Gene regulatory mechanisms controlling ageing in *Caenorhabditis elegans*

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PhD thesis

Declaration

I confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Daniel Ackerman

Abstract

Mutations in the insulin/IGF-1 signalling (IIS) pathway lead to large lifespan extensions in the nematode *Caenorhabiditis elegans.* The role of IIS in ageing is evolutionarily conserved and requires the forkhead trancription factor DAF-16.

In this thesis, I have attempted a novel strategy to identify genes involved in differential gene expression downstream of IIS in *C. elegans*. Using microarray data, I found that the H-ferritin gene *ftn-1* is strongly regulated by IIS in a *daf-16* – dependent manner. Ferritins act as intracellular iron-storage proteins. I then induced expression of a fluorescent GFP reporter of *ftn-1* by introducing a mutation in *daf-2*. I subsequently screened a library of RNAi clones targeting transcription factors in order to identify mediators of IIS-dependent gene regulation. Candidate RNAi treatments were subsequently tested for their effects on expression of endogenous *ftn-1* and on ageing. None of the genes identified had as important a role in either *ftn-1* expression or lifespan as *daf-16,* but several weaker determinants, like *mdl-1*, were identified.

During the course of my screen, I found that loss of the hypoxia inducible factor *hif-1* and its binding partner *aha-1* leads to a large increase in expression from the *ftn-1* GFP reporter, indicating that HIF-1 may act as a repressor of *ftn-1* expression. Both HIF-1 protein levels and *ftn-1* transcription are known to be responsive to iron levels. I found that regulation of the *ftn-1* reporter by iron requires *hif-1* and that repression of *ftn-1* reporter expression occurs via a 63bp iron dependent element. An unexpected role of the upstream prolyl hydroxylase EGL-9 was also identified.

I also contributed to a project aimed at identifying the consequences of over-expression of superoxide dismutases (SOD) on *C. elegans* ageing. Our efforts revealed that SOD over-expression extends lifespan by affecting signalling, not by reducing oxidative damage.

Dedicated to Marga, Vivian and Andrew,
for all their support.

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Chapter 1: Introduction

1.1. Ageing

We are all familiar with the effects of ageing, having experienced them ourselves or witnessed them in friends or family members. There is no tissue or biological system that does not suffer the effects of old age through deterioration of function and efficiency. Older people experience a number of detrimental changes, including a loss of force and elasticity in their skeletal muscles, lower glomeral filtration in their kidneys and lower pulmonary ventilation. They also often suffer from deterioration of their senses, including losses in vision and hearing, as well as loss of memory and motor coordination and a decreased capacity to maintain homeostasis of many systems. Problems with their immune system lead to both increases in autoimmune disease and a decreased ability to combat infections (Vina et al., 2007).

And yet, despite being an inevitable component of our lives, the truth is that we are fundamentally ignorant of the causes of ageing. Maybe because of its ubiquity, ageing is often seen as a natural consequence of the passage of time, but it is clear that a biological process affecting cells or tissues must underlie the many biological manifestations of ageing. Whatever its nature, this process must be a risk factor for the many diseases that increase in incidence with age. The risk of succumbing to cancer, for example, increases so dramatically that DePinho (2000) pointed out that "advancing age is the most potent of all carcinogens".

Because it can describe maturation as well as senescence, use of the word "ageing" can lead to confusion. A wine can improve as it ages, for example, and certain traits found in the elderly, such as wisdom, experience and maturity, are positive, not negative ones. In this thesis, I will use the term only to describe its deleterious effects. I will avoid using the term 'senescence' in order to avoid confusion with the process of cellular senescence.

1.1.1. Searching for a cause of ageing

To those not working in the field of ageing, the purpose of studying this particular problem is often unclear. Why does ageing remain such a mystery to biologists, when others just consider it a fact of life? For many, the ageing of organisms is comparable to that of any other objects in daily life: Old computers slow down and malfunction, cars rust and fabrics start to tear. Why should human beings be any different?

In fact, some aspects of biological ageing are very similar to everyday wear and tear. Examples include teeth that wear out after time and the exoskeletons and wings of insects, which can break. However, the ultimate cause of ageing is less clear for other biological features because there is clear evidence that different rates of ageing (estimated using maximum lifespans) are possible in very similar, and similarly sized, organisms (bats and mice are good examples (Austad and Fischer, 1991)). This strongly suggests that the ageing process is not an unalterable one.

One may even ask why we age at all. The fact that the passage of time and the number of cell divisions carried out does not in itself require a loss of function is clear, since the entire history of life on earth can be seen as an uninterrupted line of germ cell divisions. Even though no organism was created from scratch, every generation's young are born as healthy as the last. Our existence after so many million years of evolution therefore itself argues against the inevitability of degeneration with time. An unfathomable number of cell divisions occurred in the lineage of every human before birth to produce a healthy child, so why are the comparatively small number that transpire since birth and in such a short period of time sufficient to cause ageing?

Much work has gone into understanding ageing and the questions posed in the above paragraphs are partly addressed by work on the evolution of ageing. The evolutionary theory of ageing has provided possible explanations for why different rates of ageing would evolve, but this has been reviewed elsewhere and will not be discussed here (Rauser et al., 2006). What evolutionary theories cannot address are the ultimate causes of ageing, ie. what is it about old organisms that is making them more likely to succumb to disease, to lose mobility, etc. Attempts to answer this have been numerous.

1.1.2. Theories of ageing

So what causes ageing? A natural starting point is the observation that inanimate objects deteriorate with time, and more fundamentally, that in any system, disorder, or entropy, tends to increase. That ageing of organisms may also occur through simple wear and tear is a reasonable hypothesis to begin with. At what level this damage is occurring and what exactly is being damaged is the subject of numerous theories. Many propose that it is the accumulation of molecular damage that causes ageing and the most popular one states that free radicals, through their attack of macromolecules in the cell, are the cause of 'wear and tear' at the molecular level (Weinert and Timiras, 2003). Others propose that ageing occurs at the cellular or the organismal level (Jeyapalan et al., 2007). Too many theories of ageing have been proposed to discuss comprehensively, so I will present a few key ideas.

1.1.2.1. Molecular damage

Molecular damage to components of the cell, such as proteins, lipids and DNA is thought to accumulate during the course of the organism's life and lead to progressive deterioration of function. An example for a reaction that can cause damage to proteins is glycation. This is involves a condensation reaction between sugars (such as glucose) and free amino acid groups in proteins. This results in advanced glycation end products (AGE) (Chang et al., 1985) which may be deleterious to the cell and particularly to extracellular structures, like the collagen in cartilage.

Another, more frequently cited source of damage to molecules is oxidation by reactive oxygen species (ROS). ROS are produced as by-products of a number of biological processes, most notably mitochondrial respiration. The fact that damage caused by oxidation increases with age is well supported (see Chapter 6) and the oxidative stress theory of ageing predicts that this accumulation is a major cause of ageing (Harman, 1956). See Chapter 6 for a more detailed review of oxidative stress and ageing.

1.1.2.2. Somatic mutation theory

One source of damage that could be causing ageing are mutations to DNA within somatic cells. Mutations can occur spontaneously, for example via reactions with reactive oxygen species (ROS). As mutations accumulate throughout life, it is possible that increasing numbers of faulty proteins get translated with advancing age, which may lead to deterioration of cellular function.

Higher levels of DNA damage have been detected in old animals (see Chapter 6), though whether or not the accumulation of mutations during the course of an organism's life can cause sufficient damage to explain ageing remains to be seen. In one study, mice were serially cloned for six generations using somatic nuclear transfer and their rate of ageing was not affected, indicating that, at least in mice, the rate of somatic mutation is not sufficient to have major effects on ageing (Wakayama et al., 2000).

1.1.2.3. Error catastrophe theory

The error catastrophe theory of ageing is based on the knowledge that the rate of mis-incorporation of amino acids during translation is quite high (compared to, for example, the rate of error during transcription) and that large numbers of aberrant proteins are therefore constantly being produced (Rattan, 2006). Because the translational machinery is itself made of proteins, the production of faulty copies of parts of this machinery may cause an even greater rate of amino acid mis-incorporation. This theory predicts that through positive feedback, this mis-incorporation will eventually cause an 'error catastrophe' with large increases in the production of aberrant proteins and that this catastrophe is the cause of ageing. Initial experiments on amino-acid mis-incorporation during translation were ambiguous (Holliday, 1996) and at least in *C. elegans*, 2D gel analysis was not able to detect evidence of an 'error catastrophe' in old animals (Vanfleteren and De Vreese, 1994).

1.1.2.4. Gene regulation theory

The gene regulation theory of ageing predicts that ageing is caused by changes in gene expression with time. A gradual loss of fine-tuning of gene regulatory pathways would presumably lead to a dysregulation of gene expression, which could have deleterious effects. Some studies have tested the effects of ageing on gene expression using microarrays in model organisms and found remarkably little variation in gene expression in some tissues of ageing mice (Weindruch et al., 2001, 2002). Microarray studies in worms identified changes in the transcriptome that occur during ageing and which are regulated by a small set of GATA transcription factors. The authors hypothesised that this network of GATA factors has evolved to regulate gene expression during development but may become unbalanced in old animals, thereby effecting the changes in gene expression observed with age (Budovskaya et al., 2008). This drift in gene expression does have an effect on *C. elegans* lifespan, but whether it is a minor modulatory factor in ageing or a major cause of it is still unclear.

1.1.2.5. Cellular senescence theory

The finding that most cells have a limit to the number of cell divisions they can undergo in culture (Hayflick, 1965) before they enter a state of replicative senescence led to the theory that this intrinsic limit also limits organismal lifespan. Cells are restricted in the number of cell divisions they can go through by a progressive shortening of the chromosome ends, the telomeres. Immortal cells, such as stem cells and germline cells, achieve their immortality at least partly through the expression of telomerase, which extends telomere ends (Cech, 2004).

The presence of senescent cells in old tissues may affect neighbouring cells through the creation of a hazardous environment (Krtolica and Campisi, 2002). The cause for this effect on neighbouring cells is unknown, although senescent cells have been found to have increased expression of pro-inflammatory cytokines (Maier et al., 1990), which has been suggested as a possible mechanism (Weinert and Timiras, 2003), and also secrete enzymes that degrade the extracellular matrix. Although

some studies have found a correlation between the ability of cells derived from a tissue to replicate *in vitro* and age of the donor, some doubt has recently been cast on the relevance of the *in vitro* Hayflick limit to cellular senescence *in vivo* (Rubin, 2002). However, studies on baboons have demonstrated that a large percentage of skin cells from old animals are senescent (possibly more than 15%), which could have profound physiological effects (Herbig et al., 2006). No such effect was found in muscle, a post-mitotic tissue, so the degree to which senescent cells may contribute to ageing may vary in different tissues and different organisms (Jeyapalan et al., 2007).

1.1.2.6. Theories of systemic ageing

While most of the theories described above assume an intracellular cause of ageing, the complexity of multicellular organisms suggests that causes of ageing could also be found at the systemic (organismal) level.

Possible examples are human neural and endocrine systems, which are crucial for the body's ability to programme responses to environmental stimuli and to maintain an optimal functioning state. The passage of time, and possibly chronic exposure to stress, may weaken the body's capacity to respond to certain cues and may thereby cause dyshomeostasis. Ageing has also long been known to affect the immune system: older people experience a decreased resistance to infectious diseases, less protection against cancer and a decreased ability of the immune system to recognise the body's own cells. Some immune functions remain well preserved in old age, whereas others rapidly lose efficacy. The thymus, for example, reaches its largest size during puberty and then atrophies (Weinert and Timiras, 2003).

The existence of a great number of theories of ageing and the conspicuous dearth of good evidence that either clearly supports or contradicts them indicates that the causes underlying this phenomenon are not very well understood. This is not too suprising given how difficult it is to study ageing directly, especially in long lived organisms. Data on ageing therefore often consists of observations, not rigorous experiments in which a theory is put a decisive test using controlled experiments.

Early work on the oxidative stress theory of ageing is a good example for this and the question of how to best test theories of ageing is addressed in to Chapter 6. The complexity of the ageing process and the long time periods required for the effects of ageing to manifest themselves do make it clear that this biological process cannot be studied effectively in long-lived organisms, especially not in humans. It is in this context that the use of short-lived model organisms such as *C. elegans* became an attractive alternative.

1.2. *C. elegans* as a genetic model organism

In the wild, nematode *Caenorhabditis* species, including *C. elegans*, are often found in compost, rotting fruit and in the soil (Barriere and Felix, 2005). *C. elegans* are bacteriovores and are commonly fed the *E. coli* strain OP50 in the lab (Brenner 1974), although other bacterial strains can also be used. Lab cultures can be maintained on agar plates or in liquid culture. The worm's short generation time (approximately 3 days at 20^{o}C), it small size (approximately 1.2mm) and ability to grow at high densities in Petri dishes allow it to be cultured with the ease of micro-organisms such as *E. coli* or *S. cerevisiae*. Worms can also be frozen at -70^{o}C for long-term storage, which is not only convenient, but also allows for the integrity of strains to be preserved, since an aliquot of an original stock can always be thawed to remove any mutations that may have accumulated during subculture in the lab. The short lifespan of *C. elegans* (mean of 18-20 days at 20^{o}C) has led to it being extensively used in ageing research, an application that will be discussed throughout this thesis.

1.2.1. Anatomy

The adult hermaphrodite *C. elegans* contains only 959 somatic cells and has a simple anatomy. The outside boundary of the worm is called the cuticle, which is made up of collagens and is secreted by the hypodermal cells that lie just underneath. The cuticle is tough but flexible, protects the animal and acts as a type of external skeleton to which muscles are attached. *C. elegans* muscles used for movement are striated, whereas the pharyngeal muscles that pump food into the intestine and the

muscles controlling intestinal contraction and defecation are non-striated. Food is taken in through the mouth, crushed and pumped into the intestine by the pharynx (Wormatlas.org (accessed Aug 2010)). The worm's nervous system consists of 302 neurons and their connectivity has been extensively investigated by electron microscopy (White et al., 1986). The intestine is a relatively large tissue that runs from the pharynx to the anus at the tail of the worm.

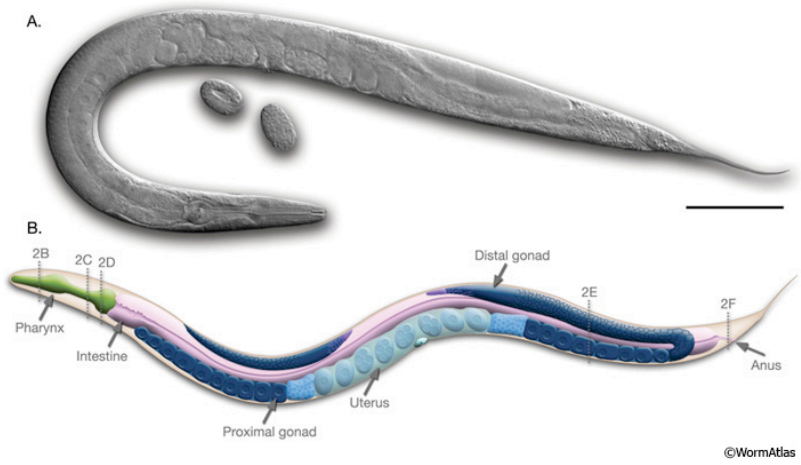


Figure 1
from Wormatlas.org (accessed Aug 2010)
A. Differential interference contrast (DIC) image of an adult hermaphrodite. Scale bar 0.1 mm.
B. Schematic drawing of *C. elegans* anatomy. See Figure 2 for cross sections at the levels 2B, 2C, 2D, 2E and 2F.

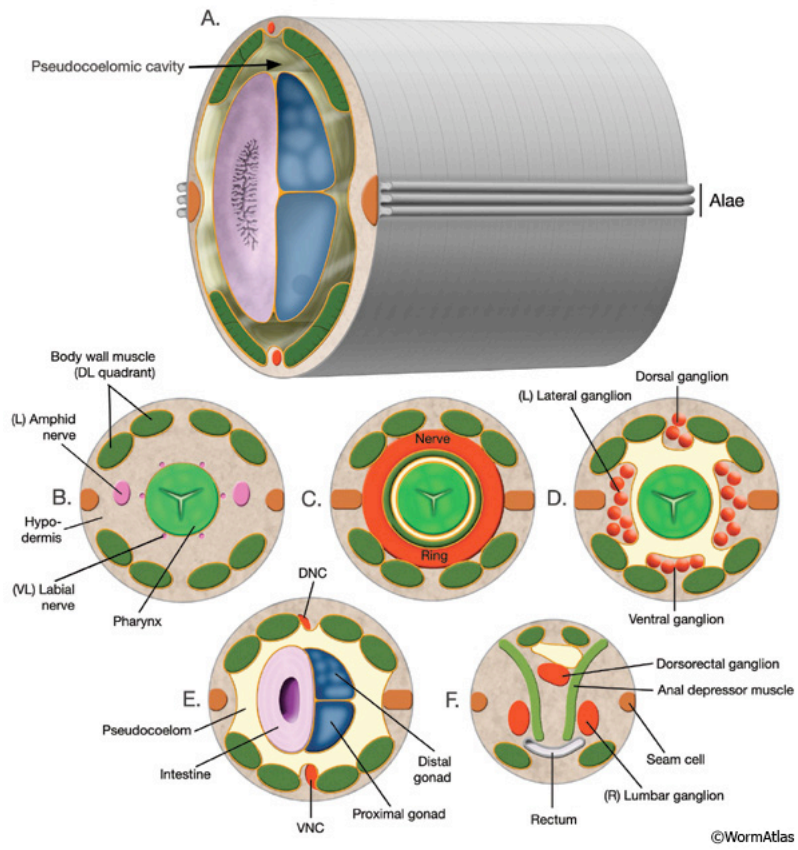


Figure 2
from Wormatlas.org (accessed Aug 2010). **A.** cross-section at level 2A in Figure 1; **B.** cross-section at level 2B in Figure 1; **C.** cross-section at level 2C in Figure 1; **D.** cross-section at level 2D in Figure 1; **E.** cross-section at level 2E in Figure 1; **F.** cross-section at level 2F in Figure 1

1.2.2. Life cycle

After hatching and under favourable conditions, *C. elegans* goes through four larval stages (named L1, L2, L3 and L4), each separated by a moult in which the collagen cuticle is shed and replaced (see Figure 3). Development from egg to reproductive adult takes approximately three days but varies with temperature. Unfavourable conditions, such as high temperature, low food availability or high population density lead worms to develop into an alternative third stage, known as the dauer larva. Under these conditions, L1s moult into an alternative L2 stage called L2d and then develop into dauers unless conditions improve. Dauer larvae are specialised in long-term survival and were named from the German verb meaning "to endure". They have thick protective cuticles, large lipid stores, are non-feeding, have an occluded mouth to protect against entry of toxic substances, and are highly

resistant to stress (Cassada and Russell, 1975). The length of time worms remain in the dauer state does not affect their post-dauer lifespan (Klass and Hirsh, 1976), possibly because advancing into the adult stage allows them to rejuvenate (Houthoofd et al., 2002).

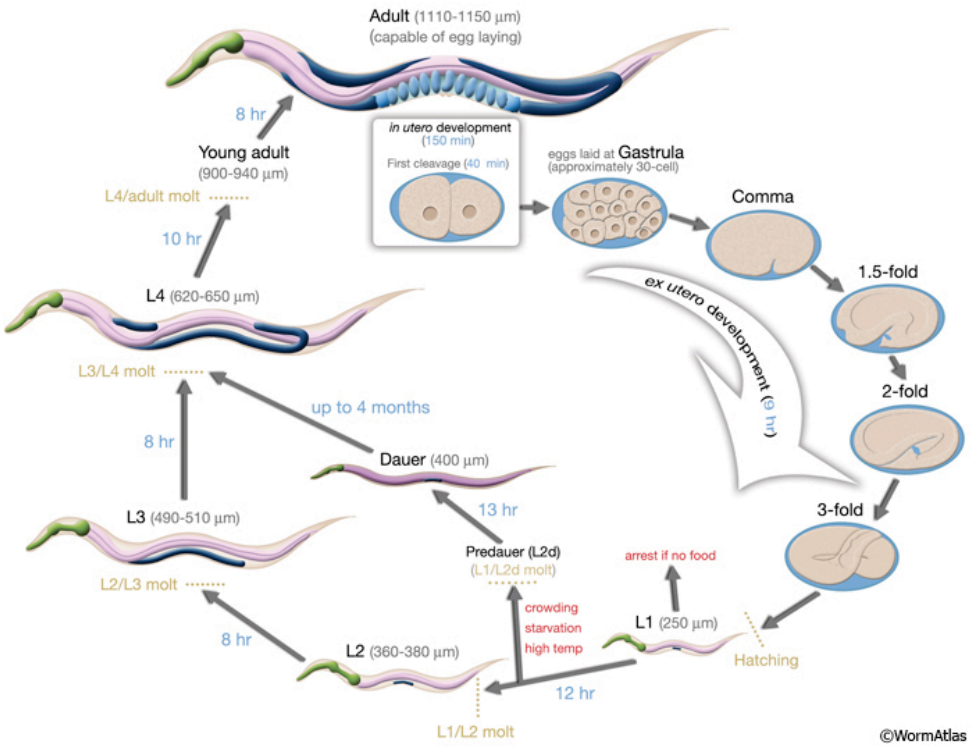


Figure 3
Life cycle of *C. elegans* at 22°C.
From Wormatlas.org (accessed Aug 2010). Development times indicated in blue next to arrows. Size of worms indicated in gray.

The entire cell lineage of *C. elegans* has been determined, making it a powerful organism to study development (Sulston and Horvitz 1976). This is aided by the fact that worms are transparents and epi-fluorescence microscopy can therefore be used to track individual cells expressing fluorescent proteins.

1.2.3. GFP reporters

The transparency of *C. elegans* allows the study of gene regulation through the expression of fluorescent reporter genes introduced via transgenesis. In most cases, a construct is made in which regulatory sequences are fused to the sequence of

GFP (green fluorescent protein), although other fluorescent proteins can be used. This construct is then micro-injected into worms, which creates a multi-copy extrachromosomal array expressing GFP from the regulatory elements included in the construct. This technique is in widespread use and projects aimed at producing a GFP reporter for every gene in the genome are underway (Dupuy et al., 2007; Dupuy et al., 2004).

Because most regulatory elements in the compact *C. elegans* genome are thought to occur within several kilobases of the start codon (Boulin et al., 2006), between 500bp and 2kb of upstream sequences fused to GFP are ofen sufficient to investigates a genes' transcriptional regulation. Constructs in which GFP expression is driven only by upstream sequences are called transcriptional reporters. The limitations of these constructs must be kept in mind, since GFP reporters can only re-iterate the expression of endogenous genes if all important regulatory elements are present. Many regulatory elements are often found within introns, especially large introns, and microRNAs can regulate expression via a gene's 3' UTR (un-translated region). A gene's coding and intronic sequences (and with a few additional cloning steps, the 3' UTR) can also be included in the reporter construct to try to mimic endogenous expression more closely. These reporters are called translational reporters. If the gene's coding sequence is fused to GFP, then this leads to the expression of a GENE::GFP chimeric protein, which is often functional and can therefore be used for rescue experiments and to investigate the effects of over-expression. GFP fusion proteins can also be useful in ascertaining a protein's sub-cellular localisation.

1.2.4. *C. elegans*: a genetic model organism

C. elegans has two sexes, which are determined by the presence or absence of a second copy of the X chromosomes: XX animals are hermaphrodites and XO animals are male. Hermaphrodites are self-fertilising but cannot cross-fertilise with other hermaphrodites. They produce both sperm and eggs, but are protandrous, meaning that their male function precedes the female function, so sperm produced during development becomes limiting during reproduction (Hodgkin and Barnes,

1991). Males are usually a rare occurrence, with a frequency of about 0.1% in progeny from a self-fertilised hermaphrodite. After mating, the progeny is 50% male, since half will not inherit an X chromosome from their fathers. Males are shorter and thinner than hermaphrodites and are easy to recognise through their characteristic tail structures.

The hermaphrodites' ability to self-fertilise makes growing isogenic populations very easy, which makes *C. elegans* particularly useful for carrying out genetic screens. When needed, males can be used to cross mutant strains with each other in order to create ones carrying multiple mutations, an important requirement for epistasis analysis.

This has made *C. elegans* a very important genetic model organism through the use of forward genetic screens. Mutations in the genome can be generated using, for example, X-ray irradiation or exposure to ethyl methane sulfonate (EMS). This allows for the investigation of the genes responsible for any biological trait. After mutagenesis, usually by EMS, animals with defects are identified and the mutations that cause the defects are mapped to a genomic locus. For example, genes responsible for egg laying could be identified by screening for mutants incapable of ejecting their eggs. After mapping, the genomic loci identified can be cloned, sequenced and their gene product can therefore be identified, which would then be known to play some role in egg laying. If several genes are known to affect the same phenotype, then epistasis analysis can be used to organise these genes into pathways. This approach has been used extensively and a very large number of mutants are now available through a central stock centre (Caenorhabditis Genetics Center).

The genome sequence of *C. elegans* was published in 1998 (*C. elegans* genome sequencing consortium, 1998) and has since been steadily improved. The genome is 102Mb long and currently thought to contain approximately 20300 protein-coding genes, or approximately 24600 if one includes alternative splice variants, although these figures are constantly being revised (wormbase.org, 2010).

While *C. elegans* was initially used primarily as a model organism for forward genetics, the availability of its genomic sequence has provided other opportunities. Possible functions of previously unexamined genes can be identified by sequence homology and the role of these genes can then be examined using deletion mutants. Extensive efforts are currently underway to obtain deletion mutants for all *C. elegans* genes by the *C. elegans* Knockout Consortium (US-Canadian

collaboration; celeganskoconsortium.omrf.org) and the National Bioresources Project (in Tokyo, Japan; www.shigen.nig.ac.jp/c.elegans). RNA interference is also being extensively used for studying the function of 'candidate' genes. Using the genome sequence, large libraries of RNAi clones were created, which target most *C. elegans* genes. The mechanisms and use of RNAi will be discussed in more detail later in this chapter. Large-scale RNAi screens, even full genome screens, have now mostly replaced forward EMS screens. However, recent advances in *C. elegans* whole genome sequencing, which allow for much faster pinpointing of mutations, may lead to a resurgence of classical forward genetics (Hobert, 2010).

1.2.5. Nomenclature

As in any field of research, the worm research community has adopted an exact nomenclature, which will be adhered to in this thesis. Genes are given names consisting of three to four letters, a hyphen, and an Arabic number and are always written in italicized lower-case letters. The proteins these genes code for have the same name, but are written in capital non-italicized letters. For example, the gene *egl-9* codes for the EGL-9 protein. The three or four letter abbreviation can refer to the phenotype observed in mutants of the gene. For example, *egl-9* mutants were identified because they are "EGg-Laying abnormal". In other cases, the name is derived from the protein product of the wild-type gene, such as *aak-2* for a subunit of the AMP-Activated protein Kinase. Phenotypes are described by three letter codes as well, but only the first letter is capitalised, so *egl-9* mutant animals can be described as appearing "Egl".

Unique names are given to alleles using a letter code, which refers to the lab they were generated at, followed by a number. If the Gems lab (allele code *wu)* produced a novel mutation that affects the *egl-9* gene, then the genotype of these worms would be written with the allele number (eg. *wu4321*) in brackets next to the gene name: *egl-9(wu4321)*. Transgenes are also given names based on the lab designation, but the letters "Ex" are added if the transgene is present as an extrachromosomal array, "Is" if it is integrated in the genome, and "Si" if it was inserted using the MosSci homologous recombination method. A number is added to create a unique allele name and the contents of the transgene can be written in square

brackets following the allele name. For example, an extrachromosomal array made by micro-injections of an *egl-9::gfp* reporter construct in the Gems lab would create the *wuEx987[egl-9::gfp]* transgene. If crossed into the *egl-9(wu4321)* genetic background, the genotype of the resulting strain would be written as *wuEx987[egl-9::gfp] egl-9(wu4321).*

Because of the extensive use of mutants in various genes in *C. elegans* research, a shorthand for refer to them is to simply state the gene name to refer to animals in which this gene is mutated. Thus, in this thesis, I will often use statements such as "*daf-2* animals were found to be long-lived" instead of specifically saying "animals with a mutation in the gene *daf-2* were found to be long lived". I will only do this when the specific allele being used has already been mentioned earlier in the text.

1.2.6. The use RNA interference (RNAi)

As described earlier, the first genetic studies of *C. elegans* employed forward genetic screens. Ever since the *C. elegans* genome was sequenced, and the phenomenon of RNAi was discovered (Fire et al., 1998), researchers have been able to knockdown transcript levels of any given gene, a much quicker approach to examine the phenotypic consequences of gene inactivation and to study gene function.

1.2.6.1. The RNAi mechanism

RNAi refers to the phenomenon where cells exposed to dsRNA respond by silencing genes with identical sequences. The bulk of this effect is achieved by a post-transcriptional mechanism in which the dsRNA is cleaved into small interfering RNAs (siRNA) by the enzyme Dicer. These siRNAs, once bound to an RNA-induced silencing complex (RISC), can base-pair to cognate mRNAs in the cell. An enzyme within RISC then cleaves the bound mRNA (see Figure 4), thus initiating its degradation (briefly reviewed in Montgomery, 2006). Although RNAi is mostly thought of as a post-transcriptional mechanism, some transcriptional effects of RNAi have also been identified (Grishok et al., 2000; Vastenhouw et al., 2006). The mechanism is thought to exist as a defence against threats such as retroviruses and

other invasive genetic elements (Ketting et al., 1999; Mourrain et al., 2000). After its discovery, researchers began to take advantage of this innate mechanism in order to specifically knock down expression of the worm's own genes. This is done by introducing dsRNA copies of parts of the gene's sequence, thereby triggering an RNAi response that degrades mRNA copies of the gene. RNAi quickly became a widespread method for quick and easy analysis of gene function in *C. elegans* and other systems.

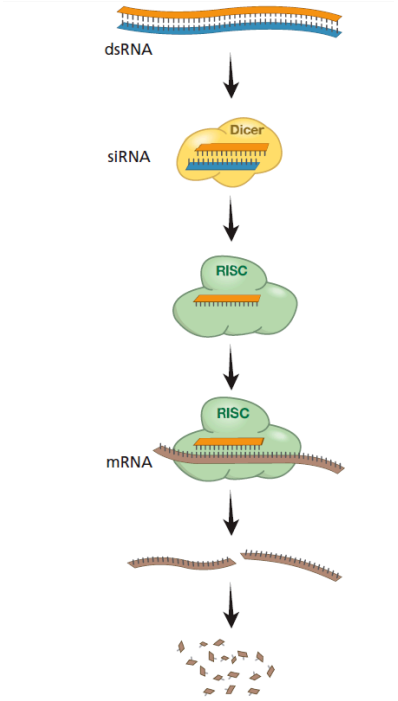


Figure 4
A schematic of the RNAi mechanism. RISC stands for RNA-induced silencing complex (nobelprize.org, accessed July 2010)

There are several methods to expose worms to the dsRNA that triggers RNAi. Initially, researchers injected the dsRNA into adult worms (Fire et al., 1998), but it was later found that soaking them in solutions of dsRNA has the same effect (Tabara et al., 1998). Even more usefully, *C. elegans* can be fed a strain of *E. coli* that has been specifically engineered to produce large amounts of dsRNA and this too leads to the knockdown of the gene in question (Timmons and Fire, 1998). A sequence specific to the gene in question is cloned into the L4440 vector and transformed into the *E. coli* strain (HT115), engineered to be RNase deficient. Effective RNAi mediated knockdown of gene expression has also been achieved through expression of dsRNA via transgenesis in worms (Tavernarakis et al., 2000).

1.2.6.2. Differences between RNAi and genetic loss of function alleles

One of the major differences between loss of gene function via RNAi or genomic mutations is that the effects of RNAi can be more variable and usually resemble hypomorphic rather than null alleles. Some genes are more difficult to target, such as those coding for proteins with long half-lives, as sufficient protein may still be present despite efficient knockdown of transcript copies (Kamath and Ahringer, 2003). In addition, the effectiveness of RNAi differs for different tissues, with neurons being particularly refractory to the effects of RNAi (Timmons et al., 2001) although this problem can be partially overcome through the use of an *rrf-3* mutant background (and a few others), which enhances the effects of RNAi (Sijen et al., 2001).

When using RNAi, the possibility of cross-reactivity with other genes should also be considered. Because gene silencing is sequence-specific, other genes with sequence similarity to the target gene may be silenced along with the targeted gene. How much similarity between genes is required for cross-reactivity to occur is uncertain. A general rule of thumb is to avoid more than 80% nucleotide identity over 200bp of recognition sequence (Fraser et al., 2000), although one recent study found that off-target effects occur when mRNA sequences contain 95% identity over as little as 40bp (Rual et al., 2007). It is therefore important to confirm RNAi phenotypes, preferably by comparing them to the phenotypes caused by deletion mutants.

1.2.6.3. The use of RNAi for large scale screens

RNAi is typically used as a quick alternative to screening for deletion mutants in reverse genetics experiments. While screening for a deletion mutant of a sequence of interest is very time-consuming, an RNAi culture for any given sequence can be created much more quickly. This has allowed the creation of RNAi libraries that contain RNAi clones targeting a large proportion of the *C. elegans* genome (Kamath and Ahringer, 2003; Rual et al., 2004), which greatly facilitates carrying out large scale RNAi screens.

Full genome screens have therefore been carried out to identify new players in a number of different processes. This approach is similar to the one used in classical forward genetic screens, except that instead of using a mutagen to identify new alleles exhibiting the coveted phenotype, worms are exposed to RNAi against a large number of genes to identify ones with the desired effect. Examples for large scale RNAi screens are numerous and range from those looking for genes required for the first two rounds of cell division (Sonnichsen et al., 2005) to screens aimed at understanding the regulation of ageing. One set of screens attempted to search for novel regulators of ageing by looking for RNAi clones that extend lifespan (Hamilton et al., 2005; Hansen et al., 2005). Another one aimed at identifying genes involved in mediating the extended lifespan of *daf-2* mutant animals by looking for clones that decreased the extended lifespan of *daf-2* (Samuelson et al., 2007).

The method of knocking down gene expression by RNAi has penetrated every area of *C. elegans* research. While the sequencing effort has revealed a large number of genes, the function of most has not been characterised. Large-scale RNAi screens are helping the *C. elegans* research community bridge this gap by identifying novel functions for many of these genes and hopefully contributing greatly to our understanding of biology.

1.3. Ferritin

In this section, I will briefly introduce the function of the iron storage protein ferritin and its regulation. This introduction is required because most of my work during the course of this PhD has been centred on understanding how a *C. elegans* ferritin gene, *ftn-1*, is transcriptionally regulated. My focus on this gene in particular will be explained in later chapters, but is based on the finding that *ftn-1* is very strongly regulated by insulin/IGF-1 signalling (IIS) in a *daf-16* -dependent manner. I have therefore used a reporter strain that expresses GFP from the promoter of *ftn-1* as a readout for IIS signalling.

1.3.1. Introduction to vertebrate ferritin and ferritin regulation

Very few studies have investigated the role of ferritins in *C. elegans*. Most of our knowledge on the function of this protein is derived from studies carried out in vertebrate cell lines. Before discussing ferritin in *C. elegans,* I will therefore briefly introduce functional studies of ferritin in vertebrates.

1.3.1.1. Ferritin and iron homeostasis

In vertebrates, the cytosolic form of ferritin consists of 24 subunits of either H or L (heavy- or light-chain) ferritin, which together form the 450kDa apoferritin molecule that can store up to 4500 atoms of iron in its hollow shell. The ratio of H to L ferritin is highly variable and differs from tissue to tissue as well as under different conditions (Torti and Torti, 2002). H ferritin contains a ferroxidase centre, which has the capacity to convert Fe(II) to Fe(III) when the iron atom enters the complex's core (Lawson et al., 1989). This ferroxidase centre is highly evolutionarily conserved.

Iron availability is required for a number of different cellular processes due to its crucial role in many enzymes. Processes that require iron include the cell cycle, electron transport, the production of deoxyribonucleotides, cellular detoxification (Hentze et al., 2004) and many others. On the organismal level, the iron in heme is of critical importance in the transport of oxygen. However, high levels of free intracellular iron is harmful to the cell, particularly due to iron's role in the Fenton reaction, which generates hydroxyl radicals from hydrogen peroxide:

$$Fe\ (II) + H_2O_2 \rightarrow Fe\ (III) + OH^- + OH^{\cdot}$$

The maintenance of iron homeostasis is therefore of great importance in the cell and ferritin, with its ability to store free iron, is capable of reducing free iron levels and thereby controlling the cell's pool of labile iron (Picard et al., 1998; Torti and Torti, 2002).

1.3.1.2. Post-transcriptional regulation in response to iron

In vertebrates, the requirement for tight regulation of labile iron levels is met with a well-defined mechanism for post-transcriptional regulation of several proteins involved in iron homeostasis. Two proteins with opposite roles, ferritin, which stores free iron, and the transferrin receptor, which imports iron from outside the cell, will be used here to illustrate this mechanism, which also affects translation of other players in the maintenance of iron homeostasis. When cellular iron levels are low, ferritin levels also need to be kept low, as more ferritin would take up the little remaining free iron. At the same time, levels of the transferrin receptor need to be increased in order to import more iron into the cell. Low iron levels would therefore be expected to lead to increased expression of the transferrin receptor and decreased levels of ferritin, which in fact has been observed ((Torti and Torti, 2002).

The regulation of these two proteins in response to iron is achieved post-transcriptionally by the actions of iron regulatory proteins (IRPs), which bind to iron responsive elements (IREs) in the mRNAs of the two genes in vertebrates. Both IRPs (IRP1 and IRP2) are active only when iron levels are low. IRP1 is only capable of binding IREs when it has assumed an open configuration, which it does when it loses the iron atoms in its iron-sulfur cluster. The IRP2 protein is degraded in conditions of iron excess. Binding of IRPs to IREs has opposite effects on the mRNAs of ferritin and the transferrin receptor. IRPs bind to IREs on the 3' UTR of the transferrin receptor mRNA and enhance the mRNA's stability, leading to increased levels of the receptor being translated. The ferritin mRNA contains an IRE in its 5' UTR and here, binding of the IRP prevents translation. When iron levels are low, high levels of IRP therefore inhibit ferritin translation, but enhance transferrin receptor translation (see Figure 5 and (Torti and Torti, 2002).

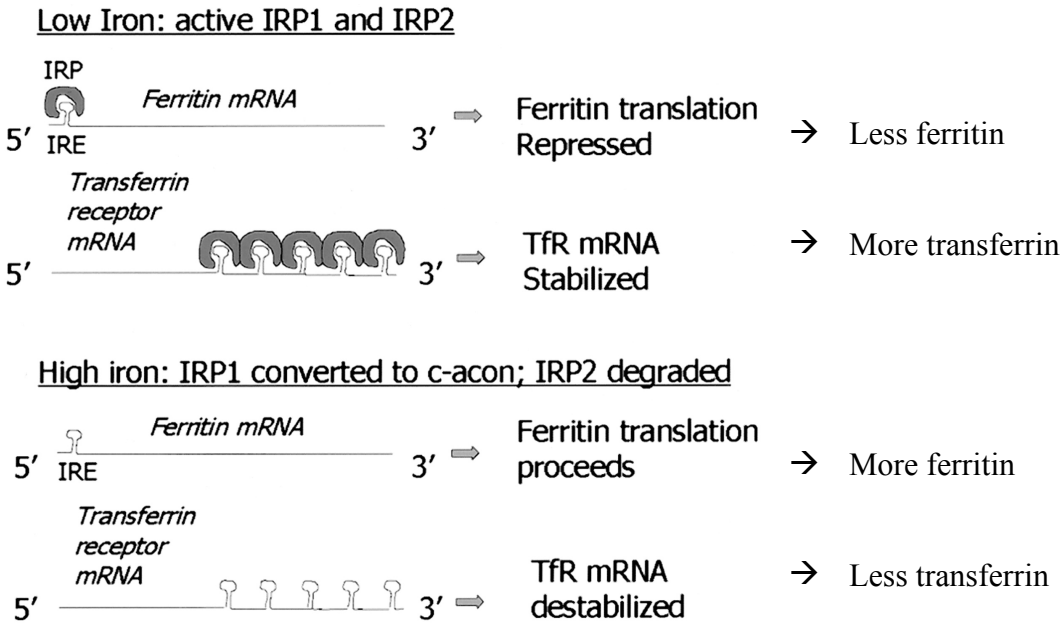


Figure 5
The post-transcriptional regulation of iron-responsive genes by IRPs. from (Torti and Torti, 2002)

In the case of ferritin, high iron levels lead to loss of binding of IRPs to the IREs in the 5' UTR and subsequent recruitment of the ferritin transcript from monosomes to polysomes (Rogers and Munro, 1987).

1.3.1.3. Transcriptional regulation of ferritin

In contrast to the post-transcriptional regulation of ferritins described above, the study of the transcriptional regulation of ferritins is much more recent and therefore a lot less well defined. It seems that ferritin transcripts are regulated in response to a number of different cues (Macdonald et al., 1994) and seems to be responsive to stress, which supports the view of ferritins as cytoprotective proteins. H ferritin regulation in response to stressors probably occurs through the binding of two stress-responsive transcription factors to an electrophile response element (EpRE) upstream of its coding sequence (Pietsch et al., 2003; Tsuji, 2005). The core sequence of the EpRE, thought to be involved in the regulation of many antioxidant genes, is 5'-TGAnnnnGC-3' (Zhang and Forman, 2010). Other regulatory responses are less well understood, such as the links between ferritin transcription and hypoxia

as well as that with cancer. In the case of cancer, it seems that both cMyc (Wu et al., 1999), which regulates cellular growth and proliferation, and the adenoviral oncogene E1A (Tsuji et al., 1993), repress ferritin expression. It is thought that iron may be a limiting factor in cellular proliferation due to its role in the synthesis of deoxynucleotides, which could explain why genes that induce proliferation would do so partly by increasing iron availability by decreasing ferritin levels. This section will briefly review the literature on the subject of transcriptional regulation of vertebrate ferritins.

1.3.1.3a Transcriptional effects through the EpRE

H and L ferritin is strongly transcriptionally up-regulated in response to a number of different oxidants and pro-oxidant xenobiotics in both humans and mice (Bosio et al., 2002; Pietsch et al., 2003; Tsuji, 2005; Tsuji et al., 2000). This induction by at least some stressors has been shown to be mediated by the two transcription factors JunD and Nrf2 through an EpRE present upstream of the human and mouse H-ferritin genes (Pietsch et al., 2003; Tsuji, 2005). The control of ferritin expression by this EpRE was initially discovered in mouse cells while searching for the mechanism by which mouse ferritin H is downregulated when cells are transformed with the adenovirus E1A oncogene (Tsuji et al., 1993). A 75bp region 4.1kb upstream of the ferritin H gene was found to be mediating the repression by E1A (Tsuji et al., 1995) and this region was found to contain an EpRE.

Nrf2 binds to the EpRE and is considered to be a mediator of the transcriptional activation of phase II detoxification enzymes in response to a number of different stressors (Itoh et al., 1997; Nguyen et al., 2000; Venugopal and Jaiswal, 1996, 1998). Nrf2 knockout mice develop normally (Chan et al., 1996), but seem to be sensitive to environmental stress (Rangasamy et al., 2004; Rangasamy et al., 2005) and have decreased ferritin H expression (Thimmulappa et al., 2002). Activation of Nrf2 by a number of different inducers occurs by the dissociation of Nrf2 from the cytosolic actin-binding protein Keap1 and the subsequent nuclear translocation of Nrf2 (Itoh et al., 1999; Zipper and Mulcahy, 2002).

JunD has been shown to bind the EpRE upstream of the human H ferritin gene and is also thought to be involved in mediating a cytoprotective response

(Gerald et al., 2004). The JunD protein is phosphorylated in response to H_2O_2 and t-BHQ treatment (Tsuji, 2005) and this phosphorylation may recruit the transcriptional co-activators p300/CBP and thus transcriptionally activate ferritin H through the EpRE (Tsuji et al., 1999). The role of both Nrf2, a transcription factor known to regulate phase II detoxification enzymes, and JunD, which may be involved in protection against ROS (Gerald et al., 2004), in the regulation of ferritin H supports the view that ferritin may be regulated in response to environmental stressors and may have a cytoprotective role in the cell. The transcriptional regulation of ferritin is summarised in Figure 6.

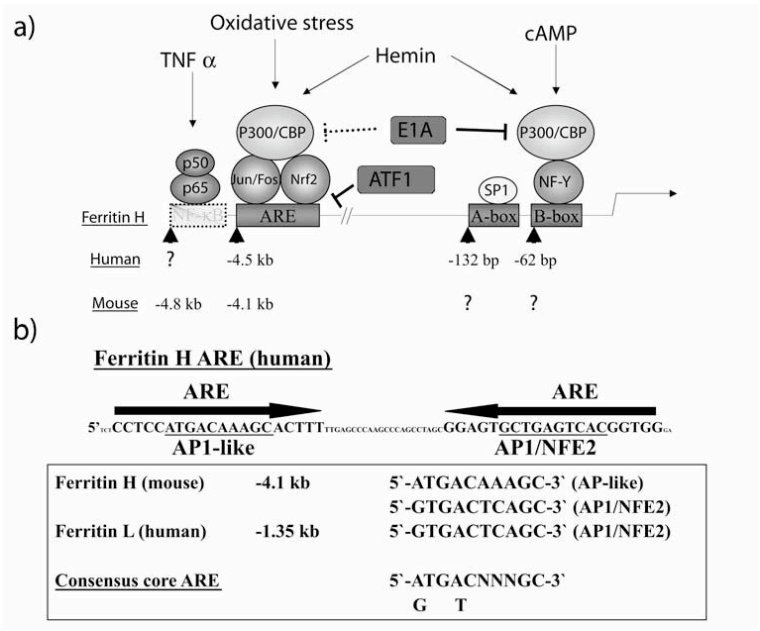


Figure 6
This figure depicts a number of different transcription factors thought to regulate H-ferritin in mice and humans. ARE refers to the "anti-oxidant response element", also termed the "electrophile response element" EpRE (Mackenzie et al., 2008).

The EpRE seems to also be the target of transcriptional repression human ferritin H expression by the transcription factor ATF1. The mechanisms for this repression are unclear, though it is possible that this occurs by preventing the binding of PIAS3, a potential activator of ferritin H (Iwasaki et al., 2007). The regulation of human L ferritin has been less well characterised, but it seems that the gene is induced by tBHQ, sulforaphane (an electrophilic isothiocyonate), hemin, and high

levels of iron through the EpRE sequence present 1.35kb upstream of the gene (Hintze and Theil, 2005).

1.3.1.3b Post-transcriptional effects

Oxidative stress can act on ferritin levels through its effect on IRPs. Reversible oxidation of IRP1 at cysteine residues can reduce binding to IREs as was shown in rat liver lysates exposed to H_2O_2 and O_2^- (Cairo et al., 1996). However, the opposite effect was seen in murine fibroblasts, in which exposure to H_2O_2 led to the activation of IRP, leading to reduced ferritin translation (Pantopoulos and Hentze, 1998).

It is possible that exposure to oxidative stress may lead to increased ferritin levels by releasing iron from iron-containing proteins. This could lead to iron-dependent increases in ferritin levels by both the post-transcriptional and transcriptional route. Increases in cellular labile iron levels would lead to decreased levels of IRP and increased ferritin translation and have also been shown to lead to the transcriptional induction of ferritin mRNA levels (White and Munro, 1988).

Multiple studies have also demonstrated that ferritin levels increase after exposure to a hypoxic environment (Cheepsunthorn et al., 2001; Chi et al., 2000; Qi and Dawson, 1994; Qi et al., 1995). There is evidence for both transcriptional and post-transcriptional regulation. In neonatal rat oligodendrocytes and human oligodendrogliomas, ferritin levels were induced by hypoxia, but H ferritin mRNA levels were unaffected (Qi and Dawson, 1994; Qi et al., 1995). A rat model for hypoxia also showed induction of ferritin levels, with an increase in both protein and mRNA levels. (Chi et al., 2000)

At least part of the response to hypoxia is mediated by altered binding of IRPs to IREs. In rat hepatoma cells, IRP1 binding activity decreased under hypoxia (Hanson and Leibold, 1998) and in mouse macrophages, hypoxia was accompanied by a decrease in IRP1 and an increase in ferritin levels. However, an increase in IRP2 activity was also detected in the macrophage cell lines in a different publication (Hanson et al., 1999). In human cell lines, hypoxia was found to induce IRP activity and decrease ferritin protein levels (Toth et al., 1999).

These effects of hypoxia on ferritin levels are not consistent with some of my own findings in *C. elegans*. During the course of my work on the transcriptional

regulation of *ftn-1*, I uncovered a major role of hypoxia signalling, although the effect seems to be the opposite of what one would predict from the findings in vertebrate systems. This will be discussed in depth in Chapter 5.

1.3.1.3c Hormonal regulation of ferritin

Insulin and IGF-1 are also thought to be involved in the regulation of ferritin. In C6 glioma cells, both insulin and IGF-1 induced H and L ferritin mRNA levels. In rat insulin-secreting pancreatic cells, glucose was shown to cause a strong induction of H ferritin mRNA and a strong decrease in the levels of L ferritin mRNA. Overall ferritin protein levels were increased in response to glucose in these same insulin-secreting cells. (Macdonald et al., 1994)

Insulin signalling has long been linked to iron levels due to the association between type II diabetes and high levels of iron stores, measured by proxy through serum ferritin levels. It was even found that frequent blood-letting, which decreases iron stores, was associated with a decreased incidence of type II diabetes in humans (Ascherio et al., 2001) and rats (Yale et al., 1988). In humans, blood letting also was shown to increase insulin sensitivity (Fernandez-Real et al., 2002). The causes for this possible link between iron and type II diabetes are unknown.

Whether there truly is a role for ferritin and iron in insulin signalling is not understood. The finding, described in Chapter 2, that mutants with reduced insulin/IGF-1 signalling (IIS) show greatly elevated levels of *ftn-1* mRNA demonstrates that an interaction between IIS and ferritin regulation exists in *C. elegans*, although it does not help explain how lower iron levels could lead to greater insulin sensitivity. The studies are also not similar enough to be able to draw any conclusions about whether the interaction is conserved: while ferritin was increased in response to glucose in one study, H- and L- ferritin responded differently. In addition, this study was performed in insulin-secreting pancreatic cells and it is unclear what the equivalent cells would be in *C. elegans*.

1.3.1.3d Ferritin and cancer

Alterations of ferritin levels in different types of cancer have been reported but both high and low ferritin levels have been found in tumours (Hazard and Drysdale, 1977) and the molecular mechanisms for the regulation of ferritin in cancer are not well understood (Torti and Torti, 2002).

The adenovirus gene E1A, an oncogene that can reprogram the regulation of cell growth to immortalise cell lines, was found to specifically repress expression of H ferritin, but not L ferritin in mouse fibroblasts (Tsuji et al., 1993). Additionally, transformation of B cells with c-Myc led to a downregulation of ferritin H and induction of IRP2 (Wu et al., 1999). c-Myc has an important role in promoting cell proliferation and transformation and the downregulation of ferritin levels were found to be a requirement for this to occur. Wu et al. found that when H ferritin was re-expressed in three different c-Myc transformed cell lines, a significant decrease in *in vitro* clonogenicity could be observed. This effect could be rescued by the addition of hemin (which carries iron), but not iron-free hemin (protoporphyrin IX), demonstrating that it is the effects of H ferritin on the intracellular iron pool that affect the clonogenicity (Wu et al., 1999). The authors hypothesise that the requirement for iron in the synthesis of dNTPs by ribonucleotide reductase (Cooper et al., 1996; Elledge et al., 1992) could explain this relationship between iron levels and proliferative capacity. However, iron is also required for other processes involved in proliferation (Hoyes et al., 1992; Kulp and Vulliet, 1996).

Further support for the idea of a requirement for low levels of ferritin for proliferation and high levels for growth arrest comes from a series of experiments showing that differentiation and cellular senescence leads to an upregulation of ferritin levels in a variety of systems (Thweatt et al., 1992; Torti et al., 1988). Additionally, overexpression of H ferritin has been shown to strongly reduce cell growth (Cozzi et al., 2000) of HeLa cells. While several pieces of evidence suggest that H ferritin is repressed by c-Myc, a microarray analysis of c-Myc responsive gene did not identify H ferritin as being differentially regulated (Guo et al., 2000).

1.3.2. Introduction to *C. elegans* ferritin

The *C. elegans* genome contains two ferritin genes, *ftn-1 and ftn-2,* which are both considered H-type ferritins due to the presence of ferroxidase centres (Kim et al., 2004). A *C. elegans* gene, *aco-1* was identified as the homologue of IRP-1, but there are a number of reasons to believe that the post-transcriptional mechanisms by which the IRPs regulate ferritin levels in vertebrates are not conserved in *C. elegans*. Beside its function as an RNA-binding protein, mammalian IRP1 is known to function as an aconitase, which catalyzes the reaction between citrate and isocitrate (Eisenstein and Blemings, 1998). While ACO-1 was shown to exhibit aconitase activity and the total aconitase activity in worms shown to be post-transcriptionally regulated by iron, ACO-1 could not be shown to bind to IREs, the 5' UTRs of *ftn-1* and *ftn-2* do not contain consensus IREs and no binding of ACO-1 to their 5' UTRs was detectable, thus arguing against the presence of non-consensus IREs in these sequences (Gourley et al., 2003). Instead, *C. elegans* ferritins seem to be transcriptionally regulated by iron, although *ftn-2* seems to be less inducible than *ftn-1* (Gourley et al., 2003; Kim et al., 2004; Romney et al., 2008).

1.3.2.1. *C. elegans* expression pattern

Using GFP reporters, *ftn-1* seems to be expressed solely in the intestine (Kim et al., 2004; Romney et al., 2008) and is expressed through all life stages beginning at gastrulation (Romney et al., 2008).

There is some ambiguity surrounding the true expression pattern of *ftn-2*, with some authors reporting that a *ftn-2::gfp* construct is not expressed in the intestine (Kim et al., 2004), while others find strong intestinal expression for a similar construct (Romney et al., 2008). The constructs used in the two publications differed in the length of the 5'-regulatory sequence used, which could explain the discrepancy. Romney et al. make a good case for intestinal expression of *ftn-2* by using a histone tagged GFP reporter and presenting microscopy of staged worms of all developmental stages clearly exhibiting nuclear GFP expression in their intestines. They also describe conserved regulatory sequences in the promoter that are sufficient to drive intestinal expression of a different construct. It is possible that without the nuclear GFP localisation, the diffuse fluorescence in the intestinal cells

was difficult to detect in the constructs used by Kim et al., especially given that the intestine is the site of worm auto-fluorescence, which makes intestinal GFP expression hard to detect. Outside of the intestine, *ftn-2::gfp* is reported to be expressed in body wall muscle, pharynx, and hypodermal cells in Kim et al. and in hypodermal cells in Romney et al. The true distribution of endogenous ferritin expression will remain unclear until this is investigated by antibody staining.

1.3.2.2. Regulation of nematode intestinal ferritin expression

Given that the post-transcriptional regulation of ferritins found in vertebrates does not seem to be conserved in worms, the mechanisms by which *C. elegans* ferritins are regulated in response to iron remain to be discovered. Romney et al. attempted to do this by analysing the promoters of *ftn-1* and *ftn-2* in *C. elegans*, as well as their two homologues in *C. briggsae* and looking for conserved sequences. They identified a conserved 63bp element containing two GATA motifs and three direct repeats (DRs). Cloning this sequence in front of a *pes-10* minimal promoter and the GFP-his sequence revealed that these 63bp are sufficient to drive GFP expression in the *C. elegans* intestine. GFP expression from this construct was responsive to iron and mutation of the three DRs abrogated this response, indicating that these 63bp represent an iron-dependent enhancer of nematode ferritin gene expression (see Figure 7). The authors named this sequence the 'iron dependent element' (IDE).

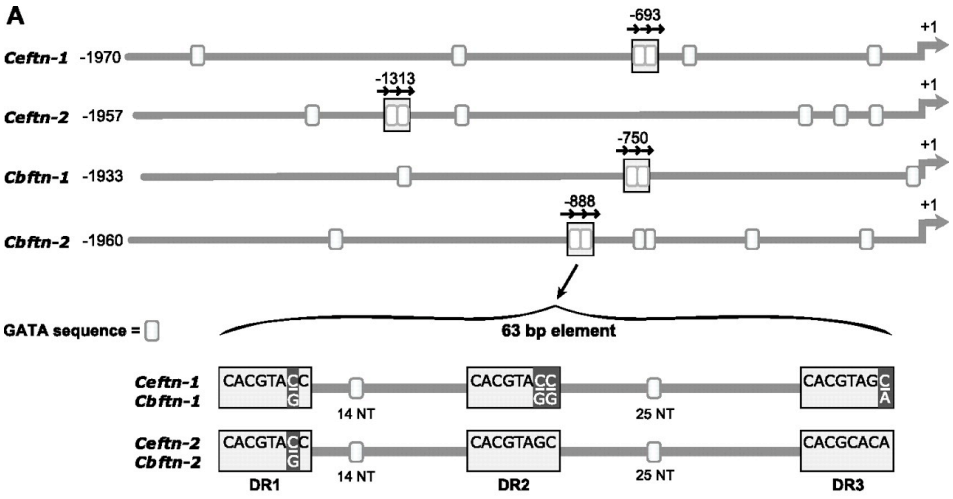


Figure 7
Location of the IDE in the 5′-upstream regions of the *ftn-1* and *ftn-2* genes in *C. elegans* and in *C. briggsae. Open boxes*, GATA motifs; *boxes with three arrows*, 63-bp element with *arrows* depicting three direct repeats (DR1–3). *Numbers* are in reference to the ATG start site (*arrow*, +1). The 63-bp element in *ftn-1* and *ftn-2* is expanded, and the nucleotide differences among the genes are indicated. (Romney et al., 2008)

Due to the presence of two GATA motifs, the authors were able to identify ELT-2 as a regulator of the IDE, which most likely binds to the GATA motifs and specifies intestinal expression. *elt-2* is thought to be the intestinal terminal differentiation factor which binds to all intestinally expressed genes (McGhee et al., 2009).

No transcription factor has so far been identified to bind the DR elements in the IDE, though Romney et al. note that the DR consensus motif shows some homology with the E-box motif, which serves as a binding site for basic helix-loop-helix transcription factors. They hypothesise that an iron-responsive transcription factor may bind to the DR elements and activate gene expression when iron levels are high, leading to increased iron storage capacity in the cell (see Figure 8).

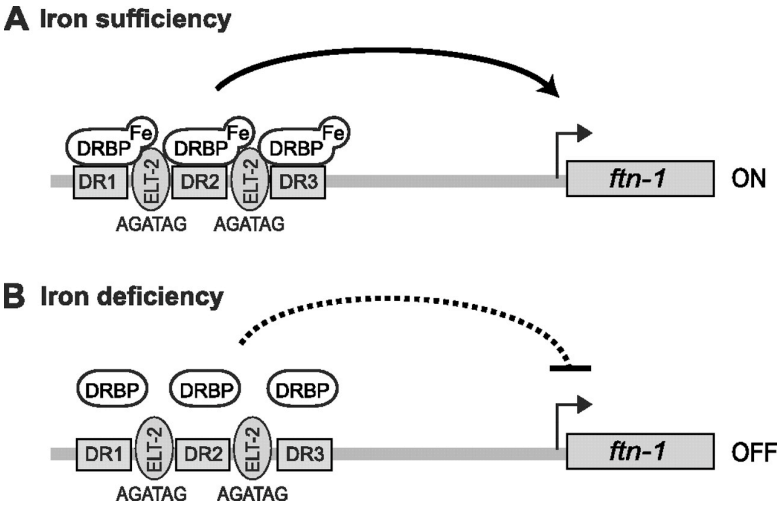


Figure 8
Proposed model for regulation of *ftn-1* transcription by iron through an iron-responsive direct repeat -binding protein (DRBP) (Romney et al., 2008).

1.4. The discovery of long-lived IIS mutants in *C. elegans*

1.4.1. Initial discovery of the role of *daf-2* and *age-1*

As described earlier in this chapter, ageing remains an unexplained biological phenomenon. Given the effectiveness of using genetic model organisms to identify genes responsible for previously unexplained phenotypes, genetic approaches were soon applied to the search for the cause of ageing. While it is clear that loss of function mutations in genes promoting longevity would shorten lifespan, performing genetic screens to identify such genes is tricky, since it is difficult to distinguish mutations in genes promoting longevity from those alleles that cause life shortening pathology unrelated to ageing. Initially, searching for long-lived mutants did not seem like a good idea, since this would require the existence of genes that reduce an organisms longevity, which one would not predict from evolutionary theories of ageing. However, an early EMS screen for long-lived mutants (Klass, 1983) revealed at least one long-lived strain, which was later found to contain a mutation in the gene *age-1* (Friedman and Johnson, 1988). A chance discovery revealed that a mutant of the gene *daf-2* were also long-lived (Kenyon et al., 1993) and the same mutant had already been known to cause a constitutive dauer formation at restrictive temperatures (the so-called Daf-c phenotype). Not all Daf-c mutants tested

subsequently showed an extended lifespan, but a very large effect was found in three different alleles of the gene *daf-2*. Testing other strains that exhibit Daf-c also identified an allele of *daf-23*, which extended lifespan (Larsen et al., 1995), but this allele was later found to be in the same gene previously described as *age-1* (Malone et al., 1996; Morris et al., 1996).

Much work had already gone into epistasis analysis of genes involved in dauer development and it was already known that the Daf-c phenotype of *daf-2* alleles required the presence of the gene *daf-16*, which has a Daf-d phenotype (Daf-d indicates that the strain is incapable, or less capable, of forming dauers than wild-type strains) (Riddle et al., 1981). This led to the finding that the longevity phenotype of *daf-2* mutant animals also requires *daf-16* (Kenyon et al., 1993). Cloning and sequence analysis of *daf-16* revealed *daf-16* to be a homologue of human fork-head transcription factors FOXO3 and FOXO4 (Ogg et al., 1997).

The discovery that single gene mutations in the insulin/IGF-1 signalling (IIS) pathway were capable of having such radical effects on *C. elegans* lifespan was unexpected since it contradicts earlier thinking about how different organisms achieve their rates of ageing. In the evolutionary theory of ageing, an organism's specific rate of ageing is the consequence of a lack of evolutionary selection against mutations that are detrimental late in life, which suggests that changes in the rate of ageing can only come from many generations of selection. The discovery that the rate of ageing can instead be modulated to a great extent by a single pathway represents an exciting departure from this view. As a consequence, much research has since been carried out on how this lifespan extension is achieved. The fact that the lifespan extension is completely dependent on a single transcription factor has given researchers the hope that the cause of ageing can be identified by investigating long-lived *C. elegans* IIS mutants and that this discovery may be within reach.

