Mechanisms of Aging and Development in the Nematode *Caenorhabditis elegans*

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ABSTRACT

Mechanisms of Aging and Development in the Nematode *Caenorhabditis elegans*

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C. elegans has a strong tradition for being used in genetic approaches to understanding the aging process. However, currently the worms are being used in a variety of other approaches, such as drug screening, disease modeling, and environmental manipulations. Collectively all these approaches are likely to provide a unique insight into the aging process. The objective of this thesis was to define the localization of peroxisomal proteins whose deficiency affects the rate of chronological aging and post-embryonic development of *C. elegans* in various tissues of this organism. The thesis focused homologs of human Sterol Carrier Protein x (hSCPx) in *C. elegans*, their roles and sub-cellular localization. P-44, nlt-1 and dhs-28 -are all members of SCP-2 sterol transfer family. My data indicate that P-44 plays vital role in the regulation of dauer formation, delayed egg-laying period, smaller body size, increased lipid accumulation and extended life-span. Nlt-1 also shows extended lifespan by 40% compared with wild type. In this thesis, I also report that P-44, nlt-1 and dhs-28 are involved in fatty acid metabolism. All of these are expressed in the intestine and appear to be peroxisomal. Both P-44 and nlt-1 seem to be required for catabolism of cyclopropane containing fatty acids. P-44 deficiency also blocks the catabolism of pristanic acid. dhs-28 (17-β-hydroxysteriod dehydrogenase), on the other hand, only blocks (affects) the pristanic acid pathway. The

data reported in this thesis support the view that there is no direct link between the amount of lipid deposits in an organism and a lifespan.

This thesis also explored the use of vital lipophilic dye Nile Red (NR) for visualizing fat storage droplets in *C. elegans* and for elucidating the role of fat regulatory genes in nematode aging. From the data analysis we can conclude that there is no correlation between the levels of triacylglycerols and a specific pattern of NR staining. Thus, NR is not suitable for monitoring body fat in *C. elegans*. Staining of animals with SBB in combination with the direct assessment of triacylglycerols by TLC is more appropriate for this purpose.

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Finally I like to dedicate this thesis to my beloved parents and younger brother Dr. Shaibal for their loving and continuous support since my arrival in Canada.

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CHAPTER 1

INTRODUCTION

1.1 *What is Caenorhabditis elegans?*

Caenorhabditis elegans is a rhabditid nematode (Blaxter, 1998). Nematodes are a group of metazoan organisms that encompasses at least 20,000 described species including numerous parasites of plants and animals. Among the human parasites are the large gut roundworm *Ascaris lumbricoides*, the blood-sucking human hookworms, and the pork trichina worm. *C. elegans* is a small (approximately 1mm in length), non-parasitic, free-living nematode found in soil across most of the temperature regions of the world. (Hope,1999). For the development, *C. elegans* requires only a humid environment, ambient temperature, atmospheric oxygen and bacteria as food. Under laboratory conditions, *C. elegans* is maintained on agar plates seeded with the slowly growing *Escherichia coli* strain, OP50 (Stienranagle, 1999). Media must be supplemented with Cholesterol, which *C. elegans* cannot synthesize (kurzchalia and Ward, 2003). The optimal temperature of the maintenance of *C. elegans* is between 16°C and 25°C. *C. elegans* ontogenesis starts in the egg. The development of the nematode to the adult stage requires 3 days at 25°C (Lewis and Fleming, 1995). During the time, worms undergo several molts (without metamorphosis) called larval stages L1, L2,L3 and L4 (Fig 1.1.1). After reaching the adult stage, *C elegans* lays eggs for the first 3 to 4 days. The mean lifespan of *C.elegans* is approximately two weeks. In the absence of food or overcrowding, L2 nematodes can transform into special dauer larvae (Riddle, 1988). During this development stage, the nematode doesn't feed and can survive for several months. Three to four hours after the dauers are presented with food, they resume feeding and reach the L4 stage after 8 hours (Lewis and Fleming, 1995).

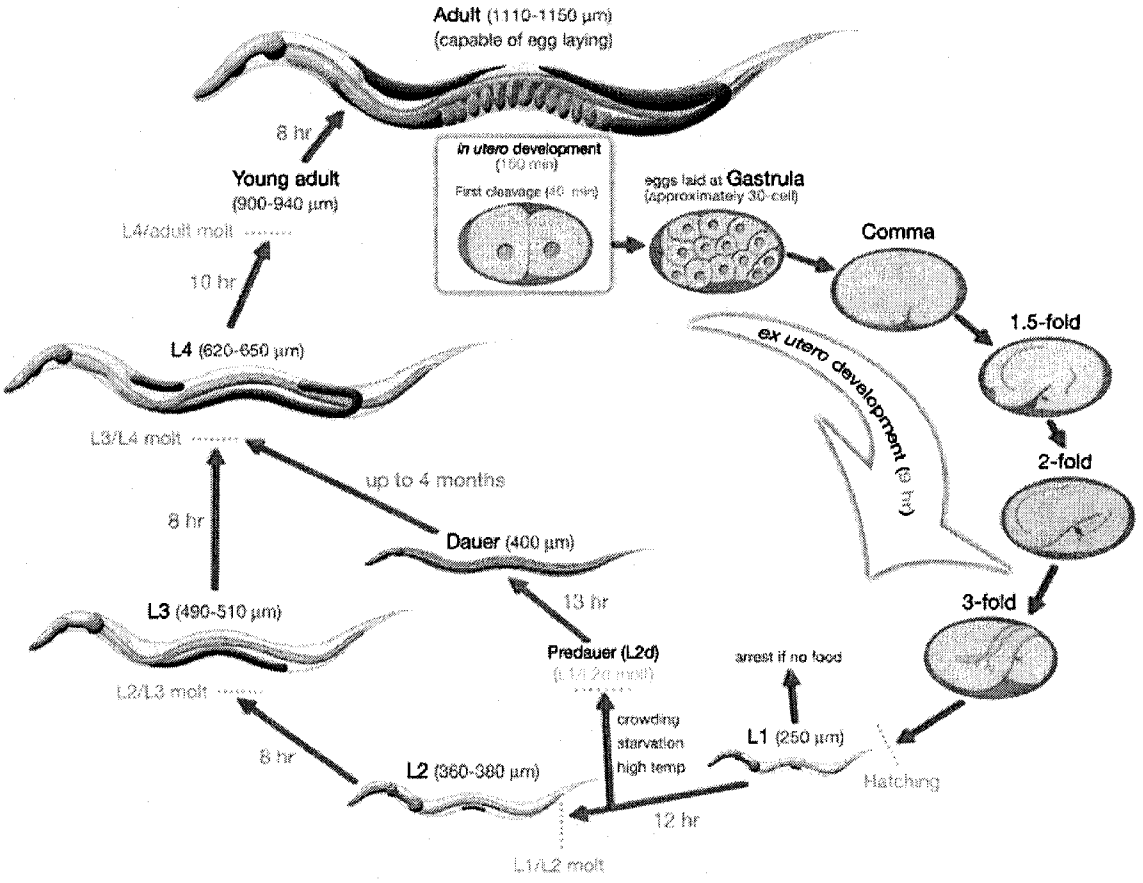


Figure 1.1.1. Life cycle of *C. elegans* at 22°C. 0 min is fertilization. Numbers in blue along the arrows indicate the length of time the animal spends at a certain stage. First cleavage occurs at about 40 min. post fertilization. Eggs are laid outside at about 150 min. post fertilization and during the gastrula stage. The length of the animal at each stage is marked next to the stage name in micrometers.

Source: http://www.wormatlas.org/handbook/anatomyintro/anatomyintro.htm

C. elegans has two sexes: self-fertilized XX hermaphrodites and XO males that differ in the arrangement of the gonad and in tail shape. The ratio between XX hermaphrodites and XO males in a wild-type population is 500:1 or less. The shape of animals does not change markedly in stages L1 through L3 and therefore they are monitored mainly by characteristic cell divisions. The entire cell lineage of *C. elegans* is known (Sulston et al., 1983). During postembryonic development, the number of somatic cells increases from 558 (hermaphrodite) or 560 (male) cells in the L1 stage to 959 (hermaphrodite) or 1031 (male) cells in adult worms (Lewis and Fleming, 1995). Almost one-third (302) of the Hermaphrodite cells are nerve cells (White *et al.*, 1986). The male has an additional 79 neurons. *C. elegans* can sense temperature, mechanical stimuli and a wide range of chemical stimuli (Hope, 1999).

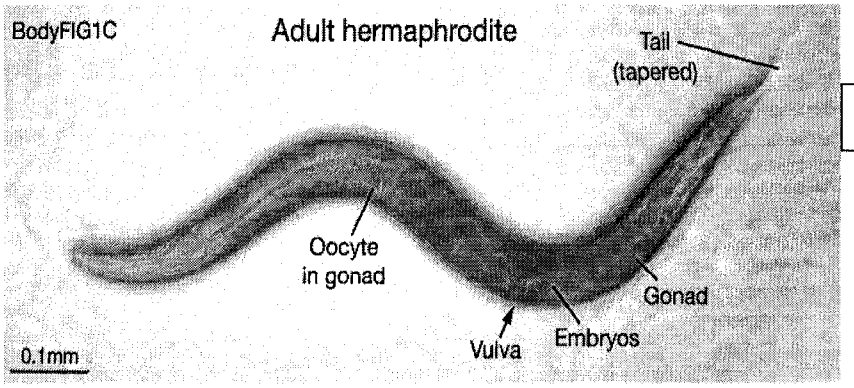


Figure 1.1.2 Adult Hermaphrodite

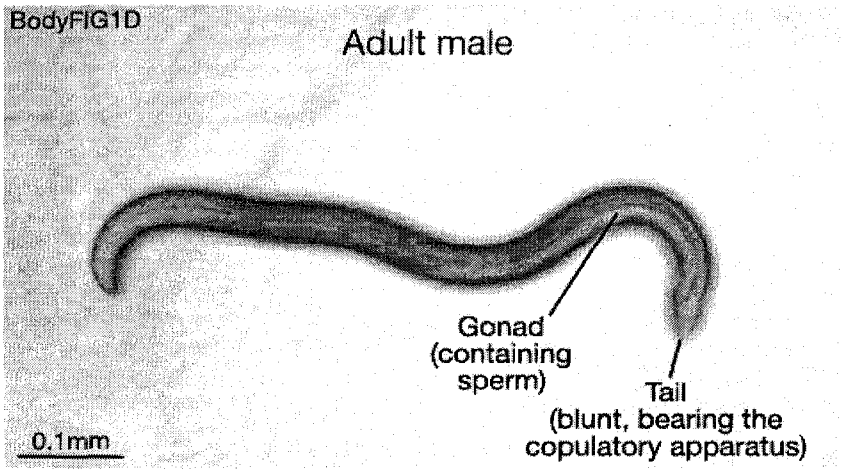


Figure 1.1.3 Adult Male

The genome is completely sequenced (The *C. elegans* Sequencing Consortium, 1998), and some 20,000 genes have been identified (Kim *et al.*, 2001). About 40% of these genes have apparent homologs in humans (strenberg, 2001). Almost all functional domains of human proteins are present in the worm.

1.2 Why *C. elegans* as a model organism for studying aging and development?

C. elegans provides several important advantages as a model system. First, the nematode is a genetically tractable organism with a sequenced genome and a well-studied lifecycle (Blaxter, 1998; Hope, 2001; Sternberg, 2001). Second, *C.elegans* has a relatively simple multicellular organization (Lewis and Fleming 1995; Hope 1999). The pedigrees of cells have been established from egg to adult organism (Huang and Sternberg 1995; Hope1999). Moreover, the transparent cuticle of the worm permits visualization of all cells, fluorescently tagged proteins and vital dyes within the live worm. Third, since the cell lineage, location, and synaptic connectivity of the *C. elegans* nervous system have been completely described (Blaxter 1998; Thomas and Lockery 1999), *C. elegans* should prove extremely useful to analyze the molecular basis underlying the impaired neuronal development and neurodegeneration observed in patients with peroxisomal disorders (Wanders and Tager, 1998; Wanders, 1999; Sacksteder and Gould, 2000; Wanders *et al.*, 2001). Lastly, although studies with cultured human fibroblasts have opened up the option of pre- or postnatal diagnosis of peroxisomal disorders (Wanders et al., 1996), they have not yet provided any prospect of therapy for these disorders. On the other hand, *C.*

elegans has been extensively used for the testing of various pharmaceutical agents and for drug discovery (Rand and Johnson, 1995; Thomas and Lockery, 1999).

1.3 What are peroxisomes?

Contemporary textbooks give us the following classical definitions of peroxisomes: "small vesicular compartments that contain enzymes utilized in a variety of oxidative reactions" (Alberts *et al.*,2002); "containers for enzymes involved in oxidative reactions" (Pollard and Earnshaw, 2002); "Peroxisomes contain at least 50 different enzymes, which are involved in a variety of biochemical pathways in different types of cells" (Cooper, 2000). Since the time when peroxisomes were first found and described in mouse kidney (Rhodin, 1954), and later also discovered in plants (Porter and Kaulfield, 1958), thousands of scientific papers have been published on the topic, and our understanding of peroxisomal functions and biogenesis has progressed significantly (Titorenko and

Rachubinski, 2001b). Interest in understanding peroxisomes is explained by findings showing peroxisomes are involved in a great variety of important cellular functions and are associated with a number of severe human diseases (Wanders et al., 2001).

1.4 Biochemical roles of peroxisomes

Microbodies, the class of subcellular compartments that includes the peroxisomes, the glyoxysomes of plants, the glycosomes of Trypanosomes and Woronin bodies in some fungi, are found in virtually all eukaryotic cells (Purdue and Lazarow, 2001). Microbodies have similar enzymatic properties, with some variations in their

methabolic pathways depending on the type of tissue or cell (Tolbert and Essner, 1981). This work is focused on peroxisomes - microbodies of eukaryotes from yeasts to metazoans, including mammals. It is well established that peroxisomes carry out the α- and β-oxidation of fatty acids and the decomposition of hydrogen peroxide. Depending on the cell type, peroxisomes also take part in the biosynthesis of plasmalogens, cholesterol and bile acids, polyunsaturated fatty acids (in metazoans), penicillin (in fungi), and lysine (in yeasts). Peroxisomes also compartmentalize the catabolism of amino acids, purines, polyamines and prostaglandins (in metazoans); the glyoxylate cycle (in fungi and yeasts); and methanol, ethanol and amine oxidation (in yeasts) (Lazarow and Moser, 1989; Purdue and Lazarow, 2001; Titorenko and Rachubinski, 2001a). In addition, peroxisomes take part in respiration. They consume oxygen while catabolizing different classes of fatty acids (Tolbert and Essner, 1981). The majority of all biochemical reactions happening in peroxisomes are catalyzed by enzymes located in the peroxisomal matrix. Due to their high concentration, some enzymes (*e.g.*, urate oxidase) can form a crystal core within the organelle (Tolbert and Essner, 1981). All peroxisomal proteins are encoded by nuclear genes and synthesized in the cytosol (Titorenko and Rachubinski, 2001b). The peroxisomal matrix is surrounded by a single, semi-permeable membrane. Morphologically, peroxisomes are very diverse. In rat liver, their shapes range from multiple isolated round vesicles to a single convoluted compartment consisting of a cup- shaped structure and torturously branched tubules (Purdue and Lazarow, 2001) or tubulo-reticular clusters (Schrader, 2001). Peroxisomes range in size from 0.1 to 1.5 μm (Tolbert and Essner, 1981). The number, size, protein composition and even the

shapes of peroxisomes vary not only depending on the organism and cell type but also on environmental conditions (Titorenko and Rachubinski, 2001a).

1.5 PEX genes and biogenesis of peroxisomes

In all organisms studied, as many as 32 different proteins that control peroxisome biogenesis and maintenance, called PEXes, are found (Table 1.5.1). In humans, there are 14 such proteins identified and designated as Pex1p, Pex2p, Pex3p, Pex5p, Pex6p, Pex7p, Pex10p, Pex11p, Pex12p, Pex13p, Pex14p, Pex16p, Pex19p and Pex26p (shaded in gray in Table 1.3.1). At least 13 of these genes, when mutated, cause lethal peroxisome biogenesis disorders in humans (Shimozawa et al., 2004).

PEX genes encode proteins belonging to various functional classes, including shuttling receptors, integral membrane proteins and ATPases (Fig. 1.5.1). The best studied PEX proteins are the receptors for peroxisomal protein import, Pex5p (Terlecky et al., 1995) and Pex7p (Marzioch et al., 1994). Both receptors are located in the cytosol when unbound by their cargo. They specifically recognize and bind proteins carrying peroxisome targeting signals (PTS). To date, PTS1 (recognized by Pex5p) and PTS2 (recognized by Pex7p) are the best defined (Rachubinski and Subramani, 1995). Another putative cytosolic receptor for peroxisomal proteins is Pex19p. Pex19p is able to recognize and bind the membrane PTS1 (mPTS1) motif found in peroxisomal membrane proteins such as Pex11p, Pex14p and PMP70 (Titorenko and Rachubinski, 2001b). The initial docking site for both the Pex5p-PTS1 and Pex7p-PTS2 complexes at the cytosolic surface of the peroxisomal membrane consists of two membrane-associated peroxins, Pex13p and Pex14p (Sacksteder and Gould, 2000; Titorenko and Rachubinski, 2001b; Fig. 1.5.1). After

docking, the PTS1 and PTS2 receptors, together with their cargoes, are transferred to other membrane-associated components of the import machinery consisting of the peroxins Pex2p, Pex10p and Pex12p (Hettema et al., 1999; Terlecky and Fransen, 2000). These components function in the translocation of cargo proteins, either alone or together with their receptors, across the peroxisomal membrane into the peroxisomal matrix (Fujiki, 2000; Subramani et al., 2000). In human cells, Pex2p, together with two cytosolic peroxins, Pex1p and Pex6p, may act to recycle the PTS1 receptor to the cytosol (Fujiki, 2000; Gould and Valle, 2000; Sacksteder and Gould, 2000). Interestingly, in humans, alternative splicing of the *pex5* gene results in the generation of the short (Pex5pS), and long (Pex5pL) forms of the PTS1 receptor. Whereas Pex5pS is implicated in PTS1 import only, Pex5pL is also involved in PTS2 import, acting in a complex with Pex7p (Braverman et al., 1998; Titorenko and Rachubinski, 2001b). The peroxin Pex16p, together with Pex3p, is an integral peroxisomal membrane protein that is inserted into the lipid bilayer upon binding to Pex19p (Sacksteder et al., 2000). This step seems to be required for the subsequent targeting of other peroxisomal membrane proteins in a Pex19p-dependent manner (Gould and Valle, 2000; Titorenko and Rachubinski, 2001b). The recently discovered peroxin Pex26p recruits the Pex1p-Pex6p AAA-ATPase complexes to peroxisomes via its binding to Pex6p (Matsumoto, et al., 2003). Finally, to maintain a stable population of peroxisomes in the dividing cell or proliferating tissue, effective control of peroxisome number is necessary. Pex11p, and its functional homologs Pex25p and Pex27p, are thought to be positive regulators of peroxisome division in the yeast *Saccharomyces cerevisiae* (Erdmann and Blobel, 1995; Smith et al., 2002; Tam et al.,

2003; Rottensteiner et al., 2003). In humans three isoforms of Pex11p, α, β, and γ are known to exist (Schrader et al., 1998; Tanaka et al., 2003), but their individual roles remain unknown.

1.6 Peroxisome targeting signals

PTSs are distinct and conserved among eukaryotes (de Hoop and AB, 1992; Blattner *et al.*, 1995). Interestingly, even viral proteins have been found to be imported into peroxisomes and to use targeting signals identical to eukaryotic PTSs (Mohan and Atreya, 2003). The majority of peroxisomal matrix proteins are targeted by PTS1. PTS1 is a carboxyl-terminal tripeptide with the consensus sequence –(S/C/A)(K/R/H)(L/M) (Subramani, 1993). The efficiency of various tripeptide combinations to function as PTS1s can be enhanced by adjacent "accessory" sequences (Purdue et al., 1996; Mullen et al., 1997; Mizuno et al., 2002; Klein et al., 2002). SKL has been found to be the most effective PTS1, and it functions in most organisms (Swinkels et al., 1992). A recent re-evaluation of the PTS1 motif (Neuberger et al., 2003a) found that the PTS1 signal is comprised of the 12 carboxyl-terminal residues of the targeted protein. The peroxin Pex5p that mediates PTS1-dependent transport is a shuttling receptor. After recognition and delivery of the cargo across the peroxisomal membrane, Pex5p is recycled to the cytosol. Dysfunctional Pex5p has been shown to cause the human peroxisome biogenesis disorder, Zellweger syndrome (Wanders, 1999; Gould and Valle, 2000). A small subset of peroxisomal matrix proteins is targeted by PTS2. The PTS2 is located at the amino terminus of proteins at variable distances from the initiating methionine. The amino terminus of

PTS2-containing proteins is sometimes cleaved from the protein molecule inside the peroxisome by a specific protease (de Hoop and AB, 1992). The consensus PTS2 was first defined as a nonapeptide with the sequence –RLX5(H/Q)L– (de Hoop and AB, 1992), which was later modified to –(R/K)(L/V/I)X5(H/Q)(L/A)– (Rachubinski and Subramani, 1995). Defective PTS2-dependent import results in the peroxisome biogenesis disorder, rhizomelic chondrodysplasia punctata (Wanders, 1999; Gould and Valle, 2000). Some peroxisomal matrix proteins contain neither a PTS1 nor a PTS2 but instead have internal targeting signals, sometimes called PTS3, which remain poorly defined (Kragler et al., 1993; Elgersma et al., 1995). Often these proteins use the PTS1-dependent targeting machinery for their import into peroxisomes (Elgersma et al., 1995). Similarly, the mPTS1 is not well defined, but two of the reported consensus sequences for the mPTS1 are (K/R)(K/R)X3-7(T/S)X2(D/E) and (Y)X3(L)X3(K/Q/N) (Titorenko and Rachubinski, 2001b).

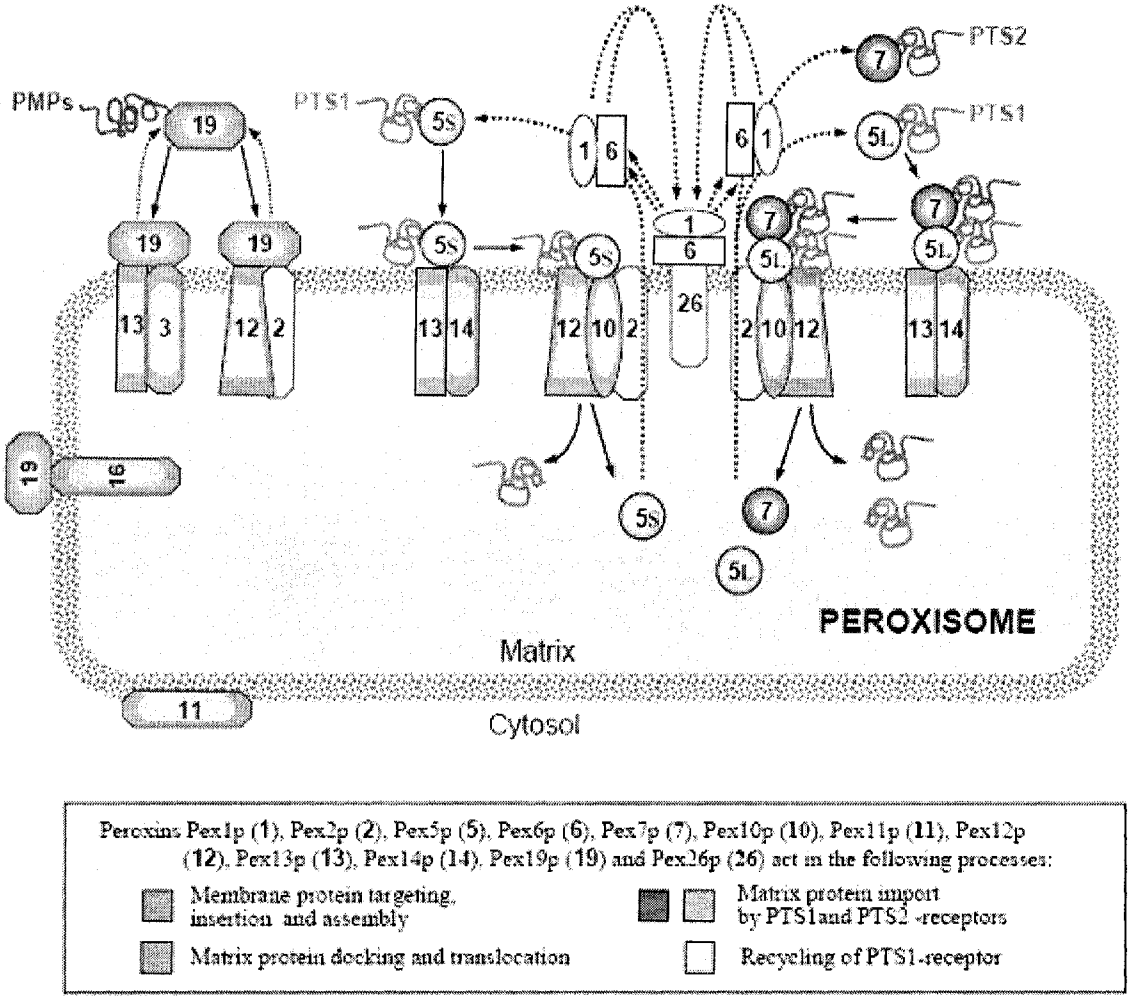


Figure 1.5.1 Peroxisomal metabolic pathways and peroxins.

Table 1.5.1 Peroxins and their functions in human and other organisms

Peroxin[1]	Function in human or other organism	Sp[2]
Pex1p	Putative AAA ATPase. Interacts with Pex6p and is required for peroxisomal protein import, genetic mutation is detected in patients with Zellweger syndrome, neonatal adrenoleukodystrophy, and infantile Refsum disease	*Hs Mm Sc Ca*
Pex2p	Peroxisomal membrane protein 3 (PXMP3). Involved in protein-peroxisome targeting and peroxisome biogenesis, increased expression may be therapeutic for Zellweger syndrome	*Hs Mm Sc*
Pex3p	A peroxisomal protein required for early stages of peroxisomal biogenesis. Mutations in the corresponding gene cause Zellweger syndrome. Docking factor for Pex19p	*Hs Mm Rn Sc Ca*
Pex4p	Ubiquitin-conjugating enzyme and a protein required for peroxisomal biogenesis	*Sc Pa Ca*
Pex5p	Peroxisome receptor 1 (PXR1), targets proteins to the peroxisome via PTS1 signal recognition. Mutations of the gene cause neonatal adrenoleukodystrophy, Zellweger syndrome, and infantile Refsum disease	*Hs Mm Sc Sp Ca*
Pex6p	Peroxisome assembly factor 2, AAA ATPase family member that interacts with Pex1p and is involved in peroxisomal protein import and peroxisome assembly; mutations correlate with peroxisome biogenesis disorders and complementation group C Zellweger syndrome	*Hs Mm Rn Sc*
Pex7p	Peroxisome receptor 2, a peroxisomal import receptor for proteins containing peroxisomal targeting signal 2 (PTS2). Mutations in PEX7 gene cause rhizomelic chondrodysplasia puncta	*Hs Mm Sc Sp Ca*
Pex8p	Peroxin involved in protein import into peroxisomes	*Sc*
Pex9p	Peroxin involved in protein import and peroxisome enlargement	*Yl*
Pex10p	Peroxin has a C3HC4 zinc finger RING motif, interacts with Pex12p, required for import of peroxisomal matrix proteins. Genetic mutation of Pex10p is detected in patients with Zellweger syndrome and neonatal adrenoleukodystrophy. Involved in import of tryptophanyl (W) tRNA synthetase predicted to be mitochondrial	*Hs Sc*
Pex11p	Peroxisomal biogenesis factor 11α. May regulate peroxisome abundance in response to external stimuli; may recruit ADP-ribosylation factor (ARF) and coatomer; may mediate peroxisome biogenesis from existing peroxisomes	*Hs Mm Rn Sp Sc*
	Peroxisomal biogenesis factor 11β. A peroxisomal integral membrane protein that is involved in regulating peroxisome proliferation and abundance through a multistep process	*Hs Mm Rn Ca*
	Peroxisomal biogenesis factor 11γ. An putative integral peroxisomal membrane protein that may have function in peroxisome division or homeostasis	*Hs Mm Rn*
Pex12p	An integral peroxisomal membrane protein that interacts with PXR1 and Pex10p and acts in peroxisomal matrix protein import downstream of receptor docking. Mutations in the zinc ring domain of Pex12p result in Zellweger syndrome	*Hs Mm Rn Sc*
Pex13p	A peroxisomal membrane protein, acts in peroxisomal matrix protein import, receptor docking, and biogenesis; deficiency is associated with peroxisome biogenesis disorder complementation group H	*Hs Mm Sc Ca*

[1] Human peroxins are shaded in gray.

[2] *Sp, species; **Hs**, Homo sapiens; **Mm**, Mus musculus; **Rn**, Rattus norvegicus; **Ca**, Candida albicans; **Sc**, Saccharomyces cerevisiae; **Sp**, Schizosaccharomyces pombe; **Yl**, Yarrowia lipolytica; **Pp**, Pichia pastoris; **Nc**, Neurospora crassa.*

Pex14p	A peroxisomal membrane protein that provides a docking target for PXR1 and is required for peroxisomal import of PTS1 and PTS2 containing proteins, altered expression is associated with neuroblastoma	*Hs Mm Rn Ca*
Pex15p	Peroxisomal integral membrane protein required for peroxisome assembly, promotes peroxisomal membrane association of Pex6p	*Sc*
Pex16p	Peroxisomal integral membrane protein involved in the initial stages of peroxisomal membrane formation during peroxisomal biogenesis; gene mutations cause Zellweger syndrome of the complementation group IX	*Hs Mm*
Pex17p	Peroxisomal peripheral membrane protein required for peroxisome biogenesis	*Sc Pp Ca*
Pex18p	Peroxin involved with Pex21p in Pex7p-mediated peroxisomal protein targeting	*Sc*
Pex19p	Peroxisomal farnesylated protein, peroxisomal protein that binds several peroxisomal membrane proteins (PMP), involved in early stages of PMP import and peroxisomal biogenesis; deficiency in Pex19p is associated with Zellweger syndrome complementation group J	*Hs Mm Sc Ca*
Pex20p	Peroxin that functions together with pex7p in PTS2-dependent protein Import	*Nc*
Pex21p	Peroxin, involved with Pex18p in Pex7p-mediated peroxisomal protein targeting, enhances the seryl-tRNA aminoacylation activity of seryl-tRNA synthetase (Ses1p) *in vitro*	*Sc*
Pex22p	Peroxisomal matrix protein import	*Sc Pp*
Pex23p	Peroxin, required for import of matrix proteins	*Yl*
Pex24p	Peroxin. Integral peroxisomal membrane protein	*Yl*
Pex25p	Peroxisomal membrane-associated protein involved in peroxisome biogenesis and peroxisomal protein import, required for regulation of peroxisome size and maintenance	*Sc*
Pex26p	Pathogenic peroxin Pex26p, a peroxisomal membrane protein. Recruits the Pex1p-Pex6p AAA ATPase complexes to peroxisomes, binds Pex6p; loss of function mutation in the corresponding gene correlates with peroxisome biogenesis disorder CG8 (CG-A)	*Hs*
Pex27p	Protein, involved in regulating peroxisome size and number	*Sc*
Pex28p	Integral peroxisomal membrane protein, required for normal peroxisome morphology and distribution	*Sc*
Pex29p	Integral peroxisomal membrane protein, required for normal peroxisome morphology and distribution	*Sc*
Pex30p	Integral peroxisomal membrane protein, functions as a negative regulator of peroxisome size	*Sc*
Pex31p	Integral peroxisomal membrane protein, functions as a negative regulator of peroxisome size	*Sc*
Pex32p	Integral peroxisomal membrane protein, functions as a negative regulator of peroxisome size	*Sc*

1.7 Peroxisomal metabolic pathways and peroxins in *C. elegans* development

The PTS2 pathway for the peroxisomal import of the matrix priteins which is found in all organisms, has been shown to be absent in *C. elegans*- instead these proteins are targeted to the peroxisome by a PTS1. Due to the lack of PTS2 import machinery and the great abundance of PTS1-dependent import machinery may be significantly higher in *C. elegans* than other organisms. Through the RNAi experiment it has been

shown that the cytosolic shuttling receptors for the peroxisomal sorting of peroxisomal membrane proteins and PTS1-targeted proteins (Pex19p and Pex5p, respectively) are essential for the development of *C. elegans* (Petriv, O. I. *et al.*, 2002). Similar experiment also showed that components of docking and translocation machineries for PTS1-containing matrix proteins (Pex13p and Pex12p, respectively) are required for the normal development of the nematode.

