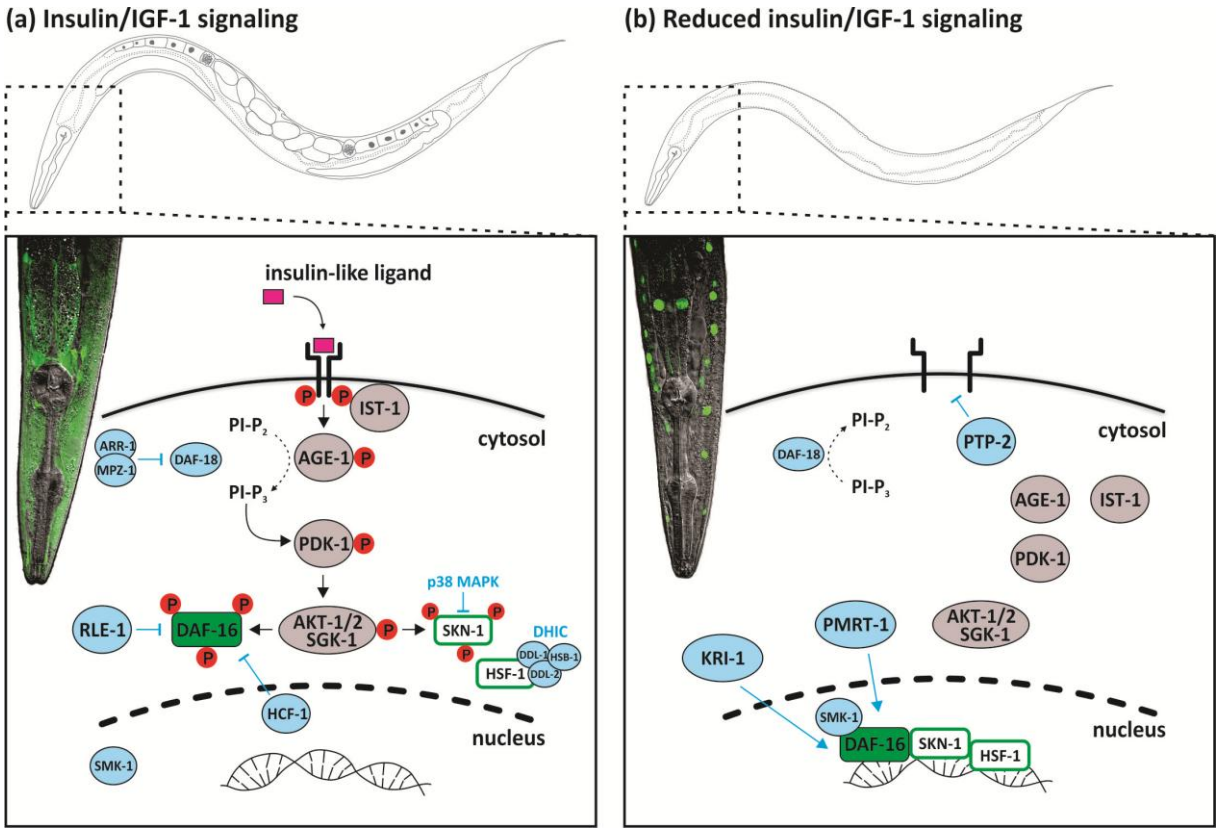


Fig. 9 Overview of insulin/IGF-1 signaling in *C. elegans.* (a) Ligand binding to DAF-2 receptor activates its tyrosine kinase activity due to autophosphorylation and dimerization and recruits AGE-1 (p110 catalytic subunit of phosphatilylinositol-3 kinase) and IST-1 (Insulin Receptor Substrate 1 orthologue). Activated AGE-1 drives the phosphorylation of phosphatidylinositol-4,5-diphosphate (PI-P_2) into phosphatidylinositol-3,4,5-triphosphate (PI-P_3). Increased levels of PI-P_3 activate PDK-1, an Akt/PKB kinase homolog, which in turn phosphorylates and activates the serine/threonine kinases AKT-1 and AKT-2 and the mammalian Serum-and Glucocorticoid-inducible Kinase orthologue SGK-1. Ultimately, AKT-1/2 and SGK-1 inactivate the Forkhead (FOXO) transcription factor DAF-16 by phosphorylation, preventing its entry into the nucleus. (b) Reduced insulin/IGF-1 signaling relieves this block and allows the translocation of DAF-16 to the nucleus, where it can regulate gene expression and longevity promoting programs. (a & b) Other mechanisms of DAF-16 regulation are indicated in blue and include (I) RLE-1, an E3 ubiquitin ligase that catalyzes ubiquitination of DAF-16 and targets it for degradation; (II) PTP-2 (Protein Tyrosine Phosphatase-2) inactivates IIS; (III) DAF-18 (PTEN) dephosphorylates and inhibits AGE-1 under reduced IIS (opposed action of AGE-1, driving the conversion from PI-P_3 to PI-P_2), but itself is inhibited by ARR-1 (human arrestin homolog) and MPZ-1 (multiple-PDZ domain containing protein); (IV) nuclear maintenance of DAF-16 is promoted by PRMT-1 (arginine methyltransferase). Additionally, transcription co-regulators modulate the DAF-16 activity and independently translocate to the nucleus. Nuclear SMK-1 (suppressor of MEK) physically interacts with DAF-16 and enhances it activities, whereas HCF-1 (host cell factor 1) negatively regulates DAF-16 access to its target genes. Lifespan is independently extended by transcription factor SKN-1, which is also activated by the IIS kinases AKT-1/2 and SGK-1. Furthermore, its subcellular location is controlled by the p38 MAPK pathway. Transcription of proteostasis-related proteins occurs in cooperation with HSF-1 (heat-shock factor-1), for which subcellular localization is controlled via the repression complex DHIC. Reviewed in (Lapierre & Hansen 2012). Confocal images indicate the subcellular localization of DAF-16::GFP under normal conditions (a) and stress conditions (b).

Other energy sensors: TOR, AAK-2 and SIR-2.1

The Target of rapamycin (TOR) is a conserved nutrient-sensing molecule and central regulator of cellular growth and metabolism in response to nutrients, growth cues and cellular status. Favorable growth conditions will activate TOR, which promotes anabolic processes including maintained ribosome biogenesis, translation initiation and nutrient import. In contrast, reduced TOR activity, due to nutrient limitation, dramatically down-regulates protein synthesis and up-regulates catabolic processes such as macroautophagy (Wullschleger *et al.* 2006). The TOR pathway, together with the IIS pathway, are essential for development, growth and survival early in life. These biological processes are tightly programmed, whereas aging and diseases are suggested to be aimless continuations of these programs that were not switched off upon their completion (Blagosklonny 2008). In this vein, Blagosklonny suggested that the TOR pathway is a pro-aging pathway. First, TOR deficiency extends lifespan in diverse organisms such as yeast, worms and flies (Vellai *et al.* 2003; Kapahi *et al.* 2004; Kaeberlein *et al.* 2005). Secondly, TOR-driven cellular hyperfunctions lead to cancer and other age-related diseases. Thirdly, administration of rapamycin, a pharmacological inhibitor of TOR, extends lifespan in all species tested and prevents age-related diseases. Fourthly, TOR is inhibited by dietary restriction, which extends lifespan (Blagosklonny 2010a).

The conserved energy-sensing enzyme AMP-activated kinase (AMPK) regulates energy metabolism, which plays a central role for the determination of lifespan. AMPK is activated via an allosteric mechanism by increases in intracellular AMP:ATP ratio, a measure of low energy levels (Hardie & Hawley 2001). In response, activated AMPK switches on catabolic pathways that generate ATP while switching off anabolic pathways that consume ATP, including biosynthesis of lipids, carbohydrates, proteins and ribosome biogenesis. This is achieved by the specific regulation of directly involved pathways, for example via suppression of TOR activity (Hardie *et al.* 2012). AMPK is a heterotrimeric complex that consists of two regulatory subunits (β,γ) and one catalytic α subunit with kinase activity. The *C. elegans* AMPK catalytic α subunit AAK-2 is found to couple lifespan to the energy state of the animal. Environmental stressors that lowers energy levels promote AAK-2 activity, which lowers fertility and underpin longevity. Additionally, increased AMP:ATP ratio is observed in

mutants with reduced IIS, which causes lifespan extension in an *aak-2*-dependent manner (Apfeld *et al.* 2004).

Yeast Sir2 (Silent information regulator 2) is a NAD-dependent protein deacetylase of the sirtuin family and a conserved regulator of longevity (Bordone & Guarente 2005). Sir2 regulates transcription via chromatin silencing and determines the lifespan of yeast mother cells (Kaeberlein *et al.* 1999). In *C. elegans*, SIR-2.1, the closest homolog to yeast Sir2, monitors the cell's metabolic state via alterations in NAD:NADH ratio (Greer *et al.* 2007). Lifespan extension, due to SIR-2.1 overexpression, depends on the transcription factor DAF-16. This observation suggests that SIR-2.1 acts in a parallel signaling cascade with the IIS pathway, which converges to DAF-16 (Tissenbaum & Guarente 2001). SIR-2.1 has been suggested to be a strong candidate to regulate dietary restriction due to its function as metabolic sensor (Guarente 2005). However, its effect on dietary restriction induced longevity is still subject to debate (Kaeberlein *et al.* 2006; Lee *et al.* 2006; Greer *et al.* 2007; Hansen *et al.* 2007; Honjoh *et al.* 2009).

Lifespan extension by dietary restriction

Dietary restriction (DR) or the reduction of nutrients without malnutrition, is a common non-genetic method to extend lifespan in organisms as diverse as yeast, nematodes, flies and rodents (McCay 1935; Lakowski & Hekimi 1998; Longo & Finch 2003). A study in rhesus monkeys showed that moderate DR results in the delayed onset of age-related diseases, such as diabetes, cancer, cardiovascular disease and brain atrophy (Colman *et al.* 2009; Colman *et al.* 2014). Although the study is ongoing, results show the significant effect of dietary restriction in increasing survival (Fig. 10) (Colman *et al.* 2014). The observed effects are similar to the results shown in rodent and invertebrate studies, illustrating the conserved effect on DR on health and survival (McDonald & Ramsey 2010).

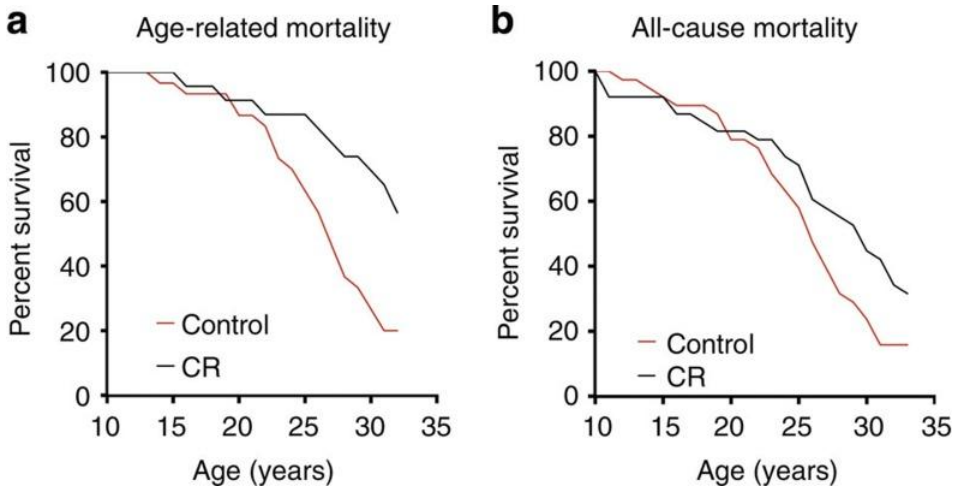


Fig. 10 Mortality curves of dietary restricted rhesus monkeys show a statistically significant effect of caloric restriction (CR=DR) in lifespan extension. Animals were given a moderate long-term ~30% restricted diet since young adults. (a) Age-related mortality: exclusion of animals that died from non-age-related causes. Of the original 76 monkeys, 63% (24/38) of the control animals died compared with only 26% (10/38) of the DR group. (b) All-cause mortality: data for all animals, not censored for cause of death (Colman *et al.* 2014).

Various DR regimens exist to extend lifespan in *C. elegans*, such as bacterial dilution (Klass 1977; Greer *et al.* 2007), peptone dilution (Hosono *et al.* 1989), dietary deprivation (Kaeberlein *et al.* 2006), intermittent fasten (Honjoh *et al.* 2009) and axenic (without bacteria) dietary restriction (Vanfleteren 1976). Different DR methods evoke different genetic pathways. This might be the consequence of the fact that different sources of calories, nutrients such as carbohydrates, amino acids or lipids, may be more limiting than others depending on the DR method. In addition, the involvement of specific nutrient sensing pathways may depend on the timing of DR-initiation, as well as tissue specificity since different DR regimens may be sensed by the intestine and others by neurons. Collectively, these observations suggest that different signaling pathways, including the energy sensing pathways IIS, TOR, AAK-2 and SIR-2.1, act together in a complex regulatory network to mediate longevity induced by dietary manipulations (Greer & Brunet 2011).

1.3.3 Alterations of proteostasis by reduced insulin/IGF-1 signaling

(1) Protein synthesis as key regulator of proteome balance

Protein synthesis allows the continuous renewal of proteins, therefore it can be seen as a major regulator in proteostasis and assumably a key node of interaction with aging-related

pathways. Surprisingly, it was found that attenuation of protein synthesis extends, rather than shortens, lifespan. For example, translation is reduced in long-lived Snell and Ames dwarf mice due to lower IIS (Hsieh & Papaconstantinou 2004; Sharp & Bartke 2005). In yeast, replicative lifespan is increased upon deletion or inhibition of ribosomal levels (Chiocchetti *et al.* 2007; Steffen *et al.* 2008) and reduction of translation initiation factors and ribosomal proteins increases lifespan in *C. elegans* (Kaeberlein et al. 2005; Hansen et al. 2007; Pan et al. 2007; Syntichaki et al. 2007). Taken together, it seems that lowering translation is a profound target for longevity pathways, including IIS. Quantitative proteomic studies confirm this idea (Depuydt et al. 2013; Stout et al. 2013) and similar findings has been reported for dietary restricted mice (Price et al. 2012). Recently, it was found that a long noncoding RNA (lncRNA), transcribed telomeric sequence 1 (*tts-1*), is highly expressed in *daf-2* IIS mutants and possibly regulates the translation of ribosomal protein mRNA (Essers *et al.* 2015). Since the expression levels of ribosomal protein mRNAs is actually higher in *daf-2* mutants compared with control animals, it can be suggested that *tts-1* lncRNA acts as a specific block to control ribosomal levels necessary for lifespan extension (Essers *et al.* 2015).

Still, the mechanistic importance of manipulating the translation machinery remains unclear. One possibility might be that lowered protein translation diminishes the load on the remainder of the proteostasis network, allowing more efficient protein folding and degradation, for example, and therefore reducing the load of aberrant and misfolded proteins (Taylor & Dillin 2011). A similar explanation might be given by the fact that IIS drives anabolic processes. Previously, it was suggested that over-activity of the IIS pathway, and its associated TOR kinase pathway, causes hypertrophy due to an excess of biosynthesis resulting in age-related pathologies later in life (Blagosklonny 2006; Blagosklonny 2008; Gems & de la Guardia 2013). In this vein, reduced IIS can promote longevity due to a better regulation of protein biomass within cells (Gems & de la Guardia 2013).

(2) Protein clearance mechanisms of the proteostasis network

Autophagy

Autophagy is an evolutionary conserved catabolic process and quality control mechanism in which cytosolic components and organelles are degraded through the lysosome to maintain

proteome integrity (He & Klionsky 2009; Vilchez *et al.* 2014). With age, lysosomal function and autophagy decrease (Terman 1995), which drives the accumulation of biological garbage (Terman *et al.* 2007). Nevertheless, the underlying mechanisms responsible for this decline remain unclear.

Autophagy plays a key role in the longevity phenotype of reduced IIS mutants, as well as in other long-lived *C. elegans* strains, such as dietary restricted worms, reduced TOR and germline-lacking mutant worms (Melendez *et al.* 2003; Gelino & Hansen 2012). Autophagy-dependent lifespan extension is independent of the DAF-16 transcription factor, suggesting that increased autophagy is not sufficient for the longevity phenotype observed in *daf-2* IIS mutants (Hansen *et al.* 2008). Intriguingly, recent data indicates a global down-regulation of bulk degradation in *daf-2*, opposing the idea of increased protein turnover in long-lived mutants (Depuydt et al., submitted). Moreover, it has been suggested that autophagy is not always beneficial to longevity. Suppression of seven autophagy genes reduces lifespan in N2, while inhibition of these genes in *daf-2* mutants further extends lifespan (Hashimoto *et al.* 2009). Therefore, autophagy might be critical for longevity, yet in a limited temporal and/or spatial manner.

Mitophagy, a selective type of autophagy targeting mitochondria for degradation, has been recently shown to play an essential role in aging and longevity (Palikaras *et al.* 2015a). The mitochondrial phosphatase and tensin (PTEN)-induced kinase 1 (nematode PINK-1 homologue) and the cytosolic E3 ubiquitin ligase Parkin (nematode PDR-1 homologue) are two conserved key components of mitophagy. First, PINK-1 phosphorlylates and recruits PDR-1 to damaged and dysfunctional mitochondria. Subsequently, PDR-1 ubiquitinates outer membrane proteins, such as the mitochondrial fusion protein FZO-1 (Mitofusin MFN1/2) and VDAC-1 (voltage-dependent anion channel 1), which signals the recruitment of the autophagic machinery (Pickrell & Youle 2015). DCT-1, a putative orthologue to the mammalian NIX/BNIP3L and BNIP3, resides on the outer mitochondrial membrane and plays a key function in the overall regulation of mitophagy. It acts as a mitophagy receptor by its interaction with the autophagosomal membrane protein LGG-1 (human LC3 homologue) to induce autophagic degradation of the targeted mitochondria (Palikaras *et al.* 2015a; Pickrell & Youle 2015). Transcription factors DAF-16 and SKN-1 control expression of DCT-1 upon low IIS and oxidative stress, respectively (Fig. 11). This coordination allows the continuous

adaption of mitochondrial content upon changing environmental conditions. The age-related decline of mitophagy might explain the uphold of mitochondrial homeostasis, driving the aging process (Palikaras *et al.* 2015a).

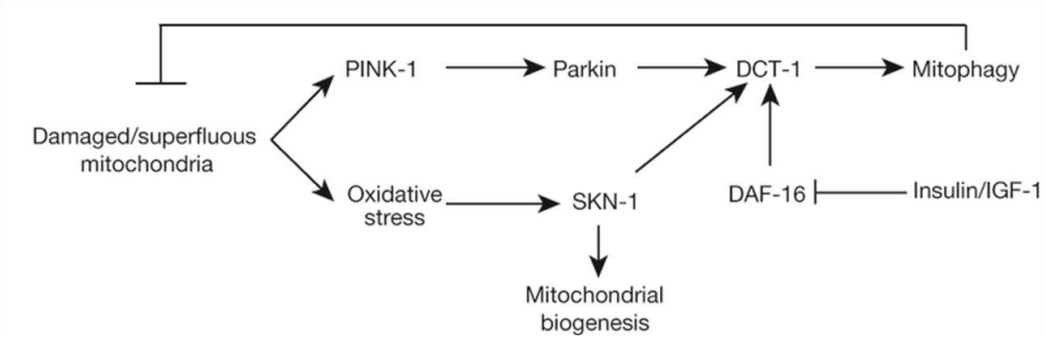


Fig.11 The mitochondrial pool is maintained via basal-level mitophagy under normal conditions. Mitophagy is triggered, depending on the metabolic state (low IIS) or stress conditions (oxidative stress) to promote cell survival (Palikaras *et al.* 2015a).

Proteasomal degradation

The ubiquitin-proteasome system (UPS) is the primary selective protein degradation system in eukaryotes (Schmidt & Finley 2014). The proteasome is a key component of the proteostasis network to handle the removal of misfolded and damaged proteins. Via ubiquitin conjugation, specific proteins are marked and recognized by the proteasome for degradation (Vilchez *et al.* 2014). The proteasome consists of a barrel-like core structure (20S) and two outer rings that control substrate entry into the catalytic cavity. Activation of 20S particles is necessary upon degradation of polyubiquitylated proteins, although free inactive 20S cores have a detectible activity independent of ATP/ubiquitination. The latter could play a crucial role in the degradation of oxidized proteins, whereby oxidative modifications triggers proteolysis by 20S (Jung *et al.* 2013; Schmidt & Finley 2014). Elimination through proteolysis is crucial, as obsolete proteins can compete with functional proteins for binding partners and they have a high propensity to associate with toxic aggregates (Goldberg 2003). Proteasomal degradation is also responsible for the maintenance of proper concentrations of many regulatory proteins, thereby playing a prominent role in cell signaling. Indeed, even components of aging signaling pathways can be targeted for breakdown by specific E3 ligases, such as VHL-1 (affecting the HIF-1

transcription factor), RLE-1 (degrading the DAF-16 transcription factor) and WWP-3 (role in dietary restriction induced longevity) (Li *et al.* 2007; Carrano *et al.* 2009; Mehta *et al.* 2009).

With age, an increase in proteasomal subunits is observed, likely representing an attempt to cope with aberrant macromolecules (Walther *et al.* 2015). It has not been proven that this up-regulation is effective *in vivo,* as the overall proteasomal function is thought to decline with age (Hipp *et al.* 2014). Nevertheless, conflicting studies have reported both increases and decreases of proteasomal activity with age (Gaczynska *et al.* 2001; Carrard *et al.* 2002; Gray *et al.* 2003; Ferrington *et al.* 2005; Zeng *et al.* 2005; Vernace *et al.* 2007; Yun *et al.* 2008). It is even more complicated as temporal and tissue-specific proteolytic activity can occur within the same organism (Vernace *et al.* 2007). It seems that the proteasome is not equally affected in different cell types, tissues and even whole organisms. Cell-specific photoconvertible reporters can address this question by measuring proteasomal activity in real time in living animals. A study in *C. elegans*, using this approach, has demonstrated a stronger decline in proteasome activity in dorsorectal neurons with age compared to body wall muscle cells (Hamer *et al.* 2010).

Several reports suggest a strong correlation between proteasome activity and lifespan. Intriguingly, long-lived humans (centenarians) show increased proteasome activity (Chondrogianni *et al.* 2000). Similar observations were made in long-lived animals such as the naked mole rat and the giant clam (Perez *et al.* 2009; Ungvari *et al.* 2013). Worms lacking essential components of the proteasome show reduced proteotoxic stress resistance and are short-lived (Ghazi *et al.* 2007; Yun *et al.* 2008). On the other hand, longevity-promoting signaling pathways increase proteome stability delaying the onset of age-related aggregopathies. Long-lived IIS mutant worms show increased proteasome activity, achieved via the DAF-16 dependent repression of *ubh-4*, a proteasome-associated deubiquitinating enzyme which probably functions as a tissue-specific proteasome inhibitor (Matilainen *et al.* 2013). Similarly, long-lived germline deficient *glp-1* mutants and dietary restricted worms, display elevated somatic proteasome activity, likely to manage proteotoxic stressors (Vilchez *et al.* 2012). Recently, enhancement of 20S core proteasome content and activation was shown to extend lifespan and to increase stress resistance in *C. elegans*. Lifespan promoting effects of this 20S up-regulation might be a potential anti-aging strategy to minimize proteostasis deficiencies (Chondrogianni *et al.* 2015).

(3) Chaperones: mediators of proteome stability

Cells express a broad collection of chaperones that contribute to the maintenance of the proteome under basal conditions. Several families exist and are named according to their molecular weight (Hsp100, Hsp70, Hsp60 and small heat shock proteins (sHSP's)). Constitutive as well as inducible chaperones are present in each family and they may localize to different subcellular compartments (Koga *et al.* 2011).

Proteins heavily rely on chaperone-dependent functions throughout their lifetime, as chaperones constantly help to maintain and regain their biologically functional conformations (Balch *et al.* 2008; Kim *et al.* 2013). Chaperones serve as sensors of alterations in protein quality and possess the ability to refold aberrant proteins and dissociate protein aggregates. A core task of sHSP's is to actively prevent protein aggregation, a process in which they buffer aggregation by binding hydrophobic regions of non-native protein species (Hipp *et al.* 2014).

The relationship between chaperones and lifespan is well-reported for many uni- and multicellular species (Lithgow *et al.* 1995; Shama *et al.* 1998; Kurapati *et al.* 2000; Walker *et al.* 2001). For example, long-lived *Daphnia pulicaria* shows constitutive induction of Hsp70 expression, which was not observed for the short-lived ecotype (Schumpert *et al.* 2014).

Lifespan extension in *daf-2* mutants requires the activation of both DAF-16 and HSF-1 transcription factors. The latter is responsible for the expression of multiple heat-shock proteins, including sHSP's (Hsu *et al.* 2003; Morley & Morimoto 2004). A recent study has indicated the role of sHSP's in the active sequestration of potentially toxic proteins into insoluble less-toxic aggregates in *daf-2* worms (Walther *et al.* 2015). Additionally, these mutants show increased levels of trehalose. It was shown that this chemical chaperone modulates proteome stability, improving overall proteostasis in this long-lived IIS mutant (Depuydt *et al*., submitted).

1.4 Study of protein turnover

1.4.1 Methods to study protein turnover

Since the proteome represents the complex functionality of a system, its analysis is crucial to obtain a comprehensive understanding of biological phenomena, including the aging process. It has been recognized that proteins in the cell are not static and indefatigable, but are in a dynamic state of turnover, a process in which they are continually degraded and renewed. The imbalance between protein synthesis and degradation can adapt the expressed protein profile in the cell upon external stimuli, developmental programs or the onset of a different physiological state, such as disease (Hinkson & Elias 2011). Hence, the information on intracellular protein concentrations is insufficient and insight on how cells adjust their proteomes is necessary to get a broader view on macromolecular expression under particular biological states.

In the late 1930's, the advent of isotopically enriched tracers initiated the quantitative analysis of protein turnover (Schoenheimer *et al.* 1938). Later, global proteome dynamics could be monitored using scintillation counting-based detection of radioactively labeled rat proteins (Arias *et al.* 1969). In the following decade, numerous studies on protein turnover in aging organisms followed using the same approach (Fig. 12a) (Zeelon *et al.* 1973; Prasanna & Lane 1977; Prasanna & Lane 1979; Sharma *et al.* 1979; Sarkis *et al.* 1988). Two-dimensional gel electrophoresis (2DE) in combination with antibody-based detection provided the capability to analyze turnover rates of isolated immuno-precipitated proteins of interest (Fig. 12b) (Larrabee *et al.* 1980; Mosteller *et al.* 1980). Another approach, which does not require radiolabeling, involves the chemical inhibition of translation using cycloheximide. By blocking protein synthesis, degradation rates of specific proteins can be measured using Western Blot (Obrig *et al.* 1971). However, this method limits high-throughput analysis and results can be inaccurate and highly variable. Moreover, inhibiting protein synthesis may indirectly affect degradation rates.

Nowadays modern proteomics have the true power to perform proteome-wide analysis of protein turnover rates at the resolution of individual proteins. The combination of Stable Isotope Labeling by Amino acids in Cell culture (SILAC) and accurate mass spectrometry technology has enabled global analysis of protein turnover in single-cells such as yeast (Pratt

et al. 2002b; Helbig *et al.* 2011), bacteria (Cargile et al. 2004; Trotschel et al. 2013) and mammalian cells (Andersen et al. 2005; Schwanhausser et al. 2009; Cambridge et al. 2011; Boisvert et al. 2012; Larance et al. 2013). The last decade has witnessed a stark increase in the number of studies of whole-organism proteome dynamics in complex organisms using the same approach (Fig. 12c) (Doherty et al. 2005; Price et al. 2010; Westman-Brinkmalm et al. 2011; Claydon & Beynon 2012; Savas et al. 2012; Reckow & Webhofer 2014).

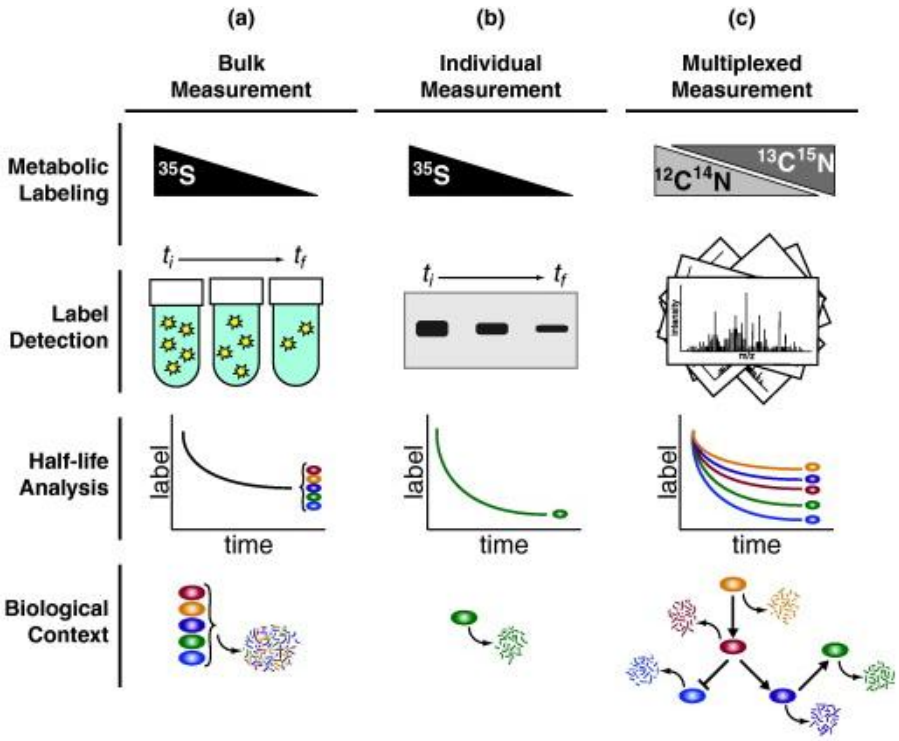


Fig. 12 Different approaches to measure turnover of metabolically labeled proteins. (a) A classical experiment in which all proteins are labeled with a radioactive element (e.g. ^{35}S). Scintillation counting enables to monitor the loss of radioactivity over time, which corresponds to bulk protein degradation. (b) All proteins are labeled with a radioactive element (e.g. ^{35}S). Biochemical purification of specific proteins combined with autoradiography can reveal turnover rates for only the assessed proteins. (c) Nearly all proteins (±96%) exist in the natural $^{12}C^{14}N$ form. Newly synthesized proteins will incorporate the stable isotopic $^{13}C^{15}N$ label during the pulse period. Using accurate mass spectrometry, loss of $^{12}C^{14}N$ (representing protein degradation) and accumulation of $^{13}C^{15}N$ (representing protein synthesis), can yield information on protein turnover dynamics of all detected proteins in a high-throughput manner (Hinkson & Elias 2011).

1.4.2 Protein turnover analysis in *C. elegans* using SILeNCe

Using *C. elegans* as a model organism in proteomics studies has some main advantages: age-synchronized worms, identical number of cells, no influence of sex in hermaphrodite cultures and strict control over feeding conditions keep the experimental variation very low compared to analyses with more complex organisms (Van Assche *et al.* 2015). This is reflected in the number of proteome studies exploring diverse aspects of *C. elegans* biology, yet protein turnover analyses remain virtually absent. To our knowledge, only few studies have been published using SILAC (strategy which involves the incorporation of the pre-labeled heavy amino acids, such as ($^{13}C_6$)-lysine or ($^{13}C_6$)-arginine, into proteins) to monitor protein turnover in *C. elegans* (Vukoti *et al.* 2015; Visscher *et al.* submitted). Vukoti *et al.* (2015) studied protein synthesis in old worms and during adult aging, while Visscher *et al.* (submitted) analyzed protein turnover in long-lived worms and models of age-related disease.

Another labeling strategy involves the uniform labeling of heavy nitrogen (^{15}N) in proteins and this metabolic labeling method has been shown to be efficient in *C. elegans* (Krijgsveld *et al.* 2003; Tops *et al.* 2010; Geillinger *et al.* 2012). Recently, this strategy was used to quantify the replenishment rates of membrane phospholipids (Dancy *et al.* 2015) and to investigate food intake (Gomez-Amaro *et al.* 2015). In this thesis, we applied ^{15}N-labeling to estimate individual protein half-lives in *C. elegans*, which we now designate Stable Isotope Labeling by Nitrogen in *Caenorhabditis elegans* or SILeNCe.

The SILeNCe strategy involves the complete ^{15}N-isotopic labeling of *E. coli* bacteria, the food source of laboratory cultured *C. elegans.* Mass spectrometry analysis is used to quantify the mass shifts and isotopomer distributions for a series of timed samples, in order to estimate individual protein turnover rates (Fig. 13).

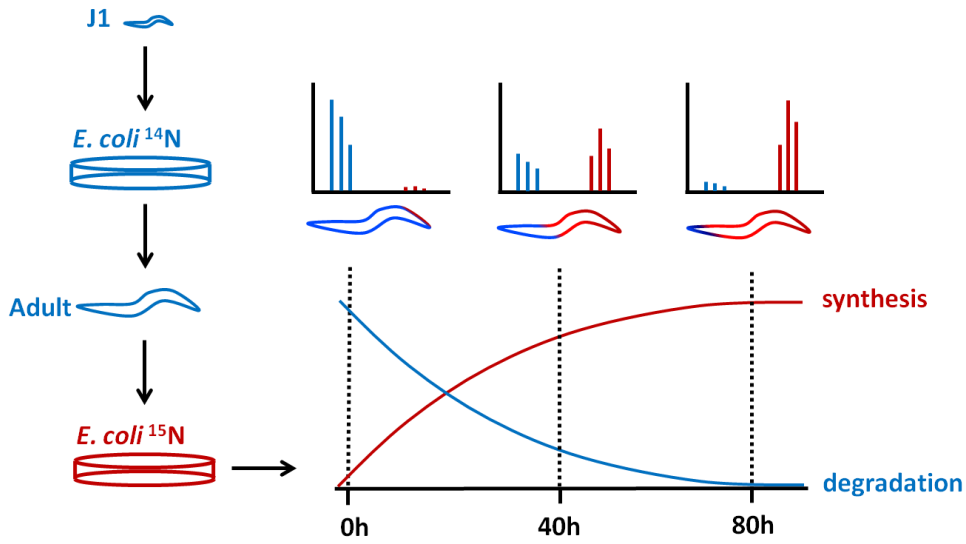


Fig. 13 Overview of the SILeNCe strategy. Stage one larvae are grown on light ^{14}N-labeled *E. coli* bacteria until young adulthood (blue). ^{15}N inorganic salts are used to make isotopically labeled media for growth of heavy ^{15}N-labeled *E. coli* bacteria (red). At day two of adulthood, worms are switched to the labeled diet and sampled over time. Through mass spectrometric analysis, time-dependent mass shifts of peptides derived from newly synthesized proteins (due to incorporation of the heavy label) can be quantified. The proportion of newly ^{15}N-labeled protein opposed to the initial ^{14}N-labeled protein is used to calculate the turnover rate of a protein.

1.4.3 *In vivo* validation of protein turnover via Dendra2

A disadvantage of the isotopic labeling techniques, including the SILeNCe approach, is the occurrence of intracellular amino acid recycling. Due to catabolic processes, free unlabeled (or labeled) amino acids can be released and recycled into the protein pool. Recycling of the light and heavy label can be a problem in measuring protein turnover rates (Claydon & Beynon 2012).

To this end, an alternative approach, which involves the photo-switchable Dendra2 protein, can be used to exclude the label recycling and to validate SILeNCe based protein turnover results *in vivo* in *C. elegans*. Dendra2, derived from octocoral Dendronephthya sp., can be irreversibly photoconverted from green to red fluorescent states in response to violet light (Gurskaya *et al.* 2006). Hence, expression of Dendra2-fused proteins allows the analysis of protein dynamics via quantification of the photoconverted fluorescent signal. This tool has been successfully applied to study protein turnover in plants (Jasik *et al.* 2013) and to monitor the activity of the ubiquitin-proteasome system along different tissues in *C. elegans* (Hamer *et al.* 2010).

1.5 Aims and outline of the thesis

Protein turnover is the continuous breakdown and re-synthesis of proteins, a mechanism essential for cellular housekeeping. Slowdown of bulk protein turnover with age is a common observation and led to the formulation of the protein turnover hypothesis in which increased dwell-time of proteins leads to progressive accumulation of altered, misfolded and non-functional proteins, likely driving the aging process. However, protein turnover seems to be drastically reduced in the long-lived *daf-2* insulin/IGF-1 signaling (IIS) mutant, consistent with the overall observed down-regulation of protein synthesis under reduced IIS.

Yet, little is known about protein turnover **at the level of individual proteins** during aging as well as under lifespan extending conditions. To this end, we aim to critically evaluate patterns of protein turnover at single-protein resolution in (1) aging *C. elegans* and (2) long-lived *daf-2* mutants using a SILeNCe labeling and quantitative proteomics approach.

The overall down-regulation of proteome turnover with age is well-documented; however, these studies on bulk turnover inform only on the weight-biased behavior of the total protein pool and likely neglect the heterogeneous nature of protein turnover among functionally and spatially different proteins. Translation and proteolytic clearance of intracellular proteins are highly selective processes, likely causing widely different protein turnover rates. In this vein, the accurate determination of protein-by-protein turnover rate is an essential dimension to understand the modulation of the global proteome dynamics under changing conditions. SILeNCe labeling, combined with accurate mass spectrometry technology, nowadays offers the possibility to reveal the existence of protein-specific turnover patterns on a proteome-wide scale. A first objective of this thesis is to extend the understanding of the changing protein pool in aging *C. elegans* cohorts. The gradual, day-by-day, alteration in single-protein turnover is studied using a multiple-pulse metabolic ^{15}N-labeling method, uncovering distinct trends between protein subsets according to their subcellular location or related cellular function with increasing chronological age. Results are presented in **chapter 2**.

Although the protein turnover hypothesis predicts enhanced turnover rates in long-lived organisms, paradoxical data challenge this concept. In **chapter 3**, the purpose is to further elucidate the modulation of proteome turnover at single-protein resolution in long-lived

worms, focusing on spatially and functionally related protein pools. Particularly, the detailed survey of turnover rates of proteostasis components, including translation and proteolytic constituents, will provide a better understanding of this longevity phenotype.

Additionally, we aim to validate our SILeNCe approach via an alternative *in vivo* method using the photoconvertible fluorescent protein Dendra2. By studying transgenically expressed Dendra2-fused proteins, issues concerning label uptake and recycling are excluded. Development, optimization and application of this reporter in aging and long-lived worms are presented in **chapter 4**.

Since reduced protein turnover seems to be a hallmark of *daf-2* longevity, we evaluate the effect of serotonin, a drug known to induce protein synthesis in *C. elegans*, on the long-life of *daf-2* worms. A preliminary lifespan experiment is discussed in **chapter 5**.

Chapter 6 provides a general discussion of the results, supplemented with some perspectives for future research.

PART II RESULTS

Chapter 2. Semi-stochastic changes of protein turnover in aging *Caenorhabditis elegans*

Personal contribution: SILeNCe labeling and sampling of nematodes

Sample preparation for mass spectrometry analysis

Functional analysis of the proteomics dataset

Writing the manuscript

Submitted for publication in Aging Cell as:

Semi-stochastic changes of protein turnover in aging *Caenorhabditis elegans.*

Ineke Dhondt[1*], Vladislav A. Petyuk[2*], Sophie Bauer[1], Richard D Smith[2], Geert Depuydt[1,3],Bart P. Braeckman[1,‡]

*Contributed equally

[1]Laboratory for Aging Physiology and Molecular Evolution, Biology Department, Ghent University, Proeftuinstraat 86 N1, 9000 Ghent Belgium.

[2]Biological Sciences Division, Pacific Northwest National Laboratory, Richland, WA 99352, USA

[3]Laboratory for Functional Genomics and Proteomics, Department of Biology, KU Leuven, Naamsestraat 59, 3000 Leuven, Belgium

2.1 Abstract

Protein turnover rates severely decline in aging organisms, including *C. elegans*. However, limited information is available on turnover dynamics at the individual protein level during aging. We followed changes in protein turnover at one-day resolution using a multiple-pulse ^{15}N-labeling and accurate mass spectrometry approach. Forty percent of the proteome shows gradual slowdown in turnover with age, while only few proteins show increased turnover. Decrease in protein turnover was consistent for only a minority of functionally related protein subsets, including tubulins and vitellogenins, while randomly diverging turnover patterns with age were the norm. Our data suggests severe dysregulation of protein turnover of the translation machinery, whereas protein turnover of UPS and antioxidant systems are well-preserved over time. Hence, we presume that maintenance of quality control mechanisms is a protective strategy in aging worms, although the ultimate proteome collapse is inescapable.

Key words: ^{15}N-metabolic labeling; aging; *C. elegans*; protein turnover; proteostasis.

2.2 Introduction

The downturn of protein homeostasis (proteostasis), including the commonly observed slowdown of protein synthesis and degradation (protein turnover), is a major hallmark of aging (Lopez-Otin et al., 2013; Ryazanov and Nefsky, 2002). Proteome mismanagement along with age-dependent protein aggregation, oxidation and misallocation, likely leads to the overall functional decline in senescent organisms (Rattan 1996).

The nematode *C. elegans* is one of the best-studied model organisms in aging research due to its relatively short lifespan and the extensive molecular toolbox available for this organism. Microarray experiments (Lund *et al.* 2002; Golden & Melov 2007) and proteomic analyses (Dong *et al.* 2007; Liang *et al.* 2014; Walther *et al.* 2015) have been used to profile the changes in gene expression and protein abundance levels of aging *C. elegans,* respectively. Overall, these studies report an age-dependent decline in ribosomal proteins and an increase of proteasome complexes and small heat shock proteins (Liang *et al.* 2014; Walther *et al.* 2015), corroborating the link between the aging process and proteostasis.