1.4.2. Other elements of the pathway

The discovery of the extended lifespan of *daf-2* mutants was found to be remarkably robust and reproducible. In one study, 15 different alleles of *daf-2* were tested and were all found to extend lifespan (Gems et al., 1998). The central role of *daf-16* in mediating the effects of reduced IIS was established using different

approaches. In addition to the *daf-16* dependence of the *daf-2* lifespan and Daf-c phenotypes (Gottlieb and Ruvkun, 1994; Larsen et al., 1995), other phenotypes of *daf-2* mutants, such as increased fat storage (Ogg et al., 1997), reduced motility, adult body shrinkage and altered gonad morphology (Gems et al., 1998) also required *daf-16*. A genetic screen using *daf-2; daf-12* (as well as *age-1; daf-12*) double mutants supported this view (Tissenbaum and Ruvkun, 1998). *daf-2; daf-12* animals are known to arrest as L1 or L2 under certain conditions, an effect that is also completely dependent on *daf-16*. This screen of 7000 genomes for suppressors of the *daf-2; daf-12* phenotype resulted in the identification of four alleles. All four mapped to the *daf-16* genomic locus, strongly indicating that *daf-16* is the major target for *daf-2* (Tissenbaum and Ruvkun, 1998).

The discovery that *age-1* codes for a phosphoinositide-3-OH kinase (PI3K) (Morris et al., 1996) led to the identification of further downstream elements in the pathway.

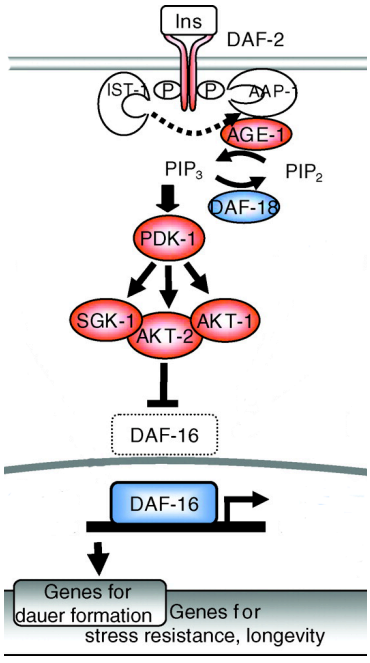


Figure 9
Adapted from (Baumeister et al., 2006)

Akt/PKB serine/threonine kinases had already been found to act as downstream effectors of PI3 kinases in other systems (Toker and Cantley, 1997), so the newly sequenced *C. elegans* genome was used to try to identify homologues in worms. The two genes *akt-1* and *akt-2* were identified and found to have a role in signalling to *daf-16*. The activating mutation *mg144* in *akt-1* was capable of fully

suppressing the Daf-c phenotype of *age-1*, as was the over-expression of *akt-1(+)*. Simultaneous inhibition of *akt-1* and *akt-2* activity led to 100% dauer arrest and this Daf-c phenotype could again be fully suppressed by a null mutation in *daf-16*, demonstrating that the two *C. elegans* Akt/PKB homologues AKT-1 and AKT -2 act redundantly to antagonise DAF-16 (Paradis and Ruvkun, 1998).

An additional member of the IIS pathway was identified in a forward genetic screen for alleles causing a Daf-c phenotype. Loss of function alleles of *pdk-1* extend lifespan and cause a Daf-c phenotype and sequence analysis revealed homology to the mammalian 3-phosphoinositide-dependent kinase-1 (PDK1). In mammals, the phospholipid products of PI3K are required for PDK1 to activate the Akt/PKB kinase via phosphorylation (Paradis et al., 1999). In *C. elegans* the Akt/PKB homologues AKT-1 and AKT-2 are also required for the phenotypes of a dominant activated form of PDK-1. Loss of function of *pdk-1* had many pleiotropic phenotypes, all of which required *daf-16*, further evidence for the important role of *pdk-1* in IIS (Paradis et al., 1999).

In mammalian cells, SGK acts in parallel to Akt/PKB kinases in response to PI3K signalling (Brunet et al., 2001). Mutations in the *C. elegans* homologue *sgk-1* were found to extend lifespan in a *daf-16*–dependent manner and SGK-1, AAK-1, and AAK-2 protein constructs were all found to be capable of phosphorylating DAF-16 in vitro. Loss of activity (by either mutation or RNAi) of PDK-1, SGK-1, AAK-1 as well as AAK-2 lead to the nuclear localisation of a DAF-16::GFP transgene, which strongly supports a role of all four of these subunits in cytoplasmic localisation of DAF-16::GFP (see review of DAF-16::GFP localisation later in this chapter). There is evidence that SGK-1, AAK-1 and AAK-2 form a multimeric complex, which presumable phosphorylates DAF-16 in the cytoplasm thus preventing nuclear localisation (Hertweck et al., 2004). For a summary of how IIS is thought to regulate DAF-16, see Figure 9.

1.4.3. Cell autonomous and non-autonomous effects of IIS

As described in the previous section, traditional forward genetic approaches combined with epistasis analysis identified a pathway with very large effects on the lifespan of *C. elegans* (see Figure 9). Since we do not know what worms typically

die of in old age, it is difficult to predict in which cells the lifespan–promoting effects of reduced IIS may be occurring. This also presents the question of whether IIS acts cell autonomously or non-autonomously, i.e. whether signalling between cells rather than just within cells plays a role or not. Given that the decision to enter into the dauer stage involves responding to environmental cues and that dauer entry is controlled by IIS, it seems likely that intercellular signalling should feed into IIS.

1.4.3.1. Tissue specific expression of IIS transgenes

One approach to identify the tissues in which IIS acts to extend lifespan is to express an IIS gene in specific tissues using transgenes in animals that lack this gene globally. If this tissue–specific expression is sufficient to rescue the mutant phenotype, this provides evidence that IIS is affecting lifespan through its actions within this tissue. This was first attempted in *daf-2* and *age-1* mutants over-expressing wild-type *daf-2* or *age-1* cDNA specifically in either neurons, the intestine or muscle cells. It was found that neuronal expression had by far the greatest effect on lifespan, with a smaller effect also seen upon intestinal expression. This study indicated that it is the activity of IIS in neurons that is extending lifespan (Wolkow et al., 2000). A similar approach by a different group (Libina et al., 2003) involved the expression of a *daf-16::gfp* transgene in *daf-16; daf-2* double mutants in order to identify the tissues in which *daf-16* acts to extend lifespan. This study also supports an important role of neurons and the intestine, although it found the magnitudes were reversed: The greater effect on lifespan followed intestinal instead of neuronal expression. In support of a major non-neuronal role of IIS, it was also found that RNAi-mediated knockdown of *daf-16*, which does not affect neuronal *daf-16* expression, was sufficient to fully eliminate the longevity of *daf-2*. The relative importance of these two tissues in IIS- mediated determination of lifespan still needs to be established and it is possible that different tissues have distinct roles in mediating different functions of the IIS pathway. For example, Libina et al. found that while the lifespan effects of reduced IIS could only be rescued to a very small extent (5%-20%) by neuronal expression of *daf-16::gfp*, the Daf-c phenotype could be fully rescued.

1.4.3.2. Evidence for cell non-autonomous effects of IIS

The study by Libina et al. also took up an additional important question: Do the effects of IIS on lifespan occur cell autonomously or non-autonomously? To address this, they used a GFP reporter of the manganese superoxide dismutase (MnSOD) gene *sod-3* as readout for *daf-16* activity. They found that tissue specific expression of *daf-16* in *daf-16;daf-2* mutants carrying the *sod-3::gfp* transgene increased GFP expression only in the tissue in which *daf-16* was expressed, demonstrating that *daf-16* regulates *sod-3* expression cell autonomously. However, when *daf-16* was expressed in the intestine of *daf-16(+)* animals carrying the *sod-3::gfp* reporter, expression was increased not only in the intestine, but also in other tissues. A similar but slightly weaker effect was found upon neuronal *daf-16* expression. This strongly suggests that DAF-16 activity in one tissue leads to the release of an intercellular signal that regulates DAF-16 activity in other tissues and argues for the existence of cell non-autonomous effects of IIS.

1.4.3.3. Role of sensory neurons

Before the discovery of the important role of the IIS pathway in ageing, many of the genes involved had already been identified as important players in the formation of the dauer stage, a non-reproductive life stage which developing worms can enter when conditions are unsuitable for reproduction. The decision of whether to enter the dauer stage or not depends on a number of environmental cues, so it is clear that mechanisms to sense these cues must feed into the decision. It was soon found that sensory neurons have a role in dauer formation as the ablation of some of them leads to dauer arrest (Bargmann and Horvitz, 1991).

The role of sensory neurons in the determination of lifespan was soon investigated: A very large number of mutations that cause impaired sensory perception were tested and all were found to extend lifespan. The same study also found that laser ablation of the two amphid sheath cells, an intervention that also leads to impaired chemosensation, also extends lifespan (Apfeld and Kenyon, 1999). This effect is presumably due to the loss of a signal secreted from sensory neurons when conditions are appropriate for reproduction, which activates IIS. Loss of this

signal through a change in the conditions would therefore lead to dauer arrest or extended lifespan in adults. The possible involvement of IIS in the lifespan extension of mutants with reduced sensory perception is supported by the finding that loss of *daf-16* mostly abolishes the lifespan effect of five such mutants. The two sensory mutations that were crossed into a *daf-2* mutant background were also not able to further extend *daf-2* lifespan. Similarly, three of these mutants were found to have a weak Daf-c phenotype, which was also completely dependent on *daf-16* (Apfeld and Kenyon, 1999).

More detailed analysis of the role of individual neurons in mediating IIS – dependent lifespan extension was performed using laser ablation. Ablation of ASI and ASG neurons was found to lead to an extension in lifespan. The effects on lifespan by ablation of the ASI neurons is completely *daf-16* dependent, indicating that a signal secreted by ASI neurons may stimulate IIS in responsive cells and that loss of it can extend lifespan via reduced IIS through DAF-16. Interestingly, the lifespan extension by ablation of ASI was found to be dependent on two other sensory neurons ASJ and ASK, indicating that some sensory neurons enhance and others may suppress IIS, although details of these interactions are not known (Alcedo and Kenyon, 2004).

1.4.3.4. *C. elegans* insulin-like peptides

There is clearly strong evidence for the existence of intercellular signalling, which affects IIS and ageing. The fact that the DAF-2 protein, which crowns the IIS pathway, is a receptor similar to the human insulin and IGF-1 receptor, suggests that identifying its ligands could be useful in understanding these interactions. Unfortunately, genetic screens carried out in search for genes involved in dauer formation did not reveal a loss of function mutant in a DAF-2 ligand (they did produce a dominant negative mutation in *daf-28* –discussed later). Bioinformatic analysis of the *C. elegans* genome performed since then revealed a very large number of genes that may code for insulin-like peptides (Pierce et al., 2001). The worm genome is believed to contain 40 such genes and this large number (compared to 7 currently identified in the human genome) suggests that functional redundancy

may be a reason they hadn't been previously identified using forward genetic approaches.

The IIS pathway regulates the developmental decision to enter the dauer stage, a decision made in response to environmental signals such as the presence of a dauer pheromone and the availability of food. Signals from sensory neurons have long been known to be required for this decision because ablation of certain sensory neurons has been shown to lead to dauer arrest (Bargmann and Horvitz, 1991). A possible model for the role of insulin-like peptides in *C. elegans* could involve the secretion of these peptides from sensory neurons under non-dauer inducing conditions to activate IIS in other tissues. When food levels in the environment are low or overcrowding causes elevated pheromone levels, loss of this signal could be the cause for decreased IIS and subsequent dauer arrest. The expression pattern of *ins* genes is consistent with a role for *ins* genes in transmitting these signals: out of 15 *ins* genes investigated in one study, 13 are expressed in at least some sensory amphid neurons. *ins-1* for example, is expressed in the ASI and ASJ neurons, as well as in some other tissues (Pierce et al., 2001).

Possible functions for a small number of these proteins are slowly being identified. Overexpression of the most conserved of the insulin-like genes, *ins-1*, was found to cause a weak Daf-c phenotype and to enhance the Daf-c phenotype of *daf-2* mutants. Another one of the *ins* genes, *ins-18,* was found to have the same effect and even expression of the human insulin gene in *C. elegans* causes Daf-c when driven by the promoter of *ins-1*. However, this result indicates that *ins-1* and *ins-18* act as antagonists of DAF-2, since it is reduced IIS that causes dauer formation (Pierce et al., 2001). As mentioned earlier, the identification of agonists of DAF-2 may be made difficult by functional redundancy among the large number of *ins* genes. However, a semidominant allele *sa191* was identified to cause a Daf-c phenotype and a 10% lifespan extension in an early screen (Malone et al., 1996) and mapped to the gene *daf-28*, which was later found to code for an insulin/IGF-1 –like peptide (Li et al., 2003). The *sa191* variant of DAF-28 is thought to reduce IIS by binding to DAF-2 without activating it, thereby preventing other, functional, agonists from binding.

As one would predict for a mutation that causes decreased IIS, the transient dauer arrest of *daf-28(sa191)* was found to be partially suppressed by mutations in

daf-16 and to cause nuclear localisation of DAF-16::GFP in larvae. The expression pattern of a *Pdaf-28::gfp* reporter were also found to be consistent with a role as an agonist of DAF-2 that is secreted by sensory neurons under reproductive growth conditions. *Pdaf-28::gfp* is expressed in ASI and ASJ neurons as well as other tissues. Expression was also found to be dramatically reduced in dauers and in mutants with impaired sensory neuron signal transduction (DAF-11/guanylyl cyclase mutants). When conditions are inappropriate for reproductive growth, less DAF-28 may be secreted, leading to reduced IIS and therefore dauer formation. The existence of the semidominant *sa191* allele of this putative DAF-2 agonist allowed for a new approach to identifying other agonists using transgenic over-expression. If the phenotype of *sa191* is caused by the binding of inactive DAF-28 to DAF-2, thereby preventing other agonists from binding, then over-expression of other agonists could suppress this phenotype by outcompeting DAF-28. Nine *ins* genes were therefore over-expressed in a *daf-28(sa191)* genetic background and two of them, *ins-4* and *ins-6,* were capable of suppressing Daf-c, indicating that the proteins they code for can functionally replace DAF-28. INS-4, INS-6 and DAF-28 may therefore be agonists of DAF-2 and at least DAF-28 seems to have a role in preventing dauer formation by activating IIS, possibly after being secreted by sensory neurons (see Figure 10).

An additional *ins* gene was identified as a possible DAF-2 agonist through microarray analysis of long-lived *daf-2* mutant animals. *ins-7* was identified as a gene down-regulated in *daf-2* mutants and RNAi –mediated knock-down of this gene led to an increase in lifespan as well as DAF-16::GFP nuclear localisation and the increased expression of a DAF-16 target, consistent with a role as an agonist of DAF-2 (Murphy et al., 2007; Murphy et al., 2003).

Further evidence was subsequently found that INS-7, like DAF-28, may be secreted by sensory neurons to activate IIS in the intestine as well as evidence for the secretion by intestinal cells to activate IIS in other cells (Kawli and Tan, 2008). INS-7 activation of IIS may also be inhibiting innate immune response, since loss of *ins-7* was found to cause a *daf-16*–dependent increase in worms' resistance to infection. *daf-2* mutant animals are also resistant to infection and this is also *daf-16* dependent (Kawli and Tan, 2008). It seems likely that further research into these DAF-2 ligands will shed light into how IIS–mediated phenotypes such as growth and stress response are regulated, how signals are transmitted across tissues and how environmental cues

are processed. This is clearly a prerequisite to understanding organismal ageing in *C. elegans*.

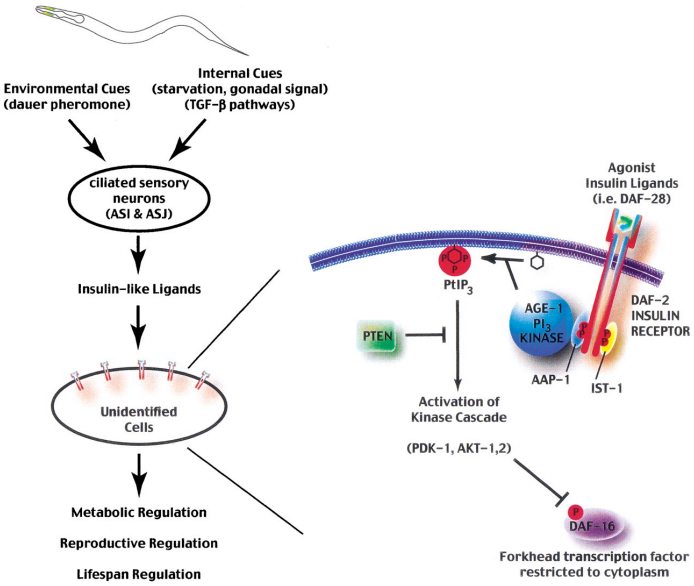


Figure 10
Representation of possible mechanism by which environmental cues could alter IIS signalling. From (Nelson and Padgett, 2003)

1.4.4. Evolutionary conservation of the role of IIS in ageing

Much research has been conducted on IIS mutants of *C. elegans*. This is often justified by pointing to human ageing related diseases, their prevalence, their human cost and their cost on society. Of course research into IIS in *C. elegans* is only relevant to humans and human diseases if the role of IIS in ageing is conserved. There is now fairly strong evidence that this is the case.

Like mutations in *C. elegans daf-2,* mutations in the *Drosophila melanogaster* insulin receptor homologue InR also greatly extend lifespan (Tatar et al., 2001), as do mutations in the downstream insulin receptor substrate *chico* (Clancy et al., 2001). Foxo, the Drosophila homologue of *daf-16,* has also been implicated in longevity assurance, since over-expression of dFoxo in the fat body also extends lifespan (Giannakou et al., 2004; Hwangbo et al., 2004). Of course, for those worried about how research on ageing in a nematode worm could ever be relevant for humans, the finding that the same processes are involved in another far removed organism might not be sufficiently reassuring. Is the role of insulin and IGF-1 signalling in ageing conserved in mammals?

The first suggestion that the effects of IIS on worms may be conserved in mice came from two mutants with hypopituitarism called the Ames and Snell mice. Both were shown to have decreased levels of IGF-1 and to be long-lived (Brown-Borg et al., 1996; Flurkey et al., 2001). More concrete evidence came from the extended lifespan of mice heterozygous for a mutation in the IGF-1 receptor (Holzenberger et al., 2003). These mutants are significantly less long-lived than Ames and Snell mice, which may indicate that the longevity phenotype of hypopituitarism may be caused by more than just decreased IGF-1. Mutants of the insulin receptor substrate Irs1 and Irs2 also exhibit delayed ageing (Selman et al., 2008a; Taguchi et al., 2007), although this second finding is controversial (Selman et al., 2008b). Deletion of the insulin receptor itself specifically in fat tissue was also found to significantly increase mouse lifespan (Bluher et al., 2003).

There is also growing evidence for a role of FOXO transcription factors in human ageing. A genome wide association study using single nucleotide polymorphisms (SNPs) in a group of 1345 participants identified SNPs near the two genes FOXO1A and FOXO3A as being associated with longevity (Lunetta et al., 2007). Other studies have supported the findings regarding FOXO3A using a candidate gene approach, in which presence of genetic variations specific genes was compared between particularly long-lived individuals and younger ones. Variations in five candidate genes, including FOXO3A and FOXO1A, were investigated in a population of Japanese-American men and a strong association was identified between FOXO3A genotype and longevity (Willcox et al., 2008). A similar study investigating only polymorphisms within the FOXO3A gene confirmed this association in German centenarians (Flachsbart et al., 2009). A study genotyping six SNPs in a Han Chinese population also identified an association between the genes FOXO1A and FOXO3A and longevity (Li et al., 2009). An additional study confirmed the association of FOXO3A in further populations and also found that the AKT1 tyrosine kinase, located downstream of insulin/IGF-1 signalling, was also associated with longevity (Pawlikowska et al., 2009).

Another interesting link between IIS in lower organisms and ageing in humans came from an Ashkenazi Jewish cohort, consisting of offspring of particularly long-lived individuals and age-matched controls. This study found that

mutations in the IGF1R gene, which codes for the IGF-1 receptor, were associated with longevity. Biochemical studies of these mutations showed that they led to a partial loss of function, suggesting that decreased IGF-1 signalling might lead to longevity in humans as well (Suh et al., 2008).

As discussed in this chapter and further discussed in Chapters 4 and 6, the mechanisms that regulate *C. elegans* ageing downstream of DAF-16 are still completely unknown and the fundamental processes responsible for ageing are also a complete mystery. However, the role of IIS in affecting longevity seems to be conserved across species, suggesting that findings in worms will be highly relevant in the understanding of human ageing related diseases.

1.5. The DAF-16 transcription factor and the search for target genes

As described above, signalling through the insulin/IGF-1 pathway plays a central role in determining the lifespan of the nematode, with decreased signalling leading to a lifespan extension. Given that the true causes of ageing have so far remained a mystery, it is no surprise that the effects of reduced IIS on ageing have become the focus of intense interest in the ageing field. While studying long-lived worms may seem esoteric to some, they are in fact an ideal model for studying the ageing process. Populations of long-lived and normal lived worms are genetically identical, except for the single mutation in the IIS pathway. If we can therefore understand which of the changes that altered IIS is causing in the worms, such as biochemical changes or changes at any other level of its physiology, are the ones responsible the lifespan extension, then the causes of ageing itself might become clear. Clearly, attempting to understand the differences between wild-type and long-lived mutant animals will be far simpler than say comparing species with different lifespans would be.

In *C. elegans*, the effects of IIS on lifespan require the actions of DAF-16, a FoxO class forkhead transcription factor. Signalling through the IIS pathway leads to the phosphorylation and cytoplasmic localisation of DAF-16 and reduced insulin signalling leads to nuclear localisation of DAF-16. Once in the nucleus, DAF-16 affects transcription of its target genes, which are assumed to extend lifespan, albeit

by unknown mechanisms. Since the effects of this pathway on ageing were discovered, much effort has gone into identifying the targets of DAF-16. Knowledge of how DAF-16 alters the cells' biochemistry could help in understanding how the ageing process itself is delayed and in fact what ageing is caused by, at least in nematodes. What follows is a brief, up-to-date summary of different approaches that have been taken to learn more about how DAF-16 acts in long-lived IIS mutants and also the approaches taken to identify its targets.

1.5.1. The role of different *daf-16* isoforms

Several different *daf-16* transcripts are expressed and some effort has gone into both characterising the expression patterns and the roles of each of the transcripts in *daf-16* –dependent phenotypes. Initially, this work was confined to the *a* and *b* isoforms (Henderson and Johnson, 2001; Lee et al., 2001; Lin et al., 2001), but an additional exon has since been discovered far upstream and a recent publication investigated the role of a third isoform, termed *d/f* (see Figure 11 (Kwon et al., 2010). This nomenclature was used because both a *d* and *f* isoform exist, but the expression patterns cannot be easily distinguished since the two forms have almost identical transcriptional start sites. Similarly, the difference between the two isoforms *a1* and *a2* was also mostly ignored since the predicted protein product only differs by two amino acids. Researchers have therefore focused their efforts on identifying the roles of the three *daf-16* transcripts *daf-16a*, *daf-16b* and *daf-16d/f*.

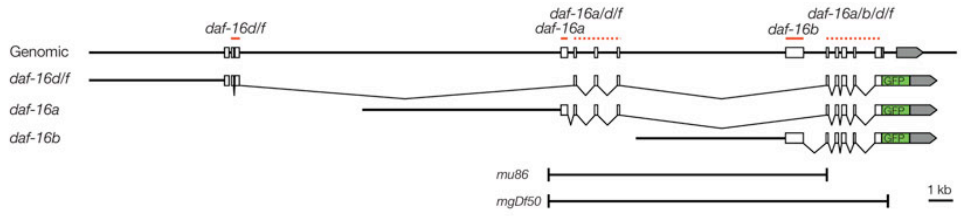


Figure 11
From (Kwon et al., 2010). Shows three different GFP reporters of *daf-16* corresponding to three different isoforms. The transgenes were used in this publication in order to characterise the expression patterns of the three different transcripts.

The nomenclature in the above figure is similar to the one used in the other publications addressing this issue and will be continued here. It should be noted that wormbase.org, the main online resource for *C. elegans* researchers, has now adopted a different nomenclature and also lists the two additional transcripts *daf-16e.1* and *daf-16e.2*. However, for simplicity's sake, I will continue to use the nomenclature used by Kwon et al., and will not be discussing the roles of *daf-16e.1* and *daf-16e.2*, since they have so far not been investigated.

Null mutations of *daf-16* fully abrogate the lifespan extension and Daf-c phenotype of *daf-2* mutants (see Section 1.4.2.) and also reduce *wild-type* lifespan by approximately 20% (Lin et al., 2001). The contribution of each transcript to these phenotypes can be investigated by using mutations in exons absent from some isoforms. Another approach is to use RNAi against these exons, although this can be less specific, since RNAi is known to be capable of affecting pre-mRNAs and therefore knock-down all isoforms of a gene, regardless of which part of the transcript was targeted (Bosher et al., 1999). Using alleles of *daf-16* that do not target the shorter *b* variant and testing the effects on *daf-2* lifespan and dauer formation revealed that expression of a functional *daf-16b* was not sufficient to rescue neither the lifespan nor the Daf-c phenotype of *daf-2* mutant worms (Lee et al., 2001). RNAi knock-down of either *daf-16a* or *daf-16d/f* also revealed that loss of neither of these forms can fully abrogate the extended lifespan of *daf-2* mutants. RNAi of both transcripts almost completely abrogates it, although additional RNAi of *daf-16b* leads to a further slight reduction in *daf-2* mutant lifespan (Kwon et al., 2010).

1.5.1.1. Investigating the role of *daf-16a* by transgene over-expression

The role of *daf-16a* in conferring the extended lifespan of *daf-2* mutants was studied by over-expressing transgenes containing this variant. Because *daf-2* mutant animals are long-lived but the double mutant *daf-16; daf-2* is not, one approach to investigate the role of *daf-16a* specifically is to express a transgene containing *daf-16a* in a *daf-16; daf-2* mutant background. It was found that this almost fully rescued the lifespan back to that of *daf-2* mutants (Kwon et al., 2010; Lin et al., 2001).

In an otherwise *wild-type* genetic background, *daf-16a* over-expression was found to have little or no effect on lifespan in some studies (Henderson and Johnson, 2001; Lin et al., 2001) and cause significant extensions in a different study (Kwon et al., 2010). In a *daf-16(-)* background, *daf-16a* was found to incompletely rescue the shortened lifespan in one case (Lin et al., 2001) but significantly extend lifespan beyond the levels of a *wild-type* strain in others (Kwon et al., 2010; Lee et al., 2001). The smaller effects of *daf-16a* over-expression in a *daf-2(+)* background are probably due to the role of IIS in repressing *daf-16* function. Unless IIS is reduced, the over-expressed DAF-16A presumably remains cytoplasmic. However, *daf-16* mutations shorten lifespan even in a *daf-2(+)* background, suggesting that even when IIS is *wild-type,* DAF-16 cytoplasmic localisation is not complete. This indicates that DAF-16 over-expression should extend lifespan even in *daf-2(+)* animals. One explanation is that only very high levels of over-expression can lead to lifespan extension in *daf-2(+)* and indeed Kwon et al. note that high-copy over-expression of *daf-16a* leads to the greatest lifespan extensions.

The role of *daf-16a* in the Daf-c phenotype of *daf-2* mutants was investigated using a similar approach, since *daf-2* mutant animals are Daf-c, but *daf-16; daf-2* animals are not. *daf-16a* expression was found to fully restore Daf-c in this background (Henderson and Johnson, 2001; Kwon et al., 2010). In a *wild-type* background, *daf-16a* over-expression was found to cause a Daf-c phenotype when worms were grown at very high temperatures. In the same study, over-expression was also shown to cause a slow growth phenotype, which is also seen in *daf-2* mutants, and this phenotype disappeared when worms were grown on *daf-16* RNAi (Henderson and Johnson, 2001). Another study observed no dauer phenotype in *daf-16a* over-expressing animals (Lin et al., 2001).

daf-2 mutant animals are also resistant to a number of different stresses (see Section 6.1.2.) and this resistance also requires *daf-16. daf-16a* expression was found to partially rescue the thermo-tolerance of *daf-16; daf-2* animals (Kwon et al., 2010). In a *wild-type* background an increase in thermo-tolerance has also been observed with *daf-16a* over-expression. A slight increase in resistance to UV was also observed (Henderson and Johnson, 2001).

1.5.1.2. Investigating the role of *daf-16b* by transgene over-expression

daf-16b GFP reporters were found to be expressed in a smaller set of cells than *daf-16a*. One study found expression in the pharynx, some neurons and the somatic gonad (Lee et al., 2001) and another found expression in the pharynx, spermathecae and some neurons (Kwon et al., 2010).

Effects of *daf-16b* on lifespan are modest. Mutation of a *daf-16b* –specific exon in a transgene expressing both *a* and *b* demonstrated that *daf-16b* was not required for restoring the lifespan extension of *daf-2* mutants by this transgene. Expression of *daf-16b* in a *daf-16* mutant background only extended lifespan by 14% compared to 65% when *daf-16a* was expressed (Lee et al., 2001). A similarly small role of *daf-16b* was also found in dauer development: the Daf-c phenotype of *daf-2* mutants did not require *daf-16b*, although dauers formed in the absence of *daf-16b* showed incomplete pharynx remodelling, indicating that this transcript plays a role in at least this aspect of dauer formation (Lin et al., 2001).

1.5.1.3. Investigating the role of *daf-16d/f* by transgene over-expression

daf-16d/f, like *daf-16a*, seems to be expressed in almost all tissues. However, within the set of tissues that show expression of these transcripts, the distribution of expression levels differs for various tissues. While *daf-16a* is enriched in muscles and neurons, *daf-16d/f* seems to be enriched in the pharynx, the hypodermis, neurons and the intestine (Kwon et al., 2010).

The *daf-16d/f* transcript seems to play a major role in the determination of lifespan. *d/f* –specific RNAi reduces both *wild-type* and *daf-2* mutant lifespan and over-expression of *daf-16d/f* leads to an extension in *daf-16* mutant lifespan that goes beyond the levels of *wild-type* lifespan. There is evidence that all three transcripts play a role in lifespan determination, since it takes knock-down of all three transcript variants to fully suppress the *daf-2* lifespan extension. However, RNAi of both *daf-16a* and *daf-16d/f* accounts for most of this effect (Kwon et al., 2010). While

expression of a *daf-16a* transgene can rescue a large part of the *daf-2* lifespan extension in *daf-16; daf-2* mutants (Kwon et al., 2010; Lin et al., 2001), the presence of a *daf-16d/f* transgene can fully rescue it. Over-expression of both *daf-16d/f* and *daf-16a* has no further effect on lifespan compared to *daf-16d/f* alone (Kwon et al., 2010), indicating that it is *daf-16d/f* that has the most important role in the determination of *daf-2* mutant longevity.

daf-16d/f also seems to play a large role in mediating other phenotypes of *daf-2* mutants. *daf-2* animals have long been known to have higher levels of fat storage than *wild-type* animals (Kimura et al., 1997) and this effect was also dependent on *daf-16*. Only the *daf-16d/f* transgene was found to be capable of fully restoring the altered fat levels in *daf-16; daf-2* double mutants. The increased thermotolerance of *daf-2* animals is thought to be due to the combined effects of *daf-16a* and *daf-16d/f* as only the combined over-expression of these two transcript could restore the phenotype in *daf-16; daf-2* animals. Similarly, the Daf-c phenotype of *daf-2* mutants also seems to be due to the joint activity of *daf-16a* and *daf-16d/f*, although at 25^{o}C, it is *daf-16a* that seems to play the greatest role (Kwon et al., 2010).

1.5.2. The contribution of each DAF-16 isoform to various IIS mutant phenotypes

It therefore seems that the *daf-16a* and the *daf-16d/f* transcripts play the largest role in the determination of most *daf-2* phenotypes and that *daf-16b* plays a far less important role. The different transcripts differ both in their expression patterns, with the *a* and *d/f* transcripts being widely expressed and the expression of *b* confined to a smaller subset of cells, as well as in their protein sequences. By swapping promoters, researchers were able to address the question of which of these differences accounts for the difference in the effects of the three transcripts in the determination of lifespan. It was found that the coding region of *daf-16b* could have the same effect on lifespan as the coding region of *daf-16a* if the *daf-16a* promoter was used to drive its expression (Lee et al., 2001). Similar results were found when the promoters of the *daf-16a* and *daf-16d/f* coding regions were swapped (Kwon et

al., 2010). This demonstrates that it is not the protein sequence of the various DAF-16 isoform expressed by these transcripts that accounts for their differing effects on lifespan but their expression pattern. Interestingly, it is the *daf-16d/f* transcript that has been shown to play the greatest role in restoring *daf-2* lifespan and it is this transcript that is enriched in the intestine (Kwon et al., 2010), a tissue thought to be particularly important in the determination of lifespan in *daf-2* mutants (see Section 1.4.3.).

1.5.3. Nuclear-cytoplasmic shuttling of DAF-16

The fluorescence observed after expression of DAF-16::GFP from transgenes is usually distributed cytoplasmically, but mutations in *daf-2* or RNAi of *daf-2* lead to nuclear localisation (Henderson and Johnson, 2001; Lee et al., 2001; Lin et al., 2001). This indicates that the inhibition of *daf-16* by IIS occurs through cytoplasmic localisation of the DAF-16 protein and that the nuclear localisation of DAF-16 under conditions of low IIS may be the cause of the *daf-16*–dependent phenotypes. Consistent with this, mutations in *daf-18*/PTEN, which suppress the longevity and dauer phenotypes of *daf-2* mutants, also inhibit the nuclear localisation of DAF-16::GFP in *daf-2* mutants (Lin et al., 2001). Mutations in the important IIS PI3-kinase *age-1* also cause nuclear localisation at elevated temperatures (Henderson and Johnson, 2001). Because DAF-16 nuclear localisation is thought to occur due to phosphorylation by AKT-1 and AKT-2 (see section 1.4.2.), the effect of RNAi – mediated knock-down of both kinases was tested and found to indeed cause nuclear localisation (Henderson and Johnson, 2001). Nuclear localised DAF-16::GFP was also observed in dauers of both *daf-2(+)* and *daf-2(-)* genotypes (Lin et al., 2001).

Since DAF-16 is thought to be a stress-responsive transcription factor, the effects of stress on localisation were also investigated. Heat stress was found to lead to rapid and dramatic nuclear localisation (Henderson and Johnson, 2001) and this move to the cytoplasm could also be blocked by a mutation in *daf-18* (Lin et al., 2001), demonstrating that this response also occurs via IIS. Oxidative stress, through exposure to juglone, also led to nuclear localisation, though this effect was less dramatic than the one caused by heat stress (Henderson and Johnson, 2001). Even the

extended time spent on the agarose pads used for microscopy in *C. elegans* led to nuclear localisation (Lin et al., 2001).

DAF-16 seems to also be very sensitive to changes in the food availability. It was found that starvation led to strong nuclear localisation of DAF-16::GFP but that this localisation was lost within ten minutes of worms being returned to plates containing food (Henderson and Johnson, 2001). Germline ablation has also been shown to extend lifespan (Hsin and Kenyon, 1999) and this effect was also found to be *daf-16*–dependent. Germline ablation also led to nuclear localisation of DAF-16::GFP although the pattern and timing was different than for IIS mutant animals and those exposed to stress (Lin et al., 2001). This supports previously presented evidence of a non-cell autonomous role of IIS (see Section 1.4.3.) and suggests that intercellular signalling acts on lifespan by changing the localisation of DAF-16.

1.5.4. Phosphorylation-defective DAF-16::GFP

Since nuclear localisation is thought to be caused by phosphorylation through AKT-1 and AKT-2, the sites at which this phophorylation takes place were identified by homology to vertebrate homologues of DAF-16 and then mutated to make the protein product of a *daf-16a::gfp* transgene phosphorylation defective. These mutations do indeed lead to nuclear localised DAF-16::GFP even in a *daf-2(+)* genetic background (Lin et al., 2001). While this mutant transgene is constitutively nuclear, Lin et al. report that its expression does not extend lifespan and has little or no effect on dauer formation, and therefore conclude that nuclear localisation of DAF-16 is not sufficient to cause the phenotypes associated with decreased IIS. Indeed, they find that *daf-2* RNAi can extend the lifespan of worms carrying this transgene in a *daf-16* mutant background, thus indicating the IIS has other effects, other than the phosphorylation of DAF-16 at these sites (Lin et al., 2001). However, a different study over-expressing phosphorylation–defective DAF-16 found a high degree of either dauer arrest or arrest at a non-dauer larval stage. This phenotype was so severe that worms of this strain had to be grown on *daf-16* RNAi to maintain the stock (Lee et al., 2001) and no lifespan assays could be carried out. This severe lethality and Daf-c phenotype contrasts sharply with the results published by Lin et al. Whether or not nuclear localisation of DAF-16 is sufficient for the lifespan effect

of IIS mutants is unclear. However, several long-lived IIS mutants show very weak and transient nuclear localisation of DAF-16::GFP (Henderson and Johnson, 2001; Patel et al., 2008), either indicating that the amount of nuclear DAF-16 required for the effects on lifespan is small enough to be undetectable through microscopy or possibly that reduced IIS is affecting gene expression in a *daf-16*–dependent manner without nuclear localisation being required.

1.5.5. Differences in nuclear localisation of different isoforms of DAF-16

The studies on the localisation of DAF-16::GFP described above were carried out using the DAF-16A isoform coded for by the *daf-16a* transcript. Investigation on the localisation of DAF-16D/F::GFP indicated that the nuclear localisation of this isoform in a *daf-2* mutant background is not as great as that of DAF-16A::GFP. As with the lifespan phenotype, the question of whether this difference was due to the expression pattern of the isoform or due to the protein itself was investigated. In this case, it was found that swapping promoters had no effect on the degree of nuclear localisation. This demonstrates that the DAF-16D/F protein itself is less readily shuttled into the nucleus when IIS is reduced than the DAF-16A isoform is, at least in these GFP-tagged reporter constructs (Kwon et al., 2010).

Kwon et al. then investigated whether the DAF-16A or DAF-16D/F isoforms were phosphorylated to the same degree by AKT-1 and AKT-2. This was done by testing the effects of mutations in either kinase on the lifespan of *daf-16* mutant animals in which the *daf-16* activity has been rescued by either a *daf-16a* or a *daf-16d/f* transgene. It was found that the effect of *akt-1* on lifespan occurs mainly through *daf-16d/f* while the effect of *akt-2* on lifespan occurs mostly through *daf-16a*.

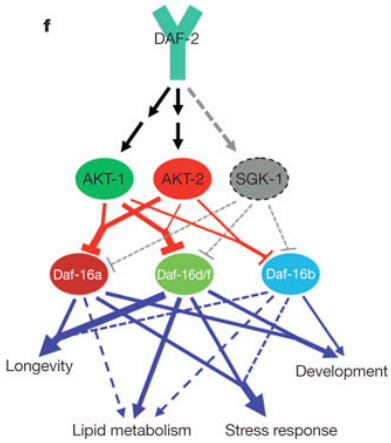


Figure 12
From Kwon et al. Depicts DAF-16 isoforms and the upstream kinases that modulate the IIS-mediated processes. The thickness of lines represents the strength of regulation. The dotted line represents minor or no regulation.

Figure 12 is a graphical representation of the strength of the interactions between DAF-2, the kinases downstream that regulate DAF-16 and the different DAF-16 isoforms. In summary, the evidence suggests different roles of the different isoforms of DAF-16 in the phenotypes regulated by IIS. While this is thought to be mediated by different expression patterns through the various promoters, evidence also supports preferential phosphorylation of different DAF-16 isoforms by the kinases.

1.5.6. Regulation of targets of IIS by the different isoforms of DAF-16

After these extensive efforts to identify the role of different isoforms of DAF-16 in regulating IIS-mediated phenotypes, an interesting question that remains is that of how target genes are regulated by different isoforms. The differences in phenotypes of worms caused by expression of different isoforms of DAF-16 suggests either that the set of target genes regulated by the isoforms differs or that the same set of genes is being regulated more or less strongly in different tissues by different isoforms. The fact that it is possible to exchange coding sequences between *daf-16a* and *daf-16b* transgenes and maintain the same phenotypes supports the latter possibility. However, DAF-16D/F is less strongly nuclear localised than DAF-16A and this was found to be due to the coding and not the promoter region. Additionally, the DAF-16A and DAF-16D/F isoforms are preferentially phosphorylated by different kinases. These two findings indicate that differences in protein sequences may very well affect the gene sets regulated by these two isoforms of DAF-16.

One experiment (Kwon et al., 2010) that addresses this question involves regulation of a *sod-3::gfp* transgene frequently used to estimate DAF-16 activity. Over-expression of both *daf-16a* as well as *daf-16d/f* led to an increase in SOD-3::GFP levels. However, *daf-16a* over-expression led to an increase in SOD-3::GFP in the muscle and *daf-16b* over-expression led to an increase in the intestine, which correlates with the tissue enrichment of the *daf-16* transcripts themselves. While this experiment only addresses the regulation of a single gene, it demonstrates at least one example where the same gene is being up-regulated in different tissues by different isoforms of DAF-16. Whether or not this is how the entire set of DAF-16 target genes is regulated by the different isoforms is unknown. An attempt to address this using qRT-PCR of various genes thought to be DAF-16 targets by Kwon et al. led to no clear pattern: some genes seemed to be co-operatively regulated by several isoforms but at least one was not (Kwon et al., 2010).

1.5.7. The search for DAF-16 targets

Besides investigating the regulation of the various isoforms of DAF-16 by IIS and the role of each of them in contributing to IIS phenotypes, much effort has also gone into identifying the downstream targets of DAF-16. Since the proximal determinants of ageing and longevity are thought to lie downstream of this transcription factor, finding genes, pathways and biological processes regulated by DAF-16 has become an important focus in the field.

1.5.7.1. Mutational and 'candidate gene' approaches

Given the *C. elegans* research community's use of this species as a genetic model organism, the obvious approach towards identifying downstream effectors would be to carry out a forward genetic screen to identify suppressors of a *daf-16*–dependent phenotype of an IIS mutant. This was done by searching for suppressors of *daf-2(e1370)* Daf-c phenotype after exposure to the mutagen EMS. In order to avoid merely identifying new alleles of *daf-16*, this screen was carried out in the presence of an additional copy of *daf-16* in the form of a functional *daf-16a::gfp* transgene (Lin et al., 2001). However, instead of identifying downstream effectors,

this screen only identified four alleles of the gene *daf-18*, which acts upstream of *daf-16*.

Another approach involves testing genes predicted to play a role in aging based on existing theories of ageing. For example, the oxidative damage theory of ageing predicts that superoxide dismutases (SODs) should play a major role in determining lifespan, since they are capable of detoxifying the harmful superoxide radical. Since reduced insulin signalling was found to lead to increased resistance against oxidative stress and the superoxide dismutase *sod-3* was identified as strongly up-regulated by *daf-16* in IIS mutants (Honda and Honda, 1999), a major role for SODs in mediating the extended lifespan of IIS mutants was often presumed. More recent work suggests that the influence of SODs in the determination of lifespan is very small or non-existent (Gems and Doonan, 2009). Evidence supporting and contradicting the oxidative stress theory of ageing is discussed in Chapter 6.

Another possible mechanism by which the longevity of IIS mutants may be achieved is through up-regulation of chaperone proteins. This is based on the prediction that misfolded proteins may contribute to pathology in old age. *age-1* mutants were indeed found to have higher levels of HSP16 protein small heat-shock protein *hsp-16* (Walker et al., 2001). Molecular chaperones are regulated by the transcription factor *hsf-1* and several studies indicate that *hsf-1*–dependent gene regulation plays an essential role in determining lifespan. Not only does loss of *hsf-1* lead to accelerated ageing (Garigan et al., 2002), but over-expression of *hsf-1* was found to extend lifespan and the extended lifespan of *daf-2* mutants was found to require *hsf-1* (Hsu et al., 2003). Elevated protein chaperone levels through altered *hsf-1*–dependent gene regulation may therefore be a mechanism by which IIS mutants delay ageing.

1.5.7.2. Identifying differentially expressed genes

However, a candidate gene approach assumes the existence of correct pre-existent ideas, by no means a certainty in ageing research (see Chapter 6). A non-biased approach was therefore needed to study the role of DAF-16. Researchers

turned to genome-level studies, with the aim of eventually fully characterising the DAF-16 –mediated changes in gene expression.

1.5.7.2a Bioinformatic approaches

Several attempts were made to identify DAF-16 target genes by searching the genome for genes linked to the *daf-16* binding element (DBE). The DBE sequence TTGTTTAC was identified *in vitro* using a method in which random oligonucleotides were allowed to bind to the purified transcription factor, DNA-protein complexes were isolated after gel electrophoresis and the oligonucleotides bound by protein were identified by sequencing (Furuyama et al., 2000). The DBE sequence strongly resembles the mammalian FOXO insulin-response element (IRE) TT(g/a)TTTT(c/g) (O'Brien et al., 1990).

One study that used the DBE sequence to search DAF-16 target genes (Lee et al., 2003) identified 947 genes that contain the DBE within 1kb of their promoters and narrowed down this list to 17 genes known to have a clear Drosophila orthologue. They studied the function of these genes by using RNAi and found that four affect lifespan and that two of these affect dauer formation. A second study took a similar approach (Ookuma et al., 2003) and identified genes which had mammalian orthologues and DBEs in their promoters. They ultimately discovered that the gene *scl-1* was upregulated by mutations in *daf-2* and seems to be required for the effects of *daf-2* mutations on fat storage as well as heat and UV resistance. Some evidence for a weak requirement for *scl-1* in mediating *daf-2* lifespan extension was also uncovered.

1.5.7.2b Microarray studies

The first attempt to characterise global changes in the gene expression of long-lived mutants used differential display RT-PCR to identify changes in the transcriptomes of *daf-2(m41*) animals compared to wild-type (Yu and Larsen, 2001). They identified nine differentially expressed genes, which were later confirmed in more comprehensive studies (McElwee et al., 2003; Murphy et al., 2003)

In an effort to gain a more complete picture of the transcriptional changes that occur in long-lived mutants, microarray technology was soon applied to the problem.

Microarrays use thousands of DNA oligonucleotide probes anchored to the surface of a chip to quantify binding of specific sequences in a DNA in or RNA sample. This can be used to quantify expression of all transcripts and to compare expression levels of individual genes in two samples. Originally this was done using so-called spotted arrays, in which genes of interest were amplified from the genome by PCR and 'spotted' onto glass. This allowed for inexpensive in-house comparison of transcript levels for any set of genes. Later technologies, called oligonucleotide arrays, used shorter probes that were directly synthesised onto the surface of the chip and were supplied by commercial manufacturers. The advantage of these arrays includes the fact that for each transcript, binding to a number of oligonucleotide probes is used to estimate expression levels, which prevents cross-hybridisation and facilitates more sensitive statistical analysis. The advantages of different platforms and the validity of comparing across platforms has been extensively discussed (Sherlock, 2005).

The first microarray study (McElwee et al., 2003) compared *daf-2* mutant animals to *daf-16; daf-2* animals in order to identify those genes that are upregulated by *daf-2* in a *daf-16* dependent manner. They identified 1646 genes to be differentially regulated, with 602 genes up-regulated and 1044 genes down-regulated in *daf-2* mutants compared to *daf-16; daf-2* double mutants. A large number of stress-response genes, including heat-shock proteins, super-oxide dismutases and cytochrome P450s were differentially regulated by IIS through DAF-16. Out of these data, 35 differentially regulated genes were tested for their effects on lifespan and dauer formation by using RNAi to knock down expression. RNAi of only three genes up-regulated in *daf-2* mutants were found to affect lifespan and RNAi of two of these led to increases, rather than decrease in *daf-2* mutant lifespan.

A second microarray study carried out by a different team (Murphy et al., 2003) focused on avoiding false positives by comparing a number of different conditions. Several long-lived mutants (three alleles of *daf-2*, one of *age-1*, and one *daf-16* over-expressing strain) were compared to wild-type animals and *daf-16; daf-2* double mutants. The authors were concerned that using a single time-point would lead to mis-identifying gene expression changes, since the two conditions would be of different biological ages (the long-lived animals age more slowly), despite being of the same chronological age. They therefore used a number of additional time-point comparisons in order to distinguish age-related changes from true *daf-2/daf-16* related changes. They used RNAi of the two genes in these comparisons.

Follow-up RNAi tests on 58 genes showed that 50/58 had significant effects on lifespan and 49/50 had effects on lifespan that matched expectation based on the expression level changes. RNAi of genes up-regulated in long-lived animals generally decreased lifespan and RNAi of down-regulated genes increased it, in most cases by a small percentage. This may indicate that each of these genes contributes to lifespan by either increasing or decreasing it and that in IIS mutants, the expression of these genes is either repressed or enhanced in a way that ultimately produces the observed Age phenotype through the sum of small effects.

A third experiment was carried out by McElwee et al. with the more sensitive Affymetrix whole genome oligonucleotide array technology (McElwee et al., 2004) on five biological replicates. They also used two *daf-2* alleles and compared *daf-2* mutants with *daf-2; daf-16* mutants to identify *daf-16*-dependent changes in expression levels. The results will be discussed in the next section.

1.5.7.2c Linking classes of differentially regulated genes from microarray studies to biological mechanisms

The analysis of microarray data is clearly a complex process given the large number of transcripts being quantified. The analysis must take into account the large amount of variation found in biological systems, subtraction of background signal, and a statistical analysis needs to be performed that takes into account the very large number of comparisons being made. Even once a set of genes that exhibit statistically significant changes in expression levels has been identified, the question of what to do next is not straightforward. Especially when a large number of differentially expressed genes have been identified, it is difficult to know which are relevant to the phenotype being investigated, especially in the case of a complex and poorly understood phenomenon such as ageing. Here one can fall back on a candidate gene approach in which one looks for genes that are involved in processes believed to be involved in the phenotype. However, this is not ideal approach in the type of situation faced by ageing researchers, since the processes causing ageing might be truly unknown. One approach used by McElwee et al. (2004), is to assign genes to different categories such as gene ontology (GO) or protein structure information and then ask the question: Which categories of genes are over-represented in the set of differentially expressed genes?

Another resource for analysing expression data is based on the fact that transcripts tend to be regulated in blocks (synexpression blocks). In other words, if a large number of microarray experiments are carried out under different conditions, using different genotypes, or different interventions, one would not expect that the sets of differentially regulated genes are different in every experiment. Instead, genes will tend to be regulated jointly with other genes in these different conditions. This could be due the transcriptional architecture regulating them, the tissue distribution of their expression or other factors. In one study, over 500 *C. elegans* microarray experiments were used to identify such sets of genes, and the data was displayed as a terrain map (topomap) in which clusters of jointly regulated genes formed 'mountains', numbered 1-43. Some of these were identified to consist of genes expressed mostly in certain tissues, such as the intestine (mount 8), genes involved in specific biological processes, such as lipid metabolism (mount 21), or those expressed during specific stages, such as the dauer (mount 15) (Kim et al., 2001). This data can now be used in the analysis of other microarray experiments in order to identify any sets of genes that are differentially regulated, which may help identify biologically relevant changes.

Tissue specificity

A large proportion of the genes identified by analyses of IIS regulated genes (McElwee et al., 2004) are intestinally expressed and map to the so-called intestinal 'topomountain', mount 8. As mentioned earlier in this chapter, IIS seems to act in both neurons and the intestine to extend lifespan and the relative importance of these tissues is not completely clear. While neuronal expression of *daf-2* was shown to be sufficient to rescue the lifespan effects of *daf-2* mutants (Wolkow et al., 2000), intestinal expression of DAF-16 was found to extend *daf-16* mutant lifespan by up to 60% (Libina et al., 2003) in a *daf-2* mutant background. In this context, the finding that intestinally expressed genes are over-represented in the set of genes differentially regulated by IIS seems to support a central role of the intestine in mediating the *daf-2* Age phenotype.

The reasons behind the central role of the intestine in the determination of lifespan are unknown, but even the effects of germline loss on lifespan were found to

be dependent on intestinal *daf-16* activity (Libina et al., 2003). It is possible that the intestine's role as the primary fat storage organ of *C. elegans* (Ashrafi et al., 2003; Kimura et al., 1997) makes it an important tissue for metabolism. This is supported by the finding that over-expression of *D. melanogaster* FoxO in fat-body is sufficient to extend fly lifespan (Giannakou et al., 2004; Hwangbo et al., 2004) and that mice lacking the insulin receptor in fat tissue were also long-lived (Bluher et al., 2003). Alternatively, if lifespan determination is in fact tightly linked to an animal's ability to deal with damage and stress, then the intestine could be of great importance due to its direct contact to food and to xenobiotics. Another explanation could be that the *C. elegans* intestine functions as a pancreas and secrets an insulin-like signal in response to food. Indeed, the mammalian pancreas and *C. elegans* intestine are both endodermal organs and the ability of the pancreas to secrete insulin is regulated by the insulin receptor (Kulkarni et al., 1999), just as IIS signalling seems to mediate an intercellular signal emitted from the *C. elegans* intestine (Libina et al., 2003; Murphy et al., 2007).

Overlap with dauer transcriptome

A number of similarities between the Daf-c and Age phenotypes of IIS mutants suggests that the same mechanisms may be causing both these phenotypes. These similarities include the fact that many, if not most, long-lived IIS mutants show a constitutive dauer phenotype, that the lifespan phenotype, like the IIS Daf-c phenotype, is completely dependent on *daf-16* and that dauers are very long-lived. These similarities strongly suggest that ageing might be delayed in adult IIS mutants due to a mis-expression of a dauer longevity program. Indeed, microarray analyses show a strong overlap between the dauer transcriptome and that of long-lived IIS mutant adults (McElwee et al., 2003; McElwee et al., 2004; Wang and Kim, 2003).

Functional genomics

Microarray data from IIS mutants is often mined for genes thought to be responsible for IIS Age based on existing theories of ageing. This includes stress response genes such as superoxide dismutases (Honda and Honda, 1999) and heat-shock proteins (Walker et al., 2001) both of which are indeed up-regulated in

microarray studies (McElwee et al., 2004; Murphy et al., 2003). However, combing through microarray data and 'fishing' for certain classes of genes from the long lists generated through microarray experiments is a flawed approach, since it invariably leads to finding support for existing theories. Given that the true causes of the lifespan extension remain unknown, expression data needs to be analysed in a more unbiased fashion (Gems and McElwee, 2003).

Using data from their own oligonucleotide array analysis (McElwee et al., 2004) and another study on dauer gene expression (Wang and Kim, 2003), McElwee et al. used EASE analysis to identify categories of genes (using Interpro protein classes, Gene Ontology categories and gene sets from other microarray experiments) that are over-represented in the differentially expressed gene set of long-lived IIS mutants and of dauers. This led them to the identification of cellular detoxification processes as up-regulated in both longevity contexts. They went on to propose that *C. elegans* can delay ageing by up-regulating a large number of enzymes with roles in removing toxins that would otherwise accumulate and cause ageing. The up-regulated enzymes include those involved in phase I and phase II cellular detoxification, such as glutathione-S-transferases, cytochrome P450s, short-chain dehydrogenases/reductases and UDP-glucuronosyltransferases. This suggested to the authors that ageing might be caused by molecular damage that is at least partly caused by toxic compounds that can be removed by enzymes involved in cellular detoxification, also called drug metabolising enzymes (DMEs) or collectively described by the term "xenobiotic metabolism". Supporting this idea, at least one glutathione-S-transferase (*gst-10)* can extend lifespan by detoxifying 4-hydroxynonenal, a product of lipid peroxidation (Ayyadevara et al., 2005a; Ayyadevara et al., 2005b). Moreover, over-expression of *skn-1,* a transcription factor known to regulate phase II detoxification enzymes, also extends lifespan and the *daf-2* Age phenotype is partly *skn-1* dependent (Tullet et al., 2008).

1.5.7.3. Identifying direct regulatory targets of *DAF-16*

Given the large number of genes that are differentially regulated by IIS, identifying the effectors of lifespan extension has proven to be difficult. In addition, given the large number of genes that probably work together to affect lifespan,

finding ways to test any emerging theory is technically challenging. This problem can be illustrated using the detoxification theory of ageing described above, based on which we can make certain predictions: If the cellular detoxification system contributes to the extended lifespan of *daf-2* mutants, then *daf-2* mutant lifespan should be reduced when the up-regulation of these enzymes is abolished. However, given the large number of detoxification genes that are up-regulated in insulin signalling mutants, one would expect that each makes an only small contribution to the extended lifespan. Given the technical limitations of even the comparatively simple method of RNAi knock-down of *C. elegans* genes, only a small number of them can be knocked-down at any time and tested for suppression of the *daf-2* Age phenotype. Clearly, only small reductions in *daf-2* lifespan would be expected from such experiments, and even here we are assuming that there is no redundancy in the roles of these enzymes in lifespan determination, which, if present, would only exacerbate the problem. Experiments to test suppression of *daf-2* longevity by RNAi of detoxification enzymes that are up-regulated in *daf-2* mutants were in fact carried out and only in a few cases could even a small reduction of *daf-2* lifespan be observed (Weinkove and Gems, unpublished results). Similarly, in earlier lifespan assays carried out using RNAi of candidate genes found to be up-regulated in *daf-2* animals compared to *daf-16; daf-2* double mutants, very few of the genes tested had any effect on lifespan, and most of those that did were not found to affect lifespan in the right direction (McElwee et al., 2003). In sharp contrast, most of the genes tested by RNAi in (Murphy et al., 2003) did seem to affect lifespan as one would expect from the change in transcript levels.

A large step forward for the field of ageing research would be to resolve the topology of the network of transcriptional regulation downstream of DAF-16. Greater understanding of how different subsets of genes are regulated would give researchers better tools to test which sets of genes affect lifespan. This could be done by up-regulating or knocking down downstream regulators or by interfering with feed-back loops or intercellular signalling. One way to make inroads into the unchartered territory downstream of DAF-16 would be to identify those differentially regulated genes that are direct targets of DAF-16. Presumably not all of the approximately 2000 genes differentially regulated by IIS (McElwee et al., 2004), are direct targets. Identifying them could make the task of finding the genes relevant for mediating *daf-2* longevity more tractable, as one would presumably be left with a

smaller number of genes with a more important role in ageing. Two attempts to do this will be discussed here.

1.5.7.3.1. Identifying direct targets by chromatin immunoprecipitation

Chromatin immunoprecipitation can be used to test for binding between a known protein and genomic DNA. This is achieved by cross-linking proteins and DNA, fragmenting the DNA through sonication, using an antibody against the protein of interest for immunoprecipitation, and then isolating the DNA that was bound to it. This was done using an antibody against endogenous DAF-16 (Oh et al., 2006) and the DNA was subsequently cloned and sequenced. This allowed the researchers to identify 130 *C. elegans* genes as linked to a DAF-16 binding site.

While there is not much overlap between the 130 genes identified by Oh et al. and lists of differentially expressed genes obtained from microarray data (9 of them up- and 1 down- regulated in data used for (McElwee et al., 2007; Schuster et al., 2010)), the authors performed qRT-PCR and found that more than half of the genes were either up- or down-regulated in *daf-16* mutant compared to wild-type animals. Knock-down of candidate genes by RNAi led to both increases and decreases in *daf-2* lifespan, in fat content and in dauer formation, but these effects rarely correlated with the changes in expression observed by qRT-PCR.

1.5.7.3.2. Dam Identification

A second approach to identifying direct targets of DAF-16 was carried out through Dam identification (DamID) using a *daf-16::dam* fusion construct (Schuster et al. 2010). The principle of this approach is to create a fusion of DAF-16 with the bacterial Dam methylase, which methylates the site GATC. Binding of this fusion to the genome causes nearby GATC sequences to be methylated, thereby creating restriction sites for the enzyme *Dpn*I. *Dpn*I digestion can then be used to isolate DNA to which the construct binds. In this study, the animals were treated with *daf-2* RNAi to ensure the nuclear localisation DAF-16::Dam and methylated DNA was subsequently hybridised to whole genome tiling arrays in order to identify areas of DAF-16 binding in the genome (see Figure 13). The authors then cross-referenced

this data to that from their previous microarray analysis in order to identify genes that are both differentially regulated by IIS and direct targets of DAF-16.

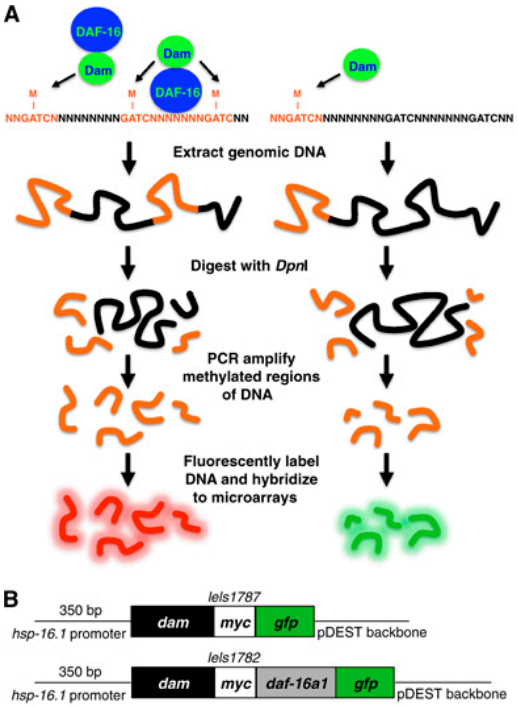


Figure 13
(Schuster et al., 2010)
Schematic describing the procedure to identify DAF-16 targets via DamID.

This study identified a large number of sites in the genome to which DAF-16 seems to bind, but only a small subset of these (a total of 65 genes) are also differentially regulated by IIS. EASE analysis of the data indicates that genes involved in somatic maintenance, including cellular detoxification enzymes such as glutathione S-transferases and cytochrome P450s, are not overrepresented among these 65. It is possible that these processes may be regulated indirectly by DAF-16 through one of its targets. The analysis also demonstrates that DAF-16 acts primarily as a transcriptional activator and not a repressor, as genes up-regulated by reduced IIS were overrepresented in the list of direct targets but down-regulated ones were not.

The 65 genes that are differentially regulated by IIS and that are also directly bound by DAF-16 define three classes of targets, although many targets fall into neither of these classes. The first group of genes up-regulated by direct binding of DAF-16 are transcription factors. This finding may help explain why such a large number of genes are differentially regulated by IIS in a *daf-16*–dependent manner while a relatively small number seem to be direct targets. It seems possible that

changes in the expression levels of even a small number of transcription factors could lead to large changes in the transcriptome of an organism. The second group are members of the IIS pathway itself, including *akt-1*, *akt-2*, the *ist-1* insulin receptor substrate, and possibly even *daf-16*. This suggests a feed-back mechanism by which sensitivity of IIS is increased by DAF-16 when IIS signalling is low. Other signalling proteins known to activate DAF-16 also seem to be up-regulated. A third set of genes over-represented in this list of 65 are those associated with carbohydrate metabolism, which fits the role of DAF-16 in the response to starvation (Weinkove et al., 2006) as well as the altered metabolism found in the dauer stage.

1.6. Using a ferritin reporter to screen for transcription factors regulating gene expression downstream of IIS

Clearly, significant progress is slowly being made in unravelling the topology of the transcriptional network downstream of DAF-16. This progress should eventually lead to an understanding of how reduced IIS extends lifespan in *C. elegans* and will hopefully also play a part in finally understanding what biochemical or physiological processes are the cause of ageing.

The total number of genes differentially regulated by DAF-16 has been estimated to be as high as 10% of the *C. elegans* genome (McElwee et al., 2004). This alone suggests that other signalling proteins (including many transcription factors) are probably involved in mediating the response. The recent finding by Schuster et al. that many direct targets of DAF-16 are transcription factors and signalling proteins supports this view (Schuster et al., 2010).