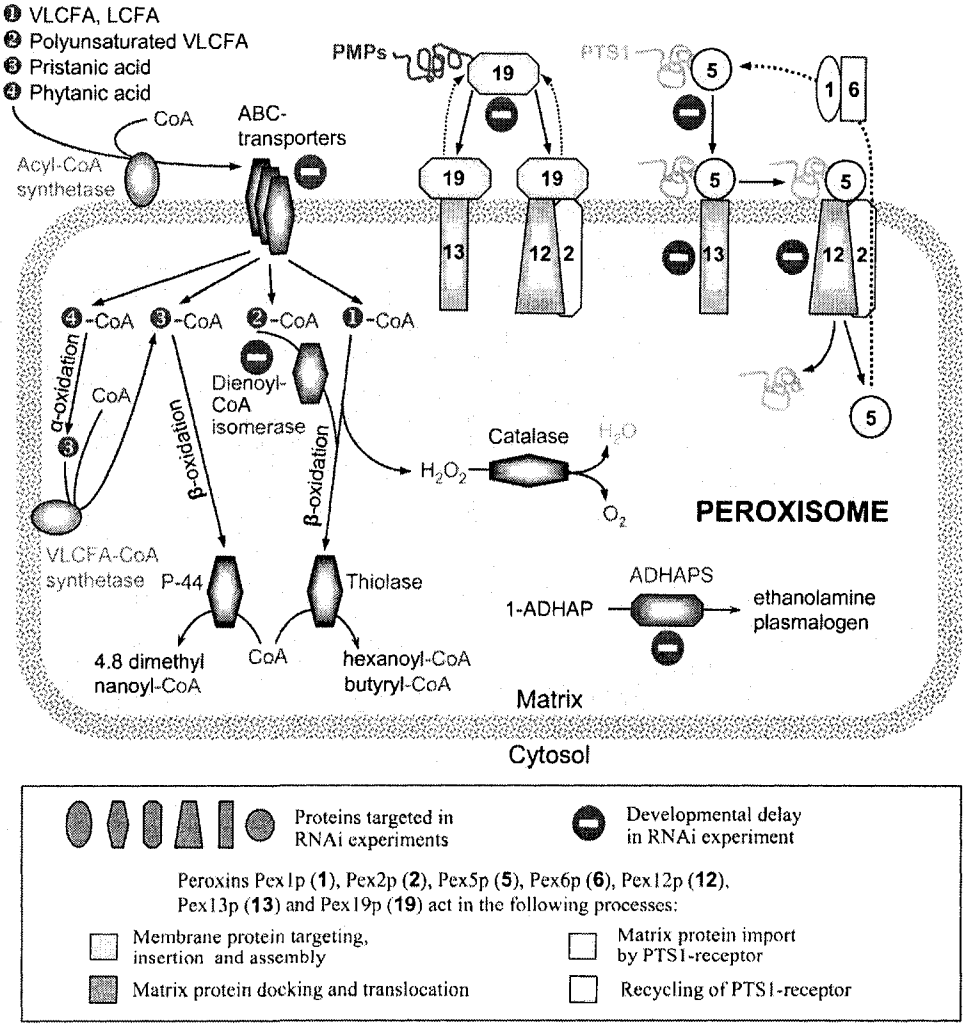


Figure 1.7.1 Peroxisomal metabolic pathways and peroxins in *C. elegans* development

Source: Petriv, O. I. et al. Physiol. Genomics 10: 79-91 2002

1.8 Theories of Aging

Unlike most biological processes, the endpoint of the aging process is degenerative in nature, which has suggested that the rate of aging is set by something that damages cells rather than something more elegant, such as the cell cycle machinery. Therefore, researchers have long been tabulating physiological and structural differences between old and young cells and postulating that one or more of these differences are actually the cause of aging. The problem with this approach is that any of the correlates of aging could be a downstream process by which a central regulatory mechanism, perhaps a more elegant mechanism, brings about cell senescence. Specific hypotheses are collectively termed the "theories of aging" (Finch 1990). Leading theories are discussed below.

1.8.1 Oxidative Damage

One theory posits that aging is caused by oxidative damage. Since different species have different life spans, this model implies that long-lived organisms are relatively more resistant to oxidative damage; e.g., they may have more effective ways of scavenging reactive oxygen species. Conversely, mutations that increase osidative damage can shorten lifespan. In *C. elegans*, mutations in ctl-1, a cytosolic catalase, shorten life span and prevent the life-span extension of *daf-2* mutants (Taub,J. *et al.* Nature 1999). *ctl-1* mutants seem to age more rapidly than normal, because they accumulate age-associated lipofuscin granules precociously. *ctl-1* expression also increases in *daf-2* mutants (Taub,J. et al. Nature 1999), as does expression of *sod-3*, which encodes a Mn-superoxide dismutase (SOD) (Vanfleteren J.R. et al 1995,

Ishii,N et al, Nature 1998). In addition, a mutation in *mev-1*, which encodes a subunit of the mitochondrial enzyme succinate dehydrogenase, accelerates the aging process (by lipofuscin granule accumulation) and shortens life-span. This mutant exhibits increased sensitivity to oxygen. Recently, a drug known as an antioxidant was shown to extend the life span of *C. elegans* up to 50% (Melov, S. et al, Science 2000).

1.8.2. Telomere Shortening

The telomeres of cultured mammalian cells undergoing senescence become shorter with consecutive cell divisions. This has suggested the possibility that telomere shortening causes aging, for example, by perturbing the expression states of genes located near the ends of chromosomes (Harley et al. 1990; Allsopp et al. 1992). Although telomere shortening may play a part in limiting the life span of cells that continue to divide, it seems unlikely to contribute to the aging process that occurs in cells that are postmitotic. The aging process is not limited to dividing cells. For example, in humans, neither muscle cells nor central nervous system neurons divide during adulthood, yet both exhibit progressive morphological signs of age during senescence. In *C. elegans*, senescence occurs without cell division, since the somatic cells of adults are post mitotic.

1.8.3. Progeny Production

Evolutionary theory posits that the rate of aging is set indirectly as a consequence of selection for reproductive success. Under conditions in which resources are limited, these resources are invested in producing progeny, rather than in maintaining the

survival of older, reproductively less fit, individuals. (If resources were not limiting, it might be possible for a species to evolve high levels of fecundity as well as a long life span.) This model is supported by the observation that some species, such as certain salmon, are programmed to undergo rapid senescence following reproduction. Some theorists also make a related but distinct assumption that there is likely to be a trade-off between fecundity and life span within a single individual; i.e., if its energy is expended on production of progeny, it will not be available for longevity. If the trade-off theory is true, one might expect a decrease in progeny production to cause increased longevity. Implicit in this hypothesis is the idea that an individual has a fixed amount of energy to use during its life span.

1.8.4. Metabolic Rate

Another theory of aging states that the length of life span is controlled by the rate of metabolism. The faster a species burns calories, the more rapidly it ages. In fact, there is a correlation between metabolic rate and life span in certain groups of animals, such as birds and mammals (excluding primates). However, the many striking exceptions to this rule argue strongly that metabolic rate cannot be the sole determinant of the rate of senescence.

1.8.5. Regulation by an Aging Program

Unlike the theories described above, which are stochastic in nature, an alternative theory is that the aging process is programmed in some way. This theory suggests that life span is determined by a timing mechanism that controls and coordinates the rates

of aging between different tissues in an organism. In principle, such a mechanism could also link the process of aging to other age-related events, such as puberty and menopause. This rate-setting mechanism would be influenced by environmental conditions and by gene activities that influence specific age-related processes (e.g., balding in humans). This model is attractive because it can explain the remarkably similar qualities shared by young, middle-aged, and old animals of many species with very different life spans. It can also account for the rapid, hormonally controlled onset of aging that occurs after reproduction in certain species of salmon and in marsupial mice (review, Russell 1987) and for the coordinated acceleration of many age-dependent phenotypes associated with aging in humans afflicted with the accelerated-aging progeria syndromes. Finally, it can explain how mutations in single genes, described below, can profoundly affect the rate of aging of an entire organism.

If there is an aging program, then there must be a regulatory mechanism that can control the rate at which the timing mechanism runs the aging process (Kenyon 1996). It is necessary to postulate this in order to explain the striking differences in life spans of different species and in aging mutants. Note that there may be a fine line between this theory and some of the other aging theories; e.g., the master regulator could be an enzyme that repairs oxidative damage at a fixed, species-specific rate. Alternatively, there could be a distinct molecular timer that is molecularly unlike anything known to be degenerative in nature, which, in turn, would bring about senescence. An example of such a timer is the *lin-14* antisense RNA timer that patterns stage-specific lineage patterns in *C. elegans* (Lee et al. 1993; Wightman et al. 1993).

1.8.6. Caloric Intake

Lifespan is regulated by diverse genetic and environmental factors (Kenyon, 2005; Kirkwood, 2005; Masoro, 2005). One of the most robust environmental manipulations of lifespan is dietary restriction (DR) (Masoro, 2005). Vertebrates such as rats can live up to 60% longer than normal if they are fed a diet low in calories. This is remarkable, since these "Dietary restricted" individuals are robust and healthy and have normal rates of metabolism. Dietary restricted animals do not produce progeny. However, once food is restored, they become fertile, even if they have reached an age at which well-fed rats would be post reproductive. Dietary restriction enhances resistance to a variety of stresses, such as heat and oxidative stress and delays onset of age-related diseases in murine models for human cancer and diabetes (Masoro, 2005). Several genetic components of the dietary restriction hasbeen identified. Foremost among these are the NAD-dependent protein deacetylase, SIR2 and other SIRTUIN family members (Guarente, 2005; Sinclair 2005), and TOR (Target of rapamycin), a protein kinase that co-ordinates cell growth in response to nutrient availability (Vellai *et al.,* 2003; Kapahi et al., 2004; Kaeberlein *et al.,* 2005).

1.9 Aging in *C. elegans*

1.9.1 Life Spans of Well-fed Adults

Even in highly inbred, presumably homozygous strains of *C. elegans*, not all individuals die at the same time; therefore, mean life span is determined by examining populations of animals. In a survey of wild strains of *C. elegans*, Johnson

and Hutchinson (1993) found that hermaphrodites of the strains they examined had similar life spans. However, in some strains, males had shorter life spans than hermaphrodites, whereas in other strains, males had longer life spans. More recently, **D. Gems and D.L. Riddle (pers. comm.)** have shown that different isolates of the N2 strain of *C. elegans* have significantly different life spans, ranging from 12 to 18 days at 20°C. It is not clear whether strain-specific differences in life spans are due to changes in a single gene or in multiple genes. Whatever the case, because the life span of *C. elegans* is somewhat strain-specific, it is important to compare the life spans of candidate mutants with those of their direct parents. In addition, in cases where mutations isolated a relatively long time ago are found to have life spans that differ from that of N2, it is safest to isolate new alleles and compare their life spans with those of the direct parental line, or at least to show that the life-span phenotype is linked genetically to the allele in question.

Unfortunately, even when a single strain is analyzed and all known variables are held constant; some degree of variability can still exist between the life-span curves observed in different experiments. This means that in all life span studies, controls must be carried out in parallel, and apparent differences between strains must be tested for reproducibility.

1.9.2 Environmental Influences

1.9.2.*1 Temperature*

The life span of *C. elegans* is influenced by temperature. For example, Klass (1977) found that the mean life span of *C. elegans* cultured in liquid media was 23 days at

16°C, but 9 days at 25°C. One might assume that the different rates of growth at different temperatures are a direct consequence of the intrinsic thermodynamic properties of chemical reaction rates. However, **Wong et al. (1995)** found that mutants defective in *clk* (clock) genes are unable to adjust their rate of growth to changes in temperature. Wild-type animals actively decrease or increase their rate of development in response to temperature changes. In contrast, *clk* mutants cultured to the two-cell stage at 15°C or 25°C and then shifted to 20°C were unable to either increase or decrease their rate of development effectively. Thus, the *clk* genes are required to reset the rate of development in response to changes in temperature.

1.9.2.2 Food

1.9.2.2.1 **Larval Arrest.**

If L1 larvae hatch in the absence of food, their growth is arrested. When they resume feeding, they grow normally to adulthood, and their subsequent adult life span is the same as that of a worm that hatches in the presence of food (**Johnson et al. 1984**).

1.9.2.2.2 **Dauer Formation.**

C. elegans has a discrete response to food limitation early in life: It enters an alternative developmental stage, the dauer. Unlike the normal feeding state, the dauer can live for many months. Dauers are an alternative L3 state. Dauer formation is potentiated by food limitation and high temperature. The dauer state is induced by a constitutively produced dauer pheromone, whose concentration increases as the animals crowd together around the remaining food. The dauer state can be induced

only in L1 and early L2 larvae. The dauer differs from the adult in many ways. Its growth is arrested, and it contains intestinal granules that are thought to store food (dauers appear dark for this reason). It is encased by a dauer-specific cuticle that is relatively resistant to dehydration. Dauers have reduced metabolic rates (O'Riordan and Burnell 1989, 1990), elevated levels of superoxide dismutase, and are relatively resistant to oxidative stress (Anderson 1982; Larsen 1993; Vanfleteren 1993). They also have elevated levels of several heat shock proteins (Dalley and Golomb 1992; **R. Shmookler Reis, pers. comm.**). Animals that exit from the dauer state resume growth and have subsequent life spans that are similar to those of animals that have not arrested at the dauer stage (Klass and Hirsh 1976).

1.9.2.2.3 ***Caloric Restriction.***

As discussed above, the life spans of healthy vertebrates can be extended dramatically by caloric restriction. Likewise, Klass (1977) reported that the life span of *C. elegans* can be extended by about 50% by growth in liquid cultures with a relatively low concentration of bacteria.

1.9.2.3 Reproduction

The most straightforward way of determining whether progeny production influences life span is to sterilize animals and measure their life spans. This has been done in several ways. First, the life spans of worms unable to make sperm have been examined and found to be similar to those of wild type. These include *fer-15* mutants, which produce defective sperm (Klass 1983; Friedman and Johnson 1988a), and *fem*

mutants, which produce oocytes instead of sperm (Kenyon et al. 1993). In addition, life span is not affected by ablation of the gonad and germ cells (Kenyon et al. 1993). Therefore, in *C. elegans*, the production of progeny does not affect life span.

One sterile mutant, *spe-26*, has been reported to have an extended life span (Van Voorhies 1992); however, in this case, the effect on life span is probably unrelated to the sperm defect, since the magnitude of the life-span extension in these mutants is not correlated with the degree of sterility in different *spe-26* alleles (**S. Ward, pers. comm.).**

It is not clear yet whether the mating itself affect life span. Van Voorhies (1992) found that mating decreased the life span of males. In fact, male life span appears to be shortened by mating, or attempted mating, with either males or hermaphrodites. When males are grown singly, their life spans are increased, an effect not seen with hermaphrodites **(D. Gems and D.L. Riddle, pers. comm.)**. In the case of hermaphrodites, Van Voorhies (1992) found that mating did not affect the life span. However, in a subsequent, more extensive study, mating with males was found to reduce hermaphrodite life span by up to one half (Gems and Riddle 1996). The act of copulation itself seems to decrease life span, because sterile males that attempt to copulate also accelerate the death of hermaphrodites.

1.9.2.4 *Oxygen Levels*

Changes in oxygen concentration perturb the life span of *C. elegans* (Honda et al. 1993). When animals are cultured in a high concentration of oxygen, their life spans

are shortened, and when they are cultured in a low concentration of oxygen, their life spans are lengthened. Furthermore, a strain with reduced levels of superoxide dismutase was more sensitive to the effects of oxygen. These findings suggest that oxidative damage can accelerate the aging process in *C. elegans* and argue that in nature, oxygen levels have a role in setting the normal life span.

1.9.2.5 *DNA Damage*

Hartman et al. (1988) questioned whether the effectiveness of DNA repair systems might determine the life expectancy of *C. elegans*. To address this issue, they asked whether there was a correlation between life span and sensitivity to three different DNA-damaging agents in recombinant inbred strains (described below) whose mean life spans range from 13 to 30 days. They found that there was no such correlation. This finding argues that the efficacy of DNA repair is not a limiting factor in determining the life span of wild-type *C. elegans*.

1.9.2.6 Relationship between Programmed Cell Death and Senescence

Mutations in *ced* genes, which alter programmed cell death, do not affect life span **(R. Horvitz, pers. comm.)**. It thus seems likely that the regulation of programmed cell death (apoptosis) is fundamentally different from the process of organismal senescence and aging.

1.10 The Search for Genes that Control the Rate of Aging in *C. elegans*

1.10.1 Life-span Mutations and Dauer Formation

Dauer larvae are developmentally arrested, sexually immature, resistant to starvation and desiccation, and long-lived. Dauer formation is normally induced when the animals reach high population density during periods of food limitation. When food is restored, dauers resume growth and become adults that have normal 2–3-week life spans (Klass and Hirsh 1976). Mutations in two genes that regulate dauer formation (Riddle et al. 1981) can also increase the life spans of active, fertile adults. These genes are *daf-2* (Kenyon et al. 1993) and *daf-23* (Larsen et al.1995). The most severe mutations at either locus produce nonconditional dauer larvae constitutively (Gottlieb and Ruvkun 1994; Riddle 1988). In addition, reduction of function of *daf-2* or *daf-23* gene activities can produce a dramatic extension of life span. The *daf-2(e1370)* mutation is a temperature-sensitive dauer-constitutive mutation. However, when these animals are cultured at the permissive temperature or shifted to the non-permissive temperature following the larval decision point for dauer formation, they become adults that live more than twice as long as wild type. The *daf-23* alleles identified by their dauer phenotype are all non-conditional mutations. However, the mutants are maternally rescued for dauer formation (Gottlieb and Ruvkun 1994), and these maternally rescued animals are also long-lived non-dauer adults (Larsen et al. 1995). The *daf-23* and *age-1* mutations have now been shown to be allelic, and the gene is now referred to as *age-1* (Malone et al. 1996; Morris et al. 1996). Because these *daf-2* and *age-1* mutations are thought to reduce gene activity, one can infer that in wild-type animals, these genes act to accelerate the aging process. The *age-1* gene has now

been cloned and found to encode a phosphatidylinositol-3-OH kinase family member (Morris et al. 1996). This implies that a phosphatidylinositol signaling pathway involving the *age-1* kinase regulates dauer formation and life span. Morris et al. suggest that the gene may be involved in neuroendocrine signaling. So far, this is the only *C. elegans* life-span gene that has been cloned. Its site of action is unknown.

The ability of *daf-2* and *age-1* mutations to extend life span is dependent on the activities of two additional genes, *daf-16* and *daf-18* (Kenyon *et al.,* 1993;Dorman *et al.*, 1995; Larsen et al. 1995). Both of these genes are thought to act downstream from *daf-2* and *age-1* in the dauer pathway because they are required for dauer formation in *daf-2* or *age-1* mutants. *daf-16* mutations do not have a marked effect on life span (Kenyon et al. 1993); however, they may cause a small but consistent decrease in life span (Larsen et al. 1995). The *daf-18* gene is defined by a single allele (Riddle et al. 1981) that also causes some animals to have grossly misshapen midbody regions and to die prematurely from internal hatching (Dorman et al. 1995; Larsen et al. 1995), and thus no firm conclusions can be drawn at this time about the wild-type role of *daf-18* in either dauer formation or life span.

In summary, four genes that act in the dauer pathway, *age-1*, *daf-2*, *daf-16*, and probably *daf-18*, also act in a single pathway for life-span control: In the wild type, *daf-2* and *age-1* activities shorten life span, possibly by down-regulating *daf-16* and *daf-18*. In contrast, *daf-16* and possibly *daf-18* function in a process that extends life span. In the regulation of the dauer state, *daf-16* activity is required for maintenance

of the dauer; it will be interesting to learn whether its activity is required continuously to maintain adult youthfulness in *daf-2* and *age-1* mutants.

Not all genes in the dauer pathwa y affect adult life span. Riddle desc ribed, the regulation of dauer formation is complex which comprises at least two parallel regulatory pathways: The group-1 pathway appears to involve the neuron ASJ and a signaling pathway similar to the photoreception signaling pathway (Thomas et al. 1993; **J. Thomas; C. Bargmann; both pers. comm**). The group-2 pathway involves a transforming growth factor-β (TGF-β)-like signaling cascade that may affect the ADF, ASI, and ASG neurons (Georgi et al. 1990; Bargmann and Horvitz 1991b; Estevez et al. 1996; Thomas et al. 1993; Ren et al. 1996; Schackwitz et al. 1996). It seems likely that the *daf-2* and *age-1* genes act downstream from these two pathways, because mutations that reduce or eliminate the functions of either *daf-2* or *age-1* trigger dauer formation in animals in which the group-1 or group-2 pathways have been put in the dauer-repressing states by mutation or by ablation of sensory neurons that act in these pathways (Thomas et al. 1993; **J. Thomas, pers. comm.)**.

Surprisingly, *daf-2* and *age-1* mutations are the only dauer-constitutive mutations that appear to extend life span. Mutations in neither the group-1 pathway (Kenyon et al. 1993) nor the group-2 signaling pathway (Kenyon et al. 1993; Larsen et al. 1995) affect life span. The discrepancy between these upstream mutants and the life-span mutants (*daf-2* and *age-1*) is particularly striking when one considers the fact that *daf-2(ts)* mutants have extended life spans even at temperatures too low to induce dauer formation. At these low temperatures, mutations in the group-1 and -2 genes do not

extend life span. Moreover, group-1 and -2 mutants have normal life spans even when they are cultured at high temperature, which is capable of inducing dauer formation very efficiently.

We know that reduction of *daf-2* or *age-1* function induces life-span extension, and thus these findings suggest that mutations in these two upstream branches of the pathway do not cause *daf-2* or *age-1* levels to fall when animals are shifted to 25°C late in development. One way to explain this is to postulate that group-1 and group-2 pathways only function in young larvae, where they specifically regulate entry into the dauer stage (Kenyon et al. 1993).

Dauer formation by dauer-constitutive mutations in the group-1 and group-2 pathways requires activity of the *daf-12* gene (Riddle et al. 1981; Vowels and Thomas 1992). When *daf-12* is missing, these mutants are unable to enter the dauer state. Is *daf-12* activity required for the life-span extension of *daf-2* mutants? When certain *daf-2*;*daf-12* double mutants are placed under dauer-inducing conditions, they go through the first stage of dauer formation, entering an alternative L2 state called L2d; however, instead of progressing to the dauer state, they arrest development as young larvae (Vowels and Thomas 1992). A plausible explanation for this is that in the absence of *daf-12*, animals cannot become dauers, but in the absence of *daf-2* activity, they cannot progress to adulthood either (Vowels and Thomas 1992; Larsen et al. 1995). It is possible to ask whether *daf-12* activity is required for the longevity of *daf-2* mutants by shifting *daf-2*, *daf-12* double mutants to the non-permissive temperature when they have passed the dauer decision point or by growing animals at 20°C.

Under these conditions, one can observe different, allele-specific effects on life span (Dorman et al. 1995; Larsen et al. 1995). In one allelic combination, dauer formation is completely suppressed, but life span extension is unaffected. Thus, the activity of *daf-12* required for dauer formation is not required for life-span extension. Nevertheless, in certain allelic combinations, *daf-12* activity does influence life span. Depending on the allele, maximum life span can be shortened, or lengthened up to fourfold over wild type.

1.10.2 Insulin/IGF-like Signaling (IIS) Pathway:

The insulin/insulin-like growth factor (IGF) signalling (IIS) pathway is evolutionary ancient and is found in species ranging from worms and flies to humans. Central to this pathway is a plasma menrane-bound tyrosine kinase(DAF-2) that acts to transducer signals to responsive tissue following activation by insulin or insulin –like ligands. In worms there are 37 genes that encode putative insulin-like (ins) molecules (Pierce et al., 2001). The IIS pathway is also comprised of several sequentially acting components, all of which are present in worms. An insulin receptor substrate(IRS-1), a phosphatidylinositol-3-kinase(AGE-1), a phosphoinosityde dependent kinase(PDK-1), a serum glucocorticoid kinase(SGK-1), two protein kinase B homologs (also known as akt) (AKT-1/2) and a forkhead transcription factor (DAF-16). A phosphatase (DAF-18 in worms and homologous to the human tumor suppressor PTEN) also acts to counter the activity of AGE-1. The role of this pathway in nematode aging has been extensively reviewed (guarente & Kenyon, 2000). Most

importantly, reducing the function of many components of this signalling module invokes prolonged lifespan and/or dauer formation.

Inhibition of daf-2 by mutation or RNA interference can dramatically increase mean life span upto 150% (Gems et al., 1998). Daf-2 is the sole member of the insulin/IGF receptor tyrosine kinase family in *C. elegans* (Rikke et al., 2000). They lack a tyrosine kinase domain and have two known functions (Dlakic, 2002) Like the mammalian insulin receptor, DAF-2, is a single pass transmembrane receptor with an extracellular ligand binding domain. The tyrosine kinase domain is well conserved between human and worm, sharing six out of eight critical catalytic residues and containing conservative substitutions at the remaining two sites. Mutation has also been identified within the kinase domain that increases life span. For example, the commonly used *daf-2(e1370)* allele is a P1465S mutation within the kinase domain (Kimura *et al.,* 1997).

Mutation of any of several downstream components in the IIS pathway also shows the rate of aging and increases mean life span. *age-1* (PI3Kinase) mutants live 65% longer (Johnson, 1990b). Similarly, alteration of pdk-1 increases mean life span by 60% (Paradis et al., 1999). *C. elegans* contains two homologues of mammalian akt, called akt-1 and akt-2. These appear to be functionally redundant (Paradis & Ruvkun, 1998). It is possible to inactivate both genes by either co-injecting double stranded RNA (dsRNA) into adult animals and examining the resulting progeny, which form dauers constitutively (Daf-c) and localize DAF-16 to the nucleus (Henderson & Johnson, 2001), or by feeding akt-2 (ok393) (a knochout allele of *akt-2*) bacteria

expressing dsRNA to *akt-1,* which extendslife-expectancy about 20% (Hertweck et al., 2004).

Recently a homologue of mammalian serum- and glucocorticoid-inducible kinase(SGK) was identified in *C. elegans.* SGK kinases are similar in sequence to AKT kinases. In mammals they are though to function in IIS through direct regulation of the mammalian homologs of DAF-16 **(Brunet et al., 2001).** Analogously, in *C. elegans*, SGK-1 may act in a complex with AKT-1/2 and function to directly phosphorylate DAF-16 and prevents its nuclear entry and shortening lifespan. Inhibition of *sgk-1* by RNA interference increased life span by approximately 70% (Hertweck et al., 2004). A major function of active IIs is to phosphorylate DAF-16, thereby excluding it from the nucleus. Converesely, inhibition of IIS causes nuclear localization of DAF-16, leading to increased stress resistance and life span (Henderson & Johnson, 2001; Lee et al., 2001; Lin et al., 2001). As mentioned before, inhibition of *daf-2* by mutation or RNAi can increase the life span more than double. But this depends on the site of mutation and/or other manipulations. Increase in both stress resistance and longevity are lost in double mutants (Arantes-Oliveira et al., 2003; Kenyon et al., 1993). This epistasis of daf-16 logically means that *daf-16* functions downstream of *daf-2.*

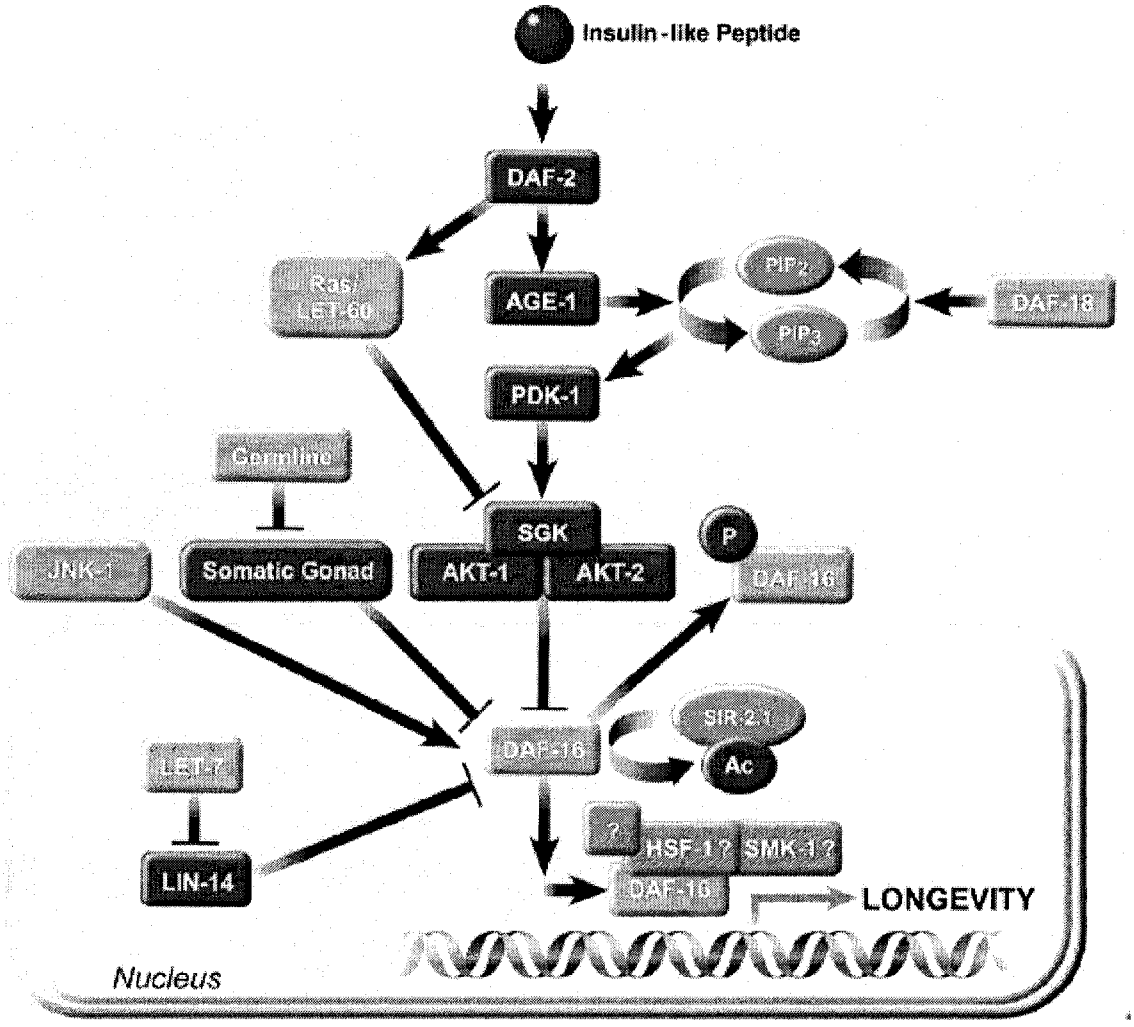


Figure 1.10.2.1 Insulin/IGF-like Signaling (IIS) Pathway

1.10.3 DAF-16

Modulating DAF-16 transcriptional activity is a major function of insulin signaling in *C. elegans*. Nuclear localization of DAF-16 plays a necessary role in increased longevity. Several different environmental stressors caused nuclear localization of DAF-16. A simple increase in the dosage of daf-16 resulted in increased stress resistance and life span with slowed growth and reproduction (Henderson and Johnson, 2001).Both these outcomes are consistent with DAF-16 functioning as a

"gerontostat", a regulator of aging. Under normal condition, DAF-16 is phosphorylated by the action of the upstream IIS elements and is consequently found in the cytoplasm. This results normal levels of stress resistance, reproduction and a normal life span. Under difficult conditions, especially reduced food concentration, DAF-16 instead moves to the nucleus and stimulates the synthesis of many transcripts leading to stress resistance and increased longevity. If these signals occur in the early stage of life, then DAf-16 stimulates dauer formation in response to environmental stress.

Researchers have attempted to identify the targets of DAF-16 by the use of DNA microarrays and computational methods. Microarray data on the upregulation of stress response genes was generally consistent. The mitochondrial superoxide dismutase *sod-3* and metallothionein *mtl-1* were recovered, confirming the earlier reports by Honda and Honda, 1999 and Barsyte et al., 2001, respectively. Besides these, Murphy et al. (2003) found that the cytosolic and peroxisomal catalases *ctl-1* and *ctl-2*, the glutathione-*S*-transferase *gst-4* and several small heat shock proteins appeared to be upregulated by DAF-16 as well. McElwee et al. (2003) reported DAF-16-dependent upregulation of heat shock proteins (HSP-16, HSP-70, HSP-90) together with an increase of cytosolic SOD-5 expression. RNAi experiments carried out by Murphy et al. (2003) showed that many of these genes are partially responsible for the lifespan extending properties of active DAF-16. The fact that longevity is associated with an enhanced stress response is in keeping with the oxidative stress theory of aging. However, two independent studies reported that *sod-3* (RNAi) did not shorten lifespan in long-lived *daf-2* mutants, suggesting that the synthesis of

mitochondrial SOD-3 per se does not increase lifespan (Hsu et al., 2003 and McElwee et al., 2003).

Murphy et al. (2003) showed that some antimicrobial genes such as the lysozymes *lys-7* and *lys-8*, and the saposin-like gene *spp-1* were upregulated in the *daf-2* Ins/IGF signaling mutant and that they contributed to its extended lifespan.

The transmembrane tyrosine kinase OLD-1 is a transcriptional target of DAF-16 and a positive regulator of longevity and stress resistance. Activity of this gene is a prerequisite to Ins/IGF-dependent longevity and it is expressed in the whole body. Its precise function and targets however, remain to be elucidated (Murakami and Johnson, 2001). The protein SCL-1 has almost identical life extending properties compared to OLD-1, except for the fact that it is a putative cysteine-rich secretory protein and thus might have extracellular signaling properties (Ookuma et al., 2003 and Patterson, 2003). This protein was also recovered in the microarray study of Murphy et al. (2003).

