

# Transcription factors regulating lifespan of *Caenorhabditis elegans*

Inaugural-Dissertation

zur

Erlangung des Doktorgrades

der Mathematisch-Naturwissenschaftlichen Fakultät

der Universität zu Köln

vorgelegt von

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Jahr der Veröffentlichung: 2021

Berichterstatter:

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Tag der Mündlichen Prüfung:

14.09.2021

## ABSTRACT

Aging is a gradual decline of physiologic function, that affects nearly all living organisms. An extensive body of research aims to unravel and understand the underlying processes and influences on aging. This research shows that aging is a complex process integrating numerous molecular pathways and biochemical interactions. Over the last decades, scientists have found conserved molecular pathways, as well as exogenous and endogenous stressors and small molecules, which regulate life span. Diet, single-gene mutations and tissues play interconnected roles in this process. The reproductive system is best known for sexual reproduction, but strongly impacts metabolism and longevity. Inhibition of germ line stem cell proliferation extends the lifespan of an organism, termed gonadal longevity. This interaction is extensively described in *Caenorhabditis elegans*, but may be conserved up to humans. Several mediators and pathways are identified and proposed to work in an integrated network enhancing homeostasis and prolonging life. Yet the map of this network is incomplete and many other factors remain to be elucidated. A comprehensive knowledge of this network could lead new approaches to attenuate the process of aging.

In this study, we use *Caenorhabditis elegans* to study gonadal longevity. Gonadal longevity can be induced by different modes, including temperature sensitive single-gene mutations that disrupt germline proliferation, such as *glp-1*/Notch, *mes-1*/tyrosine kinase or laser micro-surgical removal of the germline.

This study unravels the impact of two transcription factors on gonadal longevity. On the one hand, NHR-91, a homolog of NR6A, regulates *GLP-1/NOTCH* longevity, but not other modes of gonadal longevity. We show that loss of NHR-91 leads to a misregulation of fatty-acid desaturases and their products in *glp-1* mutants. On the other hand, ATF-5, a homolog of mammalian ATF4, is a transcriptional regulator of multiple modes of gonadal longevity. These findings indicate that the different modes of gonadal longevity can impact *Caenorhabditis elegans* through pathways or mediators that are not always

congruent. Through functional genomic screens of the ATF-5 transcriptome, we identify the ER-chaperone HSP-4 as a downstream target of ATF-5, required for lifespan extension. We further find that heat shock factor HSF-1 is an upstream regulator of ATF-5. Surprisingly, two kinases known to regulate ATF-5 expression, namely GCN-2 and PEK-1, play no obvious role in regulating gonadal longevity.

These studies indicate that gonadal longevity is governed by multiple transcription factors, with multiple modalities.

## ZUSAMMENFASSUNG

Das Altern, ein schrittweiser Verlust von physiologischen Funktionen, betrifft nahezu alle lebenden Organismen. Ein weites wissenschaftliches Feld beschäftigt sich die, dem Altern zugrundeliegenden, Prozesse und Einflüsse aufzudecken und zu verstehen. Die Alterungsforschung zeigt, dass Altern ein komplexer Prozess ist, der von zahlreichen molekularen Signalwege und biochemischen Interaktionen abhängig ist. In den letzten Jahrzehnten konnten konservierte evolutionär konservierte molekulare Netzwerke, exogene und endogene Stressoren und kleine Moleküle mit dem Altern in Verbindung gebracht werden. Heute wissen wir, dass im Alterungsprozess die Ernährung, Mutationen einzelner Gene und verschiedene Gewebe miteinander interagieren. Sexualorgane ermöglichen Fortpflanzung, stehen jedoch auch im Zusammenhang mit dem Stoffwechsel und dem Altern. Wird die Proliferation von Stammzellen der Keimbahn verhindert, so steigt die Lebensdauer. Dieser Zusammenhang wurde ausgiebig in *Caenorhabditis elegans* aufgezeigt, aber ist potentiell auch in Menschen nachweisbar. Zahlreiche Proteine und Signalwege vermitteln diesen Effekt und es wird vermutet, dass diese in einem regulatorischen Netzwerk stehen und die Homöostase unterstützen und das Leben verlängern. Bisher, so wird vermutet, sind jedoch nicht alle Mitspieler in diesem Netzwerk bekannt. Die Vervollständigung des Netzwerkes und der darin liegenden Zusammenhänge kann zu neuen Ansätzen führen den Prozess des Alterns zu verlangsamen oder negative Begleiterscheinungen abzumildern. In dieser Studie wurde *Caenorhabditis elegans* verwendet um den Einfluss der Keimbahn auf die Lebensdauer zu erforschen. Darüber hinaus kann der positive Einfluss der Keimbahn, durch Verlust der Stammzellen, auf die Lebensdauer durch verschiedene Interventionen induziert werden. Dazu zählen temperatursensitive Mutanten welche die Keimbahnproliferation unterbrechen, wie *glp-1/Notch*, *mes-1/tyrosine kinase* oder die laser-mikroskopische Entfernung der Keimbahn.

Diese Studie erforscht den Einfluss von zwei Transkriptionsfaktoren auf die Lebensdauer im Kontext der Keimbahnstammzellenablation. Auf der einen Seite steht NHR-91, ein Homolog von NR6A1, dass die Langlebigkeit von

*GLP-1/NOTCH* Mutanten beeinflusst, aber nicht die Langlebigkeit von anderen Interventionen der Keimbahnstammzellenablation. Es wird aufgezeigt, dass der Verlust von NHR-91 zu einer Fehlregulation von Fettsäuredesaturasen und ihrer Produkte in *GLP-1/NOTCH* Mutanten führt. Auf der anderen Seite steht ATF-5, ein Homolog von ATF4, welches diverse Interventionen der Langlebigkeit nach Keimbahnstammzellenablation beeinflusst. Diese Ergebnisse zeigen, dass die verschiedenen Interventionen zur Langlebigkeit nach Keimbahnstammzellenablation in *Caenorhabditis elegans* durch Signalwege oder Mediatoren reguliert werden, die nicht immer kongruent sind. Durch funktionelle, genomische Screenings des ATF-5 Transkriptom, konnten wir das ER-Chaperon HSP-4 als Mediator von ATF-5 identifizieren, welches für seinen positiven physiologischen Effekt auf Langlebigkeit benötigt wird. Darüber hinaus konnten wir zeigen, dass der heat shock factor HSF-1 wiederum ATF-5 reguliert. Überraschenderweise spielen zwei Kinasen, welche die ATF-5 Expression regulieren, namentlich GCN-2 und PEK-1, keine offensichtliche Rolle in der Regulation von Langlebigkeit nach Keimbahnstammzellenablation.

Diese Ergebnisse zeigen, dass Langlebigkeit nach Keimbahnstammzellenablation durch diverse Transkriptionsfaktoren beeinflusst wird und dieser Einfluss sich nach Art und Weise der Intervention zur Induktion dieser Langlebigkeit unterscheidet.

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# **1**

## **Introduction**

## 1.1 Genetics of Longevity and Aging

Aging is a gradual decline of cellular and organism-wide functions that ultimately lead to mortality. For a long time, aging was seen as a random and uncontrolled loss of viability. Our current understanding is that certain longevity assurance factors are critical to maintain cellular functions. During aging these factors undergo a decrease in their anti-aging capabilities, which leads to a decrease in organism health. The body is no longer able to counteract deleterious endogenous or exogenous influences, which finally leads to death. Recent aging research uncovered roles for genetic and environmental longevity factors, which indicates that defence mechanisms help stave off the process of aging. A variety of genes were identified to play a role in the regulation of longevity. First studies investigating the genetics of aging used the nematode *Caenorhabditis elegans* as model organism [Klass, 1977]. A spearheading study of a genetic regulation of longevity found mutations in single genes resulting in a modest reduction of Insulin/IGF-signaling and an enormous increase in life span ([Friedman and Johnson, 1988], [Kenyon, 1993], [Kimura et al., 1997]). These studies sparked the interest in genetics of aging and the connection between gene function and the regulation of longevity. Subsequent studies revealed numerous longevity genes related to different pathways, while making use of model organism as diverse as yeast, fruit flies or mice. Many of those genes were found to be conserved up to mammals [Lopez-Otin et al., 2013].

Research on molecular genetics of aging nowadays aims to link and validate the interaction and networks of longevity genes, and dissect their mechanism of action. Further, the current post-genomic area requires to integrate information on proteins, post-translational regulations and metabolites. Ultimately, the goal is to decelerate the process of aging, increase health span and thereby reduce the occurrence and seriousness of age-related diseases.

## 1.2 The Model Organism *Caenorhabditis elegans*

The nematode *Caenorhabditis elegans* (*C. elegans*) has a long-standing history as a model organism in biological and biomedical science, which was first established by Sydney Brenner [Brenner, 1974]. *C. elegans* is a perfect tool for aging research foremost because of its short reproductive cycle of 3-4 days and its short life span from 20-30 days. Its small size (about 1mm in length) allows the study of populations of worms and easy laboratory handling and maintenance. The genome of the worm was one of the first fully sequenced of any metazoan [Consortium, 1998]. For about 2/3 of *C. elegans* protein sequences, human homologous genes are known [Lai et al., 2000].

A broad spectrum of applicable genetic methods can be used to manipulate the genes of this multi-cellular organism. A large collection of change-of-function alleles is available and laboratories can store thousands of *C. elegans* strains by freezing. Extra-chromosomal arrays can be established to express transgenes and these arrays can be UV-integrated into the genome [Mello et al., 1991]. Recently, the CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) technology was introduced and allows the generation of targeted single basepair exchanges, larger deletions and insertions of protein tags [Paix et al., 2017]. Gene function can specifically be reduced by using RNA interference (RNAi). This approach was discovered in *C. elegans* and used short double-stranded RNA targeting a chosen gene [Fire et al., 1998]. RNAi can be induced by feeding bacteria expressing those short double-stranded RNA molecules [Timmons et al., 2001]. Large-scale RNAi screens are a powerful tool to scan the effect of a huge number of genes on a pathway, process or phenotype of interest [Boutros and Ahringer, 2008].

The nematode lives as a self-fertilizing hermaphrodite, while the occurrence of males is a relatively rare event. Through male x hermaphrodite matings, different alleles can be combined and epistatic interactions can be investigated, to order genes into pathways. As an invertebrate model organism, the laboratory use of *C. elegans* is not as strictly regulated by the government, which enables researchers to quickly set up new experiments, change

approaches in ongoing projects and reduces costs of administration and paperwork.

## **1.3 Pathways contributing to Longevity**

### **1.3.1 The Insulin/IGF signaling Pathway**

The Insulin/IGF-signaling pathway is the most studied and investigated network of proteins, genes, metabolites and small molecules in the field of aging research. The Insulin/IGF-signaling pathway was connected to longevity, when single gene mutations extending lifespan were isolated [Klass, 1983]. The mutation of a single loci of the gene *age-1* resulted in 80% lifespan extension [Friedman and Johnson, 1988] and later studies identified *age-1* as the worm ortholog of PI3K [Morris et al., 1996]. The worm ortholog of the insulin/IGF receptor *daf-2* [Kimura et al., 1997] was found to increase the lifespan of *C. elegans* up to 3-fold [Kenyon et al., 1993].

Insulin-like peptides (ILPs) bind to the Insulin/IGF receptor DAF-2 and trigger self-phosphorylation and dimerization into an active state. Activated DAF-2/IGFR recruits and activates the phosphoinositide 3-kinase AGE-1/PI3K, which phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2) and forms phosphatidylinositol 3,4,5-trisphosphate (PIP3). The phosphatase DAF-18/PTEN works in the opposite direction and is a negative regulator of the Insulin/IGF-signaling pathway. PIP3 triggers a phosphorylation cascade from PDK-1 towards AKT-1 to DAF-16/FOXO. The phosphorylated DAF-16/FOXO is excluded from the nucleus and stays in the cytoplasm. When the Insulin/IGF-signaling pathway is not activated, the nuclear localization of DAF-16/FOXO allows this transcription factor to induce its target genes, which subsequently contribute to longevity and stress resistance [Murphy et al., 2003]. In the last years it was uncovered that also SKN-1/NRF2 [Tullet et al., 2008], HSF-1 [Chiang et al., 2012] and PQM-1 [Tepper et al., 2013] are transcription factors mediating the response to Insulin/IGF-signaling and required for its protective role in cellular homeostasis. The positive effect on longevity by the Insulin/IGF-signaling pathway is conserved. In *Drosophila* ([Clancy et al., 2001], [Tatar et al., 2001]) and in mice [Blueher et al., 2003] reduced Insulin/IGF-

signaling pathway activity results in lifespan extension. In humans, polymorphisms in components of the Insulin/IGF-signaling pathway could be linked to the longevity of centenarians ([van Heemst et al., 2005], [Suh et al., 2008], [Barbieri et al., 2010]).

### **1.3.2 Target of Rapamycin (TOR) and Dietary Restriction**

The availability of nutrients is a major requirement to allow growth and reproduction. The Target of Rapamycin (TOR) pathway is a major central sensor for various nutrients and regulates metabolism and physiology. The inhibition of the TOR pathway leads to lifespan extension in a broad range of organisms, like yeast [Fabrizio et al., 2001], *C. elegans* [Vellai et al., 2003], *Drosophila* [Kapahi et al., 2004] and mice [Harrison et al., 2009].

The catalytic unit in the TOR pathway is mTOR, a protein kinase. This protein acts as a subunit in two different complexes, mTORC1 and mTORC2 in mammals. Both complexes act as sensors for nutrients, stresses or energy and can be activated by insulin, growth factors or cytokines. mTORC1 signals towards the regulation of protein synthesis and translation [Wullschlegel et al., 2006], whereas mTORC2 plays a role in proliferation and metabolism [Betz et al., 2013].

Reduction in nutrient intake in the absence of malnutrition, also termed dietary restriction, works upstream of the TOR pathway. Epistasis experiments suggest that the lifespan extension by dietary restriction often relies on components of this pathway. In yeast replicative lifespan is extended by deletions of genes from the TOR pathway [Kaeberlein et al., 2005]. In *C. elegans*, RNAi against mTOR mimics the longevity of dietary restricted worms [Hansen et al., 2007]. At least one method of dietary restriction in *Drosophila* requires 4E-BP1, a TOR target, for maximal lifespan extension [Zid et al., 2009].

The activation of TOR results in a phosphorylation of the ribosomal protein S6 kinase (S6K) [Schalm and Blenis, 2002]. Consequently, activated S6K targets a number of proteins related to translation. The initiation of translation is regulated by S6K through phosphorylation of eIF4B, a cap binding complex component [Raught et al., 2004]. S6K also activates the elongation factor 2

(eEF2) by inactivating its negative regulator eEF2K [Wang et al., 2001], and thus affects the elongation step of protein translation. The reduction of S6K activity was connected to longevity in diverse organisms, from yeast [Fabrizio et al., 2001] to mice [Selman et al., 2009]. Thus, the positive effect of reduced TOR signaling on longevity can partially be explained by reduced translation. The TOR pathway also regulates autophagy, another cellular mechanism required for maintenance, survival and longevity. Autophagy is negatively regulated by TOR signaling. mTORC1 phosphorylates and ULK1/2 and inhibits its kinase activity [Jung et al., 2009]. Upon reduced TOR signaling, ULK1/2 is no longer repressed and phosphorylates ATG13, FIP200 and itself. This induces autophagy through the formation of preautophagosomal structures with the aid of other ATG proteins [Farre and Subramani, 2016]. Autophagy was found to be required for various life extending interventions in *C. elegans* and *Drosophila* [Madeo et al., 2015]. In mammals this causal connection could not be established, because loss of autophagy factors during development is lethal [Lee et al., 2014]. Studies show that during mammalian aging, autophagic function gets reduced [Liu et al., 2013] and overexpression of pro-autophagic ATG5 extends lifespan in mice [Pyo et al., 2013]. In *C. elegans*, the overexpression of HLH-30, a transcription factor primarily targeting autophagy related genes, results in longevity [Lapierre et al., 2013]. The beneficial effect of autophagy on aging is mediated by the cytoprotective function of removing proteotoxic aggregates [Tanaka and Matsuda, 2014]. Beyond that, autophagy is required for mobilizing lipid stores that might ameliorate age-related hepatic lipid accumulation and pathologies like hepatic steatosis [Singh et al., 2009].

### 1.3.3 Respiration and Mitochondrial Longevity

Mitochondria are important hubs for cellular energy homeostasis and metabolism in all eukaryotic cells. Mitochondria are essential, but when mitochondrial function is partially reduced, positive effects on lifespan can be observed. A number of genes related to mitochondrial function have been uncovered in *C. elegans*, *Drosophila* and mice, whose modest downregulation lead to a mild decrease in mitochondrial activity and induce longevity. Several such mutations are found in genes, that play a role in the electron transport chain (ETC) of the respiratory chain. *clk-1* mutants have slower respiratory rates and are long lived. The gene encodes an enzyme required for co-enzyme Q production [Lakowski and Hekimi, 1996]. Mutations in *isp-1*, the *C. elegans* iron sulfur protein in mitochondrial complex III, have a negative effect on the electron transport chain and extend the lifespan [Feng et al., 2001]. On the other hand, mutations that impair the ETC can also shorten lifespan, such as *mev-1* mutations (*C. elegans* succinate dehydrogenase cytochrome b560 subunit of mitochondrial complex II) [Ishii et al., 1998]. These contradictory findings indicate, that further understanding has to be gained about fine tuning between the function of the ETC and longevity.

A similar effect is found in the connection of reactive oxygen levels (ROS) to longevity. ROS are produced by the respiratory chain or environmental stress and damages DNA, lipids or proteins. The free radical theory proposes that accumulation of cellular damage induced by oxidation and ROS causes aging [Harman, 1956]. Surprisingly then, long-lived mitochondrial mutants have higher levels of ROS. Similarly, mild treatment with pro-oxidant drugs such as paraquat induces ROS and extends life, and led to the mitohormesis theory [Schulz et al., 2007], which posits that mild mitochondrial derived ROS induction triggers mechanisms of cellular survival.

In response to misfolded proteins in the mitochondria or a stoichiometric imbalance of complexes of the ETC, the mitochondrial unfolded protein response (UPR<sup>MT</sup>) is activated [Martinus et al., 1996]. This pathway induces the nuclear-encoded mitochondrial chaperones, such as HSP-6 and HSP-60. UPR<sup>MT</sup> was associated with aging, because it is essential for the lifespan

extension of *isp-1* and *clk-1* mutants [Durieux et al., 2011]. However, this study was challenged by later findings that UPR<sup>MT</sup> induction by RNAi knockdown of negative UPR<sup>MT</sup> regulators does not affect lifespan in a consistent manner [Bennett et al., 2014]. The UPR<sup>MT</sup> in *C. elegans* is coordinated by ATFS-1, which is normally imported to the mitochondria [Nargrund et al., 2012]. Upon mitochondrial dysfunction the protein accumulates in the cytosol and traffics to the nucleus to facilitate the transcription of nuclear-encoded mitochondrial chaperones. A recent study suggests that mammalian ATF5 as the transcription factor is mediating the mammalian UPR<sup>MT</sup> [Fiorese et al., 2016].

### **1.3.4 Gonadal Longevity**

Different cells and organs function as coordinating hubs for metabolism, development, behavior or reproduction, but additionally can have effects on the life span. The gonad – consisting of the germ line and the somatic gonad – can influence the aging of an organism. In *C. elegans*, removal of germline precursor cells by laser-ablation extends the lifespan of *C. elegans*. It was also shown that ablation of the whole gonad or the precursor cells of the somatic gonad could not extend lifespan [Hsin and Kenyon, 1999]. Adult fruit flies lacking germ line stem cells live longer than controls [Flatt et al., 2008]. Other studies indicate that gonadal longevity is conserved in higher species. The transplantation of young ovaries into older mice led to an increased lifespan [Mason et al., 2009]. The lifespan of eunuchs is significantly longer than of non-castrated men of similar socioeconomic status [Min et al., 2012].

## **1.4 Gonadal Longevity in *C. elegans***

### **1.4.1 Mechanism and Pathways involved in Gonadal Longevity**

Studies in *C. elegans* revealed that reduction of sperm or oocyte formation has little or no effect on longevity, regardless of the savings in resources by decreased gametogenesis [Arantes-Oliverira et al., 2002]. The removal of germline precursor cells, but not of the whole gonad or the precursor cells of the somatic gonad, extends the nematode life span. These findings indicate the germline stem cells as the origin for negative effects on life span. The somatic gonad signals in an antagonistic manner and promotes longevity. These signaling pathways contradict an old theory - the so-called rate-of-living theory by Raymond Pearl [Pearl, 1928] - which proposes that reduction of fertility promotes longevity.

Germline stem cells generate haploid gametes, sperms or oocyte, which are responsible for transmitting genetic information from generation to generation. A decrease in germline stem cell number by mutation in *glp-1* or mechanical ablation leads to longevity. Overproliferation of germline stem cells, as induced by mutations in *gld-1*, leads to a reduction in life span [Arantes-Oliverira et al., 2002]. Beyond that, arrest of germline stem cell proliferation in adult worms has a positive effect on life span [Wang et al., 2008]. Similar results are obtained in *Drosophila*, where adult flies without germline stem cells live longer than controls [Flatt et al., 2008].

The positive effects of loss of germline stem cells found under laboratory conditions are logically challenged, because such traits would never be passed on by evolution. However, germ cell proliferation has to be tightly controlled and monitored in organisms in response to cues such as nutrients or pathogens. Because germline and soma must be coordinated for reproductive success, slowing germline proliferation may also signal to slow aging in the soma.

Indeed, two important studies established that important signaling pathways regulate germ line proliferation ([Michaelson et al., 2010]; [Korta et al., 2012]). The Insulin/IGF-signaling pathway, dietary restriction, TOR or S6K signaling can influence the rate of germ cell proliferation. In theory, the germline stem

cells could act as a signaling hub for unfavorable conditions and block proliferation under such challenges. The quiescent cells in parallel trigger pro-survival signals towards the somatic cells of the organism. After return to better conditions the germline stem cells are switched back to proliferation and reproduction is enabled.

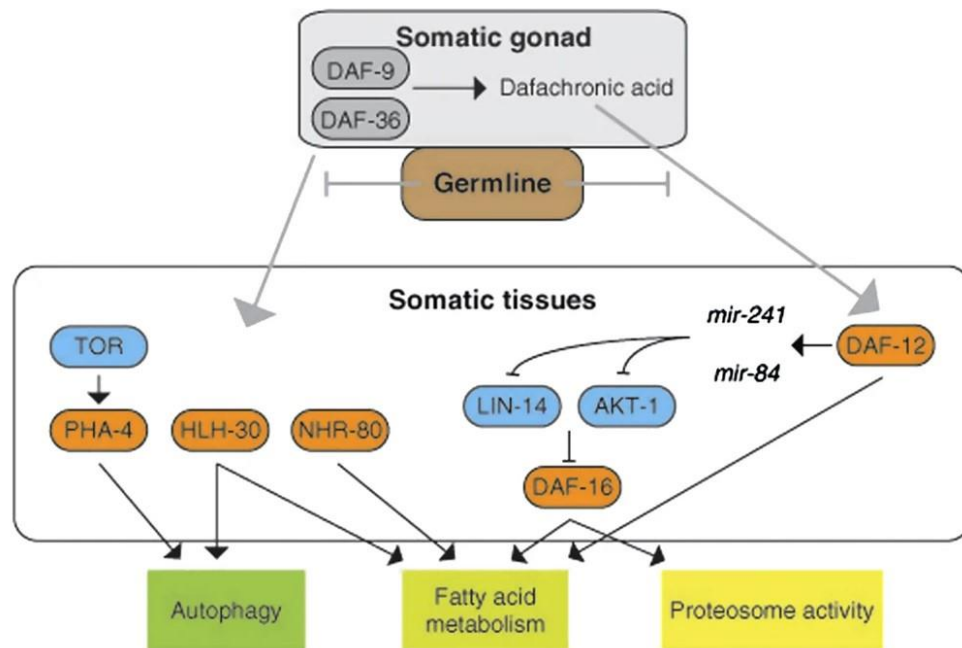


**Figure 1: Graphical Summary of the interplay of tissues and proteins in *C. elegans* gonadal longevity.** Germline elimination enhances the steroid signal dafachronic acid, which is produced in somatic gonads. DAF-12 regulates the expression of mir-84 and mir-241, and increased mir-84 and mir-241 expression results in the downregulation of two DAF-16 inhibitors (AKT-1 and LIN-14) and promotes DAF-16 nuclear localization and activation. Germline elimination also activates the transcription factors PHA-4, HLH-30 and NHR-80. The activation of these transcription factors leads to changes in fatty acid metabolism, as well as enhanced autophagy and proteasome activity. Figure and annotation taken from Uno and Nishida 2016.

In Figure 1 an graphical overview shows the interplay of tissues and proteins in *C. elegans* gonadal longevity [Uno and Nishida, 2016].

Gonadal longevity can be seen as a trade-off between loss of reproductive capability against extension of lifespan and stress survival. The ultimate goal is to extend lifespan without affecting reproduction, therefore the mechanisms, pathways and mediators underlying gonadal longevity have to be uncovered and understood.

### 1.4.2 Germline Ablation by Laser Microsurgery

There are different methods to prevent germline stem cell proliferation and extend lifespan in *C. elegans*. The outcome of these methods is similar, but the procedures are very different and explained in the following section.

Researchers can study *in vivo* cell function by eliminating cells and observe consequential developmental or behavioral abnormalities. In *C. elegans*, this is done by killing individual cells or groups of cells with a laser microbeam. Such interventions are possible, because only a few cell types are required for *C. elegans* viability [Fang-Yen et al., 2012]. The first method used to generate worms without a germline was laser-ablation of primordial germline stem cells, also termed germline laser-ablation. A laser beam focused through the objective of a microscope was used to kill specific cells, namely the Z2 and Z3 germline-precursor cells of L1 larvae [Hsin and Kenyon, 1999]. The larvae have to be immobilized for this intervention and recovered carefully after the ablation. The control group for lifespan experiments with germline laser-ablated worms are mock-ablated animals, that also underwent immobilization and recovering. This method is considered as the gold standard, when studying gonadal longevity, because no genetic intervention has to be done. However, the method is very labor-intensive, requires trained experimenters and an expensive microscope. Furthermore, researchers can only produce a limited number of germline ablated worms in a given experiment, which is sufficient for lifespan analysis, but not of sufficient throughout for the most applications in genomics, transcriptomics, proteomics or metabolomics. These limitations have led to the predominant use of other methods generating germline ablated worms. Notably, mutants of *glp-1* and *mes-1* genetically reproduce the effect of germline laser-ablation and can yield a high amount of germline ablated worms for subsequent analysis.

### 1.4.3 Germline Removal by mutations in *glp-1*

*glp-1* encodes a homolog of the Notch receptor and is expressed in germline stem cells. It binds LAG-2, a ligand produced by the somatic gonadal tip cells,

which constitutes the niche for the germ stem cell population [Henderson et al., 1997]. These interactions trigger the intracellular portion of GLP-1 to translocate to the nucleus, where it interacts with LAG-1 and blocks the transcriptional activation of GLD-1 and GLD-2 through the activation of two PUF proteins, FBF-1 and FBF-2, resulting in germline stem cells remaining in the proliferative mode [Racher and Hansen, 2012]. A decrease in GLP-1 signaling leads to active GLD-1 and GLD-2, resulting in GSC entering meiosis. There are several *glp-1* mutations identified, which can be clustered into three phenotypic classes. One class contains all non-conditional and fully penetrant alleles, which eliminate germline induction. Members of this group show the strongest *glp-1* loss-of-function phenotype and are probably null mutations. A second class is comprised of alleles with more serious effects on embryogenesis than on germline induction. The third class contains alleles that are temperature sensitive in germline induction and embryogenesis. Mutants appear wild-type at the permissive temperature (15°C), but when early hatched larvae are switched to restrictive temperature (25°C), the proliferation of germline fails and sterile adults develop. The widely used allele *glp-1(e2141ts)* is a member of this third class.

The Notch pathway is well conserved from *C. elegans* to humans and it is required in metazoans for the specification of cell fate among an equipotent cell population [Bray, 2006]. There are also an increasing number of studies, that describe Notch signaling to be required for processes in postmitotic cells such as adult brain functions like learning and memory [Costa et al., 2003].

Aberrant Notch signaling drives T cell acute lymphoblastic leukemia [Göthert et al., 2007] and Notch inhibitors are used in clinical trials for cancer treatment [Espinoza and Miele, 2013]. The second *C. elegans* Notch receptor LIN-12 plays a role in regulating neuronal physiology and animal behavior, without affecting neuronal cell fate [Chao et al., 2005]. *glp-1* is majorly expressed in the germline, but was also found to be expressed in larval somatic tissues [Austin and Kimble, 1989]. The GLP-1 ligand LAG-2 is expressed in neurons from the onset of the dauer stage and activates GLP-1/NOTCH signaling in neurons.

This signaling is required for dauer maintenance in *daf-7* mutants [Ouellet et al., 2008]. Taken together, Notch signaling is crucial for several developmental programs, with *glp-1* being a central component of germline development. Beyond that, Notch signaling also plays important roles in post mitotic cells.

#### **1.4.4 Germline Removal by mutations in *mes-1***

*C. elegans* MES-1 (maternal effect sterile 1) is a receptor tyrosine kinase, that is required for the unequal divisions of cells in the *C. elegans* embryo [Berkowitz and Strome, 2000]. Mutants of *mes-1* fail to segregate germ granules to the germline blastomere cell and instead locate germ granules to its sister cell. As a consequence, none of those cells is able to adopt a germline fate and the designated germline blastomere cell develops into a muscle precursor cell, which generates up to 20 muscle cells in place of germ cells. Most studies use the allele *mes-1(bn7)*, which is temperature sensitive. Homozygous mutants generate around 10% sterile progeny at 16°C, but about 70% sterile progeny at 25°C. In line with its role in early development, the temperature sensitive period is from fertilization to the 28-cell stage [Strome et al., 1995].

#### **1.4.5 Known Mediators of Gonadal Longevity**

In Figure 1 a graphical overview shows the interplay of tissues and proteins in *C. elegans* gonadal longevity [Uno and Nishida, 2016]. The nuclear hormone receptor DAF-12 is required for the lifespan extension in worms without a germline [Hsin and Kenyon, 1999]. The same study showed that DAF-12 can further extend gonadal longevity. The ligands of DAF-12, the bile-acid like dafachronic acids, have to be present to allow the activation of DAF-12 in germline-ablated worms. Loss of genes involved in dafachronic acid synthesis, such as *daf-36* or *daf-9*, abolishes gonadal longevity ([Gerisch, 2001], [Rottiers, 2006]). This effect is rescued by feeding dafachronic acid, whereas the loss of DAF-12 cannot be rescued [Gerisch et al., 2007]. DHS-16, a 3-hydroxysteroid dehydrogenase regulating dafachronic acid synthesis, was found to behave similarly [Wollam et al., 2012]. The target gene expression of DAF-12 is stimulated by absence of the germline, as shown

by high levels of *cdr-6* mRNA in germline-ablated worms [Yamakawi et al., 2010]. The same study showed that the additional loss of the somatic gonad reduces the *cdr-6* level back those of wild-type worms, suggesting that dafachronic acid signals emanating from the somatic gonad regulate DAF-12 signaling in the gonadal pathway.

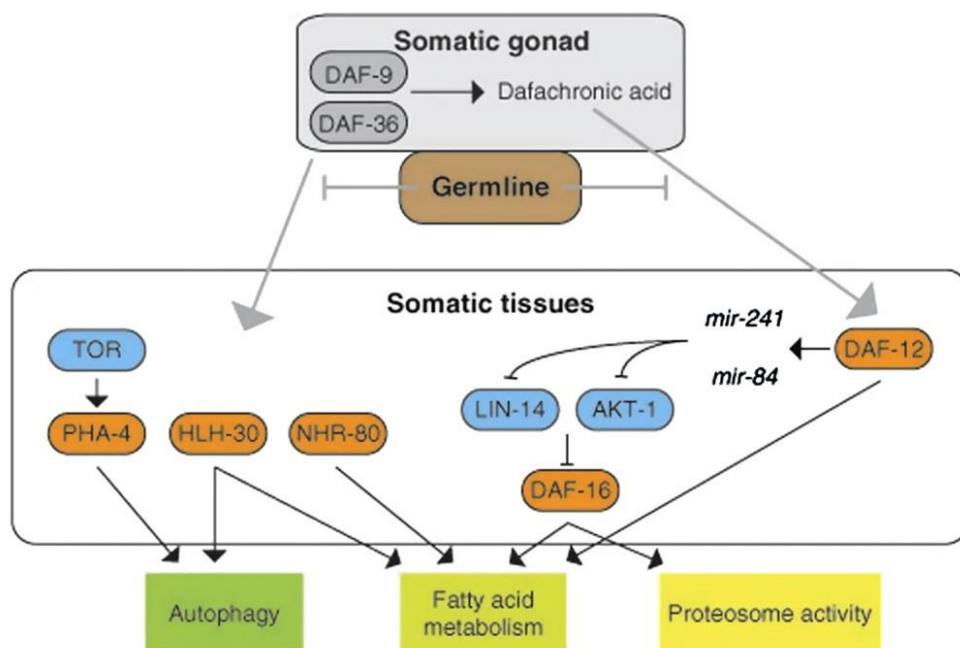


**Figure 2: Graphical Summary of the interplay of tissues and proteins in *C. elegans* gonadal longevity.** Figure and annotation taken from Uno and Nishida 2016.

DAF-16, the major transcription factor in the Insulin/IGF-1 signaling pathway, is also required for gonadal longevity [Hsin and Kenyon, 1999]. Even knockdown of DAF-16 in adult worms was sufficient to suppress gonadal longevity [Arantes-Oliverira et al., 2002]. Nonetheless, Insulin/IGF-1 signaling mediated longevity and gonadal longevity are somewhat independent, since *daf-2* and *glp-1* are additive for longevity [Hsin and Kenyon, 1999]. Other mediators of gonadal longevity, such as *kri-1* and *tcer-1*, are not required for Insulin/IGF-1 signaling mediated longevity ([Berman and Kenyon, 2006]; [Ghazi et al., 2009]). Loss of germline induces DAF-16 nuclear localization in gut cells [Berman and Kenyon, 2006], in contrast to a loss of function in DAF-2 or other genes of the Insulin/IGF-1 signaling pathway, which drives DAF-16 to the nucleus in nearly all cell types [Henderson and Johnson, 2001]. In line with these observations,

only a subset of DAF-16 targets is induced in germline-ablated worms including *sod-3* or *gpd-2* [Ghazi et al., 2009].

The heat shock factor 1 (HSF-1), a transcriptional factor mediating stress-inducible genes and protein folding homeostasis, was found in an RNAi screen to be required for the longevity of *glp-1* mutants [Hansen et al., 2005]. The transcription factor SKN-1 was also linked to gonadal longevity. Loss of germline induces SKN-1 target genes required for stress defense, extracellular matrix and lipid metabolism [Steinbaugh et al., 2015]. PHA-4, a FOXA forkhead transcription factor homolog, was initially identified as a mediator of dietary restriction [Panowski et al., 2007], but is also required for gonadal longevity. PHA-4 induces genes of the autophagic machinery, which shows a higher activity in germline-ablated worms. In those worms, autophagy and *lipl-4* mediated lipolysis are induced by mTOR and interact to allow lifespan extension of germline-ablated worms [Lapierre et al., 2011]. A network comprising of the HLH transcription factors MML-1, MXL-2 and HLH-30 is also required for gonadal longevity. These factors mutually regulate each other and share common downstream targets [Nakamura et al., 2016].

Many other factors mediating gonadal longevity remain to be identified. It still is unclear what signals the gonad responds to, and how the gonad can control the lifespan of the whole organism. Furthermore, the sequential arrangement and hierarchy of the signaling events remains unsolved, especially which is the first responding pathway and which pathways are triggered afterwards. Once activated the different pathways must be coordinated to render the animal resistant to stress and long-lived. Therefore, it is important to uncover other pathways and mediators required for gonadal longevity and complete our knowledge about this mode of extending lifespan.

## **1.5 Nuclear Hormone Receptors (NHRs)**

### **1.5.1 Function and Structure of Nuclear Hormone Receptors**

Nuclear receptors are ligand gated transcription factors, which evolved in the metazoan taxon. They are able to bind small molecules or metabolites such as vitamins, fatty acids or steroids and regulate gene transcription. Nuclear receptors are required for development, metabolism, reproduction and cellular maintenance and can act cell-autonomously or across tissues. The structure and mechanism of nuclear receptor signal transduction is conserved [Mangelsdorf et al., 1995]. An N-terminal domain of two zinc fingers comprises the DNA binding domain. The C-terminus contains a ligand binding domain, which can also bind co-activators or co-repressors, defining the activity of the transcription factor. Class 1 receptors are ligand activated, whereas Class 2 receptors act as transcriptional activators in their ligand-bound state, but repress transcription without ligand binding. A subset of nuclear receptors, which constitutively binds ligand, requires associated proteins or post-translational modifications to control activity [Gonzales, F.J., 2008]. Various modifications (phosphorylation, ubiquitination, acetylation, sumoylation) were identified among nuclear receptors. Another subset of nuclear receptors, the orphan receptors, have no known ligands [Giguère, V., 1999].

### **1.5.2 *C. elegans* Nuclear Hormone Receptors**

Humans have 48 nuclear receptors [Evans, R.M., 1988]. *C. elegans* has 284 nuclear receptors, although 269 represent an expansion and diversification of the HNF4 family [Robinson-Rechavi et al., 2005]. This expansion is also found in *C. ramenii* and *C. briggsae*, two closely related nematodes, as well as distantly related nematodes such as *Pristionchus pacificus* suggesting a primordial role in nematodes physiology. The other 15 nuclear receptors include homologs of VITD/LXR/FXR, SF1, ROR, COUP, HNF4, Rev-Erb, TLX, PNR, NGF-1, TR2/4 and GCNF. For several human nuclear receptors, like the PPARs or the retinoic acid receptor, there are no clear orthologs in *C. elegans*. Recent

studies show out that convergent evolution gave rise to proteins with analogous physiologic functions.

The nuclear receptors present in *C. elegans* play roles in various pathways and signaling cascades, are required in all stages of its development and mediate diverse responses to stress or other exogenous signals. During the development from embryo to larval stages to reproductive maturity, nuclear receptors, such as DAF-12, NHR-8 or UNC-55, act as major checkpoints to sense unfavorable conditions [Antebi, 2005]. As a response, these transcription factors can initiate or terminate developmental arrest at several states, like L1 diapause, L3 dauer diapause and an adult reproductive diapause. Several nuclear receptors are required for transitions from life stages or the control of the molt cycle. The determination of cell fate and organogenesis can also be regulated by nuclear receptors. NHR-25, NHR-67 and NHR-6 integrate inputs from growth and signaling pathways and compile that information to a positional and temporal status of the cell, which specifies the cell fate and determines the cell type ([Asahina et al., 2000], [Gissendanner et al., 2004], [Ward and Bojenala, 2013]). Animals must also be able to sense nutrient levels and react towards changes in quality or availability of their food sources. Nuclear receptors as NHR-91 or NHR-26 couple nutrient levels to growth and metabolism, whereas others like NHR-49, NHR-80 or NHR-69 have more specified roles in lipid and carbohydrate metabolism ([Van Gilst et al., 2005a], [Van Gilst et al., 2005b], [Brock et al., 2006], [Park et al., 2012]). Maintaining the balance of metabolism and detoxification also involves nuclear receptors (NHR-49 and NHR-114).

### 1.5.3 Nuclear Hormone Receptors in Aging and Longevity

The most intensively studied nuclear receptor in *C. elegans*, DAF-12, regulates a broad spectrum of processes including aging. DAF-12 is regulated by dafachronic acids [Motola et al., 2006], which are bile acid-like steroids, and cholestenoic acid [Held et al., 2006]. DAF-12 is found in all somatic cells [Antebi et al., 2000] and is required for the lifespan extension in germline-less worms [Hsin and Kenyon, 1999]. Dafachronic acids are assumed to be the life extending signal from the somatic gonad, which is sensed by DAF-12 and triggers the expression of its target genes [Yamawaki et al., 2010]. In parallel, DAF-12 regulates *let-7*-related microRNAs, which suppress inhibitors of DAF-16, *lin-14* and *akt-1*, and thereby facilitate DAF-16 nuclear localization and activation [Shen et al., 2012]. DAF-12 partially abolishes the robust longevity of *daf-2* class 1 mutants, while the pleiotropic *daf-2* class 2 mutants exhibit a synergistic life span extension upon loss of *daf-12* [Gems et al., 1998]. In addition, brief pulses of heat stress enable thermotolerance and longevity, which is *daf-12* dependent [Cypser et al., 2006].

Dietary restriction extends life span in several organisms. NHR-62 is required for the longevity of *eat-2* mutants (which ingest less food) and for lifespan extension by bacterial dilution [Heestand et al., 2003]. A major target of NHR-62 are genes related to autophagy, which are required for dietary restriction mediated longevity [Hansen et al., 2008]. NHR-62 also regulates genes involved in lipid transport and metabolism, amine metabolism and chromatin structure.

### 1.5.4 Nuclear Hormone Receptors regulating fat metabolism

NHR-80 regulates the expression of delta-9 fatty acid desaturases, namely *fat-5* and *fat-7*, and to a lesser extent *fat-6*. *Fat-5,6,7* triple mutants are lethal, while single or double mutants survive due to compensatory up-regulation of the remaining genes by *nhr-80* [Brock et al., 2006]. Loss of *nhr-80* leads to an accumulation of C18:0 stearic acid and a reduction of C18:1n9 oleic acid

[Pathare et al., 2012]. Germline loss induces *nhr-80* expression in the gut, leads to an up-regulation of *fat-6* and elevated levels of its metabolic product C18:1n9 oleic acid [Goudeau et al., 2011]. Deletion of *nhr-80* abolishes *glp-1* longevity, as well as double knockout of *fat-6* and *fat-7*. This can be overcome by supplementation with oleic acid.

NHR-49 is an HNF4-like nuclear receptor coordinating fat metabolism which functions similar to the mammalian lipid-sensing nuclear receptors PPARalpha and PPARgamma [Van Gilst et al., 2005a]. In the fed state NHR-49 induces genes required for mitochondrial beta-oxidation of fatty acids, monodesaturation of fatty acids, lipid transport and the glyoxylate cycle. NHR-49 can also repress genes involved in peroxisomal beta-oxidation and lipid binding [Van Gilst et al., 2005b]. On the one hand, the loss of *nhr-49* lowers the levels of beta-oxidation, oxygen consumption and monounsaturated fatty acids. On the other hand, it increases the levels of lysosomal-like acidic fat compartments and the levels of saturated fatty acids. As a consequence, *nhr-49* mutants have a very short life span, which can be explained by the high and lipotoxic ratio of saturated to unsaturated fatty acids [Shmookler Reis et al., 2011].