The methods to investigate the activity of DAF-16 described above all work by somehow manipulating DAF-16 and examining the downstream consequences. In the microarray studies, DAF-16 is activated by mutations in IIS and this condition is compared to a strain in which DAF-16 is inactivated. In the study using DamID, DAF-16 was tagged with a methylase and genomic locations at which DAF-16 binding sites were identified through the increased methylation found at such sites. This could be described as a 'top-down' approach: DAF-16, which crowns an IIS–dependent transcriptional network, is manipulated in order to learn more about the network downstream of it. In this project, I took a different, one could call it a

'bottom-up', approach to the problem: I identified a gene that is highly regulated by IIS in a *daf-16*-dependent manner and then carried out an RNAi screen to identify transcription factors that play a role in this induction.

1.7. Aims

The root causes of the ageing process are unknown both in simple organisms such as *C. elegans* as in humans. The existence of long-lived single gene mutants of *C. elegans* provides a powerful model for unearthing these causes. To date, important discoveries include the finding that reduction in insulin/IGF-1 signalling (IIS) extends lifespan and that the effects of these longevity-inducing IIS mutations occur through the actions of the transcription factor gene *daf-16.* Numerous attempts at identifying the downstream targets of *daf-16* have been carried out. A very large number of transcripts have been identified that are differentially regulated in long-lived mutants via *daf-16,* but it has not been possible so far to pin the effects of the IIS pathway on ageing on distinct biological processes. Studies of direct binding of the DAF-16 protein to chromatin have suggested that DAF-16 acts as a regulator of a number of key signalling proteins and therefore may exert its effect on ageing through manipulating a large number of downstream signalling cascades.

The aim of this project was to help identify transcription factors that act in concert with DAF-16 to regulate downstream targets genes. This would help shed light on the mechanisms by which DAF-16 affect the expression of so many genes and allow for a more nuanced approach to the investigation of the causes of IIS mutants' *daf-16*-mediated longevity phenotypes.

This was done by creating a transcriptional GFP transgene that is highly induced by reductions IIS in a *daf-16*-dependent manner. I induced the transgene's expression by crossing it into a *daf-2* mutant background and then monitored GFP expression or worms picked into wells of a microtitre plate using a platereader. I then carried out an RNAi screen of over 800 genes encoding putative transcription factors to identify ones that may be involved in this induction. Candidate genes could then be tested for their role in determining IIS mutant longevity.

Chapter 2: Materials and Methods

2.1 General methods and *C. elegans* stock maintenance

Worms were grown on 60mm or 100mm tissue culture plates containing nematode growth medium (NGM), an agar and nutrient mix, and seeded with the *E. coli* strain OP50 (Brenner, 1974). OP50 is a uracil auxotroph and NGM is uracil – limited, which prevents the bacterial lawn from overgrowing and obscuring the worms. When RNAi was used to knock down gene transcripts, the agar was supplemented with 1mM IPTG and 20mg/L carbenicillin and the strain of *E. coli* used was HT115, not OP50. Both HT115 and OP50 strains were obtained from the CGC (Caenorhabditis Genetics Center, University of Minnesota).

Stocks were maintained by periodically transferring 3-10 gravid adults to fresh plates or by cutting a section of agar from one plate and placing it upside down on a fresh plate (chunking), thus allowing the worms to leave the agar and crawl onto the fresh lawn of *E. coli*. The worms were transferred to plates or microtitre wells using a thin platinum wire (worm pick). When picking worms, the end of the pick is covered in *E. coli* from the bacterial lawn and is then used to lift worms off the agar.

2.1.1. Lifespan analysis

In many experiments, the effects of different genetic mutations as well as of RNAi and pharmaceutical treatments on lifespan were tested. This was done by placing 25-30 L4 animals on fresh plates and recording all deaths. Plates were monitored approximately every 2 days. Any animals that could not be made to move by repeatedly tapping the plate were directly prodded using a pick. If no movement was seen after repeated prodding, the worm was scored as dead. Animals lost, found on contaminated plates, or ones that died of causes unrelated to ageing were scored as 'censored', which was taken account of during statistical analysis. Worms that dried out on the sides of plates and those that burst from progeny production are examples of deaths that were censored.

Worms were generally transferred to fresh plates at least once a week, although the details of transferring schedules varied depending on the assay. Lifespan assays performed to test the effects of RNAi treatments on lifespan were

always transferred at least once a week to maximise RNAi efficacy. When FUdR (5-fluoro-2'-deoxy-uridine) was not used to prevent progeny formation, the animals were transferred several times during the first week of adulthood in order to eliminate progeny.

2.1.2. Drug treatment

Some lifespan assays required the use of drugs. FUdR was often added to prevent progeny production and N-acetyl cysteine (NAC) was added to test its effects on lifespan. In both cases, the drug was added topically to the solidified, cooled agar after pouring but before seeding with *E. coli.* FUdR was used at a final concentration of 10μM. NAC was added to a final concentration of either 5mM or 1mM.

2.1.3. Genetic crosses

New strains are often generated by crossing two existing strains. Males were generated either by mating mutant hermaphrodites with males from a wild-type male stock, or by heat-shocking hermaphrodites. Heat-shock increases the incidence of non-disjunction of the X-chromosome during meiosis and therefore leads to a higher incidence of males. 5-15 L4 hermaphrodites were exposed to 31^{o}C for six hours and the progeny of these animals were then screened for males. These were then mated with hermaphrodites of the same strain in order to create a population rich in males.

2.1.4. Alkaline hypochlorite treatment

This method was used frequently and for a variety of reasons. Stocks often become contaminated with foreign microorganisms, like bacteria, yeast and fungi, which need to be removed. Axenisation of stocks by alkaline hypochlorite treatment works by exposing worms to bleach, which dissolves the parent, kills any contaminants, but leaves the eggs unharmed. This is also essential when animals are transferred from OP50 bacteria to HT115 RNAi bacteria, since carrying over OP50 to RNAi plates needs to be avoided.

Protocol:

1. Gravid adults washed off plates using M9 buffer and pipetted into a 1.5mL capped tube.
2. After the adults settle (~1min), the supernatant is removed, leaving 200µl of M9 and worm pellet in the tube.
3. 125µl of a 7:8 solution of sodium hypochlorite (Sigma) and 4M NaOH is added and the contents of the tube are mixed by inversion. The tubes are left at room temperature (not held in hand) for four minutes, during which they are mixed (by gentle flicking) about once a minute.
4. After four minutes, the reaction is stopped by filling the tube with M9. Eggs are pelleted immediately by centrifuging at 1000g on a table-top centrifuge.
5. The supernatant is removed and replaced by more M9. After gentle mixing, the sample is again centrifuged at the same settings. This washing step is repeated three or four times to remove any remaining bleach.

The method is also used to purify eggs, which is necessary for some experiments, since it synchronises the progeny. However, the synchronisation is not perfect, since some of the embryos will be further developed than others. An improved synchronisation is achieved by leaving the eggs in M9 overnight. This allows all eggs to hatch, but because they lack food, the L1s cannot develop any further. These arrested L1s can then be pipetted onto seeded plates. The time left in M9 varied depending on the temperature they were incubated at. At 20^{o}C, eggs were left in M9 overnight and at 15^{o}C they were left for 36h.

2.2. Transgenesis via microinjection

Needles were pulled from borosilicate glass capillaries (Harvard Apparatus) using a P97 micropipette puller (Sutter Instrument Company). Injection pads were made by placing a drop of hot 2% agarose on a glass coverslip and then covering the drop with a second coverslip. After the agarose solidified, the coverslips were pealed apart and the pad was left in the 37°C oven overnight.

For the microinjection itself, I used an inverted microscope, model DM IRB (Leica) with Nomarski optics and a 40x Nomarski non-oil immersion objective. This microscope was equipped with a micromanipulator (Leica) and a Picospritzer II pressurized injection system (General Valve Corporation) with a needle holder. Pressurized nitrogen gas was used for injections. Worms were manipulated and placed onto the injection pads on a dissecting microscope before being moved to the inverted microscope for injection.

A drop of mineral oil (Sigma) was placed on the injection pads and single worms were placed into the oil. Young adults with few or no eggs in the uterus were placed in the oil and either left to sink to the bottom of the drop or gently pressed down using a platinum pick. Once the animals come into contact with the surface of the injection pad, they usually stick to it quite strongly.

The coverslip was then moved onto the inverted microscope. The 40x objective was used to find the syncytial gonad and the plane of focus was adjusted so that the nuclei at the periphery of the gonad arm were in focus. The needle is then brought down until it too is in focus, which ensures that the injection is occurring at the correct vertical level. The needle was then injected into one of the gonad arms and the DNA solution was injected. This was repeated for the other gonad arm if possible.

When the injection was completed, the needle was lifted out of the oil and the coverslip was moved to the dissecting microscope. There, a drop of M9 solution was quickly dropped on top of the worm and into the oil. This leads to the animal detaching from the agarose pad and reviving in the M9 solution. Animals were then picked out of the drop of M9 using a platinum pick, placed singly onto seeded NGM plates, and left to recover and lay eggs at 20°C. Around 20 – 40 animals were injected for each transgene.

Worms were injected with a mix of the reporter construct and a plasimid containing the *lin-15(+)* coding sequence. Three days after injection, F1 progeny were scored for either the absence of the Muv phenotype or the presence of the Rol phenotype. As *lin-15*(-) animals were used for injection and all *lin-15(-)* animals exhibit the Muv phenotype when grown at 20°C, non-Muv animals were transgenics expressing the *lin-15(+)* copy from the plasmid that was coinjected alongside the fusion PCR product. Not all F1 transgenics are generally capable of transmitting the transgene, so F1 animals were picked singly onto NGM plates and left to lay eggs at

20°C. Plates were screened three to four days later for non-Muv F2 animals. Once found, a new transgenic line was established.

2.3. Transgene integration

For the integration of the extrachromosomal array into the genome of *C. elegans*, around 130 animals at the L4 stage were picked onto a 10cm plate on NGM (containing 2x peptone) seeded with OP50 bacteria. These worms were then exposed to 40 Gy of X-ray radiation. This was carried out at the Department of Immunology and Molecular Pathology and Department of Infection (UCL, Windeyer Building).

X-ray irradiation of worms carrying an extrachromosomal array causes the array to become integrated in the genome of only a small fraction of their offspring. A selection process is therefore used to facilitate their identification. Most of the offspring of irradiated worms carry the transgene as an extrachromosomal array pass it on to their offspring with a transmission rate of around 60-70%, in the case of the *wuEx135* array. The few integrants among them, however, pass it on 100% of the time. This means that the integrants have 100% non-Muv offspring while the non-integrants will have mixed Muv and non-Muv offspring. Because Muv animals have reduced fertility compared to non-Muv animals, letting them compete for a number of generations should cause the integrants to become more common. Ten sets of ten irradiated animals were therefore left to compete for a number of generations. Using this selection process, non-Muv animals picked in the end were far more likely to be integrants than if selection had not been used.

After irradiation, ten non-Muv adult hermaphrodites were picked onto each of ten 10cm NGM (2x peptone) plates seeded with OP50 bacteria and left at 20°C until several generations had passed and no food (OP50 bacteria) was left on the plates. A large piece of agar from each of the ten plates, presumably containing several hundred animals, was transferred to fresh seeded 10cm plates. Once these new plates had run out of food, they too were chunked onto fresh, seeded, 10cm plates.

Fifteen adult animals from the next generation were then picked from each of the ten plates onto 150 individual seeded (6cm) NGM plates. The plates were then left at 20C. Four days later, the progeny were screened for the Muv phenotype. Integrants were identified because 100% of their progeny had a *wild-type* appearance

(100% non-Muv). Three independent integrants of the *wuEx135[Pftn-1::GFP, lin-15(+)]* transgene were identified. The two alleles *wuIs176[Pftn-1::GFP, lin-15(+)]* and *wuIs177[Pftn-1::GFP, lin-15(+)]* were outcrossed to the parent strain MT1642.

2.4. Microscopy

2.4.1. Preparing slides

2% w/v agarose was made using dH_2O. One or two drops of agarose were placed on a glass slide and quickly covered with a second slide. Spacers were used ensure that the layer of agar formed was of consistent thickness. The top slide was removed after several minutes and the microscopy pad was used immediately.
A few drops of 0.06% w/v levamisole were then added to the agarose pad and worms were picked into the drop, which immediately anaesthetised them. A coverslip was then carefully placed over the pad.

2.4.2. Fluorescence quantification

Images of GFP expressing worms were taken using a Leica DMRXA2 microscope using the 10x objective. Images were taken both using brightfield and epifluorescence using a GFP filter cube (Excitation: 470/40; Emission: 525/50) and a Hamamatsu C10600 digital camera. In order to quantify the GFP fluorescence, the Volocity software package (Improvision) was used. A protocol was set up in which the GFP image was searched for objects whose fluorescence intensity exceeded a set threshold. This threshold was manually set to fit the intensity of the fluorescent signal and fluorescence below this threshold was ignored. The strains used expressed GFP containing a nuclear localisation signal in the intestine, meaning that the GFP signal was clearly distinguishable by eye from intestinal autofluorescence. I was therefore able to adjust the threshold to a high enough level to detect only GFP, which was nuclear, and not autofluorescence. Using this protocol, the Volocity software was therefore able to recognise fluorescent objects (nuclei) in the intestine of the worm and quantify their intensity. The fluorescence intensity for all 'objects' in a worm was then added to quantify the levels of GFP expression for each worm

separately. Within any experiment, the threshold was kept at the same level, enabling me to compare fluorescence across strains and treatments.

2.5. Molecular biology

2.5.1. RNA extraction

Animals washed off the plates were then washed in M9 to remove bacteria. 200µl of worm pellet and M9 were added to 800µl of Trizol reagent in a tube of Lysing Matrix D (MP Biomedicals). The tube and its contents were then immediately frozen in liquid nitrogen. A Ribolyser (Hybaid) was then used to homogenise the samples in the lysing matrix tube. Homogenisation was carried out at setting 6.5 for two periods of ten seconds. The tubes were then left at room temperature for five minutes before 200µl of 1-bromo-3-chloropropane (BCP) was added and the tubes were vortexed for fifteen seconds. The tubes were then incubated at room temperature for three minutes, and then centrifuged at 4°C for 15 minutes at 16000g to separate phases. Sterile, RNAse free Eppendorf tubes were prepared on ice and the aqueous upper layer of the centrifuged tubes was pipetted directly into the chilled tubes.

500µl of isopropanol and 1µl of the co-precipitant GlycoBlue (Ambion) at 15mg/ml was then added, carefully mixed and then incubated at room temperature for ten minutes to precipitate RNA. RNA was then recovered by centrifugation at 4°C for ten minutes at 22000g. The aqueous solution remaining was then carefully pipetted away from the pellet. 100µl of 75% ethanol was added, the tubes were briefly vortexed to wash the pellet, and then spun at 4°C for five minutes at 6300g. The supernatant was then carefully removed and the pellet air-dried for five to ten minutes. The pellets were then dissolved in sterile water by incubating at 60°C for ten minutes.

2.5.2. RNA quantification

The Invitrogen Quant-iT ^{TM} RiboGreen RNA Assay Kit was used to quantify RNA levels for the qRT-PCR assays. This was done using the high range assay

described in the manufacturers instructions. Briefly, a standard curve was prepared using a 2μg/mL solution of ribosomal RNA. Each RNA sample was diluted 1000x to 10 000x and then added, in triplicate, to wells of a black sided, clear bottom microtitre plate. A working solution (20x dilution of stock solution) of Quant-iT RiboGreen RNA reagent was then added to the wells in equal volume to the RNA dilution. After a 5-minute incubation period, the fluorescence of these samples was measured (wavelengths: excitation 480nm, absorbance 520nm). The standard curve was used to calculate the relationship between fluorescence and RNA concentration and the concentrations of the RNA samples, so long as their fluorescence fell within the bounds of the standard curve, were calculated by interpolation. If the fluorescence of any samples did not fall within the standard curve, then the initial dilution of the RNA sample was adjusted and the assay was repeated.

2.5.3. DNAse I treatment

Amplification grade DNaseI (Invitrogen) was used to remove any contaminating genomic DNA from the isolated RNA. 1μg of RNA, manufacturer supplied reaction buffer and DNAseI were incubated at room temperature for 15min. To inactivate the DNaseI, EDTA was added to a final concentration of 2.3mM and the reaction was heated to 65^{o}C for 10min. The resulting DNA-free RNA was then used for cDNA synthesis. The samples of *daf-2* and *daf-16; daf-2* animals prepared for semi-quantitative RT-PCR were not DNAseI treated.

2.5.4. cDNA synthesis

cDNA was synthesised using SuperScript II reverse transcriptase (Invitrogen). The following components were added to a nuclease-free microcentrifuge tube: 1μl Oligo(dT) (Invitrogen) at 500μg/ml, 500ng to 2μg of total RNA (kept consistent within experiments) from RNA extraction described above, 1μl dNTP mix at 10mM each, sterile water made up to 12μl. These were then heated at 65°C for 5 minutes and then immediately chilled on ice.

Contents of the tube were then collected by centrifugation and the following components added: 4μl First Strand Buffer (Invitrogen), 2μl 0.1M DTT, 1μl RNaseOUT (Invitrogen) and 1μl of SuperScript II. Contents were then gently mixed

and incubated at 42°C for 52 minutes. The reaction was then inactivated by incubating the tubes at 70°C for 15 minutes.

2.5.5. Semi-quantitative rtPCR

Semi-quantitative PCR reactions from cDNA templates were performed to estimate the differences in expression of *ftn-1* and *sod-3* between *daf-2* and *daf-16; daf-2* mutants. Primers for these reactions were designed to anneal to exon-exon boundaries in order to exclude amplification from genomic DNA.

To estimate this change in expression, two parameters needed to be evaluated for each set of primers: the number of PCR cycles required and the template concentration. It is important to use an initial template concentration that lies within the central portion of the linear phase of the amplification reaction, as this will allow the detection of both increases in template concentration (ie.: upregulation of the gene) and decreases (downregulation). An appropriate PCR cycle number also needs to be found.

The optimal cycle numbers for the *ama-1, ftn-1* and *sod-3* reactions were established by running the PCR reactions using the same template concentration at 9 different cycle numbers from 23 to 43. The resulting bands were quantified and the linear phase established by plotting the intensities. A cycle number was picked for each primer pair (for *ama-1, ftn-1, sod-3*) that fell within the central portion of the linear phase. Next, PCR reactions using this cycle number for each of the three primer pairs were set up 8 different template concentrations. The intensities of the resulting bands were quantified and plotted on a graph. An appropriate cDNA concentration was chosen from the linear phase of this response curve for each the three primer pairs.

The changes in expression levels of *ftn-1* and *sod-3* were evaluated through the use of expression levels of *ama-1*, which codes for the large subunit of RNA polymerase II, as a reference gene. Assuming the levels of *ama-1* transcript remain constant, changes in the expression of other genes can be estimated by looking at changes in the genes' expression as a proportion of *ama-1* expression.

PCR reactions were set up with optimised template concentrations and cycle numbers for each primer pair. These factors were of course kept constant between

the two different samples (two different genotypes) of each primer pair. Two replicates of each PCR reaction were run. The product was then run on a 1% agarose gel and the band intensities were quantified. The intensities were then normalised using the intensities of the *ama-1* bands. The mean of the two replicates was used in all cases and the results graphed in Figure 19.

2.5.6. Quantification of transcripts by quantitative RT-PCR

Quantitative reverse transcription PCR (qRT-PCR) is a method to quantify a DNA template that usually takes advantage of one of several dyes that fluoresce when bound to double stranded DNA. This is achieved by monitoring the amplification of the template by measuring fluorescence during the course of the PCR reaction. Here, a system using the SybrGreen dye was used (Fast SYBR Green Master Mix –Applied Biosystems) to quantify cDNA and therefore the RNA transcript it was derived from. This was done using the 7900HT Fast Real-Time PCR System (Applied Biosciences).

cDNA was pooled from all samples being tested and this cDNA pool was then used to create a standard curve consisting of two technical replicates for each of five ten-fold dilutions. A standard curve was included with each qRT-PCR plate and each primer pair. cDNA from each sample being tested was then used to set up reactions on the same plate in triplicate. After running the reaction, the relative transcript quantities in each reaction were calculated by determining the cycle number at which the amplification curves crossed a manually set threshold (Ct value). This threshold was set manually in order to ensure that all amplification curves crossed it within their exponential phase. The Ct value for each reaction was then converted to transcript quantities using the standard curve. The mean of three technical replicates was calculated to obtain the transcript quantity for each sample.

Transcript quantities need to be normalised for the total amount of cDNA present. While this is at least partly achieved by using equal amounts of RNA for all cDNA synthesis reactions, this alone has been found to be insufficient since total RNA levels are not a good estimate of total mRNA levels (Johnson et al., 1995; Solanas et al., 2001; Spanakis, 1993). Additional normalisation is therefore required. The preferred method is to quantify other transcripts (so-called reference genes) as a

way to normalise for cDNA input. The quantity of reference gene transcript is then used as a normalisation factor and dividing the quantity of the transcript of interest with this factor then results in a more accurate estimate of the true relative expression level. The transcripts used for the normalisation needs to be stably expressed, since any variations in the reference gene levels from sample to sample would result in artefactual findings. For qRT-PCR assays performed here, four different reference genes were tested. The first is *ama-1*, which codes for the large subunit of RNA polymerase II, and was used for this purpose in the Gems lab before my arrival. The other three, Y45F10D.4, *pmp*-3, and *cdc-42*, were recommended in a study that analysed 500 *C. elegans* microarray experiments and identified a set of stably expressed genes (Hoogewijs et al., 2008).

The GeNorm algorithm (Vandesompele et al., 2002) was then used to compare the four reference genes used and rank them according to their stability. This algorithm compares the stability of expression of all reference genes tested by calculating the average pair-wise variation of each transcript with all other transcripts. The geometric mean of the three most stably expressed transcripts was then used as a normalisation factor to control for cDNA input. Relative *ftn-1* expression was calculated as a ratio of *ftn-1* transcript quantity to this normalisation factor.

qRT-PCR conditions

The basic setup of qRT-PCR reactions consisted of

5µl 0.1x cDNA

10µl 2x Fast SYBR Green Master Mix (Applied Biosystems)

x µl fwd primer

x µl rev primer

made up to 20µl with H_2O.

The amounts of primer added differed depending on the primer pair.

gene	primer	µl added (10pmol/µl)
cdc-42	DA86	0.2
cdc-42	DA87	0.2
pmp-3	DA88	0.2
pmp-3	DA89	0.2
Y45F10D.4	DA90	0.2
Y45F10D.4	DA91	0.2
ftn-1	ftn-1_fwd_RT2	0.75
ftn-1	ftn-1_rev_RT2	0.5
ama-1	ama-1_fwd	0.75
ama-1	ama-1_rev	0.5

Table 1
Primer amounts used for qRT-PCR

All qRT-PCR reactions were run using the following cycling conditions:

1. 95°C 20 sec
2. 95°C 1 sec
3. 60°C 20 sec

Stages 2. to 3. were repeated for 40 cycles

The product of a qRT-PCR reaction is never run out on an agarose gel, since the only point of interest is the rate at which the product is formed, not its size. A danger is that non-specific amplifications cannot be distinguished from the target gene cDNA. This is a possible source of inaccuracy, since different samples may have different proportions of non-specific product. This can be partly controlled for by including non-template control reactions that are run with every qRT-PCR plate. If amplification occurs when no cDNA was added, then this is clear evidence for non-specific amplification. However, adding the cDNA could change the reaction conditions sufficiently to cause non-specific amplification that would not occur otherwise, since the cDNA mix contains a number of substances, such as glycerol, salts, and EDTA, which could alter the reaction chemistry.

The presence of non-specific product can be tested after running a qRT-PCR reaction using the same detection system as used during PCR. After the 40 cycles of PCR, the sample is cooled to 60^{o}C, at which the DNA is mostly double-stranded and fluorescence is therefore at its highest. The sample is then slowly heated while fluorescence is monitored to produce a melting curve for each reaction. Since each sequence has a characteristic melting temperature, the presence of more than one PCR product can be detected. This procedure was performed for all qRT-PCR reactions and no reactions were included in the analysis in which multiple products had been amplified.

2.5.7. Single-worm PCR

A single worm was picked into a PCR tube containing 5µl of single worm lysis buffer containing 1mg/ml proteinase K. This was either stored at -80^{o}C or immediately lysed by incubating at 65^{o}C for 2h. The proteinase K was then deactivated by incubating at 95^{o}C for 15min. 1-2µl of this lysis was typically used for genotyping PCR reactions using the primers listed in section 2.6.

Genotyping PCR reactions were carried out using the ReddyMix PCR Master Mix (Thermo Scientific). Reaction volumes were typically 20µl, with primers added at a final concentration of 0.5pmol/µl, 1-2µl of worm lysis and 10µl of ReddyMix. The cycling conditions used were:

1. 94^{o}C 5 min
2. 94^{o}C 35 sec
3. 55^{o}C 30 sec (annealing temperature 55 ^{o}C typical, but varied)
4. 72 ^{o}C 45sec/kb

2. – 4. repeated 34x

5. 72 ^{o}C 7 min

2.5.8. Fusion PCR

PCR fusions of gene promoter regions to GFP were carried as described by Oliver Hobert (Hobert, 2002). Two primary PCR products were produced, one of the GFP coding region + *unc-54* 3' UTR, and the other of the fragment being fused to GFP. The first product was readily amplified from the pPD95.75 plasmid (Fire vector kit- Addgene). For this reaction, the TC12 and TC13 primers were used (see Section 2.6). For the next step, a 5' External primer (5'Ex) primer and a fusion primer was designed. The fusion primer (fus) consists of a reverse primer added to the sequence 5'-agtcgacctgcaggcatgcaagct-3', which is complementary to the GFP coding sequence. The reverse primer part of the fusion primer is designed to produce a product in a PCR reaction with the 5'Ex primer that includes the ATG start codon of the gene of interest as well as any number of codons of the coding region in the product. This can go as far as containing the whole gene, but care needs to be taken that the second (complementary to GFP) part of the fusion primer remains in frame with the start codon. The PCR product of the reaction using these two primers carries a 24bp overhang that is able to bind to the GFP PCR product generated by the first reaction.

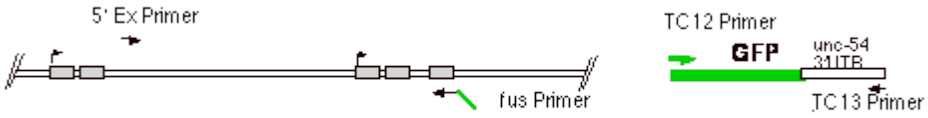


Figure 14
Adapted from Hobert et al. (2002)

A small amount of each PCR product is then added to a third PCR reaction which uses a 5' Internal (5'Int) primer nested within the gene's upstream sequence and a 3' Internal (TC14) primer nested just 5' of the TC13 primer within the GFP PCR fragment. Because the 24bp overhang causes the two sequences to anneal and the primers are located on opposite ends of either sequence, this third PCR reaction fuses the two fragments. PCR reactions were carried out using the Phusion (Finnzymes) proofreading enzyme. This fusion PCR fragment is then injected.

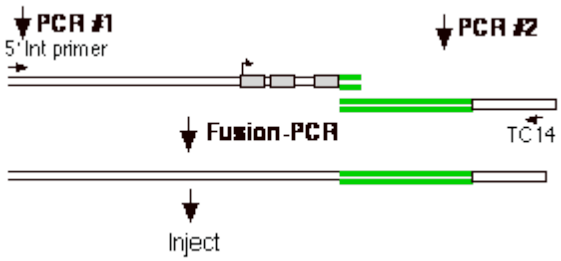


Figure 15
Adapted from Hobert et al. (2002)

PCR products were then injected into MT1642, a *lin-15(-)* strain, along with a *lin-15(+)* plasmid that rescues transformed animals from the Muv phenotype of *lin-15(-)* worms.

2.6. Reagents

2.6.1. Primers

primer name	sequence		used for …
TC12	agcttgcatgcctgcaggtcgact	5'	amplification of GFP fragment for fusion PCR
TC13	aagggcccgtacggccgactagtagg	3'ex	
TC14	ggaaacagttatgtttggtatattggg	3'in	
C06B3.4.5ex	tgccaaatcagctctgaacac	5'ex	construction of *stdh-1::gfp* by PCR fusion
C06B3.4.5in	gaaagtagtgtagcggttttattcg	5'in	
C06B3.4.3fus	agtcgacctgcaggcatgcaagctCATCAAAGGCTTATTTTCATTCTTTGCC	3'fus	
C10H11.5.5ex	ggaacaacttcccccattaactg	5'ex	construction of *ugt-27::gfp* by PCR fusion
C10H11.5.5in	ttttcaacaccgcctaccgactg	5'in	
C10H11.5.3fus	agtcgacctgcaggcatgcaagctGGTTCTCTTGATAAGCCAACGTTTC	3'fus	
F10D2.11.1	gttcacgctcggatttggagttc	5'ex	construction of *ugt-41::gfp* by PCR fusion
F10D2.11.3	aaaagacccatgttagttcacgctc	5'in	
F10D2.11.2	agtcgacctgcaggcatgcaagctATCCCGCTTTGATTTGATAGTCAA	3'fus	
dhs-9.5.ex	tgaagcatcatccagaactcg	5'ex	construction of *dhs-9::gfp* by PCR fusion
dhs-9.5.in	aactcgaagatgatgaagaagaagc	5'in	
dhs-9.3.fus	agtcgacctgcaggcatgcaagctCAATCTGCTGTTTCCAGCCCA	3'fus	
ftn-1.5'ex	tgcttactggttctgccgag	5'ex	construction of *ftn-1::gfp* by PCR fusion

ftn-1.5'in	tgtagggtttgattgtggtttg	5'in	
ftn-1.3'fus	agtcgacctgcaggcatgcaagctTTGACGAGCTAGAGACATGAC	3'fus	
cdr-3.5'ex	acctatgatagtctcggcattg	5'ex	construction of *cdr-3::gfp* by PCR fusion
cdr-3.5'in	cgacggaccatgtagaagag	5'in	
cdr-3.3'fus	agtcgacctgcaggcatgcaagctTAAGGTTCTGGTTTTGCACG	3'fus	
R06A10.1.5'ex	tacctgcttagacactttctcg	5'ex	construction of *P*R06A10.2*::gfp* by PCR fusion
R06A10.1.5'in	ccgacgatagctcggtgttag	5'in	
R06A10.1.3'fus	agtcgacctgcaggcatgcaagctGGACAACCAGGCAATCACTAC	3'fus	
sod-3_fwd	attaagcgcgacttcggttc	*sod-3*	rtPCR
sod-3_rev	ttcttccagttggcaatcttcc	*sod-3*	rtPCR
ftn-1_fwd	cgtcatgtctctagctcgtc	*ftn-1*	rtPCR
ftn-1_rev	cattgatcgaatgtacctgctc	*ftn-1*	rtPCR
ama-1_fwd	atctcgcaggttatcgcgtg	*ama-1*	rtPCR
ama-1_rev	cggtgaggtccattctgaaatc	*ama-1*	rtPCR
DA86	ctgctggacaggaagattacg	*cdc-42*	qRT-PCR
DA87	ctcggacattctcgaatgaag	*cdc-42*	qRT-PCR
DA88	gttcccgtgttcatcactcat	*pmp-3*	qRT-PCR
DA89	acaccgtcgagaagctgtaga	*pmp-3*	qRT-PCR
DA90	gtcgcttcaaatcagttcagc	Y45F10D.4	qRT-PCR
DA91	gttcttgtcaagtgatccgaca	Y45F10D.4	qRT-PCR
ftn-1_fwd_RT2	cggccgtcaataaacagattaacg	*ftn-1*	qRT-PCR
ftn-1_rev_RT2	cacgctcctcatccgattgc	*ftn-1*	qRT-PCR
DA42	tcgtagaactgaagacaagagtgtg	*hsf-1*	*sy441* genotyping PCR
DA43	tcctcggctccatcataattcg	*hsf-1*	*sy441* genotyping PCR
DA44	attttcagccgcaacaagac	*hsf-1*	sequencing primer

hif-1_ex_fwd1	gctcctcctactccacctttg	*hif-1*	*ia4* genotyping PCR
hif-1_int_rev1.2	tcggcgatggtgtcttcagtc	*hif-1*	*ia4* genotyping PCR
hif-1_ex_rev1	gtgacgagttgtgaatgcacc	*hif-1*	*ia4* genotyping PCR
rrf-3_ex_fwd1	gagttcgcatcaagtttcac	*rrf-3*	*pk1426* genotyping PCR
rrf-3_ex_rev1	tgccttcgtacatttcaacc	*rrf-3*	*pk1426* genotyping PCR
rrf-3_int_rev2	ggtatttattgcttcctgccac	*rrf-3*	*pk1426* genotyping PCR
T7	taatacgactcactataggg	pL4440 vector	identification of RNAi cultures by PCR
JJM130	gggaagggcgatcggtgcgggcc	pL4440 vector	identification of RNAi cultures by sequencing
JJM131	gcgcagcgagtcagtgagcgagg	pL4440 vector	identification of RNAi cultures by sequencing

All oligonucleotides where ordered from MWG Biotech.

2.6.2. *C. elegans* strains used

Strain	Genotype	Origin
DR1567	*daf-2(m577)*	received from the Riddle lab
GA1001	*aak-2(ok524)*	generated by Jennifer Tullet (3x outcrossed RB754)
GA134	*glp-4(bn2ts); daf-2(m577)*	generated by Josh McElwee
GA226	*wuEx122[sod-1(+), rol-6(su1006)]*	generated by David Gems
GA228	*wuEx123[sod-1(+), rol-6(su1006)]*	generated by David Gems
GA230	*wuEx122[sod-1(+), rol-6(su1006)]*	generated by David Gems
GA300	*daf-16(mgDf50); daf-2(m577)*	generated by Dhaval Patel
GA303	*rrf-3(pk1426); daf-2(m577)*	generated by Dhaval Patel
GA362	*wuEx106[stdh-1::GFP, lin-15(+)] lin-15(n765ts)*	generated by David Weinkove
GA365	*wuEx109[dhs-9::GFP, lin-15(+)] lin-15(n765ts)*	generated by David Weinkove
GA368	*wuEx112[ugt-41::GFP, lin-15(+)] lin-15(n765ts)*	generated by David Weinkove
GA379	*wuEx106[stdh-1::GFP, lin-15(+)] daf-2(m577); lin-15(n765ts)*	generated by David Weinkove
GA380	*wuIs128[ugt-27::gfp, lin-15(+)] lin-15(n765ts)*	generated by David Weinkove
GA514	*glp-4(bn2ts), daf-16(mgDf50); daf-2(m577)*	generated by Glenda Walker
GA602	*wuEx109[dhs-9::GFP, lin-15(+)] daf-2(m577); lin-15(n765ts)*	* generated by crossing GA365 and DR1567
GA604	*wuEx112[ugt-41::GFP, lin-15(+)] daf-2(m577); lin-15(n765ts)*	* generated by crossing GA368 and DR1567
GA611	*wuEx129[gst-15::GFP, lin-15(+)] lin-15(n765ts)*	* generated by micro-injection using MT1642
GA614	*wuEx135[Pftn-1::GFP, lin-15(+)] lin-15(n765ts)*	* generated by micro-injection using MT1642
GA620	*wuEx141[*PR06A10.1*::GFP, lin-15(+)] lin-15(n765ts)*	* generated by micro-injection using MT1642
GA624	*wuEx145[Pcdr-3::GFP, lin-15(+)] lin-15(n765ts)*	* generated by micro-injection using MT1642
GA630	*wuIs176[Pftn-1::gfp, lin-15(+)] lin-15(n765ts)*	* generated by X-ray irradiation of GA614, outcrossed 6x
GA631	*wuIs177[Pftn-1::gfp, lin-15(+)] lin-15(n765ts)*	* generated by X-ray irradiation of GA614, outcrossed 6x

GA633	*wuIs177[Pftn-1::GFP, lin-15(+)] daf-2(m577)*	* generated by crossing GA631 with DR1567
GA636	*wuIs177[Pftn-1::GFP, lin-15(+)] rrf-3(pk1426); daf-2(m577)*	* generated by crossing GA633 with GA303
GA639	*daf-16(mgDf50) wuIs177[Pftn-1::GFP, lin-15(+)]*	* generated by crossing GA631 with GR1307
GA641	*wuIs177[Pftn-1::GFP, lin-15(+)]*	* generated by crossing GA631 with N2
GA642	*wuIs177[Pftn-1::GFP, lin-15(+)] hif-1(ia4)*	* generated by crossing GA641 and ZG31
GA643	*wuIs177[Pftn-1::GFP, lin-15(+)] daf-16(mgDf50); daf-2(m577)*	* generated by crossing GA633 with GA300
GA644	*wuEx123[sod-1(+), rol-6(su1006)] daf-16(mgDf50)*	* generated by crossing GA228 and GR1307
GA646	*wuIs152[sod-1(+), rol-6(su1006)] hsf-1(sy441)*	* generated by crossing GA801 and PS3551
GA648	*wuIs152[sod-1(+), rol-6(su1006)] aak-2(ok524)*	* generated by crossing GA801 and GA1001
GA675	*xtEx79[pes-10::IDE::GFP-his, pha-1(+)]*	* generated by crossing UZ96 and N2
GA676	*xtEx79[pes-10::IDE::GFP-his, pha-1(+)] hif-1(ia4)*	* generated by crossing GA675 and ZG31
GA702	*wuIs128[ugt-27::gfp, lin-15(+)] daf-2(m577); lin-15(n765ts)*	generated by David Weinkove
GA800	*wuIs151[ctl-1,2,3 (+), myo-2::gfp]*	generated by Ryan Doonan
GA801	*wuIs152[sod-1(+), rol-6(su1006)]*	generated by Ryan Doonan
GA805	*wuIs156[sod-2(+), rol-6(*su1006*)]*	generated by Ryan Doonan
GA811	*wuIs152[sod-1(+), rol-6(su1006)] daf-16(mgDf50)*	generated by Ryan Doonan
GA824	*wuEx196[rol-6(su1006)]*	generated by Ryan Doonan
GR1307	*daf-16(mgDf50)*	received from CGC
GR1307	*daf-16(mgDf50)*	received from CGC
MT1642	*lin-15(n765ts)*	received from CGC
N2	*wild-type*	received from CGC
NL2099	*rrf-3(pk1426)*	received from CGC
PS3551	*hsf-1(sy441)*	received from CGC
UZ96	*xtEx79[pes-10::IDE::GFP-his, pha-1(+)] pha-1(e2123ts)*	received from Leibold lab
XA6900	*qaEx6900[Pftn-1::[delta]pes-10::GFP-his, pha-1(+)] pha-1(e2123ts)*	received from Leibold lab
XA6902	*qaEx6902[Pftn-1(delta63)::[delta]pes-10::GFP-his, pha-1(+)] pha-1(e2123ts)*	received from Leibold lab
ZG31	*hif-1(ia4)*	received from CGC

* These strains were generated by me during the course of my PhD research.

2.6.3. RNAi clones

RNAi by feeding is induced by growing *C. elegans* on HT115 bacteria containing the L4440 plasmid, into which a fragment of the gene of interest has been cloned. This vector contains two T7 promoter sites flanking the insert and in opposite orientation in order to induce transcription of the insert by the T7 RNA polymerase from both ends.

Most RNAi clones were produced as part of large-scale efforts to create genome-wide RNAi libraries. Confirming their identity was important in the later stages of this project. An RNAi culture was grown from the frozen stock in the presence of ampicillin and tetracycline. The plasmid was then purified using the QiaSpin MINIprep kit (Qiagen) and was then sent for sequencing to the Wolfson Institute for Biomedical Research (WIBR) using the JJM130 and JJM131 primers.

2.6.4. Transcription factor RNAi library

The transcription factor RNAi library used for this project was generously provided to me by the Weiqing Li (University of Washington) and contained over 1500 clones from both the Ahringer and Vidal libraries (Kamath and Ahringer, 2003; Rual et al., 2004). The presence of sequences in the genes' coding sequence that are likely to code for protein domains thought to act in DNA binding was used as a criteria for inclusion in this library. The library therefore most likely includes some genes that are not in fact transcription factors. Many clones in this library were duplicates and after some quality control, just over 800 were used for the screen. Similar libraries are now available commercially (geneservice.co.uk).

2.6.5. Buffers

NGM agar (1L in H_2O)

3g NaCl
20g Agar
2.5g Peptone

Autoclave, then add the following sterile solutions:
1mL 1M $CaCl_2$

1mL 1M $MgSO_4$
1mL 5mg/mL cholesterol
25mL 1M KH_2PO_4 (pH 6.0)

NGM IPTG agar (1L in H_2O)

3g NaCl
20g Agar
2.5g Peptone

Autoclave, then add the following sterile solutions:
400µl 50mg/mL Carbenicillin
1mL 1M IPTG
1mL 1M $CaCl_2$
1mL 1M $MgSO_4$
1mL 5mg/mL cholesterol
25mL 1M KH_2PO_4 (pH 6.0)

OP50 growth solution (1L in H_2O)

5g Tryptone
2.5g Yeast extract
Add H_2O, then autoclave.

M9 solution (1L in H_2O)

7g $Na_2HPO_4.2H_2O$
3g KH_2PO_4
5g NaCl
0.25g $MgSO_4.7H_2O$
Add H_2O, then autoclave.

Freezing medium (200mL in H_2O)

20mL 1M NaCl
10mL 1M KH_2PO_4 (pH 6.0)
60mL 100% glycerol

Add H_2O, autoclave, then add:
0.6mL sterile 0.1M $MgSO_4$

LB (Luria-Bertani) Medium (1L in H_2O)

10g Tryptone
5g Yeast Extract
10g NaCl
Add H_2O, adjust pH to 7.5 with NaOH and autoclave.

2.7. Statistical analysis

Survival analysis

C. elegans survival under various conditions and of various genotypes was monitored as described previously. Whether survival was different from that of controls was tested using both the Wilcoxon and the Log-Rank test. These tests allow for the inclusion of right-censored data, which is necessary when performing this type of experiment. Censoring is necessary when the lifespan of individual animals cannot be monitored past a certain time-point. This may occur when worms die of causes unrelated to ageing, such as by crawling up the side of the plate and drying out. In such a case, we can only say that the animal was still alive until the day it was censored, but the day it would have died of ageing-related causes can of course not be determined. Omitting these animals would not be appropriate as it would bias the data and lead to a loss of information. Log-rank and Wilcoxon p-values were obtained using JMP version 7.0 (SAS).

Two-way ANOVA

ANOVA was performed to test for statistical significance in experiments in which the effects of several treatments were compared to a single control. Before ANOVA, the data was first transformed using the natural logarithm. This was done when the data had first been normalised using the control treatment, since ratios are not usually normally distributed and ANOVA assumes a normal distribution.

Because *ftn-1* transcript levels showed large differences between experimental blocks performed on different days, two-way ANOVA was used to test the effects of the treatments as well as of the experimental blocks. All ANOVA was performed using the open-source program R (R-project.org, accessed Feb 2010).

2.8. Platereader protocols

The method by which GFP expression was measured in strains expressing Pftn-1::gfp was adapted from a published protocol to measure *sod-3::gfp* expression (Wolff et al., 2006). Worms of the appropriate age were picked into the wells of V-shaped microtitre plates (Greiner) and measured in the GeniosPlus platereader (Tecan) at the appropriate GFP wavelengths (excitation: 495; emission: 535nm). 40

adult, or 60 L4 animals were picked into each well. A constant gain was set in the platereader for each condition.

2.8.1. Protocol 1

This protocol was used for the screen itself using GA636 as well as for much of the follow up work using GA636, GA633, GA639, GA641 and GA643.

1. Worms were grown at 15^{o}C for the first generation until a large number of gravid adults were present.
2. Eggs were isolated by alkaline hypochlorite treatment.
3. Eggs were pipetted onto NGM plates seeded with OP50 bacteria and returned to 15^{o}C where they were left to develop.
4. At L4 stage, worms were washed off plates and pipetted onto RNAi plates. These plates were then incubated for two days at either 25^{o}C or 20^{o}C.
5. For each RNAi treatment, 40 two–day old adults were picked into a well of a clear microtitre plate (V-shaped wells (Greiner)), containing 50µl M9.
6. Once animals from all RNAi treatments and worms grown on control RNAi had been picked, the animals were anaesthetised by adding 10µl of 50mM sodium azide.
7. Fluorescence was measured in a GeniusPlus platereader (Tecan) at the excitation wavelength 495nm and emission wavelength 535nm.

In some experiments described in Chapter 5, the switch from 15^{o}C to 25^{o}C was not required. Instead, worms were grown at 20^{o}C throughout. The screen itself was carried out as described in Figure 16.

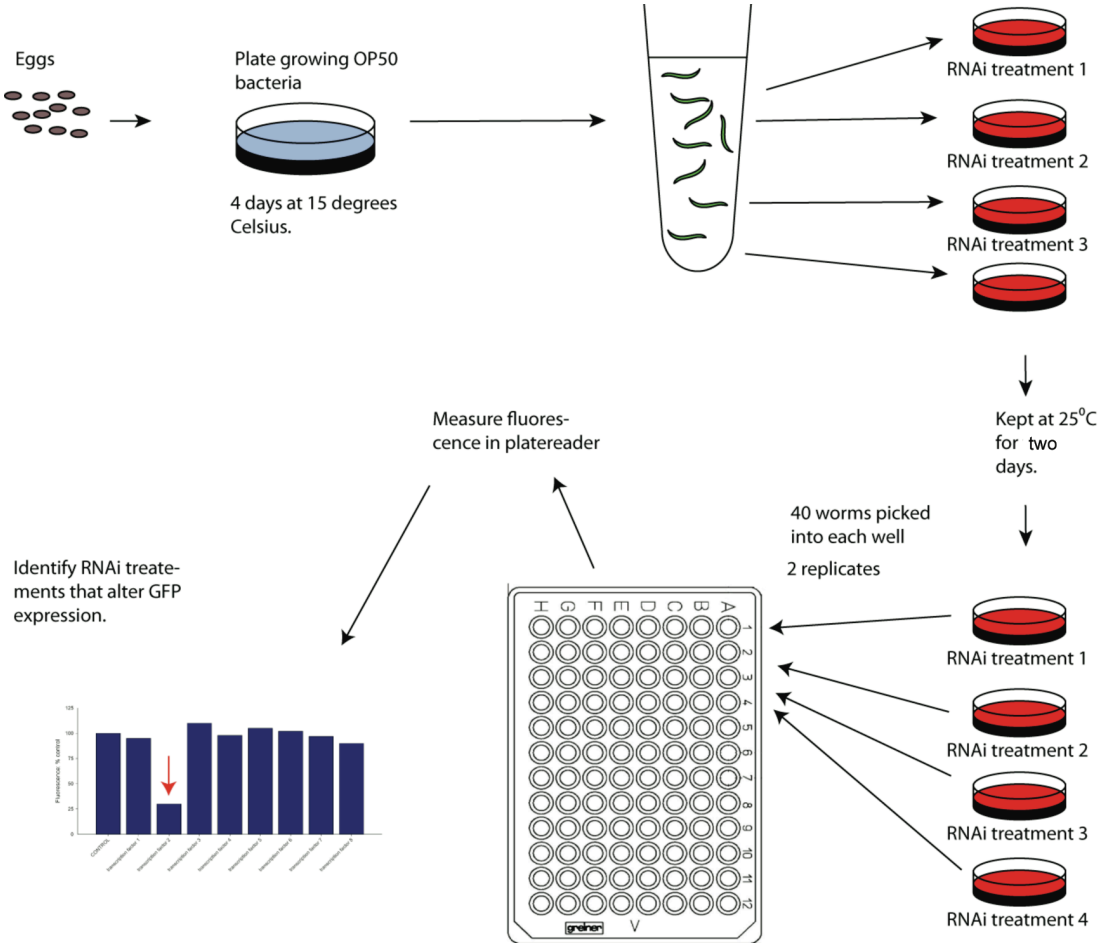


Figure 16
Protocol for finding transcription factors involved in the up-regulation of expression from reporter construct.

2.8.2. Protocol 2

1. Worms were grown at 15°C for the first generation until a large number of gravid adults were present.
2. Eggs were isolated by alkaline hypochlorite treatment.
3. Arrested L1 animals were obtained by leaving the eggs to hatch in M9 at 15°C for 36h.
4. L1s were pipetted directly onto RNAi plates and these plates were then returned to 15°C for 3 days.
5. At the L3 stage, worms were transferred to 25°C and left to incubate for a further 2 days.
6. Adult worms were subsequently either

a. picked into wells of a microtitre plate (clear, V-shaped wells (Greiner)). 40 worms were picked per well. (See protocol 1)

b. washed off plates using M9 and into 1.5mL capped tubes. Worms were left to settle, supernatant was removed, M9 replaced. This washing step was repeated four times to remove *E. coli* and L1 larvae. After final washing step, 200µl of M9 (including worms) were left in the tube and the tube was kept on ice. 800µl of Trizol reagent was then added and the contents were transferred to a ribolizer tube (manufacturer). See protocol on RNA extraction for further details.

2.8.3. Protocol 3

1. Eggs were isolated by alkaline hypochlorite treatment and were placed on RNAi plates.
2. Worms were grown at 25^{o}C for the first generation.
3. Gravid adult animals were transferred to fresh RNAi plates and returned to 25^{o}C.
4. 60 L4 animals of the second generation were picked into wells of microtitre plate.
5. Fluorescence was measured in a GeniusPlus platereader (Tecan) at the excitation wavelength 495nm and emission wavelength 535nm.

Chapter 3: The Screen

As described in Chapter 1, section 1.6, my first task was to identify a GFP reporter strain that I could use as a readout for the transcriptional consequences of altered IIS. This task was aided by the availability of microarray data. I therefore tested GFP reporters for genes found to be up-regulated in *daf-2* mutant animals compared to *daf-16; daf-2* mutants. Initially, I focused on drug metabolising enzymes (DMEs), with the aim of identifying regulators of DME metabolism, since these had been found to be up-regulated in a number of models of long-lived IIS mutants (McElwee et al., 2007). GFP reporters tested later in the project were not necessarily selected using the same criteria (eg. *ftn-1* and R06A10.1).

3.1. Testing existing reporters

Because of the potential role of drug metabolising enzymes (DMEs) as effectors of the longevity phenotype of insulin signalling mutants, GFP reporters of DMEs were tested for up-regulation in *daf-2* mutant animals compared to *daf-2* wild-type animals. Several GFP reporter strains of drug metabolising enzymes had already been created before my arrival at the Gems lab and these were the first to be tested. These first four reporter constructs I examined were so-called translational GFP fusion constructs, for which upstream and coding sequences of DME genes were fused to GFP. Induction by reduced insulin signalling was tested by crossing these constructs into a *daf-2(m577)* mutant background and then comparing the fluorescence of the two strains at 25^{o}C, the restrictive temperature of this allele.
Of these four genes, *ugt-27* and *ugt-41* code for putative UDP-glucuronosyl transferases, *dhs-9* for a putative short-chain dehydrogenase/reductase and *stdh-1* for a putative steroid dehydrogenase. The GFP expression levels of the *ugt-41*, *dhs-9*, and *stdh-1* reporters are very low, and their fluorescence was found to be below the detection threshold of the platereader method being used. This was ascertained by measuring their fluorescence alongside a non-fluorescent control strain and in none of the three cases did the fluorescence rise to detectable levels in *daf-2* mutants (Figure 17 and data not shown). None of these *daf-2(-)* reporter strains exhibited statistically significantly higher levels of fluorescence than *daf-2(+)* controls. A

GFP reporter strain for *ugt-27* showed high GFP expression, which was easily detectable above the background fluorescence. However, it showed no statistically significant increase in the *daf-2* mutant background (see Figure 17). This was surprising, since microarray data showed a highly significant, 29.6–fold difference in expression of this gene between *daf-2* and *daf-16;daf-2* mutant animals. The reasons for this discrepancy were not investigated. One possibility is that *ugt-27* is not up-regulated in response to reduced IIS, but simply requires *daf-16* to be expressed even in a wild-type background. However, RNAi of *daf-16* in a separate experiment did not statistically significantly reduce *ugt-27::gfp* expression in a *daf-2(+)* background (data not shown). It is also possible that not all of the regulatory elements required for the induction of expression in *daf-2* mutants were contained in the sequence used in this transgene.

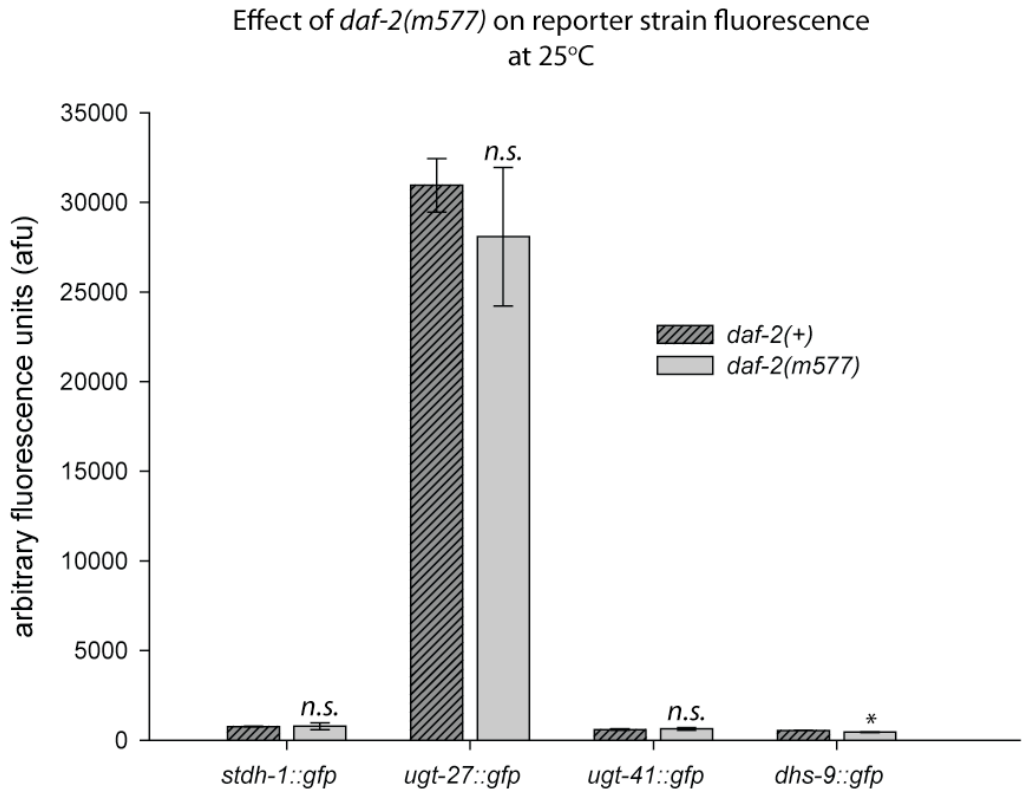


Figure 17
Gene names refer to translational GFP reporters of the gene mentioned. Error bars represent ±*s.e.m*. Statistical significance tested using Student's t-tests.

3.2. Making new reporter constructs

Four more transgenic reporter lines were made for the genes R06A10.1, *ftn-1*, *cdr-3* and *gst-15*. The *gst-15::GFP* construct was created by David Weinkove and I created the strain expressing it using microinjection; for the remaining three genes, I made the constructs and the transgenic lines. These genes were selected using a number of criteria, including whether they belonged to functional groups thought to be involved in determining lifespan (eg. DMEs), whether they were especially strongly IIS–regulated in microarray data, or for other reasons.

These were not crossed into the *daf-2(m577)* background but *daf-2* RNAi was used instead to test the effect of reduced IIS on GFP expression. This was done to avoid the time-consuming process of crossing the array into a new genetic background. Unlike the previous strains, only the genes' upstream sequences was fused to GFP for these four constructs (ie. the coding sequence was not included). All of these genes were included because they were found to be up-regulated in microarray data comparing *glp-4(bn2ts)*; *daf-2(m577)* animals to the *glp-4(bn2ts)*, *daf-16(mgDf50); daf-2(m577)* mutant animals (McElwee et al., 2004), thus indicating that they were regulated by insulin signalling in a *daf-16* dependent manner (see Table 2).

Gene name	Fold change	q	putative gene function
stdh-1	3.94	0.000367	steroid dehydrogenase
ugt-27	29.56	0	UDP-glucuronosyl transferase
ugt-41	3.38	0.00316	UDP-glucuronosyl transferase
dhs-9	2.45	0.0167	short-chain dehydrogenase/reductase
gst-15	3.55	0.00707	glutathione S-transferase
R06A10.1	5.32	0.00205	unknown
ftn-1	47.14	0	ferritin, iron-storage protein.
cdr-3	19.35	0.000103	glutathione S-transferase

Table 2
Microarray experiment was performed in (McElwee et al., 2004) and reanalysed for (McElwee et al., 2007). Fold change refers to the ratio of *glp-4(bn2ts)*; *daf-2(m577)* to *glp-4(bn2ts)*, *daf-16(mgDf50); daf-2(m577)*. q-values under 0.1 were considered statistically significant. I did not contribute to the work shown in Table 2.

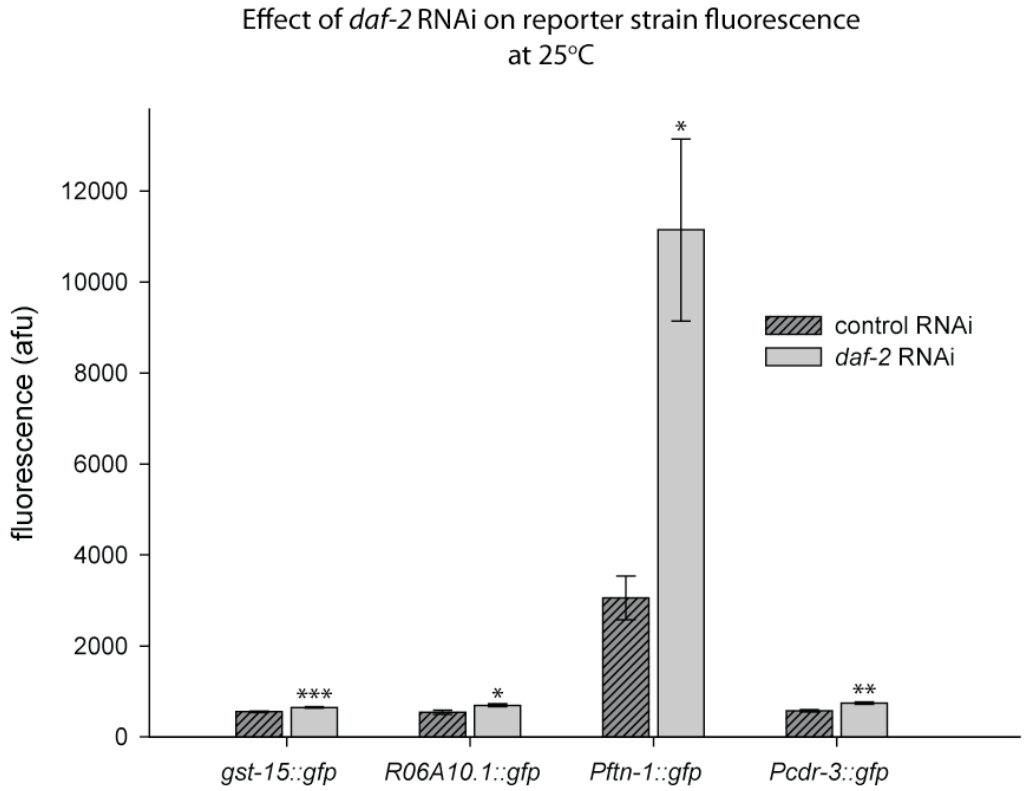


Figure 18
Effects of *daf-2* RNAi treatment on reporter strain fluorescence. Error bars represent ±*s.e.m*. Statistical significance tested using the Student's t-test.

All four of these further genes tested showed statistically significant up-regulation of their GFP transgenes upon RNAi of *daf-2*. In summary, of the eight GFP reporter strains tested here, only one, *Pftn-1::gfp*, was found to both express at sufficiently high levels and be sufficiently induced by reduced IIS to be useful for this screen. While insufficient biological replicates were tested to ascertain this with confidence, GFP expression from the other constructs may very well be increased upon *daf-2* RNAi treatment. However, this feature alone does not make them suitable for use in the screen, since the response to reduced IIS on GFP levels must be readily detectable in a platereader. This underscores one of the problems of using the fold-change of expression in microarray data to guide the making of reporter constructs: While the genes chosen on the basis of the microarray experiment (McElwee et al., 2004) all have a statistically significant increase in GFP levels in response to *daf-2* RNAi, this data gives no prediction of how high expression levels are in absolute terms. Knowledge of absolute expression levels is very important in the design of a

reporter construct for the type of high-throughput RNAi screen being prepared here because the GFP levels need to be easily detectable above the background fluorescence.

In order to illustrate this issue, Table 3 contains the same data as Figure 18, but expression is displayed as a percentage of the control RNAi –treated reporter strain. Comparison with the non-GFP control strain measured alongside it allows for an approximate estimation of the contribution of background fluorescence to the signal measured in the platereader. Comparison with the *daf-2* RNAi –treated animals demonstrates the extent to which the induction of the reporter construct is detectable using the platereader.

	non-GFP control	**control RNAi**	***daf-2* RNAi**
	%	%	%
gst-15::gfp	65.8	100	116.9
R06A10.1*::gfp*	71.3	100	128.2
Pftn-1::gfp	13.1	100	364.6
Pcdr-3::GFP	69.7	100	129.6

Table 3
Fluorescence was measured for strains containing the reporter construct and treated with both control and *daf-2* RNAi. Fluorescence of a non-GFP control strain was measured alongside the reporter strain. The fluorescence was statistically significantly higher than the non-GFP control strain for all four of these strains (t-test results: *gst-15*: p=1.27E-06; R06A10.1: p=0.0255; *ftn-1*: p=0.00521; *cdr-3*: p=0.00109)

Table 3 shows that only the *Pftn-1::gfp* reporter emits a GFP signal that is clearly distinguishable from background fluorescence, since background fluorescence only accounts for 13% of the fluorescent signal. Additionally, the induction of GFP expression by reducing IIS through *daf-2* RNAi is by far the most easily detectable in the *Pftn-1::gfp* reporter strain.

The degree to which GFP expressed from the reporter construct is detectable above background fluorescence by the platereader being used depends on both the intensity of expression and the expression pattern of the reporter. In the *Pftn-1* reporter strain, GFP is expressed in the intestine, one of the largest tissues of *C.*

elegans, and is expressed at very high levels, making it easily detectable above the low levels of background fluorescence.

3.3. Semi-quantitative rtPCR verification of microarray data

The large fold change in *ftn-1* levels detected in the microarray experiment comparing *glp-4(bn2ts)*; *daf-2(m577)* mutant animals to *glp-4(bn2ts)*, *daf-16(mgDf50); daf-2(m577)* mutant animals was confirmed in a preliminary semi-quantitative rtPCR study. The results are shown in Figure 20. Band intensities were normalised using levels of the *ama-1* transcript. *ama-1* codes for the large subunit of RNA polymerase II. Expression of *sod-3*, a superoxide dismutase gene known to be up-regulated in IIS mutants (see Chapter 1), was also quantified as a positive control.