Recently, we found a drastic decrease of protein turnover rates in aging *C. elegans* using a classical ^{35}S pulse-chase labeling (Depuydt *et al.*, submitted). Liang *et al.* (2014) profiled changes in the pool of *de novo* synthesized proteins in old versus young worms, while another recent study has used a single pulse-chase SILAC technique to estimate protein appearance at proteomic scale during adult lifespan (Vukoti *et al.* 2015). Although these studies point out shifts in protein synthesis rates in old worms, the gradual change in turnover of individual proteins with increasing chronological age has not been investigated previously. Do aging worms display a stochastic decline in proteome turnover or do specific subsets of proteins exhibit distinct trends in protein turnover with age?

To address this question, we analyzed the change of individual protein half-lives in aging worms using a multiple-pulse metabolic ^{15}N-labeling method. To this end, subsamples of an aging *C. elegans* cohort were taken daily and pulse-chased individually. These series of timed samples were subsequently analyzed with high-resolution mass spectrometry to estimate protein half-lives. Our data indicates that turnover of aging proteome slows down partially, although this pattern cannot be generalized for all proteins. Instead, it seems that most protein turnover rates are affected in a stochastic way as clear patterns are absent within

functionally or spatially related protein groups, which indicates a haphazardly disturbed proteome management in aging worms. On the other hand, some distinct protein pools do show specific tendencies in protein turnover with age, such as the proteasomal proteins which maintain their fast turnover rates and the consistent slowdown observed for all tubulins and vitellogenins. In summary, semi-stochastic changes in protein turnover occur during normal aging in *C. elegans*.

2.3 Results

Experimental design to monitor progressive changes in protein turnover

To investigate the change of protein half-lives in the aging proteome, we used the normal-lived *glp-4*(*bn2ts*) *daf-16*(*mgDf50*); *daf-2*(*e1370*) mutant strain. We chose this reference strain in line with our previous proteomic studies (Depuydt *et al.* 2013; Depuydt *et al.* 2014; Dhondt *et al.* submitted), to enable convenient comparison between the resulting data sets. The DAF-16 dependent lifespan-extending *daf-2* mutation is completely nullified by the *daf-16(mgDf50)* knockout allele present in this worm. In addition, we took advantage of working with a temperature-sensitive sterile mutant background (*glp-4*) to avoid the elimination of ^{15}N label via egg laying and to focus on the somatic cells of the aging worms. It has been shown that germline-ablated animals increase lifespan via DAF-16 activation (Hsin & Kenyon 1999; Lin *et al.* 2001; Arantes-Oliveira *et al.* 2002). Hence, longevity and other *daf-16*-dependent effects of *glp-4* will be minimal since these worms carry the *daf-16(mgDf50)* mutation.

To monitor age-dependent changes in protein turnover, we used Stable Isotope Labeling by Nitrogen in *Caenorhabditis elegans* (SILeNCe), a metabolic labeling approach that has been shown to be efficient in *C. elegans* (Krijgsveld *et al.* 2003; Geillinger *et al.* 2012; Dhondt *et al.* submitted). The experimental study design is shown in Figure 1. Synchronized L1 worms were grown at 17°C on nitrogen free agar plates seeded with ^{14}N-labeled *E. coli* bacteria. At the third larval stage, worms were switched to 24°C in liquid Fernbach culture, shaken at 120 rpm. From the first day of adulthood daily subcultures were taken and pulsed with ^{15}N-labeled *E. coli* after which samples were taken at 20 and 40 hours of incubation. Pulsing of new subcultures was continued until day 7 and the last sample was collected at day 9, which

coincides with mean lifespan of the population under these culture conditions (Fig. 1A, Table S1 in supporting information). Blocked groups based on sampling time were analyzed using accurate mass spectrometry (MS)-based quantitative proteomics in a randomized and blind manner (Fig. 1B). Molecular proportions of heavy (^{15}N) and light (^{14}N) isotope peaks of the peptides were extracted using a custom R package and the estimated protein half-lives represent the weighted average from 20- and 40-hour incorporation values.

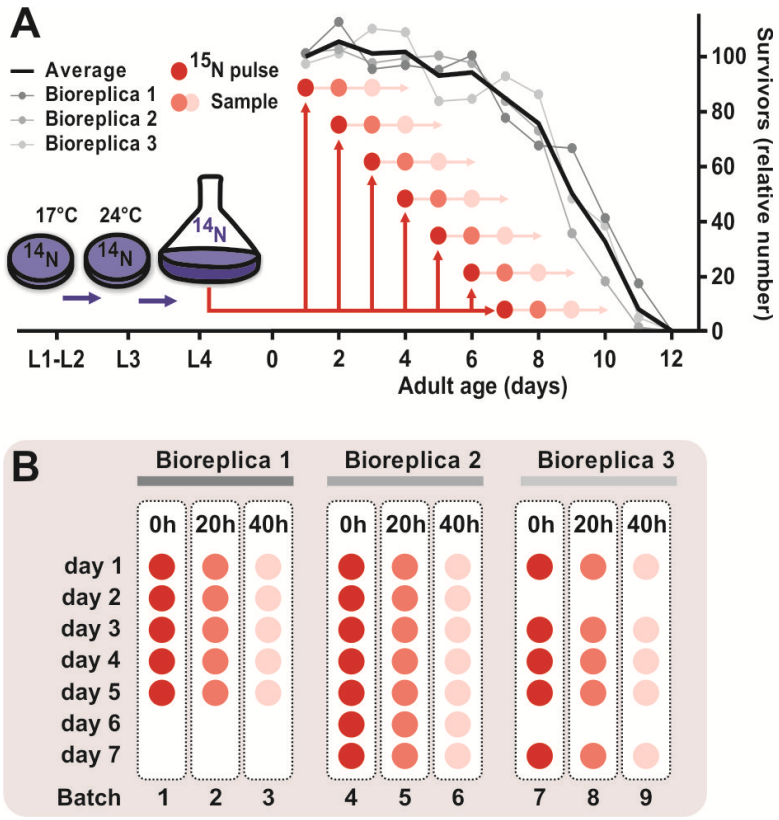


Fig. 1 Experimental study design. A multiple-pulse ^{15}N-labeling approach was performed to analyze the daily changes of individual protein half-lives in aging *C. elegans*. (A) Subsamples of three independent aging cohorts were individually pulse-chased starting from day 1 until day 7 of adulthood (old worms). Survival was estimated daily by manual counting of live worms in culture subsamples and expressed as the percentage of the initial population. (B) Samples were blocked based on the pulse-time, randomized and blindly analyzed using LC-MS/MS.

Diverse impact of aging on protein turnover

We were able to monitor the age-dependent changes in turnover of 878 peptides, corresponding to 546 unique proteins, of three independent aging cohorts. All estimated protein half-lives are listed in Table S2 (Supporting information). Median protein half-life

increases gradually with chronological age, starting from 69 hours at the first day of adulthood and reaching 132 hours at day 7 (Fig. 2). These findings are in line with previous results from classical ^{35}S pulse-chase labeling, showing decreased protein turnover in old worms (Depuydt et al., submitted). In addition, variation of protein half-lives escalates with age as is reflected by the expansion of the interquartile ranges over time.

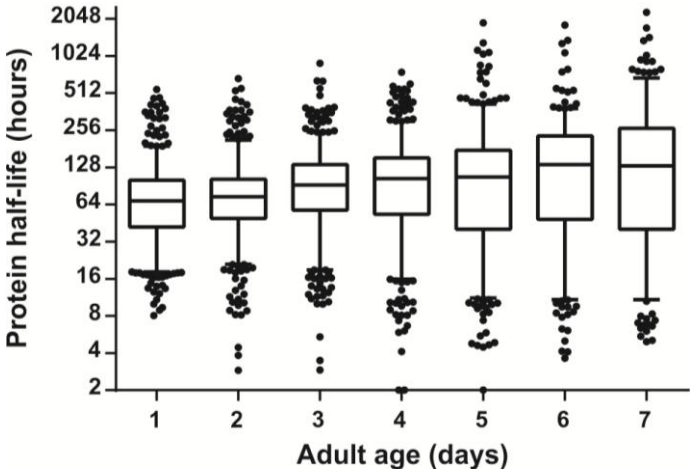


Fig. 2 Increasing variability of protein turnover in aging *C. elegans.* Boxplots showing the gradual increase in median protein half-life over time.

To investigate whether turnover rates are affected differently in several subsets of proteins according to their function or subcellular location, we discriminated proteins of which turnover was decreased or increased via Pavlidis Template Matching (PTM) (Pavlidis & Noble 2001). Turnover rate of 40 percent of the identified proteins gradually slows down with age (Fig. 3A,B), whereas only 7 percent shows an increase in turnover (Fig. 3A,C). Unmatched protein turnover trends (291 proteins) were further divided into three subgroups using the quartiles of the overall average half-life over age as cutoff values, representing proteins that have a steady slow (average half-life > 129 hours, Fig. 3A,D), median (average half-life between 55 and 129 hours, Fig. 3A,E) or fast (average half-life < 55 hours, Fig. 3A,F) turnover during aging (Table S3 for a detailed overview).

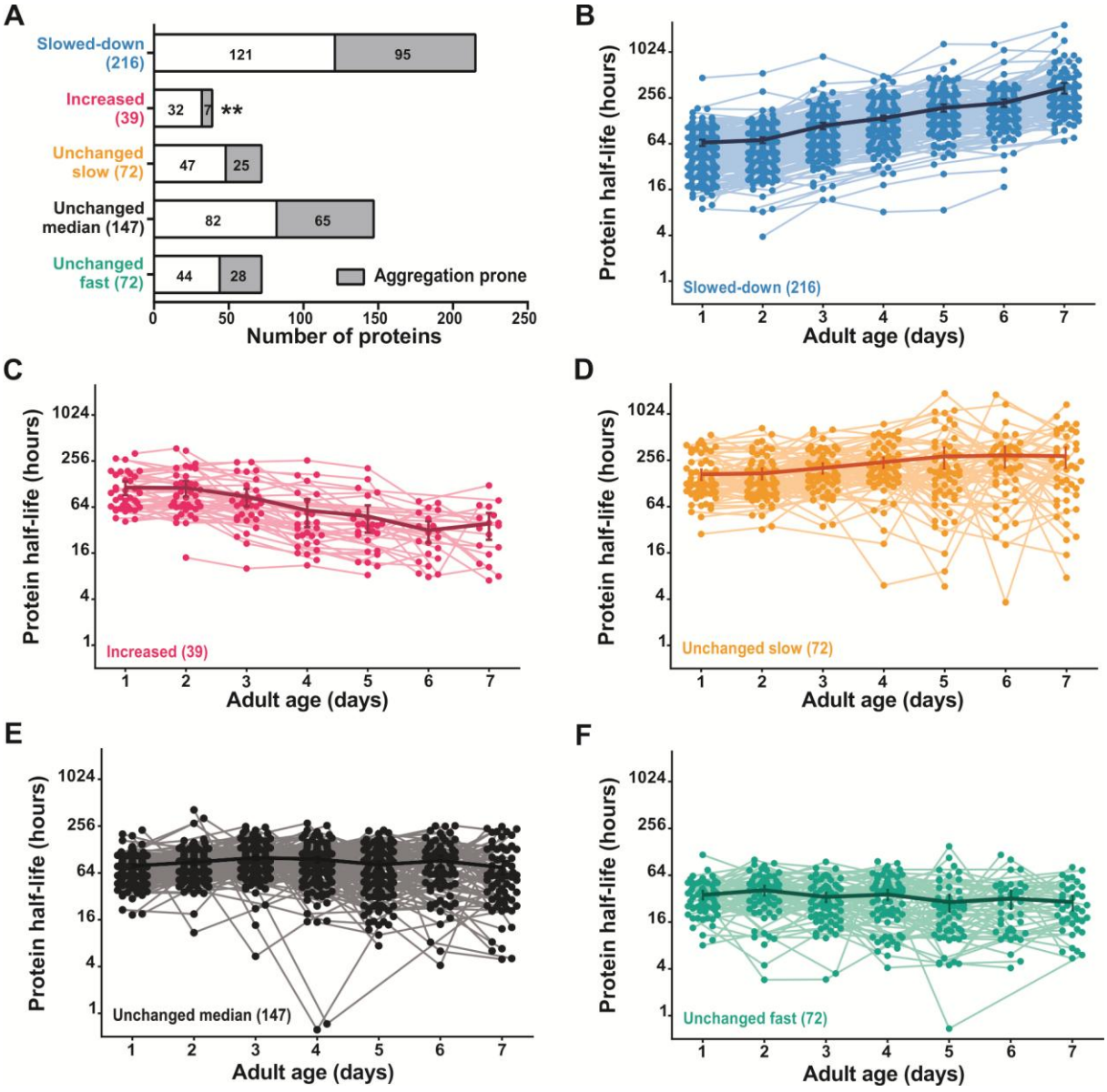


Fig. 3 Protein turnover patterns with age. (A) Overview of proteins per trend based on PTM-analysis, with indication of aggregation propensity (David *et al.* 2010). Detailed overview of proteins with slower (B), faster (C), unchanged slow (D), unchanged median (E) and unchanged fast (F) protein turnover with advancing age. Each dot represents a half-life estimation based on the weighted average of 20- and 40-hour incorporation values. Thick line with error bars (indicating SEM) visualizes the overall tendency per group. (log_2-y-axis) ** *P* = 0.003. Summary of the data from replicate experiments is included in Table S3 (Supporting information).

It has been proposed that protein aggregation actively contributes to a decrease in proteolysis with age (Grune *et al.* 2004). We therefore considered protein aggregation as a possible driving force in the slowdown of protein turnover. To evaluate this hypothesis, we compared our dataset with a list of *C. elegans* proteins prone to aggregation at advanced age (David *et al.* 2010). We found comparable aggregation propensity for proteins with unchanged and decreased protein turnover during aging, independent of their absolute

turnover rates. This finding contradicts our proposition that fast turnover is sufficient to counter late onset aggregation. However, in the small group of proteins for which turnover rate increases with age, aggregation-prone proteins are significantly underrepresented (Fig. 3A, Fisher's test, *P*-value = 0.003). This observation suggests that, in some proteins, either turnover is actively increased at advanced age to avoid aggregation, or that decreased aggregation is a side effect of these increased protein dynamics.

Proteostasis-related proteins display disparate turnover trends with age

Proteome management relies on a complex network of biological processes, including protein translation, folding and degradation by the ubiquitin/proteasomal and autophagosomal pathways (Hartl *et al.* 2011). Recent work showed increased proteome imbalance with age in *C. elegans* (Walther *et al.* 2015), possibly resulting from impaired turnover of proteostasis machinery components. To verify this idea, we performed a functional annotation analysis (Table S4). Aggregation propensity (David *et al.* 2010) of these groups was also monitored (Figure S1).

The protein synthesis machinery, including ribosomal subunits and initiation and elongation factors, display very heterogeneous patterns of turnover with age. Turnover of half of the identified ribosomal proteins slows down with age, while the other half remains unchanged (Fig. 4A,B). Half-lives of all identified ribosomal proteins in young worms are all close to 100 hours, while over about one week, these figures drastically fan out over a range of about 600 hours (Fig. 4A). The fact that 63 percent of these proteins tend to aggregate with age (Fig. S1), supports the notion of an overall dysregulation of the translation machinery in old worms.

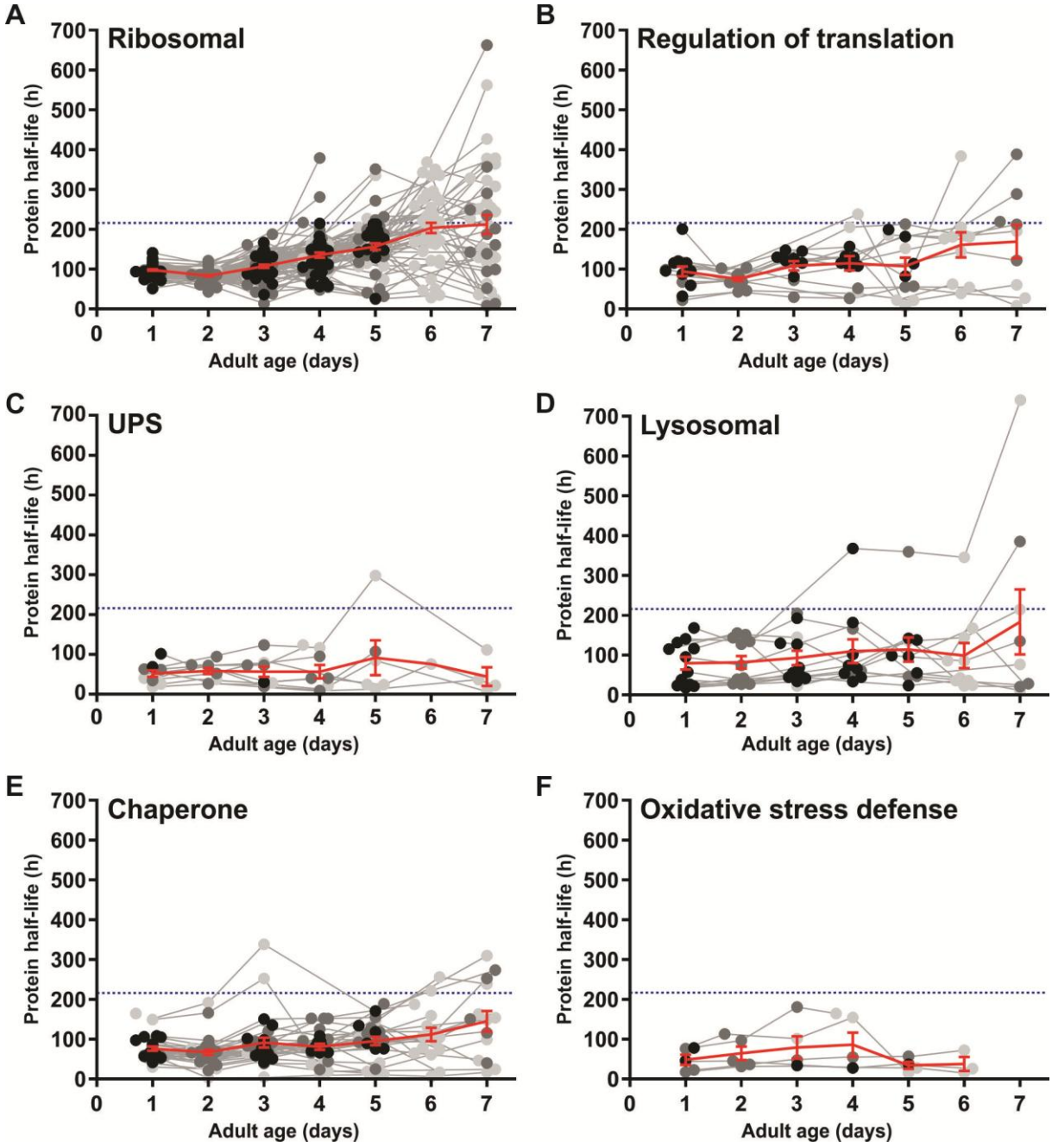


Fig. 4 Proteins of the proteostasis network show divergent protein turnover patterns with age. Patterns are shown for protein translation components, including ribosomal subunits (A) and translation regulation proteins (B); degradation-associated proteins including UPS-machinery (C) and lysosomal-related proteins (D); chaperones responsible for protein folding (E) and proteins associated with oxidative stress defense (F). Each dot represents a half-life estimation based on the weighted average of 20- and 40-hour incorporation values. Half-lives are the average of 3 (black), 2 (gray) or 1 (light gray) biological replicate(s). The average trend (± SEM) is indicated in red. Blue dashed line indicates the average lifespan of the experimental population.

A completely contrasting scenario is observed for proteins involved in proteolytic processes. The ubiquitin-proteasome system (UPS) machinery for protein degradation retains its high

turnover rate, even in old worms (Fig. 4C). The same pattern is observed for two lysosomal proteases, ASP-1 and ASP-4, although turnover of most lysosomal enzymes does slowdown when worms get older (Fig. 4D). Subunits of the V-ATPases, proton pumps responsible for lysosomal acidification, display a clear shortening in protein half-life in old animals (day 7) despite considerable variation among biological replicates. Surprisingly, more than half of the detected UPS components are consistently found in protein aggregates in aging worms, regardless of their maintained fast turnover rates. Lysosome-related proteins show less aggregation propensity, with only ASP-4 and two V-ATPase subunits being consistently found in aggregates (Fig. S1). Taken together, these observations raise the possibility that aging-induced proteome perturbations trigger increased protein turnover of proteolytic key players in an attempt to maintain a functionally intact degradation machinery with age.

Another important group of proteins implicated in proteome management comprises the chaperones, as they support protein folding and refolding under normal and stressful conditions (Hartl *et al.* 2011). Protein turnover of folding chaperones, such as the cytosolic chaperonin CCT (Chaperonin Containing TCP-1 or TRiC homolog) proteins and endoplasmic protein disulfide isomerases, resembles the pattern of the translation machinery (Fig. 4E), although with milder fanning. Likewise, 75% of these assisted protein folding components and all detected HSP70/HSP90 molecular chaperones tend to aggregate with age (Fig. S1).

Interestingly, we found that SOD-1, a superoxide dismutase known to protect cells from oxidative damage, shows increased protein turnover with age. A similar trend was detected for the peroxidase PRDX-3 in 5-day old worms. Catalases, with exception of CTL-2, display fast turnover rates, which remain steady over age (Fig. 4F). Moreover, these antioxidant enzymes are barely found in protein aggregates (Fig. S1). This suggests an overall attempt to cope with the age-associated oxidative damage accumulation.

No consistent turnover pattern of proteins involved in energy metabolism

In *C. elegans*, metabolic rate declines exponentially with age (Braeckman *et al.* 1999; Shoyama *et al.* 2007). Earlier, it was suggested that slowdown of protein turnover causes conformational changes in metabolic enzymes, resulting in an overall progressive decline in metabolic performance with age (Rothstein & Sharma 1978; Braeckman *et al.* 2000). The

relatively high aggregation propensity of these proteins (Fig. S1) points in the same direction. Nevertheless, no consistent pattern in the turnover rates of these proteins could be observed in aging worms, with highly varying half-lives with age (Fig. 5). Nevertheless, we found turnover of some proteins involved in glycolysis and the TCA cycle tend to slow down with age (Fig. 5A,C). In contrast, we found unchanged and increased protein turnover tendencies for fatty acid metabolic proteins (Fig. 5D). In line, analysis of tissue-specific expression scores (Chikina *et al.* 2009) shows an overrepresentation of proteins with increased turnover rates for the intestine, the primary site of fat metabolism (Fig. S2A).

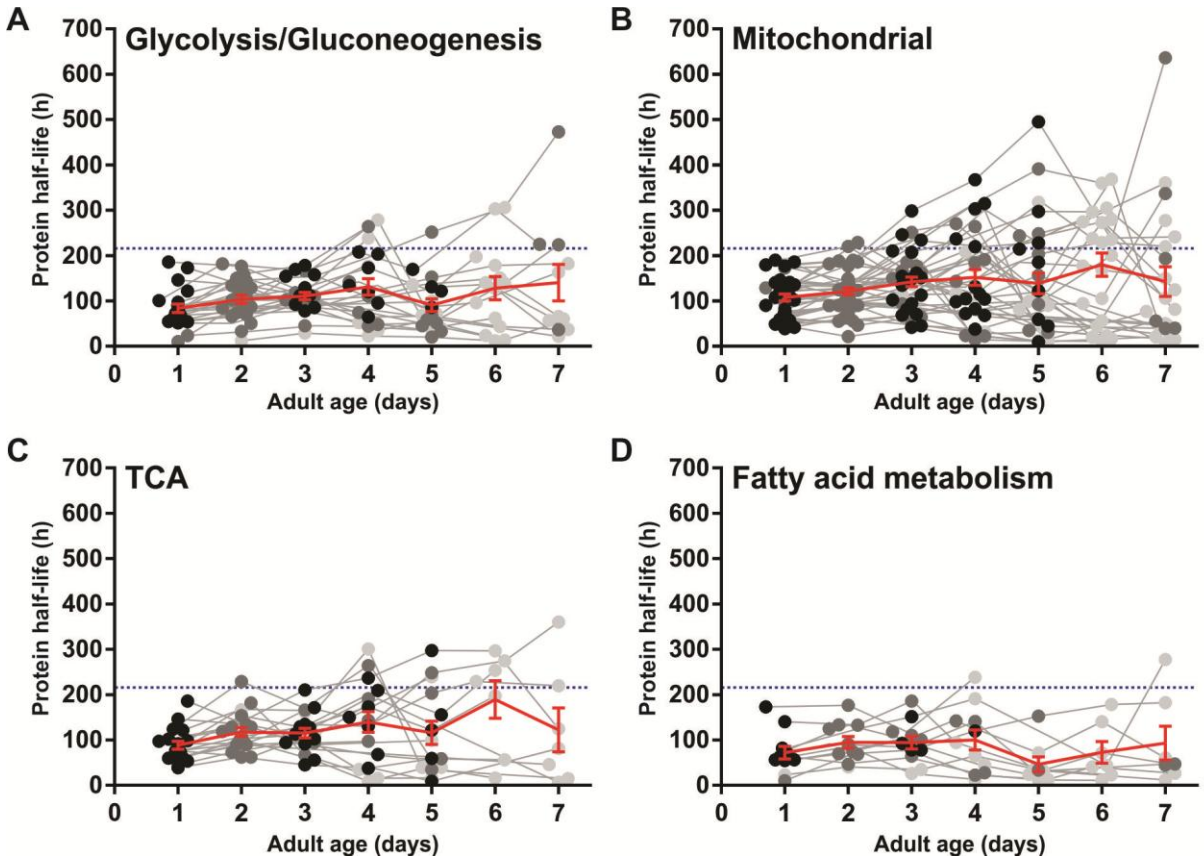


Fig. 5 One-quarter of proteins involved in energy metabolism shows slowdown in protein turnover in aging worms. Patterns are shown for enzymes involved in the glycolysis/gluconeogenesis (A), mitochondrial proteins (B), enzymes of the TCA-cycle (C) and proteins associated with fatty acid metabolism (D). Color schemes and indications are identical as in Figure 4.

Strong and consistent slowdown of tubulins and vitellogenin turnover

Although proteins of many functional groups show mixed protein turnover patterns with age, some functional groups show very consistent patterns. The basic structural units of microtubules, α- and β-tubulins, display a 10-fold increase in protein half-life in old compared to young worms (Fig. 6A). Remarkably, none of these proteins are consistently found in protein aggregates (Fig. S1). Vitellogenins (yolk proteins) show the same pattern (Fig. 6B). Although we used a germline deficient strain, these yolk proteins are detected by the mass spectrometer as they are produced in the intestine. As in fertile worms vitellogenins are translocated to oocytes, and thus considered germline proteins by the tissue prediction tool, this results in a relative over-representation of slowed-down proteins in the germline (Fig. S2). In contrast to the microtubular proteins, we found that all detected vitellogenins are likely to aggregate with age (Fig. S1). Based on these two contrasting examples, it can be argued that protein aggregation is not the determining factor for protein turnover decline during aging.

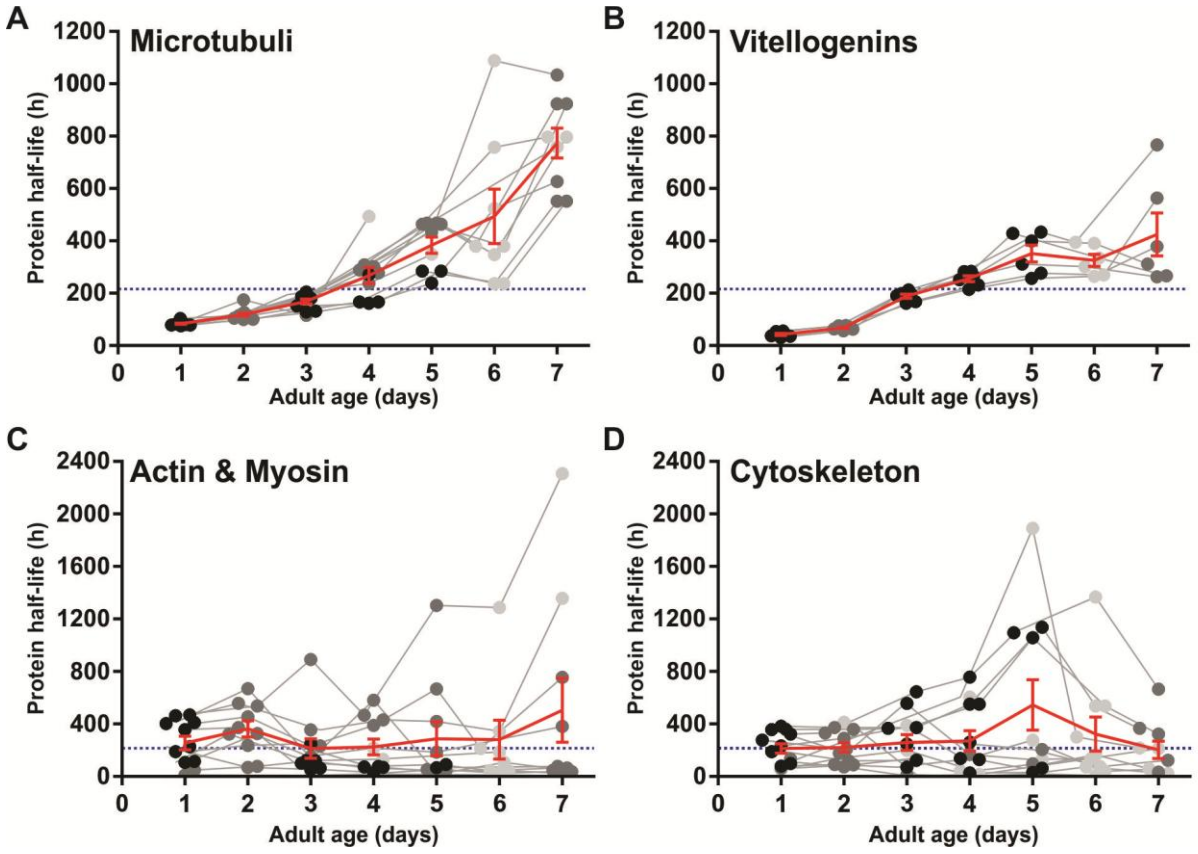


Fig. 6 Specific subsets of proteins show consistent patterns in protein turnover in aging worms. Patterns are shown for tubulins (A), vitellogenins (B), muscle-specific proteins (C) and other cytoskeletal proteins (D). Color schemes and indications are identical as in Figure 4.

Body wall muscle and the pharyngeal proteins follow distinct turnover tendencies with age

Aging worms, like humans, suffer from the progressive age-related muscle deterioration (Herndon *et al.* 2002). During aging, it might be possible that protein degradation rates exceed protein synthesis, leading to sarcopenia in old worms. Yet, no extensive alterations in protein half-lives could be observed for muscle-specific proteins (Fig. 6C), as most of them rarely turn over during lifespan (Fig. S2), which is consistent with earlier findings (See chapter 4)(Dhondt *et al*., submitted). Interestingly, we noticed strong representation of proteins with increased or fast protein turnover rates in the pharynx, while these groups are under-represented in body wall muscle (Fig. S2).

2.4 Discussion

The overall downturn in protein turnover is a common observation in senescent organisms, including nematodes (Rothstein & Sharma 1978; Rattan 1996; Ryazanov & Nefsky 2002). Due to increased protein dwell times with age, it is likely that proteins have ample time to undergo oxidation, aggregation and misallocation. It is still unclear whether the age-related decrease in protein turnover is consistent over the entire proteome or whether groups of functionally related proteins show specific patterns. Using a pulse labeling method, we have monitored the change in individual protein turnover in aging *C. elegans.* Our results are consistent with previous observations (Vukoti *et al.* 2015), yet extend the understanding of detailed protein turnover trends with age, as the changes in protein half-lives were evaluated day by day. We report the gradual slowdown of the turnover of a considerable part of the aging proteome, whereas only a minor fraction shows increased turnover with age. Disparate protein turnover changes within the majority of functional and spatially related protein groups indicate the stochastic impact of aging on protein turnover. This mirrors the randomness observed in the pathobiology of aging worms, showing heterogeneous decline between cells of the same type within individuals (Herndon *et al.* 2002). It is likely that the haphazard slowdown of protein turnover might be one of the important stochastic components underlying the random age-dependent deterioration. Conceivably, the protein synthesis machinery itself might be a key player driving this stochastic decline, as it shows disparate patterns in protein turnover as well as an escalation

in variation with age. This idea is further corroborated in overall decline in ribosomal protein abundances with age, with a prominent imbalance in the relative ribosomal subunit stoichiometry (Walther *et al.* 2015). Thus, dysregulation of protein turnover of the translation machinery in addition with slower turnover of the aggregation-prone folding chaperones (Walther *et al.* 2015), likely promote the ultimate collapse of the aging proteome.

Nevertheless, consistent trends within functional subsets of proteins do exist. Our data shows high maintenance of the degradation apparatus in aged worms, which highly contrasts the hampered turnover of the translation machinery. Moreover, an up-regulation in proteasomal subunits has been observed earlier in aging *C. elegans* (Walther *et al.* 2015). Notwithstanding their fast turnover rates, these components likely get trapped into aggregates with age. Thus, the sustained protein turnover of proteasomal subunits at advanced age is likely the ongoing endeavor to keep up the degradation machinery to further deal with the proteome imbalance. However, chronic proteotoxicity will eventually exceed the proteasomal capacity (Hipp *et al.* 2014).

No uniform slowdown in turnover of enzymes involved in energy metabolism could be observed, since only one-fourth shows a significant decline in protein turnover with age. As mitochondria are main producers of ROS, proteins with slow turnover are liable to oxidative modification, resulting in the accumulation of altered enzymes. Although the turnover of only a minor fraction of mitochondrial proteins slows down with age, these damage-prone enzymes may be the driving force of the functional decline in metabolic performance with age (Braeckman *et al.* 1999; Shoyama *et al.* 2007). Interestingly, fatty-acid β-oxidation proteins, that primarily reside in the intestine (Ashrafi 2007), exhibit increased turnover with age, which is also reflected in the tissue enrichment analysis. These findings may point to a shift from carbohydrate metabolism to fatty-acid β-oxidation in the intestine of old worms. Alternatively, the fatty-acid β-oxidation machinery may need improved turnover compared to enzymes involved in carbohydrate metabolism due to its specific location in the mitochondria and peroxisomes, which are well-known ROS generation sites that may impose excessive oxidative damage to residing proteins (Adachi *et al.* 1998; Nguyen & Donaldson 2005).

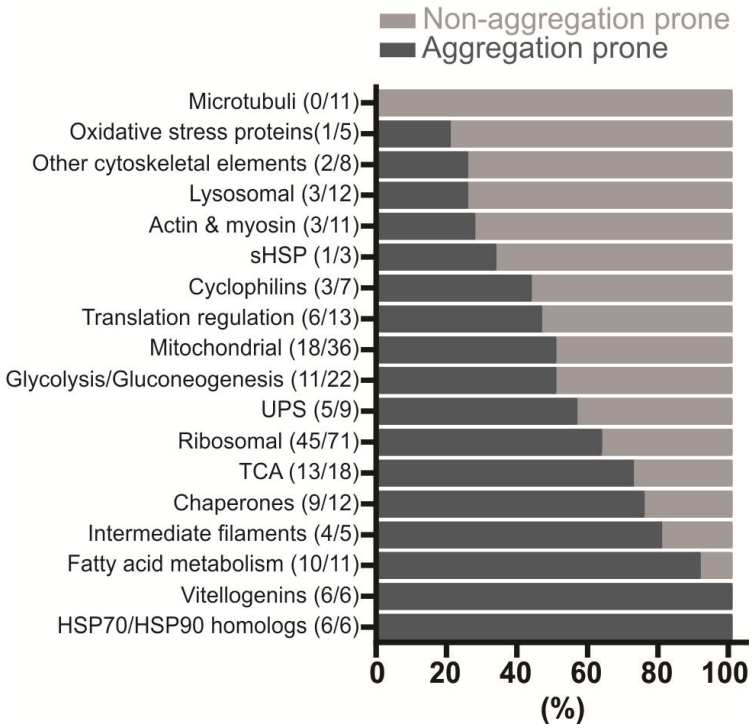
Tubulins and vitellogenins show consistent slowdown of turnover rates with age. Since the latter groups have opposite aggregation propensities, protein aggregation cannot be the sole underlying mechanism responsible for the deceleration of protein turnover with age and *vice versa*. Microtubules are responsible for a variety of functions, including cellular transport (Cooper 2000). Aged cells frequently display the accumulation of cell organelles probably due to insufficient organelle transport along disrupted microtubules (Schatten *et al.* 1999). Hence, we presume that attenuation of tubulin turnover may underlie the changing microtubule organization and dynamics during aging. On the other hand, protein aggregation might be a driving force in slowing down the turnover of vitellogenins, as these macromolecules, irrelevant to post-reproductive worms, accumulate throughout the body cavity in old worms (Herndon *et al.* 2002). Vitellogenins, trapped into accumulating protein aggregates over time, likely show increased protein half-lives with age as they become less susceptible for protein degradation in aging worms.

Aging nematodes are characterized by a progressive locomotory decline (Bolanowski *et al.* 1981). Muscle-specific proteins are very stable proteins, showing barely any turnover during a worm's lifespan (this study and Dhondt *et al.* submitted). Therefore, the lack of protein turnover may be associated with the age-related structural loss of sarcomere integrity (Herndon *et al.* 2002). Interestingly, in pharyngeal muscle cells, proteins with increased and fast turnover rates are relatively over-represented, compared to other muscle tissues. One possibility is that the pharyngeal muscle cells are, besides general deterioration, more susceptible to microbial attacks than body wall muscles (Chow *et al.* 2006). Hence, proteins with higher turnover rates might be more flexible to cope with this additional stress.

In conclusion, our data suggests that senescent *C. elegans* is characterized by mostly-stochastic changes in protein turnover. Diverging dwell times of proteins involved in translation are likely responsible for a collapse of the translation machinery over time. Intriguingly, we found that aging worms seem to preserve their (protein) quality control mechanisms, especially the UPS and antioxidant machinery, possibly to cope with the increasing proteotoxic and oxidative stress with age. However, this maintenance fights a losing battle, eventually resulting in the ultimate collapse of the proteome.

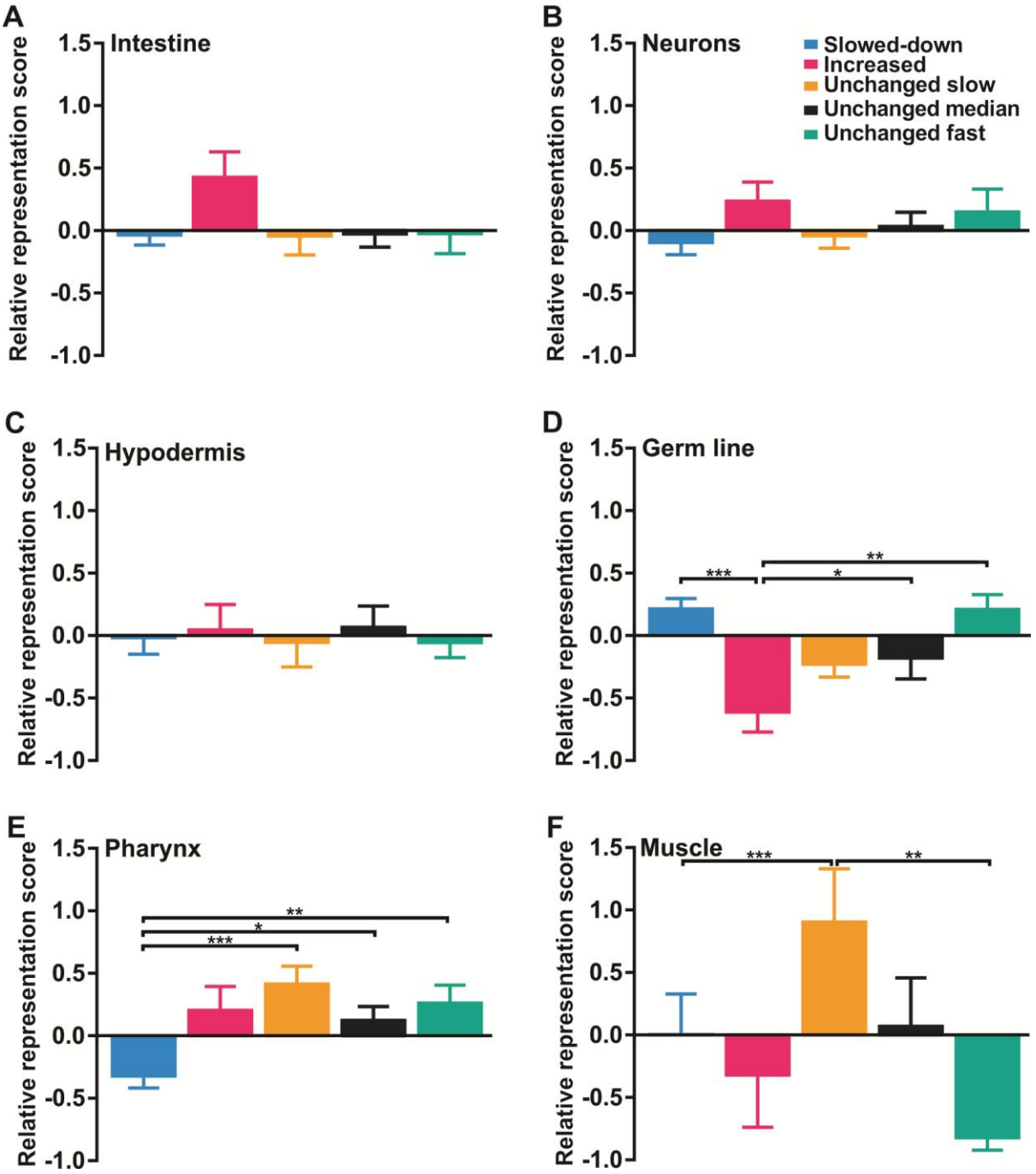
2.5 Supplementary information

Supplementary figure 1



Supplementary figure 1. Aggregation propensity differs widely between functional protein subsets (David *et al.* 2010).