### **1.5.5 The Nuclear Hormone Receptor NHR-91**

NHR-91 is little studied, even though it features has clear homologs in fruit flies (DHR4), mice and humans (GCNF) [Rottiers and Antebi, 2006]. It is an orphan nuclear receptor, since no ligand could be identified to date. NHR-91 regulates early postembryonic arrest in response to unfavorable nutrient states [Kasuga et al., 2013]. Starvation of L1 larvae results in a developmental arrest, shutdown of blast cell division and entry to a starvation and stress resistant L1 diapause. The microRNA *mir-235* is induced in L1 diapause and suppresses *nhr-91* expression under these circumstances. *Mir-235* loss results in inappropriate exit from L1 arrest, which is in part compensated for by loss of *nhr-91*, thereby preventing the onset of molting and developmental progression. NHR-91 was

identified as an interaction partner of NHR-25, a factor important for vulva development [Ward et al., 2010].

In *Drosophila* DHR4 is a negative regulator of ecdysone biosynthesis and is required for the temporal control of ecdysone pulses [Ou et al., 2011]. Its human homolog GCNF regulates Oct4 during embryonic development and triggers Oct4 repression by methylating its promotor region [Gu et al., 2011]. The repression of Oct4 by GCNF is also found in human embryonic stem cells in a dose-dependent manner. In addition, GCNF globally regulates gene expression in undifferentiated and differentiated human embryonic stem cells [Wang et al., 2016]. *Let-7* miRNAs repress GCNF after mid-gestation [Gurtan et al., 2013]. In contrast, prostate cancer cells exhibit induced GCNF levels, which implies its use as a biomarker for prostate cancer [Mathieu et al., 2013].

## 1.6 The Integrated Stress Response

### 1.6.1 Molecular Mechanisms of the Integrated Stress Response

The integrated stress response pathway can be activated in response to various stress stimuli, which enables the organism to survive and recover [Ron, D., 2002]. A central node in the integrated stress response pathway is the eukaryotic translation initiation factor 2 alpha (eIF2 $\alpha$ ), whose phosphorylation leads to a decrease in global protein synthesis and the induction of selected genes that promote cellular homeostasis and are crucial to counter unfavorable conditions, including ATF4 [Lu et al., 2004]. Figure 3 displays an graphical overview of the Integrated Stress Response [Pakos-Zebrucka et al., 2016].

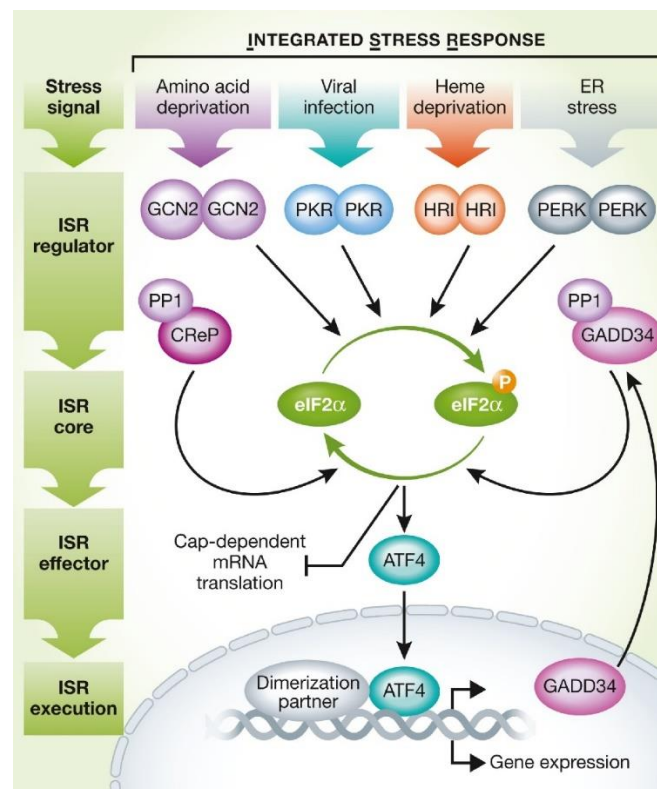


**Figure 3: Graphical Summary of the Integrated Stress Response.** ER stress, viral infection, and other cellular stress signals activate PERK, PKR, HRI, and GCN2 kinases that converge on phosphorylation of eIF2 $\alpha$ , the core of ISR. This leads to global attenuation of Cap - dependent translation while concomitantly initiates the preferential translation of ISR - specific mRNAs, such as ATF4. ATF4 is the main effector of the ISR. It forms homo - and heterodimers that bind to DNA targets to control the expression of genes involved in cellular adaptation. Termination of the ISR is regulated by the constitutively expressed CReP and stress - inducible phosphatase GADD34 that dephosphorylate eIF2 $\alpha$ . Arrows denote activation or induction, while blunt lines indicate inhibition. Figure and annotation are taken from Pakos-Zebrucka et al., 2016.

In mammals, four eIF2 $\alpha$  kinases are responsible for sensing cellular stress. The double-stranded RNA-dependent protein kinase (PKR) is activated predominantly by double-stranded RNA (dsRNA) during viral infection [Perkins and Barber, 2004]. Some studies have reported that other stresses can also activate PKR, but in a dsRNA-independent manner [Lee et al., 2007]. The heme-regulated eIF2 $\alpha$  kinase (HRI) is found in erythroid cells and coordinates the translation of globin mRNAs with the availability of heme to protect the cells against toxic globin aggregation in the case of heme deficiency [Han et al., 2001]. The PKR-like ER kinase (PERK) is located in the ER membrane and can be activated by the accumulation of unfolded proteins in the ER [Harding et al., 1999] or perturbations in cellular energy homeostasis [de la Cadena et al., 2014]. The general control non-depressible 2 (GCN2) binds to deacylated transfer RNAs (tRNAs), which occur under amino acid deprivation [Vazquez de Aldana et al., 1994]. Thus inputs to each eIF2 $\alpha$  kinases encompass a broad spectrum of stimuli.

Once triggered, the eIF2 $\alpha$  kinases dimerize and undergo autophosphorylation to become fully activated [Lavoie et al., 2014]. Thereafter, the phosphorylation of eIF2 $\alpha$ , is the core event of the integrated stress response. Together with eIF2 $\beta$  and eIF2 $\gamma$ , eIF2 $\alpha$  forms the eIF2 complex. eIF2 $\alpha$  is the main regulatory subunit, because it exhibits phosphorylation and RNA binding sites. Under unstressed conditions the eIF2 complex is a key component in the initiation of mRNA translation and recognition of the AUG start codon [Pain, V.M., 1996]. It forms a ternary complex with GTP and Met-tRNA<sub>i</sub> which binds the 40S ribosome subunit, as well as two other initiation factors eIF1 and eIF1A [Aitken and Lorsch, 2012]. These proteins form the 43S pre-initiation complex that is recruited to the 5'methylguanosine cap of mRNA by the eIF4F complex and others [Hinnebusch and Lorsch, 2012]. The interacting complexes eIF4G and eIF3 stabilize the 43S pre-initiation complex and allow its migration to the AUG start codon [Aitken and Lorsch, 2012]. The Met-tRNA<sub>i</sub> binds to the AUG codon, eIF1 dissociates from the complex and GTP on eIF2 is hydrolyzed in an eIF5 dependent-manner. The eIF2-GDP complex falls off the 40S ribosomal complex

and the exchange of GDP to GTP by eIF2B converts eIF2 into its active form again [Pain, V.M., 1996].

Upon activation of the integrated stress response pathway, the phosphorylated eIF2 $\alpha$  hinders the exchange of GDP to GTP by eIF2B and prevents the formation of the 43S pre-initiation complex. This globally reduces 5'Cap-dependent protein synthesis. The translation of mRNAs containing a short upstream open reading frame does not require cap recognition by the eIF4F complex, because it relies on a re-initiation mechanism [Rzymiski et al., 2010] or the direct recruitment of ribosomes to internal ribosome entry sites [Chen et al., 2013]. This allows for global downregulation of translation of cap dependent transcripts but selective translation of non-capped transcripts.

### **1.6.2 Translational and Post-Translational Regulation of ATF4**

The transcription factor ATF4, the mammalian homolog of *C. elegans* ATF-5, is a major component of the integrated stress response pathway [Pakos-Zebrucka et al., 2016]. The translation of ATF4 is regulated by two upstream open reading frames in the 5' leader region of its mRNA. The first upstream open reading frame facilitates ribosome scanning of the mRNA and re-initiation at downstream ORFs in the ATF4 mRNA [Hinnebusch, A.G., 1997]. Under non-stressed conditions, the ribosome scans and recognizes the second upstream open reading frame. This element is translated and overlaps with the start codon of the canonical ATF4 protein. As a result, the second upstream open reading frame acts as an inhibitory element and the ATF4 protein is not expressed. As described above, upon activation of the integrated stress response pathway, the increased probability of the ribosome to skip the start codon of the second upstream open reading frame and re-initiate translation at the canonical start codon of ATF4 protein or to internally enter at this site leads to enhanced translation of the ATF4 protein [Vattem and Wek, 2004].

ATF4 harbors domains required for the transcriptional function, regulation and degradation of the protein. The N-terminal p300 domain is important for the

stability and the transcriptional activity of ATF4. Interaction with the histone acetyltransferase p300 renders ATF4 resistant to ubiquitination. The underlying mechanisms are not fully understood, because p300-mediated ATF4 stabilization is independent of p300 catalytic activity [Lassot et al., 2005]. The oxygen-dependent degradation domain (ODDD) is recognized by the oxygen sensor prolyl-4-hydroxylase domain 3 (PHD3) and leads to the destabilization of the ATF4 protein in normoxic conditions. When oxygen levels are low, or the ODDD is genetically mutated or PHD3 is down regulated by siRNA, the ATF4 protein becomes stabilized [Koeditz et al., 2007]. The ATF4 protein can also be targeted for proteasome-mediated degradation. The protein carries a  $\beta$ TrCp recognition motif (DSGXXXS), which can be phosphorylated. Upon phosphorylation the F-box protein  $\beta$ TrCp, the receptor component of the SCF E3 ubiquitin ligase, binds to ATF4 protein, ubiquitinates it and thus targets the protein for proteasomal degradation [Lassot et al., 2001]. Located near the C-terminus of the protein, a bZIP domain is responsible for the transcriptional activity of the ATF4 protein. The basic region is responsible for the interaction with the major groove of the DNA and facilitates sequence-specific interaction [Ellenberger et al., 1994]. The leucine zipper region is required for the dimerization of two bZIP transcription factors [Vinson et al., 1989], which is critical for DNA binding. ATF4 can bind DNA as a homodimer or as a heterodimer [Podust et al., 2001]. Known dimerization partners are CEBP [Podust et al., 2001] or CHOP [Han et al., 2013]. In yeast, the ATF4 homolog Gcn4 can be activated when tRNA export from the nucleus is reduced. This occurs upon loss of the nuclear pore protein LOS1, which can be excluded from the nucleus by dietary restriction or mTOR inhibition. In this case, Gcn4 probably expresses its target genes without reduced global mRNA translation. The underlying mechanism of Gcn4 activation is not fully understood [McCormick et al., 2015].

### 1.6.3 Phenotypes of ATF4 Gain- or Loss-of-function Mutants

The role of ATF4 in mammals has been studied using mouse models. ATF4-null mice are viable and are used in various studies. Loss of ATF4 results in different defects in development. ATF4-null mice show abnormalities in eye development [Tanaka et al., 1998], defects in production of erythrocytes in fetal liver [Masuoka and Townes, 2002], bone formation [Yoshizawa et al., 2009], angiogenesis [Wang et al., 2013] and learning and memory [Costa-Mattioli et al., 2007].

ATF4-null mice also exhibit a metabolic phenotype. They are lean and resistant to age-related and diet-induced obesity. They have an increased energy expenditure, which is likely cause of the lean phenotype. Mice are hypoglycemic, connecting ATF4 to mammalian carbohydrate metabolism. Loss of ATF4 alleviates diet-induced diabetes, hyperlipidemia and hepatosteatosis [Seo et al., 2009].

ATF4 also plays an important role in cancer growth and protection. In some tumors, the integrated stress response pathway is activated and ATF4 is expressed under hypoxic or nutrient-deprived conditions. There it can promote cellular homeostasis and cancer cell survival by regulating protein synthesis, autophagy or redox balance. Inhibitors of ATF4 could be potential drugs to target cancer growth and survival [Pike et al., 2013]. ATF4 can also protect cancer cells from anoikis, a form of apoptosis that occurs after matrix detachment of cells, and contributes to their metastatic properties [Dey et al., 2015].

Only a handful of studies have investigated the role of ATF4 and its homologs and aging. Interestingly, five strains of slow aging mice show an increase in ATF4 protein levels and ATF4 target genes. Thus, the authors of this study assert that increased ATF4 levels can be seen as a hallmark of slow aging mice [Li et al., 2014]. A study in yeast found that dietary restriction or modest reduction of the mTOR pathway promotes longevity by engaging Gcn4, the yeast functional homolog of mammalian ATF4 ([Steffen et al., 2008], [McCormick et al., 2015]). In *C. elegans* the knockdown of *impt-1*, a GCN-2

inhibitor, increases lifespan. This longevity phenotype is *gcn-2* and *atf-5* dependent [Ferraz et al., 2016].

#### 1.6.4 The Integrated Stress Response in *C. elegans*

In *C. elegans*, the integrated stress response is conserved. Instead of four eIF2 $\alpha$  kinases, however, only homologs of GCN2 (*gcn-2*/Y81G3A.3) and PERK (*pek-1*/F46C3.1) are found in the worm. The activation and the consequences of the integrated stress response pathway in *C. elegans* are homologous to the mammalian pathway and result in shutdown of global protein synthesis and translation of *atf-5*/T04C10 ([Rousakis et al., 2013], [Kulalert and Kim, 2013]) (CGC\_name according to wormbase.org is *atf-4*), an ortholog of human ATF4 [Statzer et al., 2020].

*C. elegans*' *gcn-2* plays a crucial role under amino acid limitation and is required for survival under nutrient stress and the lifespan extension mediated by dietary restriction [Rousakis et al., 2013]. GCN-2 is also activated in a ROS-dependent manner by mitochondrial stress and is required for the lifespan extension associated with mitochondrial dysfunction [Baker et al., 2012]. The second *C. elegans* eIF2 $\alpha$  kinase *pek-1* responds to unfolded or misfolded proteins in the ER, which can result from heat stress or reducing conditions [Shen et al., 2001]. In *daf-28* mutants, neuronal ER stress induces *pek-1* and consequent phosphorylation of eIF2 $\alpha$  in the ASI neuron pair, which promotes entry into dauer diapause [Kulalert and Kim, 2013].

In *C. elegans* *atf-5* was found to be increased upon amino acid limitation in a *gcn-2* dependent-manner. Loss of *atf-5* could not phenocopy the effect of *gcn-2* deletion on stress responses or longevity [Rousakis et al., 2013]. In contrast, the longevity mediated by knockdown of the *gcn-2* inhibitor *impt-1* was dependent on *atf-5* [Ferraz et al., 2016].

Another controversy surrounding *C. elegans* ATF-5 originates from its upstream open reading frame structure. The first upstream open reading frame is longer than 20 amino acids, which is believed to not permit re-initiation of translation

[Hiraishi et al., 2014]. Nevertheless, stress-induced ATF-5 translation is observed in *C. elegans*, and the authors of the study state that “it will be crucial to determine the ATF-5 mRNA structure in *C. elegans* and understand the mechanism by which ATF-5 translation is induced by eIF2 inhibition”. In addition, *atf-5* was found to be a target of SKN-1, the *C. elegans* Nrf ortholog with critical roles in oxidative stress response and lifespan regulation ([Tullet et al., 2008]; [Robida-Stubbs et al., 2012]). SKN-1 binding sites were identified and regulation of *atf-5* was found upon arsenite treatment [Oliveira et al., 2009]. ChIP experiments showed binding of SKN-1 in the *atf-5* genomic region and thus confirmed a direct regulation [Glover-Cutter et al., 2013]. The same study also revealed that ER-stress induced activation of *skn-1* and activation of SKN-1 mediated oxidative stress response requires *atf-5* and other core UPR factors.

## **1.7 The Heat Shock Response**

### **1.7.1 Mechanisms of the Heat Shock Response pathway**

Proteins are one of the main functional building blocks of cells and have to be maintained to stay functional. A large number of stressors can have negative effects on the integrity and functionality of proteins. The heat shock response is triggered by such stress factors as increased temperatures, oxidative stress or heavy metals and induces heat shock proteins [Morimoto, 1993]. These molecular chaperones can prevent or reverse protein misfolding and contribute to a cellular environment that supports the integrity and functionality of proteins [Weibezahn et al., 2005].

### **1.7.2 The Heat Shock Factor (HSF-1) as a central mediator of the heat shock response**

The heat shock response is characterized by a strong induction of genes encoding heat shock proteins. A central node in this regulatory pathway is the heat shock factor-1 (HSF-1). This evolutionarily conserved transcription factor in its inactive state exists as a monomer and becomes activated via trimerization and phosphorylation [Gomez-Pastor et al., 2018]. The HSF-1 trimer accumulates in the nucleus and promotes transcription of genes harboring a 5' regulatory *cis* element in the proximal enhancer region. This motif is called heat shock responsive element (HSE) and shows a minimum of three tandem inverted repeats, TTCxxGAxxTTC (where x denotes any arbitrary nucleotide) [Amin et al., 1988].

An early study in *C. elegans* connects HSF-1 with longevity. Overexpression of *hsf-1* extends lifespan and HSF-1 is required for Insulin/IGF-signaling induced longevity. Small heat-shock proteins expressed by HSF-1 and DAF-16 together, delay the onset of polyQ-expansion protein aggregation, which is a model for age-related diseases [Hsu et al., 2003].

A transcriptome-wide study reveals that HSF-1 regulates genes involved in cytoprotection, development, metabolism and aging. HSF-1 was found to activate and repress gene expression [Brunquell et al., 2016]. The HSF-1 dependent repression of gene expression is mediated indirectly, but the exact mechanisms remain unclear. Though best known for heat activation, HSF-1 can also act heat stress-independent and has roles in development, metabolism and longevity. Heat-stress induction of HSF-1 leads to the expression of heat shock proteins like *hsp-70* and *hsp-16* family members and *hsp-4*. The heat-stress independent activation of HSF-1 shows an interaction network of aging-associated genes which, is four-fold larger than the network of aging genes that depend on heat-stress activation of HSF-1 [Brunquell et al., 2016]. These findings suggest, that HSF-1 could impact longevity in ways beyond its role in stress responses. Interestingly, it was recently shown that HSF-1 modulates mitochondrial function via mediating histone H4-dependent regulation of mtDNA gene expression and activation of the mitochondrial UPR, which consequently lead to an extension of *C. elegans* lifespan [Sural et al., 2020].

## 1.8 Aim of the study

Aging research aims to understand, characterize and finally manipulate mechanisms that contribute to the gradual decline of cellular and organism-wide functions and the progression of age-related diseases. The gonadal longevity pathway explores how the gonad can fundamentally impact the life span of metazoans. Studies revealed that single gene mutations [Hsin and Kenyon, 1999], but also complex genetic networks and interactions [Nakamura et al., 2016], play important roles in this form of longevity.

Preliminary large-scale RNAi screens carried out by my colleagues, Dr. Özlem Karalay and Dr. Shuhei Nakamura identified a number of potential mediators of gonadal longevity in *C. elegans*, including two transcription factors, NHR-91 and ATF-5.

The overall aim of my thesis work is to characterize the role of these two novel mediators of gonadal longevity, and to place them within molecular pathways to further decipher the regulatory network. My study began with validating and characterizing the role of the nuclear hormone-receptor NHR-91 in *C. elegans* gonadal longevity. This transcription factor is relatively unstudied and was not connected to aging so far. Later in my thesis, I also sought to characterize the role of ATF-5 in gonadal longevity, studying its regulation and its downstream targets in the gonadal longevity pathway. These studies have revealed new dimensions to the gonadal longevity pathway and may create promising entry points to promote healthy aging.

## **2 Materials and Methods**

## **2.1 *C. elegans* methods**

### **2.1.1 Growth, maintenance and handling of *C. elegans***

Following standard procedures, *C. elegans* strains were grown on nematode growth medium (NGM) in petri dishes seeded with the *E. coli* strain OP50 [Brenner, 1974]. OP50 bacteria, used as the food source for *C. elegans*, are uracil-auxotroph which facilitates the slow growth of the bacterial lawn. *C. elegans* strains were grown and maintained at 20°C unless otherwise noted. All strains carrying *glp-1(e2141)* allele were maintained at 15°C and shifted to 25°C during development to induce the germline-less phenotype.

For maintaining the worm strains, a cutting of NGM agar was transferred from a plate with a worm population to a fresh NGM petri dish using a sterilized spatula. Likewise, single worms of a growing population were transferred with a worm pick (a platinum wire) to fresh NGM petri dishes. Transgenic worms were maintained by selecting for the co-injection marker. Decontamination of strains from time to time prevented from contaminants or was used to eliminate contaminations. Plates were washed off with M9 buffer and the worms were collected in a micro-centrifugation tube. The tubes were briefly centrifuged to concentrate the worms at the bottom. M9 buffer was replaced by bleaching solution and incubated for around 10 minutes at room temperature. After the adult worms were dissolved, the bleach-resistant eggs were washed once with water and twice with M9. The washed eggs were seeded on fresh NGM plates.

### **2.1.2 Lifespan Analysis**

Lifespan analyses were carried out at 20°C as previously reported [Gerisch et al., 2001]. An egg-lay of 6-8 hours was done to age-synchronize the worm population. Lifespan experiments were carried out on 6 cm NGM petri dishes at the density of 20 worms per plate. Worms were scored for movement every other day and until the end of the reproductive span, worms were moved to fresh plates every day. Animals that crawled off the plates or into the agar, burst due to a ruptured vulva or showed internal hatching of their progeny were censored from the experiment. Lifespan analyses using RNAi were carried out

following the standard protocol [Kamath et al., 2001]. The data was plotted in Microsoft Excel to calculate mean and median lifespan. The log-rank (Mantel-Cox) analysis was used to determine significance between the lifespan curves (Supplement) and was executed with GraphPad Prism software.

### **2.1.3 Laser ablation**

The ablation of germline precursor cells by laser microsurgery, also called germline laser-ablation (GLA), was carried out as published [Fang-Yen et al., 2012]. 50-75 day 1 adults were allowed to lay eggs overnight (14-16h) on a 6cm NGM plate. The egg-lay was stopped by washing of the adults with M9 buffer. After one hour the hatched L1 larvae were collected by washing them of the plate with M9 buffer. The larvae were anesthetized with 1-Phenoxy 2-propanol at a final concentration of 0.25% in M9 and placed on a agar pad on a slide. A coverslip was carefully placed on pad and the cells Z2 and Z3 of the gonadal primordium were targeted and hit with the laser until damage of the nucleus was observed. The animals were rescued by removing the coverslip and adding a few drops of M9 to the agar pad. The animals were transferred with a glass pipette to a new NGM plate. After developing to young adults the worms were checked under the binocular and sorted into groups of mock-ablated and successful ablated worms for further analysis.

### **2.1.4 CRISPR and injections**

The generation of specific knock-in alleles by CRISPR/Cas9 genome editing (Figure 4) was carried out following the protocol developed by Alexandre Paix and colleagues [Paix et al., 2015]. In short, crRNA was designed according to the guidelines of the protocol and ordered from Dharmacon (GE Lifesciences) together with tracrRNA (Dharmacon #U-002000-05/20/50). The repair template was designed and ordered from IDT (single-stranded oligonucleotides, 200nt maximum size, 4nM ultramer, salt free). The sequence of the repair template used to generate the *atf-5*(dh1103) allele was

GTTTCATGACCGAGATGGGAATGAAAACCGTTACTGATGGAGCCGCTGGA  
GCTGGAAAGCCAATTCCAAACCCACTTCTTGGACTTGACTCCACCGATTA  
CAAGGATCACGATATCGATTACAAGGATGATGACGACAAGTGAACCTCAAT  
TTTTAATGTTTTTTTACTCCGTTTG. Cas9 protein was ordered from NEB (#M0646M). The injection mix was prepared as described in the protocol and directly used for injection into P0 hermaphrodites. F1 worms showing the roller phenotype (co-conversion marker) were selected and genotyped for the *atf-5*(dh1103) allele. Worms carrying the allele were used to isolate non-Roller F2 worms homozygous for *atf-5*(dh1103).

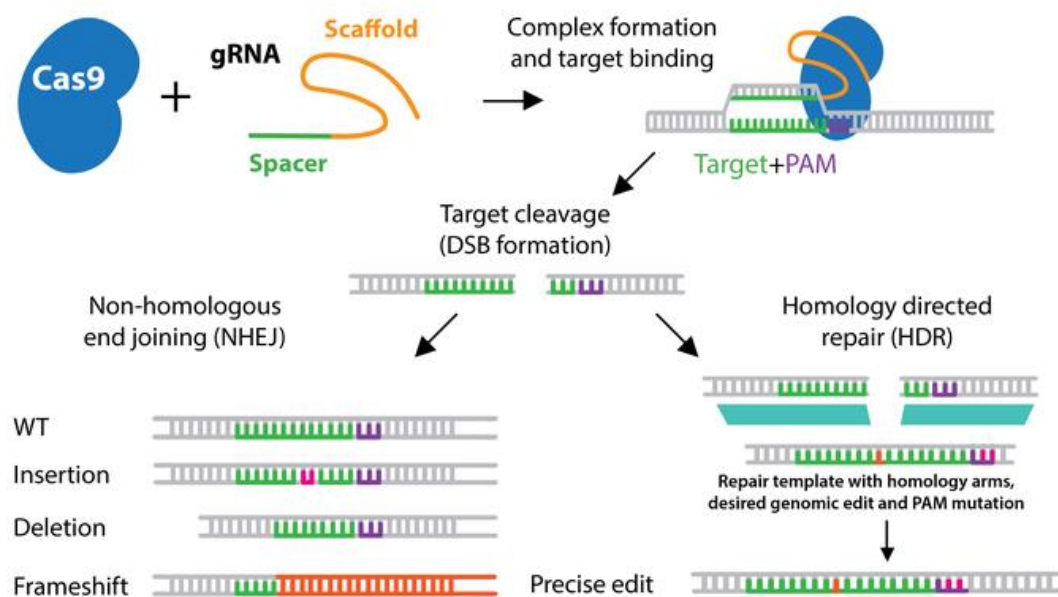


**Figure 4: An overview of CRISPR.** The Cas9 protein and the gRNA (an artificial conjunction of crRNA and tracrRNA) complex and recognize a target sequence, in which a double-strand break is induced. The presence of a repair template with homologous regions 5' and 3' of the double-strand break induces homology directed repair. The absence of a repair template causes non-homologous end joining to rejoin the cleaved DNA. This can result in a wildtype sequence, an insertion, a deletion or a frameshift. (Picture taken from <https://www.addgene.org/crispr/history/>)

## **2.2 Molecular Biology**

### **2.2.1 Western Blotting**

Worms were collected in M9, washed once with cold M9 and re-suspended in western blot lysis buffer. The samples were snap-frozen in liquid nitrogen and 3 cycles of thawing and snap-freezing were carried out. The solution was ultra-sonicated for 15 cycles and centrifuged for 15 minutes (13.000 g) on 4°C. The supernatant was collected and the protein concentration was measured using a Pierce BCA Protein Assay Kit. Prior to western blotting, an aliquot of the lysate was mixed with laemmli buffer and boiled at 95°C for 5 minutes. The samples were loaded on Precast Protein Gels and blotted on a nitrocellulose membrane using a Trans-Blot Turbo Transfer System (BioRad).

The membranes were then blocked for 30 minutes at room temperature in 5% milk or 1% Bovine Serum Albumin (BSA) in TBST and probed with a primary antibody. The antibodies were diluted in TBST with 1% BSA. Incubation was allowed overnight at 4°C, followed by washing the membrane three times with TBST. A specific secondary antibody was used at a concentration of 1:5000 in TBST with 1% BSA and incubated at room temperature for one hour. After three washes with TBST, the membranes were developed with Western Lightening Plus-Enhanced Chemiluminescence Substrate (PerkinElmer) and subsequently imaged with ChemiDoc Imager (BioRad).

### **2.2.2 Polymerase chain reaction (PCR) and gel electrophoresis**

**A.** Genomic DNA was obtained by single-worm lysis. Worms were transferred into single worm lysis buffer with 100µg/ml Proteinase K and snap-frozen. The lysis was performed in a PCR cyclor by incubating on 60°C for one hour, followed by 15 minutes on 95°C to inactive the Proteinase K.

**B.** cDNA was used as template for PCR. Therefore RNA extraction and cDNA synthesis was carried out as described in 2.2.3.

The PCR was done using Phusion Green High-Fidelity DNA Polymerase (Thermo Scientific) and following the manufacturer's instructions. The annealing temperature was adjusted to the primer set and kept for 30 seconds. The extension time was set to 30s per kb of the expected PCR product. The PCR cycles were run 35 times followed by a final extension time of 10 minutes. The agarose gel electrophoresis was carried out with 1-2% agarose gels. The gels were prepared with TAE, in which a proportional amount of agarose was dissolved. The mix was treated with 0,05 µl/ml Roti-GelStain (Roth).

### **2.2.3 RNA extraction and cDNA synthesis (reverse transcription)**

The purification of RNA required the collection of 300-400 age-matched adult worms (day1). The worms were picked into ice-cold M9, pelleted by centrifugation, TRIzol (Invitrogen) was added and snap-frozen in liquid nitrogen. The extraction of RNA was performed by using RNeasyMini Kit (QIAGEN) and following the manufactures instructions. Extracted RNA samples were analyzed by NanoDrop 2000c (peqLab) to measure the quantity of RNA. cDNA was synthesized from the extracted RNA using iScript cDNA Synthesis Kit (Bio-Rad) and used for subsequent analysis.

### **2.2.4 Real-time PCR (qPCR)**

The cDNA was pipetted along with the primers and Power SYBR Green master mix (Applied Biosystems) onto a 384-well plate using JANUS automated workstation (PerkinElmer). A ViiA7 384 Real-Time PCR System machine (Applied Biosystems) was used to carry out the reaction cycles and analysis of PCR products. The endogenous control was a PCR product displaying the levels of *cdc-42* or *ama-1* mRNA. qPCR primer sequences used in this study are described in Supplementary Materials.

### 2.2.5 RNA sequencing

The purification of RNA required the collection of 300-400 age-matched adult worms (day1). The worms were picked into ice-cold M9, pelleted by centrifugation, TRIzol (Invitrogen) was added and snap-frozen in liquid nitrogen. The extraction of RNA was performed by using RNeasyMini Kit (QIAGEN) and following the manufactures instructions. Extracted RNA samples were analyzed by NanoDrop 2000c (peqLab) to measure the quantity of RNA. polyA + mRNA was isolated from 500 ng total RNA with NEBNext Poly(A) mRNA Magnetics Isolation Module (New England Biolabs). RNA-seq libraries were prepared with the NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs). Quality and quantity was assessed at all steps by capillary electrophoresis (Agilent Bioanalyser and Agilent Tapestation). Libraries were quantified by fluorometry, immobilized and processed onto a flow cell with a cBot (Illumina) followed by sequencing-by-synthesis with TruSeq v3 chemistry on a HiSeq2500 at the Max Planck Genome Center (Cologne, Germany) under project #1823.

As input we had three replicates per sample with a sequencing depth in the range of ~33-46 million reads. Before mapping to the genome, the reads were trimmed at both ends with the FASTQ/A Trimmer of the FASTX-Toolkit ([http://hannonlab.cshl.edu/fastx\\_toolkit](http://hannonlab.cshl.edu/fastx_toolkit) by Hannon Lab). The pre-processed reads were mapped to the genome with tophat2 (v2.0.7) allowing only unique mapping. On average 99.9% of the reads could be aligned. Subsequently, differential gene expression was performed with DESeq REF. Genes with a q-value < 0.05 and fold change > |1.5| were classified as differentially expressed. (This part of the analysis was performed by Dr. Corinna Klein of the MPI Bioinformatics Facility).

### **2.2.6 Fatty acid methyl ester (FAME) analysis**

Worms were washed from the plates with M9 buffer and washed in a snap cap tube with M9 buffer two times. The worm pellet was transferred to a glass vial and six cycles of freezing in liquid N<sub>2</sub> and thawing in a 20°C water bath were carried out. The samples were sonicated thrice with a Branson 450 probe-sonicator applying 15 pulses with a duty-cycle of 70% and power output set to 5.5. The protein concentration of the worm lysate is measured using the Pierce BCA Protein Assay Kit. A volume corresponding to 150µg protein of the worm lysate is mixed with 10µl of a standard mixture (ISTD, C11:0; C13:0; C23:0, each 100ng/µl) in a glass vial. 500µl of 2,5% H<sub>2</sub>SO<sub>4</sub> in methanol is added, the mixture is vortexed and incubated for 20min at 80°C. The mixture is vortexed again, 750µl of ddH<sub>2</sub>O are added, following the addition of 350µl of cyclo-Hexane. After shaking on RT for 10min, phase separation is induced by centrifugation for 1min at 2000rpm. The top layer (cyclo-Hexane) is transferred to a glass vial with an insert, vacuum dried in a GeneVac Centrifuge and resolved in 10µl cyclo-Hexane.

The measurements were carried out by GC application specialist Christian Latza using an Agilent Technologies GC System 7890A equipped with a 30mx0,250mm DB-23 122-2332 column. The temperature program was as following: hold 60°C for 1 min // 60-180°C in 7°C/min // 180°C-200°C in 3°C/min // hold at 200°C for 1 min // 200-230°C in 10°C/min // hold at 230°C for 10min. Specimen were detected using a Flame Ionisation Detector (FID).

## 3 Results

### 3.1 A large-scale RNAi screen identified candidate genes mediating gonadal longevity

Though the role of various transcription factors in regulating lifespan and longevity in *C. elegans* has been the subject of several studies before, systematic suppressor screens to identify the transcriptional network of gonadal longevity had not been performed. In order to find novel players contributing to gonadal longevity a large-scale RNAi screen including around 600 RNAi clones of transcription factors, kinases and phosphatases was carried out by Özlem Karalay and Shuhei Nakamura in the background of sterile *glp-1* mutants. As expected, the screen revealed factors that were already connected to gonadal longevity (e.g. DAF-12, HLH-30, PHA-4, NHR-80, NHR-49), serving as positive controls. Beyond that, several novel factors were identified, namely MML-1, NHR-91 or ATF-5 (Figure 5). A project originating from MML-1 resulted in a publication, unraveling a transcription factor network important for several longevity pathways in which I was co-author [Nakamura et al., 2016].

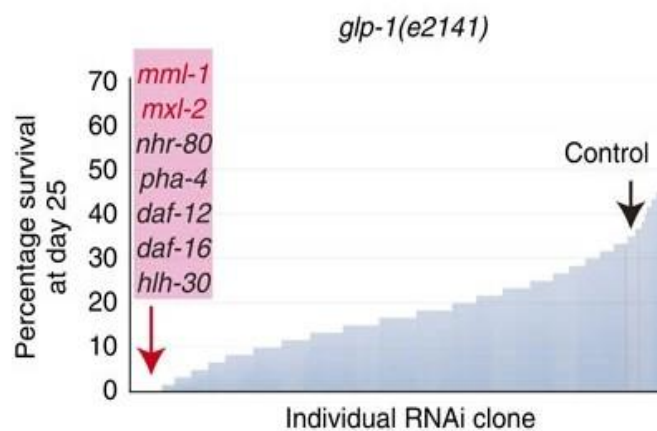


**Figure 5: Overview of RNAi clones found to reduce the survivorship at day 25 of adulthood of *glp-1(e2141ts)* mutants.** L4440 (empty vector) is used as control RNAi. Experiment and graphical presentation taken from Nakamura et al., 2016. Survivorship of 60 worms was determined and screening conducted twice.

In this thesis work, I further investigated the role of NHR-91 and ATF-5 in the gonadal longevity pathway. Therefore, these two candidates were validated by regular lifespan analysis and followed up.

### 3.1.1 Validation of RNAi-screen longevity candidate NHR-91

RNAi against *nhr-91* reduced the survivorship at day 25 of adulthood of *glp-1* mutants when compared to L4440 control RNAi in the initial screen (Figure 5). In depth lifespan analysis confirmed that *nhr-91i* suppressed *glp-1* longevity and shortened N2 life span (Figure 6). These experiments were carried out by Özlem Karalay and Shuhei Nakamura.

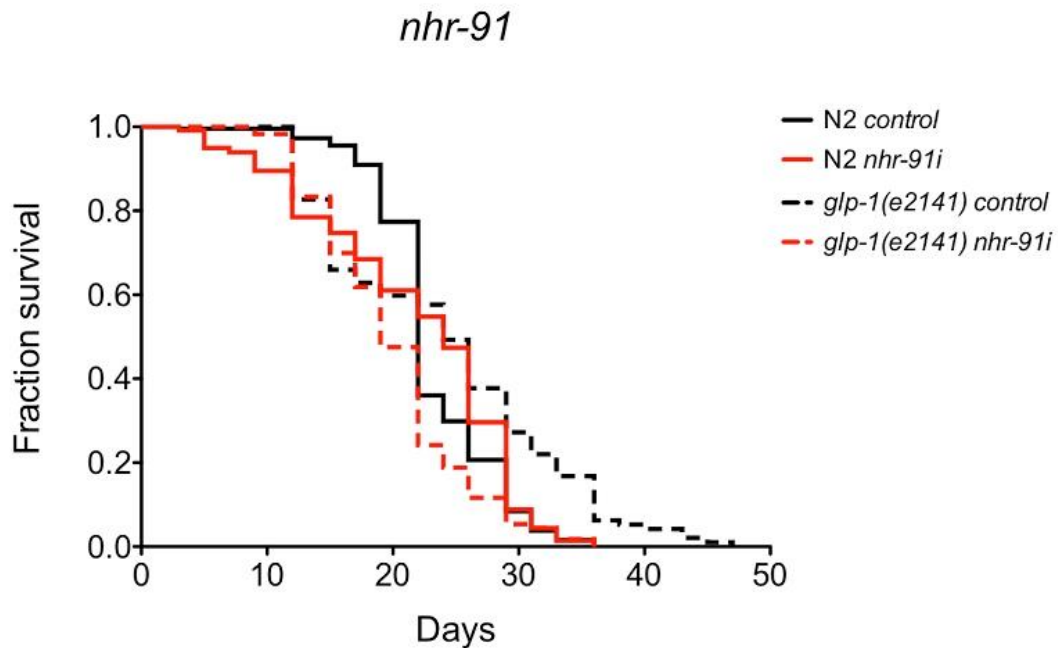


**Figure 6: *nhr-91* RNAi abolishes *glp-1* longevity.** Lifespan analysis of germlineless *glp-1* mutants and N2 wild type on *nhr-91* RNAi and L4440 empty vector control. Experiment and graphical presentation were carried out by Özlem Karalay and Shuhei Nakamura.

To validate the findings of the RNAi screen, I made use of a loss-of-function mutant available from NBRP (Japan, National BioResource Project). Allele *nhr-91(tm4713)* is a 593bp out-of-frame deletion removing part of an intron and a coding exon (Figure 7). The deletion is located in the C-terminal region of the gene and overlaps with the sequence for a putative ligand-binding domain of the nuclear hormone receptor (PROSITE annotation). The allele was outcrossed and combined with the temperature-sensitive *glp-1(e2141ts)*, the same allele used in the large-scale RNAi screen. Lifespan analysis showed that the median lifespan extension of *glp-1(e2141ts)* compared to N2 worms was 57,3% (Figure 8).

CAGTGAGAGAAGATCGCATGCCCGGCGGGAGAAATGGGAACAGTATCTATAGCAACTACAAGCAGAGGAGGAGTA  
TCATTAGGAAAACGAAAGATTATGTGGAGGAGCAGGAGAGAAGGCCGTTTCAGAGCCTTCCAAGTGGGAAAAAGT  
TAATAAAGgtgagtgcacttgcagagcgatattgtagtttttggcagccccgacactttcggggtttccata  
ccatgggtggttagtgggtcgagaccggcggaacctcgaaagtgtcgggggcttccaaacaactacatatcgcaat  
gagttgctcagccccagctctaaatgcggggctggagctgagccctcgagcccttagcagccctagtgcatt  
atgagccagttaaaaatttgaacaaattattgccttattcgattttgctatttttacagGAAGTGGTTGAGATGG  
ACTGCCCTAGACCGGCTCATCAACCTAAAAGGACTCCGAATTAATCCATCTGCAAACTGCGATATTGCCCTGCTT  
GCAAAAGGTTAACCAGAAATAGGAGATGAAATTTGGAGCAACTTGTGAGTGGACGAAAACCTTGCCGTTTTTCG  
ACGAATACCCGTAGAAGCACACACCCACCTTTTGACACAACGATGGGCCGAGCTAGTATTACTATCTGCTGGAT  
ACTACGCGTGCTCGGTCTTTACTCCGGACTCCCCCGACACAACAGAAATGATAGACGAACTGACGAGATCTCAT  
TCACAAACCCACAGGTCAACCTTCGGCTACTTCAAATCGATTATCACTGGTGTCTGGCAAAGAAATCCATTGG  
AACACGTGGCGAA

**Figure 7: Genomic sequence of *nhr-91* with the *tm4713* deletion allele.** Exons highlighted in uppercase and in orange and yellow. Intron sequence shown in lowercase. Sequence deleted in *tm4713* is underlined and bold (Sequence obtained by JBrowse).

In *nhr-91(tm4713);glp-1(e2141ts)* double mutants longevity was largely suppressed, with the median lifespan of the double-mutant *nhr-91(tm4713);glp-1(e2141ts)* not significantly different from *nhr-91(tm4713)* alone. In germline positive worms *nhr-91(tm4713)* also decreased the lifespan (N2 vs. *nhr-91(tm4713)*). This experiment that loss of *nhr-91* shortens life span and abolishes GLP-1/NOTCH mediated longevity.

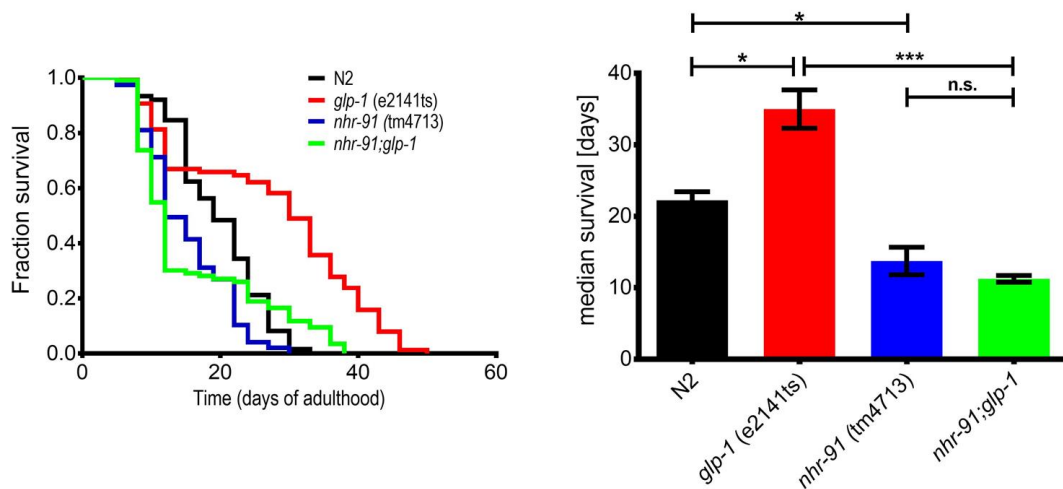


**Figure 8: Loss of *nhr-91* abolishes longevity of *glp-1(e2141ts)* mutants.** Left, representative lifespan analysis shows that *nhr-91(tm4713)* deletion mutation abolishes longevity of *glp-1(e2141ts)* worms. The deletion mutant is also short-lived in comparison to wild type N2. Right, the median lifespans are summarized. Median lifespan in N2 worms is 22 days, significantly less than in *glp-1(e2141ts)* worms, living 35 days. The median lifespan of *nhr-91(tm4713)* deletion mutants is 13.75 days and is not significantly different from the lifespan of *glp-1;nhr-91* double mutants (median lifespan 11.25 days). (N = 4). See Supplementary Section 7.1.6.1 for details and repeats. The bars represent mean  $\pm$  s.e.m., P value (\* P < 0.05 and \*\*\* P < 0.001) was determined by t-test.

