Identification of novel lipid signalling mechanisms

Marshall Plan Scholarship Paper

Joslin Diabetes Center

University of Natural Resources and Life Sciences Vienna

by Magdalena Teufl

Table of contents

1. Introduction

1.1. Caenorhabditis elegans

Caenorhabditis elegans (*C. elegans*) is a nematode, commonly used as a genetic model for understanding and answering questions connected to most areas of modern biology. *C. elegans* was proposed as a model organism by Sydney Brenner in 1963, who stated that the future of molecular research would need an extension to other fields, "notably development and the nervous system" (Brenner, 1988; Corsi et al., 2015). *C. elegans* is a tiny round-worm, which can be found on rotting vegetable matter. In the laboratory *C. elegans* is cultivated on agar plates and feeds on a bacterial lawn, most commonly an Escherichia coli strain called OP50. Due to its short life cycle of about 3 week (See Figure 1), its easy cultivation in big numbers and resulting statistical value of results as well as its simple and safe handling in the laboratory, *C. elegans* was established as a valuable model for aging research (Corsi et al., 2015). Additionally, about 60-80% of human genes have an orthologue in the *C. elegans* genome (Kaletta and Hengartner, 2006; Corsi et al., 2015), making findings in the nematode applicable and valuable to human diseases. *C. elegans* primarily exist as self-fertilizing hermaphrodites, only a small number of the population are males (Corsi et al., 2015). Because *C. elegans* is transparent, individual cells as well as organisms can be visualized using Differential Interference Contrast (DIC) microscopy and cell fate can be traced (Corsi et al., 2015).

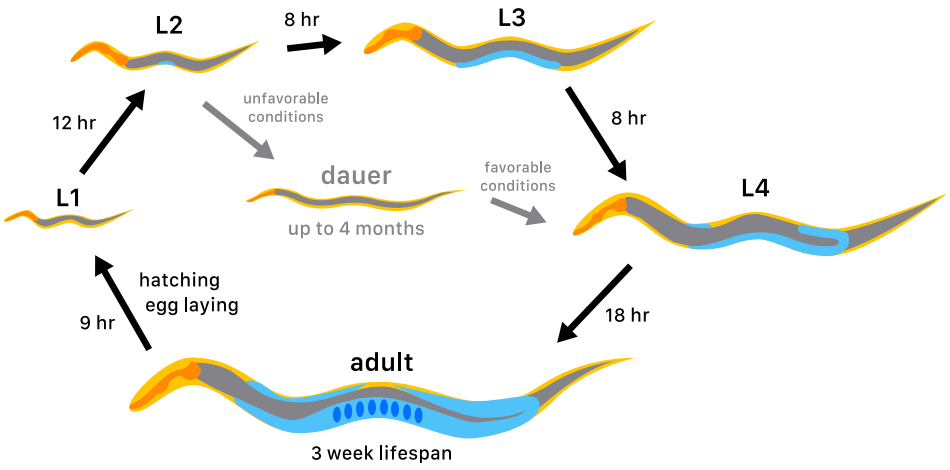

Figure 1: The C. elegans (hermaphrodite) life-cycle is shown in stages; in the laboratory hermaphrodites are used in research. The pharynx (organ for feeding) is shown in orange, the intestine is coloured grey, the gonad is coloured light blue and the embryos are shown in dark blue. Blackwell et al., Free Radic Biol Med 2015

1.2. Aging

"Aging is characterized by a progressive loss of physiological integrity, leading to impaired function and increased vulnerability to death" – this is one feasible definition of the term "aging", proposed by López-Otín in 2013.

The intense research on aging was started in 1993 by Cynthia Kenyon's paper on the *C. elegans* daf-2 (abnormal DAuer Formation) mutant, which shows a lifespan twice as long compared to the wild type (Kenyon et al., 1993). Providing proof that longevity as well as lifespan extension would also occur in lower organisms opened up new research opportunities. Since then *C. elegans*, *Drosophila melanogaster* and mice were used as model organisms in aging research to unravel the underlying molecular principles of the process considered as "aging".

In 2010 Cynthia Kenyon stated that "the aging process, like so many other biological processes, is subject to regulation by classical signaling pathways and transcription factors" and postulated seven major pathways that regulate aging: Insulin/IGF-1 signalling (IIS), TOR signaling, AMP Kinase, Sirtuins, inhibition of respiration, signals from the reproductive system and telomeres (Kenyon et al., 2010). Lifespan extension has been closely linked to stress response, which, in case of unfavorable physiological or environmental conditions, leads to physiological shift towards cell protection and maintenance (Kenyon et al., 2010).

1.3. SKiNhead-1 (SKN-1)

The *C. elegans* transcription factor SKN-1 is known to control the response to oxidative and xenobiotic stress (An and Blackwell, 2003). The following information on SKN-1 was discussed in Blackwell's review "SKN-1/Nrf, stress responses, and aging in *Caenorhabditis elegans*" (2015).

SKN-1 is orthologous to the mammalian Nrf/CNC proteins, which include Nrf1, Nrf2, Nrf3 and p45 NF-E2. Nrf2 is known as a regulator of antioxidant and xenobiotic defense, but was also shown to be involved in maintenance functions and metabolic regulation. SKN-1 on the other hand was shown to play a role in mediating longevity and stress sensitivity. Although SKN-1 and Nrf show differences in structure as well as their mode of DNA binding "the degree of functional conservation between SKN-1 and these proteins is remarkable". (Blackwell et al., 2015)

SKN-1 does not only play a role post-developmentally, but was initially discovered because of its role during embryogenesis, when it is required for tissue specification during the earliest stages of embryonic development. Its post-developmental role and activity in certain tissue is mostly investigated using translational fusion to green fluorescent protein (GFP) or any other tag. It was shown that SKN-1 location in the nematode changes with development- "zygotically expressed" SKN-1 is present in the nuclei in precursors of the intestine, while it is localized in the cytoplasm during larval and adult stages and will migrate to the nuclei in response to stress.
The SKN-1 gene has 4 predicted isoforms, 3 of which were shown to be expressed in vivo. SKN-1a has a N-terminal transmembrane segment and was shown to be localized in the intracellular plasma membranes and might therefore be the orthologue to Nrf1. Loss of Nrf1 in mice results in embryonic lethality. SKN-1c might correspond to Nrf2.

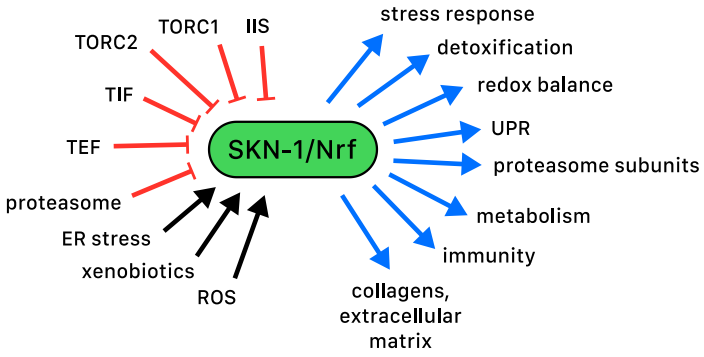

Figure 2: The complexity of SKN-1 functions is shown here. SKN-1 is activated (black arrows), repressed (red errors) by different mechanisms and provokes a wide range of effects (blue arrows). Blackwell et al., Free Radic Biol Med 2015

SKN-1 can be activated by ER stress, xenobiotics and ROS (black arrows) and supressed by several cellular processes (red bars), of which IIS is the best investigated signalling pathway (See Figure 2). "New" SKN-1 functions were described, such as the expression of C-type lectins in response to SKN-1, which localize to the intestinal cell surface and might have antimicrobial properties (Blackwell et al., 2015).

Apart from SKN-1 functions in stress response and proteostasis there is evidence that SKN-1 plays a role in lifespan extension and might be a major modulator of aging. This could be shown by lifespan extension through modest SKN-1 overexpression, SKN-1 knockdown on the other hand was shown to shorten the lifespan significantly.
Many mechanisms that promote *C. elegans* longevity were shown to require SKN-1 activity such as IIS, mTOR siganling, dietary restriction (DR) and germ cell loss. (Blackwell et al., 2015)

1.4. Longevity through germ cell loss

A lifespan extension through germ stem cells (GSC) loss seems to be evolutionary conserved (Blackwell et al., 2015) and can be observed in several species (Flatt et al., 2008; Hamilton and Mestler, 1969; Min et al., 2012). In 1999 Hsin and Kenyon discovered that laser ablation of cells, which give rise to the germ line, trigger lifespan extension in *C. elegans*. Hsin and Kenyon suggested that germline signals would act "by modulating the activity of an insulin/IGF-1 pathway" and would additionally require a putative nuclear hormone receptor, daf-12. It was thought that GSC loss extended lifespan through a mechanism (parallel to daf-2 signalling) through daf-16; removal of the germline of *daf-2* mutants was shown to further double lifespan (Hsin and Kenyon, 1999).
Investigating this matter the *glp-1* (abnormal Germ Line Proliferation) strain has served and still serves as a genetic model in which GSC proliferation is inhibited and mature germ cells are not formed at the non-permissive temperature (25°C) (Arantes-Oliveira et al., 2002; Steinbaugh et al., 2015).

Soon it was proposed that sterility per se does not lengthen lifespan but counterbalancing signals from the somatic gonad and the germ cells do (Arantes-Oliveira et. al, 2002, Hsin and Kenyon, 1999). Additional to daf-16 and daf-12, daf-9 was proposed to be required in lifespan extension through germ cell loss (Berman and Kenyon, 2006).
In 2006 Berman and Kenyon proposed the regulation of daf-16 through kri-1, an Ankyrin-repeat protein, to be required for nuclear localization in animals lacking GSC (GSC(-) animals). Further, lipophilic-hormone signalling involved in transmission of information from the reproductive system to the intestine was postulated (Berman and Kenyon, 2006).
The intestine as a site of action became important when it was shown that daf-16, present throughout the animal, enters intestinal nuclei in GSC(-) animals (Lin et al., 2001; Berman and Kenyon, 2006). This intestinal localization was believed to be important since GSC(-) animals, that expressed daf-16 solely in the intestine lived as long as GSC(-) animals that carried wt daf-16 (Libina et al., 2003; Berman and Kenyon, 2006). For these reasons, the intestine (which serves as adipose tissue in the worm) was postulated to be a "central site of action for the interpretation and execution of information coming from the reproductive system" (Berman and Kenyon, 2006).

A study by Wang et al. linked longevity through germ cell loss to fat metabolism in C. elegans. It was shown that germ cell loss could activate a specific fat lipase (K04A8.5 / LIPL-4) which in turn upregulates lipid hydrolysis. Lifespan extension could therefore be achieved by fat mobilization (Wang et al., 2008). It was further shown that, opposite to different expectations, germ line-ablated animals stored 50% less fat compared to wild type (N2) animals (Wang et al., 2008), which turned out to be caused by the difference of vital versus fixed worms. A major increase in fat content (297 ± 44 % compared to wt) in

glp-1 animals was shown by O'Rourke et al. (2009). Their conclusion was that longevity through GSC loss and subsequent changes in lipid hydrolysis would be promoted through activation of kri-1/daf-16 signalling but independent of daf-12 lipophilic hormone signalling (Wang et al., 2008).

Further, autophagy (Lapierre et al., 2011) and mir-71 acting in neurons (Boulias et al., 2012) were shown to modulate lifespan in germline-less C. elegans.