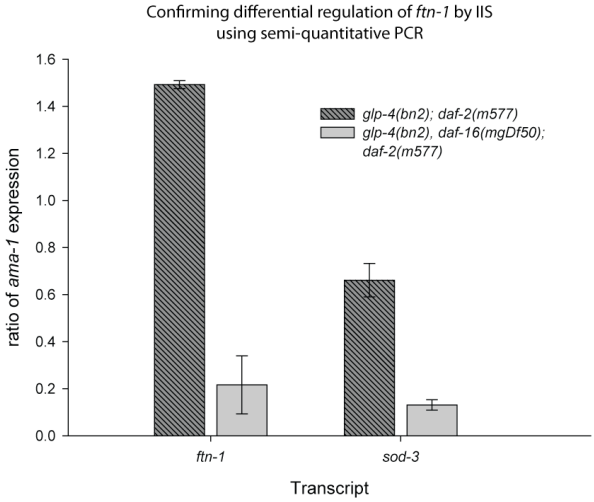
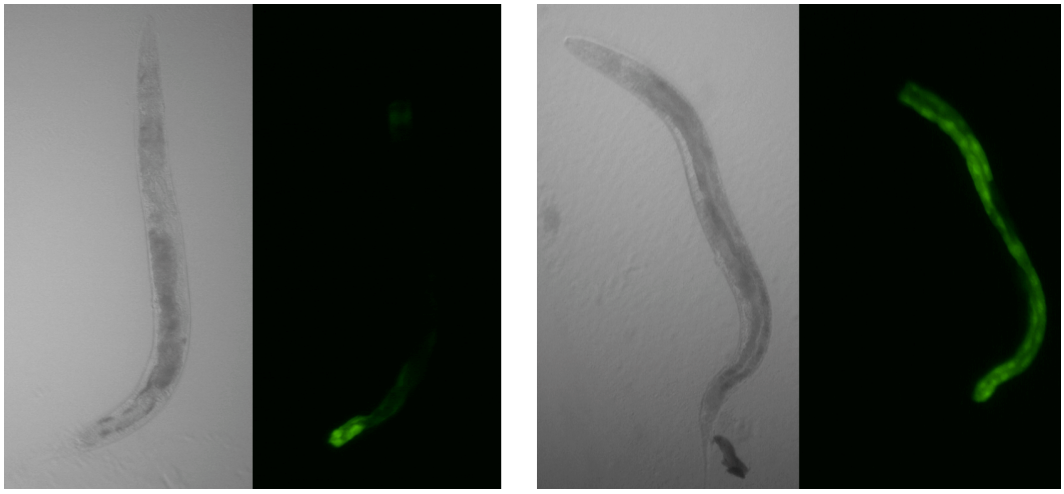


Figure 19
A *glp-4(bn2)* mutant background was used to prevent the emergence of progeny. This experiment was carried out on a single biological replicate and sqRT-PCR was carried out in two technical replicates. Error bars represent the range of these technical replicates.

The combined microarray, rtPCR and reporter gene results establish with confidence that *daf-2(m577)* mutant animals have far higher levels of the *ftn-1* transcript than *daf-16(mgDf50); daf-2(m577)* animals do. The results of changes in reporter strain fluorescence (Figure 18) suggest that this is due to up-regulation of *ftn-1* expression by *daf-2* mutations in a DAF-16 dependent manner, not by the downregulation of *ftn-1* expression in *daf-16* mutants.

3.4. The *ftn-1::gfp* reporter: A useful readout for the IIS pathway?

Several characteristics of the *Pftn-1::gfp* reporter strain make it suitable for the type of high-throughput RNAi screen I attempted in this screen. One is its intestinal GFP expression, which leads to high levels of fluorescence given large size of the intestine. Additionally, RNAi is not equally effective in different tissues of *C. elegans*, with neurons being particularly resistant to its effects. As the worms ingest the dsRNA through their food source, the intestine is likely to be exposed to higher concentrations relative to other tissues and is presumably more strongly affected. The *Pftn-1::gfp* construct was found to express GFP at particularly high levels and this made for more sensitive quantification in the platereader, which I wanted to use as a quick means of quantifying expression. Additionally, a very strong induction of GFP expression by reduced insulin was detectable using the platereader. This reporter construct, so far present in a multi-copy extra-chromosomal array, was therefore integrated in order to obtain a 100% transgenic population and subsequently out-crossed six times to the parent strain to remove mutations produced by the process of integration. The strong induction of *Pftn-1::gfp* expression by mutations in IIS can be seen in Figure 20.



daf-2(+) ***daf-2(m577)***

Figure 20
Adult *C. elegans* carrying the integrated *Pftn-1::gfp* transgene in both *daf-2* genotypes. Worms were grown at 15°C until the L4 stage and then switched to 25°C for two days before images were taken.

Other benefits of studying *ftn-1* expression can be found in the role of *ftn-1* as encoding an iron storage protein. Given that the post-transcriptional regulation of vertebrate ferritins in response to iron does not seem to be conserved in *C. elegans* and that the mechanisms involved in the transcriptional regulation of worm ferritins are unknown, an RNAi screen aimed at identifying regulators of *ftn-1* would go a long way in furthering our understanding of how iron homeostasis is maintained in *C. elegans*. Given the importance of maintaining stable labile iron pools to ensure sufficient availability for a number of different processes requiring iron, understanding how iron homeostasis is maintained should be of interest to a large number of researchers that use the worm as a model organism. In addition, given that oxidative stress has been proposed to be a primary cause of ageing (see Chapter 6), and iron catalyzes the formation of reactive oxygen species (ROS) via the Fenton reaction, there may be a direct role of *ftn-1* in ageing through its role in the maintenance of iron homeostasis.

3.5. General description of screen

The aim of the screen was to identify transcription factors that regulate *ftn-1* expression in *C. elegans*. In particular, I was interested in identifying those transcription factors that regulate the *daf-16*–dependent induction of *ftn-1* levels by reduced insulin signalling. The rationale behind using a *ftn-1* reporter and of this approach in general is discussed in section 1.6 of Chapter 1. Before beginning the screen, I first integrated the *Pftn-1::gfp* transgene into the genome by X-ray irradiation and outcrossed the resulting strain six times to remove mutations introduced through the mutagenesis. This was done in order to obtain 100% transgenic populations of worms, which greatly facilitated accurate quantification of fluorescence during the screen.

In order to induce expression, I crossed this integrated *Pftn-1::gfp* reporter described above into a *daf-2(m577)* mutant background. I also introduced the *rrf-3(pk1426)* allele in order to increase susceptibility to RNAi. Worms of the resulting *Pftn-1::gfp rrf-3(pk1426); daf-2(m577)* genotype were used to screen a library of RNAi clones. To do this, a synchronised population was grown at 15^{o}C, then switched at the L4 stage both to RNAi plates containing individual RNAi treatments

and to 25°C, the restrictive temperature for the *m577* allele. Animals were kept at 25°C for two days before fluorescence intensity was measured. This was done by picking 40 worms into the wells of V-bottom microtitre plates (Greiner) containing 50µl of M9 media. Worms were anaesthetised through the addition of 10µl of 50mM sodium azide and fluorescence was then measured at the standard wavelengths for GFP (excitation 495nm, emission 535nm). Each measurement included at least two wells of control RNAi-treated worms and one well of worms from each RNAi treatment. Two such 'batches' were measured on the same day from each set of RNAi plates. The raw data from these measurements was normalised to the mean signal from control RNAi-treated worms in the same 'batch'. This protocol was used to screen a library of RNAi clones targeting transcription factors (described in Chapter 2). In total, RNAi of 812 genes was tested for their effects on *Pftn-1::gfp* expression in *rrf-3(pk1426); daf-2(m577)* mutant animals. The results of this screen are displayed as a histogram in Figure 21, which shows how the results (normalised to fluorescence from control RNAi–treated worms) are distributed.

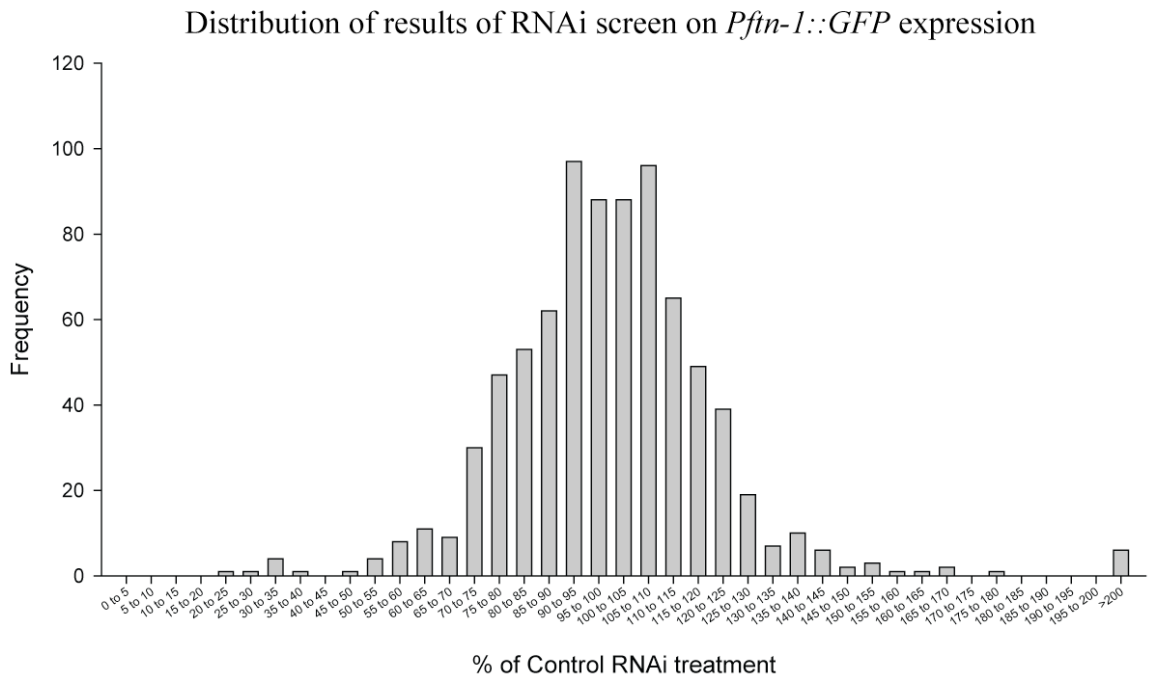


Figure 21
Screen results: Data is displayed as a histogram. The raw data was normalised to represent a percentage of the signal obtained from control RNAi-treated worms. The screen was carried out by measuring fluorescence of RNAi treated *Pftn-1::gfp rrf-3(pk1426); daf-2(m577)* animals following the protocol described in Section 2.8.1. Protocol 1.

In the central screen, treatment with many RNAi clones led to alterations in the fluorescence of my reporter strain. Most candidate genes that scored above 120% or below 80% of control were re-tested. After this re-testing phase, I focused on RNAi treatments that led to a reduction in the induced fluorescence rather than those that further increased fluorescence, since I was interested in genes involved in inducing gene expression in response to reduced IIS. Table 4 lists the 32 RNAi treatments that led to such a reduction. The mean of these measurements is displayed as a percentage of the fluorescence of control–RNAi treated worms. For RNAi treatments that led to an increase in fluorescence, see Appendix 1 and Chapter 5.

Gene	mean % of control	n	st. dev.	description
daf-16	19.7	5	3.9	
ast-1	26.8	2	9.8	determinant of DA neuron fate. ETS-box.
hsf-1	31.5	2	5.4	heat shock factor
nhr-116	45.6	2	0.2	nuclear hormone receptor
dve-1	55.9	2	0.7	*Drosophila* DVE homologue
psa-1	57.2	2	5.7	component of the SWI/SNF complex
hlh-30	58.5	2	6.2	helix loop helix factor
blmp-1	58.8	2	9.8	zinc finger protein
pbrm-1	60.6	2	14.7	polybromo 1 ortholog
mdl-1	64.3	2	7.1	similar to vertebrate MAD
nhr-66	67.4	2	11.5	nuclear hormone receptor
T07C12.11	68.5	2	10.4	unknown
nhr-34	69.6	2	9.5	nuclear hormone receptor
F44E2.7	74.6	2	2.4	unknown
nhr-36	75.8	2	6.8	nuclear hormone receptor
lin-29	76.4	2	2.9	zinc finger protein
jun-1	77.4	2	19.3	JUN homologue
lir-1	77.6	2	4.0	zinc finger protein
nhr-80	78.1	2	0.2	nuclear hormone receptor
lin-40	78.4	2	20.1	zinc finger protein
cdc-14	79.8	3	36.7	Cdc14p homologue
T26A8.4	80.7	2	14.0	Ccr4-Not deadenylase complex component
zfp-3	83.4	2	2.7	zinc finger protein
D1046.2	83.7	2	10.4	zinc finger protein
T11G6.8	84.2	2	13.8	predicted RNA-binding protein
him-8	84.6	2	25.4	zinc finger protein
C28H8.9	85.8	2	12.7	unknown
mxl-1	86.4	3	8.1	*mdl-1* binding partner
F21D5.9	87.8	2	31.0	unknown
mab-3	87.9	2	6.3	DM domain-containing
rad-26	93.5	2	30.1	predicted DNA helicase
npax-4	93.8	2	31.2	unknown

Table 4
32 genes for which knockdown led to reduced fluorescence of the *Pftn-1::gfp rrf-3(pk1426); daf-2(m577)* strain, ranked in order of severity of effect of RNAi.

In order to confirm the validity of the screen's initial findings, RNAi plasmids were purified, sequenced to confirm their identity, and subsequently re-transformed into competent HT115 *E. coli*. Several RNAi clones were discarded due to incorrect sequence of the insert. The HT115 strain of *E. coli* was obtained from the CGC and made competent using a standard $CaCl_2$ protocol (Sambrook and Russell, 2001). These re-transformed strains were used for all subsequent tests.

3.6. Testing the effects of candidate gene RNAi on ageing

The screen for transcription factors affecting *Pftn-1::gfp* expression in *daf-2* mutants revealed a large number of possible regulators, although the mechanisms by which the recorded effect on fluorescence occurs in each case is unknown. Since the ultimate aim of this project was to identify genes that contribute to the longevity of *daf-2* mutants, the next logical step was to attempt to identify whether any of the RNAi treatments were effective not only at suppressing the induced *Pftn-1::gfp* expression in *daf-2* mutants, but also at suppressing their longevity. I therefore proceeded to test the effects of RNAi of candidate genes on lifespan of *daf-2* mutants. I used the *rrf-3(pk1426); daf-2(m577)* strain and grew worms at 15^{o}C until they reached the L4 stage of development, then transferred them to RNAi plates and to 25^{o}C.

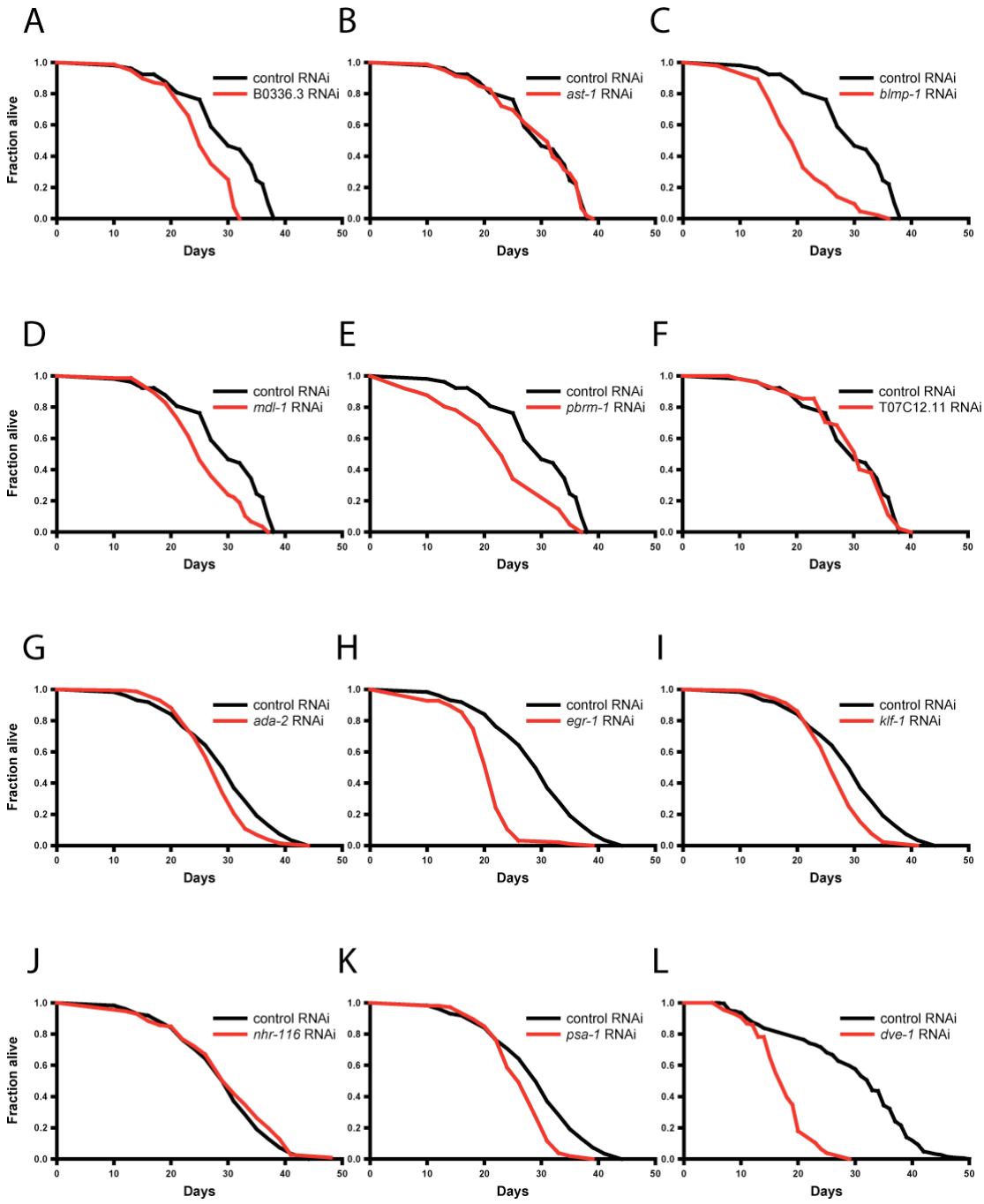


Figure 22
The effects of several candidate RNAi–treatments on lifespan of *rrf-3;daf-2* animals at 25°C was tested.

All lifespan assays were carried out on *rrf-3(pk1426); daf-2(m577)* animals

RNAi treatment [Trial number]	n	Censored	Mean Lifespan	Median Lifespan	% Change[a]	Log-Rank p-value[b]	Wilcoxon p-value[b]
Control [1]	42	10	29.3	30			
ast-1 [1]	74	7	29.3	31	none	0.7625	0.8132
B0336.3 [1]	71	8	25.3	25	-14 [-17]	<.0001	0.0011
blmp-1 [1]	44	2	20.6	19	-30 [-37]	<.0001	<.0001
mdl-1 [1]	60	16	25.9	25	-12 [-17]	0.0003	0.0056
pbrm-1 [1]	42	8	22.9	23	-22 [-23]	0.0001	0.0001
T07C12.11 [1]	46	4	29.9	31	none	0.7724	0.9493
Control [2]	231	2	29.09	29			
ada-2 [2]	139	6	27.77	29	-5 [-0]	0.0006	0.0203
egr-1 [2]	95	1	20.74	22	-29 [-24.1]	<.0001	<.0001
klf-1 [2]	132	6	26.53	26	-9 [-10.3]	<.0001	0.0002
nhr-116 [2]	110	2	29.73	31	none	0.1748	0.4519
psa-1 [2]	105	2	26.32	26	-10 [-10.3]	<.0001	0.0002
Control [3]	186	6	29.44	33			
dve-1 [3]	97	16	17.27	17	-41 [-48.5]	<.0001	<.0001
B0336.3 [3]	111	13	22.56	24	-23 [-27.3]	<.0001	<.0001
mdl-1 [3]	128	12	26.88	29	-9 [-12.1]	0.0427	0.0127
pbrm-1 [3]	87	11	24.78	25	-16 [-24.2]	0.0027	0.0006

Table 5
This table contains data corresponding to Figure 22. [a] Describes reduction in median lifespan [change in mean lifespan in brackets] compared to corresponding control RNAi–treated group. The percent reduction is only displayed when it was found to be statistically significant. [b] all statistical tests were calculated between RNAi treatment in question and the corresponding control RNAi–treated group.

The initial expectation for this set of experiments was that very few RNAi treatments would be effective at reducing the lifespan of *daf-2* mutants. This was based on previous work in the lab in which the roles of a large number of phase I and II detoxification enzymes in mediating the extended lifespan of *daf-2* mutants was tested in a similar manner (D. Weinkove, unpublished results). Another former member of the lab had similar experiences when investigating candidate genes from a microarray experiment comparing *daf-2* to *daf-16; daf-2* mutants (McElwee et al., 2003). In these two cases, as well as in a genome wide RNAi screen for genes involved in mediating *daf-2* longevity (Samuelson et al., 2007), only a very small proportion of RNAi treatments was able to reduce the lifespan of *daf-2* mutants. My aim was therefore to screen a relatively large number of 'candidate' RNAi treatments for their effects on lifespan. I was expecting to be left with a small number of genes

that both reduced *Pftn-1::gfp* expression and also reduced lifespan in *daf-2* mutants. My plan was to focus my efforts on this greatly reduced set of genes and hopefully characterise their role in mediating the longevity of *daf-2*.

Instead, I found that RNAi of most of these genes I tested reduced lifespan of *rrf-3; daf-2* from the small but statistically significant effects of *ada-2* RNAi (no reduction in median lifespan and a small reduction in mean lifespan) to the very large effect of RNAi of *dve-1*. Many of the lifespan assays were repeated and the reduction in lifespan was generally reproducible (see Table 5). This seemed to suggest that my screen had been successful in using the IIS-regulated *Pftn-1::gfp* reporter to identify factors involved in mediating *daf-2* longevity. However, it is also possible that RNAi of these genes was merely making the worms sick, thus reducing their lifespan, rather than accelerating ageing. Designing experiments that distinguish these two possibilities is quite difficult.

One approach is to test whether the reduction in lifespan is less marked or non-existent upon RNAi–mediated knockdown of the gene in question in a *wild-type* background. If yes, this would imply that the gene is contributing to *daf-2* longevity. One may also test the effects of gene knockdown on *daf-16; daf-2* lifespan to distinguish reductions in IIS-mediated longevity from simple pathology. Testing the effects of over-expressing the gene in question on lifespan is another approach, since higher levels of a gene that regulates downstream targets of IIS may itself extend lifespan. Over-expression of the important transcription factors *hsf-1, daf-16* and *skn-1* was found to extend lifespan in all three cases.

Clearly, while all of these approaches have their weaknesses, a combination of them could establish the role of any given transcription factor in mediating *daf-2* longevity. However, given the large number of RNAi treatments that had significant effects on *daf-2* lifespan and the work-intensive nature of lifespan assays, especially when creation of transgenic strains is required, finding ways to reduce the number of genes being investigated was necessary to expedite the process. I therefore decided to abort the lifespan assays before all candidate genes had been tested and to instead proceed by confirming the results of the screen before continuing, asking the question: Which of the genes identified in my screen are true regulators of *ftn-1*?

3.7. Confirming the effects of RNAi on *Pftn-1::gfp* fluorescence

In order to confirm that the genes identified earlier in this chapter were indeed true regulators of *ftn-1*, I proceeded in two stages. First, I sought to confirm that the results of my screen were accurate and that the genes in question were indeed true regulators of the *Pftn-1::gfp* transgene. Second, I confirmed that genes that have effects on *Pftn-1::gfp* expression also affect transcription of the endogenous *ftn-1* gene, thereby excluding the possibility of transgene–specific effects.

Since some of the strains required for work later in this chapter were not available in a genetic background carrying the *rrf-3(pk1426)* mutation, which would therefore need to be crossed into these strains, I simultaneously tested whether this background was even required. I therefore tested the effects of a number of different 'candidate' RNAi treatments on *Pftn-1::gfp daf-2(m577)* fluorescence in both the presence and absence of the *rrf-3* allele. Results were very similar regardless of the *rrf-3* genotype, indicating that the increased sensitivity of *rrf-3(pk1426)* mutants to RNAi is not necessary for the effect on *Pftn-1* expression. The results presented in Figure 23 were generated in an *rrf-3(+)* genetic background. See Appendix 3 for the same tests performed in the presence of *rrf-3(pk1426)*.

For this experiment, the identity of RNAi plasmids were verified by sequencing and used to transform competent HT115 bacteria that had been freshly obtained from the CGC. I grew *Pftn-1::gfp daf-2(m577)* worms at 15^{o} C until the L4 stage, then treated them with RNAi and measured their fluorescence after two days at 25^{o}C.

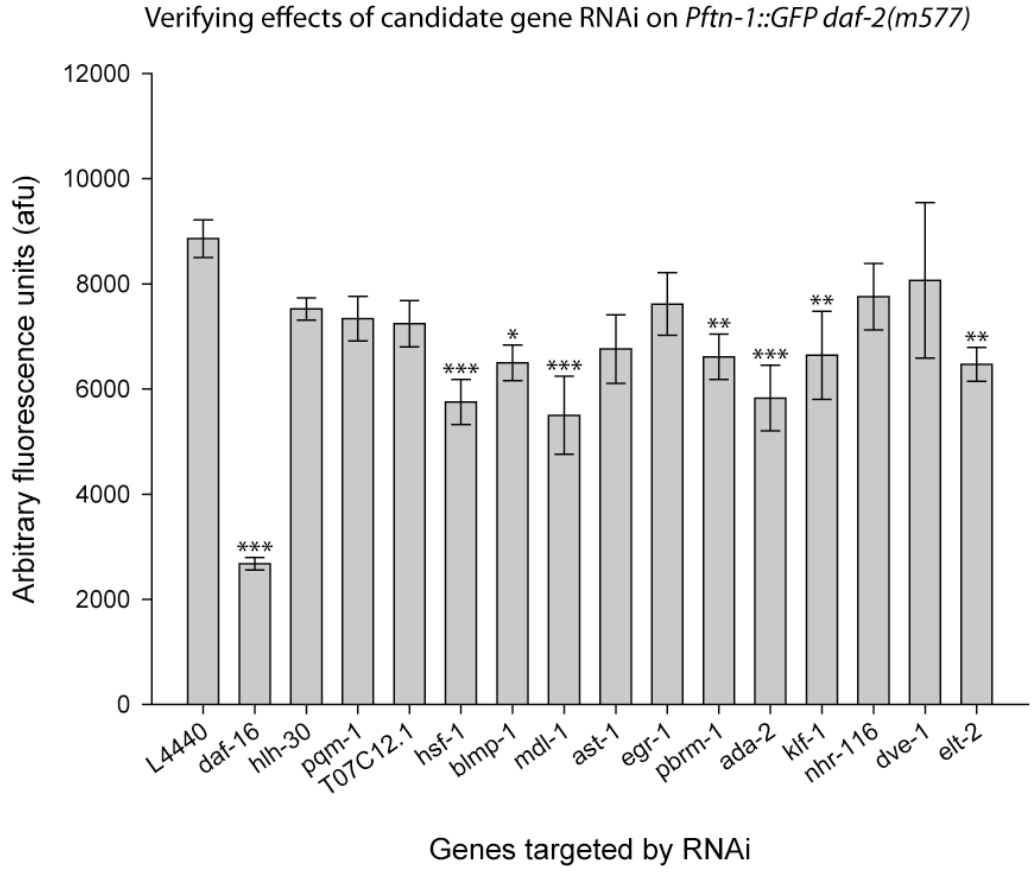


Figure 23
Shows the reduction in *Pftn-1* fluorescence in response to RNAi treatment of candidate genes in the *Pftn-1::gfp daf-2* animals. Fluorescence of RNAi-treated worms was measured following Protocol 1 (see section 2.8.1. Protocol 1). Error bars represent ±*s.e.m*. Statistical significance tested using two-way ANOVA: *: $p<0.05$, **: $p<0.01$, ***:$p<0.001$. p-values were adjusted for multiple comparisons using the Bonferroni correction.

Of these 14 genes, 7 significantly reduced *Pftn-1::gfp* expression, but none had as strong an effect as RNAi of *daf-16.* Thus, the screen did not identify any gene that acts downstream of *daf-16* to mediate all of the effects of reduced IIS on *ftn-1* expression. Weaker determinants could, however, still be important. I therefore proceeded to the second stage: confirming that the genes regulating *Pftn-1::gfp* also regulate the *ftn-1* transcript.

3.8. Using qRT-PCR to quantify *ftn-1* transcript levels in response to RNAi

Several transcription factors were confirmed to affect the transcription of GFP from a *Pftn-1::gfp* promoter, but it remained to be shown whether this effect was reproducible for the endogenous *ftn-1* transcript or whether it is specific to the regulation of the GFP reporter. Artefactual findings of changes in *Pftn-1::gfp* expression could occur if RNAi treatments were having effects on, for example, GFP protein translation rather than transcription from the *Pftn-1* promoter. Quantitative real-time PCR (qRT-PCR) is a sensitive method for the detection of differences in transcript abundance. This technique was used to monitor endogenous *ftn-1* transcript levels in worms of different genotypes that were treated with RNAi against the transcription factors in question.

However, before being able to collect the RNA samples, the protocol needed to be adjusted. Under the current protocol, worms were grown until L4 at 15°C on OP50 *E. coli*, then shifted to RNAi treatment and 25°C, and grown for two days before they were scored. This meant that the progeny of the worms being tested had already reached the late larval stages by the time the parents were ready to be scored. In the case of the platereader assay, this was not a problem, as it is a simple matter to pick only the adults into the microplate wells. However, when collecting RNA, worms needed to be washed off the plates in large numbers. In this case, a large portion of the RNA pool under examination will have been contributed by the progeny, not the adults. To circumvent this problem, the protocol was slightly altered to allow the worms to be collected two days earlier (see Section 2.8.2. Protocol 2). I confirmed that the effect of RNAi on the GFP reporter strain was preserved despite this change and found that it was (see Appendix 2)

RNAi of the following genes was tested: *daf-16, pqm-1*, T07C12.1, *hsf-1, blmp-1, mdl-1, egr-1, pbrm-1, ada-2, elt-2, klf-1*. These genes were selected because RNAi of each of them was capable of reducing *daf-2* lifespan in preliminary studies (Figure 22) and reduced *Pftn-1::gfp* expression. The three genes *pqm-1*, T07C12.1 and *egr-1* were included despite the fact that the observed small reductions in the mean fluorescence expressed from *Pftn-1::gfp* was not statistically significant (Figure 23).

3.8.1. Quantifying *ftn-1* transcript levels

Because of the high cost of performing a qRT-PCR assay with such a large number of samples, a preliminary experiment using only two biological replicates was carried out first. I collected two biological replicates of *daf-2(m577)* animals exposed to RNAi against the genes *daf-16, pqm-1*, T07C12.1, *hsf-1, blmp-1, mdl-1, egr-1, pbrm-1, ada-2, elt-2, klf-1*. The aim was to identify the true regulators of *ftn-1* expression before carrying out further work on them. I also collected samples of *wild-type, daf-16(mgDf50); daf-2(m577)* and *daf-16(mgDf50)* worms treated with the same RNAi in parallel to the *daf-2(m577)* worms. Analysis of *ftn-1* levels in these samples will be discussed in the next chapter.

RNA was isolated from these samples, DNAse I treated, cDNA was synthesised using oligodT and quantitative real-time PCR (qRT-PCR) was performed to quantify *ftn-1* mRNA transcript levels in these samples. *ftn-1* transcript levels were normalised by dividing the *ftn-1* transcript quantity by the geometric mean of three reference genes *cdc-42*, *pmp-3* and Y45F10D.4 transcript quantities (the reasons for selecting this set of reference genes will be discussed later in this chapter). The result of this analysis is shown in Figure 24.

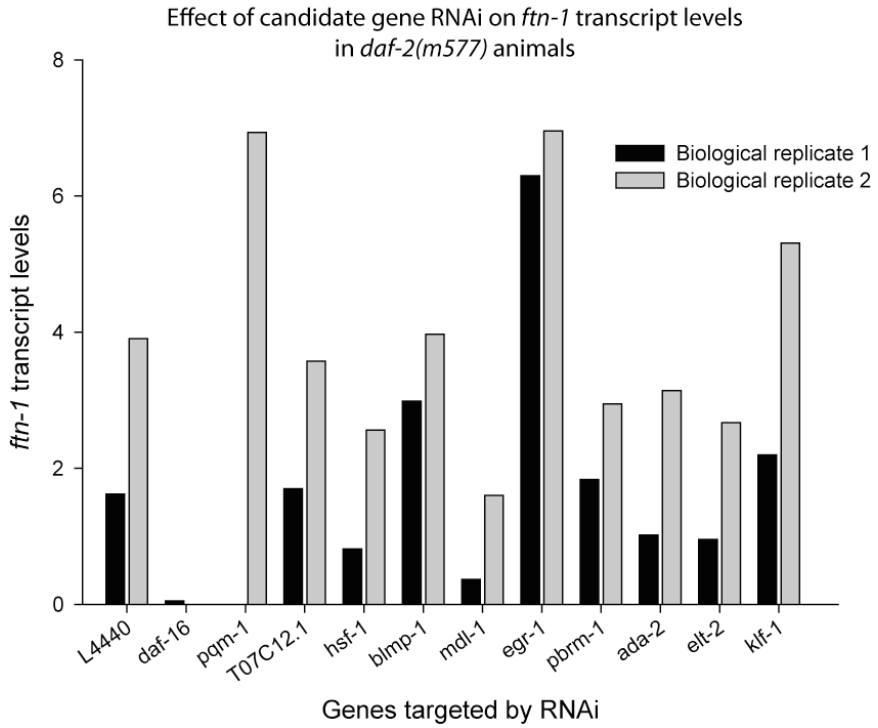


Figure 24
Preliminary screen: *ftn-1* expression levels were quantified for *daf-2(m577)* animals treated with RNAi of a number of different candidate genes. The y-axis represents the *ftn-1* expression levels when normalised to the geometric mean of *cdc-42*, *pmp-3* and Y45F10D.4 expression levels (see section 3.6.1.1a below). No statistical analysis of these results was performed as only two biological replicates were measured. However, these results were pooled with further replicates collected later and the result of that analysis is presented in Figure 26. Samples were collected following protocol 2 (see 2.8.2. Protocol 2).

Comparison of the results of fluorescence measurements performed on *Pftn-1::gfp daf-2(m577)* and quantification of *ftn-1* transcript levels in *daf-2(m577)* by qRT-PCR (see Figure 23 and Figure 24) shows that while RNAi of several genes did show the expected effects on *ftn-1* expression, most did not. Those that did were *hsf-1, mdl-1, ada-2* and *elt-2*. Many genes that had not shown a statistically significant effect on the expression of *Pftn-1::gfp* were nevertheless tested for the effects on *ftn-1* transcript. This was done because a trend towards a reduction of expression had been observed, even if this trend was not statistically significant, and because RNAi of many of these genes had reduced *daf-2* longevity. RNAi of none of these genes were found to reduce levels of *ftn-1* transcript. RNAi of the three genes *klf-1, pbrm-1* and *blmp-1* had no observable effect on transcript levels despite having led to a statistically significant reduction in GFP expression from the reporter strain. The

possible basis for the discrepancy between fluorescence and mRNA data is discussed below (see Chapter discussion).

3.8.1.1. Further work on remaining candidate regulators

While many candidate gene RNAi treatments did not result in a decrease in *ftn-1* levels, some did show the expected effect (Figure 25). However, given the high variability in *ftn-1* expression levels, more biological replicates were needed to establish whether the observed effects were statistically significant.

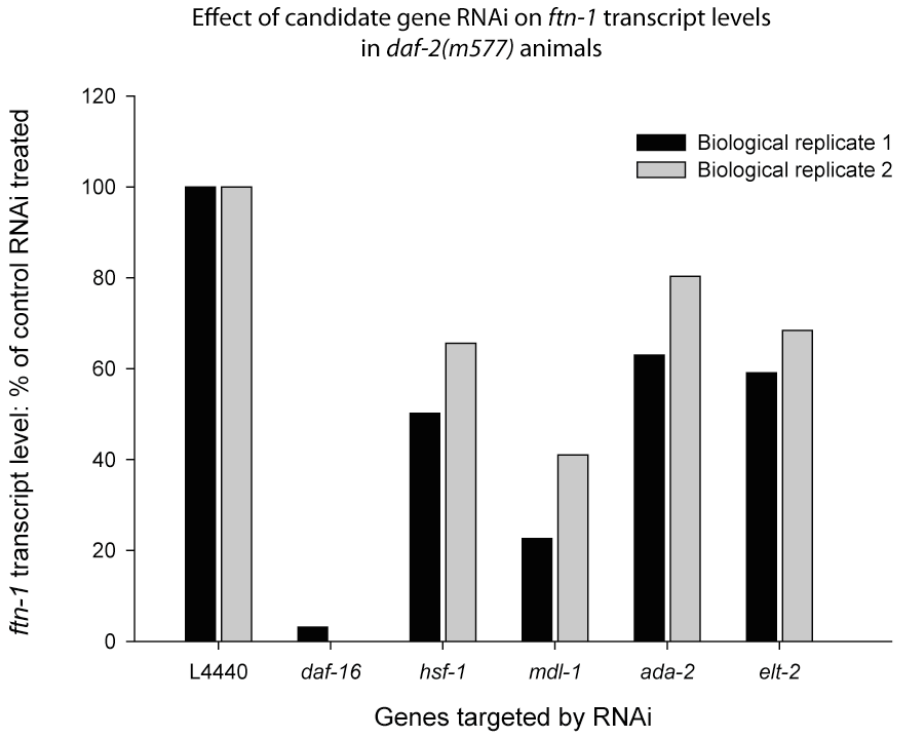


Figure 25
Reduced list of candidate regulators.
The effect of RNAi treatments is displayed as a percentage of the *ftn-1* transcript levels in control RNAi treated worms. The same data was used for this figure as for Figure 24.

Further samples were therefore prepared following the same protocol and *ftn-1* transcript abundance was measured. Due to the high variability in *ftn-1* transcript levels (compare the two biological replicates control–RNAi treated worms in Figure 24) and the uncertainty regarding the magnitude of the effects of RNAi on *ftn-1*

transcript levels, particular care was taken to select the correct reference genes to normalise the data. The following section explains how this was done:

3.8.1.1a Normalisation of qRT-PCR data

Accurate normalisation is of critical importance in the evaluation of of qRT-PCR results. After RNA extraction, the RNA was quantified and then reverse transcribed into cDNA. Despite identical amounts of RNA being introduced into the cDNA synthesis reaction, the samples cannot be assumed to contain identical concentrations of cDNA. This is partly due to the fact that most of the RNA being quantified consists of rRNA, not mRNA. The ratio of rRNA to mRNA has been shown to be highly variable in other systems and is affected by a variety of biological factors and drugs (Johnson et al., 1995; Solanas et al., 2001; Spanakis, 1993) making total RNA levels an inadequate measure of total mRNA. Additionally, the cDNA synthesis reaction is known to be of highly variable efficiency, generating different amounts of cDNA in different reactions, and thus adding additional variation in the final cDNA concentration of different samples.

Normalising quantified transcript levels to a set of internal controls is therefore necessary for the interpretation of qRT-PCR data. Reference genes should be used that are stably expressed and not affected by the experimental treatments being investigated. Since this experiment involves the use of insulin signalling mutants, which are known to differentially regulate a large fraction of expressed transcripts (McElwee et al., 2004), selecting reference genes that were not affected by insulin signalling was of particular importance. To do this I consulted a publication that compared the stability of expression of 12 different reference genes and used them to quantify superoxide dismutase expression in 6 different *C. elegans* strains: N2, *daf-2* mutants, *daf-16* mutants, *daf-16*; *daf-2* double mutants, dauer larvae, and L3 N2 larvae (Hoogewijs et al., 2008). Fortunately, this set of samples was similar to the four genotypes used in my own experiments, meaning that the authors' conclusions about the suitability of their reference genes could be applied to my own analysis. A reliable method for normalising transcript quantities in qRT-PCR is to divide the quantity of the transcript of interest by the geometric mean of three stably expressed genes. I therefore selected *cdc-42*, *pmp-3* and Y45F10D.4, the

three most stably expressed genes as identified by Hoogewijs et al., as well as *ama-1*, the reference gene mostly commonly used in the Gems' lab, as my reference genes. Transcripts of all four genes were quantified for all samples using qRT-PCR and transcript quantities were introduced into the GeNorm algorithm, which ranks the reference genes according to their stability by calculating average pairwise variations between each reference gene and all other reference genes (Vandesompele et al., 2002)**.**

3.8.1.1b. Quantifying ftn-1 transcript abundance for further biological replicates

Four reference genes were used to normalise for cDNA input: *cdc-42, ama-1, pmp-3*, and Y45F10D.4. Transcript quantities for these reference genes were analysed for the stability of expression. The result of this analysis is shown in Table 6.

Transcript	M-value
ama-1	1.189001
pmp-3	0.938806
cdc-42	1.041868
Y45F10D.4	1.05109

Table 6
M-values of four different reference genes
M describes the average pair-wise variation of each reference gene with all other reference genes. The M-values in this table were calculated using the transcript quantification of all samples that had been treated with one of the RNAi treatments in Figure 26. This include four biological replicates of four strains treated with six different RNAi treatments.

An important publication on optimal normalisation procedures for analysis of qRT-PCR results (Vandesompele 2002) recommends using only the three most stable reference genes for normalisation. The *ama-1* transcript was the least stably expressed of the four reference genes I used and was therefore omitted from the analysis. A normalisation factor was then calculated from the three remaining reference genes by taking their geometric mean. *ftn-1* expression levels were quantified by dividing the *ftn-1* transcript quantity by this value.

qRT-PCR analysis of these additional samples is summarized in Figure 26. As with the first two biological replicates (see Figure 25), a high variance in *ftn-1*

transcript levels was found even in the untreated controls. This mirrors the findings from *Pftn-1::gfp* expression in the platereader assay performed for the screen. Large variation in GFP levels in the control RNAi treated animals were found from one day to the next, even when downregulation of the GFP levels by a candidate RNAi treatment was reproducible. This suggests that *ftn-1* levels are sensitive to subtle changes in the environment. One possible source of variation is the temperature of the incubators, which could be causing large changes in *ftn-1* induction by reduced insulin signalling as the *daf-2* allele being used is very sensitive to temperature. Slightly different temperatures may lead to different levels of reduction in insulin signalling, which may translate to radically different *Pftn-1::gfp* expression as well as endogenous *ftn-1* transcript expression. Whatever the source, it seems that there is a large batch to batch variation in *ftn-1* transcript levels, which does not seem to influence the effect of the RNAi treatment of candidate regulators. The data was therefore displayed as a percentage of the control to illustrate the effects of the RNAi treatments within each batch while ignoring the differences between batches. Statistical analysis was performed using a two-way ANOVA for the same reasons, as it takes the treatment effect into account regardless of batch-to-batch differences. The two way ANOVA was performed using the RNAi treatment and the batch number as the two nominal variables and the *ftn-1* transcript level as the measurement variable (after log transformation).

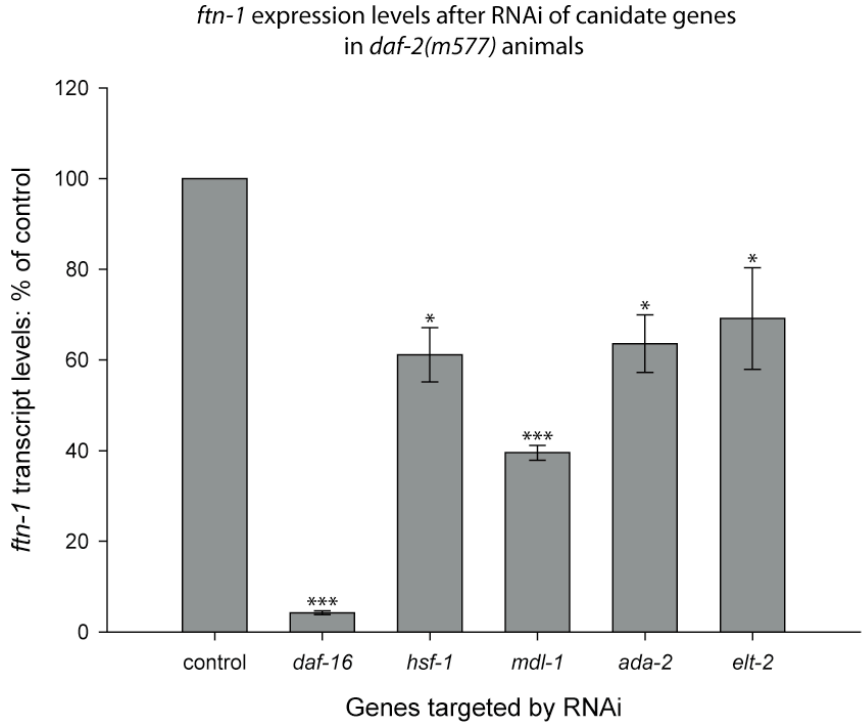


Figure 26
daf-2(m577) animals were treated with RNAi bacteria. Expression levels are expressed as a percentage of the control RNAi treatment. Error bars represent ±*s.e.m*. Statistical significance was calculated using two-way ANOVA and p-values were adjusted for multiple testing using the Bonferroni correction: *: $p<0.05$, ***:$p<0.001$.

RNAi of gene	Average *ftn-1* transcript abundance	As % of control	n	p-value
control	3.34	100	5	
daf-16	0.133	3.97	4	1.18E-13
hsf-1	1.91	57.2	5	0.0103
mdl-1	1.36	40.7	5	1.81E-05
ada-2	2.05	61.2	5	0.0204
elt-2	2.34	69.8	5	0.0420

Table 7
Summary table of qRT-PCR quantification of *ftn-1* transcript abundance following candidate gene RNAi. p-values were calculated by two-way ANOVA of log-tranformed transcript abundance values. p-values were adjusted for multiple testing using the Bonferroni correction.

As shown in Figure 26 (and Table 7), I found a statistically significant down-regulation of *ftn-1* transcript in *daf-2(m577)* animals by RNAi of *hsf-1*, *mdl-1, ada-2* as well as the known regulator of *ftn-1* transcription *elt-2* (Romney et al., 2008). This screen was therefore successful in identifying *hsf-1*, *mdl-1,* and *ada-2* as putative regulators of *ftn-1* expression. The fact that RNAi of these genes was found to reduce *ftn-1* transcript levels in *daf-2(m577)* animals demonstrates that the effect was not an artefact of transgene expression.

3.9. Chapter discussion

After very promising initial results, in which RNAi of a large number of genes led to large changes in expression of *Pftn-1::gfp* (see Table 4) and subsequent lifespan assays of these genes demonstrated that RNAi of most of them reduced *daf-2* lifespan, follow up work was unable to replicate some of the large changes in reporter gene expression found in the initial screen. Good examples for this include the genes *ast-1, nhr-116, dve-1* and *hlh-30,* RNAi of all of which led to very large decreases in *Pftn-1::gfp* expression in the initial screen but had no, or very little, effect on *Pftn-1::gfp* in later trials. One possible explanation is that the initial screen results for many of these genes had been spurious and that later tests were able to identify the true effects.

Differences in how the initial screen was carried out and the experiments performed later could also be a cause of this discrepancy. For example, after carrying out the initial screen, I purified the RNAi plasmids, sequenced them and re-introduced into the HT115 strain of *E. coli* used for RNAi. This was done after work from a colleague in the lab, who discovered that worms lived substantially longer after being fed a certain RNAi strain, only to find that this effect was unrelated to RNAi but was instead due to a spontaneous mutation in the *E. coli* host strain. The re-transformation into a fresh *E. coli* stock (obtained from the Caenorhabditis Genetic Centre) may have avoided similar bacterial strain-specific effects on expression from *Pftn-1::gfp* and therefore be a cause for some of these discrepancies.

Another possibility is that there was a loss of effectiveness of RNAi between the time the initial screen was carried out and the later assays. However, the consistently strong reduction in expression from *Pftn-1::gfp* after RNAi of *daf-16*

strongly argues against this possibility. The plasmid used for RNAi of *daf-16* was re-introduced into fresh HT115 just as the other plasmids tested in Figure 23 had been. RNAi of *daf-16* was included in every trial and robustly reduced expression both of *Pftn-1::gfp* and of the *ftn-1* transcript. The most likely explanation therefore seems to be that the large effects seen in the initial screen were spurious and probably due to the large variation in *Pftn-1::gfp* expression.

3.9.1. Explaining the discrepancy between platereader and qRT-PCR results

Surprisingly, the results of the qRT-PCR validation shown in Figure 24 demonstrate that the findings from platereader analysis of GFP expression from the *Pftn-1::gfp* construct do not, in many cases, coincide with expression changes of endogenous *ftn-1* transcript levels in the same *daf-2(m577)* background. However, a closer look at the data reveals that RNAi of the three genes *hsf-1, mdl-1* and *ada-2* that were found to cause a statistically significant reduction in *ftn-1* transcript levels were also the ones that had the largest effect on *Pftn-1::gfp* expression. RNAi of several of the other genes tested did not lead to statistically significantly reductions in *Pftn-1::gfp* in the first place, so their inclusion in the preliminary qRT-PCR screen was probably over-optimistic to begin with. RNAi of only three genes led to a clear significant down-regulation of *Pftn-1::gfp* expression but no reduction in *ftn-1* transcript in the qRT-PCR trial.

While there are several possible explanations for this discrepancy, it is possible that two biological replicates were not sufficient to detect a small decrease in expression. The preliminary results for *pbrm-1* RNAi, for example, show a slight reduction in expression in one of the two replicates, but not in the other, so further experiments may have identified an overall statistically significant but small effect on *ftn-1* transcript levels. However, given the very large number of genes found to have an effect on *Pftn-1::gfp* in the primary screen, I could not exhaustively test the effects of all RNAi treatments on the *ftn-1* transcript, especially given the high cost of qRT-PCR. The focus on investigating only genes for which RNAi knockdown leads to large effects on *ftn-1* expression may have come at the cost of losing the capacity to identify transcription factors with minor roles in *ftn-1* regulation. My

central interest, however, was not to compile a comprehensive list of transcription factors that affect *ftn-1* transcription to any degree, but to identify factors involved in the very large effects of reduced IIS on *ftn-1* expression. RNAi of major players in IIS signalling upstream of *ftn-1* regulation would be expected to have large effects on *ftn-1* expression, so this loss of sensitivity may not be a serious concern, at least in this regard. Given the large variability in the efficacy of RNAi, what cannot be excluded is that some of the RNAi treatments that had minor effects on *ftn-1* expression actually did target genes with important roles in *ftn-1* regulation but that the RNAi treatment itself was not particularly effective at knocking down expression of the target gene. Additionally, if several genes act redundantly to regulate *ftn-1* expression, knocking them down individually may not have large effects. Thus it is easy to imagine that this screen may have missed some important players in IIS regulation of *ftn-1* despite targeting such a large number of transcription factors.

Other possible explanations for the discrepancy between the results derived from RNAi of worms carrying the transgene and from qRT-PCR can be imagined, though it is beyond the scope of this project to establish the cause of the discrepancy. One possibility is that a physical change in the RNAi treated worms, such as the size of the intestinal cells themselves, led to a decrease in the fluorescent signal emitted, without actually affecting the amount of GFP expressed. Worms treated with *blmp-1* RNAi, for example, consistently appeared considerably darker than control RNAi treated worms and this phenotype may interfere somewhat with the detection of the fluorescent signal. If this is true, *blmp-1* RNAi may be producing a false positive result due to reasons unrelated to altered *ftn-1* transcription. It is also possible that some RNAi treatments lead to an altered translation, maturation or degradation of the GFP protein, which would clearly affect the intensity of the fluorescent signal. Any transcription factors involved in the transcription of elements of the translation machinery could have such an effect. While this was not investigated further, the lifespan experiments performed do show that RNAi of *klf-1, blmp-1* and *pbrm-1* all reduce *daf-2* lifespan, indicating that RNAi of these genes does have effects other than specifically on *Pftn-1::gfp.* Effects on processes unrelated to *ftn-1* transcription, such as on transcription or translation in general, could explain the reduction in GFP fluorescence as well as of lifespan found upon RNAi of *klf-1, blmp-1* and *pbrm-1*. A third explanation revolves around the absence of the endogenous *ftn-1* 3' UTR of the GFP construct, which only uses the *ftn-1* promoter to drive GFP expression. If *ftn-1*

is post-transcriptionally regulated through its 3' UTR, then this effect would be missed when the GFP construct is used to report expression. It is hard to envisage a scenario in which this would lead to false positive findings during this screen, especially since the *Pftn-1::gfp* reporter and the endogenous *ftn-1* transcript behave similarly in response to mutations in *daf-2*, *daf-16* and in double mutants of the two (see Figure 38). However, it should be noted that ferritin transcript and protein have been shown to be very tightly regulated by post-transcriptional mechanisms in other organisms, so it is far from inconceivable that the *ftn-1* transcript should be regulated via its 3' UTR.

3.9.2. Why does RNAi of no other factor reduce *ftn-1* expression as much as RNAi of *daf-16*?

As described in Chapter 1, a study on direct targets of DAF-16 (Schuster et al., 2010) concluded that DAF-16 acts as a 'regulator of regulators' by directly activating a number of signalling molecules and transcription factors. Understanding the transcriptional architecture downstream of DAF-16 would presumably go a long way in helping us understand the mechanisms by which IIS mutants exhibit such large changes in their transcriptome and such large extensions in their lifespans. My project aimed at understanding how DAF-16 acts to regulate expression of distinct sets of genes by choosing a single target, *ftn-1,* and attempting to identify if there are any transcription factors that act downstream of DAF-16 to regulate this gene. If this was occurring through a single transcription factor downstream of DAF-16, then presumably RNAi of this factor would lead to a complete or almost complete abrogation of the *ftn-1* induction by *daf-2(m577)*. However, no single gene was identified with as important a role in *ftn-1* regulation as *daf-16*.

Of course, the expectation that a transcriptional cascade exists in which DAF-16 activates a single transcription factor which itself then activates *ftn-1* could be incorrect. It is likely that the promoter of *ftn-1* binds many proteins which act in concert to regulate *ftn-1* transcription. Several of these factors may act downstream of DAF-16 to increase gene expression in IIS mutants and may do so in a redundant fashion. If this is the case, then RNAi of each of them may have an effect on *ftn-1* expression that is smaller than RNAi of *daf-16* itself. It is also possible that no RNAi treatment is effective as *daf-16* RNAi in reducing *ftn-1* expression merely because of

differences in the efficacy of the RNAi knockdown itself. Other genes may have as important a role in *ftn-1* regulation as *daf-16*, but may not be as easily silenced by RNAi.

A possible alternative explanation is that under conditions of low IIS, DAF-16 may bind to the promoter of *ftn-1* directly to activate expression. During the initial stages of this project, there were several strong indications that this is not the case. The study by Schuster et al. had not yet been published, but the preliminary data was available and no binding to the *ftn-1* promoter was detected (Eugene Schuster, personal communication). In addition, a separate publication that used Chromatin Immuno-precipitation (ChIP) to identify DAF-16 targets did not identify *ftn-1* as a candidate. Very recently however, results started to become available from a large-scale effort to identify the targets of *C. elegans* transcription factors (www.modencode.org). This was done by expressing transcription factors fused to GFP in worms and assessing binding to DNA by ChIP using an anti-GFP antibody and subsequent deep-sequencing.

The results for a DAF-16::GFP transgene recently became available and may be useful in assessing whether DAF-16 may bind to the *ftn-1* promoter directly. It should be noted that, except for carrying the DAF-16::GFP transgene, the strain used was wild-type for all elements of the IIS pathway, so it is unclear whether this data is really informative about the consequences of reduced IIS on binding of DAF-16 to its target genes. However, as depicted in Figure 27, some evidence of binding to the sequence immediately upstream of the *ftn-1* promoter was found (q=4.47E-14). A sequence resembling the *daf-16* binding element (DBE) is present 150bp upstream of the *ftn-1* start codon, which could be the site at which DAF-16 binds.

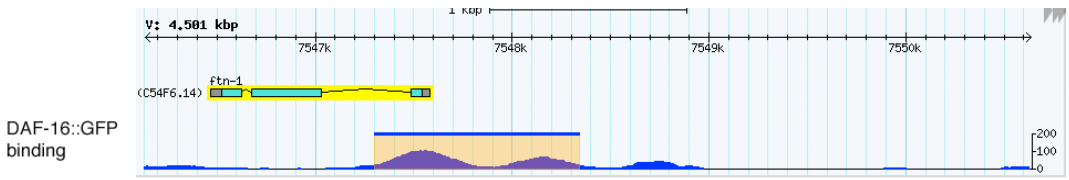


Figure 27
Possible binding of DAF-16::GFP to the *ftn-1* promoter. Figure adapted from the ModEncode project (modencode.org, accessed Jul 2010).

While much more work would need to be done to establish that transcription of *ftn-1* is indeed induced under reduced IIS by direct binding of DAF-16 to its promoter, the data from the ModEncode project at least demonstrates that DAF-16 is capable of binding *ftn-1*. This raises the prospect that the RNAi screen performed here was ineffective at identifying another gene with as important a role as *daf-16* in the regulation of *ftn-1* in *daf-2* mutants simply because this induction is occurring via direct binding of DAF-16.

At this stage in the project, two transcription factors, *hsf-1* and *mdl-1,* as well as *ada-2*, a gene thought to act as a subunit of a histone acetyl transferase complex, were identified as having important roles in the regulation of *ftn-1*. *hsf-1* has long been known to be an important player in the lifespan extension of IIS mutants (Hsu et al., 2003) and links between *mdl-1* and IIS have also been discovered (see Chapter 4). Lifespan assays on *daf-2* mutants show that RNAi of both *mdl-1* and *ada-2* statistically significantly reduce their lifespan, although the effects of *ada-2* RNAi were very modest, with no reduction in median and only a very small reduction in mean lifespan. In Chapter 4, I will therefore proceed to further characterise their role in mediating *daf-2* longevity.

Chapter 4: Further investigation of genes influencing *ftn-1* expression

4.1. The biological functions of *hsf-1, mdl-1* and *ada-2*

The work described Chapter 3 identified three genes *hsf-1*, *ada-2* and *mdl-1,* which regulate expression of *ftn-1*. RNAi knockdown of each of them leads to reductions in both *Pftn-1::gfp* expression and *ftn-1* transcript levels. In this chapter, I will investigate these genes further first by briefly introducing the literature on these genes and then describing how I attempted to test the genes' involvement in IIS in order to assess their role in the extended lifespan of *daf-2* mutants.

4.1.1. *hsf-1*

One of the genes identified in the screen is the transcription factor *hsf-1 (*heat-shock factor 1), RNAi of which greatly reduces *ftn-1* levels in *daf-2* mutants. The fact that this discovery was made as part of an unbiased, large scale screen, not a candidate gene approach, is encouraging, since *hsf-1* is one of the few transcription factors known to act downstream of IIS to regulate ageing. At the least, this finding demonstrates that the screen is capable of identifying the genes it was designed to identify, namely those involved in mediating *daf-2* longevity.

Like *daf-16, hsf-1* is absolutely required for the longevity phenotype of *daf-2* animals and its over-expression is sufficient to extend lifespan on its own (Hsu et al., 2003; Morley and Morimoto, 2004). This is thought to occur because *hsf-1* activates expression of a number of cytoprotective genes, such as small heat shock proteins (sHSPs) (Hajdu-Cronin et al., 2004), which act as molecular chaperones and prevent incorrect protein folding. *hsf-1* also has an important role in mediating the increased resistance of *daf-2* mutants to infection by pathogenic bacteria (Singh and Aballay, 2006).

4.1.2. *mdl-1*

MDL-1 (MAD- like 1) is believed to be the *C. elegans* homologue of the MAD proteins that act within the MYC/MAD/MAX network of basic helix-loop-helix (bHLH) transcriptional regulators. These genes have been extensively researched in vertebrate models due to the network's involvement in a number of important cellular processes, such as proliferation, differentiation and apoptosis. The high levels of interest in this group of regulators was driven by the tumour promoting abilities of Myc proteins, with many tumours showing enhanced levels of Myc (Dang, 1999). Both Myc and Mad proteins bind to Max to form a heterodimer, but while Myc/Max has been shown to usually activate gene expression, Mad/Max acts as a repressor antagonising the effects of Myc on proliferation. This antagonism is thought to occur via binding to common target genes and recruitment of either a histone acetyl transferase (HAT) complex in the case of Myc/Max or a histone deacetylase (HDAC) complex in the case of Mad/Max. The binding of Myc/Max or Mad/Max to common binding elements in target genes promoters (termed E-box sequences) and the predicted effects on gene regulation by recruitment of these chromatin modifying factors is depicted in Figure 28.

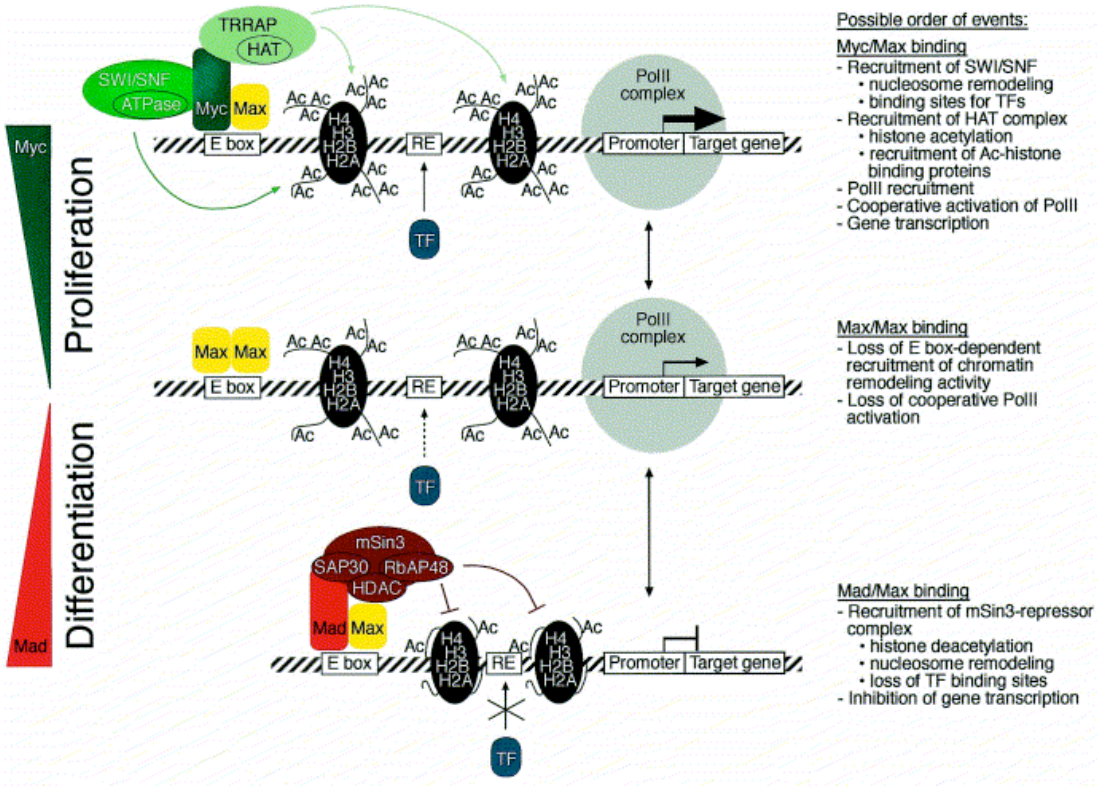


Figure 28
A model for the activity of the Myc/Max/Mad network of transcriptional regulators on common target genes (Luscher, 2001).