Supplementary figure 2



Supplementary figure 2. Worm-tissues show specific patterns of protein turnover with age.

Supplementary datasets:

Table S1 Summary of data from replicate experiments as in Fig. 1.

Table S2 Summary of the estimated protein half-lives per day as in Fig. 2.

Table S3 Summary of protein turnover patterns as in Fig. 3.

Table S4 Summary of functional annotation analysis as in Fig. 4,5,6.

2.6 Materials and methods

Strains

In this study, we used the strain GA153 *glp-4(bn2ts)I daf-16(mgDf50)I; daf-2(e1370)III* which was kindly provided by David Gems at the University College of London. Worms were maintained as described previously (Depuydt *et al.* 2013).

Metabolic ^{15}N- and ^{14}N-labeling of the *Escherichia coli* bacteria

E. coli K12 was freshly grown overnight at 37°C, shaking at 120 rpm in minimal medium, containing 42 mM Na_2HPO_4, 22 mM $KHPO_4$, 9 mM NaCl, trace elements (17 mM EDTA, 3.1 mM $FeCl_3 \cdot 6H_2O$, 0.62 mM $ZnCl_2$, 0.076 mM $CuCl_2 \cdot 2H_2O$, 0.042 mM $CoCl_2 \cdot 6H_2O$, 0.16 mM H_3BO_3, 0.0068 mM $MnCl_2 \cdot 6H_2O$), glucose 20% (w/v), 1 mM $MgSO_4$, 0.3 mM $CaCl_2$, 0.0041 mM biotin, 0.0033 mM thiamin, and 93 mM ^{15}N- or ^{14}N-containing NH_4Cl (sterilized over 0.22 µm filter). Overnight cultures were concentrated 50-fold.

Culturing and sampling of *C. elegans*

Synchronized L1 nematodes were plated on nitrogen-free agarose (1.2%) plates containing NaCl (0.3%), cholesterol (0.0005%), 1 mM $CaCl_2$, 1 mM $MgSO_4$ and 25 mM K_2HPO_4/KH_2PO_4 (pH 6.0) and a lawn of freshly grown ^{14}N-labeled *E. coli* K12 cells. Worms were grown at 17 °C until the third larval stage (L3) and then switched to 24°C for the remainder of the experiment. Worms that had freshly moulted to adults (adult day 0) were washed three times and transferred into a Fernbach flask (not exceeding 1500 worms/ml) containing S-basal (100 mM NaCl, 50 mM potassium phosphate, pH 6.0), 12.93 µM cholesterol, 75 µM 5-fluoro-2'-deoxyuridine (Acros Organics, Geel, Belgium), and ^{14}N-labeled *E. coli* K12 cells (A_{550} = 1.0). Starting from day one of adulthood, daily subcultures were collected and washed thoroughly (2x S-basal, 1x S-basal containing 2.5 mM EDTA, 2x S-basal) to remove bacteria. Next, worms were pulsed in new culture flasks containing S-basal, 12.93 µM cholesterol, 75 µM 5-fluoro-2'-deoxyuridine (Acros Organics, Geel, Belgium), and ^{15}N-labeled *E. coli* K12 cells (A_{550} = 1.0). Samples were taken at 20 and 40 hours labeling for three independent aging cohorts. Worms were washed thoroughly (2x S-basal, 1x S-basal containing 2.5 mM EDTA, 2x

S-basal) to remove bacteria, after which dead worms were removed via Percoll (Sigma-Aldrich) washing. The worm pellet was resuspended in 200 µl denaturing solution (8 M urea, 1 mM EDTA, 10 mM DTT, 50 mM TrisHCl, pH 8.0) and immediately dropwise frozen in liquid nitrogen and stored at -80°C. The sample size was chosen based on a previously published study (Depuydt *et al.* 2013).

Quantitative proteomics: Randomized study design

Samples were processed in blocked groups comprising all daily samples from one specific post-labeling time-point. Samples within a batch were analyzed blindly and in random order.

Preparation of the tryptic peptide samples

The frozen worm beads (~1500 worms in denaturing solution) were homogenized using a BioPulverizer (BioSpec, Bartlesville, USA) pre-conditioned in liquid nitrogen. The fine powder was recovered into a 1.5 ml tube, thawed, centrifuged (2 min, 5000 rpm), and sonicated for 30 s in a 5510 Branson ultrasonic water bath (Branson Ultrasonics, Soest, Netherlands). Protein concentrations were determined using Coomassie assay. For further processing steps we took the aliquots containing 150 µg of protein and readjusted the volume with denaturation buffer. The samples were subjected to cysteine alkylation by 40 mM iodoacetamide for 1 hour at 37°C in dark shaking conditions (900 rpm), 4-fold dilution with 50 mM ammonium bicarbonate (pH 7.8), and digestion with trypsin (3 µg per sample, Promega) for 12 hours at 37°C shaking conditions (400 rpm). Next, tryptic peptide samples were cleaned-up via C-18 SPE columns (Discovery DSC-18, SUPELCO, 52601-U) and concentrations were adjusted to 0.2 µg/µl (Petyuk *et al.* 2010b; Depuydt *et al.* 2013).

LC/MS analysis and peptide identification

Each individual sample was analyzed with a constant-pressure capillary HPLC system coupled online to an LTQ Orbitrap mass spectrometer (Thermo Fisher, San Jose, CA) using an electrospray ionization interface. Instrument settings were described previously by Depuydt et al. (2013). The resulting 56 datasets were converted to the mzXML format using

MSConvert (settings: --mzXML --32 -z), a part of the ProteoWizard software suite (Kessner *et al.* 2008). Peptide identification was performed by preprocessing datasets with DeconMSn (Mayampurath *et al.* 2008), DtaRefinery (Petyuk *et al.* 2010a) followed by the database search tool MS-GF+ (Kim & Pevzner 2014). The search was aimed at only light ^{14}N-peptides as we did not anticipate more than 50% label incorporation in such a short labeling pulse. The resulting mzIdentML containing, the peptide identifications, was combined with the raw mzXML file to process the 15N14N ratio (next section). The key MS-GF+ settings are: PMTolerance = 10 ppm; NumMods = 3; StaticMod = C2H3N1O1, C, fix, any, Carbamidomethyl; DynamicMod = None; EnzymeID = 1; IsotopeError = 0.1; NTT = 2; minLenght = 6; maxLenght = 50; minCharge = 1, maxCharge = 4. The results of the MS/MS searches were saved as mzIdentML files.

Processing ^{15}N/^{14}N LC-MS/MS data

The analysis of the LC-MS/MS datasets was carried out in the R computing environment (version 3.0.2), using a package N14N15 for the processing of the ^{15}N/^{14}N isotopic data (available from GitHub, https://github.com/vladpetyuk/N14N15). Two files were required as an input: (1) mzXML with raw MS spectra and (2) mzIdentML containing peptide identifications. For the peptides that passed the MS-GF+ confident identification criterion (SpecEvalue < 10^{-9}, < 1% FDR unique peptide level), extracted ion chromatograms were generated for the parent ions in the vicinity of the MS^2 spectrum that yielded identification. After chromatographic peak detection, MS^1 spectra within full width half maximum intensity were summed. Further, given the known elemental composition of the peptide, the summed MS^1 spectrum intensities were fitted with the mixed isotopic distribution assuming one natural and one non-natural proportion of the ^{15}N isotope. The peptides that fitted to the ^{14}N/^{15}N isotopic distribution were filtered based on the following criteria: (1) the proportion of ^{15}N isotope in the heavy component was constrained from 55% to 90% and (2) the R^2 value between the theoretical and experimental intensities in the isotopic envelopes (that primarily result from ~1% natural presence of ^{13}C) for light and heavy components had to be at least 0.9 and 0.5, correspondingly. Deviation of peptide mass derived from the experimental data from the theoretical was limited with 5 ppm (after zero-centering the mass measurement error histogram to account for imperfect mass spectrometer instrument

calibration). Peak picking was performed using wavelet-based approach (Du *et al.* 2006). The signal-to-noise ratio of detected peaks was required to be at least 3.

The proportion of ^{15}N-labeled peptide was log-transformed for convenience of visualization ($log2\left(\frac{P_{N15}}{P_{N14}+P_{N15}}\right)$, further denoted as x). Calculation of the protein half-lives included following steps:

We assumed that, during the two-day pulse labeling, the total protein abundance did not change significantly in the sterile adult worms, whereby protein synthesis equals degradation, collectively designated as protein turnover:

$$P_0 = P_{N14} + P_{N15} \text{ and } \frac{d[P_{N15}]}{dt} + \frac{d[P_{N14}]}{dt} = 0$$

The change in ^{14}N-labeled proteins with time is:

$$\frac{d[P_{N14}]}{dt} = -k_{dp}[P_{N14}]$$

or

$$P_{N14} = P_0 \times e^{-k_{dp} \times \Delta t}$$

In this equation, P_0 is the total protein concentration right before the labeling pulse, k_{dp} is the protein degradation kinetic constant and Δt is pulse length. According to the previous assumption, protein concentration does not change during pulse labeling. Therefore:

$$\frac{P_{N14}}{P_{N14} + P_{N15}} = e^{-k_{dp} \times \Delta t}$$

Plugging-in the proportion of ^{15}N as x and log-transforming the equations results in:

$$ln(1 - x) = -k_{dp} \times \Delta t$$

Protein half-life could be estimated using the following equation:

$$t_{1/2} = \frac{\ln(2)}{k_{dp}}$$

Protein half-life was calculated using the weighted average from 20- and 40- hour incorporation values, in which the 40 hour incorporation value was assigned double the weight as its protein half-life estimation is more accurate.

Pavlidis template matching and functional analysis

Protein turnover patterns were analyzed with Pavlidis template matching (PTM), implemented in the MultiExperiment Viewer, part of the TM4 microarray software suit (Saeed *et al.* 2003; Saeed *et al.* 2006). The dataset was searched for proteins with increasing or decreasing protein half-lives with age using a defined template profile. The PTM algorithm is based on the Pearson correlation between the template and the proteins in the dataset and protein turnover patterns were allowed to match one of the defined templates when *P*-values were lower than 0.05 (corresponding to a regression coefficient R of more than 0.75). Patterns that did not match both templates were considered not unchanged over age. *P*-values were adjusted for multiple testing using FDR-based (False Discovery Rate) correction. The Database for Annotation, Visualization, and Integrated Discovery (DAVID 2015) (Dennis *et al.* 2003) and Wormbase (Version WS246) were used for protein annotation and evaluation of functionally and spatially related proteins (Table S4). In addition, tissue specificity of expression was predicted using an online bioinformatic tool (http://worm-tissue.princeton.edu) (Chikina *et al.* 2009). Graphs and statistical tests (including tests for normality) were performed with Graphpad Prism version 6.05 for Windows (Graphpad Software, La Jolla California, USA).

Data Availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (Vizcaino *et al.* 2014) via the PRIDE partner repository with the dataset identifier PXD002317 and 10.6019/PXD002317.

Acknowledgments

We thank the Gems laboratory for providing the strain GA153. We are grateful to Caroline Vlaeminck and Renata Coopman for technical support with the SILeNCe experiments. We thank Andy Vierstraete for his technical assistance in data-processing. Preliminary experiments were performed by Dr. Filip Mathijssens, who was a postdoctoral member of the Braeckman lab. ID acknowledges a PhD grant from the fund for Scientific Research-

Flanders, Belgium (FWO11/ASP/031). Portions of this work were supported by NIH P41GM103493 (to R.D.S). The experimental work described herein was performed in the Environmental Molecular Sciences Laboratory, a national scientific user facility sponsored by the DOE and located at Pacific Northwest National Laboratory, which is operated by Battelle Memorial Institute for the DOE under Contract DE-AC05-76RL0 1830.

Chapter 3. FOXO/DAF-16 activation slows down turnover of the majority of proteins in *C. elegans*

Personal contribution: SILeNCe labeling and sampling of nematodes

Sample preparation for mass spectrometry analysis

Functional analysis of the proteomics dataset

TEM imaging

Writing the manuscript

Submitted for publication in Cell Reports as:

FOXO/DAF-16 activation slows down turnover of the majority of proteins in *C. elegans.*

Ineke Dhondt[1*], Vladislav A. Petyuk[2*], Huaihan Cai[1], Andy Vierstraete[1], Richard D Smith[2], Geert Depuydt[1,3], Bart P. Braeckman[1,‡]

*Contributed equally to this work

[1]Laboratory for Aging Physiology and Molecular Evolution, Biology Department, Ghent University, Proeftuinstraat 86 N1, 9000 Ghent Belgium.

[2]Biological Sciences Division, Pacific Northwest National Laboratory, Richland, WA 99352, USA

[3]Laboratory for Functional Genomics and Proteomics, Department of Biology, KU Leuven, Naamsestraat 59, 3000 Leuven, Belgium

3.1 Abstract

Most aging hypotheses revolve around the accumulation of some sort of damage resulting in gradual physiological decline and ultimately death. Avoiding protein damage accumulation by enhanced turnover should slow down the aging process and extend lifespan. However, lowering translational efficiency extends rather than shortens lifespan in *C. elegans*. We studied turnover of individual proteins in the conserved Insulin/Insulin-like Growth Factor (IGF-1) receptor mutant *daf-2* by combining Stable Isotope Labeling by Nitrogen in *Caenorhabditis elegans* (SILeNCe) and accurate mass spectrometry. Intriguingly, the majority of proteins displayed prolonged half-lives in this mutant, while others remained unchanged, signifying that longevity is not supported by high protein turnover. This slow-down of protein turnover was most prominent for components of the translation machinery and mitochondria. In contrast, the high turnover of lysosomal hydrolases and very low turnover of cytoskeletal proteins remained largely unchanged in *daf-2*. The slow-down of protein dynamics and decreased abundance of the translational machinery may point at the importance of anabolic attenuation in lifespan extension as suggested by the hyperfunction theory.

Keywords: ^{15}N-metabolic labeling ; Aging ; *Caenorhabditis elegans* ; *daf-2* mutant ; Protein turnover / FOXO/DAF-16

3.2 Introduction

Cellular protein quality can be maintained by eliminating damaged proteins via constitutive proteolytic activities while, at the same time, replacing them with newly synthesized copies, a process called protein turnover (Rosenberger 1991; Rattan *et al.* 1992; Stadtman 1992; Hipkiss 2006). Protein turnover rates have been estimated using SILAC (Stable Isotope Labeling by Amino acids in Cell culture) in single-cells such as yeast (Pratt *et al.* 2002b; Helbig *et al.* 2011), bacteria (Cargile *et al.* 2004; Trotschel *et al.* 2013) and mammalian cells (Andersen *et al.* 2005; Schwanhausser *et al.* 2009; Cambridge *et al.* 2011; Boisvert *et al.* 2012; Larance *et al.* 2013). The last decade has witnessed a growing interest in the analysis of whole-organism proteome dynamics in metazoans using the same approach (Doherty *et al.* 2005; Price *et al.* 2010; Westman-Brinkmalm *et al.* 2011; Claydon & Beynon 2012; Savas *et al.* 2012; Reckow & Webhofer 2014). In recent work, SILAC was applied to monitor protein synthesis throughout life in adult *Caenorhabditis elegans* (Vukoti *et al.* 2015) and to investigate food intake (Gomez-Amaro *et al.* 2015).

Progressive decrease in protein synthesis and proteolytic clearance through the autophagosomal and proteasome systems with age, results in a strong increase of protein half-life in many species, including nematodes (Young *et al.* 1975; Lewis *et al.* 1985; Grune 2000). This finding led to the formulation of the protein turnover hypothesis, stating that the increase of protein dwell-time with age results in the accumulation of damaged and misfolded proteins. The progressive decrease in general protein turnover might be responsible for the ultimate collapse of proteome homeostasis in aging cells, possibly also driving the aging process itself (Rattan 1996; Ryazanov & Nefsky 2002; Taylor & Dillin 2011). In this vein, it is expected that turnover may be a significant determinant of longevity in which increased protein turnover rates would help to maintain a young undamaged proteome and extend lifespan. However, in yeast and *C. elegans,* genetically induced attenuation of protein synthesis extends, rather than shortens, lifespan (Kaeberlein *et al.* 2005; Hansen *et al.* 2007; Pan *et al.* 2007; Syntichaki *et al.* 2007). Moreover, quantitative proteomic studies suggest that low overall protein synthesis is a hallmark of long-lived *C. elegans*, either by dietary restriction or by mutation in the insulin signaling pathway (Depuydt *et al.* 2013; Stout *et al.* 2013). Similar findings have been reported for dietary restricted mice (Price *et al.* 2012). Hence, why does reducing protein synthesis promotes

lifespan extension? And how can this be reconciled with the protein turnover hypothesis that predicts enhanced turnover rates in long-lived organisms?

The FOXO/DAF-16 transcription factor drives the increased longevity of the Insulin/Insulin-like Growth Factor (IGF-1) receptor mutant *daf-2* (Kenyon *et al.* 1993; Kenyon 2010). We hypothesized that DAF-16 dependent longevity in *C. elegans* is supported by differential protein turnover. Down regulating turnover of the majority of proteins could save much energy, which in turn could be spent at prioritized maintenance of specific proteins that are crucial to extend lifespan. To test this hypothesis, we used Stable Isotope Labeling by Nitrogen in *Caenorhabditis elegans* (which we now designate SILeNCe), a metabolic labeling method that has been shown to be efficient in *C. elegans* (Krijgsveld *et al.* 2003; Geillinger *et al.* 2012). Steady-state protein turnover was monitored by pulsing adult worms with ^{15}N-containing bacteria, and a series of timed samples were subsequently analyzed with high-resolution mass spectrometry. Of the 245 proteins that were detected in both long-lived and control strains, about half show decreased turnover in *daf-2* while the other half remains unchanged. We revealed specific turnover patterns for groups of functionally and spatially related proteins. Mitochondria show widely divergent protein half-lives with more prolonged protein half-lives for complexes of the electron transport chain. The fastest turnover rates were observed for lysosomal hydrolases and endoplasmic reticulum-associated proteins, of which only the latter have a reduced half-life in *daf-2*. Cytoskeletal elements are the most stable proteins with half-lives that even exceed the worm's lifespan and membrane-bound proteins display longer half-lives compared to free proteins. All together, we here present a SILeNCe dataset that reveals new patterns of intracellular protein dynamics in the *C. elegans* model and shifts of these patterns that occur in the long-lived *daf-2* mutant via DAF-16 activation.

3.3 Results

Worm strains used to study protein turnover in long-lived *daf-2*

To understand the role of protein turnover in DAF-16-mediated lifespan extension, we chose to compare the normal-lived *glp-4(bn2ts) daf-16(mgDf50); daf-2(e1370)* triple mutant (control strain) and the long-lived *glp-4(bn2ts); daf-2(e1370)* insulin/IGF-1 receptor mutant.

Mutation in DAF-2 causes activation of the downstream transcription factor DAF-16, which in turn activates a life maintenance program, doubling lifespan of *C. elegans*. In order to specifically study DAF-16, the major lifespan regulator, we compared two worm strains carrying the *daf-2* mutation (causing lifespan extension via DAF-16 activation) and knocked out the *daf-16* gene by mutation in the control strain, thereby nullifying the lifespan extension phenotype. Using a temperature-sensitive sterile mutant background (*glp-4*) enables us to focus on the somatic cells of the worms and avoids purging of ^{15}N label via egg laying. The *glp-4* mutation has only minimal effects on lifespan of wild-type and *daf-16* nematodes (TeKippe & Aballay 2010). However, it acts as an enhancer of *daf-2* longevity (McElwee *et al.* 2004; McColl *et al.* 2005), probably via surplus activation of DAF-16 (Hsin & Kenyon 1999; Lin *et al.* 2001; Arantes-Oliveira *et al.* 2002), which makes it an even better strain to study the DAF-16 effect on lifespan. MS proteome profiling of *glp-4(bn2ts)* revealed only modest changes relative to the N2 wild-type (Krijgsveld *et al.* 2003). The strategy of comparing *glp-4;daf-2* with *glp-4 daf-16;daf-2* control has been used earlier by others (McElwee *et al.* 2003; McElwee *et al.* 2004; Depuydt *et al.* 2013; Depuydt *et al.* 2014). Lifespan curves of these strains cultured under identical condition as in this study have been published earlier (Depuydt *et al.* 2013). For simplicity, these strains will be designated as control [*glp-4(bn2ts) daf-16(mgDf50); daf-2(e1370)*] and *daf-2* [*glp-4(bn2ts); daf-2(e1370)*] for the remainder of this article.

DAF-16 dependent increase in global proteomic half-life

In the SILeNCe experiment, worms were fed ^{15}N-labeled *E.coli* bacteria on the second day of adulthood, avoiding possible changes in relative protein composition related to development. From this moment, samples were taken at regular time points. Accurate mass spectrometry (MS)-based quantitative proteomics was performed in a randomized and blind manner. The resulting 43 MS-datasets were analyzed using a custom R package to extract the proportions of heavy (^{15}N) and light (^{14}N) isotope peaks of the peptides followed by estimation of the corresponding protein half-lives (Fig 1). Statistical differences were evaluated by using the moderated *t*-test from the *limma* R package (Ritchie *et al.* 2015). The use of a conservative pipeline enabled us to generate a very solid dataset, with the tradeoff of reduced protein diversity. Protein half-life was calculated if incorporation data of a certain

peptide was present for at least three sample time-points. Peptide half-lives were only compared between the reference strain and *daf-2* mutant strain, when at least two half-life estimations from two independent biological replicates were identified per strain. Applying this conservative pipeline, we were able to compare 345 peptide half-life calculations or 245 protein half-lives between the reference strain and *daf-2* mutant (Supplementary Figure 1A). Yet, the proteins covered in our experiment make up 30,8% of total worm mass as estimated from integrated proteomic *C. elegans* studies (Supplementary Figure 1B) (Wang *et al.* 2012; Wang *et al.* 2015).

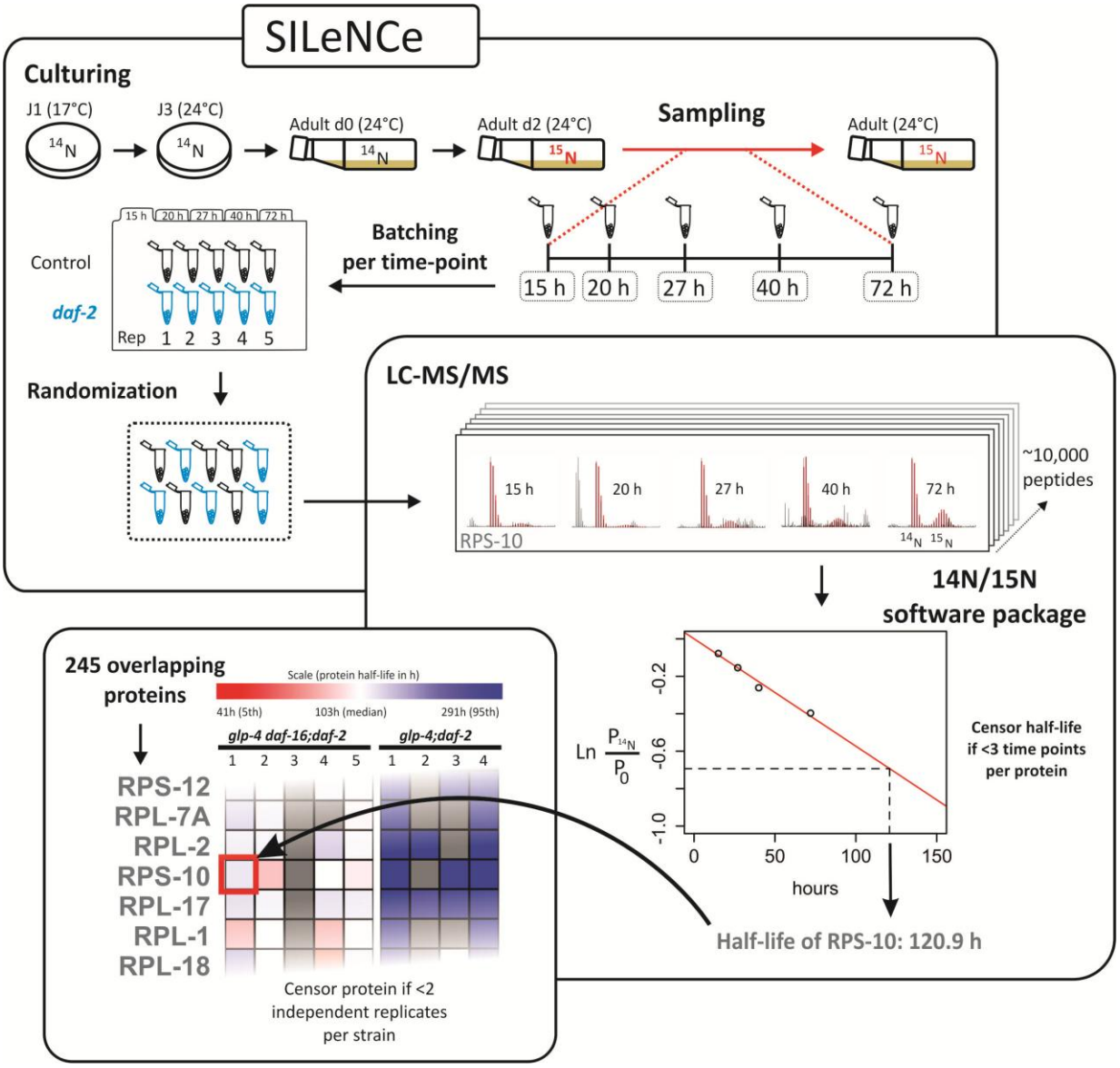


Fig. 1 A ^{15}N-metabolic labeling approach was performed to study individual protein turnover rates in long-lived *daf-2 C. elegans*. Adult worms were sampled at regular time-points after pulsing with ^{15}N-labeled *E. coli*. Samples were blocked based on the time of the pulse, randomized and blindly analyzed using LC-MS/MS. The resulting datasets were processed using a custom R package. Protein half-lives of 245 overlapping proteins between control and *daf-2* worms were estimated for five and four biological replicates, respectively.

We observed significantly decreased peptide turnover rates for 185 (i.e. 54%) of the analyzed peptides in *daf-2* compared with the control strain ($P < 0.05$). Only 5 peptides exhibit significantly faster replacement rates in *daf-2,* and turnover of 155 (i.e. 45%) peptides is unaltered (Fig 2A). Half-lives of redundant peptides from each protein were averaged. In total, we compared 245 overlapping non-redundant proteins from control and *daf-2* (Supplementary Dataset S1). Turnover rates vary widely, with half-lives spanning from 40 hours to more than 40 days. Mutation in *daf-2* leads to a shift in median protein half-life, from 103 hours to 173 hours (Fig 2B).

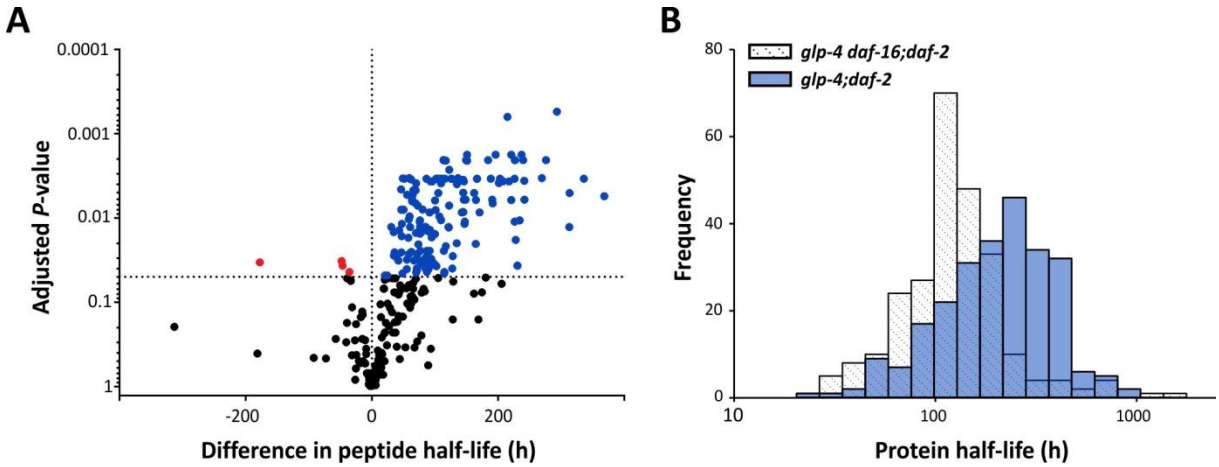


Fig. 2 Slow-down of protein turnover in *daf-2* (A) Volcano plot representing peptides with up regulated (red dots), down regulated (blue dots) and unchanged (black dots) turnover and their corresponding change in peptide half-life in *daf-2*. *P*-values were obtained from the moderated t-test implemented in the limma R package and adjusted for multiple testing according to Benjamini and Hochberg's method (Hochberg & Benjamini 1990). (B) Histogram showing the distribution of protein half-lives for control and *daf-2* worms. Median protein half-life shifts from 103 to 173 hours.

Change of protein half-life does not correlate with the change in abundance

We compared the relative changes in protein turnover with relative protein abundance shifts of the same strains published earlier by our group (Depuydt *et al.* 2013). Correlation analysis was performed on the 167 proteins in common between both datasets (Supplementary Dataset S2). Overall, the relative changes in protein abundance due to mutation in *daf-2*, do not correlate with those in protein turnover ($R^2 = 0.0054$, $P = 0.3447$ for the Pearson correlation, Fig 3). This lack of correlation indicates that the change in abundance of a particular protein is not necessarily dictated by its relative turnover. This is showcased in the fermentation enzymes SODH-1 and ALH-1: both proteins are very

responsive to stress (Bogaerts *et al.* 2010) and their mRNA and protein levels are very strongly up regulated in *daf-2* animals (McElwee *et al.* 2007; Depuydt *et al.* 2013), yet their protein half-lives are significantly increased (Fig 3 inset). This shows that certain proteins are maintained at high levels in the cell as indicated by their longer half-lives. Mitochondrial proteins show a similar pattern: their relative abundance is increased while turnover rates are decreased in *daf-2* mutants. As mitochondrial proteins represent a large fraction of the total cellular protein, this decrease in mitochondrial turnover may represent a considerable energy saving in *daf-2*. The ribosomal proteins, another family of highly abundant proteins, follow the opposite pattern: both turnover and relative abundance are decreased in *daf-2* mutants (Fig 3). Here the energy saving may be combined with an actual functional reduction as discussed below.

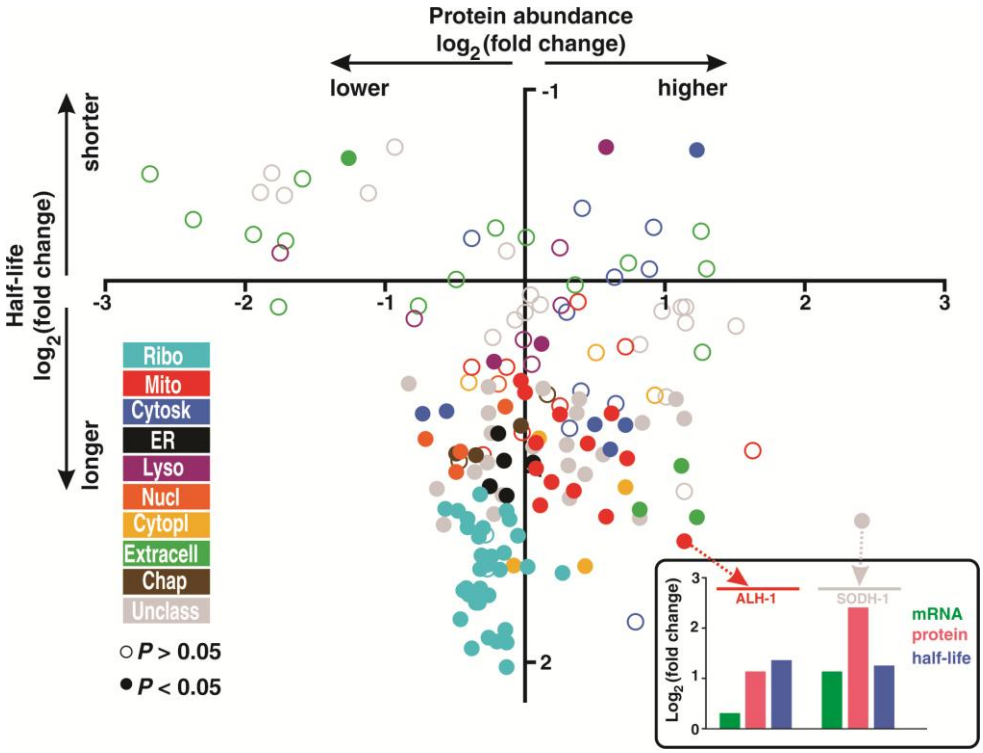
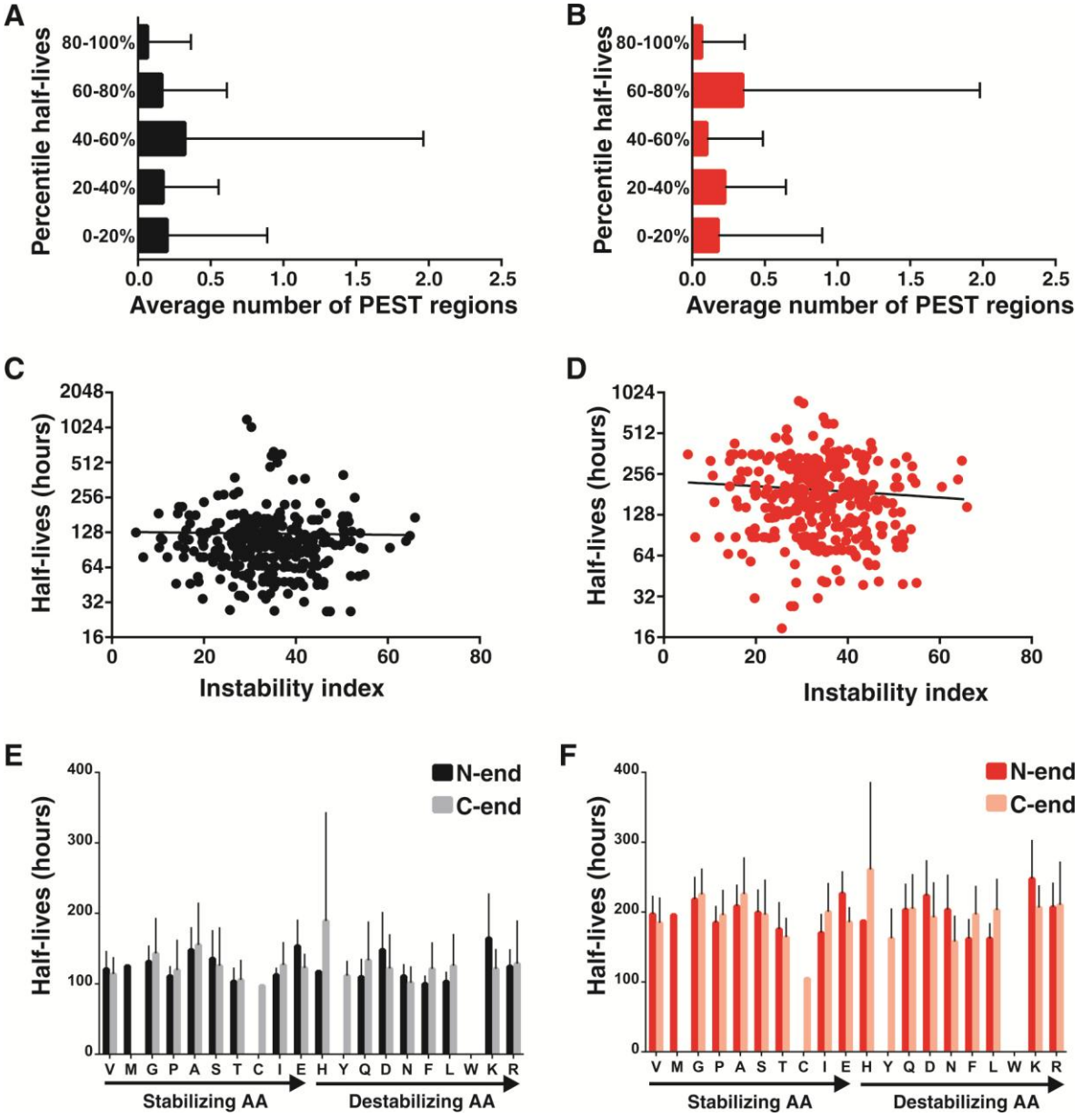


Fig. 3 Changes of protein turnover and abundance do not correlate in *daf-2*. Scatter plot illustrating the fold change (log_2) of protein abundance and protein turnover in *daf-2* relative to control (origin). Significant changes in protein turnover (*P*-value < 0.05) are signified with closed circles. *P*-values were obtained from the moderated *t*-test implemented in the *limma* R package and adjusted for multiple testing according to Benjamini and Hochberg's method (Hochberg & Benjamini 1990). Ribo: Ribosomal; Mito: Mitochondrial; Cytosk: Cytoskeletal; ER: Endoplasmic reticulum; Lyso: Lysosomal; Nucl: Nuclear; Cytopl: Cytoplasmic; Extracell: Extracellular; Chap: Chaperones; Unclass: Unclassified proteins. Inset: case study of SODH-1 and ALH-1 demonstrating discordance between transcript (McElwee *et al.* 2007) and protein (Depuydt *et al.* 2013) abundance versus protein half-life (fold change (log_2)) in *daf-2*.

Poor predictive power of proteolytic degradation signals

Proteins can be targeted for proteolytic degradation by the presence of structural protein characteristics, including the occurrence of high local concentrations of amino acids proline (P), glutamic acid (E), serine (S), threonine (T), known as PEST motifs (Rogers *et al.* 1986) and specific N-terminal amino acid sequences (Varshavsky 1997). Hence, we evaluated the relationship between these known proteolytic degradation signals and the estimated protein half-lives using online bioinformatics tools. Overall, no relationship was observed between the average number of PEST motifs and the corresponding protein half-lives (F-test, P = 0.3839 and P = 0.2585, for control and *daf-2*, respectively; Fig 4A,B). In addition, we analyzed the *in vivo* stability of proteins based on the N-end rule* (Supplementary Dataset S3).



* The N-end rule relates the regulation of the in vivo half-life of a protein to the identity of its N-terminal residue. The rule states that the N-terminal amino acid of protein determines its half-life (Varshavsky 1997).

Fig. 4 Comparison between predicted and observed protein stability. (A,B) Protein half-lives were binned using percentile ranges for control (A) and *daf-2* (B) worms. The average number of PEST regions is shown for each bin. Errors are standard deviations. (C,D) Scatterplot shows the computed instability index and experimentally observed protein half-life for each protein. Proteins with an instability index lower than 40 are predicted as stable, whereas predictions above 40 indicate unstable proteins. No evident correlation was observed for both controls (C) and *daf-2* (D) (F-test, *P* = 0.8407 and *P* = 0.1680, for control and *daf-2*, respectively). (E,F) Correlation analysis of the N- and C-terminal sequence with the experimentally observed protein half-lives for control (E) and *daf-2* (F) worms. Amino acid residues are ranked according to previously reported stability nature (Gonda *et al.* 1989; Varshavsky 1997). Errors are standard deviations.

The computed stability of proteins does not correlate with the protein half-lives observed in our study (F-test, *P* = 0.8407 and *P* = 0.1680, for control and *daf-2*, respectively; Fig 4C,D). Similar to what was found in a study on human cells (Boisvert *et al.* 2012), the amino acid identity at either the N-terminal or C-terminal positions was related to experimentally determined protein half-lives (Supplementary Dataset S3). We conclude that for both the N-terminal and C-terminal sequences, no obvious correlation with protein turnover is present (Fig 4E,F).

Turnover of the protein synthesis machinery is slowed down in *daf-2*

The half-life of proteins associated with the protein synthesis machinery is significantly increased in *daf-2* mutants. In control animals, ribosomal proteins have half-lives of approximately 100 hours, whereas in *daf-2* mutants, these figures are increased two- to fourfold. Proteins of the small (40S) and large (60S) ribosomal subunits show similar turnover rates and a comparable reduction in half-life was observed for both subunits in *daf-2* (Fig 5 and 6A). In addition, we find a similar pattern for translation factors, also involved in protein synthesis (Fig 6B).

Endoplasmic reticulum (ER)-bound ribosomes synthesize proteins, which are translocated into the ER lumen, where they are subsequently folded and assembled by aid of specialized proteins (Vazquez-Martinez *et al.* 2012). Interestingly, we found prolonged half-lives for all detected endoplasmic reticulum (ER) proteins predicted to be involved in these processes in the *daf-2* mutant (Fig 6C). The average protein half-life of ER proteins is only 86 h in control worms and shifts significantly towards 173 h in *daf-2* mutants (Two-sample Student's *t*-test,

P < 0.0001); these include the BiP/GRP78 homologs HSP-3 and HSP-4, and players in posttranslational modifications such as protein disulfide isomerase (PDI) and calreticulin (CRT-1).