Several pieces of evidence on the other hand suggested that NHR signalling and fatty acid desaturation would play a role in germline-mediated longevity. Ratnappan et al. suggested that NHR-49 would mediate the response to GSC loss by increasing the expression of genes involved in mitochondrial ß-oxidation and fatty-acid desaturation. The postulated mechanism proposes that NHR-49 is upregulated as a consequence of GSC loss in a daf-16 and tcer-1 dependent manner and subsequent enhancement of fatty acid oxidation is activated for cellular maintenance (Ratnappan et al., 2014).
Another nuclear hormone receptor, which was associated with longevity is NHR-80, which was reported to be specifically required for lifespan extension inducing depletion of the germline "through a mechanism that implicates fatty acid monodesaturation" (Goudeau et al., 2011).
Further, under dietary restriction, an enrichment of ω-6 polyunsaturated fatty acids (PUFAs) was observed and postulated to promote starvation resistance and extend lifespan in *C. elegans* (O'Rourke et al., 2013). Supplementation of *C. elegans* media with these fatty acids was shown to increase lifespan even under conditions of food abundance and explained this finding through the activation of autophagic programs (O'Rourke et al., 2013).

More evidence for the impact of fatty acids on lifespan and aging was presented in Schroeder's and Brunet's review "lipid profiles and signals for long life" (2015), see Figure 3. Reportedly lipid profiles of long-lived individuals contained a higher ratio of monounsaturated fatty acid (MUFA) to polyunsaturated fatty acids (PUFA) (Gonzalez-Covarrubias et al., 2013). This finding was explained with the fact that an excess of MUFAs would reduce oxidative stress, as PUFAs were more susceptible to oxidative stress and would increase oxidative damage (Gonzalez-Covarrubias et al., 2013; Shmookler Reis et al., 2011). Further, worms with an increased lifespan (via reduced IIS, DR or germline ablation) were reported to express higher levels of enzymes that convert saturated FAs to MUFAs (D9 desaturases) (Hansen et al., 2013; Shmookler Reis et al., 2011). These results are consistent with O'Rourke's findings that worms with an increased expression of lipases (e.g. LIPL-4) can liberate FAs from complex lipid molecules and therefore produce high levels of of ω-6 polyunsaturated fatty acids (O'Rourke et al., 2013) but also many other FAs (Folick et al., 2015), which might be important for longevity.

The search for lipid signalling molecules so far has described several molecules such as dafachronic acid, a steroid, which was shown to promote longevity in GSC(-) worms (Hansen et al., 2013). Metabolomic analysis of LIPL-4 overexpressing worms on the other hand identified high levels of oleoylethanolamide (OEA) which can interact with NHR-49 and NHR-80 (See Figure 3) to promote longevity (Folick et al., 2015). The mammalian orthologues of NHR-80 and NHR-49 are hepatocyte nuclear factor 4 (HNF4) and peroxisome proliferator-activated receptor alpha (PPAR) which are known to transcriptionally regulate lipid metabolism, inflammation and cell death in mammals (Liu et al., 2014; Zechner et al., 2010).

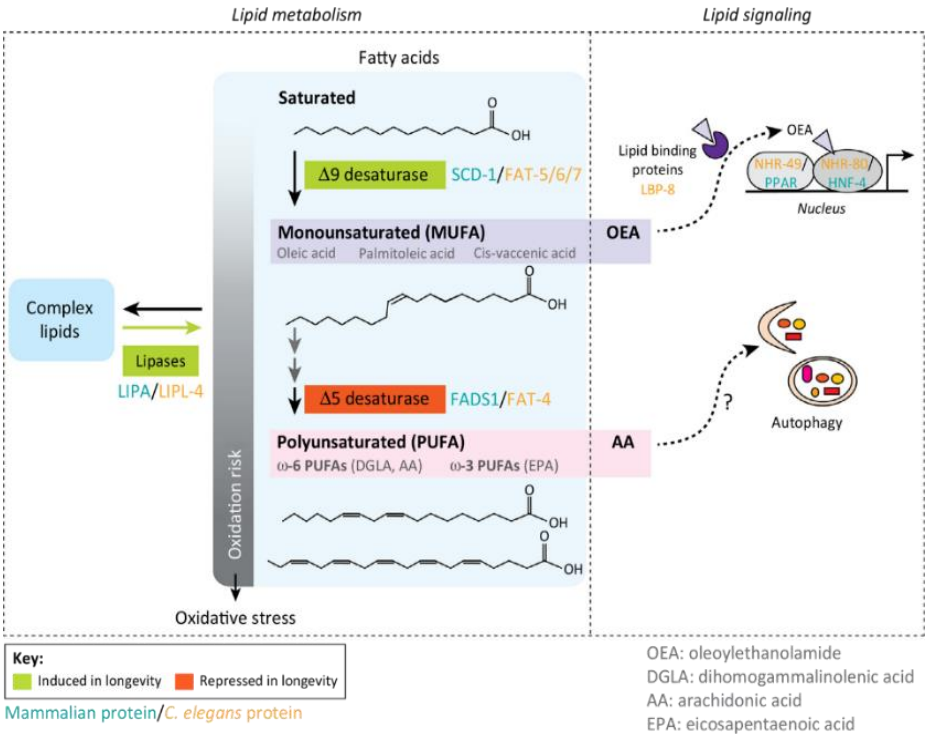

Figure 3: The risk of oxidation increases with the desaturation of FA. MUFAs, which are processed from saturated FAs by Δ9 desaturases gives rise to OEA, which was shown to activate key metabolic regulators. PUFAs were shown to activate autophagy through unknown mechanisms. Schroeder and Brunet, Trends Endocrinol Metab 2015

Recently, Michael Steinbaugh et al. have dealt with the question of how GSC loss influences the transcription factor SKN-1. He investigated the role SKN-1 plays in the context of GSC loss and has shown that GSC loss leads to a broad transcriptional reprogramming that also includes genes involved in lipid metabolism. In this context, the conserved transcription factor SKN-1 seems to activate lipid metabolism genes and reduce fat storage, therefore reducing the overall amount of fat and subsequent lipid stress. The finding that SKN-1 reduces fat storage opposes the idea that accumulation of beneficial fats on its own would promote *glp-1* longevity. SKN-1 knockdown with RNAi prevents *glp-1* longevity and reinforces the finding that SKN-1 works in a daf-16 independent mechanism (Steinbaugh et al., 2015). Analysis of RNA sequencing (RNA-seq) data of GSC(-) animals for 12,595 expressed genes revealed fivefold upregulation of 615 genes including genes involved in detoxification, immunity and metabolism, particularly FA oxidation and other lipid metabolism processes compared to GSC(+) wt animals (Steinbaugh et al., 2015). Many of those genes were found to be direct SKN-1 targets. One of these genes identified was the lipase LIPL-3, which was shown to extend C.elegans lifespan when overexpressed (O'Rourke and Ruvkun, 2013). The hypothesis postulated in this study states that the excessive fat in GSC(-) animals derives from unused yolk, which was destined for the oocytes (See Figure 4). Yolk particles which are made of lipids and proteins are produced in the intestinal cells and taken up by the developing egg cell (Grant and Hirsh, 1999). Their findings suggest, that SKN-1, which mediates lipid homeostasis, is itself activated through lipid accumulation (unused yolk) possibly through fatty acid (FA)-based signalling (Steinbaugh et al., 2015). The data provides evidence that those excessive lipid levels in the worm lead to the production of OA- and LIPL-1/3 dependent FAs which activate SKN-1 (See Figure 4). The possibility that the gonad sends additional signals was not ruled out though (Lemieux et. al, 2015).

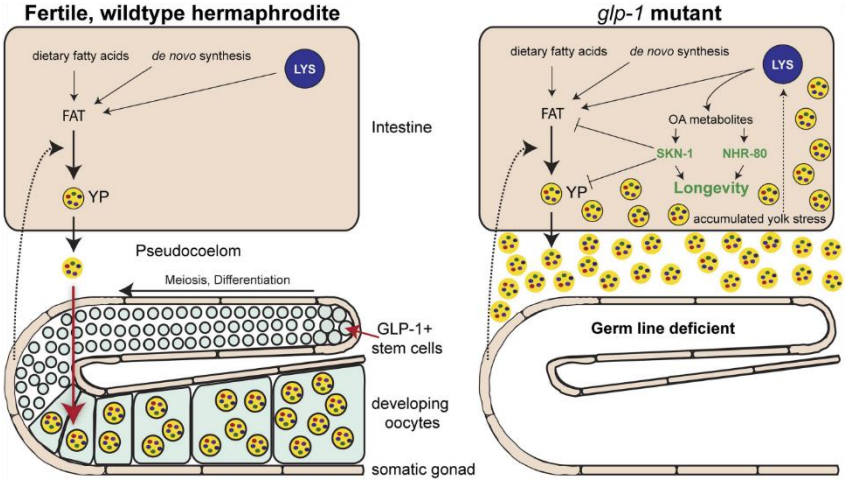

Figure 4: This presents a model of how glp-1 mutants acquire an increased fat content. On the left the wild-type (wt) hermaphrodite is shown: fat, which is used for yolk particles, can derive from several sources such as de novo synthesis, dietary FAs or the lysosome. This fat is processed into yolk particles (YP) which are taken up by developing oocytes in the somatic gonad. In the glp-1 mutant the production of YP does not stop and is proposed to be regulated by signals from the somatic gonad (dotted arrow); the YP accumulate in the body and trigger a stress response; excessive fat is processed in the lysosomes (involving lysosomal lipases, such as LIPL-3) and produces metabolites of OA, which are thought to act through SKN-1 and NHR-80 to promote longevity. SKN-1 on the other hand reduces fat storage and alleviates lipid stress. Lemieux and Ashrafi, eLife 2015

Supporting the idea of SKN-1's role in lipid homeostasis are findings in mice that have shown that mice which lack Nrf1 (a predicted SKN-1 orthologue) in the liver develop non-alcoholic fatty liver disease (NAFLD) which progresses to non-alcohohol steatohepatitis (NASH) and $Nrf2^{-/-}$ mice which develop NASH on a high fat diet (Xu et al., 2005; Okada et al., 2013; Tsujita et al., 2014). Impaired Nrf function in mammals is therefore thought to predispose to NASH by impairing hepatic stress resistance (Xu et al., 2005; Lee et al., 2013).

2. Aim

The aim of this study is to identify key players in the pathway activating SKN-1 as a response to lipid stress by high throughput RNAi screening.
As a genetic model *glp-1;gst-4p::GFP* is used which uses gst-4 as a SKN-1 target gene and GFP as a fluorescent reporter in response to SKN-1 activation. The *glp-1* strain shows a highly fluorescent phenotype, probably due to excessive fat. Knockdown of individual genes, which are required for SKN-1 activation, will show a significant reduction of GFP fluorescence and therefore help to identify genes required for SKN-1 activation through lipid stress.

Further this study shall try to reveal components involved in fatty acid (FA) desaturation and elongation, which might give rise to signalling molecules, by RNAi knockdown and subsequent rescue experiments with fatty acids.

Last, this study aimed at quantifying and verifying the effect of SKN-1 activation in *gst-4p::GFP* and skn-1::GFP through a stabilized form of oleic acid called glyceryl trioleate.

3. Materials and methods

3.1. Strain maintenance

For the experiments 3 strains were used:

- *gst-4p::GFP*
- *glp-1(bn18ts);gst-4p::GFP* (line n#5)
- *Skn-1::GFP*

The worms were grown on nematode growth medium (NGM) plates with a bacterial lawn of OP50 at 20°C. The worms were transferred every 2-3 days onto fresh NGM plates using standard techniques (Brenner, 1974).

The *glp-1(ts);gst-4p::GFP* (line n#5) was created by Lorenza Moronetti via UV integration of an extrachromosomal array of *gst-4p::GFP* (*gst-4p::GFP* ex line [CL1166] provided by Christopher Link), further crossed into the *glp-1* strain(bn18ts) and outcrossed three times.
Gst-4p::GFP is used as a readout for SKN-1 because imaging of SKN-1::GFP requires slidemounting; slidemounting is not feasible as a method in a high-throughput screen.