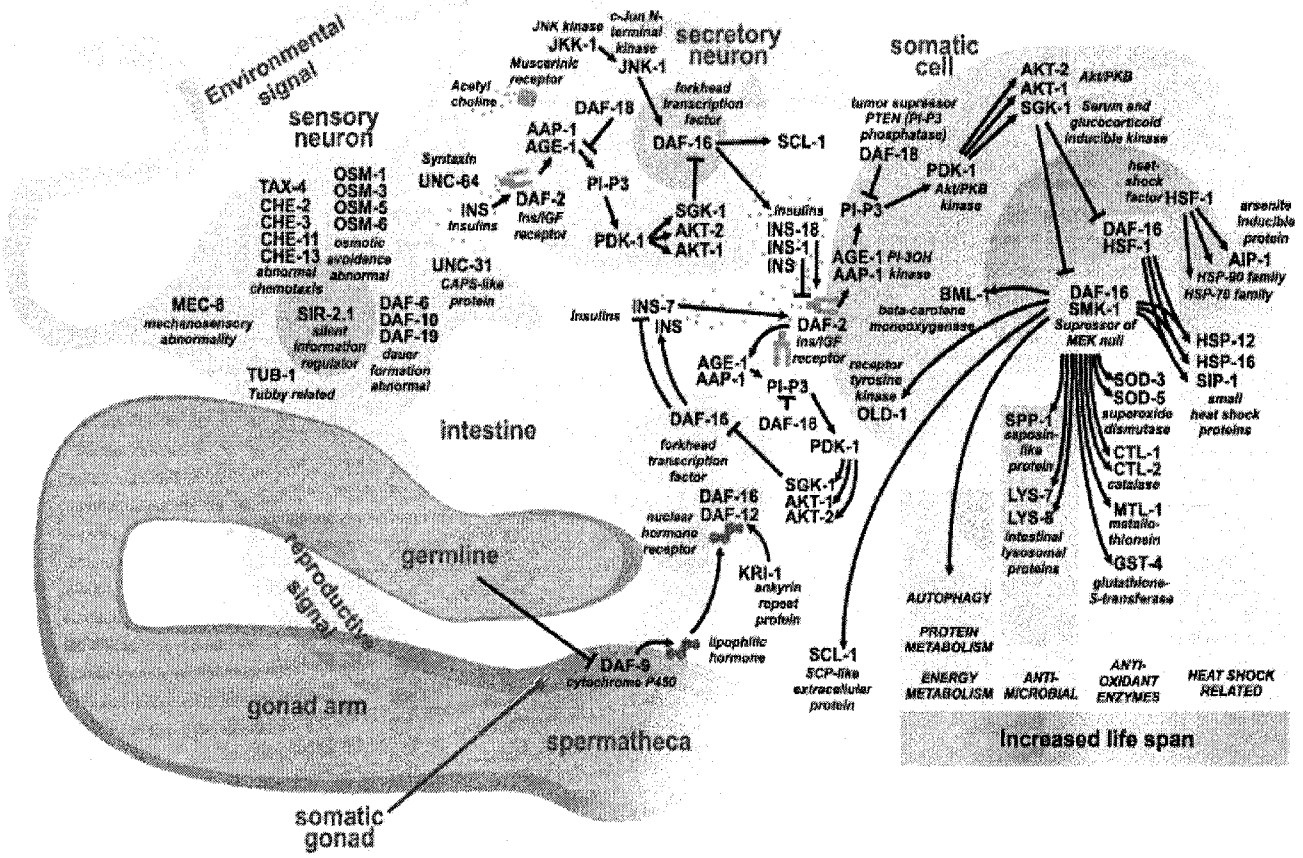


Figure 1.10.3.1 Gene interactions that influence lifespan in *C. elegans* through DAF-16 activation or inactivation

1.11 Functional dissection of the Thiolase and Sterol-Carrier Domains of SCPx of *C. elegans*

In most eukaryotic cells, the bulk of cholesterol is synthesized at the endoplasmic reticulam (ER) whereas almost 90% of the free, non-esterified fraction of this essential membrane lipid resides in the plasma membrane (B. Bloj *et al.*, 1976). Cholesterol is found mainly at the inner leaflet of the bilayer, where it limits membrane fluidity which is thought to stabilize the complex super molecular structures that are formed between lipids, receptors, adaptors proteins and the cytoskeleton at the cell surface. The cholesterol hydroxyl group forms a hydrogen bond with a phospholipids carbonyl oxygen atom whereas the bulky steroid moiety and the flexible hydrocarbon tails are directed to the hydrophobic inner portion of the membrane. Cholesterol is distributed asymmetrically in cells that make it conceivable that intracellular trafficking of cholesterol requires target-specific transport mechanisms that mediate its translocation from the site of synthesis at the ER to plasma membrane. Cholesterol transport through cytoplasm could be achieved via soluble carrier proteins that would shield the lipophilic transport substrate from the aqueous phase and harbour signals that could mediate target specificity. The best studied candidate for a soluble sterol carrier has been sterol carrier protein-2 (SCP2), also known as non-specific lipid transfer protein.

At present, the SCP2 gene family includes only four distinct members (SCP2, SCPx, D-PBE and UNC-24/hSLP-1), but it can be expected that additional homologues may be identified in the future. Apart from SCP2, which is expressed as an individual protein, the other homologues contain their SCP2 domains at the C-terminus

(Fig.1.11.1). Mammalian SCP2 is synthesized as a 143 amino acid precursor that is processed most likely in peroxisomes to the 123 amino acid mature SCP2. The human SCP2-encoding gene comprises 16 exons, which span approx. 100 kb on chromosome 1p32. Alternate transcription initiation regulates the expression of SCP2 and a second gene product that consists of 547 amino acids (named sterol carrier protein-x, SCPx). SCPx represents a fused protein consisting of a thiolase, extending from amino acid 1 to 404, and SCP2 which is located at the carboxyl terminus.

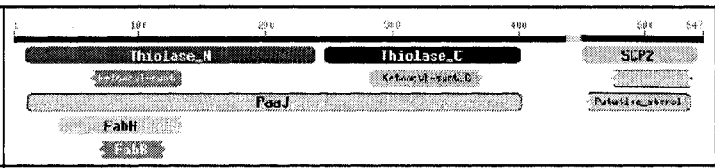
C. elegans protein function		Nematode gene	Tri-peptide at C-terminus
Thiolase type II	Score = 392 bits (1006), Expect = e-105 Identities = 298/400 (50%), Positives = 311/400 (75%), Gaps = 5/400 (1%)	P-44 (Y57A10C.6)	-SKI
SCP2	Score = 82.8 bits (203), Expect = 3e-14 Identities = 40/100 (37%), Positives = 67/100 (62%), Gaps = 2/100 (1%)	NLT-1 (ZK892.2)	-AKL
17-β-hydroxysteroid dehydrogenase	Score = 62.4 bits (150), Expect = 5e-08 Identities = 43/142 (30%), Positives = 73/142 (51%), Gaps = 5/142 (3%)	dhs-28 (M03A8.1)	-SKL
Acetyl-CoA acetyltransferase	Score = 67.0 bits (162), Expect = 3e-09 Identities = 93/380 (24%), Positives = 143/380 (37%), Gaps = 61/380 (16%)	F53A2.7	-LGL
dehydrogenase-reductase	Score = 62.8 bits (151), Expect = 4e-08 Identities = 35/88 (39%), Positives = 52/88 (59%), Gaps = 1/88 (1%)	dhs-6 (C17G10.8)	-GKL
Thiolase type I	Score = 60.5 bits (153), Expect = 2e-08 Identities = 91/407 (22%), Positives = 153/407 (37%), Gaps = 81/407 (19%)	kat-1 (T02G5.8)	-QKL
Thiolase type I	Score = 58.9 bits (141), Expect = 5e-07 Identities = 94/417 (22%), Positives = 158/417 (37%), Gaps = 84/417 (20%)	B0303.3	-YGK
Thiolase type I	Score = 52.0 bits (123), Expect = 6e-05 Identities = 87/370 (23%), Positives = 141/370 (37%), Gaps = 68/370 (18%)	T02G5.7	-KKL
Band 7 protein Stomatin	Score = 40.8 bits (94), Expect = 0.21 Identities = 24/90 (26%), Positives = 44/90 (48%), Gaps = 5/90 (5%)	unc-24 (F57H12.2)	-DWL
Thiolase type I	Score = 50.8 bits (120), Expect = 2e-04 Identities = 91/385 (23%), Positives = 155/385 (39%), Gaps = 60/385 (17%)	T02G5.4	-QKL

Figure 1.11.1 **Alignment of human SCPx and *C. elegans* homologs.** Top panel, center: amino acids 1-400 of SCPx form the thiolase domain; amino acids 450-547 form the sterol carrier domain. A typical result of the automatic detection of protein domains of SCPx using BLAST is shown at top. PaaJ, catalytic domain of acetyl-CoA acetyltransferase. Alignments: upper protein, SCPx; lower protein, C. elegans homolog. Blue denotes region of homology. Red denotes gaps. Potential peroxisome targeting signals found in C. elegans homologs of SCPx that conform or resemble the consensus PTS1 sequence, -(S/C/A)(K/R/H)(L/M) are denotes with red.

In mammals, SCPx has 3-oxoacyl-CoA thiolase activity and catalyses the thiolytic step in both the breakdown of phytanic/pristanic branched-chain fatty acids and in the formation of the CoA esters of cholic acid and chenodeoxycholic acid from di- and tri- hydroxycholestanoic acid (Antonenkov *et al.*, 1997). Both processes take place in peroxisomes. In peroxisomes, phytanic acid undergoes one round of α-oxidation to form pristanic acid (Fig. 1.11.2) (Lehninger, 2005). In turn, pristanic acid undergoes only three cycles of β-oxidation, after which the products acetyl-CoA, propionyl-CoA and 4,8- dimethylnonanoyl-CoA exit the peroxisome (Wanders *et al.*, 2000). The final step (Fig 1.11.2, Step 10) of at least the first β-oxidation cycle is catalyzed by SCPx.

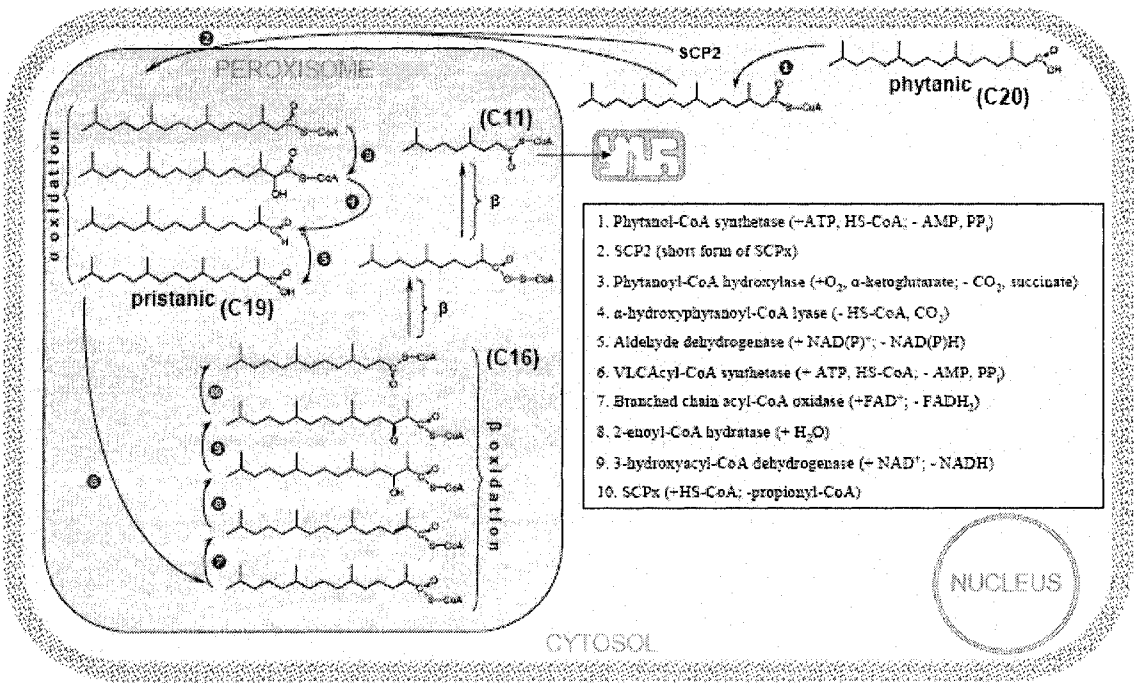


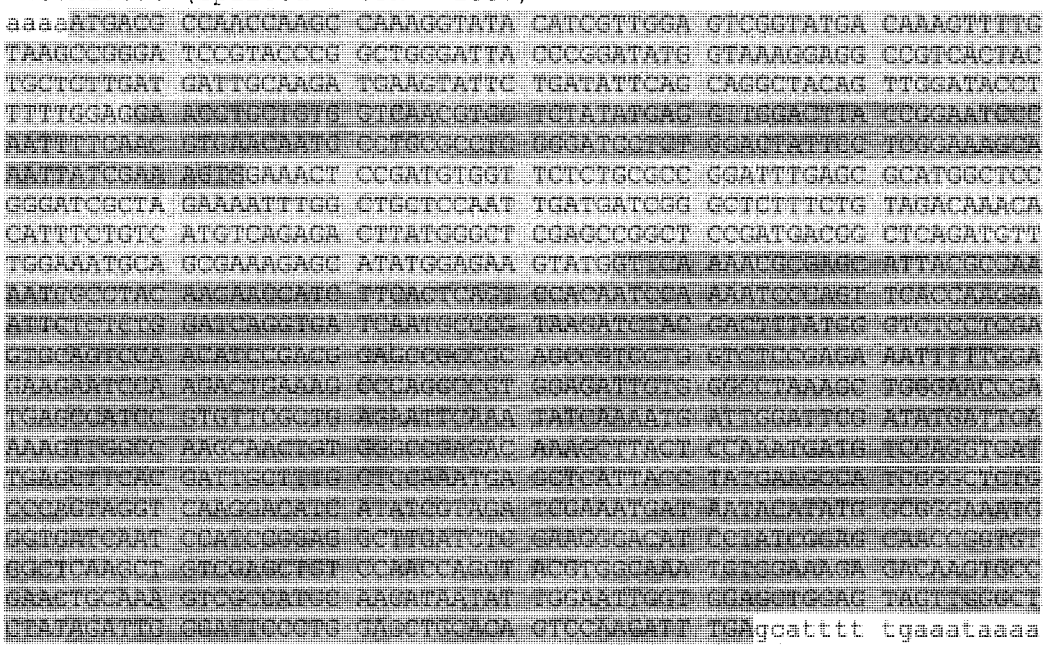
Figure 1.11.2 Catabolism of phytanic acid in humans. Reactions of first α-, and following three β -oxidation cycles take place in peroxisome. 4,8-dimethylundecanoate synthesized in the cycles is then transported to mitochondrion for further catabolism.

Proteins studied in this thesis, are all homologs of human Sterol Carrier Protein x. *C. elegans* genome encode homologs of hSCPx: P-44, nlt-1 and dhs-28.

P-44 (Sequence Name: Y57A10C.6): Peroxisomal 3-ketoacyl-CoA-thiolase P-44/SCP2. P-44 is a type II thiolase, the closest nematode homolog of mammalian SCPx protein. The length of P-44 is 412 amino acids (Nucleotide: 1239/2181 bp (Coding/transcript))

Sequence

>Y57A10C.6 (spliced + UTR - 1337)



acggaataat ttaaacattt tcttttgatt ttttttttgtg tataatagag aaacttgaat aaagtgtttt attcttt

>Y57A10C.6 (conceptual translation - 412 aa)

MTPTKPKVYI VGVGMTKFCK PGSVPGWDYP DMVKEAVTTA LDDCKMKYSD IQQATVGYLF
GGTCCGQRAL YEVGLTGIPI FNVNNACASG SSGLFLGKQI IESGNSDVVL CAGFERMAPG
SLENLAAPID DRALSVDKHI SVMSETYGLE PAPMTAQMFG NAAKEHMEKY GSKREHYAKI
AYKNHLHSVH NPKSQFTKEF SLDQVINARK IYDFMGLLEC SPTSDGAAAA VLVSEKFLEK
NPRLKAQAVE IVGLKLGTDE PSVFAENSNI KMIGFDMIQK LAKQLWAETK LTPNDVQVIE
LHDCFAPNEL ITYEAIGLCP VGQGHHIVDR NDNTYGGKWV INPSGGLISK GHPIGATGVA
QAVELSNQLR GKCGKRQVPN CKVAMQHNIG IGGAGVVGLY RLGFPGAAQS KI*

P-44 thiolase activity was also observed with 2-methylhexadecenoyl-CoA and oxo-forms of bile acids as substrates. The same catalytic activity is the characteristic of SCPx (Bun-ya et al., 1998). SCPx is also known to take part in the catabolism of methyl-branched fatty acids such as phytanic acid (3,7,11,15-tetramethylhexadecanic acid) and pristanic acid (2,6,10,14-tetramethylpentadecanoic acid) (Seedrof et al., 1998). Therefore, in analogy to SCPx, P-44 has been suggested as a candidate enzyme that could take part in branched fatty acid catabolism in nematode (Bun-ya et al., 1997; Maebuchi et al., 1999). The peroxisomal location of *P-44* and expression pattern of the gene during nematode ontogenesis also provides additional evidence that P-44 and SCPx play common physiological roles (Maebuchi et al., 1999). Thus it is expected that mutant worms deficient in P-44 will have defects in branched chain fatty acid metabolism. P-44 deficiency was found to extend the egg laying period of *C. elegans*, to cause a decrease body size and to induce the proliferation of Peroxisomes. Peroxisomes in the gut cells of P-44 mutant worms cluster in large groups and often are accompanied by unusual membranes not found in wild type worms. Moreover, Peroxisomes of P-44 mutant worms are often found in immediate proximity to unusual large lipid vesicles (Fig 1.11.3).

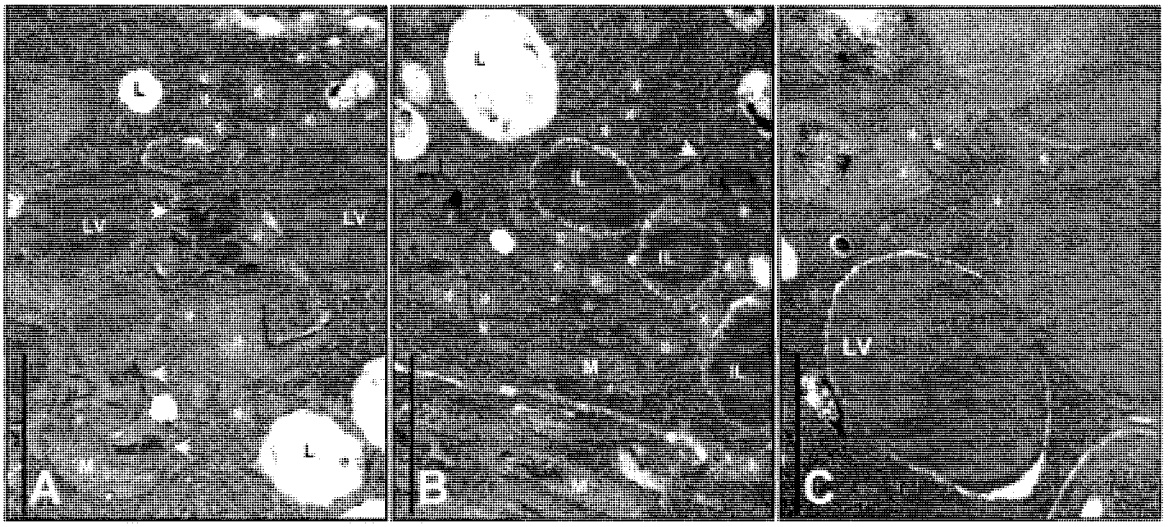


Figure 1.11.3 Electron microscopy of peroxisomes, found in P-44 mutant worms.

Clusters of peroxisomes (asterisks) and unidentified membranes (arrowheads), (A & B)

Increased lipid vesicles (LV), surrounded by peroxisomes, (C).

M, mitochondria;

L, lysosomes

IL, late intestinal lysosomes (also known as gut granules)

(Source: Clokey and Jacobson, 1986).

Nlt-1(Sequence Name: ZK892.2): Non-specific lipid transfer protein with the length of 118 amino acid [Nucleotide(coding/transcript: 357/806bp)]. Nlt-1 encoded a member of the SCP-2 sterol transfer family.

Sequence

```
>ZK892.2 (spliced + UTR - 465)
aaacgcaata aatcattttt catagtttac agcttcaaca ATGGCTTTCA AGTCCGATGT
TATTTTTGAG GAGATCAAGG AGAGAATTGC CACGTACAAA GAAATCGTGA AGAAGGTCGT
CAACTCATTT CGTATCACGA TTGCTTGAGC CGATCGAAAG ACAAAGGTCT GGAGATTCGA
TGATAAATCA GACACTTGAT ACGTCTGACA CGATTCTTCT CGACCAGTTG AGATTGAGAT
TAATATTTAG GATTCGGATT TCATTGCTAT TTCCGCCGGA AAGATGAAGC CAGATCAGGC
TTTCATGCAA GGAAAGATGA AGCTTAAGGG AAATATTGCC AAGGCAATGA AGCTCCGTAC
TATTCTCGAC CCAAAGATGC TCAAAGCCAA GCTCTAAaca aaaaccaccc attatgccat
attttgtatt aaaatcattt ccaaataaac tattattgtg gccat

>ZK892.2 (conceptual translation - 118 aa)
MAFKSDVIFE EIKERIATDK EMVKKVGTSF RMTIAGADGK TKVWTIDAKS DTPYVGDDSS
RPVEIEINIK DSDFIAIAAG KMKPDQAFMQ GKMKLKGNIA KAMKLRTILD PKMLKAKL*
```

Dhs-28(Sequence Name: M03A8.1): dhs-28 encodes an ortholog of human 17-β-hydroxysteroid DH4 (HSD17B4) which contains a C-terminal SCP-2 sterol transfer domain. Length: 436 amino acid; Nucleotide (Coding/transcript): 1311/1636 bp. Dhs-28 has Alcohol DH activity, Catalytic activity, Sterol carrier activity.