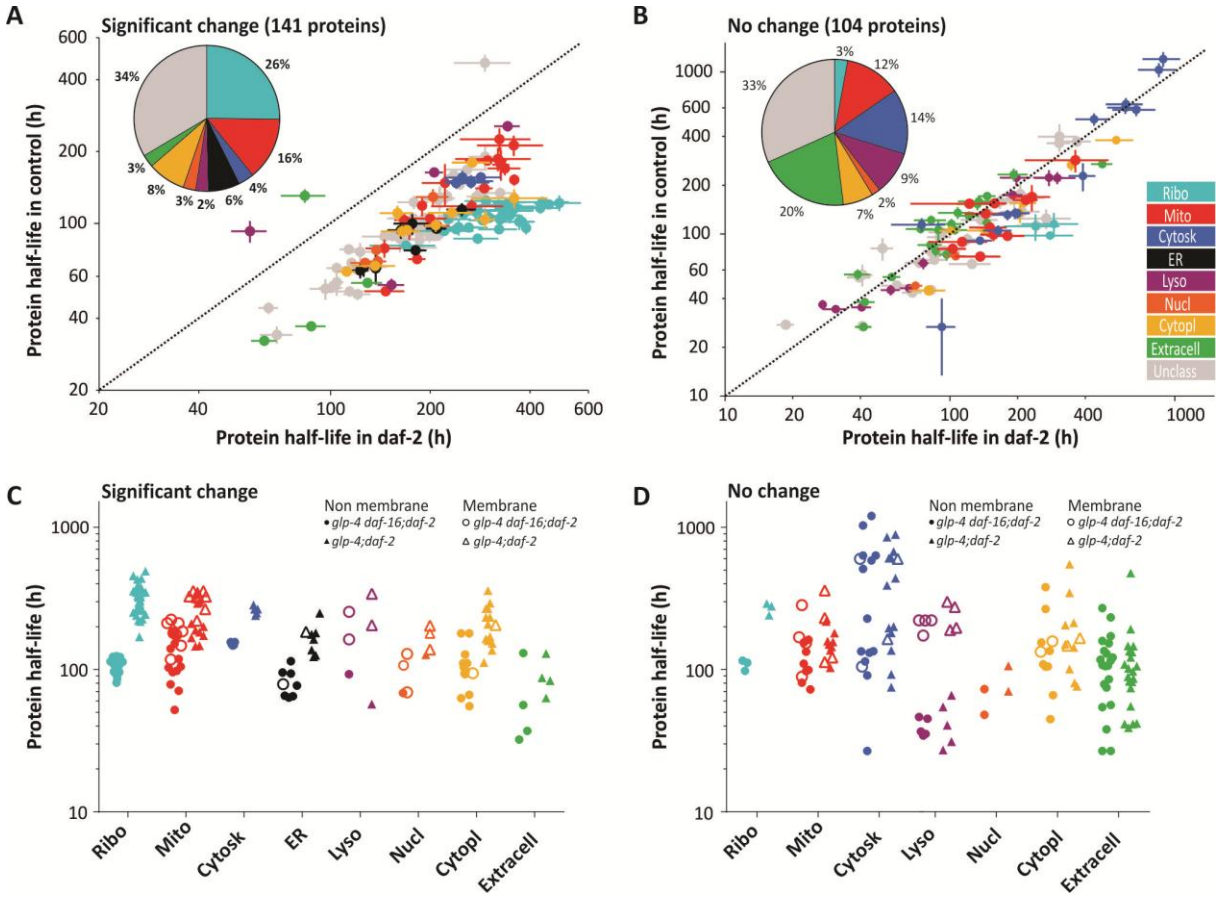


Fig. 5 Protein dynamics in subcellular compartments. (A, B) Scatterplot comparing significant (A) and non-significant (B) changes of protein half-lives in *daf-2* versus control. Functional groups are color-coded and summarized in pie-chart inserts. *P*-values were obtained from the moderated *t*-test implemented in the *limma* R package and adjusted for multiple testing according to Benjamini and Hochberg's method (Hochberg & Benjamini 1990). Errors are SEM. (C, D) Protein half-lives in different cellular compartments, with differentiation of membrane-bound proteins (open symbols) and free proteins (filled symbols). Significantly changed half-lives are represented in C (Two-sample Student's *t*-test, *P* < 0.05), non-significant changes are depicted in D. Ribo: Ribosomal; Mito: Mitochondrial; Cytosk: Cytoskeletal; ER: Endoplasmic reticulum; Lyso: Lysosomal; Nucl: Nuclear; Cytopl: Cytoplasmic; Extracell: Extracellular; Unclass: Unclassified proteins.

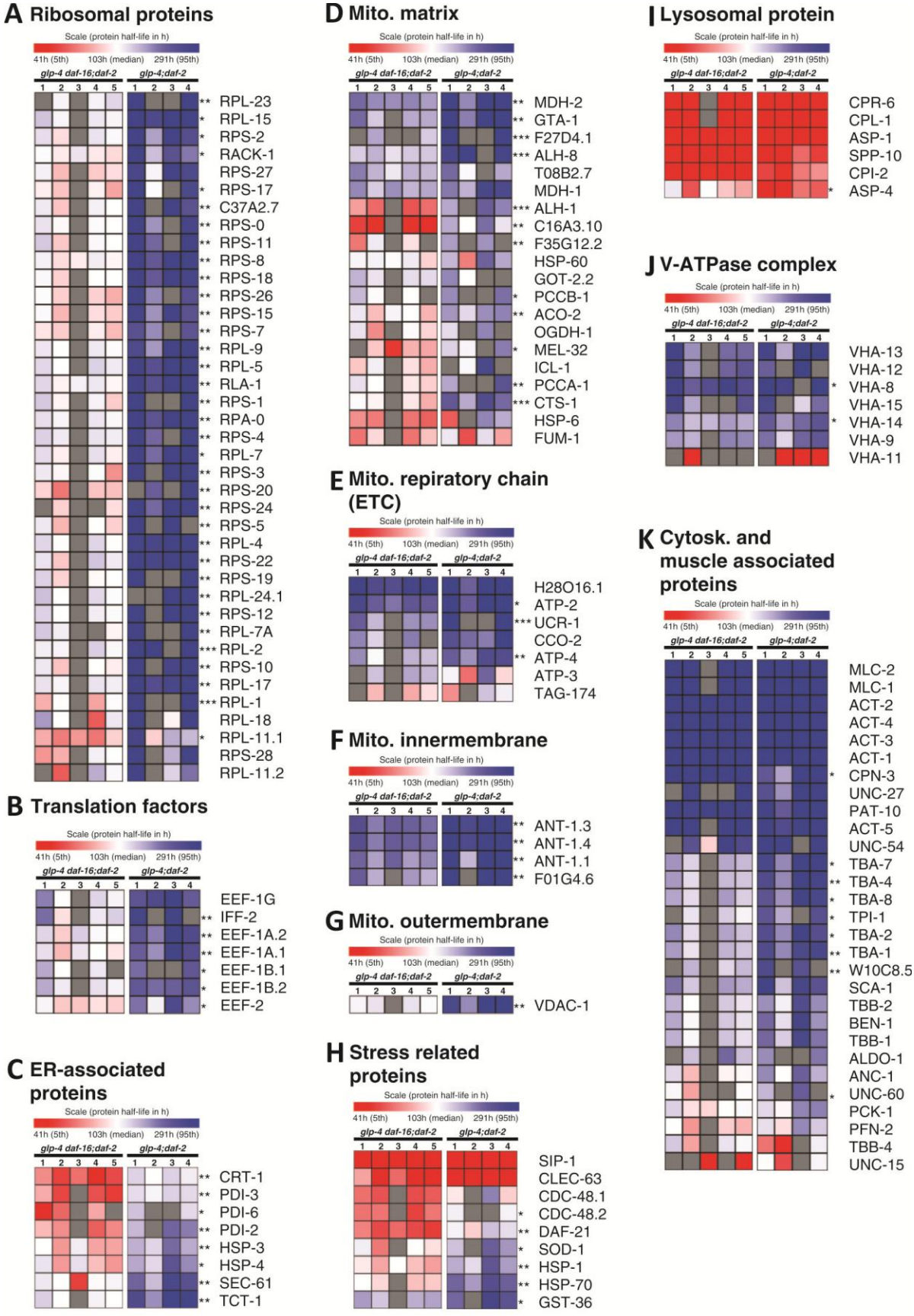

A Ribosomal proteins
Scale (protein half-life in h)
41h (5th) 103h (median) 291h (95th)
glp-4 daf-16;daf-2
glp-4;daf-2
** RPL-23
* RPL-15
* RPS-2
* RACK-1
RPS-27
* RPS-17
** C37A2.7
** RPS-0
** RPS-11
** RPS-8
** RPS-18
** RPS-26
** RPS-15
** RPS-7
** RPL-9
** RPL-5
** RLA-1
** RPS-1
** RPA-0
** RPS-4
* RPL-7
** RPS-3
** RPS-20
** RPS-24
** RPS-5
** RPL-4
** RPS-22
** RPS-19
** RPL-24.1
** RPS-12
** RPL-7A
*** RPL-2
** RPS-10
** RPL-17
*** RPL-1
RPL-18
* RPL-11.1
RPS-28
RPL-11.2
B Translation factors
EEF-1G
** IFF-2
** EEF-1A.2
** EEF-1A.1
* EEF-1B.1
* EEF-1B.2
* EEF-2
C ER-associated proteins
** CRT-1
** PDI-3
* PDI-6
** PDI-2
** HSP-3
* HSP-4
** SEC-61
** TCT-1
D Mito. matrix
** MDH-2
** GTA-1
*** F27D4.1
*** ALH-8
T08B2.7
MDH-1
*** ALH-1
** C16A3.10
** F35G12.2
HSP-60
GOT-2.2
* PCCB-1
** ACO-2
OGDH-1
* MEL-32
ICL-1
** PCCA-1
*** CTS-1
HSP-6
FUM-1
E Mito. repiratory chain (ETC)
H28O16.1
* ATP-2
*** UCR-1
CCO-2
** ATP-4
ATP-3
TAG-174
F Mito. innermembrane
** ANT-1.3
** ANT-1.4
** ANT-1.1
** F01G4.6
G Mito. outermembrane
** VDAC-1
H Stress related proteins
SIP-1
CLEC-63
CDC-48.1
* CDC-48.2
** DAF-21
* SOD-1
** HSP-1
** HSP-70
* GST-36
I Lysosomal protein
CPR-6
CPL-1
ASP-1
SPP-10
CPI-2
* ASP-4
J V-ATPase complex
VHA-13
VHA-12
* VHA-8
VHA-15
* VHA-14
VHA-9
VHA-11
K Cytosk. and muscle associated proteins
MLC-2
MLC-1
ACT-2
ACT-4
ACT-3
ACT-1
* CPN-3
UNC-27
PAT-10
ACT-5
UNC-54
* TBA-7
** TBA-4
* TBA-8
* TPI-1
* TBA-2
** TBA-1
** W10C8.5
SCA-1
TBB-2
BEN-1
TBB-1
ALDO-1
ANC-1
* UNC-60
PCK-1
PFN-2
TBB-4
UNC-15

Fig. 6 Individual protein half-lives of *daf-2* and control replicates. Heat map representation of individual protein half-lives of *daf-2* and control replicates. The colors indicates relative decrease (red) or increase (blue) of protein half-life compared to the median of the control strain (white, 103 h). Color limits signify the 5th (41 h) and the 95th (291 h) percentile. Each tile represents a biological replicate. *P*-values were obtained from the moderated *t*-test implemented in the *limma* R package and adjusted for multiple testing according to Benjamini and Hochberg's method (Hochberg & Benjamini 1990). Asterisks indicate significant differences between both strains; $^{(*)}$ $P < 0.05$, $^{(**)}$ $P < 0.01$, $^{(***)}$ $P < 0.001$, respectively.

In the same line, transmission electron microscopy (TEM) images show a loose pattern of rough ER (RER) cisternae dispersed through the cytoplasm, sparsely studded with ribosomes, in *daf-2* mutants (Fig 7). This is in contrast to controls, where abundant RER is densely stacked in Terasaki ramps to accommodate maximum protein synthesis within the confined cells (Terasaki *et al.* 2013; Heald & Cohen-Fix 2014). However, despite the dispersed RER arrangement, decreased ribosomal load and lower refreshment rates of ER-associated proteins, half-lives of RER-processed proteins (secretory proteins and lysosomal proteins), remain unchanged in *daf-2* mutants (Fig 5A,B inset).

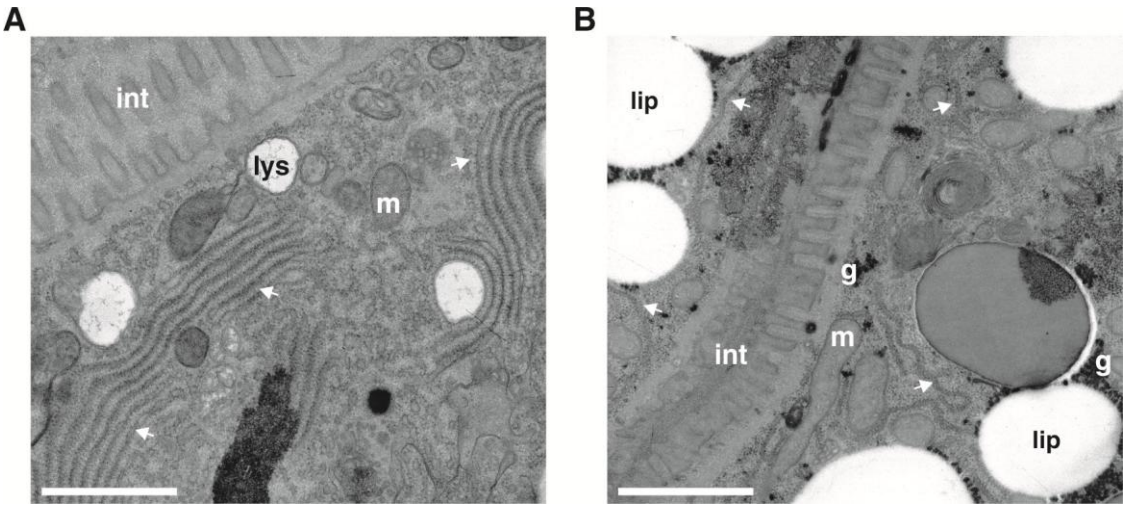


Fig. 7 Organization of rough endoplasmic reticulum (A, B) Transmission electron micrographs of intestinal cells of control (A) and *daf-2* worms (B). *daf-2* mutants maintain relatively high glycogen (g) and fat (lip) reserves, two sources of energy storage synthesized and accumulated during development. During adulthood, *daf-2* worms develop an Eat phenotype and rely on the economical use of these energy reserves (Depuydt *et al.* 2014). Arrows indicate RER; int: intestine; lys: lysosome; m: mitochondria; g: glycogen; lip: lipid. Scale bar is 1 µm.

Protein turnover in long-lived *C. elegans*

Lysosomal protein turnover: high in the lumen but low in the membrane

Autophagy is the major degradation pathway of macromolecules and organelles and this process involves lysosome-mediated catabolism (Yorimitsu & Klionsky 2005). We identified four lysosomal proteins for which protein turnover is very fast (half-life on average 38 h) and remains unchanged in controls and *daf-2* mutants, whereas protein half-life of only one hydrolase, the cathepsin ASP-4 (1.6-fold down; $P = 0.0436$) is significantly altered (Fig 6I). Acidification of the lysosomal lumen depends on the activity of proton pumps, vacuolar-type H^+ ATPase complexes, present in the lysosomal membrane (Mindell 2012). As opposed to the fast intraluminal hydrolase turnover, we found very slow replacement rates for subunits of the V1 cytosolic complex in both control and *daf-2* worms (half-life on average 225 h; Fig 6J). Only two subunits, VHA-8 and VHA-14, show a slight but significant reduction of protein half-lives in *daf-2* ($P = 0.0328$ and $P = 0.0319$, respectively). In contrast, we discovered very fast refreshment rates for VHA-11, which is the subunit C homolog of the V1 complex. This subunit has a short protein half-life in control worms (50 h, 1 replicate), and exceptionally high turnover in *daf-2* worms (half-life on average 9 h, 3 biological replicates). In general, however, we observed significantly longer protein half-lives for membrane-associated proteins compared to intraluminal proteins for both controls and *daf-2* mutants (Unpaired Student's *t*-test, $P < 0.0001$ and $P = 0.00021$, respectively). A similar distinction between membrane-bound and free proteins was observed within mitochondria.

Mitochondrial turnover is decreased in *daf-2*

Mitochondrial proteins may be vulnerable to oxidative damage due to their proximity to ROS-generating centers (Murphy 2009). Therefore it could be expected that mitochondrial proteins exhibit high refreshment rates to maintain their important vital function. Counter to prediction, 65% of the identified mitochondrial proteins exhibit longer half-lives in *daf-2* compared to the control ($P < 0.05$) while the remaining proteins do not show any significant change.

As in lysosomes, turnover of membrane-bound mitochondrial proteins is significantly slower compared to the turnover of free proteins for both strains studied, although this difference

is less prominent in *daf-2* mutants (Fig 5C-D; Two-sample Student's *t*-test, $P < 0.0001$ and $P = 0.0096$, respectively).

Widely divergent protein half-lives were found for mitochondrial matrix proteins, ranging from 52 to 183 hours in controls and 103 to 358 hours in *daf-2* mutants (Fig 6D). In addition, mitochondrial protein turnover shows limited uniformity within biochemical pathways in control worms (Supplementary Figure 2). For instance, the citric acid cycle enzymes fumarase (FUM-1) and malate dehydrogenase (MDH-1), show half-lives of 81 and 162 hours, respectively (Supplementary Figure 2B). Nevertheless, an overall reduction in protein half-life of the carbohydrate metabolic enzymes is observed in *daf-2* (Supplementary Figure 2). Proteins of the mitochondrial inner membrane, including subunits of the electron transport chain and adenine nucleotide transporters, display very slow turnover rates in both strains (Fig 6E-G). Despite the fact that the voltage-dependent anion channel 1 (VDAC-1) is the only detected outer membrane protein in our dataset, we observe significantly faster turnover for this protein compared to inner membrane proteins in controls (Unpaired Student's *t*-test, $P < 0.0001$), whereas this difference was negligible in *daf-2* mutants (Fig 6F-G).

Minimal turnover of cytoskeletal and muscle related proteins

Proteins of the muscle contractile apparatus, such as the thin filament actins and myosin class II light chain proteins, exhibit extremely long protein half-lives (on average 25 and 41 days, respectively; Fig 6K). These numbers suggest that myofilaments only undergo occasional turnover during the entire *C. elegans* lifespan. We observed slow turnover for the isoform myosin class II heavy chain protein UNC-54 (half-life on average 298 h), but we did not detect any label incorporation at all for the other three isoforms, which suggests a complete lack of turnover. On the other hand, the invertebrate-specific paramyosin UNC-15, which interacts with these heavy chain proteins, shows remarkably fast turnover in both control and *daf-2* mutants (half-life on average 66 h). Other proteins involved in muscle physiology show moderate protein half-lives in controls (140 h on average), and are somewhat delayed in *daf-2* mutants without reaching significance (202 h on average). Only the putative creatine kinase W10C8.5 and triosephosphate isomerase TPI-1 have significantly reduced turnover rates in *daf-2* ($P = 0.0062$ and $P = 0.0293$, respectively). These

proteins, involved in the supply of energy molecules in muscle cells (Ralser *et al.* 2007; Wallimann *et al.* 2011), show strong DAF-16 dependent up-regulation (Depuydt *et al.* 2013). Increased protein levels in combination with lower rates of turnover suggest an upkeep of these proteins.

Low turnover was also found for cytoskeletal proteins that reside in the cytoplasm. ACT-5, required for the formation of actin filaments in intestinal cells, displays a long protein half-life analogous to the muscle actins. The constituents of microtubules, alpha- and beta-tubulins, are less stable, with an average half-life around 141 hours in controls. Protein half-lives of beta-tubulins remain unchanged in *daf-2* (168 h on average), whereas turnover of alpha-tubulins is significantly attenuated (half-life on average 261 h; $P < 0.05$).

Reduced turnover of stress response proteins in *daf-2*

Much evidence supports the strong correlation between longevity and cytoprotective mechanisms, linking lifespan extension to an increased tolerance to a variety of stressors (Shore *et al.* 2012). We observed a wide range of stress protein half-lives, ranging from 27 to 159 hours in controls and 41 to 236 hours in *daf-2* mutants (Fig 6H). Stress-induced cytosolic chaperones, including heat shock proteins (HSPs), show fast to moderate replacement rates in controls (half-life on average 68 h). Surprisingly, these proteins show reduced protein turnover in long-lived *daf-2* mutants (half-life on average 132 h), with a significant diminution for DAF-21, HSP-1, HSP-70 and CDC-48.2. These observations are in line with the extended protein half-lives for HSPs of the ER, as described above. Several HSPs are down-regulated in *daf-2* (Depuydt *et al.* 2013), including DAF-21 and HSP-1, which implies that, counter to expectation, *daf-2* worms invest less energy in the overall presence and turnover of HSPs. In addition, other proteins involved in cytoprotection showed a similar pattern, including glutathione-S-transferase GST-36 (1.48-fold down; $P = 0.0107$), involved in xenobiotic detoxification (Ayyadevara *et al.* 2007), and superoxide dismutase SOD-1 (Fig 6H; 1.82-fold down; $P = 0.0144$). However, not all *daf-2* stress proteins show decreased turnover, e.g. turnover of SIP-1 (half-life on average 33 h), a small heat shock protein, is unaffected in *daf-2* mutants. A similar pattern was observed for CLEC-63, an antimicrobial protein involved in the worms' innate immunity (half-life on average 49 h). Our data suggest

that, to some extent, *daf-2* saves energy by reducing the turnover of specific cytoprotective proteins.

3.4 Discussion

Proteins with long dwelling times are likely to accumulate all sorts of molecular damage during aging. Thus, one could expect that by increasing protein turnover rate, cellular damage is prevented and lifespan is extended. However, several studies are in conflict with the turnover hypothesis, as they observe overall decreased protein synthesis rates in *C. elegans* longevity mutants or, vice versa, translation inhibition results in lifespan extension (Hansen *et al.* 2007; Pan *et al.* 2007; Syntichaki *et al.* 2007; Yang *et al.* 2007; Van Raamsdonk & Hekimi 2009; Depuydt *et al.* 2013; Stout *et al.* 2013). Therefore, we hypothesized that, in *daf-2* mutants, energy is saved by down-regulation of turnover of the majority of proteins, and reinvested in prioritized turnover of specific proteins that are crucial to somatic maintenance. To that end, we investigated turnover dynamics of individual proteins using a SILeNCe metabolic labeling method. Contrary to our hypothesis, we did not discover a delineated set of proteins with turnover priority in *daf-2* mutants. The majority of the detected proteins (56%) exhibit prolonged half-lives in *daf-2*, while turnover of the remaining proteins is unchanged. Only three proteins (CPN-3, ASP-4 and VIT-6) display marginally significant higher turnover rates in *daf-2*, yet, they lack a clear biological relationship.

One of our most notable observations is the drastic slow-down in turnover of the translation machinery in *daf-2* mutants. This slow-down coincides with decreased levels of ribosomal proteins and enzymes with predicted function in ribosome assembly and biogenesis that we and others observed earlier (Depuydt *et al.* 2013; Stout *et al.* 2013) and probably relates to the decreased protein synthesis rates in this mutant. Corroborating this finding, recent work suggests a specific translational block in *daf-2* by specific binding of the long non-coding RNA *tts-1* to the ribosomes (Essers *et al.* 2015).

Our observation of decreased protein turnover in *daf-2* mutants is not entirely surprising: the insulin/IGF1 signaling pathway is a main activator of anabolic metabolism, hence, it is therefore conceivable that mutants in this pathway show reduced protein turnover. This

reduction allows the worm to save much energy, which may be diverted to other processes that support longevity, such as the synthesis of trehalose, a chemical chaperone that stabilizes membranes and proteins, for which a role in *daf-2* longevity has already been shown (Honda *et al.* 2010). Additionally, the overall slow protein turnover agrees with the hyperfunction theory, stating that aging is caused by excess biosynthesis (hypertrophy) (Blagosklonny 2007b; Gems & de la Guardia 2013). Thus, inhibited insulin/IGF-1 signaling reduces anabolic pathways, which in turn lowers hypertrophy, decelerating the onset of its related pathologies.

Earlier, it has been shown that reduced insulin/IGF-1 signaling and dietary restriction rely on autophagy to extend lifespan (Melendez *et al.* 2003; Hansen *et al.* 2008). Hence, the preserved turnover rates of lysosomal proteins might be crucial to sustain autophagy in *daf-2* mutants. However, our data do not support increased bulk protein degradation as most protein half-lives are prolonged or unchanged in *daf-2*. Turnover rates of the lysosomal hydrolases are among the fastest found in the cell, which can be explained by the fact that the lysosomal lumen is a strong proteolytic environment in which accidental breakdown of lysosomal components is to be expected. Opposite to these hydrolases, subunits of the V-type ATPase proton pumps, present in the lysosomal membrane, undergo slow turnover, indicating that lysosomes, apart from their content, are not rapidly replaced as a whole. Also V-type ATPase subunits are part of large complexes, which may be intrinsically more stable and resilient to protein breakdown. One remarkable exception is the subunit C homolog VHA-11, which is a subunit that is known to reversibly dissociate from this complex in yeast cells (Iwata *et al.* 2004). It shows extremely fast turnover in controls, which is even enhanced in *daf-2* mutants. V1/V0 dissociation is thought to be an energy-conserving response (Kane 2006) and recent work shows a general role of V-type ATPase complexes in controlling metabolic programs (Zhang *et al.* 2014). It is possible that VHA-11 functions as an energy-dependent switch, down-regulating lysosomal acidification and protein degradation in the *daf-2* mutant. More detailed evaluation of a lysosomal role in the regulation of protein metabolism in *daf-2* is necessary.

Similar to lysosomes, we detected a clear discrepancy in turnover between free and membrane-bound mitochondrial proteins. In addition, half-lives of mitochondrial proteins vary widely and no uniformity is found within biochemical pathways. Furthermore, the outer

membrane protein VDAC-1 showed higher turnover rates compared to those of inner membrane proteins, an observation that was also reported for mouse cells (Kim *et al.* 2012; Lau *et al.* 2012). This variability in turnover, which is easily overseen in whole-mitochondrial turnover studies, suggests that mitophagy is not the sole or predominant process for elimination of mitochondrial proteins in *C. elegans* under normal conditions. Instead, a mixture of quality control mechanisms, including matrix proteases and extra-mitochondrial degradation mechanisms (20S proteasome), are clearly co-responsible for mitochondrial protein dynamics (Lau *et al.* 2012).

Our data disproves that *daf-2* longevity is supported by enhanced mitochondrial turnover rates, as the majority of protein half-lives in this organelle is down-regulated. One may argue that this is because *daf-2* worms activate their antioxidant defense systems (Honda & Honda 1999) making the worms less dependent on high turnover of mitochondrial components to avoid damage accumulation. However, it should be noted that levels of oxidative damage in mitochondria of young adult wild type and *daf-2* animals are similar (Brys *et al.* 2010) and that *in vivo* hydrogen peroxide production in the muscle is not altered in young adult *daf-2* (Knoefler *et al.* 2012). Moreover, expression of *sod* is not required for lifespan extension in *daf-2* mutants (Doonan *et al.* 2008). Despite their low turnover, *daf-2* mitochondria are highly abundant (Depuydt *et al.* 2013) and show enhanced coupling (Brys *et al.* 2010). Our previous experiments suggest that in this mutant, oxidative phosphorylation may be supported by anaerobic mitochondrial pathways. Although our data challenge extensive recycling of mitochondrial components in the long-lived *daf-2*, selective and specific autophagy of damaged mitochondria might be necessary for its increased lifespan as recently suggested (Palikaras *et al.* 2015a).

Proteins of the muscle contractile apparatus undergo extremely slow turnover in both controls and *daf-2* worms. Despite their high abundance, half-lives of myosin class II heavy chain proteins could not be estimated, which is indicative of rare replacement events. These results are consistent with those reported in recent work on protein synthesis during *C. elegans* lifespan (Vukoti *et al.* 2015), reporting slow appearance of newly synthesized motility-related proteins. Although reduced insulin/IGF1 signaling is often associated with muscle atrophy in mammalian systems (Sandri *et al.* 2004), our previous work showed highly preserved biomass of striated body wall muscle in *daf-2* compared to controls (Depuydt *et*

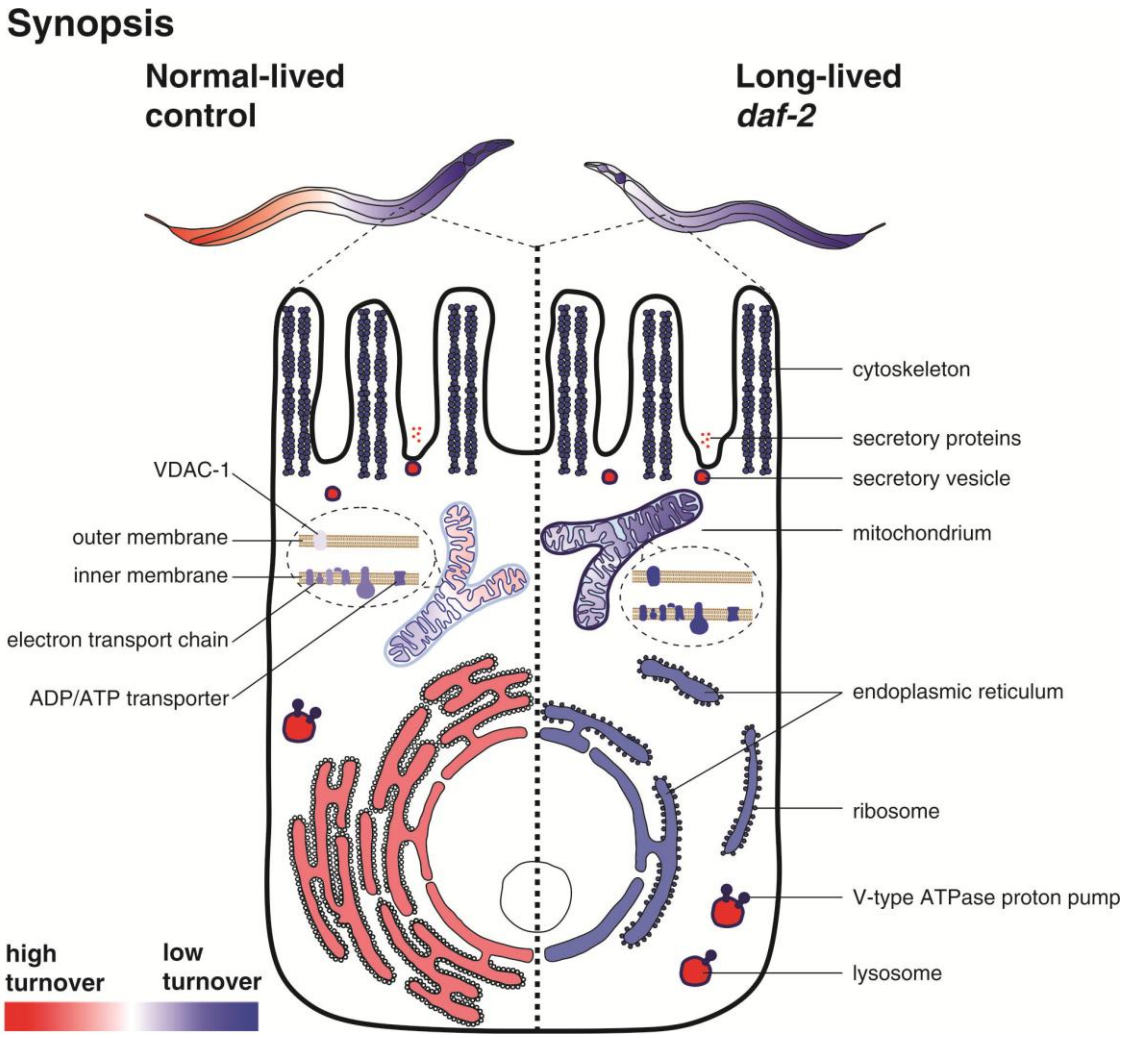
al. 2013). In addition, the upkeep of mitochondrial proteins further supports the maintenance of muscle functionality in these mutants. These findings do not exclude the possibility that non-filamental muscle proteins undergo more rapid and even enhanced turnover in *daf-2*, as was suggested in a study using a LacZ reporter (Szewczyk *et al.* 2007). Cytoskeletal components that are not restricted to muscle cells show medium turnover rates, for which only alpha-tubulins are significantly attenuated in *daf-2*, suggesting slightly reduced cellular dynamics in these mutants compared to controls.

Contrary to the extremely slow turnover observed for cytoskeletal components, fast to moderate turnover was detected for proteins involved in cytoprotective mechanisms in control worms. Although microarray studies show strong DAF-16-mediated up regulation of stress-related genes (McElwee *et al.* 2003; Murphy *et al.* 2003), several HSPs are down regulated at the protein level (Depuydt *et al.* 2013) and show a prolonged half-life in *daf-2* mutants in this study. The slow turnover of these chaperones could be related to the recent observation that small HSPs are mostly enriched in the insoluble fraction of old *daf-2* worms, playing a role in protective aggregate formation to maintain the proteome balance (Walther *et al.* 2015). However, it is currently not clear whether these phenomena would be of any significance in the young worms used in our study. Nevertheless, some cytoprotective proteins do show unchanged fast turnover, implicating the complex and specific regulation of stress proteins in *daf-2* mutants.

In summary, we observed an overall slow-down of protein turnover in long-lived *daf-2* worms using a SILeNCe approach. Most prominently, this was reflected in, and probably caused by drastic lowering of translation machinery turnover in *daf-2*. In agreement with our observations, researchers demonstrated extended ribosomal and mitochondrial half-lives in long-lived caloric restricted mice (Price *et al.* 2012; Karunadharma *et al.* 2015). Hence, it seems that high protein turnover is not essential to support lifespan extension but it is still unclear whether the observed slow-down of protein turnover is a causal factor. Recent evidence, showing that alleviation of protein synthesis inhibition in *daf-2* mutants partially rescues its longevity phenotype, strongly supports this causal relation (Essers *et al.* 2015). Reduced protein turnover may be related to some typical features of the *daf-2(e1370)* mutant, including the reduced food uptake, altered amino acid metabolism and a lower energetic flux (Fuchs *et al.* 2010; Depuydt *et al.* 2014). These *daf-2(e1370)* phenotypes

possibly support silenced anabolism and consequently, decreased global proteome turnover. However, this does not lead to an inferior proteome in *daf-2* worms, as was shown by their well-maintained muscle mass (Depuydt *et al.* 2013) and mitochondrial function (Brys *et al.* 2010), locomotive ability (Gems *et al.* 1998) and metabolic capacity upon mechanical stimulation (Braeckman *et al.* 2002b). Therefore, we believe that reduced insulin/IGF-1 signaling results in an energy-saving state along with the improved protection of proteins. The preserved somatic integrity in *daf-2* possibly relies on chemical chaperones (Honda *et al.* 2010) or small heat-shock proteins (Walther *et al.* 2015). Our findings and suggestions are also compatible with the hyperfunction theory which proposes that the attenuation of protein synthesis might reduce hypertrophy, thereby retarding the accumulation of irrelevant macromolecules that otherwise cause age-related cellular malfunction (Blagosklonny 2012; Gems & de la Guardia 2013).

Protein turnover in long-lived *C. elegans*

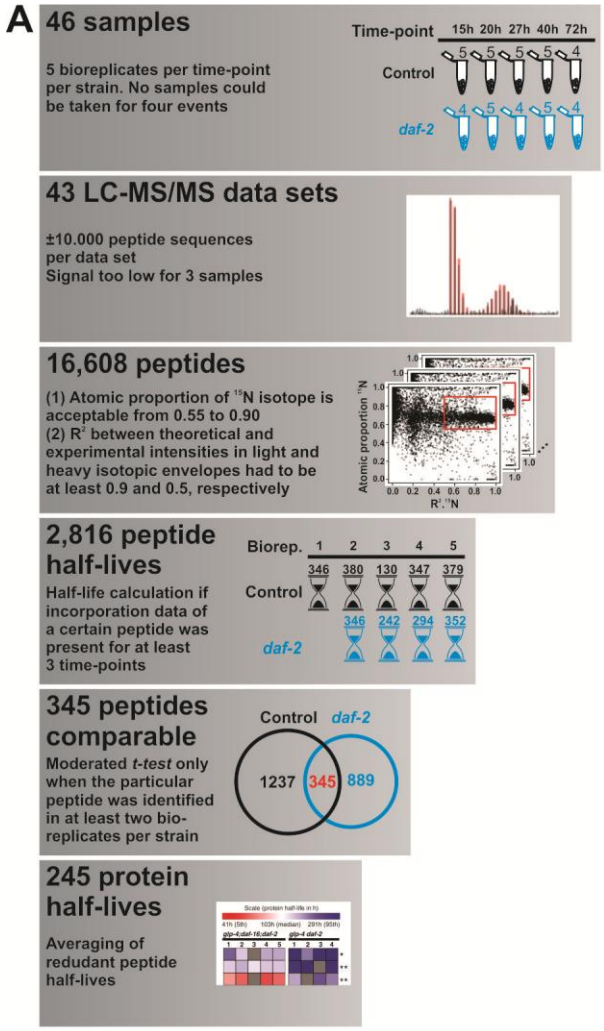


Synopsis: Stable Isotope Labeling by Nitrogen in *Caenorhabditis elegans* (SILeNCe) approach revealed individual protein turnover dynamics in the long-lived Insulin/Insulin-like Growth factor (IGF-1) receptor mutant *daf-2*.

- **Approximately 56% of the *daf-2* proteome showed decreased turnover.**
- **This decrease was most prominent for components of the translation machinery.**

Our data contradicts the protein turnover hypothesis and indicates that high protein turnover is not essential to support lifespan extension.

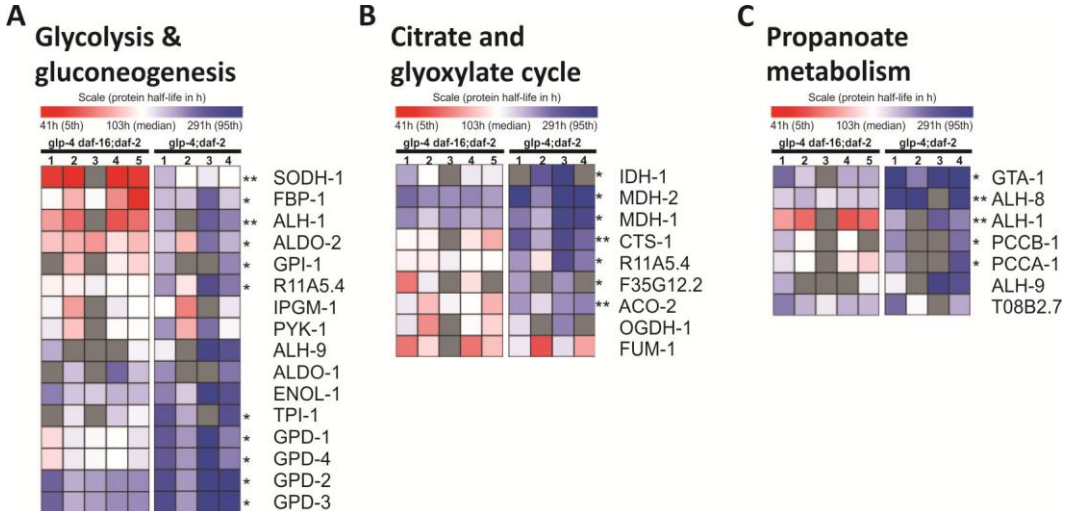
3.5 Supplementary information



Supplementary Figure 1 – Workflow and data filtering

A Schematic representation of the proteomic analysis illustrating the conservative approach to compare protein half-lives between control and long-lived *daf-2* worms.

B Abundance of proteins detected in ten integrated deep proteomic studies on *C. elegans* (gray) and this study (red). Protein abundance was determined via spectral counting and re-scaled to parts per million (datasets are available online via http://pax-db.org). Proteins in this study add up to 30.8% of total protein abundance of the integrated deep proteomics studies.



Supplementary Figure 2 - Turnover of proteins involved in carbohydrate metabolism

The color bar is limited from the 5th percentile (41 h, red) to the 95th percentile (291 h, blue) of protein half-lives determined in our data set. Median half-life (103 h) is represented in white. Each tile signifies a biological replicate. *P*-values were obtained from the moderated *t*-test implemented in the *limma* R package and adjusted for multiple testing according to Benjamini and Hochberg's method (Hochberg & Benjamini 1990). Asterisks indicate significant differences between both strains; $^{(*)}$ $P < 0.05$, $^{(**)}$ $P < 0.01$, respectively.

Supplementary Datasets:

- Table S1 presents an overview of peptide and protein half-lives detected in this study.
- Table S2 presents the comparison between protein turnover and protein abundance (data from previous study Depuydt *et al.* 2013) as in Fig. 3.
- Table S3 presents the results of N-end rule and PEST motif analysis as in Fig. 4.
- Table S4 outlines the randomized study-design.
- Table S5 summarizes the functional annotation analysis as in Fig. 5,6 and S2.

3.6 Materials and methods

Strains

The following strains were used for proteomics and TEM analysis: GA154 *glp-4(bn2ts)I;daf-2(e1370)III* (long-lived) and GA153 *glp-4(bn2ts)I daf-16(mgDf50)I; daf-2(e1370)III* (control strain) which were kindly provided by David Gems at the University College of London. Strains were maintained as described previously (Depuydt *et al.* 2013).

SILeNCe (Stable Isotope Labeling by Nitrogen in *Caenorhabditis elegans):*

Metabolic ^{15}N- and ^{14}N-labeling of the *Escherichia coli* bacteria

E. coli K12 was freshly grown overnight at 37°C at shaking at 120 rpm in minimal medium, containing 42 mM Na_2HPO_4, 22 mM $KHPO_4$, 9 mM NaCl, trace elements (17 mM EDTA, 3.1 mM $FeCl_3 \cdot 6H_2O$, 0.62 mM $ZnCl_2$, 0.076 mM $CuCl_2 \cdot 2H_2O$, 0.042 mM $CoCl_2 \cdot 6H_2O$, 0.16 mM H_3BO_3, 0.0068 mM $MnCl_2 \cdot 6H_2O$), glucose 20% (w/v), 1 mM $MgSO_4$, 0.3 mM $CaCl_2$, 0.0041 mM biotin, 0.0033 mM thiamin, and 93 mM ^{15}N- or ^{14}N-containing NH_4Cl (sterilized over 0.22 µm filter). Overnight cultures were concentrated 50-fold.