3.2. Egg preparation

The worms were grown until many gravid adult worms and few eggs could be seen on the plate. The worms were washed off a plate using M9 buffer ("medium 9", the ninth version of buffer used for transferring and suspending *C. elegans*) and disposable transfer pipets. The solution was collected in 15 ml tubes and spun down at 600 rcf for 1 min. The supernatant was removed with the use of sterile pipet tips and a vacuum. 5 ml bleach prep (1 ml 1M NaOH, 4 ml bleach, 9 ml sterile dH_2O) was added to the worm pellet for a period of 5-7 minutes, depending on the pellet size. During this period the solution was vortexed rigorously. As soon as the bodies started to dissolve (this can be observed under the microscope), 5 ml M9 buffer were added to the tube and the tube was centrifuged for 30 sec at 600 rcf. The worms were aspirated to 0.5 ml and fresh 10 ml M9 buffer were added. This washing step was repeated 3 more times. After the last wash the pellet of released eggs was transferred to a new 15 ml tube and 6 ml M9 and 6 µl cholesterol (100%) were added.

The eggs were either seeded onto NGM plates or stored on the rotator at 20°C (25°C) for an L1 arrest. By the following day L1 had hatched and were synchronized in their development.

3.3. DNA preparation

RNAi clones were sequenced to ensure the clone identity. The DNA preparation was performed using the kit SV Minipreps (PROMEGA).

3.4. Preparation of RNAi glycerol stocks

To prepare glycerol stocks from individual clones as well as duplicates of 96-well plates, bacteria were grown in Lysogeny Broth (LB) containing 12.5 µg/ml tetracycline (Tet) and 50 µg/ml carbenicillin (Carb) overnight, then mixed with 50% glycerol (1:1).

The 96-well plates were sealed with Inset Cut Sealing foil; glycerol stocks of individual clones were frozen in cryogenic vials at 1 ml volume.

3.5. Freezing worms

Worms were grown on a large (10 cm) plate of NGM and OP50 until the worms had starved and many L1 were found on the plate. Alternatively the worms were bleached and the bleached eggs were seeded on a NGM plate lacking the bacterial lawn. The worms were washed off with M9 into a 15 ml tube and spun down at 600 rcf with very low deceleration. The medium was removed, then fresh M9 and freezing media (ratio 1:1) was added to the pellet. 1 ml of this suspension was added to each vial and frozen immediately (in a Styrofoam box).

3.6. Slidemounting

A 2% agarose solution was prepared. 3 glass slides were arranged on a cool bench top, the outer 2 of which were coated with 2 stripes of tape. 40 µl of agarose solution were put onto the glass slide while a fourth glass slide was arranged across to create a flat surface of agarose.

The worms were washed off the plate with M9 and washed several times until the supernatant appeared clear; then they were transferred into an Eppendorf tube. 100 µl of 1x Tetramisol was added to the worms. After 1 min incubation time the worms were examined under the microscope to evaluate the progress of paralysis. The incubation time should not exceed too many minutes since this could provoke unspecific SKN-1 activation. 5 µl of the worm "pellet" were seeded onto the agarose pad and imaged with ZEN software 2012 on an Axio Imager.M2 microscope (Zeiss).

3.7. Supplementation of fatty acids (FA)

For this purpose oleic acid (Cayman Chemical Company, 10 g in 20 ml EtOH), linoleic acid (Cayman Chemical Company, 500 mg in 1 ml EtOH) and glyceryl trioleate (SIGMA, 10 g, >>99%) were used.

Fats were either mixed in with RNAi or OP50 bacteria (to obtain a certain fat:food ratio) or added to the liquid agar to reach a certain molar concentration in the NGM prior to pouring the plates.

Both approaches showed big variability in results.

3.8. High-throughput RNAi screening

3.8.1. Layout of the screen

The layout of the screen proposed at least 2 rounds of screening; in the first pass the whole genome will be screened in 96-well plates in duplicate. From the list of potential hits a "hit library" should be created, which might hold about 500-700 clones (depending on the stringency of cut-off). This library should be rescreened in 24-well plates to ensure higher quality and confidence. SKN-1 and the empty vector (EV) PL4440 were used as positive/negative control, respectively.

3.8.2. RNAi library

Currently 2 *C. elegans* RNAi libraries are available. The one used in this screen was originally prepared by the Vidal Laboratory and is distributed by dharmacon. Together with the library, GElifesciences offers a map of the whole library, which covers over 11.000 RNAi clones. Wells, which are empty or hold a wrong sequence are highlighted. The open reading frames (ORFs) have been cloned into the feeding vector PL4440 and transformed into the RNAi feeding bacterial strain HT115(DE3). The host carries a tetracycline marker, while the plasmid confers ampicillin/carbenicillin resistance.

3.8.3. Preparation of RNAi plates (96-well)

500 ml NGM
800 µl Cholesterol (100%)
500 µl Calcium Chloride ($CaCl_2$), 1M
500 µl Magnesium Sulfate ($MgSO_4$), 1M
12.5 ml Phosphate buffer (pH=6.0)
500 µl Carb (50 mg/ml)
500 µl Tet (12.5 mg/ml)
200 µl Isopropyl β-D-1-thiogalactopyranoside (IPTG), 1 M

The medium for RNAi plates was prepared according to the protocol above, for this purpose the NGM was kept at 55°C in the water bath. A multichanncel pipette (Finnpipette ®, ThermoLabsystems) was used to pipette 140 µl RNAi medium per well. The plates were kept in the fume hood until they were dry. For both positive and negative control 6 cm plates of the same medium were prepared. RNAi plates, stored in the cold room, could be used for up to 4 weeks after preparation.

3.8.4. Stamping out the libraries

OmniTrays™ (ThermoScientific) with LB agar (12.5 µg/ml Tet, 50 µg/ml Carb) were prepared. RNAi libraries were kept on dry ice to prevent the plates from thawing. A "hedgehog" (BOEKEL replicator) was sterilized using a series of 1 bath chlorine, 2 baths water and 1 bath ethanol, followed by subsequent inflammation. This sterilization process was carried out twice. The tips of the replicator were pressed softly on the glycerol stock and then stamped out an LB agar plate. The glycerol stock was quickly covered with a new sealing foil and transferred to the -80°C freezer.

The stamp outs were grown at 37°C overnight. The following day up to 96 individual colonies were visible on the plate; the plates were sealed with laboratory film and stored in the cold room.

3.8.5. Preparation of RNAi bacteria

Deep-well 96-well plates (Eppendorf) containing 1000 µl LB (12.5 µg/ml Tet, 50 µg/ml Carb) were inoculated from the stamp outs using the hedgehog and same sterilization procedure as stated above. The bacteria were grown overnight at 220 rpm and 37°C, closed off with airpore tape sheets.

The following day, the bacterial culture was diluted 1:1 (LB + 12.5 µg/ml Tet, 50 µg/ml Carb) and grown for another 2 hours to allow re-entry into the logarithmic growth phase. The bacteria were spun down using an Eppendorf Centrifuge 5810R at speed 3600 rcf, 20°C for 10 minutes (acceleration/deceleration = 6). The supernatant was poured off and the pellet was re-suspended in 30 µl LB (12.5 µg/ml Tet, 50 µg/ml Carb) and induced with 1 M IPTG prior to seeding. 12.5 µl of the induced bacterial culture was seeded onto 96-well RNAi plates using a multichannel pipette. The plates were dried in the fume hood until the bacterial lawn appeared dry.

For the preparation of bacterial RNAi culture of individual clones 5 ml LB (12.5 µg/ml Tet, 50 µg/ml Carb) were inoculated with a single colony from a streaked agar plate. The culture was grown overnight and diluted with 20 ml LB (12.5 µg/ml Tet, 50 µg/ml Carb) in the morning. After 6 hours of incubation the culture was spun down at 3900 rcf for 10 minutes. The supernatant was poured off and the pellet was re-suspended in 5 ml LB (12.5 µg/ml Tet, 50 µg/ml Carb) and induced with 1M IPTG, then vortexed rigorously until the pellet was dissolved. 300 µl were seeded onto 6 cm RNAi plates. The plates were dried in the fume hood until the bacterial lawn appeared dry.

3.8.6. Preparation and seeding of worms

Worms were grown on several 15 cm plates and bleached at day 1 of adulthood. The L1 arrest was incubated at 2 different temperatures (one tube was incubated at 20°C for strain maintenance, while the other tube incubated at 25°C was used for the screening).

After 1 or 2 days of incubation 10 µl (25-30 L1) were seeded onto 96-well RNAi plates already spotted with bacteria. The plates were dried until the liquid had evaporated, then the plates were incubated at 25°C in plastic boxes with a piece of wet cloth to prevent the plates from drying.

3.8.7. Scoring

The plates were incubated at 25°C for 72 hours. At 72 hours the worms were scored using a Stemi SV6 and X-Cite Series 120Q UV light. The worms were scored (according to their fluorescence) from -3 to +1. 0 indicated "no change in fluorescence", while -1 to -3 indicated a reduction in fluorescence and +1 depicted an increase in GFP fluorescence.
As additional notes "Larval arrest", "vulval defects", "sick" etc. were marked down. Wells which held less than 10 worms, were contaminated or showed no bacterial growth, were censored.

3.8.8. Analysis

The data analysis was performed in R (code written by Michael Steinbaugh) creating dataframes that could be used for gene ontology (GO) Enrichment analysis using Panther software.

Data generated in this screen was organized in an excel sheet stating ORFeomeID, score1, score2, bacterial growth and additional notes.
This information was transformed into a dataframe in R. Additional information from several sources (e.g. Panther, wormbase, Ensembl, Uniprot, InterPro) were combined in R to create a master library. The addition of supplementary information (e.g. RNAi phenotype, description, gene name, Homo sapiens homolog, Mus musculus homologs, etc.) to the dataframe enabled gaining a quick overview of potential hits or searching for key words.

The following steps of data analysis were performed in R to ensure higher reproducibility. The RNAi library holds 140 plates in total, which account for 13440 wells, only 11559 of which hold valid RNAi clones. In several consecutive steps the master file was reduced to clones, which held a reliable result.

1. Clones which showed no bacterial growth were filtered
2. Clones which were scored "L" (larva) in one or both set were filtered
3. Clones which were censored (NA) in both sets of plates were filtered
4. Clones which did not have a valid ORF according to "Cernai Feeding Library - Plate Maps" provided by dharmacon were filtered

Next, the average mean and sum of the duplicates (triplicates) were calculated.
Depending on the stringency of the test a cut-off of -2 or -3 "average mean" was used to obtain a list of "strong" or "very strong" hits. Further, a list of clones with a mean of +1 was generated to evaluate potential up-regulators of the lipid-induced stress response.

Gene ontology was performed using PANTHER. The list of potential hits was run against the list of valid clones ("background") screened so far.

4. Results

4.1. Development of a new *glp-1(bn18ts);gst-4p::GFP* line

The new *glp-1(bn18ts);gst-4p::GFP* line showed a highly fluorescent phenotype grown at the non-permissive temperature (25°C). These pictures were taken at hour 72 when a bright green fluorescent phenotype had established (See Figure 5A). SKN-1 RNAi completely knocked down GFP fluorescence (See Figure 5B).