## 3.2 NHR-91 is only required for gonadal longevity of *glp-1* mutants

### 3.2.1 NHR-91 is not required for the longevity in germline-laser ablated worms

As described in the introduction, thermo-sensitive *glp-1* mutants are only one approach to ablate the germline precursor cells. A non-genetic method used to ablate germline precursor cells is by laser microsurgery. The following experiments were designed to test the requirement of NHR-91 in gonadal longevity using this method.

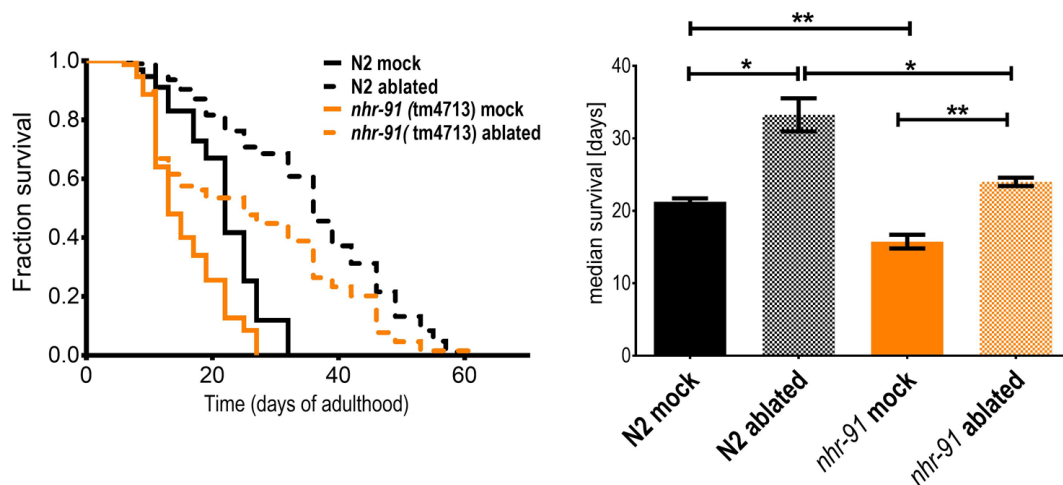


**Figure 9: Loss of *nhr-91* does not specifically abolish longevity of germline-laser ablated worms.** On the left a representative lifespan analysis shows that *nhr-91(tm4713)* deletion mutation reduces longevity of germline laser-ablated worms. The mock-ablated deletion mutant is also short-lived in comparison to mock-ablated N2. On the right side the median lifespans of 4 experiments are summarized. Median lifespan in mock-ablated N2 worms is 21.25 days, which is significantly less than ablated N2 worms, living 33.25 days. The median lifespan of mock-ablated *nhr-91(tm4713)* deletion mutants is 15.75 days. Ablated *nhr-91(tm4713)* deletion mutants display a median lifespan of 24 days, which is significantly more than of their mock-ablated control. (N = 3). See Supplementary Section 7.1.6.1 for details and repeats. The bars represent mean  $\pm$  s.e.m., P value (\* P < 0.05 and \*\* P < 0.01) was determined by *t*-test.

For this experiment germline-ablated N2 or *nhr-91(tm4713)* worms were generated by laser microsurgical removal of the germline precursors Z2 and Z3, also termed germline-laser ablation. Similar to *glp-1(e2141ts)*, N2 worms without a germline have a significantly longer median lifespan, than the un-ablated N2 control (Figure 9) and yielded a lifespan extension of 56.5%. Surprisingly, *nhr-91(tm4713)* mutants displayed a similar level of lifespan extension when their germline was ablated by laser microsurgery of 52.4%. Similar to experiments with *glp-1* mutants described above, loss of *nhr-91* reduced the lifespan of the control group. The absence of a difference in lifespan extension in the degree of germline laser-ablated worms between N2 and *nhr-91(tm4713)* mutants indicates that *nhr-91* is not specifically required for the positive effect on longevity induced by laser-ablation of germline precursor cells.

### **3.2.2 NHR-91 is not required for the longevity of *mes-1(bn7)* mutants**

To further explore the possible role of *nhr-91* in gonadal longevity, a third mode to remove the germline was used, namely *mes-1(bn7)* ts.

In this case, *mes-1(bn7)* mutants were treated with RNAi against *nhr-91* and the lifespan of fertile and sterile *mes-1* mutants was analyzed. Worms carrying this *mes-1* temperature sensitive allele produce about 70% sterile progeny at 25°C. The temperature sensitive period occurs early in embryogenesis, from fertilization to about the 28-cell stage. With luciferase RNAi controls, the mean lifespan extension of sterile *mes-1(bn7)* was 16.1% compared to fertile mutants (Figure 10). Treatment with RNAi against *nhr-91* resulted in a comparable effect; sterile *mes-1* mutants were 12.9% longer lived than the fertile cohort, indicating that loss of *nhr-91* is not detrimental for longevity of sterile *mes-1* mutants. Although the impact of *mes-1(bn7)* on lifespan is smaller than of mutations in *glp-1* or germline laser-ablation, *mes-1* longevity, nevertheless, was found to be NHR-91 dependent, similar to germline laser-ablated worms.

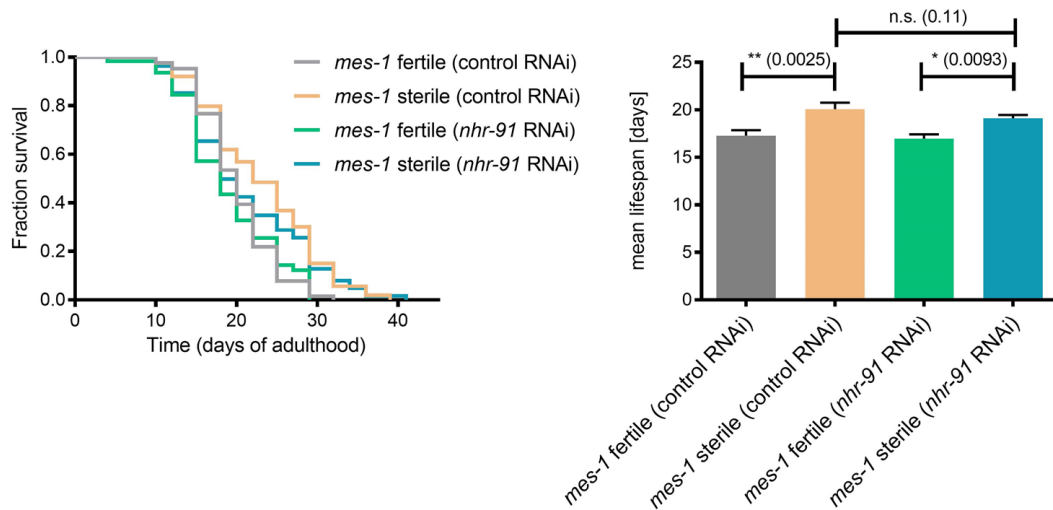


**Figure 10: RNAi against *nhr-91* does not decrease the longevity of sterile *mes-1(bn7)* mutants.** On the left a representative lifespan analysis shows that sterile *mes-1* mutants show longevity when compared to fertile mutants, independent of treatment with control RNAi or RNAi against *nhr-91*. On the right side the median lifespans of 4 experiments are summarized. On control RNAi, sterile *mes-1* mutants live 20.1 days, whereas fertile mutants live only 17.3 days. On *nhr-91* RNAi, sterile *mes-1* mutants live 19.1 days, whereas fertile mutants live only 17 days. The difference in mean lifespan between sterile *mes-1* mutants treated with control RNAi or *nhr-91* RNAi is not significant. (N = 4). See Supplementary Section 7.1.6.1 for details and repeats. The bars represent mean  $\pm$  s.e.m., P value (\*  $P < 0.05$  and \*\*  $P < 0.01$ ) was determined by *t*-test.

### 3.3 Impact of NHR-91 on Insulin/IGF-signaling, autophagy and fat metabolism in *glp-1* mutants

#### 3.3.1 mRNA levels of *nhr-91* are elevated in *glp-1* worms

The previous experiments showed, that *nhr-91* is required for *glp-1*/NOTCH longevity, but not for other interventions or mutations that remove the germline and promote gonadal longevity phenotypes. We proceeded by asking for the mechanisms and the pathways that could be responsible for our observations. In the first step, we investigated, if *nhr-91* expression was induced in *glp-1* worms. Indeed, we could detect the abundance of *nhr-91* mRNA in N2 worms and elevated levels in *glp-1* worms, as displayed in Figure 11.

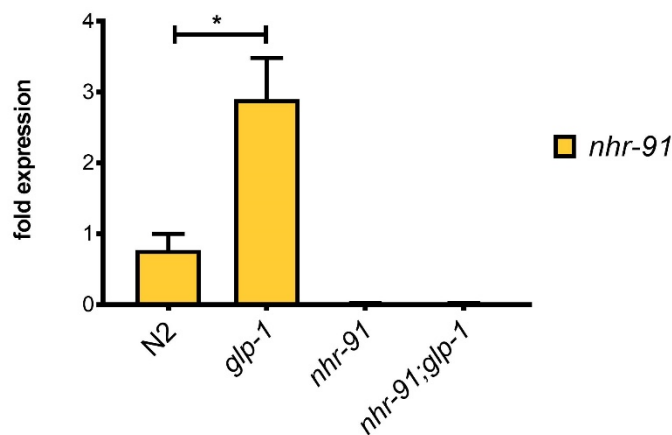


**Figure 11: mRNA levels of *nhr-91* are elevated in sterile *glp-1(e2141ts)* mutants compared to N2.** The level of *nhr-91* mRNA is 3.7-fold higher in *glp-1(e2141ts)* compared to N2. The level of *nhr-91* mRNA in *nhr-91(tm4713)* and *nhr-91(tm4713);glp-1(e2141ts)* animals is below the limit of detection. mRNA levels measured with qPCR. mRNA expression levels are shown as fold expression normalized to mRNA levels of biological replicate #1 of N2 worms. (N = 3). See Supplementary Section 7.1.2 for details and repeats. The bars represent mean  $\pm$  s.e.m., P value (\*  $P < 0.05$ ) was determined by *t*-test.

The single mutant *nhr-91(tm4713)* and the double-mutant *nhr-91(tm4713);glp-1(e2141ts)* showed only very low levels of *nhr-91* mRNA, which in both cases are below the detection limit and suggest the allele to be null. The elevated *nhr-91* mRNA levels in *glp-1* could indicate an increased expression and an increased appearance of NHR-91 protein. However, these differences mRNA levels may also arise because of differences in cell composition *per se* and not because of regulation (*cdc-42* used as housekeeping gene in qPCR experiments).

### 3.3.2 NHR-91 does not regulate Insulin/IGF-signaling in *glp-1* mutants

We reasoned that – since NHR-91 is a nuclear hormone receptor and transcription factor – it should have an effect on the levels of mRNAs. Therefore, I measured the abundance of mRNAs coding specific proteins important for longevity was measured using quantitative real-time PCR.

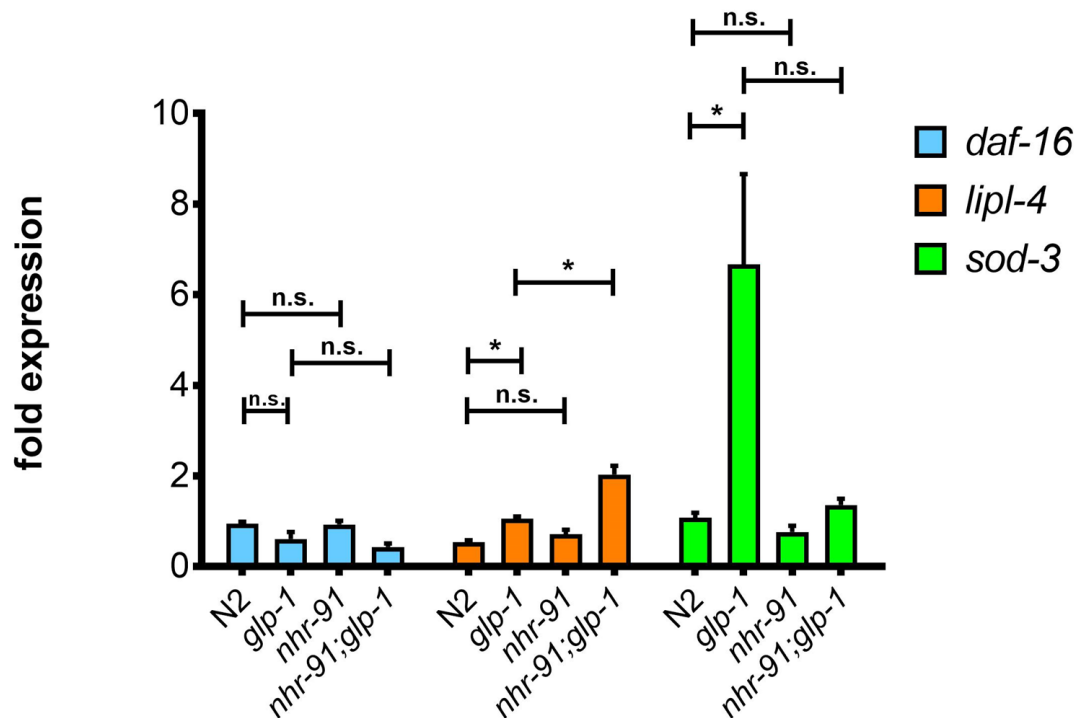


**Figure 12: *daf-16* and its target genes do not show a consistent regulation upon loss of *nhr-91*.** *daf-16* mRNA levels are not found to be regulated. mRNA levels of the DAF-16 downstream targets *lipl-4* and *sod-3* show opposing trends in *glp-1(e2141ts)* when *nhr-91* is deleted. Both genes are elevated in *glp-1(e2141ts)* mutants compared to N2. The level of *lipl-4* is significantly higher in *nhr-91(tm4713);glp-1(e2141ts)* mutants than in *glp-1(e2141ts)*, whereas the level of *sod-3* trends to be lower (no significance,  $p = 0.0567$ ) when comparing those genotypes. mRNA levels measured with qPCR. mRNA expression levels are shown as fold expression normalized to mRNA levels of biological replicate #1 of N2 worms. (N = 3). See Supplementary Section 7.1.2 for details and repeats. The bars represent mean  $\pm$  s.e.m., P value (\*  $P < 0.05$ ) was determined by *t*-test.

A major player in several longevity regimes is DAF-16/FOXO and its target genes. There was no significant difference between *daf-16* mRNA levels in N2

and *glp-1(e2141ts)* worms. Nor could we measure a significant change in *daf-16* mRNA levels in N2 or *glp-1(e2141ts)* worms, upon loss of *nhr-91* either. We further analyzed downstream targets of the DAF-16 transcription factor, which are known to be tightly regulated by DAF-16, but we did not observe a consistent trend in the expression of DAF-16 target genes in a NHR-91 dependent manner (Figure 12). Both DAF-16 Class I targets *lipl-4* and *sod-3* mRNA levels were upregulated in *glp-1(e2141ts)* in comparison with N2 worms. On the one hand, *lipl-4* was further up-regulated in *nhr-91(tm4713);glp-1(e2141ts)* compared to *glp-1(e2141ts)*. On the other hand, *sod-3* showed a tendency, but no significance, (p-value = 0.0567) to be down-regulated in *nhr-91(tm4713);glp-1(e2141ts)* compared to *glp-1(e2141ts)*. Further, there was no regulation of these DAF-16 target genes upon loss of *nhr-91*.

In summary, there was no consistent regulation of DAF-16 or its target genes upon loss of *nhr-91* in sterile *glp-1* mutants and we cannot conclusively show any direct connection of NHR-91 to DAF-16, though this issue would have to be explored further.

### **3.3.3 NHR-91 does not regulate autophagy in *glp-1* mutants**

I next examined genes involved in autophagy, a process required for longevity in multiple pathways including *glp-1*/NOTCH longevity. Notably, PHA-4 is a FOXA transcription factor, which is required for gonadal longevity and drives expression of autophagy genes.

The level of *pha-4* mRNA was up-regulated in *glp-1(e2141ts)* compared to N2, but this up-regulation was not altered in *nhr-91(tm4713);glp-1(e2141ts)* mutants (Figure 13). It was reported, that mRNA levels of *lgg-1* are elevated in *glp-1(e2141ts)* worms, but our experiments only yielded a weak trend towards a higher level when compared with N2. The loss of *nhr-91* did not change the level of *lgg-1* mRNA in *glp-1(e2141ts)* worms. We made similar observations, when examining mRNA levels of the nutrient sensor mTOR, another conserved regulator of aging. Reports showed decreased levels of mTOR mRNA in *glp-1* mutants in comparison to N2. In our hands, the level of TOR mRNA did not

change between N2, *glp-1(e2141ts)*, *nhr91(tm4713)* or *nhr-91(tm4713);glp-1(e2141ts)*. These results indicated that neither mTOR nor autophagy are major transcriptional outputs of NHR-91 in the context of *glp-1*/NOTCH longevity.

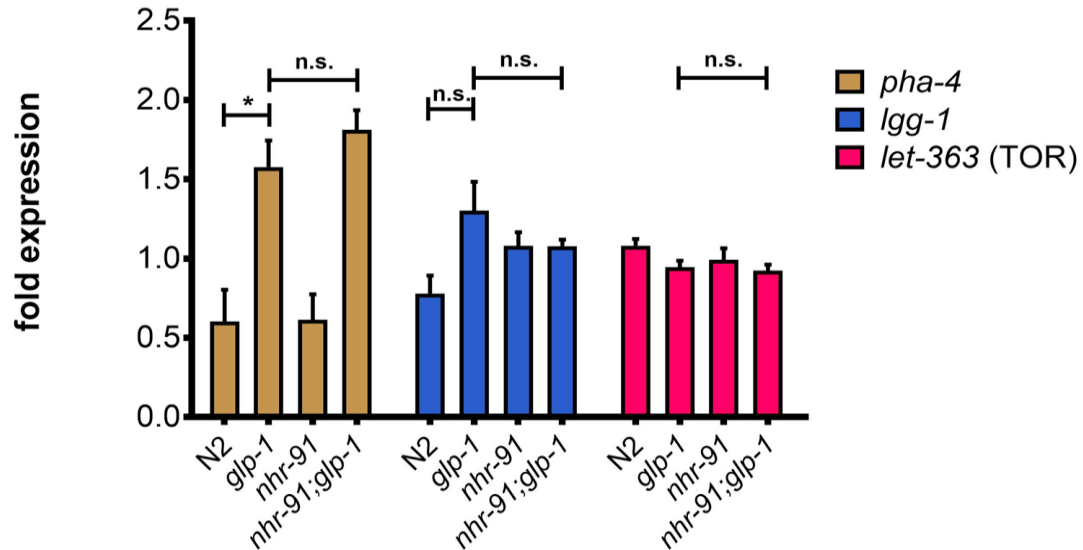


**Figure 13: mRNA levels of autophagy-related genes (*pha-4* and *lgg-1*) and *C. elegans* mTOR (*let-363*) are not affected by loss of *nhr-91* in *glp-1(e2141ts)* worms.** The mRNA level of *pha-4* is upregulated in *glp-1(e2141ts)* in comparison with N2 worms, but not changed upon loss of *nhr-91*. mRNA of *lgg-1* and *let-363* does not show different levels in any genotype investigated. mRNA levels measured with qPCR. mRNA expression levels are shown as fold expression normalized mRNA levels of biological replicate #1 of N2 worms (N = 3). See Supplementary Section 7.1.2 for details and repeats. The bars represent mean ± s.e.m., P value (\* P < 0.05) was determined by *t*-test.

### 3.3.4 NHR-91 regulates fatty-acid desaturases in *glp-1* mutants

Previous studies have shown that *glp-1*/NOTCH longevity is dependent on the fatty acid desaturase *fat-6*, which is regulated by nuclear receptor NHR-80/HNF4 [Goudeau et al., 2011]. We measured the mRNA levels of *nhr-80* and determined that no differences could be detected between *glp-1(e2141ts)* and *nhr-91(tm4713);glp-1(e2141ts)* worms (See Supplementary Section 7.1.2 for details and repeats). Moving further downstream of NHR-80, we investigated the transcript levels of various fatty acid desaturases. Induction of *fat-2*, *fat-3*, *fat-5* and *fat-6*, and reduction of *fat-7*, was observed in *glp-1(e2141ts)* in comparison to N2 (Figure 14).

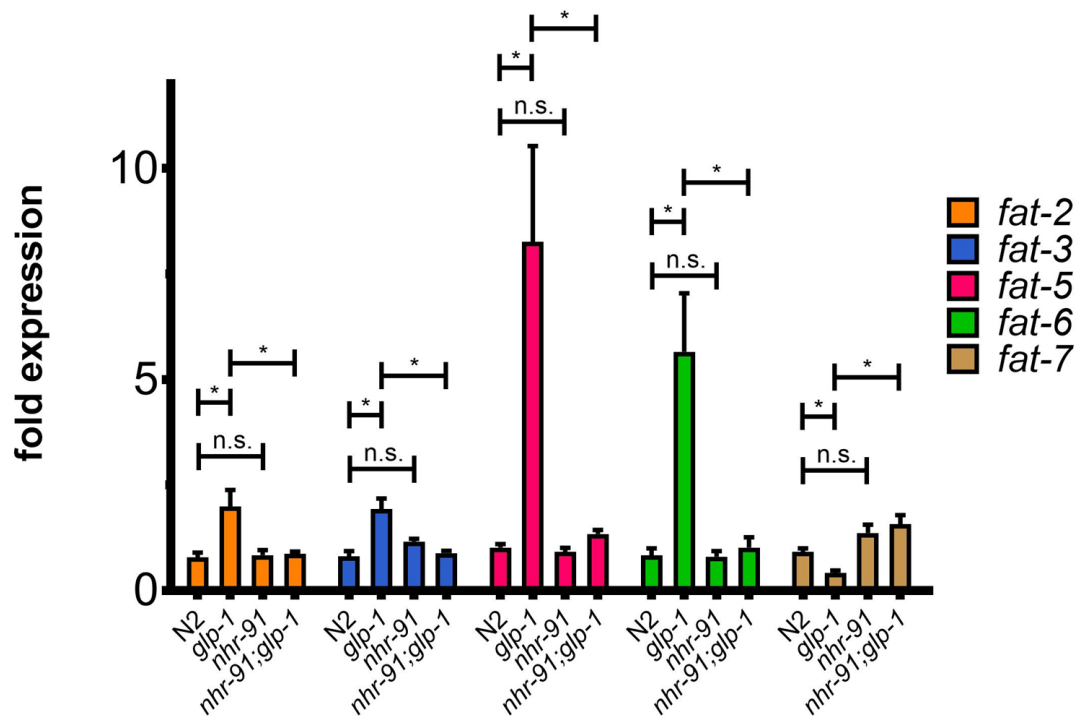


**Figure 14: mRNA levels of fatty acid desaturases are regulated upon loss of *nhr-91* in *glp-1(e2141ts)* worms.** The levels of four different fatty acid desaturases (*fat-2*, *fat-3*, *fat-5* and *fat-6*) are down-regulated in *nhr-91(tm4713);glp-1(e2141ts)* worms when compared to *glp-1(e2141ts)*. *Fat-7* is up-regulated in *nhr-91(tm4713);glp-1(e2141ts)* worms when compared to *glp-1(e2141ts)*. Loss of *nhr-91* does not affect the mRNA levels of these genes in N2 worms. mRNA levels measured with qPCR. mRNA expression levels are shown as fold expression normalized mRNA levels of biological replicate #1 of N2 worms (N = 3). See Supplementary Section 7.1.2 for details and repeats. The bars represent mean  $\pm$  s.e.m., P value (\* P < 0.05) was determined by *t*-test.

*Nhr-91* mutation had no significant effect on mRNA levels of those fatty acid desaturases in worms carrying a germline. In contrast, we observed a down-regulation of fatty acid desaturases *fat-2*, *fat-3*, *fat-5* and *fat-6* when *nhr-91* was absent in *glp-1(e2141ts)* worms. Remarkably, *fat-2*, *fat-3*, *fat-5* and *fat-6* mRNA levels were reduced to levels found in N2 worms, when measured in *nhr-91(tm4713);glp-1(e2141ts)* animals, showing a strong dependence on *nhr-91* in the *glp-1* background. The level of *fat-7* mRNA was elevated in *nhr-91(tm4713);glp-1(e2141ts)* worms when compared to *glp-1(e2141ts)*.

These results indicate that NHR-91 is required for the induction of multiple fatty acid desaturases in *glp-1* worms. The loss of NHR-91 did not affect NHR-80 mRNA levels, which may indicate a NHR-80 independent mechanism. In accord with this idea, *nhr-80* compensatory upregulation of *fat-7* expression remains intact in *nhr-91;glp-1* animals.

### **3.3.5 NHR-91 does not regulate fatty-acid desaturases in *mes-1* mutants**

The induction of fatty acid desaturases upon loss of germline can also be found in sterile *mes-1* worms. Our previous experiments showed that *nhr-91* was not required for the longevity of sterile *mes-1* worms. This leads to the question, does NHR-91 regulate the levels of fatty-acid desaturases in sterile *mes-1* worms?

The levels of *fat-2*, *fat-3* and *fat-6* were elevated in sterile *mes-1* animals in comparison to the fertile group (Figure 15). The levels of *fat-5* and *fat-7*, however, were not significantly changed between the sterile and fertile *mes-1* worms. Knockdown of *nhr-91* by RNAi did not lead to a decrease in levels of those fatty acid desaturases compared to control RNAi. Fertile worms showed no significant changes in mRNA levels of those genes. Nor were the high levels of mRNA reduced in sterile worms.

The absence of clear regulation in *mes-1* further supports the idea of a specific role of NHR-91 in *glp-1*/NOTCH longevity.

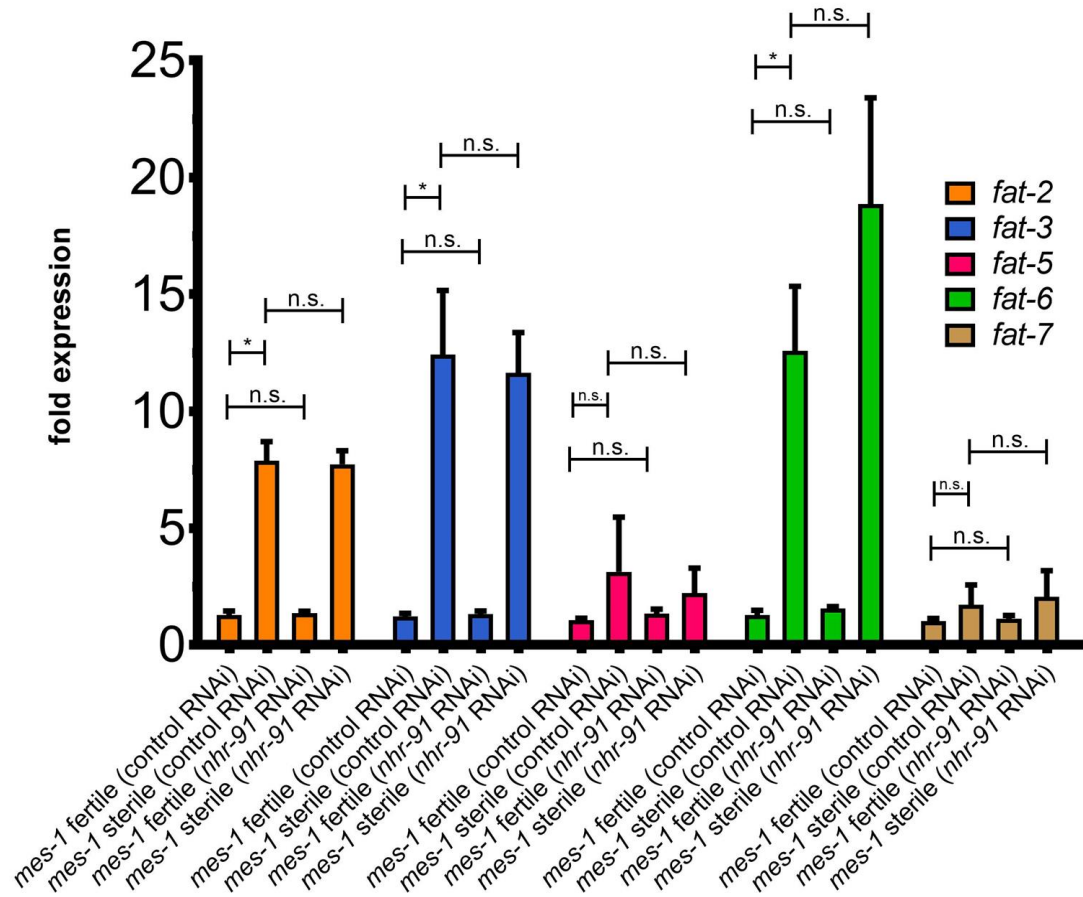


**Figure 15: mRNA levels of fatty acid desaturases are not regulated upon knockdown of *nhr-91* in *mes-1(bn7)* worms.** The levels of *fat-2*, *fat-3* and *fat-6* are elevated in sterile *mes-1* worms, when compared to fertile worms. The levels of *fat-5* and *fat-7* do not show significant change. Knockdown of *nhr-91* by RNAi does not manipulate the expression levels of those fatty acid desaturases, neither in fertile nor in sterile *mes-1* worms. mRNA levels measured with qPCR. mRNA expression levels are shown as fold expression normalized to biological replicate #1 of *mes-1* fertile (control RNAi) worms. (N = 3). See Supplementary Section 7.1.2 for details and repeats. The bars represent mean  $\pm$  s.e.m., P value (\* P < 0.05) was determined by *t*-test.

### 3.3.6 NHR-91 is required for the abundance of unsaturated fatty acids found in *glp-1* worms

To see if the regulation of fatty acid desaturases by NHR-91 results in a physiological readout, I next measured the levels the mono-unsaturated fatty acid species palmitoleic acid, oleic acid, cis-vaccenic acid and linoleic acid by FAME analysis.

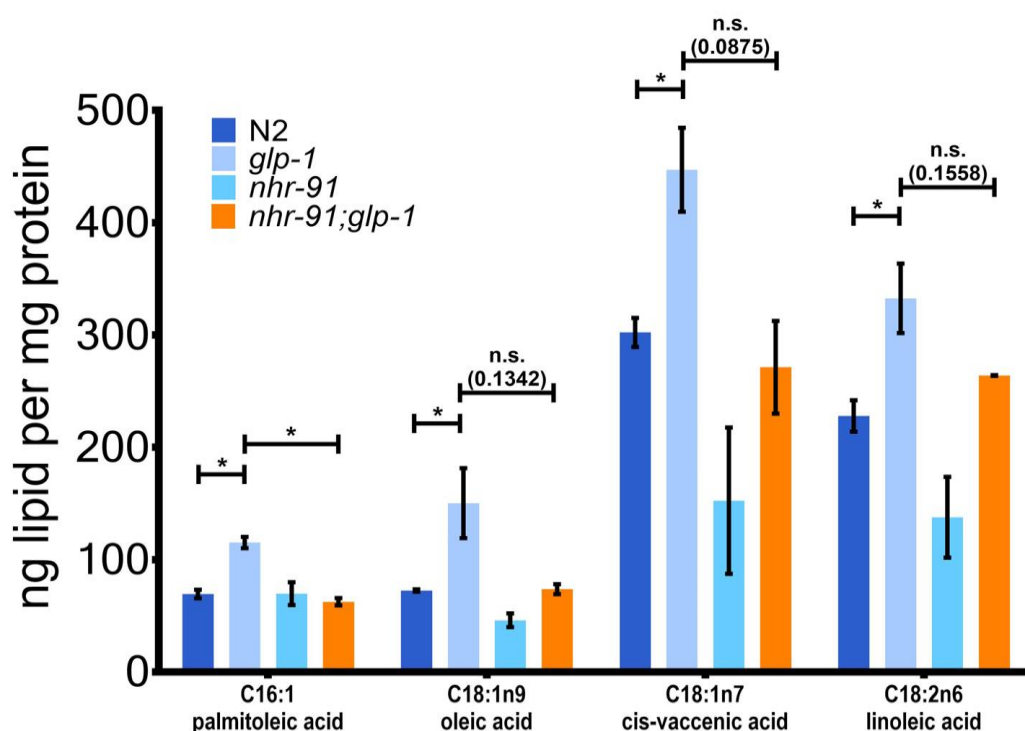


**Figure 16: Levels of mono-unsaturated fatty acids are upregulated in sterile *glp-1(e2141ts)* worms as measured by FAME analysis.** Loss of *nhr-91* reduces level of palmitoleic acid in *glp-1(e2141ts)* worms. The other fatty acid species show a trends towards reduction, but do not reach statistical significance. Levels of fatty acids are shown in ng lipid per mg protein. For N2 and *nhr-91* three repeats are analyzed. For *glp-1* and *glp-1;nhr-91* only two repeats could be analyzed. See Supplementary Section 7.1.4 for details. The bars represent mean  $\pm$  s.e.m., P value (\*  $P < 0.05$ ) was determined by *t*-test.

Levels of 4 different species of unsaturated fatty-acids were significantly more abundant in *glp-1(e2141ts)* worms than in N2 (Figure 16). Further, the level of palmitoleic acid was significantly lower in *nhr-91(tm4713);glp-1(e2141ts)* in comparison to *glp-1(e2141ts)*. Similarly, the levels of oleic acid, cis-vaccenic acid and linoleic acid were lower in *nhr-91(tm4713);glp-1(e2141ts)* in

comparison to *glp-1(e2141ts)*, but the difference did not reach statistical significance. In worm with a germline loss of *nhr-91* leads to a significant reduction of oleic acid (p-value = 0.0129), whereas the levels of palmitoleic acid, cis-vaccenic acid and linoleic acid did not show a significant reduction upon loss of *nhr-91*.

In summary, the FAME analysis shows that loss of *nhr-91* led to lower levels of mono-unsaturated fatty acids in *glp-1(e2141ts)* worms, which could be the cause of the reduction in lifespan that we observed in *nhr-91(tm4713);glp-1(e2141ts)* mutants.

### 3.4 ATF-5 a mediator of gonadal longevity in *C. elegans*

#### 3.4.1 Validation of RNAi-screen longevity candidate ATF-5

The large scale RNAi screen mentioned above showed that *atf-5* RNAi suppressed *glp-1*/Notch longevity. To validate this result, I obtained allele *atf-5(tm4397)* from the NBRP (Japan, National BioResource Project), which contains an 809bp deletion that covers part of the uORF1, the uORF2, the translational start site and the first exon, suggesting that *tm4397* is a putative null allele [Statzer et al., 2020].

The allele was outcrossed and combined with the temperature-sensitive *glp-1(e2141ts)*. As expected, median *glp-1(e2141ts)* life span was 48.3% longer than N2 worms (Figure 17). In *atf-5(tm4397);glp-1(e2141ts)* worms, this longevity was largely abolished and the median lifespan was reduced by 64.2%.

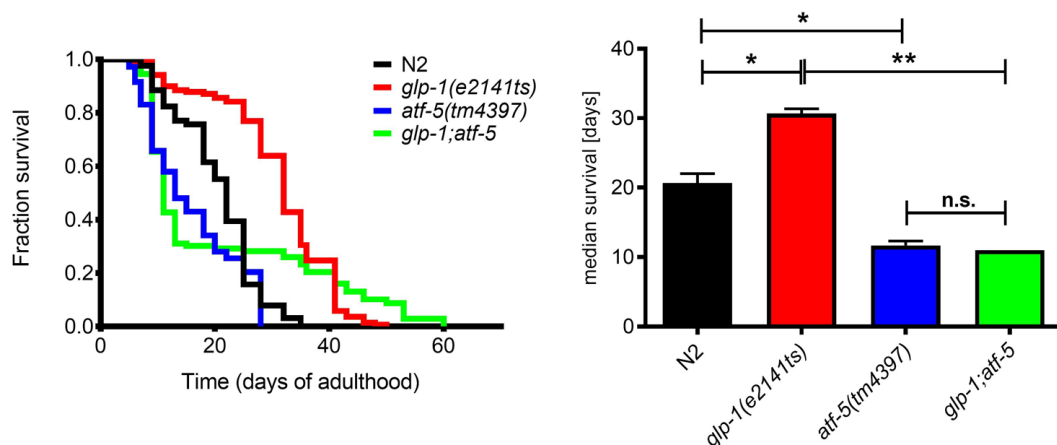


**Figure 17: Loss of *atf-5* abolishes longevity of *glp-1(e2141ts)* mutants.** On the left side, a representative lifespan analysis shows that *atf-5(tm4397)* reduces the lifespan of *glp-1(e2141ts)* mutants. On the right side, a bar graph summarizes the median lifespans of 3 independent experiments. The lifespan of N2 is 20.7 days and thus significantly lower than the 30.7 days of *glp-1(e2141ts)*. The lifespan of *atf-5(tm4397)* mutants is 11.7 days, which is significantly less than in N2, but does not differ from the lifespan of the double-mutant *glp-1(e2141ts);atf-5(tm4397)* (11 days) (N = 3). See Supplementary Section 7.1.6.1 for details and repeats. The bars represent mean  $\pm$  s.e.m., P value (\* P < 0.05 and \*\* P < 0.01) was determined by *t*-test.

The mean lifespan of the double-mutant *atf-5(tm4397);glp-1(e2141ts)* did not show a significant difference compared to *atf-5(tm4397)*. In germline positive worms the deletion of *atf-5* decreased the lifespan by 41.5% relative to N2. This experiment confirmed that loss of *atf-5* abolishes *glp-1*/NOTCH longevity.

### 3.4.2 ATF-5 is required for gonadal longevity

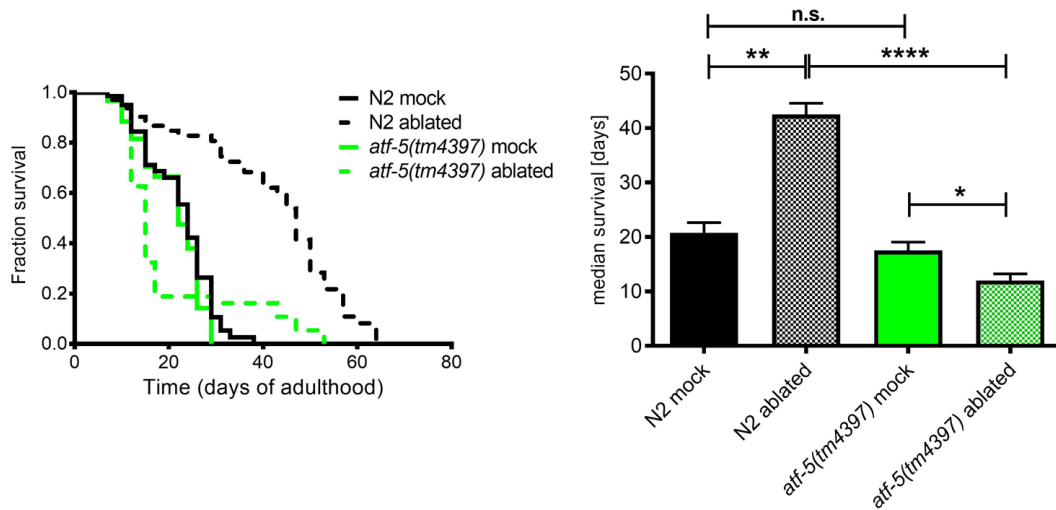


**Figure 18: Loss of *attf-5* abolishes longevity of germline laser-ablated worms.** On the left side, a representative lifespan analysis shows that *attf-5(tm4397)* reduces the lifespan of germline laser-ablated worms. On the right side, a bar graph summarizes the median lifespans of 3 independent experiments. The lifespan of mock-ablated N2 worms is 20.8 days and thus significantly lower than the 42.5 days of laser-ablated N2. The lifespan of *attf-5(tm4397)* mock-ablated is 17.5 days, which does not differ from mock-ablated N2, but does differ from the lifespan of the laser-ablated *attf-5(tm4397)* (12 days). (N = 4). See Supplementary Section 7.1.6.1 for details and repeats. The bars represent mean  $\pm$  s.e.m., P value (\*  $P < 0.05$  and \*\*  $P < 0.01$  and \*\*\*  $P < 0.001$ ) was determined by *t*-test.

As described in the previous chapter, experimenters should make use of additional treatments that generate long lived germline-less worms as models to validate an impact gonadal longevity. We used the germline laser-ablation technique on N2 and *attf-5(tm4397)* worms and monitored the lifespan of those animals.

In comparison with mock-ablated worms we obtained a robust lifespan extension in N2 animals (Figure 18) with an ablated germline. The median lifespan in laser-ablated N2 worms was extended by 104%. Loss of *attf-5* led to a dramatic decrease in median lifespan in laser-ablated worms and was reduced by 72.8% when compared to laser-ablated N2 worms. Laser-ablated *attf-5(tm4397)* worms exhibited a shorter median life span than *attf-5(tm4397)* mock-ablated controls and was reduced (31.4% reduction), yet had a longer maximum lifespan. This unusual shape of the lifespan curve of *attf-5(tm4397)* germline-ablated worms can be explained as arising from initial mortality during

the 10-15 days of adulthood followed by a short plateau phase, and an extension of the maximum life span beyond mock ablated animals, presumably due to some benefit of germline removal in a subset of the population. This curve progression was observed in 3 of 4 biological repeats and was not observed in the mock-ablated control. The mock-ablated controls of N2 and *atf-5(tm4397)* exhibited a more typical Gompertz curve progression, as well as similar median lifespans.

These experiments suggest that *atf-5* is partially required for gonadal longevity, as shown by robust suppression of the lifespan extension in two different models of gonadal longevity. In both experiments, germline-ablated worms could not establish longer lifespans than a germline-positive group. In one experiment loss of *atf-5* also reduced lifespan of germline-positive worms.

### 3.4.3 ATF-5 upstream factors GCN-2 and PEK-1 are not required for gonadal longevity

The integrated stress response is an adaptive pathway responding to several stress stimuli and is known to be a major regulator of ATF-5. The central event in this pathway is the phosphorylation of eukaryotic translation initiation factor 2 alpha (eIF2 $\alpha$ ), which modulates translation. Given the impact of ATF-5 on gonadal longevity, we next asked if the two known *C. elegans* eIF2 $\alpha$  kinases – PEK-1 and GCN-2 – were also required for the lifespan extension in germline-negative worms.

