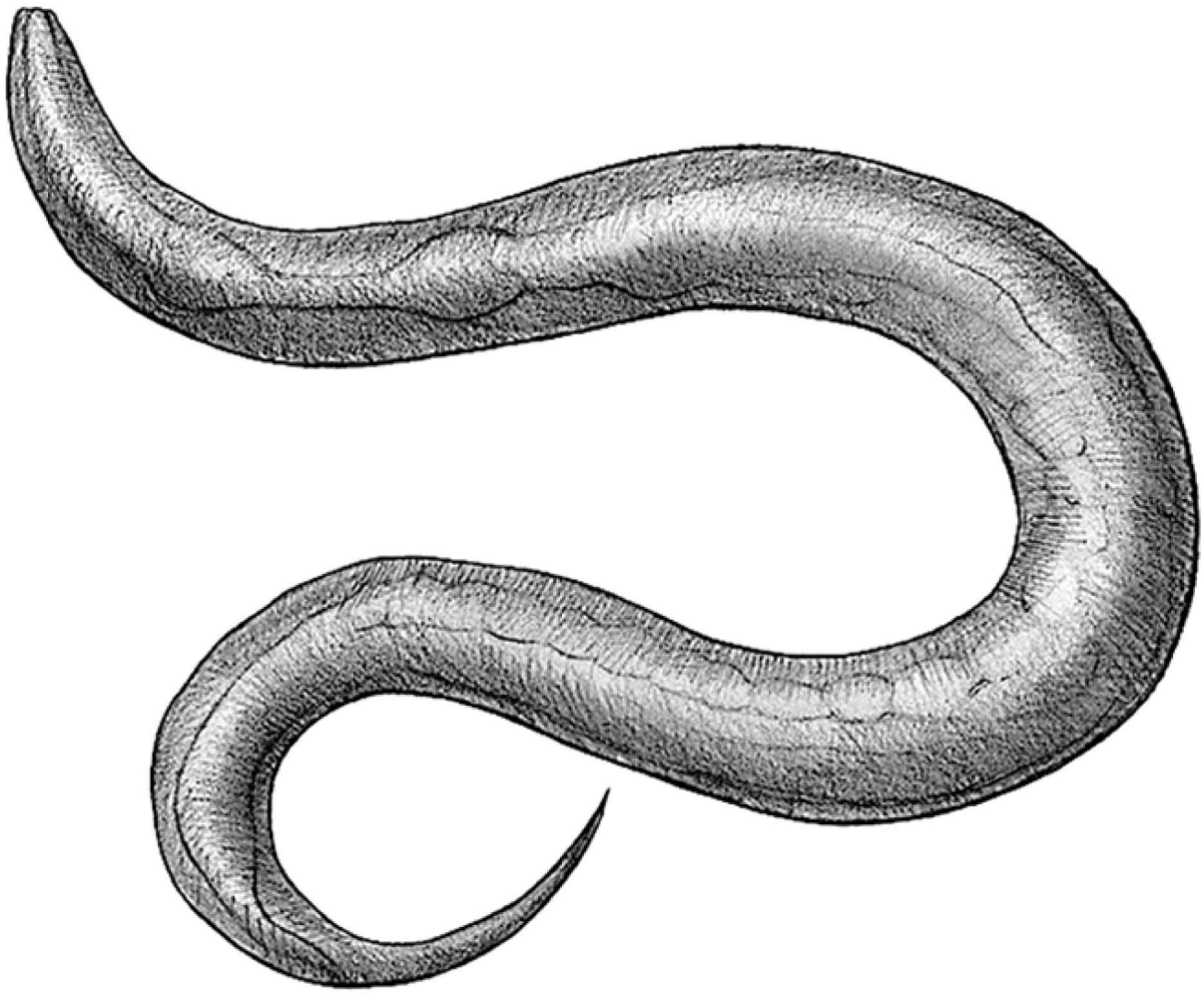


Filipa Carvalhal Marques

MECHANISMS OF AGING: NEURONAL ORCHESTRATION OF STRESS RESISTANCE AND PROTEIN HOMEOSTASIS IN THE NEMATODE *CAENORHABDITIS ELEGANS*

Tese do Programa de Doutoramento em Ciências da Saúde – ramo de Ciências Biomédicas, orientada por Doutor Henrique Manuel Paixão dos Santos Girão, co-orientada por Professor Doutor Ehud Cohen e apresentada à Faculdade de Medicina da Universidade de Coimbra.

Setembro 2015

· U C ·

Universidade de Coimbra

Filipa Carvalhal Marques

Mechanisms of aging: neuronal orchestration of stress resistance and protein homeostasis in the nematode *Caenorhabditis elegans*

University of Coimbra

Hebrew University of Jerusalem

2015

Dissertação apresentada à Faculdade de Medicina da Universidade de Coimbra para prestação de provas de Doutoramento em Ciências Biomédicas.

O trabalho apresentado nesta tese foi realizado na Faculdade de Medicina da Universidade Hebraica de Jerusalém, Israel, sob a co-orientação directa do Doutor Ehud Cohen e orientação do Doutor Henrique Girão, do Instituto Biomédico de Investigação da Luz e Imagem (IBILI) da Faculdade de Medicina da Universidade de Coimbra, ao abrigo da bolsa de Doutoramento SFRH/BD/70502/2010 (Fundação para a Ciência e a Tecnologia - FCT), no âmbito do Programa Doutoral em Envelhecimento e Degenerescência de Sistemas Biológicos Complexos da Faculdade de Medicina da Universidade de Coimbra.

ACKNOWLEDGEMENTS

To Ehud, my co-supervisor, first of all for accepting and receiving me in his lab in such a warm, welcoming way; for the amazing supervision skills that revolutionized my way of thinking; for the long conversations on politics, war, and religion that made me understand better Israel and the world; for teaching me about tolerance and coexistence; for the strong, helpful, and unforgettable words in the worst moments; in sum, for the forever-on influence in my life.

To Paulo, my previous supervisor, and to Henrique, my present supervisor, for giving me the opportunity to study, get to know, and live in Jerusalem, and also for their support and motivation, which created the environment where I gave my first and always so decisive steps in science at the University of Coimbra.

To the Programa Doutoral em Envelhecimento e Degenerescência de Sistemas Biológicos Complexos and to Fundação para a Ciência e a Tecnologia (FCT), that took me to know and do science in so many different parts of the country and the globe.

To the colleague-friends (Lorna, Yuli, Noa, Taly, Oswa, Michal, Tziona, Amir, Danielle, Tayir, Moria M., Mila, Naama, Moria L., and Lital) of Ehud's lab that, during these three years and a half, through ups and downs, created the most wonderful, fun, full of friendship and love working environment I have ever encountered and believe to be unique.

To Moria M., without whom this work would not have been possible.

To the Portuguese Family of Jerusalem, which became so large and international over the years, for all the love. Words are said daily.

To the distant friends: those from school, with me for more than half of my life; those met in my first degree; and those made in other circumstances, for actively remaining in my life despite so many years living apart.

To Su, Ki, Lu, and David, my most cherished friends, present at all times. And to David L., above all, my haven in Jerusalem.

To my family, the one I left in Portugal, for the most wonderful welcoming every time I come back. And to Gabi, old-new family member.

To my mom, whom I miss and that would be happy to know that her lessons still accompany me.
To my dad, my best friend, mentor, and most important reference.

The results presented in this thesis and obtained in the scope of the SFRH/BD/70502/2010 PhD fellowship (FCT) are under submission or in preparation for future submission to international peer-review scientific journals:

Maman, M.[*], Carvalhal Marques, F. [*], Volovik, Y., Dubnikov, T., and Bejerano-Sagie, M, Cohen E. (2013). A neuronal GPCR is critical for the induction of the heat shock response in the nematode *C. elegans*. *J Neurosci*, 33(14), 6102-11.

El-Ami, T.[*], Moll, L. [*], Carvalhal Marques, F., Volovik, Y., Reuveni, H., and Cohen E. (2014). A novel inhibitor of the insulin/IGF signaling pathway protects from age-onset, neurodegeneration-linked proteotoxicity. *Aging Cell*, 13(1), 165-74.

Volovik, Y.[*], Marques, F. C., and Cohen, E. (2014). The nematode *Caenorhabditis elegans*: a versatile model for the study of proteotoxicity and aging. *Methods*, 68(3), 458-64.

Volovik, Y.[*], Moll, L., Marques, F. C., Maman, M., Bejerano-Sagie, M., and Cohen, E. (2014). Differential regulation of the heat shock factor 1 and DAF-16 by neuronal *nhl-1* in the nematode *C. elegans*. *Cell Rep*, 9(6), 2192-205.

Carvalhal Marques, F.[*], Volovik, Y., and Cohen, E. (2015). The roles of cellular and organismal aging in the development of late-onset maladies. *Annu Rev Pathol*, 10, 1-23.

Carvalhal Marques, F.[*], El-Ami, T., Girão, H., Cohen, E. TOR-2 differentially regulates proteostasis in a tissue-specific manner in *Caenorhabditis elegans* (under preparation).

Note: the data presented in this dissertation is partially formatted according to the style of the journals of publication with minor modifications.

TABLE OF CONTENTS

INDEX OF FIGURES AND TABLES

ABBREVIATIONS

ROS	Reactive oxygen species
DR	Dietary restriction
ETC	Electron transport chain
IGF-1	Insulin/insulin-like growth factor 1
IIS	Insulin/IGF-1 signaling
PI3K	Phosphatidylinositol 3-kinase
HSF-1	Heat shock factor 1
Hsp	Heat shock protein
UPS	Ubiquitin-proteasome system
ER	Endoplasmic reticulum
HSR	Heat shock response
UPR	Unfolded protein response
AD	Alzheimer's disease
polyQ	Polyglutamine
ALS	Amyotrophic lateral sclerosis
RNAi	RNA interference
siRNA	Small interfering RNAi
dsRNA	Double stranded RNA
NGM	Nematode Growth Media
GFP	Green Fluorescent Protein
IPTG	Isopropyl β-d-1-thiogalactopyranoside
qPCR	Real-time quantitative PCR
GPCR	G protein-coupled receptor
YFP	Yellow fluorescent protein
Aβ	Amyloid β

RESUMO

O envelhecimento é um processo complexo que ocorre em todos os organismos, da levedura ao Homem. Apesar de um século de pesquisa e discussão científica, os factores subjacentes à progressão do envelhecimento permanecem por clarificar. Mas os ângulos sob os quais este processo é visto sofreram grandes mudanças com o tempo. As primeiras teorias sugeriam que o envelhecimento decorre da acumulação estocástica de danos nas macromoléculas, levando ao mal funcionamento dos organismos e, por fim, à morte dos mesmos. Esta área de investigação foi radicalmente transformada nas últimas décadas por uma série de estudos pioneiros em diferentes modelos animais que mostraram claramente que o envelhecimento pode ser alterado através da manipulação de várias vias metabólicas e genéticas. Estas descobertas sugeriram que o nível de protecção de um organismo contra danos estocásticos pode ser regulado e, consequentemente, também o período de vida durante o qual o mesmo permanece saudável. No entanto, à medida que o conhecimento acerca destes mecanismos foi maturando, tornou-se evidente que a duração de vida, a resistência a *stress* e a homeostase proteica, aspectos que são regulados pelas vias que regulam o envelhecimento, podem ser desacopladas sem se influenciarem mutuamente. Mais recentemente, o processo de envelhecimento revelou possuir um nível adicional de complexidade quando se mostrou que pode ser coordenado por diferentes tecidos ao nível do organismo.

Neste trabalho, o nosso interesse focou-se nos princípios subjacentes à orquestração do envelhecimento ao nível do organismo, bem como na dissociação entre duração de vida, resistência a *stress* e homeostase proteica. De modo a abordar estes temas, usámos o nemátode *Caenorhabditis elegans* (*C. elegans*), modelo animal que oferece inúmeras vantagens no estudo do envelhecimento. Começámos por investigar os mecanismos de comunicação entre tecidos que regulam a *heat shock response* (HSR) a nível do organismo no modelo *C. elegans*, procurando, mais concretamente, esclarecer que receptores neuronais estão envolvidos neste mecanismo de sinalização e em que neurónios desempenham a sua função. Para responder a estas questões, empregámos nemátodes geneticamente modificados que apresentam hipersensibilidade a RNA

de interferência (RNAi) no tecido nervoso e identificámos um presumível receptor acoplado a proteínas G (GPCR) como sendo um componente-chave deste mecanismo. Este gene, a que atribuímos o nome *gtr-1*, é expresso em neurónios quimiosensoriais e desempenha um papel fundamental na indução de genes que codificam proteínas de *heat shock* nos tecidos somáticos após exposição a temperaturas elevadas, apesar de não ser necessário à percepção de calor. Surpreendentemente, o *knockdown* do *gtr-1* através de RNAi tem um efeito protector em nemátodes que expressam nos músculos $A\beta_{3-42}$ (um péptido com tendência agregativa associado à doença de Alzheimer), mas não influencia a duração de vida, a resistência a outros *stresses* ou funções associadas ao desenvolvimento.

Na segunda parte deste trabalho pretendemos fazer uma caracterização mais detalhada dos elementos *downstream* à via de sinalização da insulina/IGF-1 (IIS) que estão directamente envolvidos na regulação da toxicidade proteica em *C. elegans*. Com este objectivo, procurámos genes previamente citados na literatura como reguladores da homeostase proteica e identificámos o *tor-2* como sendo regulado ao nível da transcrição pela via IIS. Nesta tese mostramos que a expressão do *tor-2* é induzida após a supressão desta via pelos factores de transcrição DAF-16 e SKN-1. Este gene revelou-se importante na resistência a temperaturas elevadas mas não na regulação do tempo de vida do animal ou na resistência a outros tipos de *stress* tais como exposição a bactérias patogénicas ou a radiação UV. Curiosamente, o *tor-2* parece ser importante no combate à toxicidade proteica em neurónios, onde se mostrou anteriormente que este gene é expresso, ao passo que o seu *knockdown* protege os nemátodes que expressam proteínas agregativas tóxicas nos músculos.

Este estudo oferece novas ideias: (1) que os neurónios quimiosensoriais desempenham um papel importante nos mecanismos que regulam a HSR no nemátode; (2) que o tempo de vida e a resistência a *heat shock* são separáveis; (3) consolida o conceito emergente de que a habilidade para responder o calor existe em detrimento da manutenção da proteostase; e (4) sugere que a homeostase proteica pode ser diferencialmente regulada de tecido para tecido por um único gene.

ABSTRACT

Aging is a complex process that occurs in organisms ranging from yeast to humans. The factors underlying the progression of aging still elude us, despite a century of scientific inquiry and discussion. Nevertheless, the angles from which aging was perceived have greatly changed over time. Early theories suggested that aging results from the accumulation of stochastic damage to macromolecules, leading to organismal malfunction and ultimately death. The field was however revolutionized over the last decades by a series of pioneering studies carried out in model organisms that showed that aging can actually be altered by the modification of several metabolic and genetic pathways. These findings suggested that the level of protection against stochastic damage can be regulated and, hence, the length of time an organism remains healthy. However, as the knowledge on these mechanisms matured, it became evident that lifespan, stress resistance, and protein homeostasis (proteostasis), aspects that are regulated by the aging-modulating pathways, can be uncoupled without influencing one another. The aging process revealed another level of complexity when it was shown to be coordinated by different tissues in an organismal-fashion.

In this work, we were interested in the principles underlying the orchestration of aging at the organismal level, as well as in the uncoupling between lifespan, stress resistance, and proteostasis. To address these questions, we employed the nematode *Caenorhabditis elegans* (*C. elegans*), which offers key advantages in the study of aging. We started by focusing on the inter-tissue communication mechanisms that regulate the heat shock response (HSR) at the organismal level in *C. elegans* and attempted to clarify which neuronal receptors are required for this signaling mechanism and in which neurons they function. To answer these questions, we employed worms that were engineered to exhibit RNA interference (RNAi) hypersensitivity in neurons and identified a putative G protein-coupled receptor (GPCR) as a novel key component of this mechanism. This gene, which we termed GPCR thermal receptor 1 (*gtr-1*), is expressed in chemosensory neurons and has no role in heat sensing but is critically required for the induction of genes that encode heat shock proteins in non-neural tissues upon exposure to heat. Surprisingly,

the knockdown of *gtr-1* by RNAi protected worms expressing the Alzheimer's-disease-linked aggregative peptide $A\beta_{3-42}$ in their body-wall muscles from protein toxicity (proteotoxicity) but had no effect on lifespan, resistance to other stresses, or developmental functions.

In the second part, we aimed at better characterizing the insulin/IGF-1 signaling (IIS)-downstream components involved in the direct regulation of protein toxicity (proteotoxicity) in the *C. elegans* model. For this, we searched for genes that are known regulators of proteostasis and identified *tor-2* as a transcriptional target of the IIS pathway. Here we show that *tor-2* is upregulated upon suppression of the IIS by both DAF-16 and SKN-1transcription factors. This gene is important for the resistance to heat shock but has no role in the determination of lifespan or in the resistance to other acute stresses such as exposure to pathogenic bacteria or to UV radiation. Interestingly, *tor-2* seems to be important to counteract proteotoxicity in neurons, previously shown to be its main site of expression, whereas its knockdown protects worms that express toxic, aggregative-proteins in their body-wall muscles.

In this work we provide several novel insights: (1) we show that chemosensory neurons play important roles in the nematode's HSR-regulating mechanism; (2) that lifespan and heat stress resistance are separable; (3) we strengthen the emerging notion that the ability to respond to heat comes at the expense of proteostasis; and (4) suggest that proteostasis can be differentially regulated in a tissue-specific manner by a sole gene.

Key words: *gtr-1*, *tor-2*, insulin/IGF-1 signaling (IIS), aging, heat shock response, proteotoxicity, proteostasis, *Caenorhabditis elegans*.

Chapter 1

RELATED LITERATURE

Chapter 1

RELATED LITERATURE

1.1. Aging

1.1.1. Aging as a regulated process

Aging occurs in organisms that range from yeast to humans. But what determines how long an organism will live? The progression of aging remains under debate despite a century of scientific inquiry. Early theories suggested that aging results from the non-adaptive accumulation of stochastic damage to macromolecules, organelles, tissues, and cells, ultimately leading to organismal malfunction and inevitable death (Longo *et al*., 2005; Carvalhal Marques *et al*., 2015). The most famous is the free radical theory, which proposes that the reactive oxygen species (ROS) formed as a result of normal metabolism are the main instigators of aging (Harman, 1956; Longo *et al*., 2005). Other potential sources of aging-promoting damage have been proposed, including random mutagenesis and environmental insults (Martin *et al*., 1996). These theories share the underlying principle that aging is an uncontrolled, random process. Notwithstanding, could it be that aging occurs mostly due to the loss of efficiency of a putative program that regulates protection and repair systems?

It was August Weismann who first introduced the idea of a programmed aging: "the causes of the duration of life must be contained in the organism itself and cannot be found in any of its external conditions or circumstances" (Weismann, 1889). In modern terms, aging must be controlled at the genetic level. Weismann suggested that the removal of aged animals that neither reproduce nor support offspring assists the evolution process by freeing resources (*e.g.* food, habitat) for younger animals. The observation that a restrictive diet, and not the opposite, extends lifespan in a diverse range of species is in disagreement with this idea (Mitteldorf, 2001). In addition, it does not provide convincing answers to major questions: how are the alleles that cause a decline in vitality late in life selected? Do they contribute to the fitness of the organism at a certain point of its early life? Can these alleles be selected because they carry benefits during early life, as suggested by the antagonistic pleiotropy theory (Longo *et al*., 2005)? Still, Weismann's

idea led to the realization that, while aging does have random aspects, it is also amenable to genetic regulation.

In the last decades, several independent and evolutionary conserved mechanisms that regulate the pace of aging and lifespan in different species were identified and characterized: dietary restriction (DR) (McCay *et al*., 1989), the mitochondrial electron transport chain (ETC) pathway (Dillin *et al*., 2001; Feng *et al*., 2001; Lee *et al*., 2003), and the insulin/insulin-like growth factor 1 (IGF-1) signaling (IIS) pathway (Kenyon *et al*., 1993). The first aging-regulating pathway to be identified was DR, shown to extend lifespan in multiple species (Fontana *et al*., 2010). The reduction of dietary intake below unlimited or *ad libitum* levels causes an increase in lifespan to an optimum point of consumption, typically around 60% of *ad libitum* food intake (Fontana *et al*., 2010). The second pathway enabling extension of lifespan acts through the disruption of the ETC function. This was first shown in the nematode *Caenorhabditis elegans* (*C. elegans*), in which a mutation or reduction of function of several mitochondrial ETC components is sufficient to extend lifespan (Dillin *et al*., 2002; Feng *et al*., 2001; Lee *et al*., 2003).

In the next sections, we will describe the advantages of using *C. elegans* in our study, as well as describe in detail the third pathway, the IIS, to which this thesis gives particular emphasis.

1.1.1.1. In search for a valuable approach to study stress resistance, proteostasis, and aging: *Caenorhabditis elegans* as a resourceful model

Invertebrate models have been widely used to further explore how homeostatic mechanisms are regulated. It is the case of the small, free-living round nematode *C. elegans* which, following its introduction as a genetic model organism in 1965, has ever since been extensively used to study a large variety of biological processes, leading the way in the aging field (Nussbaum-Krammer and Morimoto 2014; Sin *et al*., 2014). Indeed, this nematode combines a number of anatomic and genetic features that make it an advantageous model in the acquisition of fundamental insights into aging.

C. elegans is a small nematode of about 1 mm in length that feeds on bacteria, including *Escherichia coli* (*E. coli*), and exists in two sexual forms: as a hermaphrodite and a male. The

former is self-fertile, able to produce its own sperm and eggs, and is the predominant adult form. Wild type hermaphrodites are able to lay 300-350 eggs during reproductive adulthood. After hatching, it takes three days for the larvae to mature into adult worms whose mean lifespans vary between 18 to 20 days when grown at 20°C, allowing a large-scale production of animals within a short period of time. Importantly, although relatively short-lived, the worms do age and this process shares fundamental similarities with that of mammals. Among other features, aged worms suffer from muscle atrophy (sarcopenia), which leads to reduced agility and uncoordinated movement display, accumulate the autofluorescent molecule lipofuscin, and exhibit increased levels of oxidized proteins (Volovik *et al*., 2014; Rodriguez *et al*., 2013; Olsen *et al*., 2006; Nussbaum-Krammer and Morimoto, 2014).

Another major advantage of *C. elegans* is its well-dissected anatomy; the adult hermaphrodite has exactly 959 cells, of which 302 are neurons. Its transparent body further allows one to easily follow fluorescently-tagged proteins of interest within both developing and mature living animals. This feature enables the study of gene expression and protein deposition in both temporal- and tissue-specific fashions. Antibody-based labeling techniques can be also used with the drawback of requiring the death of the animal. Additionally, because *C. elegans* was the first multicellular organism to have its complete genome sequence, several highly comprehensive databases and resources are currently available online for the scientific community. We further know that roughly 83% of worm genes have a human orthologue (Volovik *et al*., 2014; Sin *et al*., 2014; Calahorro and Ruiz-Rubio, 2011).

Perhaps one of the most useful tools in *C. elegans* is the possibility of performing genetic screens. One of the key genetic screens employed is the RNA interference (RNAi) screen, which offers information on a gene following its specific and effective expression knockdown (Fire, 2007). This technique relies on feeding the worms with bacteria expressing double stranded RNA (dsRNA), which is then processed into small interfering RNA (siRNA) molecules by the nematode. This siRNA induces in turn the degradation of specific endogenous mRNAs, allowing the specific downregulation of different genes of interest (Timmons *et al*., 2001). The availability of RNAi libraries that cover the majority of the worm's genome, along with the easy applicability

of this gene-silencing technique, makes *C. elegans* a preferred organism for RNAi-based genetic screens, a trait that allows deciphering the mechanisms that regulate the aging process.

In short, *C. elegans* has shown to be crucial in giving fast answers to numerous, emerging questions, and, more importantly, useful in the transfer of knowledge to mammalian systems and, ultimately, to humans. For all of the abovementioned reasons, our study took advantage of this model.

1.1.1.2. The insulin/IGF-1 signaling (IIS) pathway

The IIS is perhaps the most prominent pathway that strongly regulates lifespan and youthfulness in both invertebrate and vertebrate species (Kenyon, 2011). In *C. elegans*, where it was first identified and well characterized, this pathway involves signaling through the Insulin/IGF-1 receptor (Fig. 1). The worm genome encodes a single receptor orthologue to the mammalian one, DAF-2, which negatively regulates the activity of a set of transcription factors through well-conserved functional components (Carvalhal Marques *et al*., 2015). So far, the forkhead (FOXO) transcription factor DAF-16 has been identified as a major downstream target of the *C. elegans* IIS pathway (Ogg *et al*., 1997; Lin *et al*., 1997; Lin *et al*., 2001. Under favorable conditions, DAF-16/FOXO is highly phosphorylated following the activation of a kinase cascade that includes the phosphatidylinositol 3-kinase (PI3K) AGE-1 (Murakami and Johnson, 1996) and is triggered by the activation of the kinase domain of DAF-2 upon its binding to insulin-like peptides (Murphy and Hsu, 2013). These post-translational modifications render DAF-16 sequestered in the cytoplasm by 14-3-3 proteins and thus inactive (Li *et al*., 2007). Accordingly, mutations that interfere with the DAF-2 downstream signaling result in reduced phosphorylation of DAF-16. Consequently, it translocates to the nucleus, whereupon it controls the expression of genes that render the worms long-lived, youthful, and resistant to a diverse array of stresses (Murphy *et al*., 2003; Hsu *et al*., 2003; Garsin *et al*., 2003; Lee *et al*., 2003; Samuelson *et al*., 2007; Volovik *et al*., 2014; Cohen *et al*., 2006; Lithgow *et al*., 1995; Honda and Honda, 1999; Murakami and Johnson, 1996; Hamilton *et al*., 2005).

In addition to DAF-16, other transcription factors act downstream of the IIS pathway. It is the case of the transcription factor heat shock factor 1 (HSF-1), which was shown to be required for longevity and stress resistance induced by reduced IIS (Hsu *et al*., 2003; Singh and Aballay,

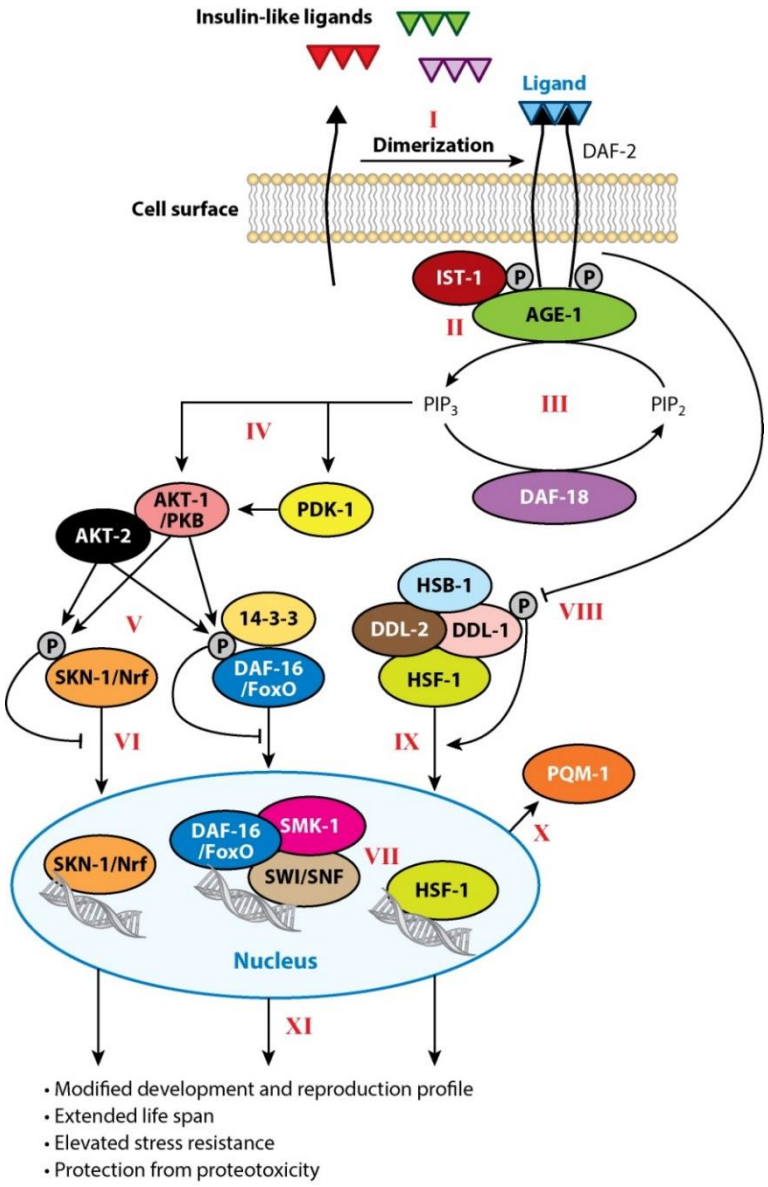


Figure 1. The insulin/insulin-like growth factor 1 (IGF-1) signaling (IIS) pathway. Upon binding of one of the ligands, the sole insulin/IGF-1 receptor of *C. elegans*, DAF-2, dimerizes and undergoes self-phosphorylation. The phosphatidylinositol 3-kinase AGE-1 and the kinase IST-1 (IRS1 ortholog) are then recruited to DAF-2 (II), whereupon AGE-1 catalyzes the generation of phosphatidylinositol 3,4,5-trisphosphate (PIP_3) (III), a molecule that activates kinases of the AKT family in a PDK-dependent manner (IV). Activated AKTs phosphorylate the transcription factor DAF-16/FoxO and mediate its binding to a 14-3-3 family member (V), preventing it from entering the nucleus.