Culturing and sampling of *C. elegans*

Synchronized L1 nematodes were plated on nitrogen-free agarose (1.2%) plates containing NaCl (0.3%), cholesterol (0.0005%), 1 mM $CaCl_2$, 1 mM $MgSO_4$ and 25 mM K_2HPO_4/KH_2PO_4 (pH 6.0) and a lawn of freshly grown ^{14}N-labeled *E. coli* K12 cells. In order to prevent *glp-4(bn2ts)I;daf-2(e1370)III* mutants to enter the dauer stage, worms were grown at 17 °C until the third larval stage (L3) and then switched to 24°C for the remainder of the experiment. Worms that had freshly moulted to adults (adult day 0) were washed three times and transferred into culture flasks (not exceeding 1500 worms/ml) containing S-basal (100 mM NaCl, 50 mM potassium phosphate, pH 6.0), 12.93 µM cholesterol, 75 µM 5-fluoro-2'-deoxyuridine (Acros Organics, Geel, Belgium), and ^{14}N-labeled *E. coli* K12 cells (A_{550} = 1.0). At day two of adulthood, worms were collected and washed thoroughly (2x S-basal, 1x S-basal containing 2.5 mM EDTA, 2x S-basal) to remove bacteria. Next, worms were pulsed in new culture flasks containing S-basal, 12.93 µM cholesterol, 75 µM 5-fluoro-2'-deoxyuridine

(Acros Organics, Geel, Belgium), and ^{15}N-labeled *E. coli* K12 cells (A_{550} = 1.0). From this moment, worms were sampled after 0, 15, 20, 27, 40 and 72 hours of ^{15}N pulsing. Animals were washed thoroughly (2x S-basal, 1x S-basal containing 2.5 mM EDTA, 2x S-basal) to remove bacteria, after which dead worms were removed via Percoll (Sigma-Aldrich) washing. The worm pellet was resuspended in 200 µl denaturing solution (8 M urea, 1 mM EDTA, 10 mM DTT, 50 mM TrisHCl, pH 8.0) and immediately dropwise frozen in liquid nitrogen and stored at -80°C. The sample size was chosen based on a previously published study (Depuydt *et al.* 2013): we sampled all the different post-pulse time-points in five biological replicates for both *glp-4(bn2ts)I daf-16(mgDf50)I; daf-2(e1370)III* and *glp-4(bn2ts)I;daf-2(e1370)III*.

Quantitative proteomics:

Randomized study design

Samples were processed in batches comprising all the biological replicates of both control and *daf-2* worms sampled at 1 time-point post-labeling. Samples within a batch were analyzed blindly and in random order (Supplementary Dataset S4).

Preparation of the tryptic peptide samples

The frozen worm beads (~1500 worms in denaturing solution) were homogenized using a BioPulverizer (BioSpec, Bartlesville, USA) pre-conditioned in liquid nitrogen. The fine powder was recovered into 1.5 ml tube, thawed, centrifuged (2 min, 5000 rpm), and sonicated for 30 s in a 5510 Branson ultrasonic water bath (Branson Ultrasonics, Soest, Netherlands). Protein concentrations were determined using Coomassie assay. For further processing steps we took the aliquots containing 150 µg of protein and readjusted the volume with denaturation buffer. The samples were subjected to cysteine alkylation by 40 mM iodoacetamide for 1 hour at 37°C in dark shaking conditions (900 rpm), 4-fold dilution with 50 mM ammonium bicarbonate (pH 7.8), and digestion with trypsin (3 µg per sample, Promega) for 12 hours at 37°C shaking conditions (400 rpm). Next, tryptic peptide samples were cleaned-up via C-18 SPE columns (Discovery DSC-18, SUPELCO, 52601-U) and concentrations were adjusted to 0.2 µg/µl (Petyuk *et al.* 2010b; Depuydt *et al.* 2013).

LC/MS analysis and peptide identification

Each individual sample was analyzed with a constant-pressure capillary HPLC system coupled online to an LTQ Orbitrap mass spectrometer (Thermo Fisher, San Jose, CA) using an electrospray ionization interface. Instrument settings were described previously by Depuydt et al. (2013). The resulting 56 datasets were converted to the mzXML format using MSConvert (settings: --mzXML --32 -z), a part of the ProteoWizard software suite (Kessner *et al.* 2008). Peptide identification was performed by preprocessing datasets with DeconMSn (Mayampurath *et al.* 2008), DtaRefinery (Petyuk *et al.* 2010a) followed by the database search tool MS-GF+ (Kim & Pevzner 2014). The search was aimed at only light ^{14}N-peptides as we did not anticipate more than 50% label incorporation in such a short labeling pulse. The key MS-GF+ settings are: PMTolerance = 10 ppm; NumMods = 3; StaticMod = C2H3N1O1, C, fix, any, Carbamidomethyl; DynamicMod = None; EnzymeID = 1; IsotopeError = 0.1; NTT = 2; minLenght = 6; maxLenght = 50; minCharge = 1, maxCharge = 4. The results of the MS/MS searches were saved as mzIdentML files. One biological replicate of *daf-2* had to be discarded due to low signal.

Processing 15N/14N LC-MS/MS data

Outlined in chapter 2. The SILeNCe pipeline is presented in Figure 1 and the filtering criteria are summarized in Supplementary Figure 1.

Functional analysis and data visualization

The UniProtKB Protein Knowledgebase (UniProt 2015), the Database for Annotation, Visualization, and Integrated Discovery (DAVID 2014) (Dennis *et al.* 2003) and Wormbase (Version WS246) were used for protein annotation and evaluation of functionally and spatially related proteins (Supplementary Dataset S5). Heatmap visualization of different protein half-lives was done with the MultiExperiment Viewer, part of the TM4 microarray software suit (Saeed *et al.* 2003; Saeed *et al.* 2006). Euclidean distances between proteins were calculated and used as a distance metric in a hierarchical clustering analysis (HCA), together with an average pairwise distance as linkage method. Graphs and statistical tests

(including tests for normality) were performed with Graphpad Prism version 6.05 for Windows (Graphpad Software, La Jolla California, USA).

Correlation analysis of protein abundance and half-lives

In total, 167 common proteins were detected comparing the SILeNCe dataset with the previously obtained proteomic dataset of Depuydt et al. (2013) (Supplementary Dataset S2). The 'dietary restriction' condition was removed from the latter and the set was re-normalized. Pearson correlation analysis was performed between $\log_2$-transformed ratios of protein abundance and protein half-lives ($\log_2$ fold change) (Graphpad Prism version 6.05 for Windows, Graphpad Software, La Jolla California, USA).

Instability index and PEST sequence analysis

ProtParam, a bioinformatics tool on the ExPASy Server (http://www.expasy.org/), was used to estimate the instability index of proteins (Guruprasad *et al.* 1990; Gasteiger *et al.* 2005). Identification of PEST regions in protein sequences was performed using the epestfind tool, available in the European Molecular Biology Open Software Suite (EMBOSS, http://emboss.bioinformatics.nl/) (Rogers *et al.* 1986). A minimal distance of 10 positively charged amino acids was used and a threshold value to discriminate weak from potential PEST motifs was set to 5. Poor and invalid PEST motifs were eliminated from the analysis.

Microscopy

Transverse sections of three young adults *glp-4(bn2ts)I;daf-2(e1370)III* and *glp-4(bn2ts)I daf-16(mgDf50)I; daf-2(e1370)III*, sampled on the second day of adulthood in a previous study (Depuydt *et al.* 2013), were reanalyzed using transmission electron microscopy (TEM) to investigate rough endoplasmic reticulum organization. Electron microscopy analysis was done using a Jeol JEM 1010 (Jeol, Tokyo, Japan) operating at 60 kV. Images were digitized using a DITABIS system (Pforzheim, Germany).

Data Availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (Vizcaino *et al.* 2014) via the PRIDE partner repository with the dataset identifier PXD002317 and 10.6019/PXD002317.

Acknowledgements

We thank the Gems laboratory for providing the strains GA153 and GA154. We are grateful to Caroline Vlaeminck and Renata Coopman for technical support with the SILeNCe experiments. We thank Andy Vierstraete for his technical assistance in data-processing. We also acknowledge Myriam Claes for assisting with TEM imaging. ID acknowledges a PhD grant from the fund for Scientific Research-Flanders, Belgium (FWO11/ASP/031). HC acknowledges a CSC PhD grant (201207650008) with BOF co-funding from Ghent University (01SC0313). Portions of this work were supported by NIH P41GM103493 (to R.D.S). The experimental work described herein was performed in the Environmental Molecular Sciences Laboratory, a national scientific user facility sponsored by the DOE and located at Pacific Northwest National Laboratory, which is operated by Battelle Memorial Institute for the DOE under Contract DE-AC05-76RL0 1830.

Chapter 4. A photoconvertible reporter to monitor *in vivo* protein turnover in *C. elegans*

Personal contribution: Design and development reporters

Confocal microscopy study

Analysis of the data

Writing the chapter

Ineke Dhondt[1*], Huaihan Cai[1], Andy Vierstraete[1], Filip Matthijssens[1,3], Geert Depuydt[1,2], Bart P. Braeckman[1,‡]

[1]Laboratory for Aging Physiology and Molecular Evolution, Biology Department, Ghent University, Proeftuinstraat 86 N1, 9000 Ghent Belgium.

[2]Laboratory for Functional Genomics and Proteomics, Department of Biology, KU Leuven, Naamsestraat 59, 3000 Leuven, Belgium

[3] Center for Medical Genetics, Department for Pediatrics, Ghent University Hospital, Ghent, Belgium

4.1 Abstract

Protein turnover has been previously monitored by stable isotope labeling and mass spectrometric-based approaches. However, estimation of protein half-life can be inaccurate due to internal recycling of the label. Moreover, these methods are incapable of direct measurement of protein turnover in living animals. To enable *in vivo* study of protein turnover and to corroborate previous SILeNCe-based protein turnover results, we used the photoconvertable fluorescent protein Dendra2. Transgenic worm lines were developed to monitor mitochondrial-targeted Dendra2 degradation in long-lived *daf-2* and control worms. Using these reporter strains, we were able to demonstrate the slowdown of protein degradation in *daf-2* worms, which confirms earlier findings. Additionally, we fused Dendra2 to specific proteins to investigate individual protein turnover. Degradation of the ribosomal protein RLA-1 was monitored in young and old worms. Results support the significant decline in ribosomal protein turnover in *daf-2* mutants. Remarkably, these long-lived worms seem to preserve their protein degradation capacity, whereas old control animals lose their ability to degrade RLA-1 protein. In conclusion, we suggest that Dendra2 is a powerful tool to study protein turnover *in vivo* in *C. elegans*.

4.2 Introduction

We developed a photoconversion-based fluorescence approach to measure and quantify protein degradation *in vivo* in *C. elegans* and to validate previous SILeNCe and MS-based results. Thereto, we used Dendra2, a fluorescent protein from the octocoral *Dendronephthya* (Gurskaya et al. 2006). This photoswitchable protein allows high contrast photoconversion from green-to-red fluorescent state by excitation of the chromophore with violet light (405 nm) (Chudakov *et al.* 2007). Hence, protein degradation can be measured by the disappearance of the red fluorescence of photoconverted Dendra2 trangsgene worms. Previously, this approach was successfully applied in *C. elegans* to monitor the activity of the ubiquitin-proteasome system *in vivo* (Hamer *et al.* 2010).

Being the main source of reactive oxygen species (ROS), mitochondrial proteins are expected to be very vulnerable to oxidative damage. In this vein, mitochondrial homeostasis is crucial and appears to be maintained by the specific autophagy of dysfunctional mitochondria, a strictly controlled process which is designated mitophagy (Lemasters 2005). We expressed Dendra2 in mitochondria of all cells to monitor its degradation in long-lived and control worms. In *C. elegans*, mitochondria are specifically recognized and targeted for degradation *in toto* via the PINK-1 (PTEN-putative kinases PINK1) – PDR-1 (cytosolic E3 ubiquitin ligase Parkin) pathway (Palikaras *et al.* 2015b). Therefore, we verified the decline of mitochondrial-targeted Dendra2 in *pink-1* and *pdr-1* knock-out mutants with impaired mitophagy machinery.

Additionally, we aimed to validate individual protein turnover in long-lived *daf-2* mutants and their corresponding control worms. Therefore, we expressed Dendra2 fused to specific proteins in the *glp-4 daf-2* mutant strain. These transgene worms were fed *daf-16* RNAi bacteria to inhibit its DAF-16 activation and consequently to reduce its long life to normal lifespan. Several expression clones were successfully generated to study protein turnover of mitochondrial (CTS-1 and ANT-1.1), endoplasmic reticulum related (PDI-2), cytoskeletal (ACT-2), lysosomal (VHA-11, VHA-12, ASP-4) and ribosomal (RLA-1) proteins. By now, two transgene lines were developed via microinjection to study single protein turnover of a lysosomal protease (*asp-4p::asp-4::Dendra2*) and a ribosomal protein (*rla-1p::rla-*

1::Dendra2). The latter was used to corroborate previous SILeNCe results in young and old worms.

4.3 Results and discussion

Dendra2 transgenic strains to study mitochondrial turnover

To evaluate mitochondrial turnover *in vivo*, we designed a unique reporter that contains a concatenate of a ubiquitous constitutive promoter of a ribosomal protein subunit, the Dendra2 gene and a mitochondrial targeting sequence of *gas-1* (coding for a subunit of complex I of the mitochondrial electron transport chain): *jrIs5[unc-119(+) rps-0p::mls::Dendra2].* Hence, this transgenic *C. elegans* line shows expression of Dendra2 in the mitochondria of all cells (Fig. 1A). In order to corroborate the SILeNCe data, we crossed the Dendra2 transgenic strain with the long-lived *daf-2* mutant. Subsequently, *daf-2(e1370)III;jrIs5[unc-119(+)rps-0p::mls::Dendra2]* transgenic worms were crossed into a *daf-16(m26)I* genetic background, resulting in *daf-16(m26)I; daf-2(e1370)III;jrIs5[unc-119(+) rps-0p::mls::Dendra2]* to serve as a control strain with normal lifespan.

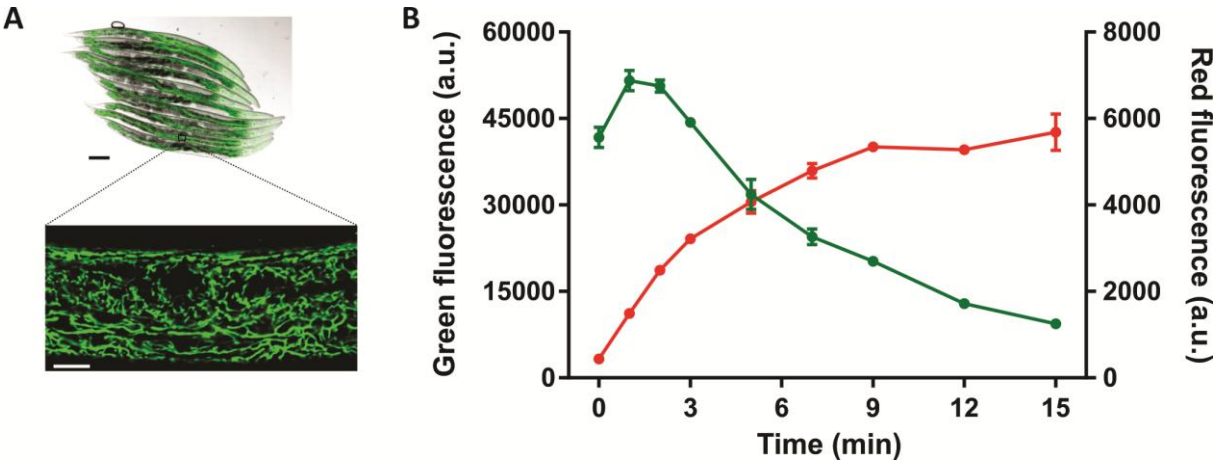


Fig. 1 (A) Confocal images of *jrIs5* transgenic worms expressing Dendra2 under the constitutive *rps-0* promotor and targeted to the mitochondria with the mitochondrial localization signal of *gas-1.* Top: overview of Dendra2-expressing worms (scale bar is 100 µm); Bottom: detail of the midsection of the body (scale bar is 10 µm). (B) Fluorescence measured at different time points during photoconversion of Dendra2 transgene worms using violet light. Errors are SEM.

No phototoxic effects of photoconversion on general well-being

Our lab has invested in the development of a LED-based device emitting intense violet light (UV and heat radiation are cut off with specific filters) for mass photoconversion of DENDRA2 transgene worms that allows us to perform high-throughput experiments to investigate mitochondrial turnover (Fig. 1B).

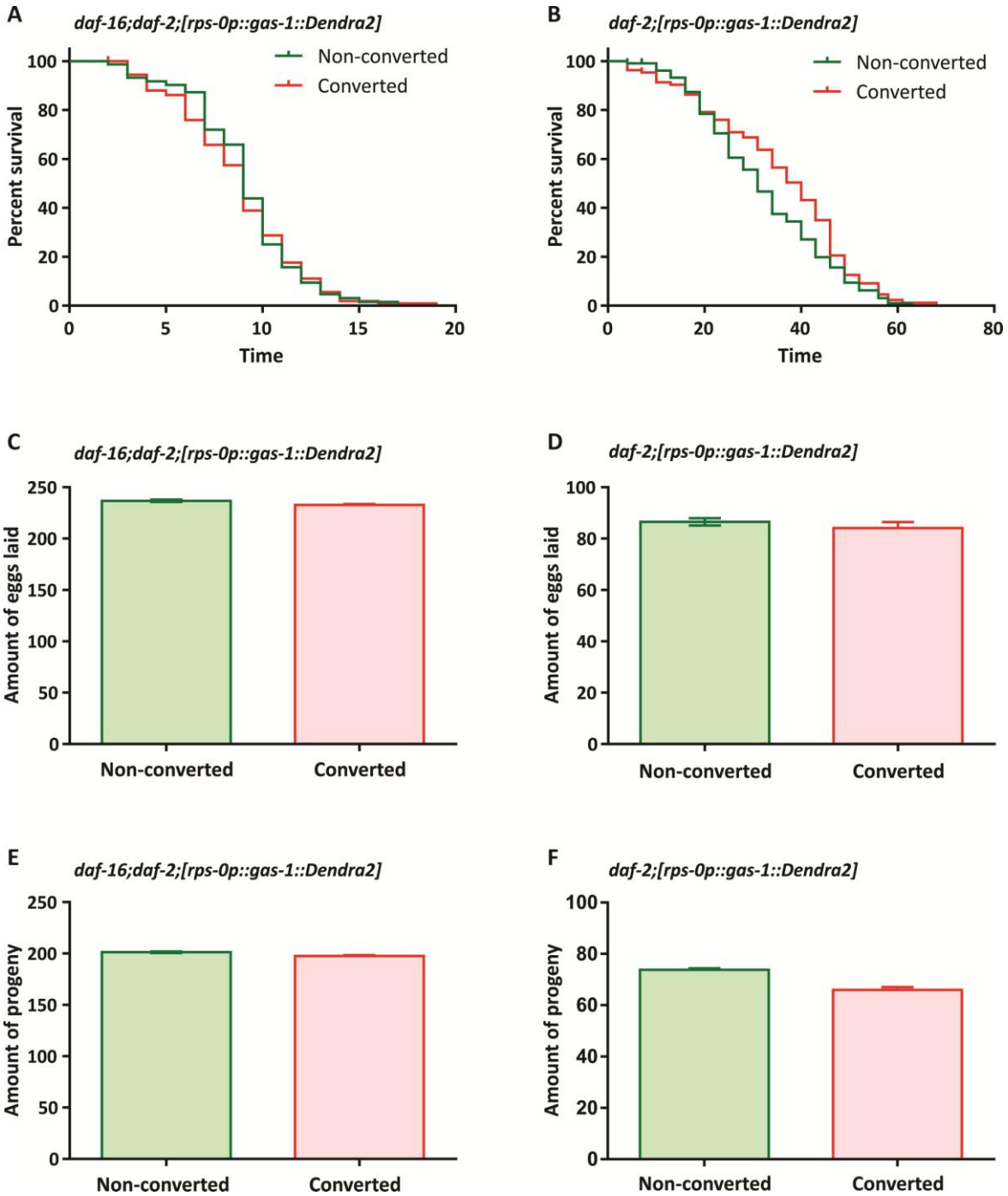


Fig. 2 No effects of photoconversion (30 min) on general well-being of day-2 treated transgenic *C. elegans* lines. (A,B) Survival of photoconverted normal lived (A) and long-lived *daf-2* (B) worms does not significantly differ. (C,D) The total amount of eggs laid does not differ after photoconversion for both strains studied. (E,F) Photoconversion has no adverse effect on the amount of hatched eggs for both strains studied. Data are represented as mean ± SEM.

Analysis of *in vivo* protein turnover in *C. elegans*

After 30 minutes of violet light exposure, worms were optimally converted to the red fluorescent form. No effect of irradiation was found on lifespan for the control strain (Log-rank Mantel Cox test, P = 0.8022). Converted *daf-2* worms show a slightly, but not significantly, increased lifespan (Log-rank Mantel Cox test, *P* = 0.0555). The amount of laid eggs was not significantly different in converted worms compared to non-converted in the studied strains (Mann-Whitney test, *P* = 0.10 and *P* = 0.70, for control strain and *daf-2* respectively). In addition, no significant difference in the amount of hatched progeny could be observed (Mann-Whitney test, P = 0.10 and P =0.10, for control strain and *daf-2* respectively). Hence, the lack of phototoxic effects allows green-to-red conversion experiments with this device.

Slow down of mitochondrial Dendra2 degradation in long-lived *daf-2*

We measured the red Dendra2 fluorescence intensity of transgene worms at different time points after photoconversion for control and *daf-2* worms using a multi-label plate reader. The decrease of the relative red signal was monitored over time as a proxy of mitochondrial protein breakdown. Consistent with our SILeNCe data, the decrease of the red fluorescence is slowed down significantly in *daf-2* mutants compared to controls (F-test, *P* < 0.0001; Fig 3A). With this method, we estimated the half-life of the mitochondrial-targeted Dendra2 protein to be 44 h in the control and 77 h in *daf-2* mutants. Mitochondrial half-life assessed in mice indicated similar turnover values in control animals (Miwa *et al.* 2008).

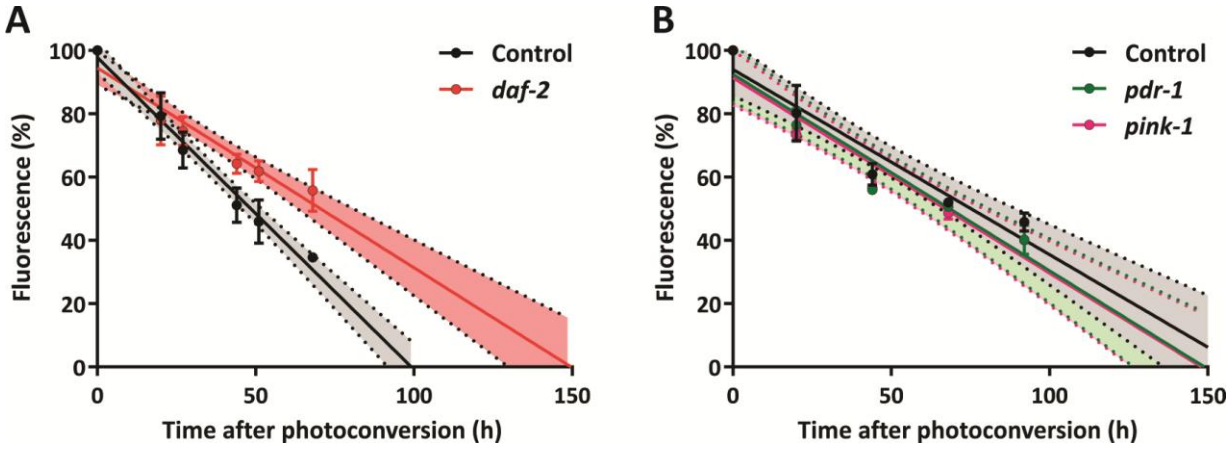


Fig. 3 Linear regression of the decline in red Dendra2 fluorescence after photoconversion in (A) control (*daf-16;daf-2;[rps-0p::gas-1::Dendra2]*) versus *daf-2* (*daf-2;[rps-0p::gas-1::Dendra2]*) strains (F-test, *P* < 0.0001) and (B) control (*rps-0p::gas-1::Dendra2*) versus mitophagy-related mutants *pdr-1 (pdr-1;[rps-0p::gas-1::Dendra2]*) and *pink-1* (*pink-1;[rps-0p::gas-1::Dendra2]*) (F-test, P = 0.9173).

Since mitophagy, the selective autophagy of dysfunctional mitochondria, is a key process in mitochondrial recycling, we evaluated the degradation of red Dendra2 in worms mutated in two mitophagy-related genes. Unexpectedly, the decline in red Dendra2 was comparable between the control strain and worms mutated in *pdr-1* or *pink-1*, which indicates a minor role of mitophagy in control worms under normal laboratory conditions. Interestingly, the turnover of Dendra2 is comparable to the fastest turnover rates observed for mitochondrial matrix resident proteins **(See Chapter 3)**.

Analysis of individual protein turnover using Dendra2

In order to investigate individual protein turnover, we fused the green-to-red photoswitchable Dendra2 to specific proteins of interest using Gateway Cloning technique (Hartley *et al.* 2000). An overview of the generated expression clones is listed in table 1.

Table 1 Expression clones generated via Gateway Cloning

Protein	Cellular location	Expression clone
• Actin ACT-2	*Cytoskeleton*	*act-2p::act-2::Dendra2*
• Ribosomal protein RLA-1 (translation)	*Ribosome*	*rla-1p::rla-1:: Dendra2*
• Protein disulfide isomerase PDI-2 (protein folding)	*Endoplasmic reticulum*	*pdi-2p::pdi-2::Dendra2*
• Mitochondrial adenine nucleotide transporter ANT-1.1	*Mitochondrium (inner membrane)*	*ant-1.1p::ant-1.1::Dendra2*
• Mitochondrial citrate synthase CTS-1 (TCA cycle)	*Mitochondrium (matrix)*	*cts-1p::cts-1::Dendra2*
• Subunit C of cytoplasmic (V1) domain of vacuolar proton-translocating ATPase (V-ATPase) VHA-11	*Lysosomal membrane*	*vha-11p::vha-11::Dendra2*
• Subunit B of cytoplasmic (V1) domain of vacuolar proton-translocating ATPase (V-ATPase) VHA-12	*Lysosomal membrane*	*vha-12p::vha-12::Dendra2*
• Aspartyl protease ASP-4	*Lysosomal lumen/membrane*	*asp-4p::asp-4::Dendra2*

Analysis of *in vivo* protein turnover in *C. elegans*

We aimed to validate individual protein turnover in control worms with normal lifespan and long-lived *daf-2* mutants via DAF-16 activation. Thereto, we chose the long-lived *glp-4(bn2ts)I daf-2(e1370)III* line to serve as genetic background. Through inhibition of DAF-16 using RNAi technique, lifespan can be easily reduced to normal (Fig. 4).

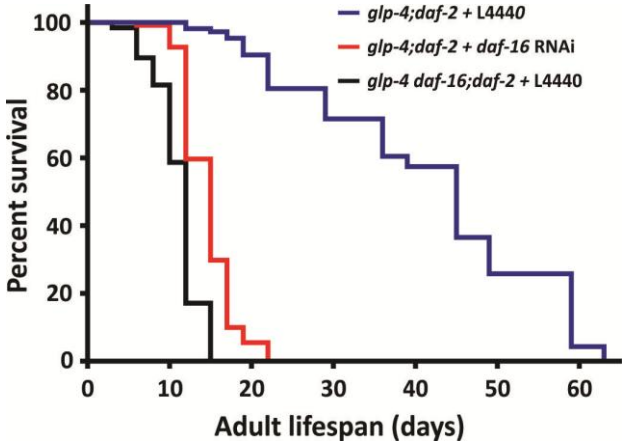


Fig. 4 Survival of *glp-4;daf-2* worms subjected to *daf-16* and empty vector (L4440) RNAi. Worms were grown at 24°C.

So far, two transgenic *C. elegans* lines have been developed via microinjection of the expression clones (Table 2).

Table 2 Transgenic *C. elegans* lines generated via microinjection

- JV17[*glp-4(bn2ts)I daf-2(e1370)III; jrEx17[rla-1p::rla-1::Dendra2]*]
- JV18[*glp-4(bn2ts)I daf-2(e1370)III; jrEx18[asp-4p::asp-4::Dendra2]*] (Fig. 5)

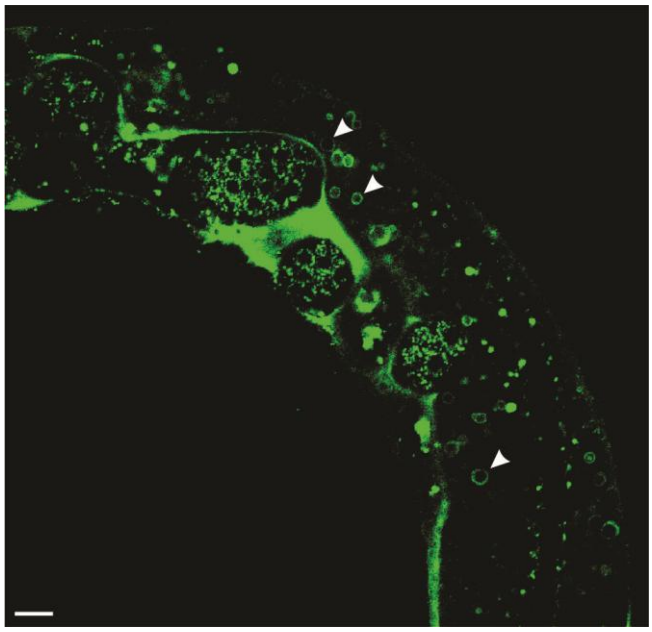


Fig. 5 Representative confocal image of *glp-4 daf-2;asp-4p::asp-4::Dendra2* transgenic worm. ASP-4::DENDRA2 is present in lysosomal membranes (arrows) and developing embryo's (scale bar is 10 µm).

Ribosomal protein turnover in young and old worms

We validated the slow-down of ribosomal turnover in worms expressing Dendra2 fused to the ribosomal RLA-1 protein. Red Dendra2 fluorescence intensity was monitored with confocal microscopy 0 and 96 hours after photoconversion. In young worms, examined between day 2 and day 6 of adulthood, we observed a significant slow-down in the decline of red fluorescence in long-lived *daf-2* compared to control worms fed with *daf-16* RNAi (Mann Whitney test, $P = 0.0043$; Fig. 6A,B,C), which confirms the reduced turnover of this ribosomal subunit upon DAF-16 activation (See discussion in chapter 3). Intriguingly, the opposite pattern is observed in older worms, monitored between day 6 and day 10 of adulthood. Whereas old worms treated with *daf-16* RNAi show no ribosomal degradation anymore, it seems that old *daf-2* worms are still capable to degrade this ribosomal protein (Mann Whitney test, $P = 0.0476$; Fig. 6D,E,F). Ribosomal protein breakdown, here shown for RLA-1, seems to slow down severely with age in control worms, while old *daf-2* worms seemingly safeguard a functional degradation machinery. Indeed, recent work shows the pronounced increase in proteasome abundance in *daf-2* animals, which suggests a preserved capacity for the clearance of proteins (Walther *et al.* 2015).

In conclusion, we presented an alternative method using Dendra2 to validate SILeNCe-based protein turnover rates *in vivo* in *C. elegans* worms. Dendra2 transgenic worms will extend our knowledge on temporal and spatial protein turnover in control and long-lived mutants, lacking the potentially confounding effect of label recycling.

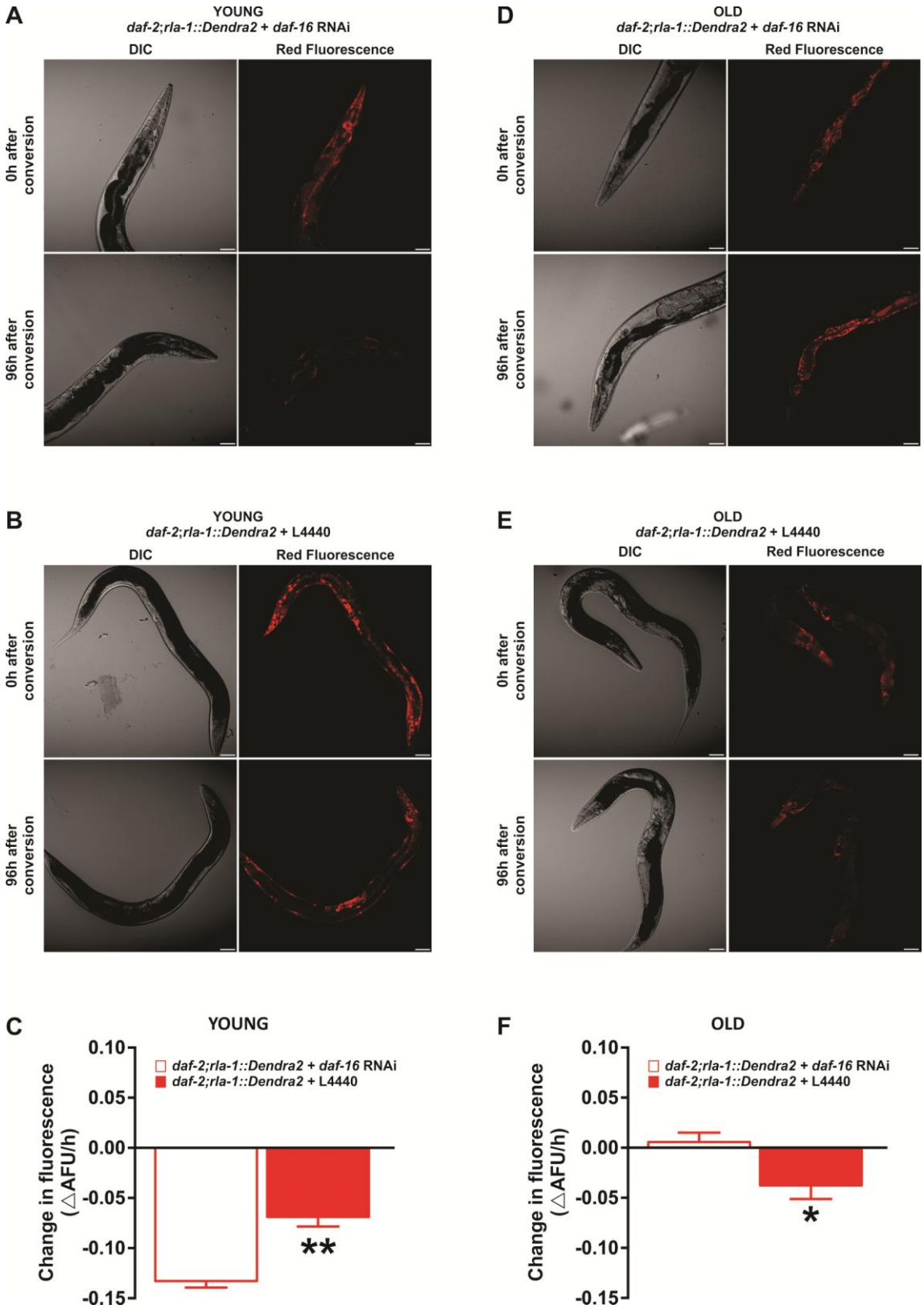


Fig. 6 Dendra2 validation experiment on ribosomal protein turnover in young (A,B,C) and old (D,E,F) control (*daf-16* RNAi) and long-lived *daf-2* worms. Representative confocal images of *glp-4 daf-2;rla-1p::rla-1::Dendra2* transgenic worms grown on *daf-16* RNAi (A,D) and empty vector L4440 (B,E), 0 h and 96 h after photoconversion (scale bars are 20 µm). Rate of decline in red RLA-1::Dendra2 fluorescence in *daf-2* worms treated with *daf-16* and empty vector (L4440) RNAi in young (C) worms (n = 6 individually tracked worms per condition, Mann Whitney t-test, *P* = 0.0043) and old (F) worms (n = 3 individually tracked worms per condition, Mann Whitney t-test, *P* = 0.0476).

4.4 Materials and methods

Dendra2 transgene worms

Stable transgenic strain *jrIs5[unc-119(+)rps-0p::mls::Dendra2]* were created using biolistic transformation (PDS-1000/He™ System, Bio-Rad, Hercules, CA, USA) to study mitochondrial turnover. This reporter contains a concatenate of a ubiquitous constitutive *rps-0* promoter (ribosomal protein subunit), the Dendra2 gene (Evrogen, Moscow, Russia) and a mitochondrial targeting sequence of *gas-1* (coding for a subunit of complex I of the mitochondrial electron transport chain). The Dendra2 transgenic strain was crossed with the long-lived *daf-2(e1370)III* mutant. Subsequently, *daf-2(e1370)III;jrIs5[unc-119(+)rps-0p::mls::Dendra2]* transgenic worms were crossed into a *daf-16(m26)I* genetic background, resulting in *daf-16(m26)I;daf-2(e1370)III;jrIs5[unc-119(+) rps-0p::mls::Dendra2]* to serve as a control strain with normal lifespan. The *pink-1(ok3538)II* and the *pdr-1(gk448)III* mutant strains were provided by the CGC and backcrossed five times by crossing wildtype N2 males with mutant hermaphrodites. Next, the outcrossed *pink-1* and *pdr-1* mutant strains were crossed with the Dendra2 transgene strain *jrIs5*, resulting in *pink-1(ok3538)II; [unc-119(+)rps-0p::mls::Dendra2] and pdr-1(gk448)III;[unc-119(+)rps-0p::mls::Dendra2],* respectively. Transgene strains were generated via microinjection of Dendra2-fused protein contructs in the genetic background *glp-4(bn2ts)I;daf-2(e1370)III* mutant in order to study individual protein turnover. *glp-4(bn2ts)I daf-2(e1370)III; jrEx17[rla-1p::rla-1::Dendra2]* and *glp-4(bn2ts)I daf-2(e1370)III; jrEx18[asp-4p::asp-4::Dendra2]* were generated to study individual protein turnover of the ribosomal protein RLA-1 and the lysosomal protease ASP-4, respectively.

Mass photoconversion

We developed and optimized a device emitting intense violet light (4 x 20W, 415-420 nm LEDs, Zhuhai Tianhui Electronics Co, Ltd., China) for mass photoconversion of worms without affecting lifespan and fecundity (Fig. 7). Intensity could be regulated by applying different distances from the LEDs and duration of the photoconversion treatment was set with a timer. In addition, ventilation vents extract heat from the

Fig. 7 Device for photoconversion.

photoconversion chamber. Residual UV and heat produced by the LEDs were eliminated using UV filters and heat mirrors, respectively.

In the experiment on gross mitochondrial turnover, day 1 adult worms were photoconverted during 30 min and the red Dendra2 fluorescence intensity was measured at different time points after this treatment using a Victor^2 1420 multilabel counter (PerkinElmer Life Sciences, Boston, MA, USA). The fluorescence was normalized to protein content as determined using a BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). The slopes, representing mitochondrial Dendra2 degradation, were evaluated in Graphpad Prism using linear regression analysis.

Ribosomal protein degradation was assayed by microinjecting a *rla-1p::rla-1::Dendra2* construct containing 2 kb upstream and 1 kb downstream sequence of the ribosomal *rla-1* gene in the *glp-4(bn2ts)I;daf-2(e1370)III* mutant. Synchronized L1 worms were grown on NGM plates seeded with freshly induced RNAi bacteria (L4440 empty vector and *daf-16*) and incubated at 17 °C until the third larval stage (L3) and then switched to 24°C for the remainder of the experiment. At the second day of adulthood, worms (n = 6 per condition) were photoconverted and immediately imaged using confocal microscopy. The photoconversion protocol was repeated in old animals at day six of adulthood (n = 3 per condition). Subsequently, worms were rescued and replaced on fresh RNAi plates. After 96 hours, these worms were re-imaged using the same camera settings specific for each animal. The mean intensity per pixel of red Dendra2 fluorescence was determined in ImageJ (Schneider *et al.* 2012).

Confocal microscopy

Confocal images were acquired using a Nikon TiE-C2 confocal microscope with objectives Plan 4x 0.10 CFI (NA 0.1, dry), 20X CFI Plan Apochromat VC (NA 0.75, dry) and Plan Apo VC 60xA WI DIC N2 (NA 1.20, water immersion) and the NIS Elements Imaging software (version 4.13.03). The Dendra2 fluorochrome was excited with a 488-nm solid state laser and the emission was collected with a 520/30-nm band pass filter.

Feeding RNAi

Worms were fed RNAi bacteria that were selected from the Ahringer library (Kamath & Ahringer 2003) and induced as described previously (Timmons *et al.* 2001).

Lifespan assay

Synchronized L1 worms were placed on NGM plates seeded with OP50 or RNAi bacteria containing the empty control vector L4440 or a vector expressing *daf-16* dsRNA. Worms were grown at 17°C until the third larval stage (L3) and then switched to 24°C for the remainder of the experiment. Adult lifespan was monitored on a daily basis and worms were regularly transferred to fresh RNAi plates. Graphpad Prism version 6.05 for Windows (Graphpad Software, La Jolla California, USA) was used for survival analysis and generation of the survival graphs. Median survival was compared using a Log-rank (Mantel Cox) test.

Fecundity assay

Synchronized worms were grown on NGM plates containing OP50 at 17°C until the third larval stage (L3) and then switched to 24°C for the remainder of the experiment. At the fourth larval stage, worms were photoconverted during 30 minutes (non-photoconverted controls were included). Individual L4 hermaphrodite were transferred to fresh plates each day until they stopped laying eggs. Eggs were counted daily during the egg-laying period of at least 10 adult hermaphrodites and 24 hours after the adult was transferred, hatched larvae were scored too. Three independent experiments were performed.