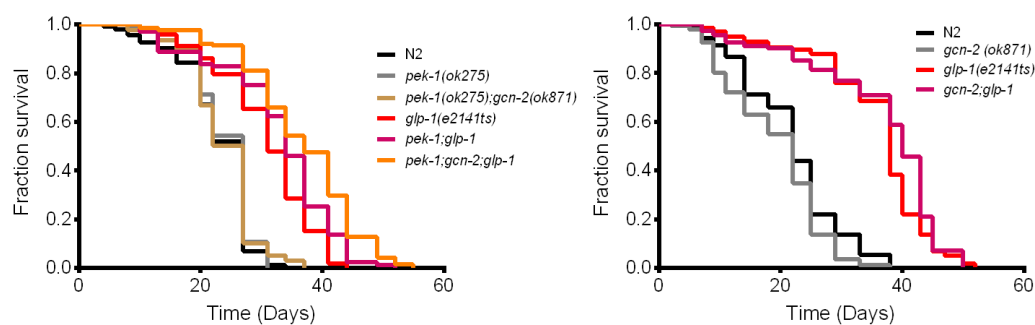


**Figure 19: Loss of ATF-5 upstream factors *pek-1* and *gcn-2* does not abolish GLP-1/NOTCH longevity.** On the left side a representative lifespan for *pek-1(ok275)* and *pek-1(ok275);gcn-2(ok871)* in *glp-1* background shows that neither loss of *pek-1*, nor loss of *pek-1* and *gcn-2* has a negative effect on the lifespan of *glp-1(e2141ts)* worms. On the right side, a representative lifespan for *gcn-2(ok871)* in *glp-1* background shows that loss of *gcn-2* has no effect on the lifespan of *glp-1(e2141ts)* worms. (N = 4). See Supplementary Section 7.1.6.1 for details and repeats.

The deletion mutant *pek-1(ok275)* carried a 2,073-bp deletion that produces a frame shift and stop codon and shows reduced levels of phosphorylated eIF2 $\alpha$  [Richardson et al., 2011]. The *gcn-2* deletion allele *gcn-2(ok871)* had a 1,481-bp in-frame deletion starting and ending in exons. It was previously shown, that this deletion results in a truncated mRNA transcript and is a loss-of-function allele, exhibiting reduction of phosphorylated eIF2 $\alpha$  levels [Rousakis et al., 2013]. We crossed the deletion alleles of both eIF2 $\alpha$  kinases into *glp-1(e2141ts)* worms to generate double-mutants. We also combined the deletions of those kinases in *glp-1(e2141ts)* worms to generate triple-mutants.

The deletion of *gcn-2* did not reduce the lifespan of N2 worms in 3 of 4 replicates significantly (Figure 19). In *glp-1* worms this deletion even modestly extended

the lifespan in 3 of 4 replicates by 9-10% (see Supplementary Section 7.1.6.1 for details). Loss of *pek-1* had no effect on N2 lifespan. We found that the deletion of *pek-1* in *glp-1(e2141ts)* worms did not reduce the longevity in 3 of 4 replicates significantly. The combination of both deletion alleles again did not affect the lifespan of N2 worms. Similar to the *gcn-2(ok871);glp-1(e2141ts)* mutant, we found surprisingly that the triple-mutant *gcn-2(ok871);pek-1(ok275);glp-1(e2141ts)* even has a longer lifespan in 3 of 4 replicates, ranging from 7-20% extension (see Supplementary Section 7.1.6.1 for details). To validate that alleles *gcn-2(ok871)* and *pek-1(ok275)* were abolishing the eIF2 $\alpha$  phosphorylation in *glp-1* mutants, a western blot analysis of phosphorylated eIF2 $\alpha$  was carried out (Figure 20). In N2 and sterile *glp-1* worms we could detect levels of phosphorylated eIF2 $\alpha$ , whereas the triple-mutant *gcn-2(ok871);pek-1(ok275);glp-1(e2141ts)* showed no detectable p-eIF2 $\alpha$ . Histone H3 was used as loading control and indicated that this effect was not due to overall reduced protein levels in the sample.

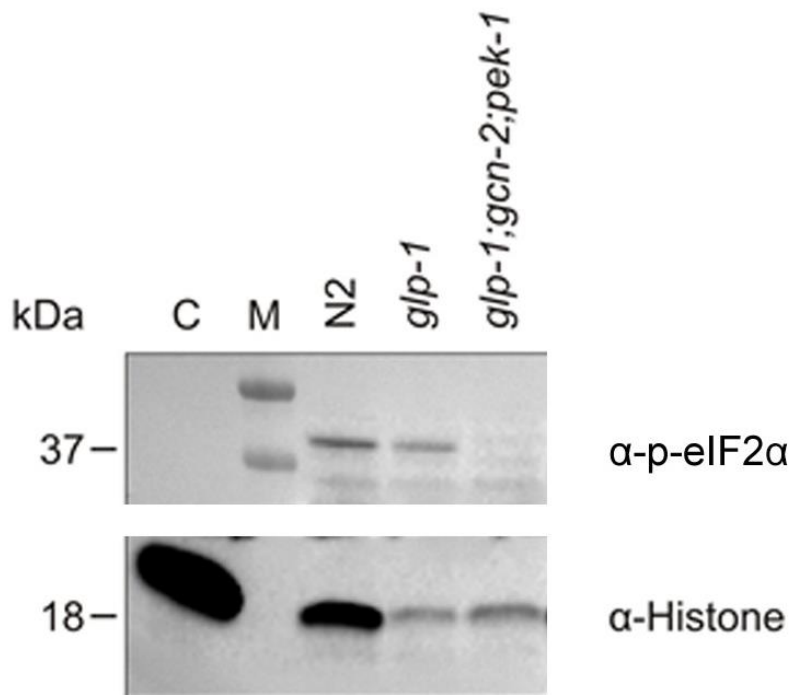


**Figure 20: Western Blot analysis of phosphorylated eIF2 $\alpha$  showed that loss of *gcn-2* and *pek-1* resulted in drastic reduction of p-eIF2 $\alpha$  in *glp-1* sterile mutants.** kDa = kilo Dalton, indicators for protein size / C = control / M = protein marker. Western blot analysis with antibodies against p-eIF2 $\alpha$  and Histone H3 was carried out.

These experiments show that the known ATF-5 upstream factors PEK-1 and GCN-2, unlike ATF-5 itself, are not required for gonadal longevity. Thus, ATF-5's role in gonadal longevity is independent from the two eIF2 $\alpha$  kinases and likely must be regulated or triggered by other factors in this context.

### 3.5 Factor and pathway downstream of ATF-5 mediating its role in gonadal longevity

#### 3.5.1 RNA-sequencing reveals targets of ATF-5 in *glp-1* mutants

As an activating transcription factor ATF-5 promotes the transcription of target genes and hence fulfills its biological role in coping with different stress stimuli. Nevertheless, the target genes of ATF-5 have not yet been identified experimentally in *C. elegans* and could only be inferred from work carried out in other organisms like yeast [Mittal et al., 2017].

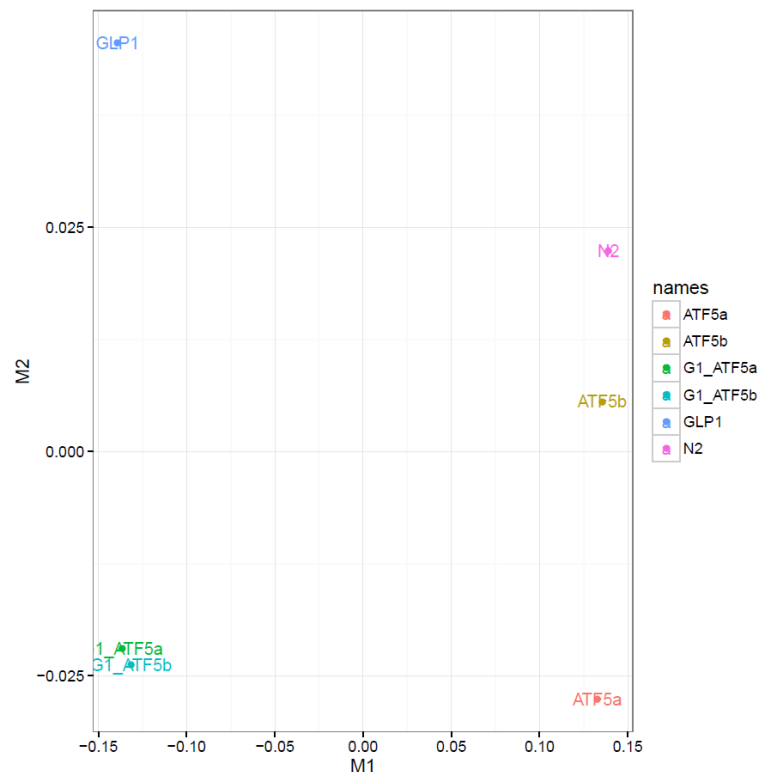


**Figure 21: Principal Component Analysis of RNAseq data.** 6 different genotypes are shown. ATF5a = *atf-5(tm4397)*, ATF5b = *atf-5(tm4212)*. Data processing and representation was done by MIPZ for quality control of sequencing data.

To elucidate the *atf-5* transcriptome and unravel target processes regulated by this transcription factor, we carried out RNA-seq experiments comparing day 1 adults of N2, *atf-5(tm4397)*, *glp-1(e2141ts)* and *atf-5(tm4397);glp-1(e2141ts)*. For each genotype we used at least 3 biological replicates. We also examined allele *atf-5(tm4212)* in these backgrounds. The *tm4397* allele is a large deletion

(806bp) and includes the first intron of *atf-5*. The allele *tm4212* is a 314bp deletion and mostly deletes a sequence in the first intron of *atf-5*.

RNA-sequencing and initial data processing was carried out at the MIPZ Genome Center (Max-Planck-Institut für Pflanzenzüchtungsforschung, Köln). Principal component analysis (Figure 21) showed a distinct allocation of the genotypes. Genotypes containing a germline, namely N2, *atf-5(tm4397)* (plot: ATF5a) and *atf-5(tm4212)* (plot: ATF5b) clustered together, and germline-free genotypes *glp-1(e2141ts)* (plot: GLP1), *atf-5(tm4397);glp-1(e2141ts)* (plot: G1\_ATF5a) and *atf-5(tm4212);glp-1(e2141ts)* (plot: G1\_ATF5b) clustered together. Evidently principle component M1 separates genotypes according to germline, and principal component M2 differentiates groups according to the presence or absence of *atf-5*.

For further analysis, the datasets obtained with genotypes carrying *atf-5(tm4397)* were used, which was based on the more distinct principal component M2 away from N2.

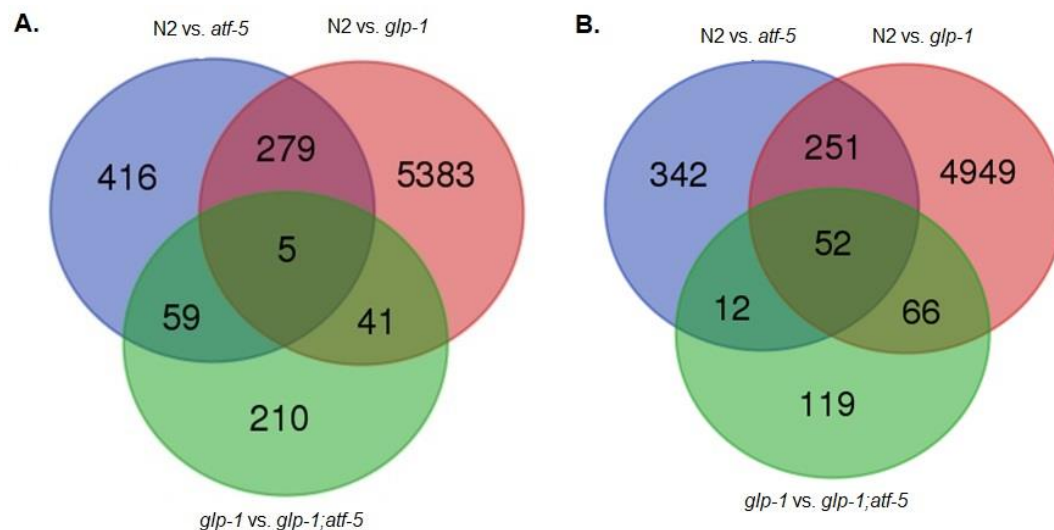


**Figure 22: Venn-Diagram representing RNAseq results of differentially regulated genes with a cut off for  $\log_2(\text{fold change}) \leq 0.25$  and  $p\text{-value} < 0.05$ . In **A**, the downregulated genes are presented. In **B**, the upregulated genes are presented. Data processing and representation was done by Torsten Bücher.**

We found 564 genes differentially regulated between *glp-1(e2141ts)* and *atf-5(tm4397);glp-1(e2141ts)*, applying the cut-offs of  $\log_2(\text{fold-change}) < 0.25$  and  $p\text{-value} < 0.05$  to our data. Among these genes, 315 were downregulated, whereas 249 were upregulated (Figure 22) in *atf-5(tm4397);glp-1(e2141ts)* compared to *glp-1(e2141ts)*.

A volcano plot showing the same data (Figure 23) figuratively depicted the significantly changed genes, highlighting the top 15 downregulated genes with the highest fold-change. Within these top hits were several heat-shock proteins (*hsp-16.11*, *hsp-16.48*, F44E5.5), two genes not yet connected to gonadal longevity (*tiar-2*, *haf-6*) and *atf-5*, which can be seen as a control.

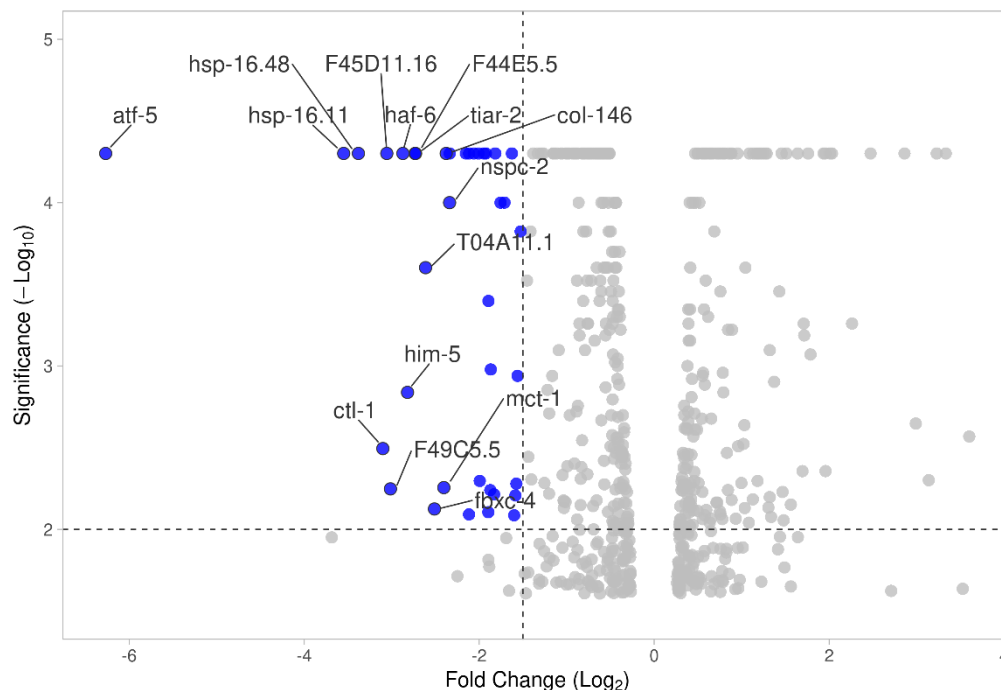


**Figure 23: Volcano plot highlighting top 15 downregulated genes in *atf-5(tm4397);glp-1(e2141ts)* compared to *glp-1(e2141ts)*.** Genes downregulated upon loss of *atf-5(tm4397)* are shown in blue. Top 15 hits are named. Cut-off for fold-change ( $\log_2$ ) is set to 1.5 and for significance ( $-\log_{10}$  of  $p\text{-value}$ ) is set to 2. Plot created with VolcanoR (<https://huygens.science.uva.nl/VolcanoR/>). Criterion for ranking hits: fold change.

Above that, a GO-Term analysis of genes downregulated in *glp-1(e2141ts)* upon loss of *atf-5* aggregated the single genes into groups related to biological pathways to identify clusters and functions that are impacted by loss of *atf-5* (Figure 24). This analysis showed an enrichment in ER-related GO-Terms such as protein refolding (GO:0042026), chaperone cofactor-dependent protein

refolding (GO:00514085) or response to heat (GO:0009408). Genes found as hits for these GO-Terms are overlapping (Data in Supplement 7.1.6/Table 3). The two GO-Terms describing protein refolding processes are highly enriched and harbor both *hsp-4*, F44E5.5, *hsp-70* and *hsp-1*. The GO-Term “chaperone cofactor-dependent protein refolding” further describes *dnj-13* as a hit. The third enriched GO-Term “response to heat” includes in addition *hsp-16* family members, *daf-21/hsp-90* and *sti-1*. These results indicated that ATF-5 is interacting with ER-related pathways and functions in the context of gonadal longevity.

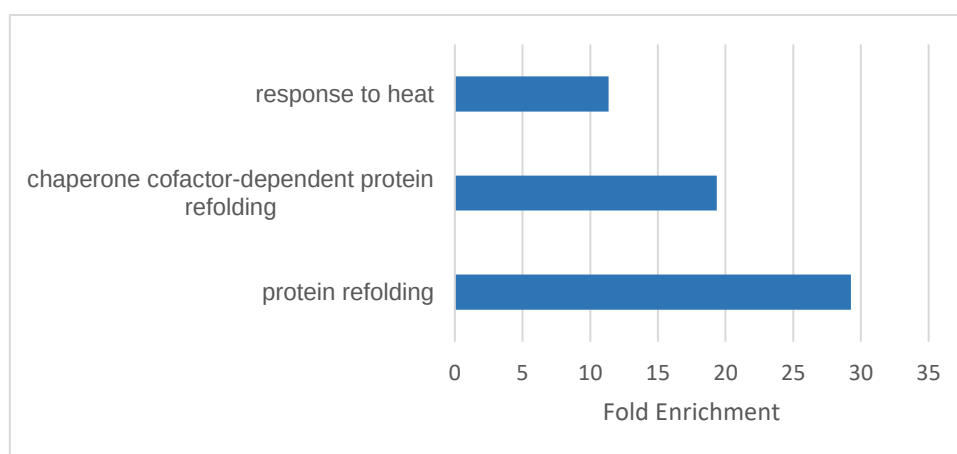


**Figure 24: GO Enrichment Analysis of downregulated genes in *atf-5(tm4397);glp-1(e2141ts)* compared to *glp-1(e2141ts)*.** Data in Supplement 7.1.7 see Table 4. Data processed with PANTHER16.0 (<http://pantherdb.org/>). Reference List: *Caenorhabditis elegans* (all genes in database). Annotation Data Set: GO biological process complete. Test Type: Fisher's Exact. Correction: Calculate False Discovery Rate.

ATF-5 is an activating transcription factor and its direct targets are found in the previously described group of downregulated genes in *atf-5(tm4397);glp-1(e2141ts)* compared to *glp-1(e2141ts)*. Nevertheless, the data obtained by RNA sequencing also displays genes that are regulated in the opposite direction. These genes were not followed up in this thesis, but the analysis is carried out for the sake of completeness. A volcano plot (Figure 25) highlights the top 15 upregulated genes with the highest fold-change. Within these top hits were several genes that are nematode specific and of yet unknown function (*pud-2.1*, *pud-1.2*, *nspc-6*, *nspc-6*). Within the top 15 upregulated genes, *ptc-2* could be of interest, because it is an ortholog of

Drosophila PATCHED (PTC) and human PTCH and could be involved in lipid transport [Shin et al., 2011].

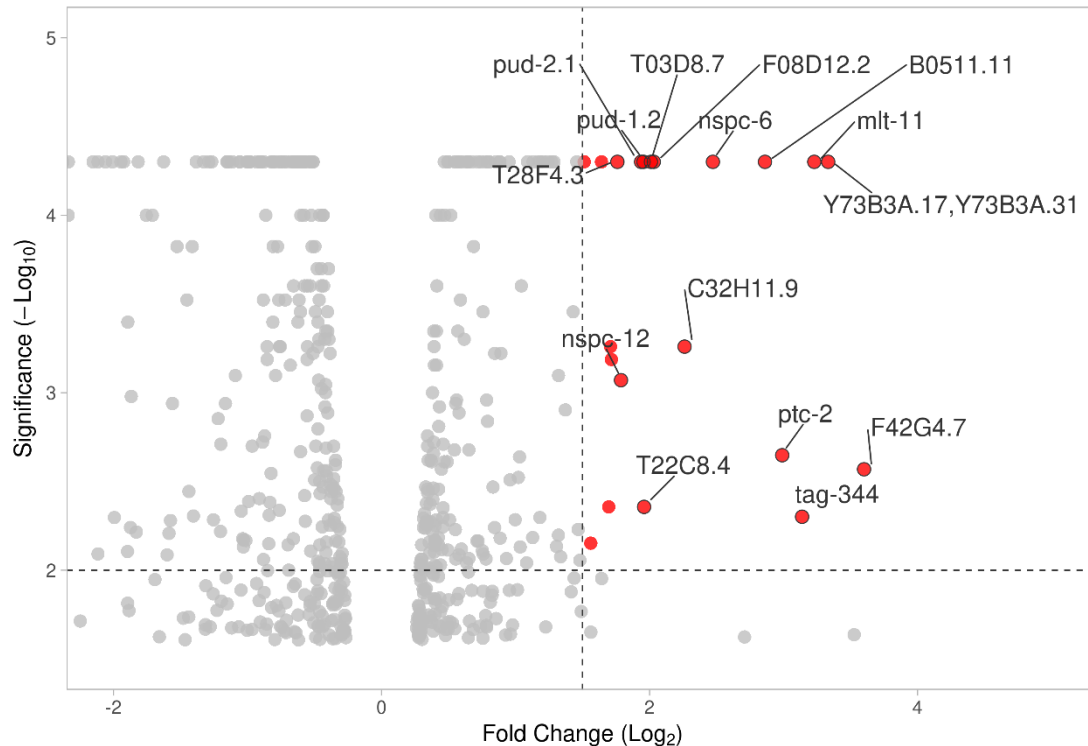


**Figure 25: Volcano plot highlighting top 15 upregulated genes in *atf-5(tm4397);glp-1(e2141ts)* compared to *glp-1(e2141ts)*.** Genes downregulated upon loss of *atf-5(tm4397)* are shown in blue. Top 15 hits are named. Cut-off for fold-change ( $\log_2$ ) is set to 1.5 and for significance ( $-\log_{10}$  of p-value) is set to 2. Plot created with VolcanoR (<https://huygens.science.uva.nl/VolcanoR/>). Criterion for ranking hits: fold change.

In addition, a GO-Term analysis of genes upregulated in *glp-1(e2141ts)* upon loss of *atf-5* aggregated the single genes into groups related to biological pathways (Figure 26) and yielded two enriched GO-Terms: response to gamma radiation (GO:0010332) and innate immune response (GO:0045087). *Parg-2* is one of four genes emerging from the GO-Term “response to gamma radiation”. Loss of Drosophila PARG is associated with neurodegeneration [Hanai et al., 2004]. For the second enriched GO-Term “innate immune response” 24 genes are accounted in the dataset. Three genes of the *dod* family (downstream of DAF-16) result from this GO-Term, namely *dod-17*, *dod-22* and *dod-24*. *Irg-4* and *irg-5*, immune effectors that contain CUB-like domains and targets of NHR-86, as well as another NHR-86 target (*drd-50*) [Peterson et al., 2019] are

associated to this enriched GO-Term (Data in Supplement 7.1.7/Table 5).

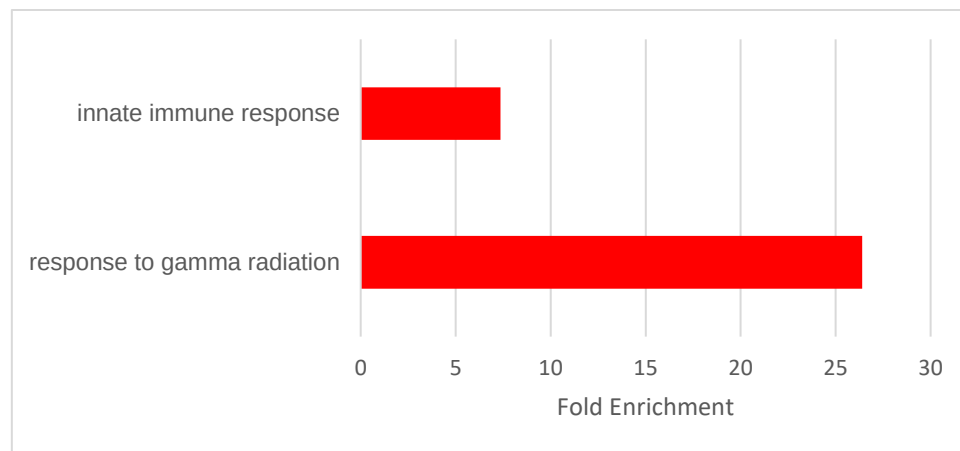


**Figure 26: GO Enrichment Analysis of upregulated genes in *atf-5(tm4397);glp-1(e2141ts)* compared to *glp-1(e2141ts)*.** Data in Supplement 7.1.7 see Table 5. Data processed with PANTHER16.0 (<http://pantherdb.org/>). Reference List: *Caenorhabditis elegans* (all genes in database). Annotation Data Set: GO biological process complete. Test Type: Fisher's Exact. Correction: Calculate False Discovery Rate.

### **3.5.2 A large scale RNAi-based screen identified genes regulated by ATF-5 to be required for GLP-1/NOTCH longevity**

To understand whether genes regulated by ATF-5 in *glp-1(e2141ts)* mutants contribute towards the regulation of longevity, we made use of a RNAi-based lifespan screen to systematically uncover their impact on life span. In particular, the genes downregulated in *glp-1(e2141ts)* upon loss of *atf-5* were further investigated, because as a transcriptional activator, these could be direct targets of ATF-5 causing the longevity phenotype.

From the 315 genes down-regulated in *atf-5(tm4397);glp-1(e2141ts)* compared to *glp-1(e2141ts)* we identified 106 genes, which were conserved and had orthologs in humans. RNAi against these genes was used to down-regulate the encoded protein in *glp-1(e2142ts)* worms and the lifespan was measured.

The screen was carried out twice, but due to a contamination one replicate had to be excluded from analysis. For each RNAi clone we used 75 animals, which were distributed on 3 NGM plates with respectively 25 adult *glp-1(e2141ts)* worms. The screen was scored in an interval of 3-5 days and the mean lifespan was calculated.

Of the genes tested, RNAi against 37 genes resulted in a decreased lifespan. Within this group 10 clones shortened the mean lifespan more than 10% and two other genes shortened the lifespan by 9,6% (*gpa-15*) and 9,2% (*sdz-24*). These genes are listed in Table 1. The complete results are displayed in Supplementary Materials 7.1.3.

To follow up these results, lifespan analysis and qPCR validation of RNAseq results of these candidates were carried out. In addition, *haf-6* and *tiar-2*, which were regulated by ATF-5 in *glp-1(e2141ts)* worms and were not described as targets of ATF-5 or its homologs before, were included in the analysis.

| Gene Name                | Blinded Clone Number | Mean Lifespan reduction compared to control |
|--------------------------|----------------------|---------------------------------------------|
| <i>hsp-1</i> /F26D10.3   | 46                   | 58,2%                                       |
| <i>hsp-4</i> /F43E2.8    | 20                   | 47,7%                                       |
| <i>npl-4.1</i> /F59E12.4 | 16                   | 44,5%                                       |
| <i>gei-4</i> /W07B3.2    | 28                   | 43,9%                                       |
| ZK177.2                  | 17                   | 24,7%                                       |
| <i>slc-36.5</i> /C44B7.6 | V6                   | 23,9%                                       |
| <i>dnj-12</i> /F39B2.10  | 7                    | 12,7%                                       |
| T20D4.7                  | 63                   | 11,8%                                       |
| F17B5.1                  | 6                    | 11,1%                                       |
| F18A11.5                 | 26                   | 10,3%                                       |
| <i>gpa-15</i> /M04C7.1   | 5                    | 9,6%                                        |
| <i>sdz-24</i> /K07E8.3   | 10                   | 9,2%                                        |

**Table 1: Top 12 Genes with the strongest negative impact on *glp-1(e2141ts)* lifespan, regulated by loss of *atf-5* in *glp-1(e2141ts)*.** Name of the genes and/or sequence name, its blinded number within the screen and the reduction of mean lifespan in percent compared to *glp-1(e2141ts)* worms treated with luciferase RNAi are displayed. See Supplementary Section 7.1.3 for details and repeats.

### 3.5.3 Several potential targets of ATF-5 from RNAseq and RNAi-based screen are not required for GLP-1/NOTCH longevity

#### 3.5.3.1 *haf-6* and *tiar-2*

We identified two genes, *haf-6* and *tiar-2*, strongly regulated by ATF-5 in *glp-1(e2141ts)* worms and were not described as targets of ATF-5 or its homologs before.

*Haf-6* encodes a ATP-binding cassette (ABC) transporter gene required for efficient RNAi and is found in intestine and germline localizing to intracellular, reticular membranes [Sundaram et al., 2006]. *Tiar-2* is an ortholog of human TIA1 and TIAL1, encodes an RNA binding protein and is involved in axon regeneration [Andrusiak et al., 2019].

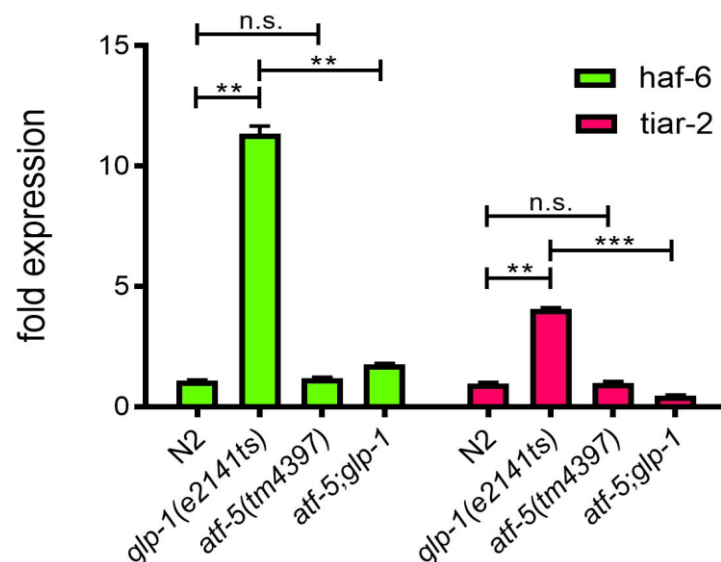


**Figure 27: Novel targets of ATF-5 are up-regulated in *glp-1* worms.** mRNA of *haf-6* is 10.6-fold induced in *glp-1(e2141ts)* and reduced to only 1.76-fold induction in *att-5(tm4397);glp-1(e2141ts)*. Loss of *att-5* does not significantly change mRNA levels of *haf-6* in N2 worms. mRNA of *tiar-2* is 4.2-fold induced in *glp-1(e2141ts)* and reduced to only 0.45-fold in *att-5(tm4397);glp-1(e2141ts)*. Loss of *att-5* does not change mRNA levels of *tiar-2* in N2 worms. mRNA expression levels are shown as fold expression normalized to mRNA levels of biological replicate #1 of N2 worms (N = 3). See Supplementary Section 7.1.2 for details and repeats. The bars represent mean  $\pm$  s.e.m., P value (\* P < 0.05) was determined by *t*-test.

We validated the result of the RNA sequencing by qPCR and found that both genes were up-regulated in *glp-1(e2141ts)* compared to N2 (Figure 27). We

measured a 10.6-fold induction for *haf-6* and a 4.2-fold induction for *tiar-2* mRNA.

This induction was abolished in *atf-5(tm4397);glp-1(e2141ts)* double-mutants. The expression of *haf-6* was reduced 6.4-fold upon loss of *atf-5*, whereas *tiar-2* was reduced 9-fold.

The data also showed that the levels of both genes did not change significantly between N2 and *atf-5(tm4397)*. The mode of regulation described above suggested both genes as potentially promising candidate mediators of the role of ATF-5 in gonadal longevity. We monitored the lifespan of *haf-6(ok3219)* mutants crossed into *glp-1(e2141ts)*. However, loss of *haf-6* did not change the lifespan of N2 worms. Nor could we see an impact of *haf-6* on the longevity of *glp-1(e2141ts)* worms (Figure 28).

Because a deletion mutant for *tiar-2* was not available, we carried out the lifespan analysis using *tiar-2* RNAi. The knockdown of *tiar-2* did not impair the lifespan of N2 worms. Similarly *tiar-2* RNAi had no effect on *glp-1(e2141ts)* longevity (Figure 28). These experiments showed that despite *atf-5* strongly regulating *haf-6* and *tiar-2* in *glp-1(e2141ts)* animals, neither gene was required to mediate *glp-1*/NOTCH longevity.

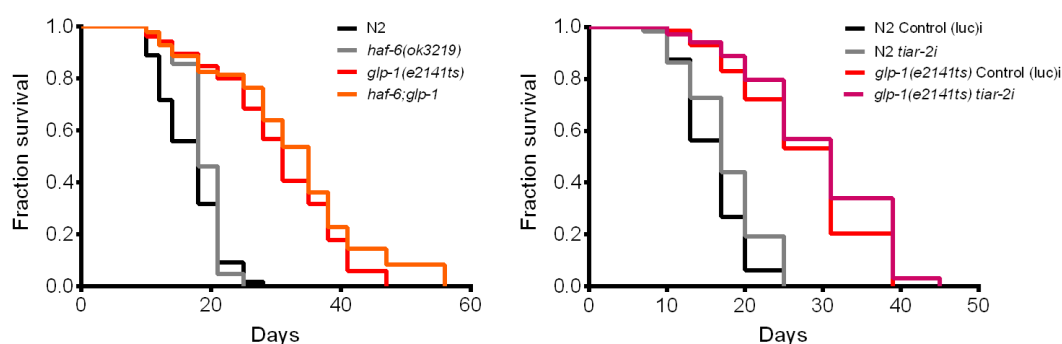


**Figure 28: Loss of ATF-5 downstream factors *haf-6* and *tiar-2* does not abolish GLP-1/NOTCH longevity.** On the left side a representative lifespan for *haf-6(ok3219)* and on the right side a lifespan using *tiar-2* RNAi in *glp-1(e2141ts)* background show, that loss of *haf-6* or *tiar-2* have neither an effect on lifespan of N2 worms, nor on GLP-1/NOTCH longevity. (each N = 2). See Supplementary Section 7.1.6.1 for details and repeats.

### 3.5.3.2 F17B5.1 and T20D4.7, orthologs of human NXNL2

We followed up on a handful of potential mediators of ATF-5 in gonadal longevity found in our RNAi-based lifespan screen for further analysis.

We first investigated F17B5.1 and T20D4.7 because both are orthologs of the same human gene, namely human NXNL2 (nucleoredoxin-like 2) and human NXN (nucleoredoxin) based on sequence homology. Nucleoredoxin is a regulator of Wnt signaling and can activate the pathway by dissociation from an essential Wnt adaptor protein upon oxygen stress [Funato and Miki, 2010].

Unfortunately, our attempt to measure the mRNA levels of these genes by qPCR was not successful, because no primer pair could yield sufficient quality for the analysis. We then went on to use the RNAi clones from the screen to validate the role of F17B5.1 and T20D4.7 in *glp-1* longevity and carried out a regular lifespan analysis including N2 worms. Unfortunately, both clones failed to replicate the effect on *glp-1* longevity or on the lifespan of N2 worms in the validation experiment (See Supplementary Section 7.1.6.1 for details and repeats).

### 3.5.3.3 *sdz-24* and *dnj-12*

*Dnj-12* and *sdz-24* are both related to different kinds of stress responses and were therefore prioritized for follow-up experiments. *dnj-12* encodes a protein containing a DnaJ domain. Such domains are found in co-chaperones recruiting HSP-70 partners and accelerate their ATP-hydrolysis [Kelley, W.L., 1998]. qPCR analysis revealed, that the levels of *dnj-12* are lower in *glp-1* animals compared to N2 (2.2-fold decrease) and loss of *atf-5* further reduced the mRNA levels of *dnj-12* in *glp-1(e2141ts)* worms, which validated the RNAseq results (Figure 29). Knockdown of *dnj-12* by RNAi had no effect on lifespan of N2 or *glp-1(e2141ts)* worms. This result was contrary to the outcome of the screen.

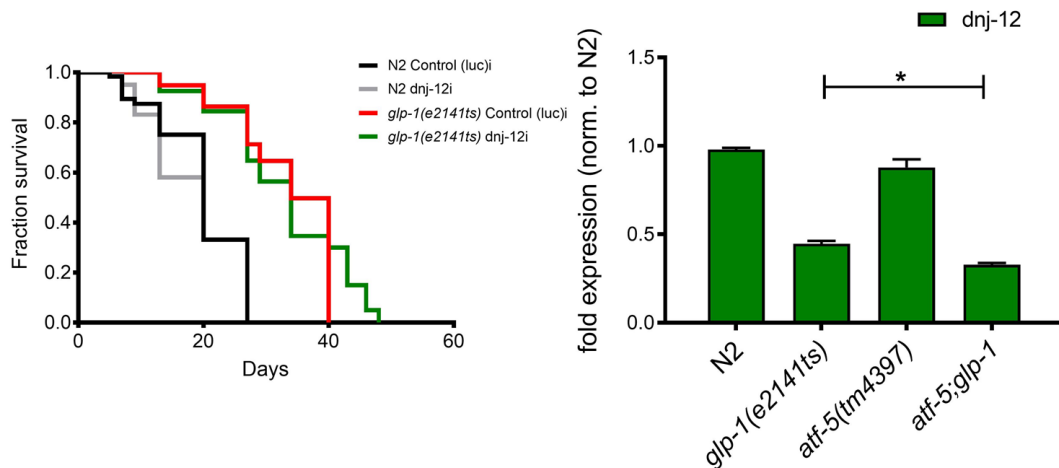


**Figure 29: Knockdown of *dnj-12* does not abolish GLP-1/NOTCH longevity.** On the left side, a representative lifespan using *dnj-12* RNAi in *glp-1(e2141ts)* background shows, that knockdown of *dnj-12* has neither an effect on lifespan of N2 worms, nor on GLP-1/NOTCH longevity (N = 2). See Supplementary Section 7.1.6.1 for details and repeats. On the right side, qPCR shows that mRNA of *dnj-12* is reduced in *glp-1(e2141ts)* vs. N2. mRNA levels are further reduced by loss of *atf-5* in *atf-5(tm4397);glp-1(e2141ts)*. Loss of *atf-5* does not change mRNA levels of *dnj-12* in N2 worms. mRNA expression levels are shown as fold expression normalized to mRNA levels of biological replicate #1 of N2 worms (N = 3). See Supplementary Section 7.1.2 for details and repeats. The bars represent mean  $\pm$  s.e.m., P value (\*  $P < 0.05$ ) was determined by *t*-test.

*sdz-24* is a homolog of human RPA1 (replication protein A1), which is a ssDNA binding protein and required for DNA metabolism, including replication but also damage repair and stress response [Zou et al., 2006].

The levels of *sdz-24* levels were up-regulated in *glp-1* animals compared to N2 (18-fold increase). Loss of *atf-5* reduced the mRNA levels of *sdz-24* around 1.9-fold in *glp-1(e2141ts)* background, but had no effect on *sdz-24* levels in N2 worms (Figure 30). This qPCR data validated the RNAseq result. Knockdown of *sdz-24* by RNAi had no effect on lifespan of N2 or *glp-1(e2141ts)* worms. This result was contrary to the outcome of the screen.

We analyzed the impact of *sdz-24* and *dnj-12* on *glp-1* longevity with regular lifespan experiments in order to establish their role as mediators of *atf-5* downstream targets in lifespan regulation. As described above, we could not reproduce the finding of the screen and could not consider *dnj-12* nor *sdz-24* as downstream targets of ATF-5, required for *glp-1*/NOTCH longevity.

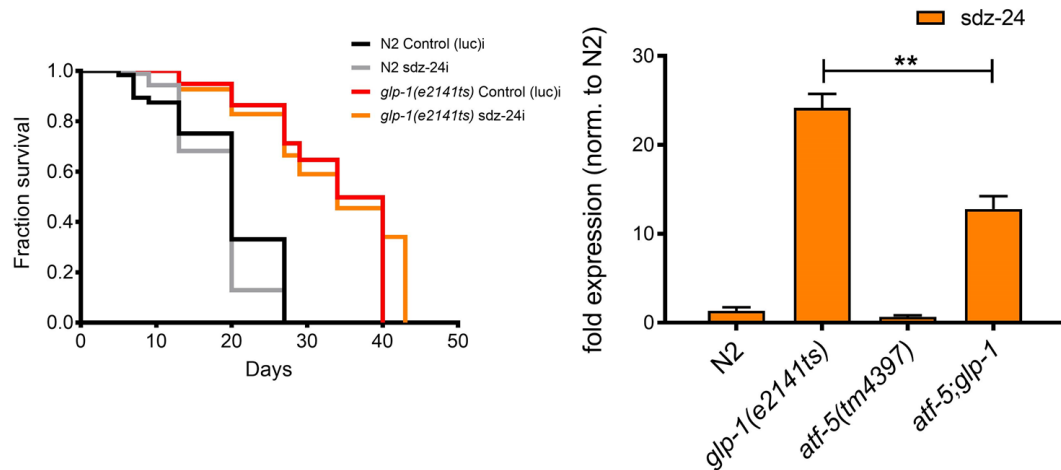


**Figure 30: Knockdown of *sdz-24* does not abolish GLP-1/NOTCH longevity.** On the left side, a representative lifespan using *sdz-24* RNAi in *glp-1(e2141ts)* background shows, that knockdown of *sdz-24* reduces lifespan of N2 worms to a minor extend, but does not significantly change GLP-1/NOTCH longevity (N = 2). See Supplementary Section 7.1.6.1 for details and repeats. On the right side, qPCR shows that mRNA of *sdz-24* is 18-fold induced in *glp-1(e2141ts)* vs. N2. mRNA levels are reduced by loss of *atf-5* in *atf-5(tm4397);glp-1(e2141ts)*. Loss of *atf-5* does not change mRNA levels of *sdz-24* in N2 worms. mRNA expression levels are shown as fold expression normalized to mRNA levels of biological replicate #1 of N2 worms (N = 3). See Supplementary Section 7.1.2 for details and repeats. The bars represent mean  $\pm$  s.e.m., P value (\*\* P < 0.01) was determined by *t*-test.

### 3.5.4 The heat-shock proteins HSP-1 and HSP-4 are required for *glp-1* longevity and regulated by ATF-5

Finally, we focused on *hsp-1* and *hsp-4* because these heat-shock proteins show the most severe impact on *glp-1* lifespan and are associated to the biological pathways, which are most enriched in *glp-1(e2141ts)* vs. *atf-5(tm4397);glp-1(e2141ts)* according to GO Term analysis of RNAseq data, namely protein refolding and response to heat.