2006 – a; Singh and Aballay, 2006 – b). The IIS negatively regulates the activity of HSF-1 by modulating the formation of a multiprotein complex containing the conserved proteins HSB-1, DDL-1, and DDL-2, which sequester HSF-1 in the cytoplasm (Chiang *et al*., 2012). The integrity of this complex is dependent on the phosphorylation status of DDL-1, itself regulated by the IIS. IIS activity dephosphorylates DDL-1, enabling the complex to form and retain HSF-1 in the cytosol. Accordingly, a reduction in the IIS promotes the phosphorylation of DDL-1, leading to the complex's disintegration and to the entry of HSF-1 to the nucleus, where it regulates its target gene network.

The *C. elegans* Nrf family transcription factor SKN-1, which regulates genes that protect against oxidative and xenobiotic stress, along with many genes involved in protein homeostasis and metabolism (An *et al*., 2005; Kahn *et al*., 2008; Oliveira *et al*., 2009; Li *et al*., 2011; Paek *et al*., 2012) , also contributes to lifespan extension in animals with reduced IIS (Tullet *et al*., 2008). When the IIS is fully functional, SKN-1 is directly phosphorylated by Akt/PKB and related kinases, being consequently sequestered in the cytoplasm. On the other hand, upon reduced IIS signaling, SKN-1 (Tullet *et al*., 2008) is allowed to translocate to the nucleus, similarly to DAF-

Figure 1. (continuation) Similarly, AKT-mediated phosphorylation retains the transcription factor SKN-1/Nrf in the cytosol (V). Thus, IIS reduction reduces the rate of AKT activity, allowing DAF-16/FoxO and SKN-1/Nrf to enter the nucleus (VI), interact with their functional partners, such as SWI/SNF and SMK-1 (VII), and regulate their target gene networks. The phosphatase DAF-18 (PTEN ortholog) acts in opposition to AGE-1, converting PIP_3 to phosphatidyl-inositol 4,5-bisphosphate (PIP_2) and thereby reducing the rate of AKT activation. The phosphatase DAF-18 (PTEN ortholog) acts in opposition to AGE-1, converting PIP_3 to phosphatidylinositol 4,5-bisphosphate (PIP_2) and thereby reducing the rate of AKT activation. The heat shock factor 1 (HSF-1) is also negatively regulated by the IIS pathway, which inhibits the phosphorylation of its interactor DDL-1 (VIII). Accordingly, IIS reduction increases the rate of DDL-1 phosphorylation and disrupts the complex (DDL-1, DDL-2, and HSB-1) that binds HSF-1, enabling its entry to the nucleus (IX). The transcriptional activator PQM-1 is also required for the IIS reduction-mediated longevity phenotype, and its cellular localization is governed by the IIS (X); however, PQM-1 and DAF-16/FoxO inhibit each other's nuclear localization. The inhibition of the IIS cascade and the resulting modified activity of its downstream transcription factors promote longevity, stress resistance, and proteostasis (XI) (from Carvalhal Marques *et al*., 2015).

16 and HSF-1, and to induce the expression of its target genes. This concerted operation contributes to the long-lived phenotype and high stress resistance typical of worms with low IIS.

1.1.1.3. Does the regulation of aging oppose cumulative damages?

The identification of many mutations that extend lifespan in model systems supports the idea of a longevity program, but it does not necessarily oppose the cumulative damage theory. It is, in fact, conceivable that these aging-regulating pathways slow the aging process by enhancing the efficiency of the organisms' mechanisms that confer resistance to both acute and chronic stresses, which in turn reduce the rate of damage accumulation. Before returning to this idea, we will provide a brief overview on the stress-responsive genes and mechanisms that contribute to the organism's ability to maintain the integrity of the proteome and on the evidences showing that the proteome suffers a dramatic deterioration with aging (Taylor and Dillin, 2011).

1.2. The proteome and its challenges

1.2.1. Facing the inevitable or how to maintain a pristine proteome

Proteins are involved in almost every biological process. Due to their structural and functional complexity, they are particularly fragile macromolecules that are only marginally stable at physiological temperature and are constantly at risk of misfolding (Hipp *et al*., 2014; Mattoo and Goloubinoff, 2014). Environmental and physiological stressors such as heat, oxidative stress, and inflammation challenge the stability of protein conformation. In addition, proteins must retain conformational flexibility to function and thus are constantly undergoing folding, assembly, disassembly, and trafficking through sub-cellular compartments, both within and outside the cell (Morimoto and Cuervo, 2014).

This poses a universal problem for all types of cells given the high macromolecular crowding in the cytosol, with little or no "free" water, which provides a friendly environment for undesirable and, ultimately, harmful interactions (Fink, 1999; Ellis, 2007). When nascent polypeptides exit the ribosome in the unfolded state, or when labile mature proteins are partially unfolded, they may achieve conformations that expose hydrophobic surfaces to the aqueous phase

that are otherwise buried in the native structure (Hinault and Goloubinoff, 2007; Hartl and Hayer-Hartl, 2011). Seeking intra-molecular stability, newly exposed hydrophobic residues tend to spontaneously associate to form polypeptide aggregates and leave solution, becoming unable to reach their native state within a biologically meaningful time-scale (Goldberg, 2003; Mattoo and Goloubinoff, 2014; Prahlad and Morimoto, 2011). These proteins can be directly cytotoxic by interacting with other cellular components and, on the other hand, can create increased demands on the cell's protein homeostasis (proteostasis) machinery, titrating away components of this network and leading to further misfolding of other proteins (Stefani and Dobson, 2003; Gidalevitz *et al*., 2006; Taylor and Dillin, 2011).

To face these harmful events, organisms have developed an exquisite network of molecular components termed proteostasis network (Balch *et al*., 2008). It comprises highly conserved, specialized sets of chaperones that assist nascent polypeptides in folding correctly and ensure their integrity, and protein degradation machineries that remove misfolded or terminally damaged polypeptides, together with a number of adaptive cellular stress response pathways that act alone or together in various sub-networks to sense and respond to protein misfolding in all cellular compartments (Labbadia and Morimoto, 2015; Ciechanover and Kwon, 2015). These constitutive and inducible protein quality control mechanisms are central to reduce damage to a minimum and maintain or reestablish proteostasis, particularly in post-mitotic cells (Gupta *et al*., 2010).

1.2.1.1. The proteostasis network: the chaperone, proteasomal, and autophagic systems

The molecular chaperones are a large family of highly conserved proteins that interact with, stabilize, and help other proteins to acquire their functionally active conformation (Hartl and Hayer-Hartl, 2011). Under normal conditions, they are expressed in adequate amounts to carry out their physiological and housekeeping functions; in contrast, during a stress such as heat-shock, some ubiquitously expressed members of these families are massively synthesized by the cell (Hinault and Goloubinoff, 2007; Gestwicki and Garza, 2012; Ellis, 2007; Gupta *et al*., 2010). Indeed, the stress-inducible nature of many molecular chaperones has led to their early

classification as heat-shock proteins (Hsps). Chaperones are usually named according to their approximate molecular weight: small Hsps (sHsps), Hsp40, Hsp60, Hsp70, Hsp90 and Hsp100. All classes share the ability to screen for proteins exposing hydrophobic residues typically concealed within the core of the protein and thus execute fundamental roles in *de novo* folding and refolding of misfolded protein (Mattoo and Goloubinoff *et al*., 2014; Morimoto and Cuervo, 2014). Notwithstanding, these classes share no apparent sequence or structural homology and in fact display large functional diversity, being involved in a multitude of proteome-maintenance functions including protein trafficking, macromolecular complex assembly, and direct cooperation with the cell's proteolytic systems upon the need of protein disposal through degradation (Hartl and Hayer-Hartl, 2011; Smith *et al*., 2015; Hipp *et al*., 2014).

Misfolded proteins can be disposed of through the ubiquitin proteasome system (UPS), the primary selective proteolytic system in the eukaryotic cell cycle and pro-survival pathways (Ciechanover and Kwon, 2015; Ortega and Lucas, 2014). It comprises two main stages: the ubiquitin-tagging of substrates and their proteolytic degradation. Proteins are flagged by sequential conjugation of ubiquitins and afterwards recognized and degraded by the proteasome, a large multicatalytic enzyme complex expressed in the cytoplasm and nucleus of all eukaryotic cells whose major assembly is the 26S complex (Shang and Taylor, 2004; Adams, 2003; Ciechanover and Kwon, 2015). Misfolded proteins can also be degraded through autophagy, a term that refers to any intracellular process resulting in the degradation of components inside lysosomes (Martinez-Vicente *et al*., 2005; Chen *et al*., 2011). Lysosomes consist of single-membrane acidic organelles with an internal pH of 5.1-5.5, fully devoted to the degradation of intra and extracellular components. These organelles contain a large assortment of hydrolases capable of degrading most naturally-occurring macromolecules (Majeski and Dice, 2004; Martinez-Vicente *et al*., 2005; Koga *et al*., 2010). Depending on the type of cargo and means of delivery to the lysosome, autophagy is classified in different modalities. The best characterized form is macroautophagy, an inducible form of autophagy responsible for the degradation of cytosolic fractions, organelles, and macromolecules (Martinez-Vicente *et al*., 2005; Salminen and Kaarniranta, 2009.

1.2.1.2. Cellular stress responses: heat shock response (HSR) and unfolded protein response (UPR)

Cells must also be responsive to acute stressful conditions (*e.g.* temperature variations) that cause proteins to unfold and increase the risk of aggregate formation. Under these conditions, the normal folding and triage capacity might be exceeded by increases in demand, requiring a dramatic up-regulation of chaperones and other proteostasis components (Taylor and Dillin, 2011). In the compartmentalized eukaryotic cells, several pathways generally called stress responses have evolved independently to ensure the integrity of the protein-folding environments in the cytosol, the endoplasmic reticulum (ER), and the mitochondria.

When facing elevated temperatures or other proteotoxic stress, cells activate a highly conserved program of stress-inducible gene expression termed the "heat shock response" (HSR) that senses protein misfolding in the cytosol (Lindquist and Craig, 1988; Morimoto, 1998) (Fig. 2). This transcriptional response is regulated by a family of heat-shock transcription factors remarkably conserved from yeast to humans (Ahn *et al*., 2005; Anckar and Sistonen, 2007; Voellmy and Boellmann, 2007). In *C. elegans*, the HSR is predominantly mediated by the ubiquitously and constitutively expressed transcription factor HSF-1, which is negatively regulated in most cell types and largely localizes in the cytoplasm in a monomeric state in normal circumstances (van Oosten-Hawle and Morimoto, 2014). In response to a cellular stress, HSF-1 senses the increase in the levels of cytosolic misfolded proteins, trimerizes, and re-localizes to the nucleus, wherein it binds with high affinity to consensus HS elements in the promoter region of its target genes. HSF-1 thus promotes the expression of Hsps together with other pro-survival factors, thereby enhancing protein folding and the assistance to client degradation (Lindquist and Craig, 1998; Morimoto, 1998; Kakkar *et al*., 2014).

The functional complement to the cytosolic HSR in the cellular compartments is the unfolded protein response (UPR), which responds to acute stress within organelles. In the ER, an important site for synthesis, folding, and modification of membrane and secretory proteins in eukaryotic cells, the UPR^{ER} is activated upon increased protein flux during development or under stresses (*e.g.* heat shock) that lead to the accumulation of unfolded proteins (Korennykh and

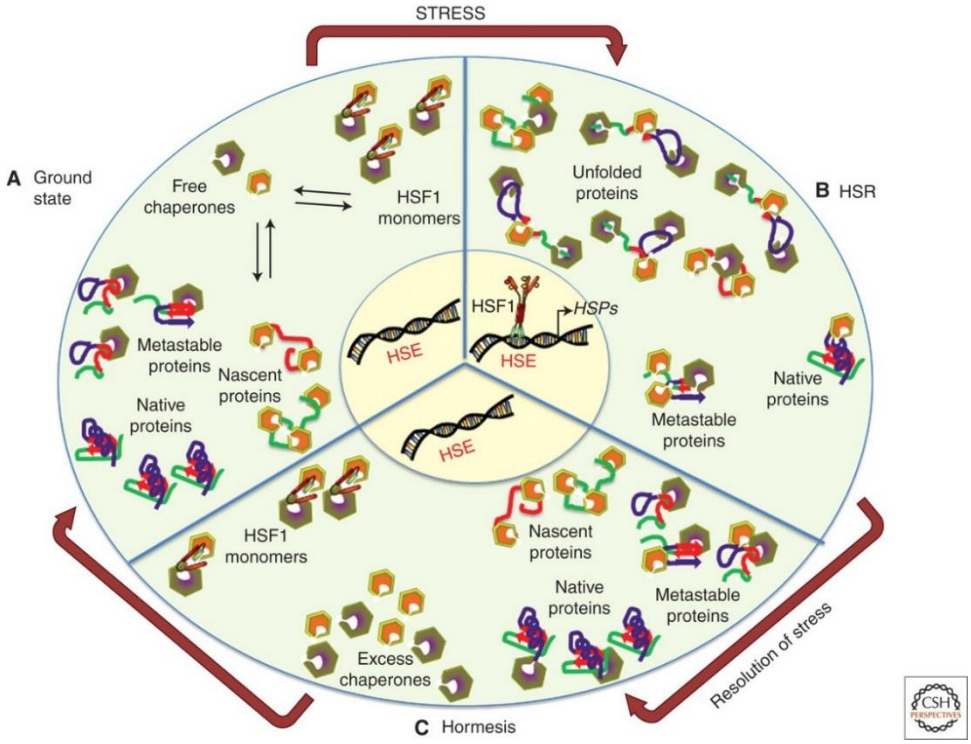


Figure 2. The heat shock response (HSR). The HSR is one of the most ancient and conserved cellular stress responses that allow cells to adapt to changing environmental conditions and ensure recovery following perturbations to proteostasis. This response is mediated at the molecular level by the transcriptional regulation of heat shock genes by the remarkably conserved heat-shock transcription factor 1 (HSF-1). (A) In unstressed metazoan cells, HSF-1 is continuously present and largely localizes in the cytoplasm in a monomeric, transiently-bound-to-chaperones state. (B) As a result of heat shock or other proteotoxic stressors, HSF-1 undergoes trimerization and re-localizes to the nucleus, where it binds to heat-shock elements (HSEs), the upstream regulatory sequences of its target genes, inducing their expression. Thus, an increased flux of misfolded and damaged proteins is countered by a corresponding increase in, among other things, chaperone levels. (C) It is unclear what happens to the excess chaperone capacity induced in the cell following the resolution of protein misfolding. It is possible that the excess of chaperones renders the cells with a hormetic state in which cells are protected from a subsequent lethal stress (from Gidalevitz *et al*.,2006).

Walter, 2012; Taylor and Dillin, 2011; van Oosten-Hawle and Morimoto, 2014). In higher eukaryotes, this pathway is induced by three upstream transmembrane sensors that coordinately detect the accumulation of unfolded proteins in the ER lumen and activate a transcriptional program with several outcomes: reduced flux of nascent chains entering the ER; increased levels

of ER luminal chaperones and protein-modifying proteins; and stimulated export and degradation of misfolded proteins (Ron and Walter, 2007; Taylor and Dillin, 2011; Jovaisaite *et al*., 2014).

Similarly to the ER, the mitochondria-specific UPR (UPR^{mt}) acts to ensure a proper protein folding environment in the mitochondrial matrix. This response copes with increased unfolded protein load by activating the transcription of nuclear-encoded mitochondrial chaperone protease genes (Jovaisaite *et al*., 2014; Haynes and Ron, 2010).

1.3. When the system starts failing: aging, loss of proteostasis, and consequences

The amount of damaged mature or newly folded proteins and the available protein quality control capacity are two sides of a carefully balanced system in our cells (Csermely *et al*., 2007). However, in certain situations, the proteostasis network cannot keep up with the rate of emergent unfolded proteins and, thus, becomes overloaded. This might occur when there is an excess of substrates or insufficient content in molecular chaperones and proteases in cells (Goldberg, 2003; Csermely *et al*., 2007; Hinault and Goloubinoff, 2007). The overload of the proteostasis network components becomes prominent in the elderly, where proteins bearing modifications (*e.g.* carbonylation and glycation) grow to be more abundant. Non-dividing cells such as neurons are particularly vulnerable to the accumulation of these modified, aggregative-prone proteins (Csermely *et al*., 2007; Calderwood *et al*., 2009). In fact, protein aggregation underlies the development of many age-related disorders collectively termed conformational disorders or proteinopathies. Among them we find the neurodegenerative diseases, including the highly abundant Alzheimer's disease (AD), the polyglutamine (polyQ) expansion disorders such as Huntington's disease, and Parkinson's disease. These disorders share two unifying features: the late age of onset and the chronic accumulation of protein aggregates in intra or extracellular deposits within the central nervous system (Takalo *et al*., 2013). This defines aging as the major risk factor for the development of these disorders and implicates that a loss in the proteostasis-maintaining machinery is inherent to the aging process. In other words, these mechanisms, highly active and thus protective in younger individuals, fail to prevent neurodegeneration late in life.

1.3.1. Aging-dependent decline in the system's homeostasis: what are the evidences? Can aging be manipulated to prevent this decline?

Recently, loss of proteostasis was indeed identified and categorized as one of the nine candidate hallmarks of aging given that, according to López-Otin *et al*., it fulfills three important criteria: (1) it manifests during normal aging; (2) its experimental aggravation accelerates aging; and (3) its experimental amelioration postpones the normal aging process and subsequently increases healthy lifespan (López-Otín *et al*., 2013). A number of animal models support the significant impairment of the protein quality control mechanism in aging, namely that of stress-induced synthesis of cytosolic and organelle-specific chaperones (Calderwood *et al*., 2009). For instance, transgenic worms (Walker and Lithgow, 2003) and flies (Morrow *et al*., 2004) overexpressing chaperones are long-lived. The overexpression of HSF-1 also promotes longevity and delays the onset of disease-linked protein aggregation in the nematode *C. elegans* (Chiang *et al*., 2012; Hsu *et al*., 2003), while amyloid-binding components can maintain proteostasis during aging and extend lifespan in the same animal model (Alavez *et al*., 2011). Conversely, mutant mice deficient in a co-chaperone of the heat shock family exhibit accelerated aging phenotypes (Min *et al*., 2008), whereas long-lived mouse strains show a marked up-regulation of some Hsps (Swindell *et al*., 2009).

Although the effects of aging in the functional capacity of both the proteasomal and autophagic systems in different tissues is not completely understood, they were observed to suffer an age-dependent decline in certain models and to be compromised in major neurodegenerative and metabolic diseases associated with aging (Lecker *et al*., 2006; Wong and Cuervo, 2010; Mizushima *et al*., 2008; Rubinsztein *et al*., 2011; Tomaru *et al*., 2010; Saez and Vilchez, 2014). Decreased expression of several autophagic genes was shown to shorten both *C. elegans* (Hars *et al*., 2007) and *Drosophila melanogaster* (*D. melanogaster*) (Simonsen *et al*., 2008) lifespans, while tissue-specific deletion of some autophagic genes precipitates aging and aging-associated phenotypes in mice (Vilchez *et al*., 2014). In agreement with this, interventions that aim at stimulating autophagy were shown to increase the lifespans of yeast, nematodes, flies, and mice (Zhang and Cuervo, 2008; Harrison et al., 2009; Wilkinson *et al*., 2012; Bjedov *et al*., 2010;

Eisenberg *et al*., 2009; Matsumoto *et al*., 2011; Soda *et al*., 2009; O'Rourke and Ruvkun, 2013). Likewise, an increase in the expression of various components of the UPS confers resistance to protein toxicity (proteotoxicity) and extends longevity in *C. elegans* (Liu *et al*., 2011; Vilchez *et al*., 2012; Chondrogianni *et al*., 2015). Kruegel *et al*. further showed that the enhancement of the UPS capacity results in improved proteotoxic stress response and increased replicative lifespan in yeast, while reduced UPS capacity has opposing consequences (Kruegel *et al*., 2011).

The correlation between aging and the loss of proteome integrity is further strengthened by the ability of several aging-regulating pathways to modulate some, when not all, elements of the proteostasis machinery in order to extend healthspan (see sections 1.1.1. and 1.1.1.2.) (Friedman and Johnson, 1988; Kenyon *et al*., 1993; Hsin and Kenyon, 1999; Dillin *et al*., 2002; Feng *et al*., 2011; Vellai *et al*., 2003; McCay *et al*., 1989).

1.3.2. Manipulating aging: what can we expect at the proteostasis level?

For more than a decade, the IIS pathway has been seen as the major candidate linking aging, proteotoxicity, and late-onset neurodegenerative diseases (Cohen and Dillin, 2008). One of the first studies that disclosed this was carried out by Morley *et al*., which created several transgenic *C. elegans* lines expressing in their body-wall muscles polyQ repeats of different lengths ranging from polyQ0 to polyQ82, all fused to the yellow fluorescent protein (polyQ0-82-YFP), to address the underlying principles of polyQ-mediated aggregation and toxicity (Morley *et al*., 2002). Using this approach, they showed that an expansion of 30-40 glutamines is associated to the appearance of protein aggregates and loss of motility, and that this is exacerbated with aging. The dynamic nature of polyQ aggregation and cytotoxicity throughout the lifetime led the authors to hypothesize that this might mirror not only the repeat length but also changes in the cellular protein-folding environment over time. To test this premise, they generated animals expressing different toxic polyQ lengths on the background of the *age-1(hx56)* mutation or *age-1* RNAi knockdown and found that these animals display reduced aggregate accumulation over time, as well as a delay in the onset of motility defects. This protective effect was shown to rely on the activity of DAF-16 (Morley *et al*., 2002). Using polyQ40-YFP-expressing animals, Hsu *et*

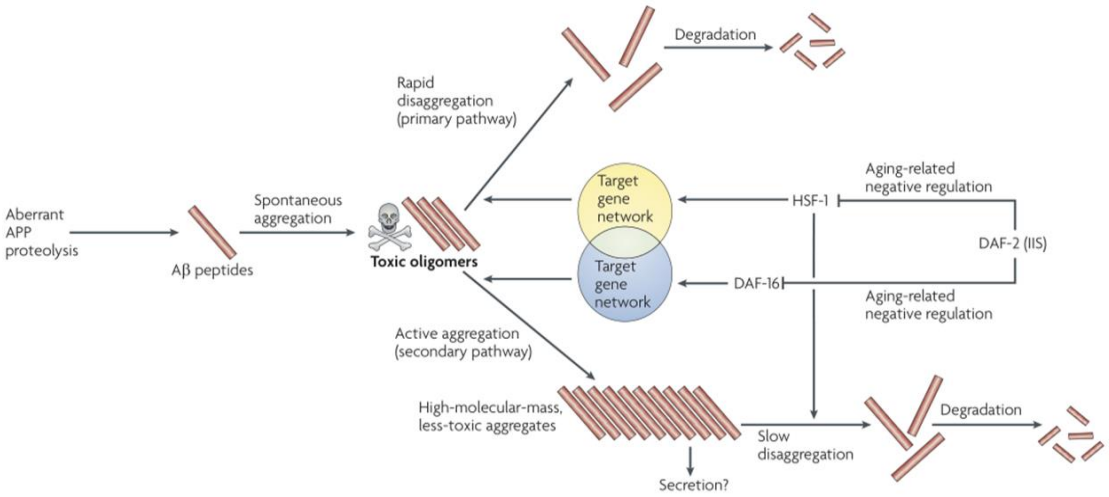


Figure 3. The IIS pathway links aging and proteotoxicity. The serial digestion of amyloid precursor protein (APP) gives rise to aggregation-prone amyloid-β (Aβ) peptides, which spontaneously form small toxic oligomers. These Aβ aggregates can be disrupted by a heat-shock factor 1 (HSF-1)-regulated disaggregation machinery, which prepares them for degradation. When this pathway is overloaded, a secondary pathway that involves active aggregation and is controlled by DAF-16 generates high-molecular-mass Aβ aggregates of lower toxicity that might subsequently undergo slow disaggregation and degradation or, alternatively, be secreted from the cell. The insulin/insulin-like growth factor 1 signaling (IIS) pathway negatively regulates HSF-1 and DAF-16, thereby compromising the activity of both protective mechanisms in an age-dependent manner (from Cohen and Dillin, 2008).

al. showed that, in addition to DAF-16, HSF-1 is important to delay the onset of polyglutamine aggregates (Hse *et al*., 2003).

Later work by Cohen *et al*. investigated the role of the aging process in the toxicity mediated by the aggregation of the AD-associated Amyloid β (Aβ) in worms expressing the human $A\beta_{3-42}$ minigene driven by the *unc-54* promoter (Cohen *et al*., 2006). These worms express the peptide in their body-wall muscles, which results in a time-dependent paralysis within the worm population (Link *et al*., 1995; McColl *et al*., 2009). By knocking down *daf-2* in these animals, thereby reducing the IIS pathway, the authors succeeded to delay the emergence of paralysis and thus, not only to show that the IIS has a broader effect in counteracting proteotoxicity, but to strengthen the idea that the toxicity deriving from aggregative proteins is not stochastic but rather highly dependent on the aging process (Cohen *et al*., 2006). This work

further showed that both HSF-1 and DAF-16 are required for the protective effect of reduced IIS against $A\beta_{3-42}$-mediated toxicity through opposing mechanisms: the HSF-1-mediated disaggregation, believed to be the preferred pathway and to precede degradation; and the DAF-16-mediated formation of less toxic high-molecular weight aggregates. The DAF-16-regulated mechanism is probably secondary but continuously assisting the HSF-1 controlled activities due to the constant overload of the latter (Fig. 3). The suppression on $A\beta_{3-42}$-induced paralysis by *daf-2* knockdown was confirmed soon after in a different *C. elegans* strain expressing the gene in the body-wall muscles under the weaker promoter *myo-3* (Florez-McClure *et al*., 2007). More importantly, this work provided an additional insight into the mechanism underlying this effect by demonstrating that it further relies on the promotion of the autophagic clearance of $A\beta_{3-42}$. The proteasome seems to be as well of great relevance, as decreased IIS was shown to increase proteasome activity in *C. elegans* in a DAF-16-dependent and cell-type-specific manner (Matilainen *et al*., 2013).

Many other studies established the reduction of the IIS pathway as powerful means to increase invertebrates' resistance to the toxicity deriving from different disease-linked proteins expressed in different tissues (Oh and Kim, 2013; Scerbak *et al*., 2014; Knight *et al*., 2014). Of foremost importance is the conservation of this effect in mammals, observed in three parallel studies (Cohen *et al*., 2009; Freude *et al*., 2009; Killick *et al*., 2009). Cohen *et al*. took advantage of two existing animal mouse models (Cohen *et al*., 2009): mice that possess a single copy of the *Igf1r* gene (*Igf1r*$^{+/-}$), the mammalian orthologue of *daf-2* (Kimura *et al*., 1997), which have reduced signaling downstream of the IGF1 receptor, are long-lived, and resistant to oxidative stress (Holzenberger *et al*., 2003); and an AD mouse model that expresses two AD-linked mutated genes and that, as a consequence, express human Aβ, exhibit amyloid plaque formation in the brain, and slow, progressive age-onset AD-like symptoms such as behavioral impairments (Jankowsky *et al*., 2004; Jankowsky *et al*., 2007; Reiserer *et al*., 2007). These animals were crossed to obtain an AD mouse model with only one *Igf1r* copy, which displayed reduced behavioral impairments, as well as lower levels of inflammation and neuronal loss, while presenting more densely packed amyloid plaques and less SDS-soluble aggregates compared to

their AD counterparts with a normal IIS pathway (Cohen *et al*., 2009). Both studies of Freude *et al*. and Killick *et al*. obtained similar results, regardless of having crossed distinct but well characterized animal models of AD and reduced IIS, reinforcing the protective role of IIS suppression towards Aβ toxicity expression in mice brains (Freude *et al*., 2009; Killick *et al*., 2009).

1.3.3. The alteration of aging is associated with elevated stress resistance

The above data concerning the (in)ability of the organisms to maintain the integrity of the proteome clearly demonstrates that aging is a genetically regulated process amenable to manipulation. The alteration of aging has, however, a much broader effect. In fact, the exposure of organisms with altered aging programs to environmental insults made evident that slowing aging robustly elevates stress resistance. IIS reduction confers protection from various stress conditions including oxidation (Honda and Honda, 1999), temperature elevation (Lithgow *et al*., 1995), ultraviolet radiation (Murakami and Johnson, 1996), and exposure to pathogenic bacteria (Singh and Aballay, 2006 – a; Singh and Aballay, 2006 – b) or toxic heavy metals (Barsyte *et al*., 2001). Similarly, DR promotes resistance to various stress conditions (Mair and Dillin, 2008).