Acknowledgements

We thank Marjolein Couvreur for the assistance with the microinjections. ID acknowledges a PhD grant from the fund for Scientific Research-Flanders, Belgium (FWO11/ASP/031). HC acknowledges a CSC PhD grant (201207650008) with BOF co-funding from Ghent University (01SC0313).

Chapter 5. Enhanced protein synthesis has no effect on *daf-2* longevity

Personal contribution: Lifespan experiment

Analysis of the data

Writing the chapter

Ineke Dhondt[1], Bart P. Braeckman[1]

[1]Laboratory for Aging Physiology and Molecular Evolution, Biology Department, Ghent University, Proeftuinstraat 86 N1, 9000 Ghent Belgium.

5.1 Introduction

Protein synthesis is a tightly regulated process to adapt the proteome composition, stability and functionality to changing environmental and physiological conditions such as aging (Charmpilas *et al.* 2015). Protein turnover enables cells to replenish aberrant proteins with new, fresh copies. In this vein, enhanced protein turnover is arguably a key process in the aging process (Ryazanov & Nefsky 2002); yet, it is the attenuation of protein synthesis that extends lifespan in many organisms, ranging from yeast to mice (Hsieh & Papaconstantinou 2004; Kaeberlein *et al.* 2005; Hansen *et al.* 2007; Pan *et al.* 2007; Syntichaki *et al.* 2007; Kaeberlein & Kennedy 2011). As a considerable quantity of the cellular energy output is used for ribosomal biogenesis and the protein translation process, the inhibition of either one of these processes can shift valuable energy resources from anabolism to repair and the activation of stress-response mechanisms (Lempiainen & Shore 2009; Charmpilas *et al.* 2015).

Reduced insulin/IGF-1 signaling (IIS) results in extensive proteome remodeling, including the dramatic reduction of ribosomal proteins and translation regulation factors (Depuydt *et al.* 2013; Stout *et al.* 2013). Corroborating these findings, we found a remarkable slowdown of the turnover of proteins involved in the translation machinery **(See chapter 3)**. Intriguingly, a recent study showed that *tts-1*, a long-noncoding RNA, acts on ribosomes in long-lived *daf-2* mutant worms, leading to the reduction in ribosomal levels. Since elevation of this block restores ribosomal levels and reduces long life of the *daf-2* mutant, *tts-1* likely plays a prominent role in the regulation of its longevity phenotype (Essers *et al.* 2015).

Essers *et al.* (2015) suggested that energy sensor AAK-2, the *C. elegans* homolog of AMP-activated kinase subunit α, plays a key role in the regulation of *tts-1* transcription. In conditions with high AMP:ATP ratios, AAK-2 is activated and probably triggers the production of higher levels of this long noncoding RNA which can interfere with the energy-costly ribogenesis. Additionally, lifespan extension caused by *daf-2* mutation, seems to be highly dependent on *aak-2* (Curtis et al. 2006).

We hypothesized that increased protein turnover, including enhanced protein synthesis, would shorten lifespan in *daf-2* mutants. To this end, we treated worms with serotonin, a

drug known to induce protein translation and ribosomal biogenesis (Gomez-Amaro *et al.* 2015).

Recent work indicates that serotonin signaling promotes pro-growth pathways through the inactivation of AAK-2 (Cunningham *et al.* 2014), hence serotonin-treatment should lower *tts-1* transcription and consequently restores ribosomal levels and protein translation in *daf-2.* An intermediate state (semi-activated, semi-inhibited state) of AAK-2 might occur under these circumstances; yet, it is uncertain to what extent this state acts on ribogenesis and protein translation (Fig. 1).

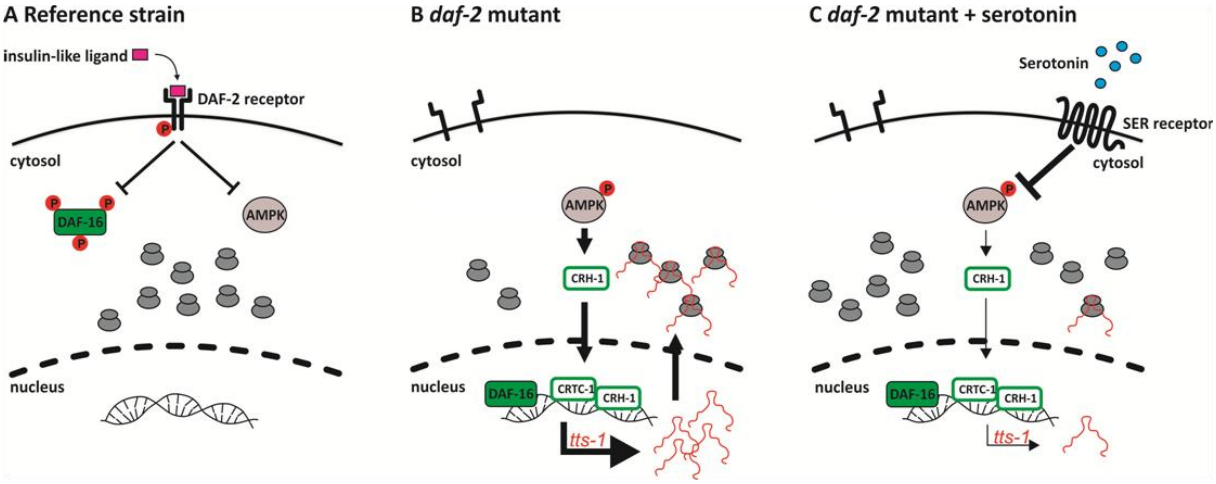


Fig. 1 Schematic overview of the hypothesis. (a) Phosphorylation of DAF-16 results in its cytosolic retention. High energy levels promote anabolic processes. (b) The elevated blockage of DAF-2 receptor will activate the energy sensor AMPK homolog (AAK-2), which stimulates the action of transcription factor CRH-1 and its co-regulator CRTC-1 (Mair *et al.* 2011). Consequently, specific transcription of translation regulatory components, probably including the long noncoding RNA *tts-1*, is initiated. *tts-1* ncRNA blocks ribogenesis and cellular energy can be shifted towards stress-response pathways, for example, via nuclear DAF-16. (c) Elevated serotonin signaling mimics the effect of inhibited AAK-2 activity (Cunningham *et al.* 2014). Therefore, it can be postulated that an intermediate state (semi-activated, semi-inhibited state) occurs in serotonin-treated *daf-2* mutants. Restored ribosomal levels and enhanced protein synthesis remains to be proven under these circumstances.

5.2 Results and future prospects

In a preliminary lifespan experiment, we found no difference in the lifespan curves of long-lived *daf-2* treated with serotonin as compared to water-treated control animals (Fig. 2, log-rank Mantel-Cox test, P = 0.2128). Thus enhanced protein synthesis possibly does not interfere with the long life of *daf-2*, although the serotonin-treatment stimulated growth,

causing the animals to be larger (data not shown). Serotonin-treated reference strain with normal lifespan shows a small, but significant reduction in lifespan compared to water-treated control worms (Fig. 2, log-rank Mantel-Cox test, $P < 0.0001$). Under low DAF-16 activity in these controls, it might be possible that enhanced protein turnover does promote aging.

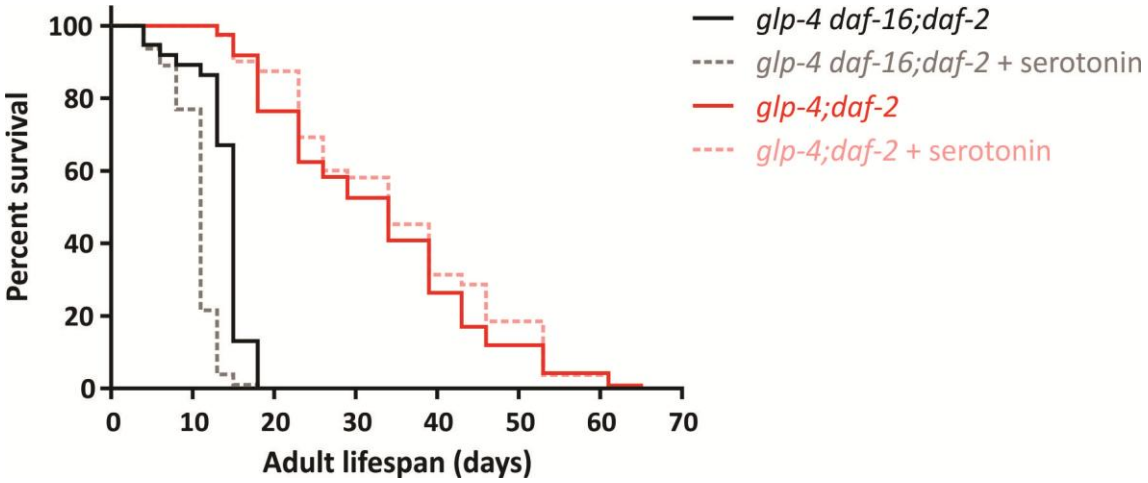


Fig. 2 No effect of serotonin on *glp-4;daf-2* mutant lifespan. Survival curves of *glp-4;daf-2* or *glp-4 daf-16;daf-2* mutant worms treated with water (full line) or 5mM serotonin (dashed line). Graph shows the result of one biological replicate (approximately 100 worms per condition).

Serotonin signaling promotes anabolic pathways, hence serotonin-treatment should reinstate protein translation in *daf-2.* Hence, the elevated attenuation of protein synthesis should reduce lifespan of long-lived *daf-2* worms. Considering this idea, the observed retained long life of serotonin-treated *daf-2* worms is rather intriguing.

Future research should confirm the reduced expression of *tts-1* in serotonin-treated *daf-2* mutants. In addition, serotonin-enhanced protein synthesis should be validated via classical ^{35}S-pulse-labeling approach and ribosomal profiling would allow verifying ribosomal levels. The use of proper controls will be necessary to further uncouple protein turnover dynamics and longevity via the administration of serotonin. For example, future experiments should include control worms with reduced expression of the serotonergic receptor *ser-7*. Several G protein-coupled receptors (GPCR) are known to play a role in serotonin-modulated behaviors in *C. elegans*, including pharyngeal pumping, egg laying, male mating and locomotion (Carre-Pierrat *et al.* 2006). Although this neurotransmitter, which act on neurons and muscles, effects a wide range of behaviors, it has been shown that serotonergic

signaling via the SER-7 receptor is specifically required to trigger food intake, nutrient absorption and consequently *de novo* protein synthesis and ribosomal biogenesis (Fig. 3) (Gomez-Amaro *et al.* 2015). In addition, treatment with the antibiotic cycloheximide, a translational elongation inhibitor, blocks mRNA translation and has been used as a suitable control treatment in the analysis of protein synthesis in *C. elegans* before (Kourtis & Tavernarakis 2009).

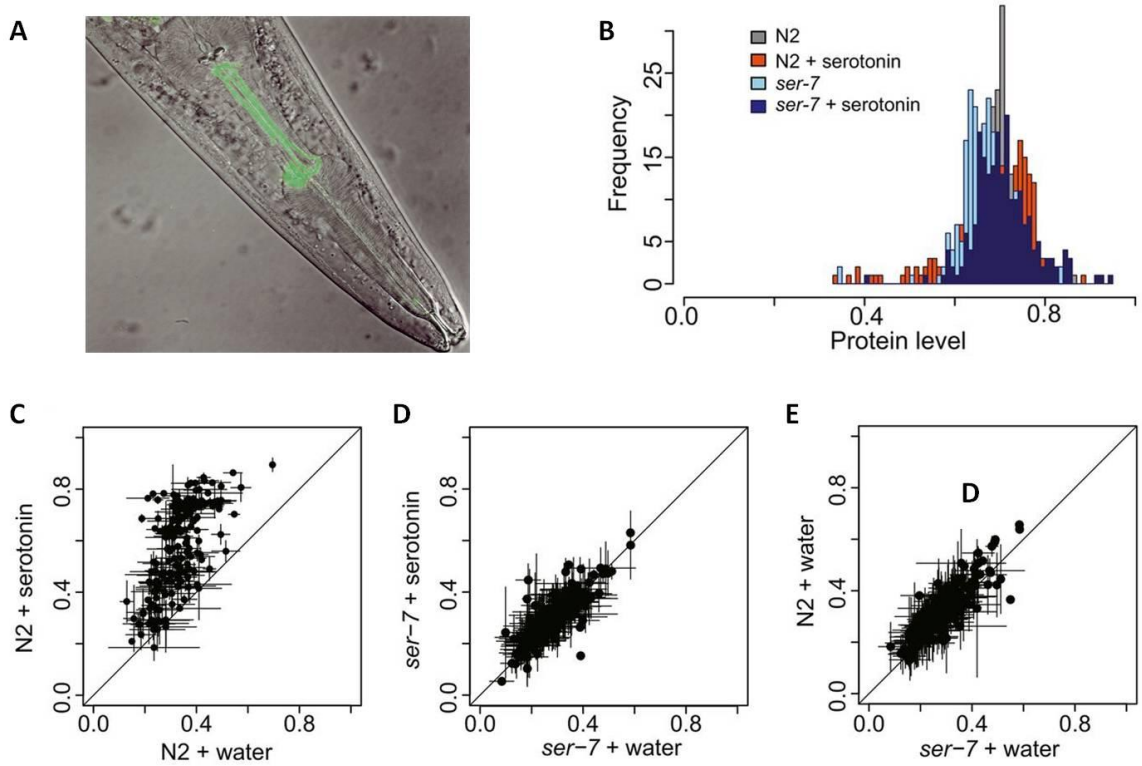


Fig. 3 Serotonin increases protein synthesis via the serotonergic SER-7 receptor in *C. elegans*. Serotonin treatment (5 mM) was initiated at day 1 of adulthood. At day 5 of adulthood, worms were pulse-labeled with ^{15}N-labeled *E. coli* and harvested at day 7 of adulthood for mass spectrometry analysis. (A) *ser-7* is constitutively and exclusively expressed in the M4 pharyngeal motorneuron (Hobson *et al.* 2006). (B) Histogram of protein levels for wild-type N2 and *ser-7* mutants treated with water or serotonin. (C) Correlation plot represents fraction of labeled proteins for wild-type N2 treated with water (x-axis) or serotonin (y-axis). Serotonin treatment increased the fraction of labeled proteins by an average of 1.9-fold compared to the water-treated control worms. (D) Correlation plot represents fraction of labeled proteins for *ser-7* mutant worms treated with water (x-axis) or serotonin (y-axis). (E) Correlation plot represents fraction of labeled proteins for *ser-7* mutant (x-axis) and wild-type N2 (y-axis) treated with water. Redrafted and adapted from Hobson *et al.* (2006) and Gomez-Amaro *et al.* (2015).

5.3 Materials and methods

Strains

In order to compare results with previous SILeNCe work, we opted for the same strains to test the effect of serotonin. GA154 *glp-4(bn2ts)I;daf-2(e1370)III* (long-lived) and GA153 *glp-4(bn2ts)I daf-16(mgDf50)I; daf-2(e1370)III* (control strain) were used in this study which were kindly provided by David Gems at the University College of London. Strains were maintained as described previously (Depuydt *et al.* 2013).

Serotonin

A stock solution of serotonin hydrochloride (Alfa Aesar) was prepared in water (250 mM, 50x) and diluted to a final concentration of 5 mM for the lifespan experiments.

Lifespan assay

Synchronized L1 worms were placed on NGM plates seeded with OP50 bacteria. Worms were grown at 17°C until the third larval stage (L3) and then switched to 24°C for the remainder of the experiment. At the fourth larval stage, serotonin (or water for control) was added to a final concentration of 5 mM. Adult lifespan was monitored on a daily basis (approximately 100 hermaphrodites per condition in one biological replicate) and worms were regularly transferred to fresh plates with or without serotonin. Graphpad Prism version 6.05 for Windows (Graphpad Software, La Jolla California, USA) was used for survival analysis and generation of the survival graphs. Median survival was compared using a Log-rank (Mantel Cox) test.

PART III DISCUSSION

Chapter 6. General discussion and future perspectives

6.1 General discussion

6.1.1 Studying proteome dynamics via SILeNCe labeling

The proteome of a particular organism, tissue or cell is far from static. Proteins are continuously being modified (by the addition of chemical constituents), degraded and re-synthesized to manage changing environmental conditions. The appearance of different isoforms (alternative splicing), mutual protein interactions and continuous flow through metabolic and degradation pathways makes the proteome a very dynamic molecular pool. Moreover, cellular proteomes may vary widely, dependent on cell type, function, developmental stage and environmental signals (Adams 2008).

Conventional quantitative proteomics compares the protein expression levels between two or more genetic, developmental or physiological states. Still, this approach cannot provide information on protein turnover, the dynamic force driving the changing protein abundances. Studying the shifts in the balance between protein synthesis and degradation is crucial to better understand changing proteome profiles (Pratt *et al.* 2002a). At present, stable isotope labeling in combination with the existing proteomics technology is a powerful strategy to determine individual protein turnover at a global scale (Claydon & Beynon 2012).

SILeNCe labeling and Dendra2-involved studies are crucial to further unravel the role of protein turnover in several biological phenomena. In the following, the potential role of protein turnover in aging and long-lived *C. elegans* will be discussed.

6.1.2 Protein turnover in aging *C. elegans*

Maintaining an undamaged, youthful proteome becomes challenging as age progresses, as the levels of damage progressively accumulates, resulting in the decline of cellular function (Rattan 1996). Cellular damage can be limited by repair mechanisms, however, the ability to fix protein damage is rather limited and most protein damage appears to be irreversible. Hence, cells rely on protein turnover as the single mechanism to replace damaged proteins with new copies (Ryazanov & Nefsky 2002). Protein turnover is determined by the combined

rates of protein synthesis and degradation. After completing development and growth, a steady-state in protein turnover is reached, in which the rate of overall protein synthesis more or less balances overall protein degradation. Age-related decline in protein turnover, both synthesis and degradation, is observed in numerous organisms ranging from yeast to humans and leads to a drastic increase in protein half-life (Prasanna & Lane 1979). In agreement with these findings, we observed a gradual increase in protein half-life for a considerable part of the aging proteome in *C. elegans*. Hence, reduction of protein turnover may be considered a major contributor to the increase of protein damage during aging (Van Remmen *et al.* 1995). In this vein, Ryazanov and Nefsky (2002) suggested that the cumulative effect of non-specific damage to various translational and proteolytic constituents underlies the decline in protein turnover. Based on this idea, they proposed their protein damage catastrophe theory, in which accumulation of protein damage negatively affects protein turnover which in turn increases levels of damaged proteins (Ryazanov & Nefsky 2002). Molecular damage may attribute to the malfunction of numerous proteins over time, yet the contribution of molecular damage to the aging process is doubtful, as indicated by numerous evidence (Gems & de la Guardia 2013). The hyperfunction provides an alternative explanation, in which a non-regulation of ongoing biosynthetic processes causes hypertrophy pathologies, ultimately causing death. Our data corroborates this idea as we observed a haphazard slowdown of protein turnover of several components involved in the protein synthesis machinery in aging worms. Correspondingly, a pronounced imbalance in the relative subunit stoichiometry of cytosolic ribosomes was found (Walther *et al.* 2015). Besides the reduced turnover, random removal of ribosomal proteins (and other proteins) from the functional protein pool through aggregation might contribute to the aging process. Protein density is high in the cytosol (macromolecular crowding, approximately 300 g/l), and likely increases with age as at least half of the aging-proteome doubles in abundance over time (Walther *et al.* 2015). Fortuitous interactions between proteins are liable under these conditions, explaining the onset of protein aggregates with age. All these processes likely drive the de-regulation of anabolism which is observed with age (Kirstein-Miles *et al.* 2013; Liang *et al.* 2014).

MicroRNAs (miRNAs), first discovered in *C. elegans*, are small noncoding RNAs (between 18-25 nucleotides) and play a key function in the regulation of protein synthesis by binding to

target mRNAs (Lee *et al.* 1993; Wightman *et al.* 1993). A single miRNA can target up to several hundred mRNA to downregulate gene expression by mRNA degradation or translational repression (Fabian *et al.* 2010). Hence, miRNAs are capable to influence the proteome by fine-tuning protein output (Baek *et al.* 2008). A release of miRNA-mediated translational repression probably enhances the observed escalation of protein abundance in senescing worms (Welker *et al.* 2007).

Concomitant with the anabolic de-regulation, an exponential decrease in metabolic activity is observed in aging worms (Braeckman *et al.* 2000). Ribosome biogenesis is a highly energy costly process, in which energy is not only necessary for the generation of ribosomal components, but also for their transport, processing and assembly into mature ribosomal subunits (Lempiainen & Shore 2009). Hence, the decline in available energy over time likely impinges on ribosome biogenesis.

In the same line, we found slower protein turnover rates for constituents of the folding machinery, which is tightly associated with the translation apparatus. Protein aggregates act as cellular sinks, not only for proteins but also for these cellular aggregation-prone chaperones, which bind these aggregates in an attempt to prevent further growth (Ravikumar *et al.* 2002). This sink effect likely attributes to the imbalance of the proteostasis network (overburdening of available chaperones) and eventually leads to the collapse of the aging proteome (Hipp *et al.* 2014).

Intriguingly, aging worms seem to safeguard their (protein) quality control mechanisms. Proteins associated with the ubiquitin/proteasomal system and antioxidant machinery preserve high turnover rates, possibly to manage the proteotoxic escalation and oxidative stress with age. Increased levels of protein aggregates, which show resistance against proteolytic degradation, potentially clog the proteolytic system as aggregate substrates can get stuck into the catalytic barrel (Hipp *et al.* 2014). The increase in misfolded proteins, together with the gradual interfered proteasomal function seems to trigger the synthesis of new proteasome components and the formation of functional proteasome degradation units with age (Aiken *et al.* 2011). This increase likely occurs through the transcriptional regulation of select proteasome subunits and their prioritized translation. Despite this attempt to maintain the degradation machinery, an overall decline in proteasomal activity is often

observed in aging organisms. Global energy becomes comprised with age, likely responsible for the lower efficiency of this ATP-dependent proteolytic complex (Hipp *et al.* 2014).

6.1.3 Protein turnover under reduced insulin/IGF-1 signaling

It is often thought that enhanced protein turnover supports somatic maintenance and preserves an undamaged, youthful proteome. In this vein, increased protein turnover seems to be a plausible strategy to retard aging and extend lifespan (Ryazanov & Nefsky 2002). However, several studies challenge this idea by the observation that attenuation of protein synthesis extends, rather than shortens lifespan in many species, including *C. elegans* (Kaeberlein *et al.* 2005; Hansen *et al.* 2007; Pan *et al.* 2007; Syntichaki *et al.* 2007). This finding is strongly reflected in our quantitative proteomics results, showing overall reduced protein turnover rates in the long-lived *daf-2* IIS mutant. The systematic downturn in protein turnover of ribosomal subunits, together with regulatory proteins involved in translation, was the most profound observation, which supports the overall diminished protein synthesis in *daf-2*. In agreement with these findings, conventional proteomics showed reduced levels of ribosomal subunits and translation regulation components (Depuydt *et al.* 2013; Stout *et al.* 2013). Recently, a causal factor was elucidated for this *daf-2* phenotype. A long noncoding RNA, transcribed telomeric sequence 1 (*tts-1*), binds ribosomes upon reduced IIS, thereby likely reducing the overall cellular translation (Essers *et al.* 2015). Despite the significant downturn of ribosomal protein levels in *daf-2* mutants, expression levels of ribosomal mRNA are actually higher in *daf-2* compared to wild-type worms (Depuydt *et al.* 2013). These observations indicate the intriguing possibility that the *tts-1* long noncoding RNA specifically regulates the translation of ribosomal mRNAs, thereby controlling ribosome biogenesis (Essers *et al.* 2015).

Regulation of mRNA translation and ribosome biosynthesis is crucial for survival upon stress (Arnsburg & Kirstein-Miles 2014). In fact, downturn of protein turnover can save much energy, which can be reallocated from anabolic processes (triggered by IIS) towards enhancement of stress response pathways (under reduced IIS) (McElwee *et al.* 2004). This idea fits well with the evolutionary theories of aging, including the Antagonistic Pleiotropy Theory and the Disposable Soma Theory **(See chapter 1 section 1.1.2)**. Inhibition of

translation initiation factor IFG-1 (eIF4G ortholog) seems to mediate the specific translation of stress genes (Rogers *et al.* 2011). Several stress-resistance systems are triggered in long-lived *daf-2*, including the resistance against proteotoxicity (Arnsburg & Kirstein-Miles 2014). For example, synthesis of trehalose, a chemical chaperone, was shown to stabilize the proteome in *daf-2* worms (Depuydt *et al.* submitted). Furthermore, reduced mRNA translation likely lowers chaperone burden by a decline of protein influx (Mehta *et al.* 2010; Kaeberlein 2013), preventing the exhaustion of the protein folding capacity. Additionally, *daf-2* worms seem to mediate proteome balance via the protective aggregate formation by small heat shock proteins. The sequestration of excess proteins into cellular aggregates might work protective, preventing proteotoxicity caused by highly interactive protein oligomers (True 2006; Walther *et al.* 2015). Taken together, a shift from protein turnover to protective proteome stabilizing strategies seems to promote the longevity phenotype in *daf-2*.

6.1.4 What can we learn from long-lived worms?

C. elegans represents a valuable tool to understand molecular mechanisms of the aging process. Many factors found being involved in the mammalian aging process are recapitulated in *C. elegans* and vice versa: accumulation of (damaged) macromolecules, progressive deterioration of structural cellular components, decline in physiological functions and increase in vulnerability to stress and infections with age (Torgovnick *et al.* 2013). However, can we extrapolate our conclusions to human aging?

The insulin signaling pathway is strongly conserved through evolution (Curran & Ruvkun 2007). Reduced insulin/IGF-1 signaling results in extended lifespan via the action of the transcription factor DAF-16/FOXO, key regulator of this pathway in worms and flies. FOXO3A represents a human homologue which is over-represented in longest-lived human individuals (Willcox *et al.* 2008; Anselmi *et al.* 2009; Flachsbart *et al.* 2009). Although this relationship between FOXO3A and human long-life is correlative, it is a strong indication for its role in human longevity.

In this thesis, we observed the systematic slowdown of individual protein turnover under reduced insulin/IGF-1 signaling, especially for proteins involved in the translation machinery.

Its seems that long-lived *daf-2* worms enter an energy-saving state along with the increased stabilization of the proteome to preserve somatic integrity (Depuydt *et al.* submitted). In this vein, the chemical chaperone trehalose, known to stabilize protein structures, is of high interest for health and longevity. Trehalose, a disaccharide of glucose, is present in a wide variety of organisms, including unicellular animals, insects, invertebrates and plants but not in humans (Elbein *et al.* 2003). Several studies in mice and human cells show its beneficial effects on protein aggregation in neurodegenerative diseases (Casarejos *et al.* 2011; Kruger *et al.* 2011). Moreover, trehalose seems to mitigate insulin resistance via its hypoinsulinemic effect (Arai *et al.* 2010). However, clear scientific studies to confirm health benefits of trehalose supplementation in humans is still missing.

Nevertheless, caution should be used in drawing conclusions to human aging. Whereas lower protein turnover seems to be beneficial in the eutelic post-mitotic *C. elegans* worms, it is likely that human tissues, which undergo frequent cell divisions, strongly rely on protein turnover. Therefore, attenuation of this critical process might have detrimental effects. In addition, laboratory-bred worms are selected to be maintained in the lab, which implies rapid reproduction, minimal stressful conditions and high food availability. This is very different from the challenging conditions human proteomes are exposed too.

6.2 Perspectives for future research

The dynamic changes of protein half-lives in aging and long-lived worms could be determined via the SILeNCe labeling technique and accurate mass spectrometry analysis. Precise estimations of protein turnover could be made due to the conserved algorithm, despite the consequential limited protein diversity. One way to increase the number of proteins is by involving a pre-labeling period starting from stage four larvae (L4). Food-uptake is high during development and in early adulthood (until day 1) and steadily decrease with age (Depuydt *et al.* 2014). Development and growth involve anabolic processes, which will result in the enhanced incorporation of the heavy label into proteins. The pre-labeling period will enable to monitor $^{15}N/^{14}N$ ratios of less abundant proteins and thus improve protein diversity. In order to monitor steady-state protein turnover, samples should be taken from day 2 of adulthood and choosing time-points with exponentially growing spans are the

most ideal if broad, proteome wide analysis of protein turnover is desired (Price & Ghaemmaghami 2014). A major drawback of the SILeNCe and other labeling methods is that they are dependent on food-uptake, label uptake, flux through multiple tissues and internal label-recycling. In this vein, Dendra2 is an elegant method to analyze protein turnover independently of label incorporation. The *in vivo* expression of Dendra2-fused proteins overcomes all these issues. Nevertheless, the generation of Dendra2-transgenic worms is time-consuming and only one protein can be studied at a time. Therefore, it is a great validation method to check certain protein half-lives of interest estimated with a high throughput isotopic labeling technique **(See chapter 4)**.

Most of experiments in this thesis are performed using the temperature-sensitive sterile *glp-4(bn2ts)I* mutant background. GLP-4 protein is required for the normal proliferation of the germline, hence mutation in *glp-4* enables us to focus on somatic cells and avoids purging of ^{15}N-label via egg laying **(See chapter 3, section 3.3)**. However, working with this mutant may have some disadvantages. First, protein turnover cannot be monitored in the somatic gonad. Secondly, loss of germline cells by laser ablation extends lifespan in *C. elegans*, while overproliferation causes rapid aging (Hsin & Kenyon 1999; Arantes-Oliveira *et al.* 2002). Hence, germline signaling play a key role in the regulation of aging and other biological processes. Although mutation in *glp-4* has only minimal effects on lifespan of wild-type and *daf-16* nematodes (TeKippe & Aballay 2010), the mutant condition may affect somatic tissues. Thirdly, it has recently been shown that *glp-4* encodes the valyl aminoacyl transfer RNA (tRNA) synthetase VARS-2, which acts in the germline and somatic tissues. At the restrictive temperature, *glp-4* mutants are partially deficient for valine-tRNA synthetase function in the soma, raising the possibility that the *glp-4* mutation causes reduced protein synthesis, which lead to the observed mutant phenotype (Rastogi *et al.* 2015). Since this finding forms a major concern, future experiments should include proper controls to study individual protein turnovers.

It becomes clear that increased protein turnover does not underlie longevity in *daf-2* **(See chapter 3)**. Still, it would be interesting to test the effect of increased protein turnover in these long-lived mutants. Protein synthesis can be stimulated by the administration of the neurotransmitter serotonin, which can induce feeding behavior in worms (Gomez-Amaro *et*

al. 2015). In a preliminary experiment, we found that long-lived *daf-2* worms, treated with serotonin, display increased biomass, probably through serotonin-induced protein synthesis. Surprisingly, lifespan of these worms was not reduced **(See chapter 5)**. Therefore, it would be interesting to measure bulk protein turnover, using classical ^{35}S-labeling, to monitor and confirm the effect of serotonin on protein synthesis and degradation. To this end, it would be possible to completely uncouple protein turnover as potential strategy for lifespan extension. Additionally, a serotonin SILeNCe experiment could reveal the effect of serotonin on the individual protein turnover rates. It would be interesting to find the specific restored protein turnover rates of distinct functional groups, including proteins of the translation machinery in serotonin-treated *daf-2*. In a preliminary experiment, serotonin could be administered to the ribosomal (RLA-1)-fused Dendra2 transgenic strain in order to provide evidence for the re-establish protein turnover of the translation machinery in serotonin-treated *daf-2*.

Additionally, it would be very interesting to analyze cell-specific age-related changes at the molecular level of proteins in *C. elegans*. Different cell types age and deteriorate at markedly different rates (Herndon *et al.* 2002). Herndon *et al.* (2002) analyzed the cellular changes in senescing worms and observed the progressive and profound age-related decline of muscle cells, while nerve cells are spared of extensive cellular deterioration. However, it is very challenging to isolate cell types by sorting or microdissection methods in small animals such as *C. elegans* worms. Recently, researches succeed to analyze protein synthesis in specific cell types *in vivo* in intact worms (Yuet *et al.* 2015). An engineered phenylalanyl-tRNA synthetase, capable of tagging proteins with the reactive unnatural phenylalanine analoge *p*-azido-*L*-Phe (Azf), was expressed in specific tissues of interest to achieve spatiotemporal selectivity in protein labeling. Cell-specific proteins could be enriched for proteomic analysis via a click chemistry approach, involving the bioorthogonal conjugation of the Azf azide side chain of the tagged proteins to probes (alkynyl or cyclooctynyl) that permit detection, purification and visualization of labeled proteins (Yuet *et al.* 2015).

Further research should also focus on the regulation mechanisms responsible for the modulation of mRNA translation. Apparently, noncoding RNAs, short (miRNAs) as well as long, are expected to play a prominent role in this process and understanding of their actions will provide more insight in the regulation of aging. In addition, translational

regulation can be distinguished and characterized via ribosome profiling or RiboSeq, a innovative technique to monitor actively translated mRNAs at a global scale (Ingolia *et al.* 2014). This method has been optimized for the application in *C. elegans* (Aeschimann *et al.* 2015) and could reveal exciting differences in the translatome between long-lived worms and control animals with normal lifespan.

Finally, it seems that cellular energy is allocated to stress-response systems, including the increased proteome stability by trehalose and beneficial aggregation mediated via sHSP. In order generate a broader understanding of the *daf-2* longevity phenotype, future studies should focus on new potential mechanisms important in the stabilization, rather than the renewal of the proteome.

6.3 Conclusion

In conclusion, this thesis outlines a detailed overview of individual protein turnover patterns during aging and longevity. Protein turnover is a dynamic process, which allows cells to regulate their protein content upon changing physiological states and environmental conditions. Our data, together with others, indicate the gradual de-regulation of anabolic processes, resulting in the excess of irrelevant biomass in post-reproductive aging *C. elegans*. The overwhelmed proteostasis network cannot manage the increasing proteotoxicity over time, whereby probably hypertrophy, and not oxidative protein damage, primary leads to death. Intriguingly, protein turnover shows an inverse relation to longevity, thereby contradicting the protein turnover hypothesis of aging and supporting the hyperthrophy hypothesis. The controlled downturn of ribosomal turnover in long-lived *daf-2* contradicts the haphazard slowdown of ribosomal proteins during aging. Apparently, reduced protein synthesis, by lowering ribosomal levels and inhibition of mRNA translation, is a key factor in *daf-2* longevity. Since reduced IIS switches off anabolic processes, the aging process seems retarded by avoiding the onset of hypertrophy-related pathologies.

List of abbreviations

AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
DNA	Deoxyribonucleic acid
ER	Endoplasmic reticulum
FRTA	Free Radical Theory of Aging
GFP	Green fluorescent protein
IIS	Insulin/IGF-1 signaling
L1/2/3/4	Larval stage 1/2/3/4
lncRNA	Long noncoding RNA
miRNA	microRNA
mRNA	Messenger ribonucleic acid
MS	Mass Spectrometry
PI3K	Phosphatidiylinositol 3-kinase
PQC	Protein quality control
PTM	Pavlidis Template Matching
RER	Rough endoplasmic reticulum
RiboSeq	Ribosome Profiling
ROS	Reactive oxygen species
sHSP	Small heat shock proteins
SILAC	Stable Isotope Labeling by Amino acids in Cell culture
SILeNCe	Stable Isotope Labeling by Nitrogen in *Caenorhabdtis elegans*
TOR	Target of Rapamycin
UPS	Ubiquitin-proteasome system

REFERENCES

Adachi H, Fujiwara Y , Ishii N (1998). Effects of oxygen on protein carbonyl and aging in Caenorhabditis elegans mutants with long (age-1) and short (mev-1) life spans. *J Gerontol A Biol Sci Med Sci*. **53**, B240-244.

Adams J (2008). The Proteome: Discovering the Structure and Function of Proteins. *Nature Education*. **1(3)**, 6.

Aeschimann F, Xiong J, Arnold A, Dieterich C , Grosshans H (2015). Transcriptome-wide measurement of ribosomal occupancy by ribosome profiling. *Methods*. **85**, 75-89.

Ahringer J (2006). Reverse genetics. *Wormbook*.

Aiken CT, Kaake RM, Wang X , Huang L (2011). Oxidative stress-mediated regulation of proteasome complexes. *Mol Cell Proteomics*. **10**, R110 006924.

Altun ZF , Hall DH (2009). Introduction. *In Wormatlas*.

Andersen JS, Lam YW, Leung AK, Ong SE, Lyon CE, Lamond AI , Mann M (2005). Nucleolar proteome dynamics. *Nature*. **433**, 77-83.

Anselmi CV, Malovini A, Roncarati R, Novelli V, Villa F, Condorelli G, Bellazzi R , Puca AA (2009). Association of the FOXO3A locus with extreme longevity in a southern Italian centenarian study. *Rejuvenation Res*. **12**, 95-104.

Apfeld J , Kenyon C (1998). Cell nonautonomy of C. elegans daf-2 function in the regulation of diapause and life span. *Cell*. **95**, 199-210.

Apfeld J, O'Connor G, McDonagh T, DiStefano PS , Curtis R (2004). The AMP-activated protein kinase AAK-2 links energy levels and insulin-like signals to lifespan in C. elegans. *Genes Dev*. **18**, 3004-3009.

Arai C, Arai N, Mizote A, Kohno K, Iwaki K, Hanaya T, Arai S, Ushio S , Fukuda S (2010). Trehalose prevents adipocyte hypertrophy and mitigates insulin resistance. *Nutr Res*. **30**, 840-848.

Arantes-Oliveira N, Apfeld J, Dillin A , Kenyon C (2002). Regulation of life-span by germ-line stem cells in Caenorhabditis elegans. *Science*. **295**, 502-505.

Arias IM, Doyle D , Schimke RT (1969). Studies on the synthesis and degradation of proteins of the endoplasmic reticulum of rat liver. *J Biol Chem*. **244**, 3303-3315.

Arking R (1998). Molecular basis of extended longevity in selected Drosophila strains. *Current Science*. **74**, 859-864.

Arnsburg K , Kirstein-Miles J (2014). Interrelation between protein synthesis, proteostasis and life span. *Curr Genomics*. **15**, 66-75.

Ashrafi K (2007). Obesity and the regulation of fat metabolism. *WormBook*, 1-20.

Ayyadevara S, Dandapat A, Singh SP, Siegel ER, Shmookler Reis RJ, Zimniak L , Zimniak P (2007). Life span and stress resistance of Caenorhabditis elegans are differentially affected by glutathione transferases metabolizing 4-hydroxynon-2-enal. *Mech Ageing Dev*. **128**, 196-205.

References

Back P, De Vos WH, Depuydt GG, Matthijssens F, Vanfleteren JR , Braeckman BP (2012). Exploring real-time in vivo redox biology of developing and aging Caenorhabditis elegans. *Free Radic Biol Med*. **52**, 850-859.

Baek D, Villen J, Shin C, Camargo FD, Gygi SP , Bartel DP (2008). The impact of microRNAs on protein output. *Nature*. **455**, 64-71.

Balch WE, Morimoto RI, Dillin A , Kelly JW (2008). Adapting proteostasis for disease intervention. *Science*. **319**, 916-919.

Bartke A (2008). Insulin and aging. *Cell Cycle*. **7**, 3338-3343.

Berman JR , Kenyon C (2006). Germ-cell loss extends C. elegans life span through regulation of DAF-16 by kri-1 and lipophilic-hormone signaling. *Cell*. **124**, 1055-1068.

Bettelheim F, Brown W, Campbell M, Farrell S , Torres O (2015). *Introduction to General, Organic and Biochemistry*: Cengage Learning.

Bierman EL (1978). The effect of donor age on the in vitro life span of cultured human arterial smooth-muscle cells. *In Vitro*. **14**, 951-955.

Blackburn EH, Greider CW, Henderson E, Lee MS, Shampay J , Shippen-Lentz D (1989). Recognition and elongation of telomeres by telomerase. *Genome*. **31**, 553-560.

Blagosklonny MV Aging is not programmed: genetic pseudo-program is a shadow of developmental growth. *Cell Cycle*. **12**, 3736-3742.

Blagosklonny MV (2006). Aging and immortality: quasi-programmed senescence and its pharmacologic inhibition. *Cell Cycle*. **5**, 2087-2102.

Blagosklonny MV (2007a). An anti-aging drug today: from senescence-promoting genes to anti-aging pill. *Drug Discov Today*. **12**, 218-224.

Blagosklonny MV (2007b). Paradoxes of aging. *Cell Cycle*. **6**, 2997-3003.

Blagosklonny MV (2008). Aging: ROS or TOR. *Cell Cycle*. **7**, 3344-3354.

Blagosklonny MV (2010a). Rapamycin and quasi-programmed aging: four years later. *Cell Cycle*. **9**, 1859-1862.

Blagosklonny MV (2010b). Revisiting the antagonistic pleiotropy theory of aging: TOR-driven program and quasi-program. *Cell Cycle*. **9**, 3151-3156.

Blagosklonny MV (2012). Answering the ultimate question "what is the proximal cause of aging?". *Aging (Albany NY)*. **4**, 861-877.

Blagosklonny MV (2013). Aging is not programmed: genetic pseudo-program is a shadow of developmental growth. *Cell Cycle*. **12**, 3736-3742.

Blagosklonny MV (2015). Rejuvenating immunity: "anti-aging drug today" eight years later. *Oncotarget*. **6**, 19405-19412.

Bogaerts A, Beets I, Temmerman L, Schoofs L , Verleyen P (2010). Proteome changes of Caenorhabditis elegans upon a Staphylococcus aureus infection. *Biol Direct*. **5**, 11.

Boisvert FM, Ahmad Y, Gierlinski M, Charriere F, Lamont D, Scott M, Barton G , Lamond AI (2012). A quantitative spatial proteomics analysis of proteome turnover in human cells. *Mol Cell Proteomics*. **11**, M111 011429.