The outcome of our RNAi-based screen showed that knockdown of *hsp-1* and *hsp-4* had the most severe impact on *glp-1* longevity (Table 1) within the group of potential ATF-5 targets. Loss of *hsp-1* completely abolished *glp-1* longevity about 58.2% (Figure 31). This gene encodes an ortholog of hsp70A, a cytosolic heat shock protein which is induced upon heat-shock 2-6 fold. In comparison, down-regulation of *hsp-1* via RNAi was found to mildly reduce the lifespan of *age-1* mutants [Morley and Morimoto, 2004].

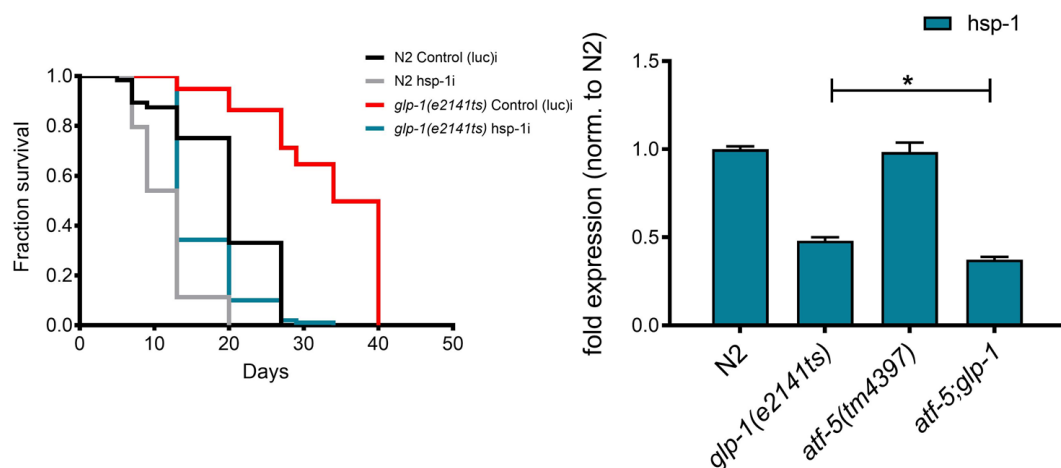


**Figure 31: Knockdown of *hsp-1* abolishes GLP-1/NOTCH longevity.** On the left side, a representative lifespan using *hsp-1* RNAi in *glp-1(e2141ts)* background shows, that knockdown of *hsp-1* reduces the lifespan of N2 worms and abolishes GLP-1/NOTCH longevity (N = 2). See Supplementary Section 7.1.6.1 for details and repeats. On the right side, qPCR shows that mRNA of *hsp-1* is reduced in *glp-1(e2141ts)* vs. N2. mRNA levels are further reduced by loss of *atf-5* in *atf-5(tm4397);glp-1(e2141ts)*. Loss of *atf-5* does not change mRNA levels of *hsp-1* in N2 worms. mRNA expression levels are shown as fold expression normalized to mRNA levels of biological replicate #1 of N2 worms (N = 3). See Supplementary Section 7.1.2 for details and repeats. The bars represent mean  $\pm$  s.e.m., P value (\* P < 0.05) was determined by *t*-test.

The mRNA level of *hsp-1* was down-regulated in *glp-1* animals compared to N2. Loss of *atf-5* reduced the mRNA levels of *hsp-1* further in *glp-1(e2141ts)* background, but had no effect on *hsp-1* levels in N2 worms (Figure 31). This qPCR data validated the RNAseq result. The difference in mRNA expression between *glp-1(e2141ts)* vs. *atf-5(tm4397);glp-1(e2141ts)* was significant, but very minor. Knockdown of *hsp-1* by RNAi had a severe effect on lifespan of N2 and *glp-1(e2141ts)* worms. It reduced the mean lifespan of N2 worms about 74%, and the mean lifespan of *glp-1(e2141ts)* worms about 115% (average of 2 biological replicates). These results validated the outcome of the screen.

The second heat shock protein found in the screen was *hsp-4*, encoding an ortholog of the endoplasmic reticulum chaperone BiP/Grp78 [Kapulkin et al., 2005].

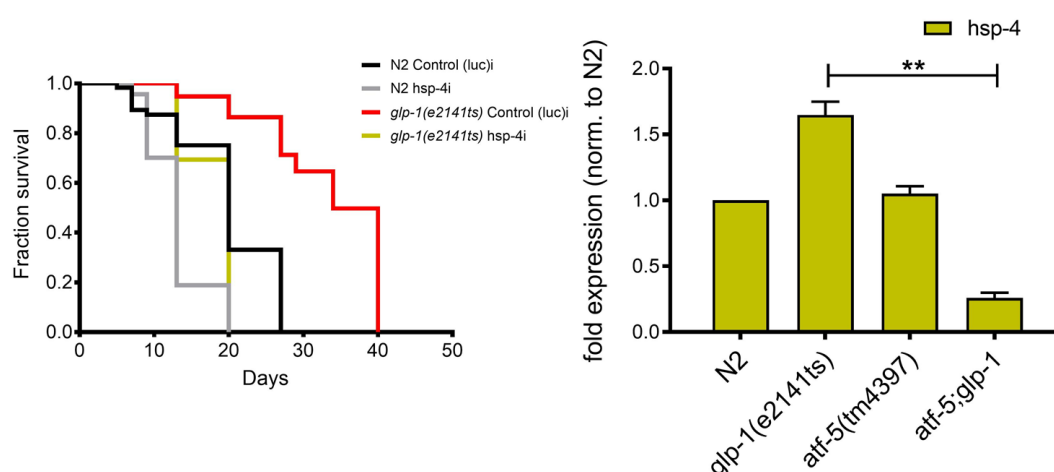


**Figure 32: Knockdown of *hsp-4* abolishes *glp-1*/NOTCH longevity.** On the left side, a representative lifespan using *hsp-4* RNAi in *glp-1(e2141ts)* background shows, that knockdown of *hsp-4* reduces the lifespan of N2 worms and abolishes GLP-1/NOTCH longevity (N = 2). See Supplementary Section 7.1.6.1 for details and repeats. On the right side, qPCR shows that mRNA of *hsp-4* is up-regulated in *glp-1(e2141ts)*. mRNA levels are 6.4-fold reduced by loss of *atf-5* in *atf-5(tm4397);glp-1(e2141ts)*. Loss of *atf-5* does not change mRNA levels of *hsp-4* in N2 worms. mRNA expression levels are shown as fold expression normalized to mRNA levels of biological replicate #1 of N2 worms (N = 3). See Supplementary Section 7.1.2 for details and repeats. The bars represent mean  $\pm$  s.e.m., P value (\*\* P < 0.01) was determined by t-test.

The mRNA levels of *hsp-4* were up-regulated in *glp-1* animals compared to N2. Loss of *atf-5* reduced the mRNA levels of *hsp-4* in *glp-1(e2141ts)* background

about 6.4-fold, but had no effect on *hsp-4* levels in N2 worms (Figure 32). This qPCR data validated the RNAseq result. Knockdown of *hsp-4* by RNAi had a severe effect on lifespan of N2 and *glp-1(e2141ts)* worms. It reduced the mean lifespan of N2 worms about 50%, and the mean lifespan of *glp-1(e2141ts)* worms about 88% (average of 2 biological replicates).

To validate and further strengthen the regulatory axis between *atf-5* and *hsp-4* in *glp-1* worms, we made use of a *hsp-4::gfp* reporter strain. In *glp-1* background the GFP/TOF ratio normalized to a luciferase RNAi control group is  $79.8\% \pm 8.2\%$  (N = 4, Data in Supplementary Materials 7.1.8, Table 6), which is a significant reduction compared to the control (*t*-Test, paired, p-value = 0.016). In unstressed N2 worms knockdown of *atf-5* did not change the GFP/TOF levels of *hsp-4::gfp* reporter strain (N = 4, Data in Supplementary Materials 7.1.8, Table 7). GFP/TOF ratio normalized to a luciferase RNAi control group is  $106.2\% \pm 46.1\%$  (*t*-Test, paired, p-value = 0.805).

Another experiment carried out by Torsten Bücher as part of his bachelor thesis. The *hsp-4::gfp* reporter strain was treated with RNAi against luciferase or *atf-5* and ER stress was induced by tunicamycin treatment for 6 hours. Tunicamycin is a known ER stress inducer and leads to the upregulation of *hsp-4* [Shen, 2001]. GFP fluorescence was checked by microscopy and representative pictures are shown in Figure 33. The non-stressed controls show basal and comparable GFP fluorescence (A + B), similar to the results obtained by worm sorter analysis (see above). Induction of ER stress by tunicamycin induced GFP fluorescence in *hsp-4::gfp* worms treated with luciferase RNAi (C). Knockdown of *atf-5* prevented the induction of GFP fluorescence in *hsp-4::gfp* worms (D).

These results validated the outcome of the screen and indicate, that *hsp-4* could be a downstream targets of ATF-5, required for its role and function in *glp-1*/NOTCH longevity. Furthermore, *hsp-4* expression in non-stressed conditions does not require ATF-5, whereas *hsp-4* induction in ER stress or in sterile *glp-1* worms is ATF-5 dependent.

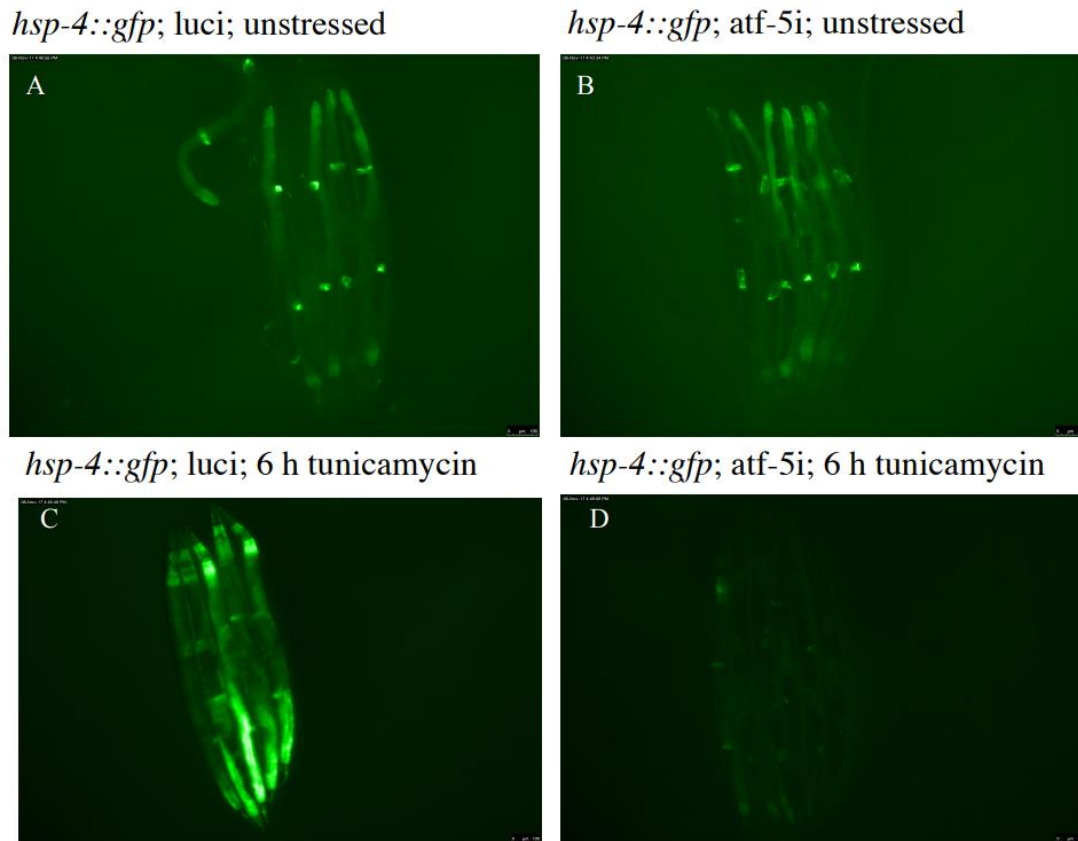


**Figure 33: The induction of *hsp-4::gfp* by tunicamycin is abolished by the *atf-5* RNAi.** **A** shows the unstressed worms grown on luciferase and **B** shows the unstressed condition grown on *atf-5* RNAi. **A** and **B** represent the basal level of *hsp-4* induction. **C** shows *hsp-4::gfp* grown on luciferase after ER stress induction with tunicamycin for 6 hours. A strong fluorescence is visible. **D** shows *hsp-4::gfp* grown on *atf-5* RNAi after ER stress induction with tunicamycin for 6 hours. Fluorescence is reduced. Results obtained by Torsten Bücher. Figure taken from BSc Thesis by Torsten Bücher.

### 3.5.5 ATF-5 regulates *hsp-4*, but not *hsp-3*, in *glp-1(e2141ts)* worms

The experiments described above showed that *hsp-4* is regulated upon loss of *atf-5* in *glp-1(e2141ts)* worms. In addition to *hsp-4*, there is another *C. elegans* BiP/Grp78 homolog, named *hsp-3*. These two genes were described as functionally redundant ([Shen et al., 2001]; [Kapulkin et al., 2005]). We therefore wondered whether *atf-5* also regulated *hsp-3*.

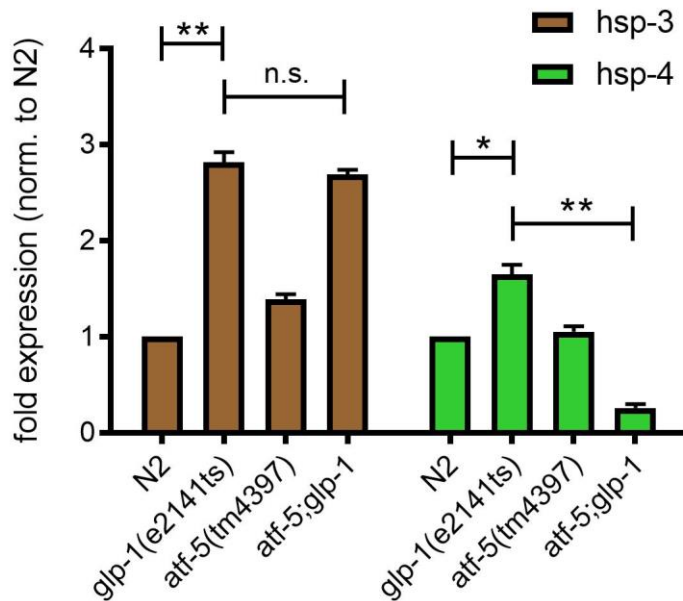


**Figure 34: Loss of *attf-5* in *glp-1(e2141ts)* worms downregulates mRNA levels of *hsp-4*, but not of *hsp-3*.** qPCR shows that mRNA levels of *hsp-3* and *hsp-4* are induced in *glp-1(e2141ts)* vs. N2. mRNA level of *hsp-4* is reduced by loss of *attf-5* in *attf-5(tm4397);glp-1(e2141ts)*, while *hsp-3* levels are stable. Loss of *attf-5* does not change mRNA levels of *hsp-4* in N2 worms, while *hsp-3* levels are mildly up-regulated. mRNA expression levels are shown as fold expression normalized to mRNA levels of biological replicate #1 of N2 worms (N = 3). See Supplementary Section 7.1.2 for details and repeats. The bars represent mean  $\pm$  s.e.m., P value (\*  $P < 0.05$  and \*\*  $P < 0.01$ ) was determined by *t*-test.

First, RNAseq data did not indicate *hsp-3* as a gene differentially regulated by ATF-5 in *glp-1(e2141ts)* worms. qPCR analysis of the levels of *hsp-3* mRNA confirmed this result (Figure 34). The regulation of *hsp-4* was as described above, but depicted again to compare better to *hsp-3* levels. Both chaperones were up-regulated in *glp-1* worms in comparison to N2. Loss of *attf-5* in N2 worms led to a minor increase in *hsp-3* mRNA levels and did not change the levels of *hsp-4* mRNA. In the double-mutant *attf-5(tm4397);glp-1(e2141ts)* we found that *hsp-3* mRNA levels were not changed, whereas the *hsp-4* mRNA levels were drastically reduced in comparison with *glp-1* worms (6.4-fold decrease). These data showed that ATF-5 impacts the expression of *hsp-4* specifically in *glp-1* worms, while it had no effect on the levels of the closely related and functionally redundant gene *hsp-3*. This indicates a specific regulation of *hsp-4* by ATF-5.

### 3.5.6 ATF-5 partially regulates small heat-shock proteins of the *hsp-16* family

The RNAseq data detected differential regulation of a number of heat shock proteins known to be downstream of HSF-1, a transcriptional regulator of stress-induced gene expression required for response to heat stress [Brunquell et al., 2016]. We validated the RNA sequencing results using qPCR and found six different small heat shock proteins from the *hsp-16* family (*hsp-16.1*, *hsp-16.2*, *hsp-16.11*, *hsp-16.41*, *hsp-16.48* and *hsp-16.49*) to be up-regulated in *glp-1(e2141ts)* in comparison to N2 (Table 2). The loss of *atf-5* in N2 animals led to a slight down-regulation of four of those six small heat shock proteins, with *hsp-16.2* and *hsp-16.41* not being differentially regulated. In the *glp-1* background loss of *atf-5* reduced the levels of all six small heat shock proteins in a robust and significant manner.

|                               | <i>hsp-16.1</i> | <i>hsp-16.2</i> | <i>hsp-16.11</i> | <i>hsp-16.41</i> | <i>hsp-16.48</i> | <i>hsp-16.49</i> |
|-------------------------------|-----------------|-----------------|------------------|------------------|------------------|------------------|
| N2/ <i>glp-1</i>              | +3.7(**)        | +4.6(*)         | +3.4(**)         | +5.3(**)         | +3.5(*)          | +3.8(*)          |
| N2/ <i>atf-5</i>              | -1.4(*)         | -1.4(ns)        | -1.5(*)          | -1.3(ns)         | -1.6(*)          | -1.6(**)         |
| <i>glp-1/<br/>glp-1;atf-5</i> | -1.7(**)        | -1.5(*)         | -1.6(*)          | -1.3(*)          | -1.7(*)          | -1.9(**)         |

**Table 2: 6 genes of the HSP-16 family are reduced in *glp-1* worms upon loss of *atf-5*.** Table shows fold expression normalized to N2. Asterisks indicate p-values, ns = not significant. mRNA level of 6 different *hsp-16* genes are significantly up-regulated in *glp-1(e2141ts)* vs. N2. Loss of *atf-5* reduces the levels of *hsp-16.1*, *hsp-16.11*, *hsp-16.48* and *hsp-16.49* in N2 worms. In *glp-1(e2141ts)* the loss of *atf-5* reduces the mRNA levels of all analyzed *hsp-16* genes. mRNA expression levels are shown as fold expression normalized to mRNA levels of biological replicate #1 of N2 worms (N = 3). See Supplementary Section 7.1.2 for details and repeats. P value (\* P < 0.05 and \*\* P < 0.01) was determined by *t*-test.

Taken together these results showed that *atf-5* positively regulates the mRNA expression levels of a number of heat-shock proteins in long-lived *glp-1* worms. The regulation appeared to be specific, since *atf-5* tightly regulated *hsp-4*, whereas *hsp-3* was not regulated, and members of the *hsp-16* family were showed clear dependence for *atf-5* to maintain high expression levels in *glp-1* worms.

### 3.5.7 Loss of *atf-5* in *glp-1* does not impact several age- and stress-related pathways

#### 3.5.7.1 Endoplasmic reticulum Unfolded Protein Response (UPR<sup>ER</sup>): *ire-1* expression and *xbp-1* splicing

Because we observed that ATF-5 was connected to the regulation of heat-shock factors, we wondered whether it regulated other protein stress signaling pathways. Another important stress signaling pathway is the Unfolded Protein Response (UPR), which governs stress signaling in the endoplasmic reticulum (ER), also termed UPR<sup>ER</sup>. One branch of the UPR<sup>ER</sup> is mediated by IRE-1 and XBP-1. Both genes have been shown to be required for the longevity of Insulin/IGF-1 signaling mutants [Henis-Korenblit et al., 2010]. IRE-1 senses misfolded proteins, homo-oligomerizes, undergoes autophosphorylation and is thereby activated. As a result, *xbp-1* mRNA alternatively spliced and the new open reading frame translates the XBP-1 transcription factor.

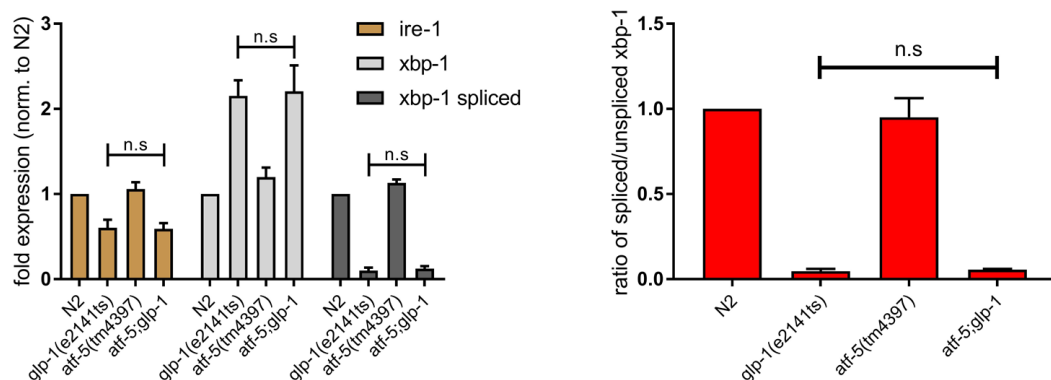


**Figure 35: The UPR factors *ire-1* and *xbp-1*/*xbp-1* spliced are not regulated by *atf-5* in *glp-1* worms.** The mRNA level of *ire-1* is reduced in *glp-1(e2141ts)* vs. N2 as determined with qPCR analysis. No significant change upon loss of *atf-5* can be detected in N2 or *glp-1(e2141ts)*. The levels of unspliced *xbp-1* are up-regulated in *glp-1(e2141ts)* vs. N2, while its the level of its spliced form are reduced. No significant change upon loss of *atf-5* can be detected in N2 or *glp-1(e2141ts)*. The ratio of spliced/unsliced *xbp-1* is 21.5-fold decreased in *glp-1(e2141ts)* compared to N2, with *atf-5* having no impact on this ratio. mRNA expression levels are shown as fold expression normalized to mRNA levels of biological replicate #1 of N2 worms (N = 3). See Supplementary Section 7.1.2 for details and repeats. The bars represent mean  $\pm$  s.e.m., P value was determined by t-test.

We monitored the mRNA levels of *ire-1* and both *xbp-1* isoforms by qPCR (Figure 35). The level of *ire-1* mRNA was reduced in *glp-1* worms in comparison to N2 worms. The deletion of *atf-5* had no effect on the levels of *ire-1* mRNA in

*glp-1* or N2 worms. The expression of unspliced *xbp-1* was elevated to the same extent in *glp-1(e2141ts)* and *atf-5(tm4397);glp-1(e2141ts)* when compared to N2 animals. The *atf-5* single mutant did not show a change in unspliced *xbp-1* mRNA level. We observed a drastic reduction of the spliced *xbp-1* isoform in *glp-1(e2141ts)* and *atf-5(tm4397);glp-1(e2141ts)* when compared to N2 animals (9.9-fold decrease). The loss of *atf-5* slightly altered the abundance of spliced *xbp-1* (1.2-fold increase). The ratio of spliced/unspliced *xbp-1* transcripts integrated these measurements and reported a decrease of 21.5-fold in *glp-1(e2141ts)* compared to N2. A recent publication stated, that proliferating germ line cells in wild-type worms can improve resistance to ER stress and modulate ER stress responses in the somatic cells non-autonomously [Deng et al., 2020], thus comparing *xbp-1* splicing of *glp-1* sterile worms to N2 animals might be not significant. Loss of *atf-5*, however did not affect the ratio of spliced/unspliced *xbp-1* transcripts in *glp-1(e2141ts)* or N2 worms.

These experiments indicate that loss of *atf-5* does not affect upstream regulators of the UPR<sup>ER</sup> in sterile *glp-1* worms or N2 animals.

### **3.5.7.2 Mitochondrial Unfolded Protein Response (UPR<sup>mt</sup>): Expression of *hsp-6* and *hsp-60***

Another mode of UPR also takes place in the mitochondria termed UPR<sup>mt</sup>. The UPR<sup>mt</sup> is typically induced in response to an imbalance of subunits of various mitochondrial protein complexes or by drugs targeting the mitochondrial ribosome. In *C. elegans* the two nuclear-encoded mitochondrion-specific chaperones, HSP-6 and HSP-60, can be used to monitor the transcriptional induction of UPR<sup>mt</sup> [Bennett et al., 2014]. In mammals UPR<sup>mt</sup> is mediated by targets of the integrated stress response CHOP, ATF4 and ATF5. In *C. elegans* ATFS-1 was found to be the functional ortholog of ATF5 [Anderson and Haynes, 2020].

We measured the levels of *hsp-6* and *hsp-60* mRNA by qPCR and found no induction in germline-less mutants compared to N2 (Supplementary Section

7.1.2 for details and repeats). The loss of *atf-5* did not affect the levels of the two mitoUPR factors in N2 or *glp-1(e2141ts)* worms.

These findings indicated that UPR<sup>mt</sup> is not induced in *glp-1* worms. Neither in wildtype nor in *glp-1* animals the loss of *atf-5* significantly affected the expression of the mitochondrion-specific chaperones *hsp-6* and *hsp-60*.

### **3.5.7.3 Insulin/IGF-signaling: Expression of *sod-3***

Longevity of germline-less worms is strongly dependent of the DAF-16/FOXO transcription factor. Notably, when germ cells are removed, the nuclear localization and activation of the DAF-16/FOXO transcription factor is triggered in the intestine, which leads to the regulation of downstream genes and lifespan extension.

The RNA sequencing did not show a particular enrichment of targets of DAF-16 among the differentially regulated genes. Nevertheless, since DAF-16/FOXO is activated in *glp-1* and turns on the expression of *sod-3*, an iron/manganese superoxide dismutase [Honda and Honda, 1999], we wondered whether *atf-5* mutation could also affect this readout. We aimed to double-check, that the insulin/IGF-1 pathway is not effected by *atf-5* in *glp-1* background and adding another gene to ongoing qPCR experiments was no great effort. As expected, our qPCR data revealed that *sod-3* is induced in *glp-1* animals (See Supplementary Section 7.1.2 for details and repeats). A small induction can also be detected in *atf-5(tm4397)* compared to N2 worms. The *atf-5(tm4397);glp-1(e2141ts)* double-mutants showed a similar induction of *sod-3* as the *glp-1(e2141ts)* mutants, suggesting that *atf-5* has no effect on this DAF-16 target gene, in line with our RNA sequencing results.

In sum, the RNA sequencing data together with the *sod-3* qPCR analysis showed to clear link of *atf-5* with DAF-16 target gene expression in *glp-1* worms.

### **3.5.7.4 Autophagy: Expression of V-ATPases and LGG-1 cleavage**

A major function essential to cellular homeostasis is the ability to degrade and recycle cellular components, which is termed autophagy. Autophagy delivers its cargo to lysosomes for degradation. This process is required for lifespan

extension in germline-less worms as well as other modes of longevity [Lapierre et al., 2011]

In our RNAseq dataset, we found the lysosomal gene *lys-7* to be down-regulated upon loss of *atf-5* in *glp-1* worms. We checked the mRNA levels again by qPCR analysis. This showed a small, but significant, induction of *lys-7* in *glp-1* worms compared to N2 worms. Loss of *atf-5* did not affect the levels of *lys-7* significantly, neither in N2, nor in *glp-1* worms (see Supplementary Section 7.1.2 for details and repeats).

To clarify the impact of ATF-5 upon autophagy, we measured the levels of other lysosomal genes. We focused on sub units of the V-ATPases, which are ATP driven proton pumps of the lysosome. We assayed the expression levels of five V-ATPases (*vha-1*, *vha-4*, *vha-12*, *vha-15*, *vha-17*) and found those genes significantly induced in *glp-1* compared to N2 worms (see Supplementary Section 7.1.2 for details and repeats). Upon *atf-5* deletion the levels of those V-ATPases did not change in N2 nor in *glp-1(e2141ts)* worms.

A third experiment was carried out to investigate the role of ATF-5 in autophagy. We crossed a strain expressing a LGG-1:GFP-fusion protein into our strains of interest. We made use of western blot techniques to measure the ratio of free GFP against the level of LGG-1:GFP-fusion protein, which is a readout of the autophagic flux (Figure 36). Compared with the LGG-1:GFP-fusion protein, free GFP increases in ageing worms and is a hallmark of ageing [Klionsky et al., 2016].

Loss of *atf-5* in worms with a germline did not result in a significant change of the ratio of cleaved GFP to LGG-1:GFP-fusion protein. In *glp-1* worms the ratio of cleaved GFP to LGG-1:GFP-fusion protein was reduced, which indicated an induction or a more active autophagic flux and referred to a more juvenile or non-aged organism. Loss of *atf-5* in *glp-1* worms did not change the ratio of free GFP to LGG-1:GFP-fusion protein.

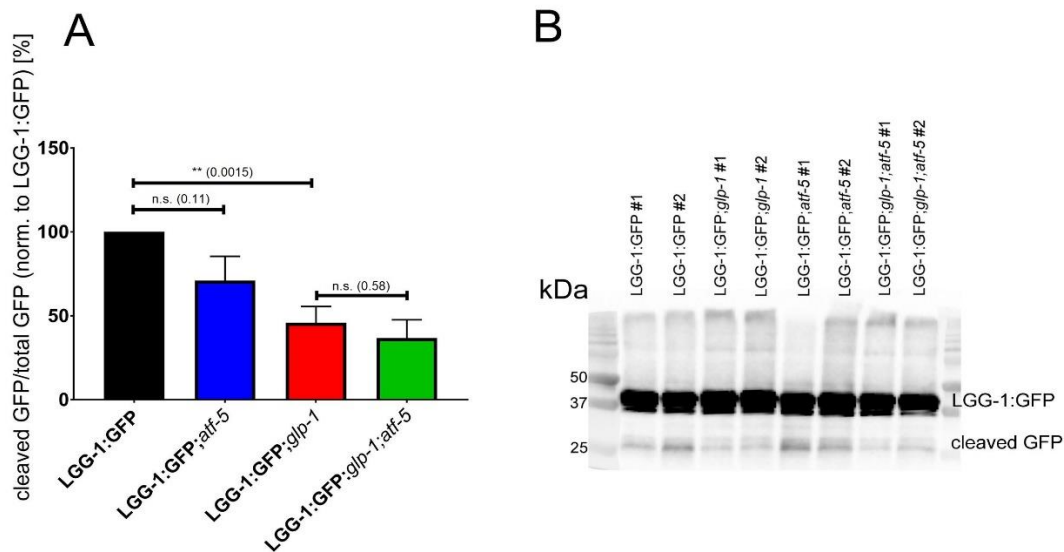


**Figure 36: Analysis of cleaved GFP/LGG-1:GFP-fusion protein shows, that loss of *atf-5* has no impact on the autophagic flux.** kDa = kilo Dalton, indicators for protein size **A.** Ratio of cleaved GFP/LGG-1:GFP-fusion protein is set to 100% in wildtypic worms (LGG-1:GFP). In *glp-1(e2141ts)* worms, the ratio drops significantly. Loss of *atf-5* does not impact the ratio in wildtypic or *glp-1(e2141ts)* worms in a significant manner (N = 3). Loading control was carried out by adjusting protein amounts before loading. See Supplementary Section 7.1.5 for details and repeats. The bars represent mean  $\pm$  s.e.m., P value was determined by *t*-test. **B.** Representative western blot using anti-GFP to detect cleaved GFP and LGG-1:GFP-fusion protein.

Our results showed that autophagy was altered in *glp-1* worms. We could not validate the impact on ATF-5 on expression of *lys-7* and in addition, two different approaches failed to connect the loss of *atf-5* to the regulation of autophagy in *glp-1* worms.

The previous findings excluded an impact of *atf-5* on several known pathways that regulate stress-responses and ageing, including the UPR<sup>er</sup>, UPR<sup>mit</sup> unfolded protein responses, Insulin/IGF-signaling or autophagy. These results suggest a function of *atf-5* as a specific regulator of heat-shock factors in *glp-1* worms, rather than playing a broader role in regulating other stress signaling pathways.

## 3.6 The regulation of ATF-5 in *C. elegans*

### 3.6.1 Promotor driven GFP transgene

The experiments carried out and described up to this point focused on loss of *atf-5* phenotypes and downstream targets of the ATF-5 transcription factor. The following experiments target on factors upstream of ATF-5, which can regulate or interact with ATF-5 to facilitate its role in gonadal longevity.

To the date of carrying out the experimental part of this thesis only a ATF-5 promoter fusion strain was generated by the Blackwell lab, but not published or made widely available. Nevertheless, we were able to receive this strain and use it for experiments. The strain is characterized as [Patf-5(uORF)::GFP::*unc-54*(3'UTR)], contains 1.8kb promoter 5' of *atf-5* including both uORFs and was cloned into pPD95.75.

We crossed the strain into our *glp-1(e2141ts)* worms and used different RNAi clones of potential upstream mediators to investigate on interactions in *glp-1* sterile worms (N = 3, Data in Supplementary Materials 7.1.8, Table 8). Knockdown of *skn-1* did not show an impact in our hands (GFP/TOF ratio normalized to a luciferase RNAi control group is 102.6 %  $\pm$  15.8 % (*t*-Test, paired, p-value = 0.8041), although SKN-1 was shown to bind to the *atf-5* promotor region and facilitate transcriptional activity [Glover-Cutter et al., 2013]. Knockdown of *gcn-2* and *pek-1* in parallel should reduce the p-eIF2 $\alpha$  levels, and in consequence translate into a reduced level of ATF-5 protein, which we could not validate using this strain in *glp-1* sterile worms (GFP/TOF ratio normalized to a luciferase RNAi control group is 98.2 %  $\pm$  8 % (*t*-Test, paired, p-value = 0.7286).

We did not further use this strains for analysis, because our experiments showed inconsistent results with expectations raised from other publications (SKN-1). Further, downregulation of the integrated stress response pathway did not alter the GFP expression driven by this ATF-5 promoter fusion construct which is not in line with the known biological function of this pathway.

### **3.6.2 Approaches to monitor ATF-5 protein levels**

#### **3.6.2.1 Extrachromosomal transgenic array**

The promotor fusion strain did not yield expected results and further investigation on interactions and the regulation of the ATF-5 protein, required the development of tools to monitor its abundance.

The expression of ATF-5 protein cannot be linked to the abundance of *atf-5* mRNA, because the translation of the canonical open reading frame is hindered by upstream open reading frames. This disqualified the use of qPCR - or other methods that detect the mRNA transcript – to represent the levels of the protein, which is the functional bio-molecule in case of a transcription factor.

Transgenes can be expressed in *C. elegans* by generating extrachromosomal transgenic arrays. These mitotically transmitted arrays are injected into the germ line and are not integrated into an endogenous chromosome, but forming long concatemers [Maine, 2010]. In order to monitor the levels of ATF-5 protein, we constructed several plasmids in which the ATF-5 canonical open reading frame is fused to a fluorescent protein (GFP or tagRFP). The plasmids were injected together with a co-injection marker into the gonads of the P0 generation. Stable lines, carrying extrachromosomal arrays of the injected plasmids, were selected by presence of the co-injection marker.

On no condition, however, we were able to detect the fusion protein ATF-5:GFP or ATF-5:tagRFP by microscopy or western blotting. Presumably this is because the ATF-5 protein is very unstable or induced only under specific stress conditions. Due to time and capacity constraints we did not follow-up on further investigation of these strains carrying extrachromosomal transgenic arrays, but switched to the novel and more promising method of generating knock-In allele by CRISPR/Cas9 gene editing.

### 3.6.2.2 CRISPR/Cas9 Genome Editing: Generation of a Knock-In Allele by homology-mediated repair

We made use of the novel CRISPR/Cas9 technique (Outline in Figure 4) to engineer an endogenously-tagged ATF-5 protein. We induced a double-strand break close to the stop-codon of the canonical ATF-5 coding sequence and integrated a DNA sequence with a V5-tag and a FLAG-tag between the last codon and the stop-codon (Figure 37). The lines carrying the knock-in allele *atf-5(dh1103)* could be identified by restriction enzyme digestion of a PCR fragment, because the integrated sequence introduces an EcoRV cut site.

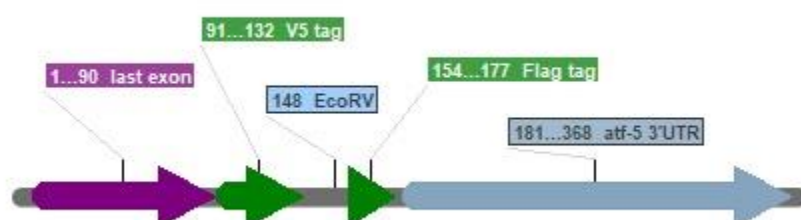


**Figure 37: Schematic representation of the allele *atf-5(dh1103)*.** A V5-tag and a FLAG-tag are integrated in frame 3' to the last coding exon of *atf-5*. A EcoRV restriction site is part of the integrated sequence to identify the allele by restriction site polymorphism.

A number of homozygous and independent *atf-5(dh1103)* lines were obtained and two were used for further experiments. We crossed out those lines four times with N2 worms to get rid of background mutations that could have been introduced by unspecific Cas9 endonuclease activity.

In the next step we verified the presence and correct position of the knock-in sequence on mRNA by PCR of cDNA (Figure 38). A primer set spanning the region of integration generated a 110bp fragment in wildtype worms. In *atf-5(dh1103)* worms the fragment was 207bp long, a shift of 97bp which corresponded with the length of the integrated sequence of the V5/FLAG-tag. A second primer set was used to validate the result. This set shared the reverse primer with the first primer set, whereas the forward primer annealed to a sequence only present in the V5/FLAG-tag. No RT-PCR product was generated in N2 worms, a 143bp long fragment only occurs in the worms carrying the V5/FLAG-tag. The quality of the primer sets was assayed using a qPCR

method. Both primer pairs showed a single major peak in the melt curve diagram and a high coefficient of determination in the standard curve.

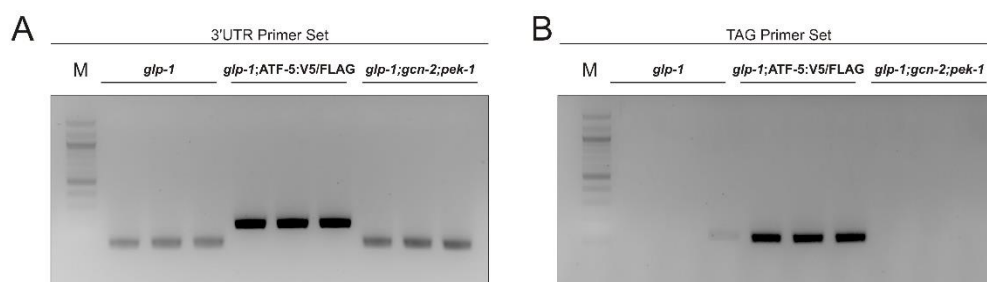


**Figure 38: PCR of cDNA obtained from *attf-5(dh1103)* shows presence of the integrated sequence.** M = marker A. A primer pair spanning the integrated sequence shows an up-shift of 97bp in length in the *attf-5(dh1103)* worms in comparison with two strains not harboring this allele. B. A primer pair detecting a 143bp region in the integrated sequence amplifies its target only in the *attf-5(dh1103)* worms in comparison with two strains not harboring this allele.

After we validated the *attf-5(dh1103)* allele on mRNA level, we made use of western blot analysis to validate the levels of the endogenous protein tagged with V5/FLAG (Figure 39). We crossed *attf-5(dh1103)* into *glp-1* worms and generated quadruple mutants of *attf-5(dh1103);glp-1(e2141ts);gcn-2(ok871);pek-1(ok275)* as another control. We checked the levels of p-eIF2 $\alpha$  in parallel to validate the status of the integrated stress response. The loss of *gcn-2* and *pek-1* drastically reduced the p-eIF2 $\alpha$  levels, which should in consequence translate into a reduced level of ATF-5 protein. Thus, we expected to detect elevated levels of ATF-5 protein in *glp-1* worms and a low abundance in the quadruple mutant.

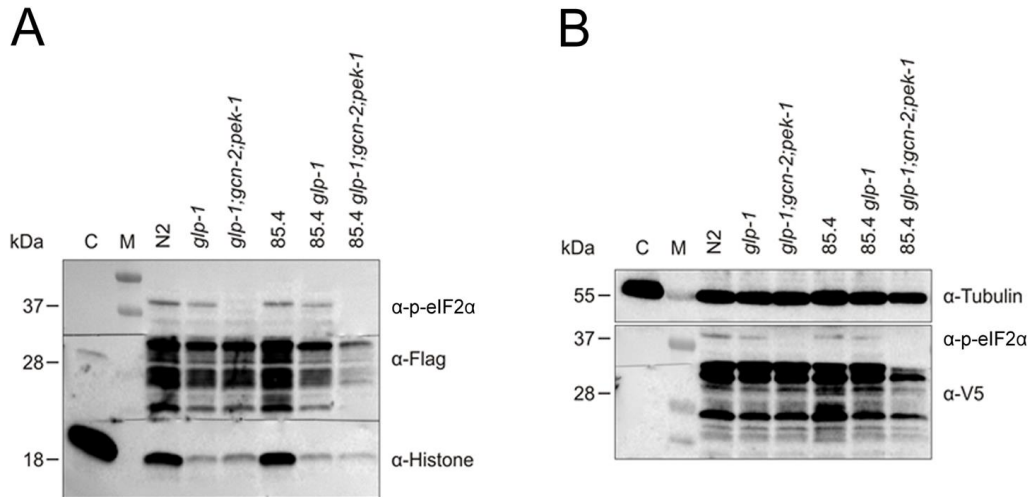


**Figure 39: Western Blot analysis could not detect ATF-5:V5/FLAG fusion protein in *attf-5(dh1103)* worms (clone 85.4), even in strains with reduced phosphorylation of eIF2α** kDa = kilo Dalton, indicators for protein size / C = control / M = protein marker / 85.4 = *attf-5(dh1103)* (clone 85.4). **A.** Western blot analysis with antibodies against p-eIF2α, FLAG and Histone H3 was carried out. **B.** Western blot analysis with antibodies against Tubulin, p-eIF2α and V5 was carried out. In both analyses the level of p-eIF2α were reduced in *glp-1* upon loss of *pek-1* and *gcn-2*. No specific band representing the ATF-5:V5/FLAG fusion protein (expected size around 27kDa) could be detected in *attf-5(dh1103)* worms using FLAG or V5 antibodies, even upon overexposure of the blot.

Unfortunately, we could not detect any ATF-5:V5/FLAG fusion protein, which has an expected size of 27kDa. We made use of antibodies against V5 and FLAG, but no specific band could be found on the western blots, even with long exposing times.