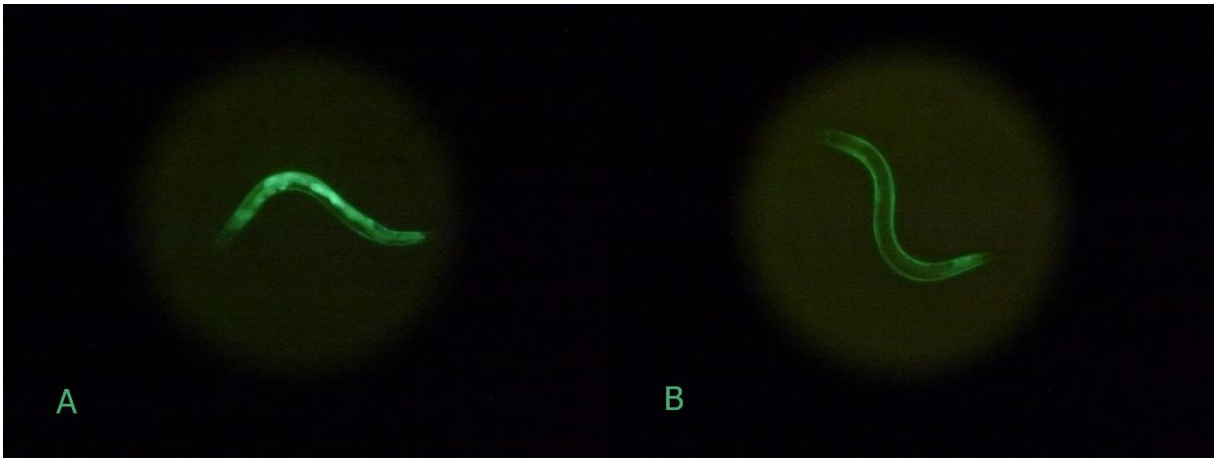

Figure 5: glp-1(bn18ts);gst-4p::GFP (line n#5) grown at 25°C at 72h (A: EV; B: SKN-1)

4.2. Supplementation of *gst-4p::GFP* with oleic acid and glyceryl trioleate

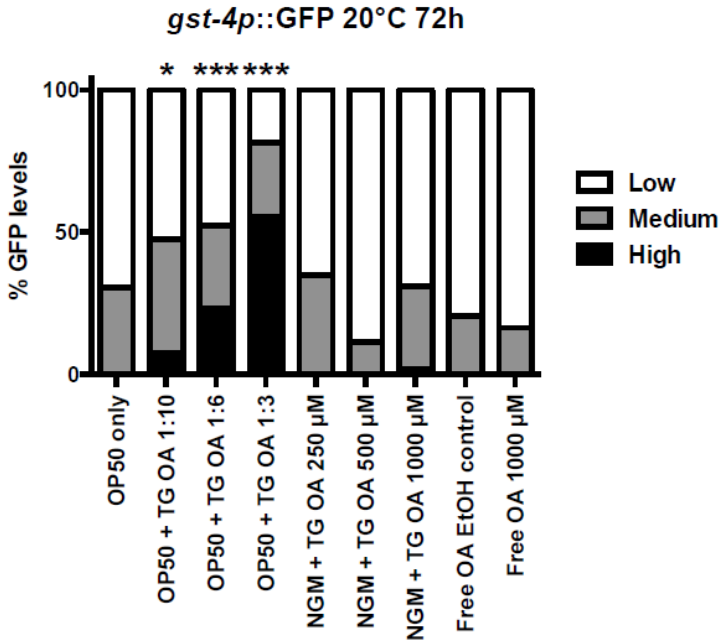

Figure 6: Chi-squared analysis (α<0.05) of gst-4p::GFP treated with glyceryl trioleate (TG OA) and OA

Chi-squared analysis with $\alpha<0.05$ (with PRISM) showed that treatment with a stabilized form of oleic acid – glyceryl trioleate (TG OA) – had a significant effect on *gst-4p::GFP* fluorescence but greatly depended on the way of application.

Supplementation of TG OA to the agar (250 µM, 500 µM, 1000 µM) did not trigger a visible activation of GFP fluorescence, while mixing TG OA with the bacterial food source OP50 provoked a significant response. The amount of glyceryl trioleate mixed into OP50 directly correlated with the significance of result. The most significant results were achieved supplementing OP50 with TG OA in a 1:6 or 1:3 ratio (See Figure 6).
Experiments using free oleic acid (which was supplemented to the agar to a molar concentration of 1000 µM) showed no significant increase in GFP fluorescence. Activation of *gst-4p::GFP* through OA was shown recently by Steinbaugh et al. (2015).

Slidemounting the fat-supplemented worms showed similar results and an apparent increase in GFP fluorescence correlating with the amount of glyceryl trioleate added to the food source OP50 could be observed (See Figure 7 & Figure 9). The control, grown on a lawn of OP50, did not show a significant increase of GFP fluorescent in the worm (See Figure 8). These results are consistent with the quantification shown above (See Figure 6).

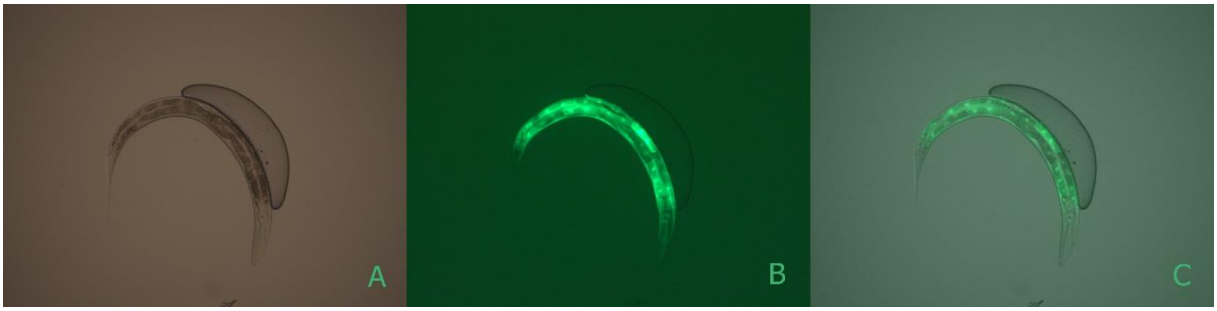

Figure 7: gst-4p::GFP with OP50 + TG OA 1:3 (A: BF, B: GFP, C: Merge), 10x

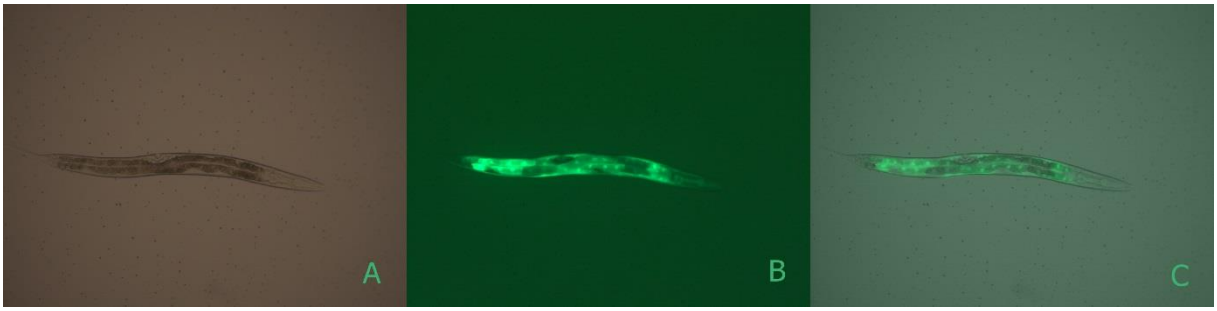

Figure 9: gst-4p::GFP with OP50 + TG OA 1:6 (A: BF, B: GFP, C: Merge), 10x

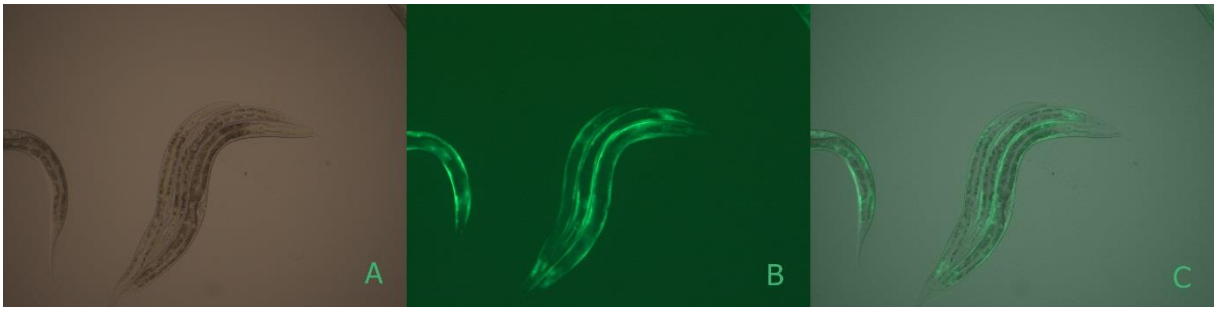

Figure 8: gst4p::GFP on OP50 (A: BF, B: GFP, C: merge), 10x

4.3. Rescue of knockdown phenotypes through supplementation of fatty acids

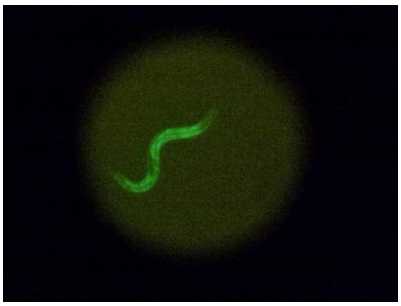

Figure 10: glp-1;gst-4p::GFP on EV RNAi (25°C, 72h)

Knockdown of GFP fluorescence by RNAi could be shown for fat-6, fat-7, fat-6+fat-7 and sbp-1 (See Figure 11). sbp-1, the SREBP1 orthologue (Yang et al., 2006) is responsible for de novo lipogenesis and was already shown to prevent SKN-1 nuclear accumulation before (Steinbaugh et al., 2015) and can be used as negative control (See Figure 11D). EV RNAi is used as positive control (See Figure 10).

Fat-3, fat-4, fat-5 RNAi failed to knock down SKN-1 activation and GFP fluorescence (not shown here). fat-6 and fat-7 (Δ9 desaturases) both catalyse desaturation from stearic acid (saturated FA) to oleic acid. fat-2 (Δ12 desaturase) catalyses the desaturation of oleic acid to linoleic acid (LA), see Figure 20; knockdown of fat-2 in *glp-1;gst-4p::GFP* was partial (data not shown here).

Though, rescuing the fat-6+fat-7 knockdown phenotype could be achieved with oleic acid as well as linoleic acid (See Figure 12). The FAs were added to the bacterial food source OP50 in a 1:6 ratio.

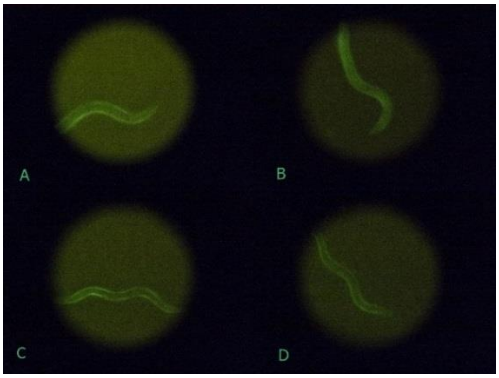

Figure 11: glp-1;gst-4p::GFP showed a knockdown phenotype on RNAi at 72 h, 25°C (A: fat-6; B: fat-7; C: fat-6+fat-7; D: sbp-1)

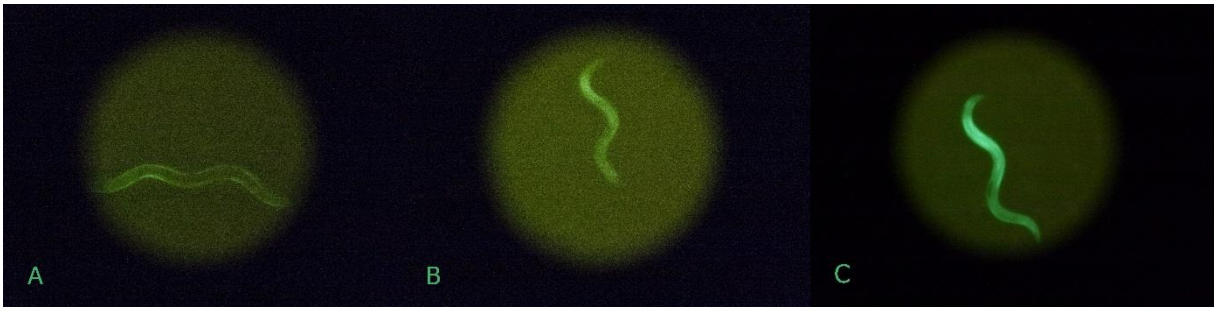

Figure 12: FA supplementation of glp-1;gst-4p::GFP on fat-6+fat-7 RNAi (A: control; B: OA; C: LA) rescued the knockdown phenotype (25°C, 72 h)