A detailed analysis showed that worms harboring a mutated, weak *daf-2* allele and animals subjected to a DR regimen (Panowski and Dillin, 2009) exhibit elevated expression levels of genes encoding protective proteins, including members of the superoxide dismutase (SOD) family of ROS-detoxifying enzymes. IIS reduction also induces the expression of genes that encode molecular chaperones of the Hsp subset (Hsu *et al*., 2003; Morimoto, 1998; Carvalhal Marques *et al*., 2015). Actually, both HSR and UPR^{ER} are regulated by the IIS pathway and are required for the longevity mechanism downstream of this pathway (Henis-Korenblit *et al*., 2010; Morley and Morimoto, 2004).

These observations are in accordance with a role of free radicals (although accumulated data from the last years strongly challenge this idea (Hekimi *et al*., 2011) and other harmful agents in causing aging-dependent damages, as previously discussed, and with the existence of a genetically programmed inactivation or decline of the stress resistance mechanisms that, thus,

fails to prevent or reduce the rate of damage accumulation. This brings up another important question: are stress resistance, proteostasis, and aging regulated in a cell-autonomous fashion or instead coordinated at the organismal level?

1.4. The old paradigm changes: regulation of aging at the organismal level

The ability of unicellular organisms and of cultured cells to induce the HSR, as well as the apparent intracellular nature of the UPR^{ER} and UPR^{mt} signaling pathways, suggested that these stress response mechanisms are regulated at a cell-autonomous level. This notion has, however, been challenged by accumulating evidence, mainly from the nematode *C. elegans*, indicating that the activation of stress response mechanisms, the maintenance of proteostasis, lifespan determination, and, consequently, the regulation of aging are coordinated at the organismal level by inter-tissue communication (Fig. 4).

1.4.1. Neuronal regulation of aging

The pivotal role of thermosensory neurons in heat sensing (Mori and Oshima, 1995) raised the prospect that in *C. elegans* the activity of these cells controls HSR activation in somatic tissues. To test this hypothesis, Prahlad *et al*. employed worms that carry a mutated *gcy-8*, a receptor-type guanylyl cyclase-encoding gene that is exclusively expressed in the AFD thermosensory neurons (Inada *et al*., 2006), and express the green fluorescent protein (GFP) under the regulation of the *hsp-70* promoter (Prahlad *et al*., 2008). They found that the inactivation of these neuronal cells abolishes the ability of the animals to induce the HSR in non-neuronal tissues. Similar results were obtained when the activity of the AFD partners, the AIY interneurons, was impaired by the knockdown of *ttx-3*, a gene that encodes an AIY-specific LIM homeodomain protein. This study showed for the first time that neurons coordinate the nematode's HSR and control the expression of Hsps in remote tissues. A follow-up study demonstrated that this communication requires serotonergic signaling (Tatum *et al*., 2015).

Neurons also regulate the innate immune response, an IIS-controlled stress-response mechanism (Singh and Aballay, 2006 – a; Singh and Aballay, 2006 – b) that enables worms to

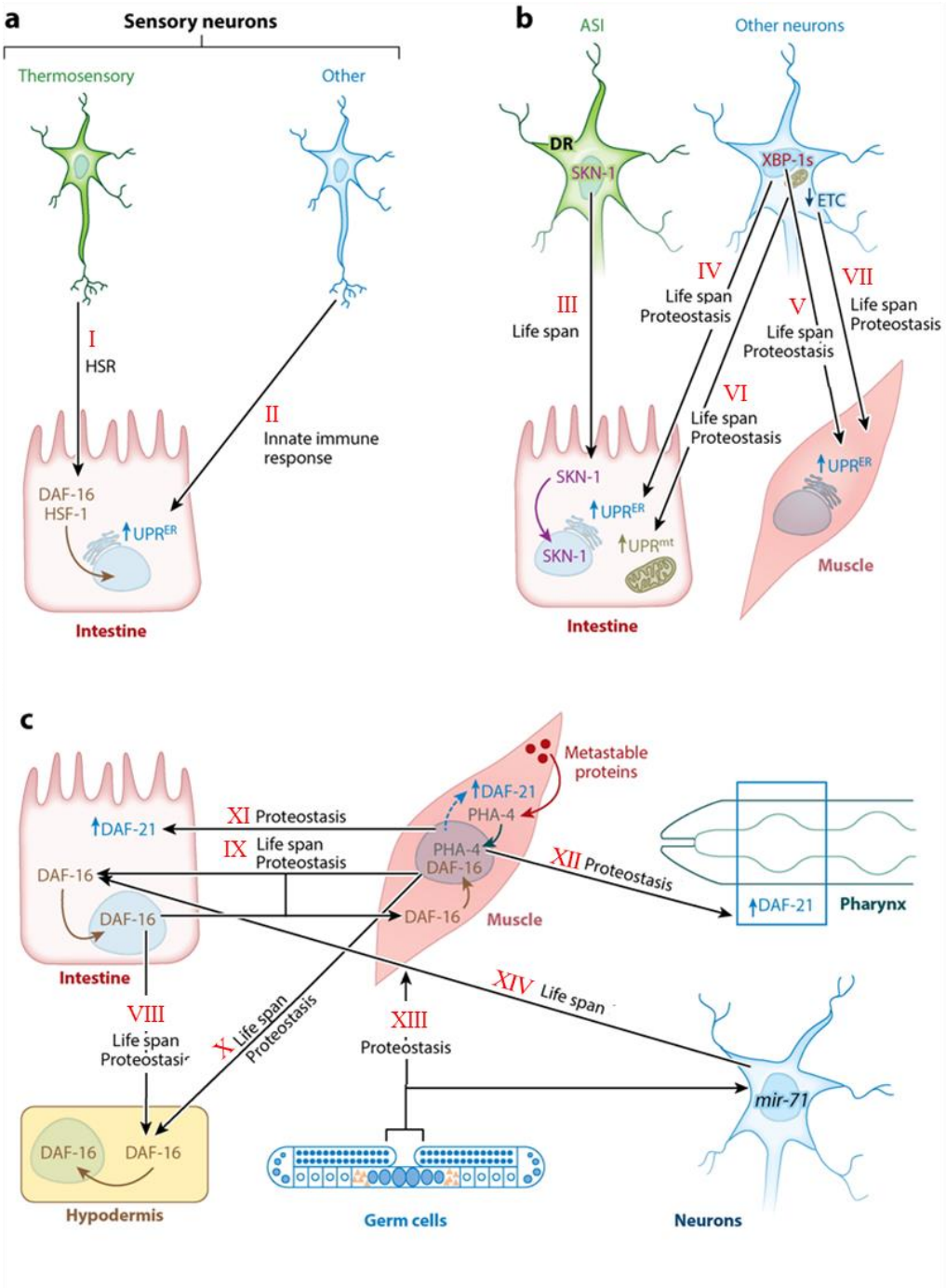


Figure 4. Inter-tissue communication regulates stress resistance, proteostasis, and life span. All three mechanisms that respond to protein misfolding – the heat shock response (HSR) and the endoplasmic reticulum (UPR^{ER}) and mitochondrial (UPR^{mt}) unfolded protein responses – are regulated in a cell non-autonomous fashion. (a) In *Caenorhabditis elegans*, thermosensory neurons (I) regulate HSR activation in remote tissues, including the intestine. Neurons also induce the nematode's innate immune response by activating the UPR^{ER} in intestinal cells (II). (b) Dietary restriction (DR)-mediated SKN-1/Nrf activation in ASI neurons increases respiration in remote tissues, extends life span, and preserves proteostasis (III). The expression of a constantly active XBP-1 (XBP-1s) in neurons activates the UPR^{ER} in intestinal cells (IV) and muscle cells

exhibit relatively extended survival while feeding on pathogenic bacteria. The knockdown of the neuronal G protein-coupled receptor (GPCR)-encoding gene *octr-1* extends the lifespan of worms fed on *Pseudomonas aeruginosa* (*P. aeruginosa*) by activating a DAF-16-independent, non-canonical UPR^{ER} mechanism in the intestine (Sun *et al.*, 2011). The surprising discovery that the UPR^{ER} is controlled by sensory neurons suggested that this stress-response mechanism may regulate lifespan and proteostasis in an inter-tissue fashion. In an attempt to reverse the loss of ER proteostasis that occurs with aging by inducing the UPR^{ER} in *C. elegans*, Taylor *et al*. found that the expression of a constitutively active *xbp-1*, a key UPR^{ER}-promoting transcription factor, can rescue the age-onset loss in UPR^{ER} function (Taylor and Dillin, 2013). Remarkably, if expressed in neurons, but not in other tissues, the *xbp-1* active form can initiate a cell non-autonomous, unidirectional response that requires neurotransmitters release from small clear vesicles (SCVs) and results in UPR^{ER} activation in the intestine and muscles, promoting ER stress resistance and substantially extending lifespan.

Figure 4. (continuation) (V), confers longevity, and enhances proteostasis. The mitigation of electron transport chain (ETC) activity in neurons activates the UPR^{mt} in the intestine (VI), extends life span, and enhances proteostasis in both intestinal and muscle cells (VII). (c) Non-neuronal tissues communicate to orchestrate the activation of stress response mechanisms. DAF-16 functions in both a cell-autonomous and a cell non-autonomous manner. The activation of this transcription factor in intestinal cells activates DAF-16-mediated transcription in the hypodermis (VIII) and muscles (IX), whereas its activation in muscle cells induces the expression of DAF-16 target genes in the intestine (IX) and hypodermis (X). Impairment of proteostasis in the body wall muscle cells by aggregation-prone proteins induces the expression of *daf-21*, which encodes the *C. elegans* HSP90 chaperone, in a PHA-4-dependent manner. This induction was observed not only in the muscles but also in the intestine (XI) and pharynx (XII), tissues that do not express these aggregative proteins. Signals that originate from the reproductive system regulate proteostasis during adulthood in muscles (XIII), as well as life span, by inducing the activation of DAF-16 in intestinal cells. Although most studies point to direct communication between the gonads and the somatic tissues, a recent study showed that the life span extension effect mediated by the activation of DAF-16 in the intestine requires the expression of *mir-71* in neurons (XIV) (adapted from Carvalhal Marques *et al.*, 2015).

Similarly to the responses to acute stresses and lifespan, neurons can also regulate proteostasis non-autonomously. UNC-30, a neuron-specific transcription factor that regulates GABA signaling, was identified in a forward genetic screen as a modulator of proteotoxicity in muscles (Garcia *et al*., 2007). Neuronal signaling seems, therefore, to be an important modulator of protein homeostasis in the post-synaptic muscle cells of *C. elegans*. These results are supported by a later study demonstrating in animals expressing either polyQ or the Amyotrophic Lateral Sclerosis (ALS)-associated mutant SOD-1^{G93A} in intestinal or muscle cells that the thermosensory neurons AFD indeed control the cellular response to these misfolded proteins through the release of calcium-activated dense core vesicles (DCV) (Prahlad and Morimoto, 2011).

In another recent study, Durieux *et al*., showed that by tissue-specific knockdown of the nuclear-encoded cytochrome c oxidase-1 subunit Vb/COX4 (*cco-1*), an ETC component, worms' lifespan extension occurs upon loss of mitochondrial function in the intestine and neurons of *C. elegans* but not in muscles (Durieux *et al*., 2011). Taking into account the role of UPR^{mt} upon different forms of mitochondrial stress (Yoneda *et al*., 2004), the authors investigated its involvement in the ETC-mediated longevity. The UPR^{mt} not only showed to be essential for this effect – this statement was later refuted by another group (Bennett *et al*., 2014) – but, surprisingly, activated in the intestine mainly in a cell non-autonomous manner that is dependent on unknown signals from the nervous system.

These studies have established neuronal signaling cascades as critical regulators of the nematode's lifespan, stress resistance, and proteostasis. Nevertheless, non-neuronal inter-tissue communication mechanisms also appear to play crucial roles in the orchestration of these functions.

1.4.2. Neuron-independent regulation of aging

The first evidence indicating that endocrine signals function to coordinate the aging program of *C. elegans* originated from studies of the IIS pathway. Using mosaic analysis in worms, it was shown that the loss of *daf-2* in only a subset of neurons results in prolonged lifespan and that the restoration of wild type *daf-2* and, thus, of the insulin signaling, in these neurons

completely abolishes the worms' long-lived phenotype (Apfeld and Kenyon, 1998). Consistently with this study, it was shown that expression of *age-1* in neurons or in the intestine rescues the longevity phenotype of *age-1* mutant animals, shortening an otherwise long lifespan back to wild type levels (Wolkow *et al*., 2000; Iser *et al*., 2007). A later study brought, however, new insights into the regulation of aging. To address the question of where does DAF-16 act to coordinate lifespan, Libina et al. created worms that bear a weak *daf-2* allele and an inactive *daf-16* and, thus, exhibit a short lifespan (Libina *et al*., 2003). The authors restored the expression of DAF-16 in these animals in a tissue-specific manner and found that this transcription factor is primarily important as a lifespan regulator in the intestine. Later, DAF-16 was found to exhibit both cell-autonomous and cell non-autonomous regulation of gene expression (Alic *et al*., 2014; Zhang *et al*., 2013). The expression of DAF-16 in intestinal cells elevates the expression of two of its target genes, *dod-11* and *hsp-12.6* (Murphy *et al*., 2003), not only in the intestine but also in muscles and in the hypodermis. Similarly, the activation of DAF-16 in muscle cells affects the expression of its target genes in the intestine and hypodermis, implying that these tissues communicate to coordinate gene expression. Zhang *et al*. further showed that, in addition to lifespan, DAF-16 acts at a distance, namely in the intestines, to protect animals from amyloid-mediated paralysis (Zhang *et al*., 2013). These results, as well as others obtained in *D. melanogaster* (Hwangbo *et al*., 2004; Giannakou *et al*., 2004), imply the requirement of endocrine longevity signals from both the nervous system and the adipose tissue, which is represented by the intestine in worms, to other tissues to allow the IIS-dependent regulation of the whole animal's aging and lifespan.

Another independent mechanism found to be aging and lifespan regulator in *C. elegans* and *D. melanogaster* depends of the reproductive system. In both organisms, the removal of the germline precursor cells, the cells that give rise to sperm and oocytes, influence the adult tissues of the whole animal, leading not only to a 40-60% increase in lifespan but also rendering them youthful and with highly functional protein quality control networks in the somatic cells (Hsin and Kenyon, 1999; Arantes-Oliveira *et al*., 2002; Broué *et al*., 2007; Flatt *et al*., 2008; Shemesh *et al*., 2013). This phenotype depends mainly on the non-autonomous regulation of the nuclear hormone receptor DAF-12, the homologue of P450, DAF-9, the oxygenase DAF-36 (both thought

to be required for the synthesis and/or modification of DAF-12 ligands), the putative transcription elongation factor KRI-1 (Russel and Kahn, 2007; Panowski and Dillin, 2009), as well as on the activation of DAF-16 in somatic tissues, especially in the intestine, whereupon it regulates the transcription of its lifespan-extending target genes (Lin *et al*., 2001; Libina *et al*., 2003). To ascertain whether signaling that originates from the reproductive system affects proteostasis, Shemesh *et al*. compared the expression levels of Hsps in 2-day-old heat-stressed nematodes harboring either functional or impaired germ cells and found that, unlike those in the control group, worms of the germ line–impaired group maintained the ability to induce the expression of Hsps during late stages of life (Shemesh *et al*., 2013). They further followed the aggregation states of metastable proteins during the worms' life cycle and confirmed that germ line impairment results in enhanced proteostasis in somatic tissues.

How the reproductive system and the somatic tissues communicate is nonetheless far from being understood. Although specific signals are exchanged directly between the gonad and distal tissues (Berman and Kenyon, 2006), neurons seem to coordinate this communication, at least in part, through microRNA-71 (*mir-71*), which acts within neurons to enable germ cell removal to extend lifespan by activating DAF-16 in the intestine (Boulias and Horvitz, 2012).

An additional important insight into the neuron-independent maintenance of proteostasis was provided by the finding that the expression of metastable proteins in muscle cells of worms activates the FoxA transcription factor PHA-4, which in turn elevates the expression of Hsp-90 (encoded by *daf-21* in *C. elegans*) in these cells. Curiously, *daf-21*'s expression was also observed in other tissues that do not express these aggregation-prone proteins (*e.g.* intestine and pharynx) (van Oosten-Hawle *et al*., 2013). The authors further show that suppression of misfolding of metastable muscle proteins can be achieved by artificially enhancing the expression of DAF-21 not only in muscle cells but also in intestinal and neuronal cells. Nonetheless, the cell non-autonomous regulation of *daf-21* seems to be independent of neuronal signaling and rather rely on direct communication between somatic tissues.

Collectively, these studies indicate that the regulation of stress resistance, proteostasis, and lifespan, known to require communication between subcellular compartments, is also

orchestrated at the organismal level by neuron-dependent and neuron-independent mechanisms (Fig. 4) and raise principal questions: what is the meaning of such mechanisms from an evolutionary point of view? How is this control performed in higher animals such as mammals? What might be the impact of such complexity in the development and applicability of therapeutic strategies?

1.5. The scope of this work

This work aimed at exploring the principals of inter-tissue communication and assessing the mutual links between the determination of lifespan, stress resistance, and proteostasis. It can be divided into two main parts. One concerns the fundamental knowledge of how stress responses are coordinated at the organismal level and addresses the recent discoveries on the neuronal regulation of these responses (Prahlad *et al*., 2008). We performed an RNAi screen and identified a putative, neuronal GPCR, which we named GTR-1, as being involved in the resistance to heat stress. Following its identification, our main aims were to analyze *gtr-1*'s expression pattern and determine the mechanisms underlying its involvement in stress resistance, assess GTR-1's role in other stress resistance pathways and lifespan, and determine its involvement in counteracting proteotoxicity.

The second part of this study stands on the emerging optimism towards aging manipulation, particularly by interfering with the IIS pathway, as a feasible approach to postpone or even prevent the emergence of conformational disorders, including the highly prevalent, late-onset neurodegenerative diseases. In fact, our lab recently reported that NT219, a novel IIS inhibitor, promotes stress resistance and protects nematodes from AD- and HD-associated proteotoxicity (El-Ami *et al*., 2014). The transposition of such data "from bench to bedside" is most probably far from being accomplished. However, a deep knowledge of the mechanistic details that underlie the IIS pathway is required in order to provide potential therapeutic approaches with means to address intrinsic difficulties of application. With this in mind, we attempted at better characterizing the IIS-downstream components involved in the direct regulation of proteotoxicity in the *C. elegans* model. For this, we searched for genes that are

known regulators of proteostasis in order to identify new effectors that are regulated by the IIS pathway. In this screen, we identified *tor-2* as a transcriptional target downstream of *daf-2*, which we show to be upregulated upon suppression of the IIS. In this project we aimed to unravel which transcription factor regulates *tor-2*'s expression, evaluate the role of the endogenous TOR-2 in the regulation of proteostasis in different tissues and the mechanisms underlying this regulation, as well as investigate TOR-2's involvement in the IIS-regulated stress pathways, lifespan, and developmental functions.

Chapter 2

RESULTS

Chapter 2

RESULTS

2.1. A neuronal GPCR is critical for the induction of the HSR in the nematode *C. elegans*

2.1.1. *gtr-1* is required for the survival of worms at elevated temperatures

We attempted to identify receptors that are expressed in neurons and are required for survival in elevated temperatures. The limited penetrance of dsRNA into neurons of *C. elegans* (Timmons *et al*., 2001) has notably restricted the efficiency of feeding RNAi bacteria as a screening technique for the identification of genes encoding products that are required for neuronal function. This technical obstacle has been, however, recently resolved by the creation of a worm strain that expresses the transmembrane protein SID-1, crucial for the uptake of dsRNA by the nematode's cells (Winston *et al*., 2002), under the regulation of the pan-neural *unc-119* promoter (strain TU3335). This expression not only enables an efficient gene-specific knockdown within neurons but also reduces the RNAi-mediated gene knockdown in other tissues (Calixto *et al*., 2010). As such, for our RNAi screen, we employed the TU3335 worms and found that animals that were fed bacteria expressing dsRNA towards *gtr-1* (encoded by F25E2.1) exhibited significantly reduced viability over time when exposed to 35°C (Fig. 5A). Four independent experiments indicated that, whereas the average survival rates of control worm populations that were fed with bacteria harboring an empty vector (EV) was of approximately 42%, only 15% of the *gtr-1* RNAi-treated worms were alive after 10 hours of exposure to heat (Fig. 5B). This survival rate was comparable to that of worms that were fed bacteria expressing dsRNA towards the neuronal *gcy-8*, previously shown to be required for HSR activation (Prahlad *et al*, 2008).

We then tested whether *gtr-1* is also required for the survival of wild type worms (strain N2) in elevated temperature. N2 worms that were treated with either *gtr-1* or *daf-16* RNAi and exposed to heat as described above exhibited similarly reduced survival rates over time compared to their untreated counterparts (EV) (Fig. 5C). These results were confirmed by three independent experiments in which the survival rate was examined after 12 h of exposure to heat (Fig. 5D).

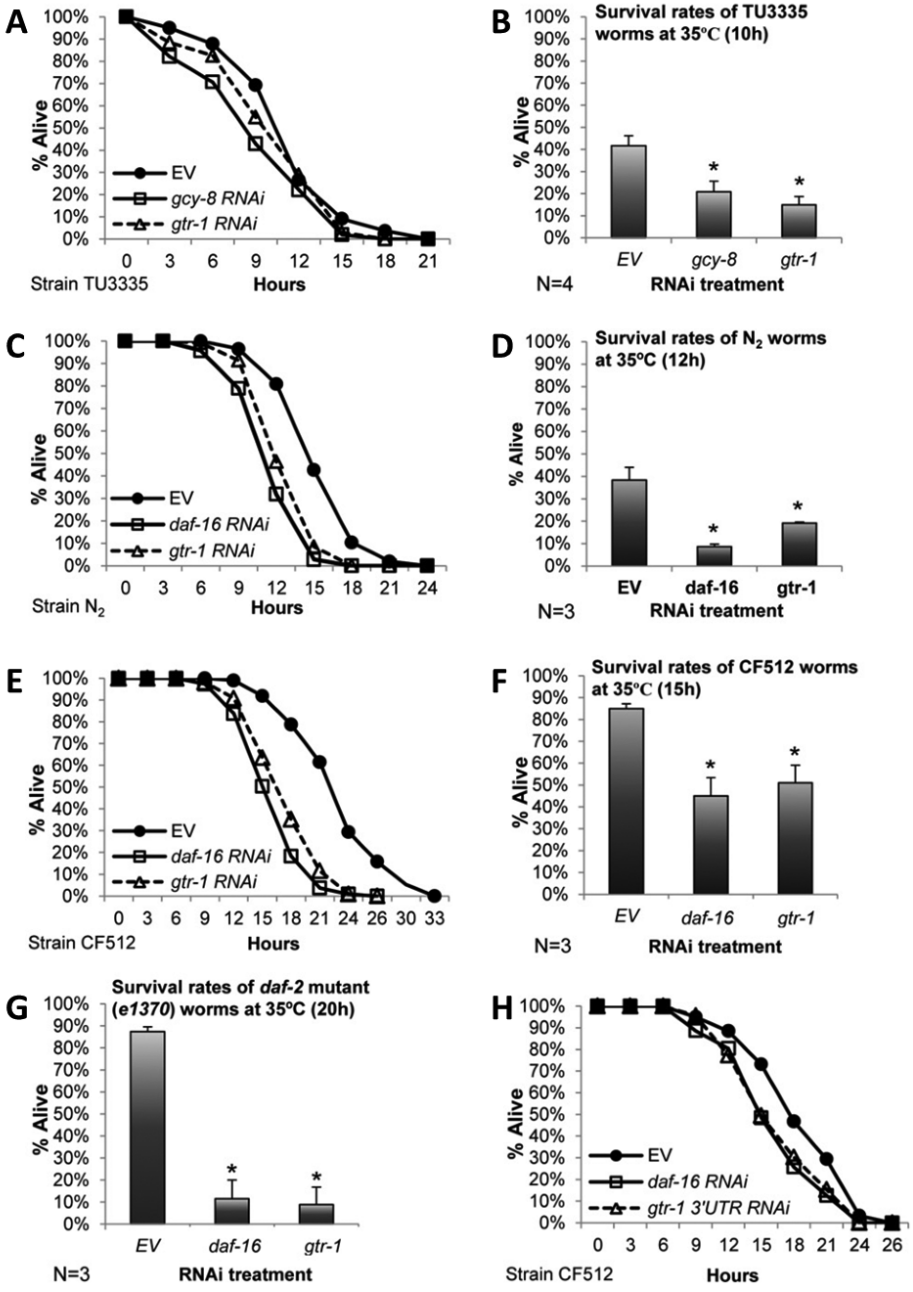


Figure 5. Knockdown of *gtr-1* sensitizes worms to heat stress. [A] Worms expressing *sid-1* under the regulation of the pan-neuronal *unc-119* promoter (strain TU3335) were treated with RNAi towards either *gcy-8* or *gtr-1* or left untreated (EV), exposed to 35°C, and their survival rates were recorded in 3 hours intervals. Much like those treated with *gcy-8* RNAi, *gtr-1* RNAi-treated animals exhibited reduced survival rates compared with their untreated counterparts. **[B]** Four independent experiments in which the RNAi-

Likewise, temperature-sensitive sterile worms (strain CF512) that were treated with either *gtr-1* or *daf-16* RNAi exhibited reduced heat stress resistance over time compared with the untreated (EV) animals (Fig. 5E), as well as in three independent experiments in which worms from the

same strain were treated with RNAi as described above and exposed to heat for 15 h (Fig. 5F). CF512 worms were exposed to 25°C during development to prevent them from having progeny, a mild treatment that was, nonetheless, shown to not activate the HSR (Volovik *et al*., 2012). In sum, these results point at *gtr-1* as being critical for the survival of three different worm strains upon heat shock.

The elevated heat stress resistance of *daf-2* mutants (Lithgow *et al*., 1995) led us to investigate whether this phenotype is dependent on the activity of *gtr-1*. For this, we used *daf-2*(*e1370*) mutant worms harboring a weak *daf-2* allele that leads to low activity of the tyrosine kinase domain of DAF-2. When grown at normal temperature, these worms are long-lived and highly stress-resistant (Kenyon *et al*., 1993). The worms were either fed control bacteria (EV) or treated with RNAi towards either *daf-16* or *gtr-1* throughout development, exposed to 35°C at day 1 of adulthood, and their survival rates were analyzed after 20 hours of exposure to heat. Three independent experiments revealed that, whereas ~85% of the untreated *daf-2*(*e1370*) mutant animals were alive, only 10% of both *gtr-1* and *daf-16* RNAi-treated worms survived after the heat insult (Fig. 5G). This further indicates that *gtr-1* is also critically required for the increased resistance of *daf-2* mutant animals to thermal stress.

To further establish our observations and to verify the specificity of the RNAi-mediated knockdown of *gtr-1*, we created an RNAi construct that is directed towards the 3′UTR of *gtr-1*.

Figure 5. (continuation) treated TU3335 worms survival rates at 35°C were recorded after a 10-hour exposure confirmed the results. **[C]** *gtr-1* RNAi-treated wild type worms (strain N2) and **[E]** CF512 animals exhibited significantly reduced survival rates at 35°C compared with their untreated counterparts (EV). Three independent single-time-point survival assays with each strain (12 and 15 hours of exposure to 35°C for N2 **[D]** and CF512 **[F]** worms, respectively) showed that *daf-16* and *gtr-1* RNAi-treated animals exhibited similarly reduced lifespan after exposure to heat compared with untreated animals. **[G]** Similarly to *daf-16*, the knockdown of *gtr-1* by RNAi abolished the elevated stress resistance of *daf-2*(*e1370*) mutant worms that were exposed to heat (35°C) for 20 hours. In **B**, **D**, **F**, and **G**, bars represent mean survival ± SEM; * $p_{value} < 0.01$. The statistical significance of the results was performed using the Student's t-test. **[H]** CF512 worms that were treated with RNAi towards the 3′UTR region of *gtr-1* and exposed to 35°C exhibited significantly reduced survival rates compared with the control group (EV).

Using CF512 worms, we found that knocking down *gtr-1* by the new RNAi construct led to an identical reduced-survival effect after exposure to 35°C observed when *gtr-1* was knocked down by the library's RNAi strain or upon *daf-16* knockdown (Fig. 5H).