This result eliminated the use of the *attf-5(dh1103)* allele to investigate ATF-5 protein levels. Our RT-PCR results clearly indicate, that the knock-in resulted in an *attf-5* mRNA with the desired coding regions for V5 and FLAG (Figure 38). Nevertheless, it was not possible to detect the tagged ATF-5 protein, even in *glp-1* worms which we hypothesized should show an induction of ATF-5 translation.

### 3.6.3 HSF-1 regulates *atf-5* and its targets in *glp-1* worms

Unfortunately, the approach to monitor ATF-5 directly could not be carried out successfully. As a consequence, we had to step back and use qPCR analysis on the ATF-5 targets *hsp-4* and *haf-6* to indirectly monitor potential regulators of *atf-5*. These genes were selected because they both showed a strong downregulation upon loss of *atf-5* in *glp-1* worms, but not in N2 worms. Beyond that they are involved in different pathways and not known to interact.

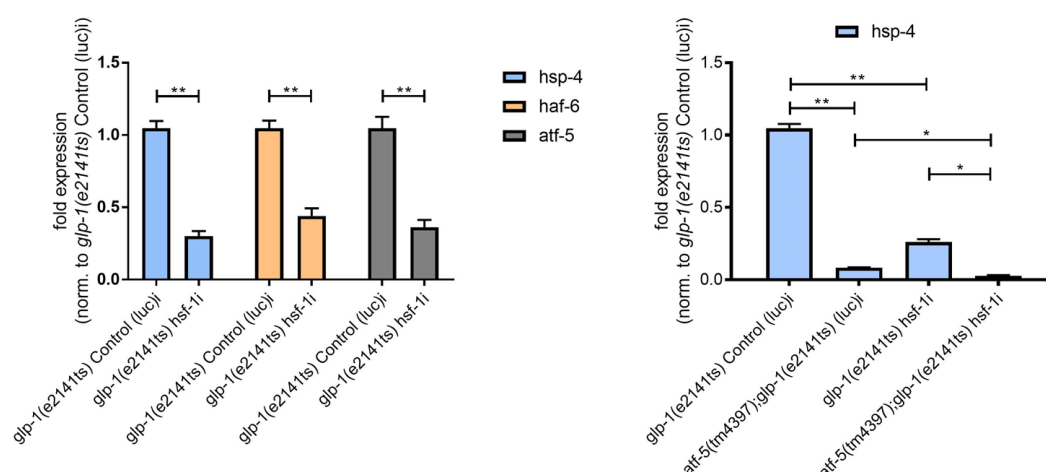


**Figure 40: Knockdown of *hsf-1* reduces the mRNA levels of *atf-5* and its targets.** **Left.** *glp-1(e2141ts)* worms treated with *hsf-1* RNAi have reduced mRNA levels of *hsp-4*, *haf-6* and *atf-5* compared to control (treated with RNAi against luciferase). **Right.** Deletion of *atf-5* or knockdown of *hsf-1* leads to drastic reduction of *hsp-4* mRNA levels in *glp-1(e2141ts)* worms. The expression of *hsp-4* is further reduced, by *hsf-1* RNAi in *atf-5(tm4397);glp-1(e2141ts)*. mRNA expression levels are shown as fold expression normalized to mRNA levels of biological replicate #1 of *glp-1(e2141ts)* worms treated with RNAi against luciferase (N = 3). See Supplementary Section 7.1.2 for details and repeats. The bars represent mean  $\pm$  s.e.m., P value (\* P < 0.05 and \*\* P < 0.01) was determined by *t*-test.

It was shown previously, that SKN-1 binds to the promotor region of *atf-5* and facilitates its transcription [Glover-Cutter et al., 2013]. To test this idea, we used RNAi against *skn-1* on *glp-1(e2141ts)* worms and measured mRNA levels by qPCR. In our experiment, we did not observe obvious transcriptional downregulation of *atf-5* or its targets *hsp-4* and *haf-6* upon knockdown of *skn-1*. We even saw a slight increase in *haf-6* levels upon knockdown of *skn-1* in *glp-1(e2141ts)*, when compared to the control group (treated with RNAi against luciferase, see Supplementary Section 7.1.2 for details and repeats).

Given the changes we saw in various heat shock proteins, we next turned our attention to HSF-1, the central transcription factor activated by heat-stress. HSF-1 is required to restore proteostasis upon heat stress and is known to induce molecular chaperones and other genes [Brunquell et al., 2016]. Among others, it regulates *hsp-4*, members of the HSP-16 family and genes required for translation. A number of HSF-1 targets were also identified in our experiments as regulated by ATF-5.

We treated worms with *hsf-1* RNAi and measured the mRNA levels of *atf-5*, *hsp-4* and *haf-6* by qPCR (Figure 40). Those three genes were down-regulated by knockdown of *hsf-1* in *glp-1(e2141ts)*. This indicated that these genes require the presence of HSF-1 to be expressed in *glp-1* worms. In addition, we revealed that the reduced levels of *hsp-4* in *atf-5(tm4397);glp-1(e2141ts)* can be further reduced by knockdown of *hsf-1*.

These experiments revealed a regulatory connection between HSF-1 and ATF-5 in *glp-1* worms. HSF-1 is required for the induction of *atf-5* and target genes selected in our experiment. The expression of *hsp-4* in *glp-1* worms required the presence of both factors.

# **4**

## **Discussion**

## 4.1 NHR-91 in gonadal longevity and lipid metabolism

NHR-91 is required for gonadal longevity induced by mutations in *glp-1*. A preliminary screen carried out by Özlem Karalay and Shuhei Nakamura yielded *nhr-91* as a potential mediator of GLP-1/NOTCH longevity. Experiments carried out in this study using a *nhr-91* deletion mutant validated the outcome of the RNAi screen. To our surprise, however, *nhr-91* was dispensable for other modes of gonadal longevity; neither germline laser-ablation nor sterile *mes-1* mutants required *nhr-91* for their lifespan extension. The consequences of those findings are discussed in the next section. We decided on follow-up experiments to uncover the pathways and mediators affected by NHR-91 in long-lived *glp-1* worms. On the one hand, we found the first gene to the best of our knowledge, which is only required for one mode of gonadal longevity and were curiosity-driven about the molecular mechanisms. On the other hand, so little was known about the role of NHR-91 in *C. elegans*, that we wanted to pursue this finding to collect more knowledge and data about one of the few conserved nuclear hormone-receptors in *C. elegans*.

The transcript levels of *nhr-91* were elevated in *glp-1* worms, when compared to N2. This finding and the fact that NHR-91 is a transcriptional repressor directed our research to investigate transcript levels of known mediators of gonadal longevity. The Insulin/IGF-signaling, autophagy or the mTOR pathway could not be identified as potential targets of NHR-91. Finally, we found that NHR-91 affected fatty acid desaturases in *glp-1* worms. The function of fatty acid desaturases is essential for the lifespan extension of *glp-1* worms. Germline-less animals require FAT-6, which turns stearic acid into oleic acid, to develop their longevity phenotype [Goudeau et al., 2011]. Our results showed that the expression levels of *fat-6* decreased in *glp-1* worms upon loss of *nhr-91*. The level of oleic acid was not significantly lower in *glp-1* worms, when NHR-91 is deleted. This could be due to the redundancy with *fat-7*, another gene encoding a stearyl-CoA desaturase [Brock et al., 2006]. We observed higher *fat-7* mRNA levels in *glp-1;nhr-91* double-mutants, indicating a

compensatory response towards the reduced level of *fat-6* in those mutants. This compensation could attenuate the reduction of oleic acid in *glp-1;nhr-91* double-mutants and explain the results of our FAME analysis. Above that, we found that *nhr-91* was required for high levels of *fat-5* and its product palmitoleic acid in *glp-1* worms. High levels of palmitoleic acid were shown to induce longevity [Han et al., 2017].

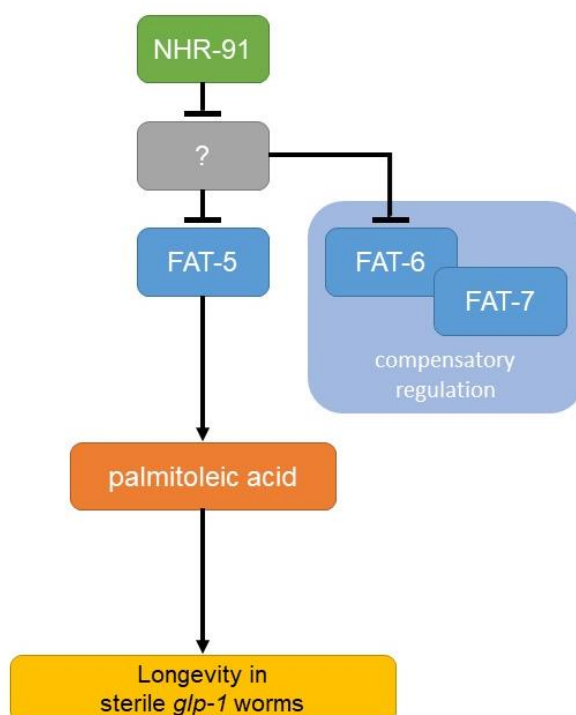


**Figure 41: A potential regulatory network of NHR-91 mediating longevity of sterile *glp-1* worms.** Induction of NHR-91 represses an unknown transcriptional repressor or a genetic network that suppresses the transcription of fatty acid desaturases in sterile *glp-1* worms, resulting in an upregulation of fatty acid desaturase. The induction of *fat-5* leads to high levels of palmitoleic acid, which have been shown to induce longevity. In *nhr-91* deletion mutants the loss of *fat-5* induction reduces palmitoleic acid levels and prevents the longevity phenotype of sterile *glp-1* worms. Reduced *fat-6* expression can be compensated by inducing *fat-7* and thus does not result in changed fatty acid levels.

In our FAME analysis we used a GC-FID, a method used in previous studies before. In the meantime, technical and analytical progress enable the use of the more advanced gas chromatography/accurate mass quadrupole time of flight mass spectrometry (GCMS-QTOF). With this machine structural confirmation and detection of FAMES without available analytical standards can be facilitated [Henry et al., 2016]. A more precise and detailed fatty acid profile

could yield a better insight into the role of NHR-91 regulating fatty acid metabolism in *C. elegans*.

These results indicated an important and novel role for NHR-91 in maintaining the high expression of fatty acid desaturases in *glp-1* worms. This maintenance is required for the longevity phenotype found in those worms. The mechanism or interactors, which connect NHR-91 to the regulation of *C. elegans*' fatty acid desaturases are yet unknown. We hypothesize that the regulation is indirect, because NHR-91 is categorized as an orphan nuclear receptor, which acts as a transcriptional repressor (Figure 41). Interestingly, recent studies implicate a role for mammalian NR6A1 in regulating lipid metabolism via mTOR [Wang et al., 2019]

NHR-91 is induced in sterile *glp-1* worms, which could be shown by qPCR. It mediates or facilitates the induction of fatty acid desaturases by a pathway yet to be determined. One hypothesis is that induction of NHR-91 represses an unknown transcriptional repressor or a genetic network that suppresses the transcription of fatty acid desaturases in sterile *glp-1* worms. This mediator acts on several fatty acid desaturases. As a result of NHR-91 induction this pathway could induce palmitoleic acid levels which are known to facilitate longevity. A potential candidate involved in mediating NHR-91 impact on fatty acid desaturases is NHR-80, which is induced in sterile *glp-1* worms. Nonetheless, our qPCR results showed no significant impact on *nhr-80* mRNA levels upon loss of *nhr-91* in sterile *glp-1* worms. It also remains possible that NHR-91 can behave as a transcriptional activator, and could promote directly the expression of desaturases, and thereby affect desaturated fatty acid production.

## 4.2 NHR-91 is a specific regulator of *glp-1*/NOTCH longevity pinpointing to disparities in the modes of gonadal longevity

Gonadal longevity has been studied for decades in the field of aging research, and *C. elegans* has been particularly useful as a model organism to study the regulation of aging by the gonad. Due to the ease of use, scientists turn to mutants, such as *glp-1*, which can be maintained as fertile a stock and induced to become sterile, in most cases facilitated by a temperature sensitive mutation.

Interestingly, this study revealed, that there are genes that impact only longevity of *glp-1*/NOTCH mutants. We observed that *nhr-91* is not required for lifespan extension of germline laser-ablated worms, nor for the longevity of sterile *mes-1* mutants. Hence the fact that different mutations or treatments result in a very similar germlineless phenotype does not, however, allow the assumption, that the different modes of gonadal longevity are the same. It has been known before, that *glp-1* is also expressed in somatic tissues [Austin and Kimble, 1989], but this factor is mainly studied for its role in development, maintenance and signaling of the gonad. In addition, the GLP-1 ligand LAG-2 was found in neurons and is involved in dauer maintenance. These findings highlight, that a somatic role of GLP-1 has to be considered and could influence the longevity phenotype of *glp-1* mutants.

One hypothesis would be that the truncated GLP-1 receptor has a negative effect on longevity or physiological function. As a response *nhr-91* is induced to overcome this detriment and supports beneficial pathways to counteract the effect of a truncated GLP-1 receptor. In particular, our studies may point to a notch specific longevity pathway, independent of germline status. Interestingly, *nhr-91* is most highly expressed in neurons and the distal tip cell<sup>1</sup>, two tissues where notch signaling is quite active. In the future it could be interesting to unravel such a pathway.

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<sup>1</sup> [http://genome.sfu.ca/gexplore/tissue\\_images/WBGene00003681.jpg](http://genome.sfu.ca/gexplore/tissue_images/WBGene00003681.jpg)

The different mutations or treatments to generate long-lived, sterile worms result in different characteristics of longevity. Germline laser-ablation resulted in 50-73% mean lifespan extension, while *glp-1(e2141ts)* mutants exhibited 22-78% mean lifespan extension. Sterile *mes-1(bn7)* mutants only showed a 11-20% longer lifespan than their fertile control. Similar observations could be made, when comparing the gene expression profiles of those different longevity modes. In this study we showed that several fatty acid desaturases are up-regulated in germline-less worms. Interestingly, *fat-5* was induced in *glp-1(e2141ts)* mutants but not in *mes-1(bn7)* mutants. The high levels of *fat-5* and its product palmitoleic acid in *glp-1(e2141ts)* required the presence of *nhr-91*. The gene, which was found to be required for *glp-1*/NOTCH longevity, but not for the longevity of sterile *mes-1* worms.

The role of lipid remodeling and the expression levels of fatty-acid desaturases on gonadal longevity was mainly studied in *glp-1* mutants and little is known about the transcriptome of germline laser-ablated worms. Sinha and Rae carried out a microarray experiment to compare wildtype to germline stem cell laser-ablated worms [Sinha and Rae, 2014]. In their dataset, *fat-1*, *fat-2*, *fat-5* and *fat-6* are up-regulated in ablated worms compared to wildtype animals, which indicates that lipid remodeling also is required for longevity of germline laser-ablated worms. In line with our experiments, *nhr-91* is not differentially regulated in their dataset, strengthening the hypothesis that *nhr-91* is not required for all modes of gonadal longevity.

The disparities in the modes of gonadal longevity found in this study call for caution and well-designed experiments, when investigating gonadal longevity with *C. elegans*. Different modes should be used when testing novel genes or compounds for their role in gonadal longevity. We emphasize to use germline laser-ablation as the gold standard in validating effects on *glp-1*/NOTCH longevity, because with this method no genetic manipulation is required. Any effects of genetically induced germline stem cell ablations can only be excluded, when using germline laser-ablation. In this study the effects of *atf-5* on gonadal longevity were validated by both methods, just like in our paper

concerning the Mondo/Max-like complex (MML-1/MXL-2) [Nakamura et al., 2016]. These genes were found in the same RNAi suppressor screen as *nhr-91* using *glp-1* worms. This brings to the fore the power of such large screens which make use of scalable and broadly available techniques as RNAi. Nevertheless, a stepwise validation with other materials (deletion mutants as control for RNAi) or other methods (germline laser ablation as control for mutants with gonadal longevity phenotypes) are required and should be carried out before further investigating molecular functions, interactions or expression patterns of candidates from large scale screens.

### 4.3 ATF-5 in gonadal longevity

The experiments carried out and described in this thesis reveal a role of *atf-5* in gonadal longevity. The first piece of evidence was generated by Özlem Karalay and Shuhei Nakamura, when *atf-5* was found in an RNAi-based screen for mediators of *GLP-1/NOTCH* longevity. In the course of our follow up experiments, we used deletion mutants to validate this finding in *glp-1* worms and additionally performed lifespan analysis in germline laser-ablated worms. In both modes of gonadal longevity, we found that loss of *atf-5* leads to a reduction of longevity. In our survival analysis of *atf-5(tm4397)* we observed a specific shape of the Kaplan-Meier curve (see Figure 17 and Figure 18). A fraction around 20% of sterile *atf-5(tm4397)* worms show an extended lifespan resulting in a plateau phase after the large fraction of this worms already died. In sterile *glp-1;atf-5(tm4397)* this fraction evens shows a longer maximum lifespan than sterile *glp-1* worms, whereas the median and mean lifespan of sterile *glp-1;atf-5(tm4397)* is significantly shorter than *glp-1*. The shape of the curve could be due to the fact that *atf-5* is more required in keeping up the molecular processes and survival in younger animals, while the surviving older fraction is not so reliant on *atf-5* anymore. A recent study on *atf-5* in *C. elegans* observed likewise shapes of the *glp-1;atf-5(tm4397)* survival curves, however the fraction alive follows the shape of N2 worms until 50% survival and flattens to show up a similar maximum lifespan as *glp-1* worms [Statzer et al., 2020]. Further analysis on health span [eg. following Rollins et al., 2017] could clarify the aging phenotype in *glp-1;atf-5(tm4397)* worms.

*C. elegans* ATF-5 was not intensively studied to the time of this thesis experimental work before and its target genes were unknown. One study described that *impt-1* activated the ISR pathway and increased lifespan and stress resistance of worms in a *gcn-2* and *atf-5*-dependent manner [Ferraz et al., 2016]. In other model organism several studies highlighted a connection between ATF-5 and longevity. Upregulation of this transcription factor has been linked to longevity in mice [Li et al., 2014] and overexpression of the

orthologous transcription factor in yeast (GCN4) extended lifespan [Mittal et al., 2017]. Therefore, we analyzed the transcriptome of *glp-1* worms upon loss of *atf-5* in order to understand what it regulates and find genes mediating its impact on gonadal longevity. In the first round of experiments we focused on two genes, *haf-6* and *tiar-2*, that were highly regulated and not well studied. Lifespan experiments showed no effect on *glp-1* longevity, when *haf-6* (deletion mutant) or *tiar-2* (RNAi knockdown) were manipulated. We therefore went back to the list of 315 potential target genes of *atf-5* in *glp-1* background and focused on evolutionary conserved genes. The remaining candidates were knocked down by RNAi and the effect on *glp-1*/NOTCH longevity was recorded in a large-scale screen. The screen was carried out twice, but the second repeat had to be neglected due to mold contaminations. This technical limitation allows false negative results. This could be overcome by repeating the screen for two or three times, which was not possible during the experimental phase of this thesis work due to time constraints. Several interesting candidates from the first repeat were selected for further validation, which included qPCR re-analysis of expression levels and regular lifespan experiments. This workflow was chosen to exclude possible false positive candidates from the screen. We found two orthologs of human NXNL2 in the screen, but their effect on longevity could not be confirmed in follow-up experiments. It is possible that the degree of knockdown was variable, and follow up experiments should monitor the level of knockdown. We also found genes that were differentially regulated in our RNAseq dataset, but this regulation could not be confirmed with qPCR. This discrepancy could be due to the broad but less specific and sensitive nature of RNAseq data. Moreover, we found *hsp-1* and *hsp-4* in our screen and both genes proved to be regulated by *atf-5* in *glp-1* sterile worms and shorten the lifespan of *glp-1* worms in follow-up experiments.

We carried out a GO-Term analysis of the data obtained by RNAseq and found ER-related GO-Terms, such as protein refolding or response to heat, enriched in the subset of genes that were downregulated upon loss of *atf-5* in *glp-1* worms. This is not surprising since ATF-4 is known as a major transcriptional

mediator of the ER stress response [Pakos-Zebrucka et al., 2016]. Together with the impact of *hsp-4* and *hsp-1* on longevity, we further investigated the role of other heat-shock proteins and chaperones, which are known to ER-related functions and pathways. We found *hsp-3* not to be regulated by *atf-5*, but several members of the cytosolic *hsp-16* family were partially regulated upon loss of *atf-5* in *glp-1* background.

We expanded our experiments to examine other players in the ER<sup>UPR</sup> regulation, but neither *ire-1*, nor *xbp-1* splicing was effected by *atf-5*. We made similar observations for mito<sup>UPR</sup>, DAF-16/FOXO signaling and autophagy; when *atf-5* was lost in *glp-1* background these pathways were not affected, at least for the tested genes. It remains possible however that other outputs of these pathways are affected.

These findings might imply a direct effect of *atf-5* on its targets, because major players acting as signaling hubs were not affected and the regulation of some *atf-5* targets, like *hsp-4*, whose regulation was strictly restricted from closely related or functionally redundant genes. Ultimately however it would be interesting to perform Chip-Seq analysis to find direct targets. From this study we concluded that in *glp-1*/NOTCH longevity, *atf-5* signals towards *hsp-4* and members of the *hsp-16* family, which are required to prevent protein aggregation and proteotoxic stress. The maintenance of proper proteostasis is crucial for longevity [Labbadia and Morimoto, 2014].

Another angle of research was to investigate how ATF-5 is regulated and what factors are working upstream of ATF-5. Another important angle would be to investigate on interaction partners of ATF-5 and their role in regulating longevity. Mammalian ATF4 is known to dimerize with other transcription factors and these heterodimers can differ in transcriptional targets and decide on the signaling outcome [Pakos-Zebrucka et al., 2016]. In *C. elegans* ATF-5 and SKN-1 are proposed to work together in regulating lifespan, but it remains unclear if those factors act as a heterodimer or work in parallel [Ferraz et al., 2016].

Upon loss of *gcn-2* or *pek-1* the levels of p-eIF2a decrease, which would prevent the preferential translation of mRNAs with 5' uORFs like *atf-5*. We expected that the inability to generate p-eIF2a would prevent ATF-5 activity. We used deletion mutants of *gcn-2* and *pek-1*, which are the two known eIF2a kinases in *C. elegans*. Neither the absence of one, nor the double knock-out of both genes had an impact on *glp-1*/NOTCH longevity. This result turned our interest on finding and identifying unknown upstream factors and pathways of ATF-5 regulation in *C. elegans*. Upstream factors could have different regulatory effects. Transcriptional regulation and translational control of ATF4 are known to be central to the integrated stress response [Dey et al., 2010]. Above that, post-translational modifications such as phosphorylation, hydroxylation or ubiquitination are known to impact the transcriptional activity and may stabilize or promote degradation of ATF4 [Pakos-Zebrucka et al., 2016].

These results obtained for ATF4 imply, that we would need a good tool to measure the regulation and activity of the ATF-5 protein. We tried to introduce extrachromosomal transgenic arrays. One approach was to use an ATF-5 promotor driving the expression of fluorescent proteins, another approach was fusing the ATF-5 protein with a fluorescent protein. We could not generate *C. elegans* strains carrying functional arrays, which we could have used for further experiments. We turned to the newly developed CRISPR/Cas9 technology, which allows to generate knock-in alleles and fuse protein tags to the endogenous sequence of the target gene. We expected that the use of the smaller V5/FLAG-tag (in comparison to GFP) would have less influence on the protein function and allow a more precise readout. We were able to introduce a V5/FLAG-tag to the 3' end of the *atf-5* sequence and could also detect the fusion mRNA product by RT-PCR. In western blot analysis we were not able to detect the fusion protein, even in conditions where we expected its translation to be highly induced, namely in sterile *glp-1* worms. This could be due to the unstable nature of the ATF-5 protein or because the tag interfered with its translation and/or stability.

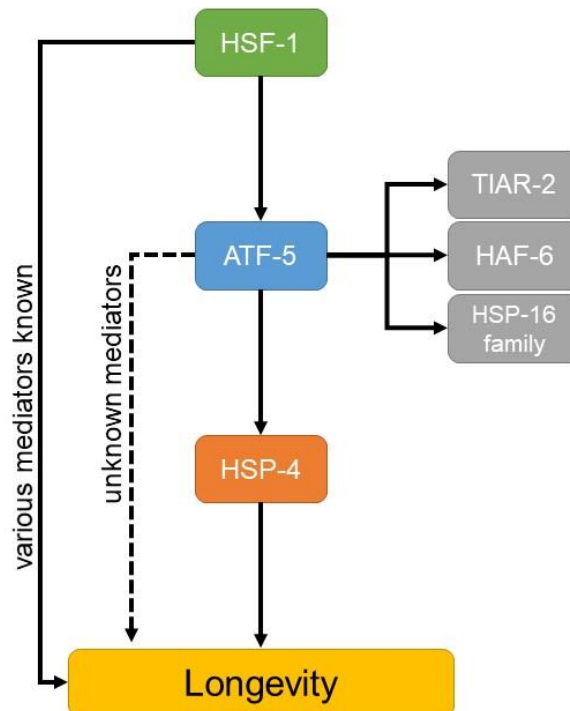


**Figure 42: A potential regulatory network of ATF-5.** In germline-less worms we found several ATF-5 targets of which HSP-4 has the strongest impact on longevity. HSF-1 is upstream of ATF-5, but is also connected to the regulation of longevity through parallel pathways.

As our approaches to directly monitor ATF-5 were not successful, we applied an indirect method by measuring the mRNA levels of its putative target genes *hsp-4* and *haf-6*. SKN-1 was reported to bind in the promotor region of *atf-5* [Glover-Cutter et al., 2013], but in *glp-1* background we could not find an impact of SKN-1 on mRNA levels of *atf-5* targets. It could require another factor to regulate *atf-5* expression in *glp-1* or there are other factors which are more potent regulators of *atf-5* in *glp-1* background.

Next we asked if HSF-1 could be a factor upstream of ATF-5. We knew that HSF-1 and ATF-5 share target genes, such as *hsp-4* or members of the *hsp-16* family [Brunquell et al., 2016]. Furthermore, HSF-1 is a central player in the regulation of heat-shock proteins and chaperones. As described in Chapter 1.7.2, HSF-1 is able to regulate ageing via various pathways, which can be independent of heat-stress induction of this transcription factor.

Our experiments showed that knockdown of *hsf-1* in *glp-1* leads to a reduction in mRNA levels of the ATF-5 targets *hsp-4* and *haf-6*, as well as *atf-5* mRNA levels itself. This placed HSF-1 upstream of ATF-5 and indicated that HSF-1 regulated *atf-5* transcription, maybe by directly binding to its promotor region. Another study found *atf-5* downregulated upon loss of *hsf-1* under normal conditions, but not differentially regulated under heat-stress conditions [Brunquell et al., 2016], which is contradictory to reports about mammalian ATF4 induces by heat stress [Zhu et al., 2012].

Interestingly, we were able to further decrease the mRNA levels of *hsp-4* in *atf-5* deletion mutants by feeding RNAi against *hsf-1* in *glp-1* worms. This showed that *hsp-4* requires HSF-1 and ATF-5 to be expressed, but that HSF-1 can also influence the expression of *hsp-4* independent of *atf-5*.

From the results described in this study, we propose an interaction map of ATF-5 in sterile *glp-1* worms (Figure 42). HSF-1 is a positive regulator of ATF-5, either directly or indirectly by facilitating its transcription, translation or post-translational modifications. The transcription factor ATF-5 targets several genes. One group of ATF-5 targets was not required for *glp-1*/NOTCH longevity including *haf-6* and *tiar-2*. Other targets like members of the HSP-16 family were only partially regulated by ATF-5 and may play a minor role in supporting longevity. We found *hsp-4* expression if *glp-1* worms to be dependent on ATF-5 and loss of *hsp-4* led to a loss of *glp-1*/NOTCH longevity. Consequently, we hypothesize that HSP-4 is the major mediator of ATF-5 in *glp-1*/NOTCH longevity. In line with this connection, HSF-1 targets *hsp-4* via ATF-5, but can also influence *hsp-4* expression through parallel pathways or a direct interaction. Additionally, there might be other ATF-5 targets that are required for *glp-1*/NOTCH longevity, but are not yet identified or validated.

After the experimental phase of this thesis work was finished the ATF-5 project was continued by Torsten Bücher as part of his bachelor thesis. Some of his results are included in this thesis, when the context and connection could lead to a better understanding and conclude outcomes and results. Above that it was reported that Torsten generated another *atf-5* deletion allele [personal

communication with Prof. Dr. Antebi], which turned out sterile. Neither the two deletion alleles used in this study, nor knockdown by RNAi showed a similar phenotype. Further investigations of this allele should clarify if the sterility is caused by the loss of *atf-5*, other genes or trans-regulatory elements that could be deleted in addition.

Recently two studies were published describing longevity phenotypes dependent on ATF-5. The insights gained by these studies can only be discussed here, but could not be integrated into the experiments, which were carried out for this thesis. Knockdown of *mprs-5* inhibits mitochondrial translation, represses cytosolic translation and extends *C. elegans* lifespan [Molenaars et al., 2020]. This cascade is *atf-5* dependent and seems to be conserved across species. Whether inhibition of mitochondrial translation and activation of ATF4 can extend lifespan in mice and humans has to be investigated. Another study found that overexpression of *atf-5* leads to lifespan extension in *C. elegans* and that longevity phenotypes observed after mTORC1 suppression are *atf-5* dependent [Statzer et al., 2020]. The study also confirmed that *atf-5* regulates heat shock proteins such as *hsp-4* or *hsp-16.2*, which were also identified as potential targets of *atf-5* in mediating *glp-1*/NOTCH longevity. In addition, the study also found that *glp-1*/NOTCH longevity was decreased by *atf-5* mutation, further confirming the results obtained in this thesis.

# **5**

## **Future Perspectives**

## 5.1 A lesson told by NHR-91

We found disparities in the modes of gonadal longevity, while investigating the role of NHR-91. We aim to publish these findings and raise awareness among aging researchers, when designing and carrying out experiments in *C. elegans* gonadal longevity. Preliminary results were presented and discussed at the 2015<sup>th</sup> International Worm Meeting. Future projects could start from this point and look for other factors, which are independent regulators of the different modes of gonadal longevity. Such research would be curiosity-driven, because it is questionable if those independent regulations are conserved in other organisms or if they are *C. elegans* specific.

Nevertheless, the difference between germline laser-ablated longevity and *glp-1*/NOTCH longevity might reveal new insights in fine-tuning mechanisms of lifespan regulation. These subtle variations could be the missing pieces of knowledge to fully understand regulatory networks in gonadal longevity and in consequence expand studies in model organisms to therapeutic approaches for increased life- and health span in humans. A first step would be to compare the transcriptomes of germline laser-ablated worms and *glp-1* worms with one method and within one experiment. The progress in technology allows to reduce the required amount of material for transcriptomic analysis and thus allows to include also germline laser-ablated worms. The results could be used in two ways. Overlapping genes would be of interest to further study gonadal longevity and could be validated with data from other model organisms to look for conserved mediators in gonadal longevity. Genes exclusively regulated in one mode of gonadal longevity could undergo a network analysis to look for pathways and functions required for the different modes of gonadal longevity.

We found NHR-91 to regulate fatty-acid desaturases and their products in *glp-1* mutants. It remains unclear how NHR-91 as an orphan nuclear receptor facilitates this regulation, which co-factors or signaling cascades are involved or how NHR-91 is regulated. Future studies could apply lipidomic or metabolomic approaches to narrow down the influence of NHR-91 on fatty-acid

desaturation or other small molecules. As mentioned above, the fatty acid profiling could be repeated using a more precise setup including GCMS-QTOF. This data could be used to define the targets of NHR-91 and the pathways and networks it is integrated. Another attempt would use ChIP-seq technology on NHR-91, which would yield its binding regions on genomic DNA. The presence of a NHR-91 binding sites in regulatory elements like promoters could reveal the direct targets of NHR-91. In addition, NHR-91 pulldowns and analysis of interacting proteins would shed light on the molecular complexes and co-factors. NHR-91 remains an interesting and promising matter of research, because it is one of the 15 conserved nuclear hormone-receptors in *C. elegans* and probably the most neglected one.

## 5.2 ATF-5 in gonadal longevity

This study provided the first results connecting ATF-5 with gonadal longevity. Factors upstream (HSF-1) and downstream (HSP-4) of ATF-5 could be identified. These findings offered several origins for new projects and experiments refining the novel insights concerning ATF-5.

While finishing up this thesis a paper was published describing a mechanism of ATF-5 regulating longevity and stress resistance in *C. elegans* [Statzer et al., 2020]. Down to the present day (30.06.2021) the paper is a preprint and has not been certified by peer review. The study found ATF-5 to promote its positive effects on lifespan and stress resistance by inducing H<sub>2</sub>S production. The mTORC1 complex was found upstream of ATF-5. The transcription factor was found to cooperate also with other longevity factors such as DAF-2 or SKN-1, but mechanism remain unclear. Findings made in this thesis were validated such as the impact of ATF-5 loss on GLP-1/NOTCH longevity and the ATF-5 targets HSP-1, HSP-4 and HSP-16.2. The study also developed two important tools for further experiments with one being a strain expressing GFP fused to the canonical uORF (to monitor its expression) and a strain with ATF-5 overexpression. The overexpression of ATF-5 led to an increased lifespan. A

major question in further analysis is if the signaling cascade HSF-1/ATF-5/HSP-4 leads to longevity via the same mechanisms. The epistatic relations between these transcription factors could be unraveled by combining deletion and rescue elements in different *C. elegans* strains and investigate the epistatic effects on longevity and integrating the recent results.

Beside another project could develop a more ATF-5-centric scope. ChIP-seq could be used to find the actual binding sites of the transcription factor and distinguish between direct and indirect targets of ATF-5. Pulldown of the protein would facilitate the identification of interaction partners and co-factors, but could also help to find post-translational modifications of ATF-5.

In this study we focused on gonadal longevity. In the future it will be of great interest to also look for the role of ATF-5 in other longevity pathways. Is ATF-5 required for lifespan extension induced by dietary restriction or reduced insulin/IGF signaling? Its requirement for the resistance against various stressors should be tested, too. A connection to heat stress or protein aggregation is obvious, but needs further investigation. Another angle would ask for the role and impact of ATF-5 on innate immunity.

All experiments mentioned and suggested above would highly benefit from a possibility to measure endogenous ATF-5 translation and activity in *C. elegans* or other model organisms. In this study we attempted to generate strains with fluorescent or small protein tags, but could not succeed. Novel techniques or new approaches using CRISPR/Cas9 would be needed to finally provide a powerful tool reporting endogenous ATF-5 translation and presence.

*C. elegans* offers a great background to study ATF-5. The insights generated in the worm could be used to ask, if those interactions are conserved and also applicable in higher organisms. ATF4 has been found to be increased in slow aging mice, but underlying mechanisms remained unclear. Cell culture experiments could be a first step to validate the conservation of what has been described in *C. elegans*. Another facet would be investigating data from human centenarian studies and ask if ATF4, its targets or inter-actors can be linked to long-lived humans.

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## **7 Supplementary Materials**

## 7.1 Supplemental Data

### 7.1.1 qPCR Primer

| Target Gene      | Primer Name  | Sequence (5'->3')            |
|------------------|--------------|------------------------------|
| <b>ama-1</b>     | ama-1 FW     | TGGA ACTCTGGAGTCACACC        |
|                  | ama-1 RV     | CATCCTCCTTCATTGAACGG         |
| <b>atf-5</b>     | atf-5 FW     | TCAATTGAGCAAAACGGATCTG       |
|                  | atf-5 RV     | TGTCAACTTCCAAATGGTTCCA       |
| <b>cdc-42</b>    | cdc-42 FW    | AGCCATTCTGGCCGCTCTCG         |
|                  | cdc-42 RV    | GCAACCGCTTCTCGTTTGGC         |
| <b>daf-16</b>    | daf-16 FW    | ATCAGACATCGTTTCCTTCGG        |
|                  | daf-16 RV    | TTAACCGTTTCTCTGGACTAGC       |
| <b>dnj-12</b>    | dnj-12 FW    | GACAGTTGTAATGGAGAAGGC        |
|                  | dnj-12 RV    | TTCTTTCACCTGCTTCTTTCC        |
| <b>fat-2</b>     | fat-2 FW     | CAGCTCGAGCAATCCCTGAGCACTGC   |
|                  | fat-2 RV     | CAGCTCGAGAGTCTTCCTCAGTAACCC  |
| <b>fat-3</b>     | fat-3 FW     | CATGGATCCACAAGAATGCCTCCGGGC  |
|                  | fat-3 RV     | CATGGATCCAATCCATCATCATGAAGC  |
| <b>fat-5</b>     | fat-5 FW     | CAGCTCGAGCGATTATATCTAAACAG   |
|                  | fat-5 RV     | CAGCTCGAGCAGCATCAGTATCCGTCC  |
| <b>fat-6</b>     | fat-6 FW     | CATGGATCCACGGCGGCCAGAGACGC   |
|                  | fat-6 RV     | CATGGATCCCAGTCCACTTGTGATGGC  |
| <b>fat-7</b>     | fat-7 FW     | CATGGATCCCTCAATACCTGTTTCATGG |
|                  | fat-7 RV     | CATGGATCCCAGCATCTGTATCAGTCC  |
| <b>haf-6</b>     | haf-6 FW     | ATAAGAAGGCATTGGAATCCG        |
|                  | haf-6 RV     | TGTGCTTCTACCAAGTTTCCT        |
| <b>hsp-1</b>     | hsp-1 FW     | TATTGGAGCCAAGAACGGA          |
|                  | hsp-1 RV     | ATCTTATCCTTGAGCTTCTCGT       |
| <b>hsp-16.1</b>  | hsp-16.1 FW  | CTCAGATGGAACGTCAATTTACTC     |
|                  | hsp-16.1 RV  | ATCTCAGAAGATTCAGATGGAGAG     |
| <b>hsp-16.2</b>  | hsp-16.2 FW  | ATATGGCTCTGATGGAACG          |
|                  | hsp-16.2 RV  | ATCTCAGAAGACTCAGATGGA        |
| <b>hsp-16.11</b> | hsp-16.11 FW | CTCAGATGGAACGTCAATTTACTC     |
|                  | hsp-16.11 RV | ATCTCAGAAGATTCAGATGGAGAG     |
| <b>hsp-16.41</b> | hsp-16.41 FW | GAGAAATGCTGATCACAACCTC       |
|                  | hsp-16.41 RV | AGACATCGAGTTGAACCGA          |
| <b>hsp-16.48</b> | hsp-16.48 FW | GAGAAATGCTGATCACAACCTC       |
|                  | hsp-16.48 RV | GAAACATCGAGTTGAACAGAG        |
| <b>hsp-16.49</b> | hsp-16.49 FW | GAGAAATGCTGATCACAACCTC       |
|                  | hsp-16.49 RV | GAAACATCGAGTTGAACAGAG        |
| <b>hsp-3</b>     | hsp-3 FW     | TTTCTCTACCGCCGCTGACAAC       |
|                  | hsp-3 RV     | CGTTCTCCTTCGAAGACCTGGATG     |
| <b>hsp-4</b>     | hsp-4 FW     | GTGGCAAACGCGTACTGTGATGA      |
|                  | hsp-4 RV     | CGCAACGTATGATGGAGTGATTCT     |
| <b>hsp-6</b>     | hsp-6 FW     | AACCACCGTCAACAACGCCG         |
|                  | hsp-6 RV     | AGCGATGATCTTATCTCCAGCGTCC    |
| <b>hsp-60</b>    | hsp-60 FW    | GATCCAACCTAAGGTGGTTCG        |
|                  | hsp-60 RV    | GACTACGCATTCCGTTGTG          |

|                             |                  |                          |
|-----------------------------|------------------|--------------------------|
| <b><i>ire-1</i></b>         | ire-1 FW         | GAAGATGTCAGACAATCTCTTGG  |
|                             | ire-1 RV         | AAGTAATAGCCGAGGGAAGC     |
| <b><i>lgg-1</i></b>         | lgg-1 FW         | ACCCAGACCGTATTCCAGTG     |
|                             | lgg-1 RV         | ACGAAGTTGGATGCGTTTTTC    |
| <b><i>lipl-4</i></b>        | lipl-4 FW        | ATGGCCGAGAAGTTCCTACATCGT |
|                             | lipl-4 RV        | GGTGAATTGGCGACCCAATCGAAA |
| <b><i>lys-7</i></b>         | lys-7 FW         | CAACTAACTGGCCAAATAACG    |
|                             | lys-7 RV         | TTAATCCGGATTGTCTGGC      |
| <b><i>mTOR</i></b>          | mTOR FW          | TATAAGAAAGCACGCGGCAC     |
|                             | mTOR RV          | CAGATACGGTATTAGTTCGCC    |
| <b><i>nhr-80</i></b>        | nhr-80 FW        | TGAGGTTTCAGGAGCCAAATAG   |
|                             | nhr-80 RV        | GAAGGAGGTGGACGATGAGA     |
| <b><i>nhr-91</i></b>        | nhr-91 FW        | ATACTTGACTGGCTTCCGG      |
|                             | nhr-91 RV        | CTTGCAAAGCAGGAATGGG      |
| <b><i>pha-4</i></b>         | pha-4 FW         | GCCAATTTTCATGCAAGGAGG    |
|                             | pha-4 RV         | GCCAGTGGTAAAACCAAGAGGT   |
| <b><i>sdz-24</i></b>        | sdz-24 FW        | AGACTCTGTTAATGTTGCTGC    |
|                             | sdz-24 RV        | AAGTAATACCACTGCTTCTCCT   |
| <b><i>sod-3</i></b>         | sod-3 FW         | CACGAGGCTGTTTCGAAAGG     |
|                             | sod-3 RV         | GAATTTTCAGCGCTGGTTGGA    |
| <b><i>tiar-2</i></b>        | tiar-2 FW        | AAGTGGAGATTCTGGAAAGC     |
|                             | tiar-2 RV        | TTACCGTAACCGTAACCGT      |
| <b><i>vha-1</i></b>         | vha-1 FW         | GCATTCTCACAATTCGCTG      |
|                             | vha-1 RV         | CATATCCTGCTCCGAGTCC      |
| <b><i>vha-12</i></b>        | vha-12 FW        | TACCCAGAGGAAATGATCCA     |
|                             | vha-12 RV        | AATTGGAATCTTCTGTCCACG    |
| <b><i>vha-15</i></b>        | vha-15 FW        | AAACTCTACGACCAACGAC      |
|                             | vha-15 RV        | TCATGTCTGTATTCATCGTGAC   |
| <b><i>vha-17</i></b>        | vha-17 FW        | CCAAAGGGACCAAACAGAG      |
|                             | vha-17 RV        | GAAGGAACACCATGATCCAG     |
| <b><i>vha-4</i></b>         | vha-4 FW         | GAAAGCTCGCTGAATTCCG      |
|                             | vha-4 RV         | ATCCTGAAGCGAGGTTACG      |
| <b><i>xbp-1</i></b>         | xbp-1 FW         | CCGATCCACCTCCATCAAC      |
|                             | xbp-1 RV         | ACCGTCTGCTCCTTCCTCAATG   |
| <b><i>xbp-1 spliced</i></b> | xbp-1 FW spliced | TGCCTTTGAATCAGCAGTGG     |
|                             | xbp-1 RV         | ACCGTCTGCTCCTTCCTCAATG   |

## 7.1.2 qPCR result

### 7.1.2.1 NHR-91

mRNA levels of input samples are normalized by using *cdc-42* mRNA abundance, if not stated otherwise.