The degree to which the function of Mad proteins is conserved in *C. elegans* is unclear. *mdl-1* was identified as the worm homologue based on sequence similarity (60% identical and 72% similar to human MAD1). Several features of the MDL-1 protein make it likely to be the homologue of Mad and not Myc or Max proteins, including the overall protein organisation, the presence of several residues specific to the Mad family and the presence of the consensus sequence for Sin3 binding (see Figure 28). Its binding partner was identified in a protein interaction screen; it showed high sequence identity as well as common protein domains with MAX and was therefore named *mxl-1* (MAX-like gene 1). In vitro assays showed binding of the MDL-1/MXL-1 heterodimer to an oligonucleotide containing the E-box sequence. Thus it seems that certain features Mad/Max are reiterated in MDL-1/MXL-1, which suggests that the genes' functions could be conserved. This is supported by the finding that MDL-1 is capable of forming a dimer with mouse MAX in vitro (Yuan et al., 1998).

However, *C. elegans* does not seem to have a clear Myc homologue. One gene *mml-1* has low sequence identity to both Myc and to another bHLH gene called Mondo (26% and 25% identity respectively). The amino acid sequence of this gene lacks Myc signature sequences but does possess certain sequences characteristic of Mondo. Its binding partner was also identified and was named *mxl-2*. MML-1 and MXL-2 were shown to form heterodimers capable of activating a luciferase reporter via E-box sequences (Pickett et al., 2007). The expression patterns of these four genes (*mdl-1, mxl-1, mml-1, mxl-2*) do overlap in intestinal and epithelial cells, so it is possible that MML-1/MXL-2 and MDL-1/MXL-1 activate and repress target genes in *C. elegans* much like Myc/Max and Mad/Max do in vertebrates. However, no target genes antagonistically regulated by these two heterodimers has thus far been identified, so there is currently no evidence for conservation of the Mad-Myc antagonism in *C. elegans*. That MML-1 seems to be more closely related to MondoA than to Myc genes suggests that it might not be. Additionally, the phenotype of mutation of *mml-1* is limited to a subtle male tail abnormality.

At least in the case of its role in the regulation of *ftn-1*, *mdl-1* has not been found to act as a repressor, since RNAi of it leads to decreased, not increased, expression. Of course, the mechanism by which MDL-1 might activate *ftn-1* expression is also unknown, so an indirect activation, for example via repression of a third regulator, is possible.

4.1.3. *ada-2*

Far less is known about the *C. elegans* gene *ada-2* since no studies have directly investigated its function. By sequence homology, *ada-2* it is thought to be a homologue of the Ada2 subunit of various histone acetyl transferase (HAT) complexes that activate gene expression by modifying chromatin via histone acetylation. Ada2 subunits form part of the three yeast complexes SAGA, ADA and HAT-A2 which acetylate H3 and H2B histones and the human PCAF complex, which acetylates H3 and H4 histones (Carrozza et al., 2003). The catalytic element of these complexes is also thought to be conserved in *C. elegans* based on sequence similarity to the gene *pcaf-1* (Poulin et al., 2005).

There is a possibility that the effects of *ada-2* and *mdl-1* RNAi are not unrelated. In vertebrates, cMyc activates gene expression by recruiting HAT complexes via the cofactor TRRAP (McMahon et al., 2000). While the human HATs containing TRRAP are not thought to also contain Ada2, yeast HATs containing the yeast homologue of TRRAP, Tra1, do contain yeast Ada2. However, uncertainty about the extent to which both the Myc/Max/Mad network and the Ada2 containing HAT complexes are conserved across these species make it difficult to predict if this potential link is likely to be real.

4.1.4. Focus on *mdl-1*

Several studies on downstream effects of reduced IIS have suggested a role of *mdl-1* in mediating phenotypes associated with reduced IIS. The first hint came from microarray studies aimed at identifying differentially regulated genes in long-lived IIS mutants (see Chapter 1). *mdl-1* was found to be up-regulated in long-lived mutants in two separate experiments (McElwee et al., 2004; Murphy et al., 2003) and this result was confirmed in a third study by qRT-PCR quantification of *mdl-1* transcript level (see Figure 29).

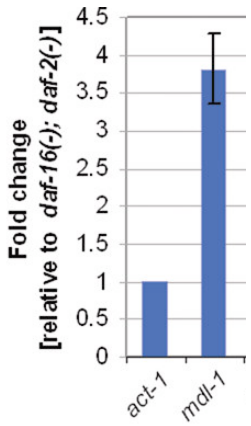


Figure 29
Figure adapted from Pinkston-Gosse et al. (2007)
The y-axis depicts the fold change in *mdl-1* transcript levels in *daf-2(-)* compared to *daf-16(-); daf-2(-)* animals, which confirms microarray experiments that found increased *mdl-1* expression in long-lived mutants (McElwee et al., 2004; Murphy et al., 2003).

Of course, given the large number of genes that have been shown to be differentially regulated in long-lived IIS mutants, evidence of increased expression alone is insufficient to demonstrate involvement in mediating IIS mutant phenotypes.

4.1.4.1. Direct binding of DAF-16 to *mdl-1* promoter

An additional and very interesting link between IIS and *mdl-1* came from the efforts of Schuster et al. to identify direct targets of DAF-16 by genome-wide chromatin profiling using DamID. They found strong evidence for direct binding of DAF-16 to the promoter of *mdl-1* (see Figure 30), indicating that the up-regulation of *mdl-1* expression by reduced IIS may occur through increased binding of DAF-16.

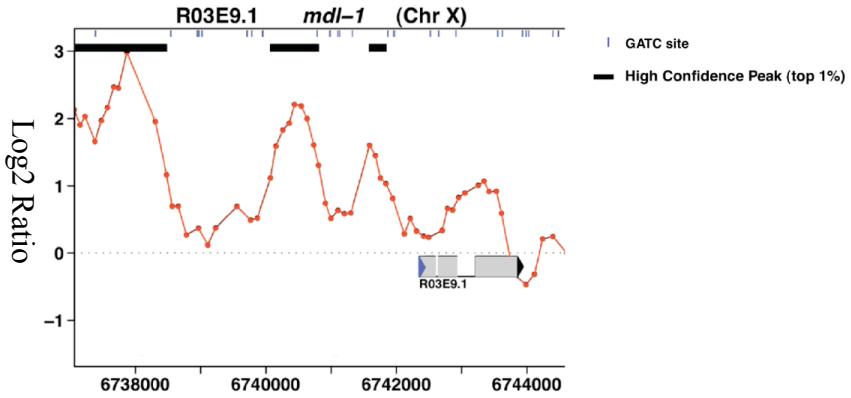


Figure 30

Adapted from (Schuster et al., 2010). DamID data indicating binding of a DAF-16 transgene to the promoter of *mdl-1*. The black bars at the top of the figure indicate areas of the promoter to which DAF-16 is likely to bind.

This result was subsequently confirmed by Jennifer Tullet and Michèle Riesen, two post-doctoral researchers in David Gems' lab. They used chromatin immuno-precipitation against endogenous DAF-16 followed by PCR (ChIP-PCR) to show direct binding to the *mdl-1* promoter (see Figure 31).

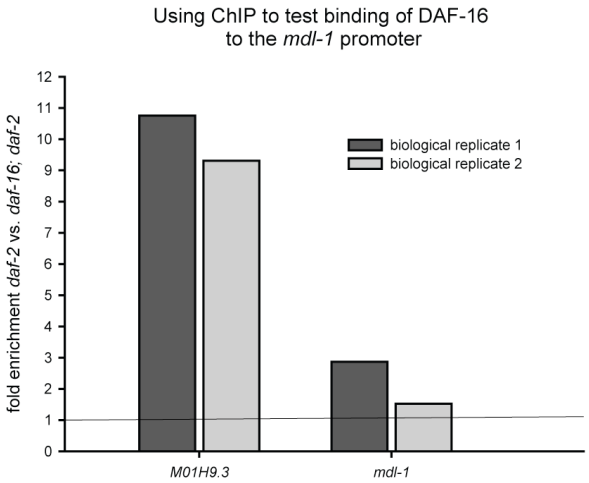


Figure 31
ChIP-PCR on two biological replicates seems to support direct binding of DAF-16 to the *mdl-1* promoter. DAF-16–target M01H9.3 was used as a positive control. These experiments were carried out by Jennifer Tullet and Michèle Riesen.

4.1.4.2. Effect on *gld-1* tumour phenotype

A possible biological function of *mdl-1* was identified in an RNAi screen on a *C. elegans* tumour model (Pinkston-Gosse and Kenyon, 2007). In worms, mutation of the tumour suppressor gene *gld-1* leads to the formation of germline tumours, which form because meiotic cells re-enter the mitotic cell cycle. While *gld-1(-); daf-2(+)* animals die of these tumours, the severity of this phenotype is greatly reduced in *gld-1(-); daf-2(-)* due to higher levels of germline apoptosis and inhibited cell proliferation within the tumour. The protection conferred by *daf-2(-)* requires *daf-16* (Pinkston et al., 2006), which makes protection from *gld-1(-)* tumours another phenotype of IIS mutants mediated by *daf-16*. Pinkston-Gosse et al. investigated which genes downstream of IIS were contributing to this effect. They compiled a list of over 700 genes thought to be regulated by IIS in microarray and ChIP experiments and tested their involvement in conferring the phenotype through RNAi. The aim was to identify RNAi treatments which lead to increased tumour lethality in the otherwise protected *gld-1(-); daf-2(-)* animals, and RNAi of *mdl-1* was one of the genes found to have this effect.

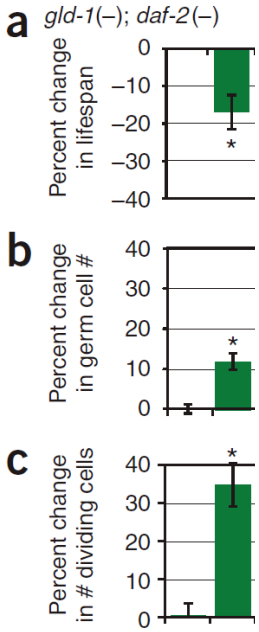


Figure 32

Adapted from Pinkston-Gosse et al. (2007). RNAi of *mdl-1* leads to an increase in tumour formation in *gld-1; daf-2* animals as well as in associated lethality.

Loss of *mdl-1* by RNAi led to an increase in tumour lethality and an increase in the number of dividing germ cells, demonstrating that *mdl-1* plays a role in conferring protection against *gld-1* tumours in *daf-2*. Given its transcriptional up-regulation in IIS mutants, the direct binding of DAF-16 to its promoter and the evidence of its involvement in conferring a protective effect of *daf-2* mutants, *mdl-1* is the prime candidate in the search for a true mediator of IIS signalling downstream of DAF-16.

4.1.4.3. Role of *mdl-1* in lifespan

The role of *mdl-1* in *daf-2* longevity was also investigated. This was first done in the follow up work to one of the microarray experiments on IIS. Murphy et al. tested a large number of differentially regulated genes on their effects on *daf-2* lifespan and found that RNAi of *mdl-1* statistically significantly reduces *daf-2(mu150)* lifespan by 9% (full genotype of strain used: *fer-15(b26); daf-2(mu150); fem-1(hc17)*) at 25°C.

Similar results were obtained in the study by Pinkston-Gosse: two trials on *daf-2(e1370)* animals found a 10% and an 8% reduction in lifespan upon *mdl-1*

RNAi. As described in Chapter 3, I obtained similar results using RNAi of *mdl-1* in *rrf-3; daf-2* animals (see Figure 22 and Table 5). This indicates that *mdl-1* may be acting downstream of *daf-2* to mediate two different phenotypes of *daf-2* mutants: The extended lifespan and the increased protection against *gld-1* tumour formation. If so, one might expect *mdl-1* RNAi to reduce *daf-2(-)* lifespan to a greater extent than *daf-2(+)* lifespan. If, on the other hand, *mdl-1* RNAi reduces lifespan to a similar extent regardless of *daf-2* genotype, then this would imply that loss of *mdl-1* shortens lifespan by a mechanism unrelated to IIS and that *mdl-1* is unlikely to be acting on lifespan downstream of DAF-16. Pinkston-Gosse et al. found that *mdl-1* RNAi caused no reduction in *daf-2(+)* lifespan in two trials, thus supporting the first of these hypotheses (see Figure 33).

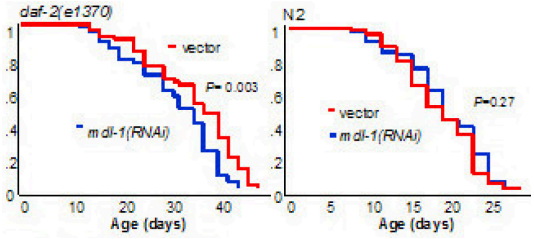


Figure 33
Figure adapted from Pinkston-Gosse (2007) and illustrates their findings on the effect of *mdl-1* RNAi on *daf-2(-)* and *daf-2(+)* lifespan.

4.1.4.4. Other possible interactions of *mdl-1*

mdl-1 may also be regulated by microRNA binding. A miRNA- microarray based study identified *mdl-1* as a target of degradation via miR-64 and miR-65, two transcripts differentially regulated in *C. elegans* models of Parkinson's disease (Asikainen et al., 2010). A different study also identified a direct interaction between the DAF-3 transcription factor and the promoter of *mdl-1* and suggested that DAF-3 acts as a suppressor of *mdl-1* in the worm pharynx during the dauer stage (Deplancke et al., 2006). Whether or not these interactions are related to the regulation of *mdl-1* by IIS has not been investigated.

4.1.4.5. Does *mdl-1* regulate *ftn-1* directly?

Very recently, the ModEncode project released data on binding of an MDL-1::GFP fusion protein. While this data was generated from L1 animals and did not test the effects of mutations in IIS, it nonetheless demonstrates that direct binding of MDL-1 to the *ftn-1* promoter may be occurring (see Figure 34).

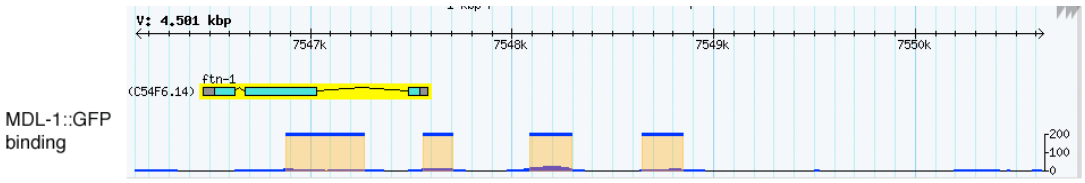


Figure 34
Figure adapted from the ModEncode project (modencode.org, accessed Jul 2010). The blue bars in the bottom track indicate statistically significant binding of MDL-1::GFP in L1 larvae.

The evidence for regulation of *mdl-1* by IIS via direct binding of DAF-16 to its promoter, for a role of *mdl-1* in effecting *daf-2* mutant phenotypes such as tumour protection and lifespan, and the possibility of direct binding of MDL-1 to the *ftn-1* promoter suggests the following model of IIS regulation:

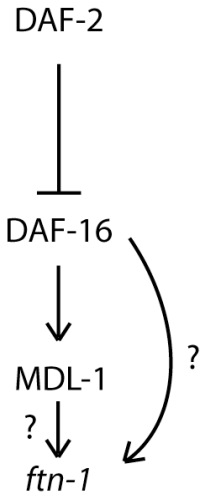


Figure 35
MDL-1 may act downstream of DAF-16 to activate *ftn-1* transcription. This may be occurring either as the only mechanism by which IIS affects *ftn-1* transcription or in addition to the possible direct activation of *ftn-1* by DAF-16.

For a direct interaction to be occurring, *mdl-1* also needs to be expressed in the intestine. In a study that used a tagged, intestinally expressed polyA-binding protein to isolate mRNA specifically enriched in this tissue, *mdl-1* was identified as

one of the intestinally expressed genes (Pauli et al., 2006). A *mdl-1* reporter gene was also found to be intestinally expressed (Yuan et al., 1998).

4.1.5. Epistasis analysis of new regulators of *ftn-1* expression

When investigating the regulation of a DAF-16–regulated gene such as *ftn-1*, one problem is that DAF-16 is responsive to many different cues, such as stress and starvation and its activity is therefore most likely affected by many different interventions. This is problematic because RNAi of many genes may indirectly affect its activity. If RNAi of a transcription factor leads to a reduction in DAF-16 activation, for example by altering a signalling pathway that DAF-16 is responsive to, this could lead to lower *ftn-1* levels. The question of whether the genes being targeted act upstream, downstream, or independently of DAF-16 to regulate *ftn-1* needs to be addressed. Since RNAi of the genes being investigated have already been shown to reduce *ftn-1* transcript levels in *daf-2* animals, I investigated whether they are also capable of doing this in *daf-16; daf-2* animals. If not, then this would provide evidence that they act upstream of *daf-16*. If they are capable of reducing *ftn-1* levels in *daf-16; daf-2,* then this possibility could be excluded.

The second approach was to investigate the role of these transcription factors in IIS mutant longevity. To do this, I tested the lifespan of *daf-2(-)* and *daf-2(+)* animals (both in an *rrf-3(pk1426)* mutant background) treated with RNAi of the gene of interest. RNAi of genes involved in mediating the longevity of *daf-2* animals would be expected to reduce lifespan in *daf-2(-)* animals to a much greater extent than *daf-2(+)* animals. This experiment has already been carried out for *mdl-1* by Pinkston-Gosse et al., but only in the context of a large-scale screen. Because the lack of a lifespan reduction by *mdl-1* RNAi in wild-type animals is such an important result, I felt it was important to establish that this finding was reproducible. The role of *hsf-1* on lifespan was not investigated further, since this transcription factor's involvement in IIS mutant longevity has already been well established.

4.2. Testing *daf-16* and *daf-2* dependence of the effects of RNAi treatment of candidate regulators

As indicated in microarray data, rt-PCR experiments and platereader experiments shown previously (see Figure 19 and Figure 18), both the endogenous *ftn-1* transcript and the *Pftn-1::gfp* reporter are strongly negatively regulated by DAF-2 and positively regulated by the FOXO transcription factor DAF-16. This screen was not primarily designed to identify general regulators of *ftn-1* expression, such as *elt-2*, but to identify transcription factors that act downstream of *daf-2* to effect the differential gene expression seen in long-lived insulin signalling mutants (McElwee et al., 2004). My hope was to identify one or several transcription factors that acted downstream of DAF-16, which would be an important step in understanding how the activation of DAF-16 is able to so strongly affect *C. elegans* lifespan.

4.2.1. The use of *elt-2* RNAi as a positive control treatment

The *elt-2* gene is thought to act as a terminal differentiation factor for the intestine and to regulate all intestinally expressed genes (McGhee et al., 2009). The direct regulation of *ftn-1* by ELT-2 was demonstrated via an electrophoretic mobility shift assay (EMSA) (Romney et al., 2008) and as expected, RNAi of *elt-2* led to large decrease in expression of a *ftn-1* reporter gene in this same study. In my hands RNAi of *elt-2* also led to decreased expression of *Pftn-1::gfp* in *daf-2* animals.

Since this gene is thought to act as a regulator of all intestinal genes, it is unlikely to be involved in the induction of *ftn-1* by reduced IIS. Instead, its regulation of *ftn-1* is likely to occur regardless of genetic background, which is supported by the fact that *elt-2* RNAi reduced *ftn-1* transgene expression in wild-type animals in Romney et al. (2008) and in *daf-2* mutants in my own hands.

In this section, I tested the effects of *hsf-1, mdl-1* and *ada-2* RNAi on *ftn-1* expression in several genetic backgrounds. The overall levels of *ftn-1* vary greatly between genotypes and it is possible that the efficacy of RNAi may also vary. Since the most likely role of *elt-2* is independent of IIS, I decided to use RNAi of this gene as a positive control in the experiments described in this section. If the efficacy of

RNAi and the sensitivity of the assays of *ftn-1* expression level are equal across all genotypes, then the effects of *elt-2* RNAi on *ftn-1* expression should also be equal. If I find that this is not the case for any of my assays, then this could be considered when analysing the results.

4.2.2. Testing the *daf-16* dependence of the candidate gene RNAi effects

The presence or absence of DAF-16 has already been shown to have large effects on expression of both *Pftn-1::gfp* and endogenous *ftn-1*. One possibility is that any of the candidate gene RNAi treatments decrease expression of *ftn-1* and *Pftn-1::gfp* by decreasing DAF-16 activity, for example by affecting elements of IIS or other pathways upstream of DAF-16 (see Figure 36).

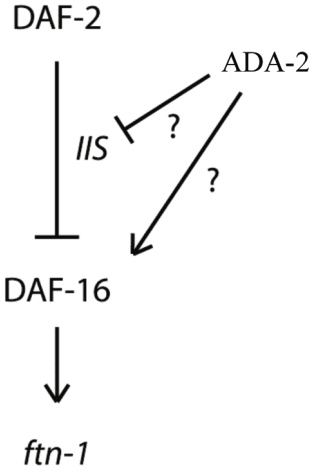


Figure 36
Model 1: Possible mechanism by which a candidate gene may be affecting *ftn-1* transcription. In this case, ADA-2 may be activating DAF-16 activity, thus affecting *ftn-1* transcription indirectly. This scenario is consistent with the finding that RNAi of *ada-2* reduces *ftn-1* transcript levels. This possibility needs to be considered for all candidate gene RNAi treatments, given the important role of DAF-16 in IIS-mediated *ftn-1* induction.

I therefore tested whether the effects of candidate gene RNAi on *ftn-1* expression were dependent on DAF-16: if any of them affected *ftn-1* expression by changing the expression or activation of DAF-16, then they should no longer do so in DAF-16 mutant animals.

4.2.1.1. Using the *Pftn-1::gfp* transgene

My first attempt to test the dependence on DAF-16 was to use the *Pftn-1::gfp* transgene to measure the effect of candidate gene RNAi in different genetic

backgrounds. I therefore crossed the *Pftn-1::gfp* construct into several different genetic backgrounds, obtaining the following strains:

Pftn-1::gfp
Pftn-1::gfp daf-2(m577)
Pftn-1::gfp daf-16(mgDf50)
Pftn-1::gfp daf-16(mgDf50); daf-2(m577)

I then tested the effect of the candidate RNAi treatments on the GFP expression from these strains. As described earlier, I used the RNAi treatment of the gene *elt-2* as a positive control for knockdown of a known regulator of *ftn-1*. As can be seen in Figure 37, despite the clear down-regulation of *Pftn-1::gfp* by *elt-2* RNAi in the *daf-2* strain, this effect was not seen in the other strains.

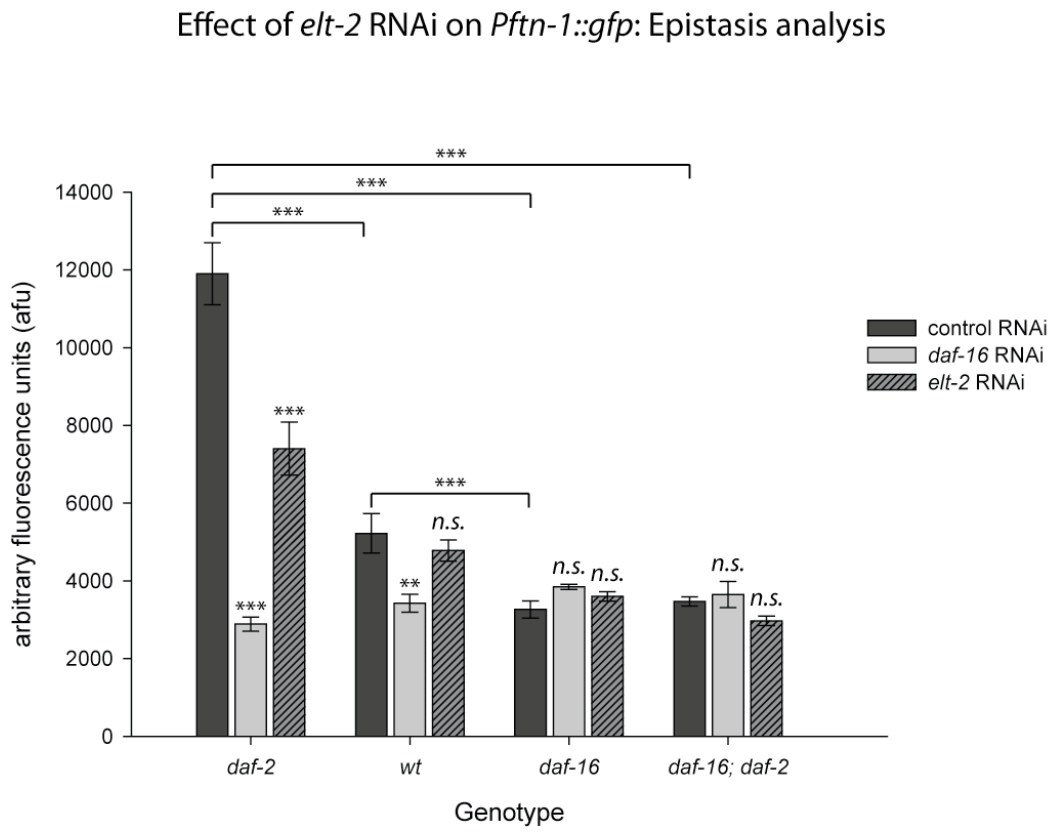


Figure 37
Use of *Pftn-1::gfp* in various genetic backgrounds.
This experiment tested the effect of *daf-16* and *elt-2* RNAi on *Pftn-1::gfp* fluorescence in various genetic backgrounds. Fluorescence was measured as described in protocol 1 (see 2.8.1. Protocol 1). Error bars represent ±*s.e.m*. Statistical significance tested using two-way ANOVA. Unless otherwise indicated, statistical significance was calculated for the comparison to control RNAi treatment of worms of the same genotype *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, *n.s.*: not significant

This could imply that the *elt-2* RNAi effect on *ftn-1* expression is DAF-16 dependent. However, this seems unlikely since *elt-2* is thought to be a general activator of intestinally expressed genes (McGhee et al., 2009). Instead, given the nature of the assay, in which fluorescence of live worms was measured in a platereader, and the large difference in signal intensity between the 'induced' levels of *Pftn-1::gfp* in the *daf-2* mutant strain and the much lower levels of GFP in the other strains, it seems likely that *Pftn-1::gfp* expression cannot be sensitively quantified in the three 'non-induced' strains. I therefore decided to use *ftn-1* transcript levels rather than *Pftn-1::gfp* fluorescence to test the *daf-16* –dependence of the effects of candidate gene RNAi.

4.2.1.2. Effect of mutations in IIS on endogenous *ftn-1* levels

After abandoning the use of *Pftn-1::gfp*, I switched to measuring endogenous *ftn-1* transcript levels by qRT-PCR on the following strains directly:

Wild-type
daf-16(mgDf50)
daf-16(mgDf50); daf-2(m577)

This was done using the same protocol as described previously for the *daf-2(m577)* samples (see Chapter 2 and 3). Worms of the four genotypes were grown simultaneously and samples were collected together for all RNAi treatments. As shown in Figure 38, *ftn-1* transcript levels responded to alterations in IIS as expected.

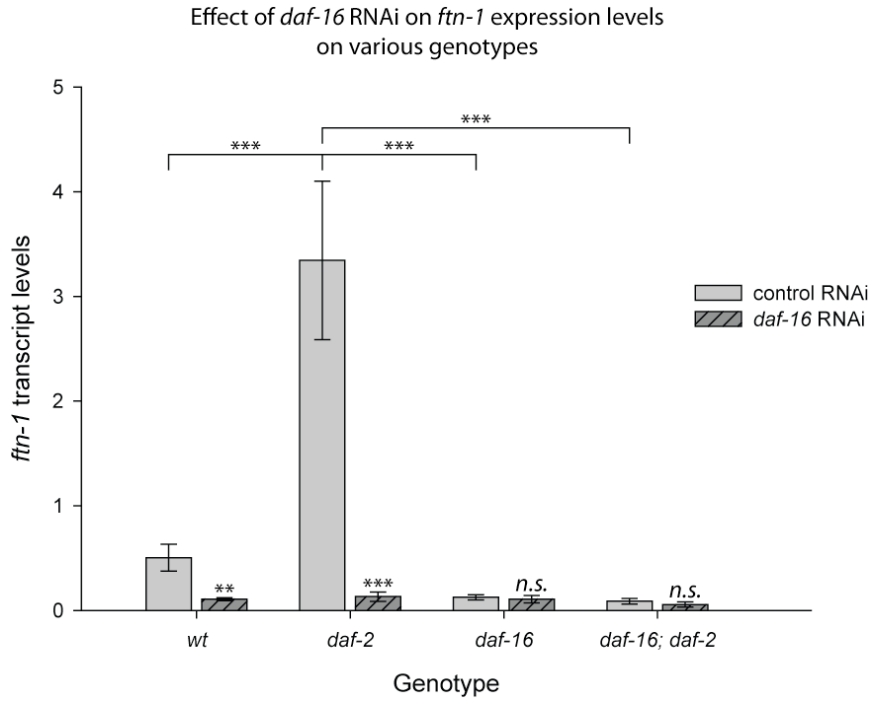


Figure 38
Error bars represent ±*s.e.m*. Transcript levels on the y-axis were calculated using a normalisation factor derived from three reference genes (see Chapter 3). Statistical significance was tested using two-way ANOVA: Unless otherwise indicated, statistical significance was calculated for the comparison to control RNAi treatment of worms of the same genotype. p-values were adjusted for multiple testing using the Bonferroni correction *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, *n.s.*: not significant

ftn-1 levels are highly upregulated in *daf-2* mutant animals and this effect is completely dependent on *daf-16*. Wild-type worms show an intermediate level of *ftn-1* transcript that is itself completely dependent on *daf-16*, indicating that some residual *daf-16* activity remains in N2 animals despite an intact IIS signalling pathway. This is consistent with the role of *daf-16* in *wild-type* lifespan and stress resistance since loss of *daf-16* in *daf-2(+)* also leads to a reduction in both these traits (Weinkove et al., 2006). This indicates that even when IIS is unperturbed, residual DAF-16 activity exists, which seems to be increasing *ftn-1* expression. This result was also seen when *Pftn-1::gfp* was used as a readout of *ftn-1* expression (see Figure 37).

4.2.1.3. Testing *daf-16* dependence

I therefore tested whether the effects of candidate gene RNAi treatments on *ftn-1* transcript levels require *daf-16*. This was done in a *daf-16; daf-2* mutant background. If any RNAi treatment was able to reduce *ftn-1* transcript levels in this genotype, then this would demonstrate that the effect of this treatment did not occur upstream of *daf-16*, for example by leading to altered DAF-16 activation (see Figure 36).

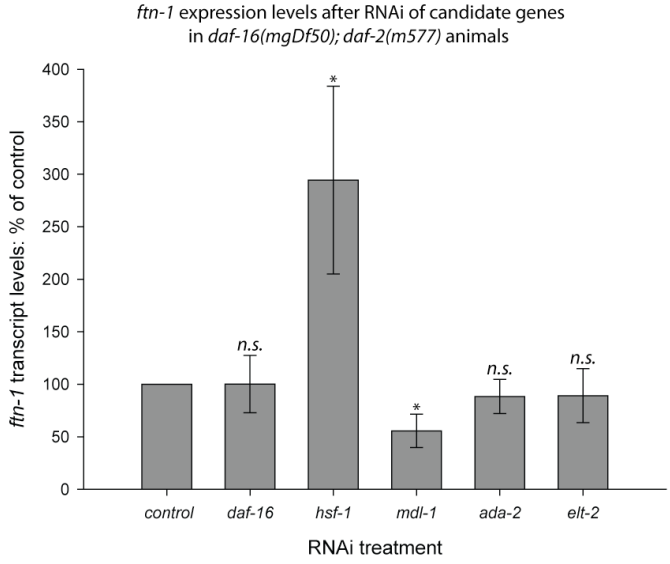


Figure 39
Expression levels are expressed as a percentage of the control RNAi treatment. Error bars represent ±*s.e.m*. Statistical significance was calculated as in Figure 38.

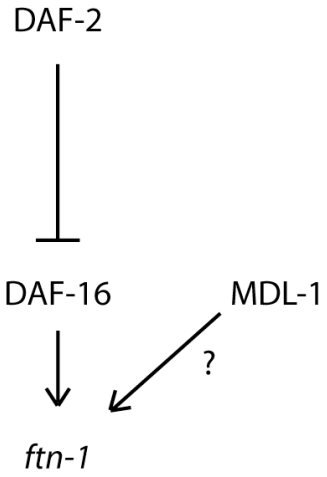
RNAi of gene	as % of control	n	p-value
control	100	5	
daf-16	100.1	4	>0.2
hsf-1	294.4	5	0.0116
mdl-1	55.6	5	0.0268
ada-2	88.3	5	>0.2
elt-2	89.2	5	>0.2

Table 8
This table contains data corresponding to Figure 39. The p-values were adjusted for multiple testing using the Bonferroni correction, but in this case this correction did not lead to a loss of statistical significance in the effects of any of the treatments.

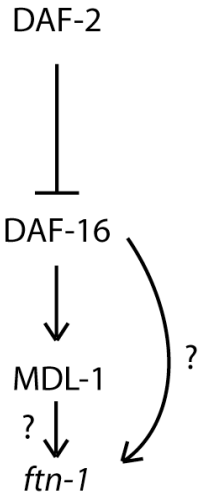
As expected, *daf-16* RNAi had no effect on this strain since it already lacks *daf-16*. *hsf-1* RNAi had the unexpected effect of increasing *ftn-1* levels, the opposite of its effect on *daf-16(+); daf-2(m577)*, in which it greatly reduced *ftn-1* levels. This implies that the effects of *hsf-1* RNAi on *ftn-1* require DAF-16. This result is consistent with previous findings on the joint action of DAF-16 and HSF-1 (Hsu et al., 2003). See the chapter discussion for a detailed interpretation of this finding.

No effects of *ada-2* or *elt-2* RNAi on *ftn-1* levels were detected in this genotype. This could merely imply that the assay's sensitivity was insufficient and more samples would be required to detect the changes. This would not be unexpected, since the effect of RNAi of these two genes had been much smaller than that of *mdl-1* RNAi in *daf-2(m577)*, both on *ftn-1* transcript and on *Pftn-1::gfp* expression levels. The absence of a statistically significant effect by RNAi of *elt-2* in particular suggests that the assay may simply not be sensitive enough, since RNAi of this GATA transcription factor has previously been shown to regulate *ftn-1* in a *daf-2* and *daf-16* wild-type background. Given its role as the intestinal terminal differentiation factor, it seems unlikely that it requires *daf-16* to regulate *ftn-1*. It is possible that including additional biological replicates would demonstrate that there is a small effect of *elt-2* RNAi on *ftn-1* levels in all genetic backgrounds, as previously reported (Romney et al., 2008). The fact that small reductions were observed in both the mean *ftn-1* expression level of worms treated with RNAi of both these genes (see Table 8) suggests that this is indeed a sensitivity issue and that collection of more replicates may make this result statistically significant.

Of the genes tested here, only *mdl-1* RNAi was capable of reducing *ftn-1* transcript levels in this genotype. Since *mdl-1* RNAi has already been found to strongly reduce *ftn-1* transcript levels in *daf-2* animals, showing that the same occurs in *daf-16; daf-2* demonstrates that *daf-16* is not required for the treatment's effects. Figure 40 depicts two possible models for the regulation of *ftn-1* by MDL-1.



Model 2: MDL-1 may be affecting *ftn-1* transcription by a mechanism unrelated to IIS. This is consistent with the results of qRT-PCR, since loss of MDL-1 by RNAi would lead to decreased *ftn-1* transcript levels regardless of IIS genotype. Given the evidence that *mdl-1* is a regulatory target of DAF-16, a role of MDL-1 in the regulation of *ftn-1* that is completely independent of DAF-16 (Model 2) seems unlikely.



Model 3: MDL-1 may act downstream of DAF-16 to activate *ftn-1* transcription. This may be occurring either as the only mechanism by which IIS affects *ftn-1* transcription or in addition to the possible direct activation of *ftn-1* by DAF-16. This scenario is also consistent with qRT-PCR data as long as MDL-1 has a role in regulating *ftn-1* even in the absence of DAF-16.

Figure 40
Schematic representation of possible involvement of 'candidate' genes in *ftn-1* regulation by IIS.

4.2.1.4. Effect of candidate gene RNAi on *daf-16(mgDf50)* and wild-type animals

In parallel, I also tested the effects of candidate gene RNAi on N2 and *daf-16(mgDf50)* animals. The levels of *ftn-1* transcript in control and *daf-16* RNAi–treated animals of all four strains can be seen in Figure 38, which demonstrates that *ftn-1* transcript levels respond to mutations in IIS as one would predict from the

experiments using *Pftn-1::gfp*. Unfortunately, none of the RNAi treatments, not even the positive control treatment against *elt-2*, had a statistically significant effect on *ftn-1* transcript levels (Figure 41). The only exception to this was RNAi against *daf-16*, which did reduce transcript levels in wild-type animals as expected.

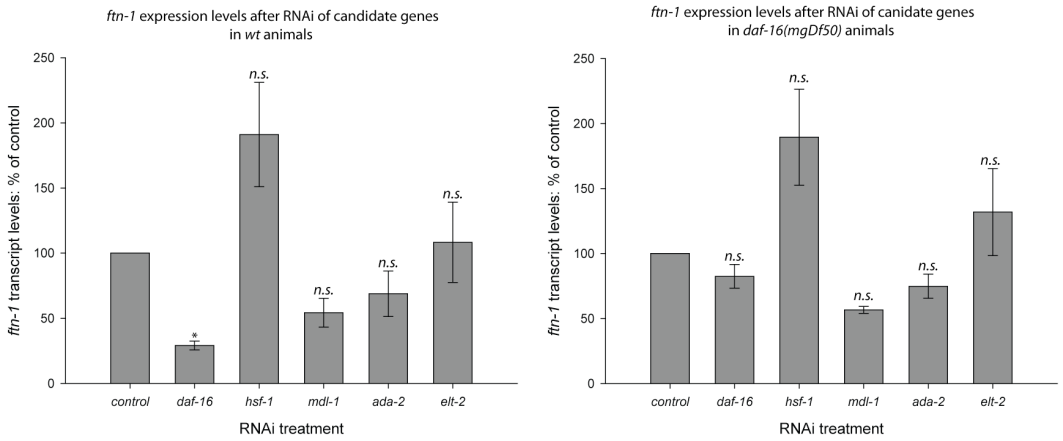


Figure 41
Expression levels are expressed as a percentage of the control RNAi treatment. Error bars represent ±*s.e.m*. Statistical significance was calculated as in Figure 38.

Wild-type animals					***daf-16(mgDf50)* animals**			
RNAi of gene	*ftn-1 transcript levels* (as % of control)	n	p-value		RNAi of gene	*ftn-1 transcript levels* (as % of control)	n	p-value
control	100	4			control	100	5	
daf-16	29.1	3	0.014		*daf-16*	82.5	4	>0.2
hsf-1	191	4	>0.2		*hsf-1*	189.5	5	0.15[a]
mdl-1	54.3	4	>0.2[a]		*mdl-1*	56.6	5	0.13[a]
ada-2	68.8	4	>0.2		*ada-2*	74.9	5	>0.2
elt-2	108.3	4	>0.2		*elt-2*	131.9	5	>0.2

Table 9
p-values calculated as described in Figure 41. Correction for multiple testing was carried out using the Bonferroni correction. [a] These p-values were <0.05 before Bonferroni correction.

The same trends were visible in these genotypes as in *daf-16; daf-2* mutants: *hsf-1* RNAi seems to cause an increase, rather than a decrease in *ftn-1* expression and

mdl-1 RNAi–treated animals show lower mean transcript levels regardless of genotype, although both effects are not statistical significant in the two genotype shown in Figure 41. Given the cost of qRT-PCR, I decided not to collect additional biological replicates to confirm these effects since the results of the analysis on *daf-16; daf-2* animals was sufficient to answer the central question about the *daf-16* – dependence of the effect. Clearly, the effect of *mdl-1* RNAi on *ftn-1* expression does not require *daf-16*. However, *daf-16* is required for the reduction in *ftn-1* transcript levels by *hsf-1* RNAi in *daf-2* mutants, since the opposite is seen in *daf-16; daf-2*. The effects of *ada-2* RNAi may also require *daf-16*, although the absence of a statistically significant result could merely reflect insufficient biological replicates.

It should be mentioned that the conservative Bonferroni correction was used to adjust p-values for multiple testing and that in the tests marked with [a], this correction caused a loss of statistical significance. Given the many comparisons being made, correcting for multiple comparisons was necessary to avoid false positive results. The reduction in the mean expression level of *ftn-1* in *mdl-1* RNAi treated animals is similar across genotypes (reduced to 39.5% of control in *daf-2*; to 55.6% in *daf-16; daf-2*; to 54.3% in *wt*; and to 56.6% in *daf-16*). This suggests that further biological replicates would likely confirm the trend that *mdl-1* RNAi reduces *ftn-1* levels regardless of IIS genotype.

Taken together with other evidence, this analysis implies that *mdl-1* is a direct regulatory target of *daf-16,* and that when *daf-2* is mutated, activation of DAF-16 further activates *mdl-1*, thereby increasing *ftn-1* expression (see Figure 35).

4.2.3. Effect of *mdl-1* and *ada-2* on *C. elegans* lifespan

While the regulation of *ftn-1* levels by *ada-2, hsf-1* and *mdl-1* is interesting, the aim of this project was to identify novel mediators of the longevity of *daf-2* mutants. Previous publications by another group (Murphy et al., 2003; Pinkston-Gosse and Kenyon, 2007), as well as my own work (see Figure 22 and Table 5), have already demonstrated that RNAi of *mdl-1* is capable of reducing *daf-2* longevity. The effect of *ada-2* RNAi on *daf-2* lifespan had not been previously described but a single experiment had shown a very slight but statistically significant reduction (-5% in *daf-2.* see Figure 22). I decided to test the effect of *ada-2* and *mdl-*

1 RNAi on *daf-2* lifespan again. I did not re-test the effect of *hsf-1* RNAi given that the role of *hsf-1* in the longevity of IIS mutants is well established (Hsu et al., 2003; Morley and Morimoto, 2004).

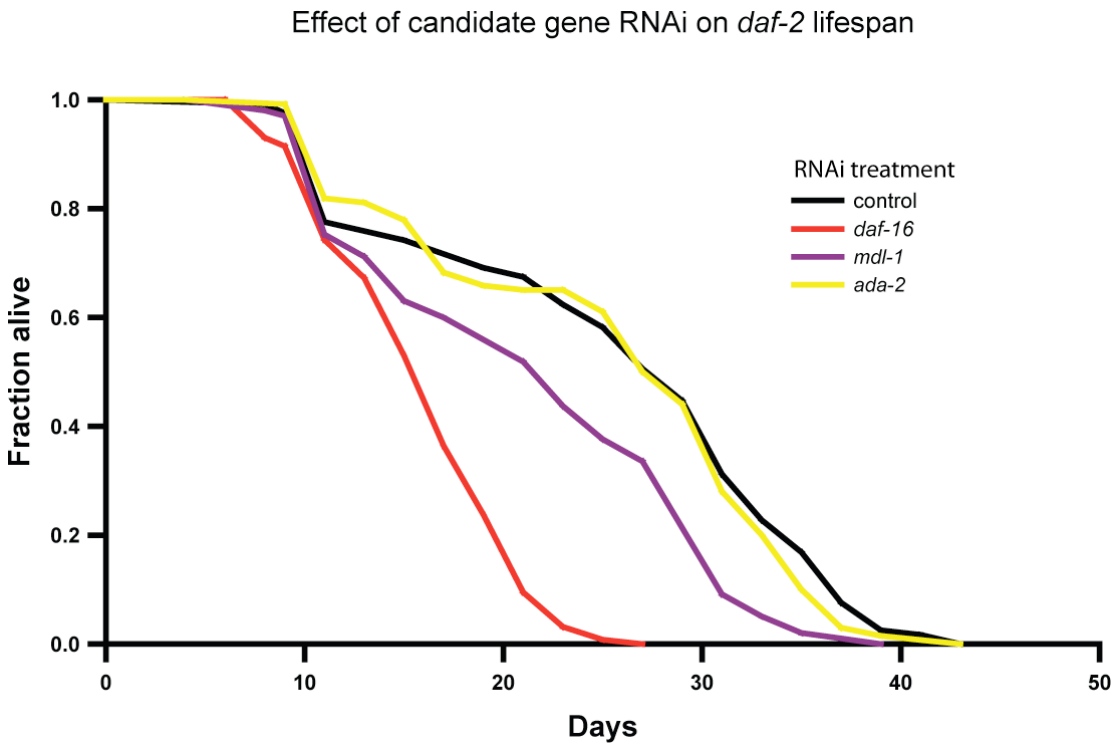


Figure 42
The effect of *ada-2* and *mdl-1* RNAi on *rrf-3(pk1426); daf-2(m577)* animals at 25°C.

RNAi treatment	n	censored	Mean Lifespan	Median Lifespan	% Change in mean [median]	Log-Rank p-value[b]	Wilcoxon p-value[b]
control	118	4	25.6	29			
daf-16	127	4	16.1	17	-37 [-41]	<0.0001	<0.0001
ada-2	108	20	25.5	29	none	0.4946	0.9693
mdl-1	99	4	21.5	23	-16 [-21]	<0.0001	0.0013

Table 10
This table contains data corresponding to Figure 42

I was able to confirm that RNAi of *mdl-1* statistically significantly reduces the lifespan of *daf-2* mutants. In combination with my previous findings (see Chapter 3) and similar findings by a different group (Murphy et al., 2003; Pinkston-Gosse and Kenyon, 2007), this demonstrated that RNAi of *mdl-1* does reduce *daf-2* longevity. The effect of *ada-2* RNAi is not robust. In the previous assay (see Chapter

3), this treatment only reduced mean *daf-2* lifespan by 5% and had no effect on the median lifespan. In this second trial, *ada-2* RNAi had no effect on *daf-2* lifespan.

The lifespan curves shown in Figure 42 look slightly unusual due to an unusual pulse of early mortality on day 11. This may be due to differences between experimenters in scoring lifespan assays as I had been unavailable to score this experiment on that particular day and a colleague had been kind enough to take over this task. Since the early mortality seems to affect all strains equally, this is unlikely to have affected the overall result, but I reanalysed this data to account for this difference (see Appendix 3). While the mean and median values calculated in this reanalysis do change, the effects of each treatment are consistent with the analysis presented here (see Table 11).

A number of different results suggest that *mdl-1* is regulated by IIS (Murphy et al., 2003; Pinkston-Gosse and Kenyon, 2007; Schuster et al., 2010), so the finding that *mdl-1* RNAi is capable of reducing *daf-2* lifespan supports a role of *mdl-1* in mediating the *daf-2* Age phenotype downstream of DAF-16. However, the reduction in *daf-2* lifespan by *mdl-1* RNAi alone is insufficient evidence for such a role, as loss of this transcription factor could simply be causing pathologies that shorten lifespan. If so, then the effect of *mdl-1* RNAi on lifespan would be completely unrelated to the lifespan extension of IIS mutants. One piece of evidence that argues against this is the finding by Pinkston-Gosse et al. that RNAi of *mdl-1* is effective at reducing *daf-2(-)* but not *daf-2(+)* lifespan. If true then this would suggest that *mdl-1* is indeed required for at least a proportion of the lifespan extension seen in *daf-2*. I therefore decided to attempt to corroborate this finding by treating *rrf-3; daf-2(+)* mutants with *mdl-1* RNAi. RNAi of *ada-2* was also tested on this strain for the same reasons.

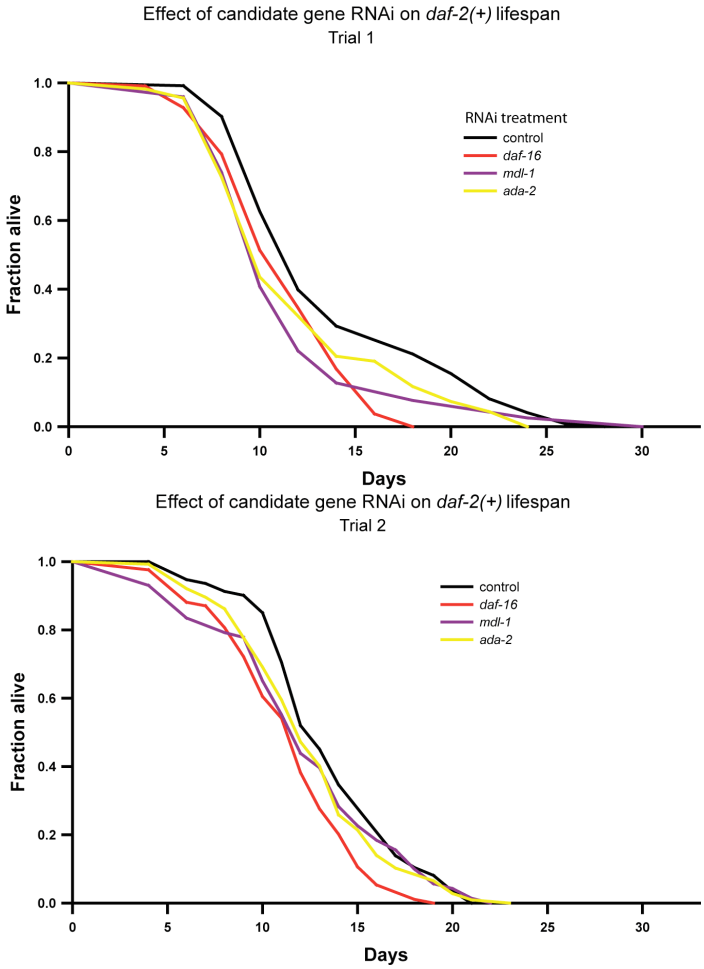


Figure 43
The effect of *ada-2* and *mdl-1* RNAi on *rrf-3* lifespan at 25°C.

RNAi treatment [Trial number]	n	censored	Mean Lifespan	Median Lifespan	% Change in mean [median]	Log-Rank p-value	Wilcoxon p-value
control [1]	123	0	13.9	12			
daf-16 [1]	109	2	11.6	12	-17 [- 0]	<0.0001	0.0049
ada-2 [1]	97	17	12.1	10	-13 [-17]	0.0052	0.0016
mdl-1 [1]	120	3	11.6	10	-17 [-17]	0.0006	<0.0001
control [2]	88	31	13.3	13			
daf-16 [2]	97	28	11.4	12	-14 [-8]	0.0002	0.0009
ada-2 [2]	114	18	12.5	12	-6 [-8]	0.2171	0.0138
mdl-1 [2]	78	37	12.2	12	-8 [-8]	0.2111	0.1013
combined data							
control [1 + 2]	211	31	13.7	12			
daf-16 [1 + 2]	206	30	11.5	12	-16 [-0]	<0.0001	<0.0001
ada-2 [1 + 2]	211	35	12.2	12	-11 [-0]	0.0009	0.0013
mdl-1 [1 + 2]	198	40	11.7	10	-15 [-17]	<0.0001	<0.0001

Table 11
This table contains data corresponding to Figure 43. All lifespan assays were carried out on on *rrf-3(pk1426)* at 25°C.

Against expectation, RNAi of *mdl-1* was also effective at reducing *wild-type* lifespan, at least to some degree. This is based on two trials: In the first, *mdl-1* RNAi reduced *daf-2(+)* mean and median lifespan by 17%. In the second trial the same trend was visible (mean and median lifespan both reduced by 8%), but this effect was not statistically significant, possibly because significantly fewer animals were used this time. When the data from the two trials is pooled, a reduction in both mean (-15%) and median (-17%) lifespan is seen (see Figure 43) and this effect is highly statistically significant ($p<0.0001$; see Table 11). This evidence contradicts the finding by Pinkston-Gosse et al. and demonstrates that loss of *mdl-1* may reduce lifespan in *daf-2(+)* as well as *daf-2(m577)* animals.

The evidence for a role of *ada-2* in mediating *daf-2* lifespan is even weaker: While *ada-2* RNAi resulted in a very small reduction in *daf-2* lifespan in one of two trials, the same treatement caused a greater reduction in *wt* lifespan. A role of *ada-2* in mediating the lifespan extension of *daf-2* animals therefore seems unlikely.

4.3. Chapter discussion

4.3.1. The use of *elt-2* RNAi as a positive control

A number of features of the *elt-2* gene suggest that it should be a good control gene for use in the qRT-PCR assay. ELT-2 has already been shown to bind to the promoter of *mdl-1* and to regulate its expression (Romney et al., 2008). Loss of *elt-2* via RNAi led to a large decrease in *ftn-1* expression. The fact that *elt-2* is thought to be required for expression of all intestinally expressed genes (McGhee et al., 2009), makes it seem unlikely that it would have different roles in IIS mutants. I therefore expected *elt-2* RNAi to reduce *ftn-1* transcript levels equally in all genotypes tested, making it a suitable positive control.

However, no significant down-regulation of *ftn-1* was detected in any of the strains other than the *daf-2* mutant line. This could indicate that RNAi is not as effective in these genotypes, that the qRT-PCR assay is not sensitive enough to detect this magnitude of change in the non-induced *ftn-1* levels of *daf-2(+)* and *daf-16; daf-2* animals, or that *elt-2* acts downstream of IIS to mediate *ftn-1* expression.

The first possibility, that RNAi is not effective in these genotypes, is unlikely, because both *mdl-1* and *hsf-1* RNAi had large effects on *ftn-1* expression in *daf-16; daf-2* mutants. It seems likely that qRT-PCR assay used here was not sensitive enough to detect these changes in *ftn-1* expression. The absence of a significant finding was presumably due to the relatively small effect of *elt-2* RNAi by feeding on *ftn-1* levels, which in the *daf-2* mutant strain only reduced *ftn-1* levels by approximately 30%, the smallest effect of any of the RNAi treatments tested. Romney et al. did find a significant reduction by *elt-2* RNAi, but this was done using a GFP reporter and RNAi was administered via injection rather than feeding, which may have been more effective. Given the large variance in *ftn-1* levels, it would have required an unreasonably high number of replicates to detect a statistically significant reduction. A better positive control treatment might have been RNAi of *ftn-1* itself.

It is also possible that *elt-2* may have a role downstream of IIS to regulate target gene expression. This seems unlikely given the function of *elt-2* in the regulation of all intestinal genes, but none of the findings in this chapter contradicts such a role. While loss of *elt-2* by RNAi had large effects on *ftn-1* expression in *daf-2(+)* animals in a different study (Romney et al., 2008), the effects on IIS mutants was not investigated.

4.3.2. The role of *hsf-1* in *ftn-1* regulation

One of the interesting findings from the qRT-PCR analysis of *daf-2* and *daf-16; daf-2* animals is the different response to knockdown of *hsf-1* in the two lines. That RNAi of *hsf-1* substantially lowers the induced *ftn-1* transcript levels of *daf-2* mutants is consistent with *hsf-1*'s established role as a co-activator with DAF-16 of longevity in *daf-2* mutants. However, the fact that this does not occur in *daf-16; daf-2* is intriguing. The involvement of *hsf-1* in the longevity phenotype of IIS mutants was demonstrated when loss of *hsf-1* was found to be sufficient to completely abrogate the lifespan extension of long-lived IIS mutants, suggesting an essential role of *hsf-1,* similar to that of *daf-16,* in mediating IIS longevity (Morley and Morimoto, 2004). However, whether this occurs through joint regulation of a common set of target genes or through independent regulation of different genes that are essential for the lifespan extension is unclear.

Evidence for the first model comes from the apparent joint regulation of small heat-shock proteins (sHSPs), which were differentially regulated in *daf-2* mutants in a manner dependent on DAF-16 (McElwee et al., 2004; Murphy et al., 2003) and are also regulated by HSF-1 (Hajdu-Cronin et al., 2004; Tonkiss and Calderwood, 2005). These heat shock proteins have been shown to be potential mediators of the longevity phenotype of IIS mutants (Morley and Morimoto, 2004). It therefore seems likely that reduced IIS leads to an up-regulation of sHSPs via the joint activities of DAF-16 and HSF-1. Other genes may be regulated by HSF-1 or DAF-16 independently and are not required for the extended lifespan of IIS mutants (see Figure 44).

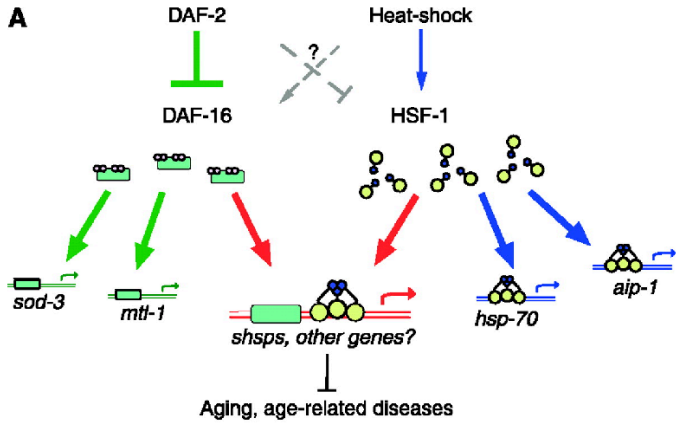
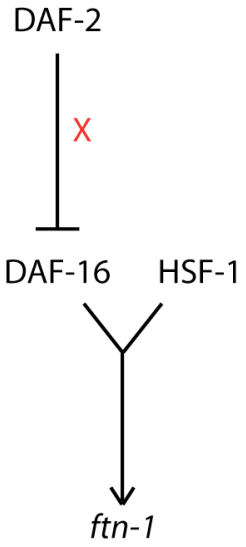
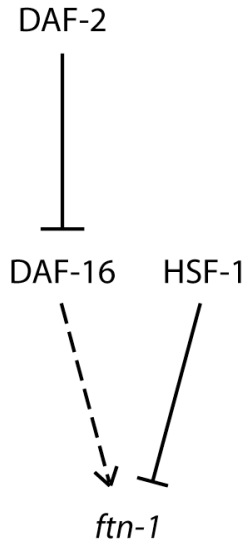


Figure 44
Adapted from Hsu et al. (2003). Model of joint regulation of target genes by HSF-1 and DAF-16.

The case of *ftn-1* may be similar: HSF-1 and DAF-16 may be acting downstream of IIS to activate *ftn-1* expression (see Figure 45: HSF-1 model 1). This would explain why RNAi of *hsf-1* reduces *ftn-1* expression in a *daf-2* mutant background. But why should it increase *ftn-1* expression in a *daf-2(+)* or *daf-16; daf-2* background? It seems possible that under normal conditions HSF-1 acts as a repressor of *ftn-1* expression and that only when under conditions of reduced IIS, DAF-16 changes HSF-1 from an inhibitor to an activator of *ftn-1* expression (see Figure 45: HSF-1 model 2).



HSF-1 model 1: HSF-1 may be acting downstream of DAF-2 and jointly with DAF-16 to regulate *ftn-1* transcription. This is consistent with the results of qRT-PCR analysis, since loss of HSF-1 was capable of reducing *ftn-1* transcript levels in *daf-2* but not *daf-16; daf-2* mutant animals.



HSF-1 model 2: When IIS is not reduced, HSF-1 may act as a repressor of *ftn-1*. DAF-16 still acts as an activator of *ftn-1* under these conditions.

Figure 45
Possible roles of *hsf-1* in the regulation of *ftn-1*

4.3.3. The role of *mdl-1* in *ftn-1* regulation

qRT-PCR of *ftn-1* transcript levels demonstrated that both *daf-2* and *daf-16; daf-2* worms treated with *mdl-1* RNAi show reduced *ftn-1* expression. Given the many studies indicating a role of *mdl-1* downstream of DAF-16, it seems likely that *mdl-1* is regulating *ftn-1* expression downstream of *daf-16*, as shown in Model 3 of Figure 40. If this is true, then RNAi of *mdl-1* should have a larger effect on *ftn-1* levels in *daf-2(-)* than in *daf-2(+)* or in *daf-16; daf-2* animals. This was in fact found to be the case: In *daf-2(m577)* animals, RNAi of *mdl-1* reduced *ftn-1* levels to 39.5% of control RNAi treated, whereas the same treatment reduced *ftn-1* levels to 55.6% of control in *daf-16; daf-2* mutants. While a role of *mdl-1* in *ftn-1* regulation that is

completely independent of IIS is possible (Model 2 of Figure 40), this and the results of other studies, in which *mdl-1* was found to be up-regulated by DAF-16 by direct binding (discussed previously in this chapter), make a role of *mdl-1* downstream of *daf-16* seem more likely. In addition, the recent finding by the ModEncode project that MDL-1 can bind to the *ftn-1* promoter also supports this model, although one should keep in mind that this study was carried out in L1 animals. While *ftn-1::gfp* transgenes are expressed at this stage (Romney et al., 2008), the regulation of *ftn-1* during early development was never investigated.

It would be very interesting to investigate further the possibility that direct binding of MDL-1 to the *ftn-1* promoter is mediating its regulation by IIS. This could be investigated using ChIP, using either the same MDL-1::GFP strain as used by the ModEncode project and an anti-GFP antibody, or ChIP of the endogenous protein could be attempted using a commercially available anti-MDL-1 antibody. The anti-MDL antibody has already been purchased and will soon be tested by Michèle Riesen. In either case, establishing whether higher levels of MDL-1 binding to the *ftn-1* promoter could be detected in *daf-2* animals compared to wild-type or *daf-2; daf-16* mutants would go a long way in explaining whether at least part of the induction in *ftn-1* by decreased IIS occurs because the long-lived mutants have increased expression of *mdl-1*.

As indicated in Chapter 3, very recent findings suggest that DAF-16 may be directly binding to the *ftn-1* promoter. This was unexpected, since *ftn-1* has not been identified in other studies of DAF-16 targets (Schuster et al. 2010). However, direct regulation of *ftn-1* by DAF-16 could explain both the very large effects that loss of *daf-16* has on *ftn-1* levels and the fact that the RNAi screen did not uncover another transcription factor with as large a role in the regulation of *ftn-1*. Further investigation of a possible direct interaction between DAF-16 and *ftn-1* promoter may clarify this issue. This could be investigated by ChIP using an anti-DAF-16 antibody by testing whether there is enhanced binding of DAF-16 to the *ftn-1* promoter in *daf-2* mutants.

4.3.4. *mdl-1* binding partner

Previous work on *mdl-1* suggests that the MDL-1 protein can only bind DNA by forming a heterodimer with MXL-1. The effects of *mxl-1* RNAi on *ftn-1* expression were investigated only in the context of the initial screen, in which a small reduction in *Pftn-1::gfp* fluorescence was detected, but the magnitude of this effect fell below the threshold that I set of a 20% reduction. I did not further investigate the role of *mxl-1*, but further work could focus on whether the effect of *mdl-1* RNAi on *ftn-1* expression requires *mxl-1*. This would help establish whether the two proteins are in fact acting as a dimer to regulate *ftn-1* levels.