4.4. Supplementation of *SKN-1::GFP* with glyceryl trioleate

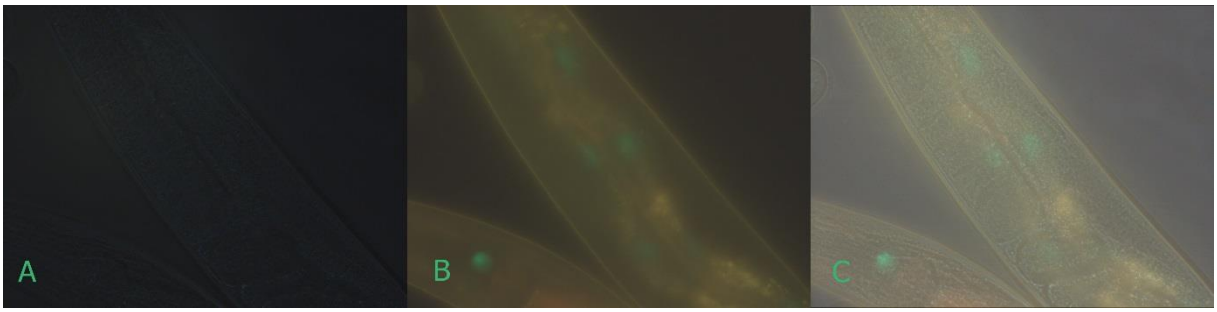

Figure 13: SKN-1::GFP with 0.2% glyceryl trioleate (0.02% NP40) in NGM (A: BF; B: GFP; C:merge), 40x

Supplementation of NGM with 0.2% glyceryl trioleate triggered nuclear accumulation of SKN-1. The response was slight and a positive control (activation through arsenite) was not available. SKN-1 nuclear accumulation can be observed as green dots with GFP imaging at 40x magnification (See Figure 13).

0.02% NP40 (a detergent) was used to increase homogenisation of the glyceryl trioleate.

4.5. High throughput RNAi screening

4.5.1. Summary

Table 1: Summary of the high-throughput RNAi screen of glp-1;gst-4p::GFP

	Number
Screened wells	6048
Censored in both wells	130
Wells which showed a larval phenotype	341
Wells with larval phenotype that held a valid clone	287
Wells which did not show bacterial growth	365
Wells lacking growth that held valid bacterial clones	94
Wells which held valid clones and were used for gene ontology after filtering	4165
“Hits down” with an average mean = -3	102
“Hits down” with an average mean <= -2	229
“Hits up” with an average mean = +1	54

In total 63 96-well plates were screened at least in duplicate which added up to a total of 6048 wells screened. 130 wells had to be censored in both set of plates for various reasons and have to be rescreened. 341 wells, of which 287 held a valid RNAi clone, showed a larval phenotype and suggested that those genes interfered with development. These RNAi clones were filtered since the questions

asked could not be answered; it might be necessary to rescreen those genes with adult worms to avoid the developmental arrest.
A total of 365 wells, of which 94 held a valid RNAi clone, did not show bacterial growth and were filtered.
After filtering, a total of 4165 genes were used for determination of hits and further gene ontology.
Depending on the stringency, a total of 102 genes (avg. mean = -3) which very strongly repressed SKN-1 expression, 229 genes with an average score of less or equal -2 and 54 genes, which appeared to upregulate SKN-1 could be found (See Table 1: Summary of the high-throughput RNAi screen of glp-1;gst-4p::GFP).
4165 genes were used for further gene ontology and several hits appeared to be promising candidates, which require further investigation and verification.

4.5.2. High confidence hits

Table 2: High confidence hits with an average score less or equal "-2"

	ORFeomeID	Score1	Score2	Score3	notes	ORF	Public name
11003@C10	11003@C10	-3	-3	NA	L?	T12G3.1	Sqst-1
10036@E11	10036@E11	-3	-2	NA	NA	ZK593.6	Lgg-2

Two of 229 genes that showed to trigger a significant reduction in SKN-1 activity in the screen were sqst-1 and lgg-2 (See *Table 2*). Sqst-1 was scored “-3” in both set of plates, while lgg-2 was scored “-3” and “-2”. Sqst-1 showed to trigger a slight larval arrest, which was not definite and will need further investigation.

sqst-1 and lgg-1 were previously mentioned in the context of autophagy and *C. elegans* longevity (Lapierre et al., 2013). sqst-1 was stated to be a HLH-30/TFEB target gene involved in autophagosome formation and autophagic flux, such as lgg-1, when fluorescently tagged sqst-1 was used to investigate elevated autophagic flux and cargo delivery to autophagosomes in *glp-1* animals (Lapierre et al., 2013).

4.5.3. Gene ontology

Functional Classification of genes classified as strong hits (avg. score <= -2) assigned hits to data annotation categories according to PANTHER terms (Mi et al., 2013).

86 of 218 tested genes were identified as cellular components, the majority of which were assigned to the category “cell part”, followed by the second biggest category “organelle” (see Figure 14).

The GO-Term “biological processes” gave 244 process hits of 218 genes with most genes belonging to the category “metabolic process”, followed by “cellular process” and “biological regulation” (See Figure 15). Level 1 of “metabolic process” revealed its major component “Primary metabolic process”, which is not shown here. Level 2 showed that the majority of genes assigned to “primary metabolic process” take part in “nucleobase-containing compound metabolic process”.

169 of 218 genes were predicted to serve a “molecular function”. According to gene ontology based on molecular function about a third of genes contributed to “catalytic activity” while another third was categorized as “binding” (See Figure 16). Amongst genes predicted to have catalytic activity the majority were categorized to have “hydrolase activity” and “transferase activity” (12 hits) (See Figure 17).

When gene ontology “Protein Class” was run on the hit list, 35 out of 141 were predicted to be “nucleic acid binding” proteins. 10 genes were assigned to the category “transferase” which correlates well with 12 hits with “transferase activity” received by doing GO analysis based on molecular function (See Figure 19). Level 1 of “nucleic acid binding” proteins showed that 26 out of 37 have predicted “RNA binding” activity (See Figure 18).

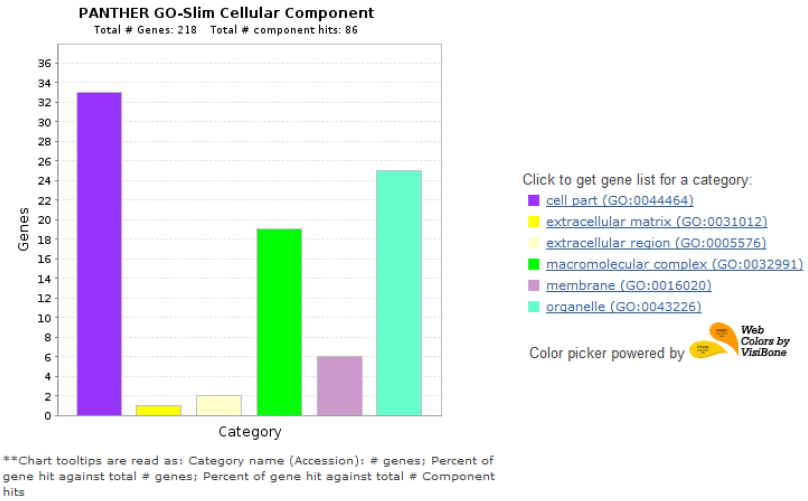

Figure 14: PANTHER Functional Classification (GO-Slim Cellular Component) of screening hits (avg. score <= -2)

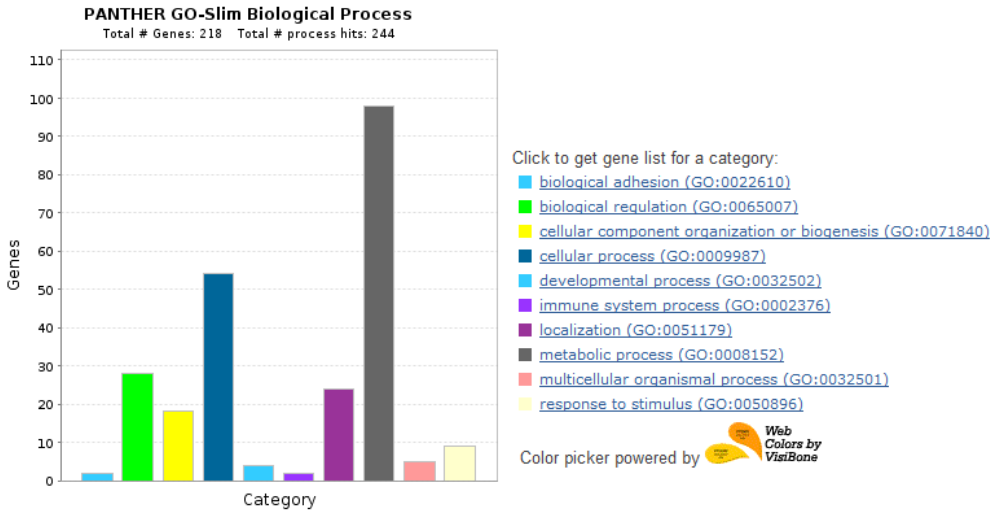

Figure 15: PANTHER Functional Classification (GO-Slim Biological Process) of screening hits (avg. score <= -2)

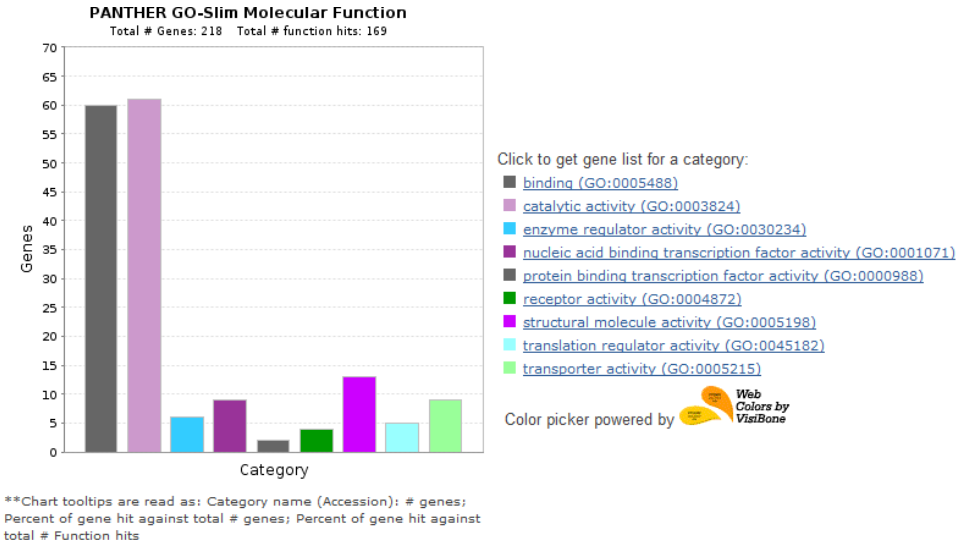

Figure 16: PANTHER Functional classification (GO-Slim Molecular Function) of screening hits (avg. score <= -2)