For expression fold change mRNA expression levels are shown as fold expression normalized to mRNA levels of biological replicate #1 of N2 worms. P-Values are calculated by *t*-test

| Genotype                      | mRNA   | expression fold change |       |       | Mean fold change from control | p-value | Reference Control |
|-------------------------------|--------|------------------------|-------|-------|-------------------------------|---------|-------------------|
| N2                            | nhr-91 | 1                      | 1     | 0,318 |                               |         |                   |
| glp-1(e2141ts)                |        | 3,71                   | 3,224 | 1,77  | 3,8                           | 0,0272  | vs N2             |
| nhr-91(tm4713)                |        | 0                      | 0,038 | 0,013 | -45,5                         | 0,0294  | vs N2             |
| nhr-91(tm4713);glp-1(e2141ts) |        | 0                      | 0,02  | 0,026 | -189,2                        | 0,0078  | vs glp-1(e2141ts) |
|                               |        |                        |       |       |                               |         |                   |
| N2                            | Daf-16 | 1                      | 0,979 | 0,867 |                               |         |                   |
| glp-1(e2141ts)                |        | 0,891                  | 0,582 | 0,361 | 0,6                           | 0,1014  | vs N2             |
| nhr-91(tm4713)                |        | 0,826                  | 1,092 | 0,876 | -1,0                          | 0,8589  | vs N2             |
| nhr-91(tm4713);glp-1(e2141ts) |        | 0,489                  | 0,525 | 0,284 | -1,4                          | 0,3552  | vs glp-1(e2141ts) |
|                               |        |                        |       |       |                               |         |                   |
| N2                            | LipL-4 | 0,614                  | 0,537 | 0,469 |                               |         |                   |
| glp-1(e2141ts)                |        | 1,135                  | 0,966 | 1,062 | 2,0                           | 0,0013  | vs N2             |
| nhr-91(tm4713)                |        | 0,907                  | 0,573 | 0,665 | 1,3                           | 0,1806  | vs N2             |
| nhr-91(tm4713);glp-1(e2141ts) |        | 1,739                  | 1,963 | 2,398 | 1,9                           | 0,0080  | vs glp-1(e2141ts) |
|                               |        |                        |       |       |                               |         |                   |

|                               |       |       |       |       |      |        |                   |
|-------------------------------|-------|-------|-------|-------|------|--------|-------------------|
| N2                            | Sod-3 | 1,00  | 1,29  | 0,94  |      |        |                   |
| glp-1(e2141ts)                |       | 6,68  | 10,12 | 3,21  | 6,2  | 0,0489 | vs N2             |
| nhr-91(tm4713)                |       | 0,97  | 0,49  | 0,83  | -1,4 | 0,1507 | vs N2             |
| nhr-91(tm4713);glp-1(e2141ts) |       | 1,34  | 1,60  | 1,14  | -4,9 | 0,0567 | vs glp-1(e2141ts) |
|                               |       |       |       |       |      |        |                   |
| N2                            | Pha-4 | 1     | 0,356 | 0,451 |      |        |                   |
| glp-1(e2141ts)                |       | 1,882 | 1,307 | 1,542 | 2,6  | 0,0202 | vs N2             |
| nhr-91(tm4713)                |       | 0,923 | 0,536 | 0,381 | 1,0  | 0,9680 | vs N2             |
| nhr-91(tm4713);glp-1(e2141ts) |       | 1,795 | 1,605 | 2,034 | 1,1  | 0,3229 | vs glp-1(e2141ts) |
|                               |       |       |       |       |      |        |                   |
| N2                            | Lgg-1 | 1     | 0,712 | 0,619 |      |        |                   |
| glp-1(e2141ts)                |       | 6,015 | 1,121 | 1,483 | 3,7  | 0,2550 | vs N2             |
| nhr-91(tm4713)                |       | 1,166 | 0*    | 0,993 | -1,1 | 0,1578 | vs N2             |
| nhr-91(tm4713);glp-1(e2141ts) |       | 1,115 | 1,124 | 0,994 | -2,7 | 0,3180 | vs glp-1(e2141ts) |
|                               |       |       |       |       |      |        |                   |
| N2                            | mTOR  | 1     | 1,103 | 1,14  |      |        |                   |
| glp-1(e2141ts)                |       | 0,879 | 1,02  | 0,937 | 0,9  | 0,0814 | vs N2             |
| nhr-91(tm4713)                |       | 0,854 | 1,097 | 1,025 | -1,1 | 0,3458 | vs N2             |
| nhr-91(tm4713);glp-1(e2141ts) |       | 0,872 | 0,999 | 0,901 | -1,0 | 0,7232 | vs glp-1(e2141ts) |
|                               |       |       |       |       |      |        |                   |
| N2                            | Fat-2 | 1     | 0,719 | 0,636 |      |        |                   |
| glp-1(e2141ts)                |       | 2,712 | 1,889 | 1,37  | 2,5  | 0,0412 | vs N2             |
| nhr-91(tm4713)                |       | 0,955 | 0     | 0,715 | -1,4 | 0,4987 | vs N2             |
| nhr-91(tm4713);glp-1(e2141ts) |       | 0,889 | 0,791 | 0,944 | -2,3 | 0,0470 | vs glp-1(e2141ts) |
|                               |       |       |       |       |      |        |                   |

|                               |                                      |             |        |       |      |             |                   |
|-------------------------------|--------------------------------------|-------------|--------|-------|------|-------------|-------------------|
| N2                            | Fat-3                                | 1           | 0,858  | 0,574 |      |             |                   |
| glp-1(e2141ts)                |                                      | 11,653<br>* | 2,177  | 1,681 | 1,6  | 0,0198      | vs N2             |
| nhr-91(tm4713)                |                                      | 1,226       | 0*     | 1,092 | -1,0 | 0,1317      | vs N2             |
| nhr-91(tm4713);glp-1(e2141ts) |                                      | 0,835       | 0,987  | 0,842 | -1,4 | 0,0130      | vs glp-1(e2141ts) |
|                               |                                      |             |        |       |      |             |                   |
| N2                            | Fat-5                                | 1           | 1,165  | 0,893 |      |             |                   |
| glp-1(e2141ts)                |                                      | 6,003       | 10,531 | 0*    | 5,4  | 0,0233      | vs N2             |
| nhr-91(tm4713)                |                                      | 1,009       | 0*     | 0,822 | -1,7 | 0,4627      | vs N2             |
| nhr-91(tm4713);glp-1(e2141ts) |                                      | 1,419       | 1,155  | 1,449 | -4,1 | 0,0264      | vs glp-1(e2141ts) |
|                               |                                      |             |        |       |      |             |                   |
| N2                            | Fat-6                                | 1           | 0,5    | 1     |      |             |                   |
| glp-1(e2141ts)                |                                      | 6,858       | 2,885  | 7,225 | 6,8  | 0,0262      | vs N2             |
| nhr-91(tm4713)                |                                      | 1,059       | 0,676  | 0,674 | -1,0 | 0,8922      | vs N2             |
| nhr-91(tm4713);glp-1(e2141ts) |                                      | 1,314       | 0,539  | 1,201 | -5,6 | 0,0303      | vs glp-1(e2141ts) |
|                               |                                      |             |        |       |      |             |                   |
| N2                            | Fat-7                                | 1           | 0,745  | 1     |      |             |                   |
| glp-1(e2141ts)                |                                      | 0,338       | 0,532  | 0,368 | 0,5  | 0,0085      | vs N2             |
| nhr-91(tm4713)                |                                      | 1,757       | 1,123  | 1,2   | 1,5  | 0,1097      | vs N2             |
| nhr-91(tm4713);glp-1(e2141ts) |                                      | 1,896       | 1,161  | 1,656 | 3,8  | 0,0067      | vs glp-1(e2141ts) |
|                               |                                      |             |        |       |      |             |                   |
| N2                            | nhr-80<br>(normalized with<br>ama-1) | 1           | 0,866  | 0,555 |      |             |                   |
| glp-1(e2141ts)                |                                      | 16,384      | 16,316 | 0*    | 13,5 | <0.000<br>1 | vs N2             |
| nhr-91(tm4713)                |                                      | 1,102       | 0,942  | 0,907 | 1,2  | 0,2895      | vs N2             |
| nhr-91(tm4713);glp-1(e2141ts) |                                      | 16,419      | 14,654 | 10,98 | 1,3  | 0,3415      | vs glp-1(e2141ts) |
|                               |                                      |             |        |       |      |             |                   |

|                                         |              |        |        |        |      |        |                                    |
|-----------------------------------------|--------------|--------|--------|--------|------|--------|------------------------------------|
| <b>mes-1 fertile<br/>(control RNAi)</b> | <b>Fat-2</b> | 1      | 1,617  | 1,216  |      |        |                                    |
| <b>mes-1 sterile<br/>(control RNAi)</b> |              | 8,884  | 6,272  | 8,494  | 6,2  | 0,0014 | vs mes-1 fertile<br>(control RNAi) |
| <b>mes-1 fertile (nhr-<br/>91 RNAi)</b> |              | 1,172  | 1,494  | 1,382  | 1,1  | 0,7430 | vs mes-1 fertile<br>(control RNAi) |
| <b>mes-1 sterile (nhr-<br/>91 RNAi)</b> |              | 6,918  | 7,394  | 8,862  | -1,0 | 0,8819 | vs mes-1 sterile<br>(control RNAi) |
|                                         |              |        |        |        |      |        |                                    |
| <b>mes-1 fertile<br/>(control RNAi)</b> | <b>Fat-3</b> | 1      | 1,478  | 1,171  |      |        |                                    |
| <b>mes-1 sterile<br/>(control RNAi)</b> |              | 16,591 | 7,254  | 13,431 | 10,2 | 0,0151 | vs mes-1 fertile<br>(control RNAi) |
| <b>mes-1 fertile (nhr-<br/>91 RNAi)</b> |              | 1,079  | 1,527  | 1,372  | 1,1  | 0,5982 | vs mes-1 fertile<br>(control RNAi) |
| <b>mes-1 sterile (nhr-<br/>91 RNAi)</b> |              | 10,386 | 15,047 | 9,467  | -1,1 | 0,8189 | vs mes-1 sterile<br>(control RNAi) |
|                                         |              |        |        |        |      |        |                                    |
| <b>mes-1 fertile<br/>(control RNAi)</b> | <b>Fat-5</b> | 1      | 1,226  | 0,951  |      |        |                                    |
| <b>mes-1 sterile<br/>(control RNAi)</b> |              | 0,781  | 5,475  |        | 2,0  | 0,3229 | vs mes-1 fertile<br>(control RNAi) |
| <b>mes-1 fertile (nhr-<br/>91 RNAi)</b> |              | 1,392  | 1,01   | 1,638  | 1,3  | 0,2263 | vs mes-1 fertile<br>(control RNAi) |
| <b>mes-1 sterile (nhr-<br/>91 RNAi)</b> |              | 1,806  | 0,57   | 4,261  | 1,1  | 0,7102 | vs mes-1 sterile<br>(control RNAi) |
|                                         |              |        |        |        |      |        |                                    |
| <b>mes-1 fertile<br/>(control RNAi)</b> | <b>Fat-6</b> | 1      | 1,684  | 1,148  |      |        |                                    |
| <b>mes-1 sterile<br/>(control RNAi)</b> |              |        | 9,831  | 15,342 | 6,6  | 0,0121 | vs mes-1 fertile<br>(control RNAi) |
| <b>mes-1 fertile (nhr-<br/>91 RNAi)</b> |              | 1,436  | 1,723  | 1,52   | 1,2  | 0,2771 | vs mes-1 fertile<br>(control RNAi) |
| <b>mes-1 sterile (nhr-<br/>91 RNAi)</b> |              | 15,581 | 27,845 | 13,148 | 2,2  | 0,3876 | vs mes-1 sterile<br>(control RNAi) |
|                                         |              |        |        |        |      |        |                                    |

|                                         |       |       |       |       |     |        |                                    |
|-----------------------------------------|-------|-------|-------|-------|-----|--------|------------------------------------|
| <b>mes-1 fertile<br/>(control RNAi)</b> | Fat-7 | 1     | 1,233 | 0,846 |     |        |                                    |
| <b>mes-1 sterile<br/>(control RNAi)</b> |       | 3,399 | 0,651 | 1,115 | 1,7 | 0,4625 | vs mes-1 fertile<br>(control RNAi) |
| <b>mes-1 fertile (nhr-<br/>91 RNAi)</b> |       | 1,002 | 1,399 | 0,969 | 1,1 | 0,6151 | vs mes-1 fertile<br>(control RNAi) |
| <b>mes-1 sterile (nhr-<br/>91 RNAi)</b> |       | 1,221 | 4,286 | 0,657 | 1,2 | 0,8251 | vs mes-1 sterile<br>(control RNAi) |

### 7.1.2.2 ATF-5

mRNA levels of input samples are normalized by using *cdc-42* mRNA abundance, if not stated otherwise.

For expression fold change mRNA expression levels are shown as fold expression normalized to mRNA levels of biological replicate #1 of N2 worms. P-Values are calculated by *t*-test

| Genotype                     | mRNA   | expression fold change |        |        | Mean fold change from control | p-value | Reference Control |
|------------------------------|--------|------------------------|--------|--------|-------------------------------|---------|-------------------|
| N2                           | haf-6  | 1                      | 1,093  | 1,134  |                               |         |                   |
| glp-1(e2141ts)               |        | 11,999                 | 11,014 | 11,035 | 10,6                          | 0,0012  | vs N2             |
| atf-5(tm4397)                |        | 1,166                  | 1,252  | 1,178  | 1,1                           | 0,0897  | vs N2             |
| atf-5(tm4397);glp-1(e2141ts) |        | 1,795                  | 1,815  | 1,672  | -6,4                          | 0,0011  | vs glp-1(e2141ts) |
| N2                           | tiar-2 | 1,00                   | 1,04   | 0,88   |                               |         |                   |
| glp-1(e2141ts)               |        | 4,05                   | 4,00   | 4,17   | 4,2                           | 0,0010  | vs N2             |
| atf-5(tm4397)                |        | 0,93                   | 0,96   | 1,11   | 1,0                           | 0,8238  | vs N2             |
| atf-5(tm4397);glp-1(e2141ts) |        | 0,42                   | 0,54   | 0,40   | -9,0                          | 0,0006  | vs glp-1(e2141ts) |
| N2                           | Dnj-12 | 1                      | 0,971  | 0,967  |                               |         |                   |
| glp-1(e2141ts)               |        | 0,421                  | 0,474  | 0,447  | -2,2                          | 0,0021  | vs N2             |
| atf-5(tm4397)                |        | 0,832                  |        | 0,924  | -1,7                          | 0,3405  | vs N2             |
| atf-5(tm4397);glp-1(e2141ts) |        | 0,332                  | 0,343  | 0,311  | -1,4                          | 0,0154  | vs glp-1(e2141ts) |
| N2                           | Sdz-24 | 1                      | 2,133  | 0,888  |                               |         |                   |
| glp-1(e2141ts)               |        | 22,51                  | 27,301 | 22,645 | 18,0                          | 0,0027  | vs N2             |
| atf-5(tm4397)                |        | 0,603                  | 0,968  | 0,429  | -2,0                          | 0,1117  | vs N2             |
| atf-5(tm4397);glp-1(e2141ts) |        | 11,292                 | 15,668 | 11,419 | -1,9                          | 0,0001  | vs glp-1(e2141ts) |
| N2                           | Hsp-1  | 1                      | 1,028  | 0,974  |                               |         |                   |
| glp-1(e2141ts)               |        | 0,447                  | 0,514  | 0,482  | -2,1                          | 0,0012  | vs N2             |
| atf-5(tm4397)                |        | 0,923                  | 1,093  | 0,936  | -1,0                          | 0,7319  | vs N2             |
| atf-5(tm4397);glp-1(e2141ts) |        | 0,358                  | 0,406  | 0,354  | -1,3                          | 0,0106  | vs glp-1(e2141ts) |

|                              |           |       |       |       |      |        |                   |
|------------------------------|-----------|-------|-------|-------|------|--------|-------------------|
| N2                           | hsp-6     | 1     | 1,243 | 1,109 |      |        |                   |
| glp-1(e2141ts)               |           | 1,107 | 1,103 | 0,772 | -1,1 | 0,4383 | vs N2             |
| atf-5(tm4397)                |           | 0,83  | 0,738 | 0,839 | -1,4 | 0,0866 | vs N2             |
| atf-5(tm4397);glp-1(e2141ts) |           | 0,795 | 0,721 | 0,668 | -1,4 | 0,0860 | vs glp-1(e2141ts) |
|                              |           |       |       |       |      |        |                   |
| N2                           | hsp-60    | 1     | 1,048 | 0,99  |      |        |                   |
| glp-1(e2141ts)               |           | 0,764 | 0,727 | 0,879 | -1,3 | 0,0675 | vs N2             |
| atf-5(tm4397)                |           | 0,995 | 1,147 | 1,006 | 1,0  | 0,3675 | vs N2             |
| atf-5(tm4397);glp-1(e2141ts) |           | 0,734 | 0,79  | 0,787 | -1,0 | 0,7050 | vs glp-1(e2141ts) |
|                              |           |       |       |       |      |        |                   |
| N2                           | hsp-16.1  | 1     | 1,616 | 1,154 |      |        |                   |
| glp-1(e2141ts)               |           | 4,091 | 5,552 | 4,149 | 3,7  | 0,0079 | vs N2             |
| atf-5(tm4397)                |           | 0,749 | 1,189 | 0,776 | -1,4 | 0,0215 | vs N2             |
| atf-5(tm4397);glp-1(e2141ts) |           | 2,014 | 4,023 | 2,129 | -1,7 | 0,0085 | vs glp-1(e2141ts) |
|                              |           |       |       |       |      |        |                   |
| N2                           | hsp-16.2  | 1     | 1,756 | 1,714 |      |        |                   |
| glp-1(e2141ts)               |           | 5,272 | 7,753 | 7,731 | 4,6  | 0,0112 | vs N2             |
| atf-5(tm4397)                |           | 0,836 | 1,21  | 1,117 | -1,4 | 0,0859 | vs N2             |
| atf-5(tm4397);glp-1(e2141ts) |           | 3,886 | 5,283 | 4,846 | -1,5 | 0,0373 | vs glp-1(e2141ts) |
|                              |           |       |       |       |      |        |                   |
| N2                           | hsp-16.11 | 1     | 1,808 | 1,348 |      |        |                   |
| glp-1(e2141ts)               |           | 4,045 | 5,587 | 4,468 | 3,4  | 0,0049 | vs N2             |
| atf-5(tm4397)                |           | 0,76  | 1,249 | 0,841 | -1,5 | 0,0479 | vs N2             |
| atf-5(tm4397);glp-1(e2141ts) |           | 2,237 | 4,267 | 2,434 | -1,6 | 0,0147 | vs glp-1(e2141ts) |
|                              |           |       |       |       |      |        |                   |
| N2                           | hsp-16.41 | 1     | 1,566 | 1,312 |      |        |                   |
| glp-1(e2141ts)               |           | 5,886 | 7,869 | 6,864 | 5,3  | 0,0053 | vs N2             |
| atf-5(tm4397)                |           | 0,842 | 1,162 | 1,013 | -1,3 | 0,0565 | vs N2             |
| atf-5(tm4397);glp-1(e2141ts) |           | 4,458 | 5,478 | 5,349 | -1,3 | 0,0286 | vs glp-1(e2141ts) |
|                              |           |       |       |       |      |        |                   |
| N2                           | hsp-16.48 | 1     | 1,616 | 1,241 |      |        |                   |
| glp-1(e2141ts)               |           | 3,699 | 5,488 | 4,468 | 3,5  | 0,0106 | vs N2             |
| atf-5(tm4397)                |           | 0,601 | 1,007 | 0,791 | -1,6 | 0,0165 | vs N2             |
| atf-5(tm4397);glp-1(e2141ts) |           | 1,981 | 3,937 | 2,241 | -1,7 | 0,0121 | vs glp-1(e2141ts) |
|                              |           |       |       |       |      |        |                   |

|                              |           |       |       |       |      |        |                   |
|------------------------------|-----------|-------|-------|-------|------|--------|-------------------|
| N2                           | hsp-16.49 | 1     | 1,532 | 1,055 |      |        |                   |
| glp-1(e2141ts)               |           | 3,923 | 5,873 | 3,691 | 3,8  | 0,0246 | vs N2             |
| atf-5(tm4397)                |           | 0,569 | 1,047 | 0,68  | -1,6 | 0,0054 | vs N2             |
| atf-5(tm4397);glp-1(e2141ts) |           | 1,588 | 3,535 | 1,845 | -1,9 | 0,0056 | vs glp-1(e2141ts) |
|                              |           |       |       |       |      |        |                   |
| N2                           | Hsp-70    | 1     | 1,575 | 0,905 |      |        |                   |
| glp-1(e2141ts)               |           | 3,736 | 5,496 | 2,845 | 3,5  | 0,0380 | vs N2             |
| atf-5(tm4397)                |           | 0,402 | 0,652 | 0,503 | -2,2 | 0,0518 | vs N2             |
| atf-5(tm4397);glp-1(e2141ts) |           | 1,523 | 2,11  | 1,483 | -2,4 | 0,0584 | vs glp-1(e2141ts) |
|                              |           |       |       |       |      |        |                   |
| N2                           | F44E5.5   | 1     | 1,571 | 1,072 |      |        |                   |
| glp-1(e2141ts)               |           | 3,955 | 5,651 | 3,141 | 3,5  | 0,0349 | vs N2             |
| atf-5(tm4397)                |           | 0,407 | 0,567 | 0,423 | -2,6 | 0,0283 | vs N2             |
| atf-5(tm4397);glp-1(e2141ts) |           | 1,402 | 1,82  | 1,541 | -2,7 | 0,0542 | vs glp-1(e2141ts) |
|                              |           |       |       |       |      |        |                   |
| N2                           | hsp-3     | 1     | 1     | 1     |      |        |                   |
| glp-1(e2141ts)               |           | 2,809 | 3,005 | 2,631 | 2,8  | 0,0035 | vs N2             |
| atf-5(tm4397)                |           | 1,288 | 1,474 | 1,404 | 1,4  | 0,0189 | vs N2             |
| atf-5(tm4397);glp-1(e2141ts) |           | 2,68  | 2,782 | 2,6   | -1,0 | 0,1478 | vs glp-1(e2141ts) |
|                              |           |       |       |       |      |        |                   |
| N2                           | hsp-4     | 1     | 1     | 1     |      |        |                   |
| glp-1(e2141ts)               |           | 1,582 | 1,512 | 1,848 | 1,6  | 0,0241 | vs N2             |
| atf-5(tm4397)                |           | 1,041 | 0,96  | 1,154 | 1,1  | 0,4554 | vs N2             |
| atf-5(tm4397);glp-1(e2141ts) |           | 0,199 | 0,241 | 0,335 | -6,4 | 0,0025 | vs glp-1(e2141ts) |
|                              |           |       |       |       |      |        |                   |
| N2                           | lys-7     | 1     | 0,906 | 1,077 |      |        |                   |
| glp-1(e2141ts)               |           | 1,182 | 1,116 | 1,334 | 1,2  | 0,0101 | vs N2             |
| atf-5(tm4397)                |           | 0,889 | 0,918 | 0,937 | -1,1 | 0,2294 | vs N2             |
| atf-5(tm4397);glp-1(e2141ts) |           | 1,165 | 1,082 | 1,278 | -1,0 | 0,0873 | vs glp-1(e2141ts) |
|                              |           |       |       |       |      |        |                   |
| N2                           | vha-1     | 1     | 1,059 | 1,015 |      |        |                   |
| glp-1(e2141ts)               |           | 2,765 | 2,611 | 2,776 | 2,7  | 0,0017 | vs N2             |
| atf-5(tm4397)                |           | 1,092 | 1,169 | 0,998 | 1,1  | 0,2603 | vs N2             |
| atf-5(tm4397);glp-1(e2141ts) |           | 2,459 | 2,603 | 2,678 | -1,1 | 0,2599 | vs glp-1(e2141ts) |
|                              |           |       |       |       |      |        |                   |

|                              |               |        |        |        |      |        |                   |
|------------------------------|---------------|--------|--------|--------|------|--------|-------------------|
| N2                           | vha-4         | 1      | 1,04   | 0,965  |      |        |                   |
| glp-1(e2141ts)               |               | 2,94   | 2,963  | 3,039  | 3,0  | 0,0006 | vs N2             |
| atf-5(tm4397)                |               | 1,055  | 1,173  | 0,993  | 1,1  | 0,1495 | vs N2             |
| atf-5(tm4397);glp-1(e2141ts) |               | 2,816  | 2,939  | 3,044  | -1,0 | 0,3468 | vs glp-1(e2141ts) |
|                              |               |        |        |        |      |        |                   |
| N2                           | vha-12        | 1      | 1,038  | 0,851  |      |        |                   |
| glp-1(e2141ts)               |               | 3,791  | 3,276  | 3,209  | 3,6  | 0,0046 | vs N2             |
| atf-5(tm4397)                |               | 0,983  | 1,007  | 0,805  | -1,0 | 0,0646 | vs N2             |
| atf-5(tm4397);glp-1(e2141ts) |               | 3,277  | 3,207  | 3,111  | -1,1 | 0,2551 | vs glp-1(e2141ts) |
|                              |               |        |        |        |      |        |                   |
| N2                           | vha-15        | 1      | 1,085  | 0,894  |      |        |                   |
| glp-1(e2141ts)               |               | 4,002  | 3,864  | 3,556  | 3,8  | 0,0013 | vs N2             |
| atf-5(tm4397)                |               | 1,08   | 1,137  | 0,868  | 1,0  | 0,3812 | vs N2             |
| atf-5(tm4397);glp-1(e2141ts) |               | 3,365  | 3,525  | 3,479  | -1,1 | 0,1622 | vs glp-1(e2141ts) |
|                              |               |        |        |        |      |        |                   |
| N2                           | vha-17        | 1      | 0,935  | 0,875  |      |        |                   |
| glp-1(e2141ts)               |               | 2,676  | 2,751  | 2,836  | 2,9  | 0,0020 | vs N2             |
| atf-5(tm4397)                |               | 0,946  | 1,095  | 0,889  | 1,0  | 0,5911 | vs N2             |
| atf-5(tm4397);glp-1(e2141ts) |               | 2,671  | 2,64   | 2,786  | -1,0 | 0,2134 | vs glp-1(e2141ts) |
|                              |               |        |        |        |      |        |                   |
| N2                           | sod-3         | 1      | 1,177  | 0,701  |      |        |                   |
| glp-1(e2141ts)               |               | 15,277 | 14,925 | 13,054 | 15,0 | 0,0018 | vs N2             |
| atf-5(tm4397)                |               | 1,259  | 1,461  | 0,837  | 1,2  | 0,0385 | vs N2             |
| atf-5(tm4397);glp-1(e2141ts) |               | 13,27  | 14,967 | 12,439 | -1,1 | 0,2905 | vs glp-1(e2141ts) |
|                              |               |        |        |        |      |        |                   |
| N2                           | xbp-1         | 1      | 1      | 1      |      |        |                   |
| glp-1(e2141ts)               |               | 2,163  | 2,331  | 1,971  | 2,2  | 0,0080 | vs N2             |
| atf-5(tm4397)                |               | 1,143  | 1,328  | 1,122  | 1,2  | 0,0944 | vs N2             |
| atf-5(tm4397);glp-1(e2141ts) |               | 2,256  | 2,483  | 1,881  | 1,0  | 0,5517 | vs glp-1(e2141ts) |
|                              |               |        |        |        |      |        |                   |
| N2                           | xbp-1 spliced | 1      | 1      | 1      |      |        |                   |
| glp-1(e2141ts)               |               | 0,128  | 0,114  | 0,062  | -9,9 | 0,0005 | vs N2             |
| atf-5(tm4397)                |               | 1,172  | 1,092  | 1,128  | 1,1  | 0,0299 | vs N2             |
| atf-5(tm4397);glp-1(e2141ts) |               | 0,126  | 0,152  | 0,096  | 1,2  | 0,2080 | vs glp-1(e2141ts) |
|                              |               |        |        |        |      |        |                   |

|                                     |                                           |              |              |             |       |             |                                        |
|-------------------------------------|-------------------------------------------|--------------|--------------|-------------|-------|-------------|----------------------------------------|
| N2                                  | spliced/<br>unspliced xbp-1<br>transcript | 1            | 1            | 1           |       |             |                                        |
| glp-1(e2141ts)                      |                                           | 0,05917<br>7 | 0,04890<br>6 | 0,0314<br>6 | -21,5 | <0.000<br>1 | vs N2                                  |
| atf-5(tm4397)                       |                                           | 1,02537<br>2 | 0,82228<br>9 | 1,0053<br>5 | -1,1  | 0,5275      | vs N2                                  |
| atf-5(tm4397);glp-1(e2141ts)        |                                           | 0,05585<br>1 | 0,06121<br>6 | 0,0510<br>4 | 1,2   | 0,2942      | vs glp-1(e2141ts)                      |
|                                     |                                           |              |              |             |       |             |                                        |
| N2                                  | ire-1                                     | 1            | 1            | 1           |       |             |                                        |
| glp-1(e2141ts)                      |                                           | 0,685        | 0,627        | 0,498       | -1,7  | 0,0189      | vs N2                                  |
| atf-5(tm4397)                       |                                           | 1,075        | 1,129        | 0,973       | 1,1   | 0,3261      | vs N2                                  |
| atf-5(tm4397);glp-1(e2141ts)        |                                           | 0,66         | 0,588        | 0,529       | -1,0  | 0,6582      | vs glp-1(e2141ts)                      |
|                                     |                                           |              |              |             |       |             |                                        |
| glp-1(e2141ts) Control (luc)i       | hsp-4                                     | 1            | 1,104        | 1,035       |       |             |                                        |
| glp-1(e2141ts) hsf-1i               |                                           | 0,261        | 0,307        | 0,332       | -3,5  | 0,0013      | vs glp-1(e2141ts) Control (luc)i       |
| glp-1(e2141ts) skn-1i               |                                           | 1,079        | 0,905        | 1,109       | -1,0  | 0,8828      | vs glp-1(e2141ts) Control (luc)i       |
|                                     |                                           |              |              |             |       |             |                                        |
| glp-1(e2141ts) Control (luc)i       | haf-6                                     | 1            | 1,104        | 1,035       |       |             |                                        |
| glp-1(e2141ts) hsf-1i               |                                           | 0,442        | 0,492        | 0,388       | -2,4  | 0,0018      | vs glp-1(e2141ts) Control (luc)i       |
| glp-1(e2141ts) skn-1i               |                                           | 1,171        | 1,324        | 1,336       | 1,2   | 0,0260      | vs glp-1(e2141ts) Control (luc)i       |
|                                     |                                           |              |              |             |       |             |                                        |
| glp-1(e2141ts) Control (luc)i       | atf-5                                     | 1            | 1,004        | 1,138       |       |             |                                        |
| glp-1(e2141ts) hsf-1i               |                                           | 0,321        | 0,35         | 0,419       | -2,9  | 0,0008      | vs glp-1(e2141ts) Control (luc)i       |
| glp-1(e2141ts) skn-1i               |                                           | 1,035        | 0,919        | 0,965       | -1,1  | 0,3428      | vs glp-1(e2141ts) Control (luc)i       |
|                                     |                                           |              |              |             |       |             |                                        |
| glp-1(e2141ts) Control (luc)i       | hsp-4                                     | 1            | 1,041        | 1,101       |       |             |                                        |
| atf-5(tm4397);glp-1(e2141ts) (luc)i |                                           | 0,08         | 0,084        | 0,087       | -12,5 | 0,0008      | vs glp-1(e2141ts) Control (luc)i       |
| glp-1(e2141ts) hsf-1i               |                                           | 0,229        | 0,254        | 0,298       | -4,0  | 0,0001      | vs glp-1(e2141ts) Control (luc)i       |
| atf-5(tm4397);glp-1(e2141ts) hsf-1i |                                           | 0,032        | 0,032        | 0,018       | -3,1  | 0,0128      | vs atf-5(tm4397);glp-1(e2141ts) (luc)i |

### 7.1.3 RNAi Lifespan Screen against potential ATF-5 downstream targets and mediators of its role in *glp-1* longevity

| Gene Name and blinded clone number | Mean Lifespan Reduction compared to luciferase control | Mean Lifespan |
|------------------------------------|--------------------------------------------------------|---------------|
| ctl-1/Y54G11A.6 (#27)              | n.a.                                                   | n.a.          |
| F22F7.7 (#56)                      | n.a.                                                   | n.a.          |
| C17B7.5 (#60)                      | n.a.                                                   | n.a.          |
| C47E8.4 (#V1)                      | n.a.                                                   | n.a.          |
| cyp-37B1/F28G4.1 (#V10)            | n.a.                                                   | n.a.          |
| mct-1/Y19D10A.12 (#V12)            | n.a.                                                   | n.a.          |
| Y97E10AR.2 (#V14)                  | n.a.                                                   | n.a.          |
| hsp-1/F26D10.3 (#46)               | 41,8%                                                  | 11,59         |
| hsp-4/F43E2.8 (#20)                | 52,3%                                                  | 14,50         |
| npl-4.1/ F59E12.4 (#16)            | 55,5%                                                  | 15,40         |
| gei-4/ W07B3.2 (#28)               | 56,1%                                                  | 15,56         |
| ZK177.2 (#17)                      | 75,3%                                                  | 20,90         |
| slc-36.5/ C44B7.6 (#V6)            | 76,1%                                                  | 21,12         |
| dnj-12/F39B2.10 (#7)               | 87,3%                                                  | 24,22         |
| T20D4.7 (#63)                      | 88,2%                                                  | 24,49         |
| F17B5.1 (#6)                       | 88,9%                                                  | 24,66         |
| F18A11.5 (#26)                     | 89,7%                                                  | 24,90         |
| gpa-15/M04C7.1 (#5)                | 90,4%                                                  | 25,08         |
| sdz-24/K07E8.3 (#10)               | 90,8%                                                  | 25,21         |
| gba-2/C33C12.8 (#13)               | 91,5%                                                  | 25,38         |
| cst-1/F14H12.4 (#84)               | 91,7%                                                  | 25,46         |
| ZC239.6 (#14)                      | 92,5%                                                  | 25,68         |
| aat-7/F54D12.3 (#11)               | 93,5%                                                  | 25,94         |
| C04E12.10 (#61)                    | 94,2%                                                  | 26,14         |
| arrd-24/F48F7.7 (#90)              | 94,7%                                                  | 26,29         |
| C53A3.2 (#67)                      | 95,0%                                                  | 26,36         |
| C50B6.7 (#75)                      | 95,2%                                                  | 26,42         |
| cpr-1/C52E4.1 (#74)                | 95,6%                                                  | 26,52         |
| twk-21/T01B4.1 (#91)               | 95,9%                                                  | 26,61         |
| F44E5.5 (#24)                      | 95,9%                                                  | 26,61         |
| T28F4.5 (#4)                       | 96,0%                                                  | 26,64         |
| k1p-18/C06G3.2 (#39)               | 96,0%                                                  | 26,65         |
| lip1-1/F54F3.3 (#76)               | 96,1%                                                  | 26,66         |
| W09C3.1 (#2)                       | 97,6%                                                  | 27,10         |
| best-5/C07A9.8 (#35)               | 97,7%                                                  | 27,11         |
| T19D2.1 (#83)                      | 97,9%                                                  | 27,16         |
| spp-8/C28C12.5 (#40)               | 97,9%                                                  | 27,17         |

|                                |        |       |
|--------------------------------|--------|-------|
| <b>T27D12.1 (#25)</b>          | 98,1%  | 27,22 |
| <b>tep-1/ZK337.1 (#8)</b>      | 98,2%  | 27,27 |
| <b>bigr-1/F37H8.3 (#23)</b>    | 98,4%  | 27,32 |
| <b>B0281.6 (#12)</b>           | 98,6%  | 27,36 |
| <b>H04D03.3 (#34)</b>          | 98,8%  | 27,41 |
| <b>mct-2/C01B4.9 (#57)</b>     | 99,0%  | 27,47 |
| <b>anmt-3/T07C12.9 (#73)</b>   | 99,0%  | 27,48 |
| <b>luciferase/CONTROL</b>      | 100,0% | 27,75 |
| <b>hil-7/C01B10.5 (#38)</b>    | 100,3% | 27,85 |
| <b>nhr-21/F21D12.1 (#19)</b>   | 100,6% | 27,93 |
| <b>ckb-2/B0285.9 (#30)</b>     | 101,4% | 28,13 |
| <b>ncx-9/C13D9.8 (#65)</b>     | 101,4% | 28,14 |
| <b>scl-20/ZK384.2 (#52)</b>    | 101,4% | 28,15 |
| <b>lipl-2/F46B6.8 (#72)</b>    | 101,9% | 28,29 |
| <b>best-5/C07A9.8 (#36)</b>    | 102,0% | 28,31 |
| <b>ZK643.2 (#33)</b>           | 102,2% | 28,35 |
| <b>Y17G7B.17 (#V3)</b>         | 102,4% | 28,42 |
| <b>C50F7.5 (#V8)</b>           | 102,5% | 28,45 |
| <b>far-3/F15B9.1 (#77)</b>     | 102,7% | 28,52 |
| <b>aagr-4/F52D1.1 (#82)</b>    | 102,8% | 28,52 |
| <b>dhs-7/E04F6.7 (#18)</b>     | 102,9% | 28,57 |
| <b>prk-1/C06E8.3 (#32)</b>     | 103,0% | 28,58 |
| <b>cest-1/T02B5.1 (#79)</b>    | 103,3% | 28,66 |
| <b>C23G10.6 (#31)</b>          | 103,4% | 28,70 |
| <b>catp-3/C09H5.2 (#V11)</b>   | 103,5% | 28,72 |
| <b>asm-3/W03G1.7 (#37)</b>     | 103,8% | 28,80 |
| <b>scav-6/F07A5.3 (#3)</b>     | 103,9% | 28,84 |
| <b>clcc-43/R07C3.1 (#9)</b>    | 104,0% | 28,85 |
| <b>swt-7/K11D12.5 (#66)</b>    | 104,5% | 29,00 |
| <b>ctl-1/Y54G11A.6 (#V15)</b>  | 104,7% | 29,05 |
| <b>twk-32/F53C11.6 (#80)</b>   | 104,8% | 29,07 |
| <b>acs-2/F28F8.2 (#47)</b>     | 104,9% | 29,12 |
| <b>F35F10.1 (#58)</b>          | 105,1% | 29,16 |
| <b>cyp-14A3/K09A11.4 (#89)</b> | 105,2% | 29,19 |
| <b>tiar-2/Y46G5A.13 (#V7)</b>  | 105,3% | 29,23 |
| <b>gly-1/F44F4.6 (#22)</b>     | 105,4% | 29,24 |
| <b>F32B5.1 (#1)</b>            | 105,4% | 29,26 |
| <b>cyp-25A2/C36A4.2 (#29)</b>  | 105,8% | 29,37 |
| <b>gst-7/F11G11.2 (#15)</b>    | 105,9% | 29,40 |
| <b>dhs-26/ZK816.5 (#85)</b>    | 106,1% | 29,43 |
| <b>ZK596.1 (#41)</b>           | 106,6% | 29,57 |
| <b>lys-7/C02A12.4 (#64)</b>    | 107,4% | 29,82 |
| <b>nape-1 (#V13)</b>           | 108,5% | 30,12 |
| <b>T28C12.4 (#69)</b>          | 108,6% | 30,13 |

|                                |        |       |
|--------------------------------|--------|-------|
| <b>lem-4/Y55F3BR.8 (#V9)</b>   | 108,7% | 30,17 |
| <b>ftn-1/C54F6.14 (#70)</b>    | 109,0% | 30,24 |
| <b>gst-29/Y53F4B.32 (#V4)</b>  | 109,7% | 30,44 |
| <b>skr-12/C52D10.6 (#45)</b>   | 109,7% | 30,45 |
| <b>chil-22/R09D1.10 (#21)</b>  | 110,3% | 30,61 |
| <b>sru-17/T04A11.10 (#43)</b>  | 110,4% | 30,63 |
| <b>frpr-1/C02B8.5 (#88)</b>    | 111,3% | 30,88 |
| <b>grl-16/Y65B4BR.6 (#V2)</b>  | 112,2% | 31,15 |
| <b>fkf-6/F31D4.3 (#53)</b>     | 112,8% | 31,30 |
| <b>nol-9/K09B11.2 (#44)</b>    | 115,2% | 31,98 |
| <b>lev-9/T07H6.5 (#86)</b>     | 115,3% | 32,00 |
| <b>F14H3.12 (#49)</b>          | 115,6% | 32,10 |
| <b>drd-5/F55E10.6 (#87)</b>    | 115,7% | 32,11 |
| <b>col-146/T06E4.6 (#71)</b>   | 116,2% | 32,26 |
| <b>F41F3.3 (#62)</b>           | 117,0% | 32,48 |
| <b>gst-13/Y53F4B.35 (#V5)</b>  | 117,2% | 32,52 |
| <b>F44E7.2 (#68)</b>           | 118,1% | 32,79 |
| <b>gst-3/K08F4.11 (#42)</b>    | 118,4% | 32,87 |
| <b>dod-3/C24B9.9 (#55)</b>     | 119,6% | 33,18 |
| <b>clcc-227/F08H9.5 (#78)</b>  | 121,2% | 33,63 |
| <b>T22F3.11 (#59)</b>          | 121,8% | 33,81 |
| <b>sti-1/R09E12.3 (#54)</b>    | 123,6% | 34,31 |
| <b>hmit-1.1/Y51A2D.4 (#51)</b> | 139,8% | 38,80 |
| <b>dgat-2/F59A1.10 (#50)</b>   | 143,1% | 39,71 |
| <b>C01G10.8 (#81)</b>          | 147,1% | 40,83 |
| <b>T26H5.8 (#48)</b>           | 150,5% | 41,77 |

#### 7.1.4 FAME Analysis

|                |                     | Mean<br>[ng/mg<br>Protein] | SD<br>[ng/mg<br>Protein] | N | p-Value |                 |
|----------------|---------------------|----------------------------|--------------------------|---|---------|-----------------|
| <b>C16:1</b>   | N2                  | 69,4                       | 6,6                      | 3 |         |                 |
|                | <i>glp-1</i>        | 115,2                      | 7,2                      | 2 | 0,0052  | vs N2           |
|                | <i>nhr-91</i>       | 69,7                       | 17,6                     | 3 | 0,9804  | vs N2           |
|                | <i>glp-1;nhr-91</i> | 62,4                       | 4,6                      | 2 | 0,0129  | vs <i>glp-1</i> |
|                |                     |                            |                          |   |         |                 |
| <b>C18:1n9</b> | N2                  | 72,4                       | 1,8                      | 3 |         |                 |
|                | <i>glp-1</i>        | 150,3                      | 44,1                     | 2 | 0,0442  | vs N2           |
|                | <i>nhr-91</i>       | 46,0                       | 10,6                     | 3 | 0,0129  | vs N2           |
|                | <i>glp-1;nhr-91</i> | 73,7                       | 6,2                      | 2 | 0,1342  | vs <i>glp-1</i> |
|                |                     |                            |                          |   |         |                 |
| <b>C18:1n7</b> | N2                  | 302,3                      | 22,4                     | 3 |         |                 |
|                | <i>glp-1</i>        | 447,1                      | 52,9                     | 2 | 0,0211  | vs N2           |
|                | <i>nhr-91</i>       | 152,4                      | 112,8                    | 3 | 0,0868  | vs N2           |
|                | <i>glp-1;nhr-91</i> | 271,3                      | 58,4                     | 2 | 0,0875  | vs <i>glp-1</i> |
|                |                     |                            |                          |   |         |                 |
| <b>C18:2n6</b> | N2                  | 227,9                      | 24,1                     | 3 |         |                 |
|                | <i>glp-1</i>        | 332,7                      | 43,7                     | 2 | 0,0371  | vs N2           |
|                | <i>nhr-91</i>       | 137,7                      | 62,3                     | 3 | 0,0794  | vs N2           |
|                | <i>glp-1;nhr-91</i> | 263,8                      | 0,5                      | 2 | 0,1558  | vs <i>glp-1</i> |

Table 3: FAME analysis of N2, *glp-1*, *nhr-91* and *glp-1;nhr-91* worms. For N2 and *nhr-91* three repeats could be analyzed. For *glp-1* and *glp-1;nhr-91* two repeats could be analyzed. P-Value was calculated by *t*-test.