4.3.5. *ada-2*

This work shows that *ada-2* influences *ftn-1* expression, but its exact role remains unclear. Loss of *ada-2* by RNAi leads to a reduction in *ftn-1* transcript levels as well as in *Pftn-1::gfp* expression in *daf-2* mutant animals. However, the same reduction could not be detected in *daf-16; daf-2* animals, implying that the effects of *ada-2* RNAi may act via *daf-16*. This would place ADA-2 upstream of DAF-16 in the regulation of *ftn-1*.

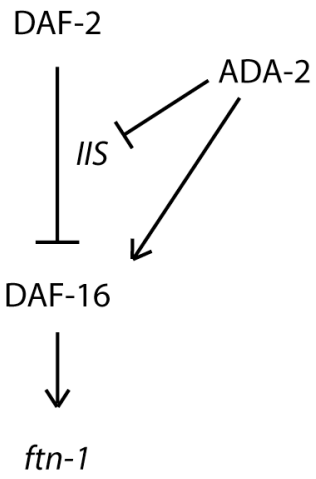


Figure 46
Schematic representation of potential role of ADA-2 in *ftn-1* regulation by IIS.

However, this conclusion is based on the finding that *ada-2* RNAi has no effect on *ftn-1* expression in *daf-16; daf-2* animals, but that the positive control used in these assays also had no effect. It therefore remains possible that a reduction in *ftn-1* levels does in fact occur upon RNAi of *ada-2* in *daf-16; daf-2* animals, but wasn't detected because of insufficient assay sensitivity.

Other approaches may be needed to investigate the role of *ada-2* in *ftn-1* regulation. One possibility is to test the involvement of *pcaf-1,* which is thought to code for the catalytic subunit of the HAT complex ADA-2 may act in. If RNAi of this gene also reduces *ftn-1* expression, then this would support a role for an ADA-2 and PCAF-1 containing complex in regulating *ftn-1*. Another approach would be to use a genetic deletion of *ada-2*. However, only one mutant allele of *ada-2* is available (*ok3151*), which only deletes one exon from one of two isoforms of the gene and unfortunately also affects an adjacent gene. Even if these problems were overcome, the allele is lethal when homozygous and would therefore be very difficult to work with. If RNAi of *pcaf-1* can be shown to affect *ftn-1* expression, then mutant alleles of *pcaf-1* could be used instead.

4.3.6. Effect on lifespan

Disappointingly, lifespan assays on worms treated with *ada-2* RNAi did not provide evidence that this gene acts downstream of IIS to extend lifespan. If this had been the case, one would expect RNAi knockdown to have reduced *daf-2* lifespan to a greater extent than wild-type lifespan. Instead, *ada-2* RNAi seems to cause an even greater reduction in wild-type lifespan than in *daf-2* lifespan.

The results for *mdl-1* are slightly more ambiguous. Based on results for microarray studies, the DamID study and the ModEncode project, the following model could be proposed: Decreased IIS activates DAF-16 and therefore increases expression of the DAF-16 target MDL-1, which in turn affects gene expression. In this model, the lifespan extension of IIS mutants is caused by a large number of transcriptional changes downstream of DAF-16, some of which are mediated by MDL-1. The model predicts that increased levels of MDL-1 are part of the reason why IIS mutants are long lived. See Figure 46 for a depiction of this model. Whether

or not loss of *mdl-1* leads to a greater reduction in lifespan in *daf-2* mutants than in *wild-type* animals is an important test of this model.

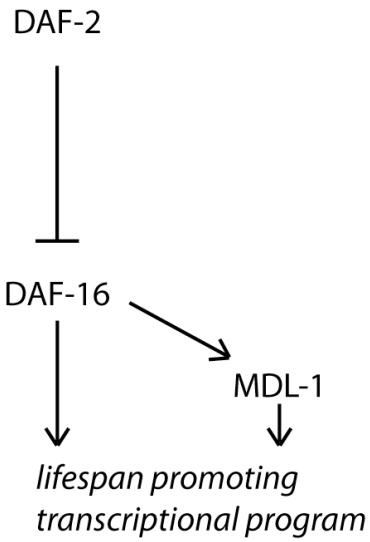


Figure 46
Possible role of MDL-1 in the longevity caused by reduced IIS.

This question currently remains unresolved, since a statistically significant reduction in *wild-type* lifespan was seen in one out of two trials. In the second trial, a trend towards reduction was seen, although this was not statistically significant, possibly partly due to a smaller sample size. If the two trials are pooled, a statistically significant reduction is seen overall. This is unexpected given that Pinkston-Gosse et al. found no lifespan reduction in *daf-2(+)* animal treated with *mdl-1* RNAi. This could be explained by a stronger RNAi effect in the assays I performed, since *rrf-3* mutant animals were used, or be due to other differences in assay conditions.

That RNAi of *mdl-1* reduces wild-type lifespan does not in itself exclude the possibility that MDL-1 may act as a mediator of *daf-2* lifespan: loss of either *hsf-1* or *daf-16* has similar effects on *daf-2(+)* lifespan. However, the fact that the magnitude of the reduction in lifespan is similar is hard to reconcile with a model in which increased levels of MDL-1 in IIS mutants is one of the causes of the *daf-2* lifespan extension. The trial described in this chapter shows that in *daf-2(m577), mdl-1* RNAi causes a 16% reduction in mean and a 21% reduction in median lifespan and

two previous trials described in Chapter 3 showed a 12% reduction in mean and 17% reduction in median lifespan in the first and a 9% reduction in mean and 12% reduction in median lifespan in the second trial. These reductions are similar in magnitude to those seen in pooled data from *daf-2(+)* animals, for which *mdl-1* RNAi causes a 15% reduction in mean and 17% reduction in median lifespan under similar conditions. Table 12 shows the results of individual trials performed in various studies on the effects of *mdl-1* RNAi on lifespan.

daf-2 genotype	lifespan change (mean)	*rrf-3* genotype	see…
daf-2(mu150)	-9%	*wild-type*	(Murphy et al., 2003)
daf-2(e1370)	-10%	*wild-type*	(Pinkston-Gosse and Kenyon, 2007)
daf-2(e1370)	-8%	*wild-type*	(Pinkston-Gosse and Kenyon, 2007)
daf-2(m577)	-12%	*pk1426*	Figure 22, Table 5
daf-2(m577)	-9%	*pk1426*	Table 5
daf-2(m577)	-16%	*pk1426*	Figure 42
daf-2(+)	+4% (*n.s.*)	*wild-type*	(Pinkston-Gosse and Kenyon, 2007)
daf-2(+)	-1% (*n.s.*)	*wild-type*	(Pinkston-Gosse and Kenyon, 2007)
daf-2(+)	-17%	*pk1426*	Figure 43
daf-2(+)	-8% (*n.s.*)	*pk1426*	Figure 43

Table 12
Summary of data on effects of *mdl-1* RNAi on lifespan. *n.s.*: not statistically significant.

It is possible that *mdl-1* may still turn out to be an important player in the longevity of IIS mutants, since the interpretation of this type of epistasis analysis using a quantitative trait such as lifespan is difficult. It is also possible that differences in the efficacy of RNAi in the two different strains may be complicating this interpretation further. To exclude possible problems with *mdl-1* RNAi treatment, an *mdl-1* deletion strain could be used to confirm the results I obtained using gene knockdown.

In fact, a colleague in the lab (Michèle Riesen) has taken over the project to illuminate the role of *mdl-1* in *daf-2* lifespan. For this purpose, she has already obtained and outcrossed an *mdl-1* deletion strain and crossed it into two different

alleles of *daf-2*. Comparing the lifespan of *daf-2* animals to *daf-2; mdl-1* animals and wild-type to *mdl-1* mutants will hopefully help answer the question of whether loss of *mdl-1* shortens *daf-2* lifespan due to a reduction in DAF-16–mediated longevity or simply due to pathology related to the loss of an important transcription factor. Using this deletion instead of RNAi will help rule out any artefacts due to changes in RNAi efficiency.

Another approach to testing this model would be to over-express MDL-1 via transgenesis, thus mimicking the effects of reduced IIS on MDL-1 levels without any genetic mutations in IIS. If *mdl-1* over-expression leads to a lifespan extension, then this would demonstrate that the genes downstream of MDL-1 are capable of extending lifespan, as the model would predict. Michèle Riesen is therefore currently working on constructing *mdl-1* over-expressing strains. Given the large effects of *mdl-1* on the expression of the IIS- regulated gene *ftn-1*, it will be fascinating to find out whether this gene does in fact act downstream of DAF-16 to extend lifespan.

Chapter 5: The regulation of *ftn-1* by iron levels is regulated by hypoxia signalling

5.1 Introduction

5.1.1. Hypoxia signalling and *C. elegans* ageing

The screen described in Chapter 3 was primarily designed to identify transcription factors with a role in promoting expression from the *Pftn-1::gfp* transgene. The aim was to identify RNAi treatments that reduce GFP fluorescence from the induced levels found in *daf-2(m577)* animals. However, treatment with a large number of RNAi clones led to increases, rather than decreases, in GFP fluorescence in this strain (see Appendix 1). For the most part, the role of these genes was not followed up, but I did decide, for several reasons, to further investigate the two transcription factors *hif-1* and *aha-1*.

The first reason that these two factors seemed particularly interesting was simply the magnitude of the effect on *Pftn-1::gfp* upon RNAi of either gene. Both led to a very strong increase in expression and a look at the literature quickly revealed that the two are known to form a dimer in *C. elegans* (Jiang et al., 2001a). Due to the high variability in the expression of *Pftn-1::gfp* and the large number of positive 'hits' identified in my screen, I was concerned that the long list of candidates identified might contain a high number of false positives. The finding that RNAi of both subunits of the HIF dimer have similarly large effects on *Pftn-1::gfp* expression suggested that this effect was not a false positive. Additionally, HIF transcription factors are known to be regulated post-translationally by a mechanism that is dependent on iron. The FTN-1 protein is predicted to be involved in iron storage and *ftn-1* mRNA levels have been shown to be responsive to iron (see Chapter 1). The discovery of a possible regulatory role of the iron responsive transcription factor HIF-1 in the regulation of the iron responsive gene *ftn-1* seemed a promising lead in discovering how worms maintain iron homeostasis. A recent publication described efforts to identify how *ftn-1* is regulated by iron (Romney et al., 2008) and identified a region of the promoter that is sufficient for the induction of *ftn-1* by iron. This 63bp 'iron dependent element' (IDE), contains three direct repeat (DR) sequences that are

required for the IDE to be responsive to iron (Romney et al., 2008). These DR sequences are similar to the E-box motif, a binding site for basic helix-loop-helix (bHLH) family of transcription factors. Since both *aha-1* and *hif-1* are bHLH transcription factors, it is possible that the HIF-1/AHA-1 dimer is repressing expression by binding to the DR sequences. This section will therefore describe my efforts to confirm the initial findings and investigate the role of hypoxia signalling in the regulation of *ftn-1* in *C. elegans*.

In vertebrates, the transcriptional response to hypoxia is regulated by the HIF transcription factors. HIF functions as a dimer consisting of one α and one β subunit. In vertebrates, the three genes HIF-1α, HIF-2α and HIF-3α code for the α subunit. In order to regulate gene expression, alpha subunits must dimerize to HIFβ, also called ARNT (the aryl hydrocarbon receptor nuclear translocator), which is ubiquitously expressed and unresponsive to hypoxia. Under normoxia, HIFα protein is hydroxylated at conserved proline residues by a group of prolyl hydroxylases (PHDs) and this modification increases the affinity of HIF for the von Hippel-Landau protein (VHL) (Kaelin, 2005). Binding of VHL targets the HIFα protein for ubiquitination and subsequent degradation by the proteosome. HIFα levels are therefore very low under normoxic conditions and increase dramatically under hypoxia. The activation of HIF in response to hypoxia occurs due to the conditions required for the PHDs to hydroxylate the HIFα proline residue. PHDs require oxygen as well as iron and 2-oxoglutarate for the hydroxylation reaction. When cells are kept under hypoxic conditions or when an iron chelator is added, the proline residue in HIFα is not hydroxylated and the HIFα protein accumulates (Mole, 2010).

5.1.2. HIFα in *C. elegans*

It is clear that not all functions of HIF in higher organisms can be conserved in *C. elegans*. An obvious example is the role of HIF-1α in angiogenesis, which accounts for a severe embryonic–lethal phenotype of HIF-1α mutant mice. In the simple nematode *C. elegans*, which lacks a circulatory system, this function of HIF will not be conserved. However, levels of HIF-1, the *C. elegans* homologue of the vertebrate HIFα genes, seem to be regulated by the same mechanisms as its vertebrate counterparts. In fact, it was in *C. elegans* that the role of the PHDs was

first discovered when the *C. elegans* PHD gene EGL-9 was identified as a gene required for the stabilisation of HIF-1 (Epstein et al., 2001).

Only one gene, *hif-1*, codes for HIFα in *C. elegans* and, like its vertebrate homologues, the HIF-1 protein is stabilised by hypoxia and degraded by normoxia. HIF-1 has been shown to bind to AHA-1, the *C. elegans* homologue of ARNT and the HIF-1/AHA-1 dimer has been shown to bind DNA sequences containing the hypoxic regulatory element (Jiang et al., 2001b). The VHL homologue VHL-1 has been shown to bind HIF-1 and *vhl-1* mutant worms have constitutively high HIF-1 levels. As mentioned above, the role of PHDs in the regulation of HIFα protein levels was identified in *C. elegans* using a candidate gene approach that ultimately identified EGL-9 as the gene responsible for targeting HIF-1 for degradation via VHL-1 (Epstein et al., 2001). The role of HIF-1 in protection against hypoxia is also conserved: animals homozygous for the strong loss-of-function allele *hif-1(ia4)* are perfectly viable and appear completely wild-type under normoxia. They are however highly sensitive to hypoxic conditions (Jiang et al., 2001b). The evolutionary conservation of the roles of HIF-1, VHL-1, EGL-9 and their homologues makes *C. elegans* a good model organism for the study of the HIF pathway.

In vertebrates, the HIF-1α and HIF-2α proteins are degraded by the proteosome in response to hydroxylation of proline residues in two oxygen–dependent degradation domains located within the central region of the protein. The hydroxylation reaction is catalysed by PHD1, 2 and 3. As discussed previously, in *C. elegans* the hydroxylation reaction is carried out by EGL-9 and targets a single proline residue at position P621 (see Figure 47). A P621G mutation in a HIF-1::Myc transgene leads to stabilisation and greatly increased HIF-1::Myc levels as measured by western blotting (Zhang et al., 2009).

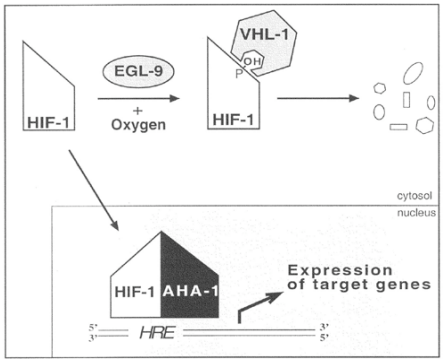


Figure 47
The hypoxia pathway in *C. elegans* (Shen and Powell-Coffman, 2003)

Other than mediating the degradation of HIF-1 by the proteosome, recent work suggests an additional role for EGL-9 in the regulation of HIF-1 target genes (Shao et al., 2009). This had already been suspected due to the extraordinarily strong induction of *hif-1* target genes in *egl-9* mutants, which far exceeds the induction found in *vhl-1* mutants (Shen et al., 2006). This second pathway (see Figure 48) is independent of the VHL-1–mediated pathway described in Figure 47. Using *egl-9* transgenes with a mutation in the hydroxylase domain, which completely abolished oxygen dependent degradation of HIF-1, Shao et al. found that the *vhl-1*–independent effects of EGL-9 do not to require hydroxylase activity .

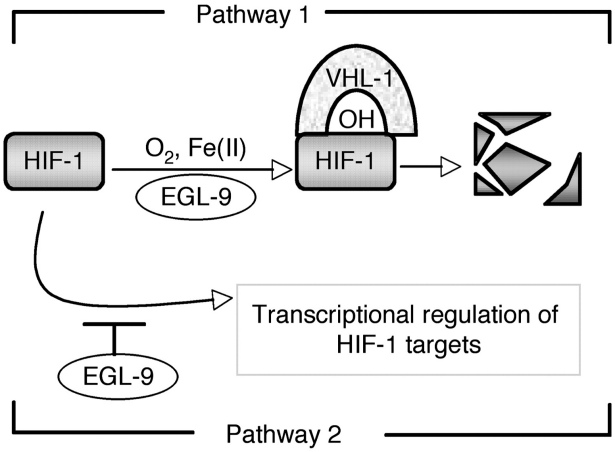


Figure 48
(adapted from Shao et al., 2009)
EGL-9 affects HIF-1 target gene expression via a second unknown. mechanism, which does not require hydroxylase activity.

5.1.3. Effect of HIF-1 over-expresson on ageing in *C. elegans*

While research into the role of *daf-16* and IIS in *C. elegans* lifespan is well established, the examination of the possible role of *hif-1* in ageing has only been carried out recently and has so far yielded contradictory results. Two reports state that higher levels of HIF-1 protein extend lifespan. Mehta et al. found that the knockdown of *vhl-1* and *egl-9* levels by RNAi, which stabilises HIF-1 levels, extends worm lifespan in a HIF-1 dependent manner and also enhances survival of two models of proteotoxicity (Mehta et al., 2009). This result was corroborated by a separate publication (Zhang et al., 2009) in which five different strains over-

expressing HIF-1::Myc were created with or without the stabilising P621G mutation. All over-expressing strains were found to be long-lived and the levels of HIF-1::Myc over-expression were found to correlate with mean lifespan.

Increases in the levels of HIF-1 therefore extend *C. elegans* lifespan, both when a HIF-1::Myc transgene is over-expressed and when endogenous HIF-1 is stabilised by knocking out VHL-1, a protein involved in targeting HIF-1 for degradation by the proteasome. However, there is some suggestion that the role of the prolyl hydroxylase EGL-9 is more complicated. Reduction of EGL-9 by RNAi was shown to extend lifespan, which fits the finding that higher levels of HIF-1 extend lifespan since reductions in EGL-9 have consistently been found to stabilise HIF-1 levels. However, two separate groups found that a strong loss of function mutation in *egl-9* did not affect lifespan (Chen et al., 2009; Zhang et al., 2009), despite leading to highly elevated levels of HIF-1. This contrasts with the findings from *vhl-1* knock-out animals, which have similar levels of HIF-1, but an extended lifespan.

However, the fact that *vhl-1* and *egl-9* should differ in their effects on lifespan is not completely unexpected, even given their joint role in the regulation of *hif-1* target genes. As discussed in the previous section, *egl-9* has pleiotropic effects on HIF-1 target gene expression and the discrepancy in the effects of *vhl-1* and *egl-9* mutations on lifespan may be due to this alternate pathway. For example, it is possible that less severe reductions in EGL-9 by RNAi would increase the levels of HIF-1 sufficiently to extend lifespan but that in severe loss-of-function mutants the lifespan extension gained through increased HIF-1 levels is lost through pleiotropic effects that occur through altered signalling of the alternate pathway.

5.1.4. HIF-1 and IIS

Because the greatest effects on *C. elegans* lifespan are caused by reductions in IIS, testing whether the effects of increased HIF-1 levels on *C. elegans* lifespan are the consequence of interaction with IIS would seem like a good idea. So far, studies of interactions between the *hif-1* pathway and IIS in the regulation of lifespan present a contradictory picture.

Given the large changes in lifespan in animals with reduced IIS and the complete dependence of this effect on the transcription factor *daf-16*, the effects of HIF-1 over–expression were tested for dependence on *daf-16* as well. Mehta et al. found that the *vhl-1* deletion mutant is still long-lived even when *daf-16* is knocked down by RNAi and Zhang et al. report that over-expression of HIF-1::Myc levels in a *daf-16* mutant background is still capable of extending lifespan. This was further supported by the finding that *vhl-1* RNAi does not lead to a nuclear localisation of a DAF-16::GFP transgene (Mehta et al., 2009). The authors of both publications therefore conclude that the effects of increased HIF-1 on lifespan are independent of *daf-16*.

However, the lifespan extension by HIF-1 over-expression would only be found to be dependent on *daf-16* or lead to the nuclear localisation of DAF-1::GFP if HIF-1 acted upstream of DAF-16 in the IIS pathway. However, as exemplified by *skn-1*, the Nrf2–like transcription factor, this is not necessary the case for all factors downstream of the insulin/IGF-1–like receptor DAF-2.

A different approach would be to test if increased levels of HIF-1 are capable of further extending the lifespan of long-lived *daf-2* animals. While Mehta et al. find that mutation of *vhl-1* further extends the lifespan of long-lived *daf-2* RNAi–treated animals, Zhang et al. report that over-expression of stabilised HIF-1::Myc cannot further extend *daf-2(e1370)* lifespan. This finding, along with suggestions in the literature that *hif-1* may be regulated by DAF-16 (Hoogewijs et al., 2007; Pocock and Hobert, 2008) led Zhang et al. to test whether reduced IIS affects the regulation of HIF-1 and its known target genes. They found no statistically significant difference in HIF-1::Myc levels in in *daf-2(e1370)* animals compared to *daf-2*(+) and no significant difference in the expression of GFP reporters of two known HIF-1 targets in the same genotypes (the mean of one seems to be about 40% higher, but measurement show a high variance, possibly explaining the lack of statistical significance). However, they note that if *daf-2* has tissue or stage–specific effects on HIF-1 levels, these may not have been detectable by Western blotting.

The relationship between HIF-1 and IIS with respect to ageing remains unclear. While no increase in HIF-1::Myc levels or in the levels of *hif-1*–target genes was detected in *daf-2(e1370)* animals, the lifespan data is more ambiguous. Whether or not the effects of HIF-1 on lifespan occur downstream of the insulin/IGF-1–like

receptor DAF-2 will hopefully be addressed with further study of the role of *hif-1* in ageing .

5.1.5. Effect of lower *hif-1* levels on lifespan

Surprisingly, loss of HIF-1 also extends *C. elegans* lifespan. Zhang et al. tested three different alleles of *hif-1* as well as RNAi of *hif-1* and in each case saw extended lifespan (at 20^{o}C). The authors note that *hif-1* mutants had previously been found to form dauers more readily at high temperatures (Shen et al., 2005), which suggests that DAF-16 may be activated in these mutants. In fact, the extended lifespan of *hif-1*(*ia4*) requires *daf-16* and also *skn-1,* which demonstrates that over-expression and knockout of *hif-1* extend lifespan by different mechanisms. This dependence on *daf-16* suggests that mutations in *hif-1* may exert their effects on lifespan by activating DAF-16. Zhang et al. also found that *hif-1*(*ia4*) was incapable of further extending *daf-2(e1370)* lifespan thus supporting this interpretation. However, even here conflicting findings can be found from a different research group. While Chen et al. can corroborate the finding that *hif-1* RNAi extends lifespan, they find that this is even the case in *daf-16(mgDf47)* animals, which would make the effects of reductions in HIF-1 levels on lifespan independent of DAF-16.

5.1.6. Possible role of HIF in iron metabolism

Iron chelators have been effectively used to stabilise HIF levels in a number of different contexts, including in *C. elegans* (Epstein et al., 2001). This occurs due to the requirement for iron as a cofactor in the hydroxylation reaction by which PHDs (EGL-9 in *C. elegans*) hydroxylate HIF, which ultimately leads to degradation by the proteasome. While exposing worms or cells to high levels of iron chelators is an extreme intervention unlikely to reflect physiologically relevant conditions, it has been found through analysis of the Km of the hydroxylation reaction in response to iron that the hydroxylation reaction should be responsive to changes in physiological iron levels (Mole, 2010). In mammals, a number of genes involved in the regulation of iron homeostasis have been identified as targets of HIF, including the transferrin

receptor (TfR) (Lok and Ponka, 1999; Tacchini et al., 1999), ceruloplasmin (Mukhopadhyay et al., 2000), hepcidin (Peyssonnaux et al., 2007) and heme oxygenase-1 (Lee et al., 1997) and ferroportin (Peyssonnaux et al., 2008). Interestingly, an iron responsive element was identified in the 5' UTR of HIF-2α mRNA and this element was found to repress translation when iron was limiting (Sanchez et al., 2007), suggesting that HIF-2α may be regulated in response to iron at the translational level as well as the post-translational level. It therefore seems likely that HIF transcription factors act as iron sensors and mediate cellular response to aberrant iron levels. In fact, there is evidence to suggest that HIF levels are responsive to iron even at physiological iron levels. For example, iron starvation leads to increased HIF-1α levels in the livers of mice (Peyssonnaux et al., 2007; Shah et al., 2009).

In vertebrates, this response is thought to be a part of the role of HIF in erythropoiesis. When oxygen levels are low, HIF levels in the kidney are stabilised and the HIF target gene erythropoietin is upregulated (Semenza and Wang, 1992) and secreted. Iron use from erythropoiesis is by far greater than from any other process and iron is the most common limiting factor in red blood cell production. Increased erythropoietin and the associated manifold upregulation of erythropoiesis would alone quickly lead to a state of iron deficiency. The concomitant upregulation of a number of proteins involved in increasing iron availability by HIF is therefore often seen as a way to cope with the increased requirement for iron from erythropoiesis. In this scenario, hypoxia in the kidney occurs either due to anaemia or to systemic hypoxia, which could for example be caused by movement to higher altitudes in which atmospheric oxygen levels are lower. To combat systemic hypoxia, erythropoiesis needs to be upregulated to improve tissue oxygenation. This is achieved when HIF is stabilised: more erythropoietin is secreted from the kidney and the bone marrow starts producing more red blood cells (Mole, 2010).

In order to supply the large amounts of iron that is now required, HIF also directly affects transcription of transferrin (Rolfs et al., 1997), which transports iron in blood, and the transferrin receptor (TfR) (Lok and Ponka, 1999; Mukhopadhyay et al., 2000; Tacchini et al., 1999), which binds transferrin and imports it into the cell using endosomes. The up-regulation of ceruloplasmin (Mukhopadhyay et al., 2000), a ferroxidase, can also be interpreted as aiding this process since transferrin can only bind Fe^{3+} and ceruloplasmin catalyses the formation of Fe^{3+} from Fe^{2+}. HIF also

activates heme oxygenase expression (Lee et al., 1997), which increases iron levels by releasing iron from heme. The net effect of HIF activation on iron metabolism is therefore an increase in iron availability through the induction of this battery of genes involved iron transport and the mobilisation of iron stores. The repression of ferritin expression by HIF would not be unexpected in this context, as lowering the cells' iron storage capacity would also lead to increased iron availability. In fact, mice with liver-specific deletions in VHL were found to have greatly reduced levels of ferritin in western blots of liver extracts, although the cause was never studied (Peyssonnaux et al., 2007).

The discovery that HIF-1 represses transcription of *ftn-1*, as measured using the *Pftn-1::gfp* transgene as a proxy, in the nematode worm *C. elegans* could have profound implications. Given the complete lack of a circulatory system or erythropoiesis in the worm, this finding strongly suggests that the evolution of a role of HIF in regulating iron availability predates the requirement for a tightly linked joint regulation of erythropoiesis and iron availability.

5.1.7. Repression of *ftn-1*: hepcidin parallel?

It should be noted that HIF has usually been found to be an activator of gene expression, not a repressor. In the context of HIF's likely role in the regulation of iron availability, a role of HIF in repressing genes that lead to decreased availability of iron would not be unexpected. Indeed, the negative regulation of another such gene, hepcidin, by HIF has already been described, although two recent publications on this subject paint a contradictory picture. Hepcidin is a peptide hormone that functions by inhibiting ferroportin (Nemeth et al., 2004). This prevents ingested iron from being secreted into the hepatic portal system by the intestinal cells that absorb iron from the diet and also prevents macrophages from releasing recycled iron (Nicolas et al., 2002; Peyssonnaux et al., 2007). Like ferritin, hepcidin therefore also serves to lower iron availability.

One study showed a strong down-regulation in hepcidin mRNA levels in mice with a liver specific deletion in VHL, which stabilises HIFα levels. The authors suggest that this effect is due to the stabilisation of HIF because $VHL^{-/-}$ $ARNT^{-/-}$ animals returned hepcidin mRNA to wild-type levels. ChIP experiments also showed

binding of HIF-1α to the hepcidin promoter in these mutants, but not in wild-type, suggesting a direct repression of hepcidin by HIF-1α. Wild-type animals treated with desferrioxamine, which also stabilises HIFα, also showed binding of HIF-1α to the promoter. Reporter genes using the human hepcidin promoter to drive luciferase expression showed a reduction in expression upon treatment with desferrioxamine, an iron chelator, and an increase in expression when the putative HIF binding site was mutated, suggesting that the role of HIF in repressing hepcidin expression is conserved from mouse to man (Peyssonnaux et al., 2007).

However, a separate study found that while iron chelators reliably reduced levels of hepcidin mRNA in cell lines, treatment with hypoxia, which is also effective at stabilising HIFα levels, did not do so. Additionally, siRNA treatment of both HIF-1α and HIF-2α did not lead to increased hepcidin mRNA levels, nor was either treatment effective at preventing repression of hepcidin by iron chelators despite being able to affect levels on known HIF targets (Volke et al., 2009).

Whether or not hepcidin is indeed directly repressed by HIF in order to increase the availability of iron has not yet been clearly established despite strong evidence from in vivo studies in mice. If further research demonstrates that this interaction is in fact taking place, then this would set a precedent for the negative regulation of a gene involved in reducing iron availability by the iron sensitive HIF. The negative regulation of *Pftn-1::gfp* by *hif-1* in *C. elegans* could be another example of such an interaction. While the repression of a gene involved in iron metabolism has not yet been unequivocally demonstrated, HIF-1α has been shown to negatively regulate other genes, such as alpha-fetoprotein, carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase (Chen et al., 2005; Mazure et al., 2002).

5.2 Results

The *hif-1* pathway has been studied primarily for its role in regulating gene expression in response to hypoxia and is of great interest to researchers due to its involvement in cancer in higher organisms. As mentioned, I identified both *hif-1* and its binding partner *aha-1* as putative repressors of *Pftn-1::gfp* expression in my screen for regulators of *ftn-1*. RNAi of either of theses elements led to large increases in the GFP expression from the already induced levels present in the *Pftn-1::gfp daf-2(m577); rrf-3(pk1426)* genotype used for this screen (Appendix 1).

This suggests that the HIF-1/AHA-1 dimer is repressing *ftn-1* expression and in this chapter, I set out to test this hypothesis. The first step was to confirm that the effects of *hif-1* and of *aha-1* RNAi on *Pftn-1::gfp* expression observed in the screen were robust and not an artefact of the *daf-2; rrf-3* mutant background used. The effect of these RNAi treatments on *Pftn-1::gfp*–expressing strains lacking these mutations was therefore tested. Next, it was important to confirm that the effects of knocking down these two genes were not independent of each other, since this would contradict the notion that they were acting as a dimer. To do this, the effect of *aha-1* RNAi was tested in the absence of *hif-1*. Given the important role of *daf-16* in the regulation of *ftn-1* (see Chapters 1, 3 and 4), it was also important to examine whether *hif-1* and *aha-1* RNAi were acting via *daf-16* to regulate gene expression and for this, the *Pftn-1::gfp daf-16(mgDf50)* strain was used.

A separate question was then addressed. As described in this chapter's introduction, the fact that *hif-1* seems to play an important role in repressing expression of a ferritin gene is particularly intriguing because of the role of ferritins in storing iron, the fact that HIF-1 is known to be responsive to iron levels and the fact that *ftn-1* transcript levels are also known to be responsive to iron levels. I therefore proceeded to test whether HIF-1 mediates the regulation of *ftn-1* by iron. To this end, I first tested whether or not *hif-1* mutants are still responsive to iron levels. In a study on *ftn-1* regulation, a 63bp iron-dependent element (IDE) was identified in the *ftn-1* promoter, which was found to be both necessary and sufficient for *ftn-1* to respond to iron levels (Romney et al., 2008). I therefore examined whether *hif-1* acts on *ftn-1* regulation via the IDE.

As described in the introduction, the responsiveness of HIF-1 levels to the oxygen and iron levels is known to be mediated by VHL-1 and EGL-9. Loss of these genes would therefore be expected to affect *ftn-1* expression, a hypothesis I tested using RNAi.

5.2.1. Confirming results from the screen

The involvement of *hif-1* and *aha-1* in *ftn-1* regulation was discovered within the context of a large-scale screen and in a genetic background containing mutations in *rrf-3* and *daf-2*. I was therefore concerned about whether the finding reflected a real role of these two genes in *ftn-1* regulation or whether it was an artefact of the experimental conditions, such as the genotype of the strain used. The fact that the screen was carried out using a single integrated *Pftn-1::gfp* transgenic reporter was another potential source of error. Since the transgene had been integrated by X-ray irradiation, it was inserted at a random location in the genome, which could, in theory, lead to artefactual results if the locus happens to be strongly regulated by these two genes. I therefore decided to test the effects of *hif-1* and *aha-1* on two separate integrants of the *Pftn-1::gfp* transgene, both of which were *wild-type* for *rrf-3* and *daf-2*.

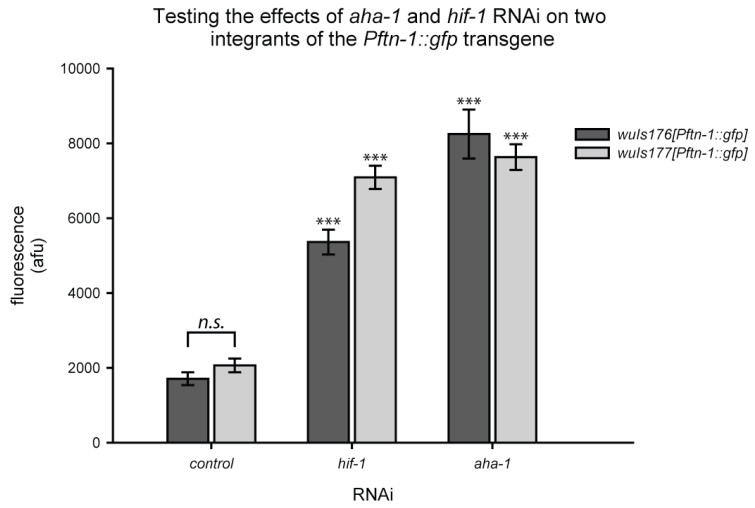


Figure 49
Worms were age-synchronised, grown at 20°C until the L4 stage, transferred to RNAi plates, and kept at 25°C for 2 days before measurements were taken. Error bars represent ±s.e.m. Unless otherwise indicated, statistical significance was calculated for the comparison to the control treatment of the same genotype. Significance was calculated by ANOVA of log-transformed values. Bonferroni correction for multiple testing was applied to p values. ***: $p<0.001$, n.s.: $p>0.05$

As shown in Figure 49, the effects of *hif-1* and of *aha-1* on *Pftn-1::gfp* expression were not specific to the *rrf-3; daf-2* background used for the screen, nor were they an artefact of the integration.

While RNAi by feeding is considered a very reliable method of gene knockdown in *C. elegans*, I was able to confirm that the RNAi treatment used throughout this chapter is effective at knocking down *hif-1*. As part of a separate collaboration with Dr. Oliver Staples from the Rayne Institute at UCL, I treated worms expressing a HIF-1::Myc fusion protein (Zhang et al., 2009) with RNAi for *hif-1* and then exposed them to hypoxia for 3h. HIF-1::Myc levels were greatly induced by hypoxia. RNAi of *hif-1* led to reductions in HIF-1::myc levels under both hypoxic and normoxic conditions (see Figure 50).

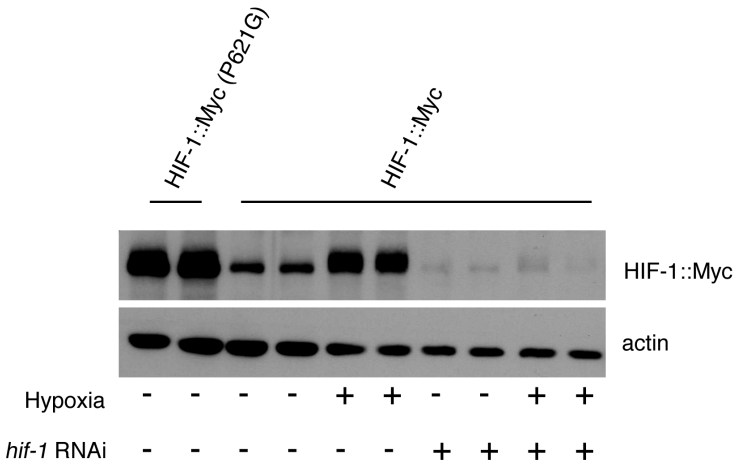


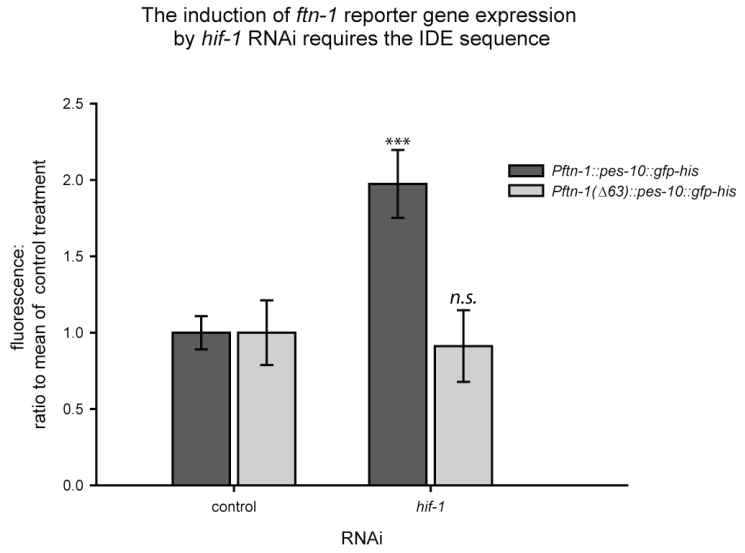
Figure 50
Western blot showing high efficacy of *hif-1* RNAi. Anti-Myc antibody was used to quantify expression from a *hif-1::myc* transgene, or one carrying the stabilising P621G mutation. Hypoxia treatment consisted of 3h of 1% O_2. The blot presented here is representative of the results of three biological replicates. This work was done in collaboration with Oliver Staples from the UCL Rayne institute. Worms were grown on RNAi plates at 20^{o}C for two generations. RNAi treatment was carried out by me, samples were prepared jointly by Oliver Staples and myself and the gels were run and blotted by Oliver.

While RNAi is clearly effective at knocking down *hif-1* gene expression, at least in the context of a *hif-1::myc* transgene, this does not exclude the possibility that *hif-1* RNAi is also having off-target effects. If genes other than *hif-1* are being targeted, it is possible that a different target is responsible for the effects on *Pftn-1::gfp* expression. This can be tested using genetic, hypomorphic mutants of *hif-1*. I therefore crossed the *Pftn-1::gfp* transgene into a *hif-1(ia4)* genetic background to see if the effect *hif-1* by RNAi is recapitulated in *hif-1* mutants. The *ia4* deletion spans over 1.2kb, deletes three exons, introduces a premature stop codon and eliminates most of the bHLH and PAS domains. These mutants are therefore thought to have a complete loss of *hif-1* function (Jiang et al., 2001b) and have been extensively used in publications studying *hif-1*. As shown in Figure 51 and throughout this chapter, *Pftn-1::gfp* fluorescence is greatly elevated in *hif-1* mutants compared to *hif-1(+)* animals, demonstrating that the effect of *hif-1* RNAi is not due to off-target effects.

5.2.2. Testing *hif-1* dependence of *aha-1* RNAi effect

Because RNAi of both *hif-1* and *aha-1* had similar effects on *Pftn-1::gfp* expression and previous studies had already shown that HIF-1 and AHA-1 act in concert (Jiang et al., 2001b), it seems likely that the HIF-1/AHA-1 dimer is working directly or indirectly to repress expression of *Pftn-1::gfp*. I tested this by treating *Pftn-1::gfp hif-1(ia4)* animals with *aha-1* RNAi. If HIF-1 and AHA-1 are acting together, then one would expect that *aha-1* RNAi should not increase expression of the transgene in the absence of *hif-1*.

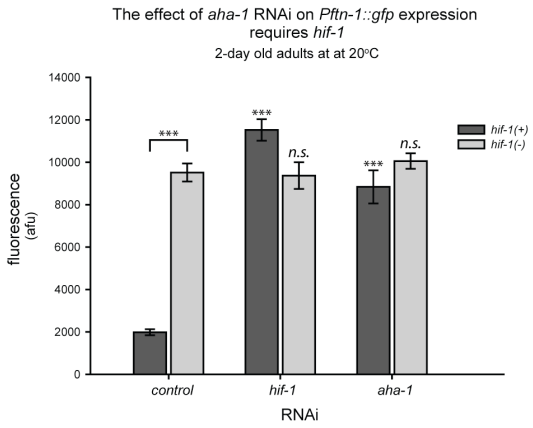
I first carried out a series of experiments in which I treated animals with RNAi for two generations, growing the second generation at 25°C and measuring the worms' fluorescence at the L4 stage of development (see 2.8.3. Protocol 3). This was done to keep experimental conditions consistent with experiments in which measurements needed to be taken at 25°C at the L4 stage (see



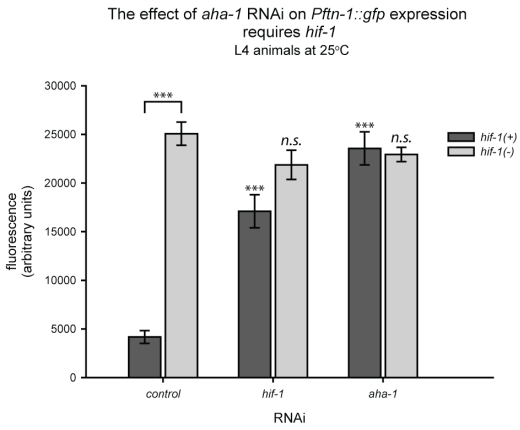
and Figure 55). However, I was concerned about the possibility of artefactual results due to developmental effects caused by RNAi and also by the fact that in my hands the *hif-1*(*ia4*) mutant animals grow much more slowly at 25°C than *hif-1*(+) animals. This phenotype not only makes the synchronisation of experiments difficult, it also raised the possibility that since some effects of *hif-1* are clearly dependent on temperature, its effects on the regulation of *ftn-1* might also be affected by temperature. I therefore additionally carried out many experiments at 20°C, transferring them to RNAi plates at the L4 stage to prevent any developmental

effects of the RNAi, and then measuring them on the second day of adulthood. In order to control for effects of temperature, I also measured the fluorescence of worms I kept at 20 °C until the L4 stage before transferring to RNAi plates and then shifting them to 25°C for two days before taking measurements (see 2.8.1. Protocol 1). 40 adult worms were used per measurement; when measuring L4 animals, I used 60 per measurement because of their smaller size. These three conditions were used in numerous instances throughout this chapter. With only a few exceptions, the results of these experiments were the same regardless of the conditions used.

A.



B.



C.

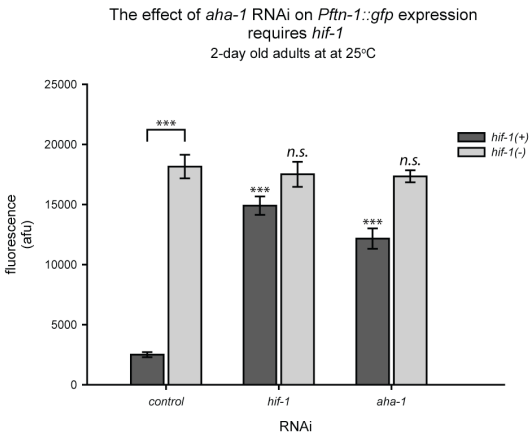


Figure 51
hif-1 and *aha-1* work together to regulate *Pftn-1::gfp*
A. Fluorescence of adult *hif-1(+)* and *hif-1(ia4)* worms carrying the *Pftn-1::GFP* transgene was measured after RNAi treatment at 20°C. **B.** L4 animals were measured after two generations of RNAi and one generation at 25°C. **C.** Adult animals were measured after two days of RNAi at 25°C. Error bars represent ±s.e.m. Statistical significance was calculated as described in Figure 49.

As shown in Figure 51, under three different experimental setups, *aha-1* RNAi greatly increases expression of *Pftn-1::gfp* in *hif-1(+)* animals and the magnitude of this effect is comparable to that seen upon *hif-1* loss by RNAi or genetic deletion. When *hif-1* is absent, *aha-1* RNAi is incapable of further increasing expression, which supports the view that *hif-1* and *aha-1* are working together to repress *Pftn-1::gfp*.

5.2.3. The effects of *hif-1* and *aha-1* RNAi on *Pftn-1::gfp* expression are independent of *daf-16*

hif-1 and *aha-1* RNAi increase *Pftn-1::gfp* expression in both *daf-2(m577)* (Appendix 1) and *daf-2(+)* (see Figure 49) genetic backgrounds. Next I tested whether this induction was *daf-16* dependent. It is conceivable that RNAi of these factors may induce *ftn-1* expression by activating *daf-16*. I therefore tested the effects of *hif-1* and *aha-1* RNAi on *Pftn-1::gfp* expression in the absence of *daf-16.*

I first exposed both *daf-16*(+) and *daf-16(mgDf50)* animals carrying the *Pftn-1::gfp* transgene to two generations of *hif-1* or *aha-1* RNAi and measured fluorescence in L4 animals of the second generation. The second generation was grown at 25^{o}C. As a positive control, worms were also grown on *daf-2* RNAi, which increases expression from the transgene in a *daf-16*(+), but not a *daf-16(mgDf50)* mutant background.

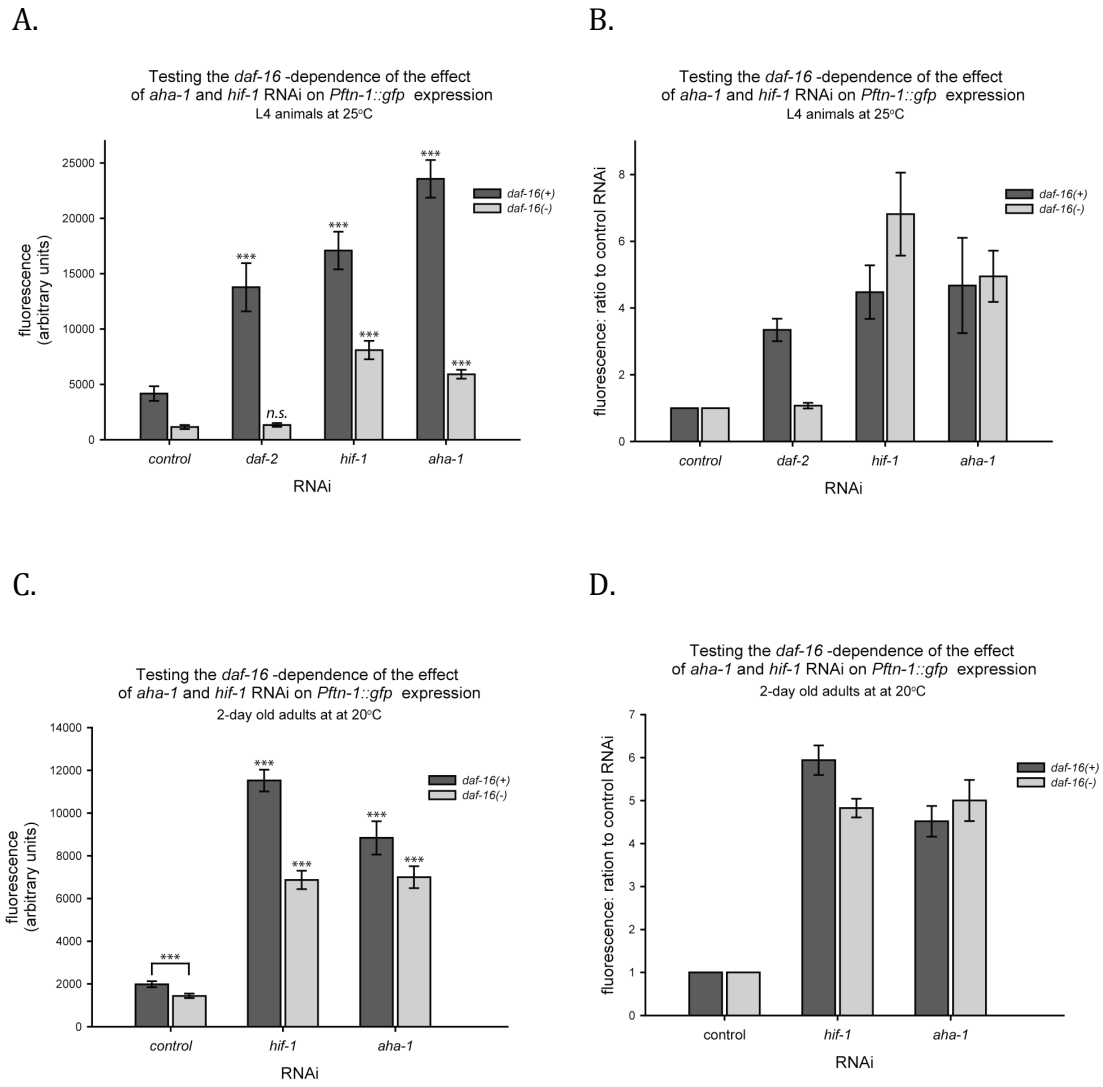


Figure 52
hif-1 and *aha-1* RNAi increase *Pftn-1::gfp* expression in the absence of *daf-16*
Fluorescence of *daf-16*(+) and *daf-16(mgDf50)* worms carrying the *Pftn-1::GFP* transgene was measured **A.** at the L4 stage after two generations of RNAi treatment and one generation at 25°C (see **B.** for the same data displayed as a ratio of the control) and **C.** at 20°C after 2 days of RNAi treatment (see **D.** for the same data displayed as a ratio of the control). Error bars represent ±s.e.m. Statistical significance was calculated as described in Figure 49.

Consistent with earlier results (see chapters 3 and 4), *daf-2* RNAi did not increase *Pftn-1::gfp* expression in *daf-16* mutants at 25°C. In contrast, both *aha-1* and *hif-1* RNAi did increase expression. The fold-change in expression induced by *hif-1* and *aha-1* was similar in both genotypes (see Figure 52 B and D). Use of both protocols resulted in the same finding: the effects of both *aha-1* and *hif-1* RNAi

occur independently of *daf-16*, thus demonstrating that RNAi of either of these genes does not lead to increased expression of *Pftn-1::gfp* via activation of DAF-16. Instead, it seems likely that hypoxia signalling acts in parallel to IIS to regulate the levels of *ftn-1* expression in *C. elegans* (see Chapter Discussion).

5.2.4. Regulation of *Pftn-1::gfp* by iron is *hif-1* dependent

The hydroxylation reaction that leads to the degradation of HIF-1 requires iron (Epstein et al., 2001). This suggests the possibility that the induction of *ftn-1* transcription by iron occurs through HIF-1. I decided to test the ability of ferric ammonium citrate (FAC) to induce *Pftn-1::gfp* expression in *hif-1*(+) and *hif-1*(*ia4*) animals. If the transcriptional response to iron requires HIF-1, then *Pftn-1::gfp* expression will not be induced in *hif-1* mutant animals. I chose to use a concentration of 25mM of FAC for this experiment, since the work of a colleague (Sara Valentini, Gems Lab, UCL), showed that this concentration is effective at inducing expression of this transgene. I also exposed animals to 0.1mM of the iron chelator 2',2-bipyridyl (BP), which should lead to a decrease in expression from the iron-responsive *Pftn-1::gfp* transgene.

Worms in their first day of adulthood and grown at 20^{o}C were exposed to either 25mM of FAC or 0.1mM of BP for 18h before measurement. In a separate experiment, worms were also grown on FAC or regular NGM plates from egg at 25^{o}C and measured at the L4 stage. BP was not used for these experiments because worms do not develop well on plates containing this chelator.

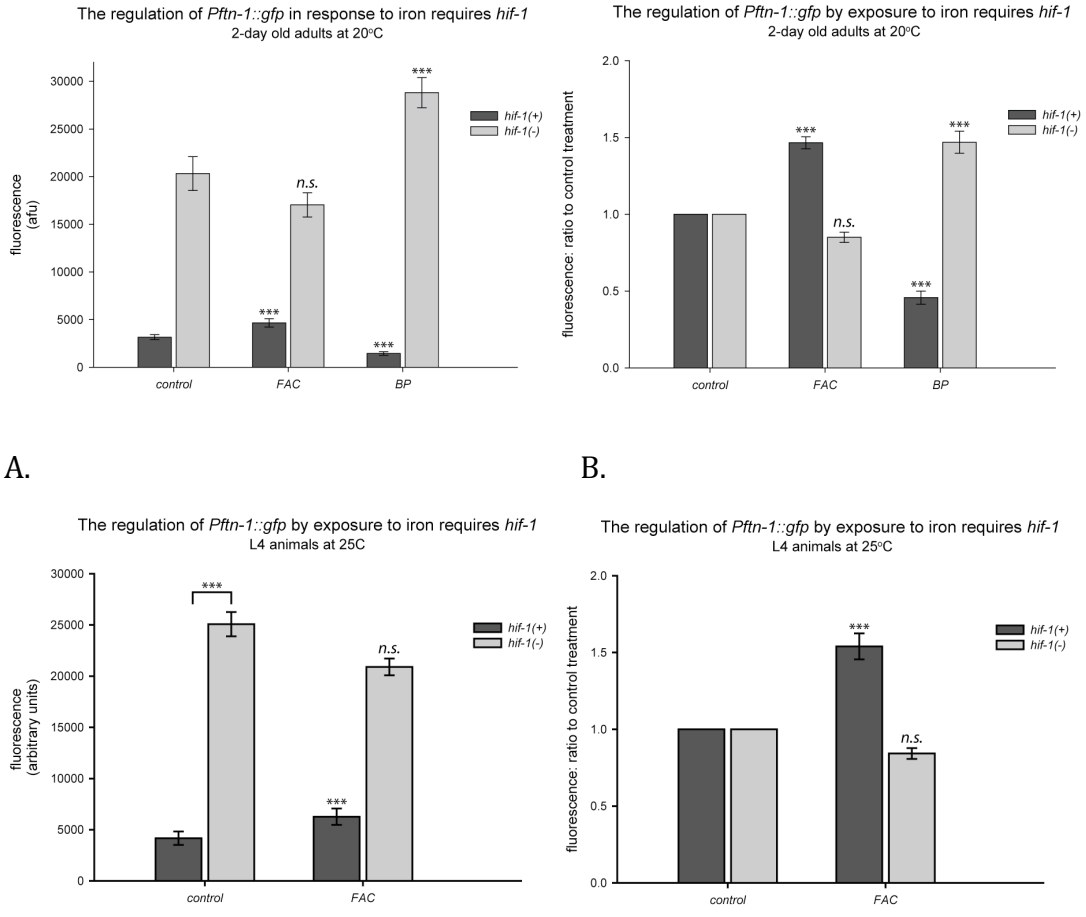


Figure 53
Induction of *Pftn-1::gfp* expression by iron requires *hif-1.*
A. Fluorescence of *hif-1(+)* and *hif-1(ia4)* adult worms carrying the *Pftn-1::gfp* transgene was measured after 18h of 25mM FAC or 0.1mM BP treatment at 20°C. **B.** The same data as shown in A. was represented as a ratio to its control treatment to illustrate the different effect of treatments in the two genotypes. **C.** *hif-1(+)* and *hif-1(ia4)* worms were exposed to FAC treatment from egg to L4 at 25°C. **D.** the same data as in C. presented as a ratio of its control. Error bars represent ±s.e.m. Statistical tests performed as described in Figure 49.

Both addition and chelation of iron had the expected effects on *Pftn-1::gfp* expression in *hif-1(+)* animals. Adding additional iron increased expression and chelating iron reduced expression. The magnitude of these effects was not as great as that of either reduced IIS or of reduced *hif-1* or *aha-1* levels, but nonetheless a 50% increase and a 50% decrease were seen with the respective treatments. This did not occur in *hif-1(ia4)* mutant animals. FAC treatment did not affect expression in this background and BP treatment did not reduce, but in fact increased, expression of *Pftn-1::gfp.* In the absence of *hif-1, ftn-1* expression is no longer be responsive to the

addition of iron, at least when measured using the *Pftn-1::gfp* transgene, indicating that *C. elegans* may require *hif-1* to respond to iron levels. The reason for the increase in *Pftn-1::gfp* expression in *hif-1(ia4)* animals upon BP treatment is unknown. However, I noticed that worms treated with BP have a sickly and thin appearance. It is possible that in the absence of the repressive effect of HIF-1, BP triggers a stress response that activates *ftn-1* expression.

What this data makes clear is that the positive link between iron levels and *Pftn-1::gfp* expression does not occur in the absence of *hif-1*, indicating that HIF-1 may have an important role in iron-dependent gene regulation. Given that a known mechanism for the regulation of HIF-1 levels by proteasomal degradation requires the presence of iron, it seems likely that changes in the environmental iron levels are leading to different levels of HIF-1, which in turn affects transcription of *Pftn-1::gfp*.

5.2.5. Testing the involvement of HIF-1 and AHA-1 in the regulation of *ftn-1* via the IDE

I next tested whether regulation of *ftn-1* by *hif-1* and *aha-1* is mediated by the IDE element of the *ftn-1* promoter. In the publication that first reported on the IDE (Romney et al., 2008), a number of transgenic reporter lines were created to characterise the role of this element. These were generously provided to me by Elizabeth Leibold (University of Utah).

The first set of experiments were carried out using a line carrying a *ftn-1* reporter very similar to the *Pftn-1::gfp* reporter used for my screen. It was constructed using 1.9kb of upstream sequence (my own reporter contains 4kb) and was fused to the minimal *pes-10* promoter and to GFP-histone. The *pes-10* promoter has no basal transcriptional activity of its own but is activated by enhancers. The use GFP-histone rather than GFP alone localises fluorescence to the nuclei, making fluorescence from transgene expression easy to distinguish from endogenous autofluorescence, which can be a problem when GFP expression is low.

A second line was created by Romney et al. in which the 63bp IDE was deleted from the *Pftn-1::pes-10::gfp-his* construct to make the *Pftn-1(Δ63)::pes-10::gfp-his* transgene. This line also exhibits GFP expression in intestinal nuclei, although the expression levels are much lower. The authors found that *Pftn-1::pes-*

10::gfp-his is responsive to iron but *Pftn-1(Δ63)::pes-10::gfp-his* is not, demonstrating that the mechanism that regulates the induction of *ftn-1* by iron acts through these 63bp.

I have hypothesised that the iron-dependent regulation of *ftn-1* is occurring via HIF-1. In this scenario, the HIF-1/AHA-1 dimer is binding and repressing *ftn-1* expression when iron is absent. In the presence of iron, more HIF-1 is hydroxylated and therefore degraded, leading to less repression and increased *ftn-1* expression. The increased levels of FTN-1 would then be able to store the excess iron. If true, then the effect of *hif-1* RNAi on *ftn-1* expression would also need to occur via the IDE. I therefore tested whether the effects of *hif-1* RNAi could be abrogated by the deletion of this element.

In the original publication, Romney et al. measured the fluorescence of these strains using the COPAS^{TM} (complex object parametric analysis and sorting) Biosort (Union Biometrica, Somerville, MA), a type of FACS sorter for worms. This instrument was not available to me, so I instead measured fluorescence by taking epifluorescent microscopic pictures of worms at the L4 stage and quantifying fluorescence using the Volocity 5.0 (Improvision) software. I chose L4 animals because little autofluorescence was seen at this stage, making GFP expression easier to quantify in strains with low levels of transgene expression, such as the *Δ63* line.

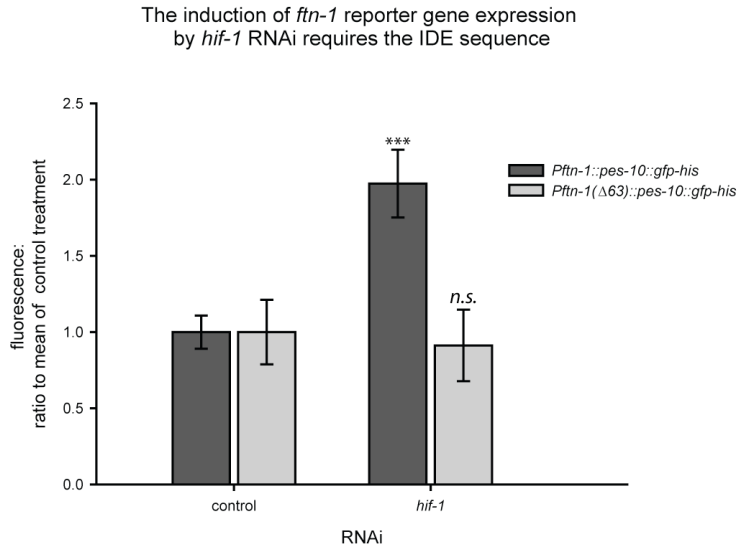


Figure 54

Two trials were combined and the mean fluorescence on control RNAi was used to control for inter-trial variation. Fluorescence of *Pftn-1(Δ63)::pes-10::gfp-his* line was much lower than the *Pftn-1::pes-10::gfp-his* line, so the data is displayed as a ratio of control. Due to the left-censored nature of this data, the non-parametric Wilcoxon test was used to compare the *hif-1* RNAi treated animals to controls of the same genotype. Bonferroni correction for multiple testing was applied to p values. ***: $p<0.001$, **: $p<0.01$, *: $p<0.05$, *n.s.*: $p>0.05$

The two trials summarised in Figure 54 very clearly show that the effect of *hif-1* RNAi occurs in this reporter strain as it does in my own *Pftn-1::gfp* strain. This alone is encouraging, since it shows that the effect was not an artefact of my construct. In support of my hypothesis on the role of HIF-1 in the regulation of *ftn-1* by iron, *hif-1* RNAi had no statistically significant effect on the expression of *Pftn-1(Δ63)::pes-10::gfp-his*. This indicates that the IDE is required for the regulation of *ftn-1* by *hif-1*.

5.2.6. The IDE is sufficient for the effects of *hif-1* RNAi on *ftn-1* expression

The next step was to identify whether the regulatory elements within the 63bp of the IDE were sufficient for the effects of *hif-1* RNAi on *ftn-1* expression. This would be expected if the regulation of *ftn-1* by iron does occur via HIF-1, since the IDE has previously been found to be sufficient for the effects of iron on *ftn-1*

expression (Romney et al., 2008). Romney et al had tested this by creating a transgene in which the IDE alone was fused to *pes-10::gfp-his*. GFP fluorescence expressed from this *IDE::pes-10::gfp-his* transgene can also be seen in intestinal nuclei, but expression is very weak. In order to investigate the role of *hif-1* in the regulation of this trangene by iron, I crossed the transgene into a *hif-1(ia4)* mutant background. I then tested the effect of loss of *hif-1* on expression. If HIF-1 really represses *ftn-1* expression via the IDE, then loss of *hif-1* should lead to increased expression of the *IDE::pes-10::gfp-his* transgene. The effect of FAC on fluorescence was also tested in these two strains, since one would predict that the reporter would not be capable of responding to FAC in the absence of *hif-1*.