2.1.2. *gtr-1* is expressed in chemosensory neurons

The fact that *gtr-1* RNAi-treated *daf-2*(*e1370*) mutant, N2, and CF512 worms show reduced heat resistance puzzled us given the low penetrance of RNAi into neurons. Two hypotheses can explain these observations: (1) either *gtr-1* is tightly regulated and thus the relatively low efficiency of RNAi-facilitated *gtr-1* knockdown in neurons is sufficient to sensitize the worms to heat or (2) it executes its heat resistance functions in a non-neural tissue. To distinguish between these two possibilities and to analyze the spatial and temporal expression patterns of *gtr-1*, we created worms that express the red fluorescent protein tdTomato (Shaner *et al*., 2004) under the regulation of the *gtr-1* promoter (3 kb upstream of the *gtr-1* open reading frame, strain EHC101).

To determine in which worm tissues *gtr-1* is expressed, we visualized EHC101 worms using fluorescent microscopy and found that the expression of tdTomato was confined to neurons (Fig. 6A). Prominent expression was observed in the head and tail neurons and along the ventral cord. Some of the head neural cell bodies appeared to be located in close proximity to the pharynx. The key role of *gtr-1* in the worm's resistance to heat and its expression in neurons that are located near the pharynx raised the prospect that, among other neuronal cells, *gtr-1* is expressed in the AFD thermosensory neurons. To examine this hypothesis, we crossed the EHC101 worms with animals that express the GFP under the regulation of the *gcy-8* promoter (strain PY1322) to obtain animals that concurrently express GFP in AFD neurons and tdTomato in *gtr-1*-expressing cells. Confocal microscopy showed distinct expression patterns for GFP and tdTomato, clearly indicating that *gtr-1* is not expressed in AFD neurons (Fig. 6B). Similarly, we crossed EHC101 with animals that express GFP under the regulation of the *ttx-3* promoter (strain OH99) and found that *gtr-1* is not expressed in the AIY interneurons (Fig. 6C).

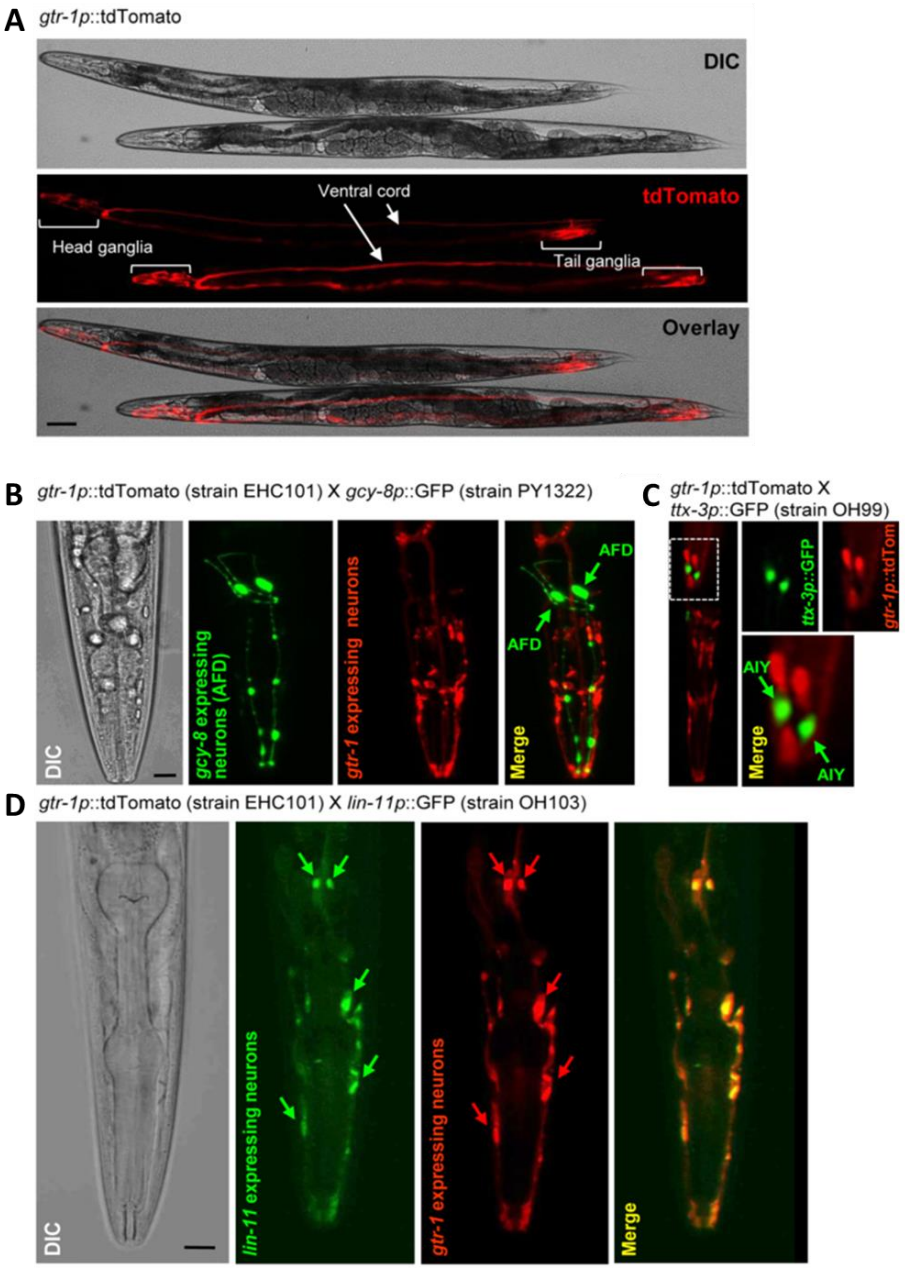


Figure 6. *gtr-1* is expressed in chemosensory neurons. [A] Fluorescent visualization of worms that express tdTomato under the regulation of *gtr-1* promoter (strain EHC101) revealed that *gtr-1* is expressed in neurons of the head ganglia, the ventral cord, and the tail. Scale bar, 50 μm. **[B–C]** *gtr-1* is expressed in neither the AFD thermosensory neurons (labeled by GFP driven by the *gcy-8* promoter, **B**) nor in AIY interneurons (labeled with GFP driven by the *ttx-3* promoter, **C**). **[D]** Visualization of worms that express GFP under the regulation of the chemosensory specific *lin-11* promoter (green channel) and tdTomato driven by the *gtr-1* promoter (red channel) showed colocalization. In **B** and **D**, scale bars, 10 μm.

We then examined whether *gtr-1* is expressed in other neurons of the thermosensory circuit (Ma and Shen, 2012). EHC101 worms were crossed with animals that express GFP under the regulation of the *lin-11* promoter (strain OH103). *lin-11* encodes a LIM homeodomain protein that is expressed in several head and tail neurons and is crucially required for the activity of the thermoregulatory neural network (Hobert *et al*., 1998). Accordingly, OH103 animals express GFP in the head chemosensory neurons ADF and ADL, as well as in the AIZ, AVG, and RIC interneurons (Hobert *et al*., 1998). Confocal visualization of these worms revealed identical expression patterns of GFP and tdTomato, indicating that *lin-11* and *gtr-1* are expressed in the same neuronal cells (Fig. 6D).

2.1.3. *gtr-1* is required for the induction of the HSR mechanisms downstream of both HSF-1 and DAF-16 but not for thermosensation

Neurons of the thermoregulatory circuit exhibit two major functions upon exposure to elevated temperature: heat sensation (Clark *et al*., 2007) and the initiation of signaling that activates the HSR in other tissues (Prahlad *et al*., 2008). With this in mind, we sought to determine in which of these neuronal functions *gtr-1* plays its roles. We started by investigating whether *gtr-1* is essential for heat sensation by performing a worm migration assay on a temperature gradient. In this set of experiments, we used *daf-2*(*e1370*) mutant worms, a strain that exhibited the most prominent reduction in heat resistance upon RNAi-mediated *gtr-1* knockdown. The worms were either grown throughout development on EV bacteria or fed with *gtr-1* RNAi bacteria and placed on temperature gradient plates (6–38°C). The plates were photographed immediately after placing the worms and 12 min thereafter. Both untreated worms (EV) (Fig. 7A, top) and *gtr-1* RNAi-treated animals (Fig. 7A, bottom) rapidly migrated to the central region of the plate where the temperature was similar to their cultivation temperatures (18–20°C). To quantify the worms' migration behavior, we divided each plate to six equal zones along the temperature gradient and counted the worms that were present in each zone at the beginning of the experiment and after 12 min in four independent experiments. Our results (Fig. 7B) confirmed the significance of the indistinguishable thermotactic behaviors of the worm groups. The migration of *gtr-1* RNAi-

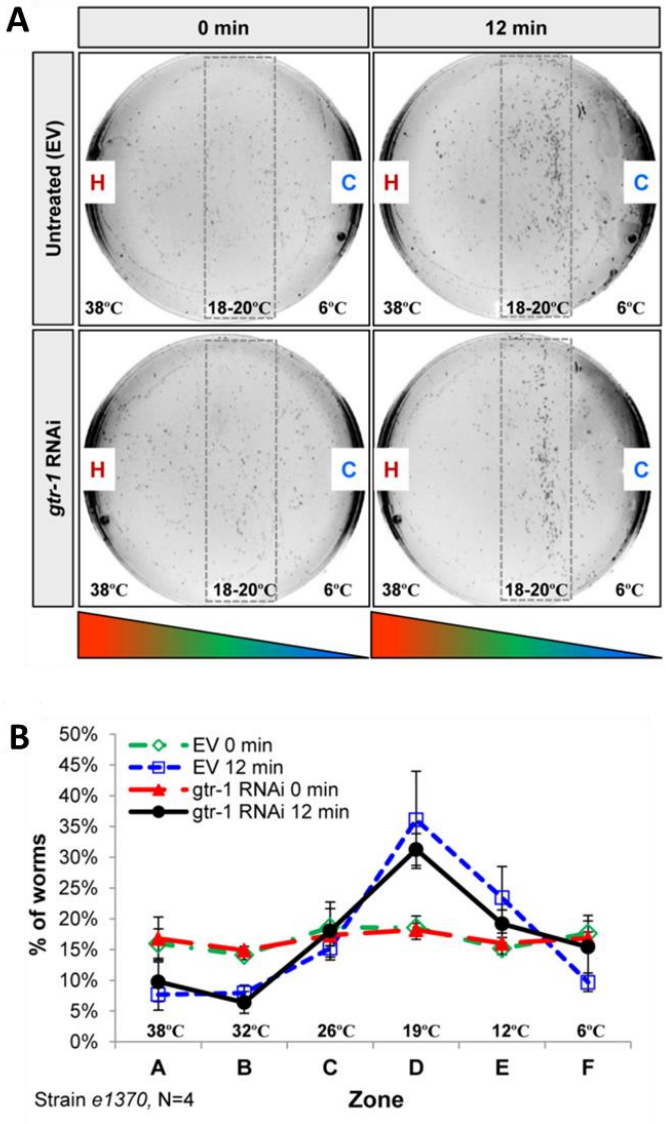
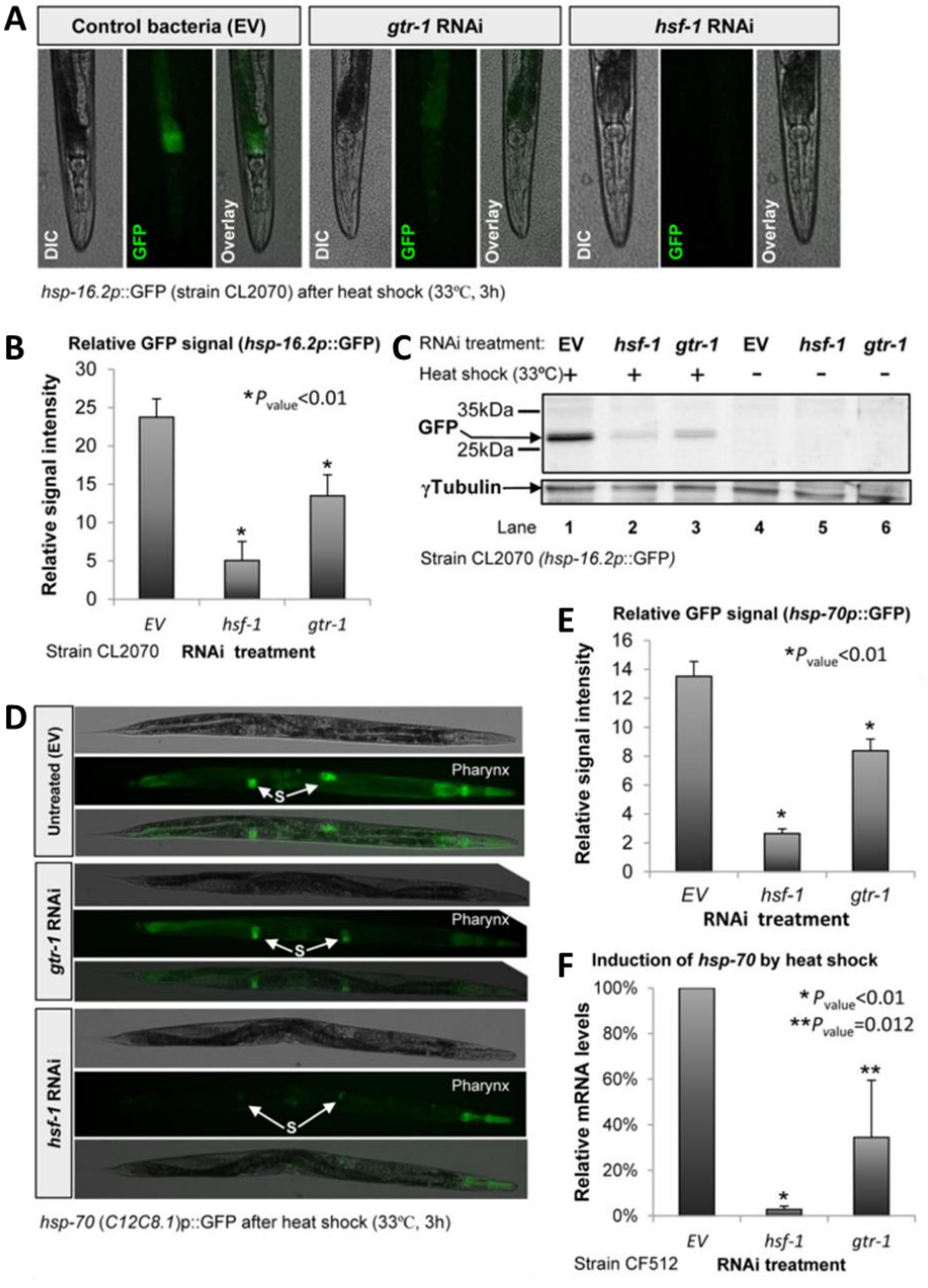


Figure 7. Knockdown of *gtr-1* by RNAi has no effect on heat sensing. [A] *daf-2*(*e1370*) mutant worms were placed on temperature gradient plates. The plates were photographed immediately after placing the worms and 12 min after. Both untreated (EV; top) and *gtr-1* RNAi-treated (bottom) animals migrated away from regions of high (~38°C) and low (~6°C) temperatures to populate the central region of the plates where the temperatures were similar to their cultivation temperature (18–20°C, rectangles). **[B]** Four independent experiments confirmed that untreated and *gtr-1* RNAi-treated worms exhibited indistinguishable thermotactic behavior.

treated worms away from the hot and cold regions indicates that this gene product is dispensable for heat sensation and suggests that it functions in the signaling mechanism that activates the HSR.

To examine the possibility that *gtr-1* is required for the HSR-activating neuronal signaling mechanism, we investigated whether the knockdown of this gene impaired the induction of heat shock proteins in remote tissues upon exposure to high temperature. First, we used worms that express GFP under the regulation of the promoter of *hsp-16.2* (strain CL2070), a well-established HSF-1 target that encodes a small heat shock protein predominantly expressed in the worm's intestine (Link *et al*., 1999). These worms were left untreated (EV) or treated with RNAi towards either *hsf-1* or *gtr-1* from hatching to the first day of adulthood. The worms were exposed



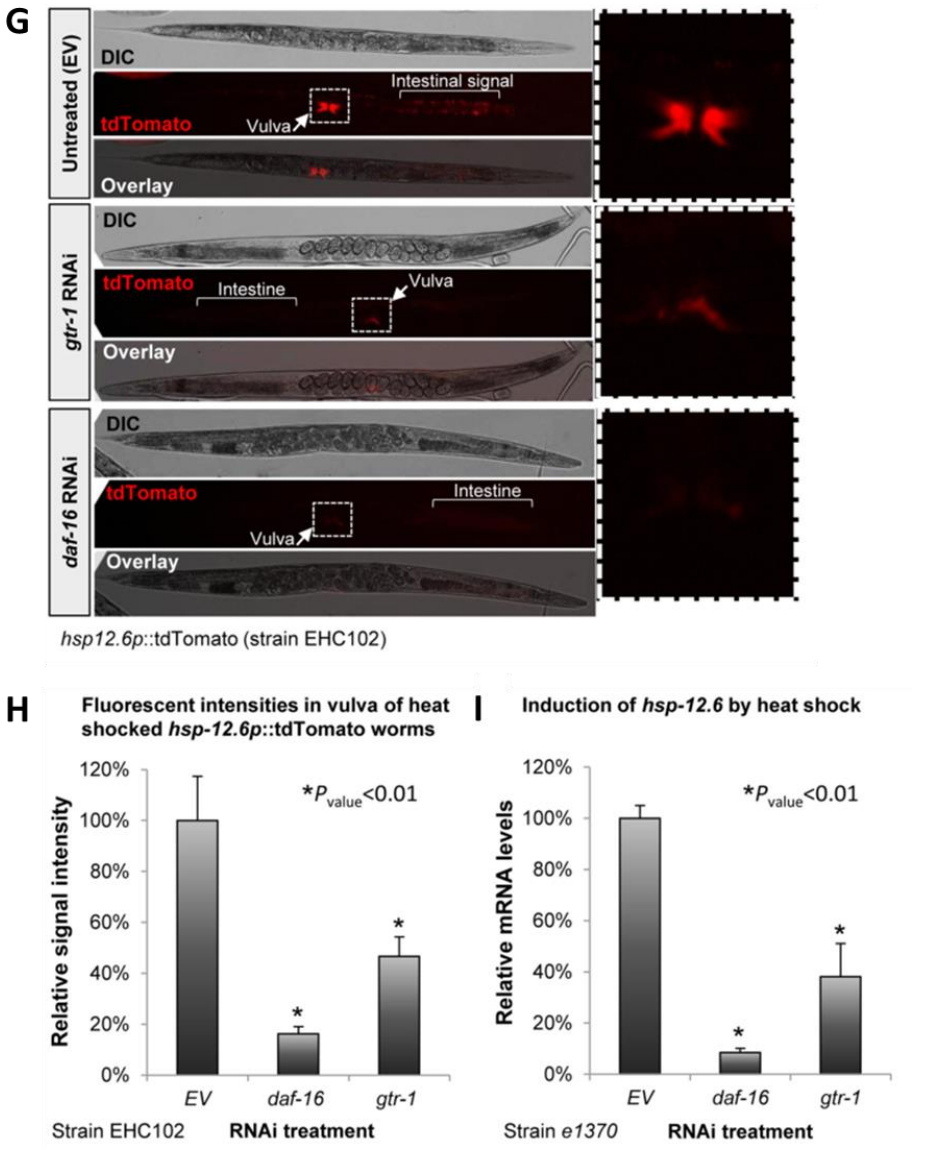


Figure 8. Knockdown of *gtr-1* prevents the induction of the HSR downstream of DAF-16. [A] Worms expressing GFP under the control of the *hsp-16.2* promoter (strain CL2070) that were treated with *gtr-1* RNAi and exposed to heat shock exhibited reduced GFP signal compared with their untreated (EV) counterparts. **[B–C]** Measurement of GFP signal intensities (**B**) and Western blot analysis using GFP antibody (**C**) indicated that knockdown of *gtr-1* resulted in remarkable reduction in the induction of *hsp-16.2* by heat. This effect was significant but less prominent than the effect of *hsf-1* RNAi; * p_{value} < 0.01. **[D–E]** Knockdown of *gtr-1* by RNAi reduces the signal intensity of worms expressing GFP under the regulation of *hsp-70* (*C12C8.1*) promoter, as visualized by fluorescent microscopy. This effect was most prominent in the pharynx (**D**) and less in the spermatheca ("S"). Signal quantification (>20 worms per group; **E**) confirmed the significance of this observation; * p_{value} < 0.01. **(F)** Knockdown of *gtr-1* by RNAi reduced the induction of *hsp-70* (*C12C8.1*) by heat shock as measured by qPCR in CF512 worms; * p_{value} < 0.01, ** p_{value} = 0.012. **[G–H]** Induction level of *hsp-12.6* was significantly reduced by the knockdown of *gtr-1* by RNAi

to heat shock (33°C) for 3 hours and the GFP was visualized by fluorescent microscopy. Although a clear GFP signal was observed in the intestine of untreated animals, *gtr-1* RNAi treatment largely abolished the induction of GFP in the worms (Fig. 8A). Signal quantification (Fig. 8B) confirmed that, despite having a less prominent effect on the expression of GFP than RNAi towards *hsf-1*, the knockdown of *gtr-1* was still significant. To further establish and quantify this phenomenon, we treated the CL2070 animals with RNAi, exposed them to heat, as described above, and homogenized them to perform a Western blot analysis of the GFP levels using a GFP antibody. Our results showed that the knockdown of *gtr-1* by RNAi resulted in a remarkably reduced GFP level compared with that seen in untreated worms (EV) (Fig. 8C). The effect of *gtr-1* RNAi treatment on the activity of the *hsp-16.2* promoter was comparable to that of *hsf-1* RNAi, though not as robust. It is plausible that the prominence of *hsf-1* RNAi in this set of experiments results from the lower efficiency of RNAi-mediated *gtr-1* knockdown in neurons (Timmon *et al*., 2001).

To further investigate the role of *gtr-1* in HSR activation, we examined whether it is required for the induction of the inducible *hsp-70*, a pivotal and well characterized HSR-induced, HSF-1 target gene in *C. elegans* that is encoded by the gene C12C8.1 (Snutch *et al*., 1988). To directly visualize directly the activity of the *hsp-70* promoter, we used worms that express GFP under its regulation (Morley and Morimoto, 2004). For this, we treated worms with the appropriate RNAi, exposed them to heat shock as previously described, and visualized the GFP signal by fluorescent microscopy. Much like the *hsp-16.2* promoter, the activity of the *hsp-70* promoter was largely reduced upon knockdown of *gtr-1*, most prominently in the pharynx (Fig. 8D, E). The key role of *gtr-1* in the induction of this gene was further confirmed by qPCR analysis of the *hsp-70* mRNA levels in CF512 worms exposed to heat shock (33°C) for 3 hours (Fig. 8F).

Figure 8. (continuation) as visualized (**G**) and quantified (**H**) in EHC102 worms that express tdTomato under the regulation of the *hsp-12.6* promoter; * $p_{value} < 0.01$. This effect was most prominent in the vulva (insets). **(I)** qPCR analysis confirmed the necessity of *gtr-1* for the induction of *hsp-12.6* in heat-shocked *daf-2*(*e1370*) worms (error bars represent ± SEM; the statistical significance of the results was performed using the Student's t-test).

We then investigated whether *gtr-1* is also required for the activation of the arm of the HSR mechanism downstream of DAF-16 upon exposure to heat shock. We focused on *hsp-12.6*, a small heat shock protein member of the α-crystalline family (Leroux *et al*., 1997) that is co-regulated by DAF-16 (Murphy *et al*., 2003) and by HSF-1 (Hsu *et al*., 2003). To visually follow the expression of *hsp-12.6*, we created worms that express the red fluorescent protein tdTomato under the regulation or the *hsp-12.6* promoter (2,697 bp upstream of the *hsp-12.6* open reading frame, strain EHC102). EHC102 worms were grown from hatching to day 1 of adulthood on bacteria expressing either *gtr-1* or *daf-16* RNAi or on EV bacteria and exposed to heat shock (33°C) for 3 hours. Similarly to the *daf-16* RNAi treatment, the knockdown of *gtr-1* reduced the tdTomato signal (Fig. 8G) in the vulva (insets) and intestine of the worms, indicative of reduced induction of the *hsp-12.6* promoter by heat shock. Both signal quantification (Fig. 8H) and qPCR analysis (Fig. 8I) revealed that, whereas the knockdown of *daf-16* reduced the induction of *hsp-12.6* by 80–90% when compared to the control counterparts, that of *gtr-1* had a less prominent but still significant effect of 60% reduction.

2.1.4. No role for *gtr-1* in the regulation of lifespan, resistance to pathogenic bacteria, or IIS downstream developmental functions

Lifespan extension is a hallmark of IIS reduction (Kenyon *et al*., 1993). Therefore, we sought to determine whether *gtr-1* plays any role in the determination of lifespan. We started by analyzing whether the knockdown of *gtr-1* has an effect on the lifespan of CF512 worms. The worms were fed with control bacteria (EV) or RNAi bacteria towards either *gtr-1* or *daf-16* throughout development and adulthood and their lifespans were recorded. Unlike the *daf-16* RNAi-treated worms, those treated with *gtr-1* RNAi exhibited no significant change in their lifespans (Fig. 9A; Table 1). We further assessed whether *gtr-1* is required for the longevity phenotype of *daf-2*(*e1370*) mutant worms by treating the animals with *daf-16* or *gtr-1* RNAi as described above and by following their lifespan. As expected, the knockdown of *daf-16* dramatically reduced the lifespan of the animals to levels similar to those of wild type animals. In

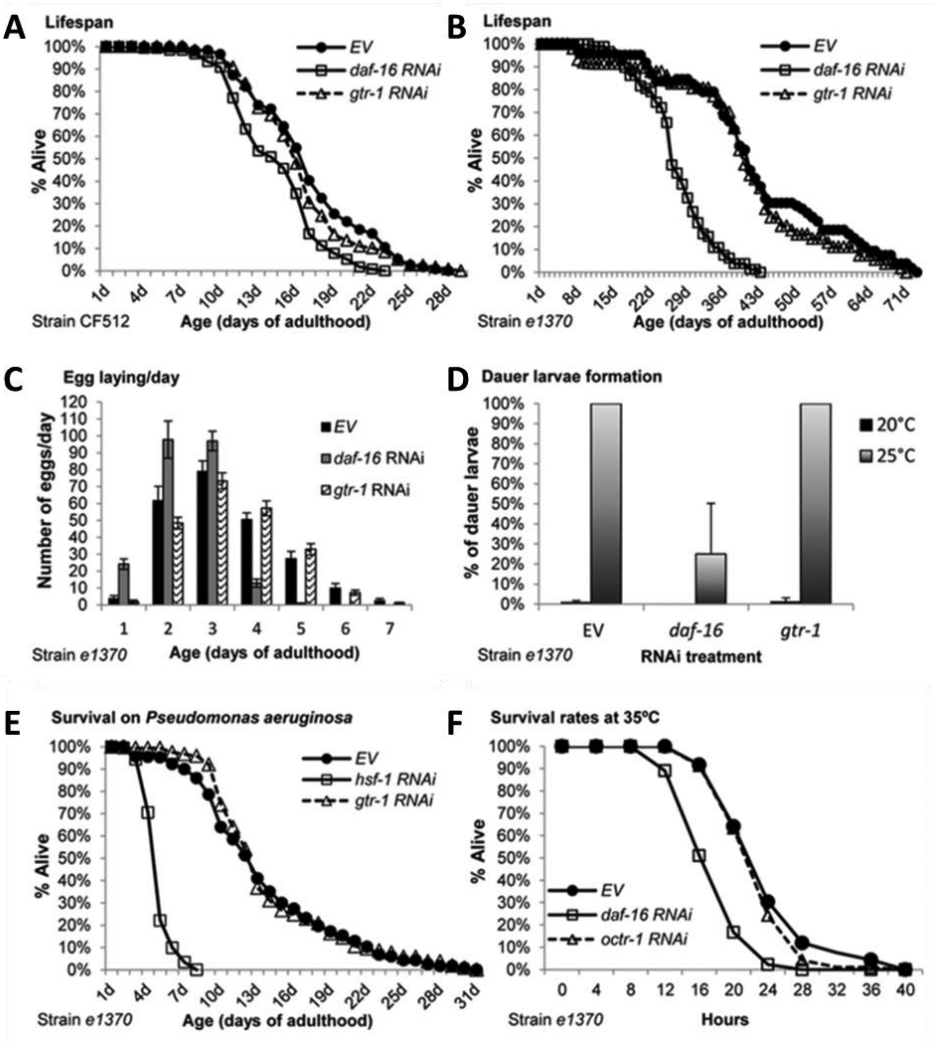


Figure 9. ***gtr-1*** **is dispensable for the determination of lifespan, for innate immunity, and for the developmental functions of the IIS. [A]** *gtr-1* RNAi-treated and untreated (EV) CF512 worms had indistinguishable lifespans. **[B]** Similarly, the knockdown of *gtr-1* by RNAi had no significant effect on the lifespans of *daf-2*(*e1370*) mutant worms. **[C]** Knockdown of *gtr-1* affected neither the egg-laying pattern of *daf-2*(*e1370*) mutant worms [**D**] nor the percentage of dauer larvae in a worm population that hatched and grew at 25°C. **[E]** In contrast to *hsf-1* RNAi, *gtr-1* RNAi treatment during larval development had no effect on the survival rates of *daf-2*(*e1370*) mutant worms that were fed with the pathogenic bacteria *P. aeruginosa* during adulthood. **[F]** Knockdown of *octr-1* by RNAi had no effect on the heat stress resistance of *daf-2*(*e1370*) mutant worms.

contrast, *gtr-1* RNAi treatment had no significant effect on the lifespan of these long-lived nematodes (Fig. 9B; Table 1).