Sequence

```
>M03A8.1 (spliced + UTR - 1402)
aaacaaaATG TCTCTTCGTT TTGACGGAAA AGTTGCTATT GTCACTGGAG CTGGAGGTGG
TCTTGGCAAG ACATATGCTC TTGAACTTGC CAAGAGAGGA TGCAAAGTTG TTGTAAACGA
TCTTGGAGGA GATAGACATG GAACTTCATC CTCTTCATCA ATGGCTGACA AGGTTGTTCA
AGAAATCAAA TCAGCTGGAG GACAAGCAGT TGCCAATTAT GACAGCGTTG AGTTTGGAGA
CAAAATCGTA AAAACCGCCA TCGACAATTT TGGAAGAATT GATATTGTTA TCAACAACGC
TGGTATCCTT CGTGACGTTT CTTTCCTCAA GATGACCGAG CTTGATTGGG ATCTTATCTT
CAAGGTCCAC GTTAAGGGAG CCTATGCTGT CACCAAAGCC GCTTGGCCAT ACATGAGAGA
TCAGAAGTAC GGGCGTATTG TGGTCACCTC TTCCAATGCC GGAGTTCACG GAAACTTTGG
ACAAGCCAAC TACGCTGCTG CCAAAAGTGC TCTCATTGGT CTGTCAAACT CTCTCGCCCA
AGAAGGTGCA AAGTACAACA TTTTGGCAAA TACCCTGGTC CCAACAGCCG GATCTAGACT
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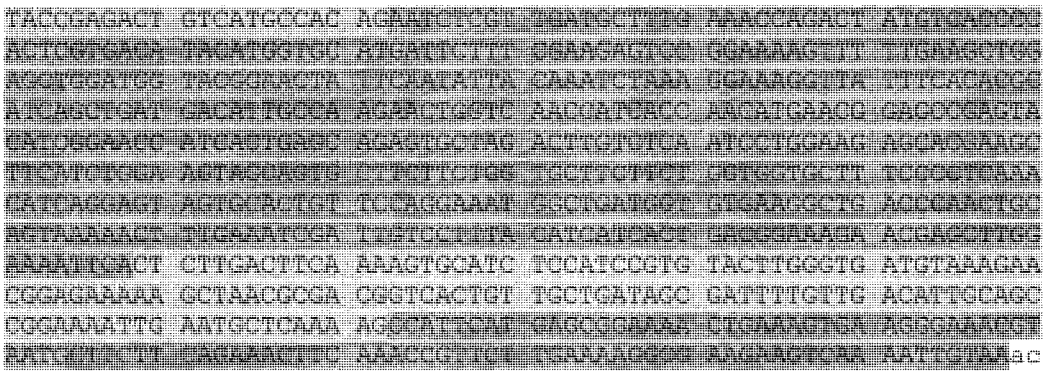
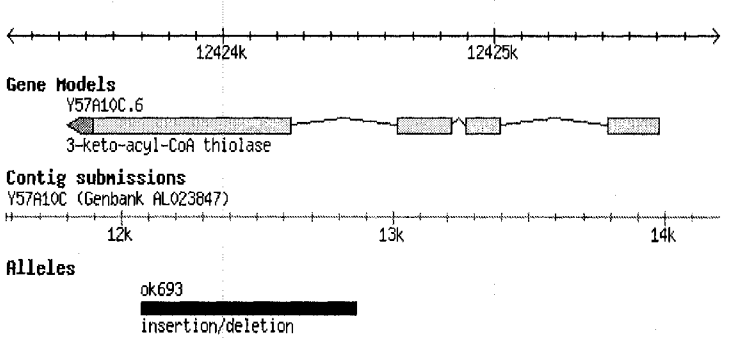


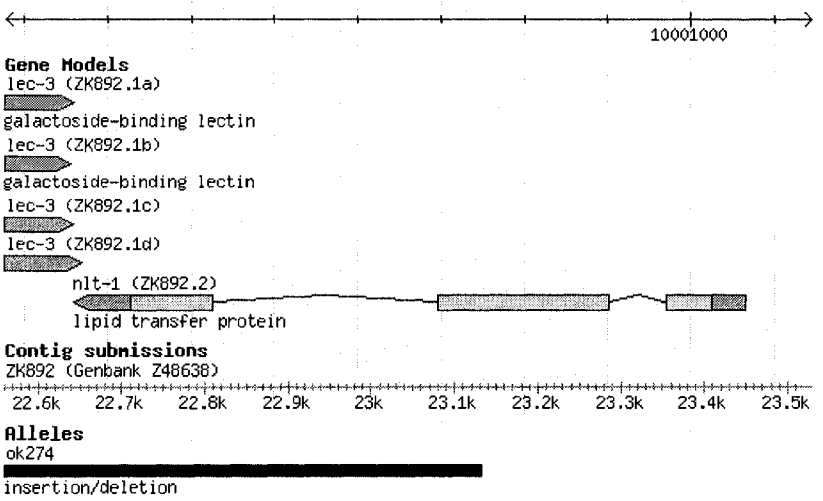
agacagaagc gttcaatttt gtaaattata ttgagtcaga atgattaatt attgatattg agtacattta ataaaacatt tc

>M03A8.1 (conceptual translation - 436 aa)
MSLRFDGKVA IVTGAGGGLG KTYALELAKR GCKVVVNDLG GDRHGTSSSS SMADKVVQEI
KSAGGQAVAN YDSVEFGDKI VKTAIDNFGR IDIVINNAGI LRDVSFLKMT ELDWDLIFKV
HVKGAYAVTK AAWPYMRDQK YGRIVVTSSN AGVHGNFGQA NYAAAKSALI GLSNSLAQEG
AKYNILANTL VPTAGSRLTE TVMPQNLVDA LKPDYVTPLV TYMVHDSFEE SGKVFEAGAG
WYGTIQYYKS KGKVISHASA DDIAKNWSTI TNMNGAEYIG TITEQSARLV SILEEHEASS
GSSSASSGAS SGGAFPSNIR SSALFQEMAD GVKADPTAVK TLKSIVLYII TDGKNELGKF
TLDFKSASPS VYLGDVKNGE KANATVTVAD SDFVDIAAGK LNAQKAFMSG KLKVKGNVML
LQKLQTVLEK AKKSKL*

A.



B.



C.

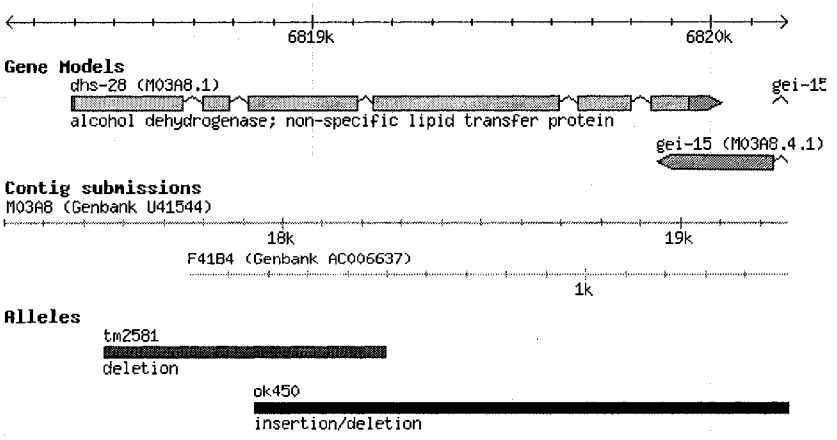


Figure 1.11.4 Location of P-44 (A), nlt-1 (B) and dhs-28 (C)

1.12 Thesis Rationale

The objective of the work in this thesis was to define the localization of peroxisomal proteins whose deficiency affects the rate of chronological aging and post-embryonic development of the *C. elegans* in various tissues of this organism. The thesis emphasized on homologs of human Sterol Carrier Protein x (hSCPx) in *C. elegans*, their role and sub-cellular localization. In mice the SCPx deficiency has a marked effect on hepatic on hepatic gene expression, peroxisome proliferation, causes hypolipidemia, impaired body weight control and neuropathy. In human, SCPx is required for metabolism of branched chains, long and very long straight chain fatty acids (VLCFAs). Oxidation of branched chain fatty acids and very long chain fatty acids and also accumulation of phytanic acids are shown to be impaired in Zellweger's syndrome(Jansen, G.A. *et al.,* 2001; Sandhir, R *et al.,* 2000) and Refsum's disease (Mihalik, S.J *et al.,* 1997). Moreover, defects of different forms of 17-β-Hydroxysteroid Dehydrogenase are found to be associated with neuronal diseases and also pathogenesis of a number of Cancers (Mindnich, R *et al.*, 2004)).This thesis reports the advantage of using C. elegans as a valuable model system to study the molecular defects underlying these human disorders. This thesis also supports the idea that often observed correlation between longevity and lipid deposits in an organism are not interrelated. It adds new information to the pool of data on the role of peroxisomes in regulation of ontogenesis and in particular, on the role of specific classes of fatty acids in aging.

Finally results reported in this thesis represent an example how the nematode model can be advantageous for solving the biochemical problems arising in a mammalian organism.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 List of chemicals and reagents

Reagent Source

2-mercaptoethanol Bioshop

3-methyl-1-butanol (isoamyl alcohol) Caledon

5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) Vector Biosystems

812 Resin Marivac

acrylamide Invitrogen

agarose, electrophoresis grade Invitrogen

ammonium persulfate [$(NH_4)_2S_2O_8$] BDH

ammonium sulfate [$(NH_4)_2SO_4$] BDH

ampicillin Sigma

Bacto agar BD

Bacto tryptone BD

benzamidine hydrochloride Sigma

Bio-Rad protein assay dye reagent Bio-Rad

bromophenol blue BDH

chloroform ($CHCl_3$) Fisher

cholesterol Sigma

Coomassie Brilliant Blue R-250 ICN

D-(+)-glucose Sigma

diethylpyrocarbonate (DEPC) Sigma

dimethylsulfoxide (DMSO) Caledon

dithiothreitol (DTT) ICN

DNA molecular size standards NEB

dodecenyl succinic anhydride Marivac

ethanol Commercial Alcohols

ethylenediaminetetraacetic acid (EDTA) Sigma

Ficoll PM400 Pharmacia

formaldehyde, 37% (v/v) BDH

FuGENE 6 Transfection Reagent Roche

glycerol BDH

hexane (*n*-haxanes, GC grade) EMD

hydrochloric acid Fisher

hydrogen peroxide solution, 30% (w/v) Sigma

isopropanol Fisher

isopropyl β-D-thiogalactopyranoside (IPTG) Vector Biosystems

leucine Sigma

leupeptin Roche

lithium acetate Sigma

methyl nadic anhydride Marivac

methanol Commercial Alcohols

N,N,N',N'-tetramethylethylenediamine (TEMED) Invitrogen

N,N'-methylene bisacrylamide Invitrogen

N-propyl gallate Calbiochem

pepstatin A Sigma

peptone Difco

phenol, buffer-saturated Invitrogen

phenylmethylsulfonylfluoride (PMSF) Roche

phytanic acid Sigma, Larodan

Ponceau S Sigma

potassium chloride BDH

potassium ferricyanide Sigma

potassium phosphate, dibasic (K_2HPO_4) Merck

potassium phosphate, monobasic (KH_2PO_4) Merck

pristanic acid Larodan, Malmö, Sweden

protein molecular size standards NEB

QIAprep Spin, Miniprep Kit (250)

QIAquick, Gel extraction Kit (250)

RNA molecular size standards NEB

Sodium Azide (NaN_3) Sigma –Aldrich Chemie Gmbh

sodium acetate BDH

sodium chloride Merck

sodium citrate BDH

sodium dodecyl sulfate (SDS) Sigma

sodium hydroxide BDH

sodium hypochlorite, 5.25% (w/w) Commercial Cleaners

sodium phosphate, dibasic (Na_2HPO_4) BDH

sodium thiosulfate Sigma

standard mixtures for gas chromatography Larodan, Malmö, Sweden

sulfuric acid (H_2SO_4) Merck

Tris-base, (tris[hydroxymethyl]aminomethane) Roche

Triton X-100 Sigma

TRIZMA hydrochloride

(tris[hydroxymethyl]aminomethane hydrochloride) Sigma

tryptone Difco

Tween 20 (polyoxyethylenesorbitan monolaurate) Sigma

xylene cyanol FF Sigma

yeast extract Difco

yeast nitrogen base without amino acids (YNB) Difco

2.1.2 List of enzymes

Enzyme Source

calf intestinal alkaline phosphatase (CIAP) NEB

proteinase, type K Sigma

restriction endonucleases NEB

ribonuclease A, DNase-free Sigma

T4 DNA ligase NEB

Taq polymerase NEB

KpnI (conc. 10u/µl): 5'...**GGTACC**...3'

3'...**CCATGG**...5'

NheI (conc. 10u/µl): 5'...**GCTAGC**...3'

3'...**CGATCG**...5'

NotI (conc. 10u/μl): 5'...**GCGGCCGC**...3'

3'...**CGCCGGCG**...5'

PstI (conc. 10u/μl): 5'...**CTGCAG**...3'

3'...**GACGTC**...5'

SacI (conc. 10u/μl): 5'...**GAGCTC**...3'

3'...**CTCGAG**...5'

XbaI (conc. 10u/μl): 5'...**TCTAGA**...3'

3'...**AGATCT**...5'

2.1.3 Plasmids

Plasmid (see also plasmid maps, Fig. 2.1.1) Source

pPD118.25 Dr. A. Fire

pGEM-T Promega

pPD118.25MCS2(pNlt-1)

pPD118.25MCS2(pdhs-28)

pPD118.25MCS2(Nlt-1)

pPD118.25MCS2(dhs-28)

pDP#MM016 Dr. D. Pilgrim

2.1.4 Strains

Strain Source

Caenorhabditis elegans variety Bristol, strain N2 CGC

Caenorhabditis elegans, strain CB4845, *unc-119(e2498)III* Dr. D. Pilgrim

Caenorhabditis elegans, strain CB1489, *him-8(e1489)IV* CGC

Caenorhabditis elegans, strain LB90 (*ua90)II* This study

Caenorhabditis elegans, strain TU2463 *ctl-1(u800) II* CGC

Caenorhabditis elegans, strain MQ130 *clk-1(qm30)III* CGC

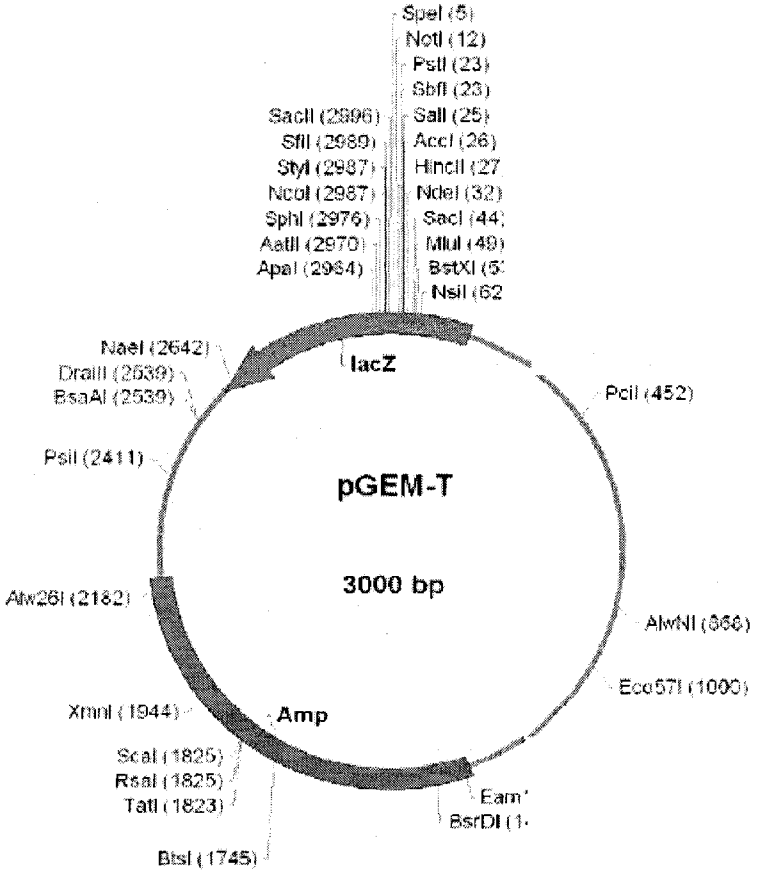
Caenorhabditis elegans, strain RB859 *Y57A10C.6(ok693) II* CGC

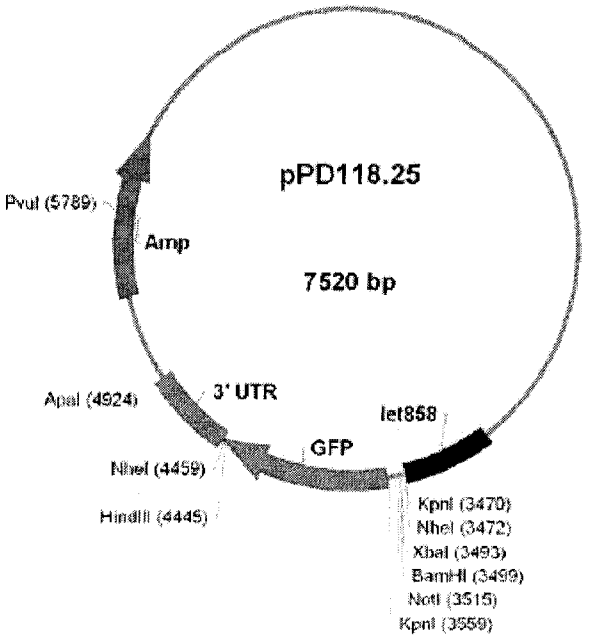
Caenorhabditis elegans, strain VC262 *dhs-28&gei-15(ok450) X* CGC

Caenorhabditis elegans, strain RB675 *pmp-4*(ok396) IV CGC

Escherichia coli OP50 Dr. D. Pilgrim

Escherichia coli DH5α, F- φ80d*lacZ*ΔM15 Δ(*lacZ*YA-*arg*F) U169

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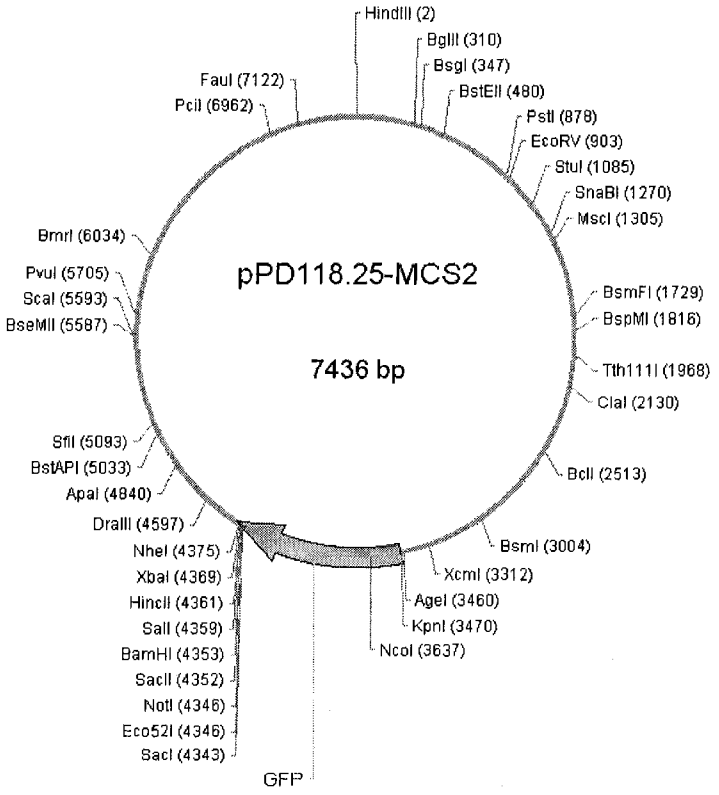
7520 bp
PvuI (5789)
Amp
ApaI (4924)
3' UTR
let858
GFP
NheI (4459)
HindIII (4445)
KpnI (3470)
NheI (3472)
XbaI (3493)
BamHI (3499)
NotI (3515)
KpnI (3559)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7436 bp
HindIII (2)
BglII (310)
BsgI (347)
BstEII (480)
PstI (878)
EcoRV (903)
StuI (1085)
SnaBI (1270)
MscI (1305)
BsmFI (1729)
BspMI (1816)
Tth111I (1968)
ClaI (2130)
BclI (2513)
BsmI (3004)
XcmI (3312)
AgeI (3460)
KpnI (3470)
NcoI (3637)
GFP
SacI (4343)
Eco52I (4346)
NotI (4346)
SacII (4352)
BamHI (4353)
SalI (4359)
HincII (4361)
XbaI (4369)
NheI (4375)
DraIII (4597)
ApaI (4840)
BstAPI (5033)
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ScaI (5593)
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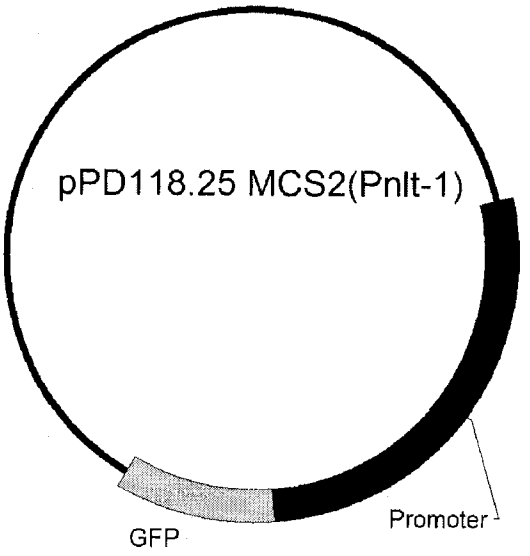
BmrI (6034)
PciI (6962)
FauI (7122)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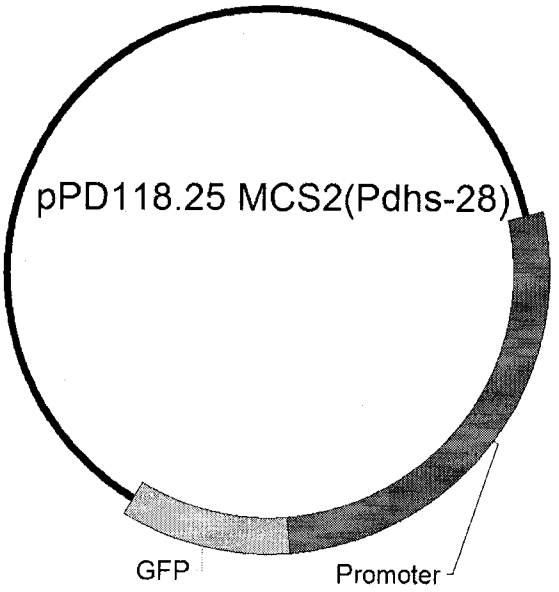
GFP
Promoter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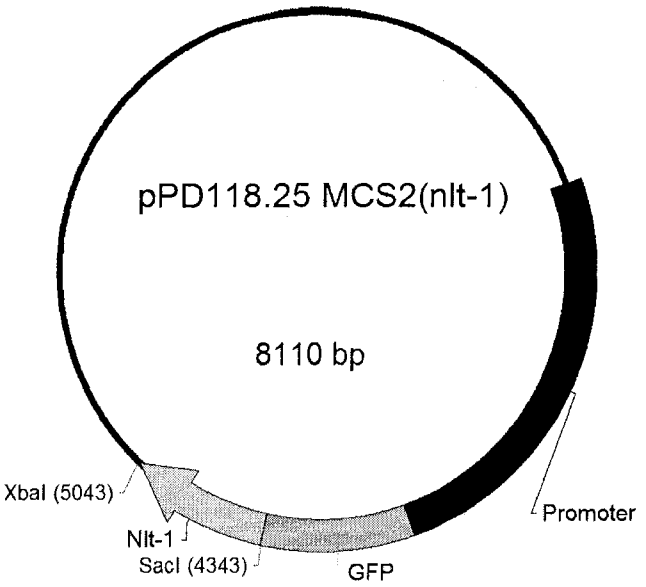
GFP
Promoter



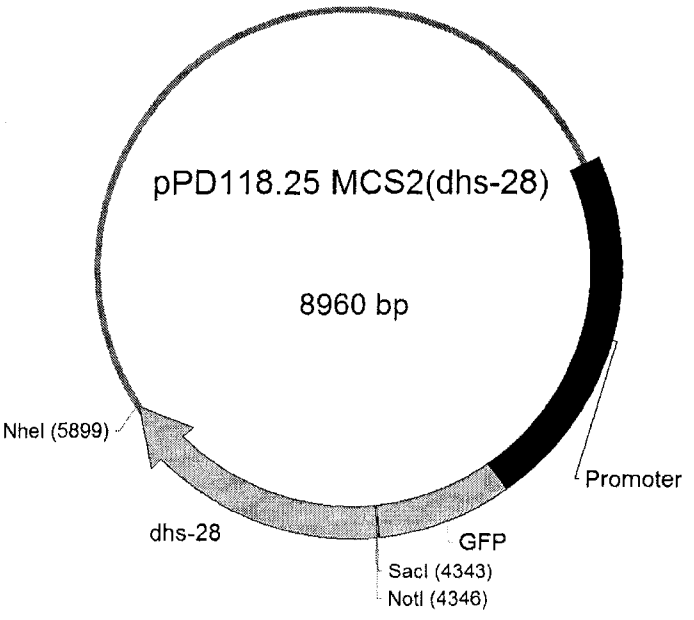


Figure 2.1.1: Different Plasmid Constructs

2.1.5 Sequences of different Plasmid Construct:

pPD118.25:

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AACTGAAAGATCGGAAAATGCACTGGATCGACGTATTCAGTATATGATTGAGACTGCAATGCAGATTC
GAAAGGACAAATTTGCGgtaaggtagaatatataaatagtttattagaaaaaaataaattagaataat
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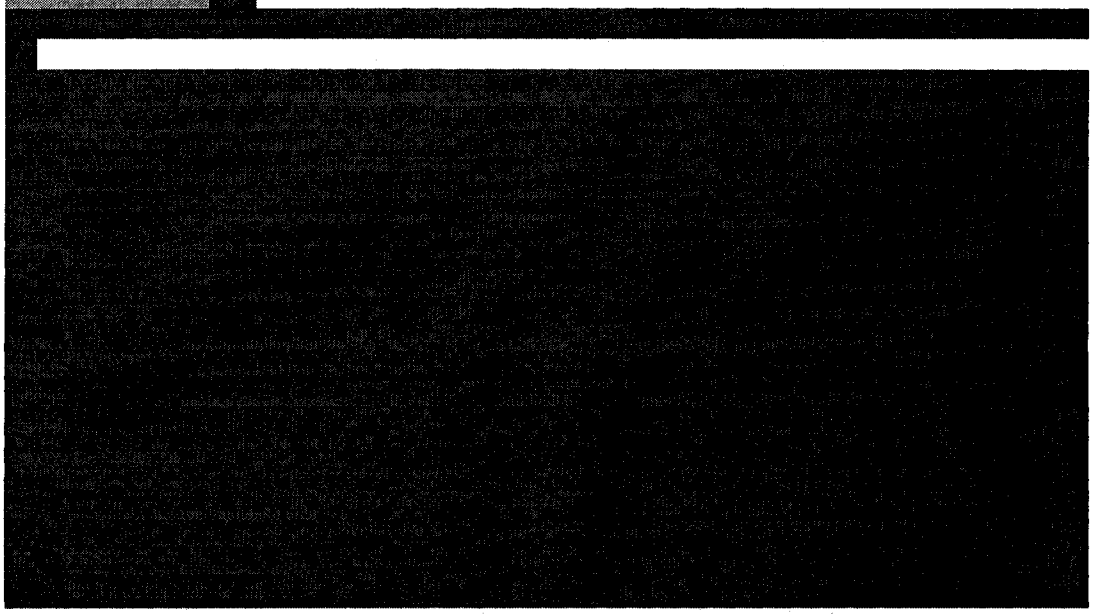


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2.1.6 Oligonucleotides

Application Sequence Name

No.	Sequence	bp	Restriction sites	Tm	Description
OP001	TAG CTA GCC **TAG TTC TGC ATC TGC TCA AAG AAC TT**	35	Nhe I	57	to PCR out APP695 from pcDNA3 plasmid from Suzuki and insert gene into pPD118.25
OP002	AGG TAC C**AT G**CT GCC CGG TTT GGC ACT GCT CCT GCT GGC CGC CTG GAC GGC TCG GGC GCT GGA AGT ACC C	70	Kpn I		to PCR out APP695 from pcDNA3 plasmid from Suzuki and insert gene into pPD118.25
OP003	ATT CGG AGG CGA CAA CGG TAT TTT CGA A	28			Sequencing of pPD118.25 (N) - forward
OP004	**TTG AAA ATT CAA CGA CGT TGG**	21		55	Sequencing of pPD118.25 (C) - reverse
OP005	AGGTACC**ATGTCCTCTTCCCCGTGGGA**	27	Kpn I	60	to PCR out hSCPxT from pcDNA3 plasmid from Mihalik and insert gene into pPD118.25
OP006	**TAGCTAGCTCAAATCTTAGACTGGGCGGCTCCCGGAAAAC C**	41	Nhe I	63	to PCR out hSCPxT from pcDNA3 plasmid from Mihalik and insert gene into pPD118.25
OP036	GCTTCCGGCTCGTATGTTGTG	21		58	pPD118.25 seq
OP037	CCAACGCGTTGGGAGCTCTCC	21		58	pGEM-T Seq
OP041	**ATGGCTTTCAAGTCCGATGTTATT**	24		57	PCR of Nlt-1 cDNA forward 357 bp
OP042	**TTAGAGCTTGGCTTTGAGCATCTT**	24		57	PCR of Nlt-1 cDNA reverse. 357 bp
OP043	**CAGCCAGTCCCATATGAATCC**	21		55	Screening of Nlt-1 deletion forward
OP044	**TGTCGTCTCTGTTTAGGCGGAT**	22		57	rev.Deletion - 1049 bp WT – 2906 bp
OP047	**TCAGCTGGAGGACAAGCAGTT**	21		56	RT-PCR of dhs-28 forward
OP048	**ACCATCAGCCATTTCCTGGAA**	21		58	RT-PCR of dhs-28 reverse 810 bp
OP051	**CATCGTTGGAGTCGGTATGACAAA**	24		59	RT-PCR of P44 forward
OP052	**CCGCCATATGTATTATCATTTCGATC**	26		58	RT-PCR of P44 reverse 984 bp
OP053	**ATGTTGTCATCGTCTGGACACG**	22		57	RT-PCR of kat-1 forward
OP054	**CTATAATTTCTGGATAACCATTCCACTTG**	29		57	RT-PCR of kat-1 reverse 1224 bp
OP055	**cgtctgcagCATCGTAATCCGACAAACATTTGC**	33	PstI	59	for nlt-1 promoter cloning
OP056	**cgaggtaccTGTTGAAGCTGTAAACTATGAAAAATGA**	37	KpnI	56	nlt-1 promoter cloning (1044)
OP057	**cgtctgcagTCTGAAACATTGTCGCTTGTTCAAC**	34	PstI	59	dhs-28 promoter cloning
OP058	**cgaggtaccTAATTTGGCAACGACTATACAAAACTC**	36	KpnI	56	dhs-28 promoter cloning (988)
OP059	**CATCACCATCTAATTCAACAAGAA**	24			pPD118.25 seq. Anneals in GFP
OP060	**CTGGTACCATGAGTAAAGGAGGAGGACTT**	29	KpnI		MCS2-forward
OP061	**GCTAGCTCTAGAATTCGTCGACGGATCCGCGGCCGCGAGC TCTTTGTATAGTTCGTCCATGCC**	63	NheI,SacI,		MCS2-reverse
OP066	**GCGTTCAACTAGCAGACCATTATC**	24			pPD118.25 seq. anneals in GFP
OP074	TTGAGCTC**ATGGCTTTCAAGTCCGATGTTATT**		SacI	57	forward for pPD118.25-MCS2 GFP-nlt-1
OP075	TATCTAGA**TTAGAGCTTGGCTTTGAGCATCTT**		XbaI	57	reverse for pPD118.25-MCS2 GFP-nlt-1
OP076	TTGCGGCCGCT**ATGTCTCTTCGTTTTGACGGAAA**		NotI	56	forward for pPD118.25-MCS2 GFP-dhs-28

OP065	TTGCTAGC**TTACAATTTTGACTTCTTCGCCTTT**	31	NheI	56	reverse for pPD118.25-MCS2 GFP-dhs-28

2.1.7 Standard media, buffers and solutions

M9 Buffer

3 gm KH_2PO_4, 6gm Na_2HPO_4, 5gm NaCl, 1ml $MgSO_4$ (1M)

Freezing Solution

5.85 gm NaCl, 6.80 gm KH_2PO_4,300gm Glycerol, 5.6ml NaOH(1N), 300µl $MgSO_4$(1M)

TBF Buffer (10X)

108gm Tris.OH, 55gm Boric Acid, 9.3gm EDTA, pH 8.3, higher resolution of smaller fragments

TAE Buffer (50X)

242gm Tris.OH, 57.1 ml Acetic acid (glacial), 37.2 gm Na_2 EDTA.2 H_2O, pH`8.5, Higher Resolution of fragments >4KB

LB:

1% tryptone, 0.5% yeast extract, 1% NaCl Maniatis *et al.*, 1982

MYOB

0.55 g TRIZMA-hydrochloride, 0.24 g Tris, 4.6 g tryptone, 8 mg cholesterol, 2 g NaCl/L Church *et al.*, 1995

DMEM

10% calf serum, penicillin and streptomycin, each at 100 units/ml Maniatis *et al.*, 1982

Worm Lysis Solution

0.1 M NaCl, 10 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS, 1% 2-

mercaptoethanol, 100 µg Proteinase K/ml Hope, P. 206

TE

1 mM Tris-HCl, pH 8.0, 1 mM EDTA Hope, P. 206

Worm Injection Buffer

2% PEG-6000, 20 mM potassium phosphate, 2mM potassium citrate, pH 7.5

Mello and Fire, 1995

TBST

20 mM Tris-HCl, pH 7.5, 100 mM EDTA, 0.05% (w/v)

Tween 20 Maniatis *et al.*, 1982

For solid media, agar was added to 2%.

2.2 Methods

2.2.1 Purification of genomic DNA from nematodes

Mixed stage animals were harvested from MYOB agar plates and washed thoroughly in M9 buffer to clean the sample of bacteria. A 10× volume of Worm Lysis Solution was added. The sample was frozen for at least 10 min at -80°C and afterwards incubated at 65°C with occasional shaking until animals were completely digested. Nucleic acids were extracted from the lysate with phenol-chloroform (25:24:1, phenol:chloroform:isoamyl alcohol followed by 24:1, chloroform:isoamyl alcohol) extraction and precipitated by the addition of 2.5 volumes of ethanol to the final aqueous phase. RNA was digested 37°C for 1 hour by DNase-free RNase A at a final concentration of 10 µg/ml. DNA was pelleted by centrifugation for 10 min at 16,000

× *g*, washed twice with 70% ethanol and dissolved in water or TE buffer.

2.2.2 Purification of mRNA from nematodes

Total RNA was isolated from the nematode using the RNAqueous Small Scale Phenol-Free Total RNA Isolation Kit (Ambion, Austin, TX) following the recommendations of the manufacturer.

2.2.3 Plasmid Extraction using QIAprep 8 Miniprep kit

Upto 20ug DNA can be purified from 1-5ml cultures of *E.coli* grown in LB(Luria-Bertani) medium. First, centrifuge in small rotor for 5 min at 5000rpm and collect all pellet in one tube and resuspend in 250µl Buffer P1 (with RNase A) and transfer to a micro centrifuge tube. Then add 250µl Buffer P2 and gently invert (no vortex) the tube 4-6 times to mix. After that add 350µl Buffer N3 and invert the tube immediately but gently 4-6 times. Centrifuge for 10min at 13,000 rpm(`17,900xg) in a big rotor. And supply the supernatants to the wells of the QIAprep 8 strips and centrifuge 30 sec at small centrifuge. Switch off vacuum and wash QIAprep 8 strips by adding 1ml buffer PB to each well and applying vacuum. Switch off vacuum again and wash QIAprep 8 strips by adding 750µl Buffer PE to each well and centrifuge 2 times 30 second each (500µl+250µl) to allow buffer PE to flow through the QIAprep 8 strips. Centrifuge for 30 seconds at 13,000 rpm in big rotor and then wait for 2-5 min for complete dry out of the alcohol. Finally, elute DNA by adding 50µl Buffer EB (10mM, Tris-Cl, pH 8.5) or water to the center of the each well of QIAprep 8

strips, let stand for 1 min, and switch on vacuum source. After elution, switch off vacuum source and ventilate the QIAvac 6S slowly.

2.2.4 DNA sequencing