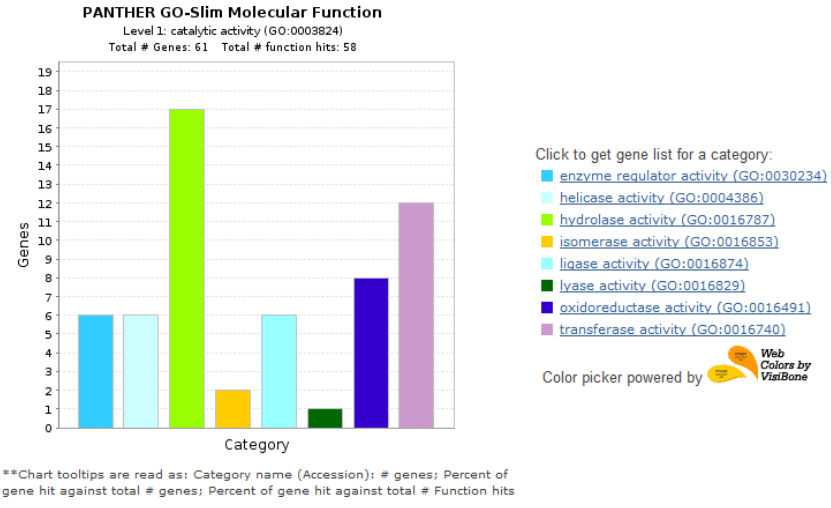

Figure 17: PANTHER Functional classification (GO-Slim Molecular Function, Level 1: Catalytic activity) of screening hits (avg. score <= -2)

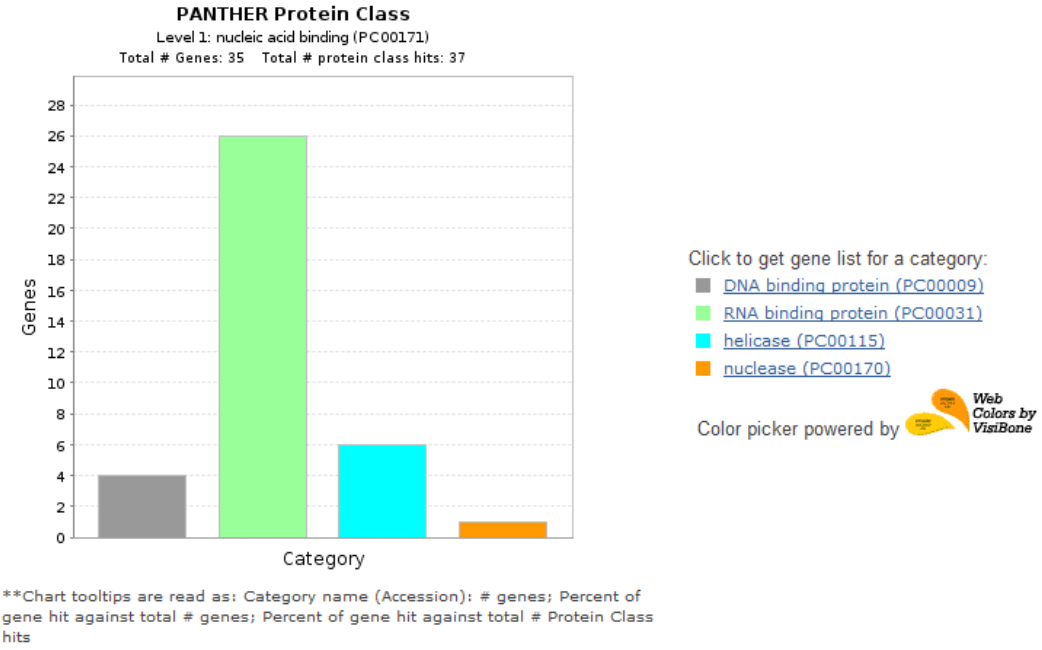

Figure 18: PANTHER Functional classification (Protein Class, Level 1: Nucleic Acid Binding) of screening hits (avg. score <= -2)

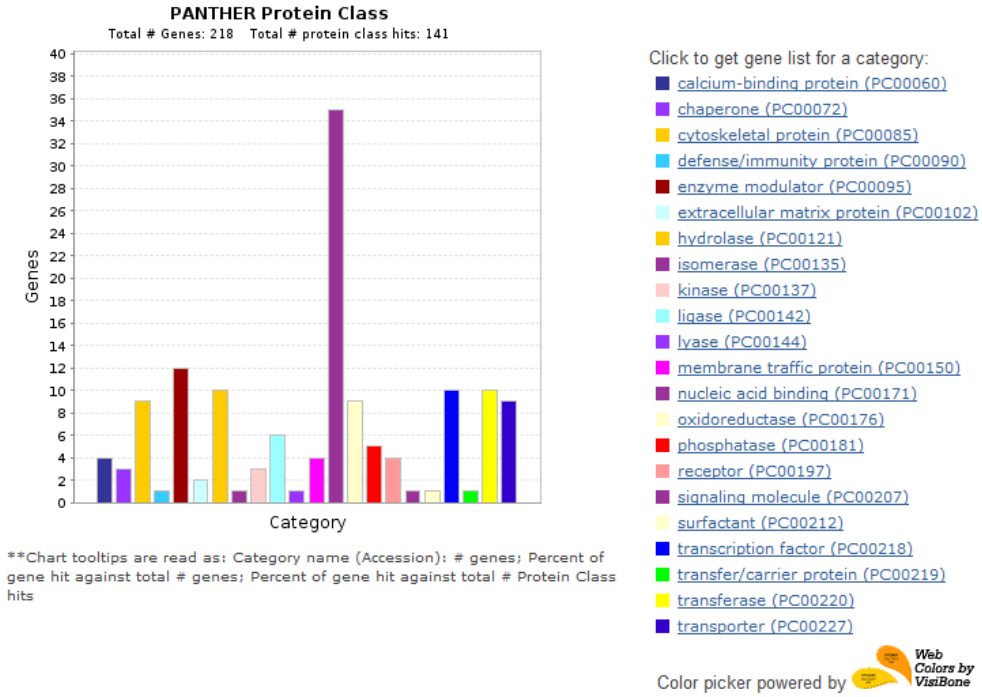

Figure 19: PANTHER Functional classification (Protein Class) of screening hits (avg. score <= -2)

The PANTHER overrepresentation test compares the number of genes assigned to a data annotation category normally present in the worm genome to the number of genes assigned to the same term present in the given data set. In this case the hits were compared against a “background” which consisted of all valid clones tested (4165), see Table 1. This measure shall ensure to avoid false positives which might be caused through an unequal distribution of genes in the dataset screened; e.g. if the majority of genes screened are developmental genes, the enrichment- when compared to the whole worm genome- could by chance be an artefact.

The Panther Overrepresentation test uses the same the GO terms as the functional classification. The first column (See Table 3-Table 6) contains the “annotation data category”, the second column contains the number of genes that belong to that data category, the third column shows the number of genes belonging to this category present in the data set which shall be analysed and the fourth column shows the number of genes predicted based on the given reference list.

The Panther Overrepresentation test “Molecular function” revealed “hydrogen ion transmembrane transporter activity “and “RNA helicase activity” to be upregulated more than fivefold with a statistical p-value < 0.05 (See Table 3). A small p-value reveals that the null-hypothesis (“There is no statistical difference between the datasets”) is rejected.

The Panther Overrepresentation test “Biological process” revealed “RNA splicing via transesterification reactions” and “RNA splicing” to be overrepresented by 4.58-fold or 4.47-fold, respectively (See Table 4). “mRNA processing”, “cellular component biogenesis” and “regulation of translation” are broad and general processes in every organism and showed roughly a threefold enrichment.

The Panther Overrepresentation Test on “Cellular Components” revealed an interesting enrichment of the “Vacuole” by more than a fivefold. In the given reference data set, 9 genes belonging to the category “vacuole” were present with a prediction of 0.50 hits in the analysed list. Though, 4 genes were found in the hit list resulting in 8-fold enrichment for the data annotation category “Vacuole”. Further the categories “tubulin complex” and “ribonucleoprotein complex” showed 7.14-fold and 3.35-fold enrichment, respectively (See Table 5).

The Overrepresentation Test “Protein Class” showed more than a fivefold enrichment for the data annotation category “tubulin” and “RNA helicase”. The category “helicase” was revealed to be overrepresented 4.13-fold while “oxidases” showed to be enriched about 3-fold (See Table 6) against the background.

Table 3: PANTHER Overrepresentation Test (GO-Slim Molecular Function) of hits (avg. score <= -2) against the background

PANTHER GO-Slim Molecular Function	#	#	expected	Fold Enrichment	+/-	P value
hydrogen ion transmembrane transporter activity (GO:0015078)	5	2	.28	> 5	+	3.24E-02
RNA helicase activity (GO:0003724)	16	5	.89	> 5	+	2.22E-03
helicase activity (GO:0004386)	26	6	1.45	4.13	+	3.69E-03
translation initiation factor activity (GO:0003743)	24	5	1.34	3.73	+	1.18E-02
translation regulator activity (GO:0045182)	31	5	1.73	2.88	+	3.12E-02
mRNA binding (GO:0003729)	44	7	2.46	2.84	+	1.26E-02
RNA binding (GO:0003723)	116	16	6.49	2.47	+	9.26E-04
protein binding (GO:0005515)	295	27	16.50	1.64	+	8.13E-03

Table 4: PANTHER Overrepresentation Test (GO-Slim Biological Process) of hits (avg. score <= -2) against the background

PANTHER GO-Slim Biological Process	#	#	expected	Fold Enrichment	+/-	P value
RNA splicing, via transesterification reactions (GO:0000375)	39	10	2.18	4.58	+	8.28E-05
RNA splicing (GO:0008380)	40	10	2.24	4.47	+	1.02E-04
protein complex biogenesis (GO:0070271)	21	5	1.17	4.26	+	6.91E-03
oxidative phosphorylation (GO:0006119)	21	5	1.17	4.26	+	6.91E-03
protein complex assembly (GO:0006461)	21	5	1.17	4.26	+	6.91E-03
mRNA splicing, via spliceosome (GO:0000398)	45	8	2.52	3.18	+	4.15E-03
mRNA processing (GO:0006397)	86	14	4.81	2.91	+	4.00E-04
cellular component biogenesis (GO:0044085)	52	8	2.91	2.75	+	9.52E-03
regulation of translation (GO:0006417)	33	5	1.85	2.71	+	3.91E-02

Table 5: PANTHER Overrepresentation Test (GO-Slim Cellular Component) of hits (avg. score <= -2) against the background

PANTHER GO-Slim Cellular Component	#	#	expected	Fold Enrichment	+/-	P value
vacuole (GO:0005773)	9	4	.50	> 5	+	1.76E-03
tubulin complex (GO:0045298)	5	2	.28	> 5	+	3.24E-02
ribonucleoprotein complex (GO:0030529)	32	6	1.79	3.35	+	9.78E-03
macromolecular complex (GO:0032991)	139	19	7.77	2.44	+	3.47E-04
nucleus (GO:0005634)	53	7	2.96	2.36	+	3.07E-02
protein complex (GO:0043234)	113	13	6.32	2.06	+	1.18E-02
organelle (GO:0043226)	288	25	16.11	1.55	+	1.95E-02
intracellular (GO:0005622)	433	33	24.22	1.36	+	4.15E-02

Table 6: PANTHER Overrepresentation Test (Protein Class) of hits (avg. score <= -2) against the background

PANTHER Protein Class	#	#	expected	Fold Enrichment	+/-	P value
tubulin (PC00228)	6	2	.34	> 5	+	4.50E-02
RNA helicase (PC00032)	16	5	.89	> 5	+	2.22E-03
helicase (PC00115)	26	6	1.45	4.13	+	3.69E-03
mRNA splicing factor (PC00148)	35	8	1.96	4.09	+	8.89E-04
mRNA processing factor (PC00147)	51	9	2.85	3.16	+	2.54E-03
oxidase (PC00175)	29	5	1.62	3.08	+	2.44E-02
microtubule family cytoskeletal protein (PC00157)	30	5	1.68	2.98	+	2.77E-02
RNA binding protein (PC00031)	200	26	11.19	2.32	+	6.27E-05
nucleic acid binding (PC00171)	416	35	23.27	1.50	+	9.51E-03

5. Discussion

5.1. Supplementation of *gst-4p::GFP* with oleic acid and glyceryl trioleate

The results showed a significant increase of GFP fluorescence in response to glyceryl trioleate added to the bacterial food source OP50. Worms grown on NGM supplemented with free oleic acid (in ethanol) or glyceryl trioleate showed no effect towards the supplementation.