We then examined whether *gtr-1* is involved in the developmental functions of the IIS and found that knocking down this gene affected neither the modified egg-laying pattern of *daf-2*(*e1370*) worms (Dillin *et al*., 2002) nor their rates of dauer larvae formation when grown at 25°C (Fig. 9C and 9D, respectively).

The recent finding that the putative neuronal GPCR OCTR-1 regulates the innate immune response of *C. elegans* (Sun *et al*., 2011), as well as the critical necessity of HSF-1 (Singh and Aballay, 2006 – a; Singh and Aballay, 2006 – b) and DAF-16 (Garsin *et al*., 2003) for this stress response, prompted us to investigate whether *gtr-1* is required for the survival of worms upon exposure to pathogenic bacteria. For this, *daf-2*(*e1370*) mutant worms were allowed to hatch and develop on EV bacteria or on bacteria harboring either *hsf-1* or *gtr-1* dsRNA. They were then transferred onto plates seeded with the pathogenic bacteria *Pseudomonas aeruginosa* and their survival rates were recorded daily. Contrarily to the animals treated with *hsf-1* RNAi, which exhibited reduced survival upon exposure to the pathogenic bacteria, worms developed on *gtr-1* RNAi bacteria, as well their control counterparts (EV), displayed similar survival rates (Fig. 9E), excluding the involvement of *gtr-1* in the innate immune response.

To further scrutinize the possible relations between heat stress resistance and the innate immune response of the nematode, we investigated whether OCTR-1 (Sun *et al*., 2011) is also required for heat stress resistance. For this purpose, *daf-2*(*e1370*) mutant animals were developed on EV bacteria or on bacteria expressing dsRNA towards either *daf-16* or *octr-1* and exposed to 35°C at day 1 of adulthood. Their survival rates were then followed in 4-hour intervals. Our results (Fig. 9F) showed no role for *octr-1* in the neuronal regulation of heat stress resistance, supporting the notion that *gtr-1* and *octr-1* function in distinct neuronal stress response mechanisms.

2.1.5. The ability to respond to heat comes at the expense of the capability to cope with proteotoxicity

In addition to their key roles in stress response, DAF-16, HSF-1, and the Hsps are also instrumental for the maintenance of proteostasis (Cohen and Dillin, 2008). Although the roles of the Hsps suggest that abolishing the worm's ability to induce the HSR will result in impaired

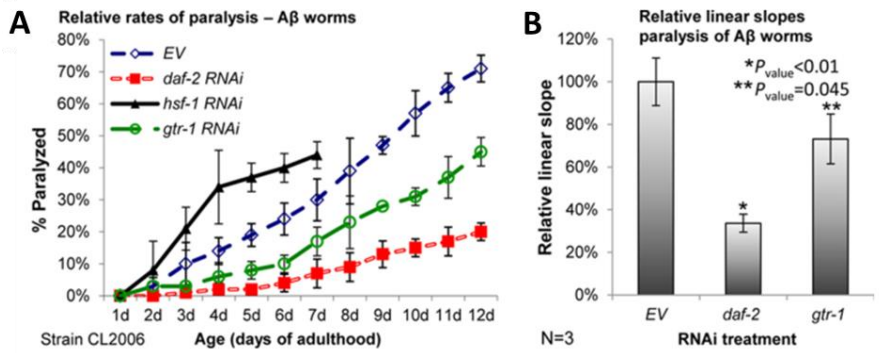


Figure 10. Knockdown of *gtr-1* partially protects from Aβ proteotoxicity. [A] CL2006 worms expressing $A\beta_{3-42}$ in their body wall muscles were either left untreated (EV) or were treated with *daf-2*, *hsf-1*, or *gtr-1* RNAi and the rates of paralysis within the worm populations were recorded daily. The rate of paralysis within the *gtr-1* RNAi-treated population was lower than that of the control group (EV), but higher than that of *daf-2* RNAi-treated worms. **[B]** The counter-proteotoxic effect of *gtr-1* RNAi treatment was confirmed by three independent paralysis assays. Bars represent the relative slopes of the paralysis graphs as in **A**; * $p_{value} < 0.01$, ** $p_{value} < 0.045$. The statistical significance of the results was performed using the Student's t-test.

proteostasis, it was reported recently that worms carrying mutated *gcy-8* or *ttx-3* are partially protected from proteotoxicity (Prahlad and Morimoto, 2011). For this reason, we investigated whether the knockdown of *gtr-1* counters proteotoxicity or if it worsens the toxic phenotype associated with protein aggregation. To clarify this, we used worms that were engineered to express the Alzheimer's-disease-associated human $A\beta_{3-42}$ peptide in their body wall muscles, hereafter called Aβ worms (strain CL2006) (Link, 1995). The expression of $A\beta_{3-42}$ (McColl *et al.*, 2009) results in progressive paralysis of the worm population, which can be alleviated by the knockdown of *daf-2* in a DAF-16- and HSF-1-dependent manner (Cohen *et al.*, 2006) – see section 1.3.2. These worms were treated throughout life with RNAi towards *daf-2*, *hsf-1*, or *gtr-1* or left untreated (EV) and their rate of paralysis was followed from day 1 of adulthood. Interestingly, *gtr-1* knockdown protected from the Aβ-derived toxicity (Fig. 10A, B), supporting the idea that abolishing the worm's ability to activate the HSR mitigates proteotoxicity.

2.2. *tor-2* differentially regulates proteostasis at the organismal level

2.2.1. *tor-2* is upregulated by the IIS pathway

We searched for genes that are known regulators of proteostasis in order to identify new effectors under the control of the IIS pathway. With this in mind, we started by testing whether the expression of these genes is regulated at the transcriptional level by the IIS. For this, we used CF512 worms fed with bacteria harboring an empty vector (EV) or expressing dsRNA towards *daf-2* and measured the mRNA levels of a previously selected set of genes by qPCR. Among these genes, we found *tor-2* mRNA levels to be upregulated around 2.5-fold in worms with reduced IIS activity compared to those with normal IIS activity (EV) (Fig. 11A). We used *sod-3*, a well-established IIS target, as a control for RNAi-mediated knockdown efficiency (Honda and Honda, 1999). The relative mRNA levels of *tor-2* and *sod-3* genes are presented after normalization to *act-1*, the house-keeping gene used as a control.

But what is *tor-2* and why did it evoke interest? *tor-2* is, together with *ooc-5* (Ozelius *et al*., 1997) and *tor-1* (Caldwell *et al*., 2003), one of the torsin-related genes previously shown to be encoded in the complete genome sequence of *C. elegans* and the one that shares higher sequence identity (~40%) with human torsinA gene (Caldwell *et al*., 2003). Torsins share amino acid sequence similarity with members of the large functionally diverse AAA+ family of proteins that include Hsps and molecular chaperones, among others (Neuwald *et al*., 1999). The Caldwell group pioneered the investigation on whether torsin proteins function in a similar capacity to molecular chaperones in facilitating the proper cellular management of misfolded proteins (Caldwell *et al*., 2003). The authors generated transgenic worms concomitantly expressing different lengths of polyQ repeats fused to GFP and the wild type TOR-2 in their body wall muscles and observed that TOR-2 dramatically reduces both the number and the size of polyQ82::GFP-containing aggregates while partially restoring the diffuse body wall muscle fluorescence. The authors further extended their study to another member of the torsin family, *ooc-5*, whose expression together with polyQ82::GFP produced identical results. Interestingly, the induction of *tor*-2's expression upon IIS suppression seems to be specific for this gene given that that of *ooc-5* remained unaltered (Fig. 11B).

Considering that *tor-2* is regulated at the transcriptional level, we aimed at determining which of the three IIS-regulated transcription factors, DAF-16, HSF-1, and SKN-1 (see chapter 1.1.1.2.), is responsible for controlling *tor-2*'s expression. For this, we used CF512 worms grown

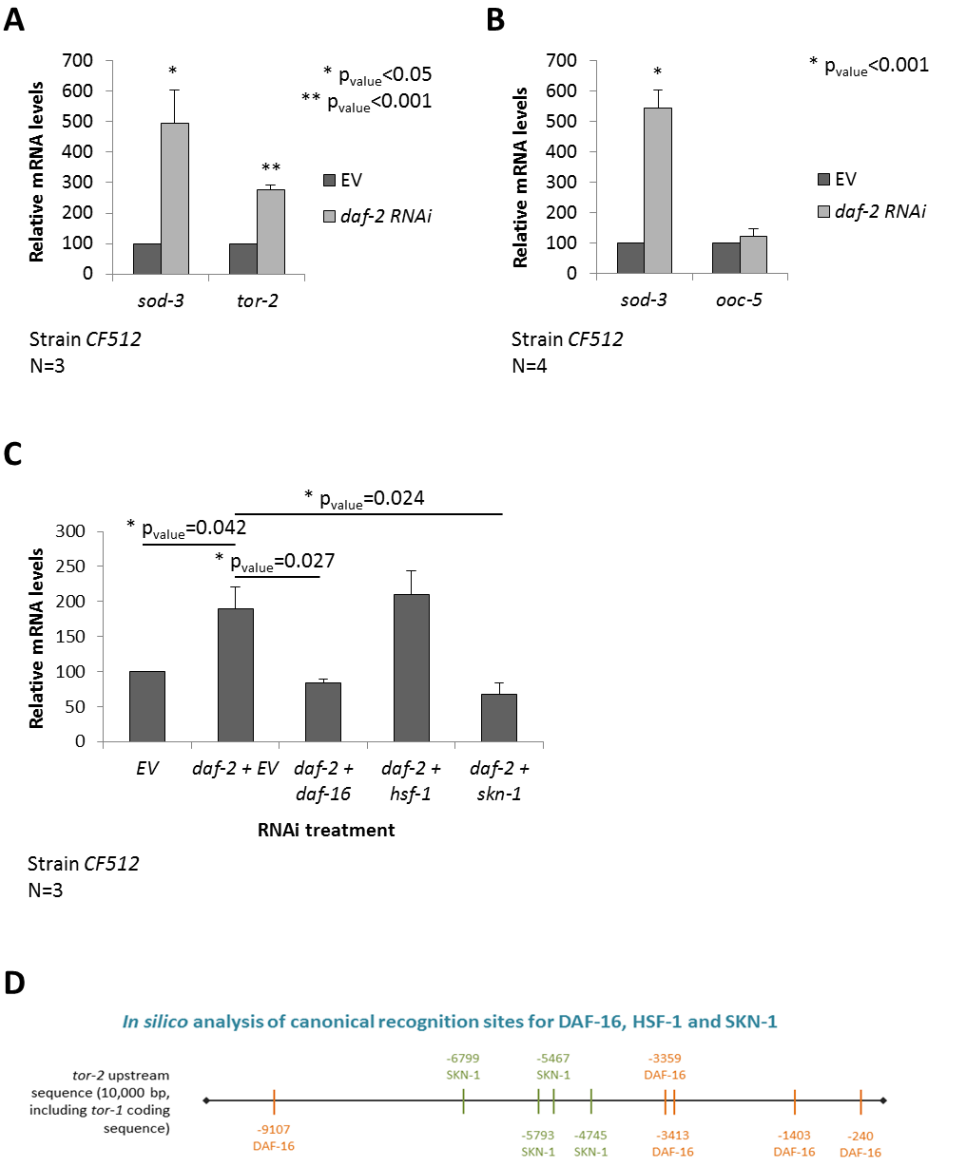


Figure 11. The IIS pathway regulates *tor-2* at the transcriptional level. [A] A qPCR analysis showed that the knockdown of *daf-2* in CF512 worms elevates the mRNA expression levels of *tor-2*. The efficiency of the RNAi treatment was confirmed by the induction of *sod-3*, known to be regulated by the IIS; * $p_{value} < 0.05$, ** $p_{value} < 0.001$. **[B]** The mRNA levels of *ooc-5* remain unaltered upon the knockdown of *daf-2*. The quantified results of **A** and **B** represent the mean relative mRNA level ± SEM of at least three independent experiments. **[C]** A qPCR analysis revealed that a concurrent knockdown of *daf-2* along with either *daf-16* or *skn-1* by RNAi abolishes the induction of *tor-2*'s

on EV bacteria or on dilutions of *daf-2* RNAi with equal amounts of either EV, *daf-16*, *hsf-1*, or *skn-1* RNAi bacteria. The worms were developed at 25ºC to ensure their sterility and, hence, the likely interference of mRNA originating from the animals' eggs in these results, and harvested for mRNA extraction at day 1 of adulthood. The mRNA levels of *tor-2* were analyzed by qPCR and normalized to those of *act-1* (Fig. 11C). Three independent experiments showed that, while the mix of *daf-2* dsRNA with EV produces an increase in the levels of *tor-2*'s mRNA when compared to the control, as observed in previous results (Fig. 11A), the knockdown of *daf-16* and *skn-1*, but not of *hsf-1*, abolishes this effect, bringing its levels to those of the control worms (EV). These results point at both DAF-16 and SKN-1 as the main transcriptional regulators of *tor-2* downstream of the IIS pathway, consistent with an *in silico* analysis of the canonical recognition sites of DAF-16 (TATTTAC and TGTTTAC – Furuyama *et al*., 2000), HSF-1 (TTCTAGAA and GGGTGTC – GuhaThakurta *et al*., 2002), and SKN-1 (AATGTCAT, AATATCAT, ATTGTCAT, ATTATCAT, TATATCAT, TATGTCAT, TTTATCAT and TTTGTCAT – Blackwell *et al*., 1994) in the genomic sequence upstream of *tor-2*'s open reading frame. We analyzed 10,000 base pairs upstream of the *tor-2* sequence (based on the cloning of Cao *et al*. (2005) for generating $P_{tor\text{-}2}$::GFP transgenic worms), which includes the *tor-1* gene, previously reported to have undetectable expression levels (Cao *et al*., 2005), and found several recognition binding sites for both DAF-16 and SKN-1 (Fig. 11D).

Given the low whole-body expression of *tor-2* and hence the lower accuracy of qPCR, we will further perform a digital droplet PCR (ddPCR), a newly developed digital PCR method in which a sample is fractioned into 20,000 individual droplets and the PCR reaction is carried out within each droplet. This method offers advantages over qPCR since it provides an absolute count of the target cDNA copies, detection of low-expression cDNA targets, and the measurement

---

Figure 11. (continuation) Our results imply that these two transcription factors are critically needed for the regulatory effect of the IIS on the expression of *tor-2*. The statistical significance of the results was tested using the Student's t-test. **[D]** Map representing 10,000 bp upstream of the *tor-2* gene (assumed to contain *tor-2*'s transcriptional regulatory regions) and the putative binding sites of all IIS-regulated transcription factors, DAF-16, HSF-1, and SKN-1, in the promoter region of *tor-2*.

of small fold differences between samples (http://www.bio-rad.com/en-sn/applications-technologies/droplet-digital-pcr-ddpcr-technology).

2.2.2. *tor-2* differentially regulates proteostasis across the worm's tissues

Our work aimed at understanding the role of the endogenous *tor-2* in the context of altered IIS activity. Differential effects of IIS reduction on lifespan, stress resistance, and proteostasis in different tissues have been reported. Thus, taking into account that *tor-2* is mainly expressed in neurons (Cao *et al*., 2005), we asked how the knockdown of *tor-2* affects proteotoxicity in different tissues. First, we investigated the phenotypic outcome of knocking down *tor-2* in a *C. elegans* model expressing polyQ67 in a pan-neuronal fashion. These worms display a pronounced age-dependent loss of motility (Vilchez *et al*., 2012). As expected, although less prominently at day 1 of adulthood, the knockdown of *tor-2* increased polyQ67 toxicity at day 3 of adulthood, reflection of the lower number of body bends per 30 seconds in liquid, whereas the knockdown of *daf-2* was protective (Fig. 12A). This suggests that TOR-2 acts within the neuronal tissues to protect against proteotoxicity.

We further tested whether *tor-2* might be involved in protecting against the toxic effects of other aggregative proteins expressed in a different tissue. For this, we grew Aβ worms (CL2006) in either *daf-2* or *tor-2* dsRNA-expressing bacteria or on the control bacteria (EV) and followed their rates of motility from day 1 to 12 of adulthood in order to avoid confounding age-related paralysis with the RNAi effects. As previously shown (Cohen *et al*., 2006), *daf-2* RNAi-treated worms were protected from Aβ-toxicity (Fig. 12B, C). Surprisingly, and contrarily to what was observed for the neuron-expressing polyQ67 worms, *tor-2* knockdown provided partial protection from the paralysis phenotype compared to the EV treatment (Fig. 12B, C).

This remarkably distinct effect of *tor-2* knockdown on the proteotoxicity of aggregative proteins expressed in different tissues, being detrimental in neurons but apparently protective in muscles (where its expression is believed to be absent), led us to hypothesize that *tor-2* might act differently in distinct tissues: be protective within its tissue of expression and detrimental in a distant tissue. Alternatively, this effect might be related to the distinct properties of the

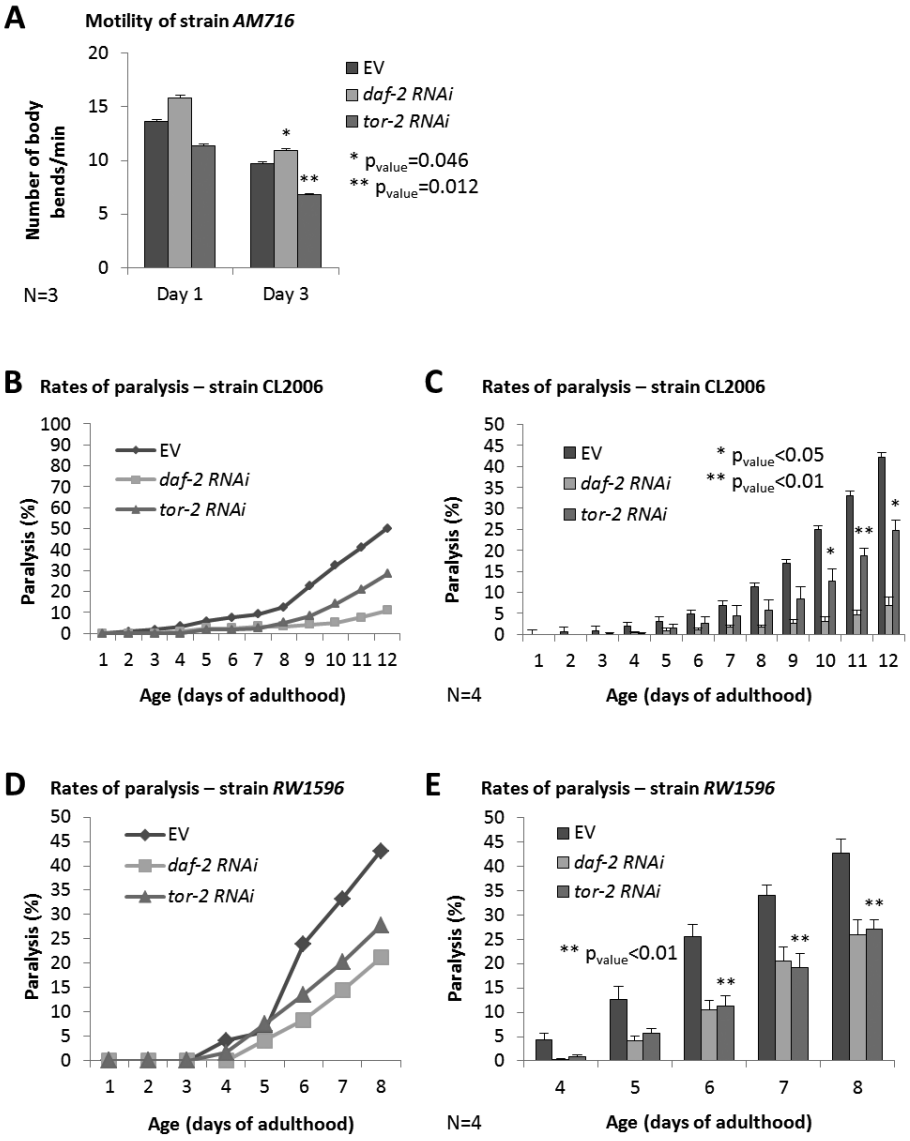


Figure 12. *tor-2* regulates proteostasis in a tissue-specific manner. [A] The knockdown of *tor-2* in worms expressing polyQ67 fused to YFP under the pan-neuronal promoter pF25B3.3 (strain AM716) aggravates the toxicity-derived reduction in the number of body bends per 30 seconds when compared to their EV-fed counterparts, whereas the knockdown of *daf-2* by RNAi ameliorates the phenotype. The quantified results represent the mean ± SEM of the number of body bends on days 1 and 3 of adulthood of at least four independent experiments; * p_{value} = 0.046, ** p_{value} = 0.012. **[B]** *tor-2* knockdown delays the progressive paralysis typical to worms that express the Alzheimer-linked Aβ peptide in their body-wall muscles (strain CL2006). The protective effect of *tor-2* RNAi is significant but less prominent than that of *daf-2* RNAi treatment. **[C]** The bars represent the mean ± SEM of the daily paralysis rates of the different worm populations of four independent experiments; * p_{value} < 0.05, ** p_{value} < 0.01. **[D]** Worms expressing MYO-3::GFP under the *myo-3* promoter (strain RW1596) were also protected by *tor-2* knockdown but exhibit a paralysis rate that resembles that of *daf-2*

aggregative proteins themselves. To discriminate between these two possibilities, we conducted the same assays employing other proteotoxicity models. We used a worm model featuring the expression in the body wall muscles GFP fused to the naturally occurring metastable myosin heavy chain A (MYO-3 ts386 – strain RW1596), which shows age-dependent misfolding and aggregation later in adulthood (Shemesh *et al*., 2013). These worms were grown at 20ºC through larval development and adulthood and their paralysis rates were scored from day 1 of adulthood. Similarly to the Aβ worms, these animals showed a motility decline that was, as expected, delayed by the *daf-2* RNAi treatment and, to the same extent, by knocking down *tor-2* (Fig. 12D, E). This confirms the protective effect of *tor-2* knockdown in muscles. Further experiments are required to establish this hypothesis. Currently, we are using other models expressing different aggregative proteins in neurons and muscles. Additionally, we will use *rde-1* mutant animals in which the wild type *rde-1* gene, which encodes an essential component of the RNAi machinery, has been rescued using tissue-specific promoters, and cross them with the different models of protein aggregation mentioned above. This will allow us to evaluate the effect of tissue-specific knockdown of *tor-2* on proteotoxicity.

2.2.3. *tor-2* is required for the resistance to elevated temperatures

Since increased survival upon exposure to high temperatures is one of the features of IIS reduction (Lithgow *et al*., 1995), we asked whether *tor-2* is also involved in this IIS-regulated function. For this, we employed *daf-2*(*e1370*) mutant worms that were grown throughout development on the control (EV) bacteria or on bacteria expressing dsRNA towards *daf-16* and *tor-2* and exposed to 35°C at day 1 of adulthood (Fig. 13A). As expected, the knockdown of *daf-16* decreased dramatically the worms' resistance to heat. *tor-2* RNAi treatment, on the other hand, seemed to have a mild, negative *effect* on the worm's ability to survive to high temperatures. The

Figure 12. (continuationRNAi treatment. **[E]** The bars represent the mean ± SEM of the daily paralysis rates of different worm populations at the ages of 4, 5, 6, and 7 of adulthood of four independent experiments; * $p_{value} < 0.05$. The statistical significance of the results was examined using the Student's t-test.

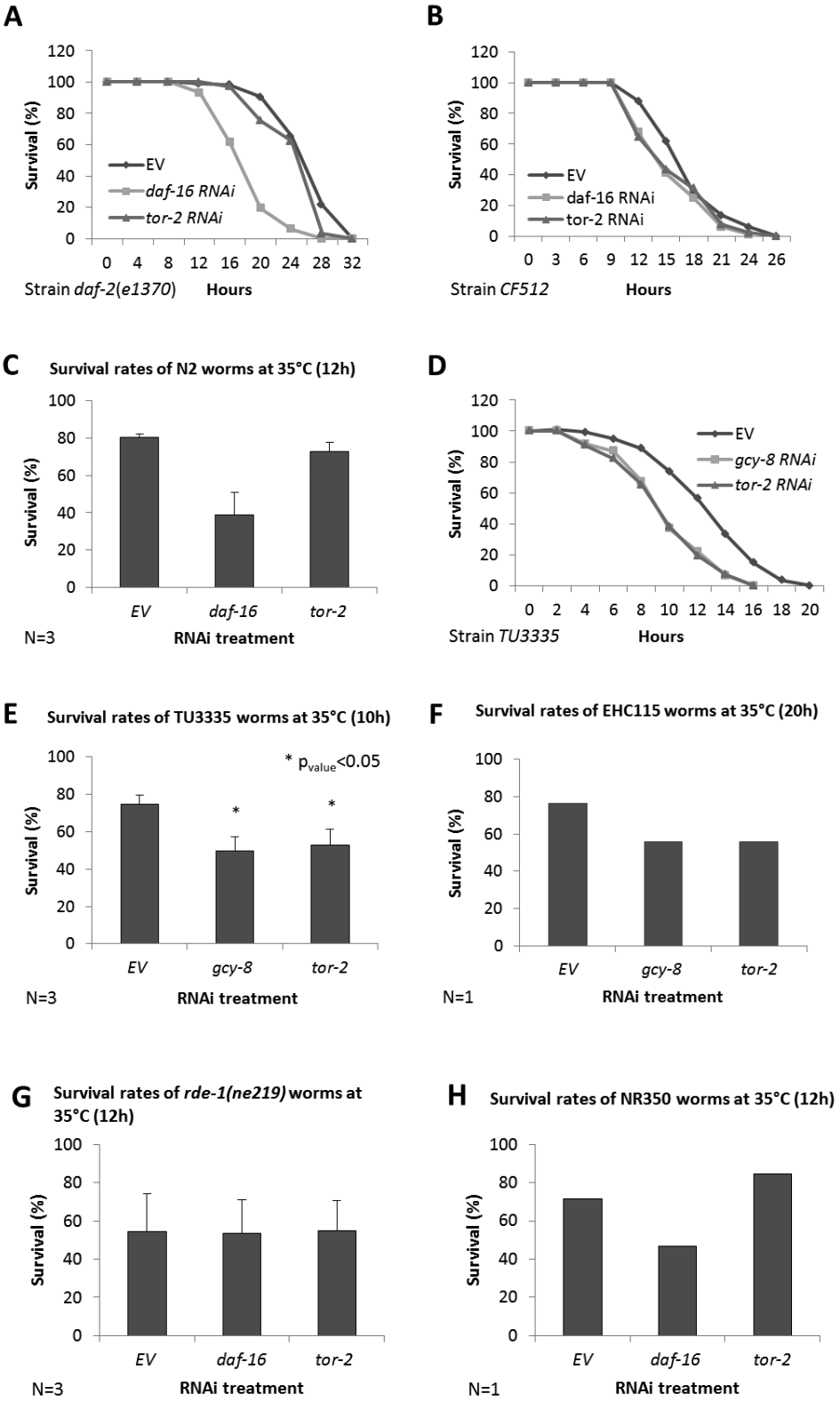


Figure 13. Neuronal *tor-2* is required of heat shock resistance. [A] The knockdown of *tor-2* by RNAi reduced the elevated stress resistance of *daf-2*(*e1370*) mutant worms that were exposed to heat (35°C), although not as prominently as that of *daf-16*. **[B]** CF512 worms fed with *daf-16* or *tor-2* RNAi bacteria show a mild, but significant,

knockdown of *tor-2* by RNAi in CF512 worms resulted in the same minor, but significant, reduction of worms' viability upon exposure to 35°C (Fig. 13B) whereas in N2 worms the effect of *tor-2* knockdown on the worms' sensitivity to elevated temperature was not perceptible after a 12-hour exposure to 35°C (Fig. 13C).

tor-2's expression is very low, as confirmed by our qPCR results, and mainly limited to a few cells comprising vulva muscles, a single cholinergic pharyngeal neuron (M1), two sensory neurons (AW class) and two interneurons (AVE) in the head, and a few neurons that are located at the tail ganglia, including a pair of PVW neurons (Cao *et al*., 2005). With this in mind, we used the TU3335 worms to confirm that neuronal *tor-2* is involved in the resistance to high temperatures. As described in section 2.1.1., these worms express the transmembrane protein SID-1 in all neurons and thus exhibit increased neuronal sensitivity to dsRNA delivered by feeding (Calixto *et al*., 2010). Animals were grown on either EV, *gcy-8* or *tor-2* dsRNA-expressing bacteria and exposed to 35°C for 10 hours at day 1 of adulthood. The number of surviving worms

---

Figure 13. (continuation) increase in sensitivity to heat compared to their untreated (EV) counterparts. **[B]** Three independent experiments using N2 worms after exposure to heat for 12 hours do not show, however, a statistically significant effect in the animals' survival. **[D]** Worms expressing *sid-1* under the regulation of the pan-neuronal *unc-119* promoter (strain TU3335) were either left untreated (EV) or treated with RNAi against *gcy-8* or *tor-2* from hatching until day one of adulthood and transferred to 35°C. Rates of survival were recorded in 2-hour intervals. Similarly to the *gcy-8* RNAi treatment, the knockdown of *tor-2* reduced the heat stress resistance of TU3335 animals, as determined by their lower rate of survival at elevated temperature. **[E]** Three independent experiments in which the survival rates of *TU3335* worms were recorded after 10 hours of exposure to 35°C confirmed the results. The quantified results represent the mean survival ± SEM; * $p_{value} < 0.05$. The statistical significance of the results was performed using the Student's t-test. **[F]** Worms resulting from the crossing of TU3335 worms with *daf-2*(*e1370*) worms (strain EHC115), fed with *tor-2* RNAi bacteria and exposed to heat (35°C) for 20 hours show greater sensitivity to heat stress when compared to *daf-2*(*e1370*) worms, stressing the importance of RNAi penetrance into neurons for the effect of *tor-2* knockdown. **[G-H]** The function of *tor-2* seems to be important in neurons but not in muscles as worms wherein the RNAi is effective only in muscles (strain NR350) do not show sensitivity to heat (35°C for 12 hours) upon knockdown of *tor-2*. *rde-1* mutants (strain WM27), which cannot process RNAi, were used as a control.

was then counted. RNAi against *gcy-8*, shown to be exclusively expressed in AFD thermosensory neurons and to be required for the viability of wild type worms under conditions of heat shock (Prahlad *et al*., 2008), was used as a control. A survival curve showed that, similarly to the *gcy-8* RNAi-treated animals, *tor-2*-RNAi treated animals have a low survival rate at 35°C compared to their EV counterparts (Fig. 13D). Three independent experiments confirmed the higher susceptibility to heat of worms fed with *gcy-8* RNAi and *tor-2* RNAi (Fig. 13E). Likewise, *daf-2*(*e1370*) mutant worms crossed with TU3335 worms (strain EHC115) and fed with RNAi against *gcy-8* or *tor-2* showed higher susceptibility to elevated temperatures than their control counterparts (EV), corroborating the requirement of neuronal *tor-2* for the response to acute heat stress (Fig. 12F). To exclude the involvement of TOR-2 that is expressed in tissues other than neurons in this effect, we performed heat shock experiments using *rde-1(ne219)* mutant animals as a control and *rde-1(ne219)* mutants in which *rde-1* was restored in the muscles by expression of wild type *rde-1* cDNA under the control of the *hlh-1* body-wall muscle promoter (strain NR350) (Qadota *et al*., 2007). *rde-1* encodes an essential component of the RNAi machinery and thus, in these animals, the RNAi is functional exclusively in the muscle cells. The animals were grown until day 1 of adulthood at 20°C and their survival rates were analyzed following 12 hours of exposure to 35°C. As expected, feeding *rde-1(ne219)* mutant worms with *daf-16* dsRNA-producing bacteria did not reduce their survival rates at elevated temperatures (Fig. 13G), whereas the specific knockdown of *daf-16* in the muscles rendered the worms more sensitive to heat (Fig. 13H). On the other hand, no effect was observed upon *tor-2* knockdown either on the *rde-1(ne219)* worms or the ones with *rde-1* expression rescued in the muscles, suggesting that muscular TOR-2 has no role in heat stress resistance (Fig. 13G, H).