Bolanowski MA, Russell RL , Jacobson LA (1981). Quantitative measures of aging in the nematode Caenorhabditis elegans. I. Population and longitudinal studies of two behavioral parameters. *Mech Ageing Dev*. **15**, 279-295.

Bordone L , Guarente L (2005). Calorie restriction, SIRT1 and metabolism: understanding longevity. *Nat Rev Mol Cell Biol*. **6**, 298-305.

Braeckman BP, Houthoofd K, Brys K, Lenaerts I, De Vreese A, Van Eygen S, Raes H , Vanfleteren JR (2002a). No reduction of energy metabolism in Clk mutants. *Mech Ageing Dev*. **123**, 1447-1456.

Braeckman BP, Houthoofd K, De Vreese A , Vanfleteren JR (1999). Apparent uncoupling of energy production and consumption in long-lived Clk mutants of Caenorhabditis elegans. *Curr Biol*. **9**, 493-496.

Braeckman BP, Houthoofd K , Vanfleteren JR (2000). Patterns of metabolic activity during aging of the wild type and longevity mutants of Caenorhabditis elegans. *J Am Aging Assoc*. **23**, 55-73.

Braeckman BP, Houthoofd K , Vanfleteren JR (2002b). Assessing metabolic activity in aging Caenorhabditis elegans: concepts and controversies. *Aging Cell*. **1**, 82-88; discussion 102-103.

Bravo-San Pedro JM , Senovilla L (2013). Immunostimulatory activity of lifespan-extending agents. *Aging (Albany NY)*. **5**, 793-801.

Brenner S (1974). The genetics of Caenorhabditis elegans. *Genetics*. **77**, 71-94.

Brieger K, Schiavone S, Miller FJ, Jr. , Krause KH (2012). Reactive oxygen species: from health to disease. *Swiss Med Wkly*. **142**, w13659.

Brown WM , Brown PM (2003). *Transcription*: Taylor & Francis.

Brys K, Castelein N, Matthijssens F, Vanfleteren JR , Braeckman BP (2010). Disruption of insulin signalling preserves bioenergetic competence of mitochondria in ageing Caenorhabditis elegans. *BMC Biol*. **8**, 91.

Butler PG, Wanamaker AD, Scourse JD, Richardson CA , Reynolds DJ (2013). Variability of marine climate on the North Icelandic Shelf in a 1357-year proxy archive based on growth increments in the bivalve Arctica islandica. *Palaeogeography, Palaeoclimatology, Palaeoecology*. **373**, 141-151.

Calahorro F , Ruiz-Rubio M (2012). Functional phenotypic rescue of Caenorhabditis elegans neuroligin-deficient mutants by the human and rat NLGN1 genes. *PLoS One*. **7**, e39277.

Cambridge SB, Gnad F, Nguyen C, Bermejo JL, Kruger M , Mann M (2011). Systems-wide proteomic analysis in mammalian cells reveals conserved, functional protein turnover. *J Proteome Res*. **10**, 5275-5284.

Cargile BJ, Bundy JL, Grunden AM , Stephenson JL, Jr. (2004). Synthesis/degradation ratio mass spectrometry for measuring relative dynamic protein turnover. *Anal Chem*. **76**, 86-97.

Carrano AC, Liu Z, Dillin A , Hunter T (2009). A conserved ubiquitination pathway determines longevity in response to diet restriction. *Nature*. **460**, 396-399.

References

Carrard G, Bulteau AL, Petropoulos I , Friguet B (2002). Impairment of proteasome structure and function in aging. *Int J Biochem Cell Biol*. **34**, 1461-1474.

Carre-Pierrat M, Baillie D, Johnsen R, Hyde R, Hart A, Granger L , Segalat L (2006). Characterization of the Caenorhabditis elegans G protein-coupled serotonin receptors. *Invert Neurosci*. **6**, 189-205.

Casarejos MJ, Solano RM, Gomez A, Perucho J, de Yebenes JG , Mena MA (2011). The accumulation of neurotoxic proteins, induced by proteasome inhibition, is reverted by trehalose, an enhancer of autophagy, in human neuroblastoma cells. *Neurochem Int*. **58**, 512-520.

Cassada RC , Russell RL (1975). The dauerlarva, a post-embryonic developmental variant of the nematode Caenorhabditis elegans. *Dev Biol*. **46**, 326-342.

Ceron J , Swoboda P (2008). Caenorhabditis elegans comes of age. *Genome Biol*. **9**, 312.

Chalfie M, Tu Y, Euskirchen G, Ward WW , Prasher DC (1994). Green fluorescent protein as a marker for gene expression. *Science*. **263**, 802-805.

Chance B, Sies H , Boveris A (1979). Hydroperoxide metabolism in mammalian organs. *Physiol Rev*. **59**, 527-605.

Charmpilas N, Daskalaki I, Papandreou ME , Tavernarakis N (2015). Protein synthesis as an integral quality control mechanism during ageing. *Ageing Res Rev*. **23**, 75-89.

Chiang WC, Ching TT, Lee HC, Mousigian C , Hsu AL (2012). HSF-1 regulators DDL-1/2 link insulin-like signaling to heat-shock responses and modulation of longevity. *Cell*. **148**, 322-334.

Chikina MD, Huttenhower C, Murphy CT , Troyanskaya OG (2009). Global prediction of tissue-specific gene expression and context-dependent gene networks in Caenorhabditis elegans. *PLoS Comput Biol*. **5**, e1000417.

Chiocchetti A, Zhou J, Zhu H, Karl T, Haubenreisser O, Rinnerthaler M, Heeren G, Oender K, Bauer J, Hintner H, Breitenbach M , Breitenbach-Koller L (2007). Ribosomal proteins Rpl10 and Rps6 are potent regulators of yeast replicative life span. *Exp Gerontol*. **42**, 275-286.

Chondrogianni N, Georgila K, Kourtis N, Tavernarakis N , Gonos ES (2015). 20S proteasome activation promotes life span extension and resistance to proteotoxicity in Caenorhabditis elegans. *FASEB J*. **29**, 611-622.

Chondrogianni N, Petropoulos I, Franceschi C, Friguet B , Gonos ES (2000). Fibroblast cultures from healthy centenarians have an active proteasome. *Exp Gerontol*. **35**, 721-728.

Chow DK, Glenn CF, Johnston JL, Goldberg IG , Wolkow CA (2006). Sarcopenia in the Caenorhabditis elegans pharynx correlates with muscle contraction rate over lifespan. *Experimental Gerontology*. **41**, 252-260.

Chudakov DM, Lukyanov S , Lukyanov KA (2007). Tracking intracellular protein movements using photoswitchable fluorescent proteins PS-CFP2 and Dendra2. *Nat Protoc*. **2**, 2024-2032.

Claydon AJ , Beynon R (2012). Proteome dynamics: revisiting turnover with a global perspective. *Mol Cell Proteomics*. **11**, 1551-1565.

Collins JJ, Huang C, Hughes S , Kornfeld K (2008). The measurement and analysis of age-related changes in Caenorhabditis elegans. *WormBook*, 1-21.

Colman RJ, Anderson RM, Johnson SC, Kastman EK, Kosmatka KJ, Beasley TM, Allison DB, Cruzen C, Simmons HA, Kemnitz JW , Weindruch R (2009). Caloric restriction delays disease onset and mortality in rhesus monkeys. *Science*. **325**, 201-204.

Colman RJ, Beasley TM, Kemnitz JW, Johnson SC, Weindruch R , Anderson RM (2014). Caloric restriction reduces age-related and all-cause mortality in rhesus monkeys. *Nat Commun*. **5**, 3557.

Cooper (2000). *The Cell: A Molecular Approach*: ASM Press.

Cunningham KA, Bouagnon AD, Barros AG, Lin L, Malard L, Romano-Silva MA , Ashrafi K (2014). Loss of a neural AMP-activated kinase mimics the effects of elevated serotonin on fat, movement, and hormonal secretions. *PLoS Genet*. **10**, e1004394.

Curran SP , Ruvkun G (2007). Lifespan regulation by evolutionarily conserved genes essential for viability. *PLoS Genet*. **3**, e56.

Curtis R, O'Connor G , DiStefano PS (2006). Aging networks in Caenorhabditis elegans: AMP-activated protein kinase (aak-2) links multiple aging and metabolism pathways. *Aging Cell*. **5**, 119-126.

Dancy BC, Chen SW, Drechsler R, Gafken PR , Olsen CP (2015). 13C- and 15N-Labeling Strategies Combined with Mass Spectrometry Comprehensively Quantify Phospholipid Dynamics in C. elegans. *PLoS One*. **10**, e0141850.

David DC, Ollikainen N, Trinidad JC, Cary MP, Burlingame AL , Kenyon C (2010). Widespread protein aggregation as an inherent part of aging in C. elegans. *PLoS Biol*. **8**, e1000450.

Demidenko ZN, Shtutman M , Blagosklonny MV (2009). Pharmacologic inhibition of MEK and PI-3K converges on the mTOR/S6 pathway to decelerate cellular senescence. *Cell Cycle*. **8**, 1896-1900.

Dennis G, Jr., Sherman BT, Hosack DA, Yang J, Gao W, Lane HC , Lempicki RA (2003). DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome Biol*. **4**, P3.

DePina AS, Iser WB, Park SS, Maudsley S, Wilson MA , Wolkow CA (2011). Regulation of Caenorhabditis elegans vitellogenesis by DAF-2/IIS through separable transcriptional and posttranscriptional mechanisms. *BMC Physiol*. **11**, 11.

Depuydt G, Shanmugam N, Rasulova M, Dhondt I , Braeckman BP (submitted). Increased protein stability and decreased protein turnover in the *C. elegans* Ins/IGF-1 *daf-2* mutant.

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

Depuydt G, Xie F, Petyuk VA, Smolders A, Brewer HM, Camp DG, 2nd, Smith RD , Braeckman BP (2014). LC-MS proteomics analysis of the insulin/IGF-1-deficient Caenorhabditis

elegans daf-2(e1370) mutant reveals extensive restructuring of intermediary metabolism. *J Proteome Res*. **13**, 1938-1956.

Dhalla NS, Temsah RM , Netticadan T (2000). Role of oxidative stress in cardiovascular diseases. *J Hypertens*. **18**, 655-673.

Dhondt *et al.* (submitted).

Dillin A, Crawford DK , Kenyon C (2002). Timing requirements for insulin/IGF-1 signaling in C. elegans. *Science*. **298**, 830-834.

Dobzhansky T (1964). Biology, Molecular and Organismic. *Am Zool*. **4**, 443-452.

Doherty MK, Whitehead C, McCormack H, Gaskell SJ , Beynon RJ (2005). Proteome dynamics in complex organisms: using stable isotopes to monitor individual protein turnover rates. *Proteomics*. **5**, 522-533.

Dominick G, Berryman DE, List EO, Kopchick JJ, Li X, Miller RA , Garcia GG (2015). Regulation of mTOR activity in Snell dwarf and GH receptor gene-disrupted mice. *Endocrinology*. **156**, 565-575.

Dong MQ, Venable JD, Au N, Xu T, Park SK, Cociorva D, Johnson JR, Dillin A , Yates JR, 3rd (2007). Quantitative mass spectrometry identifies insulin signaling targets in C. elegans. *Science*. **317**, 660-663.

Doonan R, McElwee JJ, Matthijssens F, Walker GA, Houthoofd K, Back P, Matscheski A, Vanfleteren JR , Gems D (2008). Against the oxidative damage theory of aging: superoxide dismutases protect against oxidative stress but have little or no effect on life span in Caenorhabditis elegans. *Genes Dev*. **22**, 3236-3241.

Du P, Kibbe WA , Lin SM (2006). Improved peak detection in mass spectrum by incorporating continuous wavelet transform-based pattern matching. *Bioinformatics*. **22**, 2059-2065.

Elbein AD, Pan YT, Pastuszak I , Carroll D (2003). New insights on trehalose: a multifunctional molecule. *Glycobiology*. **13**, 17R-27R.

Erdamar H, Demirci H, Yaman H, Erbil MK, Yakar T, Sancak B, Elbeg S, Biberoglu G , Yetkin I (2008). The effect of hypothyroidism, hyperthyroidism, and their treatment on parameters of oxidative stress and antioxidant status. *Clin Chem Lab Med*. **46**, 1004-1010.

Essers PB, Nonnekens J, Goos YJ, Betist MC, Viester MD, Mossink B, Lansu N, Korswagen HC, Jelier R, Brenkman AB , MacInnes AW (2015). A Long Noncoding RNA on the Ribosome Is Required for Lifespan Extension. *Cell Reports*. **10**, 339-345.

Fabian MR, Sonenberg N , Filipowicz W (2010). Regulation of mRNA translation and stability by microRNAs. *Annu Rev Biochem*. **79**, 351-379.

Ferrington DA, Husom AD , Thompson LV (2005). Altered proteasome structure, function, and oxidation in aged muscle. *FASEB J*. **19**, 644-646.

Finch CE (1998). Variations in senescence and longevity include the possibility of negligible senescence. *J Gerontol A Biol Sci Med Sci*. **53**, B235-239.

Flachsbart F, Caliebe A, Kleindorp R, Blanche H, von Eller-Eberstein H, Nikolaus S, Schreiber S , Nebel A (2009). Association of FOXO3A variation with human longevity confirmed in German centenarians. *Proc Natl Acad Sci U S A*. **106**, 2700-2705.

Friedman DB , Johnson TE (1988). A mutation in the age-1 gene in Caenorhabditis elegans lengthens life and reduces hermaphrodite fertility. *Genetics*. **118**, 75-86.

Frokjaer-Jensen C, Davis MW, Sarov M, Taylor J, Flibotte S, LaBella M, Pozniakovsky A, Moerman DG , Jorgensen EM (2014). Random and targeted transgene insertion in Caenorhabditis elegans using a modified Mos1 transposon. *Nat Methods*. **11**, 529-534.

Fuchs S, Bundy JG, Davies SK, Viney JM, Swire JS , Leroi AM (2010). A metabolic signature of long life in Caenorhabditis elegans. *BMC Biol*. **8**, 14.

Gaczynska M, Osmulski PA , Ward WF (2001). Caretaker or undertaker? The role of the proteasome in aging. *Mech Ageing Dev*. **122**, 235-254.

Gafni A (1990). Altered protein metabolism in aging. *Annu Rev Gerontol Geriatr*. **10**, 117-131.

Gasteiger E, Hoogland C, Gattiker A, Duvaud S, Wilkins MR, Appel RD , Bairoch A (2005). Protein Identification and Analysis Tools on the ExPASy Server. (JM Walker, ed^eds). Totowa: Humana Press.

Geillinger KE, Kuhlmann K, Eisenacher M, Meyer HE, Daniel H , Spanier B (2012). Dynamic changes of the Caenorhabditis elegans proteome during ontogenesis assessed by quantitative analysis with 15N metabolic labeling. *J Proteome Res*. **11**, 4594-4604.

Gelino S , Hansen M (2012). Autophagy - An Emerging Anti-Aging Mechanism. *J Clin Exp Pathol*. **Suppl 4**.

Gems D (2015). The aging-disease false dichotomy: understanding senescence as pathology. *Frontiers in Genetics*. **6**.

Gems D , de la Guardia Y (2013). Alternative Perspectives on Aging in Caenorhabditis elegans: Reactive Oxygen Species or Hyperfunction? *Antioxid Redox Signal*. **19**, 321-329.

Gems D, Sutton AJ, Sundermeyer ML, Albert PS, King KV, Edgley ML, Larsen PL , Riddle DL (1998). Two pleiotropic classes of daf-2 mutation affect larval arrest, adult behavior, reproduction and longevity in Caenorhabditis elegans. *Genetics*. **150**, 129-155.

Ghazi A, Henis-Korenblit S , Kenyon C (2007). Regulation of Caenorhabditis elegans lifespan by a proteasomal E3 ligase complex. *Proc Natl Acad Sci U S A*. **104**, 5947-5952.

Goldberg AL (2003). Protein degradation and protection against misfolded or damaged proteins. *Nature*. **426**, 895-899.

Golden TR , Melov S (2007). Gene expression changes associated with aging in C. elegans. *WormBook*, 1-12.

Gomez-Amaro RL, Valentine ER, Carretero M, LeBoeuf SE, Rangaraju S, Broaddus CD, Solis GM, Williamson JR , Petrascheck M (2015). Measuring Food Intake and Nutrient Absorption in Caenorhabditis elegans. *Genetics*.

References

Gonda DK, Bachmair A, Wunning I, Tobias JW, Lane WS , Varshavsky A (1989). Universality and structure of the N-end rule. *J Biol Chem*. **264**, 16700-16712.

Goyns MH , Lavery WL (2000). Telomerase and mammalian ageing: a critical appraisal. *Mech Ageing Dev*. **114**, 69-77.

Gray DA, Tsirigotis M , Woulfe J (2003). Ubiquitin, proteasomes, and the aging brain. *Sci Aging Knowledge Environ*. **2003**, RE6.

Greer EL , Brunet A (2011). The genetic network of life-span extension by dietary restriction. *In: Masoro E.J., Austad S.N, eds. Handbook of the Biology of Aging*.

Greer EL, Dowlatshahi D, Banko MR, Villen J, Hoang K, Blanchard D, Gygi SP , Brunet A (2007). An AMPK-FOXO pathway mediates longevity induced by a novel method of dietary restriction in C. elegans. *Curr Biol*. **17**, 1646-1656.

Greider CW (1996). Telomere length regulation. *Annu Rev Biochem*. **65**, 337-365.

Greider CW , Blackburn EH (1989). A telomeric sequence in the RNA of Tetrahymena telomerase required for telomere repeat synthesis. *Nature*. **337**, 331-337.

Gruber J, Chen CB, Fong S, Ng LF, Teo E , Halliwell B (2015). Caenorhabditis elegans: What We Can and Cannot Learn from Aging Worms. *Antioxid Redox Signal*. **23**, 256-279.

Grune T (2000). Oxidative stress, aging and the proteasomal system. *Biogerontology*. **1**, 31-40.

Grune T, Jung T, Merker K , Davies KJ (2004). Decreased proteolysis caused by protein aggregates, inclusion bodies, plaques, lipofuscin, ceroid, and 'aggresomes' during oxidative stress, aging, and disease. *Int J Biochem Cell Biol*. **36**, 2519-2530.

Guarente L (2005). Calorie restriction and SIR2 genes--towards a mechanism. *Mech Ageing Dev*. **126**, 923-928.

Gurskaya NG, Verkhusha VV, Shcheglov AS, Staroverov DB, Chepurnykh TV, Fradkov AF, Lukyanov S , Lukyanov KA (2006). Engineering of a monomeric green-to-red photoactivatable fluorescent protein induced by blue light. *Nat Biotechnol*. **24**, 461-465.

Guruprasad K, Reddy BV , Pandit MW (1990). Correlation between stability of a protein and its dipeptide composition: a novel approach for predicting in vivo stability of a protein from its primary sequence. *Protein Eng*. **4**, 155-161.

Haldane JBS (1941). New paths in genetics. *New Paths in Genetics.*

Hamer G, Matilainen O , Holmberg CI (2010). A photoconvertible reporter of the ubiquitin-proteasome system in vivo. *Nat Methods*. **7**, 473-478.

Hansen M, Chandra A, Mitic LL, Onken B, Driscoll M , Kenyon C (2008). A role for autophagy in the extension of lifespan by dietary restriction in C. elegans. *PLoS Genet*. **4**, e24.

Hansen M, Taubert S, Crawford D, Libina N, Lee SJ , Kenyon C (2007). Lifespan extension by conditions that inhibit translation in Caenorhabditis elegans. *Aging Cell*. **6**, 95-110.

Hardie DG , Hawley SA (2001). AMP-activated protein kinase: the energy charge hypothesis revisited. *Bioessays*. **23**, 1112-1119.

Hardie DG, Ross FA , Hawley SA (2012). AMPK: a nutrient and energy sensor that maintains energy homeostasis. *Nat Rev Mol Cell Biol*. **13**, 251-262.

Harley CB, Futcher AB , Greider CW (1990). Telomeres shorten during ageing of human fibroblasts. *Nature*. **345**, 458-460.

Harman D (1956). Aging: a theory based on free radical and radiation chemistry. *J Gerontol*. **11**, 298-300.

Harman D (1972). The biologic clock: the mitochondria? *J Am Geriatr Soc*. **20**, 145-147.

Hartl FU, Bracher A , Hayer-Hartl M (2011). Molecular chaperones in protein folding and proteostasis. *Nature*. **475**, 324-332.

Hartley JL, Temple GF , Brasch MA (2000). DNA cloning using in vitro site-specific recombination. *Genome Res*. **10**, 1788-1795.

Hashimoto Y, Ookuma S , Nishida E (2009). Lifespan extension by suppression of autophagy genes in Caenorhabditis elegans. *Genes Cells*. **14**, 717-726.

Hayflick L (1965). The Limited in Vitro Lifetime of Human Diploid Cell Strains. *Exp Cell Res*. **37**, 614-636.

He C , Klionsky DJ (2009). Regulation mechanisms and signaling pathways of autophagy. *Annu Rev Genet*. **43**, 67-93.

Heald R , Cohen-Fix O (2014). Morphology and function of membrane-bound organelles. *Curr Opin Cell Biol*. **26**, 79-86.

Healy K, Guillerme T, Finlay S, Kane A, Kelly SB, McClean D, Kelly DJ, Donohue I, Jackson AL , Cooper N (2014). Ecology and mode-of-life explain lifespan variation in birds and mammals. *Proc Biol Sci*. **281**, 20140298.

Helbig AO, Daran-Lapujade P, van Maris AJ, de Hulster EA, de Ridder D, Pronk JT, Heck AJ , Slijper M (2011). The diversity of protein turnover and abundance under nitrogen-limited steady-state conditions in Saccharomyces cerevisiae. *Mol Biosyst*. **7**, 3316-3326.

Herndon LA, Schmeissner PJ, Dudaronek JM, Brown PA, Listner KM, Sakano Y, Paupard MC, Hall DH , Driscoll M (2002). Stochastic and genetic factors influence tissue-specific decline in ageing C. elegans. *Nature*. **419**, 808-814.

Hinkson IV , Elias JE (2011). The dynamic state of protein turnover: It's about time. *Trends Cell Biol*. **21**, 293-303.

Hipkiss AR (2006). Accumulation of altered proteins and ageing: causes and effects. *Exp Gerontol*. **41**, 464-473.

Hipp MS, Park SH , Hartl FU (2014). Proteostasis impairment in protein-misfolding and -aggregation diseases. *Trends Cell Biol*. **24**, 506-514.

Hobson RJ, Hapiak VM, Xiao H, Buehrer KL, Komuniecki PR , Komuniecki RW (2006). SER-7, a Caenorhabditis elegans 5-HT7-like receptor, is essential for the 5-HT stimulation of pharyngeal pumping and egg laying. *Genetics*. **172**, 159-169.

Hochbaum D, Ferguson AA , Fisher AL (2010). Generation of transgenic C. elegans by biolistic transformation. *J Vis Exp*.

References

Hochberg Y , Benjamini Y (1990). More powerful procedures for multiple significance testing. *Stat Med*. **9**, 811-818.

Honda Y , Honda S (1999). The daf-2 gene network for longevity regulates oxidative stress resistance and Mn-superoxide dismutase gene expression in Caenorhabditis elegans. *FASEB J*. **13**, 1385-1393.

Honda Y, Tanaka M , Honda S (2010). Trehalose extends longevity in the nematode Caenorhabditis elegans. *Aging Cell*. **9**, 558-569.

Honjoh S, Yamamoto T, Uno M , Nishida E (2009). Signalling through RHEB-1 mediates intermittent fasting-induced longevity in C. elegans. *Nature*. **457**, 726-730.

Hosono R, Nishimoto S , Kuno S (1989). Alterations of life span in the nematode Caenorhabditis elegans under monoxenic culture conditions. *Exp Gerontol*. **24**, 251-264.

Houthoofd K, Braeckman BP, Lenaerts I, Brys K, De Vreese A, Van Eygen S , Vanfleteren JR (2002). No reduction of metabolic rate in food restricted Caenorhabditis elegans. *Exp Gerontol*. **37**, 1359-1369.

Hsieh CC , Papaconstantinou J (2004). Akt/PKB and p38 MAPK signaling, translational initiation and longevity in Snell dwarf mouse livers. *Mech Ageing Dev*. **125**, 785-798.

Hsin H , Kenyon C (1999). Signals from the reproductive system regulate the lifespan of C. elegans. *Nature*. **399**, 362-366.

Hsu AL, Murphy CT , Kenyon C (2003). Regulation of aging and age-related disease by DAF-16 and heat-shock factor. *Science*. **300**, 1142-1145.

Ingolia NT, Brar GA, Stern-Ginossar N, Harris MS, Talhouarne GJ, Jackson SE, Wills MR , Weissman JS (2014). Ribosome profiling reveals pervasive translation outside of annotated protein-coding genes. *Cell Rep*. **8**, 1365-1379.

Inoue H, Hisamoto N, An JH, Oliveira RP, Nishida E, Blackwell TK , Matsumoto K (2005). The C. elegans p38 MAPK pathway regulates nuclear localization of the transcription factor SKN-1 in oxidative stress response. *Genes Dev*. **19**, 2278-2283.

Iwata M, Imamura H, Stambouli E, Ikeda C, Tamakoshi M, Nagata K, Makyio H, Hankamer B, Barber J, Yoshida M, Yokoyama K , Iwata S (2004). Crystal structure of a central stalk subunit C and reversible association/dissociation of vacuole-type ATPase. *Proc Natl Acad Sci U S A*. **101**, 59-64.

Jasik J, Boggetti B, Baluska F, Volkmann D, Gensch T, Rutten T, Altmann T , Schmelzer E (2013). PIN2 turnover in Arabidopsis root epidermal cells explored by the photoconvertible protein Dendra2. *PLoS One*. **8**, e61403.

Johnson TE , McCaffrey G (1985). Programmed aging or error catastrophe? An examination by two-dimensional polyacrylamide gel electrophoresis. *Mech Ageing Dev*. **30**, 285-297.

Jung T, Hohn A , Grune T (2013). The proteasome and the degradation of oxidized proteins: Part II - protein oxidation and proteasomal degradation. *Redox Biol*. **2C**, 99-104.

Kaeberlein M (2013). Longevity and aging. *F1000Prime Rep*. **5**, 5.

Kaeberlein M , Kennedy BK (2011). Hot topics in aging research: protein translation and TOR signaling, 2010. *Aging Cell*. **10**, 185-190.

Kaeberlein M, McVey M , Guarente L (1999). The SIR2/3/4 complex and SIR2 alone promote longevity in Saccharomyces cerevisiae by two different mechanisms. *Genes Dev*. **13**, 2570-2580.

Kaeberlein M, Powers RW, 3rd, Steffen KK, Westman EA, Hu D, Dang N, Kerr EO, Kirkland KT, Fields S , Kennedy BK (2005). Regulation of yeast replicative life span by TOR and Sch9 in response to nutrients. *Science*. **310**, 1193-1196.

Kaeberlein TL, Smith ED, Tsuchiya M, Welton KL, Thomas JH, Fields S, Kennedy BK , Kaeberlein M (2006). Lifespan extension in Caenorhabditis elegans by complete removal of food. *Aging Cell*. **5**, 487-494.

Kaletsky R , Murphy CT (2010). The role of insulin/IGF-like signaling in C. elegans longevity and aging. *Dis Model Mech*. **3**, 415-419.

Kamath RS , Ahringer J (2003). Genome-wide RNAi screening in Caenorhabditis elegans. *Methods*. **30**, 313-321.

Kane PM (2006). The where, when, and how of organelle acidification by the yeast vacuolar H+-ATPase. *Microbiol Mol Biol Rev*. **70**, 177-191.

Kapahi P, Chen D, Rogers AN, Katewa SD, Li PW, Thomas EL , Kockel L With TOR, less is more: a key role for the conserved nutrient-sensing TOR pathway in aging. *Cell Metab*. **11**, 453-465.

Kapahi P, Chen D, Rogers AN, Katewa SD, Li PW, Thomas EL , Kockel L (2010). With TOR, less is more: a key role for the conserved nutrient-sensing TOR pathway in aging. *Cell Metab*. **11**, 453-465.

Kapahi P, Zid BM, Harper T, Koslover D, Sapin V , Benzer S (2004). Regulation of lifespan in Drosophila by modulation of genes in the TOR signaling pathway. *Curr Biol*. **14**, 885-890.

Karunadharma PP, Basisty N, Dai DF, Chiao YA, Quarles EK, Hsieh EJ, Crispin D, Bielas JH, Ericson NG, Beyer RP, MacKay VL, MacCoss MJ , Rabinovitch PS (2015). Subacute calorie restriction and rapamycin discordantly alter mouse liver proteome homeostasis and reverse aging effects. *Aging Cell*.

Katz M (2015). Genetic Methods for Cellular Manipulations in C. elegans. *Methods Mol Biol*. **1327**, 23-38.

Kenyon C, Chang J, Gensch E, Rudner A , Tabtiang R (1993). A C. elegans mutant that lives twice as long as wild type. *Nature*. **366**, 461-464.

Kenyon CJ (2010). The genetics of ageing. *Nature*. **464**, 504-512.

Kessner D, Chambers M, Burke R, Agus D , Mallick P (2008). ProteoWizard: open source software for rapid proteomics tools development. *Bioinformatics*. **24**, 2534-2536.

Kim S , Pevzner PA (2014). MS-GF+ makes progress towards a universal database search tool for proteomics. *Nat Commun*. **5**, 5277.

References

Kim TY, Wang D, Kim AK, Lau E, Lin AJ, Liem DA, Zhang J, Zong NC, Lam MP , Ping P (2012). Metabolic labeling reveals proteome dynamics of mouse mitochondria. *Mol Cell Proteomics*. **11**, 1586-1594.

Kim YE, Hipp MS, Bracher A, Hayer-Hartl M , Hartl FU (2013). Molecular chaperone functions in protein folding and proteostasis. *Annu Rev Biochem*. **82**, 323-355.

Kimble J , Sharrock WJ (1983). Tissue-specific synthesis of yolk proteins in Caenorhabditis elegans. *Dev Biol*. **96**, 189-196.

Kimura KD, Tissenbaum HA, Liu Y , Ruvkun G (1997). daf-2, an insulin receptor-like gene that regulates longevity and diapause in Caenorhabditis elegans. *Science*. **277**, 942-946.

Kipling D , Faragher RG (1999). Telomeres. Ageing hard or hardly ageing? *Nature*. **398**, 191, 193.

Kirkwood TB (1977). Evolution of ageing. *Nature*. **270**, 301-304.

Kirkwood TB , Rose MR (1991). Evolution of senescence: late survival sacrificed for reproduction. *Philos Trans R Soc Lond B Biol Sci*. **332**, 15-24.

Kirstein-Miles J, Scior A, Deuerling E , Morimoto RI (2013). The nascent polypeptide-associated complex is a key regulator of proteostasis. *EMBO J*. **32**, 1451-1468.

Klass M, Nguyen PN , Dechavigny A (1983). Age-correlated changes in the DNA template in the nematode Caenorhabditis elegans. *Mech Ageing Dev*. **22**, 253-263.

Klass MR (1977). Aging in the nematode Caenorhabditis elegans: major biological and environmental factors influencing life span. *Mech Ageing Dev*. **6**, 413-429.

Knoefler D, Thamsen M, Koniczek M, Niemuth NJ, Diederich AK , Jakob U (2012). Quantitative in vivo redox sensors uncover oxidative stress as an early event in life. *Mol Cell*. **47**, 767-776.

Koga H, Kaushik S , Cuervo AM (2011). Protein homeostasis and aging: The importance of exquisite quality control. *Ageing Res Rev*. **10**, 205-215.

Kourtis N , Tavernarakis N (2009). Cell-specific monitoring of protein synthesis in vivo. *PLoS One*. **4**, e4547.

Krijgsveld J, Ketting RF, Mahmoudi T, Johansen J, Artal-Sanz M, Verrijzer CP, Plasterk RH , Heck AJ (2003). Metabolic labeling of C. elegans and D. melanogaster for quantitative proteomics. *Nat Biotechnol*. **21**, 927-931.

Kruger U, Wang Y, Kumar S , Mandelkow EM (2011). Autophagic degradation of tau in primary neurons and its enhancement by trehalose. *Neurobiol Aging*. **33**, 2291-2305.

Kurapati R, Passananti HB, Rose MR , Tower J (2000). Increased hsp22 RNA levels in Drosophila lines genetically selected for increased longevity. *J Gerontol A Biol Sci Med Sci*. **55**, B552-559.

Lakowski B , Hekimi S (1998). The genetics of caloric restriction in Caenorhabditis elegans. *Proc Natl Acad Sci U S A*. **95**, 13091-13096.

Lant B , Storey KB (2010). An overview of stress response and hypometabolic strategies in Caenorhabditis elegans: conserved and contrasting signals with the mammalian system. *Int J Biol Sci*. **6**, 9-50.

Lapierre LR , Hansen M (2012). Lessons from C. elegans: signaling pathways for longevity. *Trends Endocrinol Metab*. **23**, 637-644.

Larance M, Ahmad Y, Kirkwood KJ, Ly T , Lamond AI (2013). Global subcellular characterization of protein degradation using quantitative proteomics. *Mol Cell Proteomics*. **12**, 638-650.

Larrabee KL, Phillips JO, Williams GJ , Larrabee AR (1980). The relative rates of protein synthesis and degradation in a growing culture of Escherichia coli. *J Biol Chem*. **255**, 4125-4130.

Larsen PL, Albert PS , Riddle DL (1995). Genes that regulate both development and longevity in Caenorhabditis elegans. *Genetics*. **139**, 1567-1583.

Lau E, Wang D, Zhang J, Yu H, Lam MP, Liang X, Zong N, Kim TY , Ping P (2012). Substrate- and isoform-specific proteome stability in normal and stressed cardiac mitochondria. *Circ Res*. **110**, 1174-1178.

Lee GD, Wilson MA, Zhu M, Wolkow CA, de Cabo R, Ingram DK , Zou S (2006). Dietary deprivation extends lifespan in Caenorhabditis elegans. *Aging Cell*. **5**, 515-524.

Lee RC, Feinbaum RL , Ambros V (1993). The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell*. **75**, 843-854.

Lee SS, Kennedy S, Tolonen AC , Ruvkun G (2003). DAF-16 target genes that control C. elegans life-span and metabolism. *Science*. **300**, 644-647.

Lemaitre JF, Berger V, Bonenfant C, Douhard M, Gamelon M, Plard F , Gaillard JM (2015). Early-late life trade-offs and the evolution of ageing in the wild. *Proc Biol Sci*. **282**, 20150209.

Lemasters JJ (2005). Selective mitochondrial autophagy, or mitophagy, as a targeted defense against oxidative stress, mitochondrial dysfunction, and aging. *Rejuvenation Res*. **8**, 3-5.

Lempiainen H , Shore D (2009). Growth control and ribosome biogenesis. *Curr Opin Cell Biol*. **21**, 855-863.

Leontieva OV, Demidenko ZN, Gudkov AV , Blagosklonny MV (2011). Elimination of proliferating cells unmasks the shift from senescence to quiescence caused by rapamycin. *PLoS One*. **6**, e26126.

Lewis SE, Goldspink DF, Phillips JG, Merry BJ , Holehan AM (1985). The effects of aging and chronic dietary restriction on whole body growth and protein turnover in the rat. *Exp Gerontol*. **20**, 253-263.

Li J, Ebata A, Dong Y, Rizki G, Iwata T , Lee SS (2008). Caenorhabditis elegans HCF-1 functions in longevity maintenance as a DAF-16 regulator. *PLoS Biol*. **6**, e233.

Li W, Gao B, Lee SM, Bennett K , Fang D (2007). RLE-1, an E3 ubiquitin ligase, regulates C. elegans aging by catalyzing DAF-16 polyubiquitination. *Dev Cell*. **12**, 235-246.

Li W, Kennedy SG , Ruvkun G (2003). daf-28 encodes a C. elegans insulin superfamily member that is regulated by environmental cues and acts in the DAF-2 signaling pathway. *Genes Dev*. **17**, 844-858.

References

Liang V, Ullrich M, Lam H, Chew YL, Banister S, Song X, Zaw T, Kassiou M, Gotz J , Nicholas HR (2014). Altered proteostasis in aging and heat shock response in C. elegans revealed by analysis of the global and de novo synthesized proteome. *Cell Mol Life Sci*. **71**, 3339-3361.

Libina N, Berman JR , Kenyon C (2003). Tissue-specific activities of C. elegans DAF-16 in the regulation of lifespan. *Cell*. **115**, 489-502.

Lin K, Dorman JB, Rodan A , Kenyon C (1997). daf-16: An HNF-3/forkhead family member that can function to double the life-span of Caenorhabditis elegans. *Science*. **278**, 1319-1322.

Lin K, Hsin H, Libina N , Kenyon C (2001). Regulation of the Caenorhabditis elegans longevity protein DAF-16 by insulin/IGF-1 and germline signaling. *Nat Genet*. **28**, 139-145.

Lithgow GJ, White TM, Melov S , Johnson TE (1995). Thermotolerance and extended life-span conferred by single-gene mutations and induced by thermal stress. *Proc Natl Acad Sci U S A*. **92**, 7540-7544.

Longo VD , Finch CE (2003). Evolutionary medicine: from dwarf model systems to healthy centenarians? *Science*. **299**, 1342-1346.

Lopez-Otin C, Blasco MA, Partridge L, Serrano M , Kroemer G (2013). The hallmarks of aging. *Cell*. **153**, 1194-1217.

Lund J, Tedesco P, Duke K, Wang J, Kim SK , Johnson TE (2002). Transcriptional profile of aging in C. elegans. *Curr Biol*. **12**, 1566-1573.

Mair W, Morantte I, Rodrigues AP, Manning G, Montminy M, Shaw RJ , Dillin A (2011). Lifespan extension induced by AMPK and calcineurin is mediated by CRTC-1 and CREB. *Nature*. **470**, 404-408.

Malone EA, Inoue T , Thomas JH (1996). Genetic analysis of the roles of daf-28 and age-1 in regulating Caenorhabditis elegans dauer formation. *Genetics*. **143**, 1193-1205.

Martin GM, Austad SN , Johnson TE (1996). Genetic analysis of ageing: role of oxidative damage and environmental stresses. *Nat Genet*. **13**, 25-34.

Massaad CA , Klann E (2011). Reactive oxygen species in the regulation of synaptic plasticity and memory. *Antioxid Redox Signal*. **14**, 2013-2054.

Matilainen O, Arpalahti L, Rantanen V, Hautaniemi S , Holmberg CI (2013). Insulin/IGF-1 signaling regulates proteasome activity through the deubiquitinating enzyme UBH-4. *Cell Rep*. **3**, 1980-1995.

Mayampurath AM, Jaitly N, Purvine SO, Monroe ME, Auberry KJ, Adkins JN , Smith RD (2008). DeconMSn: a software tool for accurate parent ion monoisotopic mass determination for tandem mass spectra. *Bioinformatics*. **24**, 1021-1023.

McCay CM (1935). Iodized Salt a Hundred Years Ago. *Science*. **82**, 350-351.

McClintock B (1939). The Behavior in Successive Nuclear Divisions of a Chromosome Broken at Meiosis. *Proc Natl Acad Sci U S A*. **25**, 405-416.

McClintock B (1941). The Stability of Broken Ends of Chromosomes in Zea Mays. *Genetics*. **26**, 234-282.

McColl G, Vantipalli MC , Lithgow GJ (2005). The C. elegans ortholog of mammalian Ku70, interacts with insulin-like signaling to modulate stress resistance and life span. *FASEB J*. **19**, 1716-1718.

McCord JM , Fridovich I (1969). Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). *J Biol Chem*. **244**, 6049-6055.

McDonald RB , Ramsey JJ (2010). Honoring Clive McCay and 75 years of calorie restriction research. *J Nutr*. **140**, 1205-1210.

McElwee J, Bubb K , Thomas JH (2003). Transcriptional outputs of the Caenorhabditis elegans forkhead protein DAF-16. *Aging Cell*. **2**, 111-121.

McElwee JJ, Schuster E, Blanc E, Piper MD, Thomas JH, Patel DS, Selman C, Withers DJ, Thornton JM, Partridge L , Gems D (2007). Evolutionary conservation of regulated longevity assurance mechanisms. *Genome Biol*. **8**, R132.

McElwee JJ, Schuster E, Blanc E, Thomas JH , Gems D (2004). Shared transcriptional signature in Caenorhabditis elegans Dauer larvae and long-lived daf-2 mutants implicates detoxification system in longevity assurance. *J Biol Chem*. **279**, 44533-44543.

Medawar PB (1952). *An Unsolved Problem of Biology: An Inaugural Lecture Delivered at University College, London, 6 December, 1951*: H.K. Lewis and Company.

Meena J, Rudolph KL , Gunes C (2015). Telomere Dysfunction, Chromosomal Instability and Cancer. *Recent Results Cancer Res*. **200**, 61-79.

Mehta R, Chandler-Brown D, Ramos FJ, Shamieh LS , Kaeberlein M (2010). Regulation of mRNA translation as a conserved mechanism of longevity control. *Adv Exp Med Biol*. **694**, 14-29.

Mehta R, Steinkraus KA, Sutphin GL, Ramos FJ, Shamieh LS, Huh A, Davis C, Chandler-Brown D , Kaeberlein M (2009). Proteasomal regulation of the hypoxic response modulates aging in C. elegans. *Science*. **324**, 1196-1198.

Melendez A, Talloczy Z, Seaman M, Eskelinen EL, Hall DH , Levine B (2003). Autophagy genes are essential for dauer development and life-span extension in C. elegans. *Science*. **301**, 1387-1391.

Mindell JA (2012). Lysosomal acidification mechanisms. *Annu Rev Physiol*. **74**, 69-86.

Miwa S, Lawless C , von Zglinicki T (2008). Mitochondrial turnover in liver is fast in vivo and is accelerated by dietary restriction: application of a simple dynamic model. *Aging Cell*. **7**, 920-923.

Morimoto RI (2008). Proteotoxic stress and inducible chaperone networks in neurodegenerative disease and aging. *Genes Dev*. **22**, 1427-1438.