The DNA sequencing and sequence analysis was done by McGill University and Genome Québec Innovation Centre Sequencing Platform. The platform uses 3730xl DNA Analyzer systems from Applied Biosystems.

2.2.5 Polymerase chain reactions (PCR)

PCR reactions were performed in a Robocycler 40 or a Robocycler 96, each equipped with a Hot Top attachment. To screen mutant libraries, Ready-To-Go PCR beads were used according to the specifications of the manufacturer (Amersham, Piscataway, NJ). Reactions were performed in a volume of 10 µl. For mutation-sensitive applications, reactions typically contained 100-300 pmol of oligonucleotide primers, 0.5 mM of each dNTP, and 5U of Taq polymerase in a 25 µl buffered reaction. The Expand Long Template PCR System (Roche) was used according to the manufacturer recommendations to make PCR products longer than 5 kilobase pairs.

2.2.6 Digestion of DNA by restriction endonucleases

DNA was digested under conditions optimal for the specific restriction enzyme according to the manufacturer's instructions. For diagnostic and preparative purposes, 1 to 5 µg of DNA was digested. Double enzyme digests were done in buffers recommended by New England Biolabs.

2.2.7 Dephosphorylation of 5' DNA ends

5' DNA ends were dephosphorylated in a buffered reaction containing 5 U of calf intestinal alkaline phosphatase for 20 min at 37° C.

2.2.8 Gel electrophoresis of DNA fragments

Samples of DNA containing an appropriate volume of 6× sample dye (0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficol PM 400) (Sambrook and Russell,

2001) were subjected to electrophoresis on 1% agarose gels containing 1× TBE buffer and 0.5 μg ethidium bromide/ml. DNA fragments were visualized by ultraviolet illumination.

2.2.9 Purification of DNA fragments from agarose

DNA fragments were excised from gel, and DNA was purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) according to the following protocol:

First, Excise the DNA fragment from the agarose gel with a clean, sharp scarpel. Then weight the gel slice in a colorless tube and add 3 volume of Buffer QG to 1 Volume of gel (100mg~100μl). Incubate at 50oC for 10 min. To help dissolve gel, mix by vortexing the tube every 2-3 min during the incubation. After the gel slice has dissolved completely, check the color of the mixture whether it is yellow. Add 500μl of QG buffer after transferring to a Qlquick spin column and centrifuge for 1 min at 13,000rpm. Add 750μl of PE and centrifuge for 1-2min. Then discard the flow

through and centrifuge the Qlquick column for an additional min at 13,000 rpm. Place the Qlquick column into a clean 1.5ml microcentrifuge tube. To elute DNA, add 20μl of EB buffer and centrifuge for 1min.

2.2.10 Ligation of DNA fragments

DNA fragments were ligated at a 1:3 to 1:5 molar ratio of **plasmid:insert** in a 10 μl buffered reaction containing T_4 DNA ligase at 4°C overnight. DNA fragments obtained by PCR were ligated into the pGEM-T shuttle vector (Promega, Madison, WI) following the manufacturer's instructions.

2.2.11 Determination of lifespan

To measure nematode lifespan, plates containing 10 to 14 worms were incubated at 20°C and scored daily for surviving animals. Worms were transferred to fresh plates every 2 to 3 days. The starting point for lifespan determination was at hatching. Lost, bagged and exploded animals were excluded from analysis.

2.2.12 Growth synchronization

Gravid *C. elegans* worms were washed with M9 buffer and bleaching solution (3.5ml ddH20, basic hypochlorite 0.25 M KOH, 1-1.5% hypochlorite) for 5 min to harvest eggs. Discard the bleaching solution (supernatant) and repeatedly wash with M9 buffer to completely remove that bleaching solution. Then distribute across fresh plates seeded with *E. coli* OP5 and incubate at room temperature for worm development.

2.2.13 Dauer Assays

For dauer assays some 500 starvation-synchronized at L1 stage worms were transferred to the regular *E. coli* OP50-seeded plates and left to clear bacterial lawn. Five days after finishing bacterial food (usually it took two days longer for P-44 mutant), worms were washed off the plates and treated for minimum 20 min with 1% SDS solution. Twitching worms were counted as SDS-resistant. Mutant worms displayed very limited movement in comparison with active wild-type worms. Close examination under microscope showed that most of SDS-resistant wild-type worms display classical dauer phenotype (Riddle DL, 1988), whereas SDS-resistant P-44 mutants did not have shrunk body diameter or constricted pharynx, and displayed well developed gut (Fig. 3.2.1B).

2.2.14 Biolistic Particle Delivery System (Bombardment procedure)

Weigh 1mg of gold in a 1.5ml Eppendrof and add 1ml of spermidin (0.80µl of spermidin in 1ml of ddH20). Vortex for 10 min with higher speed and then spin down for 10seconds. Take out 900µl of spermidin carefully and vortex for 60 seconds. Add mm016 and dhs28 or nlt-1 plasmid (DNA higher concentration) gradually by putting the tube in vortex mecine and gradually adding the DNA in the gold-spermidin solution. Keep it on ice for 10 min and in every 2min shake it by flipping to keep the gold particle always on the top. Add ddH20 to make the volume upto 360µl and then vortex for 1 min. Keep the solution on ice for 10 min and then add 100µl 1M CaCl2 dropwise to avoid clamping. Vortex and leave for 10 min at room temperature for precipitation. Centrifuge 15seconds at 13,000 rpm and remove supernatant. Wash

three times with 1ml ethanol 96% and spin down for 10sec.Take out most of the ethanol leaving behind 100-125µl mixture on the tube. Vortex properly and distribute on the plates (prepared earlier with micro carrier). Prepare the worms by washing them with Mg Buffer and distribute in 4 plates. Use the micro carrier containing plates in the Biolistic particle delivery system for shooting. After the bombardment allow the alive worms to get recovery for 10-15 mins and then transfer them by washing with Mg buffer to plate containing OP50 bacterial growth.

2.2.15 Fluorescent microscopy

Worms were washed from plates with M9 buffer, fixed on slide through the mixture of water and NaN_3. Then they were ready to examine with a Zeiss Axioplan microscope (Carl Zeiss Inc, Germany). The microscopic images were captured with a Spot Insight Color camera.

2.2.16 Single worm PCR

A nested PCR method (Plasterk, 1995) was used for single worm PCR. Individual worms were put in a 96-well format plate in 2.5 µl of worm lysis solution (Table 2.1.7), lysed by incubation for 90 min at 65°C, and then maintained for 30 min at 90°C to inactivate proteinase K. The PCR master mix containing an external pair of primers for nested PCR was dispersed into wells (10 µl each). The PCR reaction was performed on a

Robocycler 96 machine equipped with a Hot Top. After the first round of PCR (35 cycles), the reaction mixture was removed from the wells, and PCR master mix

containing an internal pair of primers was dispersed, and the cycling was again performed. The resulting PCR products were analyzed by electrophoresis on an agarose gel.

2.2.17 Lipid extraction and thin-layer chromatography (TLC)

Lipid extraction from the nematodes was performed as described (Ashrafi *et al.*, 2003). Plates-grown nematodes were washed off with M9 buffer, rinsed several times to remove bacteria and immersed into 5 ml of ice-cold methanol : chloroform : formic acid 10:10:1 mixture and frozen overnight at -20 °C. Next day 2.2 ml of 1M KCl in 0.2M H_3PO_4 was added to the samples and chloroform phase recovered. Samples were concentrated under nitrogen. TLC was done using silica gel plates (Whatman) in chloroform : methanol : formic acid (80:20:0.5) system. The lipid standards used were: triolein (Sigma), tripalmitolein (Larodan), diolein (Avanti), oleic acid (Sigma). Lipids were detected with phosphomolybdic acid (Landing *et al.*, 1952).

2.2.18 Nile red and Sudan black Staining

Lipid droplets were stained as described elsewhere (Ashrafi et al., 2003; McKay et al., 2003). For Nile red staining, worms were grown on MYOB plates supplemented with Nile red (Sigma) at final concentration 1 ng/ml. For Sudan black staining worm culture was harvested with M9 buffer, fixed in 1% paraformaldehyde, freeze-thawed 3 times, dehydrated in consequent concentrations of ethanol and stained in 50% saturated solution of Sudan black (Sigma) in 70% ethanol overnight. Before examination under microscope stained worms were re-hydrated and washed with M9 buffer.

2.2.19 Gas chromatography

To analyze the overall composition of fatty acids in the nematode, a mixed population of worms was washed from agar plates, rinsed several times with water, submerged in 1 ml of 2.5% H_2SO_4 in methanol and incubated at 80°C for 1 hour. 200 µl of hexane and 1.5 ml of water was then added to extract methyl esters. The solution was concentrated under vacuum. Samples were analyzed by GC-MS according to the method of Watts and Browse (Watts and Browse, 2002) using a Hewlett-Packard (HP) Gas Chromatograph 5890 Series II equipped with a DB-225 column. The initial temperature was 50°C, and the gas chromatograph was programmed to increase temperature at 10°C/min to 180°C, to hold for 5 min, and then increase temperature to 220°C at 5°C/min, and then to hold for 5 min.

2.2.20 Supplementation of the nematode diet with pristanic acid

Pristanic acid was found to be catabolized by *E. coli.* Therefore, bacteria had to be removed from all experiments involving the supplementation with external pristanic acid. Worms grown on agar plates and fed OP50 *E. coli* were rinsed off with M9 buffer and cleaned of remaining bacteria by several consecutive washes with M9 buffer. Cleaned worms were resuspended in 250 µl of M9 buffer in a capped glass vial 1.5-cm in diameter and with a flat bottom. 2 µl of pure pristanic acid was added to the vial, and worms were left at room temperature for 16 hours. Worms were washed thoroughly with M9 buffer and then water and subjected to GC-MS analysis.

2.2.21 Catalytic hydrogenatio n of FAMEs

After extraction of FAMEs from nematode they were dried and dissolved in methanol:chlorophorm (1:1, v/v), and incubated for 4 hours with stirring under 50 psi of hydrogen at room temperature with a presence of 10% Pd on charcoal (Sigma). Cyclopropane-containing fatty acids are not affected during this procedure, although unsaturated bonds in FAMEs are converted to saturated ones.

CHAPTER 3.1

Expression and sub-cellular localization of *C. elegans* homologs of hSCPs (P-44, nlt-1 and dhs-28)

3.1.1 Expression and Sub-cellular localization of *C. elegans* homologs of hSCPs

In order to study the function of *C. elegans* homologs of hSCPs, their expression and subcellular localization should be identified. It has already been shown by another research group that P-44 is expressed mainly in intestine **(Maebuchi, M *et al.*, 1999)** and it is peroxisomal **(Maebuchi, M *et al.*, 1999)**. From our experimental data, we have tried to characterize the expression and sub-cellular localization of both nlt-1 and dhs-28. For achieving that goal, we constructed several expression vectors. A pair of vectors expressed GFP gene under the control of the nlt-1 or dhs-28 promoter. The plasmid constructs are known as **pPD118.25MSC2(pnlt-1)** and **pPD118.25MSC2(pdhs-28)** , respectively. They are also named as **nlt-1::GFP** and **dhs-28::GFP,** respectively. There is no artificial PTS1 (peroxisomal targeting signal Type1) present in any of these vectors. The second pair of vector was constructed with GFP is fused at the N-terminal of the nlt-1 and dhs-28 proteins. The C-terminas of both nlt-1 and dhs-28 proteins contained consensus sequence for a peroxisomal targeting signal type1. This signal is found in most peroxisomal proteins and targets them from cytoplasm to the peroxisomes. These plasmid constructs are termed as **pPD118.25MSC2(nlt-1)** and **pPD118.25MSC2(dhs-28)** , respectively. Here we will present them as let858::GFP::nlt-1 and let858::GFP::dhs-28, respectively.

In case of nlt-1::GFP and dhs-28::GFP plasmid constructs, only intestinal cells show expression of GFP because these promoters are only active in intestinal cells (Fig 3.1.1, Fig 3.1.2, Fig 3.1.3, Fig 3.1.4). This also suggests that the nlt-1 and dhs-28 promoters are only expressed in intestinal cells. The expression pattern of GFP is rather diffused pattern of fluorescence which is characteristic of proteins localized to

the cytoplasm. Based on the GFP expression expression at different stages of worm lifecycle we can conclude that these genes are expressed early in development, starting from embryo (Fig 3.1.1, Fig 3.1.4).

The expression results of recombinant constructs let858::GFP::nlt-1 and let858::GFP::dhs-28 show that these proteins appear to be peroxisomal as they show a punctuate pattern of fluorescence, which is characteristic of peroxisomal localization. The GFP is targeted into peroxisomes by dhs-28 and nlt-1 Fig 3.1.7 and Fig 3.1.8 respectively. Confocal microscopic image (Fig 3.1.9) using nlt1-GFP and YFP-SKL confirms peroxisomal localization of nlt-1. The worm expressing GFP-nlt-1 construct (green, Fig 3.1.9B) was fixed and subjected to immunostaining with anti-SKL antibodies which are labeled with rhodamine (red, Fig 3.1.9A). These antibodies recognize SLK-containing proteins (mostly peroxisomal). The patterns of anti-SKL antibodies and GFP-nlt-1 overlap (yellow, Fig 3.1.9 C). This clearly indicates that GFP-nlt-1 is really in peroxisomes.

Expression results of Nlt-1::GFP plasmid construct

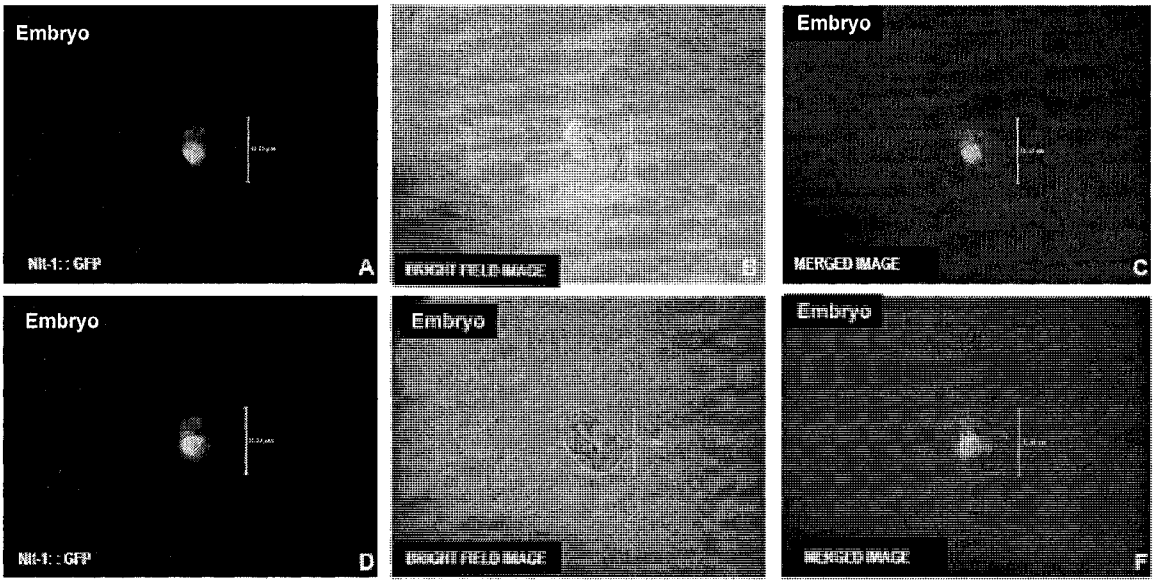


Figure 3.1.1 Pattern of fluorescence generated by nlt-1::GFP plasmid construct. The diffused pattern of fluorescence is the characteristic of **cytoplasm**. GFP expression is seen in **intestinal** cells of embryos. A: GFP expression in embryo (Day 0). B: Same image in Bright field. C: Merged image of A & B. D-F: Same as A-C. This figure also reflects that *nlt-1* gene is expressed in early stage of lifecycle.

Expression results of Nlt-1::GFP plasmid construct

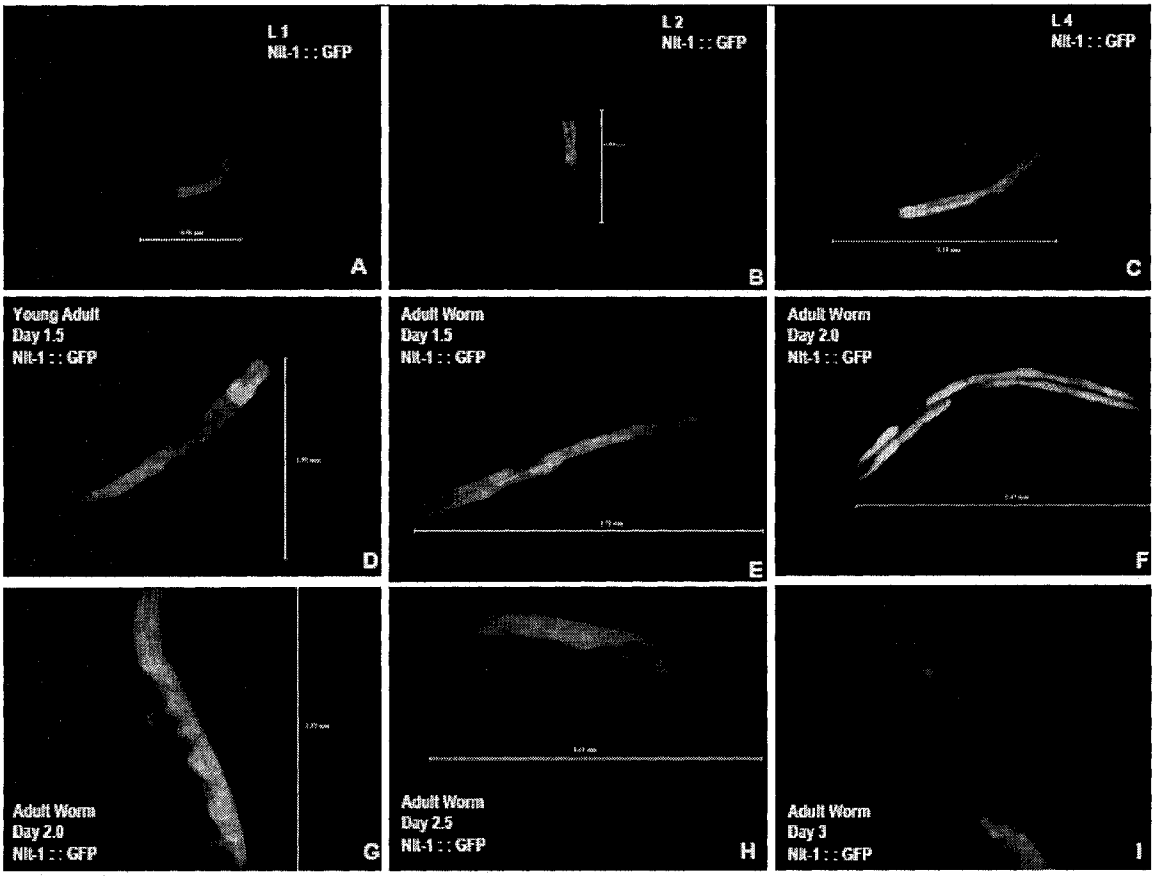


Figure 3.1.2 Pattern of fluorescence generated by nlt-1::GFP plasmid construct at different stages of the lifecycle. The diffused pattern of fluorescence is characteristic of localization of the cytoplasm. GFP expression is seen in intestinal cells of L1 (Day 0 hr 4), L2 (Day 0 hr 12), L4 (Day 1 hr 4), young Adult (Day 1 hr 14) and adult worms (>Day 1 hr 22) at different body positions.

Expression results of Nlt-1::GFP plasmid construct

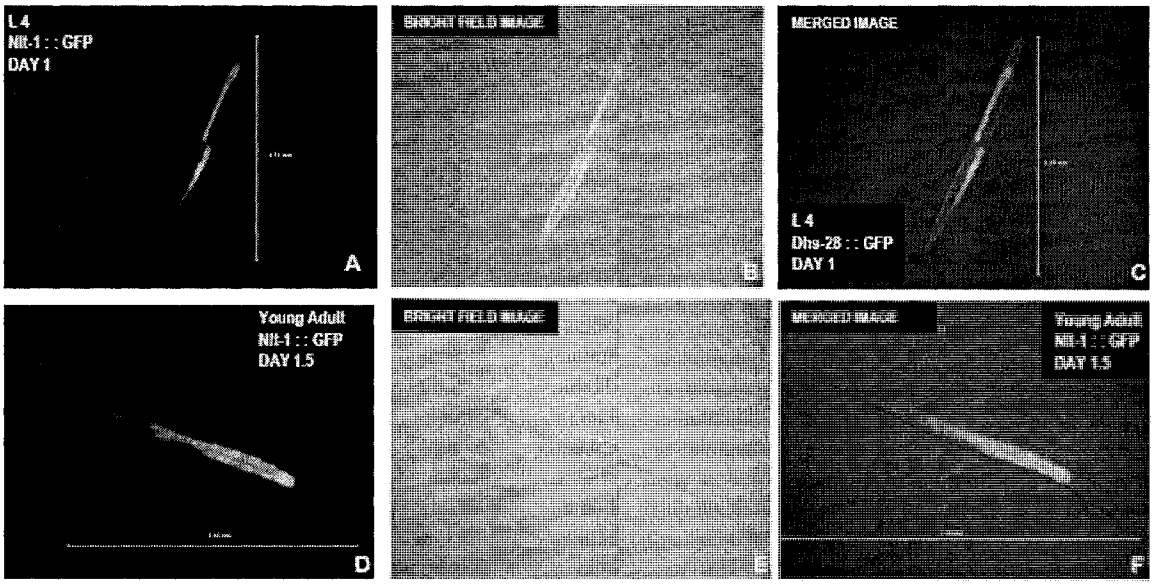


Figure 3.1.3 Pattern of fluorescence generated by nlt-1::GFP plasmid construct. The diffused pattern of fluorescence is seen in intestinal cells of worms at different stages of the lifecycle. A: GFP expression in L4 (Day 1). B: Same image in Bright field microscope. C: Merged image of A & B. D: GFP expression in Young Adult worm (Day 1.5). E: Same image in Bright field microscope. F: Merged image of D & E.

Expression results of dhs-28::GFP plasmid construct

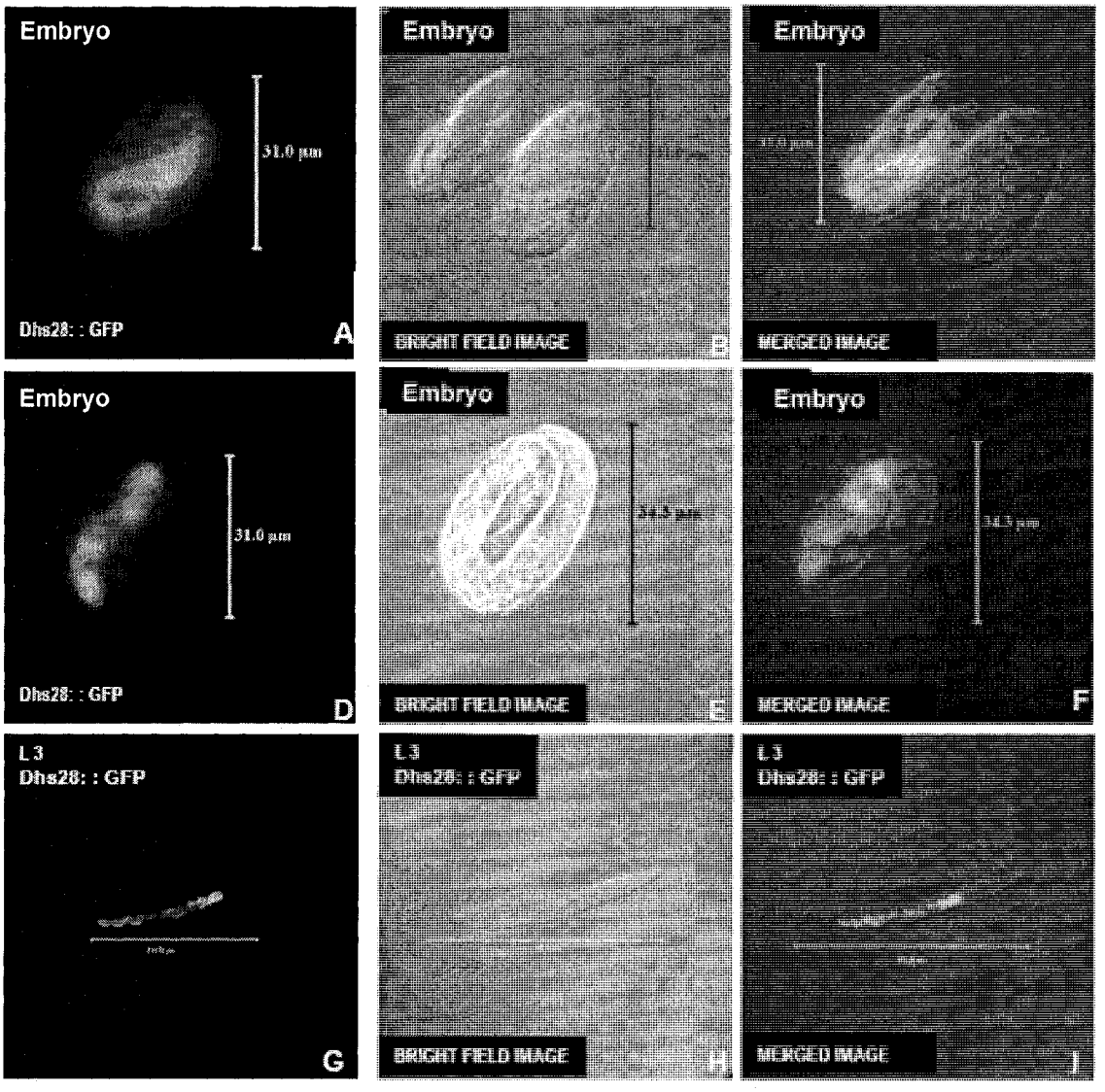


Figure 3.1.4 Pattern of fluorescence generated by dhs28::GFP plasmid construct. The diffused pattern of fluorescence is seen in intestinal cells of worms at different stages of lifecycle. This figure also reflects that dhs-28 gene is expressed in early stage of lifecycle. A: GFP expression in embryo (Day 0). B: Same image in Bright field microscope. C: Merged image of A & B. D-F: Same type of result in a different embryo. G: GFP expression in worm at L3 stage. H: Same image in Bright field microscope. I: Merged image of G & H.