Altogether, these results are consistent with those observed for *gtr-1* and with the seminal work from Morimoto's laboratory showing that thermosensory neuronal mutants are able to suppress aggregation and toxicity in muscles while being highly sensitive to acute heat shock (Prahlad and Morimoto, 2011).

2.2.4. *tor-2* is not required for lifespan, resistance to pathogenic bacteria, or exposure to UV

As mentioned in section 1.1.1.2., lifespan extension is one of the hallmarks of IIS reduction (Kenyon *et al*., 1993). With this in mind, we investigated the possible involvement of *tor-2* in the determination of lifespan. We first assessed whether its knockdown affects the lifespan of CF512 worms. For this, we fed the worms throughout development and adulthood with control bacteria (EV) or with bacteria expressing dsRNA towards either *tor-2* or *daf-16* and recorded their lifespans. We observed that, while *daf-16* RNAi dramatically reduced their survival, *tor-2* had no significant effect on the worms' lifespans (Fig. 14A; Table 2). We then examined whether the knockdown of *tor-2* shortens the long lifespan of *daf-2*(*e1370*) mutant worms. To overcome the difficult penetrance of RNAi into neurons, the worms were grown on RNAi for two generations and the lifespans of the worms of the second generation were followed. Contrarily to *daf-16* RNAi, *tor-2* RNAi had no impact on the longevity of these worms (Fig. 14B; Table 2). Finally, we employed worms that express *sid-1* in neurons (strain TU3335), fed them from hatching with control bacterial (EV) or with bacteria expressing dsRNA against *gcy-8* or *tor-2*, and followed their lifespans. As observed for both *daf-2*(*e1370*) mutant and wild type worms, *tor-2* knockdown did not impact the animals' survival (Fig. 14C; Table 2). In sum, these results point to the absence of a role of *tor-2* in the determination of lifespan and further establish the theme that stress resistance and lifespan are not necessarily coupled.

As previously mentioned, Sun *et al*. showed that sensory neurons control the activity of a non-canonical UPR^{ER} pathway required for innate immunity in *C. elegans* (Sun *et al*., 2011). A recent study reported that resistance to heat stress and pathogenic bacteria are interrelated (Ermolaeva *et al*., 2013). This was corroborated by results from our group showing that the neuronal protein NHL-1 is required for resistance to both stresses (Volovik *et al*., 2014) Together with the necessity of *tor-2* for heat stress resistance, these obervations have led us to anticipate that *tor-2* might be involved in the innate immune response. To test this, we used *daf-2*(*e1370*) mutant worms, fed them from hatching with control bacteria (EV) or bacteria harboring dsRNA against *daf-16* or *tor-2* for two generations and transferred them into plates seeded with *P.*

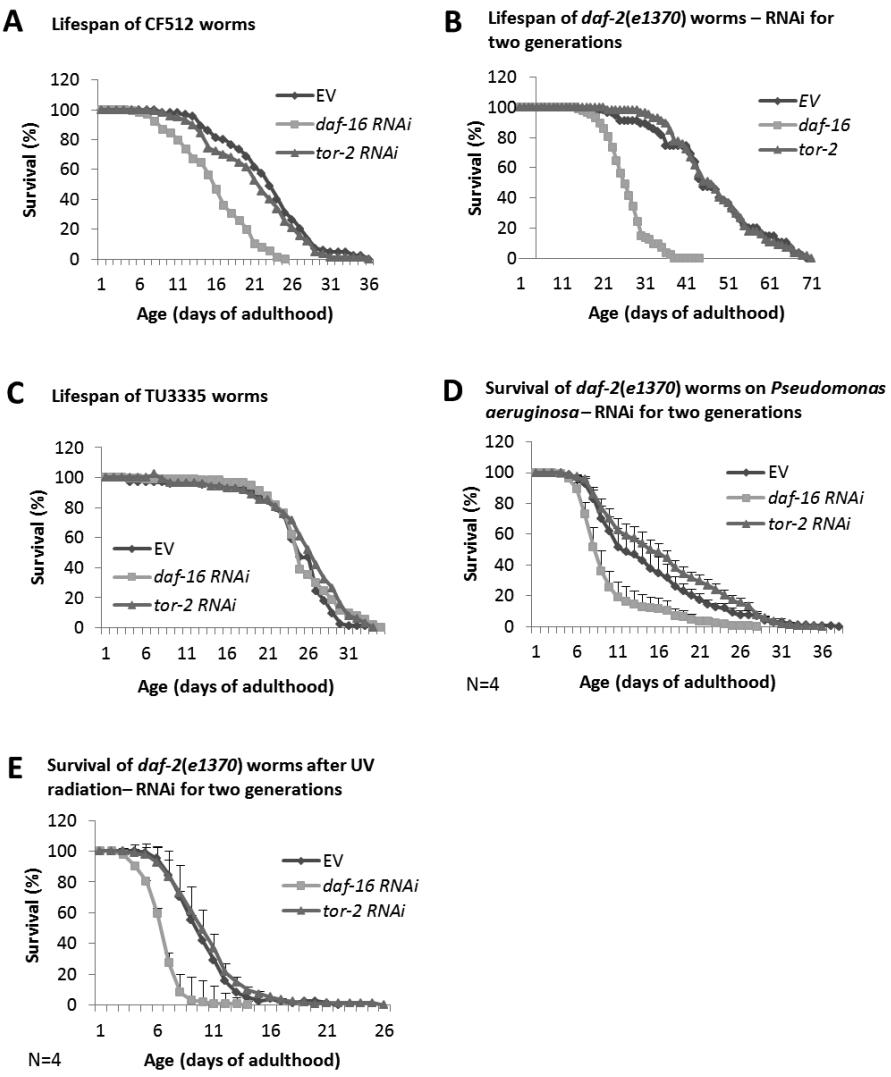


Figure 14. Lifespan and resistance to UV or pathogenic bacteria are not regulated by *tor-2*. [A] CF512 worms were treated throughout development and adulthood with *daf-16* or *tor-2* RNAi or left untreated (EV) and their lifespan were recorded. *tor-2* knockdown had no significant effect on the worms' lifespan. **[B]** *tor-2* showed to be dispensable for the longevity phenotype of *daf-2*(*e1370*) mutant worms grown on *tor-2* RNAi for two generations, as well as for **[C]** the lifespan of TU3335 worms. **[D]** *tor-2* is also dispensable for the increased resistance of *daf-2*(*e1370*) worms grown on *tor-2* RNAi for two generations to the pathogenic bacteria *P. aeruginosa* and **[E]** to DNA damage inflicted by UV. The graphs **D** and **E** represent the mean survival ± SEM of four independent experiments.

aeruginosa at day 1 of adulthood. Unlike the worms that were treated with *daf-16* RNAi, those grown on *tor-2* RNAi bacteria showed no significant difference in survival compared to their

control counterparts, indicating that *tor-2* has no role in resistance to pathogenic bacteria (Fig. 14D).

Given that one neuronal protein can be involved in the activation of some but not all stress responses in remote tissues (Volovik *et al*., 2014), we further sought to determine whether *tor-2* is also involved in the resistance to UV stress, whose enhancement is another feature of reduced IIS (Murakami and Johnson, 1996). For this, synchronized day 1 *daf-2*(*e1370*) mutant worms grown for two generations on EV or on *daf-16* or *tor-2* RNAi were exposed to a sub-lethal dose of UV radiation, and their survival rates were followed. Four independent experiments show a remarkable effect of *daf-16*'s knockdown on the worms' survival, mirrored by the pronounced decrease in the animals' average survival. Conversely, the animals fed on *tor-2* RNAi exhibited a survival curve identical to that of the control worms (EV) (Fig. 14E), advocating the absence of a role for *tor-2* in the regulation of resistance to DNA damage inflicted by UV exposure.

Chapter 3

DISCUSSION

Chapter 3

DISCUSSION AND CONCLUSIONS

3.1. Neuronal *gtr-1* plays key roles in the orchestration of HSR activation in distal tissues

In this study, we identified *gtr-1* as a new critical component of the neuronal signaling mechanism that is required for HSR activation in non-neural tissues. *gtr-1* (F25E2.1) is located on chromosome X and holds eight exons that encode a 329 aminoacids protein predicted to be a seven-transmembrane GPCR with close homologs in other nematodes. The role of *gtr-1* as a GPCR has, however, not been established and thus one cannot exclude that it might play other functions.

gtr-1 is co-expressed with *lin-11* in chemosensory neurons (Hobert *et al.*,1998), but has no apparent role in thermotaxis. This finding indicates that *gtr-1* is not required for heat sensing and suggests that this putative GPCR is solely needed for the induction of the neural signaling that activates the HSR in remote tissues upon exposure to heat. Interestingly, the similar effects of RNAi towards *gtr-1* and *gcy-8* on the worms' survival after heat shock (Fig. 5A, B) suggest that the thermosensory and chemosensory neurons are equally important for HSR induction. Although it is unclear how this signaling mechanism acts, it was reported recently that the worm's thermotactic behavior is dependent upon the activity of HSF-1 and the estrogen signaling pathway (Sugi *et al*., 2011). In the light of this study, it will be interesting to determine whether GTR-1 is also functionally interrelated with the estrogen signaling pathway. It would be further important to characterize the neuronal secretion mechanism that is influenced by GTR-1; does it affect dense core vesicle secretion mechanism? Is it perhaps involved in mediating neurotransmitter release?

It was reported previously that the AFD neurons are pivotal for the activation of the HSR in remote tissues (Prahlad *et al*., 2008). In the present study, we show that the activation of the HSR is not exclusively controlled by this pair of neurons, but rather is also dependent upon the activity of additional components of the thermosensory circuit, the chemosensory neurons. This finding raises the key questions of how neurons of this circuit communicate to integrate environmental cues and how the decision to activate the HSR is made at the cellular and inter-

neuronal levels. It will be crucial to determine whether the chemosensory neurons are involved in sending the HSR-activating signal or if they play their roles exclusively in the decision making process.

3.2. *tor-2* acts downstream of the IIS and is required for resistance to heat stress

In the second part of this thesis, we identified *tor-2* as a new IIS-regulate gene by showing that it is transcriptionally regulated upon suppression of the pathway by both DAF-16 and SKN-1 (Fig. 11A, C). This observation points at *tor-2* as a possible effector of the functions downstream of this pathway. This putative chaperone had been previously reported to counteract proteotoxicity stemming from the expression of an aggregative protein in *C. elegans* muscles upon overexpression (Caldwell *et al*., 2003). The authors observed that the expression of TOR-2, as well as that of its human orthologue, the early-onset torsion dystonia-associated protein torsinA, concurrently with that of polyQ82::GFP, dramatically reduced the number and size of GFP-containing puncta in an age-persistent manner. They further showed that TOR-2 localized to the sites of putative aggregation in a ring-like formation that surrounded the inclusions (Caldwell *et al*., 2003). Later, the same group reported that the overexpression of TOR-2 or torsinA in dopaminergic neurons significantly elevated the resistance of these neurons to the dopamine-selective neurotoxin 6-OHDA and also conferred protection against α-synuclein-induced neurodegeneration (Cao *et al*., 2005). TorsinA, shown to co-localize with Lewy bodies (Shashidharan *et al*., 2000; Sharma *et al*., 2011) and to suppress α-synuclein toxicity in cell cultures (McLean *et al*., 2002), was in fact shown recently to display molecular chaperone activity *in vitro* (Burdette *et al*., 2010), suggesting a similar role for TOR-2. As a direct target of the IIS pathway, in which functions is the endogenous *tor-2* involved? Is it involved in other functions or is it just a proteostasis modulator and, if so, how does it exert its effects under the regulation of the IIS?

We considered the likely involvement of *tor-2* in other IIS-regulated functions and discovered that, similarly to *gtr-1*, it is required for the worms' survival under heat stress. This effect was more prominent in worms with a more efficient RNAi penetrance into neurons (Fig.

13D, E) and barely perceptible in both wild type (Fig. 13B, C) and *daf-2*(*e1370*) mutant worms (Fig. 13A). These findings, together with the absence of effect of the knockdown of *tor-2* in muscles on the worms' resistance to heat (Fig. 13H), are consistent with the fact that the expression of *tor-2* is mainly confined to a few neurons (Cao *et al*., 2005). Indeed, by crossing the *daf-2*(*e1370*) mutant worms with the neuronal RNAi sensitive TU3335 worms, we observed an increased sensitivity of the resulting worm strain to heat when the animals were fed with *tor-2* RNAi bacteria (Fig. 13G).

Among other neurons, *tor-2* was reported to be expressed in two sensory neurons of the AW class (Cao *et al*., 2005). It is possible that *tor-2* is specifically expressed in the amphid AWC neurons, which, together with the AFD and ASI neurons, are required for the worm's thermosensation (http://www.wormatlas.org/). However, contrarily to AFD neurons, the major thermosensors and also coordinators of the HSR in somatic cells (Prahlad *et al*., 2008), AWC neurons display a supportive rather than primary role in thermosensation. This might offer an explanation for the fact that worms treated with *tor-2* RNAi are not as dramatically affected upon exposure to heat as those fed with *gtr-1* RNAi. This, however, needs further clarification. Among other things, it will be also crucial to investigate the mechanism through which *tor-2* plays its role in the response to acute heat stress: is it involved in the activation of the HSR in distal tissues, similarly to *gtr-1*? Is it just required for the sensing of heat and subsequent avoidance behavior?

3.3. The ability to respond to heat comes at the expense of the capability to cope with proteotoxicity

Interestingly, while protecting against heat stress, both *gtr-1* (Fig. 5) (Maman, Carvalhal Marques *et al*., 2013) and *tor-2* (Fig. 13) seem to be less favorable when it comes to counteract proteotoxicity in the muscles given that, upon knockdown of both genes, worms are more protected against the expression of different aggregative proteins (Fig. 10A, B; Fig. 12B, C; respectively). This phenomenon was first observed in *C. elegans* expressing different protein misfolding reporters in distinct tissues, including polyQ44 in the intestine, or the ALS-associated mutant SOD-1^{G93A} in muscle cells (Prahlad and Morimoto, 2011). In sequence of their previous

work (Prahlad *et al*., 2008), the authors hypothesized that, if the HSR is regulated at the organismal level by thermosensory neurons, neuronal activity could also be responsible for the response of the organism to chronic protein misfolding. Surprisingly, the loss of function of either AFD sensory neurons or AIY interneurons, whose activity is essential for the initiation of the HSR, resulted in a cell non-autonomous, HSF-1-dependent chaperone up-regulation that suppressed aggregation and toxicity in distal tissues such as the intestine and muscles (Prahlad and Morimoto, 2011). In view of these results, Prahlad and Morimoto proposed that the fine-tuned neuronal control of chaperone expression in *C. elegans* allows tissues within the organism to maintain optimal levels of chaperones for normal function, being able to respond to acute environmental stresses by transiently upregulating chaperones but protecting inefficiently against chronic accumulation of protein damage. The work presented in this thesis, together with that of Volovik *et al*., wherein the knockdown of *nhl*-1, a putative E3 ligase that is expressed in chemosensory neurons and regulated by the IIS, was shown to decrease stress resistance in distal tissues while conferring protection against proteotoxicity (Volovik *et al*., 2014), comes in line with this discovery. Having *tor-2* a chaperone-like behavior, it is also tempting to compare the counter-proteotoxic effect seen in muscle cells stemming from its knockdown and the results in a recent report showing that either enhancing or suppressing the levels of the HSP90 chaperone within a single tissue has complementary effects on the induction of the HSR in distal tissues of *C. elegans* (van Oosten-Hawle *et al*., 2013). The authors used living animals to monitor the effects of local HSP90 overexpression on the organismal HSR and observed a reduction in the heat shock inducibility of three representative HS genes in multiple tissues. Conversely, by reducing the tissue-specific expression of HSP90, the inducibility of *hsp70* was restored in distinct individual tissues (van Oosten-Hawle *et al*., 2013). Hence, it is plausible that, by reducing the levels of TOR-2 in neurons, a compensatory mechanism is activated in muscles and/or other tissues.

3.4. TOR-2 regulates proteostasis in a different manner across different tissues

Curiously, the knockdown of *tor-2* by RNAi seems to be detrimental to worms expressing an aggregative protein in neurons (Fig. 12A). If proven to be consistent, our results open a new

possibility in the proteostasis field: can a protein protect against proteotoxicity wherein it is expressed and be disadvantageous in distal tissues? This possibility has to be critically evaluated to assess the therapeutic potential of inter-tissue manipulations for the treatment of age-onset maladies. The use of additional proteotoxicity models expressing other metastatic or aggregative-proteins in distinct tissues (*e.g.* neurons, muscles, intestine) is still required to support this notion. Likewise, it would be of great importance to test the outcome of the tissue-specific knockdown of TOR-2 on the toxicity deriving from the expression of an aggregative protein in a different tissue (*e.g.* neuron-specific knockdown of *tor-2* in worms expressing $A\beta_{3-42}$ peptide in muscles).

TOR-2 and torsinA were shown to be ER-resident proteins (Caldwell *et al*., 2003; Jungwirth *et al*., 2010). More recently, the Caldwell group discovered that the presence of torsinA reduces the ER stress within the same tissue by suppressing the UPR^{ER} (Chen *et al*., 2010). This led them to propose that torsinA increases the overall cellular threshold to which misfolded proteins or other stressors may induce dysfunction. More importantly, torsinA is able to attenuate the enhancement in the ER stress response caused by the expression of the ALS-associated $SOD1^{G85R}$ mutant in neurons, as well as to rescue these animals from the resulting locomotive defects (Thompson *et al*., 2014). The fact that the knockdown of *tor-2* by RNAi exacerbates the movement impairment of worms expressing polyQ67 in neurons seems to be in accordance with the findings of the Caldwell group (Chen *et al*., 2010; Nery *et al*., 2011; Thompson *et al*., 2014) given that *tor-2*'s expression is mainly confined to neurons (Cao *et al*., 2005). Thus, it is expected that the knockdown of *tor-2* results in a worsening of the polyQ67-resulting phenotype due to lack of protection against chronic ER stress, a known early event in polyglutamine toxicity (Duennwald and Lindquist, 2008). Despite this, we need yet to confirm that *tor-2*'s expression is indeed confined mainly to a few neuronal cells and absent in other tissues by generating worms expressing a fluorescent protein (*e.g.* tdTomato) under the control of the *tor-2* promoter. It will be also critical to comprehensively test the possibility that *tor-2* has roles in controlling the activation of the UPR and, if so, the relation between this effect and its differential regulation of proteotoxicity across different tissues.

3.5. Stress resistance, longevity, and counter-proteotoxic mechanisms are separable

This work further strengthens the notion that stress resistance, longevity, and protection from proteotoxicity are not necessarily coupled. Indeed, despite required for resistance to heat stress, both *gtr-1* and *tor-2* play no role in lifespan determination, as extensively confirmed in wild type (Fig. 9A; Fig. 14A; respectively), *daf-2*(*e1370*) mutant (Fig. 9B; Fig. 14B, C; respectively), and RNAi neuronal sensitive worms (Fig. 14D), in the resistance to other stresses such as exposure to pathogenic bacteria (Fig. 9E; Fig. 14E; respectively) or UV (Fig. 14F).

The mutual relations of stress resistance, proteostasis, and lifespan have been previously addressed in our laboratory by testing whether IIS reduction can execute its counter-proteotoxic functions in midlife, when it can no longer extend lifespan (Cohen *et al*., 2010). By using the Aβ worm model and conditional *daf-2* knockdown, we discovered that this treatment can, in fact,

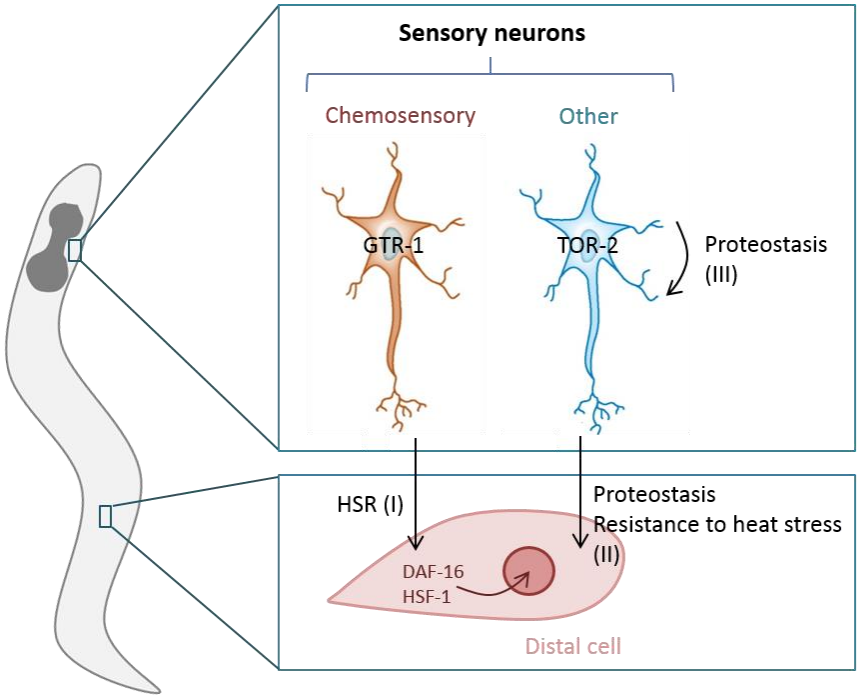


Figure 15. The model. In *Caenorhabditis elegans*, chemosensory neurons regulate the heat shock response (HSR) in remote tissues, including the intestine. The putative G-coupled protein receptor GTR-1 seems to play an important role in this regulation (I). TOR-2 that is expressed in neurons coordinates the response to heat shock in distal tissues through an unknown mechanism (II). It further regulates proteostasis in a tissue-specific manner, being detrimental in distal tissues, such as muscles (II), but protective within its tissues of expression (neurons – III).

alleviate Aβ proteotoxicity in midlife, thereby demonstrating that the lifespan-regulating and counter-proteotoxic functions downstream of the IIS pathway are separable. Another study questioned whether the long-lived phenotypes observed in worms subjected to DR or with a low IIS activity are conferred by an elevation in the ability to resist oxidative stress stemming from the transcriptional regulation of *sod* family members in both aging-regulating pathways (Van Raamsdonk and Hekimi, 2009). Being this the case, one would expect that the deletion of genes of the *sod* family would shorten lifespan. Surprisingly, the deletion of *sod-1*, *sod-3*, *sod-4*, or *sod-5* has no effect on lifespan, whereas worms without the *sod-2* gene, which encodes for the mitochondrial SOD of the worm, exhibit longer lifespan than their wild type counterparts (Van Raamsdonk and Hekimi, 2009). More recently, the deletion of all five *sod* genes was found to hypersensitize the nematode to oxidative stress but to have no effect on lifespan (Van Raamsdonk and Hekimi, 2012). Our lab further identified *nhl-1* as a co-factor of DAF-16 that is required for resistance against heat, oxidative, and immune stresses while having no impact on lifespan (Volovik *et al*., 2014). More striking is ours and others discovery that the knockdown of neuronal genes that abolish the nematode's ability to activate a full HSR provides partial protection from proteotoxicity, as extensively described above (Prahlad *et al*., 2011; Maman, Carvalhal Marques *et al*., 2013). In sum, these studies reveal that the functions promoted by aging-regulatory pathways are not necessarily linked. The present work supports this idea by showing that the ability to cope with heat stress and proteotoxicity does not always impact lifespan.

3.6. Concluding remarks

This study offers new comprehensive data regarding how stress response mechanisms and proteostasis are regulated at the organismal level (Figure 15), highlighting that each component cell in a multicellular organism works in an integrated and interdependent way, consenting for a highly regulated division of labor that is required for proper physiological function and homeostasis. It further supports the idea that the functions promoted by aging-regulatory pathways, lifespan, stress resistance, and proteostasis, are not necessarily linked. These insights might point at new research avenues for the development of systemic therapies that will

target signaling mechanisms to specifically modulate inter-tissue communication and preserve proteostasis through late stages of life. This is more clearly the case of the research on TOR-2, whose human orthologue, TorsinA, is involved in the development of the genetic disorder torsion dystonia when defective (Ozelius *et al*., 1997). This work further highlights the attractiveness of the alteration of aging as a therapeutic strategy, suggesting that protection from late-onset disorders may be achieved without extending lifespan.

Chapter 4

METHODS

Chapter 4

METHODS

C. elegans strains and growth conditions

CF512 (*fer-15*(*b26*)II*;fem-1*(*hc17*)IV), N2 (wild type, Bristol), *daf-2*(*e1370*) mutant, CF1844 (*fer-5*(*b26*) II*; daf-2*(*mu150*) III*; fem-1*(*hc17*) IV), CL2006 (dvIs2 [*pCL12* (*unc-54/human Aβ peptide 1-42 minigene*) + *pRF4*]), RW1596 (stEx30[*myo-3p::GFP* + *rol-6*(su1006)], TU3335 (uIs57 [*unc-119p::YFP* + *unc-119p::sid-1* + *mec-6p::mec-6*]), PY1322 (*gcy-8p*::*GFP*), OH103 (*lin-11p*::*GFP*), OH99 (*ttx-3p*::*GFP*), CL2070 (*hsp-16.2p*::*GFP*), WM27 (*rde-1*[*ne219*]), and NR350 (*rde-1*[*ne219*]; kzIs20[pDM#715(*hlh-1p::rde-1*) + pTG95(*sur-5p*::*nls*::*GFP*)] *C. elegans* strains were obtained from the *Caenorhabditis* Genetic Center. Strain AM716 (rmIs284 [*pF25B3.3::Q67::YFP*]) was a gift from Andrew Dillin's lab. All strains were grown at 20°C (unless indicated otherwise) on nematode growth media (NGM) plates and fed with *E. coli* HT115 bacteria. CF512 are heat sensitive and were routinely grown at 15°C. To avoid progeny, CF512 worms were hatched at 20°C and L1 larvae transferred to 25°C for 48 hours and back to 20°C until harvested. Standard *C. elegans* techniques were used to maintain the strains (Stiernagle, 2006).