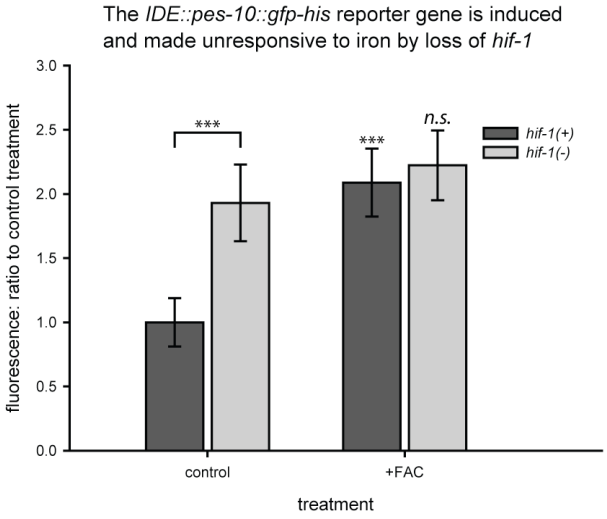


Figure 55

No statistically significant increase in fluorescence was seen when worms carrying the *IDE::GFP hif-1(ia4)* mutation were treated with FAC. In order to pool multiple trials, I normalised the fluorescence of each worm using the mean fluorescence of all *hif-1(+)* animals on NGM plates from the same trial. Statistical analysis was performed as described in Figure 54.

Loss of *hif-1* led to a large increase in expression, indicating that the role of *hif-1* in *ftn-1* regulation does in fact occur through the IDE. Additionally, the clear induction in expression after to iron seen in the *hif-1(+)* line did not occur in the *hif-1(ia4)* mutants, strongly suggesting that the iron-dependent regulation of *ftn-1* through the IDE occurs via the actions of *hif-1*.

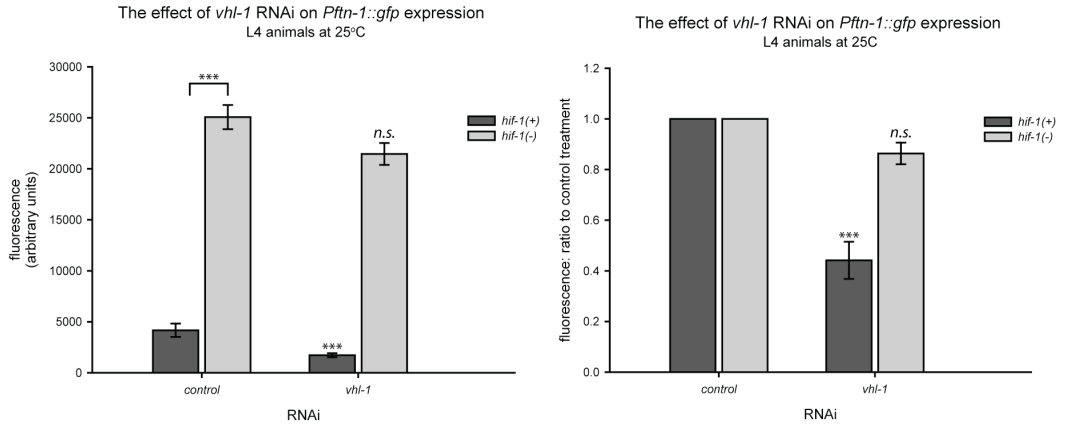
It should be noted that before the Bonferroni correction was applied, the difference in fluorescence in *hif-1(-)* animals on FAC to those on NGM was statistically significant (p=0.032). While correcting for multiple testing is necessary, it is possible that a statistically significant increase would have been detected had a larger sample size been used. In any case, the fluorescence in *hif-1(+)* animals by

FAC treatment was 2.09-fold higher than in controls, whereas the potential increase in *hif-1(-)* animals was only 1.15-fold (109% increase compared to a 15% increase). Any *hif-1*–independent component to the *ftn-1* induction by iron is likely to be of minor importance compared to the role of *hif-1*.

5.2.7. *vhl-1* RNAi reduces *Pftn-1::gfp* expression

The regulation of HIF-1 by VHL-1 and EGL-9 was discussed in this chapter's introduction. In brief, EGL-9 is a prolyl hydroxylase that hydroxylates the P621 residue of HIF-1 in the presence of O_2 and iron (and 2-oxoglutarate). When HIF-1 is hydroxylated, VHL-1 binds and this targets HIF-1 for degradation by the proteosome (see Figure 47). Since it is this EGL-9 and VHL-1–mediated degradation of HIF-1 that makes it responsive to iron levels, I decided to investigate its role in the *hif-1*–dependent regulation of *ftn-1*.

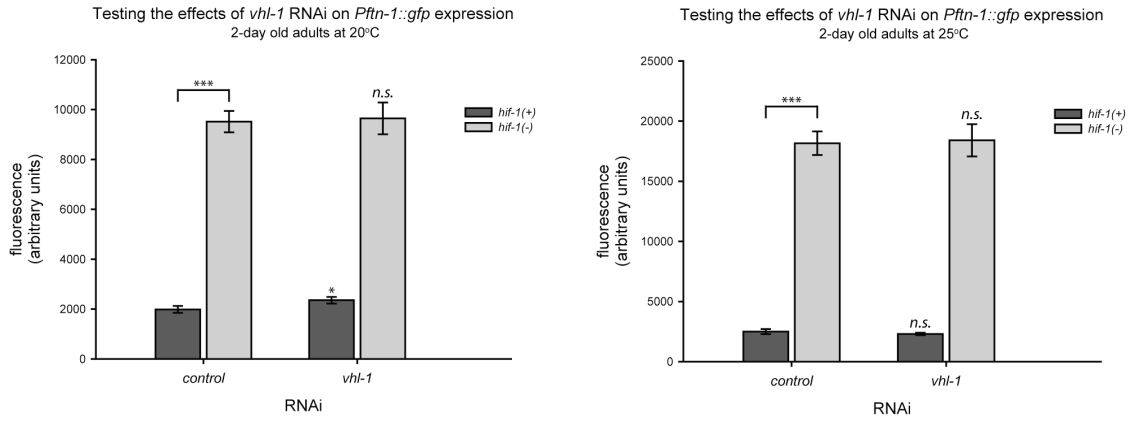
Loss of *vhl-1* leads to increased HIF-1 levels. If it is correct that HIF-1 represses transcription of *Pftn-1::gfp,* then RNAi of *vhl-1* should lead to a decrease in expression from *Pftn-1::gfp*, since HIF-1 levels should be increased. I therefore tested the effects of *vhl-1* RNAi on *Pftn-1::gfp* expression.



A. B.

Figure 56
A. Fluorescence of *hif-1(+)* and *hif-1(ia4)* animals carrying the *Pftn-1::gfp* transgene was measured at the L4 stage after two generations on *vhl-1* or control RNAi and one generation at 25°C. **B.** The same data as A. but presented as a ratio to the control treatment. Error bars represent s.e.m. Statistical tests performed as described in Figure 49.

Worms were exposed to two of *vhl-1* RNAi at 25°C. This reduced GFP expression, presumably because *vhl-1* knockdown leads to increased HIF-1 levels, which represses transcription from the *Pftn-1::gfp* transgene. Consistent with this, no repression was seen in a *hif-1* mutant background. However, when worms were subjected to RNAi for a shorter period, two days of adulthood, this decrease was not seen. In fact, at 20°C, a small (+20%), but statistically significant increase in expression was observed instead. At 25°C, no increase or decrease was seen.



A B

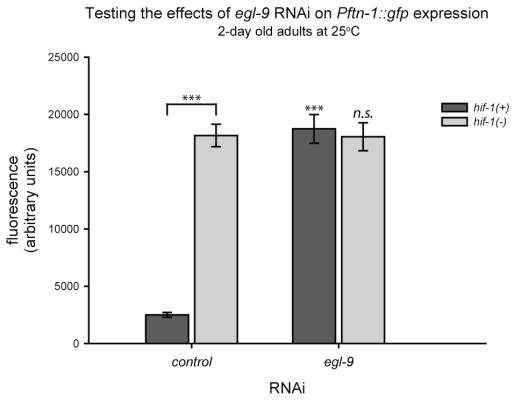
Figure 57
Effect of 2 days of *vhl-1* RNAi at 20°C and 25°C on *Pftn-1::gfp* fluorescence
A. Fluorescence of *hif-1(+)* and *hif-1(ia4)* animals carrying the *Pftn-1::gfp* transgene was measured after 2 days of RNAi at 20°C **B.** Fluorescence of *hif-1(+)* and *hif-1(ia4)* animals carrying the *Pftn-1::gfp* transgene was measured after 2 days of RNAi at 25°C. Error bars represent ±s.e.m. Statistical tests performed as described in Figure 49.

The simplest explanation for the slightly discrepant results is that the RNAi treatment for *vhl-1* is slightly less effective than other treatments used here and requires a longer exposure. VHL-1 protein may perdure for a long time, so RNAi for two days may still leave active VHL-1 in play.

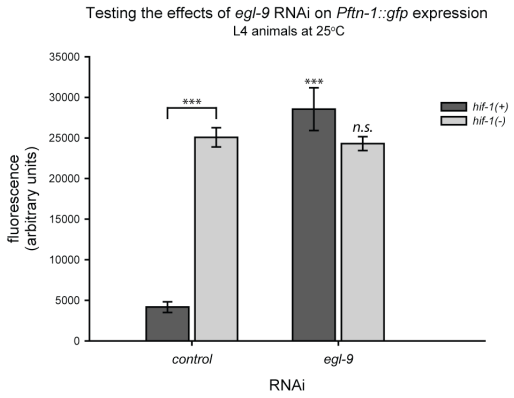
5.2.8. Examining the effect of *egl-9* RNAi on *Pftn-1::gfp* expression

The prolyl hydroxylase EGL-9 functions upstream of VHL-1 in targeting HIF-1 for proteosomal degradation (see Figure 47). RNAi of *egl-9*, like *vhl-1*, should therefore lead to increased levels of HIF-1 and therefore reduced levels of GFP expression from *Pftn-1::gfp*.

A.



B.



C.

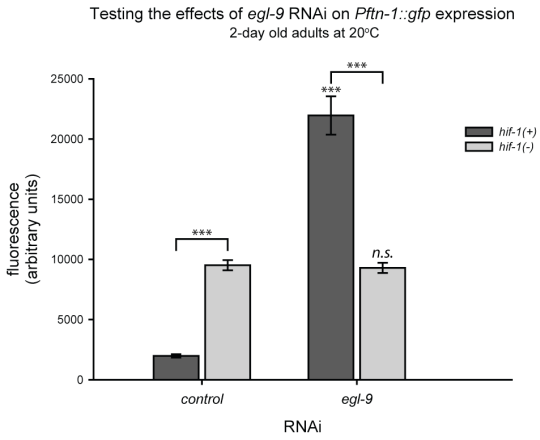


Figure 58
2 days of *egl-9* RNAi at 20°C and 25°C **A.** Fluorescence of L4 *hif-1(+)* and *hif-1(ia4)* animals carrying the *Pftn-1::gfp* transgene grown at 25°C **B.** Fluorescence of *hif-1(+)* and *hif-1(ia4)* animals carrying the *Pftn-1::gfp* transgene was measured after 2 days of RNAi at 25°C. **C.** Fluorescence of *hif-1(+)* and *hif-1(ia4)* animals carrying the *Pftn-1::gfp* transgene was measured after 2 days of RNAi at 20°C. Error bars represent ± s.e.m. Statistical tests performed as described in Figure 49.

Instead of the expected decrease in expression from *Pftn-1::gfp* upon RNAi of *egl-9*, I saw a large increase. The extent of the increase differs depending on the conditions used. In both experiments carried out at 25°C, *egl-9* RNAi leads to an increase similar to that seen in *hif-1* mutant animals or upon treatment with *hif-1*

RNAi. At 20°C however, the increase is much greater, such that the level of GFP expression is double that found in *hif-1* mutant or *hif-1* RNAi–treated animals.
Under both conditions, the increase in expression is completely dependent on *hif-1*, since *egl-9* RNAi never leads to an increase in *ftn-1* reporter gene expression in *hif-1* mutant animals. Given that all evidence so far indicates that HIF-1 acts as a repressor of *ftn-1*, the consequences of *egl-9* RNAi on *Pftn-1::gfp* expression indicate that *egl-9* acts here as an activator rather than a repressor of HIF-1. This is unexpected, since it is not consistent with the known action of PHDs on HIF.

5.2.9. Is the effect of *egl-9* RNAi due to activation of DAF-16?

One possible explanation could again lie with *daf-16*: *egl-9* RNAi might activate DAF-16, thereby inducing expression of *Pftn-1::gfp*. I therefore tested the effects of these two treatments in *daf-16* mutants. However, both effects were independent of *daf-16*.

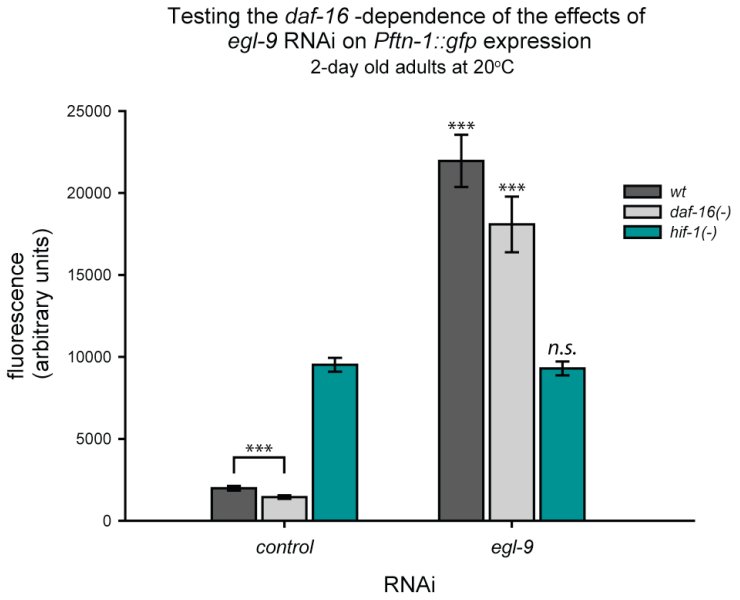


Figure 59
Fluorescence of *wt, hif-1(ia4)* and *daf-16(mgDf50)* animals carrying the *Pftn-1::gfp* transgene was measured after 2 days of *egl-9* or control RNAi at 20°C. Error bars represent ±s.e.m. Statistical tests performed as described in Figure 49.

The effect of *egl-9* RNAi is therefore not due to an activation of *daf-16*, since it can still occur in the absence of *daf-16*. Similar results were seen at 25^{o}C (data not shown).

5.3. Chapter discussion

An RNAi screen for regulators of *ftn-1* expression led me to the discovery that expression of *ftn-1* is directly or indirectly repressed by *hif-1* and *aha-1,* as measured via reporter gene expression. This is potentially an important finding, since the mechanism by which *ftn-1* is regulated in response to iron in *C. elegans* is unknown and HIF-1 itself is also regulated by iron levels. This role of HIF-1 in repressing *ftn-1* expression matches its role in increasing iron availability found in vertebrate systems, because lower ferritin levels should lead to more free iron in the cell. While most studies focus on the regulation of ferritin levels at the translational level in response to iron (see Chapter 1), transcriptional up-regulation of ferritin after iron treatment has also been described (White and Munro, 1988). Understanding how ferritin is transcriptionally regulated in response to iron in *C. elegans* may therefore be useful in the study of iron regulation in higher organisms.

RNAi of both *hif-1* and *aha-1* led to large increases in expression from my reporter of *ftn-1* transcription. *hif-1* and *aha-1* seem to act together, since there is no additive effect of *aha-1* RNAi and *hif-1* deletion. As discussed previously (see chapter 1 and 2) the expression of the *Pftn-1::gfp* reporter re-iterates the expression pattern of *ftn-1* found by other groups, accurately predicts expression of endogenous *ftn-1* mRNA levels in response to mutations in IIS and, like *ftn-1* mRNA, is also inducible by exposure to iron, all of which strongly supports its reliability as a proxy for detecting changes in *ftn-1* expression. Additionally, RNAi of *hif-1* also leads to increased expression of the *Pftn-1::pes-10::GFP-his* reporter provided to me by Elizabeth Leibold (see Figure 54). I confirmed that RNAi was effectively targeting *hif-1* by western blotting of a strain carrying a HIF-1::Myc construct. I also showed that the effects of *hif-1* RNAi are not due to off-target effects, as a deletion in *hif-1*

shows a very similar effect on *Pftn-1*::expression and *hif-1* RNAi has no additional effect on expression when *hif-1* is deleted.

There are hints that the role of HIF in the regulation of ferritin may extend to higher organisms. In a renal carcinoma cell line that carries a mutation in the VHL gene and thus increased levels of HIFα, both L- and H-ferritin transcript levels were decreased compared to cells transfected with a wild-type VHL clone (Alberghini et al., 2005). Similarly, mice with liver-specific deletions of VHL were also found to express lower levels of ferritin (Peyssonnaux et al., 2007). Neither study investigated the HIFα dependence of the down-regulation of ferritin. Contradictory results have also been found. A study using a human monocyte/macrophage cell line to study the effects of cigarette smoke found an up-regulation of H-ferritin mRNA in response to the combined exposure to a cigarette smoke condensate and hypoxia. This induction was abrogated when cells were treated with siRNA for HIF-1α, which suggests a role of HIF in inducing, rather that repressing, ferritin expression in response to this set of stimuli (Goven et al., 2010). Similarly, northern blot analysis following a microarray study on renal and non-renal cell lines seems to suggest that L-ferritin may be induced by hypoxia and by mutation in VHL although again, the mechanisms by which this occurs remain unclear (Wykoff et al., 2004).

5.3.1. Interplay between IIS and hypoxia signalling

During the course of my PhD, I was able to show that both IIS and hypoxia signalling, two important stress response pathways, regulate *ftn-1* expression. While interactions between these two pathways have not been extensively studied in *C. elegans,* there have been suggestions that they may cooperatively regulate common targets. *daf-2* mutants, for example, are resistant to hypoxia (Mabon et al., 2009) and this resistance is *daf-16* dependent (Scott et al., 2002), demonstrating that IIS-regulated genes have a role in protection against hypoxia. Whether *hif-1* is involved in this resistance is not known.

Another interesting link between *hif-1* and IIS comes from the microarray study on *daf-2* and *daf-16; daf-2* by McElwee et al. (see Chapter 1). The authors analysed sequences 1kb upstream of differentially regulated genes in search for

transcription factor binding elements enriched in these sequences. They found that the *hif-1* response element was overrepresented in the set of IIS-regulated genes, suggesting that *hif-1* and *daf-16* may act together to regulate gene expression. The authors hypothesise that genes regulated jointly by *hif-1* and *daf-16* may be involved in protection against hypoxia, thus explaining the resistance against hypoxia seen in *daf-2* mutants (McElwee et al., 2004). However, given recent findings on the role of *hif-1* in the determination of lifespan, it is also possible that the IIS- and *hif-1*–mediated effects on ageing are mediated by a set of common targets. The question of whether the effects of the two pathways on lifespan are independent remains unresolved. As described in the introduction, the data seems to suggest independent effects of the two pathways, since the effects of hypoxia signalling on lifespan are independent of *daf-16* (Mehta et al., 2009; Zhang et al., 2009).

A study investigating axon guidance in *C. elegans* found that HIF-1 activation leads to axon-pathfinding defects (Pocock and Hobert, 2008). Mutations in *daf-2* were able to rescue these defects and this suppression of the effects of HIF-1 activation was dependent on *daf-16.* Because the suppressive effect of *daf-2* mutations was absent when HIF-1 activation was achieved via stabilising mutations, the authors predict that DAF-2 acts upstream of HIF-1 to affect axon guidance. Because *daf-16* is known to regulate anti-oxidant enzymes, Pocock and Hobart tested if the changes in ROS levels could account for the interaction between IIS and HIF-1, since in human cell lines, the elevated ROS produced by mitochondria during hypoxia leads to increased HIF levels (Brunelle et al., 2005). They found that loss of the antioxidant enzyme *sod-1* did lead to the same axon guidance defects seen upon HIF-1 activation. They conclude that IIS may be interacting with hypoxia signalling by altering the levels of cellular ROS and thereby affecting HIF-1 (see Figure 60). Similar examples of possible regulation of HIF through alterations in ROS levels can be found in vertebrate systems as well (Gerald et al., 2004).

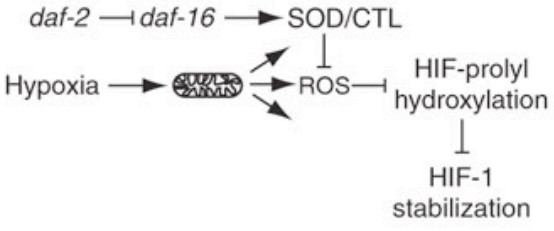


Figure 60
Adapted from Pocock and Hobert (2008). Possible mechanism by which IIS and hypoxia signalling interact to affect axon guidance.

A study into the role of HIF in type-2 diabetes found that mice with a deletion of HIF-1a in pancreatic β-cells are glucose intolerant and exhibit β-cell dysfunction. Treating mice on a high-fat diet with an iron chelator led to an amelioration of glucose intolerance and improved insulin secretion in wild-type, but not HIF-1a null mice, indicating that HIF can protect against glucose intolerance (Cheng et al., 2010). Studying the regulation of *ftn-1* by insulin/IGF-1 signalling and hypoxia signalling might be a useful approach to further our understanding of the dynamics of interactions between the two pathways. Expression of the *Pftn-1::gfp* transgene can be used as a readout for signalling from both pathways, allowing for interactions to be tested by epistasis analysis.

One possible explanation for the effects of *hif-1* RNAi on *Pftn-1::gfp* expression is that loss of this gene (or of *aha-1*) leads to an activation of DAF-16, possibly by causing stress. If so, then this alone could explain the elevated expression of *Pftn-1::gfp,* since *daf-16* is clearly a powerful regulator of *ftn-1* (see Figure 61).

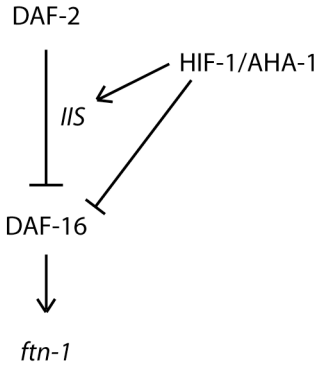


Figure 61
Model A: The regulation of *ftn-1* by HIF-1 requires DAF-16.

I therefore investigated whether the effects of *hif-1* required the presence of *daf-16* and found that they did not. This excludes Model A.

The fact that *hif-1* and *aha-1* RNAi are capable of increasing *Pftn-1::gfp* expression in a *wild-type, daf-2* mutant and *daf-16* mutant background suggests that the role of HIF-1/AHA-1 in *ftn-1* regulation is completely independent of IIS.

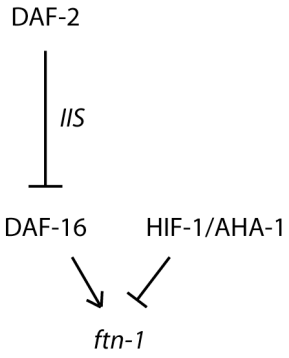


Figure 62
Model B: The regulation of *ftn-1* by HIF-1 is independent of IIS.

However, the hypoxia resistance of *daf-2* mutants, the over-representation of putative *hif-1* binding elements in IIS targets and the interactions between *hif-1* and IIS in axon pathfinding (see above) suggests that *hif-1* may act downstream of *daf-2*. Two such scenarios are possible:

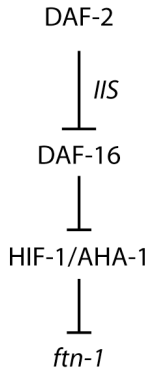


Figure 63
Model C: *hif-1* acts downstream of IIS and *daf-16* to regulate *ftn-1* expression.

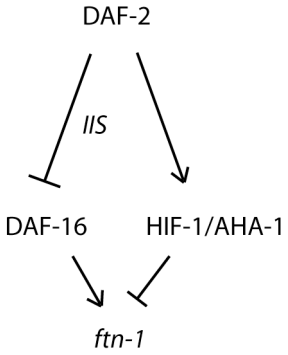


Figure 64
Model D: DAF-2 regulation of DAF-16 and HIF-1 are two parallel mechanisms of *ftn-1* regulation.

Whether or not HIF-1 acts downstream of DAF-2 to regulate *ftn-1* could be tested by treating *Pftn-1::gfp hif-1(+)* and *Pftn-1::gfp hif-1(ia4)* animals to *daf-2* and control RNAi. If *daf-2* RNAi is equally effective at inducing transgene expressions in both genotypes, then this would strongly support an independent role of IIS and hypoxia signalling. However, if *daf-2* was incapable of inducing expression in *hif-1* mutants, then models C and D would need to be considered.

In model C, the role of HIF-1 lies downstream of DAF-16, for example following the regulatory model proposed by Pocock and Hobert (see Figure 60). In their model, the inhibition of HIF-1 by DAF-16 is occurring through reduced ROS

levels by the DAF-16 –mediated up-regulation antioxidant enzymes. Since ROS are capable of increasing HIF-1 levels, reduced ROS would lead to less HIF-1 protein and therefore higher levels of *ftn-1* expression. If *hif-1* is indeed found to act downstream of *daf-16,* then the mechanism proposed by Pocock and Hobert could be tested using *sod-1(0)* mutants. If DAF-16 is regulating *ftn-1* expression at least partly by increasing SOD-1 levels, thereby decreasing ROS and inhibiting HIF-1 (see Figure 60), then *daf-2* RNAi should be less effective at increasing *ftn-1* expression in *sod-1(0)* mutants.

Alternatively, HIF-1 may act downstream of DAF-2 but in parallel to DAF-16 to regulate *ftn-1* levels (model D). This seems unlikely, as one would expect that, in this scenario, *ftn-1* induction by mutations in *daf-2* would be partially dependent on *daf-16* and partially dependent on *hif-1*, but I have shown that the effect of mutation of *daf-2* on *ftn-1* expression is completely *daf-16*–dependent.

5.3.2. *hif-1*: A new regulator of *C. elegans* iron homeostasis

Why would iron homeostasis be regulated in response to the levels of both iron and oxygen? One possibility is that there is a biological requirement for a closely linked regulation of iron homeostasis and oxygen levels. Because of *C. elegans'* small size and the ease with which molecular oxygen diffuses through membranes, it is unclear whether oxygen availability is mediated by simple diffusion or whether active processes exist to transport and store oxygen. 30 genes are thought to code for globins in *C. elegans* and though their role is not well understood, at least one could potentially have a role in O_2 binding and storage. This gene, *glb-1*, is induced by hypoxia (Geuens et al., 2010). Since globins require iron to store and transport O_2, it is possible that *hif-1* mediates a joint regulation of iron availability and globin expression in order to effectively respond to hypoxia. In this scenario HIF-1 activates globin expression, which increases the requirement for iron, and decreases *ftn-1* expression to meet this requirement. This joint regulation is similar to that seen in vertebrates, where HIF stimulates erythropoesis, which requires high levels of iron, as well as a battery of genes involved in increasing iron availability (see Chapter Introduction).

Another possibility is that the levels of free iron are tightly regulated partly due to the extreme toxicity of the hydroxyl radical produced by Fe^{2+} from hydrogen peroxide in the Fenton reaction. It is possible that iron levels are regulated in response to the availability of oxygen as well as iron because the damage caused by reactive oxygen species is greatly exacerbated when levels of free iron are high (Mole, 2010). Under conditions of high oxygen levels, increased ferritin expression due to HIF-1 degradation may contribute to reducing ROS by sequestering iron, thus detoxifying the noxious combination of O_2 and free iron.

5.3.3. The unexpected effects of *egl-9* RNAi

Given what is known about the hypoxia pathway in *C. elegans*, I expected that knock-down of both *egl-9* and *vhl-1* would have the same effect on HIF-1 levels: Since both are required for its proteosomal degradation, RNAi of either should lead to a large increase in HIF-1 levels. Because HIF-1 appears to be acting as a repressor of *Pftn-1::gfp*, both RNAi treatments should therefore lead to a decrease in *Pftn-1::gfp* expression. While this is in fact what I found after two generations of *vhl-1* RNAi treatment, *egl-9* RNAi has the opposite effect and leads to very large increases in *Pftn-1::gfp* expression.

One possibility is that this finding is artefactual. I confirmed the identity of the *egl-9* RNAi clone by sequencing, so the correct clone was used for these experiments. It is possible that off-target effects of RNAi could be responsible, so it would be important to test genetic mutants of *egl-9* to exclude this possibility. That *egl-9* RNAi is in fact causing an increase in HIF-1 levels could be confirmed by treating worms expressing HIF-1::Myc to *egl-9* RNAi and measuring protein levels by western blotting. If this confirms that *egl-9* RNAi is having the expected effects on HIF-1::Myc levels, i.e. increasing them, then this would pose the interesting question of how increased expression from the *Pftn-1::gfp* transgene can be associated with both increases and decreases in HIF-1 levels.

Alternatively, these results may indicate that *egl-9* acts by more than one pathway to influence *ftn-1* expression and that *egl-9* has *vhl-1*–independent effects on the regulation of *ftn-1*. As mentioned in the chapter introduction, a recent publication found that *egl-9* can have effects on gene expression that are independent of *vhl-1*

(Shao et al., 2009). The authors were able to demonstrate that the induction of *hif-1*–target genes can occur even when *hif-1* is constitutively stabilised by a mutation in P621 (P621G). Moreover, neither mutations in *vhl-1* nor the inhibition of EGL-9 hydroxylase activity increases *hif-1* target gene expression to the extent that strong loss-of-function alleles of *egl-9* can. Further support for a dual role of EGL-9 in the regulation of *hif-1* target genes comes from their use of an *egl-9* transgene in which hydroxylase activity is abolished through a mutation in the Fe(II) binding pocket. While the mutated transgene is incapable of destabilising HIF-1, it is still able to strongly reduce expression of a *hif-1* target gene, thus demonstrating that the *vhl-1*–independent alternative pathway by which *egl-9* regulates the expression of *hif-1* target genes does not require hydroxylase activity.

If the same *vhl-1*–independent pathway of HIF-1 target gene regulation by EGL-9 is in fact responsible for the differing effects of *egl-9* and *vhl-1* RNAi on expression from my transgene, then the fact that it does not require hydroxylase activity is also of great interest since it is the hydroxylation reaction of *hif-1* that requires iron.

If the effects of *egl-9* RNAi on *ftn-1* expression can be verified, a hypothetical model could be proposed in which EGL-9 can both promote and repress expression of *ftn-1*. In this model, EGL-9 promotes the expression of *ftn-1* through the VHL-1–dependent degradation of HIF-I, which acts as a repressor of *ftn-1* expression. This degradation presumably occurs at different rates depending on the concentration of iron present. By a second mechanism, EGL-9 can also repress *ftn-1* expression and interestingly, this alternative pathway also requires HIF-1. The *hif-1*–dependence of the effects of *egl-9* RNAi on *Pftn-1::gfp* expression has already been shown (see Figure 59).

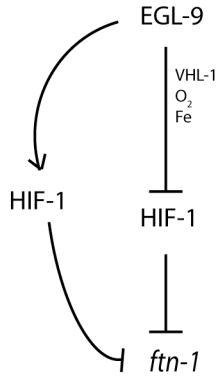


Figure 65
EGL-9 may regulate *ftn-1* by two separate pathways.

While the existence of an alternative pathway by which *egl-9* regulates gene expression may help explain how RNAi of *vhl-1* and *egl-9* could have different effects on gene regulation, it should be noted that Shao et al. do not report that the *vhl-1*-dependent and –independent pathways have opposite effects on gene expression. The two genes used in their publication as *hif-1*–target genes are both induced by *hif-1* and the activities of both *vhl-1* and *egl-9* act to reduce their expression. Clearly, the involvement of EGL-9 in the regulation of *Pftn-1::gfp* is more complex than expected.

5.3.4. Further steps

Because both hypoxia signalling and IIS have such dramatic effects on *ftn-1* expression and these two pathways are thought to interact in some cases (see Chapter Introduction), it could be important to test whether *ftn-1* just happens to be a common target or whether their joint regulation of *ftn-1* is evidence of a specific interaction between these two pathways. I have proposed several models of regulation and some of these could be directly tested. I was already able to exclude a scenario according to model A, in which the regulation *ftn-1* by hypoxia signalling is the consequence of increased activation of DAF-16. Alternatively, HIF-1 could be acting downstream of DAF-2 to repress *ftn-1* expression, either downstream of DAF-16 (Model C) or in parallel (Model D). Model D seems unlikely given the absolute requirement for DAF-16 in IIS regulation of *ftn-1,* but model C is possible and has been proposed as a possible explanation for effects of IIS on HIF-1 mediated axon-guidance defects in *C. elegans* neurons (Pocock and Hobert, 2008). If IIS–mediated regulation of *ftn-1* does occur through *hif-1,* then reduced IIS should not have an effect on *Pftn-1::gfp* expression *hif-1(ia4)* mutants. This could be tested using *daf-2* RNAi on the *Pftn-1::gfp hif-1(ia4)* line. If no induction of GFP expression occurs when IIS is reduced, or if the magnitude of the effect is less than in *hif-1(+)* animals, then IIS may be acting through HIF-1 to regulate *ftn-1*. If the induction is similar in both backgrounds, then this would strongly support model B, in which the regulation of *ftn-1* by IIS and hypoxia signalling occurs independently.

The strong effects of *hif-1* and *aha-1* on the regulation of *ftn-1* are clear, that this occurs through direct binding remains uncertain. My interest in *hif-1* and *aha-1* was partly motivated by the fact that Romney et al. had identified three direct repeat (DR) sequences in the IDE of the *ftn-1* promoter that resembled an E-box motif, leading them to the hypothesis that the iron-dependent transcription factor regulating *ftn-1* is likely to be a bHLH factor. Indeed my experiments on the regulation of the IDE demonstrate that the effects of *hif-1* on *ftn-1* expression do occur through these 63bp. That they act through the three DR sequences within the IDE seems likely, but needs to be tested further. This could be examined by crossing the *IDE(123m)::pes-10::gfp* line created by Romney et al, which has mutations in each of the three DR sequences, into a *hif-1(ia4)* background. Since loss of *hif-1* leads to an induction of *IDE::pes-10::gfp* (see Figure 55), demonstrating that this does not occur when the DR sequences have been mutated would show that *hif-1* is repressing expression from the IDE via the DR sequences.

HIF-1 and AHA-1 are two bHLH transcription factors that regulate *ftn-1* transgenes via a 63bp sequence that contains three sequences very similar to the E-box sequence. While this suggests that *ftn-1* might be directly repressed by the binding of the HIF-1/AHA-1 dimer, none of the work described in this chapter was designed to demonstrate direct binding. I did try to express the HIF-1 and AHA-1 proteins *in vitro* in order to test binding to the IDE using an electrophoretic mobility shift assay. However, my attempts to express the HIF-1 protein were unsuccessful (data not shown). In their publication on the effects of HIF-1 on lifespan, Zhang et al. describe a HIF-1::Myc-expressing transgene used to over-express HIF-1. I successfully used this strain to confirm the efficacy of *hif-1* RNAi (see Figure 50). Since, like mutations in *vhl-1*, this transgene is capable of extending lifespan, the HIF-1::Myc protein appears to be functional. This was confirmed to me by the authors, who found that the transgene was able to rescue *hif-1* mutant phenotypes (Jo-Anne Powell-Coffman, personal communication). Anti-Myc antibodies could therefore be used to perform chromatin immuno-precipitation followed by qRT-PCR to test whether the HIF-1/AHA-1 dimer is binding the *ftn-1* promoter. The assay could be performed using worms expressing low levels of HIF-1::Myc and comparing them to ones in which HIF-1::Myc was stabilised, for example by mutation of *vhl-1*.

That loss of *egl-9* leads to an increase rather than a decrease in *Pftn-1::gfp* expression indicates that a *vhl-1*–independent pathway may be regulating *ftn-1* antagonistically to the *vhl-1*–dependent pathway (see Figure 65). It would be fascinating to investigate the nature of this hypothetical second pathway using the reagents created by the Powell-Coffman group. Whether or not the effect of iron on *Pftn-1::gfp* expression requires EGL-9 hydroxylase activity could be investigated using the transgene expressing hydroxylase–deficient EGL-9. A HIF-1(P621G)::Myc transgene (Zhang et al., 2009) could also be used.. In *hif-1(P621G)::myc hif-1(ia4)* animals, all HIF-1 would be expressed from the transgene and would therefore not be susceptible to EGL-9 hydroxylation. If *ftn-1* levels are still responsive to iron, then this would be strong evidence for a role of the *vhl-1* independent pathway in iron-dependent *ftn-1* regulation. If not, then it is possible that the *ftn-1* induction by iron occurs solely through *vhl-1*–dependent pathway and the *vhl-1*–independent pathway may serve to respond to different cues.

5.3.5. Concluding remarks

During my PhD research, I investigated the regulation of *ftn-1* by means of an RNAi screen. The aim was to identify transcription factors that act downstream of DAF-16 to regulate the differential gene expression of IIS mutants and ultimately to identify novel genes that may play a role in the longevity of IIS mutants.

While RNAi of many genes affected regulation of a *Pftn-1::gfp* transgene, the screen was less successful in identifying novel mediators of IIS longevity, since the effects of *hsf-1, hif-1* and even *mdl-1* on ageing had already been described. However, little is known about the function of *mdl-1* in *C. elegans* and none of its target genes have been identified. Its involvement in the regulation of *ftn-1* may help identify other targets and further test its role downstream of DAF-16. *mdl-1* therefore remains interesting and is being pursued further.

Rather than identifying novel genes, the screen was successful in defining a complex gene regulatory network controlling *ftn-1*, which shows integration of IIS, *hsf-1* and *hif-1* pathways. The identification of *hif-1* as an important regulator of *ftn-1* expression is especially interesting given that HIF-1 levels are modulated by iron. I was subsequently able to show that that the regulation of a *Pftn-1::gfp* transgene by

iron requires *hif-1*. This work will hopefully be useful to other researchers interested in how iron homeostasis in maintained and to those investigating hypoxia signalling.

Chapter 6: Investigating the role of superoxide dismutases in determining *C. elegans* lifespan

6.1. Chapter Introduction

During the course of my PhD studies at the Gems lab, other laboratory members had been working on investigating the role of reactive oxygen species (ROS) in ageing in *C. elegans*. This is a subject that greatly interested me, despite being far removed from my own research into the regulatory architecture downstream of IIS. During the course of one of my colleague's research, he uncovered evidence for an atypical activation of DAF-16, which seemed to be the consequence of alterations in the levels of reactive oxygen species (ROS). I decided to get involved in this project as it allowed me to study the role of oxidative stress in ageing within the context of my previous work on the role of DAF-16 and IIS in *C. elegans* ageing.

Oxidative stress has long been thought to play a role in determining the rate of ageing (Harman, 1956). The theory proposes that ageing is caused by exposure to ROS, which leads to the oxidation of macromolecules and eventual loss of cellular function. This links ageing to mitochondrial metabolism, since superoxide (O_2^-) production by the electron transport chain is the greatest source of ROS in cells (Brand, 2000). Mitochondria produce O_2^- by electron leakage from bisemiquinone (QH^-) or from other electron carriers in the ETC. The oxidative stress theory has been popular in the ageing field partly because it provides a mechanism with which to explain how different species achieve different lifespans, since differences in their metabolic rates could cause differences in O_2^- formation. The underlying idea that animals with fast metabolism live shorter than those with a slower one is also prevalent (the so-called "rate of living" theory). These theories seem consistent: selection of different metabolic rates in different organisms leads to different rates of O_2^- production and therefore, different rates of ageing (Gems, 2009; Sohal and Weindruch, 1996). However, this long-held view of a direct relationship between metabolic rate and ROS production is now seen as naïve, given the extent to which these phenomena are uncoupled (Brand, 2000). Whether differences in metabolic rate control lifespan or not, the theory that ageing is caused by oxidative stress from

ROS is widespread but also highly controversial in the field of ageing research. Some of the work on the relationship between oxidative damage and ageing will therefore be briefly reviewed.

In general, three different types of investigations can be carried out on this subject. The first is to identify a simple correlation: Do older animals have more damage? The second approach is to ask whether interventions that extend lifespan also protect against damage. Last, one can attempt to ask whether interventions that specifically alter the levels of oxidative stress also affect lifespan. This last approach is the most effective at testing causality. I will briefly review the work done using these three approaches before proceeding to describe my contribution to this project.

6.1.1. Does oxidative damage increase with age?

If the oxidative stress theory of ageing is true and ageing really is the consequence of an accumulation of damage caused by the presence of ROS, then clearly one should be able to detect higher levels of damage in older animals compared to younger ones. Much work has therefore gone into testing for an increase in damage with age. As discussed in Chapter 1, not all damage is caused by oxidation (eg. see glycation), but this chapter will focus on oxidative damage. While looking for increases in oxidative damage with old age can only yield correlative data, this approach seems like a good place to start testing the oxidative stress theory of ageing: Do older animals actually have higher levels of damage? This has been addressed by looking for changes in the levels of damage to lipids, proteins and DNA.

Lipid peroxidation has been studied because the polyunsaturated fatty acid component of the phospholipids in membranes are thought to be particularly sensitive to attack by ROS (Bokov et al., 2004). A number of studies (Sohal and Weindruch, 1996) have found that older animals seem to have higher levels of lipid peroxidation than young animals. For example, a study on levels of F_2-isoprostanes, a product of free radical attack on membranes, in young and old rats, found substantial increases with age (Roberts and Reckelhoff, 2001).

Proteins can also be damaged by ROS, both irreversibly (eg. by the formation of carbonyl derivatives) as well as reversibly (eg. the formation of methionine sulfoxide). The most widely studied alteration is the formation of carbonyl groups in

amino acids, which can be measured using antibodies in a western blot. Increases in protein carbonyl levels with age have been detected in specific proteins as well as in the total protein content of animals (Bokov et al., 2004). This includes samples from human fibroblast cell lines derived from individuals of different ages (Oliver et al., 1987), from rat livers (Starke-Reed and Oliver, 1989), aged fly mitochondria (Sohal and Dubey, 1994) and the skeletal muscle of rhesus monkeys (Zainal et al., 2000). Age increases in protein carbonyl levels have also been detected in worms (Adachi et al., 1998).

Damage to DNA by ROS is often measured by quantifying the by-product 8-oxo-2-deoxyguanosine (oxo8dG), which can be accurately and sensitively quantified using high-performance liquid chromatography (HPLC). Both nuclear and mitochondrial DNA has been found to contain more oxo8dG in old compared to young rats (Fraga et al., 1990), though contradictory findings have also been published (Anson et al., 1999). One confounding factor is that phenol (used in DNA extraction) can cause DNA oxidation during extraction (Hamilton et al., 2001a), so more recent publications often use alternative reagents. One such study, for example found large increases in oxo8dG levels with age in various rat tissues when NaI was used instead of phenol for DNA extraction procedures (Hamilton et al., 2001b)

The prediction that older animals should show increases in damage has therefore in general been found to be true. But the question remains: Is this damage the cause of ageing or is it merely correlated with age?

6.1.2. Do manipulations that extend lifespan also protect against oxidative stress?

There are several interventions known to extend lifespan in lower organisms. These include dietary/caloric restriction and genetic interventions that cause reductions in IIS. It is possible that these interventions lower the rate of accumulation of oxidative damage, thereby slowing down ageing. This prediction has been tested in a variety of systems.

6.1.2.1. CR in rodents

Many studies have found that rodents fed under a caloric restriction (CR) regimen that extends lifespan also have reduced levels of lipid, protein and DNA damage (Bokov et al., 2004). One study found that CR led to increased levels of antioxidant enzymes in most tissues, but decreased levels in others (Xia et al., 1995). CR may also protect against damage by decreasing the generation of ROS. Indeed, mitochondria isolated from hearts and livers of CR –treated rats generated less ROS than from normally fed (Gredilla et al., 2001). In short, there is evidence that CR treatment in rodents may lead to decreased levels of damage, which is possibly due to decrease generation of ROS in older, CR –treated animals, and is therefore at least consistent with the oxidative stress theory of ageing.

6.1.2.2. IIS mutations in *C. elegans*

Mutations affecting IIS extend the lifespan of *C. elegans* (see Chapter I). IIS and other long-lived mutants are resistant to a number of different stresses, including heat, UV-irradiation, and ROS (Johnson et al., 2002). This has been taken as evidence that oxidative stress resistance itself extends lifespan, presumably because the increased antioxidant defenses that cause this resistance would also reduce the oxidative damage that causes ageing (Bokov et al., 2004). *daf-2* mutants have elevated expression levels of a number of cytoprotective genes (McElwee et al., 2004; Murphy et al., 2003), including the MnSOD *sod-3* (Honda and Honda, 1999; Vanfleteren and De Vreese, 1995). Of course the fact that long-lived worms have increased levels of antioxidant enzymes does not demonstrate a causative relationship. Given the very large number of genes thought to be up-regulated in *daf-2* mutants, searching for genes with a specific function from the list of up-regulated ones is likely to lead to confirmation of existing theories and represents a weak approach to hypothesis testing (see Chapter 1).

IIS mutants of *C. elegans* have not only been found to express higher levels of cytoprotective genes, but have also been found to have decreased levels of damage. Along with the expected increases in protein carbonyl damage with age, one study (Adachi et al., 1998) found lower levels of protein carbonyls in *age-1* mutants

compared to *wild-type* controls. The same group reported similar results in other publications (Yanase et al., 2002; Yasuda et al., 1999). Because of the *C. elegans* research community's history of using this organism mostly as a genetic model, biochemical techniques have not been widely used in worms. If *C. elegans* is to be effectively used to study oxidative stress and damage, more sophisticated measurements of how the interventions being used affect ROS and damage levels may be required. Of course, oxidative damage to proteins can be identified by western blotting and recently, expression of a transgenic H_2O_2 –sensitive fluorescent protein has been used to detect intracellular oxidative stress in vivo (Back and Vanfleteren, 2010 -unpublished). Hopefully these, and other similar techniques, will make *C. elegans* a more effective model for the study of molecular damage in the future.

6.1.2.3. *Drosophila melanogaster*

Mutations in IIS genes also extend lifespan in fruit-flies (see Chapter I). Like *C. elegans* IIS mutants, *chico* flies have increased SOD activity and females are slightly resistant to oxidative stress (Clancy et al., 2001).

Several interesting selection–based experiments were carried out in Drosophila that indirectly address the question of whether ageing is caused by oxidative stress. Long term selection for late life reproductivity led to the creation of several long-lived lines with elevated antioxidant activity (Arking et al., 2000). Selection based on paraquat resistance also led to strains of flies that are long-lived (Vettraino et al., 2001). In another experiment, flies selected for their resistance to starvation were found to have gained resistance against oxidative stress, but none of them showed an increase in lifespan (Harshman et al., 1999). These studies support the idea that there is a strong link between oxidative stress resistance and longevity, but also suggest that stress resistance alone is not sufficient to extended lifespan.

6.1.2.4. Mouse

Effects of altered insulin/IGF-1 signalling on the regulation of anti-oxidant genes have also been found in mice. Ames dwarf mice (see Chapter 1), which are long-lived and have reduced IGF-1 levels (Bartke et al., 2001a; Bartke et al., 2001b; Flurkey et al., 2001), have increased levels of catalase (Brown-Borg and Rakoczy, 2000) and superoxide dismutase (Hauck and Bartke, 2000) and also show lower levels of protein oxidation (Brown-Borg et al., 2001). Embryonic fibroblast cells derived from long-lived Snell dwarf mice (see Chapter I) were also found to be resistant to a number of stressors (Murakami et al., 2003). Female mice heterozygous for a mutation in the IGF-I receptor were also shown to be long-lived and were also resistant to paraquat toxicity (Holzenberger et al., 2003). Embryonic fibroblast cells from these animals were also more resistant to hydrogen peroxide than controls. As in Drosophila and *C. elegans*, mice with alterations in IGF-1 signalling seem to be both long-lived and protected against oxidative stress.

6.1.3. Do induced alterations in ROS levels lead to changes in lifespan?

In summary, evidence from several model organisms supports the view that long-lived animals have increased protection against oxidative stress. But does the protection against stress cause the lifespan extension? The data discussed so far mainly serves to answer two questions: Do animals have higher levels of oxidative damage at old age than in their youth? and: Do interventions that extend lifespan also lead to reduced damage from oxidative stress? On both counts the evidence is generally consistent with the oxidative damage theory of ageing. However, the interpretation of this data is complicated by findings that show that in humans, a number of pathologies themselves cause increased oxidative damage (Halliwell and Gutteridge, 1989). Two different explanations for the correlation between oxidative damage and age could therefore be proposed: oxidative stress itself could be the ultimate cause of ageing, or ageing could cause a higher incidence of pathologies that lead to oxidative damage.

An example that could support the latter view is the finding that damaged mitochondria produce more ROS than healthy ones (Droge and Schipper, 2007). One

may imagine a scenario in which ageing causes a deterioration of mitochondrial health, possibly through the failure of cellular repair processes that ensure mitochondrial maintenance. This could therefore cause an increase in ROS formation and consequently an increase in cellular damage with age, without any causal link between this damage and the ageing process. In this scenario, any intervention that delays ageing would also be expected to reduce ROS at any age, simply because it is ageing that is causing increased mitochondrial damage. Slower ageing would therefore delay the occurrence of this damage and reduce the increase in ROS and oxidative damage. This criticism applies to all interventions shown to reduce oxidative damage, including mutations in IIS and caloric restriction: If they delay ageing and it is ageing-related pathologies that are causing increased oxidative stress, then the fact that these interventions extend lifespan as well as reduce oxidative stress is uninformative.

A similar scenario may be applied to the long-lived animal model mutants, which are resistant to oxidative stress, presumably through the up-regulation of a number of cytoprotective genes such as superoxide dismutases and catalases. This link might also be coincidental. *C. elegans*, for example, is capable of forming an alternate developmental stage, termed the 'dauer' larva, under conditions of high stress, starvation, or high population density. Dauers are very long-lived (Klass and Hirsh, 1976) and very resistant to a number of different stresses (Cassada and Russell, 1975). The same IIS pathway that regulates ageing also regulates entry to the dauer stage and most of the genes involved in the ageing- phenotype are also involved in dauer formation. In fact, many of the genes known to be involved in the IIS pathway were first discovered due to their role in dauer formation (the daf- prefix of many of these genes refers to genes that when mutated cause worms to have abnormal dauer formation). This is also true for *daf-16*, the transcription factor required for lifespan extension by reduced IIS. *daf-16* mutant animals are also incapable of forming dauers, implying that it is involved in the regulation of genes involved in dauer formation. In fact, microarray analyses reveal large overlaps between transcriptional changes of IIS mutant worms and dauers (McElwee et al., 2004).

It seems possible that the longevity of IIS mutants reflects the misexpression of a dauer longevity program regulated by *daf-16*. If DAF-16 is regulating genes important in dauer animals, stress–response genes could be up-regulated by DAF-16

simply because of the role of dauer larvae in ensuring survival until conditions become more favourable for reproduction. A long lifespan is useful for dauers for similar reasons: the animals must survive for however long it takes for the adverse conditions to pass. If regulatory programs producing both these dauer traits are being regulated by DAF-16, then the fact that IIS mutants are both long-lived and protected against ROS would be no surprise, since both traits might be independent requirements of dauer larva. While this is also a hypothetical scenario, it too serves to illustrate the problems with drawing conclusion from what may merely be the co-occurrence of two traits.

A better approach to testing the oxidative stress theory of ageing would be to test whether specific interventions that decrease the levels of ROS in organisms lead to lifespan extensions. A number of different approaches have been used in several different organisms and these will be summarised in the next section.

6.1.4. Testing the oxidative stress theory of ageing via interventions into ROS levels

The most direct approach to testing the oxidative stress theory of ageing is to directly influence the levels of ROS and to test the effects of these alterations on lifespan. Based on the theory, one should be able to make straightforward predictions: increases in the levels of ROS should exacerbate the damage caused by physiological ROS levels and therefore accelerate ageing. Decreasing levels of ROS would be expected to extend lifespan, since the same levels of damage that cause normal ageing would now be occurring at a later time-point.

6.1.4.1. Non-genetic interventions in *C. elegans*

One of the ways in which the levels of ROS formation can be altered is by increasing the concentration of ambient oxygen from 21% to 60%, which shortens *C. elegans* lifespan, or decreasing it to 1%-2%, which lengthens it slightly (Adachi et al., 1998; Honda et al., 1993). Similarly, a number of pro-oxidant compounds have been found to shorten lifespan, including H_2O_2 (Larsen, 1993), arsenite (Olahova et

al., 2008), *tert*-butylhydroperoxide (An and Blackwell, 2003), paraquat and juglone (Van Raamsdonk and Hekimi, 2009; Vanfleteren, 1993). These findings are consistent with the theory, but inconclusive, since many bioactive compounds could be found to be toxic at some concentration.

The addition of antioxidant compounds represents the opposite approach. The oxidative stress theory of ageing implies that the addition of compounds that mop up oxidants to reduce the damage caused by ROS should thereby extend lifespan. Studies using antioxidants have yielded contradictory results. Some antioxidants, like N-acetylcysteine and vitamin C, had no effect on lifespan (Harrington and Harley, 1988; Schulz et al., 2007), while others, like α-lipoic acid (Benedetti et al., 2008) and a mixture of tocotrienols (Adachi and Ishii, 2000), did. Other antioxidant compounds increased lifespan in some studies but not others (Gems and Doonan, 2009).

The effects of the SOD mimetics EUK-8 and EUK-134, which catalyze the removal of O_2^-, on *C. elegans* lifespan have have also been tested (Gems and Doonan, 2009). It has been found, for example, that 2mM paraquat shortens lifespan and generates O_2^- and that certain concentrations of EUK-8 can rescue this shortened lifespan. These same concentrations of EUK-8 however, are not capable of extending lifespan in the absence of paraquat. This strongly suggests that O_2^- levels do not usually limit worm lifespan (Keaney et al., 2004). Treatment with higher doses of SOD mimetics actually shortens *C. elegans* lifespan and EUK-8 has also been shown to cause increases in oxidative damage in *E. coli* (Gems and Doonan, 2009). It is possible that strongly increased SOD activity actually increases overall ROS by increasing levels of H_2O_2 (Buettner et al., 2006). This further complicates the interpretation of this type of study.

The central problem with this approach is the uncertainty concerning the *in vivo* efficacy of interventions, such as the addition of antioxidant compounds or the over-expression of antioxidant enzymes, in actually reducing the levels of ROS. Improved methods that allow for a better characterisaton of the efficacy of compounds in reducing ROS levels or damage from ROS, would help advance this direction of research. Recent work using a fluorescent H_2O_2-sensitive reporter to measure *in vivo* ROS levels is a promising step in this direction (Back and Vanfleteren, 2010 -unpublished).

6.1.4.2. Loss of antioxidant genes

Two genes code for Mn-SOD in *C. elegans*: *sod-2* and *sod-3,* which are thought to be expressed in the mitochondrial matrix (Doonan et al., 2008). Simultaneous deletion of both increases levels of protein oxidation and severely sensitises worms to oxidative stress (Doonan et al., 2008; Honda et al., 2008; Van Raamsdonk and Hekimi, 2009; Yen et al., 2009) confirming that the deletions lead to weakened antioxidant defences. This complete loss of Mn-SOD does not reduce lifespan, strongly implying that whatever damage is caused in the mitochondrial matrix by the O_2^- radical does not contribute to ageing.

The loss of *sod-1*, the main Cu/Zn SOD, present in the cytosol and intermembrane space, leads to only a small reduction in lifespan in some studies and none at all in others (Doonan et al., 2008; Van Raamsdonk and Hekimi, 2009; Yang et al., 2007). Simultaneous deletion of both intracellular Cu/Zn SOD genes has no additional effect on lifespan and deletion of the extracellular Cu/Zn SOD gene *sod-4* also did not reduce lifespan (Doonan et al., 2008; Honda et al., 2008; Van Raamsdonk and Hekimi, 2009; Yen et al., 2009).

It has been suggested that the longevity phenotype of IIS mutant worms is attributable to increased expression of SOD enzymes (Larsen, 1993; Vanfleteren, 1993; Vanfleteren and De Vreese, 1995). Loss of the main Cu/Zn SOD *sod-1* leads to a small reduction in *daf-2* longevity as in wild-type (Doonan et al., 2008; Yang et al., 2007) and loss of the other Cu/Zn SOD *sod-5* also had little effect (Doonan et al., 2008). Loss of the main Mn-SOD *sod-2* has been found to have either no effect in some studies (Doonan et al., 2008; Van Raamsdonk and Hekimi, 2009) or a very small effect in another (Honda et al., 2008). Deletion of *sod-3* led to no reduction in *daf-2* mutant lifespan in two studies (Doonan et al., 2008; Honda et al., 2008). There is therefore evidence for at most a small role of reductions in cytosolic O_2^- in the longevity of IIS mutants, but this can by no means explain the large lifespan extension seen in these mutants. Similar findings with dietary restricted worms suggest that this lifespan extension is also not due to reduced levels of O_2^- levels (Yen et al., 2009).

The H_2O_2 produced by SOD from O_2^- is removed by both catalases and peroxiredoxins. Deletion of the peroxisomal catalase *ctl-2* or of the peroxiredoxin

prdx-2 shortens lifespan (Olahova et al., 2008). However, in *ctl-2* mutants, it is unclear whether or not the shortened lifespan is due to increased levels of damage, as protein carbonyl levels were found to be lower in old *ctl-2* mutant animals than in controls (Petriv and Rachubinski, 2004).

6.1.5. Over-expression of Cu/Zn SOD in *C. elegans*

The discovery that over-expression of *sod-1*, the main *C. elegans* Cu/Zn SOD, modestly extends lifespan (Doonan et al., 2008) provided an opportunity to test the role oxidative stress in ageing. The fact that deletion of *sod-1* slightly reduces lifespan and over-expression extends it would argue for at least a small role of cytosolic O_2^- toxicity in limiting the lifespan of *C. elegans*. This would support a model under which oxidative stress from respiration is one of many sources of damage that ultimately cause ageing.

However, an additional unpublished observation by my colleague (Ryan Doonan) led to an alternative hypothesis. In two preliminary trials, Ryan found that the lifespan extension by *sod-1* over-expression was dependent on the transcription factor *daf-16*. This is not consistent with the oxidative stress theory of ageing, as one would not expect the lifespan extension via reduced O_2^- toxicity to be dependent on genetic background. Of course, *daf-16* is responsible for the lifespan extension by reductions in IIS, which implies that the lifespan extension seen in *sod-1* over-expressing animals may similarly be due to the induction of a *daf-16*–dependent longevity program.

The role of DAF-16 in protection against H_2O_2, the ability of exposure to H_2O_2 to induce the nuclear localisation of DAF-16 (Weinkove et al., 2006) and the role of SOD enzymes in the dismutation of O_2^- into H_2O_2 suggested the hypothesis that over-expression of *sod-1* leads to an overproduction of H_2O_2, which activates DAF-16 and extends lifespan via mechanisms similar to those found in IIS mutants. While a lifespan extension by exposure to a stressor may sound paradoxical, there is a precedent for this type of effect, hormesis. This occurs when exposure to sub-lethal levels of stress induces a stress response, thereby protecting against a later exposure to an otherwise lethal stress. Interestingly, hormetic doses of stress can increase lifespan in *C. elegans*.

6.1.6. Hormesis and ageing in *C. elegans*

While several definitions of hormesis exist, the one that will be used here is that of "stress-response hormesis" (Gems and Partridge, 2008) which describes a beneficial response to an exposure to sub-lethal levels of a stressor. Examples are plentiful across a number of different biological systems (Calabrese and Baldwin, 1999; Le Bourg and Minois, 1997; Yanase et al., 1999). The ageing field's interest in this phenomenon lies in the fact that alongside the commonly observed resistance against the original stressor, hormetic responses can also extend lifespan in model organisms. In worms, exposure to short periods of thermal stress (Lithgow et al., 1995) and 100% oxygen (Cypser and Johnson, 2002) lead to extensions in lifespan. This response to hormetic stress is commonly interpreted as being due to an up-regulation of cytoprotective genes. It is thought that the consequently higher levels of cytoprotective proteins lead to an extended lifespan once animals are returned to the standard maintenance conditions.

A possible example could be the finding that after a brief hormetic heat-shock, stochastic variability in expression of the heat-stress responsive chaperone gene *hsp-16.2* is a predictor of lifespan (Rea et al., 2005). This suggests that genes co-regulated with *hsp-16.2* are likely to be responsible for the lifespan extension found in heat-shocked animals. Although no further attempt was made to identify the effectors of the lifespan extension, *hsp-16.2* is known to be regulated by *hsf-1*, which is itself known to be required for lifespan extension by reduced IIS (Hsu et al., 2003) and is also known to regulate other chaperones (see Chapter I). The correlation of *hsp-16.2* expression with lifespan after hormetic heat stress and the known involvement of *hsf-1* in the regulation of *hsp-16.2* suggests that the cytoprotective genes regulated by *hsf-1* have a role in the extended lifespan after heat stress.

Not much research has been carried out to further understand the mechanisms by which hormesis extends lifespan. One such experiment did find that lifespan extension by hormesis requires several genes which are also required for dauer formation, including *daf-16* (Cypser and Johnson, 2003). This suggests that the mechanisms of lifespan extension are the same in worms subjected to a hormetic stress as in worms with reduced IIS and supports our hypothesis concerning the longevity of *sod-1*.

6.1.7. Can elevated levels of SOD lead to ***in vivo*** increases in H_2O_2?

Since DAF-16 is known to be both required for protection against H_2O_2 (Weinkove et al., 2006), a simple explanation for our observations could be that increases in SOD-1 levels are increasing intracellular H_2O_2, thereby activating DAF-16. The idea that higher levels of SOD would increase H_2O_2 levels may seem intuitive since the enzyme catalyses a reaction that produces H_2O_2. However, this assertion is highly controversial (Buettner et al., 2006; Liochev and Fridovich, 2007) since the traditional view is that higher levels of SOD should have either no effect on H_2O_2 levels or even decrease them. This is based on the fact that $O_2^{\bullet -}$ can lead to the creation of H_2O_2 by several different routes (Buettner et al., 2006):

Case 1: This is the dismutation reaction of $O_2^{\bullet -}$, which can occur spontaneously or can be catalysed by SOD at a faster rate. This reaction leads to the creation of one H_2O_2 for every two $O_2^{\bullet -}$.

Case 2: This is the reaction of $O_2^{\bullet -}$ with electron donors

$O_2^{\bullet -} + \text{donor-H} + H^+ \rightarrow H_2O_2 + \text{donor}^{\bullet}$
In this case, 1 H_2O_2 is produced for every $O_2^{\bullet -}$.

Case 3: Participation of $O_2^{\bullet -}$ in chain reactions that produce more than one H_2O_2. There are several examples of this scenario, one of which is the oxidation of 6-hydroxydopamine (6-OHDA) to its semiquinone ($SQ^{\bullet -}$) and quinone (Q) described in Buettner et al. (2006):

$\text{6-OHDA} + Fe^{3+} \rightarrow SQ^{\bullet -} + Fe^{2+}$ (Initiation)
$SQ^{\bullet -} + O_2 \rightarrow Q + O_2^{\bullet -}$ (Superoxide formation)
$\text{6-OHDA} + O_2^{\bullet -} \rightarrow SQ^{\bullet -} + H_2O_2$ (Chain propagation)
$SQ^{\bullet -} + O_2^{\bullet -} + 2H^+ \rightarrow Q + H_2O_2$ (Chain termination)
$2SQ^{\bullet -} + 2H^+ \rightarrow \text{6-OHDA} + Q$ (Chain termination)
$O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ \rightarrow O_2 + H_2O_2$ (Termination)

The prediction that increased levels of SOD should decrease levels of H_2O_2 is based on the fact that the dismutation reaction (Case 1) leads to the least amount of H_2O_2 being formed. Increasing levels of SOD would increase the rate of the dismutation reaction, thereby allowing Case 1 to outcompete Cases 2 and 3. It would therefore follow that increased levels of SOD would decrease the amount of H_2O_2 generated.