Expression results of dhs-28::GFP plasmid construct

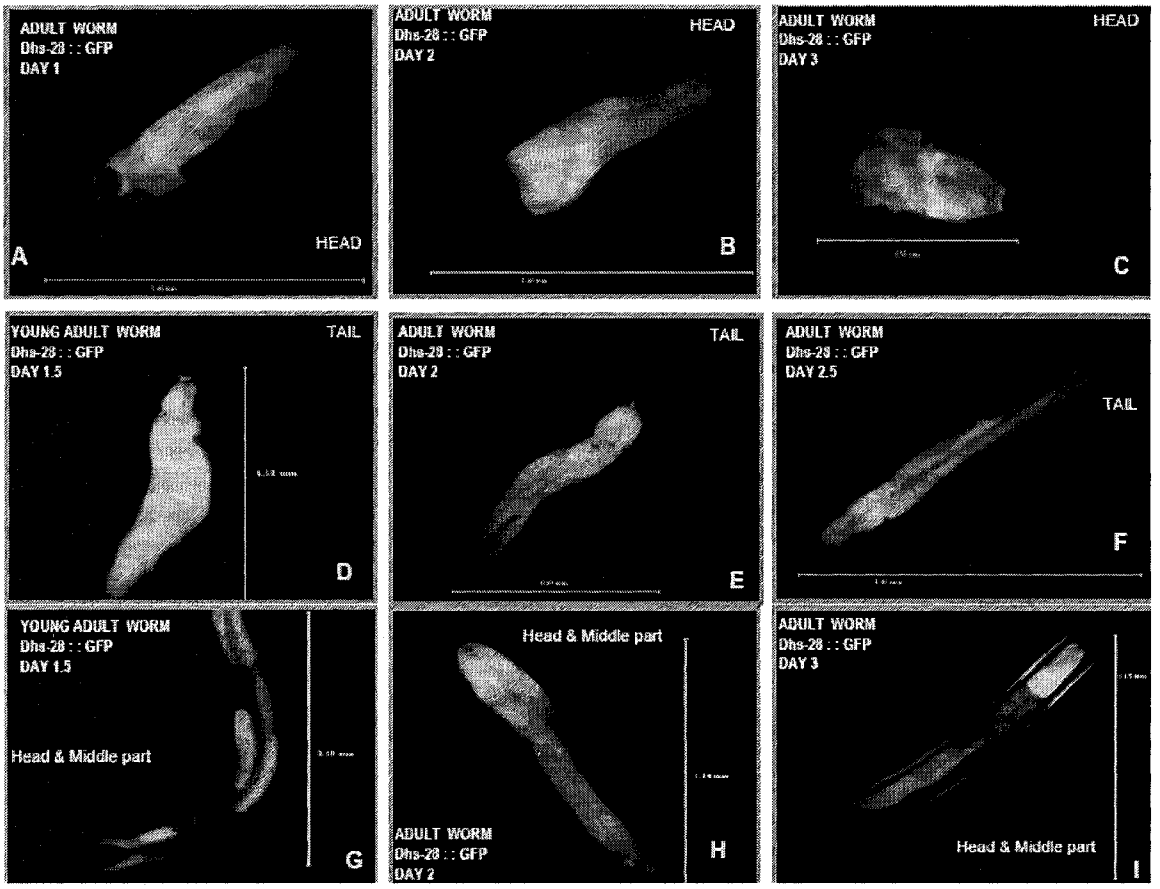


Figure 3.1.5 Pattern of fluorescence generated by dhs28::GFP plasmid construct. The cytosolic pattern of fluorescence is seen in intestinal cells of worms at yound adult and adult stage (Day1.5, Day2 and Day 3) of lifecycle at different body positions (Head, Tail, Head+Middle body part). A: GFP expression in adult nematode head (Day 1). B: Same image in Bright field microscope. C: Merged image of A & B. D-F: Same type of result in a different young adult in tail (Day 1.5). G: GFP expression in worm at L3 stage. H: Same image in Bright field microscope. I: Merged image of G & H.

Expression results of dhs-28::GFP plasmid construct

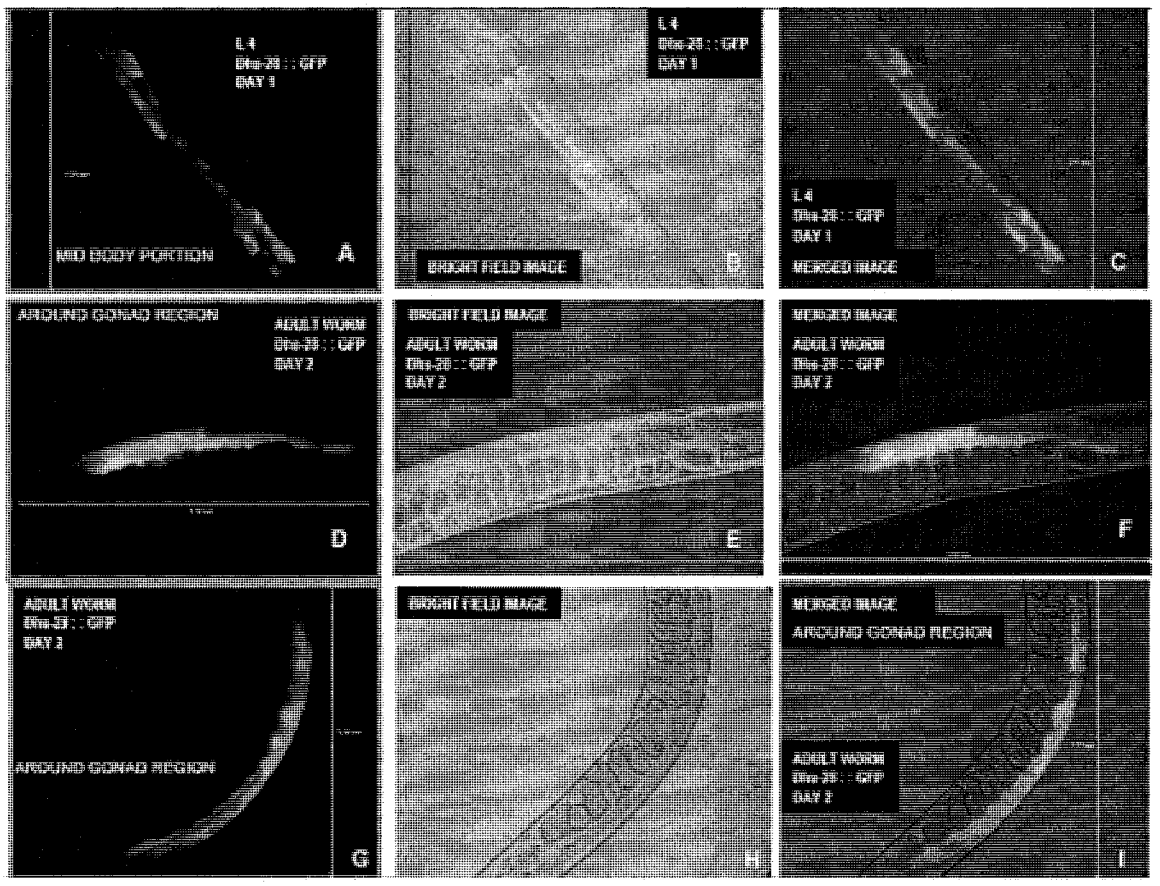


Figure 3.1.6 Pattern of fluorescence generated by dhs28::GFP plasmid construct. The cytosolic pattern of fluorescence is seen in intestinal cells of worms at L4 and Adult stage (Day1 & Day2) of lifecycle at different body positions.

Expression results of let858::GFP::nlt-1 and let858::GFP::dhs-28 plasmid construct

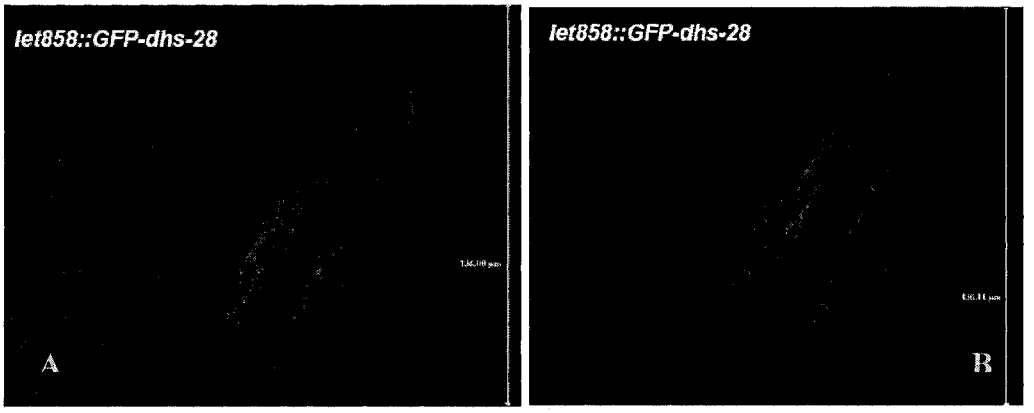


Figure 3.1.7 Pattern of fluorescence generated by let858::GFP::dhs-28 plasmid constructs. A punctuate localization pattern of fluorescence is found, which is characteristic of Peroxisome. GFP expression is seen in intestinal cells of the adult worm. A-B: punctuate localization of GFP expression in the adult worm using let858::GFP::dhs-28 construct.

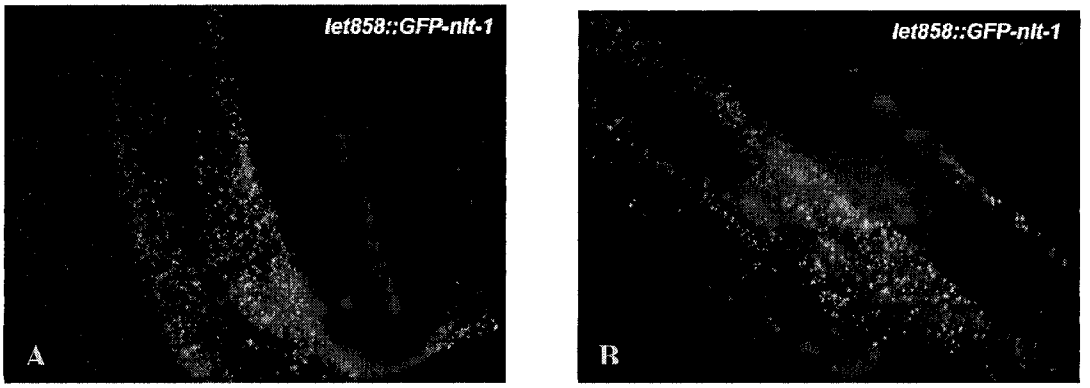


Figure 3.1.8 Pattern of fluorescence generated by *let858::GFP::nlt-1* plasmid constructs. The punctuate structure pattern of fluorescence is found which is the characteristic of **Peroxisome**. GFP expression is seen in **intestinal** cells of Adult worm. A-B: punctuate localization of GFP expression in Adult worm using let858::GFP::nlt-1 construct.

3.1.2 Peroxisomal Localization of *nlt-1*:

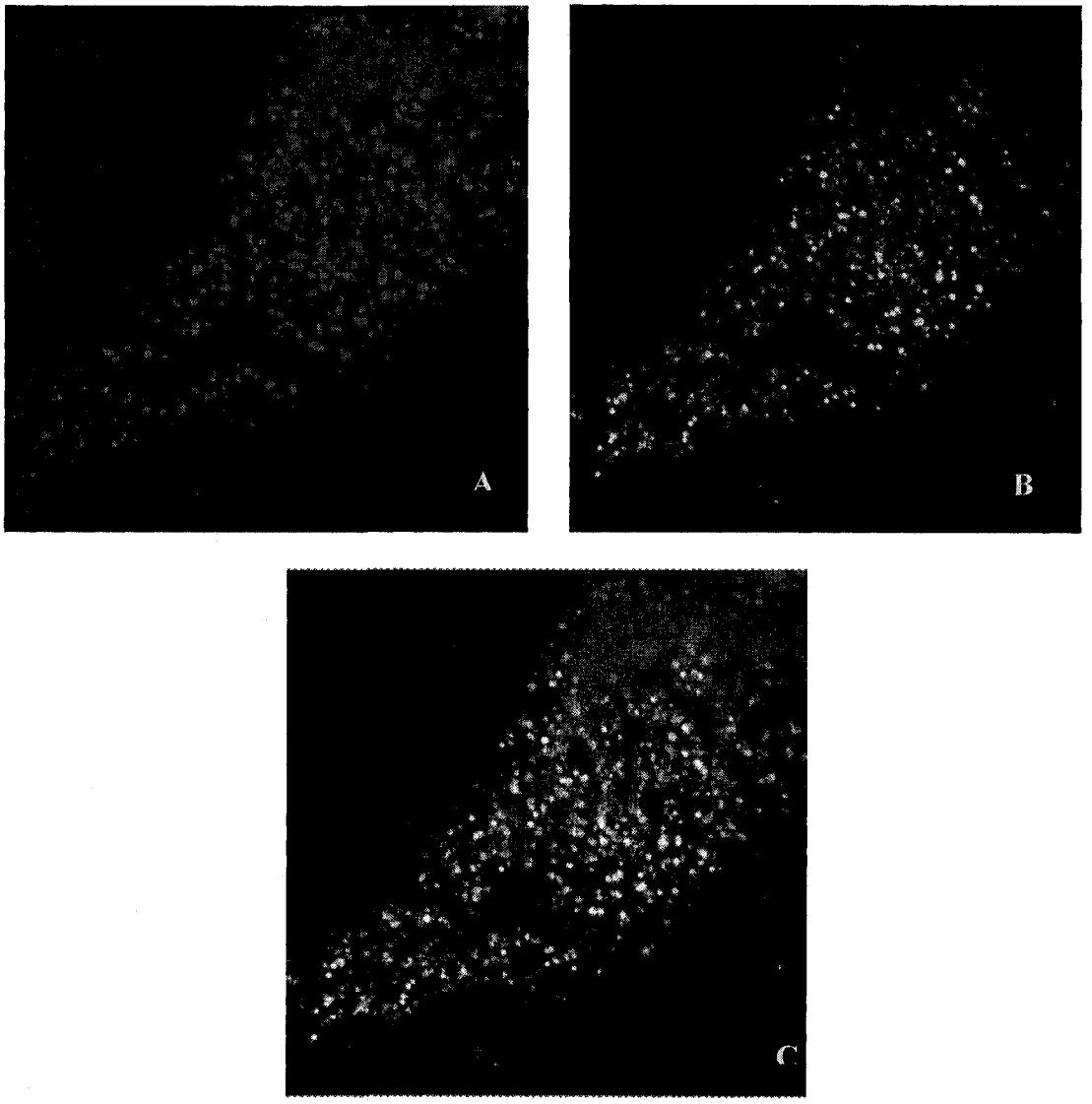


Figure 3.1.9 Confocal microscopic analysis for peroxisomal localization: the punctuate fluorescence is the pattern of peroxisomes. A. Red - YFP-SKL (detected using anti-SKL antibodies); B. Green - GFP-nlt-1; C. Yellow-Combined of A & B image. This result proves that nlt-1 is exclusively peroxisomal.

3.1.3 Discussion

The intestine is one of the major organs in *C. elegans* and is largely responsible for food digestion and assimilation as well as the synthesis and storage of macromolecules. In addition, the intestine is emerging as a powerful experimental system in which to study such universal biological phenomena as vesicular trafficking, biochemical clocks, stress responses and aging. P-44, nlt-1 and dhs-28 are all members of the SCP-2 sterol transfer family. They are thought to be involved in the transport of various lipids. P-44 is expressed largely in the intestine and is located within the peroxisome matrix (Maebuchi et al., 1999). P-44, nlt-1 and dhs-28 are also involved in the fatty acid metabolism. *C. elegans*, like mammals, appears to have two sites of β-oxidation of fatty acids: mitochondria and peroxisomes (Gurvitz et al., 2000), and most *C. elegans* peroxisomes are found in the intestine (Togo et al., 2000;Yokota et al., 2002). All the data presented in this study show that using different plasmid constructs confirm that both nlt-1 and dhs-28 aremainly expressed in the intestinal cells following differentiation in early stage of the life cycle.The results also show that nlt-1 and dhs-28 are expressed in embryonic cells.

GFP localization in figure 3.1.9 shows that the expression of nlt-1 is exclusively localized to the peroxisome, which are apparently present in higher numbers in intestinal cells and plays role in peroxisomal oxidation of Fatty acids. Although my preliminary data using let858::dhs-28::GFP plasmid construct shows dhs-28 is probably localized to the peroxisome (Fig 3.1.7), a confocal microscopic approach similar to that used by nlt-1 should be taken to confirm that .

CHAPTER 3.2

CAENORHABDITIS ELEGANS ORTHOLOG OF HUMAN STEROL CARRIER PROTEIN X PLAYS AN ESSENTIAL ROLE IN NEMATODE DEVELOPMENT

3.2.1 Summary:

Caenorhabditis elegans possesses only one type II thiolase, P-44 which is an ortholog of the human sterol carrier protein x (SCPx). SCPx is known to be required for the metabolism of variety of 3-oxoacyl-CoA esters including the ones with α-methyl branched carbon chains, long- and very-long straight chains. SCPx deficiency in mice has a marked effect on hepatic gene expression, peroxisome proliferation. In addition SCPx deficiency also causes hypolipidemia, impaired body weight control and neuropathy. P-44 deficiency in nematodes results in defective dauer formation, delayed egg-laying period, smaller worm body size and increased lipid accumulation. The lifespan of the P-44 deficient nematodes appears to be extended. I analyzed fatty acid (FA) composition in wild-type and *ΔP-44* nematodes and found that P-44 is required for normal metabolism of variety of minor forms of FAs including iso-FAs, cyclopopane-containing FAs, some unsaturated and hydroxy-FAs. P-44 deficiency also blocks catabolism of pristanic acid in the nematode. A similar phenotype is found for worms carrying a defect in 17-β-hydroxysteroid dehydrogenase, dhs-28, suggesting dhs-28 and P-44 have roles in the same biochemical pathway. We propose several possible interpretations for the observed effects. Non-specific lipid transfer protein, nlt-1 deficient worms have an extended lifespan relative to that of wild type worms. It is also involved in the fat metabolism. Our data also support views that there is no direct link between amount of lipid deposits in an organism and a lifespan.

3.2.2 Phenotypical characterization of *C. elegans* worms defective in type II 3-oxoacyl-CoA thiolase:

P-44 deficiency affects worm size, dauer formation, egg laying, peroxisome morphology, and causes accumulation of enormously increased lipid droplets in the worm. Mutant worms are 25% shorter in length than the wild-type worms 4 days after hatching and remain smaller onwards (Fig. 3.2.1). They fail to enter effectively dauer stage when induced by food deprivation and overpopulation. While one-quarter of wild-type worms enters dauer stage, only 8% of the P-44 deficient nematodes display a dauer-like phenotype under comparable conditions. These worms do not display all classical features of dauer phenotype (See *Experimental Procedures*) (Fig. 3.2.2). Mutant worms lay similar numbers of eggs as wild-type worms, but the egg laying period of P-44 deficient nematodes is longer than that of wild-type worms (Fig. 3.2.3). The egg laying by mutant worms starts 12 hours later and finishes 24 hours later in comparison with the wild-type.

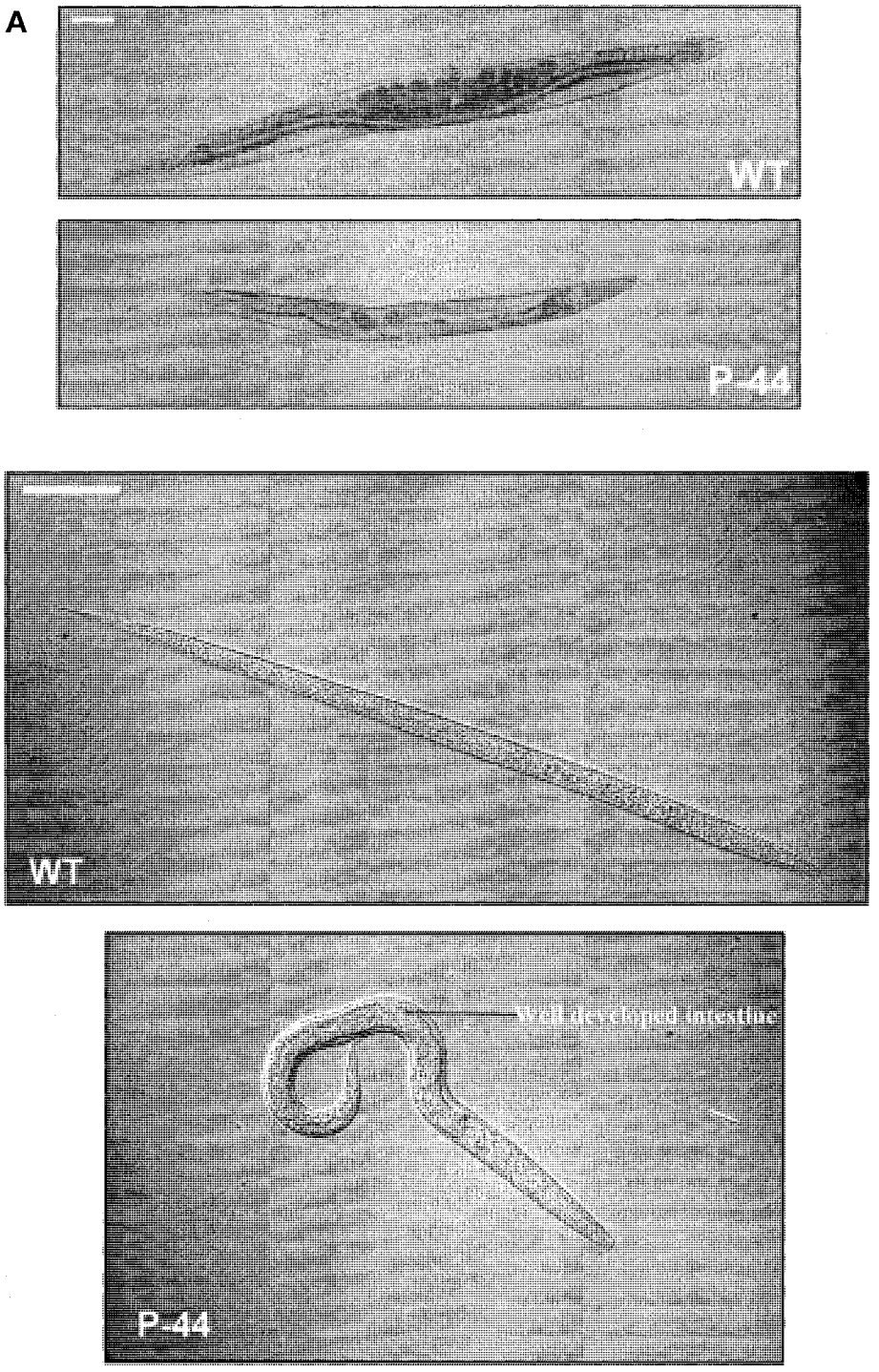


Figure 3.2.1 Sizes of age-matched wild-type and P-44 mutant adults and dauer larvae. Bar, 0.1 mm. *A,* The average length of wild-type worms was 1.57 ± 0.06 mm and of P-44 mutant worms, 1.18 ± 0.14 mm. *B*, SDS-resistant ΔP-44 worms do not have contracted pharynx and display a well-developed intestine unlike in wild-type dauer larvae.

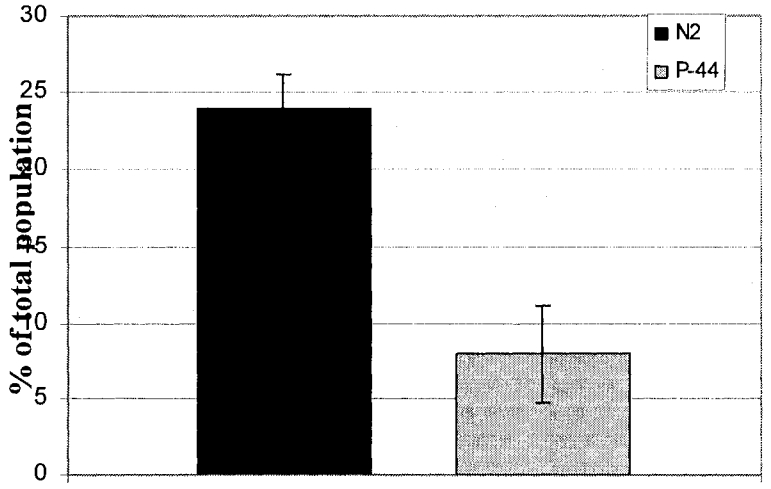
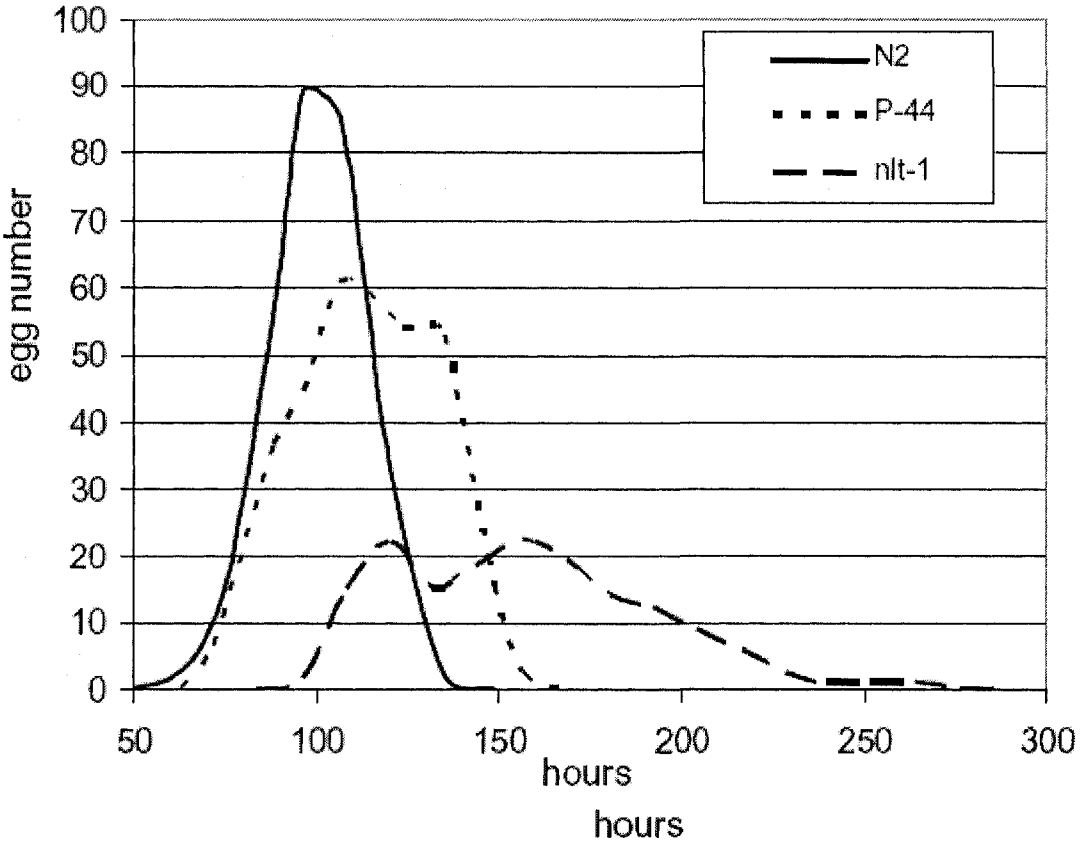


Figure 3.2.2 Dauer phenotype expression in P-44 mutant vs W/T (N_2)



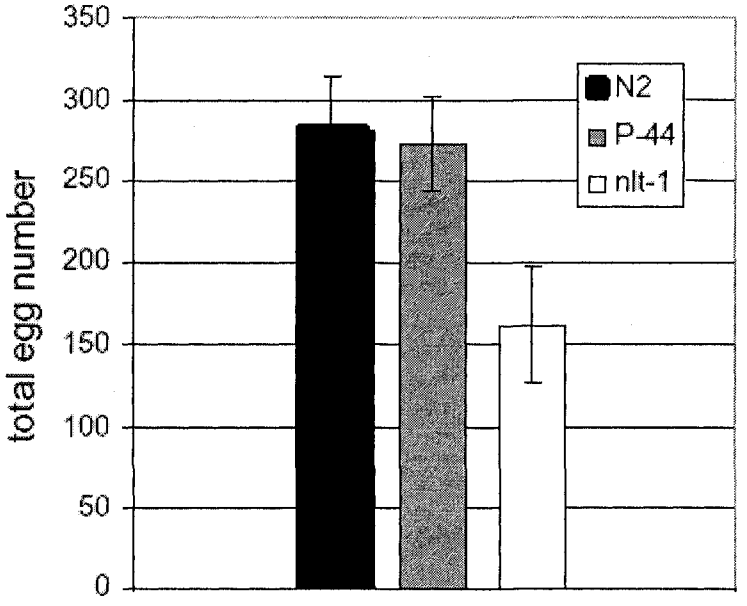


Figure 3.2.3 Egg Laying Assays with W/T (N2), ΔP-44 and Δnlt-1 mutants

Peroxisomes in P-44 mutants are irregularly shaped and abnormally distributed (Fig. 3.2.4 *A-C*). Electron microscopy studies demonstrated that peroxisomes in the gut cells of P-44 mutant worms cluster in large groups and often are accompanied by unusual membranes not found in wild-type nematode. Moreover, peroxisomes of P-44 mutant nematodes are found in immediate proximity to unusually large lipid vesicles (Fig. 3.2.4 *C*). Accumulation of enlarged lipid vesicles in P-44 mutant was also demonstrated using Nomarski phase-contrast imaging and two dies specific for neutral lipids, Nile red and Sudan black (Fig. 3.2.4 *D-F*).

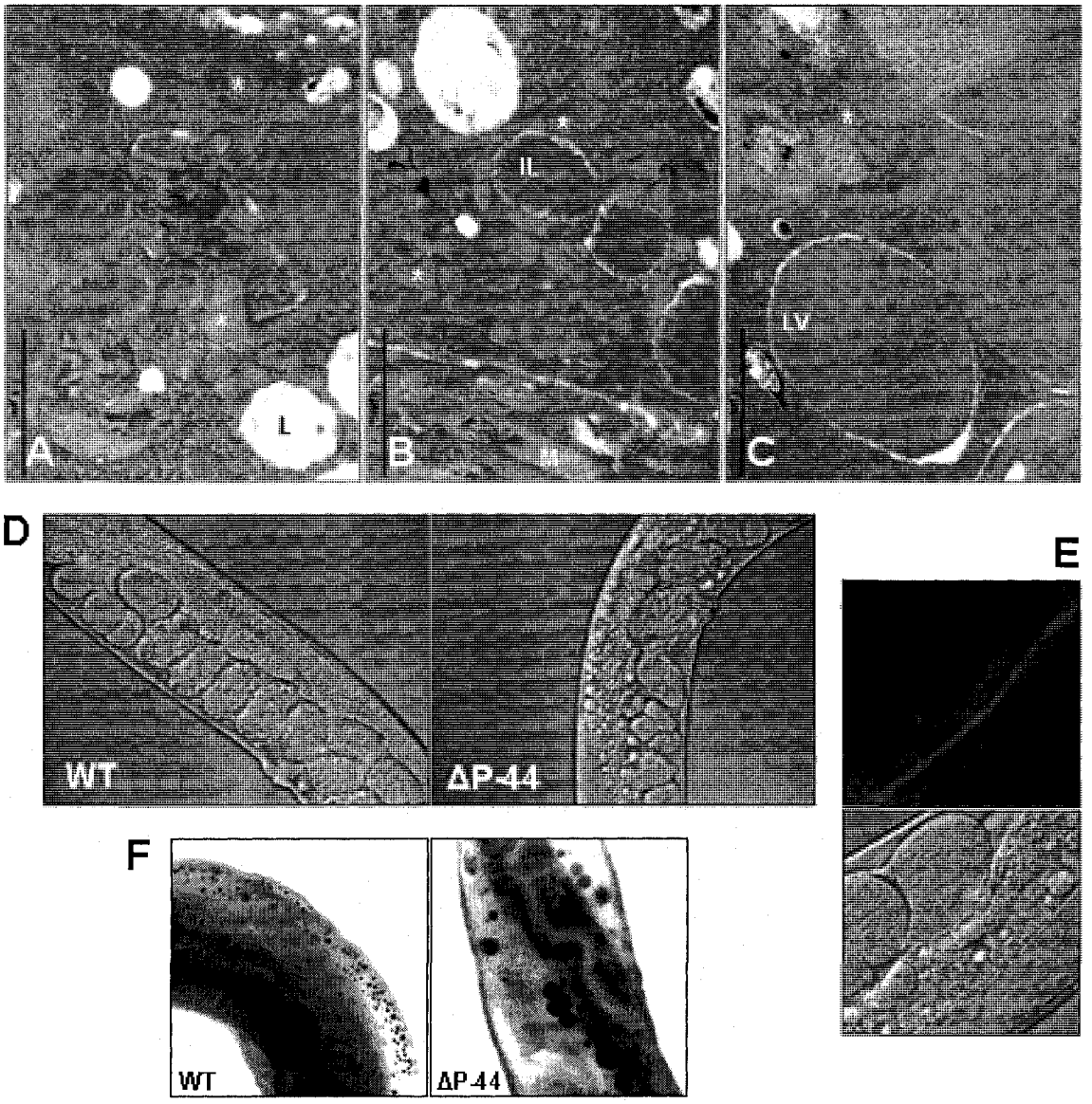
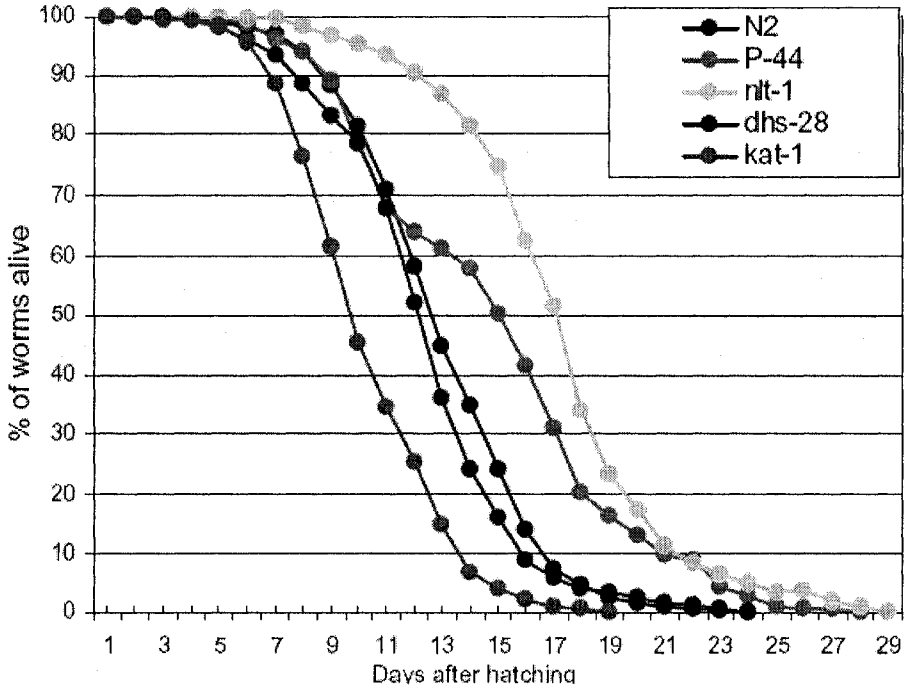


Figure. 3.2.4 Electronic microscopy study for Peroxisomes in Gut cells of P-44 mutants and W/T worms (A-C). Visualization of lipid vesicles by Normarski Phase-contrast imaging and dye specific for Neutral lipids-Nile Red and Sudan Black (D-F)

In addition, P-44 worms were found to have increased levels of triacylglycerols (Fig. 3.2.7). In light of available data (Van Gilst MR et al, 2005; Harrison, DE *et al*, 1984) that report a negative correlation between the amount of fat in an organism and lifespan, i expected to see decrease of longevity of P-44 mutant worms. Interestingly, from our data, the mean lifespan of P-44 mutant worms is 20.7 % longer than in wild-type worms (Fig. 3.2.5 A).

A.



B.

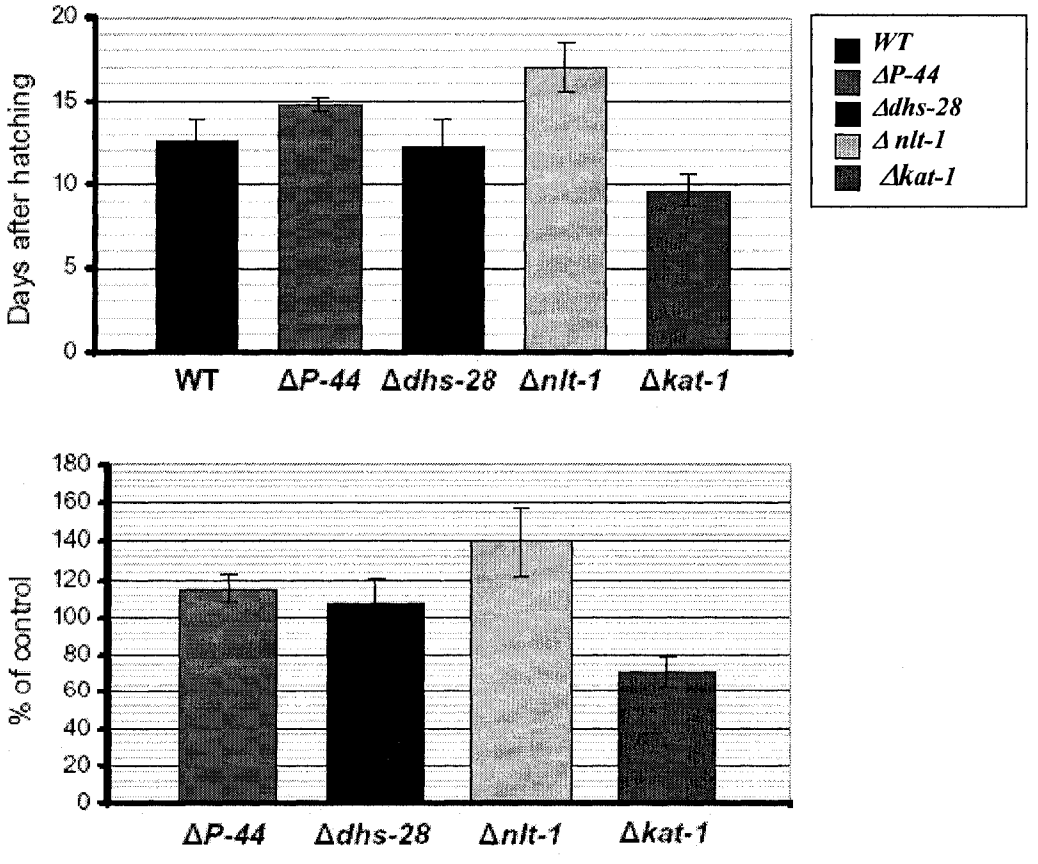


Figure 3.2.5 Lifespan Analysis of WT and different Mutant worms

3.2.3 Phenotypical characterization of nlt-1 and dhs-28 mutants:

The lifespan of nlt-1 deficient worms is 40% longer than in the wild-type worms (Figure 3.2.5). Δnlt-1 mutant has later onset and extension of egg-laying period (significant developmental delay), and lays significantly less eggs in comparison with the wild-type.

The dhs-28 deficient worms result in identical changes in the composition of triacylglycerols and FAs as in P-44 mutant (Fig. 3.2.6 and Fig. 3.2.8). An increased accumulation of lipid vesicles and higher levels of triacylglycerols were found in *dhs-28* mutant worms (Fig. 3.2.7). This suggests that dhs-28 is functioning in the same metabolic pathway as P-44. The lifespan of dhs-28 deficient worms was not different from the control (Fig. 3.2.5).

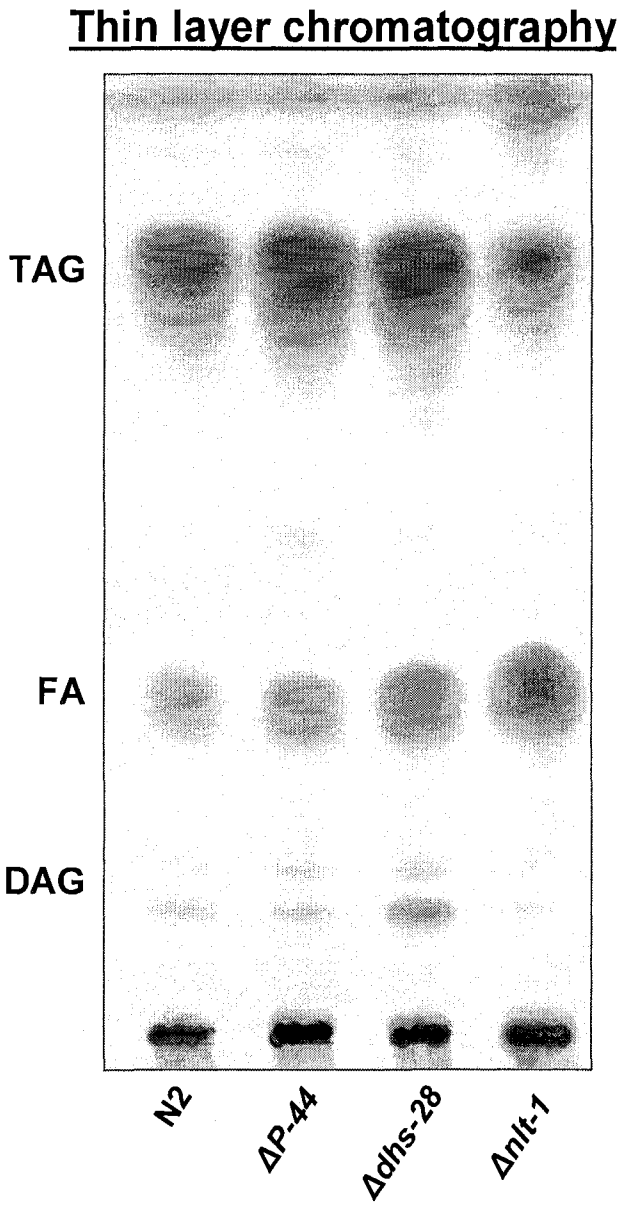


Figure 3.2.6 Thin-layer Chromatography (TLC) analysis for measuring body lipid of wild type, ΔP-44, Δdhs-28, Δnlt-1mutants

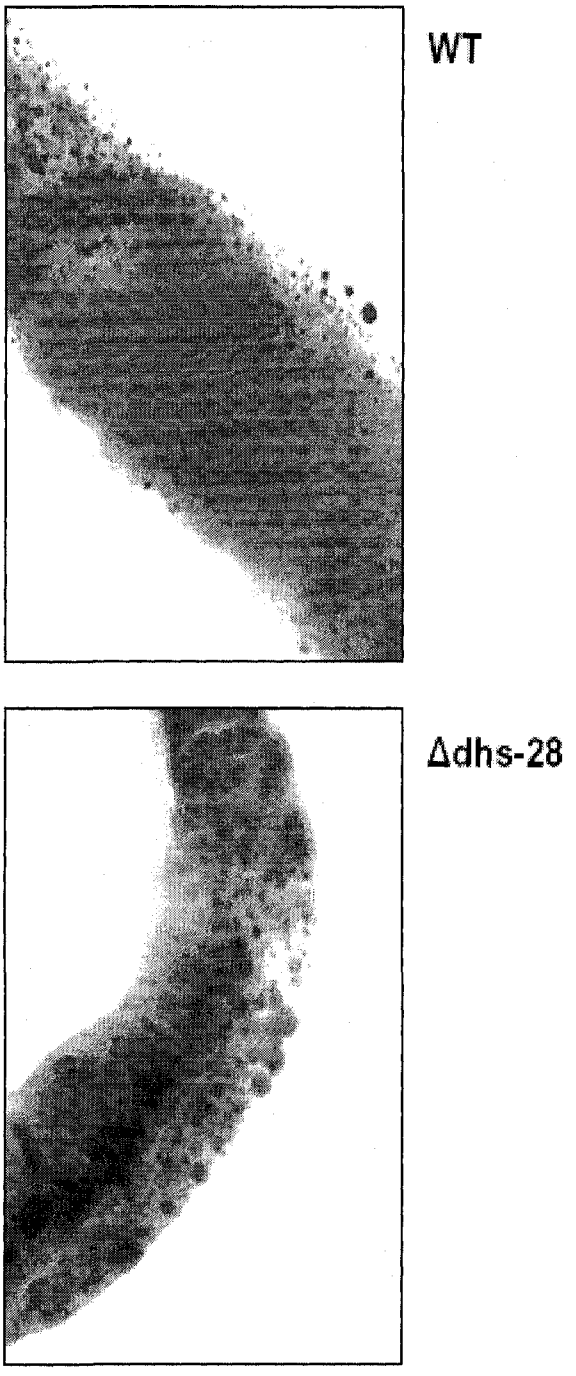


Figure 3.2.7 Analysis of lipid vesicles and TAG by Sudan Black staining between wild type and dhs-28 mutants

GC-MS of total lipid extract from wild-type, *ΔP-44* and *Δdhs-28* mutants

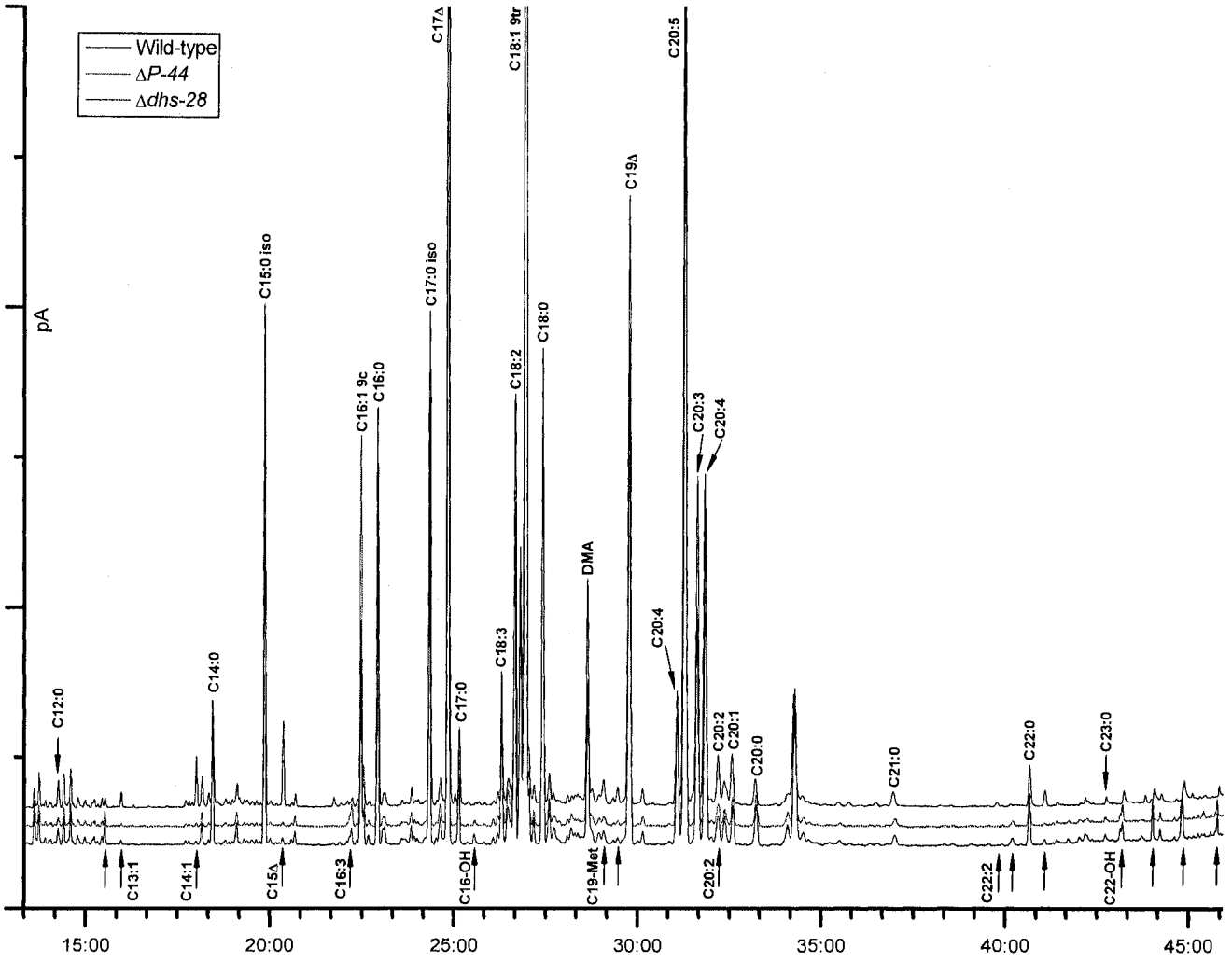


Fig. 3.2.8 Gas chromatogram spectra of the FAMEs prepared from total lipids extracted from wild-type, *ΔP-44* and *Δdhs-28* mutant worms. DB-5 column was used for separation.

The levels of dauer formation was assayed in these mutant worms. I found that the nlt-1 mutant gave a high tendency of dauer formation when compared with the wild type (Fig. 3.2.9). Nlt-1 shows phenotype-c (whereas P-44 and dhs-28 show phenotype-d) which means nlt-1 can form dauer even in non-inducing condition. Both P-44 and dhs-28 were unable to form dauers.

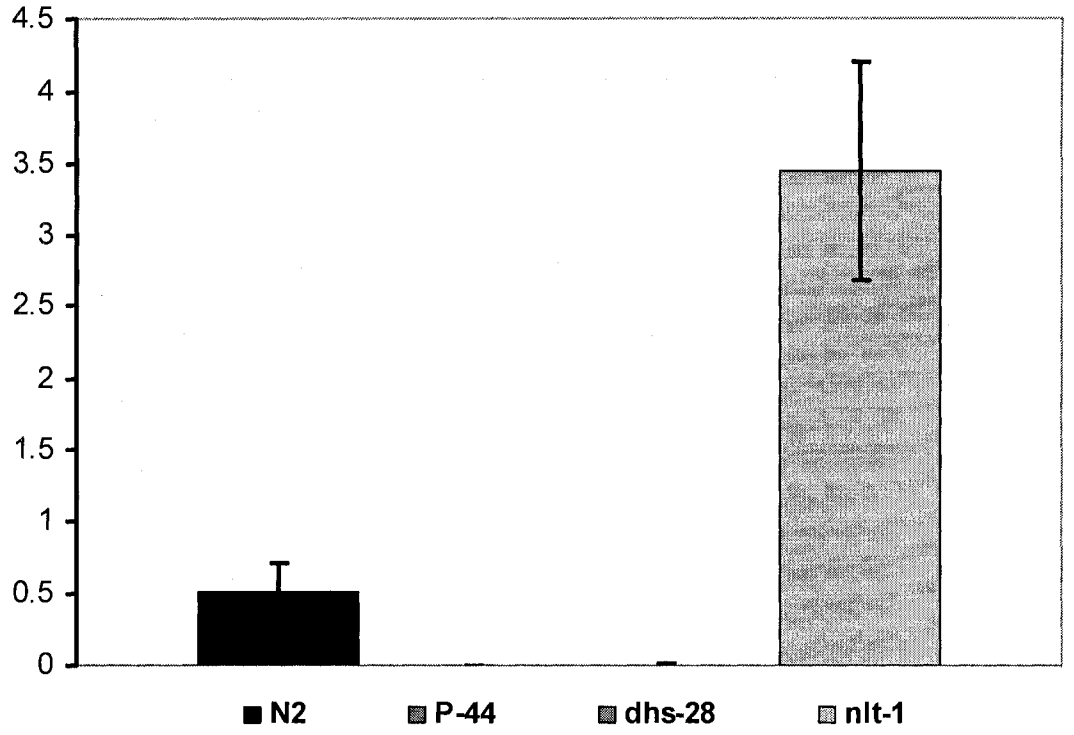


Figure 3.2.9 Dauer formation assay by the mutant and wild type strain

3.2.4 Fatty acid composition of P-44 mutant and wild-type *C. elegans*

P-44 is reported as a type II thiolase (Bun-ya *et al.* 1998). It is the closest nematode homolog of the mammalian SCPx protein. In contrast to type I thiolase, which act only on straight-chain substrates, P-44 thiolase catalytic activity was also observed with the 2-methylhexadecenoyl-CoA and oxo-forms of bile acids as substrates. The same catalytic activity is characteristic of SCPx (Bun-ya *et al.* 1998). SCPx is also known to take part in the catabolism of methyl-branched FAs such as phytanic acid (3,7,11,15-tetramethylhexadecanoic) and pristanic acid (2,6,10,14-tetramethylpentadecanoic) (Seedorf, U, *et al*, 1998)). Therefore, in analogy to SCPx, it

has been suggested that P-44 acts on branched fatty acid catabolism in the nematode (Bun-ya *et al.* 1997, Maebuchi, M. *et al,* 1999). The peroxisomal location of P-44 and expression pattern of the gene during nematode ontogenesis also suggest that P-44 and SCPx play common physiological roles (Maebuchi, M. *et al,* 1999). Thus, it was expected that mutant worms deficient in P-44 will have defects in branched FAs metabolism.

Gas chromatography-mass spectroscopy (GC-MS) spectra of total lipid extracts from P-44 mutant worms fed *E. coli* strain OP50 differ from the spectra obtained from wild-type worms in abundance of major and minor forms of FAs (Fig. 3.2.10; Table 3.2.1 for the nomenclature of fatty acids). In comparison with wild type, ΔP-44 mutants accumulate higher levels of C18:1 (n9) FA, which is the main component of nematode lipids. Similar difference between P-44 and N2 were observed for C18:2, C15:0 iso, C17:0 iso, C18:0 and 2-hydroxy-C22:0 FAs. Decreased levelsrelative to N2 were found for C13Δ, C14:1, C15Δ, C17Δ, DMA (derivative of prostaglandins), C19:0 Met (methylated, not iso- or a iso-, form of C19:0), C19Δ, poly unsaturated C20:5, C20:4, C20:2, C22:2, and 2-hydroxy-C22:0.

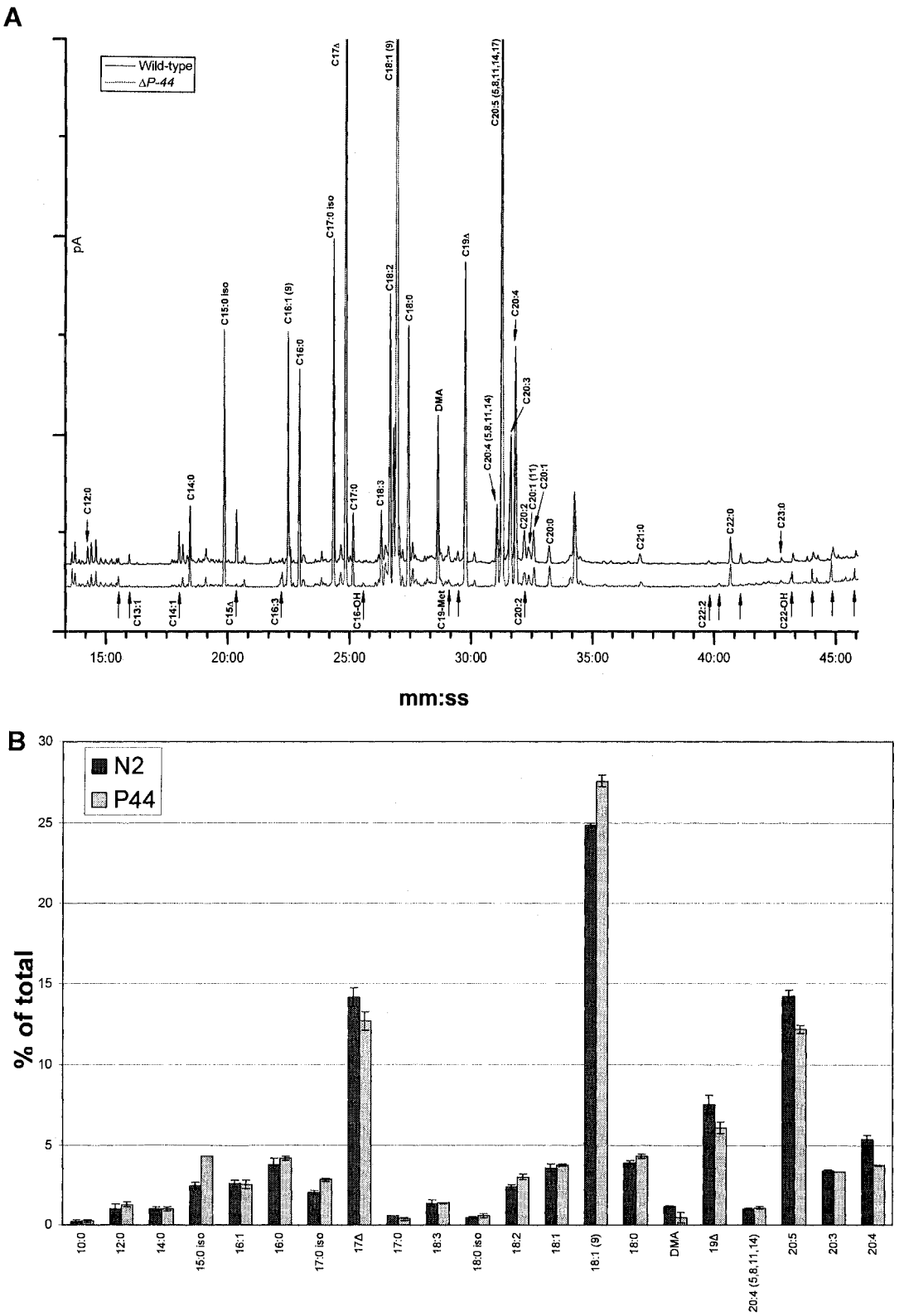
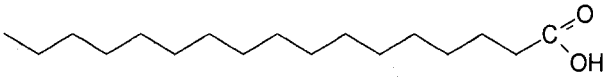
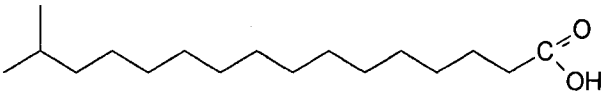
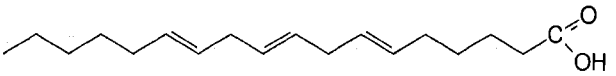
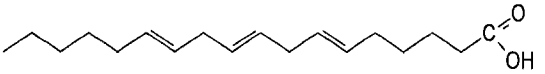
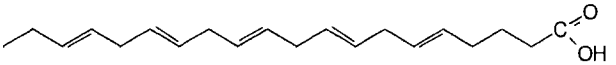
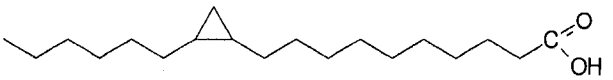
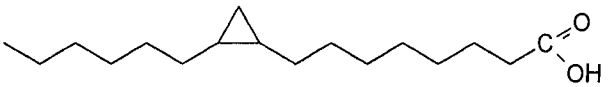
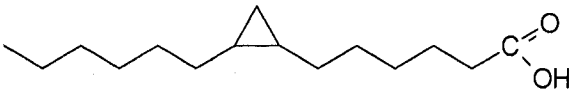
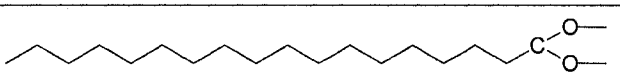
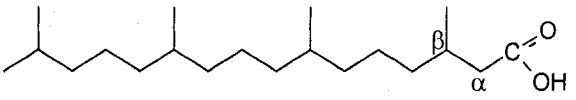
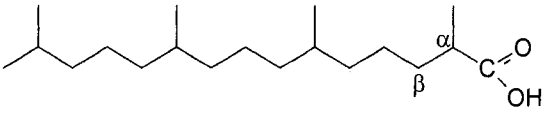
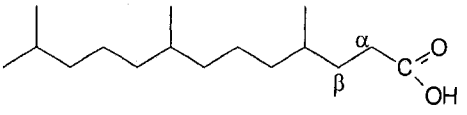


Figure 3.2.10 GC-MS spectra of total lipid extracts from P-44 mutants and W/T

Table 3.2.1 Structure of some fatty acids and their derivatives

Abbreviation Used in text	Structure	Chemical name of acid
C17:0		heptadecanoic
C17:0 iso		15-methyl hexadecanoic
C18:3		6,9,12-octadeca-trienoic
C16:3		6,9,12-hexadeca-trienoic
C20:5		5,8,11,14,17-eicosa-pentanoic
C19Δ		11,12-methylene-octadecanoic
C17Δ		9,10-methylene-hexadecanoic
C15Δ		7,8-methylene-hexadecanoic
DMA[1]		octadecanal-dimethyl acetal
phytanic		3,7,11,15-tetramethyl-hexadecanoic
pristanic		2,6,10,14-tetramethyl-pentadecanoic
TMTD		4,8,12 trimethyl-tridecanoic

Surprisingly, total lipid extracts from wild-type or P-44 mutant *C. elegans* (grown on *E. coli* plate) did not reveal any branched-chain fatty acid except for mono-methyl

[1] DMA is a derivative of plasmalogens formed during esterification of phospholipids.

branched, mostly iso- and cyclopropane-containing forms. Next I inverstigated the effect of pristanic acid (branched FA) supplimenation on the diet of wild-type and P-44 mutant worms. When the diet of *C. elegans* was supplemented with pristanic acid, it was detected among the other FAs found in the nematode. In addition, the intermediates of pristanic acid catabolism, 4,8,12 trimethyltridecanoate, could be detected in wild-type worms but not in P-44 mutant worms (Fig. 3.2.11; Fig. 3.2.12 for mass spectra of pristanate and TMTD).

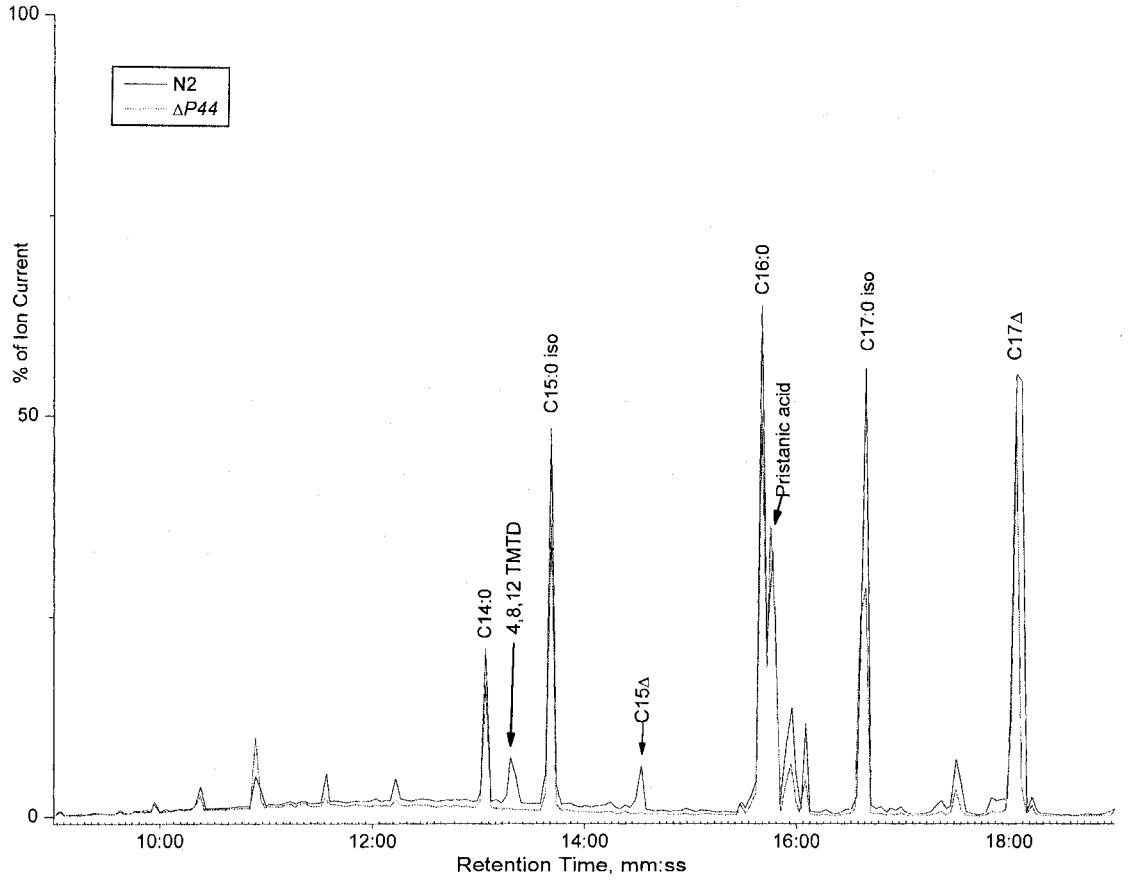


Figure 3.2.11 Mass spectra of pristanic, and 4,8,12 trimethyltridecanoic (TMTD) acids from *C. elegans* lipid extracts

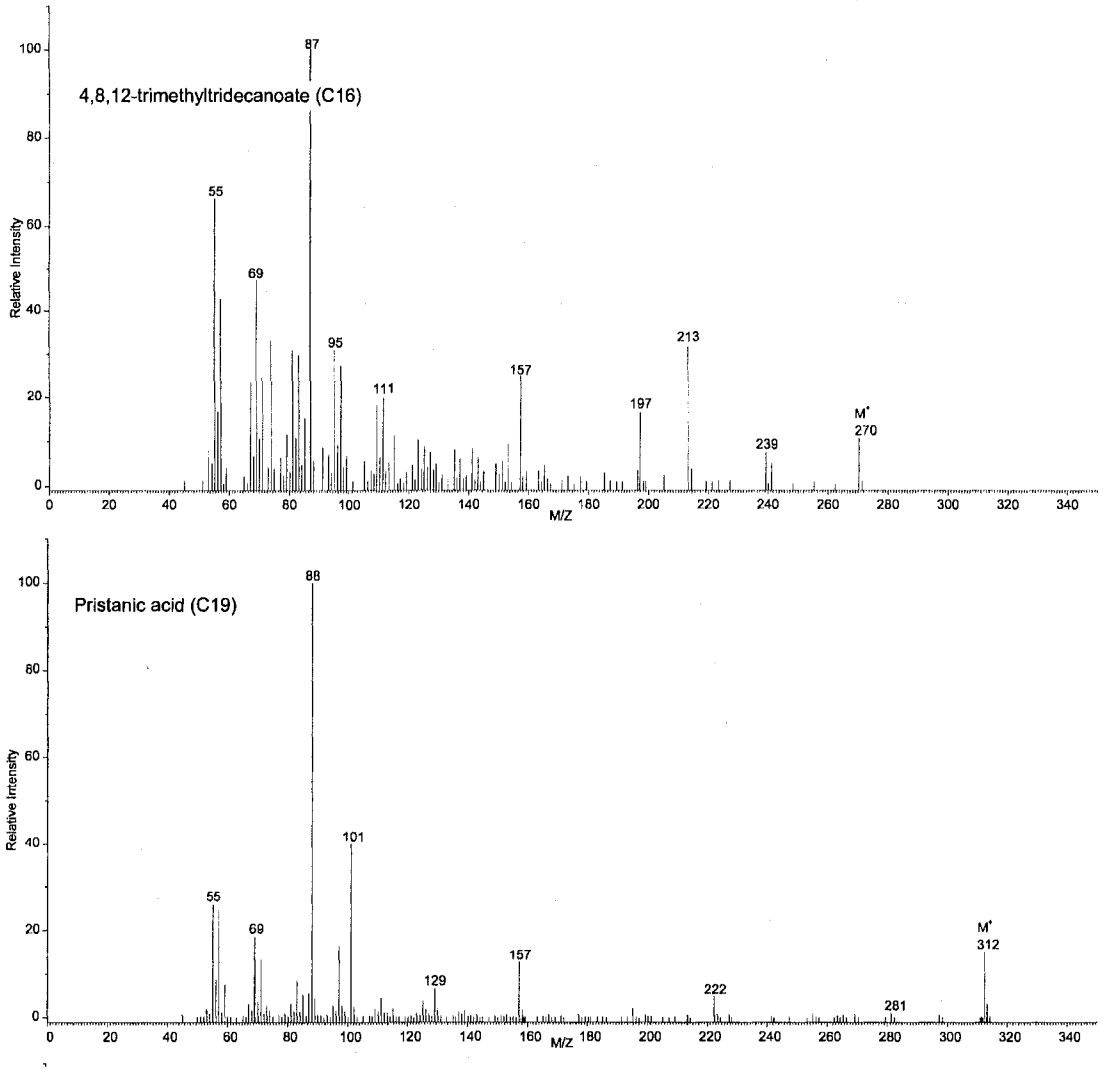


Figure 3.2.12 Ion impact mass spectra of pristanic, and 4,8,12 trimethyltridecanoic (TMTD) acids from *C. elegans* lipid extracts. Molecular ions, M^+, represent the molecular mass of a compound.

Growth of wild-type and P-44 mutant *C. elegans* was significantly retarded when grown on *E. coli* OP50 seeded agar plates enriched with phytanic acid (Fig. 3.2.13A).

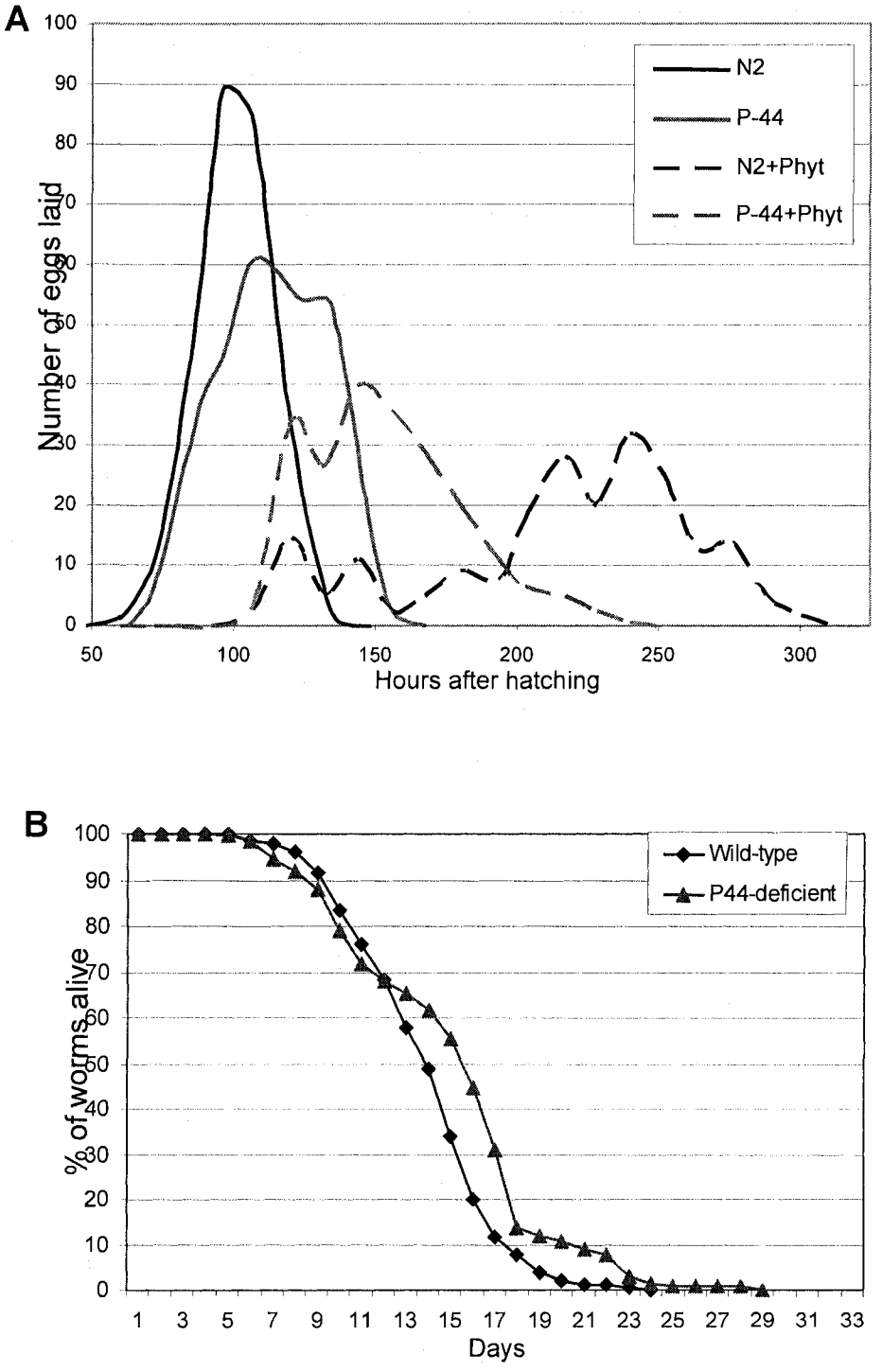


Figure 3.2.13 Comparative lifespan analysis of p-44 mutants vs. wild type worms with and without phytanic acid enriched media

3.2.5 Fatty acids composition of nlt-1 mutant and dhs-28 mutant *C. elegans*

Δnlt-1 nematode contains less C18:1 (n7), C20:5 (n3) and C20:4 (n3) fatty acids, but significantly more C17 iso, C18:2, C20:4 (n6) and C20:2 (n6) FAs (Fig. 3.2.14).