RNAi

All RNAi experiments were carried out on NGM-ampicillin plates seeded with cultures of *E. coli* harboring the appropriate RNAi clone grown in LB overnight at 37 ºC and supplemented with 100 mM isopropyl β-d-1-thiogalactopyranoside (IPTG – ~4 mM final concentration) to induce the expression of the dsRNA. All RNAi clones were obtained from the libraries generated in the laboratories of Dr. Julie Ahringer (Kamath *et al*., 2003) and D. Marc Vidal (Rual *et al*., 2004) and sent for verification by sequencing prior using. *E. coli* carrying an empty vector (EV) were used as a control.

Creation of worm strains and RNAi constructs

The following primers were used to amplify the promoter region of *gtr-1* (including restriction sites): forward-CAGAAGCTTCCCCACTCTCTACCCAACG; reverse-TGACCCGGGTGAAA ATGTGTTCTGAAAAAAAAAACGAATTCGATA. The PCR product was cloned into the plasmid vector pUC118 between BamH1 and XmaI restriction sites and upstream of tdTomato. The final construct was microinjected into N2 worms. The promoter region of *hsp-12.6* was amplified using the following primer set (including restriction sites): forward-CAAGTCGACAATTTGTTTATGTAAATGCGTTTTAGTGTG; reverse–ACGGGATCCATC ATCTTGGCAAAAGTTTTTGGG. The PCR product was cloned upstream of tdTomato and the resulting plasmid was injected into N2 worms. Selection was performed using fluorescence microscopy. The RNAi construct towards the *gtr-1* 3′UTR was generated using gBlock synthesis (Integrated DNA Technologies): AGCGAGCTATTTACCAACTTTTCCCCTTTTTTGCT GTGTTTAATTTTATCACTTTTGTAAATCTAAAGATCTCATTTTCATTCAATTC ACGGTTATTTTAATAAATATTCTGTTCGGCTAGCTCG. The gBlock construct was digested with NheI and SacI and cloned into the pL4440 plasmid.

RNA isolation, quantitative real-time PCR (qPCR)

Synchronized worm populations were grown in four or more NGM-ampicillin plates until day one of adulthood, harvested in M9 buffer, and washed in RNase-free water. The worm pellet was resuspended in 2 volumes of QIAzol (catalog #79306; Sigma) and frozen overnight. Chloroform was used to separate RNA from protein and other materials. Total mRNA was then extracted using the RNeasy Lipid Tissue Mini Kit (catalog #74804; Qiagen) and quantified using a NanoDrop 2000c spectrophotometer. Random-primed cDNAs were generated by reverse transcription of the total RNA samples with iScript^{RT} Advanced cDNA Synthesis Kit for RT-PCR (catalog #170–8891; Bio-Rad) per manufacturer's protocol. Quantitative real-time PCR (qPCR) analyzes were performed with EvaGreen SuperMix (catalog #172–5204; Bio-Rad) and normalized to levels of *act-1* cDNA. Primer sequences are available on request. The qPCR reactions for each gene were performed, at least, in triplicate.

Lifespan assays

Eggs were isolated by bleaching worms in order to obtain an age-synchronized worm population. The worms were grown in big NGM-ampicillin agar plates seeded with the appropriate dsRNA-carrying bacteria and supplemented with 100 mM IPTG (~4 mM final concentration) until the first day of adulthood at 15 or 20°C, according to the maintenance specifications of the strain. 120 animals were assayed for each condition. For this, they were transferred into small NGM-ampicillin plates seeded with the appropriate bacteria (12 worms per plate) and moved to new plates every two days of their reproductive period, after which they were transferred every three days. Lifespan was monitored every day and death was stipulated as the total movement irresponsiveness to gentle mechanical stimulation with a platinum wire.

Stress resistance assays

All assays were performed using age-synchronized, day-1 adult animals developed on NGM-ampicillin plates seeded with *E. coli* harboring the appropriate RNAi clone or control and supplemented with 100 mM IPTG (~4 mM final concentration). The worms were developed and maintained at 15 or 20°C, according to the strain. Following the treatment, the death scores of 120-worm populations were scored every day as for the lifespan assays.

Heat-stress

For heat-stress assays, 120 day-1 adult animals were transferred onto fresh plates (12 animals per plate) spotted with RNAi bacteria and exposed to 35°C. Survival rates were recorded after 10, 12, 15, or 20 hours, in conformity with the strain. Survival curves were obtained by counting the worm survival rates in 2-3 hours intervals, as indicated.

UV-stress

For the UV-stress assays, *daf-2*(*e1370*) worms were transferred to unseeded plates and irradiated with 800 Joules for 40 seconds, after which the worms were moved back to plates (12 per plate) spotted with the bacteria harboring the desired RNAi clone or control.

Immune stress

To evaluate resistance to pathogenic bacteria (innate immunity), *daf-2*(*e1370*) worms grown on the appropriate RNAi bacteria until day 1 or adulthood were transferred onto small NGM plates (12 animals per plate) seeded with *P. aeruginosa*. The survival rates were scored daily.

Microscopy and signal quantification

Synchronous worms were washed twice with phosphate buffer saline (PBS) and snap frozen in liquid nitrogen. Fluorescent images were obtained using a Nikon AZ100 microscope and NIS elements software. Quantitative fluorescence analysis was performed using ImageJ software. Neural expression patterns were tested using confocal microscopy. The worms were washed twice with M9, anesthetized using 18 mM of sodium azide (#S-2002; Sigma) and placed on an agar pad for visualization. Images were obtained using a Zeiss LSM 710 laser scanning microscope.

Thermotaxis assay

Synchronized, 1-day-old, *daf-2*(*e1370*) mutant worms that were developed on the indicated RNAi bacteria were placed on fresh NGM-ampicillin plates and exposed to the temperature gradient. The temperatures of each zone of the plate were monitored using a laser-guided, infrared thermometer. Photographs were taken before exposing the worms to the temperature gradient and 12 min thereafter.

SDS-PAGE and Western blot analysis

Twelve thousand CL2070 worms were treated with RNAi bacteria as indicated and homogenized using a dounce homogenizer. The worm homogenates were centrifuged for 3 minutes at 850 × g (3,000 rpm in a desktop centrifuge) to sediment debris. The post-debris supernatants were collected, protein amounts were measured (catalog #500–0006; Bio-Rad), supplemented with loading buffer (10% glycerol, 125 mM Tris base, 1% SDS), boiled for 10 minutes, and 10 μg of total protein was loaded into each well. Proteins were separated on polyacrylamide gels, transferred onto PVDF membranes (Pierce), and probed with either GFP antibody (catalog #2956;

Cell Signaling Technology) or anti γ-tubulin antibody clone GTU-88 (catalog #T-6557; Sigma). HRP-conjugated secondary antibody, a chemiluminescence system, and a luminescent image analyzer (Las-3000; Fujifilm) were used to detect protein signals.

Egg-laying assay

To follow the reproductive profiles of *daf-2*(*e1370*) mutant animals, synchronized eggs were placed on NGM-ampicillin plates seeded with the appropriate RNAi bacteria. At the L4 larval stage, 15 animals were transferred onto new plates seeded with the same RNAi strain (one worm per plate). The worms were transferred onto new plates in 24 hours intervals and the progeny number in each plate was counted 72 hours thereafter.

Dauer formation assay

Eggs of *daf-2*(*e1370*) mutant animals were transferred onto plates seeded with RNAi bacteria, as indicated, and were incubated at either 20°C or at 25°C. Dauer larvae and adult worms were counted 3 days after.

Behavioral assays

For the paralysis assay, synchronized CL2006 eggs were placed on NGM-ampicillin plates seeded with the desired bacteria and supplemented with 100 mM ampicillin (~4 mM final) and allowed to develop until day 1 of adulthood. 120 animals were then transferred to small NGM plates seeded with the respective *E. coli* culture. The score of paralyzed worms was recorded daily by tapping the worms' noses with a platinum wire. A paralyzed animal was defined as an animal than can move its head but is unable to crawl away ("windshield wiper" phenotype). The assays were terminated at the age of 12 of adulthood, when the wild type animals start exhibiting age-related paralysis. The same assay was performed with the RW1596 strain. The trashing rate was determined by transferring individual animals in a drop of M9 buffer at day 1 and 3 of adulthood, allowing 30 seconds for adaptation and counting the number of body bends for 30 seconds. A body bend was defined as the change in direction of the bend in the mid-body of an animal

(Brignull *et al*., 2006; Chai *et al*., 2002). 20 animals per treatment per independent experiment were used.

Statistical analysis and software

The results are presented as the mean ± SEM of three or more independent, biological repeats of each experiment. Statistical significance of differences was assessed by Student's *t* test. Both statistical analysis and plotting of the data were performed using Microsoft Excel.

REFERENCES

Adams, J. (2003). The proteasome: structure, function, and role in the cell. *Cancer Treat Rev, 29 Suppl 1*, 3-9.

Ahn, S. G., Kim, S. A., Yoon, J. H. and Vacratsis, P. (2005). Heat-shock cognate 70 is required for the activation of heat-shock factor 1 in mammalian cells. *Biochem J, 392(Pt 1)*, 145-52.

Alavez, S., Vantipalli, M. C., Zucker, D. J., Klang, I. M., and Lithgow, G. J. (2011). Amyloid-binding compounds maintain protein homeostasis during ageing and extend lifespan. *Nature, 472(7342)*, 226-9.

Alic, N., Tullet, J. M., Niccoli, T., Broughton, S., Hoddinott, M. P., Slack, C., Gems, D., and Partridge, L. (2014). Cell-nonautonomous effects of dFOXO/DAF-16 in aging. *Cell Rep, 6(4)*, 608-16.

An, J. H., Vranas, K., Lucke, M., Inoue, H., Hisamoto, N., Matsumoto, K., and Blackwell, T. K. (2005). Regulation of the Caenorhabditis elegans oxidative stress defense protein SKN-1 by glycogen synthase kinase-3. *Proc Natl Acad Sci U S A, 102(45)*, 16275-80.

Anckar, J. and Sistonen, L. (2007). Heat shock factor 1 as a coordinator of stress and developmental pathways. *Adv Exp Med Biol, 594*, 78-88.

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

Arantes-Oliveira, N., Apfeld, J., Dillin, A., and Kenyon, C. (2002). Regulation of life-span by germ-line stem cells in Caenorhabditis elegans. *Science, 295(5554)*, 502-5.

Balch, W. E., Morimoto, R. I., Dillin, A. and Kelly, J. W. (2008). Adapting proteostasis for disease intervention. *Science, 319(5865)*, 916-9.

Barsyte, D., Lovejoy, D. A., and Lithgow, G. J. (2001). Longevity and heavy metal resistance in daf-2 and age-1 long-lived mutants of Caenorhabditis elegans. *FASEB J, 15(3)*, 627-34.

Bennett, C. F., Vander Wende, H., Simko, M., Klum, S., Barfield, S., Choi, H., Pineda, V. V. and Kaeberlein, M. (2014). Activation of the mitochondrial unfolded protein response does not predict longevity in Caenorhabditis elegans. *Nat Commun*.

Berman, J. R. and 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(5)*, 1055-68.

Bjedov, I., Toivonen, J. M., Kerr, F., Slack, C., Jacobson, J., Foley, A., and Partridge, L. (2010). Mechanisms of life span extension by rapamycin in the fruit fly Drosophila melanogaster. *Cell Metab, 11(1)*, 35-46.

Blackwell, T. K., Bowerman, B., Priess, J. R., and Weintraub, H. (1994). Formation of a monomeric DNA binding domain by Skn-1 bZIP and homeodomain elements. *Science, 266(5185)*, 621-8.

Boulias, K. and Horvitz, H. R. (2012). The C. elegans microRNA mir-71 acts in neurons to promote germline-mediated longevity through regulation of DAF-16/FOXO. *Cell Metab, 15(4)*, 439-50.

Brignull, H. R., Moore, F. E., Tang, S. J., and Morimoto, R. I. (2006). Polyglutamine proteins at the pathogenic threshold display neuron-specific aggregation in a pan-neuronal Caenorhabditis elegans model. *J Neurosci, 26(29)*, 7597-606.

Broué, F., Liere, P., Kenyon, C., and Baulieu, E. E. (2007). A steroid hormone that extends the lifespan of Caenorhabditis elegans. *Aging Cell, 6(1)*, 87-94.

Burdette, A. J., Churchill, P. F., Caldwell, G. A., and Caldwell, K. A. (2010). The early-onset torsion dystonia-associated protein, torsinA, displays molecular chaperone activity in vitro. *Cell Stress Chaperones, 15(5)*, 605-17.

Calahorro, F. and Ruiz-Rubio, M. (2011). Caenorhabditis elegans as an experimental tool for the study of complex neurological diseases: Parkinson's disease, Alzheimer's disease and autism spectrum disorder. *Invert Neurosci, 11(2)*, 73-83.

Calderwood, S. K., Murshid, A. and Prince, T. (2009). The shock of aging: molecular chaperones and the heat shock response in longevity and aging--a mini-review. *Gerontology, 55(5)*, 550-8.

Caldwell, G. A., Cao, S., Sexton, E. G., Gelwix, C. C., Bevel, J. P., and Caldwell, K. A. (2003). Suppression of polyglutamine-induced protein aggregation in Caenorhabditis elegans by torsin proteins. *Hum Mol Genet, 12(3)*, 307-19.

Calixto, A., Chelur, D., Topalidou, I., Chen, X., and Chalfie, M. (2010). Enhanced neuronal RNAi in C. elegans using SID-1. *Nat Methods, 7(7)*, 554-9.

Cao, S., Gelwix, C. C., Caldwell, K. A., and Caldwell, G. A. (2005). Torsin-mediated protection from cellular stress in the dopaminergic neurons of Caenorhabditis elegans. *J Neurosci, 25(15)*, 3801-12.

Carvalhal Marques, F., Volovik, Y., and Cohen, E. (2015). The roles of cellular and organismal aging in the development of late-onset maladies. *Annu Rev Pathol, 10*, 1-23.

Chai, Y., Shao, J., Miller, V. M., Williams, A. and Paulson, H. L. (2002). Live-cell imaging reveals divergent intracellular dynamics of polyglutamine disease proteins and supports a sequestration model of pathogenesis. *Proc Natl Acad Sci U S A, 99(14)*, 9310-5.

Chen, P. M., Gombart, Z. J. and Chen, J. W. (2011). Chloroquine treatment of ARPE-19 cells leads to lysosome dilation and intracellular lipid accumulation: possible implications of lysosomal dysfunction in macular degeneration. *Cell Biosci*.

Chen, P., Burdette, A. J., Porter, J. C., Ricketts, J. C., Fox, S. A., Nery, F. C., Hewett, J. W., Berkowitz, L. A., Breakefield, X. O., Caldwell, K. A., and Caldwell, G. A. (2010). The early-onset torsion dystonia-associated protein, torsinA, is a homeostatic regulator of endoplasmic reticulum stress response. *Hum Mol Genet, 19(18)*, 3502-15.

Chiang, W. C., Ching, T. T., Lee, H. C., Mousigian, C., and Hsu, A. L. (2012). HSF-1 regulators DDL-1/2 link insulin-like signaling to heat-shock responses and modulation of longevity. *Cell, 148(1-2)*, 322-34.

Chondrogianni, N., Georgila, K., Kourtis, N., Tavernarakis, N., and Gonos, E. S. (2015). 20S proteasome activation promotes life span extension and resistance to proteotoxicity in Caenorhabditis elegans. *FASEB J, 29(2)*, 611-22.

Ciechanover, A. and Kwon, Y. T.. (2015). Degradation of misfolded proteins in neurodegenerative diseases: therapeutic targets and strategies. *Exp Mol Med*.

Clark, D. A., Gabel, C. V., Gabel, H., and Samuel, A. D. (2007). Temporal activity patterns in thermosensory neurons of freely moving Caenorhabditis elegans encode spatial thermal gradients. *J Neurosci, 27(23)*, 6083-90.

Cohen, E. and Dillin, A. (2008). The insulin paradox: aging, proteotoxicity and neurodegeneration. *Nat Rev Neurosc, 9(10)*, 759-67.

Cohen, E. and Dillin, A. (2008). The insulin paradox: aging, proteotoxicity and neurodegeneration. *Nat Rev Neurosci, 9(10)*, 759-67.

Cohen, E., Bieschke, J., Perciavalle, R. M., Kelly, J. W., and Dillin, A. (2006). Opposing activities protect against age-onset proteotoxicity. *Science, 313(5793)*, 1604-10.

Cohen, E., Du, D., Joyce, D., Kapernick, E. A., Volovik, Y., Kelly, J. W., and Dillin, A. (2010). Temporal requirements of insulin/IGF-1 signaling for proteotoxicity protection. *9(2)*, 126-34.

Cohen, E., Paulsson, J. F., Blinder, P., Burstyn-Cohen, T., Du, D., Estepa, G., Adame, A., Pham, H. M., Holzenberger, M., Kelly, J. W., Masliah, E., and Dillin, A. (2009). Reduced IGF-1 signaling delays age-associated proteotoxicity in mice. *Cell, 139(6)*, 1157-69.

Csermely, P., Söti, C. and Blatch, G. L. (2007). Chaperones as parts of cellular networks. *Adv Exp Med Biol, 594*, 55-63.

Dillin, A., Crawford, D. K., and Kenyon, C. (2002). Timing requirements for insulin/IGF-1 signaling in C. elegans. *Science, 298(5594)*, 830-4.

Duennwald, M. L. and Lindquist, S. (2008). Impaired ERAD and ER stress are early and specific events in polyglutamine toxicity. *Genes Dev, 22(23)*, 3308-19.

Durieux, J., Wolff, S., and Dillin, A. (2011). The cell-non-autonomous nature of electron transport chain-mediated longevity. *Cell, 144(1)*, 79-91.

Eisenberg, T. et al. (2009). Induction of autophagy by spermidine promotes longevity. *Nat Cell Biol, 11(11)*, 1305-14.

El-Ami, T., Moll, L., Carvalhal Marques, F., Volovik, Y., Reuveni, H., and Cohen E. (2014). A novel inhibitor of the insulin/IGF signaling pathway protects from age-onset, neurodegeneration-linked proteotoxicity. *Aging Cell, 13(1)*, 165-74.

Ellis, R. J. (2007). chaperones, Protein misassembly: macromolecular crowding and molecular. *Adv Exp Med Biol, 594*, 1-13.

Ermolaeva, M. A., Segref, A., Dakhovnik, A., Ou, H. L., Schneider, J. I., Utermöhlen, O., Hoppe, T. and Schumacher, B. (2013). DNA damage in germ cells induces an innate immune response that triggers systemic stress resistance. *Nature, 501(7467)*, 416-20.

Feng, J., Bussière, F., and Hekimi, S. (2001). Mitochondrial electron transport is a key determinant of life span in Caenorhabditis elegans. *Dev Cell, 1(5)*, 633-44.

Fink, A. L. (1999). Chaperone-mediated protein folding. *Physiol Rev, 79(2)*, 425-49.

Fire, A. Z. (2007). Gene silencing by double-stranded RNA. *Cell Death Differ, 14(12)*, 1998-2012.

Flatt, T., Min, K. J., D'Alterio, C., Villa-Cuesta, E., Cumbers, J., Lehmann, R., Jones, D. L., and Tatar, M. (2008). Drosophila germ-line modulation of insulin signaling and lifespan. *Proc Natl Acad Sci U S A, 105(17)*, 6368-73.

Florez-McClure, M. L., Hohsfield, L. A., Fonte, G., Bealor, M. T., and Link, C. D. (2007). Decreased insulin-receptor signaling promotes the autophagic degradation of beta-amyloid peptide in C. elegans. *Autophagy, 3(6)*, 569-80.

Fontana, L., Partridge, L., and Longo, V. D. (2010). Extending healthy life span--from yeast to humans. *Science, 328(5976)*, 321-6.

Freude, S., Hettich, M. M., Schumann, C., Stöhr, O., Koch, L., Köhler, C., Udelhoven, M., Leeser, U., Müller, M., Kubota, N., Kadowaki, T., Krone W, Schröder H, Brüning JC, Schubert M. (2009). Neuronal IGF-1 resistance reduces Abeta accumulation and protects against premature death in a model of Alzheimer's disease. *FASEB J, 23(10)*, 3315-24.

Friedman, D. B. and Johnson, T. E. (1988). Three mutants that extend both mean and maximum life span of the nematode, Caenorhabditis elegans, define the age-1 gene. *J Gerontol, 43(4)*, B102-9.

Furuyama, T., Nakazawa, T., Nakano, I., and Mori, N. (2000). Identification of the differential distribution patterns of mRNAs and consensus binding sequences for mouse DAF-16 homologues. *Biochem J, 349(Pt 2):*, 629-34.

Garcia, S. M., Casanueva, M. O., Silva, M. C., Amaral, M. D., and Morimoto, R. I. (2007). Neuronal signaling modulates protein homeostasis in Caenorhabditis elegans post-synaptic muscle cells. *Genes Dev, 21(22)*, 3006-16.

Garsin, D. A., Villanueva, J. M., Begun, J., Kim, D. H., Sifri, C. D., Calderwood, S. B., Ruvkun, G., and Ausubel, F. M. (2003). Long-lived C. elegans daf-2 mutants are resistant to bacterial pathogens. *Science, 300(5627)*, 1921.

Gestwicki, J. E. and Garza, D. (2012). Protein quality control in neurodegenerative disease. *Prog Mol Biol Transl Sci, 107*, 327-53.

Giannakou, M. E., Goss, M., Jünger, M. A., Hafen, E., Leevers, S. J., and Partridge, L. (2004). Long-lived Drosophila with overexpressed dFOXO in adult fat body. *Science, 305(5682)*, 361.

Gidalevitz, T., Ben-Zvi, A., Ho, K. H., Brignull, H. R. and Morimoto, R. I. (2006). Progressive disruption of cellular protein folding in models of polyglutamine diseases. *Science, 311(5766)*, 1471-4.

Gidalevitz, T., Prahlad, V. and Morimoto, R. I. (2011). The stress of protein misfolding: from single cells to multicellular organisms. *Cold Spring Harb Perspect Biol, 3(6)*.

Goldberg, A. L.. (2003). Protein degradation and protection against misfolded or damaged proteins. *Nature, 426(6968)*, 895-9.

GuhaThakurta, D., Palomar, L., Stormo, G. D., Tedesco, P., Johnson, T. E., Walker, D. W., Lithgow, G., Kim, S., and Link, C. D. (2002). Identification of a novel cis-regulatory element involved in the heat shock response in Caenorhabditis elegans using microarray gene expression and computational methods. *Genome Res, 12(5)*, 701-12.

Gupta, S., Deepti, A., Deegan, S., Lisbona, F., Hetz, C. and Samali, A. (2010). HSP72 protects cells from ER stress-induced apoptosis via enhancement of IRE1alpha-XBP1 signaling through a physical interaction. *PLoS Biol*.

Hamilton, B., Dong, Y., Shindo, M., Liu, W., Odell, I., Ruvkun, G., and Lee, S. S. (2005). A systematic RNAi screen for longevity genes in C. elegans. *Genes Dev, 19(13)*, 1544-55.

Harman, D. (1956). Aging: a theory based on free radical and radiation chemistry. *J Gerontol, 11(3)*, 298-300.

Harrison, D. E., Strong, R., Sharp, Z. D., Nelson, J. F., Astle, C. M., Flurkey, K., Nadon, N. L., Wilkinson, J. E., Frenkel, K., Carter, C. S., Pahor, M., Javors, M. A., Fernandez, E. and Miller, R. A. (2009).

Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. *Nature, 460(7253)*, 392-5.

Hars, E. S., Qi, H., Ryazanov, A. G., Jin, S., Cai, L., Hu, C. and Liu, L. F. (2007). Autophagy regulates ageing in C. elegans. *Autophagy, 3(2)*, 93-5.

Hartl, F. U., Bracher, A. and Hayer-Hartl, M.. (2011). Molecular chaperones in protein folding and proteostasis. *Nature, 475(7356)*, 324-32.

Haynes, C. M. and Ron, D. (2010). The mitochondrial UPR - protecting organelle protein homeostasis. *J Cell Sci, 123(Pt 22)*, 3849-55.

Hekimi, S., Lapointe, J., and Wen, Y. (2011). Taking a "good" look at free radicals in the aging process. *Trends Cell Biol, 21(10)*, 569-76.

Henis-Korenblit, S., Zhang, P., Hansen, M., McCormick, M., Lee, S. J., Cary, M., and Kenyon, C. (2010). Insulin/IGF-1 signaling mutants reprogram ER stress response regulators to promote longevity. *Proc Natl Acad Sci U S A, 107(21)*, 9730-5.

Hinault, M. P. and Goloubinoff, P. (2007). Molecular crime and cellular punishment: active detoxification of misfolded and aggregated proteins in the cell by the chaperone and protease networks. *Adv Exp Med Biol, 594*, 47-54.

Hipp, M. S., Park, S. H., and Hartl, F. U. (2014). Proteostasis impairment in protein-misfolding and -aggregation diseases. *Trends Cell Biol, 24(9)*, 506-14.

Hobert, O., D'Alberti, T., Liu, Y., and Ruvkun, G. (1998). Control of neural development and function in a thermoregulatory network by the LIM homeobox gene lin-11. *J Neurosci, 18(6)*, 2084-96.

Holzenberger, M., Dupont, J., Ducos, B., Leneuve, P., Géloën, A., Even, P. C., Cervera, P., and Le Bouc, Y. (2003). IGF-1 receptor regulates lifespan and resistance to oxidative stress in mice. *Nature, 421(6919)*, 182-7.

Honda, Y. and 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(11)*, 1385-93.

Hsin, H. and Kenyon, C. (1999). Signals from the reproductive system regulate the lifespan of C. elegans. *Nature, 399(6734)*, 362-6.

Hsu, A. L., Murphy, C. T., and Kenyon, C. (2003). Regulation of aging and age-related disease by DAF-16 and heat-shock factor. *Science, 300(5622)*, 1142-5.

Hwangbo, D. S., Gershman, B., Tu, M. P., Palmer, M., and Tatar, M. (2004). Drosophila dFOXO controls lifespan and regulates insulin signalling in brain and fat body. *Nature, 429(6991)*, 562-6.

Inada, H., Ito, H., Satterlee, J., Sengupta, P., Matsumoto, K., and Mori, I. (2006). Identification of guanylyl cyclases that function in thermosensory neurons of Caenorhabditis elegans. *Genetics, 172(4)*, 2239-52.

Iser, W. B., Gami, M. S., and Wolkow, C. A. (2007). Insulin signaling in Caenorhabditis elegans regulates both endocrine-like and cell-autonomous outputs. *Dev Biol, 303(2)*, 434-47.

Jankowsky, J. L., Fadale, D. J., Anderson, J., Xu, G. M., Gonzales, V., Jenkins, N. A., Copeland, N. G., Lee, M. K., Younkin, L. H., Wagner, S. L., Younkin, S. G., Borchelt, D. R. (2004). Mutant

presenilins specifically elevate the levels of the 42 residue beta-amyloid peptide in vivo: evidence for augmentation of a 42-specific gamma secretase. *Hum Mol Genet, 13(2)*, 159-70.

Jankowsky, J. L., Slunt, H. H., Ratovitski, T., Jenkins, N. A., Copeland, N. G., and Borchelt, D. R. (2001). Co-expression of multiple transgenes in mouse CNS: a comparison of strategies. *Biomol Eng, 17(6)*, 157-65.

Jovaisaite, V., Mouchiroud, L. and Auwerx, J. (2014). The mitochondrial unfolded protein response, a conserved stress response pathway with implications in health and disease. *J Exp Biol, 217(Pt 1)*, 137-43.

Jungwirth, M., Dear, M. L., Brown, P., Holbrook, K., and Goodchild, R. (2010). Relative tissue expression of homologous torsinB correlates with the neuronal specific importance of DYT1 dystonia-associated torsinA. *Hum Mol Genet, 19(5)*, 888-900.

Kahn, N. W., Rea, S. L., Moyle, S., Kell, A., and Johnson, T. E. (2008). Proteasomal dysfunction activates the transcription factor SKN-1 and produces a selective oxidative-stress response in Caenorhabditis elegans. *Biochem J, 409(1)*, 205-13.

Kakkar, V., Meister-Broekema, M., Minoia, M., Carra, S. and Kampinga, H. H. (2014). Barcoding heat shock proteins to human diseases: looking beyond the heat shock response. *Dis Model Mech, 7(4)*, 421-34.