Morimoto RI , Cuervo AM (2009). Protein homeostasis and aging: taking care of proteins from the cradle to the grave. *J Gerontol A Biol Sci Med Sci*. **64**, 167-170.

Morley JF , Morimoto RI (2004). Regulation of longevity in Caenorhabditis elegans by heat shock factor and molecular chaperones. *Mol Biol Cell*. **15**, 657-664.

References

Mosteller RD, Goldstein RV , Nishimoto KR (1980). Metabolism of individual proteins in exponentially growing Escherichia coli. *J Biol Chem*. **255**, 2524-2532.

Muller HJ (1938). The remaking of chromosomes. *Collect. Net*. **13**, 1181-1198.

Murakami S , Johnson TE (1996). A genetic pathway conferring life extension and resistance to UV stress in Caenorhabditis elegans. *Genetics*. **143**, 1207-1218.

Murphy CT, McCarroll SA, Bargmann CI, Fraser A, Kamath RS, Ahringer J, Li H , Kenyon C (2003). Genes that act downstream of DAF-16 to influence the lifespan of Caenorhabditis elegans. *Nature*. **424**, 277-283.

Murphy MP (2009). How mitochondria produce reactive oxygen species. *Biochem J*. **417**, 1-13.

Nguyen AT , Donaldson RP (2005). Metal-catalyzed oxidation induces carbonylation of peroxisomal proteins and loss of enzymatic activities. *Arch Biochem Biophys*. **439**, 25-31.

Obrig TG, Culp WJ, McKeehan WL , Hardesty B (1971). The mechanism by which cycloheximide and related glutarimide antibiotics inhibit peptide synthesis on reticulocyte ribosomes. *J Biol Chem*. **246**, 174-181.

Ogg S, Paradis S, Gottlieb S, Patterson GI, Lee L, Tissenbaum HA , Ruvkun G (1997). The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in C. elegans. *Nature*. **389**, 994-999.

Olovnikov AM (1971). [Principle of marginotomy in template synthesis of polynucleotides]. *Dokl Akad Nauk SSSR*. **201**, 1496-1499.

Olovnikov AM (1996). Telomeres, telomerase, and aging: origin of the theory. *Exp Gerontol*. **31**, 443-448.

Orgel LE (1963). The maintenance of the accuracy of protein synthesis and its relevance to ageing. *Proc Natl Acad Sci U S A*. **49**, 517-521.

Palikaras K, Lionaki E , Tavernarakis N (2015a). Coordination of mitophagy and mitochondrial biogenesis during ageing in C. elegans. *Nature*. **521**, 525-528.

Palikaras K, Lionaki E , Tavernarakis N (2015b). Coupling mitogenesis and mitophagy for longevity. *Autophagy*. **11**, 1428-1430.

Pan KZ, Palter JE, Rogers AN, Olsen A, Chen D, Lithgow GJ , Kapahi P (2007). Inhibition of mRNA translation extends lifespan in Caenorhabditis elegans. *Aging Cell*. **6**, 111-119.

Parker J, Flanagan J, Murphy J , Gallant J (1981). On the accuracy of protein synthesis in Drosophila melanogaster. *Mech Ageing Dev*. **16**, 127-139.

Partridge L , Gems D (2002). Mechanisms of ageing: public or private? *Nat Rev Genet*. **3**, 165-175.

Pavlidis P , Noble WS (2001). Analysis of strain and regional variation in gene expression in mouse brain. *Genome Biol*. **2**, RESEARCH0042.

Pearl R (1928). *The rate of living: being an account of some experimental studies on the biology of life duration*: A.A. Knopf.

Perez VI, Buffenstein R, Masamsetti V, Leonard S, Salmon AB, Mele J, Andziak B, Yang T, Edrey Y, Friguet B, Ward W, Richardson A , Chaudhuri A (2009). Protein stability and resistance to oxidative stress are determinants of longevity in the longest-living rodent, the naked mole-rat. *Proc Natl Acad Sci U S A*. **106**, 3059-3064.

Petyuk VA, Mayampurath AM, Monroe ME, Polpitiya AD, Purvine SO, Anderson GA, Camp DG, 2nd , Smith RD (2010a). DtaRefinery, a software tool for elimination of systematic errors from parent ion mass measurements in tandem mass spectra data sets. *Mol Cell Proteomics*. **9**, 486-496.

Petyuk VA, Qian WJ, Smith RD , Smith DJ (2010b). Mapping protein abundance patterns in the brain using voxelation combined with liquid chromatography and mass spectrometry. *Methods*. **50**, 77-84.

Pickrell AM , Youle RJ (2015). The roles of PINK1, parkin, and mitochondrial fidelity in Parkinson's disease. *Neuron*. **85**, 257-273.

Pierce SB, Costa M, Wisotzkey R, Devadhar S, Homburger SA, Buchman AR, Ferguson KC, Heller J, Platt DM, Pasquinelli AA, Liu LX, Doberstein SK , Ruvkun G (2001). Regulation of DAF-2 receptor signaling by human insulin and ins-1, a member of the unusually large and diverse C. elegans insulin gene family. *Genes Dev*. **15**, 672-686.

Pitt JN , Kaeberlein M (2015). Why is aging conserved and what can we do about it? *PLoS Biol*. **13**, e1002131.

Prasanna HR , Lane RS (1977). Impaired Protein-Synthesis in Aged Turbatrix-Aceti. *Federation Proceedings*. **36**, 292-292.

Prasanna HR , Lane RS (1979). Protein degradation in aged nematodes (Turbatrix aceti). *Biochem Biophys Res Commun*. **86**, 552-559.

Pratt JM, Petty J, Riba-Garcia I, Robertson DH, Gaskell SJ, Oliver SG , Beynon RJ (2002a). Dynamics of protein turnover, a missing dimension in proteomics. *Mol Cell Proteomics*. **1**, 579-591.

Pratt JM, Robertson DH, Gaskell SJ, Riba-Garcia I, Hubbard SJ, Sidhu K, Oliver SG, Butler P, Hayes A, Petty J , Beynon RJ (2002b). Stable isotope labelling in vivo as an aid to protein identification in peptide mass fingerprinting. *Proteomics*. **2**, 157-163.

Price JC , Ghaemmaghami S (2014). Analysis of proteome dynamics in mice by isotopic labeling. *Methods Mol Biol*. **1156**, 111-131.

Price JC, Guan S, Burlingame A, Prusiner SB , Ghaemmaghami S (2010). Analysis of proteome dynamics in the mouse brain. *Proc Natl Acad Sci U S A*. **107**, 14508-14513.

Price JC, Khambatta CF, Li KW, Bruss MD, Shankaran M, Dalidd M, Floreani NA, Roberts LS, Turner SM, Holmes WE , Hellerstein MK (2012). The effect of long term calorie restriction on in vivo hepatic proteostatis: a novel combination of dynamic and quantitative proteomics. *Mol Cell Proteomics*. **11**, 1801-1814.

Promislow DE (1993). On size and survival: progress and pitfalls in the allometry of life span. *J Gerontol*. **48**, B115-123.

References

Qin B, Cartier L, Dubois-Dauphin M, Li B, Serrander L , Krause KH (2006). A key role for the microglial NADPH oxidase in APP-dependent killing of neurons. *Neurobiol Aging*. **27**, 1577-1587.

Raices M, Maruyama H, Dillin A , Karlseder J (2005). Uncoupling of longevity and telomere length in C. elegans. *PLoS Genet*. **1**, e30.

Ralser M, Wamelink MM, Kowald A, Gerisch B, Heeren G, Struys EA, Klipp E, Jakobs C, Breitenbach M, Lehrach H , Krobitsch S (2007). Dynamic rerouting of the carbohydrate flux is key to counteracting oxidative stress. *J Biol*. **6**, 10.

Rastogi S, Borgo B, Pazdernik N, Fox P, Mardis ER, Kohara Y, Havranek J , Schedl T (2015). Caenorhabditis elegans glp-4 Encodes a Valyl Aminoacyl tRNA Synthetase. *G3 (Bethesda)*.

Rattan SI (1996). Synthesis, modifications, and turnover of proteins during aging. *Exp Gerontol*. **31**, 33-47.

Rattan SI, Derventzi A , Clark BF (1992). Protein synthesis, posttranslational modifications, and aging. *Ann N Y Acad Sci*. **663**, 48-62.

Ravikumar B, Duden R , Rubinsztein DC (2002). Aggregate-prone proteins with polyglutamine and polyalanine expansions are degraded by autophagy. *Hum Mol Genet*. **11**, 1107-1117.

Reckow S , Webhofer C (2014). Analysis of individual protein turnover in live animals on a proteome-wide scale. *Methods Mol Biol*. **1156**, 147-154.

Reiss U , Rothstein M (1974). Age-Related Change in Specific Activity of Isocitrate Lyase from Turbatrix-Aceti. *Federation Proceedings*. **33**, 1308-1308.

Riddle DL , Albert PS (1997). Genetic and Environmental Regulation of Dauer Larva Development.

Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W , Smyth GK (2015). limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res*. **43**, e47.

Rogers AN, Chen D, McColl G, Czerwieniec G, Felkey K, Gibson BW, Hubbard A, Melov S, Lithgow GJ , Kapahi P (2011). Life span extension via eIF4G inhibition is mediated by posttranscriptional remodeling of stress response gene expression in C. elegans. *Cell Metab*. **14**, 55-66.

Rogers S, Wells R , Rechsteiner M (1986). Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. *Science*. **234**, 364-368.

Rosenberger RF (1991). Senescence and the accumulation of abnormal proteins. *Mutat Res*. **256**, 255-262.

Rothstein M (1979). The formation of altered enzymes in aging animals. *Mech Ageing Dev*. **9**, 197-202.

Rothstein M (1982). Biochemical approaches to aging. *Academic Press, New York.* .

Rothstein M (1989). An overview of age-related changes in proteins. *Prog Clin Biol Res*. **287**, 259-267.

Rothstein M , Sharma HK (1978). Altered enzymes in the free-living nematode, Turbatrix aceti, aged in the absence of fluorodeoxyuridine. *Mech Ageing Dev*. **8**, 175-180.

Rual JF, Ceron J, Koreth J, Hao T, Nicot AS, Hirozane-Kishikawa T, Vandenhaute J, Orkin SH, Hill DE, van den Heuvel S , Vidal M (2004). Toward improving Caenorhabditis elegans phenome mapping with an ORFeome-based RNAi library. *Genome Res*. **14**, 2162-2168.

Rudolph KL, Chang S, Lee HW, Blasco M, Gottlieb GJ, Greider C , DePinho RA (1999). Longevity, stress response, and cancer in aging telomerase-deficient mice. *Cell*. **96**, 701-712.

Rueppell O, Bachelier C, Fondrk MK , Page RE (2007). Regulation of life history determines lifespan of worker honey bees (Apis mellifera L.). *Experimental Gerontology*. **42**, 1020-1032.

Russell JB , Cook GM (1995). Energetics of bacterial growth: balance of anabolic and catabolic reactions. *Microbiol Rev*. **59**, 48-62.

Ryazanov AG , Nefsky BS (2002). Protein turnover plays a key role in aging. *Mech Ageing Dev*. **123**, 207-213.

Saeed AI, Bhagabati NK, Braisted JC, Liang W, Sharov V, Howe EA, Li J, Thiagarajan M, White JA , Quackenbush J (2006). TM4 microarray software suite. *Methods Enzymol*. **411**, 134-193.

Saeed AI, Sharov V, White J, Li J, Liang W, Bhagabati N, Braisted J, Klapa M, Currier T, Thiagarajan M, Sturn A, Snuffin M, Rezantsev A, Popov D, Ryltsov A, Kostukovich E, Borisovsky I, Liu Z, Vinsavich A, Trush V , Quackenbush J (2003). TM4: a free, open-source system for microarray data management and analysis. *Biotechniques*. **34**, 374-378.

Sandri M, Sandri C, Gilbert A, Skurk C, Calabria E, Picard A, Walsh K, Schiaffino S, Lecker SH , Goldberg AL (2004). Foxo transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy. *Cell*. **117**, 399-412.

Sarkis GJ, Ashcom JD, Hawdon JM , Jacobson LA (1988). Decline in protease activities with age in the nematode Caenorhabditis elegans. *Mech Ageing Dev*. **45**, 191-201.

Savas JN, Toyama BH, Xu T, Yates JR, 3rd , Hetzer MW (2012). Extremely long-lived nuclear pore proteins in the rat brain. *Science*. **335**, 942.

Schatten H, Chakrabarti A , Hedrick J (1999). Centrosome and microtubule instability in aging Drosophila cells. *J Cell Biochem*. **74**, 229-241.

Schmidt M , Finley D (2014). Regulation of proteasome activity in health and disease. *Biochim Biophys Acta*. **1843**, 13-25.

Schneider CA, Rasband WS , Eliceiri KW (2012). NIH Image to ImageJ: 25 years of image analysis. *Nature Methods*. **9**, 671-675.

Schneider EL , Mitsui Y (1976). The relationship between in vitro cellular aging and in vivo human age. *Proc Natl Acad Sci U S A*. **73**, 3584-3588.

Schoenheimer R, Rittenberg D, Foster GL, Keston AS , Ratner S (1938). The Application of the Nitrogen Isotope N15 for the Study of Protein Metabolism. *Science*. **88**, 599-600.

References

Schug TT (2010). mTOR favors senescence over quiescence in p53-arrested cells. *Aging (Albany NY)*. **2**, 327-328.

Schumpert C, Handy I, Dudycha JL , Patel RC (2014). Relationship between heat shock protein 70 expression and life span in Daphnia. *Mech Ageing Dev*. **139**, 1-10.

Schwanhausser B, Gossen M, Dittmar G , Selbach M (2009). Global analysis of cellular protein translation by pulsed SILAC. *Proteomics*. **9**, 205-209.

Scialo F, Sriram A, Naudi A, Ayala V, Jove M, Pamplona R , Sanz A (2015). Target of rapamycin activation predicts lifespan in fruit flies. *Cell Cycle*, 0.

Shama S, Lai CY, Antoniazzi JM, Jiang JC , Jazwinski SM (1998). Heat stress-induced life span extension in yeast. *Exp Cell Res*. **245**, 379-388.

Sharma HK, Prasanna HR, Lane RS , Rothstein M (1979). The effect of age on enolase turnover in the free-living nematode, Turbatrix aceti. *Arch Biochem Biophys*. **194**, 275-282.

Sharp ZD , Bartke A (2005). Evidence for down-regulation of phosphoinositide 3-kinase/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR)-dependent translation regulatory signaling pathways in Ames dwarf mice. *J Gerontol A Biol Sci Med Sci*. **60**, 293-300.

Shay JW , Wright WE (2011). Role of telomeres and telomerase in cancer. *Semin Cancer Biol*. **21**, 349-353.

Shaye DD , Greenwald I (2011). OrthoList: a compendium of C. elegans genes with human orthologs. *PLoS One*. **6**, e20085.

Sherman PW , Jarvis JUM (2002). Extraordinary life spans of naked mole-rats (Heterocephalus glaber). *Journal of Zoology*. **258**, 307-311.

Shore DE, Carr CE , Ruvkun G (2012). Induction of cytoprotective pathways is central to the extension of lifespan conferred by multiple longevity pathways. *PLoS Genet*. **8**, e1002792.

Shoyama T, Ozaki T, Ishii N, Yokota S , Suda H (2007). Basic principle of the lifespan in the nematode C. elegans. *Mech Ageing Dev*. **128**, 529-537.

Snelgrove RJ, Edwards L, Rae AJ , Hussell T (2006). An absence of reactive oxygen species improves the resolution of lung influenza infection. *Eur J Immunol*. **36**, 1364-1373.

Sohal RS , Weindruch R (1996). Oxidative stress, caloric restriction, and aging. *Science*. **273**, 59-63.

Sonnichsen B, Koski LB, Walsh A, Marschall P, Neumann B, Brehm M, Alleaume AM, Artelt J, Bettencourt P, Cassin E, Hewitson M, Holz C, Khan M, Lazik S, Martin C, Nitzsche B, Ruer M, Stamford J, Winzi M, Heinkel R, Roder M, Finell J, Hantsch H, Jones SJ, Jones M, Piano F, Gunsalus KC, Oegema K, Gonczy P, Coulson A, Hyman AA , Echeverri CJ (2005). Full-genome RNAi profiling of early embryogenesis in Caenorhabditis elegans. *Nature*. **434**, 462-469.

Sorce S , Krause KH (2009). NOX enzymes in the central nervous system: from signaling to disease. *Antioxid Redox Signal*. **11**, 2481-2504.

Stadtman ER (1988). Protein modification in aging. *J Gerontol*. **43**, B112-120.

Stadtman ER (1992). Protein oxidation and aging. *Science*. **257**, 1220-1224.

Starke-Reed PE , Oliver CN (1989). Protein oxidation and proteolysis during aging and oxidative stress. *Arch Biochem Biophys*. **275**, 559-567.

Steffen KK, MacKay VL, Kerr EO, Tsuchiya M, Hu D, Fox LA, Dang N, Johnston ED, Oakes JA, Tchao BN, Pak DN, Fields S, Kennedy BK , Kaeberlein M (2008). Yeast life span extension by depletion of 60s ribosomal subunits is mediated by Gcn4. *Cell*. **133**, 292-302.

Stout GJ, Stigter EC, Essers PB, Mulder KW, Kolkman A, Snijders DS, van den Broek NJ, Betist MC, Korswagen HC, Macinnes AW , Brenkman AB (2013). Insulin/IGF-1-mediated longevity is marked by reduced protein metabolism. *Mol Syst Biol*. **9**, 679.

Suh Y, Atzmon G, Cho MO, Hwang D, Liu B, Leahy DJ, Barzilai N , Cohen P (2008). Functionally significant insulin-like growth factor I receptor mutations in centenarians. *Proc Natl Acad Sci U S A*. **105**, 3438-3442.

Sulston JE, Schierenberg E, White JG , Thomson JN (1983). The embryonic cell lineage of the nematode Caenorhabditis elegans. *Dev Biol*. **100**, 64-119.

Syntichaki P, Troulinaki K , Tavernarakis N (2007). Protein synthesis is a novel determinant of aging in Caenorhabditis elegans. *Ann N Y Acad Sci*. **1119**, 289-295.

Szewczyk NJ, Peterson BK, Barmada SJ, Parkinson LP , Jacobson LA (2007). Opposed growth factor signals control protein degradation in muscles of Caenorhabditis elegans. *EMBO J*. **26**, 935-943.

Takahashi R , Goto S (1988). Fidelity of aminoacylation by rat-liver tyrosyl-tRNA synthetase. Effect of age. *Eur J Biochem*. **178**, 381-386.

Tanaka M, Kim YM, Lee G, Junn E, Iwatsubo T , Mouradian MM (2004). Aggresomes formed by alpha-synuclein and synphilin-1 are cytoprotective. *J Biol Chem*. **279**, 4625-4631.

Taylor RC , Dillin A (2011). Aging as an event of proteostasis collapse. *Cold Spring Harb Perspect Biol*. **3**.

TeKippe M , Aballay A (2010). C. elegans germline-deficient mutants respond to pathogen infection using shared and distinct mechanisms. *PLoS One*. **5**, e11777.

Terasaki M, Shemesh T, Kasthuri N, Klemm RW, Schalek R, Hayworth KJ, Hand AR, Yankova M, Huber G, Lichtman JW, Rapoport TA , Kozlov MM (2013). Stacked endoplasmic reticulum sheets are connected by helicoidal membrane motifs. *Cell*. **154**, 285-296.

Terman A (1995). The effect of age on formation and elimination of autophagic vacuoles in mouse hepatocytes. *Gerontology*. **41 Suppl 2**, 319-326.

Terman A, Gustafsson B , Brunk UT (2007). Autophagy, organelles and ageing. *J Pathol*. **211**, 134-143.

Thompson O, Edgley M, Strasbourger P, Flibotte S, Ewing B, Adair R, Au V, Chaudhry I, Fernando L, Hutter H, Kieffer A, Lau J, Lee N, Miller A, Raymant G, Shen B, Shendure J, Taylor J, Turner EH, Hillier LW, Moerman DG , Waterston RH (2013). The million

mutation project: a new approach to genetics in Caenorhabditis elegans. *Genome Res*. **23**, 1749-1762.

Timmons L, Court DL , Fire A (2001). Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in Caenorhabditis elegans. *Gene*. **263**, 103-112.

Tissenbaum HA , Guarente L (2001). Increased dosage of a sir-2 gene extends lifespan in Caenorhabditis elegans. *Nature*. **410**, 227-230.

Tops BB, Gauci S, Heck AJ , Krijgsveld J (2010). Worms from venus and mars: proteomics profiling of sexual differences in Caenorhabditis elegans using in vivo 15N isotope labeling. *J Proteome Res*. **9**, 341-351.

Torgovnick A, Schiavi A, Maglioni S , Ventura N (2013). Healthy aging: what can we learn from Caenorhabditis elegans? *Z Gerontol Geriatr*. **46**, 623-628.

Trotschel C, Albaum SP , Poetsch A (2013). Proteome turnover in bacteria: current status for Corynebacterium glutamicum and related bacteria. *Microb Biotechnol*. **6**, 708-719.

True HL (2006). The battle of the fold: chaperones take on prions. *Trends Genet*. **22**, 110-117.

Tschopp J (2011). Mitochondria: Sovereign of inflammation? *Eur J Immunol*. **41**, 1196-1202.

Tullet JM, Hertweck M, An JH, Baker J, Hwang JY, Liu S, Oliveira RP, Baumeister R , Blackwell TK (2008). Direct inhibition of the longevity-promoting factor SKN-1 by insulin-like signaling in C. elegans. *Cell*. **132**, 1025-1038.

Ungvari Z, Csiszar A, Sosnowska D, Philipp EE, Campbell CM, McQuary PR, Chow TT, Coelho M, Didier ES, Gelino S, Holmbeck MA, Kim I, Levy E, Sonntag WE, Whitby PW, Austad SN , Ridgway I (2013). Testing predictions of the oxidative stress hypothesis of aging using a novel invertebrate model of longevity: the giant clam (Tridacna derasa). *J Gerontol A Biol Sci Med Sci*. **68**, 359-367.

Van Assche R, Broeckx V, Boonen K, Maes E, De Haes W, Schoofs L , Temmerman L (2015). Integrating -Omics: Systems Biology as Explored Through C. elegans Research. *J Mol Biol*.

Van Raamsdonk JM , Hekimi S (2009). Deletion of the mitochondrial superoxide dismutase sod-2 extends lifespan in Caenorhabditis elegans. *PLoS Genet*. **5**, e1000361.

Van Raamsdonk JM , Hekimi S (2010). Reactive Oxygen Species and Aging in Caenorhabditis elegans: Causal or Casual Relationship? *Antioxid Redox Signal*. **13**, 1911-1953.

Van Raamsdonk JM, Meng Y, Camp D, Yang W, Jia X, Benard C , Hekimi S (2010). Decreased energy metabolism extends life span in Caenorhabditis elegans without reducing oxidative damage. *Genetics*. **185**, 559-571.

Van Remmen H, Ward D, Sabia R , Richardson A (1995). Gene Expression and Protein Degradation. *Compr Physiol 2011, Supplement 28: Handbook of Physiology, Aging*, 171-234.

Van Voorhies WA, Khazaeli AA , Curtsinger JW (2004). Testing the "rate of living" model: further evidence that longevity and metabolic rate are not inversely correlated in Drosophila melanogaster. *J Appl Physiol (1985)*. **97**, 1915-1922.

Vanfleteren JR (1976). Large scale cultivation of a free-living nematode (Caenorhabditis elegans). *Experientia*. **32**, 1087-1088.

Vanfleteren JR , Braeckman BP (1999). Mechanisms of life span determination in Caenorhabditis elegans. *Neurobiol Aging*. **20**, 487-502.

Vanfleteren JR , De Vreese A (1994). Analysis of the proteins of aging Caenorhabditis elegans by high resolution two-dimensional gel electrophoresis. *Electrophoresis*. **15**, 289-296.

Varshavsky A (1997). The N-end rule pathway of protein degradation. *Genes Cells*. **2**, 13-28.

Vaughan WJ , Calvin M (1977). Electrophoretic analysis of brain proteins from young adult and aged mice. *Gerontology*. **23**, 110-126.

Vazquez-Martinez R, Diaz-Ruiz A, Almabouada F, Rabanal-Ruiz Y, Gracia-Navarro F , Malagon MM (2012). Revisiting the regulated secretory pathway: from frogs to human. *Gen Comp Endocrinol*. **175**, 1-9.

Vellai T, Takacs-Vellai K, Zhang Y, Kovacs AL, Orosz L , Muller F (2003). Genetics: influence of TOR kinase on lifespan in C. elegans. *Nature*. **426**, 620.

Vernace VA, Arnaud L, Schmidt-Glenewinkel T , Figueiredo-Pereira ME (2007). Aging perturbs 26S proteasome assembly in Drosophila melanogaster. *FASEB J*. **21**, 2672-2682.

Vilchez D, Morantte I, Liu Z, Douglas PM, Merkwirth C, Rodrigues AP, Manning G , Dillin A (2012). RPN-6 determines C. elegans longevity under proteotoxic stress conditions. *Nature*. **489**, 263-268.

Vilchez D, Saez I , Dillin A (2014). The role of protein clearance mechanisms in organismal ageing and age-related diseases. *Nat Commun*. **5**, 5659.

Visscher M, Wildschut MHE, De Henau S, van Es RM, Michels H, Kemmeren P, Nollen EA, Burgering BM, Vos HR , Dansen TB (submitted). Proteome-wide changes in protein turnover rates in *C. elegans* models of longevity and age-related disease.

Vizcaino JA, Deutsch EW, Wang R, Csordas A, Reisinger F, Rios D, Dianes JA, Sun Z, Farrah T, Bandeira N, Binz PA, Xenarios I, Eisenacher M, Mayer G, Gatto L, Campos A, Chalkley RJ, Kraus HJ, Albar JP, Martinez-Bartolome S, Apweiler R, Omenn GS, Martens L, Jones AR , Hermjakob H (2014). ProteomeXchange provides globally coordinated proteomics data submission and dissemination. *Nat Biotechnol*. **32**, 223-226.

von Zglinicki T (1998). Telomeres: influencing the rate of aging. *Ann N Y Acad Sci*. **854**, 318-327.

Vukoti K, Yu X, Sheng Q, Saha S, Feng Z, Hsu AL , Miyagi M (2015). Monitoring Newly Synthesized Proteins over the Adult Life Span of Caenorhabditis elegans. *J Proteome Res*.

Waaijers S , Boxem M (2014). Engineering the Caenorhabditis elegans genome with CRISPR/Cas9. *Methods*. **68**, 381-388.

References

Wadhwa R, Takano S, Kaur K, Aida S, Yaguchi T, Kaul Z, Hirano T, Taira K , Kaul SC (2005). Identification and characterization of molecular interactions between mortalin/mtHsp70 and HSP60. *Biochem J*. **391**, 185-190.

Walker GA, White TM, McColl G, Jenkins NL, Babich S, Candido EP, Johnson TE , Lithgow GJ (2001). Heat shock protein accumulation is upregulated in a long-lived mutant of Caenorhabditis elegans. *J Gerontol A Biol Sci Med Sci*. **56**, B281-287.

Wallimann T, Tokarska-Schlattner M , Schlattner U (2011). The creatine kinase system and pleiotropic effects of creatine. *Amino Acids*. **40**, 1271-1296.

Walther DM, Kasturi P, Zheng M, Pinkert S, Vecchi G, Ciryam P, Morimoto RI, Dobson CM, Vendruscolo M, Mann M , Hartl FU (2015). Widespread Proteome Remodeling and Aggregation in Aging C. elegans. *Cell*. **161**, 919-932.

Wang M, Herrmann CJ, Simonovic M, Szklarczyk D , von Mering C (2015). Version 4.0 of PaxDb: Protein abundance data, integrated across model organisms, tissues, and cell-lines. *Proteomics*.

Wang M, Weiss M, Simonovic M, Haertinger G, Schrimpf SP, Hengartner MO , von Mering C (2012). PaxDb, a database of protein abundance averages across all three domains of life. *Mol Cell Proteomics*. **11**, 492-500.

Welker NC, Habig JW , Bass BL (2007). Genes misregulated in C. elegans deficient in Dicer, RDE-4, or RDE-1 are enriched for innate immunity genes. *RNA*. **13**, 1090-1102.

Westman-Brinkmalm A, Abramsson A, Pannee J, Gang C, Gustavsson MK, von Otter M, Blennow K, Brinkmalm G, Heumann H , Zetterberg H (2011). SILAC zebrafish for quantitative analysis of protein turnover and tissue regeneration. *J Proteomics*. **75**, 425-434.

Wightman B, Ha I , Ruvkun G (1993). Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. *Cell*. **75**, 855-862.

Willcox BJ, Donlon TA, He Q, Chen R, Grove JS, Yano K, Masaki KH, Willcox DC, Rodriguez B , Curb JD (2008). FOXO3A genotype is strongly associated with human longevity. *Proc Natl Acad Sci U S A*. **105**, 13987-13992.

Williams GC (1957). Pleiotropy, Natural-Selection, and the Evolution of Senescence. *Evolution*. **11**, 398-411.

Wilson DL, Hall ME , Stone GC (1978). Test of some aging hypotheses using two-dimensional protein mapping. *Gerontology*. **24**, 426-433.

Wolff S, Ma H, Burch D, Maciel GA, Hunter T , Dillin A (2006). SMK-1, an essential regulator of DAF-16-mediated longevity. *Cell*. **124**, 1039-1053.

Wolkow CA, Kimura KD, Lee MS , Ruvkun G (2000). Regulation of C. elegans life-span by insulinlike signaling in the nervous system. *Science*. **290**, 147-150.

Wright WE, Piatyszek MA, Rainey WE, Byrd W , Shay JW (1996). Telomerase activity in human germline and embryonic tissues and cells. *Developmental Genetics*. **18**, 173-179.

Wullschleger S, Loewith R , Hall MN (2006). TOR signaling in growth and metabolism. *Cell*. **124**, 471-484.

Yang W, Li J , Hekimi S (2007). A Measurable increase in oxidative damage due to reduction in superoxide detoxification fails to shorten the life span of long-lived mitochondrial mutants of Caenorhabditis elegans. *Genetics*. **177**, 2063-2074.

Yasuda K, Adachi H, Fujiwara Y , Ishii N (1999). Protein carbonyl accumulation in aging dauer formation-defective (daf) mutants of Caenorhabditis elegans. *J Gerontol A Biol Sci Med Sci*. **54**, B47-51; discussion B52-43.

Yorimitsu T , Klionsky DJ (2005). Autophagy: molecular machinery for self-eating. *Cell Death Differ*. **12 Suppl 2**, 1542-1552.

Young VR, Steffee WP, Pencharz PB, Winterer JC , Scrimshaw NS (1975). Total human body protein synthesis in relation to protein requirements at various ages. *Nature*. **253**, 192-194.

Yuet KP, Doma MK, Ngo JT, Sweredoski MJ, Graham RL, Moradian A, Hess S, Schuman EM, Sternberg PW , Tirrell DA (2015). Cell-specific proteomic analysis in Caenorhabditis elegans. *Proc Natl Acad Sci U S A*. **112**, 2705-2710.

Yun C, Stanhill A, Yang Y, Zhang Y, Haynes CM, Xu CF, Neubert TA, Mor A, Philips MR , Ron D (2008). Proteasomal adaptation to environmental stress links resistance to proteotoxicity with longevity in Caenorhabditis elegans. *Proc Natl Acad Sci U S A*. **105**, 7094-7099.

Zeelon P, Gershon H , Gershon D (1973). Inactive enzyme molecules in aging organisms. Nematode fructose-1,6-diphosphate aldolase. *Biochemistry*. **12**, 1743-1750.

Zeng BY, Medhurst AD, Jackson M, Rose S , Jenner P (2005). Proteasomal activity in brain differs between species and brain regions and changes with age. *Mech Ageing Dev*. **126**, 760-766.

Zhang CS, Jiang B, Li M, Zhu M, Peng Y, Zhang YL, Wu YQ, Li TY, Liang Y, Lu Z, Lian G, Liu Q, Guo H, Yin Z, Ye Z, Han J, Wu JW, Yin H, Lin SY , Lin SC (2014). The lysosomal v-ATPase-Ragulator complex is a common activator for AMPK and mTORC1, acting as a switch between catabolism and anabolism. *Cell Metab*. **20**, 526-540.

Zhang Y, Dawson VL , Dawson TM (2000). Oxidative stress and genetics in the pathogenesis of Parkinson's disease. *Neurobiol Dis*. **7**, 240-250.

Curriculum vitae

Personal information

Name	Ineke Dhondt
Email	Ineke.Dhondt@UGent.be
Date of birth	June 9, 1987
Nationality	Belgian

Education

2010-present PhD in Biology, Lab of Aging Physiology and Molecular Evolution, Ghent University, Belgium

2008-2010 Master in Biology (Major Functional Biology and Major Evolution), with greatest distinction, Ghent University, Belgium.

Master Dissertation: Stem cell dynamics during homeostasis and regeneration in *Macrostomum lignano* (Platyhelminthes)
Department of Biology, Nematology, Ghent University, Belgium

2005-2008 Bachelor in Biology, with distinction, Ghent University

Bachelor project: The effect of FUdR on the stem cell population in *Macrostomum lignano*
Department of Biology, Nematology, Ghent University, Belgium

Awards and honors

- Runner Up in the poster awards at ProteoMMX^3 meeting by the British Society for Proteome Research (2014)
- FWO PhD scholarship (2011-2015)
- 'Pierre Verkerk' award for best Master Dissertation Biology (2010).

Research visit abroad

Pacific Northwest National Laboratory (PNNL, Richland WA, USA): June-September 2013
Subject: Turnover rates in the protein pool of *C. elegans* with extreme lifespan
Outcome: Stable Isotope Labeling by Nitrogen in *Caenorhabditis elegans* (SILeNCe) labeled samples were analyzed and quantified using high resolution mass spectrometry technologies at PNNL. Accurate estimations of protein half-life could be extracted from the MS dataset.

A1 publications

- **Dhondt I.**, Petuyk V.A., Bauer S., Smith R.D., Depuydt G., Braeckman B.P. (Submitted and under review at Aging Cell). Semi-stochastic changes of protein turnover in aging *Caenorhabditis elegans*.
- **Dhondt I.**, Petuyk V.A., Cai H., Vierstraete A., Smith R.D., Depuydt G., Braeckman B.P. (Submitted and under review at Cell Reports). FOXO/DAF-16 activation slows down turnover of the majority of proteins in *C. elegans*.
- Depuydt G., Shanmugam N., Rasulova M., **Dhondt I.**, Braeckman B.P. (Submitted and under review at Journal of Gerontology). Increased protein stability and decreased protein turnover in the *C. elegans* Ins/IGF-1 *daf-2* mutant.
- Wang W., McReynolds M.R., Goncalves J.F., Goncalves J.F, Shu M., **Dhondt I.**, Braeckman B.P., Lange S.E., Kho K., Detwiler A.C., Pacella M.J., Hanna-Rose W. (2015). The Journal of Biological Chemistry. Comparative metabolomic profiling reveals that dysregulated glycolysis stemming from lack of salvage NAD+ biosynthesis impairs reproductive development in C. elegans. J Biol Chem. 290, 26163-26179.
- Castelein N., Muschol M., **Dhondt I.**, Cai H., De Vos WH., Dencher N.A., Braeckman B.P. (2014). Mitochondrial efficiency is increased in axenically cultured *Caenorhabditis elegans*. Exp Gerontol. 56, 26-36.
- Depuydt G., Xie F., Petyuk V.A., Shanmugam N., Smolders A., **Dhondt I.**, Brewer H.M., Camp D.G., Smith R.D., Braeckman B.P. (2013). Reduced insulin/insulin-like growth factor-1 signaling and dietary restriction inhibit translation but preserve muscle mass in *Caenorhabditis elegans*. Mol Cell Proteomics. 12, 3624-39.
- Mouton S., Verdoodt F., Willems M., **Dhondt I.**, Crucke J., Braeckman B.P., Houthoofd W. (2010). Establishing a flatworm ageing model. Belgian Journal of Zoology, 140 (Suppl.). 195-197.
- Verdoodt F., Willems M., **Dhondt I.**, Houthoofd W., Bert W., De Vos W. (2012). Measurement of S-phase duration of adult stem cells in the flatworm *Macrostomum lignano* by double replication labelling and quantitative colocalization analysis. Cell Biol Int. 36, 1251-1259.

Conference abstracts

- Cai H., Rasulova M., Meagher L. M., **Dhondt I.**, Braeckman B. P. Lifespan extension by axenic dietary restriction is independent of the mitochondrial unfolded protein response and mitohormesis in *C. elegans*. Cold Spring Harbor Asia Conferences – Molecular Basis of Aging and Disease (China) 14.-18. September 2015.
- **Dhondt I.**, Petuyk V.A., Cai H., Smith R.D., Depuydt G., Braeckman B.P. SILeNCe is golden: slow-down of protein turnover in the long-lived *Caenorhabditis elegans daf-2*

mutant. 19th International *C. elegans* meeting, Los Angeles (USA), 24. – 28 June 2015.

- **Dhondt I.**, Petuyk V.A., Cai H., Smith R.D., Depuydt G., Braeckman B.P. SILeNCe is golden: slow-down of protein turnover in the long-lived *Caenorhabditis elegans daf-2* mutant. Dutch worm meeting, Wageningen (Netherlands), 29. March 2015.
- **Dhondt I.**, Petuyk V.A., Depuydt G., Smith R.D., Braeckman B.P. Analysis of individual protein turnover in long-live*d daf-2 C. elegans.* Aging, Metabolism, Stress, Pathogenesis and Small RNAs in *C. elegans* (Madison, Wisconsin), 10. – 13. July 2014.
- **Dhondt I.**, Petuyk V.A., Depuydt G., Smith R.D., Braeckman B.P. Analysis of individual protein turnover in the long- lived daf-2 mutants of *Caenorhabditis elegans*. Dutch worm meeting, Groningen (Netherlands), 20. February 2015.
- **Dhondt I.**, Petyuk V.A., Depuydt G., Smith R.D., Braeckman P.B. (2014) Analysis of individual protein turnover in long-lived *Caenorhabditis elegans*. ProteoMM^{X3}, Chester (UK), 25. – 27. March 2014.
- **Dhondt I**., Depuydt G., Cai H., Staal J., Borghi A., Verstrepen L., Baten L., Beyaert R., Braeckman B.P. Proteostasis in the aging model *Caenorhabditis elegans.*19th International C. elegans meeting, Los Angeles (USA), 26. – 30 June 2013.
- **Cai H.**, Castelein N., **Dhondt I.,** Rasulova M., Braeckman B.P. Genetic Screening of genes for lifespan extension effect in axenic medium in *C. elegans*. CSHasia 2013, China, 9. – 13. September 2013.
- **Dhondt I.,** Depuydt G., Cai H., Matthijsens F., Braeckman P.B. (2012) Mitophagy and the control of lifespan in *C. elegans.* MiPsummer 2012 - FEBS Workshop, Cambridge (UK), 7. – 13. July 2012.
- **Dhondt I**., Verdoodt F., De Vos W., Houthoofd W., Willems M. (2010) Stem cell dynamics during homeostasis and regeneration in *Macrostomum lignano* (Platyhelminthes). 17th Benelux Congress of Zoology, Ghent (Belgium), 22. – 23. October 2010.
- Verdoodt F., **Dhondt I.**, De Vos W., Mouton S., Houthoofd W., Willems M. (2010) Estimation of S-phase duration during homeostasis and regeneration in *Macrostomum lignano* (Platyhelminthes). 4th International Macrostomum Meeting, Basel (Switzerland), 12. – 14. November 2010.
- Mouton S., Verdoodt F., Willems M., **Dhondt I.,** Crucke J., Braeckman B.P., Houthoofd W., (2009) *Macrostomum lignano*: an emerging model for studying the reciprocal influence between tissue homeostasis and ageing. 3rd International Macrostomum Meeting, Diepenbeek (Belgium), 31 July 2009.

Teaching related activities

Supervision undergraduate students

Master thesis:

2012-2013	Lenore Baten: Expression analysis of mitophagy-related genes via single-copy insertion in *Caenorhabditis elegans.*
2011-2012	Huaihan Cai: Study of mitochondrial function and turnover in the ageing model *Caenorhabditis elegan*s .
2010-2011	Md. Kamrul Islam: Optimization of mitochondrial turnover experiments in *Caenorhabditis elegans* using the photoconvertible fluorescence protein Dendra2.

Bachelor thesis:

2014	Lisa Vanderstraeten & Ellen Boven: Study of individual protein turnover in the model organism *C. elegans* via western blot
2011	Stef Heindryckx: The use of liposomes and the effect on the lifespan of *Caenorhabditis elegans*.

Internships:

2015	HoGent (16 students): Project using *C. elegans* as model organism.
2013-2014	Stephanie Caveye: The paradox between mitochondrial dysfunction and longevity in *C. elegans*.
2012-2013	Nazanin Joon: Function of MALT-1 in protein aggregation clearance in *C. elegans*
2011-2012	Esther Lezcano Fernández: Mitochondrial metabolism in two mitophagy related mutants: *pink-1* and *pdr-1* of the nematode *Caenorhabditis elegans*.
2011	Marek Adamowicz: Construction of roGFP2-Orp1 vectors via Gateway Cloning.

Practical courses

2010-2014	Introducing animal physiology (3rd Bachelor Biology)
2013	Nematodes as model organisms: practical course in Basic Bioinformatics (1st Master Nematology)
2012-2014	Model organisms: Regeneration in the flatworm *Macrostomum lignano*(2nd Bachelor Biomedical Sciences).
2011-2012	Terrestrial internships (Field practical training): Regulation of mass specific metabolic rate in different species in sandhill habitats (3rd Bachelor Biology)
2011	Marine internship (Field practical training): Analysis of stem cell distribution in marine species (3rd Bachelor Biology)