Assertions to the opposite rest on the possibility that alterations in [SOD] affects the reaction that creates $O_2{\cdot}^-$ by oxidation of the semiquinone of CoQ ($CoQ{\cdot}^- + O_2 \rightleftharpoons CoQ + O_2^-$) in the electron transport chain. This reaction creates $O_2{\cdot}^-$, but is constantly occurring as both a forward and back reaction. Increased removal $O_2{\cdot}^-$ by dismutation to H_2O_2 would therefore lead to a replacement of a reaction removing $O_2{\cdot}^-$ without creating H_2O_2 (the back reaction) for a reaction that does create H_2O_2 (the dismutation reaction), thus increasing H_2O_2 levels. Whether or not this would indeed lead to increased formation of H_2O_2 is a controversial issue in the field (Buettner et al., 2006; Liochev and Fridovich, 2007). In practice, while it has been shown that increased levels of SOD leads to increased levels of H_2O_2 in some systems (Buettner et al., 2006; Scott et al., 1987), the opposite result has also been found (Teixeira et al., 1998).

6.1.8. Aims of this study

At the point at which I joined this project, it was already known that over-expression of *sod-1* extended lifespan and there were strong indications that the same was true for *sod-2*. Several preliminary trials had also demonstrated that the *sod-1* lifespan effect was dependent on *daf-16*. My role in this project was to confirm that the *sod-1* lifespan effects are *daf-16* dependent and to test whether they require *hsf*-1 and *aak-2*, two other mediators of lifespan and hormesis. Since it is possible that the lifespan phenotype of these lines is due to increased H_2O_2 levels activating DAF-16, I also contributed to efforts to test this hypothesis. All data presented here in the form of figures and tables was obtained only from my own work. In most cases, my colleagues replicated my findings, but their data is only referred to and not presented here in any figure or results table.

6.2. Results

6.2.1. Testing dependence on genetic background

6.2.1.1. Verifying *daf-16*–dependence of effects of *sod-1* over-expression on lifespan

I first verified whether the effects of *sod-1* on lifespan were indeed *daf-16* dependent. I crossed a line of *sod-1* over-expressing animals that carry their *sod-1*(+) transgene in an extra-chromosomal array (*wuEx123(sod-1(+))* into a *daf-16(mgDf50)* mutant background in order to independently verify the previous result. Two trials were performed, one using the extra-chromosomal *wuEx123 daf-16* line I created (see **Figure 66**) and another one using the integrated line (*wuIs152 daf-16)* tested in previous trials (see Figure 67).

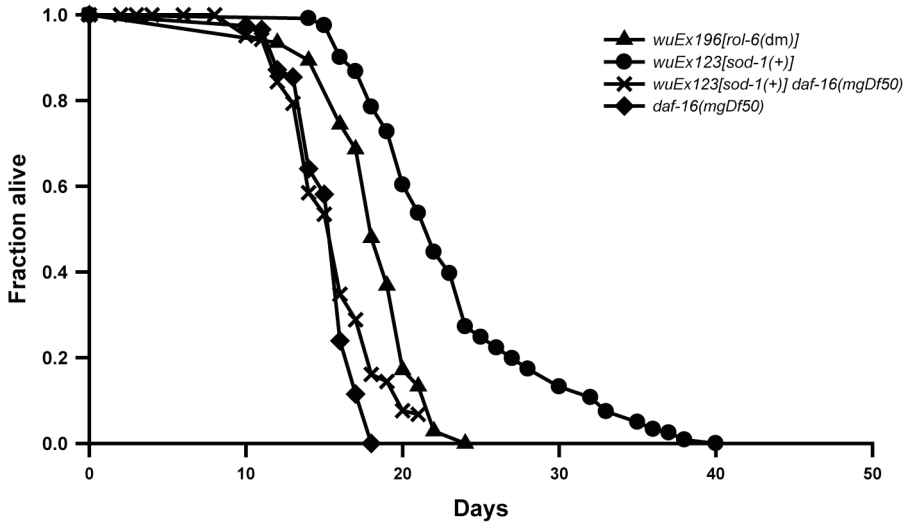


Figure 66

Testing the effects of *sod-1* over-expression on lifespan at 20°C via multi-copy extra-chromosomal array. Lifespan effects are *daf-16* dependent. Several additional trials using the same strains were carried out by Ryan Doonan and support the conclusions presented here.

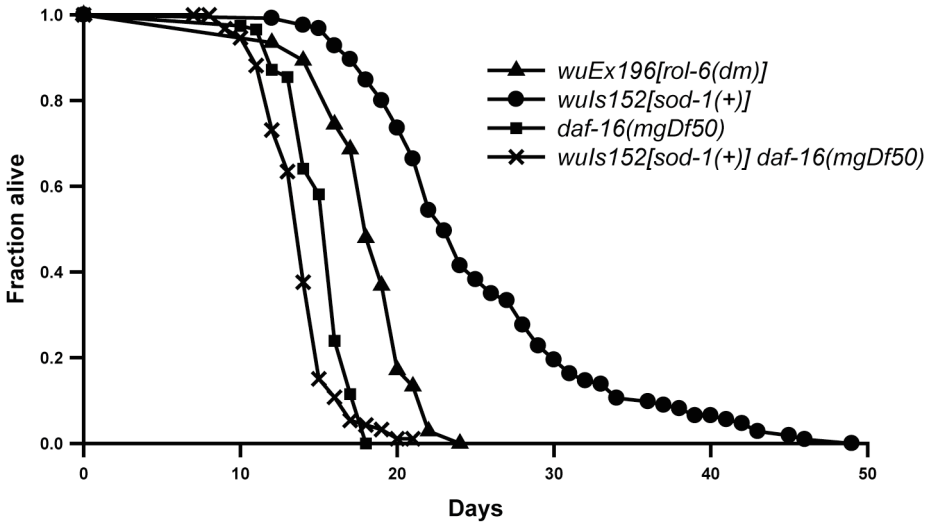


Figure 67
Testing the effects of *sod-1* over-expression on lifespan at 20°C via an integrated multi-copy array. Lifespan effects are *daf-16* dependent.

Genotype	n	censored	Mean lifespan	Median lifespan	% Change in mean [median]	Log-Rank p-value[a]	Wilcoxon p-value[a]
*rol-6(*dm*)*	117	6	18.3	18			
wuIs152(sod-1(+))	123	2	25.3	23	+38 [+28]	<0.0001	<0.0001
wuEx123(sod-1(+))	121	0	23.2	22	+27 [+22]	<0.0001	<0.0001
daf-16(mgDf50)	116	1	15.2	16			
wuEx123(sod-1(+)) daf-16(mgDf50)	119	15	15.9	16	+5 [+0]	0.0157	0.48
wuIs152(sod-1(+)) daf-16(mgDf50)	92	3	13.9	14	-9 [-13]	<0.0001	<0.0001

[a] calculated by comparing to non-over-expressing control strain

Table 13
This table contains data corresponding to **Figure 66** and Figure 67.

The finding that *sod-1* over-expression causes a lifespan extension was found to be robust, with a slightly greater effect seen in the integrated line. The lifespan extension by *wuIs152* completely required *daf-16*, as *wuIs152* did not extend lifespan in a *daf-16(mgDf50)* mutant background. In fact a small reduction in lifespan was seen. The extra-chromosomal *wuEx123* transgene showed a small 5% increase in mean lifespan in *daf-16* mutants and this change was statistically significant using the Log-Rank, but not the Wilcoxon test. The increase in mean lifespan is solely due to a significantly longer 'tail' in the lifespan curve, so while the mean lifespan was slightly higher, the median was unchanged. The magnitude of this increase in mean

lifespan is only 5%, much less than the 27% increase seen when *daf-16* is present, demonstrating that the lifespan extension is at least mostly, and most likely completely, dependent on *daf-16.* The effects of the *wuIs152* transgene are completely *daf-16*–dependent.

This requirement for the transcription factor DAF-16, which mediates stress response and longevity programs, suggests that the lifespan extension caused by *sod-1* overexpression does not occur as a consequence of a reduction in oxidative damage via the antioxidant activity of SOD-1. An alternative possibility is that *sod-1* over-expression is stressful and extends lifespan via hormesis. As discussed earlier, *daf-16* is required for hormetic effects on lifespan in various contexts. Other pathways have also been implicated in hormetic effects on lifespan and I therefore tested their involvement in longevity induced by *sod-1* over-expression.

6.2.1.2. The *hsf-1* transcription factor

I also tested whether the effect of *sod-1* over-expression on lifespan was dependent on other stress response pathways found to have effects on worm lifespan. I tested the *hsf-1* transcription factor, which strongly affects lifespan in *C. elegans* (Hsu et al., 2003) and is thought to act together with *daf-16* to control a subset of genes critical for ageing (see Chapter 3). The fact that expression of an *hsp::gfp* reporter was found to be predictive of future lifespan (Rea et al., 2005) was another reason for the inclusion of *hsf-1*, since heat-shock proteins are known to be regulated by this transcription factor.

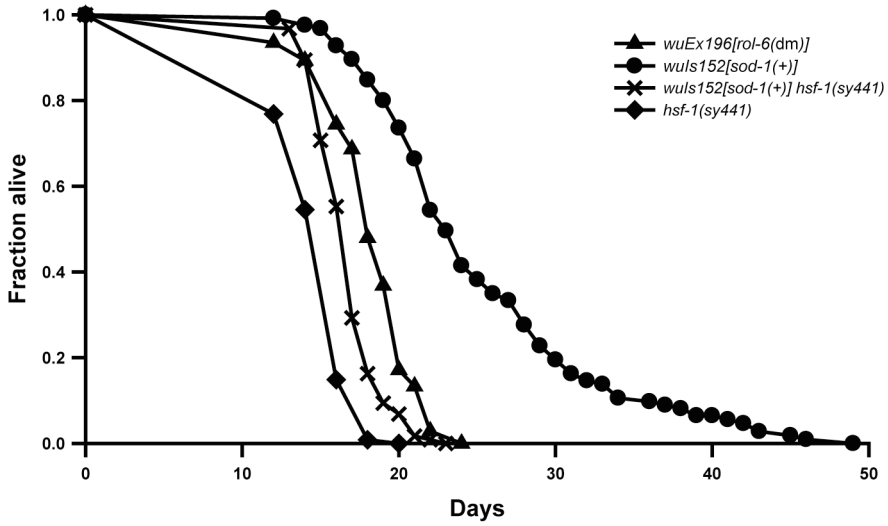


Figure 68

sod-1 over-expression via multi-copy integrated array. Lifespan effects are partially dependent on *hsf-1.* A second trial using the same strains was carried out by Ryan Doonan and supports the conclusions presented here.

Genotype	n	censored	Mean Lifespan	Median Lifespan	% Change in mean [median]	Log-Rank p-value[a]	Wilcoxon p-value[a]
*rol-6(*dm*)*	117	6	18.3	18			
wuIs152(sod-1(+))	123	2	25.3	23	+38 [+28]	<0.0001	<0.0001
hsf-1(sy441)	121	0	14.9	16			
wuIs152(sod-1(+)) hsf-1(sy441)	122	1	16.8	17	+13 [+6]	<0.0001	<0.0001

[a] calculated by comparing to non-over-expressing control strain

Table 14
This table contains data corresponding to Figure 68

In *hsf-1* mutant animals *wuIs152* transgene extended mean lifespan by 13% and median lifespan by 6%, compared to a 38% increase in mean and a 28% increase in median lifespan found in *hsf-1(+)* animals. This indicates that *sod-1* over-expression partially requires *hsf-1,* since the increase in mean and median lifespan was greatly reduced in its absence.

6.2.1.3. AMP-activated protein kinase

Like *hsf-1,* AMPK (AMP-activated protein kinase) is required for the lifespan extension caused by reduced IIS. Loss of the AMPK α-subunit *aak-2* suppresses *daf-2* Age and over-expression of *aak-2* also extends lifespan (Apfeld et al., 2004). *aak-2* was also of interest here because of the results of a study of lifespan extension caused by glucose restriction in worms. This effect was found when worms are treated with 2-deoxy-D-glucose (DOG), which blocks glycolysis. The cause of the lifespan extension is thought to be increased levels of ROS from the mitochondria, which triggers a hormetic response. In this system, the lifespan extension from this 'mitohormesis' was found to require *aak-2* (Schulz et al., 2007), which presumably acts as a stress sensor and initiates downstream signalling. Given that our hypothesis for the mechanisms for lifespan extension in *sod-1* over-expressing animals rests on a similar premise, I tested for dependence on *aak-2* as well.

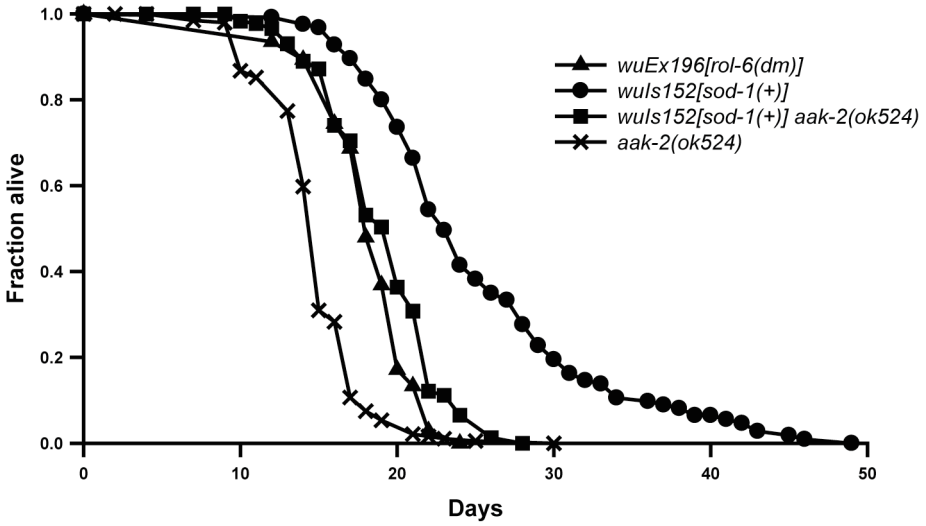


Figure 69
sod-1 over-expression via multi-copy integrated array. Lifespan effects are not dependent, or only very partially dependent, on *aak-2*. A second trial using the same strains was carried out by Ryan Doonan and supports the conclusions presented here.

Genotype	n	censored	Mean Lifespan	Median Lifespan	% Change in mean [median]	Log-Rank p-value[a]	Wilcoxon p-value[a]
rol-6(dm)	117	6	18.3	18			
wuls152(sod-1(+))	123	2	25.3	23	+38 [+28]	<0.0001	<0.0001
aak-2(ok524)	119	9	15.9	15			
wuls152(sod-1(+)) *aak-2(ok524)*	67	41	18.8	20	+18 [+33]	<0.0001	<0.0001

[a] calculated by comparing to non-over-expressing control strain

Table 15

This table contains data corresponding to Figure 69.

The result of this experiment seems to suggest that the effects of *sod-1* over-expression are independent of *aak-2,* since the increase in median lifespan through *sod-1* over-expression was not reduced when *aak-2* was absent (28% vs 33% increase). However, it is clear from the lifespan curves (see Figure 69) that the maximum lifespan is much greater in *aak-2(+) wuIs152* than in *aak-2(-) wuIs152*, which suggests a possible partial dependence. This would be reflected in the extension in mean lifespan, which in fact is much greater in *aak-2(+)* than in *aak-2(ok524)* animals (38% vs 18%). Since the mean lifespan is not considered a reliable statistic and the maximum lifespan is highly variable, the 75th percentile, the day at which 75% of animals used in this trial had died, can be used instead. Here we also see that *sod-1* over-expression causes a greater lifespan extension in an *aak-2(+)* than in an *aak-2(-)* genetic background (45% vs 25%). While these results are difficult to interpret with certainty, they suggest that the effects of *sod-1* over-expression are partially dependent on *aak-2*.

6.2.2. Testing the role of H_2O_2

This dependence on *daf-16* and partial dependence on *hsf-1* suggest a hormetic cause for the lifespan extension through *sod-1* over-expression. We wondered whether SOD over-expression was causing an increase in the levels of H_2O_2, which can activate DAF-16 (Weinkove et al., 2006) and could thereby extend lifespan. This was tested in *sod-1* over-expressing animals through the co-over-expression of catalase (using a transgene containing copies of *ctl-1, ctl-2* and *ctl-3)*, which converts H_2O_2 to water. However, the extent of lifespan extension by *sod-1* over-expression remained similar whether catalase was over-expressed or not (Cabreiro et al., 2011 -unpublished; Doonan et al., 2008). This suggests that H_2O_2 does not cause the lifespan extension.

6.2.2.1. Can the lifespan extension be suppressed by addition of N-acetyl cysteine?

In a previous study describing the phenomenon of mito-hormesis, in which mitochondrial stress through glucose restriction led to a lifespan extension via and *aak-2* –dependent mechanism, the anti-oxidant N-acetyl cysteine (NAC) was shown to rescue the lifespan effects (Schulz et al., 2007). I therefore tested whether NAC could abrogate the lifepan extension seen in our *sod-1* over-expressing animals.

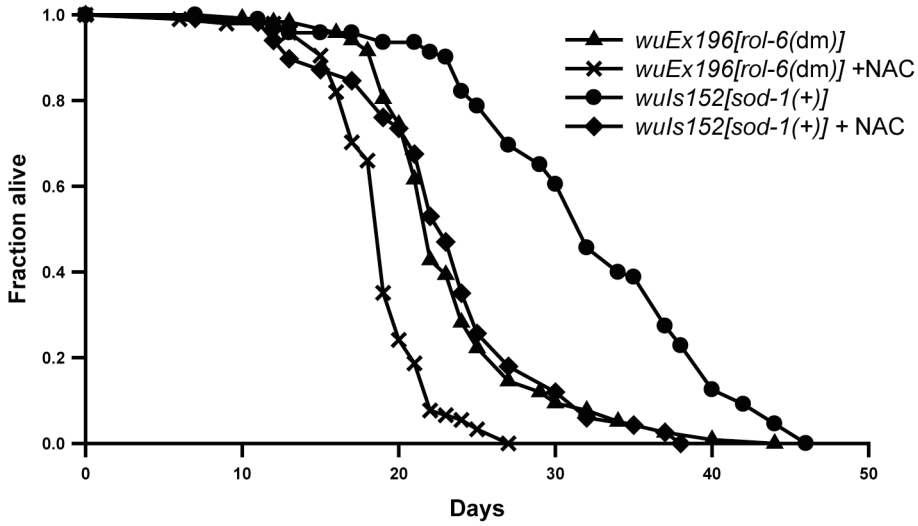


Figure 70

The effects of 5mM NAC on the lifespan of *wild-type* and *sod-1* over-expressing worms was tested. This concentration of NAC shortened lifespan in both strains, suggesting that it is mildly toxic.

Genotype	n	censored	Mean Lifespan	Median Lifespan	% Change in mean [median]	Log-Rank p-value[a]	Wilcoxon p-value[a]
rol-6(dm)	117	7	23.5	22			
rol-6(dm) +NAC	93	4	18.9	19	-20 [-14]	<0.0001	<0.0001
wuIs152(sod-1(+))	88	9	32.3	32			
wuIs152 +NAC	116	1	23.2	23	-28 [-28]	<0.0001	<0.0001

[a] calculated by comparing to non-over-expressing control strain

Table 16
This table contains data corresponding to Figure 70.

Despite the atypically long lifespan in control worms in this trial (for reasons that are unclear), the lifespan extension by *sod-1* over-expression was robust (p=0.0001 for both statistical tests; lifespan extension +37% [+45%] increase in mean [median]). While the NAC did reduce the lifespan of *sod-1* over-expressers, it also markedly shortened the lifespan of the control animals, suggesting that the NAC concentration used was mildly toxic. After consultation with the authors of the original publication, I repeated the experiment using 1mM NAC.

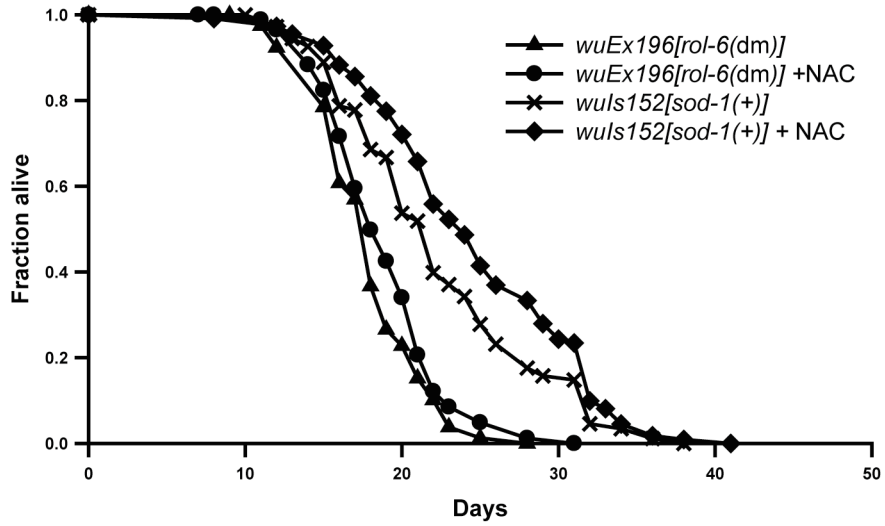


Figure 71
The effects of 1mM NAC on the lifespan of *wild-type* and *sod-1* over-expressing worms was tested. 1mM NAC does not abrogate the lifespan extension by over-expression of *sod-1*

Genotype	n	censored	Mean Lifespan	Median Lifespan	% Change in mean [median]	Log-Rank p-value[a]	Wilcoxon p-value[a]
*rol-6(*dm*)*	79	1	17.9	18			
*rol-6(*dm*)* +NAC	83	5	18.9	18		0.1164	0.1582
wuIs152(sod-1(+))	105	6	22.4	22			
wuIs152 +NAC	111	2	24.6	24	+10 [+9]	0.0183	0.0077

[a] calculated by comparing to non-over-expressing control strain

Table 17
This table contains data corresponding to Figure 71.

1mM NAC treatment also did not abrogate the lifespan extension by *sod-1* over-expression, which is consistent with the result of the catalase over-expression studies. Instead, a small increase in lifespan was seen in *sod-1* over-expressers, but not wild-type animals, treated with NAC. While an elimination of the lifespan extension by NAC treatment would have indicated that a mechanism similar to the one termed 'mitohormesis' might be at play, NAC treatment does not represent a direct approach to reducing H_2O_2 specifically. Instead, NAC is thought to generally mimic the ROS-scavanging effect of reduced glutathione (Schulz et al., 2007) and may therefore not have been specific enough for this test.

6.2.2.2. The effects of catalase co-over-expression on MnSOD over-expressing lines

Clearly the more direct approach to testing the role of H_2O_2 in triggering the hormetic response is the co-over-expression of catalase, which should reduce the possibly elevated H_2O_2 levels. Using *sod-1* over-expressing animals, this intervention was not found to eliminate the lifespan extension either (Cabreiro et al., 2011 - unpublished; Doonan et al., 2008), suggesting that H_2O_2 is not involved in this phenotype of *sod-1* over-expression. An alternative explanation is that the levels of H_2O_2 production in this strain are too great to be eliminated by the catalase over-expressing construct used in these experiments. While one would expect catalase over-expression to cause at least a partial elimination of the longevity if H_2O_2 did play a role and this was not observed, it is also possible that H_2O_2 levels above a certain threshold have no additional effect on lifespan. Reductions in H_2O_2 by catalase expression could therefore not cause a change in lifespan if the magnitude of the reduction is insufficient.

Over-expression of the Mn-SOD gene *sod-2* also extends lifespan, albeit to a lesser extent (Cabreiro et al., 2011 -unpublished). The increase in SOD protein levels is less pronounced in the *sod-2* over-expressing strains than in the *sod-1* strains, so the increase in H_2O_2 production may also be reduced. If catalase over-expression was capable of abrogating the lifespan extension in the case of *sod-2*, then this would provide at least some evidence for the role of H_2O_2 in causing a lifespan extension via DAF-16. A small preliminary study by a colleague (Ryan Doonan) had suggested that this could be the case. I therefore carried out a lifespan assay to test whether catalase co-over-expression is capable of abrogating the lifespan extension by *sod-2* over-expression.

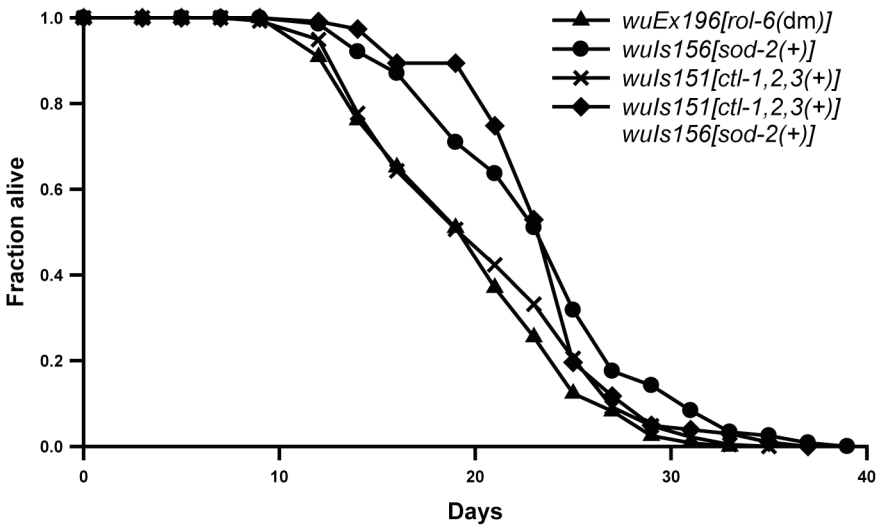


Figure 72
The effect of catalase over-expression on the lifespan of *sod-1* over-expressing animals was tested. An additional trial by Filipe Cabreiro using the same strains supported the overall findings of this trial.

Genotype	n	censored	Mean Lifespan	Median Lifespan	% Change in mean [median]	Log-Rank p-value[a]	Wilcoxon p-value[a]
*rol-6(*dm*)*	125	15	20.0	21			
wuIs151(ctl-1,2,3 (+))	235	180	20.6	21		0.1786[a]	0.3670[a]
wuIs156(sod-2(+))	128	17	23.7	25			
wuIs151(ctl-1,2,3 (+)) wuIs156(sod-2(+))	106	24	23.8	25		0.5426[a]	0.6076[a]
Additional statistical tests							
rol-6 vs wuIs156				+19 [+19]	<0.0001[b]	<0.0001[b]	
wuIs151 vs wuIs151 wuIs156				+15 [+19]	0.0003[b]	<0.0001[b]	

[a] calculated by comparing to corresponding *ctl-1,2,3 (+)* non-over-expressing control strain
[b] calculated by comparing to corresponding sod-2 (+) non-over-expressing control strain

Table 18
This table contains data corresponding to Figure 72.

As with *sod-1*, co-over-expression of *ctl-1,2,3* in *sod-2* does not eliminate the lifespan extension of *sod-2* lines. Thus the data from catalase and *sod* over-expression studies and the results of NAC treatment provide no evidence for a role of H_2O_2 in lifespan extension by *sod* over-expression.

6.2.3. Investigating possible DNA damage in SOD-1 over-expressing animals

While creating the strains used to investigating the roles of *daf-16*, *hsf-1* and *aak-2* in the longevity of *sod-1* over-expressing worms, I discovered that a large number of *sod-1* over-expressing worms were sterile. This was only apparent during the process of crossing the strain into other genetic backgrounds because the sterility was not complete and strains are usually maintained by transferring a large number of adult worms onto fresh plates. The sterility of a fraction of them presumably went unnoticed because their non-sterile siblings produced enough progeny to maintain the strain. During a *C. elegans* cross, individual worms are frequently picked onto fresh plates to self-fertilise and produce progeny. During the cross to create the *wuIs152 aak-2* line, for example, individual hermaphrodites of the F2 generation were isolated, allowed to lay eggs, and then genotyped for *aak-2* in order to identify homozygotes of the *aak-2* deletion. During this process, I noticed that some F2 animals were sterile and decided to investigate this phenotype further.

I was initially concerned that the *sod-1* OE line had acquired a recessive mutation that caused sterility. Such a mutation could easily remain unnoticed in the genetic background since homozygous animals would not pass the mutation on and heterozygous ones would appear wild-type. However, after observing the strain for many generations and attempting several crosses, the pattern of inheritance of the sterility phenotype was not consistent with a recessive mutation causing sterility. For example, the severity of the trait was found to increase with passing generations, and there was marked variability in the number of sterile animals from different fertile parents.

I investigated the possibility that the phenotype was not due to a separate mutation but was instead due to the SOD-1 over-expression itself. This alternative hypothesis was made with the knowledge that the *sod-1* OE line shows an increase in oxidative damage of proteins (Cabreiro et al., 2011 -unpublished). If *sod-1* over-expression was causing damage to DNA as well, it is possible that this would cause an cumulative loss in fertility with passing generations. This phenotype has already been described in *C. elegans* and is called the MoRTal germline (Mrt) phenotype.

6.2.3.1. Introduction to the Mrt phenotype

A number of genes have been found to be required for the maintenance of an immortal germline in *C. elegans* since their mutation causes a decrease in fertility with passing generations (the Mrt phenotype). These genes all have some role in the maintenance of DNA, with the best characterised of the Mrt genes having roles specifically in telomere maintenance. These include the *C. elegans* telomerase *trt-1* as well as *hus-1*, *mrt-2* and *hpr-17*, all three of which are part of the 9-1-1 complex. This complex is recruited to sites of DNA damage has also been involved in the extension of telomeres (Ahmed and Hodgkin, 2000; Boerckel et al., 2007; Harris et al., 2006; Hofmann et al., 2002).

rfs-1 is thought to play a role in the repair and reinitiation of stalled replication forks and is also involved in homologous recombination. The Mrt phenotype of *rfs-1* mutants could also be related to telomeres, since recombination is thought to be one of the mechanisms by which they are extended (Yanowitz, 2008).

The Mrt phenotypes of other genes have no obvious link to telomere maintenance. Besides being Mrt, *him-6, dog-1* and *msh-2* mutants also have an increased mutation rate, which suggests that the slow accumulation of mutations leads to eventual sterility (Cheung et al., 2002; Degtyareva et al., 2002; Grabowski et al., 2005; Youds et al., 2006).

In Mrt mutants with impaired telomere maintenance (eg. *trt-1* and *mrt-2*), the cause of sterility in is thought to be the dramatic changes in karyotype observed in these mutants. DAPI staining of the germline of mutants with impaired telomere maintenance reveals the occurrence of chromosome fusions, which leads to a gradual reduction in chromosome number with passing generations (Ahmed and Hodgkin, 2000). These changes in chromosome number are likely to cause failures in mitosis and meiosis.

6.2.3.2. Testing the Mrt hypothesis

I decided to test whether *sod-1* over-expression causes a Mrt phenotype by observing worms for many generation and looking for loss of fecundity. This was done by transferring single animals to new plates each generation, which prevented the sterility of single animals from being masked by the presence of their fertile

siblings. At each generation the size of the brood size was roughly evaluated by eye using four categories: wild-type, medium, small and sterile. In order to adequately assess whether *sod-1* over-expression causes Mrt and to fully exclude the possibility of second-site mutations caused by the X-ray integration of the SOD-1 over-expressing transgene, three separate extra-chromosomal arrays carrying multiple copies of the genomic *sod-1(+)* gene were used.

A separate strain of worms carrying only the *rol-6(su1006)* co-injection marker in an extra-chromosomal array was used to control for this genetic background in the *sod-1* over-expressors. Additionally, I considered the possibility that a sterility-inducing mutation (which could theoretically even be a Mrt mutation) had been present in the wild-type strain we used to generate all our *sod-1* over-expressing lines. Since the *rol-6* control line was generated at a different time and may therefore not carry this hypothetical mutation, this would lead to incorrectly finding that *sod-1* over-expression leads to a sterility phenotype. Because the *sod-1* over-expression array in these lines is extra-chromosomal, it is not inherited at a 100% frequency. It was therefore possible to control for the genetic background by creating a line derived by picking non-transgenic (non-Rol) siblings of one of the *sod-1* over-expressing lines. Over several months, these lines were therefore transferred every generation and brood sizes were evaluated. The workload on this project initially shared between Daniel Jones, an undergraduate summer project student, and me. In the later stages, the project was taken over by the post-doctoral researcher Caroline Araiz (Gems Lab, UCL).

The results were initially encouraging, with all three extra-chromosomal sod-1 over-expressing lines showing a large degree of sterility by generation 15 and all controls remaining fertile. However, by approximately generation 20, the *rol-6* control line began showing some sterility and eventually showed nearly as much as the *sod-1* over-expressing lines. It is unknown what could be the cause of this sterility in the transgenic control worms, which only over-express a cuticle protein used as a co-injection marker, could be is unknown. Possibly, the presence of an extra-chromosomal repetitive array might itself cause genomic instability, although this has never been observed in this laboratory despite extensive use of multi-copy arrays. Whatever the cause, it became clear that we would be unable to distinguish the sterility caused by *sod-1* over-expression from the sterility presumable being caused by the presence of any extra-chromosomal array.

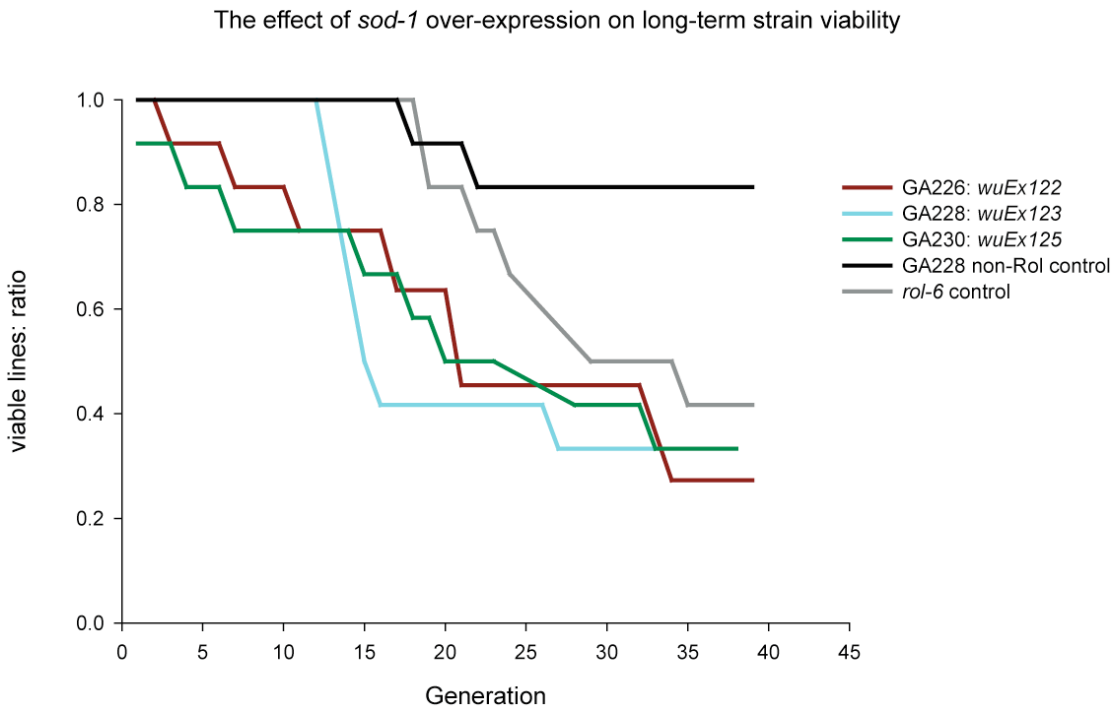


Figure 73
Three lines over-expressing *sod-1* were used and non-transgenic control animals from one of these lines were also tested (see GA228 non-RoI control) to control for the genetic background. The y-axis represents the fraction of lines being tested that are still viable at each generation.

While the data does show an earlier onset of sterility in the strains over-expressing *sod-1*, this result cannot be interpreted to indicate that *sod-1* causes Mrt. The reason for this is that the assumed mechanism by which the Mrt phenotype occurs is through the accumulation of DNA damage, by telomere erosion or possibly through the accumulation of inherited mutations. If the *rol-6* control line and the three *sod-1* over-expressing lines had all been outcrossed several times to the same genetic background before this experiment was started, then one could have assumed that the earlier occurrence of sterility could be caused by a faster rate of accumulation of DNA damage. However, this was not done and therefore it is possible that the *sod-1* over-expressing lines had already accumulated some DNA damage and were therefore further along the track to sterility, before the experiment had even begun. The experimental design therefore only allows us to ask whether the strains show a Mrt phenotype or not. The fact that the control, *sod-1* non-over-expressing, line does exhibit a Mrt phenotype makes the assay uninformative.

This outcome cannot be used to demonstrate that *sod-1* over-expression does not cause DNA damage; we were simply unable to distinguish sterility caused *sod-1* over-expression from that caused by carrying a multi-copy array. There are few alternatives to multi-copy arrays for over-expression studies in *C. elegans*, so this problem is a very fundamental one and over-expression by other mean does not seem like a viable option. The experiments were therefore aborted after approximately 40 generations. Daniel Jones and I also looked for chromosomal fusion in late-generation *sod-1* over-expressing lines by DAPI–staining and microscopy but did not see any (data not shown).

6.3. Chapter Discussion

6.3.1. Overview of results

The initial finding that led to the initiation of this project was that over-expression of Cu/Zn SOD leads to an extension in lifespan. Given the large amounts of contradictory findings in the supplementation of *C. elegans* with anti-oxidants, the lack of an effect on lifespan when SOD enzymes were removed, and the inherent weakness of the large amount of correlative data, the fact that the up-regulation of an anti-oxidant enzyme leads to a lifespan extension seemed like a rare opportunity to test the oxidative stress theory directly.

In light of the theory, the lifespan extension by *sod-1* or *sod-2* over-expression could be explained by an increase in O_2^- detoxification and a subsequent reduction in oxidative damage to the cell. However, given the results from this study, this now seems unlikely. Moreover, Filipe Cabreiro found that *sod-1* over-expression leads to increased production of H_2O_2 and increased levels of protein oxidation along with the already reported reduction in resistance to oxidative stress (Doonan et al., 2008) (Cabreiro et al., 2011 -unpublished). The finding that these long-lived animals have increased protein oxidation itself strongly argues against the theory.

Instead, we were able to establish that the lifespan effect requires the actions of the transcription factor *daf-16* and may also partially require *hsf-1*. This leads us to the intriguing possibility that the lifespan extension is due to a hormetic response:

low levels of stress caused by *sod-1* over-expression lead to an activation of DAF-16 (and possibly HSF-1), thereby causing a lifespan extension. Filipe Cabreiro was able to exclude the alternative possibility that *sod-1* is no longer being effectively over-expressed in a *daf-16(mgDf50)* mutant background by showing that SOD-1 expression and activity were unaffected by this allele. This strongly suggests that SOD-1 activity is in fact affecting signalling in a way that activates DAF-16. This is not without precedent, as another interaction of SOD-1 with a signalling pathway was described in a recent study on the role of ROS in MAPK signalling in *C. elegans* oocyte maturation pathways (Yang et al., 2010). The authors find that ROS have a role in promoting phosphorylation of MPK-1 and that SOD-1 has a role attenuating this phosphorylation. The MnSOD genes *sod-2* and *sod-3* have also been reported to modulate the Age and Daf-c phenotype of IIS mutants, although the mechanisms by which this occurs remain unclear (Honda et al., 2008). Cu/Zn SOD and Mn SOD over-expression can also extend lifespan in *Drosophila* (Sun and Tower, 1999). Interestingly, lifespan extension by Mn SOD over-expression has been shown to be associated with changes in the transcriptional profile that resemble those found in *C. elegans* when IIS is reduced. This suggests that a similar mechanism of lifespan extension by alteration in signalling may be occurring in SOD over-expressing flies as in worms (Curtis et al., 2007).

While it is tempting to speculate that the mechanism of lifespan extension occurring through *daf-16* is the same as the one occurring when IIS is reduced, there are however at least some signs that they are different. A separate study that investigated transcriptional changes in these same *sod-1* over-expressing lines found that two well characterised *daf-16* target genes (*sod-3* and *mtl-1* (Honda and Honda, 1999)) were not significantly up-regulated (Back et al., 2010). Since reduced IIS leads to nuclear localisation of DAF-16::GFP transgenes (see Chapter 1), similar changes could be expected from *sod-1* over-expression, if it was activating DAF-16. No obvious changes were observed in a DAF-16::GFP line carrying the *wuIs152* transgene. This is not too surprising, since *daf-16*–mediated increases in lifespan can occur without increases in DAF-16 localisation being detectable (Henderson and Johnson, 2001). These observations suggest that DAF-16 promotes longevity in *sod-1* and *sod-2* over-expressing lines either by a mechanism distinct from that in IIS mutants or at least via an atypical activation that does not fully reiterate the transcriptional changes mediated by DAF-16 in IIS mutants.

The mechanism by which *sod-1* over-expression affects signalling remains unidentified. Given the known role of DAF-16 in protection against H_2O_2 (Weinkove et al., 2006) and the role of SODs in the formation of H_2O_2, we were intrigued by the possibility that SOD over-expression was leading to increased levels of H_2O_2, thus activating DAF-16 and extending lifespan. Despite a large amount of controversy about whether over-expression of SODs would lead to increased formation of H_2O_2 (Buettner et al., 2006; Liochev and Fridovich, 2007), we detected increased levels of H_2O_2 in lysates of worms over-expressing Cu/Zn SOD. While the *in vitro* nature of these assays may make them unsuitable for getting an accurate picture of the consequences of SOD over-expression within *C.elegans* cells, our collaborators (Vanfleteren group in Ghent, Belgium) were able to cross our *sod-1* over-expressing construct into a genetic background expressing an H_2O_2–sensitive fluorescent protein (Hyper). This in vivo assay allows them to ascertain that H_2O_2 levels, which they found were slightly elevated in the over-expressing line (Cabreiro et al., 2011 -unpublished).

We therefore proceeded to test whether H_2O_2 was in fact extending lifespan via DAF-16 by treating the transgenic animals with the anti-oxidant N-acetyl cysteine (NAC) and by co-over-expressing catalase. The aim of this approach was to reduce the elevated levels of H_2O_2 back to normal levels and to examine the effects on the extended *sod-1* over-expressers. Neither of these approaches suppressed longevity, indicating that despite its elevated levels in SOD over-expressing worms, H_2O_2 may not be responsible for the *daf-16*–dependent lifespan extension. Other explanations are possible: the lack of specificity of NAC could make it unsuitable for use in this case and the increased levels of catalase may be insufficient to act as an sink for the excess H_2O_2 generated by SOD over-expression.

In conclusion, this study demonstrates that the effect of *sod-1* and *sod-2* over-expression on lifespan is unlikely to be a consequence of lowered levels of O_2^- and therefore of reduced oxidative damage. Instead, the lifespan extension is dependent on *daf-16,* which indicates that the over-expression may be causing alterations in signalling which activate a DAF-16-controlled longevity program. The mechanism of this activation is still completely unknown. While we were able to detect that SOD over-expression causes elevated levels of H_2O_2 and DAF-16 is known to be responsive to H_2O_2, we currently have no evidence that DAF-16 activation by H_2O_2 is responsible for the extended lifespan.

6.3.2. The implications for the oxidative stress theory of ageing

The co-occurrence of a lifespan extension and of increased levels of protein carbonylation seems to itself argue against the oxidative stress theory of ageing. However, whilst an alternative explanation for the effect on lifespan was identified, what the increase in H_2O_2 and protein damage demonstrate in particular is that use of lines over-expressing SOD is not an informative test of the theory. In order to test whether ROS play a role in determining lifespan, we would need to artificially decrease the levels of oxidative stress and then examine effects on lifespan, which was the initial purpose of over-expressing Cu/Zn SOD. Finding that this over-expression is not reducing, but increasing, the levels of damage, does not help us address the question. It merely means that the lines do not fulfil the role that they initially were designed for.

A lesson that could be drawn from this project, in which the over-expression of an anti-oxidant enzyme was found to increase, rather than decrease, oxidative damage to proteins, is that the effects of interfering with the cellular redox balance are hard to predict. Clearly, interventions used to reduce ROS need to be tested to confirm their efficacy, either using existing methods (eg. the oxy-blot) or through the development of new techniques (eg. HyPer). This will allow for a more meaningful study of the effects of ROS levels on lifespan.

6.3.3. What causes ageing?

There is a great deal of new data on how interventions in ROS levels affect lifespan in *C. elegans* from various research groups. An in depth discussion of how this data affects the oxidative stress theory of ageing has been presented elsewhere (Gems and Doonan, 2009). Gems and Doonan list three possibilities concerning the validity of the oxidative stress theory of ageing in light of recent studies in *C. elegans*. Briefly, these possibilities are that: 1. The theory is broadly correct but the details need to be adjusted. In this "neo-oxidative damage theory", oxidative stress and anti-oxidant defences remain the primary determinants of ageing, but O_2^- and SOD are not the critical forms of pro-oxidant and anti-oxidant activity. 2. The theory is a 'half-truth' and oxidative damage is one of many types of molecular damage that

lead to ageing. In this "molecular damage theory of ageing", changes in lifespan could be achieved in a number of different ways, for example by alterations in the levels of phase I, II, and III detoxification systems, which would presumably reduce diverse sources of damage, thus extending lifespan (McElwee et al., 2004). Both 1. and 2. imply a weaker mechanistic link between ageing and the 'rate of living', since respiration would now only cause a fraction of the damage that leads to ageing. The last possibility is 3. The oxidative damage theory is wholly wrong: molecular damage is correlated with ageing, but not a cause of it.

Concerning this last possibility, could it be that ageing is not caused by the accumulation of damage? Intuitively, it is easy to understand that the maintenance of any complex system requires high levels of investment in repair mechanisms and that these mechanisms can be more or less well suited for the job. Given the lack of selective pressure on providing systems that maintain organisms long past the age of reproduction, we may be right in thinking that the deterioration of our bodies, once they are past this age, is due to the changes that occur due to insufficient maintenance. A city, for example, is constantly being maintained: roads are re-paved, water pipes are replaced and old buildings are either expensively restored or replaced by new ones. Ageing organisms may therefore be like insufficiently maintained cities: as time passes, pipes begin leaking, buildings crumble and bridges collapse. It is an appealing idea that ageing is caused by the accumulation of some sort of damage, whether it takes place at a molecular level, or at the level of cells, tissues or organ systems.

But what if ageing has nothing to do with damage? It is jarring to imagine how our familiarity with our physical world may be biasing our thinking of biological systems. We are used to objects decaying over time and man-made machines accumulating faults that eventually lead them to fail. In trying to explain ageing, we may simply be applying the intuition we have gained from our personal experiences to a sub-cellular world that we, as biologist, may sometimes believe to understand quite well, but whose physical realities are well beyond the limits of our 'common sense'. Intuitive explanations that, in retrospect, reveal more about the false preconceptions of the historical period in question and the lack of information available at the time can be found throughout the history of science. An example could be found in Aristotelian physics, which was taught for thousands of years but which described all matter as being made up of the five elements earth, air, fire,

water and aether. This now seems silly. In science, it may be prudent to be wary of our 'common sense'. Clearly, damage does accumulate with age, but since we currently have no clear evidence that it is causing ageing, it might be advisable to adopt a conservative, or agnostic, attitude toward the question: What causes ageing? Answer: We don't know.

Without clear evidence, what can really be said about the possible causes of ageing? Theories of the evolution of ageing predict that selection acts more weakly on traits that occur after reproduction, and therefore that the deterioration in health late in life is unlikely to have been actively selected for during evolution. Instead, it is thought to be either the consequence of the lack of selection against changes that have deleterious effects late in life (mutation accumulation theory), or the selection for changes that have beneficial effects early in life, but deleterious ones late in life (antagonistic pleiotropy). Despite our ignorance of the mechanisms that may be causing ageing, we could thus conservatively state that ageing is caused by destructive forces. One of these may be oxidative stress (model 1., see above), or molecular damage in general (model 2.), or they could possibly even be completely unrelated to damage (model 3.). It has, for example, been proposed that the cause of ageing may be cellular hyper-function (Blagosklonny, 2008); this is a good example of a theory that does not rely on the steady accumulation of damage to explain ageing. Cancer could be seen as another example. Although cancers are initially caused by mutations (usually), which are a form of damage, the lethality of cancer is due to uncontrolled proliferation of tumour cells, another destructive force different from the slow accumulation of damage.

The example of cancer helps illustrate the complexity of the task ahead: The frequency of cancer increases with age in humans, making it an important destructive force in human ageing. *C. elegans,* however, do not get cancer, so while the forces that cause ageing in *C. elegans* may also be relevant to humans, it is naive to think that we will be able to find a single destructive force that explains ageing in all organisms. In the search of a universal cause of ageing in organisms like *C. elegans*, we may identify forces that can lead to a deterioration of health with time, but the degree to which each force contributes to ageing can vary greatly from organism to organism. Thus, our focus should not solely lie in identifying a single cause of ageing, but in identifying which processes contribute to ageing and to test the extent

to which they contribute to the ageing-related pathologies that we are trying to cure in humans.

These questions will undoubtedly be discussed for a long time. *C. elegans* is clearly an excellent model for testing theories of ageing, although the vast differences between worms and higher organisms need to be kept in mind. We may establish that O_2^- plays no role in *C. elegans* ageing only to later discover that it does play an important role elsewhere, so testing whether any mechanisms of ageing are public or private is clearly a priority. That said, if theories of ageing are to be tested directly, this will always need to be carried out in well defined biological systems. Demonstrating the veracity of a theory should require making specific predictions and testing them experimentally in such a way as to unambiguously verify or falsify the theory. Hopefully, experimental work on *C. elegans* and other organisms will help identify the causes of ageing and facilitate the development of treatments for human age-related disease.

Appendix 1

The screen described in Chapter 3 was designed to identify RNAi treatments that lead to a reduction in the expression of a *Pftn-1::gfp* transgene from the induced levels seen in a *daf-2* mutant. However, a large number of treatments were also found to lead to increased expression.

Gene	mean % of control	n	st. dev.	description
aha-1	317.1	2	214.4	ortholog of human ARNT
hif-1	309.5	2	94.9	hypoxia –inducible factor α homolog
unc-62	258.2	3	87.9	enocodes Meis-class homeodomain protein
irx-1	195.3	2	10.9	encodes homeodomain protein
fkh-10	182.3	2	63.6	forkhead transcriptional regulator
let-607	177.9	2	31.4	CREB/ATF family transcription factor
ceh-60	177.8	2	17.1	PBX family of homeodomain transcription factors
ceh-18	166.1	2	19.8	POU-class homeodomain transcription factor
nhr-165	149.3	2	38.6	nuclear hormone receptor
gei-17	148.6	2	9.0	encodes zinc finger protein
pqn-21	148.5	2	14.3	contains Q/N-rich domain
repo-1	146.2	2	26.1	putative splicing factor
fkh-9	142.8	2	16.1	forkhead transcriptional regulator
mep-1	142.0	2	14.1	encodes zinc finger protein
ztf-23	139.7	2	14.9	encodes zinc finger protein
Y60A9.3	137.1	2	7.6	encodes zinc finger protein
pqn-75	136.9	2	18.7	contains Q/N-rich domain
ztf-22	133.0	2	16.5	*none available*
cey-4	131.9	2	6.9	encodes Y-box containing protein
ceh-52	131.5	2	11.8	encodes homeobox protein
tbx-36	131.3	2	10.8	T-box factor
F47G4.6	130.7	2	4.6	*none available*
ehn-3	130.3	2	20.5	encodes zinc finger protein
nhr-7	128.3	2	9.9	nuclear hormone receptor
nhr-265	127.2	2	14.1	nuclear hormone receptor
nhr-141	127.2	2	13.4	nuclear hormone receptor
klf-3	126.1	2	13.3	Krueppel -like factor
isw-1	121.3	2	15.5	homolog of chromatin remodelling ATPase ISW1

Table 19
28 genes for which knockdown led to increased fluorescence of the *Pftn-1::gfp rrf-3(pk1426); daf-2(m577)* strain, ranked in order of severity of effect of RNAi.

Appendix 2

In order to quantify endogenous ftn-1 levels using qRT-PCR, the protocol under which worms were treated with RNAi needed to be altered. I therefore tested whether the suppression of Pftn-1::GFP by the various RNAi treatments still occurred under the new protocol. As clear from Figure A1 and Table A1 below, despite an increased baseline of GFP expression using protocol 2, the effects of the RNAi treatments remained very similar.

	protocol 1		protocol 2	
RNAi target gene	mean % of control	n	mean % of control	n
L4440	100.0	17	100.0	5
daf-16	30.2	17	15.9	5
pqm-1	82.8	6	85.7	2
T07C12.1	81.8	3	77.8	2
hsf-1	64.9	5	47.2	4
blmp-1	73.3	4	80.5	1
mdl-1	62.1	5	67.2	1
egr-1	86.0	4	52.9	1
pbrm-1	74.6	4	61.3	3
ada-2	65.8	5	59.7	3
elt-2	73.0	4	47.9	1

Table 20
Data from Figure 74 displayed as percentage of control.

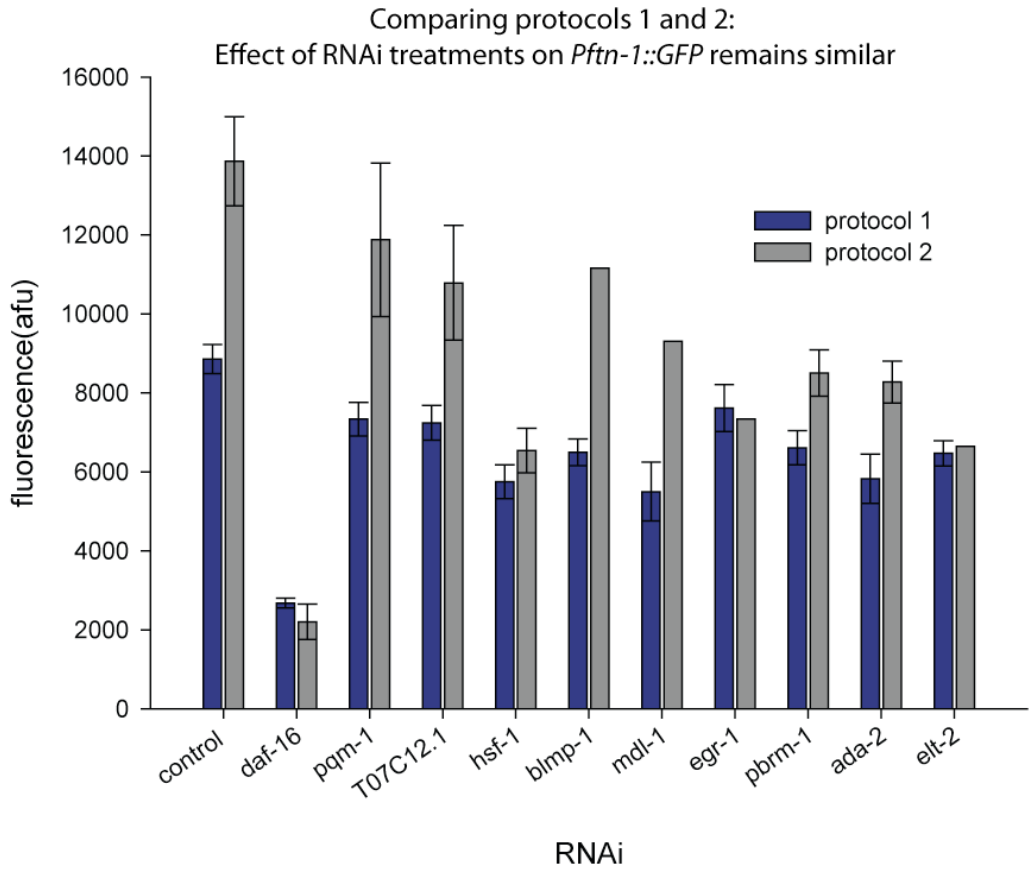


Figure 74
Pftn-1::GFP daf-2(m577) treated with various RNAi using two different protocols. Error bars represent ±*s.e.m*.

Appendix 3

From my previous experience in working with this particular strain (GA303: *rrf-3; daf-2* (see Figure 22 and Table 5)), I would not expect to see such any deaths at all (or very few) so early in the lifespan. In discussions with my colleague, he agreed that we use different criteria when establishing whether or not a worm is dead. I therefore reanalysed this lifespan experiment under the assumption that all worms that my colleague scored as dead on day 11 would have been scored as alive if I had scored them myself. This involves right-censoring of the data.

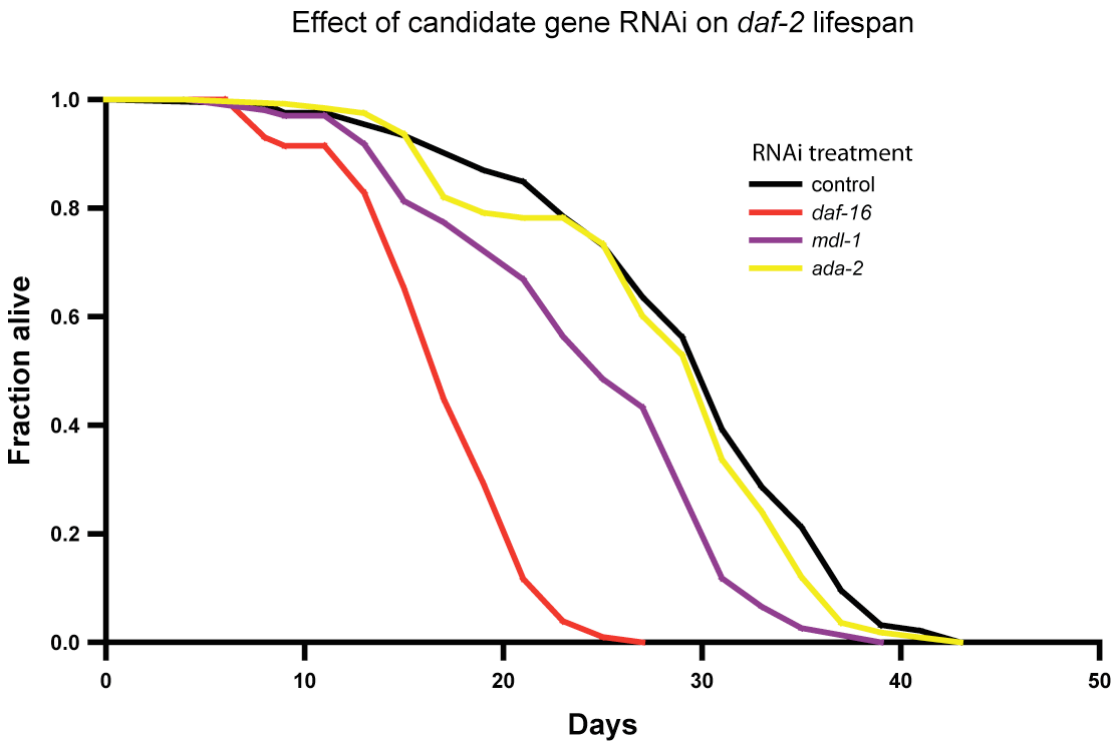


Figure 75
The effect of *ada-2* and *mdl-1* RNAi on *rrf-3(pk1426); daf-2(m577)* animals at 25°C.

lifespan assay was carried out on *rrf-3(pk1426); daf-2(m577)* animals at 25°C

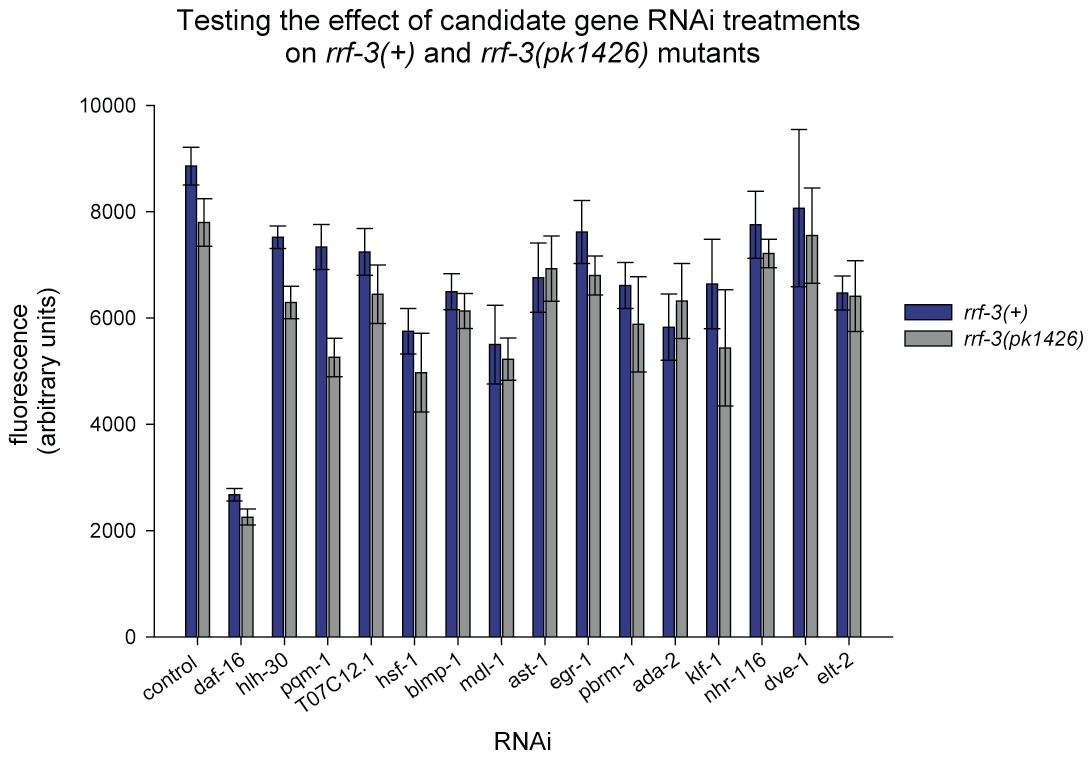
RNAi treatment	n	censored	Mean Lifespan	Median Lifespan	% Change in mean [median]	Log-Rank p-value[a]	Wilcoxon p-value[a]
control	94	28	29.4	31			
daf-16	105	26	17.4	17	-41 [-45]	<0.0001	<0.0001
ada-2	87	41	28.4	31	none	0.2402	0.4307
mdl-1	77	26	24.6	25	-16 [-19]	<0.0001	<0.0001

This table contains data corresponding to Figure 75.

After this modification, the lifespan curves now show a more typical rectangular shape. This reanalysis had no major effect on the interpretation of the treatment effects, with animals treated with RNAi of *daf-16* and of *mdl-1* still showing statistically significant lifespan reduction, while RNAi of *ada-2* has no effect.

Appendix 4

Loss of *rrf-3* does not greatly affect the effect of most RNAi treatments on *Pftn-1::gfp; daf-2* fluorescence.



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