Kamath, R. S., Fraser, A. G., Dong, Y., Poulin, G., Durbin, R., Gotta, M., Kanapin, A., Le Bot, N., Moreno, S., Sohrmann, M., Welchman, D. P., Zipperlen, P., and Ahringer, J. (2003). Systematic functional analysis of the Caenorhabditis elegans genome using RNAi. *Nature, 421(6920)*, 231-7.

Kenyon, C. (2011). The first long-lived mutants: discovery of the insulin/IGF-1 pathway for ageing. *Philos Trans R Soc Lond B Biol Sci, 366(1561)*, 9-16.

Kenyon, C., Chang, J., Gensch, E., Rudner, A., and Tabtiang, R. (1993). A C. elegans mutant that lives twice as long as wild type. *Nature, 366(6454)*, 461-4.

Killick, R., Scales, G., Leroy, K., Causevic, M., Hooper, C., Irvine, E. E., Choudhury, A. I., Drinkwater, L., Kerr, F., Al-Qassab, H., Stephenson, J., Yilmaz, Z., Giese, K. P., Brion, J. P., Withers, D. J., and Lovestone, S. (2009). Deletion of Irs2 reduces amyloid deposition and rescues behavioural deficits in APP transgenic mice. *Biochem Biophys Res Commun, 386(1)*, 257-62.

Kimura, K. D., Tissenbaum, H. A., Liu, Y., and Ruvkun, G. (1997). daf-2, an insulin receptor-like gene that regulates longevity and diapause in Caenorhabditis elegans. *Science, 277(5328)*, 942-6.

Knight, A. L., Yan, X., Hamamichi, S., Ajjuri, R. R., Mazzulli, J. R., Zhang, M. W., Daigle, J. G., Zhang, S., Borom, A. R., Roberts, L. R., Lee, S. K., DeLeon, S. M., Viollet-Djelassi, C., Krainc, D., O'Donnell, J. M., Caldwell, K. A., and Caldwell, G.A. (2014). The glycolytic enzyme, GPI, is a functionally conserved modifier of dopaminergic neurodegeneration in Parkinson's models. *Cell Metab, 20(1)*, 145-57.

Koga, H., Kaushik, S. and Cuervo, A. M. (2010). Inhibitory effect of intracellular lipid load on macroautophagy. *Autophagy, 6(6)*, 825-7.

Korennykh, A. and Walter, P. (2012). Structural basis of the unfolded protein response. *Annu Rev Cell Dev Biol, 28*, 251-77.

Kruegel, U., Robison, B., Dange, T., Kahlert, G., Delaney, J. R., Kotireddy, S., Tsuchiya, M., Tsuchiyama, S., Murakami, C. J., Schleit, J., Sutphin, G., Carr, D., Tar, K., Dittmar, G., Kaeberlein, M.,

Kennedy, B. K. and Schmidt, M. (2011). Elevated proteasome capacity extends replicative lifespan in Saccharomyces cerevisiae. *PLoS Genet, 7(9)*.

Labbadia, J. and Morimoto, R. I. (2014). Proteostasis and longevity: when does aging really begin? *F1000Prime Rep.*

Lecker, S. H., Goldberg, A. L., and Mitch, W. E. (2006). Protein degradation by the ubiquitin-proteasome pathway in normal and disease states. *J Am Soc Nephrol, 17(7)*, 1807-19.

Lee, S, S., Lee, R. Y., Fraser, A. G., Kamath, R. S., Ahringer, J., and Ruvkun, G. (2003). A systematic RNAi screen identifies a critical role for mitochondria in C. elegans longevity. *Nat Genet, 33(1)*, 40-8.

Leroux, M. R., Ma, B. J., Batelier, G., Melki, R., and Candido, E. P. (1997). Unique structural features of a novel class of small heat shock proteins. *J Biol Chem, 272(19)*, 12847-53.

Li, J., Tewari, M., Vidal, M., and Lee, S. S. (2007). The 14-3-3 protein FTT-2 regulates DAF-16 in Caenorhabditis elegans. *Dev Biol, 301(1)*, 82-91.

Li, X., Matilainen, O., Jin, C., Glover-Cutter, K. M., Holmberg, C. I., and Blackwell, T. K. (2011). Specific SKN-1/Nrf stress responses to perturbations in translation elongation and proteasome activity. *PLoS Genet, 7(6)*.

Libina, N., Berman, J. R., and Kenyon, C. (2003). Tissue-specific activities of C. elegans DAF-16 in the regulation of lifespan. *Cell, 115(4)*, 489-502.

Lin, K., Dorman, J. B., Rodan, A., and Kenyon, C. (1997). daf-16: An HNF-3/forkhead family member that can function to double the life-span of Caenorhabditis elegans. *Science, 278(5341)*, 1319-22.

Lin, K., Hsin, H., Libina, N., and Kenyon, C. (2001). Regulation of the Caenorhabditis elegans longevity protein DAF-16 by insulin/IGF-1 and germline signaling. *Nat Genet, 28(2)*, 139-45.

Lindquist, S. and Craig, E. A. (1988). The heat-shock proteins. *Annu Rev Genet, 22*, 631-77.

Link, C. D. (1995). Expression of human beta-amyloid peptide in transgenic Caenorhabditis elegans. *Proc Natl Acad Sci U S A, 92(20)*, 9368-72.

Link, C. D., Cypser, J. R., Johnson, C. J., and Johnson, T. E. (1999). Direct observation of stress response in Caenorhabditis elegans using a reporter transgene. *Cell Stress Chaperones, 4(4)*, 235-42.

Lithgow, G. J., White, T. M., Melov, S., and Johnson, T. E. (1995). Thermotolerance and extended life-span conferred by single-gene mutations and induced by thermal stress. *Proc Natl Acad Sci U S A, 92(16)*, 7540-4.

Liu, G., Rogers, J., Murphy, C. T., and Rongo, C. (2011). EGF signalling activates the ubiquitin proteasome system to modulate C. elegans lifespan. *EMBO J, 30(15):*, 2990-3003.

Longo, V. D., Mitteldorf, J., and Skulachev, V. P. (2005). Programmed and altruistic ageing. *Nat Rev Genet, 6(11)*, 866-72.

López-Otín, C., Blasco, M. A., Partridge, L., Serrano, M. and Kroemer, G. (2013). The hallmarks of aging. *Cell, 153(6)*, 1194-217.

Ma, X. and Shen, Y. (2012). Structural basis for degeneracy among thermosensory neurons in Caenorhabditis elegans. *J Neurosci, 32(1)*, 1-3.

Mair, W. and Dillin, A. (2008). Aging and survival: the genetics of life span extension by dietary restriction. *Annu Rev Biochem, 77*, 727-54.

Majeski, A. E. and Dice, J. F. (2004). Mechanisms of chaperone-mediated autophagy. *Int J Biochem Cell Biol, 36(12)*, 2435-44.

Maman, M., Carvalhal Marques, F., Volovik, Y., Dubnikov, T., and Bejerano-Sagie, M, Cohen E. (2013). A neuronal GPCR is critical for the induction of the heat shock response in the nematode C. elegans. *J Neurosci, 33(14)*, 6102-11.

Martin, G. M., Austad, S. N., and Johnson, T. E. (1996). Genetic analysis of ageing: role of oxidative damage and environmental stresses. *Nat Genet, 13(1)*, 25-34.

Martinez-Vicente, M., Sovak, G., Cuervo, A. M. (2005). Protein degradation and aging. *Exp Gerontol, 40(8-9)*, 622-33.

Matilainen, O., Arpalahti, L., Rantanen, V., Hautaniemi, S., and Holmberg, C. I. (2013). Insulin/IGF-1 signaling regulates proteasome activity through the deubiquitinating enzyme UBH-4. *Cell Rep, 3(6)*, 1980-95.

Matsumoto, M., Kurihara, S., Kibe, R., Ashida, H., and Benno, Y. (2011). Longevity in mice is promoted by probiotic-induced suppression of colonic senescence dependent on upregulation of gut bacterial polyamine production. *PLoS One, 6(8)*, e23652.

Mattoo, R. U. and Goloubinoff, P. (2014). Molecular chaperones are nanomachines that catalytically unfold misfolded and alternatively folded proteins. *Cell Mol Life Sci, 71(17)*, 3311-25.

McCay, C. M., Crowell, M. F., and Maynard, L. A. (1989). The effect of retarded growth upon the length of life span and upon the ultimate body size. 1935. *Nutrition, 5(3)*, 155-71.

McColl, G., Roberts, B. R., Gunn, A. P., Perez, K. A., Tew, D. J., Masters, C. L., Barnham, K. J., Cherny, R. A., and Bush, A. I. (2009). The Caenorhabditis elegans A beta 1-42 model of Alzheimer disease predominantly expresses A beta 3-42. *J Biol Chem, 284(34)*, 22697-702.

McLean, P. J., Kawamata, H., Shariff, S., Hewett, J., Sharma, N., Ueda, K., Breakefield, X. O., and Hyman, B. T. (2002). TorsinA and heat shock proteins act as molecular chaperones: suppression of alpha-synuclein aggregation. *J Neurochem, 83(4)*, 846-54.

Min, J. N., Whaley, R. A., Sharpless, N. E., Lockyer, P., Portbury, A. L., and Patterson, C. (2008). CHIP deficiency decreases longevity, with accelerated aging phenotypes accompanied by altered protein quality control. *Mol Cell Biol, 28(12)*, 4018-25.

Mitteldorf, J.. (2001). Can current evolutionary theory explain experimental data on aging? *Sci Aging Knowledge Environ*.

Mizushima, N., Levine, B., Cuervo, A. M., and Klionsky, D. J. (2008). Autophagy fights disease through cellular self-digestion. *Nature, 451(7182)*, 1069-75.

Mori, I. and Ohshima, Y. (1995). Neural regulation of thermotaxis in Caenorhabditis elegans. *Nature, 376(6538)*, 344-8.

Morimoto, R. I. (1998). Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. *Genes Dev, 12(24)*, 3788-96.

Morimoto, R. I. and Cuervo, A. M. (2014). Proteostasis and the aging proteome in health and disease. *J Gerontol A Biol Sci Med Sci, 69*.

Morley, J. F. and Morimoto, R. I. (2004). Regulation of longevity in Caenorhabditis elegans by heat shock factor and molecular chaperones. *Mol Biol Cell, 15(2)*, 657-64.

Morley, J. F., Brignull, H. R., Weyers, J. J., and Morimoto, R. I. (2002). The threshold for polyglutamine-expansion protein aggregation and cellular toxicity is dynamic and influenced by aging in Caenorhabditis elegans. *Proc Natl Acad Sci U S A, 99(16)*, 10417-22.

Morrow, G., Samson, M., Michaud, S., and Tanguay, R. M. (2004). Overexpression of the small mitochondrial Hsp22 extends Drosophila life span and increases resistance to oxidative stress. *FASEB J, 18(3)*, 598-9.

Murakami, S. and Johnson, T. E. (1996). A genetic pathway conferring life extension and resistance to UV stress in Caenorhabditis elegans. *Genetics, 143(3)*, 1207-18.

Murphy, C. T. and Hsu, P. J. (2013). Insulin/insulin-like growth factor signaling in C. elegans. *WormBook, 26*, 1-43.

Murphy, C. T., McCarroll, S. A., Bargmann, C. I., Fraser, A., Kamath, R. S., Ahringer, J., Li, H., and Kenyon, C. (2003). Genes that act downstream of DAF-16 to influence the lifespan of Caenorhabditis elegans. *Nature, 424(6946)*, 277-83.

Nery, F. C., Armata, I. A., Farley, J. E., Cho, J. A., Yaqub, U., Chen, P., da Hora, C. C., Wang, Q., Tagaya, M., Klein, C., Tannous, B., Caldwell, K. A., Caldwell, G. A., Lencer, W. I., Ye, Y., and Breakefield, X. O. (2011). TorsinA participates in endoplasmic reticulum-associated degradation. *Nat Commun, 2*, 393.

Neuwald, A. F., Aravind, L., Spouge, J. L., and Koonin, E. V. (1999). AAA+: A class of chaperone-like ATPases associated with the assembly, operation, and disassembly of protein complexes. *Genome Res, 9(1)*, 27-43.

Nussbaum-Krammer, C. I. and Morimoto, R. I. (2014). Caenorhabditis elegans as a model system for studying non-cell-autonomous mechanisms in protein-misfolding diseases. *Dis Model Mech, 7(1)*, 31-9.

Ogg, S., Paradis, S., Gottlieb, S., Patterson, G. I., Lee, L., Tissenbaum, H. A., and Ruvkun, G. (1997). The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in C. elegans. *Nature, 389(6654)*, 994-9.

Oh, K. H. and Kim, H. (2013). Reduced IGF signaling prevents muscle cell death in a Caenorhabditis elegans model of muscular dystrophy. *Proc Natl Acad Sci U S A, 110(47)*, 19024-9.

Oliveira, R. P., Porter Abate, J., Dilks, K., Landis, J., Ashraf, J., Murphy, C. T., and Blackwell, T. K. (2009). Condition-adapted stress and longevity gene regulation by Caenorhabditis elegans SKN-1/Nrf. *Aging Cell, 8(5)*, 524-41.

Olsen, A., Vantipalli, M. C., and Lithgow, G. J. (2006). Using Caenorhabditis elegans as a model for aging and age-related diseases. *Ann N Y Acad Sci, 1067*, 120-8.

O'Rourke, E. J. and Ruvkun, G. (2013). MXL-3 and HLH-30 transcriptionally link lipolysis and autophagy to nutrient availability. *Nat Cell Biol, 15(6)*, 668-76.

Ortega, Z. and Lucas, J. J. (2014). Ubiquitin-proteasome system involvement in Huntington's disease. *Front Mol Neurosci*.

Ozelius, L. J., Hewett, J. W., Page, C. E., Bressman, S. B., Kramer, P. L., Shalish, C., de Leon, D., Brin, M. F., Raymond, D., Corey, D. P., Fahn, S., Risch, N. J., Buckler, A. J., Gusella, J. F., and Breakefield, X. O. (1997). The early-onset torsion dystonia gene (DYT1) encodes an ATP-binding protein. *Nat Genet, 17(1)*, 40-8.

Paek, J., Lo, J. Y., Narasimhan, S. D., Nguyen, T. N., Glover-Cutter, K., Robida-Stubbs, S., Suzuki, T., Yamamoto, M., Blackwell, T. K., and Curran, S. P. (2012). Mitochondrial SKN-1/Nrf mediates a conserved starvation response. *Cell Metab, 16(4)*, 526-37.

Panowski, S. H. and Dillin, A. (2009). Signals of youth: endocrine regulation of aging in Caenorhabditis elegans. *Trends Endocrinol Metab, 20(6)*, 259-64.

Prahlad, V. and Morimoto, R. I. (2011). Neuronal circuitry regulates the response of Caenorhabditis elegans to misfolded proteins. *Proc Natl Acad Sci U S A., 108(34)*, 14204-9.

Prahlad, V., Cornelius, T., and Morimoto, R. I. (2008). Regulation of the cellular heat shock response in Caenorhabditis elegans by thermosensory neurons. *Science, 320(5877)*, 811-4.

Qadota, H., Inouem, M., Hikita, T., Köppen, M., Hardin, J. D., Amano, M., Moerman, D. G., and Kaibuchi, K. (2007). Establishment of a tissue-specific RNAi system in C. elegans. *Gene, 400(1-2)*, 166-73.

Reiserer, R. S., Harrison, F. E., Syverud, D. C., and McDonald, M. P. (2007). Impaired spatial learning in the APPSwe + PSEN1DeltaE9 bigenic mouse model of Alzheimer's disease. *Genes Brain Behav, 6(1)*, 54-65.

Rodriguez, M., Snoek, L. B., and De Bono, M., and Kammenga, J. E. (2013). Worms under stress: C. elegans stress response and its relevance to complex human disease and aging. *Trends Genet, 29(6)*, 367-74.

Ron, D. and Walter, P. (2007). Signal integration in the endoplasmic reticulum unfolded protein response. *Nat Rev Mol Cell Biol, 8(7)*, 519-29.

Rual, J. F., Ceron, J., Koreth, J., Hao, T., Nicot, A. S., Hirozane-Kishikawa, T., Vandenhaute, J., Orkin, S. H., Hill, D. E., van den Heuvel, S., and Vidal, M. (2004). Toward improving Caenorhabditis elegans phenome mapping with an ORFeome-based RNAi library. *Genome Res, 14(10B)*, 2162-8.

Rubinsztein, D. C., Mariño, G., and Kroemer, G. (2011). Autophagy and aging. *Cell, 146(5)*, 682-95.

Russell, S. J. and Kahn, C. R. (2007). Endocrine regulation of ageing. *Nat Rev Mol Cell Biol, 8(9)*, 681-91.

Saez. I. and Vilchez, D. (2014). The Mechanistic Links Between Proteasome Activity, Aging and Age-related Diseases. *Curr Genomics, 15(1)*, 38-51.

Salminen, A. and Kaarniranta, K. (2009). Regulation of the aging process by autophagy. *Trends Mol Med, 15(5)*, 217-24.

Samuelson, A. V., Klimczak, R. R., Thompson, D. B., Carr, C. E., and Ruvkun, G. (2007). Identification of Caenorhabditis elegans genes regulating longevity using enhanced RNAi-sensitive strains. *Cold Spring Harb Symp Quant Biol, 72*, 489-97.

Scerbak, C., Vayndorf, E. M., Parker, J. A., Neri, C., Driscoll, M., and Taylor, B. E. (2014). Insulin signaling in the aging of healthy and proteotoxically stressed mechanosensory neurons. *Front Genet*.

Shaner, N. C., Campbell, R. E., Steinbach, P. A., Giepmans, B. N., Palmer, A. E., and Tsien, R. Y. (2004). Improved monomeric red, orange and yellow fluorescent proteins derived from Discosoma sp. red fluorescent protein. *Nat Biotechnol, 22(12)*, 1567-72.

Shang, F. and Taylor, A. (2004). Function of the ubiquitin proteolytic pathway in the eye. *Exp Eye Res, 78(1)*, 1-14.

Sharma, N., Hewett, J., Ozelius, L. J., Ramesh, V., McLean, P. J., Breakefield, X. O., and Hyman, B. T. (2011). A close association of torsinA and alpha-synuclein in Lewy bodies: a fluorescence resonance energy transfer study. *Am J Pathol, 159(1)*, 339-44.

Shashidharan, P., Good, P. F., Hsu, A., Perl, D. P., Brin, M. F., and Olanow, C. W. (2000). TorsinA accumulation in Lewy bodies in sporadic Parkinson's disease. *Brain Res, 877(2)*, 379-81.

Shemesh, N., Shai, N., and Ben-Zvi, A. (2013). Germline stem cell arrest inhibits the collapse of somatic proteostasis early in Caenorhabditis elegans adulthood. *Aging Cell, 12(5)*, 814-22.

Simonsen, A., Cumming, R. C., Brech, A., Isakson, P., Schubert, D. R. and Finley, K. D. (2008). Promoting basal levels of autophagy in the nervous system enhances longevity and oxidant resistance in adult Drosophila. *Autophagy, 4(2)*, 176-84.

Sin, O., Michels, H., and Nollen, E. A. (2014). Genetic screens in Caenorhabditis elegans models for neurodegenerative diseases. *Biochim Biophys Acta, 1842(10)*, 1951-1959.

Singh, V. and Aballay, A. (2006). Heat shock and genetic activation of HSF-1 enhance immunity to bacteria. *Cell Cycle, 5(21)*, 2443-6 (a).

Singh, V. and Aballay, A. (2006). Heat-shock transcription factor (HSF)-1 pathway required for Caenorhabditis elegans immunity. *Proc Natl Acad Sci U S A, 103(35)*, 13092-7 (b).

Smith, H. L., Li, W., and Cheetham, M. E. (2015). Molecular chaperones and neuronal proteostasis. *Semin Cell Dev Biol, 40*, 142-152.

Snutch, T. P., Heschl, M. F., and Baillie, D. L. (1988). The Caenorhabditis elegans hsp70 gene family: a molecular genetic characterization. *Gene, 64(2)*, 241-55.

Soda, K., Dobashi, Y., Kano, Y., Tsujinaka, S., and Konishi, F. (2009). Polyamine-rich food decreases age-associated pathology and mortality in aged mice. *Exp Gerontol, 44(11)*, 727-32.

Stefani, M. and Dobson, C. M. (2003). Protein aggregation and aggregate toxicity: new insights into protein folding, misfolding diseases and biological evolution. *J Mol Med (Berl), 81(11)*, 678-99.

Stiernagle, T. (2006). Maintenance of C. elegans. *WormBook, 11*, 1-11.

Sugi, T., Nishida, Y., and Mori, I. (2011). Regulation of behavioral plasticity by systemic temperature signaling in Caenorhabditis elegans. *Nat Neurosci, 14(8)*, 984-92.

Sun, J., Singh, V., Kajino-Sakamoto, R., and Aballay, A. (2011). Neuronal GPCR controls innate immunity by regulating noncanonical unfolded protein response genes. *Science, 332(6030)*, 729-32.

Swindell, W. R., Masternak, M. M., Kopchick JJ, Conover CA, Bartke A, Miller RA. (2009). Endocrine regulation of heat shock protein mRNA levels in long-lived dwarf mice. *Mech Ageing Dev, 130(6)*, 393-400.

Takalo, M., Salminen, A., Soininen, H., Hiltunen, M., and Haapasalo, A. (2013). Protein aggregation and degradation mechanisms in neurodegenerative diseases. *Am J Neurodegener Dis, 2(1)*, 1-14.

Tatum, M. C., Ooi, F. K., Chikka, M. R., Chauve, L., Martinez-Velazquez, L. A., Steinbusch, H. W., Morimoto, R. I. and Prahlad, V. (2015). Neuronal serotonin release triggers the heat shock response in C. elegans in the absence of temperature increase. *Curr Biol, 25(2)*, 163-74.

Taylor, R. C. and Dillin, A. (2011). Aging as an event of proteostasis collapse. *Cold Spring Harb Perspect Biol., 3(5)*.

Taylor, R. C. and Dillin, A. (2013). XBP-1 is a cell-nonautonomous regulator of stress resistance and longevity. *Cell, 153(7)*, 1435-47.

Thompson, M. L., Chen, P., Yan, X., Kim, H., Borom, A. R., Roberts, N. B., Caldwell, K. A., and Caldwell, G. A. (2014). TorsinA rescues ER-associated stress and locomotive defects in C. elegans models of ALS. *Dis Model Mech, 7(2)*, 233-43.

Timmons, L., Court, D. L., and Fire, A. (2001). Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in Caenorhabditis elegans. *Gene, 263(1-2)*, 103-12.

Tomaru, U., Takahashi, S., Ishizu, A., Miyatake, Y., Gohda, A., Suzuki, S., Ono, A., Ohara, J., Baba, T., Murata, S., Tanaka, K. and Kasahara, M. (2010). Decreased proteasomal activity causes age-related phenotypes and promotes the development of metabolic abnormalities. *Am J Pathol, 180(3)*, 963-72.

Tullet, J. M., Hertweck, M., An, J. H., Baker, J., Hwang, J. Y., Liu, S., Oliveira, R. P., Baumeister, R., and Blackwell, T. K. (2008). Direct inhibition of the longevity-promoting factor SKN-1 by insulin-like signaling in C. elegans. *Cell, 132(6)*, 1025-38.

van Oosten-Hawle, P. and Morimoto, R. I. (2014). Transcellular chaperone signaling: an organismal strategy for integrated cell stress responses. *J Exp Biol, 217(Pt 1)*, 129-36.

van Oosten-Hawle, P., Porter, R. S., and Morimoto, R. I. (2013). Regulation of organismal proteostasis by transcellular chaperone signaling. *Cell, 153(6)*, 1366-78.

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

Van Raamsdonk, J. M. and Hekimi, S. (2012). Superoxide dismutase is dispensable for normal animal lifespan. *Proc Natl Acad Sci U S A, 109(15)*, 5785-90.

Vellai, T., Takacs-Vellai, K., Zhang, Y., Kovacs, A. L., Orosz, L., and Müller, F. (2003). Genetics: influence of TOR kinase on lifespan in C. elegans. *Nature, 426(6967)*, 620.

Vilchez, D., Morantte, I., Liu, Z., Douglas, P. M., Merkwirth, C., Rodrigues, A. P., Manning, G., and Dillin, A. (2012). RPN-6 determines C. elegans longevity under proteotoxic stress conditions. *Nature, 489(7415)*, 263-8.

Vilchez, D., Saez, I. and Dillin, A. (2014). The role of protein clearance mechanisms in organismal ageing and age-related diseases. *Nat Commun*.

Voellmy, R. and Boellmann, F. (2007). Chaperone regulation of the heat shock protein response. *Adv Exp Med Biol, 594*, 89-99.

Volovik, Y., Maman, M., Dubnikov, T., Bejerano-Sagie, M., Joyce, D., Kapernick, E. A., Cohen, E., and Dillin, A. (2012). Temporal requirements of heat shock factor-1 for longevity assurance. *Aging Cell, 11(3)*, 491-9.

Volovik, Y., Marques, F. C., and Cohen, E. (2014). The nematode Caenorhabditis elegans: a versatile model for the study of proteotoxicity and aging. *Methods, 68(3)*, 458-64.

Volovik, Y., Moll, L., Marques, F. C., Maman, M., Bejerano-Sagie, M., and Cohen, E. (2014). Differential regulation of the heat shock factor 1 and DAF-16 by neuronal nhl-1 in the nematode C. elegans. *Cell Rep, 9(6)*, 2192-205.

Walker, G. A. and Lithgow, G. J. (2003). Lifespan extension in C. elegans by a molecular chaperone dependent upon insulin-like signals. *Aging Cell, 2(2):*, 131-9.

Weismann, A. (1889). *Essays upon heredity and kindred biological problems.* Oxford: Clarendon Press.

Wilkinson, J. E., Burmeister, L., Brooks, S. V., Chan, C. C., Friedline, S., Harrison DE, Hejtmancik, J. F., Nadon, N., Strong, R., Wood, L. K., Woodward, M. A., and Miller, R. A. (2012). Rapamycin slows aging in mice. *Aging Cell, 11(4)*, 675-82.

Winston, W. M., Molodowitch, C., and Hunter, C. P. (2002). Systemic RNAi in C. elegans requires the putative transmembrane protein SID-1. *Science, 295(5564)*, 2456-9.

Wolkow, C. A., Kimura, K. D., Lee, M. S., and Ruvkun, G. (2000). Regulation of C. elegans life-span by insulinlike signaling in the nervous system. *Science, 290(5489)*, 147-50.

Wong, E. and Cuervo, A. M. (2010). Integration of clearance mechanisms: the proteasome and autophagy. *Cold Spring Harb Perspect Biol, 2(12)*, a006734.

Yoneda, T., Benedetti, C., Urano, F., Clark, S. G., Harding, H. P. and Ron, D. (2004). Compartment-specific perturbation of protein handling activates genes encoding mitochondrial chaperones. *J Cell Sci, 117(Pt 18)*, 4055-66.

Zhang, C. and Cuervo, A. M. (2008). Restoration of chaperone-mediated autophagy in aging liver improves cellular maintenance and hepatic function. *Nat Med, 14(9)*, 959-65.

Zhang, P., Judy, M., Lee, S. J., and Kenyon, C. (2013). Direct and indirect gene regulation by a life-extending FOXO protein in C. elegans: roles for GATA factors and lipid gene regulators. *Cell Metab, 17(1)*, 85-100.

Cover: http://www.dicyt.com/viewItem.php?itemId=23324

APPENDIX

Table 1. Lifespans of CF512 and *daf-2*(*e1370*) mutant worms on *gtr-1* RNAi

Strain	RNAi treatment	Mean lifespan ± SEM (hours)	Number of animals /initial number	Mean lifespan % of control	P_{value} against control
CF512	EV	17.02 ± 0.42	114/124	-	-
	daf-16	14.41 ± 0.33	115/125	85%	1.94E-06
	gtr-1	16.19 ± 0.38	118/122	95%	0.128
e1370	EV	42.30 ± 1.80	64/120	-	-
	daf-16	26.24 ± 0.66	61/121	62%	2.47E-16
	gtr-1	39.74 ± 1.84	86/125	94%	0.321

Table 2. Lifespans of CF512, TU3335, and *daf-2*(*e1370*) mutant worms on *tor-2* RNAi

Strain	RNAi treatment	Mean lifespan ± SEM (hours)	Number of animals /initial number	Mean lifespan % of control	P_{value} against control
CF512	EV	22.40 ± 0.64	83/120	-	-
	daf-16	15.13 ± 0.53	82/120	68%	1.33E-15
	tor-2	20.65 ± 0.60	93/120	92%	2.52E-02
TU3335	EV	25.44 ± 0.35	83/120	-	-
	daf-16	25.79 ± 0.54	53/120	101%	2.89E-01
	tor-2	26.48 ± 0.53	65/120	104%	0.053
e1370	EV	46.33 ± 1.73	55/120	-	-
	daf-16	26.40 ± 0.51	93/120	56%	3.62E-25
	tor-2	47.82 ± 1.41	56/120	103%	0.5