The simplest reason, which might explain this result, is the big difference in the amount added. A molar concentration of 1000 µM of glyceryl trioleate in NGM accounts to 5 µl TG OA in a 6 cm plate, compared to 50 µl glyceryl trioleate added to 300 µl OP50, preparing a 1:6 ratio of "fatty food".
Further, the way of application might have had an influence on the result too. Since glyceryl trioleate was shown to significantly induce fluorescence when mixed with OP50 prior to seeding the plates, it is fairly plausible that the agar was too hot when adding the fatty acids, which are known to be sensitive towards heat treatment.

Another reason that could explain the difference in result is the unpredictability of the assay. This experiment was repeated but the results could not be reproduced. One way to explain this is the handling and usage of FAs in the laboratory. The plates were incubated at 25°C for several days and sometimes appeared "crystallized" under the microscope. FAs, which are sensitive towards oxidation, might not be stable enough in this experimental setup. Possibly, it might work best to make plates supplemented with FAs shortly before usage and transfer worms to freshly prepared plates every other day. Also, this might explain the positive result using glyceryl trioleate while supplementation with OA, which was previously shown to activate gst-4 (Steinbaugh et al., 2015), could not be repeated. Glyceryl trioleate, which consists of oleic acid in a triglyceride backbone, might be more stable than free OA.

Unpredictability also applies to the way but especially rate of administering the chemicals. Since the fatty acids are not evenly distributed throughout the bacterial lawn or NGM, it is impossible to predict the amount of FAs the worms take in.
One idea that could be useful in this context is the use of oleic acid – biotin or fluorescently labelled fatty acids, although it would need to be evaluated whether the increased size of the molecule has an impact on uptake rate and speed.
In mammalian cell culture the problem concerning the distribution of lipids in media can be solved using carrier molecules, emulsion, microemulsions or liposomes although these techniques might not be applicable to the work with *C. elegans*. The use of detergents (e.g. NP40) to dissolve and reach homogenisation of fat might be suitable but should be monitored closely in order not to falsify the results.

Last, an elegant approach to administer the fatty acid of interest would be transient or stable transformation of OP50 (the commonly used food source) to express the fatty acid of interest, ensuring reproducible ingestion and digestion.

5.2. Rescue of knockdown phenotypes through supplementation of fatty acids

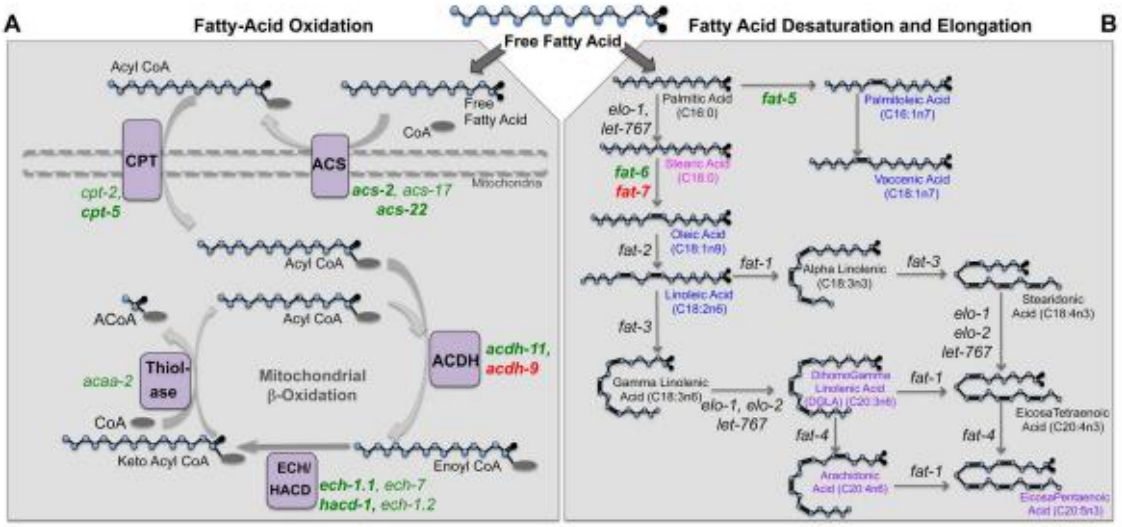

Figure 20: In panel B steps along fatty acid desaturation and elongation are shown which process saturated FAs to MUFAs and ultimately to PUFAs. Genes upregulated in glp-1 through nhr-49 are marked in green, genes repressed are coloured red. Ratnappan et al., PLOS Genetics 2014

Knocking down fat-6 (Δ9) or fat-7 (Δ9), which catalyse desaturation of stearic (18:0) to oleic acid (18:1n9), via RNAi showed a sharp decrease in GFP fluorescence, which indicates a disruption of SKN-1 activity. Knocking down fat-2 (Δ12) via RNAi showed a partial reduction of fluorescence. These results suggest that the signalling molecule/derivate activating SKN-1 might derive from oleic acid.

Though, rescuing experiments of fat-6/fat-7 knockdowns revealed that both oleic acid as well as linoleic acid (18:3n6) could restore GFP fluorescence, suggesting that a molecule further downstream of fat-2 might function as a signalling molecule.

fat-3, fat-4 and fat-5 RNAi did not show a knockdown phenotype suggesting that the products of those enzymes (palmitoleic acid, gamma linolenic acid, stearidonic acid, arachidonic acid (AA), eicosapentaenoic acid (EPA)) are not relevant for SKN-1 activation (data not shown here).

These results are controversial since derivatives of C_{20} FAs, such as eicosanoids and prostaglandins, are known to function as lipid signalling molecules in several processes. Eicosanoids were shown to play a role in metabolic syndrome (MetS), a constellation of diseases which include obesity, diabetes, hypertension, dyslipidemia, hypertriglyceridemia, and hypercholesterolemia and are associated with Nonalcoholic fatty liver disease (NAFLD) (Hardwick et al., Eicosanoids in Metabolic Syndrome). MetS triggers an inflammatory response, which includes the production of eicosanoids to recruit immune cells. Therefore eicosanoids were linked not only to persistent inflammation but also to alterations in the carbohydrate and lipid metabolism (Hardwick et al., Eicosanoids in Metabolic Syndrome).

Also, ω-6 PUFAs were shown to extend lifespan (O'Rourke et al., 2013). This lifespan extension was explained with an upregulation in autophagy by O'Rourke et al. (2013). ω-6 PUFAs include linoleic acid, EPA and AA amongst others.

Together, these results suggest that longevity in *glp-1* animals might be caused by several different mechanisms, which might be activated through (distinct) lipid signalling molecules.

5.3. Supplementation of *SKN-1::GFP* with glyceryl trioleate

Activation of gst-4 (gst-4 is a SKN-1 target gene) in response to free oleic acid (OA) has already been shown by Steinbaugh et al. (2015). Nuclear accumulation of SKN-1 in response to glyceryl trioleate could be shown in one case, but will need further repetitions to verify the result.
As *gst-4p::GFP* activation through glyceryl trioleate (See 124.2) could be shown the result seems plausible.
High GFP fluorescence of the background (yellowish colour) might be delusive, therefore additional washing steps or computational subtraction of the background might be necessary to get a better result.

5.4. High-throughput RNAi screening

5.4.1. Summary

A lot of valuable information can be gained through a whole-genome screen, though there are constraints to this method which have to be kept in mind. Certain genes, which are crucial for development, had to be filtered from the pool of genes as an adult phenotype is required for scoring. This implicates that a total of 287 genes (larval phenotype with a valid clone) got "lost" from this set. It will be necessary to grow the worms until L4 stage or adulthood before they are transferred onto RNAi plates. Though, this approach might fail as fat has already built up in the worm at this stage and knockdown through RNAi might not be stringent enough to disrupt lipid signalling and subsequent SKN-1 activation.

Next, there are some limitations to the specificity of this screen. SKN-1 is activated or repressed through a wide variety of signals (see Chapter 1.3. SKiNhead (SKN-1)) which might not necessarily be caused by fats or lipid stress.
Originally, it was aimed at using both a genetic as well as biochemical approach and screening both *glp-1;gst-4p::GFP* animals as well as *gst-4p::GFP* worms, which were supplemented with olive oil at the L4 stage. This approach failed due to high variability in administration of oil (and probably the oil itself) but was promising to give lipid-specific results. Another approach to show the specificity of the hits obtained from this screen, is treatment with known SKN-1 inducers such as Arsenite. If the induction is successful, this suggests that SKN-1 is not inactivated in general but explicitly through knocking down a lipid-associated gene; if the induction fails on the other hand, this suggests that the gene knocked down is broadly required for SKN-1 activity and is not specific for lipid stress.

Last, since SKN-1 is induced and repressed through several mechanisms, it is very important to keep the conditions stable, which is not easy in a 96-well format. Starvation of single wells is a common problem in 96-well plates but dietary restriction (DR) is also modulating SKN-1 activity (Blackwell et al., 2015). These limitations should be kept in mind to ensure high-quality data.

5.4.2. High confidence hits

Sqst-1 (and presumably lgg-2) are genes involved in autophagosome formation and autophagic flux. Knockdown of certain autophagy genes were shown to prevent lifespan extension in LIPL-4 overexpressing *C. elegans* (Lapierre et al., 2011). In general, autophagic events were shown to be upregulated in GSC(-) animals, which supports the idea of transcriptional reprogramming (Lapierre et al., 2011). So far it is not known how autophagy would influence lifespan and aging, but "presumably it occurs through benefical turnover of as-yet-unidentified-cargo." (Lapierre et al., 2012).

Recently, it was shown that autophagy can hydrolyse lipids through a process termed “lipophagy” (Lapierre et al., 2012). Lysosomal acid lipases (LALs), which correspond to the *C. elegans* LIPL enzymes, were proposed to catabolize lipid-droplet fat stores through lipophagy (Czaja et al., 2009; O’Rourke et al., 2013).

The screen revealed that sqst-1 and lgg-2 strongly supress SKN-1 activity in *C. elegans,* which could possibly be explained with the connection of lipid homeostasis and lipophagy. If SKN-1 is activiated by lipid signalling molecules, as proposed in the introduction, disruption of autophagy/lipophagy might disturb the processing (hydrolyzation?) of fat (lipid droplets) stored in *glp-1* animals. This result further proposes that signalling molecules might derive from triglycerides stored in lipid droplets which have to be hydrolysed through a process as lipophagy.
It would be necessary to further investigate how autophagy effects lipid metabolism and vice versa. Further, it might be interesting to see whether sqst-1 or lgg-2 knockdown in *glp-1* worms reveal a higher fat content compared to *glp-1* animals. Also, it might be interesting to see whether overexpression of lipophagy-associated genes will induce SKN-1 and decrease lipid stress and fat content.

5.4.3. Gene ontology

PANTHER functional classification assigned hits to data categories. Although the visualization of genes is illustrative, not a lot of information can be gained from those graphs. The *C. elegans* RNAi screening library is arranged in 140 96-well plates, therefore it is possible that all genes belonging to one category were screened at first and possibly showed up as the category containing the highest number of hits. Overrepresentation tests present a more efficient approach to identify classes of genes which are enriched among the hits.

PANTHER overrepresentation tests revealed two interesting categories which were overrepresented in the given data set of 229 hits, which strongly decreased SKN-1 activitiy, namely “oxidase” and “vacuole”. It should be kept in mind though, that results might be artefacts especially if the numbers of hits are small (e.g. If 2 genes are found to belong to a category that was predicted to only hold 0.5, a 4-fold increase is the result, which still might not be significant after all). Though, using the list of genes already screened as a reference increases the reliability of the results.