Just like P-44, nlt-1 seems to be required for catabolism of cyclopropane-containing FAs.

The spectra obtained from *Δdhs-28* mutant worms are similar with the lipid spectra extracted from P-44 defective nematodes (Fig. 3.2.8).

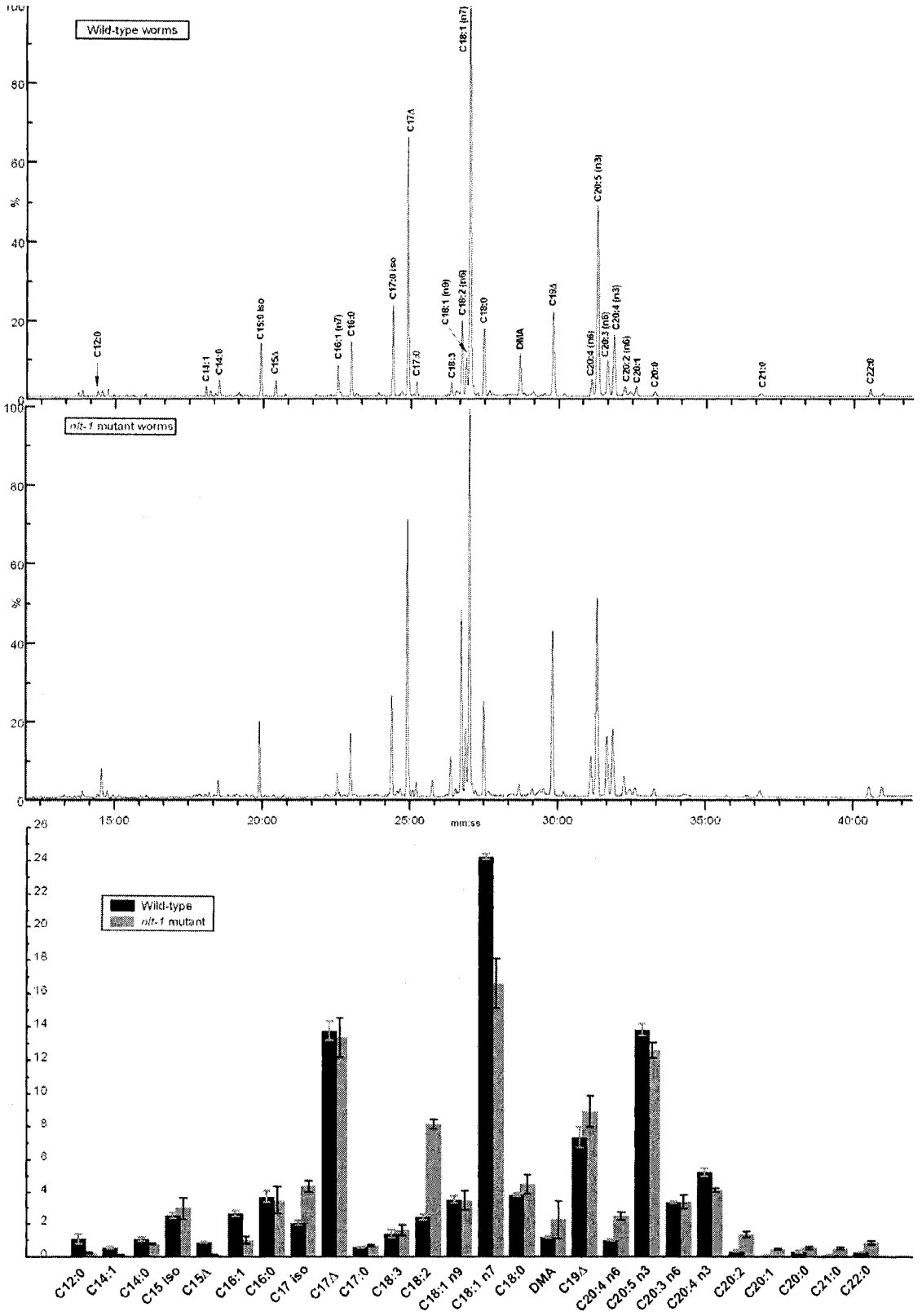


Figure 3.2.14 GS_MS spectra of total lipid extracts from *Δ*nlt-1 mutants and W/T (N2) *C elegans*

3.2.6 Discussion:

A number of developmental processes are impaired in P-44 mutants of *C. elegans*: dauer formation (Daf-d phenotype), egg laying, lifespan, body size and lipid accumulation.

The transition of *C. elegans* to the dormant dauer stage under conditions of scarce food supply and overcrowding requires daumone – a pheromone that is secreted by worms into their environment (Thomas JH *et al*, 1993). The daumone is a fatty acid derivative - (3,5-dihydroxy-6-methyltetrahydropyran-2-yloxy) heptanoic acid, the metabolism of which is unknown (Jeong et al, 2005). The P-44 thiolase can potentially be involved in biosynthesis of the daumone, or downstream effectors of daumone receptor.

Enormously increased lipid bodies and accumulation of triacilglycerols were found in worms with deficiency in P-44 thiolase (Fig. 3.2.4*D-E*, Fig. 3.2.7). The lifespan of these mutant nematodes in comparison with the wild-type was extended by 20.7% (Fig. 3.2.5*A*). Often a negative correlation between the amount of fat in worms or mice and their lifespan is observed (Van Gilst *et al*, 2005; Harrison *et al*, 1984; Bluher *et al*, 2003). Nevertheless, it is now becoming clear that low fat amount in an organism does not necessary directly increase lifespan (Sinclair *et al*, 2005), and our data present an additional case where these factors are completely unrelated. Extended longevity of ΔP-44 worms can be explained in several ways- 1) The delay in the development of this mutant (Fig. 3.2.5) is responsible for their extended lifespan. 2) P-44 mutant worms have 25% shorter body length (Fig. 3.2.1). Since there is a dependency of longevity on body mass for mammals (Van Heemst *et al*, 2005, Liang *et al,* 2003). However, no direct dependency of lifespan on body size has been reported for *C. elegans* (McCulloch *et al*, 2003). Yet, the correlation between

these two factors is not uncommon (Ventura et al, 2005; Lanjuin et al, 2002) and is attributed to involvement of insulin/IGF-I signaling pathway that controls entry to the dauer larval stage and adult longevity (McCulloch *et al*, 2003). Both programs are affected in ΔP-44 worms. A positive correlation between the decrease in unsaturated/saturated FAs ratio and extension of lifespan under caloric-restriction (CR) conditions was reported (Merry *et al*, 2002 review). *C. elegans* was already proposed as a convenient model for exploring the genetics of fat storage (McKay et al, 2003). Therefore, increased fat deposits in *C. elegans* do not mean increase in the number of fat storing cells.

Absence of P-44 thiolase in nematode resulted in significant changes in the levels of a variety of other fatty acids. Potentially this could also be a cause of documented irregularities in dauer transition, lipid accumulation and aging in the mutant worms.

CoA esters that are substrates of thiolase type II include intermediates of peroxisomal α-oxidation of tetramethyl-branched phytanic acid (C20), 2-hydroxytetracosanoic (cerebronic, C24) acid, as well as very-long chain fatty acids (VLCFAs) such as C24:0, C26:0 and C24:6 (n3) (Wanders, R.J. *et al,* 2001; Ferdinandusse, S. *et al*, 2004; Ferdinandusse, S. *et al*, 2004; Ferdinandusse, S. *et al*, 2001; Hashimoto, T. *et al*, 1999). During α-oxidation, phytanic acid is shortened to pristanic acid, which then enters peroxisomal β-oxidation, with the last step catalyzed by thiolase type II (Wanders, R.J. *et al,* 2001; Wanders RJ. *et al*, 1997). P-44 was previously demonstrated to exhibit thiolase activity toward branched substrates such as oxo-forms of the bile acid precursors as well as straight chain substrates in vitro

(Bun-ya, M. *et al,* 1997; Bun-ya, M. *et al*, 1998). At the same time, in mammals, a human counterpart of P-44, SCPx has been shown to possess 3-oxoacyl-CoA thiolase activity and catalyses the thiolytic step in both the breakdown of phytanic/pristanic branched-chain fatty acids and in the formation of the CoA esters of cholic acid and chenodeoxycholic acid from di- and tri-hydroxycholestanoic acid (Antonenkov, V.D. *et al*, 1997). SCPx deficient mice have marked changes in hepatic gene expression, increased peroxisome proliferation, and 10-fold elevated levels of phytanic acid (Seedorf, U. *et al*, 1998). Therefore, observed absence of tetramethyl-branched fatty acids in wild-type and even P-44 mutant *C. elegans* worms is rather unexpected. Most likely, it is due to the specific laboratory conditions of the nematode culture. The dietary source of phytanic acid for mammals is phytol - side chain of chlorophyll and isopreniods that are ingested with plants or certain bacteria (Verhoeven *et al,* 2001; Rontani *et al*, 2002). In case of humans sources of phytol also include dairy products and ruminant fats. Ruminants accumulate phytanic acid after phytol is released and oxidized to phytanate in their digestive system by gastrointestinal microorganisms (Verhoeven *et al,* 2001). Most likely, in a natural environment, the sources of phytol and hence branched fatty acids for *C. elegans* are organic remains of animals, plants, and soil microorganisms, particularly cyanobacteria. Under laboratory conditions, *C. elegans* are fed E. coli OP50 (Stiernagle, T. 1999). *E. coli,* like most other gram-negative bacteria, do not synthesize branched fatty acids (Smirnova et al, 2001; Lu Y.J *et al*, 2004). GC-MS analysis of total lipids from E. coli OP50 identifies only straight chain, hydroxy-containing, and cyclopropane-containing fatty acids (Fig. 3.2.8). Therefore, most likely, *E. coli* can not be considered an abundant natural

source of compounds containing the isoprenoid chain, such as benzoquinones, naphthoquinones, carotenes, tocopherols, farnesol, geranylgeranol and phytol (Lehninger, Principles of Biochemistry). Nevertheless, supplementation of *C. elegans* diet with additional source of pristanic acid proves that the P-44 nematode protein and mammalian SCPx have similar biochemical functions. Accumulation of an intermediate of pristanic acid catabolism, TMTD, in wild-type worms, but not in P-44 mutant worms fed pristanic acid, indicates that P-44 thiolase does take part in the β-oxidation of this α-methyl branched FA (Fig. 3.2.11).

SCPx was found to be implicated in the conversion of the CoA esters of different derivatives of cholestanoic acid (Antonenkov *et al*, 1997). *C. elegans* is found to have very low amounts of cholesterol in membranes, but it is also known that the nematode development is regulated by sterol-derived signaling molecules (Kurzchalia et al, 2003). P-44 may be involved in the biosynthesis of these specific forms of signaling molecules that regulate dauer transition, egg laying or lifespan.

P-44 and dhs-28 mutant worms also lack C13/15Δ fatty acids (Fig. 3.2.8). Cyclopropane-containing fatty acids are seldom found in eukaryotic lipids (Grogan et al, 1997; Body *et al,* 1972). Feeding of nematodes with FT17 *E. coli* strain defective in the synthesis of cyclopropane fatty acids shows that they are not synthesized *de novo* in *C. elegans*. C13/15Δ FAs are therefore most likely products of the β-oxidation of dietary C17/C19Δ lipids. Cyclopropane rings in the carbohydrate chains of delta-fatty acids put them into a class of lipids that is distinct from straight-chain FAs. The catabolism of delta-fatty acids has so far only been studied in microorganisms and their biological function is elusive even in bacteria (Tipton et al,

1972). We suggest that type II 3-oxoacyl CoA thiolase, P-44, as well as 17-β-hydroxysteroid dehydrogenase, dhs-28, catalyze intermediate steps in the β-oxidation of the dietary pristanic and C17/19Δ fatty acids in nematodes. Our observation shows that Cyclopropane fatty acids are dispensable for nematode development, as *C. elegans*, feeding on bacteria *(E. coli* strain OP50) that do not synthesize delta-fatty acids, develop normally to adult stage.

P-44 mutant worms are found to contain elevated levels of iso- C15 and iso-C17 FAs (Fig. 3.2.10*B*). These monomethyl branched FAs were reported previously to be essential for growth and development of *C. elegans* (Kniazeva *et al*, 2004). Their role in humans has become an object of investigation only recently, but some datas already report involvement of some monomethyl branched FAs, in particular C15:0 iso in cancer progression and apoptosis regulation (Yang *et al*, 2000). The levels of C17:0 iso FAs were found to correlate with the levels of C18:2 (n6) and C18:1 (n7) during nematode development (Kniazeva *et al*, 2004). We observed similar positive correlation between the levels of iso- C15/17:0 and C18:2 (n6) in worms at L4 stage (Fig. 3.2.10*B*). No observation was made for C18:1 (n7) as it significantly overlaps with C18:1 (n9) on the chromatogram.

The biochemical processes affected by P-44 deficiency causing depletion and/or accumulation of other minor forms of fatty acids in mutant nematodes seen at GC-MS spectra (Fig. 3.2.10*A*) require further detailed investigation. Similarly, the lower levels of C17/19Δ, C20:5 and C20:4 FAs as well as DMA in P-44 mutant are difficult to interpret.

A difference from the wild-type phenotype of peroxisomes was also observed in experiments with RNAi to P-44 (type 2 peroxisomal thiolase) or the three homologs of type 1 thiolases plus P-44 (Petriv O.I. *et al*, 2002). Whereas peroxisomes in worms injected with control dsRNA were round in shape, of uniform size and randomly distributed in the organism, the injection of dsRNA to P-44 caused the accumulation of clusters of peroxisomes that were fewer in number, irregular in shape and of variable size (Fig. 4*M* in Petriv O.I. *et al*, 2002), with most peroxisomes being larger than those found in worms injected with control dsRNA. A similar phenomenon has been observed in human, mammalian and yeast mutant cells deficient in other enzymes of β-oxidation. In these mutant cells, loss of the enzymatic activity of acyl-CoA oxidase (Fan *et al,* 1998; Chang et al, 1999; Van Roermund *et al*, 2000), fatty acyl-CoA synthetase (Van Roermund *et al*, 2000), and/or another peroxisomal β-oxidation enzyme, 2-enoyl-CoA hydratase/*D*-3-hydroxyacyl-CoA dehydrogenase (Chang et al, 1999; Smith *et al*, 2000), resulted in significant changes in peroxisome size and/or number. The primary targets for this so called metabolic control of peroxisome abundance (Chang et al, 1999) are likely the levels of other peroxisomal β-oxidation enzymes that are dramatically increased by the loss of acyl-CoA oxidase (Kniazeva *et al*, 2004) or 2-enoyl-CoA hydratase/*D*-3-hydroxyacyl-CoA dehydrogenase (Smith *et al*, 2000) enzymatic activity. The resultant overproduction of abundant matrix proteins leads to a significant change in peroxisome size (Chang et al, 1999; Van Roermund *et al*, 2000; Smith *et al*, 2000) and/or number (Kniazeva *et al*, 2004; Smith *et al*, 2000).

Interestingly, spontaneous peroxisome proliferation and significant alteration of gene expression in liver are early events in SCPx/SCP2-deficient (Ellinghaus, P. *et al*, 1999) and acyl-CoA oxidase-deficient (Klenk, E. *et al*, 1963) mice. These effects can be attributed to the biological activities of the SCPx and acyl-CoA oxidase substrates, phytanic acid and very-long chain fatty acids. They are biological ligands for the peroxisome proliferator-activated receptor α (PPARα) (Seedorf, U. *et al*, 1998; Kniazeva M. *et al*, 2004; Ellinghaus, P. *et al*, 1999). Although GS-MS analysis did not detect these forms of fatty acids in P-44 mutant, nlt-1 mutant and dhs-28 mutant worms fed *E. coli*, an increased concentration of these molecules in mutant worms cannot be ruled out. In fact, the biological effect of dietary phytanic acid on the nematode resembles that of P-44 deficiency but is more prominent. When P-44 mutant worms or wild-type worms were fed phytanic acid added to the *E. coli* lawn, development of both strains was severely affected as indicated by enormously extended egg-laying period of both strains (Fig. 3.2.13 *A*). Nlt-1 may play role of the substrate-binding factor for the enzyme by converting 18:2 (n6) into c18:1 (n9).

No human disorder has been described in which either peroxisomal thiolase I or thiolase II (SCPx) is deficient (Wanders, R.J. *et al,* 2001). Nevertheless, an accumulation of phytanic acid and VLCFAs was detected in patients suffering from Refsum disease (Klenk, E. *et al*, 1963), some cases of Zellweger's syndrome, Rhizomelic chondrodysplasia punctata, Sjögren-Larsson syndrome, and α-methylacyl-CoA epimerase deficiency (Mukherji, M. *et al*, 2003). Mammalian SCPx protein consists of two functional domains, the thiolase domain and the SCP2 (sterol carrier protein 2) domain (Ohba, T. *et al*, 1994). As the *SCPx* gene has two independent

promoters that control transcription from alternative start sites, both the SCPx (thiolase domain fused to SCP2) and the SCP2 (displaying only sterol carrier activity) proteins are synthesized in mammals (Ohba, T. *et al*, 1995). SCP2 was found to be involved in the formation of a substrate complex for the enzyme phytanoyl-CoA 2-hydroxylase (PhyH) (Mukherji, M. *et al,* 2002) and also proposed that SCP2 may form substrate complexes with all enzymes of the α-oxidation pathway (Mukherji, M. *et al,* 2003). PhyH is defective in patients with Refsum's disease (Mihalik, S.J. *et al,* 1997). Some data also indicate that SCPx, together with the thiolase type I, is involved in the oxidation of the very-long straight-chain fatty acids C24:0, C26:0 (Ferdinandusse, S. *et al*, 2004) and C24:6n-3 (Ferdinandusse, S. *et al*, 2001). According to immunoblot analysis, SCPx knockout mice appear to lack both proteins, SCPx and SCP2 (Seedorf, U. *et al*, 1998). No animal model organism having SCP2 and the thiolase domains of SCPx in different genes has been reported to date. Interestingly, the thiolase and SCP2 domains of SCPx have nematode homologs that are encoded by two different genes, *p-44* and *nlt-1* (ZK892.2), respectively, in the *C. elegans* genome (WORMBASE).

These results show how the nematode model can be advantageous for resolving the biochemical problems arising in a mammalian organism.

CHAPTER 3.3

THE ACCUMULATION OF THE VITAL DYE NILE RED IN THE INTESTINE-SPECIFIC "LIPOFUSCIN GRANULES" MAKES IT UNSUITABLE FOR VISUALIZING FAT STORAGE DROPLETS IN LIVING Caenorhabditis elegans

3.3.1 Summary

Fat deposition and mobilization in the nematode *Caenorhabditis elegans* are important determinants of longevity. In recent years, the vital lipophilic dye Nile Red (NR) has been employed for visualizing fat storage droplets in living nematodes and for elucidating the role of fat regulatory genes in nematode aging. Here, we directly measured the levels of triacylglycerols, the main form of fat storage in *C. elegans*, by thin-layer chromatography (TLC). Using TLC for a quantitative assay of triacylglycerols in several nematode mutants known to display enhanced, reduced or unchanged (as compared to that in wild type) NR fluorescence, we found no correlation between the levels of triacylglycerols and a specific pattern of NR staining. Thus, NR is not suitable for monitoring body fat in *C. elegans*. Our analysis of the spatial distribution of NR in wild-type animals and in short- or long-lived nematode mutants revealed that a substantial amount of this dye accumulated in autofluorescent secondary lysosomes. These intestine-specific secondary lysosomes are called "lipofuscin granules" because they accumulate the wear-and-tear pigment lipofuscin, which is considered as a biomarker of age-related decline. Although the number of "lipofuscin granules" in the intestine of wild-type and mutant animals increased with their chronological age, we found no correlation between the mean lifespan of a strain and the number of the granules it accumulated. Hence, the number of "lipofuscin granules" is not a reliable biomarker of nematode aging.