### 7.1.5 LGG-1:GFP Western Blot analysis

P values were calculated by the *t*-test

For each experiment two independent lanes per group were analyzed. Each lane was loaded with the same protein amount.

For each experiment the ratio of free GFP/LGG-1:GFP was set to 1 for lane #1 of N2 worms.

|                    | <b>Mean of<br/>free GFP /<br/>LGG-1:GFP</b> | <b>s.e.m of<br/>free GFP /<br/>LGG-1:GFP</b> | <b>N</b> | <b>p-Value</b> |                 |
|--------------------|---------------------------------------------|----------------------------------------------|----------|----------------|-----------------|
| N2                 | 106.9 %                                     | 31.7 %                                       | 3        |                |                 |
| <i>atf-5</i>       | 70.9 %                                      | 10.9 %                                       | 3        | 0.1092         | vs N2           |
| <i>glp-1</i>       | 41.6 %                                      | 19.3 %                                       | 3        | 0.0015         | vs N2           |
| <i>glp-1;atf-5</i> | 34.1 %                                      | 24.1 %                                       | 3        | 0.5791         | vs <i>glp-1</i> |

## 7.1.6 Lifespan Analysis

### 7.1.6.1 Statistical Data

\* number of dead animals/total number of animals analyzed, P values were calculated by the Mantel-Cox Log Rank test.

LS = lifespan, diff. = difference, RC = Reference Control

| Strains/Treatment or Condition | Rep-licate | Mean LS [d] | Mean diff. from RC | Animals* | Maximum LS | Max diff. from RC | p-value  | RC                |
|--------------------------------|------------|-------------|--------------------|----------|------------|-------------------|----------|-------------------|
| N2                             | 1          | 18,89       |                    | 69/120   | 22         |                   |          |                   |
| glp-1(e2141ts)                 |            | 32,23       | 70,6 %             | 82/120   | 58         | 75,8 %            | < 0,0001 | vs N2             |
| nhr-91(tm4713)                 |            | 13,41       | -29 %              | 91/120   | 33         | 0 %               | < 0,0001 | vs N2             |
| nhr-91(tm4713);glp-1(e2141ts)  |            | 17,31       | -46,3 %            | 106/120  | 58         | 0 %               | < 0,0001 | vs glp-1(e2141ts) |
| N2                             | 2          | 18,66       |                    | 67/120   | 33         |                   |          |                   |
| glp-1(e2141ts)                 |            | 26,32       | 41 %               | 83/120   | 50         | 51,5 %            | < 0,0001 | vs N2             |
| nhr-91(tm4713)                 |            | 14,11       | -24,4 %            | 66/120   | 30         | -9,1 %            | < 0,0001 | vs N2             |
| nhr-91(tm4713);glp-1(e2141ts)  |            | 14,70       | -44,1 %            | 105/120  | 38         | -24 %             | < 0,0001 | vs glp-1(e2141ts) |
| N2                             | 3          | 22,24       |                    | 54/120   | 34         |                   |          |                   |
| glp-1(e2141ts)                 |            | 27,14       | 22 %               | 88/120   | 61         | 79,4 %            | 0,0023   | vs N2             |
| nhr-91(tm4713)                 |            | 17,85       | -19,7 %            | 75/120   | 31         | -8,8 %            | < 0,0001 | vs N2             |
| nhr-91(tm4713);glp-1(e2141ts)  |            | 16,66       | -38,6 %            | 106/120  | 43         | -29,5 %           | < 0,0001 | vs glp-1(e2141ts) |

|                                 |   |       |         |         |    |         |          |                                |
|---------------------------------|---|-------|---------|---------|----|---------|----------|--------------------------------|
| N2                              | 4 | 22,20 |         | 83/150  | 45 |         |          |                                |
| glp-1(e2141ts)                  |   | 39,48 | 77,9 %  | 71/150  | 62 | 37,8 %  | < 0,0001 | vs N2                          |
| nhr-91(tm4713)                  |   | 15,75 | -29,1 % | 86/150  | 34 | -24,4 % | < 0,0001 | vs N2                          |
| nhr-91(tm4713);glp-1(e2141ts)   |   | 16,58 | -58 %   | 141/150 | 52 | -16,1 % | < 0,0001 | vs glp-1(e2141ts)              |
| N2/mock-ablated                 | 1 | 19,13 |         | 56/90   | 33 |         |          |                                |
| N2/germline-ablated             |   | 28,63 | 49,7 %  | 68/75   | 61 | 84,8 %  | < 0,0001 | vs N2/mock-ablated             |
| nhr-91(tm4713)/mock-ablated     |   | 14,33 | -25,1 % | 79/105  | 33 | 0 %     | < 0,0001 | vs N2/mock-ablated             |
| nhr-91(tm4713)/germline-ablated |   | 22,19 | -22,5 % | 80/103  | 54 | -11,5 % | < 0,0001 | vs nhr-91(tm4713)/mock-ablated |
| N2/mock-ablated                 | 2 | 19,06 |         | 72/133  | 33 |         |          |                                |
| N2/germline-ablated             |   | 32,93 | 72,8 %  | 105/125 | 68 | 106,1 % | < 0,0001 | vs N2/mock-ablated             |
| nhr-91(tm4713)/mock-ablated     |   | 16,22 | -14,9 % | 70/119  | 31 | -6,1 %  | 0,0091   | vs N2/mock-ablated             |
| nhr-91(tm4713)/germline-ablated |   | 18,00 | -45,3 % | 90/106  | 48 | -29,4 % | < 0,0001 | vs nhr-91(tm4713)/mock-ablated |
| N2/mock-ablated                 | 3 | 20,82 |         | 70/135  | 32 |         |          |                                |
| N2/germline-ablated             |   | 34,50 | 65,7 %  | 87/127  | 60 | 87,5 %  | < 0,0001 | vs N2/mock-ablated             |
| nhr-91(tm4713)/mock-ablated     |   | 14,73 | -29,2 % | 55/105  | 27 | -15,6 % | < 0,0001 | vs N2/mock-ablated             |
| nhr-91(tm4713)/germline-ablated |   | 16,00 | -53,6 % | 70/100  | 62 | 3,3 %   | < 0,0001 | vs nhr-91(tm4713)/mock-ablated |
|                                 |   |       |         |         |    |         |          |                                |

|                                 |   |       |         |         |    |         |          |                                 |
|---------------------------------|---|-------|---------|---------|----|---------|----------|---------------------------------|
| N2/mock-ablated                 | 4 | 18,83 |         | 103/135 | 36 |         |          |                                 |
| N2/germline-ablated             |   | 31,48 | 67,2 %  | 103/127 | 60 | 66,7 %  | < 0,0001 | vs N2/mock-ablated              |
| nhr-91(tm4713)/mock-ablated     |   | 15,55 | -17,4 % | 89/135  | 29 | -19,4 % | 0,0007   | vs N2/mock-ablated              |
| nhr-91(tm4713)/germline-ablated |   | 19,65 | -37,6 % | 91/118  | 51 | -15 %   | < 0,0001 | vs nhr-91(tm4713)/mock-ablated  |
| mes-1 fertile Control (luc)i    | 1 | 18,98 |         | 72/120  | 32 |         |          |                                 |
| mes-1 sterile Control (luc)i    |   | 22,01 | 16 %    | 60/120  | 39 | 21,9 %  | 0,0011   | vs mes-1 fertile Control (luc)i |
| mes-1 fertile nhr-91i           |   | 17,85 | -6 %    | 70/120  | 29 | -9,4 %  | 0,4157   | vs mes-1 fertile Control (luc)i |
| mes-1 sterile nhr-91i           |   | 20,14 | -8,5%   | 68/120  | 41 | 5,1 %   | 0,2878   | vs mes-1 sterile Control (luc)i |
| mes-1 fertile Control (luc)i    | 2 | 16,91 |         | 54/80   | 27 |         |          |                                 |
| mes-1 sterile Control (luc)i    |   | 18,82 | 11,3 %  | 41/67   | 33 | 22,2 %  | 0,1066   | vs mes-1 fertile Control (luc)i |
| mes-1 fertile nhr-91i           |   | 15,69 | -7,2 %  | 60/80   | 27 | 0 %     | 0,2138   | vs mes-1 fertile Control (luc)i |
| mes-1 sterile nhr-91i           |   | 18,81 | -0,1 %  | 40/68   | 37 | 12,1 %  | 0,924    | vs mes-1 sterile Control (luc)i |
| mes-1 fertile Control (luc)i    | 3 | 16,98 |         | 85/120  | 23 |         |          |                                 |
| mes-1 sterile Control (luc)i    |   | 19,98 | 17,7 %  | 53/120  | 35 | 52,2 %  | 0,0059   | vs mes-1 fertile Control (luc)i |
| mes-1 fertile nhr-91i           |   | 16,95 | -0,2 %  | 39/120  | 26 | 13 %    | 0,9147   | vs mes-1 fertile Control (luc)i |
| mes-1 sterile nhr-91i           |   | 18,58 | -7 %    | 42/98   | 33 | -5,7 %  | 0,299    | vs mes-1 sterile Control (luc)i |
|                                 |   |       |         |         |    |         |          |                                 |

|                              |   |       |         |         |    |         |          |                                 |
|------------------------------|---|-------|---------|---------|----|---------|----------|---------------------------------|
| mes-1 fertile Control (luc)i | 4 | 16,28 |         | 56/150  | 25 |         |          |                                 |
| mes-1 sterile Control (luc)i |   | 19,46 | 19,5 %  | 85/150  | 43 | 72 %    | 0,0062   | vs mes-1 fertile Control (luc)i |
| mes-1 fertile nhr-91i        |   | 17,34 | 6,5 %   | 52/150  | 25 | 0 %     | 0,1351   | vs mes-1 fertile Control (luc)i |
| mes-1 sterile nhr-91i        |   | 18,93 | -2,7%   | 99/150  | 35 | -18,6 % | 0,621    | vs mes-1 sterile Control (luc)i |
| N2                           | 1 | 19,58 |         | 70/160  | 35 |         |          |                                 |
| glp-1(e2141ts)               |   | 30,00 | 53,2 %  | 139/160 | 50 | 42,9 %  | < 0,0001 | vs N2                           |
| atf-5(4397)                  |   | 14,89 | -24 %   | 68/160  | 28 | -20 %   | 0,0002   | vs N2                           |
| atf-5(4397);glp-1(e2141ts)   |   | 19,22 | -35,9 % | 108/160 | 60 | 20 %    | 0,0003   | vs glp-1(e2141ts)               |
| N2                           | 2 | 20,19 |         | 72/160  | 37 |         |          |                                 |
| glp-1(e2141ts)               |   | 28,61 | 41,7 %  | 100/160 | 44 | 18,9%   | < 0,0001 | vs N2                           |
| atf-5(4397)                  |   | 10,84 | -46,3 % | 70/160  | 29 | -21,6 % | < 0,0001 | vs N2                           |
| atf-5(4397);glp-1(e2141ts)   |   | 23,03 | -19,5%  | 72/120  | 61 | 38,6 %  | 0,0084   | vs glp-1(e2141ts)               |
| N2                           | 3 | 17,47 |         | 104/160 | 32 |         |          |                                 |
| glp-1(e2141ts)               |   | 28,91 | 65,5 %  | 126/160 | 44 | 37,5 %  | < 0,0001 | vs N2                           |
| atf-5(4397)                  |   | 12,60 | -27,9 % | 76/160  | 23 | -28,1 % | < 0,0001 | vs N2                           |
| atf-5(4397);glp-1(e2141ts)   |   | 19,14 | -33,8 % | 114/160 | 58 | 31,8 %  | 0,0024   | vs glp-1(e2141ts)               |
|                              |   |       |         |         |    |         |          |                                 |

|                                |   |       |         |       |    |         |          |                               |
|--------------------------------|---|-------|---------|-------|----|---------|----------|-------------------------------|
| N2/mock-ablated                | 1 | 21,37 |         | 41/75 | 38 |         |          |                               |
| N2/germline-ablated            |   | 41,08 | 92,2 %  | 47/70 | 64 | 68,4 %  | < 0,0001 | vs N2/mock-ablated            |
| atf-5(tm4397)/mock-ablated     |   | 20,00 | -6,4 %  | 25/75 | 29 | -23,7 % | 0,2393   | vs N2/mock-ablated            |
| atf-5(tm4397)/germline-ablated |   | 18,50 | -55 %   | 47/81 | 53 | -17,2 % | < 0,0001 | vs atf-5(tm4397)/mock-ablated |
| N2/mock-ablated                | 2 | 20,90 |         | 45/90 | 33 |         |          |                               |
| N2/germline-ablated            |   | 40,66 | 94,5 %  | 40/89 | 60 | 81,8 %  | < 0,0001 | vs N2/mock-ablated            |
| atf-5(tm4397)/mock-ablated     |   | 16,45 | -21,3 % | 27/75 | 29 | -12,1 % | 0,0039   | vs N2/mock-ablated            |
| atf-5(tm4397)/germline-ablated |   | 20,31 | -50,1 % | 44/68 | 53 | -11,7 % | < 0,0001 | vs atf-5(tm4397)/mock-ablated |
| N2/mock-ablated                | 3 | 17,27 |         | 57/90 | 29 |         |          |                               |
| N2/germline-ablated            |   | 37,59 | 117,7 % | 59/80 | 68 | 134,5 % | < 0,0001 | vs N2/mock-ablated            |
| atf-5(tm4397)/mock-ablated     |   | 15,17 | -12,1 % | 46/90 | 36 | 24,1 %  | 0,2198   | vs N2/mock-ablated            |
| atf-5(tm4397)/germline-ablated |   | 16,94 | -54,9 % | 48/90 | 40 | -41,2 % | < 0,0001 | vs atf-5(tm4397)/mock-ablated |
| N2/mock-ablated                | 4 | 15,95 |         | 58/90 | 30 |         |          |                               |
| N2/germline-ablated            |   | 31,25 | 95,9 %  | 38/69 | 48 | 60 %    | < 0,0001 | vs N2/mock-ablated            |
| atf-5(tm4397)/mock-ablated     |   | 16,12 | 1,1 %   | 23/90 | 33 | 10 %    | 0,8364   | vs N2/mock-ablated            |
| atf-5(tm4397)/germline-ablated |   | 13,74 | -56 %   | 75/83 | 53 | 10,4 %  | < 0,0001 | vs atf-5(tm4397)/mock-ablated |
|                                |   |       |         |       |    |         |          |                               |

|                             |   |       |         |         |    |        |          |                   |
|-----------------------------|---|-------|---------|---------|----|--------|----------|-------------------|
| N2                          | 1 | 20,79 |         | 49/150  | 38 |        |          |                   |
| glp-1(e2141ts)              |   | 34,78 | 67,3 %  | 88/150  | 52 | 36,8 % | < 0,0001 | vs N2             |
| gcn-2 (ok871)               |   | 18,06 | -13,1 % | 98/150  | 38 | 0 %    | 0,0422   | vs N2             |
| gcn-2(ok871);glp-1(e2141ts) |   | 35,62 | 2,4 %   | 53/150  | 50 | -3,8 % | 0,203    | vs glp-1(e2141ts) |
| N2                          | 2 | 16,38 |         | 74/150  | 30 |        |          |                   |
| glp-1(e2141ts)              |   | 30,37 | 85,4 %  | 100/150 | 43 | 43,3 % | < 0,0001 | vs N2             |
| gcn-2 (ok871)               |   | 16,05 | -2 %    | 81/150  | 33 | 10 %   | 0,8866   | vs N2             |
| gcn-2(ok871);glp-1(e2141ts) |   | 33,03 | 8,8 %   | 121/150 | 43 | 0 %    | 0,0154   | vs glp-1(e2141ts) |
| N2                          | 3 | 20,72 |         | 86/120  | 35 |        |          |                   |
| glp-1(e2141ts)              |   | 30,70 | 48,2 %  | 92/150  | 49 | 40 %   | < 0,0001 | vs N2             |
| gcn-2 (ok871)               |   | 21,80 | 5,2 %   | 89/150  | 35 | 0 %    | 0,0694   | vs N2             |
| gcn-2(ok871);glp-1(e2141ts) |   | 33,83 | 10,2 %  | 98/150  | 55 | 12,2 % | 0,0083   | vs glp-1(e2141ts) |
| N2                          | 4 | 19,05 |         | 81/150  | 37 |        |          |                   |
| glp-1(e2141ts)              |   | 29,36 | 54,1 %  | 89/150  | 47 | 27 %   | < 0,0001 | vs N2             |
| gcn-2 (ok871)               |   | 19,03 | -0,1 %  | 91/150  | 37 | 0 %    | 0,9438   | vs N2             |
| gcn-2(ok871);glp-1(e2141ts) |   | 32,27 | 9,9 %   | 105/150 | 50 | 6,4 %  | 0,0009   | vs glp-1(e2141ts) |
|                             |   |       |         |         |    |        |          |                   |

|                                          |   |       |        |         |    |        |          |                   |
|------------------------------------------|---|-------|--------|---------|----|--------|----------|-------------------|
| N2                                       | 1 | 21,97 |        | 108/150 | 34 |        |          |                   |
| glp-1(e2141ts)                           |   | 29,76 | 29,4 % | 116/150 | 44 | 29,4 % | < 0,0001 | vs N2             |
| pek-1(ok275)                             |   | 22,93 | 4,4 %  | 117/150 | 31 | -8,8 % | 0,3559   | vs N2             |
| pek-1(ok275);glp-1(e2141ts)              |   | 31,77 | 6,8 %  | 98/150  | 52 | 18,2 % | 0,0034   | vs glp-1(e2141ts) |
| gcn-2(ok871);pek-1(ok275)                |   | 22,77 | 3,6 %  | 105/150 | 37 | 8,8 %  | 0,442    | vs N2             |
| gcn-2(ok871);pek-1(ok275);glp-1(e2141ts) |   | 35,68 | 19,9 % | 89/150  | 55 | 25 %   | < 0,0001 | vs glp-1(e2141ts) |
| N2                                       | 2 | 22,08 |        | 101/150 | 39 |        |          |                   |
| glp-1(e2141ts)                           |   | 28,53 | 29,2 % | 104/150 | 47 | 20,5 % | < 0,0001 | vs N2             |
| pek-1(ok275)                             |   | 21,92 | -0,7 % | 104/150 | 36 | -7,7 % | 0,9397   | vs N2             |
| pek-1(ok275);glp-1(e2141ts)              |   | 30,48 | 6,8 %  | 101/150 | 54 | 14,9 % | 0,022    | vs glp-1(e2141ts) |
| gcn-2(ok871);pek-1(ok275)                |   | 20,95 | -5,1 % | 94/150  | 36 | -7,7 % | 0,1974   | vs N2             |
| gcn-2(ok871);pek-1(ok275);glp-1(e2141ts) |   | 34,15 | 19,7 % | 88/150  | 58 | 23,4 % | < 0,0001 | vs glp-1(e2141ts) |
|                                          |   |       |        |         |    |        |          |                   |

|                                          |   |       |         |         |    |         |          |                                  |
|------------------------------------------|---|-------|---------|---------|----|---------|----------|----------------------------------|
| N2                                       | 3 | 16,94 |         | 90/150  | 33 |         |          |                                  |
| glp-1(e2141ts)                           |   | 34,5  | 103,7 % | 96/150  | 61 | 84,8 %  | < 0,0001 | vs N2                            |
| pek-1(ok275)                             |   | 16,56 | -2,2 %  | 86/150  | 28 | -15,2 % | 0,4248   | vs N2                            |
| pek-1(ok275);glp-1(e2141ts)              |   | 34,27 | -0,7 %  | 78/150  | 56 | -8,2 %  | 0,9019   | vs glp-1(e2141ts)                |
| gcn-2(ok871);pek-1(ok275)                |   | 15,36 | -9,3 %  | 88/150  | 33 | 0 %     | 0,0858   | vs N2                            |
| gcn-2(ok871);pek-1(ok275);glp-1(e2141ts) |   | 29,57 | -14,3 % | 108/150 | 53 | -13,1 % | 0,0013   | vs glp-1(e2141ts)                |
| N2                                       | 4 | 18,42 |         | 116/150 | 30 |         |          |                                  |
| glp-1(e2141ts)                           |   | 28,28 | 53,5 %  | 110/150 | 41 | 36,7 %  | < 0,0001 | vs N2                            |
| pek-1(ok275)                             |   | 19,53 | 6 %     | 112/150 | 30 | 0 %     | 0,1166   | vs N2                            |
| pek-1(ok275);glp-1(e2141ts)              |   | 22,09 | -21,9 % | 86/150  | 37 | -9,8 %  | < 0,0001 | vs glp-1(e2141ts)                |
| gcn-2(ok871);pek-1(ok275)                |   | 19,34 | 5 %     | 106/150 | 27 | -10 %   | 0,3454   | vs N2                            |
| gcn-2(ok871);pek-1(ok275);glp-1(e2141ts) |   | 30,38 | 7,4 %   | 87/150  | 47 | 14,6 %  | 0,0399   | vs glp-1(e2141ts)                |
| N2 Control (luc)i                        | 1 | 19,26 |         | 71/150  | 27 |         |          |                                  |
| glp-1(e2141ts) Control (luc)i            |   | 32,44 | 68,4 %  | 78/150  | 50 | 85,4 %  | < 0,0001 | vs N2 Control (luc)i             |
| N2 tiar-2i                               |   | 22,25 | 15,5 %  | 54/150  | 33 | 22,2 %  | 0,0003   | vs N2 Control (luc)i             |
| glp-1(e2141ts) tiar-2i                   |   | 31,24 | -3,7 %  | 81/150  | 50 | 0 %     | 0,3973   | vs glp-1(e2141ts) Control (luc)i |
|                                          |   |       |         |         |    |         |          |                                  |

|                               |   |       |         |         |    |         |          |                                  |
|-------------------------------|---|-------|---------|---------|----|---------|----------|----------------------------------|
| N2 Control (luc)i             | 2 | 14,99 |         | 104/150 | 25 |         |          |                                  |
| glp-1(e2141ts) Control (luc)i |   | 26,59 | 77,4 %  | 111/150 | 39 | 56 %    | < 0,0001 | vs N2 Control (luc)i             |
| N2 tiar-2i                    |   | 16,74 | 11,7 %  | 57/150  | 25 | 0 %     | 0,0074   | vs N2 Control (luc)i             |
| glp-1(e2141ts) tiar-2i        |   | 28,63 | 7,10%   | 125/150 | 45 | 15,4 %  | 0,0411   | vs glp-1(e2141ts) Control (luc)i |
| N2                            | 1 | 15,82 |         | 97/150  | 28 |         |          |                                  |
| glp-1(e2141ts)                |   | 29,38 | 85,7 %  | 102/150 | 47 | 67,9 %  | < 0,0001 | vs N2                            |
| haf-6(ok3219)                 |   | 17,82 | 12,6 %  | 86/150  | 25 | -10,7 % | 0,0052   | vs N2                            |
| haf-6(ok3219);glp-1(e2141ts)  |   | 31,85 | 8,4 %   | 92/150  | 56 | 19,1 %  | 0,0458   | vs glp-1(e2141ts)                |
| N2                            | 2 | 14,15 |         | 108/150 | 27 |         |          |                                  |
| glp-1(e2141ts)                |   | 41,26 | 191,6 % | 111/150 | 60 | 122,2 % | < 0,0001 | vs N2                            |
| haf-6(ok3219)                 |   | 19,56 | 38,2 %  | 124/150 | 36 | 33,3 %  | < 0,0001 | vs N2                            |
| haf-6(ok3219);glp-1(e2141ts)  |   | 40,89 | -0,9 %  | 133/150 | 55 | -8,3 %  | 0,1816   | vs glp-1(e2141ts)                |
| N2 Control (luc)i             | 1 | 19,95 |         | 41/150  | 25 |         |          |                                  |
| glp-1(e2141ts) Control (luc)i |   | 27,86 | 39,6 %  | 104/150 | 48 | 92 %    | < 0,0001 | vs N2 Control (luc)i             |
| N2 F21B5.1i                   |   | 18,24 | -8,6 %  | 36/150  | 25 | 0 %     | 0,04     | vs N2 Control (luc)i             |
| glp-1(e2141ts) F17B5.1i       |   | 31,16 | 11,8 %  | 21/150  | 43 | -10,4 % | 0,167    | vs glp-1(e2141ts) Control (luc)i |
|                               |   |       |         |         |    |         |          |                                  |

|                               |   |       |        |         |    |         |          |                                  |
|-------------------------------|---|-------|--------|---------|----|---------|----------|----------------------------------|
| N2 Control (luc)i             | 2 | 18,83 |        | 67/150  | 27 |         |          |                                  |
| glp-1(e2141ts) Control (luc)i |   | 32,32 | 70,7 % | 52/150  | 40 | 48,1 %  | < 0,0001 | vs N2 Control (luc)i             |
| N2 F21B5.1i                   |   | 21,26 | 12,3 % | 41/150  | 27 | 0 %     | 0,0071   | vs N2 Control (luc)i             |
| glp-1(e2141ts) F17B5.1i       |   | 30,71 | -5 %   | 116/150 | 46 | 15 %    | 0,0533   | vs glp-1(e2141ts) Control (luc)i |
| N2 Control (luc)i             | 1 | 19,95 |        | 41/150  | 25 |         |          |                                  |
| glp-1(e2141ts) Control (luc)i |   | 27,86 | 39,6 % | 104/150 | 48 | 92 %    | < 0,0001 | vs N2 Control (luc)i             |
| N2 T20D4.7i                   |   | 18,51 | -7,2 % | 20/150  | 25 | 0 %     | 0,1895   | vs N2 Control (luc)i             |
| glp-1(e2141ts) T20D4.7i       |   | 33,88 | 21,6 % | 6/150   | 43 | -10,4 % | 0,0081   | vs glp-1(e2141ts) Control (luc)i |
| N2 Control (luc)i             | 2 | 18,83 |        | 67/150  | 27 |         |          |                                  |
| glp-1(e2141ts) Control (luc)i |   | 32,32 | 70,7 % | 52/150  | 40 | 48,1 %  | < 0,0001 | vs N2 Control (luc)i             |
| N2 T20D4.7i                   |   | 17,72 | -6,4 % | 48/150  | 27 | 0 %     | 0,4461   | vs N2 Control (luc)i             |
| glp-1(e2141ts) T20D4.7i       |   | 35,76 | 10,6 % | 62/150  | 48 | 20 %    | 0,0078   | vs glp-1(e2141ts) Control (luc)i |
| N2 Control (luc)i             | 1 | 19,95 |        | 41/150  | 25 |         |          |                                  |
| glp-1(e2141ts) Control (luc)i |   | 27,86 | 39,6 % | 104/150 | 48 | 92 %    | < 0,0001 | vs N2 Control (luc)i             |
| N2 dnj-12i                    |   | 21,05 | 5,5 %  | 27/150  | 25 | 0 %     | 0,1568   | vs N2 Control (luc)i             |
| glp-1(e2141ts) dnj-12i        |   | 29,58 | 6,2 %  | 37/150  | 48 | 0 %     | 0,3958   | vs glp-1(e2141ts) Control (luc)i |
|                               |   |       |        |         |    |         |          |                                  |

|                               |   |       |         |         |    |         |          |                                  |
|-------------------------------|---|-------|---------|---------|----|---------|----------|----------------------------------|
| N2 Control (luc)i             | 2 | 18,83 |         | 67/150  | 27 |         |          |                                  |
| glp-1(e2141ts) Control (luc)i |   | 32,32 | 70,7 %  | 52/150  | 40 | 48,1 %  | < 0,0001 | vs N2 Control (luc)i             |
| N2 dnj-12i                    |   | 17,98 | -5 %    | 45/150  | 27 | 0 %     | 0,6131   | vs N2 Control (luc)i             |
| glp-1(e2141ts) dnj-12i        |   | 32,03 | -0,9 %  | 60/150  | 48 | 20 %    | 0,6654   | vs glp-1(e2141ts) Control (luc)i |
| N2 Control (luc)i             | 1 | 19,95 |         | 41/150  | 25 |         |          |                                  |
| glp-1(e2141ts) Control (luc)i |   | 27,86 | 39,6 %  | 104/150 | 48 | 92 %    | < 0,0001 | vs N2 Control (luc)i             |
| N2 sdz-24i                    |   | 18,70 | -6,3 %  | 89/150  | 29 | 16 %    | 0,0014   | vs N2 Control (luc)i             |
| glp-1(e2141ts) sdz-24i        |   | 27,68 | -0,6 %  | 121/150 | 41 | -14,6 % | 0,4466   | vs glp-1(e2141ts) Control (luc)i |
| N2 Control (luc)i             | 2 | 18,83 |         | 67/150  | 27 |         |          |                                  |
| glp-1(e2141ts) Control (luc)i |   | 32,32 | 70,7 %  | 52/150  | 40 | 48,1 %  | < 0,0001 | vs N2 Control (luc)i             |
| N2 sdz-24i                    |   | 17,43 | -7,9 %  | 85/150  | 27 | 0 %     | 0,027    | vs N2 Control (luc)i             |
| glp-1(e2141ts) sdz-24i        |   | 32,31 | 0 %     | 61/150  | 43 | 7,5 %   | 0,9428   | vs glp-1(e2141ts) Control (luc)i |
| N2 Control (luc)i             | 1 | 19,95 |         | 41/150  | 25 |         |          |                                  |
| glp-1(e2141ts) Control (luc)i |   | 27,86 | 39,6 %  | 104/150 | 48 | 92 %    | < 0,0001 | vs N2 Control (luc)i             |
| N2 hsp-1i                     |   | 11,79 | -40,9 % | 54/150  | 12 | -52 %   | < 0,0001 | vs N2 Control (luc)i             |
| glp-1(e2141ts) hsp-1i         |   | 12,81 | -47,9 % | 31/150  | 25 | -47,9 % | < 0,0001 | vs glp-1(e2141ts) Control (luc)i |
|                               |   |       |         |         |    |         |          |                                  |

|                               |   |       |         |         |    |         |          |                                  |
|-------------------------------|---|-------|---------|---------|----|---------|----------|----------------------------------|
| N2 Control (luc)i             | 2 | 18,83 |         | 67/150  | 27 |         |          |                                  |
| glp-1(e2141ts) Control (luc)i |   | 32,32 | 70,7 %  | 52/150  | 40 | 48,1 %  | < 0,0001 | vs N2 Control (luc)i             |
| N2 hsp-1i                     |   | 10,53 | -44,4 % | 125/150 | 20 | -25,9 % | < 0,0001 | vs N2 Control (luc)i             |
| glp-1(e2141ts) hsp-1i         |   | 15,20 | -53 %   | 122/150 | 34 | -15 %   | < 0,0001 | vs glp-1(e2141ts) Control (luc)i |
| N2 Control (luc)i             | 1 | 19,95 |         | 41/150  | 25 |         |          |                                  |
| glp-1(e2141ts) Control (luc)i |   | 27,86 | 39,6 %  | 104/150 | 48 | 92 %    | < 0,0001 | vs N2 Control (luc)i             |
| N2 hsp-4i                     |   | 13,77 | -31 %   | 35/150  | 20 | -20 %   | < 0,0001 | vs N2 Control (luc)i             |
| glp-1(e2141ts) hsp-4i         |   | 15,19 | -45,5 % | 103/150 | 20 | -58,3 % | < 0,0001 | vs glp-1(e2141ts) Control (luc)i |
| N2 Control (luc)i             | 2 | 18,83 |         | 67/150  | 27 |         |          |                                  |
| glp-1(e2141ts) Control (luc)i |   | 32,32 | 70,7 %  | 52/150  | 40 | 48,1 %  | < 0,0001 | vs N2 Control (luc)i             |
| N2 hsp-4i                     |   | 12,03 | -36,5 % | 103/150 | 20 | -25,9 % | < 0,0001 | vs N2 Control (luc)i             |
| glp-1(e2141ts) hsp-4i         |   | 16,85 | -47,9 % | 103/150 | 20 | -50 %   | < 0,0001 | vs glp-1(e2141ts) Control (luc)i |

### 7.1.7 GO Term Analysis of RNA Sequencing

| GO biological process                          | GO Term    | C. elegans REF | Genes found | Fold enrichment | raw P Value | FDR      | Genes                                                                                  |
|------------------------------------------------|------------|----------------|-------------|-----------------|-------------|----------|----------------------------------------------------------------------------------------|
| protein refolding                              | GO:0042026 | 9              | 4           | 29,26           | 3,16E-05    | 2,45E-02 | hsp-4, F44E5.5, hsp-70, hsp-1                                                          |
| chaperone cofactor-dependent protein refolding | GO:0051085 | 17             | 5           | 19,36           | 1,55E-05    | 1,92E-02 | hsp-4, F44E5.5, hsp-70, hsp-1, dnj-13                                                  |
| response to heat                               | GO:0009408 | 58             | 10          | 11,35           | 6,55E-08    | 4,06E-04 | daf-21, tiar-2, mtl-1, hsp-16.41, hsp-70, sti-1, hsp-1, hsp-16.11, hsp-16.48, hsp-16.2 |

Table 4: Results of GO Enrichment Analysis of downregulated genes in *atf-5(tm4397);glp-1(e2141ts)* compared to *glp-1(e2141ts)*.

| GO biological process       | GO Term    | C. elegans REF | Genes found | Fold enrichment | raw P Value | FDR      | Genes                                                                                                                                                                                                        |
|-----------------------------|------------|----------------|-------------|-----------------|-------------|----------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| response to gamma radiation | GO:0010332 | 13             | 4           | 26,39           | 3,63E-05    | 1,88E-02 | parg-2, K08D8.5, clec-47, mul-1                                                                                                                                                                              |
| innate immune response      | GO:0045087 | 280            | 24          | 7,35            | 1,32E-13    | 8,16E-10 | C17H12.8, irg-5, sysm-1, F08F3.4, dod-17, C49G7.10, K08D8.5, elt-2, irg-4, gst-38, dod-24, C32H11.4, drd-50, K08D8.4, dct-17, dod-22, H20E11.1, F55G11.8, C17H12.6, clec-186, cpr-3, spp-18, ugt-44, clec-66 |

Table 5: Results of GO Enrichment Analysis of upregulated genes in *atf-5(tm4397);glp-1(e2141ts)* compared to *glp-1(e2141ts)*.

## 7.1.8 Wormsorter Data

### 7.1.8.1 *Phsp-4::GFP* transgene

|               |          | BR1   | BR2   | BR3   | BR4   | Mean  |
|---------------|----------|-------|-------|-------|-------|-------|
| <i>atf-5i</i> | GFP      | 129   | 116   | 177   | 87.4  |       |
|               | TOF      | 1549  | 1080  | 1094  | 898   |       |
|               | GFP/TOF* | 88.4% | 82.5% | 79.6% | 68.7% | 79.8% |
| luci          | GFP      | 152   | 133   | 236   | 126   |       |
|               | TOF      | 1613  | 1021  | 1161  | 890   |       |
|               | GFP/TOF* | 100%  | 100%  | 100%  | 100%  | 100%  |

**Table 6:** Worm Sorter Data of *hsp-4::gfp;glp-1* treated with *atf-5i* or control RNAi (luc).  
\* GFP/TOF-ratio is normalized to results of luc RNAi for each biological replicate.

|               |          | BR1    | BR2     | BR3     | BR4     | Mean    |
|---------------|----------|--------|---------|---------|---------|---------|
| <i>atf-5i</i> | GFP      | 58.1   | 225     | 207     | 259     |         |
|               | TOF      | 1117   | 1335    | 1363    | 1137    |         |
|               | GFP/TOF* | 47.1 % | 100.4 % | 119.5 % | 157.9 % | 106.2 % |
| luci          | GFP      | 127    | 201     | 184     | 150     |         |
|               | TOF      | 1149   | 1197    | 1448    | 1040    |         |
|               | GFP/TOF* | 100%   | 100%    | 100%    | 100%    | 100%    |

**Table 7:** Worm Sorter Data of *hsp-4::gfp* treated with *atf-5i* or control RNAi (luc). \* GFP/TOF-ratio is normalized to results of luc RNAi for each biological replicate.

### 7.1.8.2 *Patf-5::GFP* transgene

|                                  |          | BR1    | BR2    | BR3     | Mean    | p-value |
|----------------------------------|----------|--------|--------|---------|---------|---------|
| <i>skn-1</i>                     | GFP      | 109    | 157    | 110     |         |         |
|                                  | TOF      | 965    | 888    | 697     |         |         |
|                                  | GFP/TOF* | 99.7 % | 88.4 % | 119.6 % | 102.6 % | 0.8041  |
| <i>pek-1i</i> +<br><i>gcn-2i</i> | GFP      | 91.3   | 220    | 96      |         |         |
|                                  | TOF      | 884    | 1140   | 681     |         |         |
|                                  | GFP/TOF* | 91.2 % | 96.5 % | 107 %   | 98.2 %  | 0.7286  |
| luc                              | GFP      | 129    | 224    | 109     |         |         |
|                                  | TOF      | 1139   | 1120   | 826     |         |         |
|                                  | GFP/TOF* | 100%   | 100%   | 100%    | 100%    |         |

**Table 8:** Worm Sorter Data of *Patf-5::gfp;glp-1(e2141ts)* treated with RNAi against *hsf-1*, *ife-2*, *skn-1* and *pek-1+gcn-2* or control RNAi (luc). \* GFP/TOF-ratio is normalized to results of luc RNAi for each biological replicate. p-Value is calculated by paired *t*-test and compares each RNAi condition to the control RNAi (luc).

## 8 Appendix

### 8.1.1 Acknowledgment

I want to thank Prof. Dr. Adam Antebi for being my PhD supervisor and for giving me the opportunity to be part of his department for more than three amazing years. I also want to thank Prof. Dr. Aleksandra Trifunovic, Prof. Dr. Jan Riemer and Dr. Orsolya Symmons for taking over responsibilities in my thesis committee. Moreover, I thank Dr. Orsolya Symmons and Dr. Martin Denzel for feedback and constructive reviewing during my studies.

Above that, a big THANK YOU goes out to my colleagues at the A-Team. I enjoyed the time we could do great science and great fun activities together. I also want to mention the great support from all members of the Max-Planck-Institute for Biology of Ageing.

Für die jahrelange Unterstützung, das unermüdliche Motivieren und viele aufmunternde Worte möchte ich meiner gesamten Familie und meinen Freunden danken. Ganz besonders ohne meine wunderbare Frau Michaela, die mir zu jeder Zeit zur Seite steht, wäre ich nicht so weit gekommen. Besonderer Dank gilt auch meinen Eltern, die mit steter und konstanter Motivation geholfen haben. Ich danke auch denjenigen, die nicht mehr unter uns weilen um diesen Moment mit mir zu teilen, aber zu ihren Lebzeiten stets für mich da waren. Danke Oma, danke Opa.

### 8.1.2 Work Contributions

Work Contributions are marked in figure legends and described in text.

Özlem Karalay and Shuhei Nakamura carried out the large RNAi suppressor screen for mediators of *glp-1* longevity (Figure 5). The lifespan analysis for *nhr-91* RNAi on *glp-1* worms (Figure 6) was also carried out by Özlem Karalay and Shuhei Nakamura.

Torsten Bücher did the data analysis and data representation for the Venn-Diagram (Figure 22) of RNAseq results for differentially expressed genes upon loss of *atf-5* in *glp-1* worms. I also used data from Torstens Bachelor Thesis in Chapter 3.5.4 (*Phsp-4::GFP* reporter strain, ER stress assay).

### 8.1.3 Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit – einschließlich Tabellen, Karten und Abbildungen –, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie – abgesehen von unten angegebenen Teilpublikationen – noch nicht veröffentlicht worden ist, sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde.

Die Bestimmungen der Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. Adam Antebi betreut worden.

A handwritten signature in black ink, appearing to read 'P. Jäger', with a long horizontal stroke extending to the right.

Philipp Sebastian Jäger

Bonn, 2021

## 8.1.4 Curriculum Vitae

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**Laborleiter (2017-2019)**  
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#### **Akademische Laufbahn**

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**Doktorarbeit Molekulare Genetik (2013-2017)**  
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Prof. Adam Antebi – Molecular Genetics of Ageing

**Life and Medical Science (M.Sc.) (2011-2013)**  
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**Biologie (B.Sc.) (2008-2011)**  
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**Abitur (1998-2007)**  
Gymnasium am Ölberg Oberpleis

## **Kompetenzen**

---

### **Lebenswissenschaften**

Molekularbiologie, Biochemie und Mikroskopie

### **Analytische Chemie**

Elementanalytik (ICP/MS)

Quecksilberanalytik (Kaldampf AAS)

Ionenchromatografie

Photometrische Bestimmungen

### **Management**

Mitarbeiterführung

Projektleitung

Lean Management

### **Sprachen**

Deutsch (Muttersprache)

Englisch (fließend)

Spanisch (Grundkenntnisse)

### **Workshops und Fortbildungen**

Wissenschaftliches Schreiben

Projektmanagement

Strategisches Management

### **Software**

Microsoft Office (Word, Excel, Powerpoint)

Microsoft Project

GraphPad Prism

## Wissenschaftliche Publikationen

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Nakamura S\*, Karalay Ö\*, Jäger PS, Horikawa M, Klein C, Nakamura K, Latza C, Templer SE, Dieterich C, Antebi A. (2016). Mondo complexes regulate TFEB via TOR inhibition to promote longevity in response to gonadal signals. Nature Communications 7, 10944.

Gosejacob D, Jäger PS, Vom Dorp K, Frejno M, Carstensen AC, Köhnke M, Degen J, Dörmann P, Hoch M. Ceramide Synthase 5 Is Essential to Maintain C16:0-Ceramide Pools and Contributes to the Development of Diet-induced Obesity. (2016) Journal of Biological Chemistry 291, 6989-7003.



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