The category “oxidase” was overrepresented among the list of hits, proposing that oxidation of certain substrates might be important for SKN-1 activation.
Ratnappan et al. (2014) have previously proposed that *C. elegans* might adapt to the loss of reproductive potential through enhancement of fatty-acid oxidation and desaturation through nhr-49. In fact oxidation of fatty acids creates free radicals (Schroeder et al., 2015), which are known to activate SKN-1 (Blackwell et al., 2015). SKN-1 on the other hand is known to respond to elevated lipid levels by activating genes involved in β-oxidation, lipolysis, fatty acid desaturation, elongation, and transport (Blackwell et al., 2015).
Steinbaugh et al. (2015) have further shown that GSC(-) animals showed a distinct upregulation of genes involved in metabolism and particularly FA oxidation and other lipid metabolism processes compared to GSC(+) animals (See page 5).
Alternatively, lipid oxidation in fact does not only describe the process of energy generation and oxidation to cytotoxic products, but also defines the process which creates signalling molecules such as eicosanoids through oxidation of C_{20} FAs such as AA and EPA (Dicfalusy, 1994).
Taken together these results either suggest that the oxidation of lipids might be a crucial step in the lipid-stress response and subsequent SKN-1 activation through free radicals or further supports the hypothesis of lipid-derived signalling molecules. It would be interesting to see whether reactive oxygen

species (ROS) are elevated in *glp-1* animals and subsequently cause SKN-1 activation. If SKN-1 shows to be activated through ROS, it will need to be investigated if this is a lipid-specific response. The use of a ROS-scavenger might be useful to prove SKN-1 activation through ROS in *glp-1* animals.

The vacuole is the cell organelle which has been associated with autophagy and shows many similarities to mammalian lysosomes (Li and Kane, 2009). Autophagy literally means “self-eating” and includes all processes which deliver cytoplasmic material to the lysosome for degradation. Lysosomes have previously been associated with the metabolism of excessive fat and lipid-based signalling from the lysosome to the nucleus (Steinbaugh et al., 2015).
In *C. elegans* there are at least three types of autophagy, chaperone-mediated autophagy, microautophagy and macroautophagy; most studies have focused on this last form of autophagy (Meléndez and Levine, 2009).
As discussed above, lipid-associated autophagy, lipophagy might be of crucial importance for lipid turnover and SKN-1 activation. Overrepresentation of vacuolar genes in this test further emphasizes the hypothesis that autophagy might play a role regarding SKN-1 activation (See page 25).

6. References

1. An JH and Blackwell TK. 2003. SKN-1 links C. elegansmesendodermal specification to a conserved oxidative stress response *Genes Dev.* 17(15):1882-93.
2. Arantes-Oliveira N, Apfeld J, Dillin A, Kenyon C. 2002. Regulation of life-span by germ-line stem cells in Caenorhabditis elegans. *Science* 295:502–505.
3. Berman JR and Kenyon C. 2006. Germ-cell loss extends C. elegans lifespan through regulation of DAF-16 by kri-1 and lipophilic-hormone signaling. *Cell* 124: 1055–1068.
4. Blackwell TK, et al. 2015. SKN-1/Nrf, stress responses, and aging in Caenorhabditis elegans. *Free Radic. Biol. Med.* 88(Pt B):290-301.
5. Boulias K and Horvitz HR. 2012. The C. elegans microRNA mir-71 acts in neurons to promote germline-mediated longevity through regulation of DAF-16/FOXO. *Cell Metab.*15, 439–450.
6. Brenner S. 1974. The genetics of Caenorhabditis elegans. Genetics77:71–94.
7. Brenner, S. 1988. Foreword. The Nematode Caenorhabditis elegans, edited by W. B. Wood, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
8. Corsi AK, Wightman B and Chalfie M. 2015 A Transparent window into biology: A primer on Caenorhabditis elegans. *GENETICS* 200: 387-407.
9. Czaja MJ, Cuervo AM. 2009. Lipases in lysosomes, what for?. *Autophagy* 5:866–867.
10. Flatt T, Min KJ, D'Alterio C, Villa-Cuesta E, Cumbers J et al. 2008. Drosophila germ-line modulation of insulin signaling and lifespan. *Proc Natl Acad Sci USA* 105(17):6368-73.
11. Folick A, Oakley HD, Yu Y, Armstrong EH, Kumari M et al. 2015. Aging. Lysosomal signaling molecules regulate longevity in Caenorhabditis elegans. *Science* 347(6217):83-6.
12. Gonzalez-Covarrubias V. 2013. Lipidomics in longevity and healthy aging. *Biogerontology* 14: 663–672
13. Goudeau J, Bellemin S, Toselli-Mollereau E, Shamalnasab M, Chen Y, Aguilaniu H. 2011. Fatty acid desaturation links germ cell loss to longevity through NHR-80/HNF4 in C. elegans. *PLOS Biology* 9:e1000599.
14. Hamilton JB and Mestler GE. 1969. Mortality and survival: comparison of eunuchs with intact men and women in a mentally retarded population. *J. Gerontol*.24: 395–411.
15. Hansen M, Flatt T, Aguilaniu H. 2013. Reproduction, fat metabolism, and life span: what is the connection?. *Cell Metab.* 17(1):10-9.
16. Hardwick JP, Eckman K, Kwang Lee Y, Abdelmegeed A, Esterle A et al. 2013. Eicosanoids in Metabolic Syndrome. Adv Pharmacol. 66: 157–266.
17. Hsin H and Kenyon C. 1999. Signals from the reproductive system regulatethe lifespan of C. elegans. *Nature* 399:362–366.
18. Kaletta T and Hengartner MO. 2006. Finding function in novel targets: C. elegans as a model organism. *Nat. Rev. Drug Discov.* 5: 387-398.
19. Kenyon CJ, Chang J, Gensch E, Rudner A and Tabtiang R. 1993. A C. elegans mutant that lives twice as long as wild type. *Nature*: 366:461-464
20. Kenyon CJ. 2010. The genetics of ageing. *Nature*: 464:504–512.
21. Lapierre LR, De Magalhaes Filho CD, McQuary PR, Chu CC, Visvikis O et al. 2013. The TFEB orthologue HLH-30 regulates autophagy and modulates longevity in Caenorhabditis elegans. *Nat Commun.* 4:2267.
22. Lapierre LR, Gelino S, Meléndez A and Hansen M. 2011. Autophagy and lipid metabolism coordinately modulate life span in germline-less C. elegans. *Curr. Biol.* 21, 1507–1514.
23. Lapierre LR, Meléndez A, Hansen M. 2012. Autophagy links lipid metabolism to longevity in C. elegans. *Autophagy*. 8(1):144-6.
24. Lee CS, Ho DV, Chan JY. 2013. Nuclear factor-erythroid 2-related factor 1 regulates expression of proteasome genes in hepatocytes and protects against endoplasmic reticulum stress and steatosis in mice. *The FEBS Journal* 280:3609–3620.
25. Lemieux GA and Ashrafi K. 2015. Stressing about misplaced fat is a key to longevity. *Elife*. 2015;4. doi: 10.7554/eLife.10161.
26. Li SC and Kane PM. 2009. The Yeast Lysosome-like Vacuole: Endpoint and Crossroads. *Biochim Biophys Acta*. 1793(4): 650–663.
27. Libina N, Berman JR and Kenyon C. 2003. Tissue-specific activities of C. elegans DAF-16 in the regulation of lifespan. *Cell* 115: 489–502.
28. Lin K, Hsin H, Libina N and Kenyon C. 2001. Regulation of the Caenorhabditis elegans longevity protein DAF-16 by insulin/IGF-1 and germline signaling. *Nat. Genet.* 28: 139–145.

29. Liu S, Alexander RK, Lee CH. 2014. Lipid metabolites as metabolic messengers in inter-organ communication. *Trends Endocrinol. Metab.* 25:356–363
30. López-Otín C, Blasco MA, Partridge L, Serrano M and Kroemer G. 2013. The hallmarks of aging. *Cell* 153(6):1194-217
31. Meléndez A and Levine B. 2009. Autophagy in C. elegans, WormBook , ed. The C. elegans Research Community, WormBook, http://www.wormbook.org
32. Mi H, Muruganujan A, Casagrande JT, Thomas PD. 2013. Large-scale gene function analysis with the PANTHER classification system. *Nat Protoc*. 8(8):1551-66.
33. Min KJ, Lee CK and Park HN. 2012. The lifespan of Korean eunuchs. *Curr. Biol.*22: R792–R793.
34. O'Rourke EJ, Kuballa P, Xavier R, Ruvkun G. 2013. Omega-6 Polyunsaturated fatty acids extend life span through the activation of autophagy. *Genes & Development* 27:429–440.
35. Okada K, Warabi E, Sugimoto H, Horie M, Gotoh N et al. 2013. Deletion of Nrf2 leads to rapid progression of steatohepatitis in mice fed atherogenic plus high-fat diet. *Journal of Gastroenterology* 48:620–632.
36. O'Rourke EJ and Ruvkun G. 2013. MXL-3 and HLH-30 transcriptionally link lipolysis and autophagy to nutrient availability. *Nat Cell Biol.* 15(6):668-76.
37. O'Rourke EJ, Kuballa P, Xavier R, Ruvkun G. 2013. ω-6 Polyunsaturated fatty acids extend life span through the activation of autophagy. *Genes Dev.* 27(4):429-40.
38. O'Rourke EJ, Soukas AA, Carr CE, Ruvkun G. 2009. C. elegans major fats are stored in vesicles distinct from lysosome-related organelles*. Cell Metab.* 10(5):430-5.
39. Ratnappan R, Amrit FR, Chen SW, Gill H, Holden K. 2014. Germline signals deploy NHR-49 to modulate fatty-acid β-oxidation and desaturation in somatic tissues of C. elegans. *PLoS Genet.* 10(12):e1004829.
40. Schroeder EA and Brunet A. 2015. Lipid Profiles and Signals for Long Life*. Trends Endocrinol Metab*.: 26(11):589-92.
41. Seo M, Seo K, Hwang W, Koo HJ, Hahm JH et al. 2015. RNA helicase HEL-1 promotes longevity by specifically activating DAF-16/FOXO transcription factor signaling in Caenorhabditis elegans*. Proc Natl Acad Sci U S A* 112(31):E4246-55.
42. Shmookler Reis RJ, Xu L, Lee H, Chae M, Thaden JJ et al. 2011. Modulation of lipid biosynthesis contributes to stress resistance and longevity of C. elegans mutants. *Aging* 3:125–147
43. Steinbaugh MJ, Narasimhan SD, Robida-Stubbs S, Moronetti Mazzeo LE, Dreyfuss JM et al. 2015. Lipid-mediated regulation of SKN-1/Nrf in response to germ cell absence. Elife. doi: 10.7554/eLife.07836.
44. Tsujita T, Peirce V, Baird L, Matsuyama Y, Takaku M, Walsh SV, Griffin JL, Uruno A, Yamamoto M, Hayes JD. 2014.Transcription factor Nrf1 negatively regulates the cystine/glutamate transporter and lipid-metabolizing enzymes. *Molecular and Cellular Biology* 34:3800–3816
45. Wang MC, O'Rourke EJ, Ruvkun G. 2008. Fat metabolism links germline stem cells and longevity in C. elegans. *Science* 322(5903):957-60.
46. Xu Z, Chen L, Leung L, Yen TS, Lee C, Chan JY. 2005. Liver-specific inactivation of the Nrf1 gene in adult mouse leads to nonalcoholic steatohepatitis and hepatic neoplasia. *Proceedings of the National Academy of Sciences of USA* 102:4120–4125.
47. Yang F, Vought BW, Satterlee JS, Walker AK, Jim Sun ZY et al. 2006. An ARC/Mediator subunit required for SREBP control of cholesterol and lipid homeostasis. *Nature* 442:700–704.
48. Zechner R, Zimmermann R, Eichmann TO, Kohlwein SD, Haemmerle G. 2012. FAT SIGNALS--lipases and lipolysis in lipid metabolism and signaling. *Cell Metab.* 15(3):279-9
49. Diczfalusy U. 1994. β-oxidation of Eicosanoids. *Prog. Lipid Res*. Vol. 33, No. 4, pp. 403-428