3.3.2 Results:

Fat metabolism in *Caenorhabditis elegans* plays an important role in lifespan determination. In fact, the key longevity genes regulate the biosynthesis and degradation of neutral lipids deposited in fat storage droplets (Kimura *et al.*, 1997; Ashrafi *et al.*, 2003; Lee *et al.*, 2003; Murphy *et al.*, 2003; Mukhopadhyay *et al.*, 2005; Van Gilst *et al.*, 2005; Mak *et al.*, 2006; McElwee *et al.*, 2006; Oh *et al.*, 2006; Taubert et al., 2006; Brock *et al.*, 2007).

In recent years, the vital lipophilic dye Nile Red (**NR**), whose fluorescence is considered as an indicator of neutral lipid content (Ashrafi *et al.*, 2003), has been used for visualizing fat storage droplets in living nematodes and for elucidating the role of fat regulatory genes in nematode aging (Ashrafi *et al.*, 2003; Lee *et al.*, 2003; Mukhopadhyay *et al.*, 2005; Van Gilst *et al.*, 2005; Mak *et al.*, 2006; Oh *et al.*, 2006; Taubert et al., 2006; Brock *et al.*, 2007). A recent study showed a significantly increased NR fluorescence in aging *kat-1* mutant worms to be due to their markedly increased body fat (Mak *et al.*, 2006). However, we found that the observed staining pattern is not due to the excessive accumulation of fat storage droplets in *kat-1* animals and thin-layer chromatography (TLC) to separate total lipids and failed to reveal any significant difference in triacylglycerol levels between wild-type (WT) and *kat-1* animals (Fig. 3.3.1; Fig. 3.3.2). Triacylglycerols are the main constitutuent of fat storage in *C. elegans* (Ashrafi *et al.*, 2003). Moreover, staining of fixed animals with Sudan Black B (**SBB**), a dye that has been previously (but not in recent years)

employed to visualize fat storage droplets (Kimura *et al.*, 1997), revealed no difference in body fat between WT and *kat-1* animals (Fig. 3.3.1). These observations prompted me to use TLC to measure directly triacylglycerols level (as quantitative assessment) in several nematode mutants. These mutants are known to display enhanced, reduced or unchanged NR fluorescence compare to that in WT. We found no correlation between triacylglycerol levels and the intensity of NR staining (Fig. 3.3.1; Fig. 3.3.2). In contrast, SBB staining intensity of fixed animals completly correlated with triacylglycerol levels (Fig. 3.3.1). Thus, NR is not suitable for monitoring body fat in *C. elegans*. It seems that staining of animals with SBB in combination with the direct assessment of triacylglycerols by TLC is more appropriate for this purpose.

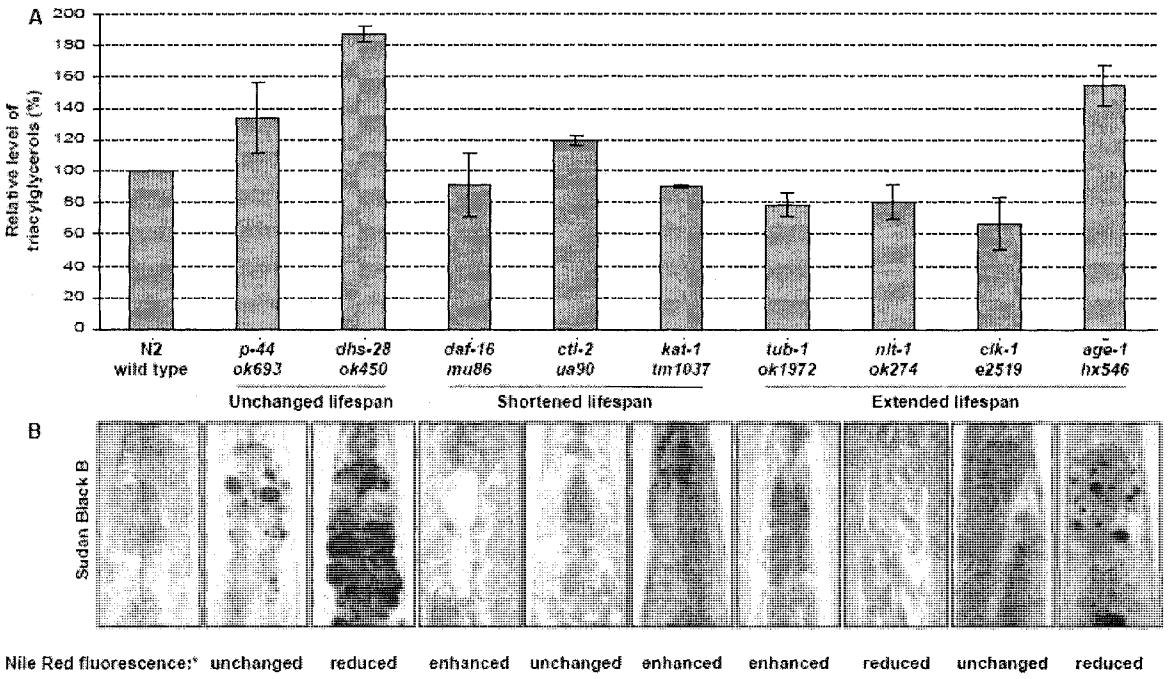


Figure 3.3.1 The levels of triacylglycerols in wild-type (WT) and mutant worms, which were monitored using thin-layer chromatography (TLC), correlate with the intensities of staining of fixed animals with Sudan Black B (SBB). (A) Lipids that were extracted from equal amounts of total protein recovered from wild-type and mutant animals were separated by TLC and visualized using 5% phosphomolybdic acid in ethanol. Triacylglycerols were quantitated by densitometric analysis of TLC plates as described previously (Guo et al., 2007). (B) SBB staining of WT and mutant animals. Each picture displays the anterior part of animal's gut. Pharynx is positioned upwards. All animals were analyzed 3 days after they reached the reproductive state. *The relative intensity of Nile Red fluorescence for each of the mutant animals, as compared to that for wild-type worms, is shown at the bottom.

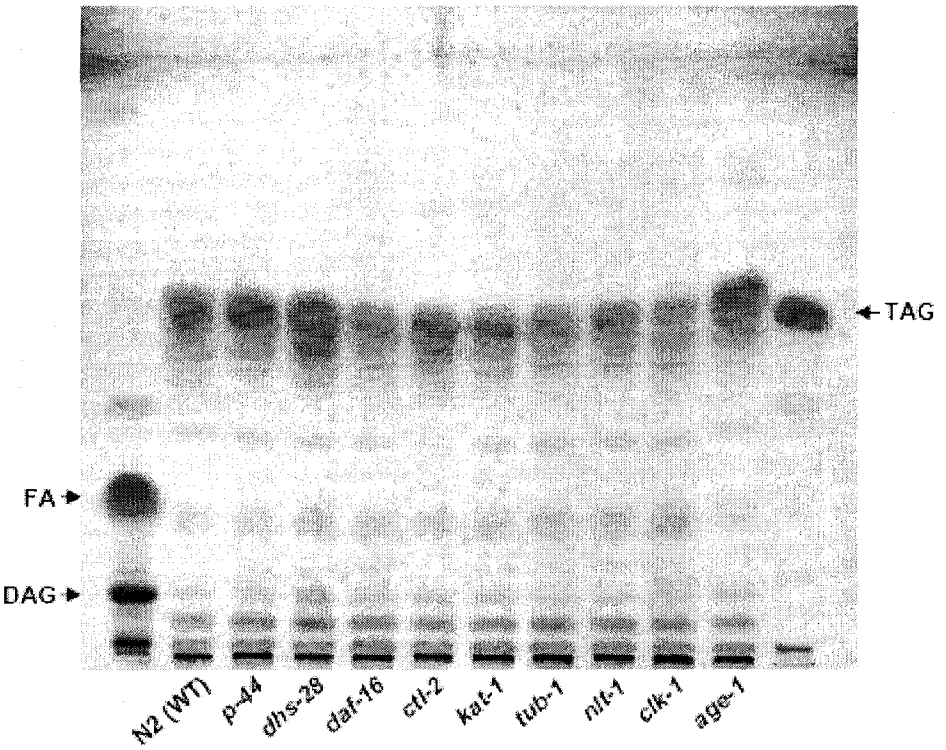


Figure 3.3.2 Spectra of neutral lipids extracted from wild-type and mutants

Extracted total lipids were separated by thin-layer chromatography (TLC) and visualized using 5% phosphomolybdic acid in ethanol. Lipids that were extracted from equal amounts of total protein recovered from wild-type and mutant animals were spotted on a silica gel plate for T LC and developed in the hexane/diethyl ether/acetic acid (80:20:0.5, v/v) solvent system. *Abbreviations*: DAG, diacylglycerol; FA, fatty acid; TAG, triacylglycerol.

Our analysis of the spatial distribution of NR in *C. elegans* revealed that a substantial amount of this dye in WT animals accumulated in distinct autofluorescent and birefringent granules that are visible with polarization optics (Fig. 3.3.3A). These granules represent secondary lysosomes located exclusively in the intestine, the major fat storage tissue (Clokey and Jacobson, 1986; Kostich *et al.*, 2000). They are also called "lipofuscin granules" due to their ability to accumulate the wear-and-tear autofluorescent pigment lipofuscin (Clokey and Jacobson, 1986; Kostich *et al.*, 2000). Lipofuscin is considered as an endogenous biomarker of age-related decline (Braeckman *et al.*, 2002; Gerstbrein *et al.*, 2005; Hütter *et al.*, 2007). These granules accumulate another vital dye called FM4-64 (Chen *et al.*, 2006) besides NR (Fig. 3.3.3).

But Sudan Black B (SBB) accumulation in animals correlated with their triacylglycerol levels (Fig. 3.3.1) and fully blocks the immense autofluorescence produced by "lipofuscin granules" (Romijn *et al.*, 1999).

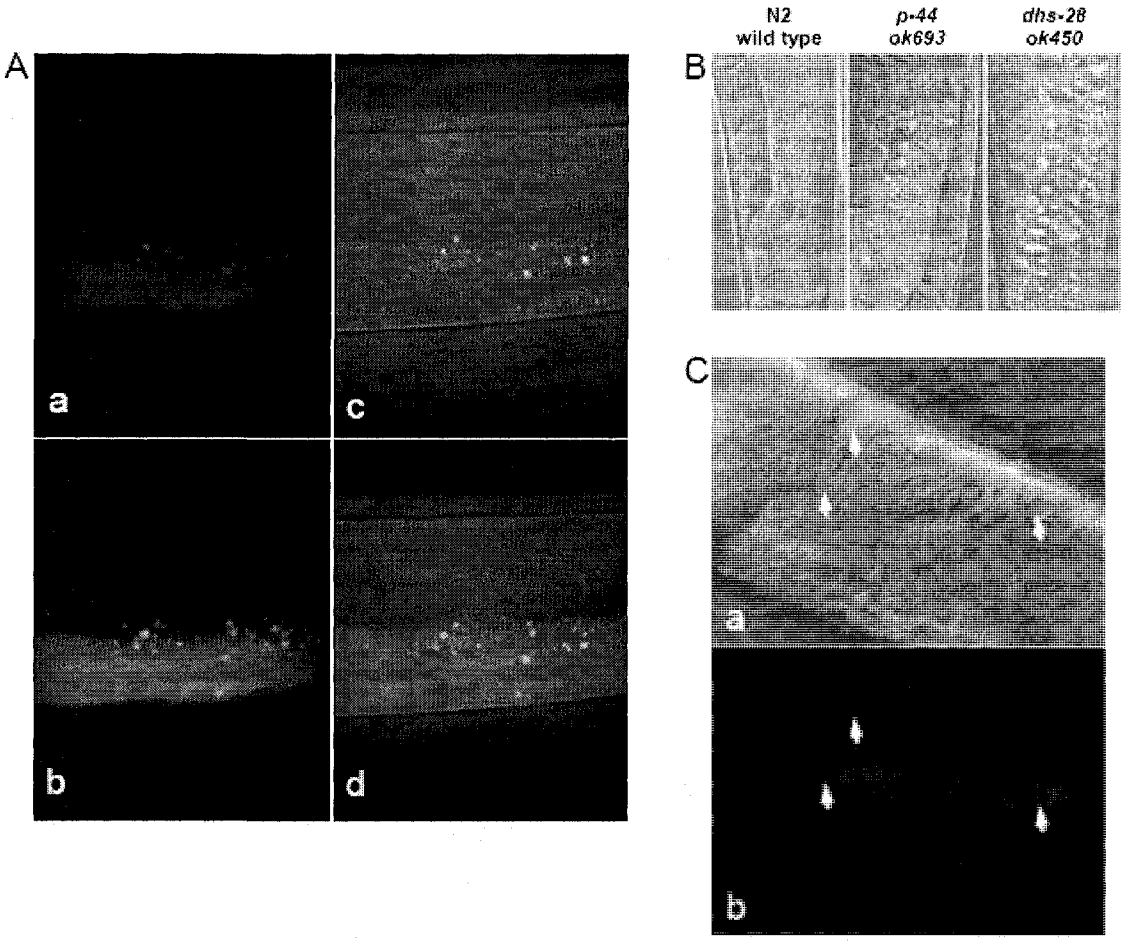


Figure 3.3.3 Nile Red accumulates in the intestine-specific autofluorescent and birefringent "lipofuscin granules" more efficiently than in fat storage droplets of *C. elegans*. (A) Nile Red staining of wild-type (WT) worms: (a) Fluorescence in the Rhodamine excitation channel using the Green H 546 filter; (b) Fluorescence in the DAPI excitation channel using the UV-G 365 filter; (c) Birefrigent granules as seen in polarized light; and (d) Merge of images b and c. Images (a) and (b) were taken in all three (*i.e.*, red, green and blue) emission channels in order to record broad-range emission spectra of the specimen. (B) Bright-field images of WT, *p-44* and *dhs-28* worms. Greatly enlarged fat storage droplets are seen in both mutants. (C) Nile Red staining of the *dhs-28* mutant worm: (a) Bright-field image; and (b) Nile Red fluorescence. Arrows indicate the positions of fat storage droplets.

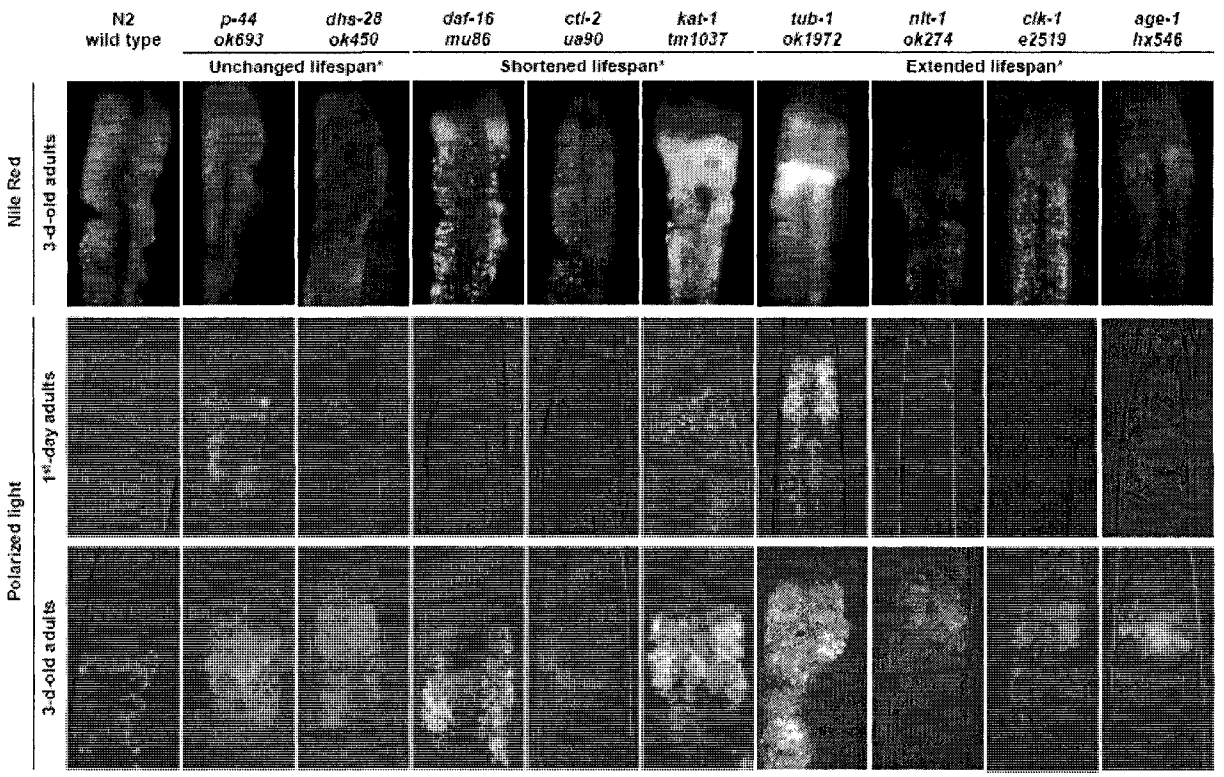


Figure 3.3.4 The intensity of Nile Red (NR) fluorescence correlates with the number of autofluorescent "lipofuscin granules" in wild-type (WT) worms and in all of the mutant animals tested. NR fluorescence (*upper row*) and birefringent autofluorescent granules visualized with polarization optics (*middle and lower rows*) of WT and mutant animals are shown. Animals were analyzed when they reached the reproductive state (*middle row*) or 3 days after that (*upper and lower rows*). Each picture displays the anterior part of animal's gut. Pharynx is positioned upwards.

*The names of mutant animals and of the mutant gene alleles they carried, as well as the relative lifespan for each of the mutant animals used (as compared to that for WT) are shown at the top.

3.3.3 Discussion

These findings suggest that the significant increase in NR fluorescence seen in aging *daf-16*, *kat-1* and *tub-1* animals (Fig. 3.3.4; Mukhopadhyay *et al.*, 2005; Mak *et al.*, 2006) was not due to the excessive accumulation of fat storage droplets. In contrast, this staining pattern could be attributed to the increased number of autofluorescent "lipofuscin granules" that are capable of accumulate NR (Fig. 3.3.3A) in the intestine, the major fat storage tissue. Our hypothesis is supported by the observation that the number of these intestine-specific "lipofuscin granules" in aging *daf-16*, *kat-1* and *tub-1* animals considerably exceeded that seen in WT (Fig. 3.3.4; Gerstbrein *et al.*, 2005). Notably, the excessively proliferated fat storage droplets found in some mutant worms showed a diminished ability to accumulate NR.

In fact, *p-44, dhs-28* and *age-1* animals had greater levels of storage triacylglycerols (as compared to that seen in WT) that were directly assessed by TLC and visualized using SBB (Fig. 3.3.1). These mutants did not show a significant increase in the total NR fluorescence (Fig. 3.3.4). They also had built up giant fat storage droplets that could be easily seen in visible light (Fig. 3.3.3B) and that accumulated NR much less efficiently than the intestine-specific autofluorescent "lipofuscin granules" surrounding them (Fig. 3.3.3C).

Importantly, although the number of "lipofuscin granules" in the intestine of WT and mutant animals increased with their chronological age (Fig. 3.3.4), we found no correlation between the mean lifespan of a strain and the number of "lipofuscin granules" it accumulated. Microscopical monitoring of "lipofuscin granules" with polarization optics revealed that the number of these granules in aging *p-44* and *dhs-*

28 mutants substantially exceeded that seen in aging WT animals (Fig. 3.3.4). But their mean lifespans were very similar to that of WT (Table 3.3.1).

The number of these granules in prematurely aging *daf-16* and *kat-1* mutants (Fig. 3.3.5; Table 3.3.1) considerably exceeded that seen in aging WT animals, while the short-lived *ctl-2* mutant had fewer "lipofuscin granules" than the WT animals (Fig. 3.3.4).

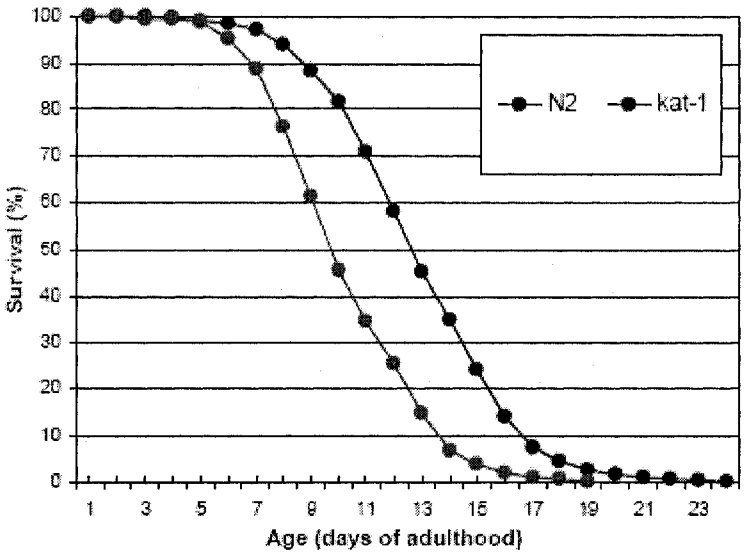


Figure 3.3.5 The *kat-1 (tm1037)* mutation significantly shortens the lifespan of *C. elegans*

The *kat-1 (tm1037)* mutation significantly shortens the lifespan of *C. elegans*. The mean lifespan of *kat-1 (tm1037)* worms (8.8 ± 1.0 days) is 68.4 ± 3.8% of the mean lifespan (13.6 ± 0.6 days) of wild-type worms. Average of 4 independent experiments ($p \leq 0.05$).

Table 3.3.1 Mutant strains of *C. elegans* used in this study and their mean lifespans relative to that for the wild-type strain N2.

Gene name (ORF name)	STRAIN (*mutant allele*)	Mean lifespan as compared to that for the wild-type strain N2
p-44 (Y57A10C.6)	RB859 (*ok693*)	Unchanged
dhs-28 (M03A8.1)	VC262 (*ok450*)	Unchanged
daf-16 (R13H8.1)	CF1038 (*mu86*)	Shortened (Larsen *et al.*, 1995)
ctl-2 (Y54G11A.5)	LB90 (*ua90*)	Shortened (Petriv and Rachubinski, 2004)
kat-1 (T02G5.8)	TM1037 (*tm1037*)	Shortened (Fig. 3.2.5)
tub-1 (F10B5.4)	RB1600 (*ok1972*)	Extended (Mukhopadhyay *et al.*, 2005)
nlt-1 (ZK892.2)	RB1428 (*ok274*)	Extended
clk-1(ZC395.2)	CB4876 (*e2519*)	Extended (Wong *et al.*, 1995)
age-1 (B0334.8)	TJ1052 (*hx546*)	Extended (Friedman and Johnson, 1988)

On the other hand, the long-lived *tub-1* mutant accumulated excessive amount of these autofluorescent granules, but the long-lived *nlt-1*, *clk-1* and *age-1* mutants did not (Fig. 3.3.4).

We therefore conclude that the number of autofluorescent "lipofuscin granules" in the intestine of worms is not a reliable biomarker of their chronological lifespan.

CHAPTER 4

CONCLUSION

&

FUTURE PROSPECTS

4.1.1 Conclusion:

The main objective of this research was to define the localization of *C. elegans* orthologs of Human Sterol Carrier Protein x (hSCPx) whose deficiency affects the rate of chronological aging and post-embryonic development in various tissues of this organism and in different neurons. Peroxisomal thiolase P-44 is the main nematode thiolase or at least it has no redundant homologs. This plays important role in the regulation of dauer formation, egg-laying period, worm body size and lipid accumulation. The lifespan of the P-44 mutant appears to be extended (by 20%). The nlt-1 mutantio also extends lifespan by 40%in compare with wild type. P-44, nlt-1 and dhs-28 are expressed mostly in intestinal cells and localized to the peroxisome. They are involved in the fatty acid metabolism. Both P-44 and nlt-1 seem to be required for catabolism of cyclopropane containing fatty acids. P-44 deficiency also blocks the catabolism of pristanic acid. Dhs-28 (17- β-hydroxysteriod dehydrogenase) on the otherhand also shows same biochemical pathway. Often a negative correlation between amount of fat in worms or mice and their lifespan is observed (Van Gilst MR *et al.,* 2005; Harrison, DP *et al,* 1984; Bluher M, *et al.,* 2003). Nevertheless it is now becoming clear that low fat amount in an organism does not necessary directly increase lifespan (Sinclair et al., 2005 Review). Our data also support views that there is no absolute link between amount of lipid deposits in an organism and a lifespan.

We also checked the use of vital lipophilic dye Nile Red (NR) for visualizing fat storage droplets in living nematodes and for elucidating the role of fat regulatory genes in nematode aging. We also directly measured the levels of triacylglycerols, the main form of fat storage in different *C. elegans* mutants known to display enhanced, reduced or unchanged TAG metabolism(as compared to that in wild type) by thin-layer chromatography (TLC). We found no correlation between the levels of triacylglycerols and the pattern of NR staining. Thus, NR is not suitable for monitoring body fat in *C. elegans*. It seems that staining of animals with SBB in combination with the direct assessment of triacylglycerols by TLC is more appropriate for this purpose.

4.1.2 Future Research Prospect:

My findings have opened several directions for the future studies. In particular, I would like to use functional proteomics in order to define the network of protein components whose synthesis and degradation are regulated by the peroxisome-associated proteins controlling aging and development of the nematode.

We can also make double mutant (nlt-1/daf-16 and P44/daf-16) to find out whether nlt-1 and daf-16 are working in the same pathway. In case of double daf-16/Nlt-1 mutant we need to measure lifespan to see whether Δnlt-1/Δdaf-16 has a shorter lifespan as Δdaf-16. If so, then daf-16 will be downstream of nlt-1. If double mutant

keep living long like Δnlt-1 mutant – then nlt-1 is downstream of daf-16 or in an alternative pathway. If not then we need to move to 2nd option- to measure lifespan of Δnlt-1 and ΔP44 at the conditions of caloric restriction. nlt-1 and P-44 has already extended lifespan- if DR increases the lifespan even further then they work in a pathway that is parallel to the DR pathway. The 3rd choice is to make double mutant that has impaired peroxisomal respiration and measure the lifespan of ΔP44 and Δnlt-1 mutants in a strain with defective mitochondrial respiration. Using this analysis we can place P-44 and nlt-1 proteins within the regulatory network contents nematode lifespan.

The other direction of my future research will be to employ the mass spectrometry based lipidomics for analyzing the chronological changes in steady-state levels of various lipid species in subcellular organelles, cells and tissues of the short- and long-lived mutants that I have identified so far. Taken together, the above described directions of my future research will establish the hierarchy of events that occur during the remodeling of the metabolic pathways, inter-organellar communications and signal transduction networks in the aging nematode.

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